DOCTORAL THESIS

Characterising and Determining the Botanical Origin of Estonian Honeys

Evelin Kivima

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for a doctoral degree at Tallinn University of Technology, has not been previously submitted for a doctoral or equivalent academic degree.

Evelin Kivima

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Eesti mete iseloomustamine ja botaanilise päritolu määramine

EVELIN KIVIMA

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LIST OF PUBLICATIONS

This dissertation is based on the following publications:

I Kivima, E.; Seiman, A.; Pall, R.; Sarapuu, E.; Martverk, K.; Laos, K. (2014). Characterization of Estonian honeys by botanical origin. *Proceedings of the Estonian Academy of Sciences, 63 (2)*, 183-192.

II Seisonen, S.; Kivima, E.; Vene, K. (2015). Characterization of the aroma profiles of different honeys and corresponding flowers using solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry. *Food Chemistry, 169*, 34-40.

III Kivima, E.; Tanilas, K.; Martverk, K.; Rosenvald, S.; Timberg, L.; Laos, K. (2021). The composition, Physicochemical Properties, Antioxidant Activity, and Sensory Properties of Estonian honeys. *Foods*, *10*, 511.

AUTHOR'S CONTRIBUTION TO PUBLICATION

I. In Publication I, the author performed the experimental study and interpreted the data in collaboration, wrote the manuscript and is the corresponding author.

II. In Publication II, the author performed the experimental work in collaboration, participated as a GC-O assessor and is a co-author of the manuscript.

III. In Publication III, the author was one of the performers of the experimental work, interpreted the results in collaboration, wrote the manuscript and is the corresponding author.

INTRODUCTION

Honey is a sweet substance produced by bees, has been consumed since ancient times and is widely known for its beneficial properties. The compositions and properties can vary greatly depending on the botanical origin of the honey, as well as the geographical origin and other factors. The largest proportion of honey is made up of two monosaccharides: glucose and fructose. Honey also contains other components, including vitamins, minerals, phenolic compounds, enzymes and amino acids. The basic method to determine the botanical origin of honey is pollen analysis, but other analytical methods have been successfully used to complement it. These include the determination of physico-chemical parameters and sensory analysis. Sometimes pollen analysis is not enough to determine whether a honey is monofloral or not. In order to classify honey, it is important to identify marker compounds, i.e. a certain component that occurs only in a certain type of honey.

The current honey directive lays down the requirements for the quality and description of honeys when marketing. There are various types of honey fraud, for example dilution with syrups, harvesting of immature honey, and masking or mislabelling the geographical and/or botanical origin. In recent years, more work and discussions at the European Union level have taken place regarding two main concerns: the detection of honey authenticity and correct honey labelling when indicating botanical and geographical origin. It is important for the consumer that the contents of the product correspond to what is stated on the package label of the honey. Thus, determining the authenticity and detection of the origin is of major importance, in addition to honey quality.

Since the production and consumption of honey is also very important in Estonia, more attention must be paid to its authenticity in terms of domestic consumption and exports. It is important to know what Estonian honeys are like and what their compositions are, which allows for better identification of the authenticity and origin. There have been some studies focused on different analyses related to Estonian honeys, but there is a lack of comprehensive data that makes it possible to create connections between different properties and compositions of honeys. The results of this study can be used to determine the botanical origins of honeys and to determine the quality of honeys. The data obtained from this work makes a major contribution to the knowledge of and future work related to the honeys originating in Estonia. This thesis points out the pros and cons of various methods and offers possible solutions for a better understanding of typical Estonian honeys. Physico-chemical parameters are evaluated routinely for honey quality control but this thesis focuses on other effective methods to determine the origins of honeys and on finding specific marker compounds.

The purpose of this thesis was to characterise typical Estonian honeys by their compositions and properties, and to differentiate between them by botanical origin. The objective was to use various methods of analysis to determine the most effective approach(es) to honey analysis. The goal was to find specific characteristics or marker compounds that can be used to describe honeys of certain botanical origins. Botanical origins were determined by using melissopalynological analysis, which made it possible to estimate nectar content by pollen percentage and the presence of certain plants. Various methods were used to determine the characteristic compositions and properties of honeys. Physico-chemical parameters, such as electrical conductivity, moisture content, free acidity, hydroxymethylfurfural, diastase and invertase activity and sugar content, were measured. Those parameters are used in routine analysis for evaluating

honey quality but some can also be used for honey classification. In addition, honeys were differentiated based on amino acid content, total polyphenol and total flavonoid content, polyphenol identification, antioxidant activity and fluorescence spectra. Honeys were characterised by their aroma profiles, and the connection between aroma compounds of honeys and of certain plants most affecting their botanical origins were detected. For flavour and odour evaluations, such attributes as berry-like, fruity, floral, woody, herbal, spicy, sweet and animal-like were used for description and the identification of differences. Statistical analysis was used to interpret the data.

ACRONYMS

a*	colour parameter (redness/greenness)
ACL	lipid-soluble antioxidants
ACW	water-soluble antioxidants
AHC	Agglomerative Hierarchical Clustering
b*	colour parameter (blueness/yellowness)
CA	Correspondence Analysis
EC	electrical conductivity
EM	excitation range
EX	emission range
F/G	fructose/glucose ratio
GC	gas chromatography
GC-O	gas chromatography-olfactometry
G/W	glucose/water ratio
HMF	hydroxymethylfurfural
HPLC-RI	high-pressure liquid chromatograh refractive index detector
L*	colour parameter (lightness/darkness)
LC-MS	liquid-chromatography-mass-spectometry
LC-UV	liquid-chromatograph Ultraviolet
MIR	mid-infrared spectroscopy
NIR	near-infrared spectroscopy
NMR	nuclear magnetic resonance
PARAFAC	Parallel Factor Analysis
PCA	Principal Component Analysis
PLC	photochemiluminescence method
SFS	spectral fluorescence signatures
SPE	solid-phase extraction
SPME	solid-phase microextraction
TFC	total flavonoid content
TPC	total polyphenol content
UPLC	ultra-performance liquid chromatography
VOCs	volatile organic compounds

1. LITERATURE REVIEW

1.1 Bee products

Honey is a naturally sweet substance produced by honeybees (*Apis mellifera*). During maturation, the bees turn nectar, a thin and easily spoiled sweet liquid, into a stable, high-density and high-energy food (White & Doner, 1980). A honey may be classified as floral when it is produced by honeybees from the nectar of plants, and non-floral (e.g. honeydew) when it is derived from secretions of plants or excretions of plant-sucking insects (Anklam, 1998). The properties and composition of a honey depend on the plant the nectar is derived from, but also on bee species, climate, storage and even harvest technology and, as there are so many plant species, each honey is unique (Popek, 2002; Persano Oddo & Bogdanov, 2004; Tosi et al., 2004; Kaškonienė & Venskutonis, 2010; Drivelos et al., 2021; Yayinie et al., 2021). The compositions of some components, such as water, carbohydrates, trace organic acids, amino acids, pollen and wax, result from the maturation of honey. Some components are added by bees and some come from plants (Anklam, 1998).

Besides honey, there are other essential products obtained from the beehive, including beebread and bee pollen (Figure 1). Bee pollen consists of pollens that have been packed by the worker honeybees into granules called pollen balls, with added honey and nectar (Abdulrahaman et al., 2013).

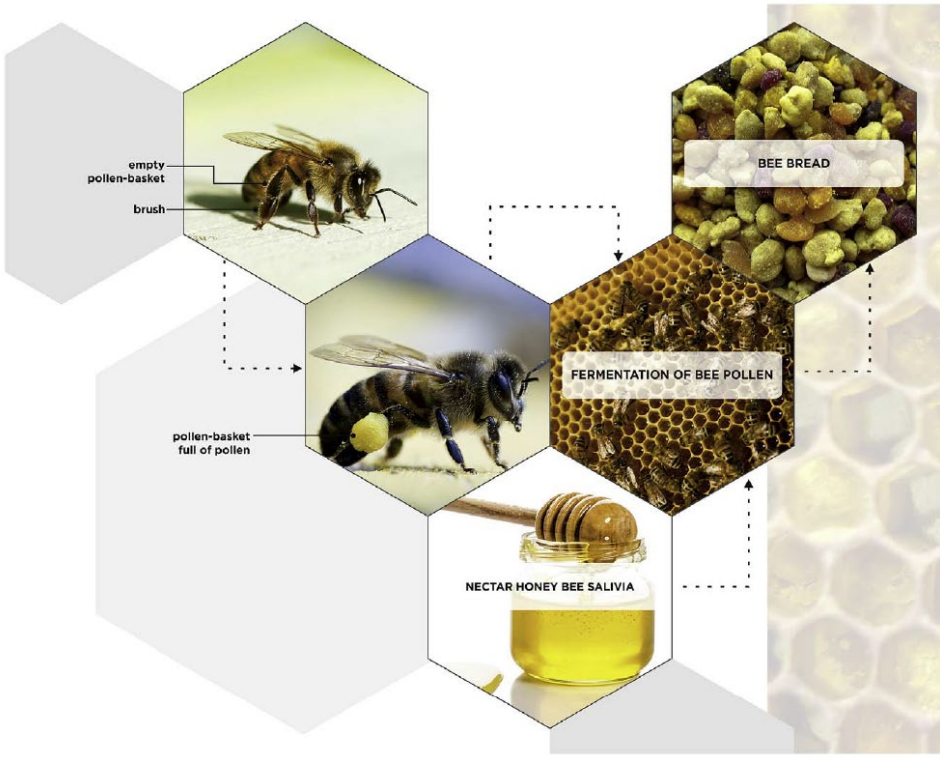

Figure 1. *The process of making beebread (Kieliszek et al., 2018).*

Beebread is a compound of pollens collected by the bee to which honey, enzymes and organic acids that are contained in the secretions of the salivary glands of bees are added. The beebread is then stored inside the beehive usually for fifteen days, where it undergoes lactic acid fermentation (Tammet, 2007). Beebread is the primary source of protein for bees and has a richer chemical composition than bee pollen (Kieliszek et al., 2018). For both bee pollen and beebread, the compositions vary depending on the botanical origin (Campos et al., 2008; Al-Kahtani et al., 2020).

1.2 Classical methods of honey specification

1.2.1 Melissopalynology

Melissopalynology is an official method of determining the floral origin (Kaškonienė et al., 2010; Čeksterytė et al., 2013; Puusepp & Koff, 2014; Puścion-Jakubik et al., 2020) and geographical origin (Dimou et al., 2014; El Sohaimy et al., 2015) of a honey. It is a time-consuming method that is based on the identification and counting of pollen grains and honeydew elements in honey sediment by microscopic analysis (Anklam, 1998).

Pollen is the main source of proteins (González Paramás et al., 2006; Al-kahtani et al., 2020), fatty substances (Čeksterytė et al., 2014), vitamins and minerals (Soares de Arruda et al., 2013) for bees. The number of pollen grains in a honey depends on many factors, including plant morphology and physiology, the action of foraging bees, the distance of the hive from the forage source, the harvesting season and beekeeping practices (Crane, 1975; Bilisik et al., 2008; Al-Kahtani et al., 2020). The pollen may get into the honey through the nectar of the plant that contains the pollen, or from bees, to whose legs and antennae the pollen adheres. It is then later regurgitated with the collected nectar (Salonen et al., 2009). In addition, some pollen may get into honeys during the extraction process or from the air (Von der Ohe et al., 2004).

The mass and morphology of pollen have to be considered, because when bees fly back to the hive they move vast amounts of pollen from the nectar sources, and bees are able to remove larger pollens more efficiently than smaller ones (Bryant, 2001). The plants that produce large pollen grains do not produce large amounts of nectar and are under-represented. On the other hand, smaller pollens are usually over-represented in honey and, because of their smaller size, they are only partially filtered out in the honey stomachs of honeybees (Bryant & Jones, 2001).

Bees gather nectar and pollen from different plants and sometimes the presence of even a small percentage of a certain plant type can affect the properties of a honey (Persano Oddo & Bogdanov, 2004). When the amount of a dominant type of pollen (e.g. rape) decreases, then the amount of another pollen (e.g. willow, dandelion or clover) increases (Kaškonienė & Venskutonis, 2010). It is quite unlikely for a honey to originate from only one plant, and the term “monofloral” may be used to describe honeys that are produced mostly from one plant source (Anklam, 1998). Such honeys have higher market demand, which means that they also have a higher commercial value for producers than honeys from mixed botanical sources, and thus can be considered premium products (Feás et al., 2010).

Usually honey is considered monofloral when the relative frequency of the pollen from a certain plant is above 45% (Maurizio, 1975). Since pollen types in honey can be either under- or over-represented, the percentages of pollens in different monofloral honeys can vary greatly (Table 1).

Table 1. *Representation of pollen types in honey.*

Under-represented pollen	Over-represented pollen
Taraxacum officinale Weber (5-40%)[1], (>15%)[4]	*Brassica napus* L. (>60%) [1,3] (>80%)[4]
Calluna vulgaris Hull. (10-77%)[1,2], (2-90%)[4]	*Salix alba* L. (>70%) [3, 5]
Fagopyrum esculentum L. (>30%)[4]	*Castanea sativa* Miller (> 86%)[1], (>90%)[5, 6]
Borago officinalis L. (>10%)[5]	*Myosotis* spp. (>90%)[6]
Tilia spp. (>20%)[5]	*Eucalyptus* spp. (>83%)[1]
Citrus spp. (10-20%)[6], (2-42%)[1]	

[1]Von dr Ohe et al., 2004; [2]Bryant & Jones, 2001; [3]Bryant, 2001; [4]Puścion-Jakubik et al., 2020, [5]van de Ham et al., 1999, [6]Louveaux et al., 1970

In the case of monofloral honeys with under-represented pollen, the amount of nectar actually involved in the formation of a honey is greater than that resulting from the number of pollen grains, and usually the minimum percentage of the taxon that gives a honey its name is 10-20% or 20-30% (Mateo & Bosch-Reig, 1998; Von der Ohe et al., 2004). Examples include heather (Escuredo et al., 2013; Puścion-Jakubik et al., 2020), buckwheat (Pasini et al., 2013), linden (Puścion-Jakubik et al., 2020) and thyme (Rodopoulu et al., 2017) honeys. In the case of over-represented pollen, the minimum percentage of the taxon that gives the honey its name is 70-90% (Mateo & Bosch-Reig, 1998). For example, the minimum taxa present in monofloral rape honey is considered to be over 60% (Persano Oddo et al., 2006) or over 80% (Bodó et al., 2020; Puścion-Jakubik et al., 2020), and chestnut honey is at least 90% (Puścion-Jakubik et al., 2020). The normally represented pollens are, for example, *Trifolium repens* L. and *Rubus idaeus* L., with pollen representations of over 45% (Bryant & Jones, 2001).The differences in the nectar- and pollen-producing ability of plants and the coexistence of over-represented and under-represented pollen grains unfortunately often lead to false results regarding the melissopalynological analysis (Rodopoulu et al., 2017).

In the case of honeydew honeys, the content of honeydew elements is evaluated by microscopy, and the ratio of honeydew elements to pollen from the nectar of plants should be >3 to qualify as a honeydew honey (Mateo & Bosch-Reig, 1998). Honeydew elements include microalgae (usually species of green algae (*Chlorophyceae*)) and fungi spores; sooty moulds (*Fungi imperfecti*), sometimes visible as dark brown or black coats of leafs, needles and bark, are typical in honeydew honeys (Rybak-Chmielewska et al., 2013).

Since the interpretation of pollen percentages may be difficult, the pollen analysis should be combined with other analyses (Feás et al., 2010; Rodopoulu et al., 2017) or interpreted by statistical analysis (Herrero et al., 2002; Corbella & Cozzolino, 2008; Aronne & de Micco, 2010).

The main plants that produce nectar and/or pollen in Estonia are Rosaceae, Brassicaceae, Apiaceae, Fabaceae, Asteraceae, Poaceae, *Salix*, *Trifolium*, *Fagopyrum*, *Frangula* and *Calluna* (Puusepp & Koff, 2014). The typical Estonian honey is polyfloral (Tammet, 2007). Under favourable weather conditions, heather, dandelion and raspberry rape are monofloral honey-producing plants (Salonen et al., 2011).

1.2.2 Physico-chemical parameters

The physico-chemical parameters for honeys are laid down in Codex Alimentarius Standard 12-1981 and Council Directive 2001/110/EC. The analytical methods used to classify honeys are usually the same as used in the routine control of honey. The composition criteria for honeys include the sugar content, moisture, water-insoluble

content, electrical conductivity, free acid, diastase activity and hydroxymethylfurfural (HMF). The criteria are set for blossom or honeydew honeys and there are some specific criteria for various honey types. A list of the average compositions of blossom and honeydew honeys is presented in Tables 2 and 3.

Table 2. *Physico-chemical parameters of multifloral honeys and honeydew honeys.*

	Blossom honey	**Reference**	**Honeydew honey**	**Reference**
Electrical conductivity (mS/cm)	< 0.8	Dir 2001/110/EC	> 0.8	Dir 2001/110/EC
	0.47	Bentabol Manzanares et al., 2011	1.20	Pasias et al., 2017
	0.68		1.00	Popek et al., 2002
	0.49	Popek et al., 2002	1.70	Miłek et al., 2021
	0.59	Miłek et al., 2021	1.03	Nešović et al., 2020
	0.37	Nešović et al., 2020	1.00	Esriche et al., 2014
	0.55	Esriche et al., 2014 Bertoncelj et al., 2011	1.14	Rybak-Chmielewska et al., 2013
Free acidity (mmol/kg)	< 50	Dir 2001/110/EC	< 50	Dir 2001/110/EC
	46.10	Rodrigues et al., 2019	88.60	Terrab et al., 2002
	29.80	Terrab et al., 2002	31.80	Miłek et al., 2021
	19.15	Miłek et al., 2021	29.84	Nešović et al., 2020
	27.18	Nešović et al., 2020	25.10	Geană et al., 2020
	29.50	Geană et al., 2020	37.88	Iglesias et al., 2004
	28.14	Iglesias et al., 2004	27.60	Rybak-Chmielewska et al., 2013
Diastase (Schade Unit)	> 8	Dir 2001/110/EC	> 8	Dir 2001/110/EC
	7.00	Pasias et al., 2017	11.90	Pasias et al., 2017
	16.20	Rodrigues et al., 2019	11.20	Terrab et al., 2002
	27.60	Terrab et al., 2002	31.64	Nešović et al., 2020
	34.14	Nešović et al., 2020	39.20	Iglesias et al., 2004
	29.70	Iglesias et al., 2004	20.52	Esriche et al., 2014
	19.74	Esriche et al., 2014	8.40	Rybak-Chmielewska et al., 2013
Moisture content (%)	< 20	Dir 2001/110/EC	< 20	Dir 2001/110/EC
	18.40	Finola et al., 2007	16.10	Popek et al., 2002
	16.21	Popek et al., 2002	20.30	Terrab et al., 2002
	17.60	Rodrigues et al., 2019	17.10	Escuredo et al., 2014
	16.90	Salonen et al., 2011	18.43	Miłek et al., 2021
	17.59	Terrab et al., 2002	15.96	Nešović et al., 2020
	19.19	Miłek et al., 2021	15.31	Esriche et al., 2014
pH	–	Dir 2001/110/EC	–	Dir 2001/110/EC
	4.10	Bogdanov, 1997	4.24	Popek et al., 2002
	3.78	Popek et al., 2002	4.28	Terrab et al., 2002
	3.72	Terrab et al., 2002	4.32	Miłek et al., 2021
	4.08	Miłek et al., 2021	4.40	Geană et al., 2020
	4.25	Geană et al., 2020	4.46	Escuredo et al., 2019
	3.90	Iglesias et al., 2004	4.70	Iglesias et al., 2004
HMF (mg/kg)	< 40	Dir 2001/110/EC	< 40	Dir 2001/110/EC
	14.8	Finola et al., 2007	2.4	Pasias et al., 2017
	7.67	Bentabol Manzanares et al., 2011	31.7	Terrab et al., 2002
	7.6		20.5	Miłek et al., 2021
	8.6	Pasias et al., 2017	0.69	Nešović et al., 2020
	2.33	Rodrigues et al., 2019	4.6	Geană et al., 2020
	6.10	Nešović et al., 2020 Esriche et al., 2014	3.91	Esriche et al., 2014
Water-insoluble content (g/100g)	< 0.1	Dir 2001/110/EC	< 0.1	Dir 2001/110/EC
	0.03	Andrade et al., 1999	0.05	Küçük et al., 2007
	0.085	Küçük et al., 2007		

1.2.2.1 Carbohydrates

Sugars, of which glucose and fructose form the base, are the main constituents of a honey, and make up 95% of its dry weight (Devillers et al., 2004; Finola et al., 2007; Bogdanov, Ruoff & Persano Oddo, 2004; Ouchemoukh et al., 2010) (Table 3).

Table 3. *Content of various sugars in polyfloral and honeydew honeys.*

	Blossom honey		**Honeydew honey**	
Sugar	**Value (g/100g)**	**Reference**	**Value (g/100g)**	**Reference**
Glucose	39.80 31.65 30.56 27.50 28.90	Kaškonienė et al., 2010 Ouchemoukh et al., 2010 Nešović et al., 2020 Geană et al., 2020 Iglesias et al., 2004	23.20 27.07 29.48 27.60 27.22	Escuredo et al., 2014 Victorita et al., 2008 Nešović et al., 2020 Geană et al., 2020 Iglesias et al., 2004
Fructose	35.97 38.93 36.21 37.80 36.35	Kaškonienė et al., 2010 Ouchemoukh et al., 2010 Nešović et al., 2020 Geană et al., 2020 Iglesias et al., 2004	32.90 27.07 36.35 36.40 32.80	Escuredo et al., 2014 Victorita et al., 2008 Nešović et al., 2020 Geană et al., 2020 Iglesias et al., 2004
F+G	**> 60**	**Dir 2001/110/EC**	**>45**	**Dir 2001/110/EC**
Sucrose	**<5**	**Dir 2001/110/EC**	**<5**	**Dir 2001/110/EC**
	2.19 1.35 1.43 2.05 0.95	Popek et al., 2002 Ouchemoukh et al., 2010 Nešović et al., 2020 Dos Santos Scholz et al., 2020 Bentabol Manzanarez et al., 2011	3.89 0.50 0.19 1.62 3.10	Popek et al., 2002 Escuredo et al., 2014 Victorita et al., 2008 Nešović et al., 2020 Przybyłowski & Wilczyńska, 2001
Maltose	0.15- 1.94 1.72 1.09 1.50 5.25	Kaškonienė et al., 2010 Ouchemoukh et al., 2010 Nešović et al., 2020 Geană et al., 2020 Bentabol Manzanarez et al., 2011	1.40 2.48 0.74 2.40 3.20	Escuredo et al., 2014 Victorita et al., 2008 Nešović et al., 2020 Geană et al., 2020 Rybak-Chmielewska et al., 2013
Melezitose	0.18 0.15 0.33 1.27	Ouchemoukh et al., 2010 Nešović et al., 2020 Devillers et al., 2004 Bentabol Manzanarez et al., 2011	0.14 4.23 0.27 3.20	Escuredo et al., 2014 Victorita et al., 2008 Nešović et al., 2020 Rybak-Chmielewska et al., 2013
Raffinose	0.05 0.22	Ouchemoukh et al., 2010 Devillers et al., 2004	0.35	Victorita et al., 2008
Erlose	0.35 0.33	Ouchemoukh et al., 2010 Devillers et al., 2004	0.53	Victorita et al., 2008
Melibiose	0.00 0.06	Ouchemoukh et al., 2010 Nešović et al., 2020	0.05	Nešović et al., 2020
Trehalose	0.01 0.29 1.67	Ouchemoukh et al., 2010 Nešović et al., 2020 Bentabol Manzanarez et al., 2011	0.74 2.70 1.89	Victorita et al., 2008 Rybak-Chmielewska et al., 2013 Bentabol Manzanarez et al., 2011
Turanose	0.96 0.65 1.46	Ouchemoukh et al., 2010 Nešović et al., 2020 Bentabol Manzanarez et al., 2011	2.10 0.80 1.80	Victorita et al., 2008 Nešović et al., 2020 Rybak-Chmielewska et al., 2013
Isomaltose	0.79 0.68 0.84	Ouchemoukh et al., 2010 Nešović et al., 2020 Bentabol Manzanarez et al., 2011	0.49 1.11	Nešović et al., 2020 Bentabol Manzanarez et al., 2011

The sugars in a honey affect its energy value, viscosity, hygroscopicity and granulation, density, tendency to absorb moisture from the air and immunity from some types of spoilage (White & Doner, 1980; Cavia et al., 2002; Escuredo et al., 2014). The amounts of fructose and glucose should be at least 60g/100g and at least 45g/100g for blossom honeys and honeydew honeys, respectively (Council Directive 2001/110/EC).

The sugar content of a honey depends on the sugar content of the nectar of the plant, which is composed mainly of three sugars: sucrose, fructose and glucose; the proportion varies depending on the plant type (Mateo & Bosch-Reig, 1997; Krömer et al., 2008; Escuredo et al., 2013). Sucrose is the dominant sugar in nectar, and its different proportions affect the sweetness of the nectar and thus influence the preference of bees in the flowers they choose to visit (Nardone et al., 2013). Enzymes from the bee's hypopharyngeal glands are added to the nectar in the bee's crop and these enzymes break down the sugars of the nectar into simple forms of sugars, which are easier for the bees to digest. Those enzymes also protect honey from bacteria during storage (Bryant, 2001).

The compositions and variations of sugars can be used as indicators to identify different monofloral honeys. For example, the average ratio of fructose to glucose (F/G) is about 1.2 (Anklam, 1998; Bentabol Manzanares et al., 2014; de la Fuente et al., 2011), but some monofloral honeys are exceptions, with glucose as the dominant sugar; these include dandelion, rape, goldenrod and sunflower honeys (Persano Oddo & Piro, 2004; Escuredo et al., 2013; Kaškonienė & Venskutonis, 2010; Escuredo et al., 2014; Ratiu et al., 2020). The F/G ratio for honeydew is usually over 1.5 (Gleiter et al., 2006; Geană et al., 2020). Compared to blossom honeys, the monosaccharide content is lower in honeydew honey (Iglesias et al., 2004; Kaškonienė et al., 2010).

Depending on the fructose and glucose ratio (F/G), honeys with higher glucose content (F/G usually under 1.14) start to crystallise more quickly (Tosi et al., 2004; Al et al., 2009; Smanalieva & Senge, 2009) because glucose is less water soluble than fructose (Laos et al., 2011). However, honeys with F/G ratios over 1.58 do not crystallise (Tosi et al., 2004). Another useful indicator to evaluate the rate of honey crystallisation is glucose to water ratios (G/W) (Manikis & Thrasivoulou, 2001; Bogdanov, Ruoff & Persano Oddo, 2004; Escuredo et al., 2014). The honey has no or slow crystallisation with the G/W ratio under 1.7 and fast with the G/W value over 2 (Dobre et al., 2012).

Besides the two main monosaccharides, honey contains much smaller amounts of disaccharides (sucrose and maltose) and much smaller concentrations of tri- and oligosaccharides (Kaškoniene et al., 2010; Ouchemoukh et al., 2010; de la Fuente et al., 2011; Salonen et al., 2011; Escuredo et al., 2013; Elamine et al., 2019). The level of sucrose differs according to the maturity degree and origin of the nectar compound of the honey (Kahraman et al., 2010) and is a very important indicator of honey authenticity, as standards require its maximum content to be 5% in honey (Council Directive 2001/110/EC). A higher content of sucrose can also indicate an early harvest of honey, which means that the sucrose has not been converted into fructose and glucose (Gomes et al., 2010).

In addition, blossom honey and honeydew honey show variations in sugar compositions. For instance, higher levels of oligosaccharides, mainly melezitose, raffinose and melibiose, can be found in honeydew honey but are almost non-existent in blossom honey (Bogdanov, Ruoff & Persano Oddo, 2004; Victorita et al., 2008; Escuredo et al., 2014; Nešović et al., 2020; Vasi et al., 2020). Higher concentrations of oligo- or

polysaccharides in blossom honey can sometimes indicate that the honey has been adulterated with sweeteners (Anklam, 1998; Megherbi et al., 2009).

Bearing all of this in mind, sugar content can only be used for honey classification in the case of monofloral honeys with very high amounts of the dominant plant, because when the percentage of the nectar source in the dominant plant is reduced, the interpretation of the results of the measurement of sugars becomes more difficult and almost useless in determining the floral origin of such honeys (Kaškonienė & Venskutonis, 2010).

1.2.2.2 Moisture and water activity

Moisture content is an important quality parameter that affects the shelf life of honey, the physiological parameters, such as viscosity and crystallisation, the colour and the taste (Conforti et al., 2006; Bulut & Kilic, 2009). European Directive 2001/110/EC has set the maximum value at 20% for moisture content in general honeys and not more than 23% for heather and baker's honey (Directive 2001/110/EC). The moisture content is mainly dependent on the moisture content of the nectar, the harvesting season and the degree of maturity reached in the hive (Alvarez-Suarez et al., 2010; Kahraman et al., 2010; Escuredo et al., 2013), as well as the processing and storage conditions of the honey (Subramanian et al., 2007). The moisture content of a honey is highly important in contributing to its stability against fermentation (Nair & Chitre, 1980; Gleiter et al., 2006; Prica et al., 2015). Fermentation of honey is caused by the action of osmotolerant yeasts upon the sugars fructose and glucose, resulting in the formation of ethyl alcohol and carbon dioxide; the alcohol in the presence of oxygen then may break down into acetic acid and water; as a result, the fermented honey tastes sour (Chrife et al., 2006). It is well-known that properly ripened honey is not susceptible to spoilage by microorganisms, with the exception of osmopholic yeasts, and then only above moisture contents of 17% (White et al., 1961).

Water is mainly fixed to sugars via hydrogen bonding, and during crystallisation the water bound to the glucose is set free, which increases the water activity (Gleiter et al., 2006; Abramovi et al., 2008; Bentabol Manzanares et al., 2014). Sometimes honeys with higher moisture contents separate into two different layers: a crystallised layer at the bottom and a liquid layer on top (Gleiter et al., 2006). Since honey contains fructose and glucose in large quantities and the moisture content is low, the water activity value is usually under 0.6, which is enough to inhibit the growth of osmotolerant yeasts (Chirife et al., 2006).

The difference in water activity between liquefied and crystallised honeys is higher in flower honeys than in honeydew honeys (Abramovi et al., 2008). The results of Rybak-Chmilewska (2013) show relatively low water content in honeydew honeys.

1.2.2.3 Electrical conductivity

Electrical conductivity can be used to determine the quality of honey and it can also be used instead of ash content analysis, since those two parameters correlate well with each other (Popek, 2002; Kropf et al., 2008; Silva et al., 2009; Elamine et al., 2019). Two variations of electrical conductivity levels are set for blossom honeys and honeydew honeys, or blends of honeydew honeys and blossom honeys. Blossom honeys have lower levels (not more than 0.8 mS/cm) than honeydew honeys (not less than 0.8 mS/cm), with some exceptions, such as honeys of ling heather (*Calluna*), bell heather (*Erica*), strawberry tree (*Arbutus unedo*), lime (*Tilia* spp.), manuka and lime (*Lepospermum*)

(Directive 2001/110/EC). Furthermore, electrical conductivity is connected with the concentrations of organic acids, mineral salts and proteins, which differ in their values depending on the botanical origins of the honeys (Popek, 2002; Bentabol Manzanares et al., 2011; Alves et al., 2013; Pertretto et al., 2015; Oroian & Sorina, 2017). As a result, a higher level of electrical conductivity shows that a honey contains more organic acids and inorganic matter (Alves et al., 2013, Yadata, 2014).

1.2.2.4 Hydroxymethylfurfural

Honey sugars, particularly glucose and fructose, are affected by temperature during extracting, liquefying or clarifying, or by ageing during storage, and the result is the production of 5-hydroxymethyl furfuraldehyde (HMF) (Abu-Tarboush et al., 1993; Ajlouni & Sujirapinyokul, 2010). The European Directive states that the content of HMF in fresh honeys should not be more than 40 mg/kg (Directive 2001/110/EC). Fresh honeys do not contain HMF or contain only minimal amounts, and during storage HMF forms slowly and naturally but increases over time and when heated; therefore, it is considered a parameter for honey quality (Karabournioti & Zeravalaki, 2001; Bogdanov, Ruoff & Persano Oddo, 2004; Bulut & Kilic, 2009; Pasias et al., 2017). HMF can also form due to fructose degradation in an acidic environment (Crane, 1980).

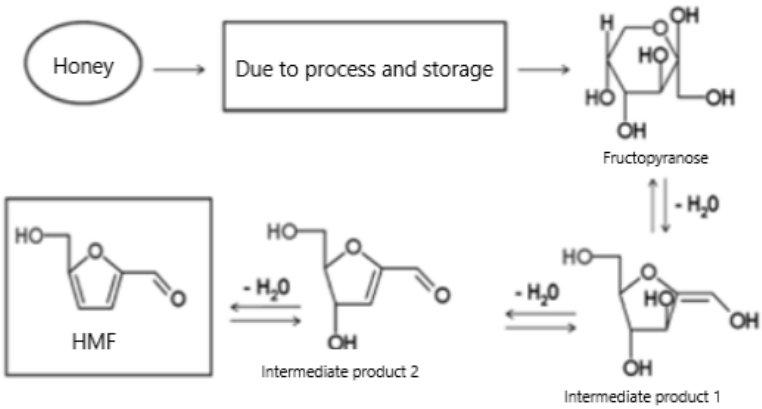

Figure 2. *The formation of HMF from glucose and fructose (Islam et al., 2014).*

The reactivity of fructose is higher than that of glucose, which can form a stable ring, and thus the enolisation rate of fructose is higher. Enolisation is the rate-determining step for HMF formation. Fructose forms an equilibrium of mixtures of difructose and dianhydrides and thus internally blocks the most reactive groups, leading to the formation of some by-products (Figure 2). Glucose forms true oligosaccharides, which contain reactive-reducing groups, resulting in a greater risk of cross-polymerisation with reactive intermediates and HMF (Islam et al., 2014).

1.2.2.5 Diastase and Invertase activity

The origin of diastase and invertase is attributed to the bee, as those enzymes are contained in its saliva, and are then added to honey in different proportions (Persano Oddo et al., 1999). Diastase and invertase are used as measures of honey freshness and as parameters of unheated honeys (Karabournioti & Zervalaki, 2001).

Diastase is the most resistant enzyme in honey and is formed by a group of amylolytic enzymes that include α- and β-amylases (Abu-Tarboush et al., 1993). This enzyme transforms starch to other carbohydrates, such as oligo-, di- and monosaccharides (Kowalski et al., 2012). The higher the diastase activity value, the more biologically active the honey (Semkiw et al., 2010), and the minimum value set by Directive 2001/110/EC is 8 Schade units. Diastase is strongly correlated with invertase (Persano Oddo et al., 1999).

Invertase is an enzyme that turns sucrose into two monosaccharides: glucose and fructose (Crane, 1975). Its decomposition is very fast and starts at 35 °C, a temperature which in many countries occurs during summer (Karabournioti & Zervalaki, 2001). In honeys heated to 45 °C, the invertase activity slightly decreases, but in overheated honeys (at 70 °C) the decrease is rapid (Serra Bonvehi et al., 2000). For invertase activity, the suggested level is at least 50 U/kg for fresh untreated honeys (Liitmaa & Sõukand, 2009).

Besides invertase and diastase, honeys contain other enzymes, including glucose oxidase, catalase and acid phosphatase (Persano Oddo et al., 1999).

1.2.2.6 Free acidity and pH

The free acidity of honey can be explained by the presence of organic acids in equilibrium with their corresponding lactones or internal esters, and some inorganic ions, such as phosphate (Finola et al., 2007), sulfate and chloride (Alves et al., 2013). Organic acids are derived from sugars by enzymes secreted by honeybees when transforming nectar into honey or when obtained directly from nectar (Cherchi et al., 1994). The acids make up less than 0.5 percent of honey dry matter (Semkiw et al., 2010). The main acid found in honey is gluconic acid, which arises from glucose through the action of an enzyme called glucose oxidase. Other acids in honey are formic, acetic, butyric, lactic, oxalic, siccinic, tartaric, maleic, pyruvic, pyroglutamic, glycollic, citric, malic, 2- or 3- phosphoglyceric acid, α- or β-glycerophosphate and glucose 6-phosphate (White & Doner, 1980; Tezcan et al., 2011). Lactones are internal esters of organic acids and do not contribute to a honey's active acidity but they hydrolyse over time, therefore increasing the honey's free acid. The total acidity is the sum of free acid and lactones (Terrab et al., 2002; Orioan et al., 2016). High acidity can also indicate the fermentation of sugars into organic acids (Alvarez-Suarez et al., 2010; Gomes et al., 2010).

Honeys are acidic, with an average pH from 3.5 to 5.5, which is due to the presence of organic acids in honey (Bogdanov, Ruoff & Persano Oddo, 2004). The pH value may vary depending on the botanical origin of honey, but the variations are relatively small (Bogdanov, 1997). Nevertheless, honeydew honey, with a darker colour, shows higher pH and higher acidity (Devillers et al., 2004; Bentabol Manzanares et al., 2011; Oroian et al., 2016), which may be associated with a higher concentration of acetic acid (Manyi-Loh et al., 2011). In addition, the pH value is affected by the mineral content, as honeys rich in ash generally have high pH values (Crane, 1975; Terrab et al., 2004).

1.2.3 Ash

The ash content represents the total mineral content in honey. It mostly results from the soil composition, the geographical origin of the honey (Pasquini et al., 2014; Di Bella et al., 2015; Bodó et al., 2020) and the botanical origin (Lachman et al., 2007; Chudzinska & Baralkiewicz, 2010; Mračević et al., 2020; Vasić et al., 2020), and is one of the properties to be considered in the evaluation of a honey's nutritional value (Alves et al., 2013). Lighter honeys (e.g. rape honey) tend to contain less mineral content than darker ones,

such as chestnut or heather honey (Alvarez-Suarez et al., 2010; Kaškonienė et al., 2010; Chudzinska & Baralkiewicz, 2010; Fernández-Torres et al., 2005). Blossom honey contains less mineral content than honeydew honey (Finola et al., 2007; Lachman et al., 2007; Madejczyk & Baralkiewicz, 2008; Vanhanen et al., 2011; Orioan & Sorina, 2017).

Table 4. *Minerals in honey.*

Element	mg/kg	Reference
K	1346.00	Chudzinska & Baralkiewicz, 2010
	672.33	Elamine et al., 2019
	1520.70	Escuredo et al., 2013
	681.26	Kaygusuz et al., 2016
	1150.10	Silva et al., 2009
	679.00	Terrab et al., 2004
Na	24.80	Chudzinska & Baralkiewicz, 2010
	64.83	Elamine et al., 2019
	70.00	Escuredo et al., 2013
Mg	18.50	Chudzinska & Baralkiewicz, 2010
	32.82	Elamine et al., 2019
	74.00	Escuredo et al., 2013
	50.10	Lachman et al., 2007
	35.57	Silva et al., 2009
	77.00	Terrab et al., 2004
Ca	44.40	Chudzinska & Baralkiewicz, 2010
	145.52	Elamine et al., 2019
	118.00	Escuredo et al., 2013
	80.18	Kaygusuz et al., 2016
	64.90	Lachman et al., 2007
	59.88	Silva et al., 2009
Al	10.50	Chudzinska & Baralkiewicz, 2010
	7.20	Lachman et al., 2007
Mn	4.96	Chudzinska & Baralkiewicz, 2010
	0.90	Elamine et al., 2019
	2.95	Kaygusuz et al., 2016
	4.43	Lachman et al., 2007
Fe	12.43	Elamine et al., 2019
	7.00	Escuredo et al., 2013
	3.34	Kaygusuz et al., 2016
Ni	0.24	Bogdanov et al., 2007
	0.43	Chudzinska & Baralkiewicz, 2010
	0.43	Lachman et al., 2007
Cu	0.66	Bogdanov et al., 2007
	0.82	Chudzinska & Baralkiewicz, 2010
	1.59	Elamine et al., 2019
	2.10	Escuredo et al., 2013
	0.39	Kaygusuz et al., 2016
	0.42	Lachman et al., 2007
Zn	1.04	Bogdanov et al., 2007
	3.22	Chudzinska & Baralkiewicz, 2010
	1.56	Elamine et al., 2019
	1.20	Escuredo et al., 2013
Cd	0.03	Bogdanov et al., 2007
	0.02	Chudzinska & Baralkiewicz, 2010

Honey contains a variety of minerals, of which the most abundant is potassium, forming about one-third of the total mineral content (Table 4). Minerals and trace elements can be either from natural sources (soil and plants) or from anthropogenic sources (air or soil contaminants). Trace elements, such as Cd and Pb, are toxic, but the levels in honey are low (Bogdanov et al., 2007).

1.2.4 Sensory properties

Sensory analysis is used to establish the organoleptic profile of a honey, and plays an important role in determining its quality, as it is based upon the assessment and scoring of the organoleptic properties of visual, olfactory, gustatory and tactile perceptions (Marcazzan et al., 2018). A honey's quality is estimated by detecting certain defects, such as impurities, off-odours, off-flavours and fermentation (Castro-Vázquez et al., 2012). Sensory evaluation makes it possible to determine the geographical and seasonal conditions (Castro-Vázquez et al., 2010; Stolzenbach et al., 2011) and, most importantly, the botanical origin of the honey (González-Viñas et al., 2003; González et al., 2010; Marcazzan et al., 2014), through analytical methods (Anupama et al., 2003; Kaakeh et al., 2005; González Lorente et al., 2008; Bertoncelj et al., 2011; Belay et al., 2015) and pollen analysis (Stolzenbach et al., 2011; Rodopoulu et al., 2017).

The sensory characteristics are closely interrelated with aroma-active components (Mannaş & Altuğ, 2007; Castro-Vázquez et al., 2008; Castro-Vázquez et al., 2009; Castro-Vázquez et al., 2010; Ruisinger & Schieberle, 2012). So the presence of a small component of a strongly flavoured nectar may easily change the sensory characteristics of a light honey, while larger amounts of a light nectar may have no or little effect on a strong flavoured honey (Persano Oddo & Piro, 2004). Each monofloral honey type has a characteristic odour, taste and aroma (Castro-Vázquez et al., 2009; González et al., 2010; Bertoncelj et al., 2011; Marcazzan et al., 2014), but the polyfloral honeys can greatly differ in attributes depending on the types and concentrations of the nectar the bee has foraged (Bertoncelj et al., 2011).

For the assessment of botanical origin, assessors use a qualitative method based on the ability of the assessor to evaluate the correspondence of a declared monofloral honey to a standard that they have memorised. This assessment takes into account the physico-chemical and melissopalynological results (Marcazzan et al., 2018). In order to conduct a proper sensory analysis, general factors have to be taken into account: the test room, the selection and training of the assessor, product storage, and the preparation and presentation of the samples (Marcazzan et al., 2018). Beekeepers determine the botanical origins of their honeys mainly on the basis of organoleptic characteristics (e.g. colour, taste, smell and consistency), on the basis of bee flight patterns and on the flowering time of honey plants (Puścion-Jakubik et al., 2020).

To assess honey flavour and odour and to establish the organoleptic profile of a honey, an odour and aroma wheel can be used (Figure 3). This contains a sufficiently wide range of terms to describe all of the possible variations of the product. The wheel is divided into sectors (families) and sub-sectors (sub-families) that correspond to one or more actual references (Piana et al., 2004).

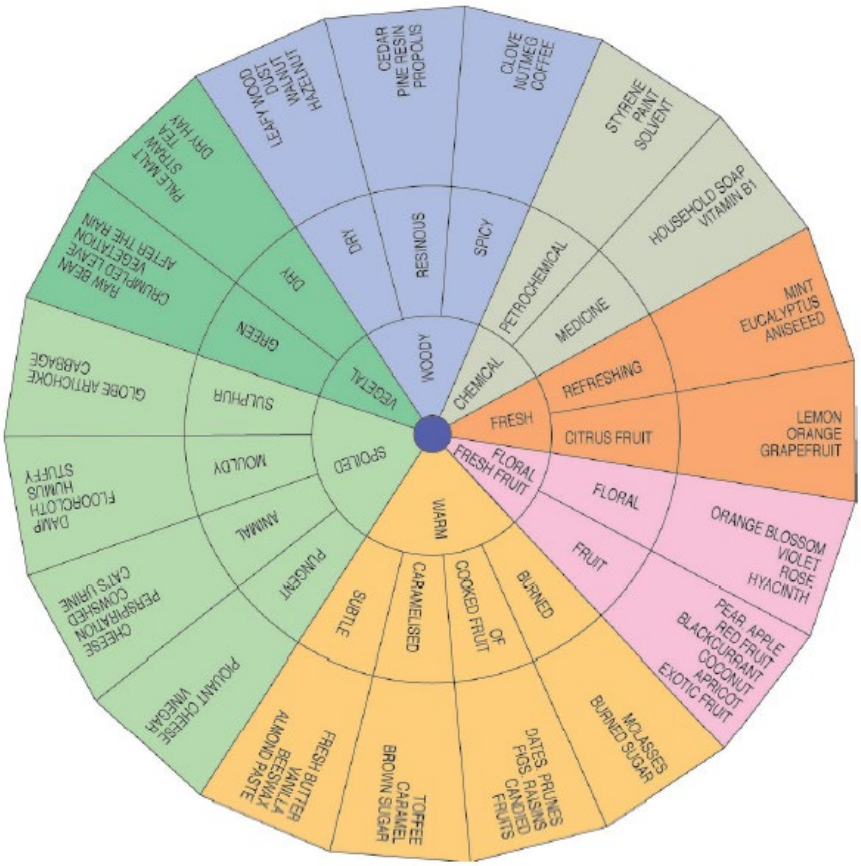

Figure 3. *Odour and aroma wheel (Piana et al., 2004, IHC, 2001).*

Flowery and fruity notes are considered more pleasant and are used as quality parameters (Anupama et al., 2003). For example, honeydew honey has been characterised as being less sweet and floral and strongly flavoured (Marcazzan et al., 2014), and heather honey has a ripe fruit and spicy aroma (Castro-Vázquez et al., 2009). Those honeys usually have higher pH, electrical conductivity and ash percentages, which are all related to the content of minerals, organic acids and amino acids, and thus give more diverse bitterness and less sweetness to the honeys (González et al., 2010; Semkiw et al., 2010; Batista de Sousa et al., 2016). Citrus honey, on the other hand, has high sweetness, a fresh fruit quality and lacks bitterness and saltiness (Castro-Vázquez et al., 2009; Marcazzan et al., 2014). Rape honey is described as mainly sweet, woody and waxy, with no sensory peculiarities (Siegmund et al., 2018).

The sensory properties of a honey are correlated with its colour, as lighter honeys have milder characteristics, and darker honeys (e.g. honeydew) tend to have stronger ones (Gonzales et al., 1999; Bertoncelj et al., 2011). Honeys with darker and more reddish colours often have higher intensities of caramelised notes (Anupama et al., 2003).

1.3 Alternative methods of honey specification

1.3.1 Colour analysis

The colour of a honey is the feature that the consumer notices first and that affects the acceptance of the honey; colour is a parameter used as an indication of quality and the content of minerals and phenolic compounds, and thus antioxidant properties (Alvarez-Suarez et al., 2010; Escuredo et al., 2019; Starowicz et al., 2021). During storage, the colour changes and an unnatural colour of honey may indicate inappropriate technological processes or long-term storage (Piotraszewska-Pająk & Gliszczyńska-Świgło, 2015); it may also be due to the reaction of reducing sugars with substances containing amino acids, polypeptides or proteins (Maillard reaction), or instability of the fructose in acid solutions (caramellisation) (Crane, 1975; Starowicz et al., 2021). During storage, the colour of honey gets lighter and this results from the crystallisation process (Piotraszewska-Pająk &Gliszczyńska-Świgło, 2015; Tappi et al., 2021). The crystal size affects the degree of lightening, the finest crystals imparting the lightest shade (Crane, 1975). With increasing temperatures, honey gets darker (Gonzales et al., 1999; Bulut & Kilic, 2009; Mouhoubi-Tafinine et al., 2018). Although fructose is more susceptible than glucose to browning, the darkening of honey has no correlation to those sugars since the variability is relatively small in honey, and has been attributed to a Maillard reaction (Gonzales et al., 1999).

The range of colour is very broad, varying from light white to almost black and containing many possible hues, including shades of green, red and bright yellow (Bertoncelj et al., 2011; Tuberosi et al., 2014; Dos Santos Scholz et al., 2020; Ratiu et al., 2020). This may be influenced by such pigments as carotenoids and flavonoids (Stalikas, 2007; Saxena et al., 2010).

There is no official method for determining honey colour, although it is an indicator of the quality (González-Miret et al., 2007) and botanical origin (Bertoncelj et al., 2007; Kuś et al., 2014; Tuberoso et al., 201;, Szabó et al., 2016; Escuredo et al., 2019). Still, there are different informal ways to determine colour, one of which is the CIELab method, which sets colour coordinates, such as L* (lightness), a*(greenness/redness) and b* (blueness/yellowness), which uniformly cover the full human visible spectrum.

Such honeys as rape and willow have lighter colours (Bodó et al., 2020; Jerković et al., 2014; Kuś et al., 2014; Starowicz et al., 2021). Heather and buckwheat honeys have darker colours, with reddish hues (Kuś et al., 2014; Tuberoso et al., 2014; Piotraszewska-Pająk &Gliszczyńska-Świgło, 2015; Starowicz et al., 2021). Honeydew honey has a very distinctive colour: dark, with greenish and mildly opalescent tones, and during crystallisation it turns lighter, having greenish-grey or brownish-greenish hues unique to honeydew honeys (Rybak-Chmielewska et al., 2013).

1.3.2 Amino acids

Amino acids are components that are produced by broken down proteins during chemical or digestion processes. Amino acids are the “building blocks” of proteins and form about 1% (w/w) of the constituents of honey depending on the botanical (Hermosín et al, 2003; Cotte et al., 2004; Janiszewska et al., 2012; Łozowicka et al., 2021) or geographical (Cometto et al., 2003; Stremel Azevedo et al., 2017) origin of the honey.

The most dominant free amino acid is proline, making up 50-85% of all the amino acids in honey (Iglesias et al., 2004; González Paramás et al., 2006; Pérez et al., 2007;

Łozowicka et al., 2021). This is due to the fact that it originates mostly from honeybees, as it is secreted mainly in bee saliva during the conversion of nectar to honey (Cotte et al., 2004; da Silva et al., 2016). The amount of proline has also been used as a standard for quantifying the total amino acid content and as an indication of honey quality, as it shows the honey's adulteration with sugar when the value is below 183 mg/kg (Bogdanov, 1999) or 200 mg/kg (Hermosín et al., 2003). During storage, the proline content decreases, and thus the proline level may also be used to indicate honey ripeness (Czipa et al., 2012). The proline concentration is higher in honeydew honey, with the dominant amino acids being glutamic acid, asparagine, glutamine, glutamic acid, alanine, leucine and tyrosine, while in blossom honeys the major amino acids, besides proline, are phenylalanine, glutamic acid and tyrosine (Iglesias et al., 2004; Pérez et al., 2007; Carratù et al., 2011; Czipa et al., 2012). If proline is not taken into account, a higher amount of phenylalaine is found in lavender honey (Cotte et al., 2004), glutamic acid in rape honey (Rebane & Herodes, 2008), asparagine and aspartic acid in raspberry honey (Janiszewska et al., 2012), GABA in chestnut honey (González Paramás et al., 2006), leucine in buckwheat honey (Janiszewska et al., 2012) and arginine in heather honey (Rebane & Herodes, 2008; Janiszewska et al., 2012).

1.3.3 Phenolic compounds

Plants are the main sources of honey phenolic compounds since they are plant-derived secondary metabolites (Bogdanov, Ruoff & Persano Oddo, 2004), biosynthesised mainly for protection against stress and oxidative damage and transferred via the nectar to the honey (Ciulu et al., 2016). The bioactive substances are transferred from the plant to the nectar and from propolis and pollen to honey, and for this reason the properties of the final product depend on the floral source (Kaškonienė & Venskutonis, 2010; Jasicka-Misiak et al., 2012; Kuś et al., 2014; Gašic et al., 2017). The typical phenolic compounds in honey are classified as phenolic acids, with their related derivatives (e.g. abscisic, ellagic, p-coumaric, gallic, caffeic, chlorogenic, vanillic and ferullic acids) and flavonoids (e.g. quercetin, pinocembrin, chrysin, kaempferol, luteolin, apigenin, myricetin, naringenin and hesperetin) (Bertoncelj et al., 2007; Isidorov et al., 2009; Socha et al., 2009; Socha et al., 2011; Lo Dico et al., 2019; Cheung et al, 2020; Nešović et al., 2020). Flavonoids are formed from the aromatic amino acids phenylalanine, tyrosine and malonate, and are the most common pigments besides chlorophyll and carotenoids (Stalikas, 2007). A positive correlation between total polyphenol and total flavonoid content has been found (Al et al., 2009; Khalil et al., 2012; Escuredo et al., 2013; Habryka et al., 2020).

The botanical origin of a honey is determined by identifying certain phenolic compounds unique to certain honey types, e.g. certain monofloral honeys can contain distinguishable marker compounds (Table 5).

Some phenolic compounds (e.g. pinocembrin, pinobanksin, crysin, galangin, apigenin, abscisic acid, kaempferol and caffeic acid) are specific compounds of propolis because they are derived from the bee metabolism and are present in every honey. Thus they cannot be considered markers but in some cases the compounds can be of nectar origin and the content of compounds has to be taken into consideration (e.g. kaempferol and quercetin occur in greater volumes in rapeseed honeys) (Soler et al., 1995; Tomás-Barberán et al, 2001; Schievano et al., 2013).

A strong correlation between the content of phenolics and colour has been found (Bertoncelj et al., 2007; Alves et al., 2013; Kuś et al., 2014; Alqarni et al., 2016; Wesołowska

& Dżugan, 2017). The total polyphenol content is highest in darker honeys, such as heather and buckwheat (Socha et al., 2011; Jasicka-Misiak et al., 2012; Escuredo et al., 2013; Kuś et al., 2014; Starowicz et al., 2021), and lower in lighter honeys (e.g. rape) (Socha et al., 2011). In addition, compared to the average blossom honey, higher total polyphenols are found in honeydew honeys, especially with higher levels of flavonoids (Al et al., 2009; Escuredo et al., 2013).

Table 5.*Phenolic compounds as markers for some monofloral honeys.*

Honey type	**Marker compound**	**Reference**
Heather	Abscisic acid	Jasicka-Misiak et al., 2012, Ferreres et al., 1994, Natić et al., 2016, Tomás-Barberán et al., 2001
	Hesperitin	Sergiel et al., 2014
	Ellagic acid	Ferreres et al., 1994, Natić et al., 2016, Soler et al., 1995
	Benzoic acid	Salonen et al., 2017
Rape	Rutin	Sergiel et al., 2014
	Ellagic acid	Wang et al., 2014
Lavender	Luteolin	Ferreres et al., 1994
Citrus	Hesperetin	Ferreres et al., 1994, Petrus et al., 2011, Tomás-Barberán et al., 2001, Soler et al., 1995, Escriche et al., 2014, Ferreres et al., 1993
Sunflower	*p*-coumaric acid	Orioan & Sorina, 2017
	quercetin	Tomás-Barberán et al., 2001
Buckwheat	Hydroxybenzoic acid	Jasicka-Misiak et al., 2012, Pasini et al., 2013
	Ferulic acid	Jasicka-Misiak et al., 2012, Pasini et al., 2013
	p-coumaric acid	Salonen et al., 2017, Pasini et al., 2013
Acacia	Chlorogenic acid	Wang et al., 2014
Thyme	Vanillic acid	Cheung et al., 2019
Eucalyptus	qercetin	Tomás-Barberán et al, 2001
Honeydew	Protocatechuic acid	Trautvetter et al., 2009
Beebread	*p*-coumaric acid	Baltrušaitytė et al., 2007, Isidorov et al., 2009
	kaempferol	Baltrušaitytė et al., 2007, Isidorov et al., 2009

Bee pollen contains a higher amount of phenolics than honey in variable amounts depending on the botanical origin, and those compounds affect the bioactive characteristics of pollen (Leja et al., 2007; Rzepecka-Stojko et al., 2015), as well as colour, taste and odour (Kielszek et al., 2018).

1.3.4 Antioxidant activity

The antioxidant properties of honey result from the presence of several substances, such as enzymatic (e.g. catalase, peroxidase and glucose oxidase) and non-enzymatic substances (e.g. organic acids, amino acids, phenolic compounds, flavonoids, ascorbic acid, tocopherols (α-tocopherol), catechins, carotenoids and Maillard reaction products) (Meda et al., 2005). The water-soluble antioxidants are flavonoids, amino acids and ascorbic acid, and the lipid-soluble antioxidants are tocopherols, tocotrienols and carotenoids (Wesołowska & Dżugan, 2017). Given the fact that nectars, from which honeys derive, are relatively high in water content (ranging from 30% to 90%), the probability is high that the majority of antioxidant honey constituents are water soluble (Frankel et al., 1998). Antioxidant substances can act against oxidants and free

radicals by limiting the molecular damage that can compromise the functioning of essential lipids, proteins and nucleic acids (Petretto et al., 2015).

The antioxidant activity of honey is variable and depends on the plant the nectar is derived from (Alvarez-Suarez et al., 2010; Socha et al., 2011; Dżugan et al., 2018; Starowicz et al., 2021), as well as on environmental factors and the processing and storage of the honey (Chaikham & Prangthip, 2015; Wesołowska & Dżugan, 2017). Antioxidant activity is correlated with increased browning in honey, which can be a consequence of the formation of compounds with different levels of antioxidant activity at various stages of Maillard reactions, depending on treatment temperatures (Turkmen et al., 2006). Consequently, heat treatment at lower temperatures does not seem to affect antioxidant activity (Kowalski, 2013; Šarić et al., 2013), and may even increase its level (Kowalski, 2013; Elamine et al., 2020; Sulaiman & Sarbon, 2020).Antioxidant activity has a strong correlation with polyphenol content, which means that polyphenolic compounds are the main components affecting the antioxidant activity of honeys (Bertoncelj et al., 2007; Krishna Kishore et al., 2011; Sarmento Silva et al., 2013; Kuś et al., 2014). As a result, darker honeys have higher antioxidant properties than light honeys (Bertoncelj et al., 2007; Alves et al., 2013; Wesołowska & Dżugan, 2017). For example, rape (Socha et al., 2011; Salonen et al., 2017; Dżugan et al., 2018) and willow honeys have relatively low values (Jerkovic et al., 2014) compared to buckwheat and honeydew (Dżugan et al., 2018; Starowicz et al., 2021).

1.3.5 Aroma-active compounds

The aroma profile is an important feature of honeys for organoleptic quality and authenticity (Radovic et al., 2001). Volatile compounds are associated with the aroma and flavour of honey, and are affected by geographical (Radovic et al., 2001; Mădas et al., 2019) and botanical origin (Kaškonienė et al., 2008; Castro-Vázquez et al., 2009; Soria et al., 2011; Ruisinger & Schieberle, 2012), as well as seasonal conditions (Castro-Vázquez et al., 2010). Many of the volatile compounds of honey come from the nectar or plant source, from the transformation of plant compounds by the metabolism of a bee, from heating or handling during honey processing and storage, or from microbial or environmental contamination (Manyi-Loh et al., 2011). For the above-mentioned reasons, monofloral honeys have distinctive patterns of volatile composition that should be taken into consideration when differentiating them from honeys of other floral origins (Table 6). Although some volatile compounds appear in minor concentrations in honey, they may have major impacts on distinct aromas (Siegmund et al., 2018).

Table 6. *Aroma-active compounds as markers for some monofloral honeys.*

Honey type	Marker compound	Reference
Heather	Phenylacetic acid	Guyot et al., 1999
Ericaceae	Cinnamic acid	Guyot et al., 1999
Rape	Dimethyl disulphide absence of 2-methyl-propanol	Kaškonienė et al., 2008, Radovic et al., 2001 Kaškonienė et al., 2008, Radovic et al., 2001
Dandelion	nitrile derivatives	Soria et al., 2003, Piasenzotto et al., 2003
Buckwheat	3-methylbutanoic acid	Pasini et al., 2013
Acacia	*cis*-linalooloxide absence of phenylacetaldehyde absence of Dimethyl disulphide	Radovic et al., 2001 Radovic et al., 2001 Radovic et al., 2001
Lavender	heptanal	Radovic et al., 2001
Citrus	Lilac aldehyde	Alissandrikis et al., 2007 Castro-Vázquez et al., 2009
Rosemary	kaempferol	Esriche et al., 2014
Eucalyptus	3-Hydroxy-2-butanone dimethyldisulfide	Castro-Vázquez et al., 2009 Bouseta et al., 1996
Chestnut	linalool	Bonvehi & Coll, 2003

More than 600 volatile compounds have been identified as honey aroma compounds originating from different floral origins. Aroma compounds are present in honey at very low concentrations as complex mixtures of volatile components of different chemical families: monoterpenes, norisoprenoids, benzene compounds, alcohols, esters, fatty acids, ketones, terpenes and aldehydes, furan, pyran and hydrocarbons (Pontes et al., 2007; Kaškonienė et al., 2008; Manyi-Loh et al, 2011; Tahir et al., 2016). The character of the resulting aroma depends upon a number of factors: the availability and structure of the reagents, the participation of fat, amino acids and saccharides, and reaction conditions (temperature, duration, water activity, pH and oxygen level) (Plutowska & Wardenski, 2007). Some natural volatile compounds, such as carboxylic acids, alcohols, carbonyl compounds and lactones, originate along metabolic pathways of amino acids and fats, but other volatiles, such as terpenes, esters and ethers, come from ripening. Those above-mentioned compounds are responsible for the desired aromas and characteristics of certain origins of the raw materials (Plutowska & Wardenski, 2007). Other compounds, such as some alcohols, branched aldehydes and furfural derivatives, may be related to the microbial purity of the processing and storage conditions of honey (Pontes et al., 2007).

1.3.6 Fluorophores

The presence of fluorophores in honey makes front-face fluorescence spectroscopy a promising method to determine the botanical (Ruoff et al., 2006a; Karoui et al., 2007; Lenhardt et al., 2014; Sergiel et al., 2014) or geographical (Cebrero et al., 2020) origins of honeys. This method can also be used to detect honey adulteration, because the differences in the fluorescence of natural and adulterated honey samples are extremely significant (Lenhardt et al., 2015; Dramianin et al., 2018). The main advantages of molecular fluorescence spectroscopy are its sensitivity and selectivity, in addition to the

ease of use (i.e. little sample preparation), instrumental versatility, speed of analysis and its non-destructive character (Airado-Rodríguez et al., 2011; Lenhardt et al., 2014).

Natural fluorophores include aromatic amino acids, enzymes and proteins, phenolic compounds, vitamins, cofactors and Maillard reaction products, which can be detected on the basis of fluorescence emission (EM) and excitation (EX) spectra (Ghosh et al., 2005; Lenhardt et al., 2014; Sergiel et al., 2014; Parri et al., 2020). The spectra of fluorophores are obtained from simultaneous scans of excitation and emission wavelengths (λ_{ex}, λ_{em}) and ratios, as peak intensities provide spectral parameters (Parri et al., 2020).

The amino acids consist of three fluorescents, tryptophane, tyrosine and phenylalanine, and by the changes in their spectra it is possible to evaluate the structural changes in proteins (Karoui et al., 2007), for which the emission spectra occur from 280 to 480 nm following excitation at 250 nm (Trifkovi et al., 2017). Tryptophane, aromatic amino acids and nucleic acids provide information about the tertiary structure of proteins, while Maillard reaction products (furosine and HMF) provide information on the degree of lipid oxidation (Karoui et al., 2007). The fluorescent emission is mainly caused by tryptophane at EM:350/EX:280 nm and secondary tryptophane at EM:330/EX:230-235 nm. Maillard reaction products, such as HMF and furosine, show fluorescence values at around EM:305-520/EX:375-440 nm (Kulmyrzeav & Dufour, 2002; Karoui et al., 2007; Lenhardt et al., 2015; Dramianin et al., 2018). Phenolic compounds are strong fluorophores, and exhibit in the range of EM: 360-420/EX:250-335 (Rodriguez Delgado et al., 2001; Karoui et al., 2007; Lenhardt et al., 2015; Parri et al., 2020). The vitamins B9 and B6 exhibit at spectra of around EM:400-640/EX: 380-450 (Sikorska et al., 2009; Trifković et al., 2017), respectively.

Since the differences between the samples are very slight, they may be difficult to distinguish; the analytical data contained in the fluorescence spectra can be extracted by using various multivariate analysis techniques that relate several analytical variables to the properties of the analytes (Sádecká & Tóthová, 2007; Lenhardt et al., 2015; Dramicanin et al., 2018).

1.3.7 Other novel methods

The properties and botanical origins of honeys can be determined by various analyses (Figure 4). Physico-chemical parameters make up part of the routine control in honey analyses; chromatographic methods and mass spectrometry are nowadays widely used for the detection and identification of certain compounds in honey. In recent years, the number of new methods used has increased.

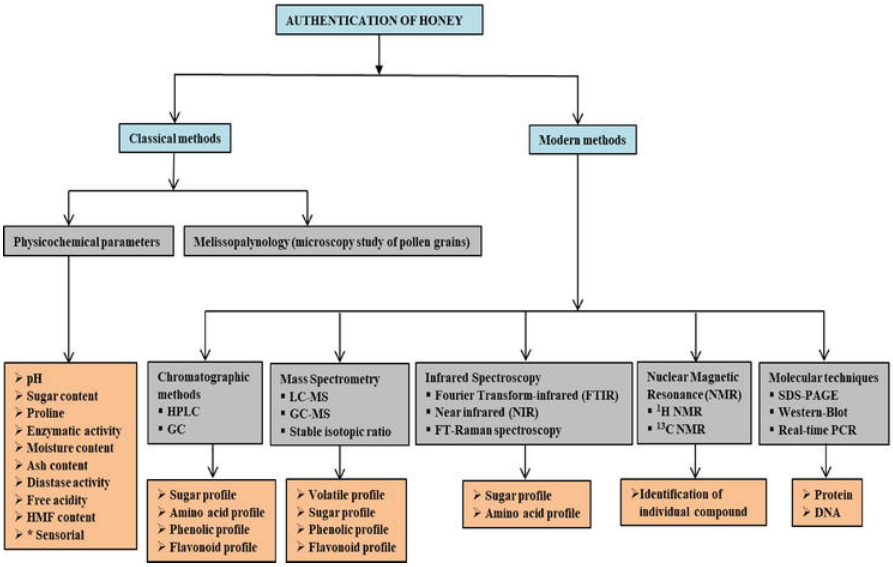

Figure 4. *Analytical methods of honey authentication (Chin & Sowndhararajan, 2020)*

Different ranges of infrared absorptions can be used for the determination of food parameters. Infrared radiation is the region of the electromagnetic spectrum between the visible and the microwave wavelengths; the nominal range of wavelengths for near-infrared (NIR) is between 750 and 2500 nm, and for mid-infrared (MIR) it is 2500 to 25,000 nm (Cozzolino et al., 2011). Near-infrared spectroscopy allows for the use of more concentrated samples and longer optical paths than those used in mid-infrared spectroscopy (MIR). The main advantages of near-infrared spectroscopy for food analysis are its speed, the absence of, or reduced need for, sample pretreatment, and the absence of the use of chemicals (García-Alvarez et al., 2000; Herrero Latorre et al., 2013). Since honey is a very complex matrix and the samples used for calibration should represent the whole matrix of variations in the concentration of the components and honey types, distinguishing between monofloral and polyfloral honeys remains problematic (Ruoff et al., 2006b; Etzold & Lichtenberg-Kraag, 2008).

Nuclear magnetic resonance spectroscopy (NMR) allows for the analysis of samples in all physical states, providing detailed information at the molecular level (Consonni & Cagliani, 2015). NMR can determine botanical origin by providing the identity of reliable marker compounds, a crucial step not only in characterising the particular type of honey (Simova et al., 2012), but also in exposing possible adulterations (Schievano et al., 2013). The advantages of this technique are its easy and simple sample preparation, high reproducibility and sensitivity, and quick data acquisition, which is a major advantage over other spectroscopic methods (Trifković et al., 2017).

DNA bar-coding is based on specific DNA markers of plant species that identify botanical origins of honeys in which the species composition of mixed matrices is determined by comparing sequences of the same DNA region with a reference database (Bruni et al., 2015). This is an effective method for analysing honeys of various plant species, can be easily applied to large-scale studies (Valentini et al., 2010), and has advantages in terms of rapidity, sensitivity and specificity (Soares et al., 2015; Hawkins et al., 2015). This method does not require the high level of taxonomic expertise required for microscopic examination (Hawkins et al., 2015).

2. AIMS OF THE DISSERTATION

The main objective of this dissertation was to carry out a comprehensive study of various Estonian honeys, on which little scientific data is available. The intention was to provide a good overview of the composition and properties of the honeys, and to find marker compounds characteristic of certain honeys. The thesis aims to distinguish between specific multifloral honeys from various botanical origins by using the data from physico-chemical analysis and front-face fluorescence spectroscopy, together with PCA and PARAFAC models (Publication I). In addition, the goal was to determine marker compounds of aromas specific to honeys of certain botanical origins and to find out whether there is a connection between aroma compounds of different honey types and their corresponding blossoms (Publication II), as well as to characterise Estonian honeys by their quality parameters, amino acid content, phenolic components, antioxidant activities and sensory properties, and to use all of this data to find specific marker compounds that could be used to determine the botanical origins of honeys (Publication III)

3. MATERIALS AND METHODS

3.1 Materials

Honey samples for analysis were collected directly from beekeepers who operate in different areas of Estonia and are members of the Estonian Beekeepers Association. Samples analysed in Publication I (n=18) and in Publication II (n=13) were collected in the same harvesting season and were of the same batches (Figure 5), whereas samples in Publication III (n=30) and additional samples (n=7) were from different seasons.

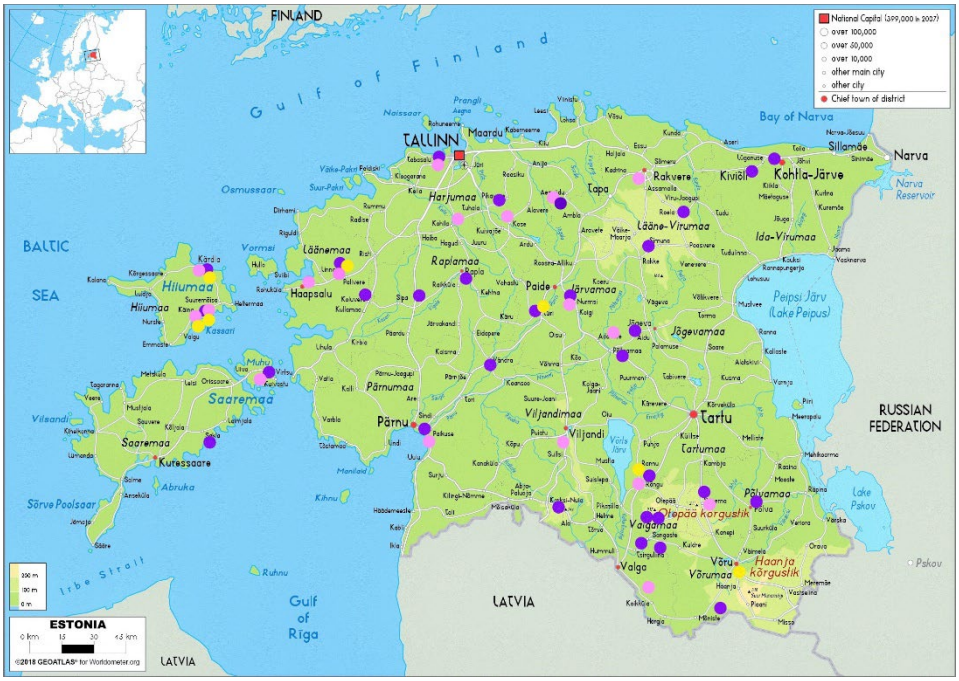

Figure 5. . *Harvested honey samples from different regions of Estonia. Pink (Publications I and II), violet (Publication III) and yellow (supplementary analyses) circles represent batches of honey samples harvested in different seasons.*

The honey samples were stored for further analysis at a controlled temperature of 18±2 °C in a climate chamber in darkness (Publications I and III) and at 4 °C for sensory analysis (Publication II). Seven samples for the analysis of changes in total polyphenol and total flavonoid content in storage time were put in a freezer at -18°C for further analysis. Samples were held there for two years until further analysis was conducted. Bee bread (n=5) and bee pollen (n=4) samples were collected directly from beekeepers. The samples were stored in a freezer at -18°C for further analysis.

3.2 Methods

3.2.1 Melissopalynological analysis

Pollen analysis (Publications I-III) was carried out according to a non-acetolytic method. 10 g of honey was dissolved in 20 mL of distilled water. The solution was centrifuged and the remaining liquid was removed. The sediment was transferred to a microscope slide

and covered with glycerine jelly and then with cover-glass. The relative frequency was found by counting at least 500 pollen grains. The pollen in honey was identified by the species level whenever possible, but in other cases by the type of structure, genus or family level. Percentages refer to pollen from nectar plants.

3.2.2 Physico-chemical parameters

Physico-chemical parameters, such as electrical conductivity, moisture content, diastase activity, free acidity, invertase activity and hydroxymetyhlfurfural, were determined according to harmonised methods of the International Honey Commission (2009) (Publication III). Electrical conductivity was measured by using conductivity meter (IHC method 2), moisture content by digital refractometer (IHC method 1), free acidity by titration to pH 8.3 (IHC method 4.1), diastase photometrically at 620 nm (IHC method 6.2) and invertase activity photometrically at 400 nm (IHC method 9). Hydroxymethylfurfural was measured by using UPLC.

For glucose and fructose determination, the HPLC-RI method was used. Honey samples were diluted with water, filtered and injected into the system. An Aminex HPX-87H 300 mm x 7.8 mm column (Publication I) and a Zorbax Carbohydrate Analysis Column (Publication III) were used.

3.2.3 Colour

The colours of honey were determined by using the CIELab method, using a spectrophotometer CM-700d (Publication III), where L*-, a*- and b*-coordinates were determined. Readings were taken against a white background.

3.2.4 Mineral content

The mineral compositions were determined by using an ion chromatograph system (Publication I). Honey samples were diluted with water, filtered and injected into the chromatograph. An IC-Pac 3.9mm x 150 mm Cation Column 432 was used. Minerals, such as sodium, potassium, magnesium and calcium, were detected.

3.2.5 Fluorophores

Fluorescence measurements were performed using an Instant Screener® (ISC) Analyzer (Publication I). Spectral fluorescence signatures (SFS) were measured at excitation wavelengths from 230 to 350 nm, and at emission wavelengths from 250 to 565 nm, with 5 nm intervals in both directions.

3.2.6 Total polyphenol content

The determination of total phenolic content (TPC) of each sample was carried out using the Folin-Ciocalteu method (Publication III). A UV-Vis Spectrophotometer was used for measurements and the absorbance of the mixture was measured at 760 nm against a methanol blank. Gallic acid (0–200 mg/L) was used as the standard. The sample preparations for honey, bee bread and bee pollen were different. Detailed descriptions of the honey preparations are available in Publication III. A sample of bee bread or bee pollen (2 g) was crushed and 20 mL of ethanol 80% (*v/v*) was added. The mixture was put in darkness for 24 h at +18 °C. Then the mixture was centrifuged and, to 0.1 mL of that mixture, 4.9 mL of 0.2 N Folin-Ciocalteu reagent was added. Then 4 mL of 7.5 g/L sodium carbonate (Na_2CO_3) solution was added. After incubation in the dark at room temperature for 2 h, the reaction mixture absorbance was measured at 760 nm against a methanol blank.

3.2.7 Total flavonoid content

The total flavonoid content (TFC) was found by using a UV-Vis Spectrophotometer (Publication III), and the absorbance of the mixture was measured at 415 nm against a methanol blank. Quercetin (0–50 mg/L) was used as the standard. The sample preparations for honey, bee bread and bee pollen were different. Detailed descriptions of the honey preparations are available in Publication III. The determination of the total flavonoid content of the bee bread and bee pollen was carried out by adding 20 mL of methanol 80% (*v/v*) to the crushed sample (2 g). The mixture was then put in the darkness for 24 h and then centrifuged for 10 min. 0.1 mL of that solution was mixed with 4.9 mL of methanol 80% (*v/v*). In addition, 3 mL of the given solution was then mixed with 3 mL of $AlCl_3$ (2% W/v) in methanol. The mixture was homogenised and allowed to stand for 30 min in the dark. The absorbance was measured at 415 nm.

3.2.8 Polyphenol identification

For the identification of polyphenols in honey (Publication III), a liquid-chromatography-mass-spectrometry (LC-MS) method developed at the Centre of Food and Fermentation Technologies was used. Polyphenols were separated using an ACQUITY UPLC HSS C-18 1.8 µm (2.1 x 150 mm) column (Waters). Mass spectra signal intensities were used to assess the compounds' indirect abundance in the sample. The detected polyphenols and their derivatives were the following: shikimic acid, gallic acid, protocatechuic acid, protocatechuic acid, gentisic acid D_1, chlorogenic acid, chlorogenic acid D_1, catechin, 4-hydroxybenzioc acid, gentisic acid, caffeic acid, caffeic acid D_1, coumaric acid, coumaric acid D_1, ferulic acid, ferulic acid D_1, myricetin, morin, salicylic acid, abscisic acid, abscisic acid D_1, abscisic acid D_2, abscisic acid D_3, luteolin, luteolin and kaempferol D_1, quercetin, cinnamic acid D_1, cinnamic acid D_2, apigenin, naringenin, naringenin D_1, kaempferol, chrysin, chrysin D_1 and galangin (Publication III, Table 1).

3.2.9 Antioxydativity

The antioxydant activity of the honeys was evaluated by using the photochemiluminescence (PLC) method, together with a Photochem device (Publication III). Commercial standard sets of total water-soluble antioxidant capacity (ACW) and total lipid-soluble antioxidant capacity (ACL) were used. For ACW, the honeys were dissolved with distilled water and mixed with a ready reagent. The process for ACL was the same, except honeys were dissolved in methanol.

3.2.10 Amino acid content

Free amino acids were determined by using LC-UV methodology (Publication III). The samples, derivatised with AccQ-Fluor, were loaded on an AccQ-Tag Ultra column before separating by gradient of AccQ-Tag Ultra eluents A and B, which were then detected by photodiode array detector.

3.2.11 Sensory properties

The sensory analysis (Publication III) was composed of various steps, starting with sample preparation, which was done differently for gustatory and olfactory assessments. A sampling container with a twist-off cap was used for flavour evaluation, and honey diluted in 1:1 portions with drinking water put in a sniffing glass and covered with a lid was used for odour evaluation. Two training sessions were conducted before a sensory analysis was conducted, which were necessary to identify odours and flavours. Scales of

0-15 were used in assessing honey samples. The attributes berry-like, fruity, floral, herbal, woody, nutty, spicy, sweet, earthy and animal-like were chosen to describe the flavours and odours of honeys. The overall intensities of flavours, aromas and sour taste were also determined.

3.2.12 Aroma analysis

Aroma analysis (Publication II) was carried out on honeys (n=13) and on blossoms (n=4). Different blossoms, such as raspberry, heather, alder buckthorn and rape, were first chosen according to the results of a pollen analysis, picked during blooming time and immediately put in 20 mL SPME vials. Honeys were diluted with water (50% w/w) and 1 mL of this dilution was mixed with 1 g NaCl and placed into 20 mL SPME vials. Only for the honey samples, a glass covered magnetic stirrer was used. Three assessors participated in the evaluations. For GC-O, two replications were made of both blossom and honey samples and one for GC-MS. For GC-MS data analysis, a NIST05 library was used. The Kovats retention indices and standard compounds were used for GC-MS data analysis. The GC-MS data were used to identify the odour-active compounds detected by GC-O assessors. A detection frequency method was used to interpret the GC-O data, and the results were expressed in percentages. Three assessors took part in the GC-O and each sample was assessed in two parallels.

3.2.13 Statistical analysis

A statistical analysis was conducted according to the results obtained from the data. Principal Component Analysis (PCA) was carried out to visualise data from different honey samples and to identify their similarities and differences (Publications I and III). In Publication II, Correspondence Analysis (CA) and Agglomerative Hierarchical Clustering (AHC) were applied to map samples and flavour descriptions, and to cluster samples based on dissimilarities, respectively. For chemometric analysis of the fluorescence spectra, the PARAFAC algorithm was applied. Pearson correlation coefficients were calculated on the basis of the measurements (Publications I and III). For sensory analysis, the mean values were calculated for all of the sensory attributes over two sessions and 10 assessors.

4. RESULTS AND DISCUSSION

This dissertation is based on three publications. The main results are presented as a summary and divided into sections. Detailed discussions are found in Publications I-III.

4.1 Classical methods of honey specification

4.1.1 Pollen analysis

The honeys analysed in this thesis were collected in three different harvesting seasons. The eighteen honeys in Publication I and thirteen in Publication II were from the same year, but for Publication II some polyfloral honeys were set aside and only potential monofloral ones were chosen for analysis. For Publication III, honeys (n=30) from another year were collected. In the third harvesting season, seven samples were gathered.

In most analysed honey samples, the pollen families Cruciferae (mainly *Brassica napus*) and Rosaceae (mainly the *Rubus* type) were represented in various amounts, with maximum contents of 77% and 79%, respectively. Only a few of the observed honeys lacked the above-mentioned taxa. A detailed table of pollen content is presented in Figure 6, where the main pollen types occurring in the honey samples are listed (n=55). In addition to the above-mentioned rape and raspberry, there were small amounts of frequently occurring pollens of the following plant types: white clover (*Trifolium repens* L.), willow (*Salix spp*.), maple (*Acer spp*.), dandelion (*Taraxacum officinale* Weber), heather (*Calluna vulgaris* Hull), red clover (*Trifolium pratense* L.), alder buckthorn (*Frangula alnus* Miller) and meadowsweet (*Filipendula ulmaria* Maxim).

The occurrence of such pollen species as *Frangula* and *Calluna* is worth noting, as they played an important role in monofloral honey classification (Publications I-III). The pollen of those plants was under-represented in honeys, yet there was enough to give the honeys distinctive sensory properties or other parameters measured by analytical methods. The maximum of *Calluna* and *Frangula* pollen in honey was 29% and 42%, respectively. The honeys with *Frangula* pollen present originated only from western Estonia, mostly from the islands of Hiiumaa and Saaremaa. Other plants were not found to be specific to any regions. Rape, raspberry, willow, alder buckthorn, heather and clover are all the most common plants in Estonia that provide nectar and pollen. Willow is one of the first plants to bloom in spring, with alder buckthorn and raspberry soon following (late spring and early summer), and rape, clovers and dandelion blooming throughout the summer (Tammet, 2007). No pollens originating from foreign plants were found, which affirms the authenticity of the honeys.

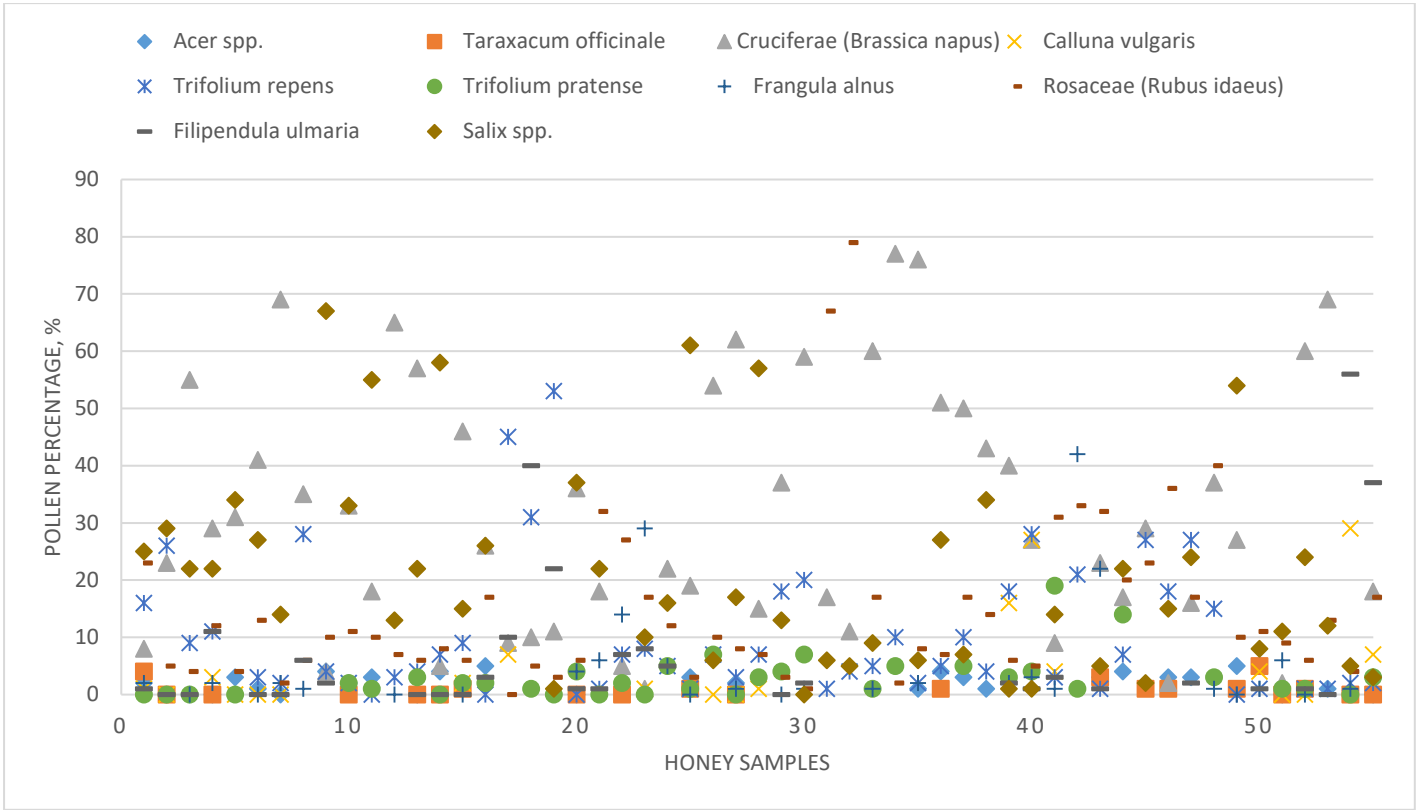

Figure 6. *The occurrence of pollen taxa in analysed honey samples (n=55)*

In Publication I, the honeys were classified as monofloral or polyfloral. This classification was made by taking into account the pollen types and amounts in honeys. Usually a honey is considered monofloral when the pollen frequency of a certain taxon is above 45% but, depending on the floral origin, the pollen in honey can also be over-represented or under-represented (Von Der Ohe et al., 2004). Therefore, for a proper interpretation, the botanical origin of the plant should first be considered. For instance in rape honey the percentage of pollen should be over 60% (or even over 80%) to be considered monofloral (Bodó et al., 2020). For heather honey, only 10% is needed (Von Der Ohe et al., 2004), and for raspberry honey, 45% (Bryant & Jones, 2001).

Based on the pollen analysis, the potential monofloral honeys, rape, raspberry and heather honeys, were determined (Publication I, Table 1). Since not much data is available on *Frangula* monofloral honeys, those were not considered monofloral, although they had distinct properties. In Publication III, the pollen analysis was conducted according to physico-chemical properties, antioxidant activity and sensory properties. All honeys, except for one, were determined to be polyfloral. Heather honey, with a specific taxon (*Calluna,* 7%), was identified as monofloral and this was confirmed by other analytical methods. Pollen analyses were conducted on all honey samples and they indicated that most samples were polyfloral, which meant that no pollen of a specific plant dominated in those honeys. Of course, there is no firm line between a honey being monofloral or polyfloral, and the pollen percentage does not always match the exact nectar content in honey. In addition, sometimes even the smallest amount of a nectar of a specific plant type can give a honey characteristic properties. Taking this into account, the results of other analyses were used together with pollen analysis, with the object of verifying the botanical origins of the honeys. Monofloral honeys are relatively rare in Estonia and this complicated the goal of finding specific marker compounds, but even with the small number of samples of the same honey type, clear conclusions could be reached Those results were consistent with the literature. Some monofloral honeys, including rape, raspberry, alder buckthorn and heather, were identified As for pure honeydew honey, none was gathered in the various harvesting seasons. Nevertheless, two polyfloral honeys contained traces of honeydew elements, enough to change the content and properties of the honey so that it could be differentiated from the rest of the samples.

The reason why some analytical methods used in the honey differentiation showed poor results may lie in the low number of samples and small amounts of monofloral honeys collected. More monofloral honey samples were needed to draw comprehensive conclusions. Still, this thesis gives a good overview of what was possible to achieve and indicates what needs further examination.

4.1.2 Physico-chemical properties

Certain compositional requirements that determine the quality of honeys are set by Council Directive 2001/110/EC, relating to honey. Table 7 shows the results of physico-chemical parameters expressed as ranges in all of the analysed honey samples examined (n=48), and compares them with the values set by the Directive. Detailed results are available in Publication I (Table 2) and Publication III (Table 2).

The parameters for the sum of glucose and fructose, diastase activity and hydroxymethylfurfural (HMF) met the quality norms, but there were some minor deviations in some physico-chemical properties, such as water content and free acidity.

The moisture content in quality honey should be lower than 20%, but for four honeys it was slightly higher. As for free acidity, one honey had a slightly higher level than the norms require.

Table 7. *Comparison of the results of quality parameters of analysed honey samples with the ones set by Council Directive 2001/110/EC relating to honey.*

Quality parameter	**Composition criteria**	**Range (n=48)**
Glucose+Fructose (g/100g)	> 60	62.5 - 84.6
Moisture content (%) (in general/heather honey)	<20 / <23	15.6 - 20.9/18.2-20.4
Electrical conductivity (mS/cm) (in general/honeydew)	<0.8 / >0.8	0.10 - 0.8/0.5
Free acidity (mEKV/kg)	<50	12 - 54
Diastase activity (schade scale)	>8	15.4 - 58.8
Hydroxymethylfurfural (mg/kg)	<40	<1 - 19.5

Most honeys contained raspberry and rape pollen in various amounts, thus indicating that these two plants play a big part in their botanical origin. The electrical conductivity of honeys was rather low, with an average of 0.33 mS/cm, but it was higher in heather and alder buckthorn honeys than in other polyfloral honeys. Electrical conductivity is a good indicator of the difference between honeydew honey and blossom honey, as honeydew honey usually has electrical conductivity above 0.8 mS/cm (Directive 2001/110/EC). For this thesis, we managed to gather only two honeys that contained traces of honeydew elements, and thus the electrical conductivity value did not exceed the norm required to be classified as honeydew honey. Electrical conductivity shows high correlations with mineral content (r=0.95) and so can be used in place of it: the mineral content of honey increases with increasing levels of electrical conductivity.

Glucose and fructose levels varied depending on the honey type. In rape honeys, glucose was the dominant sugar, while in alder buckthorn and heather honeys fructose was dominant. The monosaccharide content was above the 60 g/100g level set by the Directive and in most cases fructose was the dominant sugar. The fructose/glucose ratio ranged between 0.89 and 1.41. The lower levels indicated the impact of rape origin in a honey and the higher levels the impacts of heather and alder buckthorn.

The pH levels measured in honey samples (n=18) showed that only two honeys, related to alder buckthorn origin, showed remarkably higher levels than other analysed samples (5.12 and 4.52), which was due to the presence of organic acids in the honeys (Bogdanov, Ruoff & Persano Oddo, 2004). Apart from these two levels, the range for all other honeys was 3.38-3.80.

Invertase activity is not standardised in Estonia; it can vary greatly but the suggested level is at least 50 U/kg for fresh unheated honeys. In this thesis, the level (n=30) was in all cases above this.

The thesis showed that, in order to classify honeys, the results of other analytical or sensory analysis have to be taken into account. Although physico-chemical parameters are used in the routine control of honey quality, the results showed that especially electrical conductivity, and glucose and fructose content can be successfully used as supplementary parameters in determining the botanical origin of a honey. Other parameters, such as hydroxymethylfurfural, diastase and invertase activity, and free acidity, show whether a honey has been heated and whether it is of good quality.

Principal Component Analysis was used to differentiate honeys according to their physico-chemical parameters. In Figure 7, the first and second components contained 37.1% and 22.5% of the data variance, respectively.

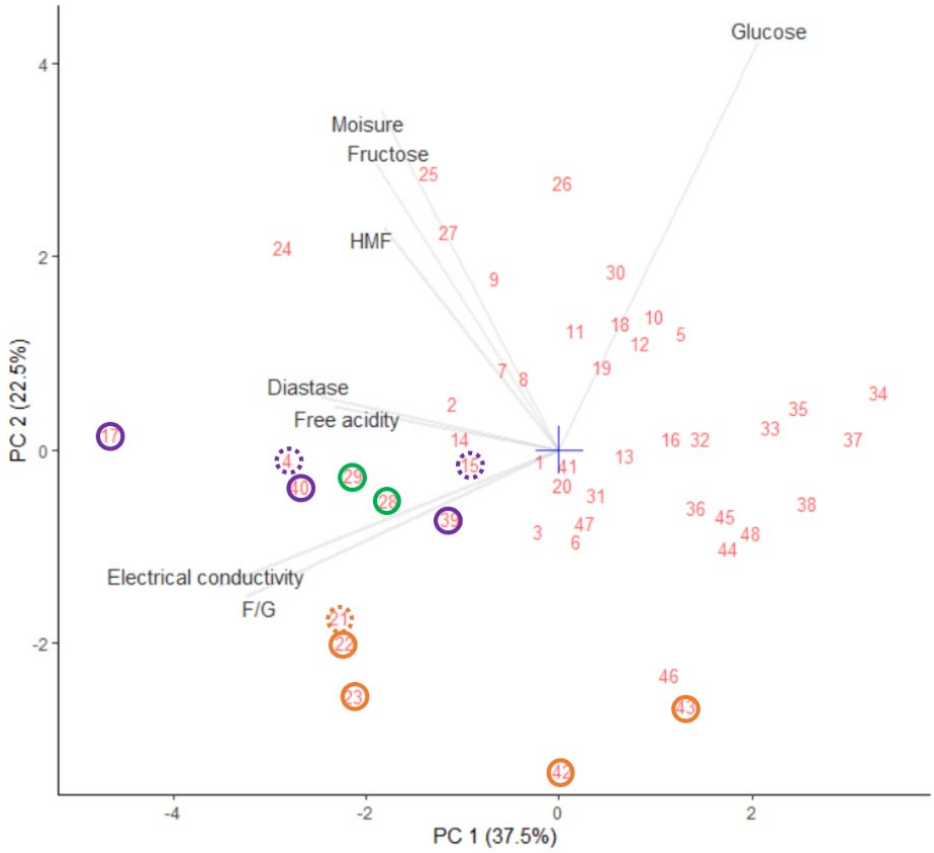

Figure 7. *PCA plot of physico-chemical parameters of analysed honeys (n=48). Honeys with high alder buckthorn pollen (orange circles), heather pollen (violet circles) and honeys containing traces of honeydew elements (green circles). The dotted circles mark small quantities of certain pollens.*

Three honey types (heather, alder buckthorn and honeydew) were grouped separately from most polyfloral honeys. Those honeys correlated positively with electrical conductivity levels and fructose/glucose ratio, and negatively with glucose content. Since rape and raspberry form the basis of pollen percentage in most honeys in different variations, most honeys in the PCA plot were scattered and it was not possible to determine any specific groups.

4.1.3 Sensory properties

The evaluation of honey flavour and aroma indicated that floral, sweet and berry-like characteristics were dominant, and the honeys lacked spicy, woody and animal-like notes (Publication III, Figure 2). The over-all odours and flavours were mild, which may have been affected by the amounts of rape and raspberry pollen, since the honeys of those botanical origins have mild flavours and odours (Persano Oddo & Piro, 2004). Since the

majority of the honeys were of mixed botanical origin, with rape and raspberry pollen occurring in all samples, the similarities in flavour and aroma are understandable. Besides the botanical origin, even when the pollen content is about the same, other factors, such as weather conditions, honey harvesting and storage, can affect the sensory properties and so no two honeys have the same sensory properties (Tahir et al., 2016; Stolzenbach et al., 2011).

The honeys with higher herbal, woody and spicy notes had stronger flavours and odours. Those characteristics had an influence on stronger intensities of sensory properties. More specifically, woody and animal-like notes had the highest correlations (r=0.63 and r=0.54, respectively). Sour taste had the largest effect on the over-all intensity of taste (r=0.61). This is associated with higher acid content in honeys. When honey taste and odour characteristics were compared, correlations above 0.5 were found, which showed that when a certain odour characteristic was detected, with high probability it was recognised during tasting and vice versa. Spicy characteristics were observed to be most related (r=0.93).

Sensory evaluation made it possible to detect honeys that had significant distinctive properties. In terms of flavour and odour characteristics, more animal-like, spicy, woody and herbal notes were perceived and the overall intensity was much higher. There were only two such honeys, heather honey and the honey containing the highest amounts of alder buckthorn (29%). Berry-like and sweet tastes were the least detected.

The two honeys that contained honeydew elements did not differ as much in their sensory properties as the aforementioned honeys did because they were not entirely honeydew honeys, showing higher scores in woody and animal-like characteristics.

The colour of honey is the first thing one notices when assessing honey. The calculated correlations (the correlations in both cases were over r=0.55) indicated that the darker and more reddish tones a honey had, the more woody and sour characteristics it had.

Notably, colour is a parameter that gives information on the flavour and odour of a honey. Most honeys were rather light in colour, and had more floral, sweet and berry-like characteristics, and the honeys lacked spicy, woody and animal-like notes. On the other hand, darker honeys had the opposite characteristics. Few honey types could be classified based on sensory attributes (only honeydew, heather and alder buckthorn), as most honeys had similar attributes, with some variations.

4.2 Alternative methods of honey specification

4.2.1 Colour

The majority of honey samples were very similar in colour parameters, with some variations. Examples of visual colour observations (n=30) can be seen in Figure 8. It was evident that by only looking two different types of honeys could be detected by their darker colours: heather honey (no 17) and honeys that contained traces of honeydew elements (nos. 28 and 29). This was also confirmed by measurements by a spectrophotometer, which gave specific L*-, a*- and b*-coordinates (L* stands for lightness/darkness, a* for redness/greenness and b* for blueness/yellowness), which are presented as numerical values in Table 2 of Publication III.

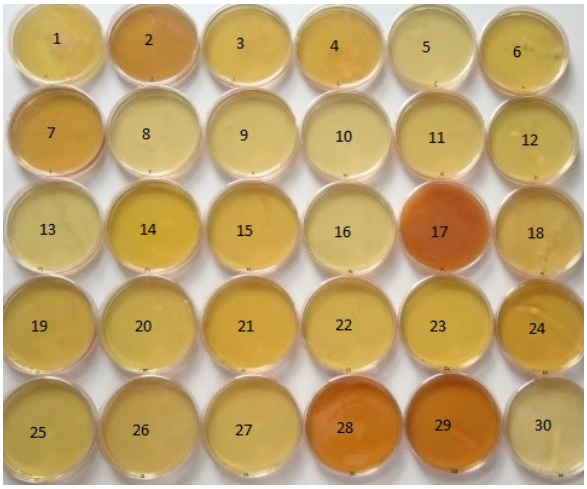

Figure 8. *Visual observation of honey colour (n=30).*

Most of the honeys were relatively light, with a higher L*-value, and had red, yellow and slightly green tones (Publication III, Table 2). Heather honey stood out for having the darkest colour, higher reddish tones, the lowest L*- values (65.3) and higher a*-values (9.1). Reddish and yellowish tones were most dominant in honeys containing honeydew elements, with the highest a*- and b*-values (12.5 and 12.3 for a*-values, and 60.3 and 58.7 for b*-values, respectively). The details of all the colour coordinates are presented in Table 2 of Publication III. The calculated correlations indicated that the darker the honey, the more reddish tones it had (Publication III, Table 3).

The colour of a honey proved to be a good indicator of the honey's composition and properties. Good correlations were found between colour coordinates and bioactive components. The results showed that darker and reddish tones in a honey indicated higher levels of total polyphenol and total flavonoid content (r above 0.8), as well as antioxidant activity (Publication III, Table 3).

4.2.2 Mineral content

It was observed that the mineral content was dependent on the botanical origin of the honey, with honeys rich in heather or alder buckthorn having the highest values (Publication I, Table 4). The study showed that potassium was most abundant in all samples, with great variability. Other minerals, such as calcium, magnesium and sodium, seemed to occur at slightly more uniform levels. Generally, the mineral level in a honey is relatively low, but by visual observation alone one can make preliminary assumptions about its content. The darker the honey, the more minerals it contains. Honeys with either heather or alder buckthorn pollen appeared to be of darker colour, which was confirmed by the above-mentioned results. Furthermore, the total mineral amounts were found to correlate well with the levels of electrical conductivity (r=0.949), which therefore can successfully be used as an indicator of mineral content. Focusing on the results of the electrical conductivity of different honeys from Publication III, we notice that the above-mentioned honey types had the highest levels and that indicates higher mineral levels. The colours of those two honeys were darker as well.

4.2.3 Amino acids

Since polyfloral honeys contain pollen and nectar derived from various floral sources, the levels of amino acids can vary. Proline (Pro) was the most predominant in all honey samples, ranging from 257-1328 mg/kg. These levels show the authenticity of honeys for which the proline content must be at least 200 mg/kg. Other amino acids, such as phenylalanine (Phe), lysine (Lys), glutamic acid (Glu) and glutamine (Gln), showed relatively higher levels compared to others, but were still much lower than proline. Proline was identified as the only amino acid to differentiate honeydew honeys from blossom honeys by their higher content (1023 and 1328 mg/kg). No other amino acids could be used for detecting the botanical origins of honeys.

Amino acids affect the antioxidant activity of honey, correlating well with water-soluble antioxidants (ACW). Mostly alanine (Ala) and proline (Pro), and to a lesser extent glutamic acid (Glu), glycine (Gly), threonine (Thr) and valine (Val), were observed to have the largest influence on antioxidant properties by calculated correlations (Publication III, Table 5).

The amino acid determination did not show any specific markers, which was probably due to the fact that many amino acids originate from the bee or plant and most honeys were polyfloral, rich in rape, clover, willow and raspberry pollens. Proline and phenylalanine were highest in honeys containing honeydew elements. Higher proline levels also occurred in heather honey. In general, the honeys were of good quality, which could be estimated by proline content.

4.2.4 Phenolic compounds

The honey samples (n=30) were analysed for the levels of total polyphenol (TPC) and total flavonoid (TFC) content, which were in the range of 26.2-88.7 mgGAE/100g and 1.9- 6.4 mgQE/100g, respectively (Publication III, Table 2). The amount of TPC varied more than that of TFC and a high correlation was found between these levels (r=0.88). The calculated correlations between TFC or TPC and colour were high, which means that honeys with darker and more reddish tones had higher levels of TFC or TPC (Publication III, Table 3). For example, the correlations of lightness (L*-value) and redness (a*-value) with TPC levels were r= -0.93 and r=0.85, respectively. In seven honey samples, the change in TFC and TPC values was measured during storage at -18˚C for two years and the levels of both increased (Table 8). The increase in the above-mentioned levels was probably due not to an actual increase but to the breakdown of phenolic molecules of higher molecular weight as the honeys aged (a result of enzymatic reactions and/or Maillard reactions), freeing chemical substituent groups with reduced power, which in turn react to a greater extent (compared to the original molecules) with the chemical reagents used in the spectrophotometric methods applied (Soares et al., 2017). Such reducing substances can be ascorbic acid, reducing sugars and amino acids, which can lead to an overestimate of the TPC values and interfere with Folin-Ciocalteu reactions (Zarei et al., 2019). Freezing causes cell breakdown, allowing enzymatic reactions to occur and, therefore, phenolic compounds can degrade (Khattab et al., 2015). Thus phenolic markers have limited usefulness as indicators for determining the botanical origins in honeys that have been stored for long periods before consumption (Šaric et al., 2020).

Table 8. *Change in total flavonoid content (TFC) and total polyphenol content (TPC) after two-year storage.*

Sample no	TFC 1st year (mg QE/100g)	TFC 3rd year (mg QE/100g)	TPC 1st year (mg GAE/100g)	TPC 3rd year (mg GAE/100g)
1	5.33 ± 0.14	8.44 ± 0.22	41.27 ± 0.31	45.53 ± 0.70
2	3.59 ± 0.05	5.64 ± 0.05	28.24 ± 0.13	32.29 ± 1.28
3	5.49 ± 0.00	7.53 ± 0.13	29.91 ± 0.44	41.57 ± 0.19
4	6.07 ± 0.00	7.68 ± 0.04	42.97 ± 0.84	47.12 ± 2.20
5	4.03 ± 0.00	5.54 ± 0.16	26.79 ± 0.29	29.55 ± 0.81
6	6.47 ± 0.12	9.39 ± 0.09	84.18 ± 0.91	87.27 ± 1.14
7	6.88 ± 0.00	9.20 ± 0.35	46.03 ± 0.15	51.14 ± 0.61

The highest levels of TFC and TPC were observed in heather honey (29% *Calluna* pollen).

Compared to the TPC and TFC levels in honey, beebread (n=5) and bee pollen (n=4) showed much higher levels (Table 9). The total flavonoid level of bee pollen (BP) is higher than that of beebread (BB), in the ranges of 979.3-1184.1 mgQE/100g and 335.1-919.5 mgQE/100g, respectively.

Table 9. *Total flavonoid content (TFC) and total polyphenol content (TPC) in bee pollen and bee bread.*

Sample no	Bee pollen		Bee bread	
	TFC (mg QE/100g)	TPC (mg QE/100g)	TFC (mg GAE/100g)	TPC (mg GAE/100g)
1	1184.10 ± 10.20	2278.35 ± 12.30	335.10 ± 11.60	1368.30 ± 97.10
2	817.27 ± 20.10	1439.21 ± 21.10	488.90 ± 10.20	1429.10 ± 15.92
3	881.13 ± 11.50	1399.33 ± 50.10	506.20 ± 32.10	1556.10 ± 87.4
4	979.33 ± 13.80	1717.11 ± 12.40	637.80 ± 18.30	1823.30 ± 13.90
5	-	-	919.50 ± 15.60	1688.40 ± 18.70

At the same time, the total polyphenol levels were about the same in BP and BB samples, within the ranges of 1399.3-2278.4 mgGAE/100g and 1368.3-1823.3 mgGAE/100g, respectively. Those results are in accordance with the ones obtained by Pełka and others (2021), showing that there is little difference between the values of TPC in BB and BP.

4.2.4.1 Polyphenol identification in honeys

The identified polyphenols, such as caffeic acid, coumaric acid and abscisic acid, and their derivatives had the highest levels in the analysed honey samples, followed by shikimic acid, 4-hydroxybenzoic acid, salicylic acid, quercetin, kaempferol, ferulic acid and its derivative. By TPC and TFC content and by identification of polyphenols by their levels, it was possible to classify honeys by distinct botanical origin and to identify their marker compounds (Publication III, Figure 1). For instance, heather honey had the highest TPC content and the highest levels were found in cinnamic acid, myricetin and abscisic acid derivatives D_2 and D_3. This was true of all honeys that contained heather pollen, even if it was minimally represented.

Protocatechuic acid is a known marker compound for honeydew honeys (Trautvetter et al., 2009), and the results showed that this polyphenol was present in honeys which contained only traces of honeydew elements.

4.2.5 Antioxidant activity

The honey samples (n=30) were analysed for their levels of water-soluble antioxidants (ACW) and lipid-soluble antioxidants (ACL), which were in the ranges of 37.8-311.2 mgAAE/100g and 14.4-60.7 mgTE/100g, respectively (Publication III, Table 2). Strong correlations were seen with colour parameters, as darker tones indicated higher ACL values (r=-0.85), while reddish tones indicated higher ACW values (r=0.92). This means that the darker honeys had higher antioxidant properties. The highest ACW value was in honeys containing traces of honeydew elements, and ACL was nearly twice as high in heather honey than in any other honeys.

4.2.6 Aroma-active compounds

Odour-active compounds of honey (n=13) and certain blossoms (raspberry, alder buckthorn, rape and heather) were detected and characterised (Publication II, Table 2). Only those compounds that had detection frequencies over 33% were taken into account and all together forty-six compounds were determined. It was found that the odour of honey was more intense and that it contained more aroma-active compounds than blossoms did. The aroma profiles of various honeys were rather similar and mostly characterised by floral, honey-like and green notes. Leather, mushroom, metallic and urine notes were also found, and in many cases candy and vanilla notes were used to describe odour-active components. With GC-O, in the case of some honeys an odour-active compound might have been present but was below the odour threshold value and for this reason was not detected.

The compounds present in all of the honey samples were butyric acid (cheesy odour), methional (potato odour), oct-1-en-3-one (mushroom odour), camphene (camphor odour), phenylacetaldehyde (honey odour), 2-hydroxy-benzaldehyde (medicinal odour), (Z)-linalool oxide (floral odour), 3,5-dimethyl-2-ethylpyrazine (coffee odour), (E,Z)-2,6-nonadienal (green odour), benzoic acid (urine odour), phenylacetic acid (honey odour), carvone (green odour), hydrocinnamic acid (floral odour), hexyl hexanoate (apple odour), (E)-β-damascenone (apple odour), vanillin (vanilla odour) and δ-decalactone (coconut odour). Eugenol (clove odour) and geranyl acetone (floral odour) were present in most of the samples.

Using GC-O made it possible to find distinct aroma-active compounds characteristic of certain honey types or their corresponding blossoms. For instance, in heather honey, most aroma-active compounds were detected and mostly sweet and candy-like notes were used in descriptions. The characteristic compounds found in all honeys that contained heather pollen, to different extents, were isophorone (candy odour) and 2-Methylbutyric acid (potato chip odour). Linalool (floral odour) was the one compound missing in heather honeys and blossoms (Publication II, Figure 2).

On the other hand, for rape, raspberry and alder buckthorn honeys, no characteristic compounds could be found. Rape honey had the least odour-active compounds detected. Raspberry honeys were mostly characterised by green notes and a lack of honey notes (Publication II, Figure 2), while alder buckthorn honeys had floral and honey notes and fewer green and sweet notes.

A hierarchical cluster analysis (HCA), based on dissimilarities, was conducted and this indicated that it was possible to classify honey types of different botanical origins (Publication II, Figure 1). Heather honeys had the most distinct odour profiles and were more similar to alder buckthorn honeys. Meanwhile raspberry and rape honeys had

similarities which may have resulted from the fact that in all of the honeys rape and raspberry pollens were present in various amounts.

The honeydew honey samples were not included in the analysis, and thus there are no data on its volatile markers.

4.2.7 Fluorophores

Front-face-fluorescence spectroscopy was used to classify the honey samples and, as a result, unique characteristics of honey samples were detected (Publication I, Figure 2). In the measured excitation (EX) and emission (EM) ranges, a typical SFS signal of measured honey samples contained three fluorescence peaks, with varying intensities. In EX: 270-290/EM: 320-350, the most prominent peak was detected in all honey samples which indicated aromatic amino or nucleic acid and mainly included tryptophane residuals. The honeys with the highest percentages of rape pollen had the highest peaks, but these peaks were detected in all honey samples, because all of the honeys contained rape pollen to some extent. Secondary peaks of tryptophane corresponded to the area of EX: 230/EM: 320-335, and higher peaks were detected in the area of EX: 330-350/EM: 380-440, indicating vitamins (riboflavin and vitamin A). This third peak was highest in heather honey, but varied greatly among all samples.

The PARAFAC model (Publication I, Figure 3) showed that the differences between spectra that corresponded to various groups of honey were small but some conclusions could be reached. For example, the honey samples with higher amounts of heather pollen could be distinguished from other honeys. Sometimes the low phenolic spectra for heather honey were due to scattering, reflection and interference effects resulting from the numerous air bubbles present (Ruoff et al., 2006).

4.3 Markers for the differentiation of the botanical origins of Estonian honeys

A conclusive information based on all the methods and results of analysis in this thesis is presented in Table 10. This data gives an understanding, which methods are best to use in describing and determining monofloral honey types and which methods have given the best results. It gives an opportunity for mapping honeys based on their specific characteristics and components, but also gives a base for future work in differentiating different Estonian honey types.

For example, monofloral heather honey can be detected best by polyphenol identification, as higher intensities of cinnamic acid, myricetin and abscisic acid derivatives are characteristic to that honey type. The characteristic aroma-active components are 2-Methylbutyric acid and isophorone. Honeydew honeys are characterised by higher intensities of protocatechuic acid. Other analyses, such as of physicol-chemical parameters, minerals, proline content, phenolic acids and antioxydativity levels, can be used as supplementary ways of describing honeys. Those levels show many similarities and dissimilarities between different honeys. As for amino acids, no marker compounds specific to any honeys were found, except that both heather honeys and honeydew-blossom honeys have relatively higher Proline contents. Typical fluorophores in rape and heather honeys were tryptophane and vitamins, respectively. Rape honeys and alder buckthorn honeys differed in terms of physico-chemical parameters, but no other identifying characteristics were found.

***Table 10**. Specific markers for the differentiation of the botanical origins of Estonian honeys.*

	Heather	Alder buckthorn	Rape	Honeydew-blossom
Physico-chemical parameters	↑ F/G ↑ EC	↑ pH ↑ F/G ↑ EC	↓ F/G ↓ EC	↑ EC
Mineral content	↑	↑	↓	N/A
Front-face fluorescence	↑ vitamins	ND	↑ tryptophane	N/A
Colour	↓ L*, ↑ a*	ND	ND	↓ L*, ↑ a*b*
Amino acids	↑ Proline	ND	ND	↑ Proline ↑ Phenylalanine
Polyphenol	•cinnamic acid •myricetin •abscisic acid derivatives	ND	ND	•protocatechuic acid
TPC, TFC	↑	ND	ND	↑
ACW	↑	ND	ND	↑
ACL	↑	ND	ND	ND
Odour/flavour	↑spicy,woody, herbal	↑spicy,woody, herbal	ND	↑woody, animal-like
Odour-active components	•2-Methylbutyric acid •Isophorone	ND	ND	N/A

• marker compound, ↑ higher value, ↓ lower value, N/A-not applicable, ND-specificies not detected (average value).

In conclusion, two honey types, heather honey and honeydew-blossom honey, gave good results on specific characteristics measured by different methods. For all of the different analysed honey types, more data is needed to confirm these results.

5. CONCLUSIONS

All forty-eight analysed honey samples met the quality standards set by Council Directive 2001/110/EC relating to honey. Those quality parameters include moisture content, electrical conductivity, invertase activity, free acidity, diastase, hydroxymethylfurfural and sugar content. Only minor excesses on certain parameters were detected in four samples. These findings indicate that Estonian honey is of good quality.

The melissopalynological analysis showed that the main pollen and nectar representatives in Estonian honeys were from the following plants: *Brassica napus* (rape)*, Rubus idaeus* (raspberry)*, Salix spp*. (willow)*, Frangula alnus* (alder buckthorn)*, Trifolium pratense* (red clover)*, Trifolium repens* (white clover) and *Calluna vulgaris* (heather). The pollen analysis indicated that polyfloral honeys are more common in Estonia and that monofloral honeys are quite rare. The thesis found that usually rape, raspberry, heather and alder buckthorn produce good monofloral honeys. Determining whether to classify a honey as monofloral or polyfloral is a challenge, as several aspects have to be taken into consideration. The pollen percentage does not always correspond to the exact amount of nectar in a honey, especially when the pollen percentage is on the borderline of what is counted as monofloral. Combining the results of melissopalynological analysis with other analytical data makes it easier to interpret the results and to differentiate between specific honey types. Typically, physico-chemical analysis is used to determine honey quality but some parameters are more useful for the determination of the botanical origins of monofloral honeys. The data produced by physico-chemical analysis are best interpreted by statistical analysis, as PCA was successfully used in this thesis. Honey types of the same botanical origin could be distinguished from polyfloral honeys and grouped together. The best parameters for differentiating honeys seemed to be electrical conductivity and fructose/glucose ratio. This information clearly indicates that by the routine analysis of quality parameters, combined with statistical analysis, different honey types can be distinguished.

Although the number of monofloral honeys in this thesis was small, it was possible by using some specific methods to differentiate certain honeys by botanical origin. The best results were achieved by the analysis of aroma-active compounds and by the identification of polyphenols by their levels. Polyphenol identification proved to be the most effective method of determining the botanical origin of a honey; in addition, marker compounds were found. Protocatechuic acid was considered a marker for honeydew honey, which distinguished it from other blossom honeys. Cinnamic acid, myricetin and abscisic acid derivatives were considered marker compounds for heather honey.

By using GC-O, the aroma profiles of various honeys were found to be rather similar and mostly characterised by floral, honey-like and green notes. The odour--active components characteristic of heather honeys were 2-Methylbutyric acid and isophorone. Aroma-active compounds characteristic of heather honey and its blossom were also found. No other honey types could be connected to their corresponding blossoms based on aroma-active compounds.

In addition front-face fluorescence spectroscopy was used to differentiate among honey types, although the differences between spectra that corresponded to specific honeys were small. Heather honey contained less tryptophane and more vitamins than rape honeys and polyfloral honeys.

An amino acid analysis showed that proline was the amino acid with the highest content in all honeys and was above the level indicating quality honeys. Such amino acids as phenylalanine and glutamine were present in significant amounts. The proline content

was highest in honeys that contained traces of honeydew and in heather honey, but no other clear conclusions regarding various honey types could be reached based on other amino acids.

The results of various analyses were combined, and as a result good correlations were found. For example, the total polyphenol and flavonoid content correlated well with such physico-chemical parameters as electrical conductivity and free acidity. The higher the level, the higher the total polyphenol and flavonoid content. By using the colour coordinates L*, a* and b*, conclusions regarding honey composition could be reached The polyphenol concentration increased with decreasing honey lightness and with increasing honey redness. Similarly, darker and more reddish honeys contained higher levels of lipid-soluble and water-soluble antioxidants. The darker the honey, the more reddish tones it likely had.

Most analysed honeys were rather light in colour and had higher floral, sweet and berry-like odours and flavours. Spicy, woody and animal-like characteristics and over-all intensity were detected in darker honeys, particularly in alder buckthorn and heather honeys. In addition, it was determined that honeys with dark and reddish colours had stronger sour flavours.

For analysing the large amounts of data, statistical analysis was most useful. It was helpful when classifying honeys by their various parameters and grouping similar ones together. For example PCA was successfully used to interpret the results of sensory analysis, polyphenol identification and physico-chemical data. The PARAFAC model was used for front-face fluorescence data and AHC for grouping honeys of various botanical origins based on their aroma profiles.

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ABSTRACT

Characterising and determining the botanical origins of Estonian honeys

This thesis investigates Estonian honeys and samples were collected from different regions of Estonia. Various analyses were conducted, with the purpose of thoroughly characterising the properties and compositions of honey and their botanical origins. One of the goals was to detect the marker compounds specific to certain honey types.

The botanical origins of honeys were determined by using pollen analysis, which provided an overview of the pollinating areas of bees. The botanical origins of honeys vary greatly, and thus honeys are very different from each other in their compositions and properties. Physico-chemical parameters, such as electrical conductivity, moisture content, free acidity and HMF content, and diastase and invertase activity, as well as the content of fructose and glucose were examined for quality evaluation. In addition, the levels of amino acids, fluorophores, total polyphenol and total flavonoid were examined. Polyphenols were identified by their intensities. Furthermore, aroma-active compounds were investigated and aroma and flavour characteristics were assessed by using "fruity", "floral", "berry-like", "herbal", "woody", "spicy", "sweet" and "animal-like" as the main attributes. The colours of honeys were measured by using L*-, a*- and b*-coordinates.

The results of this thesis show that the typical Estonian honey is polyfloral, which means that it originates from many plants, of which the main ones are rape, raspberry, clover, willow, alder buckthorn and heather. Monofloral honeys (i.e. originating mostly from only one plant) are not very common. Of all the analysed samples, only heather and alder buckthorn could be differentiated from the rest as monofloral. The pollen analysis revealed two honeys containing honeydew elements; they were not honeydew honeys, but still had specific characteristics. Most of the honeys contained rape and raspberry pollen to various degrees and lacked clearly recognisable characteristics.

Based on the results of physico-chemical parameters, it was possible to conclude that Estonian honey is of good quality. The most effective methods for determining the botanical origins of honeys were polyphenol identification and the study of aroma-active components. Those methods made it possible to find marker compounds, i.e. compounds present in a certain honey that can be used for the identification of the botanical origin of the honey. For example, cinnamic acid, myricetin and abscisic acid derivatives are characteristic to heather honey, and protocatechuic acid is characteristic to honeydew honey. Again, such aroma-active compounds as 2-methylbutyric acid and isophorone were only found in heather honey. The analysis of amino acids indicated higher proline concentrations in heather honey, and they were highest in honeys that contained traces of honeydew elements. This amino acid is considered a marker for honeydew honey. The determined proline contents in honeys correlated well with physico-chemical data, as the proline content indicated honey quality. The colour, flavour and aroma characteristics varied greatly. In general, it can be concluded that heather and honeydew honeys were darker in colour, with deep reddish tones. The tastes and aromas of such honeys were rather intense, with spicy, woody, herbal and animal-like characteristics. Furthermore, the total polyphenol and flavonoid levels and antioxidative properties were higher. The darker and more reddish the colour of a honey, the higher bioactive properties it had.

Alder buckthorn honey was lighter in colour than the above-mentioned honeys, but the sensory characteristics were the same. This honey differed from the rest of the analysed honeys by its higher pH value. Due to the fact that most honeys contained both rape and raspberry pollens to various extents (polyfloral honeys), it was not possible to determine specific characteristics related to those pollens. Generally, those honeys were not as dark, and had milder flavours and aromas, with sweet, floral and berry-like characteristics.

KOKKUVÕTE

Eesti mete iseloomustamine ja botaanilise päritolu määramine

Käesolevas doktoritöös uuriti Eesti mett ning selleks koguti kokku meeproovid erinevatest Eesti piirkondadest. Mee koostise ja omaduste põhjalikuks iseloomustamiseks ning botaanilise päritolu määratlemiseks viidi läbi erinevaid uuringuid. Üheks eesmärgiks oli erinevate analüütiliste meetodite kasutamisel tuvastada markerühendid, mis vastaks teatud mee tüübile.

Mee botaanilise päritolu määramiseks kasutati õietolmuanalüüsi, mis andis ülevaate mesilaste korjemaast. Kuna mee botaaniline päritolu on vägagi varieeruv, siis sellest tulenevalt erinevad meed nii oma koostise kui ka omaduste poolest. Kvaliteedi hindamiseks analüüsiti füüsikalis-keemilisi parameetreid nagu elektrijuhtivus, niiskuse sisaldus, vabade hapete ja HMF sisaldus, diastaasi ja invertaasi aktiivsus, samuti ka glükoosi ja frukstoosi kogus. Lisaks määrati meeproovides aminohapete sisaldused, fluorofooride, polüfenoolide ja flavonoidide üldkogused, sh identifitseeriti polüfenoolid nende intensiivsuste põhjal. Samuti tuvastati meeproovides aroomiühendid ning assessorid hindasid mee maitset ja lõhna. Mee värvus määrati L*-, a*- ja b*-koordinaatide abil.

Uurimustöö tulemustest selgus, et tüüpiline Eesti mesi on polüfloorne, st pärineb mitmetelt erinevatelt taimedelt, millest peamiselt olid esindatud raps, vaarikas, ristik, paju, paakspuu ja kanarbik. Monofloorne mesi, mis koosneb peamiselt ainult ühe taime liigist, on Eestis vähem levinud ning uuritud proovidest eristusid teistest paakspuu ja kanarbiku meed. Samuti tuvastati õietolmuanalüüsiga kahes mees lehemee elementide sisaldus, mistõttu need meed olid samuti eristuvad. Suurem enamus meedest sisaldasid rapsi ja vaarika õietolmu varieeruvates kogustes, seega konkreetseid erisusi nende puhul ei olnud välja võimalik tuua.

Tuginedes uurimustöös esitatud füüsikalis-keemiliste parameetrite tulemustele, on võimalik öelda, et Eesti mesi on kvaliteetne, st, et see vastab sätestatud kvaliteedikriteeriumitele. Valitud analüüsimeetoditest osutusid mee botaanilise päritolu tuvastamisel ja mee erisuste rõhutamisel kõige efektiivsemateks aroomianalüüs ning polüfenoolide identifitseerimine. Need analüüsimeetodid võimaldasid leida ka teatud meetüüpide markerühendid, st ühendid, mida leidub ainult teatud kindlas meesordis ja mida saaks edaspidi kasutada mee päritolu tuvastamisel. Näiteks kanarbikumett on võimalik tuvastada kaneelhappe, müritsetiini ja abstsiishappe derivaatide abil ning lehemett protokatehoolhappe järgi. Aroomikomponentidest on kanarbikumeele iseloomulikud 2-metüülbutüürhape ja isoforoon. Aminohapete analüüs näitas kõrgemat proliini sisaldust kanarbikumees, kuid kõrgeim oli see meedes, mis sisaldasid lehemee elemente. Seda aminohapet loetakse lehemee markeriks. Ühtlasi hinnatakse proliini sisalduse põhjal ka mee kvaliteeti ning analüüsitud meeproovide tulemused kinnitasid füüsikalis-keemiliste kvaliteediparameetrite tulemusi. Ka meede värvus ning maitse- ja lõhnaomadused olid väga varieeruvad. Üldiselt võib välja tuua, et kanarbiku ja lehemee elemente sisaldavad meed olid tumedaimad ning domineeris punakas toon. Selliste meede lõhna- ja maitseomadused olid intensiivsemad ning rohkem esines vürtsikat, puidulikku, taimset ja loomalikke nüansse. Need meed olid kõrgema polüfenoolide ja flavonoidide sisaldusega ning samuti kõrgemate antioksüdatiivsete omadustega. Mida tumedam ja punakama tooniga on mesi, seda kõrgemad on selle bioaktiivsed omadused.

Paakspuu meed olid küll oma värvuselt heledamad, kuid sarnaste sensoorsete omadustega võrreldes eelpool mainitutega ning erinesid teistest oma kõrgema pH poolest. Kuna suurem osa analüüsitud metest sisaldasid nii rapsi kui ka vaarika õietolmu varieeruvates kogustes, siis nende puhul eristuvaid omadusi polnud võimalik esitada. Üldiselt on kõik meed pigem heledama tooniga, maitselt mahedad, kus domineerivad magusad, lillelised ning marjased maitse- ja lõhnaomadused.

APPENDIX 1

PUBLICATION I

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Characterization of Estonian honeys by botanical origin

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Abstract. This study characterizes Estonian honeys based on their physico-chemical properties and chemical composition. Melissopalynological analysis was carried out to determine the botanical origin of each honey. According to pollen analysis, 39% of the honeys analysed appeared to be unifloral rape (*Brassica napus*), raspberry (*Rubus idaeus*), or heather (*Calluna vulgaris*) honeys.

Fluorescence spectroscopy was used to estimate both the physico-chemical parameters and floral content of each honey sample by comparing these estimates with experimental data measured using standard techniques. The r^2 correlation between estimated values and experimental data was above 0.7 for several parameters, including free acidity with an r^2 of 0.919.

Key words: honey, physico-chemical properties, melissopalynology, front-face fluorescence.

1. INTRODUCTION

Honey is a complex product composed of monosaccharides such as glucose and fructose and other components, including amino acids, proteins, minerals, enzymes, and vitamins (White, 1975). The exact composition of any given honey depends mainly on the plant sources it is derived from, but also on the weather, soil, and other factors; therefore no two honeys are identical (Crane, 1980).

Quality parameters of honey are specified in a European Directive, which brings out the physico-chemical criteria for honey, such as moisture content, electrical conductivity, free acidity, diastase activity, hydroxymethylfurfural (HMF) content, ash content, and sugar content (EU, 2002). These parameters, together with melissopalynological analysis, can be used to authenticate the botanical origin of honey. In recent years the botanical origin of honey has also been determined using front-face fluorescence spectroscopy because the spectra obtained using this method contain a large amount of information regarding the chemical content of honey. Natural fluorophores in honey include aromatic amino acids, nucleic acids, HMF, furosine, and phenolic compounds. The concentrations of these fluorophores can vary to a large degree depending on the geographical and floral origin of the honey (Ruoff et al., 2006).

A library of knowledge of honey types allows one to discern honeys from different regions in Europe and those that originate from other continents (Maurizio, 1975). Pollen analysis of multifloral honeys indicates their botanical composition, as represented by the spectrum of pollen variability, and can also be used to determine if a honey is a blend of different honeys, and if so allows one to approximate the blending ratios (Agashe and Caulton, 2009). Precise identification of the discrimination point between multifloral and unifloral honeys can nevertheless be difficult. However, there are specific physico-chemical properties that can be used to confirm the results of microscopical analysis.

Considering the number of floral sources visited by the bees and small areas of certain plant types during the flowering period, pure unifloral honeys can rarely be obtained in Estonia, with the most common exception being rape honeys. The most widespread plants in Estonia that provide both pollen and nectar are willow,

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dandelion, white clover, raspberry, red clover, willow-herb, fruit trees, and berry bushes, in addition to heather, which is one of the most highly valued honey plants (Tammet, 2007).

Domestic honey is highly appreciated in Estonia, and several quality analyses are performed every year; however, very few scientific studies have analysed the relationships between the botanical origin of honey and its physico-chemical properties. Furthermore, studies that make use of front-face fluorescence spectroscopy to classify honey are scarce. Consequently, the aim of this study is to characterize unifloral and multifloral honeys of Estonian origin and thus contribute to the growing library of characteristics and botanical origins of honeys from around the world.

2. MATERIALS AND METHODS

2.1. Honey samples

Eighteen honey samples were collected directly from beekeepers who operate in different areas of Estonia. These honeys were stored at 18 ± 2 °C in air-tight glass containers until further analysis.

2.2. Melissopalynological analysis

Melissopalynological analysis was carried out according to the non-acetolytic method described by Louveaux et al. (1978). The pollen counts were expressed as percentages after counting 500–600 pollen grains. The identification of the pollen types was based mainly on the reference collection of the Department of Food Processing of Tallinn University of Technology and data provided by Ricciardelli D'Albore (1997). An Olympus CX21 (Japan) binocular light-microscope with 40×15 magnification was used.

2.3. Physico-chemical parameters

Physico-chemical properties, such as moisture content, pH, free acidity, electrical conductivity, diastase activity, and HMF were determined according to the official Estonian methods (EVS, 1997).

The fructose and glucose content were both determined using high-performance liquid chromatography (HPLC) (Waters, USA). The chromatograph was equipped with Alliance Separations Module 2695 (Waters, Milford, MA, USA), Aminex HPX-87H 300 mm × 7.8 mm column (BioRad, Philadelphia, PA, USA), and Refractive Index Detector 2414 (Waters, Milford, MA, USA). Each sample contained 0.4 g of honey dissolved in 50 mL of Milli-Q water, filtered through a 0.2 μm Millipore filter, and diluted in addition 10 times with an HPLC eluent (0.05 M H_2SO_4). The injection volumes of the samples were 20 μL, with a flow rate of 0.6 mL/min (isocratic). The HPLC sample peaks were identified by comparing the retention times obtained from standards. Triplicate injections were performed, and the average peak areas from these technical replicates were used for peak quantification.

Mineral content analysis was carried out using an ion chromatograph system (Waters, Milford, MA, USA) that consisted of Conductivity Detector 432, Isocratic HPLC pump 1515, and IC-Pac 3.9 mm × 150 mm Cation Column 432 (Waters, Ireland). Honey samples of 5 g were dissolved in 50 mL of Milli-Q water, and this solution was filtered through a 0.2 μm Millipore filter. The injection volume of the samples was 20 μL with a flow rate of 1 mL/min. For data analysis we used Breeze software (Waters, Milford, MA, USA).

2.4. Front-face fluorescence spectroscopy

Fluorescence measurements were performed using an Instant Screener® (ISC) Analyzer (LDI Ltd., Tallinn, Estonia). This compact spectro-fluorometer has a 10 mL optical cell and is equipped with a 5 W pulsed Xenon lamp capable of generating excitation emission matrixes (EEM) or spectral fluorescence signatures (SFS). The SFSs were measured in a front-face optical layout (35°) from the surface at excitation wavelengths from 230 to 350 nm and at emission wavelengths from 250 to 565 nm with 5 nm intervals in both directions.

Raw spectral data were rearranged into three-dimensional data arrays, with each dimension corresponding to the sample array, emission data, and excitation data. Data were decomposed and analysed in three dimensions using an algorithm implemented in the N-way toolbox, Matlab (Andersson and Bro, 2000).

2.5. Statistical analysis

Statistical analysis was performed using Matlab (Mathworks, Natick, MA, USA). Principal component analysis (PCA) was carried out in order to visualize data from different honey samples and to identify their similarities and differences. The analysis was made on the basis of physico-chemical properties such as moisture content, pH, free acidity, electrical conductivity, diastase activity, mineral content, and sugar composition (glucose and fructose). In addition, Pearson correlation coefficients were calculated between all measurements.

3. RESULTS AND DISCUSSION

3.1. Melissopalynological analysis

The most numerous pollen types identified in the samples were rape (*Brassica napus*), white clover (*Trifolium repens*), melilot (*Melilotus officinalis*), raspberry (*Rubus idaeus*), and willow (*Salix* spp.) (Table 1).

Table 1. Content of pollen types in honey samples, %. Percentages in boldface refer to unifloral honeys; the plus sign (+) stands for minor pollen (<1%)

Pollen type	Honey samples																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Aceraceae																		
Acer spp.		+			1	4	3	1					3	4	+	3	3	
Betulaceae	2	1	+		+		1	+		+	+		1	1		1	2	
Boraginaceae																		
Echium vulgare	+		+			+					3					2		
Compositae																		
Centaurea cyanus			+					+										
Taraxacum officinale						1		+					3	+	1	1	+	
Cruciferae																		
Brassica napus s.l.	17	11	**60**	**77**	**76**	51	50	43	40	27	9	+	23	17	29	2	16	37
Ericaceae	1										3							
Calluna vulgaris									**16**	**27**	4							
Fabaceae																		
Galega officinalis			2	5			2	+					5	6	17			3
Lathyrus pratensis s.l.									2		3							
Gramineae									1		+							
Hippocastanaceae																		
Aesculus hippocastanum				+		1		+				+		+		+		
Hydrophyllaceae																		
Phacelia tanacetifolia														3			2	
Leguminosae																		
Melilotus officinalis s.l., *Trifolium repens* s.l.	1	4	5	10	2	5	10	4	18	28	3	21	1	7	27	18	27	15
Trifolium pratense s.l.	+		1	5	+	+	5	+	3	4	19	1	+	14	+		+	3
Menyanthaceae																		
Menyanthes trifoliata											1							
Onagraceae																		
Epilobium angustifolium										+								
Ranunculaceae	+								+									
Rhamnaceae																		
Frangula alnus			1		2				2	3	1	**42**	**22**	+	+		1	
Rosaceae																		
Rubus idaeus s.l.	**67**	**79**	17	2	8	7	17	14	6	5	31	33	32	20	23	36	17	40
Filipendula ulmaria			+	+			+		2	1	3		1			+	2	
Salicaceae																		
Salix spp.	6	5	9	+	6	27	7	34	1	1	14	+	5	22	2	15	24	
Umbelliferae	+	+	1	+	+	1	+	+	+	+	1	+		1	+	2	1	

All of these plant species are relatively common in Estonia. Two of the honey samples analysed contained pollen of alder buckthorn (*Frangula alnus*) and also traces of honeydew elements. Three of the honey samples contained heather (*Calluna vulgaris*) pollen.

Usually, honey is considered unifloral when 45% of the relative frequency of all pollen counted is identified as belonging to a single taxon. However, because of the numerous over- or under-represented pollen types, pollen percentages can vary considerably between different unifloral honeys. Therefore, to correctly interpret the botanical origin of a honey, sensory and physico-chemical data should also be taken into account. Because the pollen of rape in honey is over-represented, honey samples with over 60% rape pollen are considered unifloral. In contrast, as the pollen of heather is under-represented, even honeys with 10% pollen identified within this taxon may be considered unifloral (Von Der Ohe et al., 2004). This view is further supported by the work of Salonen et al. (2009). In accordance with Bryant and Jones (2001), we classified honey samples as being unifloral raspberry when 45% of the pollen distribution originated from *Rubus idaeus*. Taking the pollen types into account, 7 of the 18 analysed honey samples (Table 1) are potentially unifloral raspberry (1 and 2), rape (3, 4, and 5), and heather (9 and 10) honey varieties.

3.2. Physico-chemical parameters

The acidity of honey is an important parameter during the extraction and storage of honey, because it influences the texture, stability, and shelf life (Terrab et al., 2004). All honey samples analysed were acidic and found to range between pH 3.38 and 5.12 (Table 2). Two of the honey samples have higher pH values relative to the others (the pH values of samples 12 and 13 were 5.12 and 4.52, respectively). This may be due to a higher content of alder buckthorn pollen and/or the presence of honeydew elements (Table 1).

Moisture content is an important quality parameter that influences the shelf life of honey (Bogdanov et al., 2004). It depends on various factors, including the harvesting season, the degree of maturity reached in the hive, and climate factors (Finola et al., 2007). The moisture content of all 18 honey samples ranged between 16.1% and 18.9%. These percentages are below the upper limits of ≤20% and ≤23% for heather honeys set by the relevant EU directive (EU, 2002).

Diastase is a starch digesting enzyme whose activity is used as an indicator of honey freshness because it becomes denatured during heat treatment; it has reduced activity in heated or old honeys (White, 1975). The diastase numbers (DN) of the 18 honey samples ranged between 16.2 and 32.9, and are thus all higher than the minimum of 8 DN set by European legislation (EU, 2002).

Table 2. Physico-chemical parameters of honey samples

Sample	pH	Moisture, %	Diastase (DN)	HMF, mg/kg	Electrical conductivity, mS/cm	Free acidity, mmol/kg
1	3.41	16.7	28.0	5.8	0.2	29
2	3.55	18.3	25.8	<1	0.1	20
3	3.52	17.5	19.4	3.8	0.2	23
4	3.51	17.3	20.7	<1	0.1	20
5	3.56	17.4	26.9	1.9	0.1	21
6	3.72	17.9	21.3	1.9	0.3	23
7	3.59	16.1	26.0	<1	0.1	19
8	3.68	17.1	16.2	<1	0.2	21
9	3.53	18.2	28.5	3.8	0.4	43
10	3.79	18.9	32.9	2.9	0.6	54
11	3.38	17.0	25.7	3.8	0.3	35
12	5.12	16.8	28.0	<1	0.4	17
13	4.52	17.3	17.6	<1	0.4	14
14	3.48	17.8	23.0	<1	0.2	22
15	3.75	17.0	21.9	<1	0.2	22
16	3.69	16.1	25.1	<1	0.2	22
17	3.80	18.9	29.1	1.9	0.3	25
18	3.53	16.1	22.5	1.9	0.1	16
Mean	3.73	17.4	24.4		0.2	25
SD	0.43	0.86	4.36		0.14	10.00
Range	3.38–5.12	16.1–18.9	16.2–32.9	max. 5.8	0.1–0.6	14–54

The HMF, a compound that is formed by the decomposition of fructose in the presence of an acid, is also an important indicator of honey quality because the amount of HMF increases in honey that is subjected to higher temperatures (Crane, 1980). The amount of HMF found in all honey samples was below 5.8 mg/kg, and well below the limit of 40 mg/kg, stated by European legislation (EU, 2002). This indicates that these honey samples had not been overheated.

Electrical conductivity is a good indicator of the botanical origin of honey and is currently used routinely instead of measuring the ash content (Bogdanov et al., 2000). The electrical conductivity of the 18 honey samples ranged from 0.1 to 0.6 mS/cm, which indicates their floral origin because all were below the limit of 0.8 mS/cm for blossom honeys and mixtures of blossom and honeydew honeys (EU, 2002; Ouchemoukh et al., 2007). All three honeys that contained heather pollen had higher values of electrical conductivity (0.3–0.6 mS/cm), which agrees with a measurement of 0.73 mS/cm for pure heather honey by Persano Oddo and Piro (2004). The honey samples that contained pollen of alder buckthorn also had higher electrical conductivity (0.4 mS/cm), although this could also be due to honeydew. Rape honey samples had the lowest electrical conductivity (0.1–0.2 mS/cm), which is in accordance with values reported by Persano Oddo and Piro (2004).

The free acidity of honey may be explained by the presence of organic acids in equilibrium with their corresponding lactones, or internal esters, and some inorganic ions, such as phosphate (Finola et al., 2007). Free acidity values ranged between 14 and 54 mmol/kg. All honey samples, except for heather honey of sample 10 with a free acidity of 54 mmol/kg, met the relevant EU standard being under 50 mmol/kg (EU, 2002), which indicates the absence of unwanted fermentation. Also Persano Oddo and Piro (2004) state that honey samples that contain heather pollen have high values of free acidity (see honey samples 9, 10, and 11 in Table 2).

Glucose and fructose are the main sugars in honey and their actual proportion depends largely on the source of the nectar (Anklam, 1998). Normally, fructose predominates slightly, with some exceptions being rape and dandelion honeys (Crane, 1980). In our study 72.2% of the honey samples analysed had fructose as the dominating sugar with a mean value of 36.53 g/100g (Table 3). Glucose values were lower with a mean value of 34.79 g/100g. Samples that contained mostly rape pollen had the highest concentration of glucose (see honey samples 3–8 in Table 3).

The fructose/glucose ratio ranged between 0.89 and 1.20, indicating their floral origin because it is known that flower honeys have a fructose/glucose ratio of

Table 3. Fructose and glucose content of the analysed honeys (g/100g) and fructose/glucose ratio (F/G)

Sample	Glucose	Fructose	F/G
1	34.83	38.15	1.10
2	36.16	38.29	1.06
3	37.18	36.30	0.98
4	40.32	35.78	0.89
5	37.86	36.64	0.97
6	35.00	35.10	1.00
7	38.64	36.78	0.95
8	36.96	35.46	0.96
9	32.44	37.77	1.16
10	32.99	39.53	1.20
11	35.99	39.36	1.09
12	28.84	33.61	1.17
13	30.22	33.08	1.09
14	34.19	35.31	1.03
15	34.97	37.16	1.06
16	30.83	35.27	1.14
17	33.40	35.88	1.07
18	35.42	38.02	1.07
Mean	34.79	36.53	1.06
SD	2.99	1.79	0.09
Range	28.84–40.32	33.08–39.53	0.89–1.20

about 1 while in honeydew honeys the ratio ranges between 1.5 and 2.0 (Gleiter et al., 2006). Five of the honey samples analysed had fructose/glucose ratios under 1, an effect caused by their having a higher content of rape pollen (Table 1). According to Persano Oddo and Piro (2004), the fructose/glucose ratio of unifloral rape honey is lower than 1, which agrees with our findings.

The mineral composition of the honey samples is presented in Table 4. Minerals such as sodium, potassium, magnesium, and calcium were identified. The mineral content of honey is generally small and depends on the composition of the nectar of the plants that dominate its makeup (Felsner et al., 2004). Light blossom honey has a lower mineral content than dark honey such as honeydew and heather (Bogdanov et al., 2007). Potassium was quantitatively the most important mineral, whose content ranged from 125.79 to 2854.78 mg/kg, where the lower values are for rape honeys and the higher values are for honey samples that either originated from heather or contained alder buckthorn pollen. The sodium and magnesium contents in the samples were lower with average values of 15.46 mg/kg and 19.37 mg/kg, respectively. The calcium content ranged from 20.37 to 100.33 mg/kg, with heather honeys having the highest content. Generally, the results of this study confirmed that light-coloured honeys have a lower mineral content (rape and raspberry honeys) than darker honeys (alder buckthorn and heather honeys).

Table 4. Mineral content of the analysed honeys, mg/kg

Sample	Na	K	Mg	Ca
1	9.62	292.69	20.85	53.88
2	9.65	237.14	17.73	36.54
3	13.18	459.87	24.87	50.40
4	13.82	126.37	14.65	46.27
5	6.64	253.80	18.53	40.17
6	6.85	578.88	21.34	63.65
7	5.67	262.90	17.24	49.98
8	9.04	517.93	25.23	53.02
9	40.72	1271.23	20.79	76.17
10	62.55	2854.78	24.96	100.33
11	24.22	902.75	20.27	56.82
12	19.44	1235.58	23.46	39.80
13	8.17	1381.53	16.50	20.37
14	8.11	257.64	14.95	42.86
15	11.70	569.73	25.49	55.73
16	13.35	485.75	5.53	39.42
17	10.79	862.22	24.28	56.63
18	4.77	125.79	12.05	29.20
Mean	15.46	704.25	19.37	50.62
SD	14.50	667.78	5.35	17.85
Range	4.77–62.55	125.79–2854.78	5.53–25.49	20.37–100.33

3.3. Front-face fluorescence spectroscopy

3.3.1. Description of fluorescence spectra of honey samples

Fluorescence spectroscopy is a useful tool to fingerprint or classify honey samples because a large number of different substances can affect their spectral signature (Ruoff et al., 2006). To record fluorescence data we applied SFS technology, which was found to provide extra information compared to normal emission or excitation spectra. Examples of unique fingerprints of honey samples of various unifloral origin are presented in Fig. 1. The peaks of the spectra of unifloral heather, rape, and raspberry honey samples vary by shape and height.

In the measured excitation and emission range, a typical SFS signal of measured honey samples contains three fluorescence peaks with varying intensities. The maximum of the most informative peak is located in the area EX:270–290/EM:320–350. This area corresponds to aromatic amino or nucleic acids and mainly includes tryptophane residuals. This peak is very common not only in honey but in various samples of biological origin. Although the tryptophane peak is detected in all honey samples, it serves as a sensitive contribution to the fingerprint of honey samples because its emission spectra change in accordance to its local environment. This peak is higher in honey samples that contain more rape pollen, although this conclusion is not quite straightforward because almost all samples contain rape pollen to some degree.

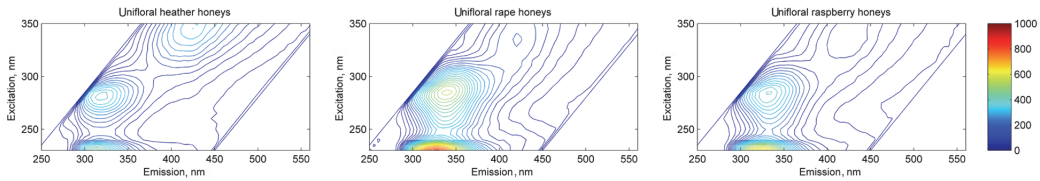

Fig. 1. Three typical peaks in unifloral honey samples.

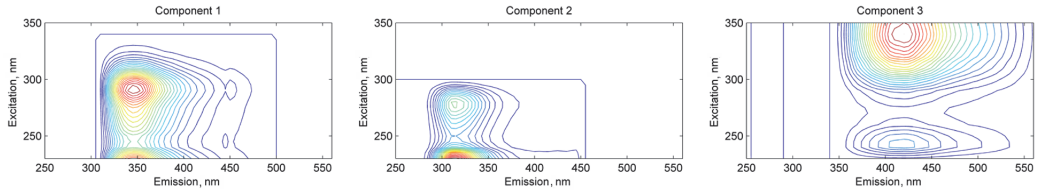

Fig. 2. Reconstructed spectra of three components of the PARAFAC model corresponding to three peaks typically found in honey spectra.

The maxima of the highest peak are located around EX:230/EM:320–335. For samples with lower intensities, the peak maxima are located towards lower emission wavelengths, as observed in honey samples that contain heather pollen (samples 9–11). This peak is located in the area that typically corresponds to secondary peaks of tryptophane.

The third obvious peak in typical SFS spectra of honey samples is located in the area of EX:330–350/EM:380–440 and corresponds to vitamins (FADH/NADH, riboflavin, and vitamin A). Compared to the two peaks described previously, the intensity of the third peak showed the most variation between honey samples. The highest values for the third peak were observed in honey samples that contain heather pollen.

3.3.2. Chemometric analysis of SFS

For chemometric analysis of fluorescence spectra, the PARAFAC algorithm was applied to decompose the SFS data into a number of trilinear components that can be presented as scores that directly relate to the relative concentration of components whose emission and excitation spectra are described with factor loadings. To enable for easier physical interpretation of the results, PARAFAC was applied using non-negativity constraints in all three modes.

The SFS data for honey were first modelled using PARAFAC with 1 to 6 factors. Comparison of core consistency values revealed that three factors should be suitable to model this particular kind of data. This was confirmed using split-half analysis.

Three factors of the model in Fig. 2 correspond roughly to the same three peaks described above, although the separation is not perfect, the peaks overlap, and components often contain traces of information from several of the peaks described. Nonetheless, the first component corresponds to the so-called tryptophane peak but contains traces of the secondary tryptophane peak. In contrast, the second component corresponds to the secondary tryptophane peak but contains traces of the primary peak. The third component corresponds to the vitamin peak, although there are traces that describe the area between two peaks of tryptophane. Therefore, it can be assumed that the third component also contains information regarding changes in the shape of the tryptophane peak.

A plot of the scores that result from the PARAFAC model (Fig. 3) reveals that differences between spectra that correspond to various groups of honey are rather small. Honey samples that contain heather pollen are distinguished from the rest of the group by a high score of the third PARAFAC component and a low score of the first PARAFAC component. This is evidence that these samples contain less tryptophane. The third typical peak corresponds to the concentration of vitamins or other substances corresponding to the third typical peak in honey, as described above. Compared to all other spectra, raspberry honey samples have SFS spectra with lower intensity. Therefore, these samples form a distinct group from the rest of the samples in the plot of the PARAFAC model scores. The rest of the honey samples correspond mainly to unifloral rape, honeys with a high amount of rape, and the rest of the multifloral honeys. All these have rather similar spectra and therefore cannot be classified using the scores plot.

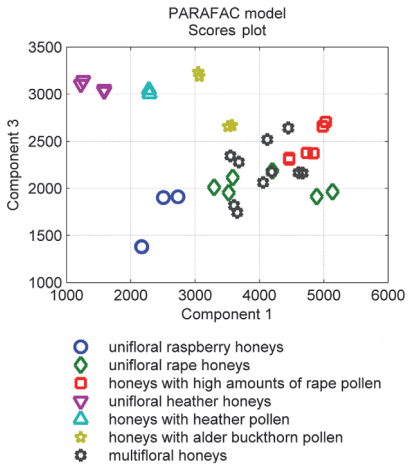

Fig. 3. Scores plot of the PARAFAC model.

Besides PARAFAC modelling, chemometric analysis was applied to determine whether SFS spectra can be used to estimate various physico-chemical parameters of honey samples or their floral content (Table 5). This was done using various multivariate calibration models (PCR, PLS, N-PLS, PARAFAC model scores). The measured spectra were divided randomly into calibration and validation sets (25 and 11 spectra each, splitting ratio roughly 70 : 30).

The calibration set was used to generate calibration models, which later were applied to estimate values of various physico-chemical parameters or the floral content of validation set samples. To compare the results obtained with various calibration techniques, correlation coefficients were calculated between the measured values and model estimations found using the validation set. Because the number of spectra in the validation set was relatively small, the results depend on the selection of the spectra. To prevent against systematic bias, the validation process was repeated ten times using spectra that were randomly divided between calibration and validation sets. Average values from these ten validation steps were used to compare calibration models and evaluate the potential use of the SFS method to estimate the physico-chemical parameters or floral content. The average results from the validation process are reported in Table 5. The PCR and PLS methods provide the most reliable results. The correlation between SFS estimations and real data for free acidity is 0.919. For electric conductivity and contents of minerals and glucose or fructose, the Pearson correlation coefficient is around 0.8. For seven plants in the table the correlation between SFS estimations and experimental data is between 0.717 and 0.829. These results were achieved using available SFS spectra. We expect that with a larger and more diverse calibration dataset better correlation between model predictions and measured values could be achieved.

Table 5. Comparison of tested calibration models for estimating values of physico-chemical parameters and floral content

	Model			
	PCR (12 components)	PLS (12 components)	N-PLS (10 components)	PARAFAC (2 components)
Physico-chemical property[a]				
pH	0.739	0.780	0.457	0.178
Free acidity	0.919	0.853	0.823	0.306
Electrical conductivity	0.859	0.861	0.792	0.675
Glucose content	0.852	0.873	0.314	0.076
Fructose content	0.775	0.822	0.162	0.251
Mineral content	0.776	0.838	0.785	0.713
Floral content[a]				
Acer spp.	0.736	0.717	0.599	0.154
Taraxacum officinale	0.704	0.718	0.770	0.099
Cruciferae (*Brassica napus* s.l.)	0.786	0.763	0.747	0.061
Calluna vulgaris	0.811	0.828	0.731	0.565
Menyanthes trifoliata	0.772	0.762	0.422	0.171
Frangula alnus	0.788	0.800	0.644	0.194
Rosaceae (*Rubus idaeus* s.l.)	0.787	0.829	0.648	0.216

[a] The table contains results where the correlation (r^2) between experimental results and estimations was at least 0.7.

3.4. Statistical analysis

The results of principal component analysis (PCA) are shown in Fig. 4. The first component (PC1) contained 43.0% of the data variance and was positively related to the glucose content, electrical conductivity, and free acidity, and negatively related to fructose content. The second component (PC2) contained 28.7% of the data variance and was positively related to pH, and negatively related to both fructose and mineral content. All honey samples that contain heather pollen (9, 10, and 11) have positive PC1 values, while honey samples with alder buckthorn (12, 13) have highly positive PC2 values. Most multifloral honeys appear in the centre of the graph and have similar physico-chemical properties, whereas unifloral rape honey samples (3, 4, and 5) and honey samples that contain high amounts of rape pollen are slightly separate from the other honeys under the group of multifloral honey cluster having negative PC1 and PC2 values. In addition, one sample of unifloral raspberry honey is located close to the honey that contains heather pollen in small amounts, while the other raspberry honey sample is more closely related to unifloral rape honeys. Because the physico-chemical

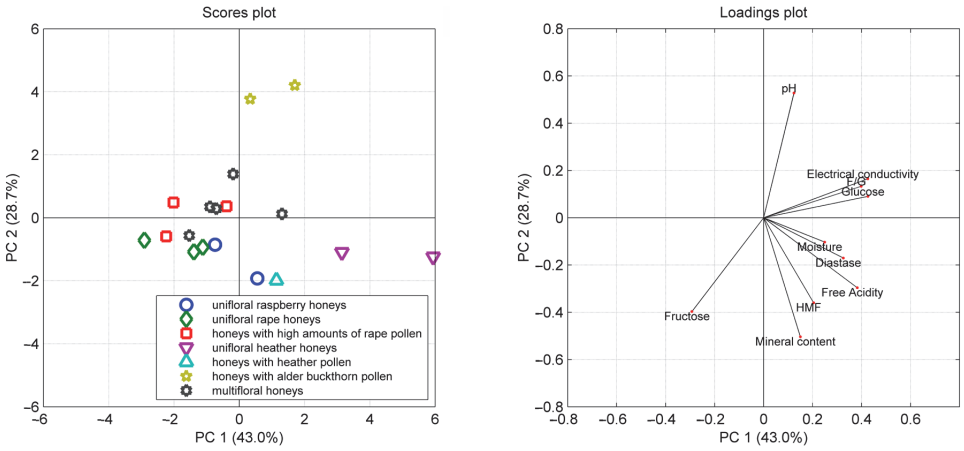

Fig. 4. PCA plot for physico-chemical properties of analysed honeys.

Table 6. Correlation matrix between physico-chemical parameters

Variables	Moisture	pH	Free acidity	El. conductivity	Diastase	HMF	Glucose	Fructose	F/G
pH	−0.045								
Free acidity	0.519	−0.271							
El. conductivity	0.491	0.480	0.651						
Diastase	0.320	0.003	0.622	0.381					
HMF	0.176	−0.386	0.590	0.248	0.383				
Glucose	−0.086	−0.690	−0.120	−0.675	−0.265	0.048			
Fructose	0.160	−0.646	0.650	−0.008	0.501	0.570	0.355		
F/G	0.173	0.376	0.512	0.715	0.587	0.264	−0.844	0.196	
Mineral content	0.502	0.394	0.723	0.949	0.456	0.204	−0.534	0.158	0.671

F/G – fructose/glucose ratio.

parameters of the two raspberry honey samples are similar, except for free acidity, the difference in the scores is related to this latter property.

The Pearson correlation matrix is presented in Table 6. It can be seen that the highest correlation is between electrical conductivity and mineral content ($r^2 = 0.949$), which is not surprising because the electrical conductivity depends on the mineral content and free acidity in honey: the higher their values, the higher the resulting conductivity (Bogdanov, 2002). As a result of the previous strong correlation, a correlation was also found between free acidity and mineral content ($r^2 = 0.723$). Similar correlations were reported by Feás et al. (2010) and Saxena et al. (2010), who reported correlation coefficients of 0.995 and 0.980, respectively. This dependence might be explained by the observation that a higher mineral content in honey corresponds to a higher salinized fraction of the acids present (Finola et al., 2007). A relatively good correlation was found between the fructose/glucose ratio and electrical conductivity ($r^2 = 0.715$).

4. CONCLUSIONS

In conclusion, the results of physico-chemical analysis indicate that all samples of Estonian honeys are of good quality and meet the requirements of the relevant European Directive (2001/110/EC) for all parameters, with one exception where a heather honey was found to have free acidity slightly exceeding the regulated limit. The mineral content was higher in honey samples that contain heather or alder buckthorn pollen. In all honey samples potassium was the most abundant mineral. The honey samples that were classified as blossom honeys with small traces of honeydew elements and various unifloral honeys, such as rape, raspberry, and heather honeys, were identified by their physico-chemical

properties, mineral content, front-face fluorescence spectroscopy, and basic melissopalynological analysis.

PARAFAC analysis of the measured fluorescence spectra revealed a similar grouping between the different samples as was found by PCA analysis of the physico-chemical parameters. Moreover, various calibration models were used to estimate the physico-chemical parameters and floral content according to fluorescence spectra. For several parameters, the results were promising because with a very limited calibration set, the correlation (r^2) between experimental data and estimated values was higher than 0.8 (0.919 for free acidity). It is expected that with a more extensive calibration set better correlation may be obtained. Nevertheless, more extensive research should be conducted with unifloral and multifloral honeys to more precisely characterize them.

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Eesti mee iseloomustamine botaanilise päritolu järgi

Evelin Kivima, Andrus Seiman, Raili Pall, Evelyn Sarapuu, Kaie Martverk ja Katrin Laos

On iseloomustatud Eesti meesorte füüsikalis-keemiliste omaduste põhjal. Uuriti nende happesust, niiskussisaldust, vabade hapete sisaldust, elektrijuhtivust ja diastaasi aktiivsust. Samuti tehti hüdroksümetüülfurfuraali (HMF), glükoosi, fruktoosi ja erinevate mineraalainete (naatrium, kaalium, magneesium, kaltsium) koguste analüüs. Mee botaanilise päritolu määramiseks viidi läbi õietolmuanalüüs, mille kohaselt olid 39% uuritud meesortidest rapsi (*Brassica napus*), vaarika (*Rubus idaeus*) või kanarbiku (*Calluna vulgaris*) monofloorsed meed.

Tulemused näitasid, et Eesti meed on hea kvaliteediga ja vastavad Euroopa direktiivi (2001/110/EC) nõuetele. Mineraalainete sisaldus oli suurem kanarbiku ja paakspuu (*Frangula alnus*) õietolmu sisaldavas mees.

Mee füüsikalis-keemiliste omaduste ja taimse koostise hindamiseks kasutati fluorestsentsspektroskoopiat. Fluorestsentsspektrite järgi arvutatud tulemuste ja eksperimentaalsete mõõtmiste vaheline determinatsioonikordaja r^2 oli mitme parameetri korral suurem kui 0,7.

APPENDIX 2

PUBLICATION II

Seisonen, S., Kivima, E., Vene, K.

Characterization of the aroma profiles of different honeys and corresponding flowers using solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry.

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Characterisation of the aroma profiles of different honeys and corresponding flowers using solid-phase microextraction and gas chromatography–mass spectrometry/olfactometry

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ABSTRACT

The aroma profiles of thirteen different honey samples from four botanical origins: heather (*Calluna vulgaris*), raspberry (*Rubus idaeus*), rape (*Brassica napus*), alder buckthorn (*Frangula alnus*) and the blossoms of the four corresponding flowers were investigated to find odour-active compounds exclusively representing specific honeys based on odour-active compounds from the blossoms. Gas-chromatography–mass spectrometry (GC–MS) and gas-chromatography–olfactometry were used to determine and identify the odour-active compounds. Data was analysed using agglomerative hierarchical clustering and correspondence analysis. Honeys from the same botanical origin clustered together; however, none of the identified compounds were exclusive to a particular honey/blossom combination. Heather honey had the flavour profile most different to the others. Isophorone and 2-methylbutyric acid were found only in heather honeys. Heather honey was characterised by having more "sweet" and "candy-like" notes, raspberry honeys had more "green" notes, while alder buckthorn had more "honey" and "floral" notes.

1. Introduction

Honey is a highly regarded food product in all parts of the world. The main parameters of honey quality, which also influence its price, are derived from its botanical origin. Several articles have been published on marker compounds from the volatile fraction, which could be used to identify the floral origin (Castro-Vazquez, Diaz-Maroto, Gonzalez-Vinas, & Perez-Coello, 2009; Castro-Vazquez, Diaz-Maroto, & Perez-Coello, 2007; de la Fuente, Sanz, Martinez-Castro, Sanz, & Ruiz-Matute, 2007; Guyot, Bouseta, Scheirman, & Collin, 1998; Guyot, Scheirman, & Collin, 1999; Jerković, Tuberoso, Marijanović, Jelić, & Kasum, 2009; Piasenzotto, Gracco, & Conte, 2003). Instrumental analysis has also been combined with descriptive sensory analysis, where, for example, heather honey was described with attributes "ripe fruit", "spicy", "woody" and "resin" (Castro-Vazquez et al., 2009). Cuevas-Glory, Pino, Santiago, and Sauri-Duch (2007) reviewed volatile analytical methods for determining the botanical origin of honey, pointing out extraction methods, fibres and extraction conditions used.

Solid-phase microextraction (SPME) as an aroma extraction method eliminates the use of (toxic) organic solvents, allows the quantification of a large number of molecules, requires little or no manipulation/preparation of samples, substantially shortens the time of analysis and, moreover, it is simple (Pontes, Marques, & Cámara, 2007). SPME has been widely used in analysis of different food products including honey (Piasenzotto et al., 2003; Plutowska, Chmiel, Dymerski, & Wardencki, 2011; Wolski, Tambor, Rybak-Chmielewska, & Kędzia, 2006).

SPME sampling can be performed in three basic modes: direct extraction, headspace extraction (HS) and extraction with membrane protection. The main advantage of the HS analysis is that it is carried out on an untreated sample (Piasenzotto et al., 2003) and the profile of the isolated volatiles is closely associated with sensory perception (Kaškoniene, Venskutonis, & Čeksteryte, 2008).

Heather honey has been previously characterised by a relatively high content of phenolic compounds, such as guaiacol, p-anisaldehyde and propylanisole (Castro-Vazquez et al., 2009). Phenylacetic acid was found exclusively in *Calluna vulgaris*

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(heather) honey (Guyot et al., 1999). Radovic et al. (2001) analysed 43 authentic honey samples of different botanical and geographical origins by means of dynamic headspace GC–MS, in order to assess marker compounds (if/when existing) of both botanical and geographical origin. Honey samples were of nine different botanical origins (seven acacia, nine chestnut, three eucalyptus, eight heather, two lavender, four lime, four rape, two rosemary and four sunflower) and from eight different countries (one from Denmark, ten from Germany, thirteen from Italy, eight from France, four from The Netherlands, two from Spain, two from Portugal and three from England). Radovic et al. (2001) identified phenylacetaldehyde as a characteristic compound to heather honeys.

According to Radovic et al. (2001) the authenticity of rape honeys could be confirmed by the absence of 2-methyl-1-propanol; however, this compound was absent also in one of the seven acacia honeys analysed, therefore it was emphasised that the simultaneous presence of dimethyl disulphide is necessary in order to confirm the authenticity of rape honeys. Plutowska et al. (2011) determined volatiles from popular Polish honeys (rape, acacia, linden, buckwheat, heather, polyfloral and honey-dew) by HS–SPME and found that the presence of dimethyl disulphide is not a peculiar feature of rape honey and can also be found in other honeys. Authors also emphasised that a much more significant feature to rape honeys is the lack or much lower concentrations of characteristic volatile compounds occurring in other honeys, e.g., linalool oxides, furfural and phenylacetaldehyde, which were present in most honey samples of different botanical origins. Kaškoniene et al. (2008) also found in their study that dimethyl disulphide was present only in six rape honeys out of eleven, while 2-methyl-1-propanol was absent in all of them.

Raspberry honey is characterised by the presence of 2-ethenyl-2-butenal, 3-methylhexane, 3-methylnonane, 3-pyridinemethanol, β-myrcene, cyclopentanemethanol, norbornane, and undecanal (Špánik et al., 2013), while tere is no literature available on volatile fraction of alder buckthorn honey.

Not all volatile compounds have significant impact on honey aroma due to different odour thresholds and interactions between compounds. GC–olfactometry (GC–O) can be used to select key odour-active compounds affecting the aroma of the honey. There is very limited information available about GC–O analysis of honey. Pino (2012) carried out a study on black mangrove honey using aroma extraction dilution analysis (AEDA) complemented by quantitative analysis and calculation of odour activity values. It was concluded that (*E*)-β-damascenone, nonanal and decanal are primarily responsible for the distinctive and characteristic aroma of black mangrove honey.

Alissandrakis, Tarantilis, Pappas, and Pashalis (2011) and Amtmann (2010) have conducted studies on volatile compounds present in honey and flower using GC–MS. It was found that relatively high percent of volatile compounds were overlapping in flowers and honeys, which allowed on floral markers to be proposed. However, since many of the compounds were common in the plant kingdom, they were present in various plants and honeys.

The aim of the present study was to determine floral markers influencing the aroma profile of honeys from different botanical origins by using HS–SPME–GC–O. Additionally, blossoms from representing plants were studied to find odour-active compounds that are carried over from the blossom to the honey. To the authors' best knowledge, GC–O has not been used on this purpose before.

2. Materials and methods

2.1. Materials

Honey samples were collected from local beekeepers in Estonia. Thirteen different honey samples were analysed. Samples 1 and 2 were unifloral raspberry honeys, 3–5 unifloral rape honeys, 6–8 honeys with high rape pollen content, 9–10 unifloral heather honeys, 11 honey with high heather pollen content and 12–13 honeys with high alder buckthorn pollen content. Samples 12–13 could also be unifloral honeys, but there is no literature available determining the content of pollen of alder buckthorn in unifloral honey. Visually samples 12 and 13 were rather different from other samples because of their dark colour and liquid consistency. Honey samples were stored at 4 °C until analysis. Plant blossoms were chosen according to the honey pollen analysis and harvested at the time of blossoming.

2.2. Melissopalynological analysis

Melissopalynological analysis was carried out according to the non-acetolytic method described by Louveaux, Maurizio, and Vorwohl (1978). The pollen counts were expressed as percentages after counting 500–600 pollen grains (Table 1). The identification of the pollen types were based mainly on the reference collection of the department of Food Processing in Tallinn University of Technology and data provided by Ricciardelli ÓAlbore (1997). An Olympus CX21 (Japan) binocular light microscope with 40 x 15 magnification was used. Required pollen contents to consider honeys unifloral can be found from previous research carried out by Kivima et al. (2014).

Table 1
The main pollen types of honey samples (%). Percentages in boldface refer to unifloral honeys; the plus sign (+) stands for minor pollen (<1%).

Pollen type	Honey samples												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Cruciferae *Brassica napus* s.l.	17	11	**60**	**77**	**76**	51	50	43	40	27	9	+	23
Ericaceae *Calluna vulgaris*	1								**16**	**27**	3 4		
Leguminosae *Melilotus officinalis* s.l., *Trifolium repens* s.l.	1	4	5	10	2	5	10	4	18	28	3	21	1
Trifolium pratense s.l.	+		1	5	+	+	5	+	3	4	19	1	+
Rhamnaceae *Frangula alnus*			1		2				2	3	1	**42**	**22**
Rosaceae *Rubus idaeus* s.l.	**67**	**79**	17	2	8	7	17	14	6	5	31	33	32
Salicaceae *Salix* spp.	6	5	9	+	6	27	7	34	1	1	14	+	5

2.3. Chemicals

Pure standards (GC grade) of furfural, eugenol, (*E*)-β-damascenone, vanillin, linalool, methional, furaneol and acetone were purchased from Merck (Darmstadt, Germany). Phenylacetaldehyde, benzoic acid, hexane, ethyl acetate, methylene chloride and NaCl were from Sigma–Aldrich (St. Louis, MO). Ethanol (96.6%) was acquired from Rakvere Piiritustehas (Rakvere, Estonia). Kovats retention indices were determined using a C_8–C_{22} mix from Sigma–Aldrich.

2.4. Sample preparation for solid-phase microextraction (SPME)

50% w/w dilution with water was made for all honey samples. Diluted honey (1 mL) and 1 g of NaCl were measured into a 20-mL SPME vial with a glass covered stirrer. Blossoms were placed into 20-mL SPME vial immediately after harvesting depending on the size of the blossoms, covering approximately 1 cm above the bottom of the vial. In order to apply the same headspace volume to all the samples and to avoid cutting the flowers, volume was used instead of weight for the samples. Two replications of each sample were done for GC–O for each assessor (three assessors in total) and one sample for GC–MS. All vials were capped with PTFE-silicon septa and placed in an autosampler tray at room temperature. Samples were brought one-by-one into magnetic stirring chamber for volatile extraction using a method described in the next section. Magnetic stirring was used for honey samples but not for blossoms. After the extraction process the fibre was injected into the GC inlet for desorption for 10 min (either GC–MS (TOF) or GC–O), followed by the oven temperature program described in the next section.

2.5. Parameters for GC–MS and GC–O

For SPME, 30/50 μm DVB/Car/PDMS Stableflex 2-cm long fibre from Supelco (Bellefonte, PA) was used. The GC column for both GC–MS (Agilent 6890; Agilent Technologies, Santa Clara, CA) and GC–O (Agilent 7890) was a DB5-MS (30 m × 0.25 mm × 1.0 μm; Agilent). The GC–MS inlet was a PTV, while the GC–O inlet was split/splitless using a Merlin Microseal (Agilent), and both were run in splitless mode. A 0.75 mm i.d. liner at 250 °C was used in both injectors. Carrier gas was He, 1.0 mL/min for GC–MS and 2.0 mL/min for GC–O. The GC–MS was equipped with a time-of-flight detector (Waters, Manchester, UK) and the GC–O was equipped with a flame ionisation detector (Agilent) and odour detection port (Gerstel, Mülheim an der Ruhr, Germany). For GC–MS data analysis the NIST05 library was used. The oven temperature programme for best separation of volatiles, and SPME extraction time and temperature for best sensitivity were previously optimised. An incubation time of 5 min at 60 °C for honey and 35 °C for blossoms with an extraction time of 20 min (250 rpm) and desorption time 10 min were chosen as optimum. The oven program for both GC–MS and GC–O was from 35 °C, 45 °C/min to 85 °C, 9 °C/min to 200 °C, 45 °C/min to 280 °C holding time 1 min (total 16.67 min). For identification of the odour-active compounds, the results of GC–MS and GC–O were correlated using Kovats retention indices.

2.6. Statistical analysis

For GC–O data, detection frequency method was used and results were inverted into percent values. Detection frequency method estimates the odour intensity based on recording detected odours from a number of sniffers. More than 33% was counted as a signal, meaning the odour was detected at least two times out of six analyses. Odour descriptions were generated by assessors. Compounds with similar odour descriptions were summed for statistical analysis. Mapping of samples and flavour descriptions was carried out using correspondence analysis (CA) (XLStat, Addinsoft, New York, NY). Correlations between attributes were found using Pearson correlation coefficient ($p = 0.05$). Agglomerative hierarchical clustering (XLStat) based on dissimilarities was used to explain the results based on clustering.

3. Results and discussion

3.1. Odour-active compounds

Forty-six odour-active compounds which had detection frequency more than 33% were found using GC–O. Compounds were extracted by a DVB/CAR/PDMS fibre that was selected according to research carried out by Plutowska et al. (2011) and which showed the best efficiency and repeatability. Table 2 shows the presence of each compound according to GC–O data. Compared to blossoms more odour-active compounds were detected in honey samples and the odours were generally more intense. GC–MS data were used for the tentative identification of odour compounds detected by GC–O assessors. For GC–MS data analysis, Kovats retention indices and standard compounds were used. Due to co-elution, where higher intensity compounds were masking some low intensity compounds, we could have missed some odour-active compounds. Moreover, according to Table 2, the absence of a specific compound means that the compound was not detected using GC–O; it still might occur in the sample, but below the odour threshold value.

The compounds present in all the honey samples were butyric acid (cheesy), methional (potato), oct-1-en-3-one (mushroom), camphene (camphor), phenylacetaldehyde (honey), 2-hydroxybenzaldehyde (medicinal), (*Z*)-linalool oxide (floral), 3,5-dimethyl-2-ethylpyrazine (coffee), (*E*,*Z*)-2,6-nonadienal (green), benzoic acid (urine), phenylacetic acid (honey), carvone (green), hydrocinnamic acid (floral), hexyl hexanoate (apple), (*E*)-β-damascenone (apple), vanillin (vanilla) and δ-decalactone (coconut). Eugenol (clove) and geranyl acetone (floral) were present in most of the samples.

Guyot et al. (1999) investigated marker compounds of heather honeys by isolating aroma compounds by extraction with dichloromethane, followed by a Likens–Nickerson steam distillation/solvent extraction. They suggested *p*-anisaldehyde as a marker compound for heather honeys. In this study *p*-anisaldehyde was also detected in Estonian heather samples, but it was present also in all the other investigated honey samples. Guyot et al. (1999) also found that the presence of benzoic acid and isophorone indicated floral origin within the heather family; this corresponded well with the current study, where these compounds played an important role in heather honey aroma profile according to GC–O results. In this study isophorone was exclusively found in heather honeys and benzoic acid was present in all of the heather honey samples. 2-Methylbutyric acid was found exclusively in heather honey. Additionally, linalool was absent in both heather honey and heather blossom; it was also not detected in heather honey by Castro-Vazquez et al. (2009) and Wolski et al. (2006). Castro-Vazquez et al. (2009) used extraction with dichloromethane followed by simultaneous distillation–extraction, while Wolski et al. (2006) used SPME for isolation of the aroma compounds.

Robertson, Griffiths, Woodford, and Birch (1995) investigated volatiles at various stages of inflorescence development, bud formation, flowering, fruit formation and ripening of a red raspberry. The samples were entrained on the porous polymer Tenax TA and analysed by thermal desorption-GC–MS. Robertson et al. (1995) found (*Z*)-3-hexenyl acetate and *E*-β-ocimene to be major volatile compounds in raspberry flower. In our research we could not detect these compounds with GC–O, which could be explained

Table 2
Compounds and their detection frequencies according to GC–O (1–2 raspberry honeys, 3–8 rape honeys, 9–11 heather honeys, 12–13 alder buckthorn honeys; Ras – raspberry, Al – alder buckthorn, Rap – rape, He – heather).

Compound	Kovats RI[a]	Observed RI	Ident.[b]	Fl. Description	1	2	3	4	5	6	7	8	9	10	11	12	13	Ras	Al	Rap	He
Unknown		808		Potato															66		
Butyric acid	820	808	L,	Cheesy	100	100	100	100	100	100	100	100	83	66	83	83	83	33			
Furfural	829	835	L, St	Sweet				33	33	33		33	33	33	33				66		
(*E*)-2-hexenal	844	844	L	Grass														33	100		66
2-Methylbutyric acid	873	866	MS, L	Potato chips									33	50	33						
Methional	909	904	St, L	Potato	33	33	66	33	83	33	33	66	33	50	50	83	50	50	66	50	33
2-Acetylfuran	893	912	MS, L	Candy									33	33	33						
Unknown		912		Whiskey	83	66	66	66	100	100	100	100					66		66	66	
Unknown		944		Lemonade	33					33			33	33	33		33				
1-Octen-3-one	976	968	MS, L	Mushroom	100	100	33	66	83	83	83	83	66	66	83	100	50	100	100	50	66
Camphene	953	972	MS, L	Camphor	33	33	33	66	50	33	50	66	33	33	33	50	50	100	50	66	66
Dimethyl trisulphide	974	982	MS, L	Sulphur	100	66	66	100	100	66	100	100	83	66	83					100	
2-Ethyl-5-methylpyrazine	993	991	L	Sweet														50	83		66
2-Ethyl-1-hexanol	1032	1044	L	Grass																100	
Phenylacetaldehyde	1049	1046	St, MS, L	Honey	100	100	83	100	100	83	66	100	100	100	100	100	66		50		
2-Hydroxybenzaldehyde	1041[p]	1064	MS, L	Medicinal	33	33	33	33	33	33	33	33	33	33	33	66	33				
Furaneol	1064	1071	St, L,	Caramel, honey					66	50	33	33	66	33	33	33	33				
Acetophenone	1065[p]	1081	MS, L	Green	66	33	33				33	33	66	50	66			50	83		33
(*Z*)-linalool oxide	1070	1088	MS, L	Floral	66	83	50	83	83	66	66	66	50	50	66	83	66	33	83		50
Linalool	1100	1092	L, St	Floral	50	66		33	50	33	33	100				50	50	83	50		
3,5-Dimethyl-2-ethylpyrazine	1083	1094	L	Coffee	66	66	50	83	83	66	33	50	50	50	66	83	83				
2-Phenylethylalcohol	1118	1118	MS, L	Floral	66	33	33	50	83	50	50		50	50	50	83	50		66		33
Isophorone	1118[p]	1126	MS, L	Candy									50	66	33						
Unknown		1129		Green	33																
(*E*,*Z*)-2,6-nonadienal	1154	1148	L	Green	50	50	50	33	83	33	50	33	33	33	33	33	33		66		
Octanoic acid	1179[p]	1156	L	Leather														83	83	66	50
Lilac aldehyde A	1155[p]	1169	MS, L	Honey	33		33		33				66	66	50	83	33				
Benzoic acid	1276[n]	1178	St, MS, L	Urine	33	33	50	66	50	66	66	66	33	50	66	33	33				
Ethyl benzoate	1185	1186	MS, L	Green	83	50	50	33	33	50	33	50	33	33					33		
Unknown		1198		Leather														50			
Phenylacetic acid	1262	1236	MS, L	Honey	66	66	83	66	83	66	100	100	66	66	66	100	50	66	66	33	33
Ethyl-2-phenylacetate	1244[p]	1246	MS, L	Honey		33							33	50							
DL-carvone	1253	1259	MS, L	Green	33	66	33	33	33	33	66	66	33	33	50	33	33				
p-Anisealdehyde	1252[p]	1273	MS, L,	Honey			33	66	33		50	33	33	50	33	33	33		66		
γ-Butyrolactone	1299	1288	L	Honey	50		33		33				50	33	33	100	33	66			
Hydrocinnamic acid	1321	1321	MS, L	Floral	100	100	83	100	100	83	100	100	83	83	83	100	83		33		33
Methyl 2-methoxybenzoate	1340[p]	1345	L	Honey	50	33	33	33			50	33	33		33						
Ethyl dihydrocinnamate	1351	1351	MS, L	Floral		33			66	33	33	50	33	50	33		33				
Eugenol	1364	1358	St, MS, L	Clove	66	66	50	50	33	33		66	66	33	33	83	50		66		50
Hexyl hexanoate	1379	1369	MS, L	Apple	83	100	33	33	33	33	33	50	83	66	66	33	33				
β-Damascenone	1386	1385	St, MS, L	Apple	66	66	50	83	66	50	100	100	50	50	50	100	83	66			
Vanillin	1410	1403	St, MS, L	Vanilla	66	83	33	66	83	50	66	66	83	66	50	83	50				
Geranyl acetone	1448	1444	MS, L	Floral	50	50	33	33	33	50	33	50	83	33	50			66	66		
δ-Decalactone	1469	1459	MS, L	Coconut	83	83	33	50	50	33	100	100	50	66	66	50	50	33	33	50	
Unknown		1505		Sweet	33	33							33	66	33		33				
Unknown		1536		Sweet	33	33															

[a] www.flavornet.org.
[p] www.pherobase.com.
[n] NIST 2.0.
[b] Identification based on MS – GC–MS, St – standard, L – literature, RI.

by the different methods used and the high odour threshold values of both compounds.

As literature about alder buckthorn honey is absent, any links with previous researches could not be made. Also, it was not possible to highlight any compounds specific to raspberry or to alder buckthorn honeys.

Rape honey has been characterised by the presence of dimethyl disulphide (Radovic et al., 2001), which was not found during this study. Instead there was dimethyl trisulphide found in rape blossoms and in all honey samples except for alder buckthorn.

3.2. Clustering of honey samples

The data matrix was subjected with odour descriptions to hierarchical cluster analysis (HCA) based on dissimilarities. As seen from the Fig. 1, honey samples from the same botanical origins have clustered together. Heather honeys have the most similar flavour profiles. Raspberry honeys have similarities with rape honeys, which could be explained by small amounts of raspberry pollen found in rape honeys. Heather honeys group together with alder buckthorn. Aliferis, Tarantilis, Harizanis, and Alissandrakis (2010) used HCA on GC–MS data and also obtained very good classification results of different honeys according to their botanical origin.

3.3. Aroma profiles

The aroma profiles of different honeys were rather similar. The most commonly used descriptors were floral and honey-like, and also green. Typical non-herbal aromas were leather, mushroom, metallic and urine. Many compounds also had sweet aromas, like candy and vanilla. Figs. 2 and 3 show the correlation of honey samples and blossoms with flavour characteristics from GC–O,

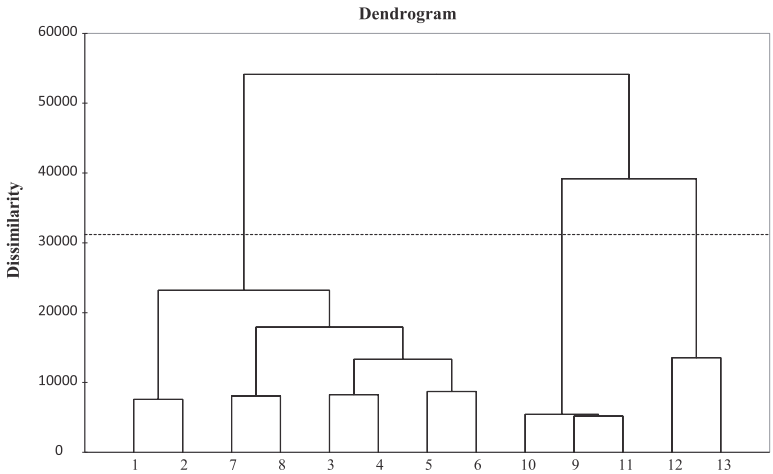

Fig. 1. Agglomerative hierarchical clustering (AHC) of 13 investigated honey samples; 1–2 raspberry honeys, 3–8 rape honeys, 9–11 heather honeys, 12–13 alder buckthorn honeys.

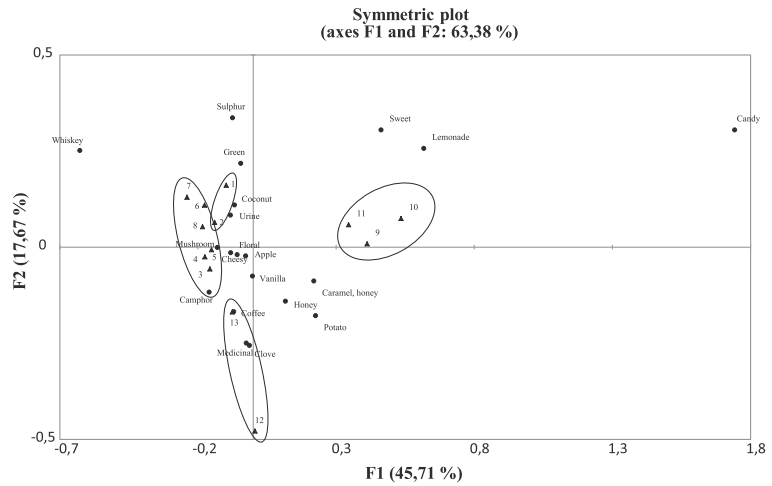

Fig. 2. Correspondence analysis of 13 honey samples (1–2 raspberry, 3–8 rape, 9–11 heather, 12–13 alder buckthorn) and flavour characteristics from GC–O.

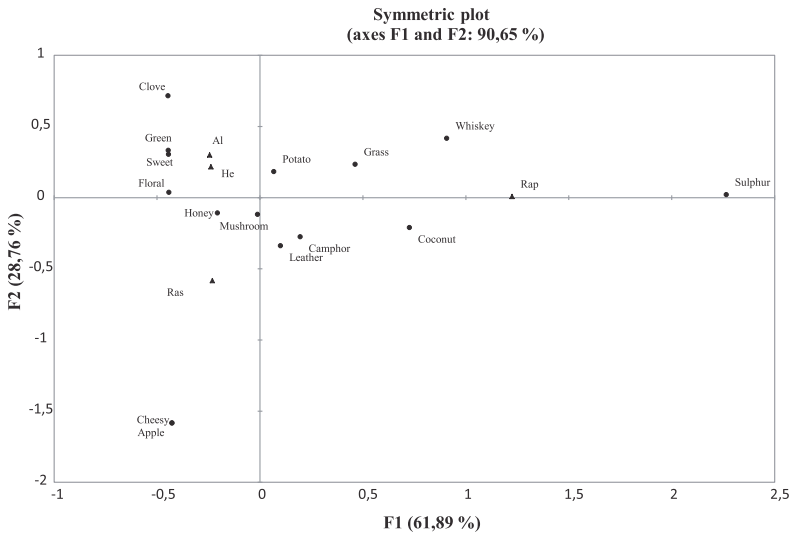

Fig. 3. Correspondence analysis of 4 blossoms (rape, heather, raspberry, alder buckthorn) and flavour characteristics from GC–O.

accordingly. Heather honeys had more odour-active compounds than other investigated samples and could be described as having more sweet candy-like aromas. Raspberry honey can be characterised by a larger number of green notes and lack of honey notes. Rape honey has the poorest aroma profile without many characteristic notes as also mentioned by Plutowska et al. (2011). The only important feature in rape honeys as well as blossom is sulphur content. Rape blossom seems to be the source for sulphur and all the samples contain rape pollen to some extent, which explains sulphur in the aroma profiles of most of the samples. Alder buckthorn honeys tend to have more floral and honey notes and less green and sweet/candy characteristics. Additionally, sulphur was not present (over threshold), unlike the other honey samples.

4. Conclusions

In terms of this research, no marker compounds were common to the honey and the corresponding blossom; no volatiles were found which are coming from a specific blossom to the specific honey. The most important compounds indicating the botanical origin of heather honeys are the presence of isophorone and 2-methylbutyric acid and the absence of linalool. Dimethyl trisulphide refers to the content of rape pollen in the honey. Flavour profiles of heather, rape, raspberry and alder buckthorn honeys are rather similar. There are some nuances in flavour composition and intensities which make the honeys from the same botanical origin cluster together. Heather honey has the biggest differences due to odour-active compounds which were not present in the other honeys. Heather honey can be characterised by having more "sweet" and "candy like" notes, raspberry honey "green" notes, alder buckthorn "honey" and "floral" notes and rape honey has the poorest profile, without any characteristic peaks.

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APPENDIX 3

PUBLICATION III

Kivima, E., Tanilas, K., Martverk, K., Rosenvald, S., Timberg, L., Laos, K.

The Composition, Physicochemical Properties, Antioxidant Activity, and Sensory Properties of Estonian honeys.

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Article

The Composition, Physicochemical Properties, Antioxidant Activity, and Sensory Properties of Estonian Honeys

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Abstract: Thirty honey samples from different regions of Estonia were investigated to determine the chemical compositions, physicochemical properties, bioactive compounds, and sensory characteristics of typical honeys from a northern climate. The physicochemical parameters, such as electrical conductivity, moisture content, free acidity, hydroxymethylfurfural, diastase, and invertase activity were measured. The color was measured and expressed by L*-, a*-, and b*-coordinates. Sensory parameters were determined by using "fruity", "floral", "berry-like", "herbal", "woody", "spicy", "sweet", and "animal-like" as the main odor and flavor attributes. The total polyphenol and flavonoid contents were in the range of 26.2–88.7 mg gallic acid equivalents (GAE) per 100 g and 1.9–6.4 mg quercetin equivalents (QE) per 100 g, respectively. The identified polyphenols showed the highest intensities of caffeic acid, coumaric acid, and abscisic acid and its derivatives. The protocatechuic acid intensity was highest in honeys containing traces of honeydew elements and of cinnamic acid and myricetin in heather honey. The water-soluble antioxidant values were 37.8–311.2 mg ascorbic acid equivalents (AAE) per 100 g and the lipid soluble antioxidant values were 14.4–60.7 mg Trolox equivalents (TE) per 100 g. The major amino acid in the analyzed honeys was proline, with variable values depending on the honey's botanical source. Correlations were calculated based on the results obtained. It was revealed that the typical Estonian honey has floral, berry-like, sweet, and rather mild sensory characteristics. Most of the honeys lacked stronger spicy, woody, and animal-like attributes. The typical color of Estonian honey is quite light.

Keywords: honey; polyphenols; flavonoids; antioxidant activity; amino acids; sensory analysis; flavor; aroma; CIELAB

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1. Introduction

Honey is a natural product containing about 600 different constituents [1]. It consists mainly of carbohydrates and water, and traces of other components, such as vitamins, minerals, and aromatic substances [2]. Honey is also rich in enzymatic (e.g., glucose oxidase and catalase) and non-enzymatic antioxidants, such as flavonoids (chrysin, pinocembrin, pinobanksin, quercetin, kaempferol, luteolin, galangin, apigenin, hesperetin, and myricetin), phenolic acids (caffeic, coumaric, ferulic, ellagic, and chlorogenic), organic acids, ascorbic acid, amino acids, proteins, Maillard reaction products, α-tocopherol and carotenoids [3–7]. Darker honeys have higher total polyphenol and flavonoid values [5,8], and the polyphenol level in honey is directly associated with flower nectar, propolis, and pollen [9]. High correlations between antioxidant activity and total polyphenol and flavonoid contents have been found in several studies [3,10,11]. Honey properties and compositions depend, above all, on the chemical content of the nectar of the plant that the

honey is derived from, as well as on the geographic area, as soil and weather determine melliferous flora, bee species, and even storage mode [12].

From the consumer's point of view, honey sensory properties, such as flavor, aroma, and color, are most important, and those parameters are determined by the honey's botanical origin. In addition to chemical and pollen analysis, sensory analysis also provides an opportunity to evaluate the honey's quality, making it possible to detect the presence of such defects as impurities, off-flavors and odors, which are indicators of changes happening during storage or heating during pasteurization [13,14]. Natural honey variability can make its sensory characterization complicated because in mixed botanical origin honeys the strong sensory characteristics of one botanical source, even in minor amounts, can affect the milder characteristics of another botanical source and change the overall sensory profile [15]. Nevertheless an analysis can reveal the presence of botanical components not picked up by other analytical systems (physicochemical or melissopalynological) [14].

Total honey production in Estonia is approximately 1100 tons per year [16]. Polyfloral honey is most common, as unifloral honey production in Estonia is challenging due to the short summers, small areas of certain plant types during the flowering period, and changing weather conditions. The most widespread plants in Estonia that provide both pollen and nectar are rapeseed (*Brassica napus*), white clover (*Trifolium repens*), melilot (*Melilotus officinalis*), raspberry (*Rubus idaeus*), and willow (*Salix* spp.), in addition to heather (*Calluna vulgaris*), which is one of the most highly valued honey plants [17].

There have been a few scientific studies on Estonian honeys investigating pesticide residues [18,19], pollen analysis [20], amino acid analysis [21], physicochemical properties [17,22], and crystallization behavior [23]; however, there has been no diverse and comprehensive survey on the quality, nutritional properties, and sensory characterization of typical Estonian honeys. Therefore, this work aims to determine the physicochemical properties, antioxidant activity, bioactive attributes, amino acid compositions, and sensory quality of honeys from different areas of Estonia.

2. Materials and Methods

2.1. Honey Samples

Honey samples were provided directly by beekeepers from all Estonian counties and were stored for further analysis in a climate chamber (+18 °C) in the absence of light. All beekeepers were Estonian Beekeepers Association members. The honey samples were harvested from June to September. One honey (harvested at the beginning of October) was identified by the beekeeper as a unifloral heather honey. Honey sample botanical origins were confirmed by melissopalynological analysis.

2.2. Melissopalynological Analysis

Harmonized melissopalynology methods [24] were used in order to determine the honeys' botanical origins. Honey (10 g) was dissolved in 20 mL of distilled water. The solution was centrifuged and the remaining liquid was removed. The sediment was used to make the microscope preparation. The relative frequency was found by counting at least 500 pollen grains.

2.3. Physicochemical Parameters

The physicochemical parameters (electrical conductivity, moisture content, diastase activity, free acidity and invertase activity, and hydroxymethylfurfural) were determined using harmonized International Honey Commission methods [25]. The glucose and fructose levels were determined using an in-house developed HPLC-RI method. Briefly, the honey samples were diluted with water (25×), filtered, and injected into the HPLC system (Waters). A Zorbax Carbohydrate Analysis column (Agilent Technologies Inc., Santa Clara, CA, USA), a temperature of 30 °C, and isocratic elution with acetonitrile/water (75/25 *v/v*) were used to separate the sugars. The data were processed using Empower software (Waters, Milford, MA, USA).

The honey colors were measured by the CIELAB method using spectrophotometer CM-700d (Konica Minolta Inc., Osaka, Japan). The honey samples were heated to 50 °C, poured into petri dishes, covered with lids and left at room temperature for 30 min before the measurements. The measured honey was 1 cm thick. The L*-, a*-, b*-parameters were determined against a white background, readings were taken from three different points and the averages were calculated.

2.4. Amino Acids

Free amino acids were determined by the LC-UV methodology (AccQ•Tag™ Ultra Derivatization Kit; Waters, Milford, MA, USA) developed by Waters. Honey samples were dissolved in water, vortexed, and filtered (0.2 µm). The samples were derivatized with an AccQ-Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) and then loaded on an AccQ-Tag Ultra column. Amino acids were separated using a gradient of AccQ-Tag Ultra eluents A and B. These were detected with a photodiode array detector, and data were processed with Empower 2 software (Waters, Milford, MA, USA).

2.5. Bioactive Compounds

2.5.1. Total Polyphenol Content and Identification

The total phenolic content (TPC) of each sample was determined using the Folin-Ciocalteu method, according to Meda et al. [26]. Each honey sample (5 g) was diluted to 50 mL with distilled water and filtered through Whatman No. 1 paper. This solution (0.5 mL) was then mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich Chemie, Steinheim, Germany) for 5 min and 2 mL of 75 g L^{-1} sodium carbonate (Na_2CO_3) (Sigma-Aldrich Chemie, Steinheim, Germany) solution was then added. After incubation in the dark at room temperature for 2 h, the reaction mixture absorbance was measured at 760 nm against a methanol blank (Spectronic Helios Gamma UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Gallic acid (Sigma-Aldrich Chemie, Steinheim, Germany) (0–200 mg L^{-1}) was used as the standard to produce the calibration curve. The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE) per 100 g of honey.

To identify the polyphenols in the honey, a liquid-chromatography-mass-spectrometry (LC-MS) method developed in the Center of Food and Fermentation Technologies was used. Polyphenols were isolated and pre-concentrated from honey samples using a solid-phase extraction (SPE) procedure, as described by Michalkiewicz et al. [27], with modifications. Briefly, honey samples were extracted with formic acid (pH < 2) and concentrated using an SPE column (Oasis HLB, Waters, Milford, MA, USA). The adsorbed compounds were eluted with methanol and dried using a SpeedVac evaporator at 30 °C. A methanol: water (1:1) mixture was used to reconstitute dried samples. Polyphenols were separated using an ACQUITY UPLC HSS C-18 1.8 µm (2.1 × 150 mm) column (Waters, Milford, MA, USA). Elution was carried out using water + 0.1% formic acid (*v/v*) (A) and a 0.1% acetonitrile (*v/v*) + 0.1% formic acid (*v/v*) (B) gradient: initial 86%A/14%B (*v/v*), 0–8 min 70%A/30%B (*v/v*), 8–18 min 55%A/45%B (*v/v*), 18–21 min 20%A/80%B (*v/v*), 21–22 min 100%B, 22–23 min 100%B, and 86%A/14%B (*v/v*) at a 0.25 mL min^{-1} flow rate. Mass spectrometry analysis was carried out in a negative electrospray ionization mode. Data were collected and reprocessed using MassLynx 4.1 software (Waters, Milford, MA, USA).

The detected polyphenols and their derivatives (D) (*m/z*) were numbered from 1 to 34 (Table 1). To evaluate the compounds' indirect abundance in the honey samples, their mass spectra signal intensities were used.

Table 1. The detected polyphenols and their derivatives (D) mass-to-charge ratios (m/z).

Number	Polyphenol	(M-H)-	Number	Polyphenol	(M-H)-
1	Shikimic acid	173.05	18	Salicylic acid	137.02
2	Gallic acid	169.01	19	Abscisic acid	263.13
3	Protocatechuic acid	153.02	20	Abscisic acid D_1	263.13
4	Protocatechuic and gentisic acid D_1	153.02	21	Abscisic acid D_2	263.13
5	Chlorogenic acid	353.09	22	Abscisic acid D_3	263.13
6	Chlorogenic acid D_1	353.09	23	Luteolin	285.05
7	Catechin	289.08	24	Luteolin and kaempferol D_1	285.05
8	4-hydroxybenzoic acid	137.02	25	Quercetin	301.03
9	Gentisic acid	153.02	26	Cinnamic acid D_1	147.05
10	Caffeic acid	179.03	27	Cinnamic acid D_2	147.05
11	Caffeic acid D_1	179.03	28	Apigenin	269.05
12	Coumaric acid	163.04	29	Naringenin	271.07
13	Coumaric acid D_1	163.04	30	Naringenin D_1	271.07
14	Ferulic acid	193.05	31	Kaempferol	285.04
15	Ferulic acid D_1	193.05	32	Chrysin	253.05
16	Myricetin	317.03	33	Chrysin D_1	253.05
17	Morin	301.05	34	Galangin	269.05

2.5.2. Total Flavonoid Content

The total flavonoid content (TFC) was determined by the method described by Bueno-Costa et al. [28]. A honey solution (100 mg mL^{-1}) was prepared with methanol 50% (*v/v*), previously homogenized, and filtered through a quantitative filter. Honey solution (5 mL) was mixed with 5 mL $AlCl_3$ (2% *w/v*) in methanol. The mixture was homogenized and allowed to stand for 30 min in the dark. The absorbance was measured at 415 nm (Spectronic Helios Gamma UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The total flavonoid content was determined using a standard curve with quercetin (Sigma-Aldrich Chemie, Steinheim, Germany) (0–50 mg L^{-1}) as a standard. A three-reading mean was used and expressed as mg of quercetin equivalents (QE) per 100 g of honey.

2.5.3. Antioxidant Activity

To evaluate the antioxidant activity, the photochemiluminescence (PLC) method, together with a Photochem device (Analytik Jena AG, Jena, Germany), was used. Commercial standard sets of total water-soluble antioxidant capacity (ACW) and total lipid soluble antioxidant capacity (ACL) and a method by Wesolowska and Dżugan [29] were used.

A honey solution (10 g L^{-1}) dissolved in distilled water for ACW and in methanol for ACL was used; 20 µL of suitable solution was mixed with ready reagents (ACW or ACL) according to the attached instructions. The prepared mixture was placed in a Photochem device equipped with PCL Soft 5.1 software (Analytik Jena AG, Jena, Germany). The results were calculated on the basis of standard curves into mg ascorbic acid (AA) equivalents per 100 g of honey for ACW and mg Trolox equivalents (TE) per 100 g of honey for ACL.

2.6. Sensory Analysis

2.6.1. Sample Preparation

Guidance for the sample preparation was taken from Piana et al. [14]. Honey preparation was done differently for gustatory and olfactory assessment. For flavor evaluation, about 30 g of honey was put in sampling containers (one for each assessor) and covered with twist-off caps. For odor evaluation, honey was diluted in a 1:1 portion by weight with odorless drinking water, and 20 mL of the honey-water mixture was put in sniffing glasses and covered with lids. The prepared samples were kept at room temperature for at least an hour before analyses to allow the headspace to equilibrate. All of the assessments

were done between 10 a.m. and 12 p.m., and the room temperature was 21 ± 1 °C during the evaluations.

2.6.2. Training of Assessors

Honey sample sensory evaluation was conducted under standardized conditions in a sensory room [30]. The panel consisted of 10 expert panelists between the ages of 25 and 40 from the Center of Food and Fermentation Technologies. All of the assessors had previous experience in sensory analysis, meeting the requirements described in ISO 8586:2012 [31]. The assessors participated in two training sessions to become familiar with the samples and took part in choosing identifying odors and flavors by using terminology from the odor and aroma wheel described by Piana et al. [14] and the Honey Flavor Wheel (UC Davis, Honey, and Pollination Center). During the discussion, the assessors were trained to use the given scales (0–15) and vocabulary based on EN ISO 13299:2016 [32].

2.6.3. Sensory Evaluation

For both the honey flavor and odor evaluations, the following attributes were chosen to describe the samples: "berry-like", "fruity", "floral", "herbal", "woody", "spicy", "sweet", and "animal-like". Besides overall flavor and aroma intensities, sour taste levels were determined. A 0 to 15 scale was used for all assessments. The olfactory characteristics were evaluated first.

Water and crackers were used to cleanse the palate between sample evaluations. In each session, only six honey samples were analyzed to avoid fatigue. Sensory analyses were carried out in duplicate, for a total of 10 sessions. Average scores were calculated over two sessions and 10 panelists.

2.7. Statistical Analysis

For data analysis and visualization, Principal Component Analysis (PCA) was used and RStudio 1.0.136 (Boston, MA, USA) was applied. The data were normalized before carrying out the analysis. Pearson correlation coefficients were calculated from the measurements. Mean values were calculated for all sensory attributes over two sessions and 10 assessors. For statistical analysis, the R software packages FactoMineR and Factoextra were used (R 3.4.0.). Before the analysis, all data were auto-scaled.

3. Results and Discussion

3.1. Melissopalynological Analysis

All honeys, except for one, were determined to be polyfloral honeys and the pollen types were variable. The most dominant pollen types detected in honey samples were Cruciferae (mainly *Brassica napus*) and Rosaceae (mainly *Rubus* type). In addition, the pollens of willow (*Salix* spp.), clover (*Trifolium*), and alder buckthorn (*Frangula alnus*) occurred in noticeable amounts. By melissopalynological analysis, two honeys were observed to contain numerous honeydew element traces (numbers 28 and 29). The unifloral heather honey (number 17) identified by beekeepers was confirmed to be such by pollen analysis (*Calluna vulgaris*, 7%).

3.2. Physicochemical Parameters

Certain limits have been set on physicochemical quality parameters to avoid honey adulteration and to guarantee safe and good quality honey on the market [33]. The analyzed honeys' physicochemical parameters are presented in Table 2. The moisture content varied from 15.6% to 20.9%, four honeys exceeding the 20% level set by Council Directive 2001/110/EC. This higher percentage may have resulted from processing techniques or storage conditions [2]. A higher heather honey moisture content (20.4%) is allowed [33].

Table 2. Physicochemical parameters and antioxidant activity of honey samples. HMF—hydroxymethylfurfural, F/G—fructose/glucose ratio, TPC—total phenolic content, TFC—total flavonoids content, ACW—water-soluble antioxidants, ACL—lipid-soluble antioxidants, L*—lightness/darkness, a*—greenness/redness, b*—blueness/yellowness.

Sample	Electrical Conductivity (mS cm^{-1})	Moisture (%)	Invertase Activity (U kg^{-1})	Free Acidity (mmol kg^{-1})	Diastase (Schade Unit)	HMF (mg kg^{-1})	Fructose (g 100 g^{-1})	Glucose (g 100 g^{-1})	F/G	TPC (mg GAE 100 g^{-1})	TFC (mg QE 100 g^{-1})	ACW (mg AAE 100 g^{-1})	ACL (mg TE 100 g^{-1})	L*	a*	b*
1	0.5	18.2	188.0	17.0	32.2	3.7	38.0	35.7	1.1	37.5	3.0	84.5	23.9	85.4	−0.3	43.4
2	0.4	19.8	126.0	40.0	33.8	6.8	36.5	34.8	1.1	53.6	3.4	118.4	31.2	72.8	5.8	44.0
3	0.3	16.8	133.0	20.0	29.2	8.4	38.1	33.1	1.2	38.3	3.2	65.1	20.1	84.9	1.3	38.6
4	0.6	20.0	206.0	35.0	48.6	6.1	37.8	32.1	1.2	53.9	3.5	55.5	17.3	82.3	2.3	42.3
5	0.3	20.0	124.0	18.0	22.0	5.0	37.3	37.8	1.0	28.3	2.5	37.8	18.4	88.1	−1.7	26.7
6	0.4	15.6	132.0	21.0	26.0	7.2	39.2	34.0	1.2	41.1	3.1	56.0	20.1	84.3	0.3	39.2
7	0.4	19.1	167.0	26.0	34.4	8.2	38.5	35.4	1.1	50.4	4.3	200.4	40.7	78.4	6.2	48.9
8	0.2	18.0	199.0	25.0	40.8	6.9	39.8	36.2	1.1	28.8	1.9	81.4	16.5	88.4	−1.1	29.1
9	0.3	19.5	50.6	38.0	19.6	19.5	36.8	35.6	1.0	34.8	3.3	66.5	16.9	85.6	−0.7	31.6
10	0.2	20.3	118.0	16.0	21.6	6.0	38.7	36.4	1.1	27.9	3.1	77.0	17.2	88.6	−1.6	27.5
11	0.2	19.8	114.0	22.0	26.3	11.5	36.5	35.3	1.0	30.9	3.1	96.8	20.5	85.4	−0.1	33.5
12	0.3	18.3	93.5	20.0	22.5	8.9	39.2	36.9	1.1	35.2	2.8	84.8	18.2	86.6	−0.6	36.2
13	0.3	16.4	63.9	18.0	21.1	11.8	38.3	35.5	1.1	29.8	2.1	78.4	18.1	88.0	−1.4	30.9
14	0.5	20.4	168.0	25.0	24.6	5.0	38.4	33.0	1.2	49.0	3.9	140.6	31.5	83.5	1.6	51.8
15	0.4	19.1	153.0	27.0	30.9	6.5	37.8	32.9	1.1	48.4	3.2	135.3	27.0	83.8	2.7	43.5
16	0.2	17.4	82.8	12.0	17.7	9.1	38.8	35.4	1.1	26.2	2.4	69.2	19.4	90.4	−1.1	29.6
17	0.7	20.4	114.0	39.0	58.8	7.8	39.7	31.6	1.3	88.7	6.4	245.3	60.7	65.3	9.1	37.8
18	0.2	18.5	74.1	21.0	25.6	9.2	39.5	37.0	1.1	33.7	3.5	87.0	20.7	84.4	0.3	35.8
19	0.3	19.3	119.0	23.0	24.9	7.2	37.5	36.8	1.0	40.3	3.3	113.9	21.4	85.4	2.0	44.4
20	0.4	17.6	184.0	16.0	28.1	5.6	38.4	34.2	1.1	38.7	3.0	87.5	17.8	86.3	0.4	40.6
21	0.7	17.8	182.0	23.0	22.5	9.4	38.5	30.2	1.3	52.6	4.3	105.9	22.8	81.2	3.0	46.5
22	0.5	18.2	102.0	20.0	15.4	5.1	41.1	29.2	1.4	40.2	3.8	85.7	14.4	85.5	0.6	40.8
23	0.8	15.6	168.0	14.0	21.1	6.5	41.5	29.9	1.4	46.4	4.2	82.0	16.6	83.5	1.9	46.6
24	0.7	19.9	172.0	31.0	39.1	9.2	44.5	37.4	1.2	50.7	5.7	176.9	31.9	82.8	3.8	47.6
25	0.3	20.7	145.0	23.0	26.4	8.4	46.4	37.9	1.2	35.6	2.8	105.4	25.2	87.1	−0.2	36.9
26	0.3	20.9	124.0	17.0	16.0	5.9	44.9	39.7	1.1	30.8	2.4	138.3	27.7	86.2	0.6	34.9
27	0.3	20.0	189.0	21.0	25.4	7.8	45.9	36.9	1.2	34.7	2.7	96.6	22.8	86.7	0.1	36.1
28	0.5	18.3	50.4	43.0	35.5	5.9	37.6	32.6	1.2	68.6	5.3	299.3	32.5	76.8	12.5	60.3
29	0.5	18.9	231.0	35.0	36.2	10.0	37.4	32.6	1.1	56.5	4.9	311.2	37.0	76.7	12.3	58.7
30	0.2	19.2	228.0	23.0	37.1	3.5	40.7	38.8	1.0	26.8	2.6	73.7	17.1	88.3	−1.2	25.6
Average	0.4	18.8	140.0	24.3	28.8	7.7	39.4	34.8	1.1	41.9	3.5	115.2	24.2	83.7	1.9	39.6
SD	0.2	1.4	49.8	8.2	9.7	3.0	2.7	2.7	0.1	13.8	1.0	67.9	9.6	5.3	3.8	8.8
Min.	0.2	15.6	50.4	12.0	15.4	3.5	36.5	29.2	1.0	26.2	1.9	37.8	14.4	65.3	−1.7	25.6
Max.	0.8	20.9	231.0	43.0	58.8	19.5	46.4	39.7	1.4	88.7	6.4	311.2	60.7	90.4	12.5	60.3

The analyzed honeys' measured electrical conductivities were all under 0.8 mS cm^{-1}. The highest values (0.7 mS cm^{-1} and 0.8 mS cm^{-1}, respectively) were observed in the heather honey (number 17) and honey number 23 (containing the highest alder buckthorn pollen level: 29%). The honeydew honeys' electrical conductivity should be no less than 0.8 mS cm^{-1}, and, in this case, the lower values of electrical conductivity of the honeys containing traces of honeydew elements (numbers 28 and 29) were probably due to their too small amounts. Free acidity is related to the decrease in honey quality as the level increases over 50 mmol kg^{-1} [33]. The investigated honeys' free acidity levels were in the range of 12.0 to 43.0 mmol kg^{-1}, which met the quality honey requirements. Invertase activity is not standardized in Estonia and can vary greatly, especially in summer honeys; however, the suggested level is at least 50 U kg^{-1} for fresh unheated honeys [34]. The invertase activity of the studied honey samples ranged from 50.4 U kg^{-1} to 231.0 U kg^{-1}, which is within the fresh honey range.

The analyzed honeys' diastase activity varied from 15.4 to 58.8 (Schade units) and the highest level was found in heather honey. However, all honeys met the quality norms.

One of the most important quality indicators of honey is its hydroxymethylfurfural (HMF) level. All analyzed honeys proved to be of high quality, as the HMF concentrations were under 19.5 mg kg^{-1}.

The total fructose and glucose levels in all honey samples were above 60 g per 100 g and, thus, met the quality requirements. The fructose content was higher in all honey samples, with an average of 39.4 g per 100 g, than the glucose content, with an average of 34.8 g per 100 g. These results are similar to those of a survey conducted in our previous study [17]. In terms of fructose and glucose (F/G) ratio, honeys with levels of about 1.0 can be considered blossom honeys [35]. However, the heather honey F/G was 1.3, which is comparable to the heather honeys analyzed in our previous study [17] and by other authors [36,37]. Honey color is associated with phenolic compounds, pollen and mineral element contents [13], and depends directly on the plants the nectar is derived from [38]. The L*-coordinates, which indicate honey lightness or darkness, ranged from 65.3 to 90.4. The a*-coordinates (redness/greenness) and b*-coordinates (blueness/yellowness) were in the range of −1.7 to 12.5 and 25.6 to 60.3, respectively. Generally, most honeys were rather light in color and had red, yellow, and mildly green tones. The heather honey had the lowest L*-value and highest a*-value, which meant that it was the darkest and one of the most reddish honeys of the analyzed samples. The two honeys containing honeydew elements differed greatly from the other honeys. These two honeys were slightly lighter than the heather honey but tended to be the most reddish and yellowish, with the highest a*- and b*-values. Based on the calculated correlation coefficient between the L*- and a*-value (Table 3), it is clear that, as expected, the darker the honey, the more reddish tones it had.

Table 3. Calculated correlations. EC—electrical conductivity, M—moisture, IA—invertase activity, FA—free acidity, D—diastase, HMF—hydroxymethylfurfural, F/G—fructose/glucose ratio, TPC—total polyphenol content, TFC—total flavonoids content, ACW—water-soluble antioxidants, ACL—lipid-soluble antioxidants, AA—amino acids, L*—lightness/darkness, a*—greenness/redness, b*—blueness/yellowness.

	EC	M	IA	FA	D	HMF	F/G	TPC	TFC	ACW	ACL	AA	L*	a*
M	−0.08													
IA	0.28	0.09												
FA	0.34	0.36	−0.04											
D	0.41	0.23	0.40	0.64										
HMF	−0.18	−0.05	−0.43	0.25	−0.17									
F/G	0.68	−0.21	0.20	−0.01	0.03	−0.17								
TPC	0.77	0.12	0.07	0.72	0.66	−0.06	0.41							
TFC	0.78	0.13	0.04	0.60	0.51	0.03	0.43	0.88						
ACW	0.42	0.24	0.06	0.62	0.46	0.01	0.17	0.73	0.75					
ACL	0.46	0.37	0.06	0.56	0.61	−0.02	0.14	0.80	0.72	0.80				
AA	0.39	0.07	0.04	0.73	0.54	−0.04	0.12	0.74	0.71	0.86	0.62			
L*	−0.62	−0.18	−0.02	−0.74	−0.65	−0.01	−0.24	−0.93	−0.80	−0.71	−0.85	−0.66		
a*	0.55	0.11	0.08	0.73	0.54	−0.02	0.23	0.85	0.80	0.92	0.75	0.89	−0.85	
b*	0.65	−0.06	0.20	0.47	0.24	−0.12	0.36	0.69	0.69	0.71	0.46	0.71	−0.57	0.79

3.3. Amino Acids

The UV chromatograms of amino acids of standard and honey sample (number 1) are shown in the Supplementary material (Figure S1). Honey quality, maturity, and natural origin are estimated by the proline content, which can also be considered an indicator of the total amounts of amino acids in honey [26]. In all of the honey samples, the proline concentration was higher than any other amino acid, followed by phenylalanine and glutamine (Table 4). The proline content ranged from 257 mg kg^{-1} to 1328 mg kg^{-1}, which indicated good quality honeys, meeting the general requirement of the proline content being above 200 mg kg^{-1} [39].

Table 4. The amino acid composition of honeys. Ala—alanine, Asp—aspartic acid, GABA—gamma aminobutyric acid, Gln—glutamine, Glu—glutamic acid, Gly—glycine, Ile—isoleucine, Leu—leucine, Lys—lysine, Phe—phenylalanine, Pro—proline, Ser—serine, Thr—threonine, Tyr—tyrosine, Val—valine.

Sample	Ala	Asp	GABA	Gln	Glu	Gly	Ile	Leu	Lys	Phe	Pro	Ser	Thr	Tyr	Val
1	9	7	4	18	19	2	4	6	13	27	334	8	5	8	8
2	11	11	5	26	20	3	7	13	22	73	552	11	5	9	9
3	12	15	6	48	27	3	9	13	19	98	512	15	8	9	13
4	10	14	2	23	18	3	3	4	20	17	622	12	7	7	8
5	4	6	2	28	9	0	2	1	10	26	257	5	2	8	4
6	9	7	4	30	13	2	6	8	18	283	426	10	5	33	8
7	11	10	1	42	23	2	18	28	21	92	643	11	6	22	12
8	8	6	5	24	15	2	4	6	20	19	543	9	5	11	7
9	10	13	6	53	22	3	8	8	20	36	399	10	5	8	6
10	7	10	4	31	15	2	4	5	19	15	290	9	5	4	7
11	8	8	5	29	10	3	4	4	21	16	389	10	5	6	6
12	8	9	5	35	15	3	6	11	18	83	367	12	6	9	8
13	8	11	4	44	20	2	7	8	15	20	350	12	7	7	10
14	11	9	4	35	18	3	11	27	15	230	447	10	8	18	12
15	12	10	7	42	20	4	25	43	20	27	480	12	10	38	15
16	6	7	4	24	12	2	4	0	15	19	307	9	5	6	7
17	24	19	14	28	32	7	9	11	31	33	956	20	14	13	16
18	8	8	6	35	15	4	5	9	23	36	430	11	5	8	7
19	12	11	6	44	24	4	7	8	36	49	638	13	7	10	10
20	9	13	2	39	15	2	4	5	15	22	492	11	5	5	8
21	13	19	3	13	22	2	3	5	11	22	661	12	5	4	7
22	10	12	3	9	16	3	3	5	8	18	471	11	4	6	6
23	10	9	3	3	21	2	2	5	2	16	375	8	3	8	4
24	18	46	4	41	49	7	9	10	26	31	757	31	13	13	15
25	8	13	3	33	14	2	4	5	21	30	475	11	4	6	7
26	9	15	6	37	18	1	4	3	23	13	525	12	5	4	8
27	7	7	4	24	12	2	4	4	14	36	320	8	4	9	6
28	26	26	9	56	37	7	16	32	33	294	1328	28	11	28	19
29	18	20	6	46	29	4	9	20	30	205	1023	18	7	18	13
30	9	16	5	36	22	3	15	22	33	42	589	15	6	19	11

According to Crane [40], the proline content is high in honeydew honeys. In this study, very high proline values were observed for the two honeys in which honeydew elements were found (numbers 28 and 29): 1328 mg kg^{-1} and 1023 mg kg^{-1}, respectively. Although the heather honey contained less proline than the ones containing honeydew elements (956 mg kg^{-1}), its content was still higher than in any of the other analyzed honeys. Pollen is considered the main source of amino acid; however, bees also contribute to the free amino acid content, which results in high variability of these components in honey, even from the same botanical origin [41]. Another amino acid that was found in honeys in noticeable amounts, but with great variation, was phenylalanine (Phe): in the range of 13 mg kg^{-1} to 294 mg kg^{-1}, with the higher levels resulting in the existence of honeydew elements in honey. Higher glutamine (Gln), lysine (Lys), and glutamic acid (Glu) levels were observed

in honeys containing major Cruciferae pollen amounts. These three amino acids, as well as arginine (Arg) and histidine (His), are known to be characteristic of rapeseed honeys [42].

3.4. Bioactive Compounds

3.4.1. Polyphenols and Flavonoids

Polyphenols come to honey through plant nectar, propolis, and pollen [43]. The average total polyphenol value of the analyzed honeys was 41.9 mg GAE per 100 g, and the average total flavonoid content was 3.5 mg QE per 100 g (Table 2). The total polyphenol variability was much higher than total flavonoid variability. The total polyphenol content of polyfloral honeys was generally much lower than that of heather honey. The total polyphenol and flavonoid content in heather honey was almost twice as high as the average: 88.7 mg GAE per 100 g and 6.4 mg QE per 100 g, respectively. The average total polyphenol and flavonoid levels in honeys that contained honeydew elements were 62.5 mg GAE per 100 g and 5.1 mg QE per 100 g, respectively. The polyphenol content of the aforementioned honeys has also been found to be higher by other researchers [8,26].

A high correlation between total polyphenol and total flavonoid content was found (Table 3), which is consistent with the results of Escuredo et al. [43], A-Rahaman et al. [44] and Khalil et al. [45].

The electrical conductivity and free acidity levels seemed to have stronger connections to polyphenols by calculated correlations than any other physicochemical honey property. Moreover, a high correlation was found between polyphenol content and honey color. The polyphenol concentration increased with decreasing honey lightness (L*) and with increasing honey redness (a*). This is in agreement with Kuś et al. [11] and Bertoncelj et al. [3]. Of all the amino acids, mostly alanine (Ala) and proline (Pro), and to a lesser extent glutamic acid (Glu), glycine (Gly), threonine (Thr), and valine (Val), most affected the antioxidant honey properties (Table 5). The highest correlation was found between alanine and total polyphenol content. The identified polyphenols showed the highest intensities of caffeic acid, coumaric acid, and abscisic acid, and its derivatives. Higher intensities were also detected in shikimic acid, 4-hydroxybenzoic acid, salicylic acid, quercetin, kaempferol, ferulic acid, and its derivatives.

Table 5. Calculated correlations between amino acids and TPC, TFC, ACW, ACL. TPC—total polyphenol content, TFC—total flavonoids content, ACW—water-soluble antioxidants, ACL—lipid-soluble antioxidants.

Amino Acids	Ala	Asp	GABA	Gln	Glu	Gly	Ile	Leu	Lys	Phe	Pro	Ser	Thr	Tyr	Val
TPC	0.88	0.47	0.54	0.07	0.63	0.73	0.33	0.41	0.33	0.37	0.77	0.58	0.71	0.37	0.67
TFC	0.86	0.67	0.42	0.09	0.77	0.78	0.27	0.33	0.32	0.34	0.74	0.71	0.69	0.27	0.63
ACW	0.85	0.54	0.51	0.40	0.66	0.67	0.47	0.55	0.56	0.49	0.87	0.70	0.63	0.42	0.74
ACL	0.72	0.38	0.59	0.22	0.54	0.61	0.39	0.42	0.46	0.28	0.63	0.50	0.68	0.33	0.66

Protocatechuic acid can be considered a marker for honeydew honey, and distinguishes honeydew honey from polyfloral honeys [46]. This study showed that even if a honey contained only traces of honeydew elements, the protocatechuic acid intensities were significantly higher than in the other honeys analyzed (Figure 1).

Heather honey differed in terms of higher levels of cinnamic acid, myricetin, and abscisic acid derivatives D_2 and D_3. The levels of these components were also higher in those honeys that contained only minor heather pollen levels (honeys numbers 4 and 15, with 3% and 2% pollen, respectively). Therefore, higher cinnamic acid, myricetin, and abscisic acid derivative levels seemed to be characteristic of the heather honeys, and that is in accordance with other research [9,39].

Honey number 30 had higher intensities of flavonoids, such as galangin and chrysin, the latter indicating the presence of propolis in honey [47].

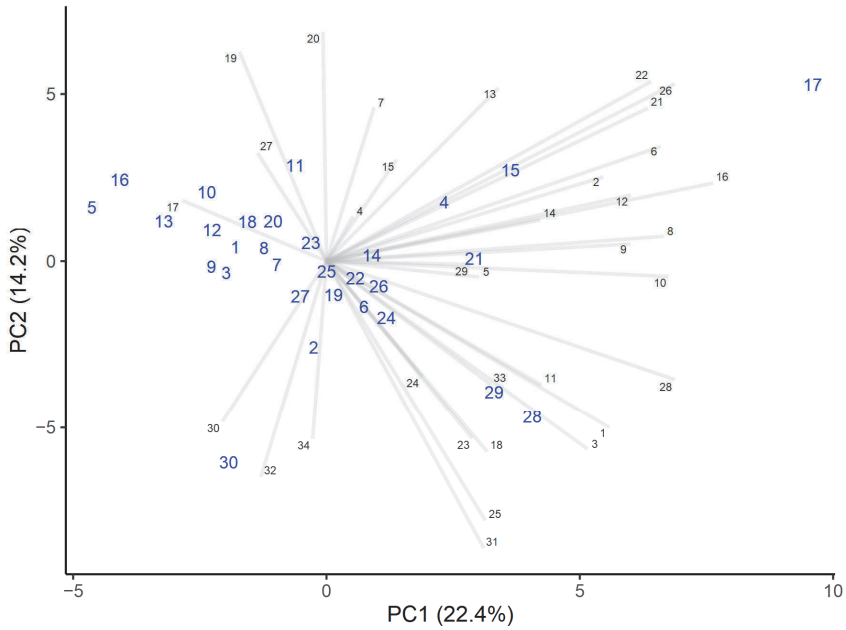

Figure 1. PCA analysis of the intensities of polyphenols. Honey samples are marked by larger blue numbers and polyphenols by smaller numbers marked black.

Small apigenin levels have been found only in rapeseed honeys and polyfloral honeys [5], and quercetin and kaempferol only in *Brassica* honeys [47], as botanical origin markers. Apigenin was present in all of the analyzed honeys, although in small amounts, and quercetin and kaempferol were found in similar amounts, which was because all honeys consisted of *Brassica* pollen to some extent.

3.4.2. Antioxidant Activity

The levels of water-soluble antioxidants (ACW), such as flavonoids, ascorbic acid and amino acids, and lipid-soluble antioxidants (ACL), such as tocopherol, carotenoids, and tocotrienols [29], were determined. The average water-soluble antioxidant level was 115.2 mg AAE per 100 g, and the average lipid-soluble antioxidant level was 24.2 mg TE per 100 g (Table 2). The analysis showed that the water-soluble antioxidant composition was dominant over the lipid-soluble antioxidant composition. The ACW of different honeys varied greatly and the highest levels were in the two honeys that contained honeydew elements or heather honey—299.3 mg AAE per 100 g, 311.2 mg AAE per 100 g, and 245.3 mg AAE per 100 g—indicating higher antioxidant properties. However, the highest ACL content (60.7 mg TE per 100 g), which was twice as high as in other honeys, was again associated with heather honey. The ACW and ACL values correlated with the total polyphenol content of honey (Table 3), which indicated that phenolic compounds might be the principal components that affect honey antioxidant properties. This correlation has also been found by other researchers [3,11,29]. In addition, a high correlation has been found between antioxidant activity and honey color. The lightness of honey correlated well with ACL value, while the redness correlated better with ACW value and amino acid content.

3.5. Sensory Evaluation

Aroma and taste are important honey characteristics and depend on specific complex substances derived from its plant sources [40]. The sensory analysis results are presented in Figure 2. The first axis accounts for 26.8% of the variance and is positively related to berry-like and fruity but negatively related to spicy, woody, herbal, and animal-like. The overall

odor intensity was influenced by woody and animal-like characteristics, which showed slightly higher correlations (r = 0.63 and r = 0.54, respectively). Meanwhile, the second axis explains the variance of 16.7% and is loaded positively for floral and sweet, and thus negatively for fruity, sour, and overall intensity in flavor. Overall taste intensity had the highest correlation with sour taste (r = 0.61).

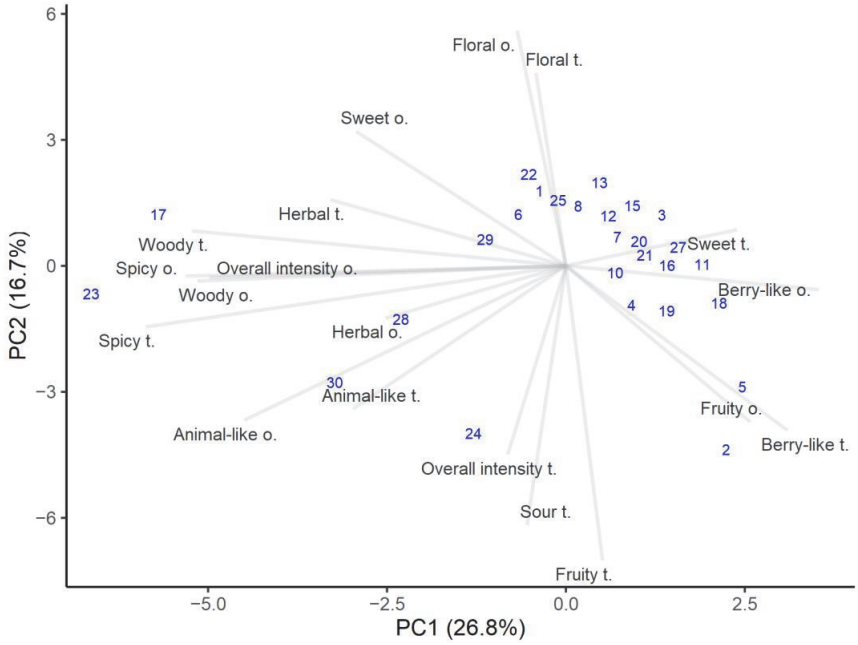

Figure 2. PCA analysis of the sensory attributes of honey samples. The number in parenthesis shows variance explained by the principal component.

The sensory evaluation indicated that most of the honeys were grouped in the right top corner, showing higher floral, sweet, and berry-like characteristics. Honey sweetness can vary because sugars in honey have different sweetness levels [40]. These are typical quality honeys attributes, and floral and fruity notes are valued as more pleasant notes [48]. However, the overall odor and taste intensity tended to be lower for fruity samples compared to the samples with herbal woody and spicy notes. Most of the honey samples lacked of spicy, woody, and animal-like characteristics. Even if different honeys have the same floral source and the same number of pollen grains, the sensory characteristics of those honeys can be quite different. This could mean that honey properties and composition not only depend on the plant species that provide the nectar, but also on other factors, such as different locations, storage conditions, and even harvesting technology and conditions [49,50].

It is said that flavor is closely related to aroma [51] and this was corroborated by the calculated correlations, which for all flavor and odor attributes were above 0.5, except for sweetness. This means that when a certain odor characteristic was detected, with high probability it was recognized during tasting and vice versa. The highest correlation was found between spicy flavor and aroma notes (r = 0.93). It was interesting that the calculated correlation between overall aroma and flavor intensities was very low (r = 0.15), which means that these two attributes were independent of each other, depending on the honey composition.

Although most of the honeys had quite similar sensory profiles, some stood out for their distinct aroma and taste. Heather honey (number 17) and honey containing the highest amount of alder buckthorn pollen (number 23) had the highest spicy, woody, herbal, and animal-like notes. At the same time, the overall intensity was higher for both odor

and flavor. Those honeys tasted less sweet than the others, and had the least berry-like aroma. Honeys containing traces of honeydew elements (numbers 28 and 29) showed high scores in animal-like and woody attributes, which is exactly characteristic of honeydew honeys [36].

The color of honey is related to its taste and darker honeys usually have stronger flavors [51]. In the present study, it was found that woody and sour attributes were most associated with lightness/darkness and redness/greenness. Thus, honeys with dark and reddish colors had stronger woody and sour flavors (the correlations in both cases were above $r = 0.55$).

4. Conclusions

The analysis of Estonian honeys indicated that the botanical origins are diverse and those honeys are polyfloral, with the most dominant pollen types being Cruciferae and Rosaceae, Salix, Trifolium, and Frangula alnus. The physicochemical values met all of the quality norms set by Directive 2001/110/EC, with some exceptions in moisture content. Among all of the analyzed honeys, heather honey and two polyfloral honeys stood out for their color, amino acid content, bioactive compounds, and organoleptic parameters. Although those polyfloral honeys contained only traces of honeydew elements, those traces still had significant influence on the honey properties.

Those different honeys provide a good basis for comparing and evaluating typical polyfloral Estonian honeys. The total polyphenol and flavonoid contents, as well as antioxidant activity, varied greatly among the honey samples analyzed. These properties are strongly connected with honey color. Most analyzed honeys tended to be lighter in color, had rather mild flavor and aroma characteristics, had higher floral, sweet, and berry-like notes, and minimal spicy, woody, and animal-like notes. The overall intensity was quite low. Those honeys had relatively low bioactive and antioxidant properties. On the other hand, such properties were observed as being much higher in heather honey and honeys containing honeydew element traces. Those honeys were darker, more reddish and yellowish, had higher flavor and odor intensities, and had higher spicy, woody, herbal, and animal-like notes.

Honey lightness was strongly correlated with polyphenols and lipid soluble antioxidants. Honey redness seemed to be connected with water-soluble antioxidants and amino acid content. The honeys with higher electrical conductivity and free acidity levels tended to be richer in polyphenol content. Moreover, darker honeys tended to have stronger flavors, such as woody, and sour attributes were most associated with lightness/darkness and redness/greenness. Woody and animal-like attributes had the strongest effect on overall odor intensity, while sour attributes most affected overall flavor intensity.

Although more samples are needed, the results from of only a few distinctive honeys provide a good basis for further research and give primary knowledge of marker compounds for identifying the honeys that can be found, for example, among amino acids and polyphenols.

Supplementary Materials: The following are available online at https://www.mdpi.com/2304-8158/10/3/511/s1, Figure S1: UV chromatograms of amino acids of standard (black line) and honey sample number 1 (blue line).

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