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Differentiation of Blossom Honey and Honeydew Honey from Northwest Spain

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Abstract: The knowledge of important parameters for honey characterization is an increasing requirement of consumers and the honey industry. In this respect, differentiation between blossom honeys and some honeydew honeys is still an unresolved task. This study includes the results of physicochemical and melissopalynological analysis of 86 honey samples from north-western Spain. The relationship between the microscopic elements in honey, such as *Metschnikowia* cells and fungal spores from plant pathogens, together with their physicochemical parameters were analysed. A cluster analysis was performed to differentiate blossom honey samples from honeydew samples. *Metschnikowia* cells and certain fungal spores were found to be good variables to enable differentiation between blossom honeys and honeydew honeys.

1. Introduction

The composition, flavour and colour of honey vary considerably depending on its botanical source. European legislation (Council Directive 2001/110/EC of 20 December 2001 relating to honey) defines various honey types and the requirements for labelling. Furthermore, the directive allows honey packaging to include information about the product's regional, territorial or topographical origin, floral or vegetable origin, and even specific quality-related criteria. Consequently, there is an increasing

commercial interest in determining the botanical origins of honeys, particularly to distinguish between blossom honeys and honeydew honeys. Improved capabilities for classifying honeys could prevent commercial frauds. The ability to be able to make these distinctions, especially for honeys with similar physicochemical characteristics, has intrigued the scientific community [1].

The study of the microscopic elements present in honey, aims primarily to recognise the pollen types in honey and their relationship to the botanical and geographical provenance of the honey [2]. Pollen grains are the most commonly studied element in honey; however, fungal spores, yeast, or other microscopic elements are less well known. Only a small number of studies has attempted to classify these biotic elements [1,3–11]. Fungal spores and yeast can contact honeys at different points during their harvest. These elements can come from primary sources (the honeydew or nectar), as a secondary contamination during post-harvest, or once the honey begins to deteriorate [12].

Honeybees collect honeydew from the green parts of plants and at the same time, with honeydew, they may collect other attached structures, such as the hyphae or fungal spores of plant pathogens and microalgae. Additionally, nectar itself has a microbial community associated with bees. Yeasts are the most frequent inhabitants of floral nectar and were familiar to microbiologists more than a century ago [13], indicating the presence of yeast in floral nectaries. More recent research has examined the presence of the microorganisms in nectar, their ecology and their classification [14–23]. The principal group of microorganisms are Ascomycetes, particularly *Metschnikowia*, *Candida*, *Zygosaccharomyces*, *Debariomyces*, *Starmerella* and Basidiomycetes, such as *Cryptococcus* sp. and *Cystofilobasidium* sp.

It has been known that physicochemical analysis could be used to differentiate between honeydew honey and some blossom honey [24–26]. However, no studies include detailed information on fungal structures found in the sediment of honey and its physicochemical characteristics. In this respect, the main objective of this study was to establish the relationship between these structures (main *Metschnikowia* cells and fungal elements) and the source of honey.

2. Results

2.1. Microscopic Characteristics

The quantitative analysis of samples led to the identification of honeys with a high quantity of pollen and fungal elements and others very poor in these elements. Pollen content varied between 1274 and 130,832 pollen grains per gram of honey, with a standard deviation of 25,585.

The qualitative pollen analysis identified 90 different pollen types in the samples. Table 1 lists the most commonly occurring pollens. This table includes 16 pollen types from ten botanical families, along with their corresponding maximum values, the standard deviation, and the range and the number of honeys in which the various pollen types reached a determined percentage level.

Table 1. Principal pollen types, their frequency classes and their representation in the samples.

Family	Pollen type	(%)	P (0–1%)	R (1–3%)	I (3–15%)	A (15–45%)	D >45%	Max.	St. Dv.
			Frequency classes [a]						
Fagaceae	*Castanea*	100.0	0.0	7.0	9.3	48.8	34.9	87.9	21.55
Leguminosae	*Cytisus* t.	100.0	18.6	25.6	52.3	2.3	1.2	48.6	6.81
Rosaceae	*Rubus*	100.0	3.5	3.5	29.1	34.9	29.1	83.6	21.31
Ericaceae	*Erica*	97.7	23.3	23.3	43.0	8.1	0.0	35.5	6.13
Leguminosae	*Trifolium* t.	87.2	40.7	29.1	17.4	0.0	0.0	9.6	1.87
Myrtaceae	*Eucalyptus*	86.0	22.1	18.6	20.9	12.8	11.6	81.0	22.81
Boraginaceae	*Echium*	76.7	45.3	20.9	10.5	0.0	0.0	9.8	1.82
Fagaceae	*Quercus*	66.3	52.3	10.5	2.3	1.2	0.0	27.8	3.03
Salicaceae	*Salix*	59.3	43.0	9.3	7.0	0.0	0.0	10.9	1.55
Rosaceae	*Crataegus monogyna* t.	40.7	33.7	5.8	1.2	0.0	0.0	3.6	0.61
Campanulaceae	*Campanula* t.	39.5	33.7	4.7	1.2	0.0	0.0	3.9	0.59
Umbelliferae	*Conium maculatum* t.	34.9	25.6	8.1	1.2	0.0	0.0	7.0	0.89
Rhamnaceae	*Frangula alnus*	31.4	23.3	5.8	2.3	0.0	0.0	13.7	1.72
Leguminosae	*Lotus* t.	20.9	17.4	2.3	1.2	0.0	0.0	3.7	0.45
Boraginaceae	*Lithodora*	16.3	12.8	1.2	2.3	0.0	0.0	4.3	0.60
Boraginaceae	*Myosotis*	11.6	10.5	0.0	1.2	0.0	0.0	5.6	0.61
	Others	100.0	18.6	53.5	27.9	0.0	0.0	14.3	2.20

(%): percentage of samples in which the pollen type was identified; [a] Percentage of samples in which the pollen type was identified in each marked range, according Louveaux *et al.* (1978); P: present pollen; R: minor pollen; I: important pollen; A: accompanying pollen; D: dominant pollen.

Castanea sativa, *Cytisus* type and *Rubus* were present in 100% of the samples, whereas *Erica* was present in 97.7% and *Eucalyptus* in 86%. These pollens were the principal types found in the honeys from north-western Spain. *Echium*, *Quercus* and *Trifolium* pollens were also frequently found. Pollens from *Salix*, *Crataegus monogyna* type, *Campanula* type, *Conium maculatum* types, *Frangula alnus*, *Lotus* type, *Lithodora* and *Myosotis* comprised more than 3% of pollen spectra and might also be important in honeys. Fifty pollen types were present only in a few honeys (less than 10%) and were included in other pollen type groups.

Regarding the botanical classification of honeys, 46 samples contained pollens from a variety of sources and were classified as polyfloral. Another 25 samples contained more than 45% *Rubus* pollens and were classified as blackberry monoflorals. Honeys containing more than 70% *Castanea sativa* pollen were classified as chestnut honeys (9 samples). The remaining 6 samples were *Eucalyptus* monofloral honeys with a percentage higher than 70%. Pollens from *Erica* plants were common, but none of the samples contained enough *Erica* pollen to be classified as heather monofloral.

Further microscopic analysis identified additional elements in the sediments from samples. Table 2 shows a descriptive analysis of principal fungal elements sorted by frequency. *Cladosporium* conidia were the best represented elements (occurring in 97.7% of samples), with a mean of 325 spores per gram of honey. Spores from Myxomycetes and *Penicillium* were also very abundant, with mean content of 135 and 151 spores/g, respectively. More than 40% of the samples contained these elements.

The second-most common element was *Metschnikowia* yeast, present in 79.1% of the honeys studied. These cells have appeared in high content in some samples, with a maximum value of 46,217 cells per gram of honey.

Table 2. Descriptive analysis of microscopic elements per g of honey.

Fungal elements	Max.	Min.	Mean	St. Dv.	Rep. (%)
Cladosporium	1,934	0	325	346	97.7
Metschnikowia [a]	46,217	0	2,244	5,998	79.1
Leptosphaeria	613	0	42	84	51.2
Myxomycete	3,815	0	135	443	47.7
Penicillium	1,594	0	151	324	41.9
Basidiospores	679	0	20	78	32.6
Stemphylium	251	0	19	46	32.6
Urediniospores	454	0	25	67	30.2
Alternaria	91	0	6	18	15.1
Bipolaris	92	0	3	14	5.8
Torula	241	0	4	27	4.7
Fusicladium	56	0	1	8	4.7
Curvularia	71	0	2	10	3.5
Botrytis	51	0	1	6	2.3
Pleospora	151	0	2	16	2.3
Fern spores	1,126	0	20	135	2.3
Helminthosporium	71	0	1	8	1.2
Sporidesmium	19	0	0	2	1.2
Drechslera	109	0	1	12	1.2
Unidentified	761	0	22	86	27.9
HDE/P	0.63	0	0.16	0.09	100

[a] cells; Rep. (%): percentage of representation; HDE/P: honeydew index.

Fungal spores produced by plant pathogens, such as *Leptosphaeria*, *Stemphylium*, Urediniospores, *Alternaria*, *Pleospora* and *Botrytis*, were also counted. Of these, *Leptosphaeria* was the most common (more than 50% of samples contain its spores), with a mean of 42 spores/g and a maximum of 613 spores/g. The next most common spores found were *Stemphylium* and Urediniospores (present in more than 30% of samples), with means of 19 spores/g and 25 spores/g, respectively. Other elements from plant pathogens were uncommon (occurring in less than 15% of the samples), with mean values of 6, 2 and 1 spores/g for *Alternaria*, *Pleospora* and *Botrytis*, respectively.

Basidiospores, which include spores from Basidiomycota other than yeasts, were present in 32.6% of samples with a maximum of 679 spores/g. A high number of spores from ferns (Pterydophyta) were present in two samples, probably because the bees collected them as a protein source. In relation to the honeydew index, the highest value was 0.63 and the lowest was 0.00, with a standard deviation of 0.09.

2.2. Physicochemical Characteristics

Table 3 shows the descriptive analysis for physicochemical parameters. The honeys had a low level of hydroxymethylfurfural (HMF), indicative of fresh honeys. The mean value was 0.3 mg/100 g; in

fact, only three samples had more than 1 mg/100 g of HMF. Diastase varied between 6.1 Shade and 31.9 Shade, and invertase varied between 4.3 IN and 35.9 IN. Electrical conductivity and pH were two parameters widely used to distinguish between nectar and honeydew honeys. The pH varied between 3.5 and 5.0, values higher than 5.0 were not detected. The electrical conductivity varied between a minimum value of 0.224 mS/cm and a maximum value of 1.168 mS/cm. The humidity content had a mean value of 17.3 ± 0.8%. Colour varied between 39 and 150 mm, with a mean value of 95 ± 27 mm. Light amber colour (34–85 mm) accounted for 38.3% of honeys; amber colour (85–114 mm) for 41.9%; and dark amber colour (more than 114 mm) for 19.8%. The studied honeys were representative of the honeys produced in this geographical area, in which medium-high coloured honeys with medium or high electrical conductivity predominate.

Table 3. Descriptive analysis of physicochemical parameters.

Parameters	Max.	Min.	Mean	St. Dv.
Humidity (%)	19.8	15.5	17.3	0.8
pH	5.0	3.5	4.2	0.3
EC (mS/cm)	1.168	0.224	0.615	0.218
Colour (mm pfund)	150	39	95	27
Invertase (IN)	35.9	4.3	17.5	5.7
Diastase (Shade)	31.9	6.1	16.5	5.7
HMF (mg/100 g)	1.6	0.0	0.3	0.3
Potassium (mg/100 g)	312.1	32.8	138.7	62.8
Calcium (mg/100 g)	16.6	4.4	9.0	2.8
Iron (mg/100 g)	1.1	0.0	0.3	0.2
Magnesium (mg/100 g)	30.7	1.4	7.8	7.0
Sodium (mg/100 g)	26.7	0.9	5.6	4.8
Phosphorus (mg/100 g)	31.5	3.0	8.8	5.5
Zinc (mg/100 g)	0.4	0.1	0.2	0.1
Copper (mg/100 g)	3.7	0.0	0.4	0.8
Total mineral (mg/100 g) [a]	387.4	47.9	170.8	73.4

[a] Contains the sum of all minerals identified; EC: electrical conductivity.

The most abundant mineral in the honeys was potassium, with a mean value of 138.7 ± 62.8 mg/100 g. The phosphorus content varied between 31.5 mg/100 g and 3.0 mg/100 g, with a mean value of 8.8 mg/100 g. The average amount of magnesium was 7.8 ± 7.0 mg/100 g, and the calcium level was 9.0 ± 2.8 mg/100 g. Other mineral elements were present in low quantities.

2.3. Spearman's Linear Rank Correlation between Microscopic and Physicochemical Variables

A linear rank correlation analysis was used to examine the relationships between the variables studied. We used a non-parametric method, in which a linear correlation coefficient was calculated using Spearman's linear rank correlation analysis. In this procedure, the variables with the strongest relationship to the provenance of the honey were used.

Table 4 includes the value of the correlation coefficient and the significance ($P < 0.05$, $P < 0.01$, $P < 0.001$) of its relationship with the variables obtained by microscopic analysis, that could be related

to the source of the honey. The presence of *Metschnikowia* cells had a positive correlation with *Eucalyptus* and *Rubus* pollen content ($P < 0.05$) and a negative correlation with *Cytisus* t. ($P < 0.01$) and *Erica* pollen content ($P < 0.001$). The first taxon produced blossom honeys, and the other two were numerous in dark honeys.

Table 4. Significant correlation coefficients for the microscopic analysis variables, based on Spearman's test.

	Castanea	*Eucalyptus*	*Rubus*	*Cytisus* t.	*Erica*
Metschnikowia	−0.048	0.240 *	0.239 *	−0.317 **	−0.544 ***
HD spores	0.051	−0.390 ***	0.264 *	0.043	−0.041

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. HD: plant pathogen spores.

The group called HD spores included the levels of *Alternaria*, *Leptosphaeria*, *Stemphylium*, *Botrytis*, *Pleospora* and Urediniospores. These plant pathogen fungi constitute an important subgroup of the identified fungal elements. This group had a negative correlation with the *Eucalyptus* pollen content ($P < 0.001$) and a positive correlation with *Rubus* pollen ($P < 0.05$).

Metschnikowia cells had negative correlation coefficients with most of the physicochemical variables ($P < 0.001$) other than pH (no significance) and humidity (positive correlation $P < 0.01$) (Table 5).

Table 5. Significant correlation coefficients among the microscopic analysis and physicochemical parameter variables, based on Spearman's test.

	Diastase	**Invertase**	**pH**	**EC**	**Humidity**	**Colour**	**Total mineral**
Metschnikowia	−0.329 **	−0.353 ***	−0.179	−0.663 ***	0.298 **	−0.662 ***	−0.592 ***
HD spores	0.376 ***	0.441 ***	0.468 ***	0.476 ***	−0.240 *	0.399 ***	0.436 ***
Castanea	0.141	0.038	0.105	0.247 *	0.165	0.195	0.156
Eucalyptus	−0.386 ***	−0.344 **	−0.346 **	−0.520 ***	0.197	−0.491 ***	−0.456 ***
Rubus	0.232 *	0.207	0.167	0.057	−0.179	0.053	0.043
Cytisus t.	0.251 *	0.163	0.050	0.285 **	−0.069	0.347 **	0.231 *
Erica	0.161	0.122	−0.237 **	0.310 **	0.111	0.437 ***	0.231 **

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; EC: electrical conductivity.
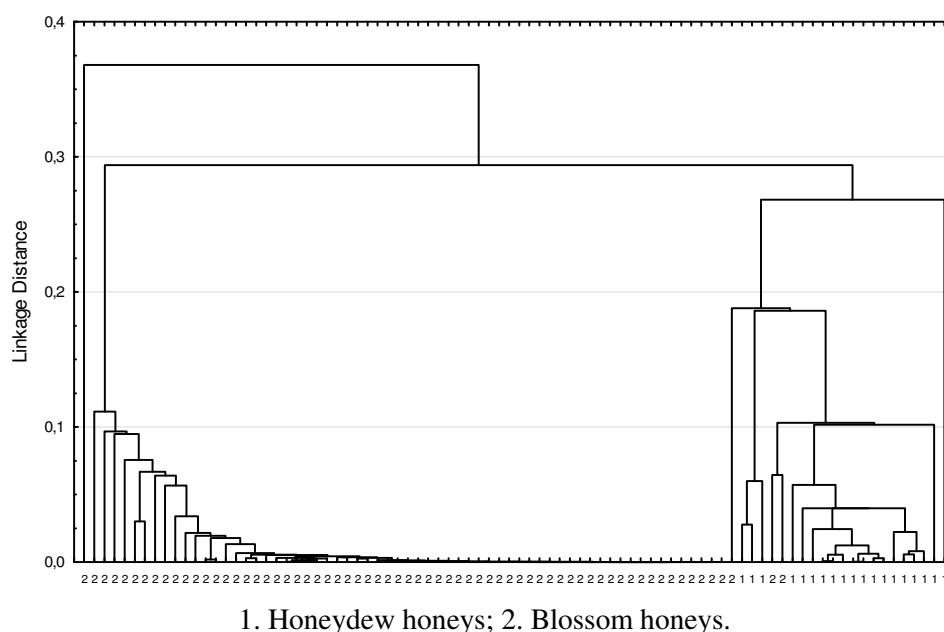
The presence of other fungal elements in honeys correlated positively with all physicochemical variables except humidity. HD spores in particular had a positive correlation ($P < 0.001$) with all the physicochemical parameters except humidity, with which it was negatively correlated ($P < 0.05$).

Regarding the honeys' pollen content, *Eucalyptus* pollen had similar correlations to those of nectar yeast. Pollens from *Cytisus* and *Erica* types had positive correlations ($P < 0.01$) with electrical conductivity, mineral content ($P < 0.05$, $P < 0.01$, respectively) and colour ($P < 0.01$, $P < 0.001$, respectively). *Cytisus* t. also had positive correlations ($P < 0.05$) with diastase content, and *Erica* had negative correlations with pH ($P < 0.01$). *Castanea* pollen and *Rubus* pollen had positive correlation with electrical conductivity ($P < 0.05$) and diastase ($P < 0.05$), respectively.

2.4. Cluster Analysis with the Main Microscopic Variables

The cluster analysis was performed with the main variables obtained by microscopy as independent variables. These were *Metschnikowia* cells, HD spores, *Castanea* pollen, *Eucalyptus* pollen, *Rubus* pollen, *Cytisus* pollen and *Erica* pollen. The groups (honeydew honeys or blossom honeys) were established according to the characteristics of the samples, as previously pointed out. The cluster (Figure 1) represents the groups established in the analysis. Sixty four samples belonged to the blossom honey group (polyfloral honeys, *Eucalyptus* honeys, *Rubus* honeys and *Castanea* honeys) and 22 to the honeydew honey group (including blend honeys and honeydew samples).

Figure 1. Cluster analysis of the studied samples.

1. Honeydew honeys; 2. Blossom honeys.

2.5. T Test and Levene's Test of the Homogeneity of the Variance

The groups established by the cluster analysis were compared using a t-test for the equality of the means. Levene's test examined the homogeneity of the variances by performing a one-way ANOVA on the absolute deviation scores.

Table 6 shows the descriptive statistical analysis of each group. Blossom honeys (N group) had a mean electrical conductivity of 0.540 ± 0.2 mS/cm, lower than that of the HD group (mean: 0.830 ± 0.2 mS/cm). Colour was clearer for nectar honeys (85 ± 20 mm pfund) than for honeydew honeys (124 ± 21 mm pfund). Mineral content was generally lower in blossom honeys, particularly for potassium content (N group mean: 120.4 ± 50.7 mg/100 g *vs.* HD group mean: 192.1 ± 65.2 mg/100 g), as was phosphorus content (7.4 ± 3.2 mg/100 g *vs.* 13.2 ± 8.2 mg/100 g). Even the enzymatic activity was high in the nectar samples. The subindex of the variables indicates the significance levels between the groups. Some parameters led to differentiation between the groups. These were enzymatic activity, *Metschnikowia* cells and *Cytisus* pollen type with $p < 0.05$, electrical conductivity, humidity, colour, potassium, calcium, magnesium, phosphorus, total mineral content, *Eucalyptus* pollen, *Erica* pollen and HD spores content with $p < 0.01$.

Table 6. Descriptive statistical analysis of the groups established by cluster analysis.

Parameters	N group (N = 64)				HD group (N = 22)			
	Max.	Min.	Mean	St. Dv.	Max.	Min.	Mean	St. Dv.
Pollen grain per gram	90,232	1,632	24,659	23,006	130,832	1,274	26,071	32,561
Diastase content (Shade) [b]	31.8	7.5	15.7	5.4	30.9	6.1	18.8	5.9
Invertase content (IN) [b]	35.9	4.3	16.8	5.8	26.0	10.1	19.7	4.8
HMF (mg/100 g)	1.6	0.0	0.4	0.3	0.9	0.0	0.3	0.3
pH	5.0	3.5	4.2	0.3	4.9	3.5	4.3	0.4
EC (mS/cm) [a]	0.920	0.224	0.540	0.2	1.168	0.482	0.830	0.2
Humidity (%) [a]	19.8	16.0	17.5	0.7	17.8	15.5	16.7	0.6
Colour (mm pfund) [a]	118	39	85	20	150	79	124	20.9
Potassium (mg/100 g) [a]	239.0	32.8	120.4	50.7	312.0	65.5	192.1	65.2
Calcium (mg/100 g) [a]	13.7	4.7	8.4	2.3	16.6	4.4	10.6	3.4
Iron (mg/100 g)	1.1	0.03	0.3	0.2	0.7	0.1	0.3	0.1
Magnesium (mg/100 g) [a]	18.7	1.6	5.8	4.4	29.2	1.4	13.5	9.8
Sodium (mg/100 g)	26.7	1.2	5.7	4.9	17.8	0.9	5.3	4.7
Phosphorus (mg/100 g) [a]	17.1	3.3	7.4	3.2	31.5	2.9	13.2	8.2
Zinc (mg/100 g)	0.4	0.1	0.2	0.1	0.4	0.1	0.2	0.1
Copper (mg/100 g)	3.7	0.02	0.4	0.8	0.7	0.1	0.3	0.5
Total mineral (mg/100 g) [a]	280.3	47.9	148.6	55.9	387.4	95.2	235.4	80.8
Castanea	86.2	1.0	36.2	20.9	87.9	2.5	41.5	23.3
Rubus	83.6	0.3	29.7	21.6	65.8	7.1	31.5	20.8
Erica [a]	15.3	0.0	3.7	3.9	35.5	1.3	8.9	9.2
Eucalyptus [a]	81.0	0.0	17.9	4.6	14.2	0.0	2.7	4.3
Cytisus t. [b]	31.4	0.1	4.0	25.2	26.8	0.3	7.7	10.6
Mestchnikowia (cells/g) [b]	46217	17.1	3013	6796	41	0	4.7	286
HD spores (spores/g) [a]	406	0	61	84	1159	5	193	11.5

[a] differences among both groups (*P* < 0.01); [b] differences among both groups (*P* < 0.05); N group: blossom
honeys; HD group: honeydew honeys; HMF: hydroxymethylfurfural; EC: electrical conductivity.

3. Discussion

Some physicochemical parameters indicate the presence of honeydew in honey, as is the case for the electrical conductivity. European legislation has established a minimum of 0.800 mS/cm for honeydew honeys, chestnut honeys and their blends. Only 18.6% (16 samples) of the studied honeys had higher electrical conductivity and all of them show a significant amount of *Castanea* pollen in the pollen spectra (6 samples more than 70%). In general, dark honeys had the highest mineral content [27,28], nevertheless *Erica* honeys and *Castanea* honeys frequently have similar mineral content to honeydew honeys [29]. Another physicochemical parameter used for honey typification was colour; dark honeys or amber dark honeys were frequently associated with honeydew but as occurs with electrical conductivity, *Castanea* honeys or *Erica* honeys, among others, have dark or amber dark colour. Related to sensorial attributes honeydew honeys were described by González *et al.* [30] as dark caramelized liquid honeys, without crystallization, of intense fruitiness with certain floral notes, spicy with a woody fresh odor and very greasy in the mouth. Again nectar contributions of *Castanea* could

confuse the origin of honeys by their sensorial perceptions since this type of honey has woody olfactory perceptions but with strong intensity [29].

The interpretation of the pollen spectra of the honeys is an important tool in its characterization. This analysis together with some sensorial attributes and physicochemical parameters are frequently necessary to typify honeys. Also a high ratio of HDE (spores, hyphae or algae) to P (number of pollen grains), being the honeydew index [31], has been used to indicate the presence of honeydew. Pine honeys from Greece had a higher HDE/P ratio, with levels higher than 1.5 [1]; this high HDE content was reported also by Persano-Oddo *et al.* [29] for honeydew honeys. In the case of the honeys studied in this work the mean HDE/P ratio of 0.16 was considered very low. As a result, the honeydew index was not found to be a useful way to classify honeydew honeys from this region.

However considering the range of honeydew elements that can be found in honey, it has been possible to established a relation between some of these elements and the provenance of the honey (nectar or honeydew). The results showed the importance of the microscopic analysis for typification of the studied honey. Yeast with an airplane-cell configuration has been found in higher quantities in some honey samples. These structures, specific to floral nectar [32–34] and identified as *Metschnikowia,* could be indicative of blossom honeys. Other important fungal elements identified in the honeys from northwest Spain belonging to the fungus kingdom were: spores, hyphae or conidia. The most abundant conidia were *Cladosporium*, Myxomycete, and *Penicillium*. These grow frequently in both indoor and outdoor environments [35,36] and can be passed to honey during the different stages of production or as a secondary contamination [10]. Some fungal spores, produced by plant pathogens, were also found, albeit of low content. The most important were *Leptosphaeria*, *Stemphylium* and Urediniospores, present in more than 30% of the studied samples. They grow over the leaves and green parts of plants, causing various diseases. Fungal elements can be introduced into honeys when the worker bees collect honeydew from the plant. As expected, the presence of fungal pathogens could be indicative of the presence of honeydew.

Nectar honeys are produced during the spring and the early summer, normally over short periods of time. In our region, the eucalyptus (*E. globulus*) honey production period starts in winter (January) and stops in April. The nectar secretion pattern is very abundant and fast; thus, honey production is completed quickly. These honeys have low enzyme content. Conversely, when honey production takes longer, then enzyme content is higher. This occurs with honeys in the honeydew group, which probably began as blossom honeys early in spring but were mixed with honeydew secretions toward the end of summer (August and September). This fact was supported by the values for the principal pollen taxa in each group. In this instance, honeys with high *Metschnikowia* yeast had less electrical conductivity, lighter colour, higher humidity, minor enzymatic content and lower mineral content, which are all common features of blossom honeys [26,29]. On the other hand, honeys with significant quantities of HD spores were dark or dark amber honeys with high electrical conductivity, relatively high pH, high enzymatic activity, high mineral content and low humidity content. These characteristics are common to honeydew honeys or blends [25,37,38].

4. Material and Methods

In this study 86 honey samples were analysed from Galicia (Northwest Spain), collected during the years 2008 and 2009 directly by the beekeepers and analysed in the Laboratory of Aerobiology and Beekeeping of the Faculty of Sciences of Ourense (University of Vigo). Each sample (1 kg) was separated into two parts. One of them was used for physicochemical analysis and was stored at room temperature; the other was frozen at −30 °C for further analysis. All the determinations were performed in duplicate.

4.1. Microscopic Analysis

Pollen and the different fungal elements were analysed, identified and counted with a modified version of the method described by Louveaux *et al.* [31]. The quantitative analysis was made by a volumetric method using two aliquots of 10 µL taken from 2 mL of sediment obtained by centrifuging 10 g of honey. The total number of pollen grains and fungal elements were counted by microscopy in each aliquot and were expressed as an average. Pollen spectra were constructed by counting a minimum of 800 pollen grains in two 100 µL aliquots of the sediment obtained previously. The amounts of fungal spores and yeasts were also calculated from a 100 µL aliquot, with the number of pollen grains on the slide as a reference. The results were expressed as the quantity of the different fungal elements per gram of honey. Also, the relation between the number of fungal spores and the number of pollen grains (HDE/P) named as the honeydew index (HDE) was calculated.

4.2. Physicochemical Analysis

The principal parameters of honey quality were determined using the methods adopted by the International Honey Commission [39].

Hydroxymethylfurfural (HMF) content was determined by UV absorbance of HMF at 284 nm. To avoid interference from other components at this wavelength, we determined the difference between the absorbencies of a clear aqueous honey solution and the same solution with added bisulphite, as described by White [40].

Invertase activity was determined by spectrophotometry of 4-Nitrophenyl-alpha-D-glucopyranoside decomposition at 400 nm. The samples were incubated at 40 °C. The invertase activity was expressed as an invertase number (IN). Diastase activity was based on the rate of starch hydrolysis by diastase, present in a honey buffer solution. The endpoint of this reaction was determined by measuring samples of the mixture at different time intervals using a UV-VIS spectrophotometer at 660 nm. The time required to reach an absorbance of 0.235 was calculated using linear regression, and the results were expressed using a Gothe scale. The UV-VIS absorbance was measured with a spectrophotometer (Jenway 6505).

The percentage of moisture was determined by refractometry, using an Abbe 325 refractometer. The pH was measured with a pH meter (Crison micropH 2001) of honey dissolved in bidistilled water. The electrical conductivity was determined with a conductivity meter (Knick 913 C), and the results were expressed as mS/cm. All measurements were performed at 20 °C. The colour was measured with a

Hanna Honey Colour C221 digital instrument, and the results were expressed in millimetres using the Pfund scale.

The quantitative determination of minerals, such as potassium, calcium, iron, magnesium, sodium, phosphorus, zinc and copper, in the honey was conducted with an atomic absorption spectrophotometer (Varian SpectrAA-220). Samples were digested in a microwave before their mineral content was evaluated.

4.3. Statistical Analysis

Physicochemical variables, mineral content, the most frequently occurring pollen types, the yeast content and the fungal content were statistically compared using Spearman's rank correlation analysis.

A cluster analysis was applied to differentiate the honeys according to their botanical origin (either blossom honeys or honeydew honeys). The variables used were principal pollen types, *Metschnikowia* cells and a group of spores of plant pathogens called HD spores. Finally, the two groups established by the cluster analysis were compared with an independent samples *t*-test, which determines the presence of statistically significant differences between-groups.

All statistical analyses were performed with the SPSS Statistic 17.0 software for Windows.

5. Conclusions

Some biotic elements were identified in the sediment of the honeys. The presence of these structures can be used to distinguish between blossom and honeydew honeys. The statistical analysis led to a differentiation between these two groups of honeys. The parameters showing clear differences were enzymatic content, electrical conductivity, humidity, colour, mineral content (especially potassium, calcium, magnesium and phosphorus) and microscopic elements, such as, fungal spores from plant pathogens, yeast and some common pollen grains in the honeys.

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