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An overview of the challenges when analysing pollen for monofloral honey classification

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ABSTRACT

The complexity of the pollen analysis, even for accredited laboratories, and the absence of standardized guidelines for a routine method to determine the monoflorality of a honey is a challenge. To expose the reality of this situation, the aim of this work is to discuss how the information and the quantification of the honey pollen content results are presented, considering the reports provided by four of these laboratories. Noticeable differences in how the data is presented is observed; not only in the number of types of pollens (main and accompanying) which percentage is reported but also for the criteria followed in naming the same pollen, applying different taxonomic levels (family, genus, type, specie, etc.). Although there is one common consensus by all laboratories based on discarding the pollens from non-melliferous plants, in general the discrepancies observed are important. The problem is more evident when there is a presence of over-represented pollen and the target pollen is under-represented as is the case of *Citrus*. Thus, the simultaneous presence of under-represented and over-represented grains of pollen may frequently lead to misleading results. The lack of standardization in pollen analysis to be applied equally by all laboratories significantly contributes to the discrepancies. Only by having a common criterion to interpret the pollen spectrum and, obviously, ensuring the adequate experience of the analyst and the knowledge of the related flora is when the results can be considered reliable.

1. Introduction

The technique widely used to classify monofloral honey is the melissopalynological analysis which focuses on the identification of the pollen grains morphology of the different botanical species, and their quantification. The presence of pollen in honey is logical since bees are impregnated with pollen when they visit the flowers to collect nectar. However, the melissopalynologically analysis is a complex procedure that requires, among other things, highly skilled analysts, which are very few perhaps due to the demanding preparation in this field. This analyst must first locate under the microscope at least 500 grains of pollen in the honey sediment (Louveaux, Maurizio, & Vorwohl, 1978). Then, they must identify the morphology of the different pollens present with the aim of attributing their belonging to a certain botanical species. Once this stage is complete, the relative frequency with respect to the total number of pollen grains counted (expressed as the respective percentage) for each pollen type must be calculated. However, the calculation of this percentage, is not always straightforward, since certain aspects must be considered. For instance, some types of pollens, although present, must be excluded from the count, because they stem from botanical species that are not nectar producers (nectarless taxa) and therefore do not contribute to the formation of honey. To complicate the issue even further, there is a lack of agreement when considering if a plant can be nectariferous or not (Ricciardelli D'Albore, 1998).

With respect to the pollen grains count, when the sediment contains a high percentage of over-represented pollen (such as *Myosotis*, *Castanea*, *Eucalyptus*, among others), some analysts disregard these types of pollen, while others do not (Von Der Ohe, Oddo, Piana, Morlot, & Martin, 2004). It is noteworthy to mention that the analyst occasionally faces the problem of observing a presence of abnormally high amounts of pollen in the sediment, having to pay special attention in the interpretation of melisopalynological results (Rodopoulou et al., 2018). These pollens could be from different origins: the so-called secondary sources of enrichment (pollen stored by the bees in the hive itself), tertiary (pollen stored in the brood chamber extracted during the honey extraction process) and quaternary (carried by the bee by air) (Von Der

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Ohe et al., 2004). All these factors prove that the analyst needs time (usually 30 min to 1 h for each sample) depending upon the complexity of the pollen spectrum. In addition to the fact that the analyst is required to give a real time result (immediately after counting), it is obvious that the subjective component is a factor that could condition the result. However, this problem is minimized when the technician who performs the honey pollen analysis is an expert (Aronne & De Micco, 2010; Bryant & Jones, 2001).

Another issue is related to the monofloral classification of a honey. When the analyst must determine whether the honey is monofloral and if so, define the monofloral type it belongs to, their final decision will be based on the resulting percentage of the pollen count (previously explained) and the sensory characteristics, sometimes together with some specific physicochemical parameters (Rodopoulou et al., 2018; Von Der Ohe et al., 2004). In addition, there is no consensus on the percentage required of the predominant pollen for each monofloral type, since there is lack of a commonly agreed international standards in this matter. Countries (based on the limits established by certain laboratories) apply their own classification criteria of monofloral honeys. As a result, finding a true definition of what a monofloral honey is, creates uncertainties that affects regulatory agencies, beekeepers and agents involved in commercial transactions and even the consumer. This provokes the discrepancies among the different European countries with regards to their national regulation in terms of the minimum percentage of pollen required for the characterization of a monofloral honey. Taking this into consideration, it is worth mentioning the study carried out by Thrasyvoulou et al., 2018, in which, the minimum percentage of pollen required for the characterization of more than twenty monofloral honeys in five European countries (considering their national legislation or guidelines) were compared. A significant discrepancy is observed both due to the absence of criteria in some countries for most monoflorals and when regulation is present there are significant differences in the mandatory minimum percentages for some honeys. As an illustrative example, it can be cited the case of citrus honey, whose pollen is under-represented and its classification presents an added difficulty. For this type of honey, the minimum *Citrus* pollen content is: Germany (20%); Italy (10%); Croatia (10% or 5% with characteristic smell, taste and color organoleptic properties of honey) or Greece (3%) (Thrasyvoulou et al., 2018; Greek Decision 127, 2004). In the case of Spain there is no national regulation whatsoever. However, in the Valencian region (Spain) the guidelines stipulate a minimum 10% of *Citrus* pollen to be labelled as citrus honey (DOGV, 2002; Juan-Borrás, Periche, Domenech, & Escriche, 2015).

Furthermore, the absence of international standardized criteria on the correct way to count pollen makes matters worse and influence the results provided by different laboratories. The method described by Louveaux et al. (1978), laid the foundation for the pollen analysis of honey and currently is a viable method in performing this type of routine analysis in most European laboratories (Von Der Ohe et al., 2004). However, the efforts made over the last decades to coordinate, implement, and validate it have proven insufficient since the laboratories are continuously adapting this method as they deem appropriate, with the repercussions this may entail (Bicudo de Almeida-Muradian et al., 2020; Bryant & Jones, 2001).

Therefore, it is apparent the way in which the pollen analysis of honey is being carried out requires a thorough revision. Nevertheless, the reality of the situation must be put forward. Hence, the aim of this work is to discuss how the information and the quantification of the honey pollen content results are presented based on examples of the reports provided by different accredited laboratories.

2. Material and methods

2.1. Honey samples

The honey samples used in the present study were provided by different Spanish beekeepers (collected in 2020 and 2021) either directly or through *Ministry of Spanish Agriculture and Fishing, Food and Environment* thanks to an agreement (characterization of the main Spanish monofloral honeys, B.O.E., 2018) with the *Institute of Food Engineering for Development* where the laboratory in charge of this study (LABMIEL: *Laboratory* of *Quality Control of honey and Bee Products* at Universitat Politècnica de València, Spain) is located. Thirteen honey samples in addition to being melissopalynologically analysed in LABMIEL (Lab 4), were sent with the same purpose to three different European laboratories. Due to data protection regulations, in this work they will be referred to as Lab 1, Lab 2 and Lab 3. The four laboratories are accredited as per ISO 17025 (ISO/IEC 17025:2017, 2017) and well-known experts in this field. Therefore, in the present study a total of 52 reports were assessed.

2.2. Melissopalynological analysis in LABMIEL

LABMIEL has more than 20 years' experience in providing this type of analytical support to companies of the apiculture sector, in addition to being at the forefront of honey research. It also participates every year in all possible Proficiency Tests Schemes specifically designed for pollen and other quality parameters analysis in honey such as BIPEA (Bureau Interprofessionel d'Etudes Analytiques, Gennevilliers, France-Proficiency Testing Scheme; http://www.bipea.org).

More specifically, in this laboratory the pollen was analysed following the International Commission for Bee Botany recommendations with slight modifications (Von Der Ohe et al., 2004). This entails identifying the morphology of the different pollens present to attribute their belonging to a certain botanical species and establishing the percentage of the most abundant pollen types compared with the total percentage. The sample honey (10 g) was dissolved in acidulated water (H_2SO_4, 5%) at 40 °C. Thereafter, it was centrifuged at 1860g (Centronic II, B.P. Selecta, Barcelona, Spain), the supernatant decanted, and the precipitate suspended in distilled water (10 mL). After a second centrifugation, the supernatant decanted off and water (0.2 mL) added to the precipitate. After stirring, 0.2 mL were placed on a slide and dried at room temperature. As a last step, a drop of glycerine was used to seal the coverslip. A count of at least 500 pollen grains through 10 equidistant parallel lines (from one edge of the field of vision to the other) or 10 more lines (if the slide is poor in plant elements) in order to obtain a total count of 500 of pollen grains. This was performed observing (at × 400–1000 magnifications) using a light optical microscope (Zeiss Axiolab, Göttingen, Germany) coupled with a digital camera (Axiocam 305 Color, Zeiss). The grains of pollen were classified by an expert analyst according to pollen morphology (Carretero, 1989; PalDat, 2022; Saenz-Laín & Gómez-Ferreras, 2000). The labelling and annotation of each type of pollen was carried out by a new specific image software, developed by the Institute of Industrial Computing and Control Systems (AI2) at the Univèrsitat Politècnica de València. The pollen identification was always done by an expert analyst of Lab 4 and the software was simply an auxiliary tool for labelling and annotation.

3. Results and discussion

3.1. Variability according to how the information is presented

To show the complexity on how the information of pollen analysis is presented by the different laboratories, the honey samples were melissopalinologically studied. Following the methodology of classifying monofloral honeys, these samples were in first instance analysed by LABMIEL and, after, with the same purpose in mind they were send to the other accredited laboratories. They received for each sample the information that the beekeeper previously declared about the supposed attribution of each one of them to a certain monofloral variety. This is a common practice when a honey sample is submitted to a laboratory for its cataloguing as monofloral, and only if the information is available.

Table 1 shows the detailed information of the pollen analyses provided by these laboratories. All these sample reports included the pollen percentage corresponding to the botanical species declared by the beekeeper, although it was not always among the most abundant pollen according to the laboratory's criteria: sample 1 to sample 5 (citrus honey: *Citrus* sp.); sample 6 (eucalyptus honey: *Eucalyptus* sp.); sample 7 (thyme honey: *Thymus* sp.); sample 8 (raspberry honey: *Rubus* sp.); sample 9 (acacia honey: *Robinia pseudoacacia*); sample 10 (coriander honey Umbelliferae/*Coriandrum sativum*); Sample 11 (rape honey: Brassicaceae); Sample 12 (linden honey: *Tilia* sp.); Sample 13 (sunflower honey: *Helianthus annuus*). In general, when an analyst performs the pollen analysis of a honey, they primarily look for the pollen corresponding to the information given by the beekeepers, even if it is not among the most abundant pollen according to the laboratory's criteria. It was decided to analyse a larger number of citrus honey samples since this type of honey entails an additional difficulty due to its pollen being underrepresented.

At first glance, it can be observed that there are noticeable differences in how the data is presented by the laboratories. Here, it is interesting to highlight that only in one specific laboratory the results are presented with two distinct interpretations (two different columns in Table 1). Another dissimilarity is that in some cases, only the percentage of the main pollens is reported, while in "other", the percentage of the least present is also added. As an example, observing sample 1, Lab 4 only reports the percentage of the major pollen specie (18% *Citrus* sp), identifying the rest of the pollens as "other" (with a global figure of 82), and without specifying the percentage of each one. On the contrary, Lab 2 reports up to 11 percentages, at different levels (species, type and in some cases only family). Another illustrative example could be sample 6 where Lab 4 reports the percentage of the major pollen species, *Eucalyptus* sp (77%), followed by *Castanea sativa* (17%) and others (6%), whereas Lab 2 reports up to 8 different percentages and levels including the most abundant (Myrtaceae/*Eucalyptus* sp: 75.7%). In general, Lab 1 and Lab 3 tend to opt for an intermediate solution, giving the percentage of pollen that they consider being the predominant, together with those assessed as relevant. However, this is not the general rule for these two laboratories since in some cases Lab 3 provide more detailed information than Lab 4, but not as thorough as Lab 2 (e.g. sample 9 or 10).

In this regard, when the purpose of pollen analysis is to determine the monoflorality of a honey, providing the information of the main pollen is sufficient. For this reason, some laboratories do not usually give more details on the percentage of the other accompanying pollens, unless the client specifically asks for this information. However, when the country of origin of a honey needs to be stated it is essential to identify the accompanying pollens (regardless of its amount) since they provide additional information about the surrounding flora where the honey was harvested (Baño-Breis, 1990; Karabagias, Karabournioti, Karabagias, & Badeka, 2020; Moraes et al., 2019; Sniderman, Matley, Haberle, & Cantrill, 2018; Terrab et al., 2022). For instance, the presence of pollens such as *Vitex negundo* var. heterophylla is attributed to China (Song, Yao, & Yang, 2012).

When dealing with the discrepancy in how the results are reported, it is also important to take into consideration the criteria followed by the different laboratories to name the same pollen, since they could refer to it, applying different taxonomic levels (family, genus, type, specie, etc.). As an example, for the case of sample 1, only Lab 2 and Lab 3 give pollen percentages referring to families, although in a different way. More concretely, Lab 3 reports Asteraceae (11%), while Lab 2 does the same with Compositae (equivalent to Asteraceae) but identifying specifically the specie *Taraxacum* sp. (Compositae/*Taraxacum* sp.; 1.9%). Furthermore, it should be noted that when referring to the same family, these two laboratories use different nomenclatures (Compositae = Asteraceae).

3.2. Variability of pollen percentage information in honey samples

To demonstrate the variability of the percentage values provided, Fig. 1 represents for each sample, the information of those pollens for which all the laboratories specified a percentage, considering the main and the accompanying pollens. This figure by itself is able to show the discrepancy of the pollen percentage results. It is remarkable the implication of the two ways of presenting the result by one specific laboratory, as shown in Table 1 for samples 1 to 7 and codified as Lab 1 and Lab 1*.

The information referred to Lab1* is only provided by this laboratory, in which all the pollens observed in the slide are considered in the count both from melliferous and non-melliferous plants (from sample 8 to 13, there are no values for Lab1* because this laboratory does not report non-melliferous pollens for these samples). When considering non-melliferous pollens in the calculation, the final percentage of the target pollen for evaluation will decrease. For instance, in this case (Lab 1*), samples 1 and 4 present 2% and 18% of *Citrus* sp. pollen, respectively. Obviously, this information can lead to some confusion when looking at the report. Only expert technicians can assess whether or not these results are valid for commercial purposes.

It is true that the application of some kind of recalculation of the pollen count is a common practice carried out by all the laboratories. This issue lies in the criteria followed by each one, which somehow are evident in their reports. The only type of recalculation criteria applied by all laboratories is: do not consider in the count the pollens from non-melliferous plants, because they do not provide nectar. This is the only criteria followed by Lab 2, Lab 3 and Lab 4. However, Lab 1, apart from applying this same criterion, is also disregarding those pollens from over-represented species (although they come from melliferous plants but are not abundant enough to define a specific monoflorality).

Based on the results provided, it can be concluded that the criteria followed by all the laboratories to consider a plant as non-melliferous has been followed equally by all of them although there is not always consensus on this aspect as mentioned in the introduction section. Lab 4 does not include nectarless pollen in the count, but it provides this data in the "other" section for information purpose.

For the above-mentioned example (samples 1 and 4), in this case (Lab 1) the *Citrus* sp. pollen percentage are 18% and 38%, more in line with the rest of the laboratories. This greater coincidence is due to the fact that in both samples the compensated pollens come only from the non-melliferous category and in which, as it was explained before, all laboratories agree. Therefore, when over-represented pollen species (such as *Castanea sativa*) are present, the discrepancy of Lab 1 with respect to the other laboratories increases. For instance, in the case of sample 6, Lab 1 reports 99% Eucalyptus sp. whereas the other three are more in line with respect to each other (75.7, 79 and 77%). This because the first laboratory eliminated *Castanea* from the count, reporting cero pollens for this specie. However, it is evident that these pollens were observed by the analyst as this laboratory confirms its presence (31%) when all the pollens seen in the slide are considered (Lab1*).

In short, part of the discrepancy among the results is likely caused by some laboratories not considering in the count certain over-represented pollens even if they originate from melliferous species. Nevertheless, they are taken into account by all the other laboratories. Therefore, when there is a greater presence of this type of pollen in the sample, this inconsistency is more noticeable.

It is noteworthy that although all the laboratories follow the same recalculation criteria (because the sample does not have over-represented pollens), significant disparities in the percentages are reported: e.g. sample 1 for *Citrus* sp: 30% by Lab 3, almost 4 times lower by Lab 2 (7.9%), whereas an intermediate result (18%) was reported by Lab 1 and Lab 4. On the contrary, when the content of the same pollen is very high the results obtained are homogeneous (such is the case of Brasicaceae in sample 11: 99%, 95.1%, 99% and 99%). These observations are in line with what was reported by Von der Hoe, 2004, who stated

Table 1
Detailed information of the pollen analyses provided in 13 honey samples by four accredited laboratories. Beekeeper Information declared about the supposed attribution of each sample to a certain monofloral variety is shown between brackets (the corresponding pollen taxonomic level is highlighted in red). Type of recalculation applied: A (Discarding the pollens from non-melliferous plants), B (Discarding the pollens from over-represented species although they come from melliferous plants but are not in enough quantities to define a specific monoflorality), C: all the pollens observed in the slide are considered in the count both from melliferous and non-melliferous plants). Laboratory codes and correction criteria: Lab1 (A + B); Lab1* (C); Lab 2(A), Lab 3(A) and Lab 4(A). Lab 4 = LABMIEL where this study was carried out.

SAMPLE 1 (Beekeeper attribution: Citrus honey)

Family/Type	% Pollen applying recalculation (A & B)	% Pollen applying recalculation (C)	Family/Type	% Pollen applying recalculation (A)
Laboratory 1	Lab1	Lab1*	**Laboratory 2**	Lab 2
Olea (Olive)	0	60	Leguminosae/*Cytisus* sp., *Genista* sp.	18.7
Quercus ilex (Holm Oak)	0	29	Leguminosae/*Lotus* sp.	1.4
***Citrus* sp. (Orange)**	**18**	**2**	Cruciferae/ *Brassica* sp., *Raphanus* sp.	3.7
			Cruciferae/ *Diplotaxis* sp.	58.4
			Rosaceae/*Rubus* sp.	1.9
			Rosaceae/*Prunus* sp.(including *Crataegus* sp.)	1.4
			Compositae/*Taraxacum* sp.	1.9
			Salicaceae/*Salix* sp., *Populus* sp.	1.4
			Rutaceae/*Citrus* sp.	**7.9**
			Campanulaceae	1.4
			other	1.9
	% Pollen applying recalculation (A)			**% Pollen applying recalculation (A)**
Laboratory 3	Lab3		**Laboratory 4**	Lab 4
Citrus	**30**		***Citrus* sp.**	**18**
Echium	22		other: Umbelliferae, *Pistacia*	82
Asteraceae	11		*Quercus* sp., *Olea* sp., *Echium* sp., *Erica* sp.,	
Myrtaceae	8		*Hieracium* sp., *Taraxacum* sp.	

SAMPLE 2 (Beekeeper attribution: Citrus honey)

Family/Type	% Pollen applying recalculation (A & B)	% Pollen applying recalculation (C)	Family/Type	% Pollen applying recalculation (A)
Laboratory 1	Lab1	Lab1*	**Laboratory 2**	Lab2
Olea (Olive)	0	35	Leguminosae/*Medicago* sp.	2
Genista (Petty Whin)	38	24	Leguminosae/*Cytisus* sp., *Genista* sp.	12.9
Ulex (Gorse)	15	10	Leguminosae/*Vicia* sp.	5
Brassicaceae (Crucifers)	**18**	**11**	Fagaceae/*Castanea sativa*	1.5
Rosmarinus (Rosemary)	8	5	**Cruciferae/*Brassica* sp., *Raphanus* sp.**	**7.4**
Trifolium pratense (Red Clover)	10	6	**Cruciferae/*Diplotaxis* sp.**	**19.8**
Quercus ilex (Holm Oak)	0	2	Lamiaceae/*Rosmarinus officinalis*	2
***Citrus* sp. (Orange)**	**1**	**1**	Borraginaceae/*Echium* sp.	6.4
			Rosaceae/*Rubus* sp.	7.9
			Rosaceae/*Prunus* sp.(including *Crataegus* sp.)	18.3
			Compositae/*Taraxacum* sp.	2.5
			Salicaceae/*Salix* sp., *Populus* sp.	2
			Rutaceae/*Citrus* sp.	**10.4**
			other	2
	% Pollen applying recalculation (A)			**% Pollen applying recalculation (A)**
Laboratory 3	Lab3		**Laboratory 4**	Lab4
Echium	16		***Citrus* sp.**	**7**
Brassicaceae	**13**		**Brassicaceae**	**15**
Prunus	12		other: *Prunus dulcis*, *Echium* sp., *Rosmarinus officinalis*, *Castanea sativa*, *Anthyllis* sp. *Salix* sp., *Olea europaea*, *Thymus* sp., Type *Carduus*, Leguminosae	78
Citrus	**9**			
Rosmarinus	6			
Castanea	4			
Salix	4			
Genista	3			

SAMPLE 3 (Beekeeper attribution: Citrus honey)

Family/Type	% Pollen applying recalculation (A & B)	% Pollen applying recalculation (C)
Laboratory 1	Lab1	Lab1*
Rubus	**36**	**22**
Quercus Ilex	0	19
Olea	0	17
Citrus	**12**	**8**
Onobrychis	8	5
Cistaceae	0	2

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Leguminosae/*Cytisus* sp., *Genista* sp.	31
Vicia sp.	12.4
Lotus sp.	1.6
Anthyllis sp.	5.8
Cruciferae/*Brassica* sp., *Raphanus* sp.	6.2
Diplotaxis sp.	4.7
Rosaceae/ *Eriobotrya* / *Rubus* sp.	**11.2**
Prunus sp.(including *Crataegus* sp.)	6.2
Helianthus annuus, C. arvensis	1.6
Rutaceae/*Citrus* sp.	**11.2**
Foeniculum sp.	1.9
Myrtaceae/*Eucaliptus* sp.	1.6
Oxalidaceae/*Oxalis* sp.	3.1

	% Pollen applying recalculation (A)
Laboratory 3	Lab3
Rubus	**29**
Brassicaceae	16
Citrus	**14**
Onobrychis	12
Asteraceae	5
Lamiaceae	5

	% Pollen applying recalculation (A)
Laboratory 4	Lab4
***Citrus* sp.**	**12**
***Rubus* sp.**	**21**
other: Brassicaceae, *Onobrychis* sp., *Hypecoum* sp., *Thymus* sp., *Ceratonia siliqua*, *Taraxacum* sp., Asteraceae, *Erica* sp.	67

SAMPLE 4 (Beekeeper attribution: Citrus honey)

Family/Type	% Pollen applying recalculation (A & B)	% Pollen applying recalculation (C)
Laboratory 1	Lab1	Lab1*
Quercus Ilex	0	33
Cistaceae	0	19
Citrus	**38**	**18**
Ericaceae	**5**	**3**
Helianthus	3	1

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Ericaceae/*Erica* sp.	**5.1**
Calluna sp.	
Leguminosae/*Cytisus* sp., *Genista* sp.	40.4
type *Vicia* sp.	1.3
type *Lotus* sp.	
Cruciferae/*Brassica* sp., *Raphanus* sp. *Diplotaxis* sp.	3.8
Boraginaceae/*Echium* sp.	1.7
Rosaceae/*Rubus* sp.	3.8
Prunus sp.(including *Crataegus* sp.)	1.3
Compositae/ *Helianthus annuus*, C. arvensis	3.4
Rutaceae/*Citrus* sp.	**36.2**
Oxalidaceae/*Oxalis* sp.	1.7

	% Pollen applying recalculation (A)
Laboratory 3	Lab3
Citrus	**46**
Rhamnaceae	10
Asteraceae	7
Arecaceae	6
Erica	**4**
Lamiaceae	4

	% Pollen applying recalculation (A)
Laboratory 4	Lab4
***Citrus* sp.**	**34**
***Erica* sp.**	**7**
Cytisus sp.	27
other: *Brassicaceae*, *Helianthus annuus*, *Lotus* sp., *Rosmarinus officinalis*, *Rhamnus* sp., *Prunus* dulcis, Asteraceae	32

SAMPLE 5 (Beekeeper attribution: Citrus honey)

Family/Type	% Pollen applying recalculation (A & B)	% Pollen applying recalculation (C)
Laboratory 1	Lab1	Lab1*
Brassicaceae	**53**	**37**
Olea	0	25
Citrus	**12**	**8**
Echium	**0**	**5**
Helianthus	6	4
Prunus	6	4
Hypericum	0	1

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Leguminosae/*Cytisus* sp., *Genista* sp.	3.6
Lotus sp.	1.3
Cruciferae/*Brassica* sp., *Raphanus* sp.	**31.4**
***Diplotaxis* sp.(Brassicaceae)**	**9.4**
Boraginaceae/*Echium* sp.	**6.7**
Rosaceae/*Prunus* sp.(including *Crataegus* sp.)	15.2
Compositae/*Centaurea* sp.	<1
Carduus sp., *Galactites* sp.	1.3
Helianthus annuus, C. arvensis	4.5
Rutaceae/*Citrus* sp.	**16.1**
Myrtaceae/*Eucalyptus* sp.	1.3
Oxalidaceae/*Oxalis* sp.	6.7

	% Pollen applying recalculation (A)
Laboratory 3	Lab3
Brassicaceae	**42**
Citrus	**18**
Echium	**13**
Asteraceae	5
Pyrus/Prunus	5
Arecaceae	4

	% Pollen applying recalculation (A)
Laboratory 4	Lab4
***Citrus* sp.**	**9**
***Echium* sp.**	**20**
Brassicaceae	**42**
other: *Prunus* dulcis, Leguminosae, *Helianthus annuus*, Umbelliferae, *Rosmarinus officinalis*	29

SAMPLE 6 (Beekeeper attribution: Eucalyptus honey)

Family/Type	% Pollen applying recalculation (A & B)	% Pollen applying recalculation (C)
Laboratory 1	Lab1	Lab1*
***Eucalyptus* sp.**	**99**	**68**
***Castanea sativa* (Chestnut)**	**0**	**31**

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Leguminosae/*Cytisus* sp., *Genista* sp.	1.9
Fagaceae/*Castanea sativa*	**14.6**
Cruciferae/*Brassica* sp., *Raphanus* sp.	1
Cruciferae/*Diplotaxis* sp.	1
Borraginaceae/ *Echium* sp.	1.9
Rosaceae/*Prunus* sp.(including *Crataegus* sp.)	1.5
Compositae/*Taraxacum* sp.	1
Myrtaceae/*Eucalyptus* sp.	**75.7**

	% Pollen applying recalculation (A)
Laboratory 3	Lab3
Eucalyptus	**79**
Castanea	**17**

	% Pollen applying recalculation (A)
Laboratory 4	Lab4
***Eucalyptus* sp.**	**77**
Castanea sativa	**17**
other: *Astragalus* sp., Liliaceae, Brassicaceae, *Rosmarinus officinalis*	6

SAMPLE 7 (Beekeeper attribution: Thyme honey)

Family/Type	% Pollen applying recalculation (A & B)	% Pollen applying recalculation (C)
Laboratory 1	Lab1	Lab1*
Cistaceae	0	24
***Thymus* (Thyme)**	**47**	**29**
Onobrychis (Sainfoin)	5	3
Ericaceae	14	8
Quercus ilex (Holm Oak)	0	15
Brassicaceae (Crucifers)	**7**	**4**
Genista (Petty Whin)	4	3
Ceratonia (St.John's Bread)	5	3

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Ericaceae/*Erica* sp.	9.5
Leguminosae/*Cytisus* sp./*Genista* sp.	31.7
Leguminosae/ *Vicia* sp.	9
Cruciferae/*Brassica* sp., *Raphanus* sp.	**9**
Lamiaceae/*Rosmarinus officinalis*	3.6
Lamiaceae/ *Thymus* sp.	**21.7**
Lamiaceae /*Lavandula* sp.	1.8
Rosaceae/*Prunus* sp.(including *Crataegus* sp.)	5
Compositae/*Centaurea* sp.	1.8
Rutaceae/*Citrus* sp.	1.4
other	1.8
Caryophyllaceae/*Silene* sp.	3.6

	% Pollen applying recalculation (A)	
Laboratory 3	Lab3	Lab4
***Thymus* (Thyme)**	**45**	
Ericaceae	6	
Echium	5	
Asteraceae	3	
Brassicaceae	**3**	
Onobrychis	3	
Citrus	3	

	% Pollen applying recalculation (A)
Laboratory 4	Lab4
***Thymus* sp.**	**37**
Rosmarinus officinalis	2
Brassicaceae	**5**
other: *Onobrychis* sp., *Borago* sp., *Citrus* sp., *Anthyllis* sp., Leguminosae, *Echium* sp.	56

(Beekeeper attribution: Raspberry honey)

SAMPLE 8

Family/Type	% Pollen applying recalculation (A & B)
Laboratory 1	Lab1
***Rubus* (Raspberry)**	**73**
Brassicaceae (Crucifers)	**11**
Trifolium repens (White Clover)	5
Apiaceae (Umbellifers)	2
Salix sp. (Willow)	5

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Leguminosae/*Medicago* sp.	3.4
Leguminosae/*Cytisus* sp./*Genista* sp.	18.6
Leguminosae/*Sophora* sp.	14
Cruciferae/*Brassica* sp., *Raphanus* sp.	**3**
Cruciferae/*Diplotaxis* sp.(Brassicaceae)	**8.5**
Rosaceae/*Rubus* sp.	**44.5**
Rosaceae/*Prunus* sp.(including *Crataegus* sp.)	3.4
Salicaceae/*Salix* sp., *Populus* sp.	1.3
other	3.4

	% Pollen applying recalculation (A)	
Laboratory 3	Lab3	
Rubus	**63**	
Brassicaceae	**15**	

	% Pollen applying recalculation (A)
Laboratory 4	Lab4
***Rubus* sp.**	**59**
Brassicaceae	**8**
other: *Salix* sp., Leguminosae	33

SAMPLE 9 (Beekeeper attribution: Acacia honey)

Family/Type	% Pollen applying recalculation (A & B)
Laboratory 1	Lab1
***Robinia pseudoacacia* (False Acacia)**	**42**
Salix sp. (Willow)	17
Amorpha fruticosa (false Indigo)	15
Gleditsia (Honey locust)	3
Brassicaceae (Crucifers)	**5**
Pirus/Prunus (Fruit Blossom)	6
Rubus (Raspberry)	2

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Leguminosae/*Cytisus* sp./*Genista* sp.	14
Leguminosae/*Robinia pseudoacacia*	**14**
Leguminosae/*Sophora* sp.	34.9
Cruciferae/ *Brassica* sp., *Raphanus* sp.	**3.3**
Rosaceae/*Rubus* sp.	6
Rosaceae/*Prunus* sp.,including *Crataegus* sp.)	16.7
Compositae/*Taraxacum* sp.	2.3
Celastraceae	3.3
other	5.6

Family/Type	% Pollen applying recalculation (A)
Laboratory 3	Lab3
Robinia	**26**
Amorpha	12
Prunus	10
Salix	7
Gleditsia	5
Acer	5
Brassicaceae	**4**

Family/Type	% Pollen applying recalculation (A)
Laboratory 4	Lab4
Robinia pseudoacacia	**29**
Brassicaceae	**5**
Helianthus annuus	9
other: Asteraceae, *Prunus* sp.	57

SAMPLE 10 Beekeeper attribution: Coriander honey

Family/Type	% Pollen applying recalculation (A & B)
Laboratory 1	Lab1
Coriandrum sativum	**31**
Brassicaceae (Crucifers)	**17**
***Helianthus* (Sunflower)**	**23**
Cyanus (Cornflower)	11
Robinia pseudoacacia (False Acacia)	7

Family/Type	% Pollen applying recalculation (A)
Laboratory 2	Lab2
Leguminosae/*Lotus* sp.	2
Leguminosae/*Robinia* pseudoacacia	3.5
Leguminosae/*Sophora* sp.	5
Cruciferae/*Brassica* sp., Raphanus sp.	**54**
Borraginaceae/*Echium* sp.	1.5
Rosaceae/*Prunus* sp.(including *Crataegus* sp.)	7
Compositae/*Centaurea* sp.	1.2
Compositae/*Helianthus annuus*/C. arvensis	**12.4**
Umbelliferae/ *Foeniculum* (same type *Coriandrum*)	**8.7**
Polygonaceae/*Fagopyrum* sp.	3.5
other	1.2

Family/Type	% Pollen applying recalculation (A)
Laboratory 3	Lab3
Coriandrum sativum	**26**
Helianthus	**23**
Brassicaceae	**17**
Robinia	7
Centaurea cyanus	6
Lotus	4

Family/Type	% Pollen applying recalculation (A)
Laboratory 4	Lab4
Umbelliferae (*Coriandrum sativum*)	**29**
Brassicaceae	**5**
Helianthus annuus	**9**
other: *Centaurea cyanus*, *Robinia pseudoacacia*, *Lotus* sp., *Tilia* sp.	57

SAMPLE 11 — Beekeeper attribution: Rape honey

Family/Type	% Pollen applying recalculation (A & B)	Family/Type	% Pollen applying recalculation (A)
Laboratory 1	Lab1	**Laboratory 2**	Lab2
Brassicaceae (Crucifers)	**99**	Leguminosae/*Sophora* sp.	1.3
		Cruciferae/*Brassica* sp./ *Raphanus* sp.	**95.1**
		Salicaceae/*Salix* sp./*Populus* sp.	1.3
		other	1.3
	% Pollen applying recalculation (A)		**% Pollen applying recalculation (A)**
Laboratory 3	Lab3	**Laboratory 4**	Lab4
Brassicaceae	**99**	**Brassica sp.**	**99**
		other: *Prunus* sp.	1

SAMPLE 12 — (Beekeeper attribution: Linden honey)

Family/Type	% Pollen applying recalculation (A & B)	Family/Type	% Pollen applying recalculation (A)
Laboratory 1	Lab1	**Laboratory 2**	Lab2
Tilia (Linden)	**41**	Leguminosae/*Cytisus* sp., *Genista* sp.	11.1
Helianthus (Sunflower)	**27**	Leguminosae/*Sophora* sp.	3.1
Trifolium repens (White Clover)	10	Borraginaceae/*Echium* sp.	7.6
		Rosaceae/*Prunus* sp.(including *Crataegus*	
Rubus (Raspberry)	5	sp.)	4.4
Serratula (Thistle), Brassicaceae (Crucifers)	3	Compositae/*Carduus* sp., *Galactites* sp.	2.2
		Compositae/*Helianthus annuus*/C. arvensis	**15.6**
		Malvaceae/*Tilia* sp.	**53.3**
		other	2.7
	% Pollen applying recalculation (A)		**% Pollen applying recalculation (A)**
Laboratory 3	Lab3	**Laboratory 4**	Lab4
Tilia	**59**	***Tilia sp.***	**38**
Helianthus	**28**	***Helianthus annuus***	**35**
		Rubus sp.	2
		other: Brassicaceae, *Thymus* sp., *Centaurea* sp., *Robinia pseudoacacia*	25

SAMPLE 13 — Beekeeper attribution: Sunflower honey

Family/Type	% Pollen applying recalculation (A & B)	Family/Type	% Pollen applying recalculation (A)
Laboratory 1	Lab1	**Laboratory 2**	Lab2
***Helianthus* (Sunflower)**	**92**	Leguminosae/*Sophora* sp.	8.2
Brassicaceae (Crucifers)	3	Rosaceae/*Rubus* sp.	3.1
Apiaceae (Umbellifers)	1	Compositae/*Centaurea* sp.	4.1
		Compositae/*Helianthus annuus*/C. arvensis	**39.8**
		Foeniculum sp.	6.1
		Caprifoliaceae/*Lonicera* sp.	5.1
		Polygonaceae/*Fagopyrum* sp.	24.5
		Malvaceae/*Tilia* sp.	4.1
		other	5.1
	% Pollen applying recalculation (A)	**Laboratory 4**	**% Pollen applying recalculation (A)**
Laboratory 3	Lab3		Lab4
Helianthus	**92**	**Helianthus annuus**	**81**
		other: Brassicaceae, Umbelliferae, Type *Taraxacum*, *Ambrosia* sp., *Zea mays*	19

that the higher the frequency of a pollen specie the better the repeatability and reproducibility of the results provided by experienced pollen analysts. However, this trend was not always observed in all laboratories e.g. *Helianthus annus* in sample 13 (92%, 39.8%, 92%, 81%). The opposite usually happens when these frequencies are very low as is the case, among others, for *Citrus* sp. or *Lavandula latifolia* (Von Der Ohe et al., 2004).

Fig. 1 also shows that not only differences in the percentages of the pollen species that would define the monoflorality of the honey (according to the information declared by the beekeeper) were found. Also, other dissimilarities have been observed in the percentage of the other accompanying pollens. Obviously, the case of these pollens itself is not

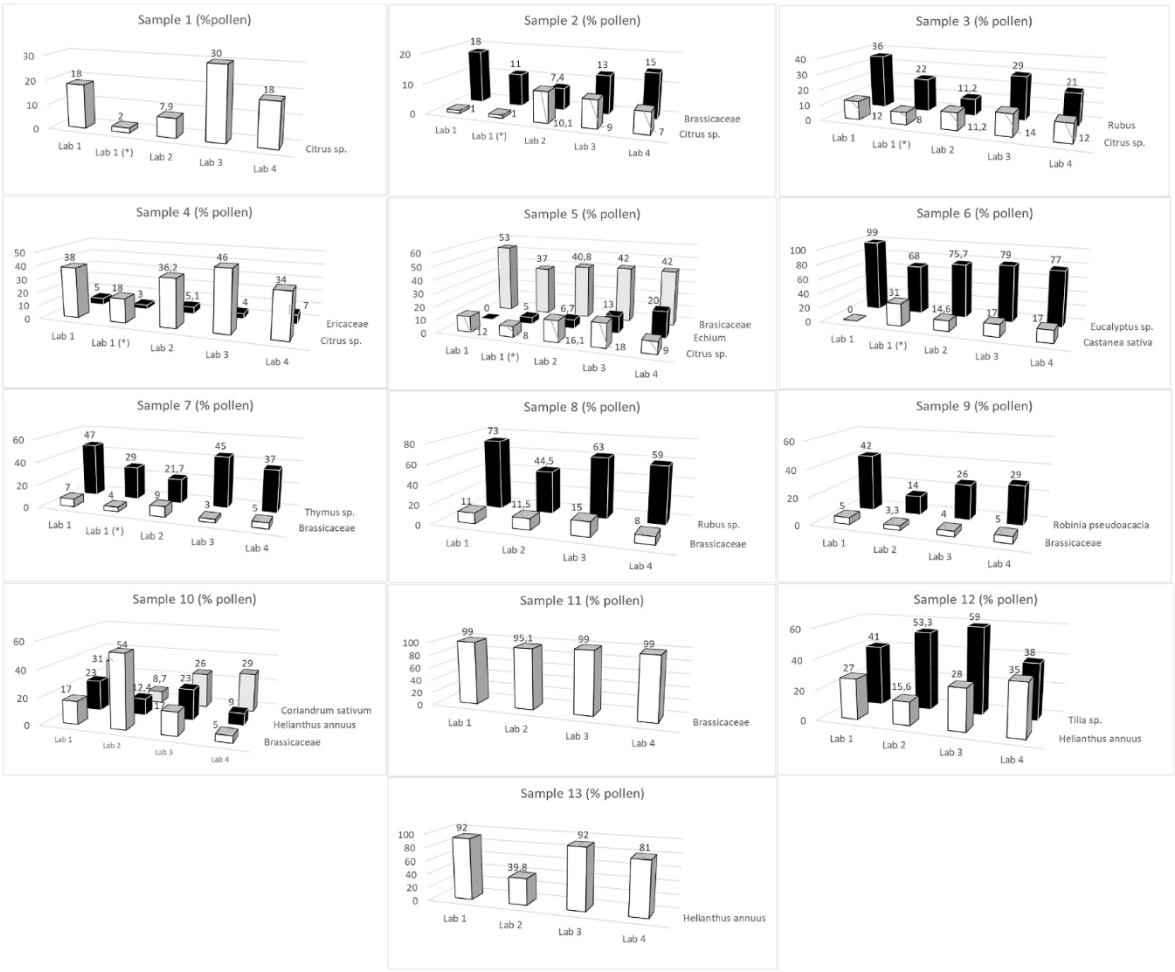

Fig. 1. Percentage of the pollen types identified by all four accredited laboratories in the 13 honey samples. [Type of recalculation applied to determine the percentage of pollen: A (Discarding the pollens from non-melliferous plants), B (Discarding the pollens from over-represented species although they come from melliferous plants but are not in enough quantities to define a specific monoflorality), C (all the pollens observed in the slide are considered in the count both from melliferous and non-melliferous plants). Laboratory codes and recalculation criteria: Lab1 (A + B); Lab1* (C); Lab 2(A), Lab 3(A) and Lab 4(A). Lab 4 = LABMIEL where this study was carried out.].

relevant, but this situation could somehow influence the percentage of target pollen which defines the monoflorality. In other words, since the result of each taxon is expressed as the percentage of the total pollen taxa, these values are interdependent. A clear example of this is observed in sample 10, where the reported percentage of Brassicaceae varies from 5% (Lab 4) to 54% (Lab 2), causing the target pollen (*Coriandrum sativum*) to vary from 8.7% to 31% between these two laboratories. Another example is shown in sample 6 in which, as mentioned before, the accompanying pollen *Castanea sativa* (varies from 0% to 17%) definitively affects the assignation of the percentage value of the main pollen *Eucalyptus* sp. (from 75.7% to 99%) and therefore its monoflorality.

As it has been observed in the present work and was reported by others authors the simultaneous presence of under-represented and over-represented grains of pollen in honeys may frequently lead to misleading results and discrepancies among the reports provided among laboratories (Rodopoulou et al., 2018). A greater problem occurs for honeys with under-represented pollens, of commercial importance such as thyme or citrus honey, in which the inconsistencies could exist among experts to the point that the same batch can be considered monofloral or not depending on the laboratory that issues the report (Rodopoulou et al., 2018). With this respect, when a high percentage of over-represented pollen is present, the International Honey Commission recommends to carry out a second count, ignoring the over-represented pollen to determine more exactly the relative abundance. However, they do not provide further details regarding the percentage that should be taken into consideration. (Von Der Ohe et al., 2004). Some authors also consider that in the case of presence of over-represented pollens, they should be excluded from the count when are present in percentages of up to 30% (Rodopoulou et al., 2018).

It is evident that the melisopalinological analysis presents important difficulties, both in the realization and subsequent interpretation, currently a necessary step to accurately catalogue the monofloral honeys. This fact represents serious damage o the beekeeping sector, since the price of monofloral honeys (higher than the cost of the polyflorals) is agreed in commercial transactions based mainly on the pollen content. It could be that for the same batch of honey, the buyers and sellers have different findings from two different accredited laboratories. This situation can lead to important economic inconveniences, both for beekeepers and marketers.

4. Conclusion

It has been proven that important complications arise when analysing pollen in honey even when it is carried out by laboratories accredited in this methodology. The problem is more evident when the target pollen is under-represented and there is a presence of over-represented pollens. It is true that this analytical method is

conditioned by the obvious variability of any analytical technique, but even more because it is a manual process. However, only technicians with proven experience in pollen analysis and with adequate knowledge of the flora of the area where the honey is collected, can provide reliable results. Furthermore, there is a lack of standardized criteria that implies additional difficulties for the expert technicians to provide consistent results. The honey pollen analyst is alone when interpreting a sample, using disparate internal laboratory procedures that lack international regulation. Needless to say, there is an urgent need to unify and establish harmonized criteria outlining the analysis protocols, both the denomination of pollen types and the guidelines for counting. This standardization is of interest for all parties involved (beekeepers, traders, researchers, laboratories) and subsequently, it will be the final customer who will be best served, and it will ensure a fair commercial transaction of monofloral honeys.

The intention of this work is not to judge the pollen analysis carried out by accredited laboratories nor question their ability but to give visibility to this anomalous situation and to encourage all the stakeholders (regulatory bodies, scientists, accredited laboratories, etc.) to work together and develop the necessary international regulations to which all can adhere to. Furthermore, it would be advisable to propose complementing the pollen quantification with analyses of both sensory and certain physicochemical parameters, including marker volatile compounds to correctly attribute the monoflorality, especially to honeys with pollen under-represented.

CRediT authorship contribution statement

Isabel Escriche: Funding acquisition, Project administration, Conceptualization, Supervision, Writing – review & editing. **Marisol Juan-Borrás:** Validation, Formal analysis, Writing – original draft. **Mario Visquert:** Investigation, Formal analysis, Writing – original draft. **José Miguel Valiente:** Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the present work.

Data availability

The data that has been used is confidential.

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