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Volatile markers as a reliable alternative for the correct classification of citrus monofloral honey

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ABSTRACT

The pollen analysis to classify monofloral honey is an unresolved challenge specially when the pollen is under-represented as the case of citrus honey. Thus, this study assesses the validity of the volatile fraction to differentiate types of honey, with special attention to markers compounds of citrus honey that could permit their distinction. Unsupervised analysis (PCA and HCA) showed that the volatile fraction of honey containing *Citrus* sp. pollen, undoubtedly differentiates it from other types of honey. An OPLS model focused on citrus honey selected 5 volatile compounds (of the 123 found in all samples by GC–MS) as significant predictors of the currently used value of methyl anthranilate obtained by HPLC. The joint detection of 4 lilac-aldehydes and the volatile methyl-anthranilate has the advantage of providing more precise information. Therefore, it could be proposed as a consistent marker to ensure the correct classification of citrus honey, fostering its labelling reliability.

1. Introduction

Agri-food fraud continues to be of great concern in the EU, with a 20% increase in cases compared to 2019 as reflected in the 2020 Annual report "*The EU Agri-Food Fraud Network and the Administrative Assistance and Cooperation System*" (European Union Commission, 2021). In this document, honey continues to be one of the most affected food categories after fats/oils and fish/fishery products with 51%, 34% and 25%, respectively. This situation is reflected in the annual reports of the Rapid Alert System for Food and Feed and specially in emerging situations, such as the COVID-19 pandemic and Brexit (Brooks, et al., 2021) where, for the specific case of honey, the notifications are mostly due to the incorrect labelling. Consequently, the consumer may in fact feel cheated or misinformed with what it is shown on the label (Machado et al., 2022). Sometimes fraud can be "easily recognized" when there is a legislation or regulation in place. An example of this may be the regulation adopted by some nations that requires detailing on the packaging all the countries of origin where the honey comes from (B.O.E., 2020). However, oddly enough, there are other aspects included on the label, such as the monoflorality (of great commercial importance), where the consumer must accept the given without the support of an international standardized regulation, with exceptions of local regulations or quality marks (D.O.G.V., 2002). To make matters worse, the procedure for the monofloral classification of a honey is an unresolved challenge. This is because the melissopalynological analysis, by optical microscopy, which it is carried out is far from being considered a routine method. This method based on the identification and quantification of the pollen grains of the different botanical species, is laborious, tedious, time consuming and requires expert analysts making its analysis extremely complex, resulting in its high price in accredited laboratories. Furthermore, this technique has a high level of subjectivity making analysis even more complicated in some monofloral honeys in which the target pollen is under-represented such as citrus honey. For this reason, the search for objective techniques to find a true definition of a monofloral honey is more than a challenge, it is a necessity. This would provide regulatory agencies, beekeepers and agents involved in commercial transactions with an objective tool that would easily clarify the uncertainties associated with the incorrect classification of the monofloral honeys. Furthermore, it will ensure the application of correct labeling procedures and therefore, more transparency for consumers. Therefore, it is advisable to focus the efforts on the optimization of objective analytical techniques that could unequivocally define its monoflorality (Karabagias, et al., 2020).

Honey is a very complex matrix that contains a vast number of

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Table 1
Detailed information of the pollen analysis of honey samples including the percentage of the main pollen and other accompanying pollens that are also present in the samples. Sample codes were assigned considering the first three letters and its corresponding percentage of the most abundant pollen followed by the three letters of the second most abundant pollen present.

Sample Codes	Detailed information of the pollen analysis
	Main pollen: *Citrus* sp. (Methylantranilate presence confirmated**)**
Cit 5-Bras	5% *Citrus* sp., 18% Brassicaceae, Umbeliferae, *Echium* sp., *Prunus dulc*is, *Rosmarinus officinalis*.
Cit 8-Bras	8% *Citrus* sp., 12% Brassicaceae, Umbelliferae, *Helianthus annuus*, *Prunus dulcis*, *Rosmarinus officinalis.*
Cit 24-Bras	24% *Citrus* sp., 19% Brassicaceae, *Echium* sp., *Castanea sativa*, *Taraxacum* type, Leguminosae, Umbelliferae, *Prunus dulcis*, Ericaceae.
Cit 10-Bras	10% *Citrus* sp., 8% Brassicaceae, *Echium* sp., *Rosmarinus officinalis*, *Taraxacum* type, *Prunus dulcis.*
Cit 13-Bras	13% *Citrus* sp., 10% Brassicaceae, *Echium* sp., *Prunus dulcis*, *Anthyllis* sp.
Cit 7-Prun	7% *Citrus* sp., 5% *Prunus dulcis*, *Echium* sp., *Rosmarinus officinalis*, *Castanea sativa*, *Anthyllis* sp., *Salix* sp., *Olea europaea*, *Thymus* sp., Brassicaceae, *Carduus* type, Leguminosae.
Cit 16-Ech	16% *Citrus* sp., 21% *Echium* sp., Umbelliferae, *Eucalyptus* sp., Palmaceae, *Helianthus annuus*.
Cit 6-Ech	6% *Citrus* sp., 14% Echium sp., Rosaceae, Umbelliferae, *Eucalyptus* sp., Palmaceae, Brassicaceae, *Taraxacum* type, *Helianthus annuus*, *Rosmarinus offi*cinalis, Leguminosae.
Cit 3-Ech	3% *Citrus* sp., 31% *Echium* sp., *Helianthus annuus*, Brassicaceae, *Taraxacum* type, *Carduus* type, *Lavandula stoechas*.
Cit 20-Bras	20% *Citrus* sp., 18% Brassicaceae, *Taraxacum* type, *Echium* sp., *Prunus dulcis*, Leguminosae, *Rosmarinus officinalis*, *Erica* sp., Umbelliferae.
Cit 15-Bras	15% *Citrus* sp., 21% Brassicaceae, *Ceratonia siliqua*, *Lavandula stoechas*, *Prunus dulcis*, *Rosmarinus officinalis,* Leguminosae, *Thymus* sp., *Taraxacum* type, *Helianthus annuus*, *Carthamus sp*., *Erica* sp., Rosaceae.
Cit 7-Bras	7% *Citrus* sp., 11% Brassicaceae, *Taraxacum* type, *Echium* sp., *Prunus dulcis*, Leguminosae, *Rosmarinus officinalis*, *Erica* sp., Umbelliferae.
Cit 25-Ech	15% *Citrus limon*, 10% *Citrus* sp., 26%*Echium* sp., Brassicaceae, *Anthyllis* sp., Palmaceae, *Ceratonia siliqua*, *Taraxacum* type, *Carduus* type.
Cit 3-Eric	3% *Citrus* sp., 12% *Erica* sp., Leguminosae, *Hypecoum* sp., *Prunus dulcis*.
Cit 8-Bras	8% *Citrus* sp., 23%Brassicaceae, *Taraxacum* type, Leguminosae, *Prunus dulcis*, *Rosmarinus officinalis*, *Carduus* type.
Cit 16-Gen	16% *Citrus* sp., 9% *Genista* type, Brassicaceae. Palmaceae, *Taraxacum* sp.
Cit 12-Bras	12% *Citrus* sp., 8% Brassicaceae, *Taraxacum* sp.
Cit 42-Ech	42% *Citrus* sp., 15% *Echium* sp., Leguminosae
Cit 18-Umb	18% *Citrus* sp., 11% Umbelliferae, *Pistacia* sp., *Quercus* sp., *Olea* sp., *Echium* sp., *Erica* sp., *Hieracium* sp., *Taraxacum* sp., *Echium* sp.
Cit 2-Ech	2% *Citrus* sp., 27% *Echium* sp., *Helianthus annuus*, Brassicaceae, Leguminosae, *Eucalyptus* sp., *Erica* sp., Umbelliferae, *Carduus* type.
Cit 1-Ech	1% *Citrus* sp., 29% *Echium* sp., Brassicaceae, Leguminosae, *Erica* sp.
	Main pollen: *Helianthus annuus*
Hel 18-Xan	18% *Helianthus annuus*, 7% *Xanthium* sp., *Onobrychis* sp., Leguminosae, *Thymus* sp.
Hel 34-Ono	34% *Helianthus annuus*, 4% *Onobrychis* sp., *Thymus* sp., *Xanthium* sp., Leguminosae, *Centaurea cyanus.*
Hel 35-Ono	35% *Helianthus annuus*, 8% *Onobrychis* sp., *Thymus* sp., *Xanthium* sp., Leguminosae
Hel 37-Ono	37% *Helianthus annuus*, 9% *Onobrychis* sp., Leguminosae, *Thymus* sp., *Xanthium* sp., Centaurea cyanus
Hel 17-Xan	17% *Helianthus annuus*, 9% *Xanthium* sp., *Onobrychis* sp., Leguminosae, *Thymus* sp.
Hel 30-Lav	30% *Helianthus annuus*, 2% *Lavandula latifolia*, *Taraxacum* type, *Genista* type, *Thymus* sp., *Centaurea* sp., Brassicaceae, Liliaceae, *Prunus* sp., *Rosmarinus officinalis*, *Citrus* sp.
Hel 31-Leg	31% *Helianthus annuus*, 12% Leguminosae, *Xanthium* sp., *Onobrychis* sp., *Thymus* sp., Brassicaceae, Rosaceae, Asteraceas, Labiatae
	Main pollen: *Lavandula latifolia*
Lav 2-Hel	2% *Lavandula latifolia*, 8% *Helianthus annuus*
Lav 7-Car	1% *Lavandula latifolia*, 8% *Carduus* sp., *Rubus* sp., *Cytisus* sp., *Helianthus annuus*, *Taraxacum* type.
Lav 5-Hel	5% *Lavandula latifolia,* 5 *% Helianthus annuus, Rubus* sp., *Rosmarinus officinalis*
Lav 8-Hel	1% *Lavandula latifolia,* 3% *Helianthus annuus*
	Main pollen: *Echium* sp.
Ech 63-Hel	63% *Echium* sp., 6% *Helianthus annuus*, Leguminosae, *Erica* sp., *Rubus* sp., *Centaurea* sp.
Ech 53-Hel	53% *Echium* sp., 6% *Helianthus annuus*, Leguminosae, *Erica* sp., *Rubus* sp., *Centaurea* sp.
Ech 58-Hel	58% *Echium* sp., 13% *Helianthus annuus*, 2% *Lavandula latifolia*, *Genista* type, *Ceratonia siliqua*, Brassicaceae, *Thymus* sp., *Taraxacum* type, *Erica* sp., *Prunus dulcis*, *Carthamus* sp.
Ech 50-Hel	50% *Echium* sp., 17% *Helianthus annuus*, 3% *Lavandula latifolia*, *Ceratonia siliqua, Trifolium* sp., *Genista* type, *Thymus* sp., *Erica* sp., *Carthamus* sp., *Lavandula stoechas*
Ech 58-Lav	58% *Echium* sp., 1% *Lavandula latifolia, Rubus* sp., *Carthamus* sp., *Thymus* sp., *Lavandula stoechas*, *Helianthus annuus*
Ech 49-Rub	49% *Echium* sp., 25% *Rubus* sp., 1% *Lavandula latifolia,* Leguminosae, *Rosmarinus officinalis, Erica* sp., *Thymus* sp., *Lavandula stoechas,* Brassicaceae, *Prunus dulcis, Helianthus annuus*
Ech 30-Hel	30% *Echium* sp., 19% *Helianthus annuus,* Leguminosae, *Erica* sp., *Lavandula latifolia*
Ech 83-Hel	83% *Echium* sp., 6% *Helianthus annuus,* Brassicaceae, Umbelliferae, *Lavandula stoechas,* Asteraceae, *Prunus dulcis, Centaurea jacea*
Ech 89-Hel	*89% Echium* sp., *5%* Brassicaceae, *Carduus type, Erica* sp., *Prunus dulcis, Helianthus annuus,* Umbelliferae, *Lavandula latifolia*
Ech 72-Hel	72% *Echium* sp., 4% *Helianthus annuus,* Umbelliferae, Brassicaceae, *Trifolium* sp., Asteraceae, *Erica* sp.
Ech 71-Ros	71% *Echium* sp., 9% *Rosmarinus officinalis, Anthyllis* sp., Brassicaceae, *Lavandula stoechas, Thymus* sp., *Helianthus annuus, Prunus dulcis*
Ech 71-Hel	71% *Echium* sp., 9% *Helianthus annuus, Trifolium* sp., *Lavandula stoechas, Carduus type, Campanula* sp., *Centurea cyanus, Eryngium* sp., *Carduus type*
Ech 80-Lav	80% *Echium* sp., 1% *Lavandula stoechas, Trifolium* sp., *Castanea sativa, Lotus* sp., *Carduus* type, *Taraxacum* type, *Eucalyptus* sp., *Genista* type
Ech 78-Eric	78% *Echium* sp., 2% *Erica* sp., *Trifolium* sp., *Castanea sativa, Lotus* sp., *Carduus* type, *Taraxacum* type, *Helianthus annuus.*
Ech 66-Hel	66% *Echium* sp., 1% *Helianthus annuus, Astragalus* sp., *Eucalyptus* sp., Umbelliferae, Brassicaceae, *Centaurea* sp., Leguminosae, *Prunus* sp.
Ech 56-Euc	56% *Echium* sp., 13% *Eucalyptus* sp., 4% *Helianthus annuus*, Brassicaceae, Umbelliferae, *Centaurea* sp., *Taraxacum* type, *Lavandula stoechas*, *Carduus* type
Ech 56-Bras	56% *Echium* sp., 12 % Brassicaceae
Ech 68-Lav	68% *Echium* sp., 6% *Lavandula stoechas,* Leguminosae, *Taraxacum* type, Brassicaceae, Umbelliferae, *Helianthus annuus, Carduus type, Trifolium* sp.
Ech 57-Hel	57% *Echium* sp., 8% *Helianthus annuus, Eucalyptus* sp., Brassicaceae, Leguminosae, *Lavandula stoechas, Castanea sativa, Citrus* sp.
Ech 93-Tri	93% *Echium* sp., 2% *Trifolium* sp., *Cytisus* sp., *Carduus* type, *Taraxacum* type, *Helianthus annuus, Lavandula stoechas,* Brassicaceae
Ech 72-Hel	72% *Echium* sp., 3% *Helianthus annuus,* 2% *Citrus* sp., Le, Leguminosae, *Eucalyptus* sp., *Erica* sp., Umbelliferae, *Carduus* type
	Main pollen: *Thymus sp*.
Thy 19-Ono	19% *Thymus* sp., 12% *Onobrychis* sp., *Centaurea cyanus, Genista* type, *Prunus* sp., *Hypecoum* sp., *Rosmarinus officinalis, Erica* sp., Brassicaceae, *Lavandula stoechas,* Leguminosae
Thy 22-Ono	22% *Thymus* sp., 20% *Onobrychis* sp., *Cytisus* sp., *Centaurea cyanus,* Leguminosae, Brassicaceae, *Erica* sp., *Rosmarinus officinalis,* Asteraceae
Thy 10-Ono	10% *Thymus* sp., 5% *Onobrychis* sp., *Vicia* type, Brassicaceae, *Cytisus* sp., *Centaurea cyanus, Prunus* sp., *Rosmarinus officinalis,* Leguminosae
Thy 7-Lav	7% *Thymus sp*., 2% *Lavandula latifolia*, 14% *Helianthus annuus*, Leguminosae, *Ceratonia siliqua*, *Rosmarinus officinalis*, Brassicaceae.
Thy 5-Lav	5% Thymus sp., 3% *Lavandula latifolia*, Leguminosae, *Helianthus annuus*, *Ceratonia siliqua*, *Rosmarinus officinalis*, Brassicaceae.
Thy 37-Rom	37% *Thymus* sp., 2% *Rosmarinus officinalis, Onobrychis* sp., Brassicaceae, *Borago* sp., *Citrus* sp., *Anthyllis* sp., Leguminosae, *Echium* sp.

(*continued on next page*)

Table 1 (*continued*)

Sample Codes	Detailed information of the pollen analysis
Thy 17-Ono	17% *Thymus* sp., 10% *Onobrychis* sp., Brassicaceae, *Rosmarinus officinalis,* Rosaceae, *Hypecoum* sp., *Taraxacum* type, Asteraceae, *Lavandula stoechas*
Thy 12-Lav	12% *Thymus* sp., *1% Lavandula latifolia, Echium* sp., *Hypecoum* sp., Brassicaceae, *Helianthus annuus,* Umbeliferae, *Citysus* sp., *Eucalyptus* sp., Leguminosae
Thy 4-Vic	4% *Thymus* sp., 2% *Vicia* type, *Echium* sp., Leguminosae, *Rosmarinus officinalis*, Brassicaceae, *Helianthus annuus, Centaurea cyanus, Castanea sativa, Lavandula latifolia, Carthamus* sp.
Thy 18-Lav	18% *Thymus* sp., 6% *Lavandula latifolia*, *Onobrychis* sp., *Rubus* sp., *Echium* sp., *Vicia* type, *Genista* type, *Erica* sp., *Carduus* type, *Prunus* sp.
Thy 8-Lav	8% Thymus sp., 2% *Lavandula latifolia, Carthamus* sp., *Cytisus* sp., *Helianthus annuus,* Rosaceae, *Onobrychis* sp., Leguminosae, Brassicaceae, *Centaurea* sp.
Thy14-Vic	14% *Thymus* sp., 7% *Tipo Vicia, 4% Carthamus* sp., *3% Lavandula latifolia, Onobrychis* sp., *Rosmarinus officinalis, Cytisus* sp., Leguminosae, *Helianthus annuus,* Brassicaceae, *Echium* sp., *Citrus* sp.
Thy 11-Lav	11% *Thymus* sp., 1% *Lavandula latifolia, Onobrychis* sp., Brassicaceae, Leguminosae, *Helianthus annuus, Carthamus* sp., *Rosmarinus officinalis, Centaurea cyanus,* Rosaceae **Main pollen: *Eucalyptus* sp.**
Euc 71-Ret	71% *Eucalyptus* sp., 9% *Retama sphaercarpa, Echium* sp., *Rosmarinus officinalis, Erica* sp., Umbelliferae
Euc 58-Ech	58% *Eucalyptus* sp., 3% *Echium* sp., *Lotus* sp., *Trifolium* sp., *Helianthus annuus, Anchusa* sp.
Euc 56-Leg	56% *Eucalyptus* sp., 16% Leguminosae
Euc 77-Cas	77% *Eucalyptus* sp., 17% *Castanea sativa*, *Astragalus* sp., Liliaceae, Brassicaceae, *Rosmarinus officinalis*
Euc 64-Bra	64% *Eucalyptus* sp., 10% Brassicaceae, *Salix* sp., *Thymus* sp., *Centaurea cyanus*, *Rosmarinus officinalis*, *Artemisia* sp., Umbeliferae, Asteraceae, *Hypecoum* sp., *Lotus* sp., *Echium* sp.
Euc 39-Ech	39% *Eucalyptus* sp., 23% *Echium* sp., 8% *Helianthus annuus*, Brassicaceae, *Trifolium* sp., Umbelliferae, *Lavandula stoechas*, *Taraxacum* type, Leguminosae, *Thymus* sp., *Erica* sp., *Xanthium* sp., *Fragaria vesca.*
Euc 26-Ech	26% *Eucalyptus* sp., 18% *Echium* sp., 3% *Helianthus annuus*, Umbeliferae, *Astragalus* sp., Brassicaceae, *Trifolium* sp., *Taraxacum* type, *Lavandula stoe*chas, *Carduus* type, Leguminosae, Rosaceae
Euc 50-Cas	50% *Eucalyptus* sp., 23% *Castanea sativa*, 4% *Helianthus annuus*, *Echium* sp., *Hypecoum* sp., Brassicaceae, *Prunus dulcis*, Asteraceae, Leguminosae. **Main pollen: *Rosmarinus officinalis***
Ros 32-Bra	32% *Rosmarinus officinalis,* 7% Brassicaceae, *Erica* sp., *Genista* type, *Ceratonia siliqua, Prunus dulcis, Hypecoum* sp., *Thymus* sp., *Citrus* sp.
Ros 12-Bra	12% Rosmarinus sp., 12% Brassicaceae.
Ros 17-Pru	17% *Rosmarinus officinalis*, 9% *Prunus dulcis*, 1% *Thymus* sp., *Echium* sp., Leguminosae, Brassicaceae, *Helianthus annuus,* Umbelliferae, *Lavandula stoechas*
Ros 17-Cer	35% *Ceratonia siliqua,* 17% *Rosmarinus officinalis*, Brassicaceae, *Erica* sp., *Prunus* type, *Helianthus annuus*, *Trifolium* sp., *Echium* sp.
Ros 12-Pru	32% *Prunus dulcis,* 12% *Rosmarinus officinalis, Anthyllis* sp., Brassicaceae, *Prunus* sp., Leguminosae, *Hypecoum* sp., Asteraceae
Ros 23-Thy	23% *Rosmarinus officinalis*, 9% *Thymus* sp., *Echium* sp., *Onobrychis* sp., Brassicaceae, *Prunus* sp., *Lavandula stoechas*, *Centaurea cya*nus, Asteraceae, *Lavandula latifolia*, *Citrus* sp.
Ros 22-Pru	22% *Rosmarinus officinalis*, 11% *Prunus dulcis*, Brassicaceae, Leguminosae, *Erica* sp., *Echium* sp., *Thymus* sp., *Eucalyptus* sp., Asteraceae
Ros 18-Pru	18% *Rosmarinus officinalis*, 10% *Prunus dulcis*, Brassicaceae, Leguminosae, Thymus sp., *Eucalyptus* sp., Asteraceae
Ros 28-Thy	28% *Rosmarinus officina*lis, 2% *Thymus* sp., Brassicaceae, *Anthyllis* sp., Leguminosae, *Erica* sp., *Lavandula stoechas*, *Prunus dulcis*, *Hypecoum* sp., *Trifolium* sp., *Citrus* sp.
Ros 93-Thy	93% *Rosmarinus officina*lis, 1% *Thymus* sp., Brassicaceae

compounds, including among others phenolics (Liang, et al., 2009) and volatiles (Escriche et al, 2017; Karabagias, et al., 2017). Many of these volatile compounds are characteristic of the basic aroma/flavor of this foodstuff and, therefore, are common in almost all types of honey. Besides, certain monofloral honeys have attributed characteristic odour and flavour nuances, that can be related to other own specific volatile compounds. Hence, it would be very interesting to find the appropriate markers that unmistakably define a precise monofloral honey; a task not resolved yet despite it has been the subject of research in recent years (Zhao, et al., 2022).

Methyl anthranilate is an important compound in the case of citrus honey, as it is found in the citrus blossom nectar. However, this compound is not considered in any legislation although it is used as a "bargaining chip" in the trade for this variety of honey, without a consensus of a minimum level required (Juan-Borrás, et al., 2015). It should also be noted that, despite the problems associated with the low presence of *Citrus* sp. pollen in citrus honey (being this type of pollen underrepresented), there is a double taxation in its commercial transaction requirements, demanding a minimum citrus pollen content (between 10 and 20%), together with a specific methyl anthranilate level (not lower than 2 mg kg^{-1}). This value of methyl anthranilate is commercially mandatory although it has been proven that citrus honeys from some countries usually do not reach this level (Papotti et al., 2009, Juan-Borrás, et al., 2015). Nevertheless, methyl anthranilate does not have to be the only useful compound to characterize citrus honey since in the organoleptic perception other sensory nuances are perceived different from those of this compound (da Costa, et al., 2018; Seraglio, et al., 2021). The issue is objectively discerning these compounds from the large number of those that may be present in the volatile fraction of any other honey. That is why the objective of this study is to estimate the validity of the volatile fraction in the differentiation of types of honeys, with special attention to citrus honey and the target compounds that could allow for their distinction.

2. Material and methods

2.1. Honey samples

A total of 104 different honeys were used in the present study. In 2020 and 2021, 84 samples were collected from different Spanish beekeepers. Honeys with predominant pollen of *Eucalyptus* sp. came from the North of the country, *Helianthus annuus, Lavandula latifolia, Echium* sp. and *Thymus* sp. from central Spain and those of *Rosmarinus officinalis* and *Citrus* sp. were harvested in the South and in the East. The latter having been provided by *Melazahar Cooperativa Apícola* and by experimental beehives located in citrus fields property of *Sant Vicent Ferrer de Benaguasil Cooperativa* in Pedralba (Valencian Region). The pollen content of all samples was analyzed in LABMIEL (*Quality Control of honey and Bee Products Laboratory of Universitat Politecnica de Valencia, Spain),* where the present study was carried out. This is an accredited laboratory in pollen analysis methodology as per ISO 17025 (ISO/IEC 17025:2017, 2017), in addition to being experienced in providing this type of service to companies of the beekeeping sector. Another set of 20 samples collected in 2022 were provided by the experimental beehives mentioned before and the Ministry of Spanish Agriculture and Fishing, Food and Environment, after they were classified as monofloral of citrus honey following the standard pollen criteria by the laboratory of this Ministry. The purpose of these last samples from 2022 was to verify the validity of what was observed for citrus honeys with the samples from 2020 and 2021. Both institutions have an agreement dealing with the characterization of the most important Spanish monofloral honeys (B.O.E. 2018).

The data of pollen analyses obtained by LABMIEL from each sample are shown in Table 1, which includes the information corresponding to the percentage of the main pollen, together with other accompanying pollens that are also present in the samples. Each sample has been assigned a code considering the first three letters and its corresponding

percentage of the most abundant pollen followed by the three letters of the second most abundant pollen present. For example, the code Euc 71-Ret, can be read as "Euc" refers to *Eucalyptus* sp., 71 represents the percentage of this pollen and "Ret" represents *Retama sphaercarpa*. In this table it can be seen as: Euc 71-Ret (71% *Eucalyptus* sp., 9% *Retama sphaerocarpa*, *Echium* sp., *Rosmarinus officinalis, Erica* sp., Umbelliferae). The 84 honey samples analysed were classified into seven groups considering the criterion of the predominant pollen present in each of them. The only exception was for the groups *Citrus* sp. and *Lavandula stoechas,* which received special consideration since these pollens are underrepresented. This is mainly due to the extended use of hybrid varieties of citrus trees and lavender plants, which are characterized by their small amounts of pollen production (Persano-Oddo and Piro, 2004; Escriche et al., 2011; Juan-Borrás et al., 2015; Escriche et al., 2017).

In the specific case of *Citrus* sp. group, in this study, all the honey samples in which this type of pollen was present were included along with the confirmation of the presence of methyl anthranilate analysed by HPLC (MA-HPLC) at whatever level. Referring to *Lavandula stoechas* group, honeys that contained this pollen were included. However, in most cases very little quantity of this type of pollen was observed (although all these honeys had the typical organoleptic characteristics of this type of honey). With all this, in no way do these seven groups represents the monoflorality of the samples, their only purpose (based on precise criteria) is to assign each sample a reference code.

2.2. Melissopalynological analysis

In LABMIEL, the pollen analysis was performed following the International Commission for Bee Botany recommendations (Von der Ohe, et al., 2004). The specific method is detailed by Escriche, et al. (2023). Briefly, after dissolving the honey in acidulated water, centrifuging twice, and decanting the supernatant, a small amount of the precipitate was placed on a slide, dried and sealed with glycerin. At least 500 pollen grains were counted, at magnifications of × 400–1000, in an optical microscope (Zeiss Axio Imager, Göttingen, Germany) with Axiocam 305 Color, Zeiss camera. The analyst identified and attributed the different pollen grains morphology to a specific botanical species (Carretero, 1989; Saenz-Laín & Gómez-Ferreras, 2000). After, the percentage of each type of pollen was calculated considering the total number of pollen grains counted.

A specific image software (HoneyApp), engineered by the Institute of Industrial Computing and Control Systems (AI2) at the Univèrsitat Politècnica de València, was used to assist in the labelling and annotation of the different pollen grains.

2.3. Methyl anthranilate analysis

Methyl anthranilate was analyzed and validated by HPLC as described by Juan-Borrás et al., 2015. An acid hydrolysis, followed by a solid phase extraction with copolymer cartridges (500 mg, 6 mL) Extrabond Polymeric EBH (Scharlab, Spain) was performed. The HPLC analysis was conducted using an Agilent 1100 series HPLC system equipped with: auto-sampler, inline degasser, quaternary pump, diode-array detector (DAD) and the ChemStation software. The mobile phase was water MilliQ quality (solvent A) and acetonitrile HPLC-Grade Prolabo VWR, Darmstadt, Germany (solvent B). The gradient was: an isocratic step with 30% B (from 0 to 3.1 min), then 42% B at 5.5 min, 90% B (2 min), and the re-equilibration of the column in 3 min. A flow rate of 1.0 mL/min was applied and 5 μL of sample were injected. The chromatographic separation was performed using a Kinetex C18 column (150 × 4.6 mm, 5 μm) (Phenomenex) at 30 °C. The methyl anthranilate (purity > 99%, Merck, Darmstadt, Germany) was monitored at 335 nm. Quantification was performed applying matrix calibration curves obtained from spiked fortified blank samples, that is, the standards were added into the matrix before the extraction. The blanks were polyfloral samples honey with proved absence of methyl anthranilate and *Citrus* sp. pollen.

Before each batch of samples, a quality control was injected (a spiked blank sample with a final concentration of 2 mg/kg) to check the quality of the results as well as the stability of the analytical procedure. The stock standard solution of methyl anthranilate (1 mg/mL) and the working standard solution (0.1 mg/mL in H_2SO_4 1 M) were stored at −20 °C + 4 °C, respectively.

2.4. Volatile compounds analysis

The analysis was carried out using solid-phase micro-extraction (SPME) and gas chromatography/mass spectrometry (GC/MS) as described by Escriche et al. (2021). The honey volatile compounds were trapped (30 min, 50 °C and 250 rpm) in a fiber (DVB/CAR/PDMS, divinyl benzene/carboxen/polydimethylsiloxane, 50/30 μm), Subsequently, the fiber was placed into the injection port of the GC/MS chromatograph (Agilent Intuvo 9000 gas chromatograph coupled to an Agilent 7000 Series GC/TQ triple quadrupole detector and an electron ionization source at 70 eV) with a DB WAX column (Agilent HP-5MS, 30 m × 0.25 mm × 0.25 μm). The mass spectra were acquired in TIC mode (mass range *m/z* 40–280). The MassHunter Workstation software was used for the data acquisition and analysis.

The identification of the compounds was done considering: mass spectra, retention times, calculated relative retention indices (RI) and where possible authentic standards (Sigma-Aldrich, St Louis, MO; Acros Organics, Geel, Belgium and Fluka Buchs, Switzerland). When authentic standards were not available, a tentative identification was carried out by comparing their mass spectra (considering at least a match factor 80%) with the spectral data from the National Institute of Standards and Technology 2002 library, the linear retention indices and the data published in the literature (Shimoda, et al., 1996; Bianchi, et al., 2007; Goodner, 2008). These indices were obtained injecting a mixture of a homogenous series of alkanes (C8-C20, Fluka Buchs, Schwiez, Switzerland). The deconvolution base peak area (average value for three replicates), and not the concentration, was considered to estimate the abundance of each volatile compound (Verzera, et al., 2014).

2.5. Statistical analysis

Principal component analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied to identify underlying patterns in the set of volatile compounds and relationships among groups of samples. The initial data matrix contained the areas of the 123 volatile compounds identified in the 84 honey samples. Only volatile compounds were included in this data matrix. This data set was imported into the statistical environment R (v4.2.1) to perform the PCA and HCA analyses. Previously, the area values were scaled by z-score standardization. HCA was performed from PCA scores to facilitate the identification of groups by means of uncorrelated variables. Squared Euclidean distance was used as similarity measure, and Ward's conglomeration method were selected for the identification of clusters.

Subsequently, Orthogonal Partial Least Squares (OPLS) analysis was carried out by SIMCA® version 17 software (Umetrics, Umea, Sweden, https://umetrics.com) in the subset of 21 citrus honey samples collected during 2020 and 2021. OPLS, a supervised learning method, was used for analyzing the regression model between a selection of 84 volatile compounds with non-zero variability in citrus and methyl anthranilate-HPLC, applying a default 7-fold internal cross-validation. To improve the generalizability of these results, an additional OPLS model was estimated using another data matrix consisting of the 21 citrus samples collected during 2020–2021 (training set) and another 20 citrus samples collected during 2022 (test set). In both cases, performance was expressed by the $R^2(x)$ parameter, which represents goodness of fit, and by the Q^2, the predictive ability parameter. Variable Importance Projection (VIPs) was assessed to evaluate predictive ability of markers. This is a widely used procedure for variable selection not associated with

statistical significance values (Chong & Jun 2005). VIPs are dimensionless (without units of measurement), since they are calculated as the weighted sum of squares of the PLS weights, divided by the amount of variance explained in each latent variable or dimension. VIP plots show the bars with the VIP values sorted in descending order and confidence intervals derived from Jack-Knifing (Umetrics, Umea, Sweden, https://umetrics.com). The regularization of the three components of the VIP vector (predictive VIP, orthogonal VIP and total VIP) allows the selection of variables, with VIP > 1, as those with the greatest influence on Y (MA-HPLC in this work) (Farrés, et al., 2015; Giannetti, et al., 2017; Biancolillo, et al., 2022). Additionally, to confirm the reliability of these models, two-hundred-permutation-tests were also carried out as well as cross–validation ANOVA (Lindgren, et al., 1996; Triba et al., 2015).

3. Results and discussion

3.1. Clustering of honey samples by unsupervised analysis

In the volatile fraction of the 84 honey samples (from 2020 and 2021) 123 volatile compounds were identified (Table S1). In this table, these samples were clustered into seven groups considering the criterion of the predominant pollen as previously explained in Material and Methods. The data (mean, standard deviation, maximum, minimum) were expressed as deconvolution base peak areas. Figure S1 shows an example of the typical chromatograms (TICs) volatile profiles of the seven groups of honeys considered. With a glance, considerable variations can be observed among these chromatograms as a consequence of the presence or absence of certain chromatographic peaks, as well as differences in their relative levels. This reveals that the volatile fraction of each type of honey has its own peculiar characteristics, hence, a further in-depth analysis of this fraction compounds could be useful for differentiating types of honey.

Principal Component Analysis (PCA) was initially performed to assess the relationship between the set of volatile compounds found in the honey samples. The first four components (PC1, 13.6%; PC2, 11.5%; PC3, 8.3% and PC4, 6.9%) explained the amount of variability present in the raw data and the identification of outliers. The volatile compound scores graph presented in Fig. 1(a) shows that all honeys are distributed along the first component placing thyme samples at negative values and those of citrus at positive values, whereas the rest of the samples are located around the zero mark. The loading plot shows that certain compounds are to some extent responsible for this differentiation (Figure S2).

HCA (Hierarchical Cluster Analysis) was also performed (Fig. 1b) for unsupervised analysis as an exploratory tool to reveal natural groupings among the honey samples using PCA scores, as previously explained in Material and Methods section. This figure shows the relative frequencies or proportions of the different honey groups. According to the corresponding dendrogram, 3 clusters were identified (cluster sizes: 21, 7 and 55 samples, respectively). A well differentiated first cluster consisting of all the *Citrus* sp. samples (100%) was obtained. The second cluster is mainly composed by 71.4% of the *Thymus* sp. samples, 14.3% of the

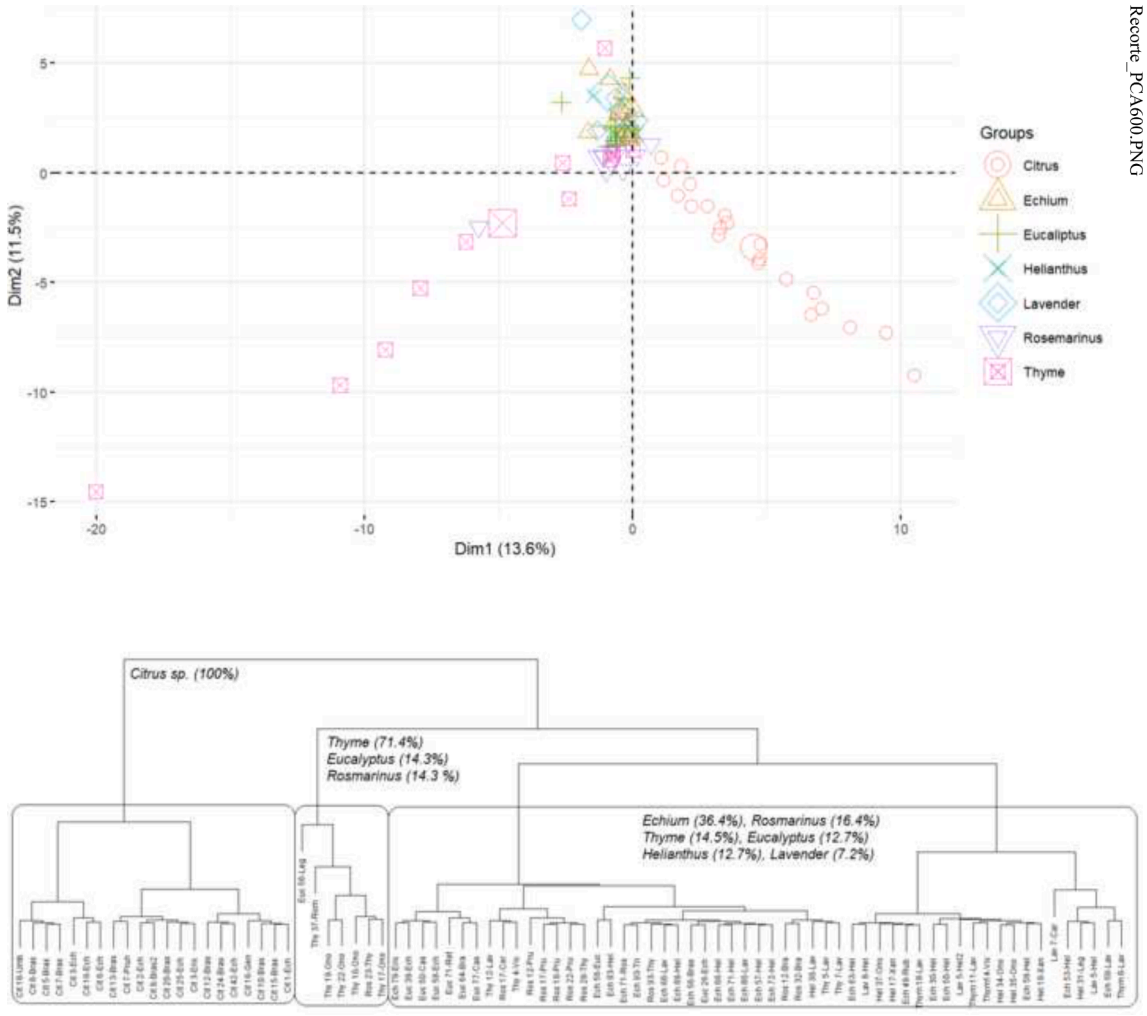

Fig. 1. (a) Volatile compound scores from Principal Component Analysis (PCA). (b) Dendrogram from Hierarchical Cluster Analysis (HCA).

Eucalyptus sp. and 14.3% of the *Rosmarinus officinalis*; while in the third cluster there was no clear predominant presence of any of the six groups (36.4% *Echium* sp., 16.4% *Rosmarinus officinalis,* 14.5%, *Thymus* sp. 12.7% *Eucalyptus* sp. 12.7% *Helianthus annuus*, 7.2% *Lavandula latifolia*). The independence contrast between "*cluster of classification*" and the "*predominant pollen criteria*" confirmed that both variables were significantly dependent (X^2 2 = 101.15, df = 12, $p < 0.001$). Therefore, the proportion of honey samples classified according to their predominant pollen content varies significantly depending on the cluster. These differences are especially remarkable for citrus honey samples (100% in cluster 1 vs. 0% in clusters 2 and 3). Both PCA and HCA have proven to be useful to reveal the predominant influence of the volatile fraction on the differentiation of this type of honey rather than others. Recently, these chemometric methods have been successfully applied together for the classification of different food matrices such as citrus fruits (Jahani et al., 2022) or aromatic herbs (Rivera-Pérez et al., 2022) according to their volatile fraction.

The above results demonstrate that the volatile fraction of the honey that contains *Citrus* sp. pollen has clearly differentiating characteristics from other types of honey attributable to the nectar collected (to a greater or lesser extent) by the bees from the orange blossom trees. This finding could be especially useful to correctly attribute the monoflorality of citrus honey, since its pollen is underrepresented, which makes it difficult to catalogue based solely on the percentage of this type of pollen (Escriche et al, 2023). In this sense, it is worth exploring the identification of specific compounds of its volatile fraction since it is directly related to its organoleptic perception.

3.2. Markers for identification of citrus samples

The relationship between methyl anthranilate obtained by HPLC (MA-HPLC) and the nectar of the citrus blossom is manifested in the fact that only the samples that contain pollen of *Citrus* sp. present values significantly different from zero in this parameter in contrast to the rest of the groups defined according to Table S1(ANOVA: F = 39.35, p ~ 0.000). A further step in the present work was to examine whether there is a relationship between the value of MA-HPLC and the compounds identified in the volatile fraction of citrus samples (among which the methyl anthranilate has also been identified). For this, an OPLS analysis has been carried out for MA-HPLC (dependent variable) and for the volatile fraction of citrus (matrix of predictor variables). As a result of this model, two components were identified that provided acceptable performance (*Goodness of prediction*: $Q^2 = 0.604$, *goodness of fit*: R^2 Y = 0.802). Fig. 2 shows the biplot analysis where the code of each citrus sample includes the pollen percentage of *Citrus* sp. pollen, as indicated in Table 1, and its calculated MA-HPLC value in brackets. Citrus samples

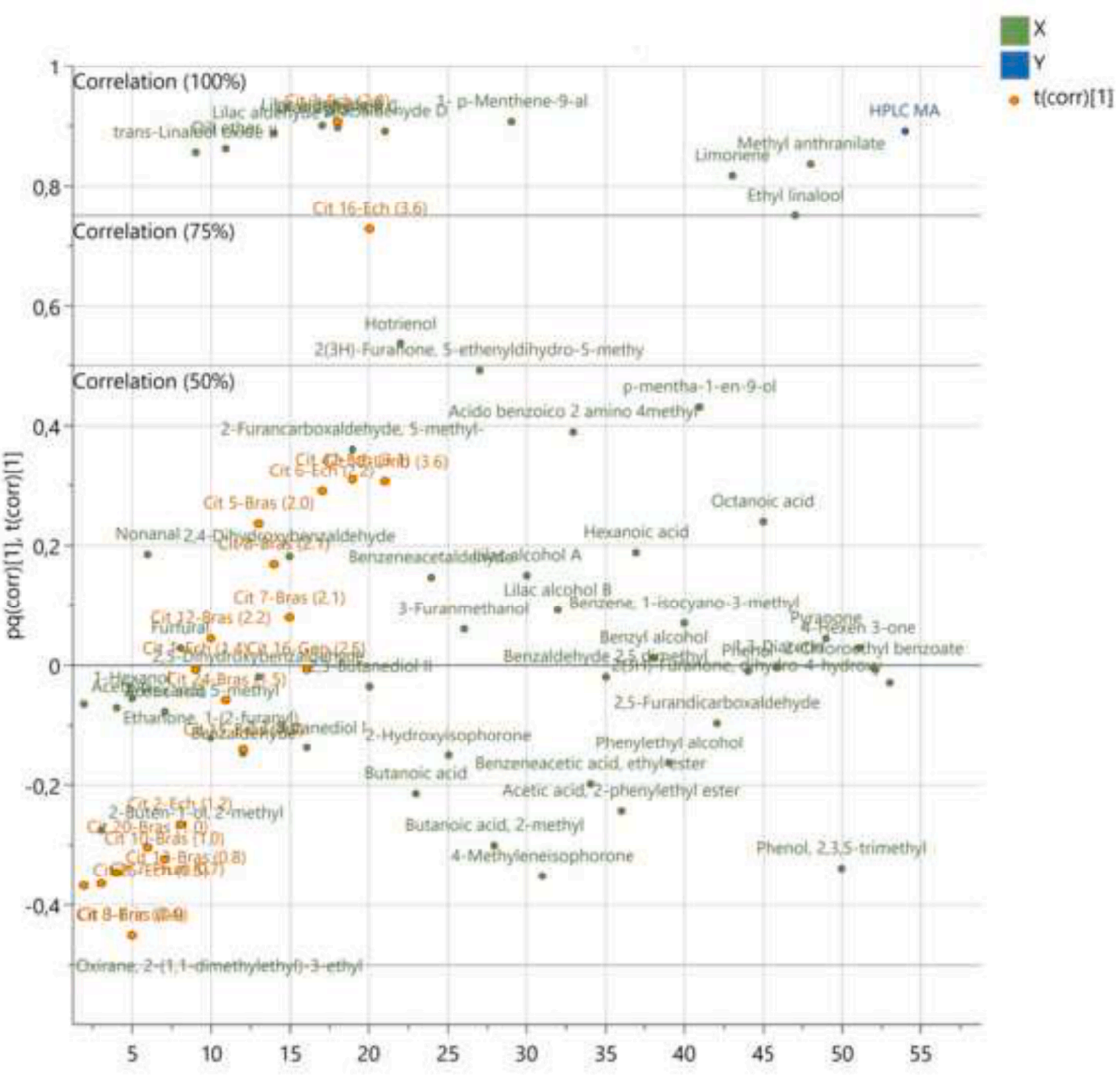

Fig. 2. OPLS biplot of honey samples (with pollen of *Citrus* sp.) and volatile compounds identified as relevant predictors of methyl anthranilate (HPLC). The code of each sample shows the pollen percentage of *Citrus* sp. and the MA-HPLC value.

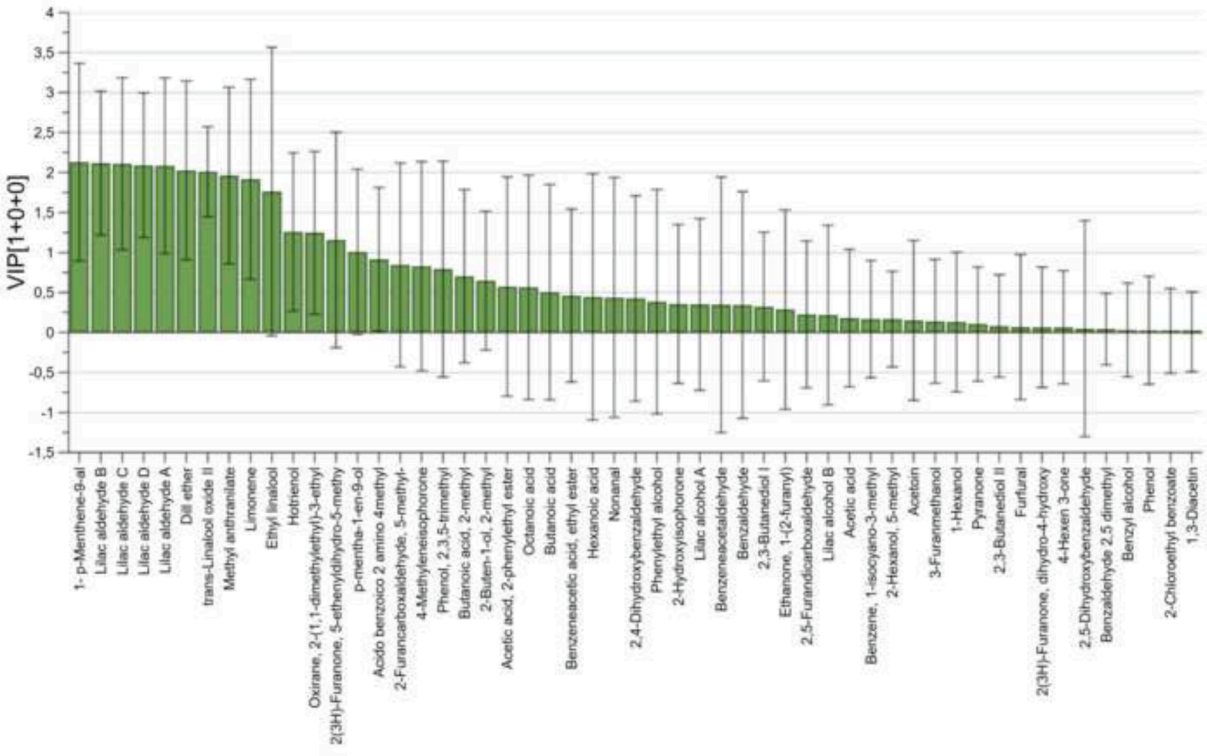

Fig. 3. Variable Importance Projection (VIP) of the identified volatile compounds from the OPLS model for methyl anthranilate (HPLC) prediction.

with high MA-HPLC values are at the top of the graph, while samples with low MA-HPLC values are at the bottom. On the other hand, Fig. 3 shows the volatile compounds ordered according to the Variable Importance Projection (VIP) obtained from the model and allows identifying 14 compounds as reliable markers for citrus honey (VIP score > 1.00): including 1-p-menthene-9-al; lilac aldehyde B; lilac aldehyde C; lilac aldehyde D; lilac aldehyde A; dill ether; *trans*-linalool oxide II; methyl anthranilate (volatile); limonene; ethyl linaool; hotrienol; oxirane 2-(1,1-dimethylethyl)-3-ethyl; 2(3H)-furanone 5-ethenyldihydro-5-methyl and p-mentha-1-en-9-ol). It is noteworthy that 8 of these 14 compounds have VIP values near 2 (VIP ~ 2), among which is the methyl anthranilate preceded by 7 of them.

Permutation plots were examined to prove that the values of the quality parameters (R^2 and Q^2) did not depend highly on the subset of citrus samples (2022) used for validation. The plot obtained with this test (Figure S3.a) showed the correlation coefficient between the original and the permuted MA-HPLC versus the cumulative R^2 and Q^2. The permutations test confirmed that there was no superposition of the results since the intercept of the Q^2 line took negative values (Q^2: −1.39). In addition, the points generated by the random permutations in the left section of the graph were always below the original values in the right sector of the chart. It is evident that considering all these compounds to characterize a citrus monofloral can provide additional information to that obtained exclusively with MA-HPLC.

3.3. Markers validation of citrus samples

The previous section has confirmed not only the relevance of methyl anthranilate (volatile) but also that of the other 7 compounds previously highlighted. However, the generalizability of the importance of these compounds as citrus markers cannot be guaranteed beyond the citrus subsample collected in this study in 2020–2021. Therefore, a new model was carried out that included samples from 2022. Only samples that met the Spanish commercial transaction requirement were included: 2 ≥ mg/kg of MA-HPLC and *Citrus* sp. pollen ≥ 10%. Therefore, validating the model with honey samples that meet this requirement favors the generalization of the results when applying it to other citrus honey samples and other harvest years.

Therefore, this new OPLS model was carried out using another data matrix consisting of 10 citrus samples collected during 2020–2021 (training set) and another 9 citrus samples collected during 2022 (test set). Of the 14 volatile compounds identified as VIPS in 2020–2021, 8 of them (methyl anthranilate; lilac aldehydes A, B, C and D; trans linalool II; dill ether and ethyl linalool) were detected again in the volatile fraction of the samples collected in 2022. The identification of these 8 compounds in both harvests (2020/2021 and 2022) confirm their relevance as potential citrus markers. Therefore, these eight compounds were considered as predictor variables of MA-HPLC in this OPLS model.

It is not surprising that there are small differences in the identified compounds among years. It can be attributed to the natural variability of the composition of the nectar. This is influenced by the flowering period of the main plant (orange citrus tree in this case) and the flowers of the plants that grow near them (up to 3 km which is the distance a bee can go to look for nectar). These variations in the period and type of flowering are mainly due to environmental factors such as climate, temperature, rainfall, which in a certain way affects the composition of the nectar, even if the honey has been collected in the same season and period of the year (Erickson, 1975; Deiana, et al., 2022).

Fig. 4 shows the OPLS biplot with the simultaneous relationship between scores and loadings expressed using correlation scaling. The code of each sample shows the pollen percentage of *Citrus* sp. and its MA-HPLC value in brackets. The high proximity between MA-HPLC and volatile methyl anthranilate points out the strong correlation between both variables. In addition, it is noteworthy that both are in the 100% correlation zone where the sample with the highest levels of MA-HPLC (4.5) is also located. In the 50–75% correlation zone, the four lilac aldehyde are positioned together with citrus samples with high but slightly lower values of MA-HPLC (up to 4.1). In general, samples with lower MA-HPLC are located in the lower correlation zone, where the other 3 compounds are placed.

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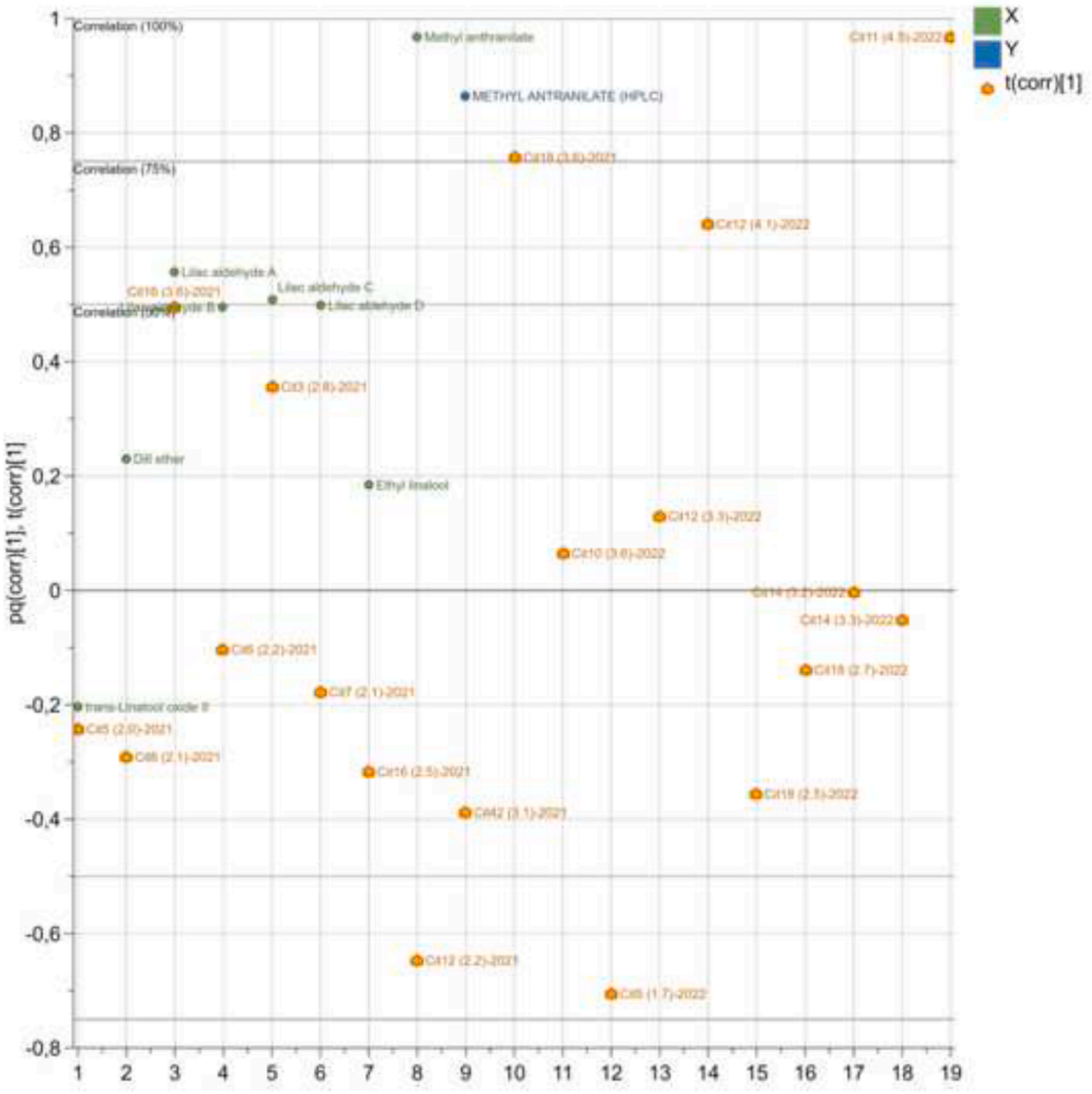

Fig. 4. OPLS biplot of honey samples in accordance with commercial requirements (pollen of *Citrus* sp. ≥ 10% and MA-HPLC ≥ 2 mg/kg) and volatile compounds validated as relevant predictors. The code of each sample shows the pollen percentage of *Citrus* sp. and the MA-HPLC value.

Fig. 5 shows the contribution of each compound to this OPLS model through VIP scores. Methyl anthranilate (volatile) and lilac aldehydes (A, C, D and B) were highlighted by a VIP score > 1.00. Correlations between these VIP markers and MA-HPLC were significant in all cases (p-values < 0.05) with correlation coefficients between 0.43 and 0.46 for lilac aldehyde compounds and 0.84 for methyl anthranilate compound (volatile). In contrast, compounds with VIP < 1.00 (trans linalool oxide II, dill ether and ethyl linalool) did not significantly correlate with MA-HPLC (p-values ~ 0.50). This model explained a cumulative 93.2% of the total variance and consisted of one predictive component and two orthogonal components with high-quality performance parameters (R^2Y = 0.748, Q^2 = 0.566). The validation plot obtained after two hundred permutations of this model showed that the Q^2 line had a negative intercept on the y axis (Q^2: −1.39) (Figure S3.b). In addition, the values of the cumulative R^2 and Q^2 generated by random permutations on the left were lower than the original values on the right. Therefore, overfitting could be discarded, and the model was reliable (Li et al., 2022).

Along with the cross-validation procedure applied to the OPLS model, the p-CV-ANOVA is user friendly and quick to estimate its significance. In this case, the p-value of 0.074 can be considered acceptable since the model was built using a reduced number of samples and this test has low statistical power with small datasets as previously reported by Eriksson, et al., (2008). Nevertheless, it can confirm the reliable predictive ability of the OPLS model for MA-HPLC in further citrus samples. As a result, a total of 5 of the 123 initially considered volatile compounds, play a crucial role to guarantee the correct classification of a monofloral citrus honey. The presence of these five selected compounds in this type of honey has been previously reported (Castro-Vázquez et al., 2009; Escriche et al., 2011). Other authors also found high proportions of lilac isomers in citrus honeys from Morocco, Greece, Egypt and Spain, although these compounds could not help the geographic discrimination of these honeys (Karabagias, et al., 2017). This reaffirms the fact that these compounds have to do more with the blossom citrus itself than with its botanical and geographical environment. More recently, Karabagias, et al. (2020) found Greek citrus honey to contain an important presence of certain specific aldehydes, such as the lilac aldehydes, and the ester methyl anthranilate. These findings are in line with what other authors already claimed regarding methyl anthranilate being an undisputed marker of citrus honey, as it is

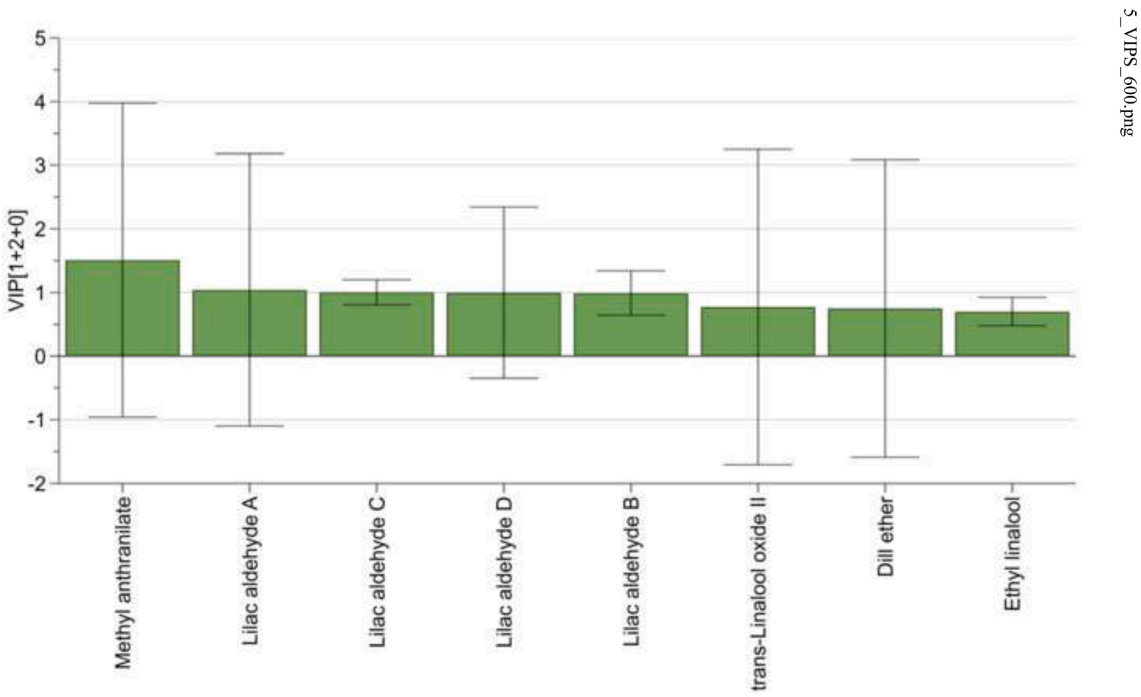

Fig. 5. Variable Importance Projection (VIP) of the validated volatile compounds obtained from the OPLS model for methyl anthranilate (HPLC) prediction.

identified only in this monofloral honey (Juan-Borrás et al., 2015).

Despite the proven importance of these compounds in the volatile fraction of citrus honey, is the first time that their joint relevance as a marker group for this monofloral honey has been proposed.

4. Conclusions

This paper has outlined the importance of the information provided by the volatile fraction as an objective tool in the differentiation of types of honey, particularly when they contain pollen of *Citrus* sp. This outcome could be especially useful to correctly attribute the monoflorality of citrus honey, since its underrepresented pollen makes its cataloguing difficult using the conventional criteria for the rest of monofloral honeys (based on the percentage of pollen). The predictive model built for citrus honey samples, demonstrates the possibility of using as a marker a set of 5 volatile compounds (4 lilac aldehydes and the volatile methyl anthranilate) determined by means of the GC–MS as the ultimate non-subjective analytical technique. The joint detection of these compounds would provide broader information in contraposition with a single compound (methyl anthranilate by HPLC). The strong correlation among them confirms the suitability of this proposal, without contradicting the current use of MA HPLC. These results highlight the need to rethink the exclusive use of the methyl anthranilate as commercial standard for the marketing of citrus honeys. The findings obtained so far shed new light on the gap in scientific knowledge about adequate and accurate markers for monofloral honeys. To guarantee the generalization of the conclusions based on the goodness of fit and the predictability of the model, it would be recommendable to incorporate more samples of citrus honey collected in the coming years, as well as different geographical areas. This will allow the reconsideration of the here proposed chemical markers and even determine the feasibility of including new ones.

CRediT authorship contribution statement

Isabel Escriche: Funding acquisition, Project administration, Conceptualization, Supervision, Writing – review & editing. **Andrea Conchado:** Formal analysis, Methodology, Writing – original draft. **Ana María Peral:** Investigation, Resources. **Marisol Juan-Borrás:** Investigation, Formal analysis.

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 work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112699.

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