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Improving honey identification: Stable isotope ratios variability of mono and polyfloral honeys from the citrus growing area of Salto/Concordia and from Uruguayan coastal areas

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ABSTRACT

Concordia in Argentina and Salto in Uruguay are two neighbouring regions famous for the production of a precious citrus honey, whose valorisation passes through the ability to guarantee its geographical origin. In this study the influence of the different botanical origins (monofloral and polyfloral) within the same region of origin on the different stable isotope ratios was evaluated. Moreover, the effectiveness of the stable isotope ratio parameters $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$, $\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$ to discriminate between honeys produced in the internal border regions between Argentina and Uruguay (namely, Concordia and Salto) and honeys sampled in the two different Uruguayan coastal regions (Canelones and Maldonado) was tested on 82 monofloral and polyfloral honey samples. The results show that the different stable isotope ratios, except that of nitrogen, are not significantly influenced by the botanical origin of the samples. The sulphur isotopic ratio of proteins and carbon of honey are the most significant parameters for discriminating the geographical origin of the honeys considered. Applied a Principal Component Analysis, the first two factors overall explain 63.5% of the total variance, while the Discriminant Analysis provided optimal discrimination between the three origins, reaching a minimum of 96.7% correct reclassification.

1. Introduction

The authenticity and traceability of food are worldwide recognised requirements for consumers and producers. However, the production of a particular food product in a specific site has been overcome by food industry globalisation. In turn, with the increase of world trade, food is more regulated, resulting in the need to develop new methods and techniques to discriminate among different food origins and sources and also different agricultural systems (Zhang et al., 2020). Among food products, honey is one of those whose added value is related to a defined geographical origin and floral composition. Moreover, the chemical composition of honey is generally associated with its botanical origin and, to a lesser extent, with the geographical area of production, since the environment (soil and climate) determines the honey flora (Santos-Buelga, González-Paramás., 2017)

In Uruguay (30° and 35° south latitude and 53° and 58° west longitude), the honey production reaches an annual amount of 12,000 tons, 90% of which is exported mainly to Spain, United States and Germany (Situacion y Perspectivas Del Sector Agro-rural Paraguayo., 2007; Bianchi and Carrau, 2021).

Since 2006, to guarantee honey quality of the country production, a traceability system has been implemented, including all production stages: extraction, transportation, trade, and distribution of honey and of other products from the hive (IMPO, 2006). The system is managed by the State official bodies and it is designed and executed following the guidelines and recommendations of the most demanding markets (EU, USA) (FAO., 2021).

The main types of honey produced in Uruguay can be classified as meadow honey (from clover *Trifolium L.*, lotus *Lotus corniculatus L.* and alfalfa *Medicago sativa L.* flowers), citrus blossoms honey (from flowers

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of orange *Citrus sinensis L.*, mandarin *Citrus reticulata Blanco*, 1837 and lemon *Citrus limon L.* trees), native forest honey and finally honey obtained in reforestation areas of sugar cane fields or other fast-growing crops such as eucalyptus *Eucalyptus saligna Sm.*

The Concordia-Salto regions, located in the northwest part of the country, at the border between Uruguay and Argentina, are traditional citrus production areas having extensive plantations dedicated to the production of oranges and mandarins, which constitute a source of nectar for the hives here installed by beekeepers of both countries (Tamaño, 2009). The Citrus honey produced in the mentioned area is highly appreciated and the attribution of a significant added value to this product comes from the capability to certify its geographical origin. Notably, honeys originating from citrus sources present a promising opportunity for enhanced valuation due to their distinct properties as previously reported (Fratianni et al., 2023; Seraglio et al., 2021). It is important to note that this information is in the process of being generated and is currently in the research phase, representing novel insights for Uruguay. This step has already been completed for the valorisation of lemon blossom honeys in Tucuman Province (Argentina) (Dominguez, 2023), and can be followed for the honeys from the Concordia/Uruguay area studied in this work. These regions share the banks of the Uruguay River, and present a similar productive structure, dominated by citrus crop varieties commonly defined as sweet (oranges and mandarins).

The geographical origin has a direct influence on the botanical composition of honey. Indeed, the type of soil and the climate determine the type of plants that the bees will use to produce honey. Generally, the botanical origin of any monofloral honey is verified by melissopalynological analysis, based on the identification of pollen by microscopic examination, which requires experienced analysts. This analytical approach has a fundamental role when it comes to commercialising monofloral honeys (Louveaux et al., 1970). The main problem associated with citrus honey characterization is due to the features of the citrus species involved, as they are not very polliniferous. Therefore, the number of pollen granules found in honey is usually low, which makes it very difficult to determine its botanical origin considering this parameter only. Melissopalinological analysis of monofloral honey is often supported by sensory analysis. In this contest, it is worth pointing out that a considerable amount of physico-chemical characteristics has to be determined for a complete characterization of honey (Cuevas-Glory et al., 2007, 2008; Serrano et al., 2004)

Due to the limitations of the commonly used authentication techniques, more reliable modern analytical methods have been used to determine botanical and geographical origins of honey. The studies included measuring carbohydrate (sugar) profiles, mineral content, phenolic and flavonoid compositions, aroma profile and amino acid composition using advanced analytical tools like chromatographic techniques, mass spectrometry (MS)-based techniques, vibrational spectroscopy like infrared (IR) and Raman techniques, nuclear magnetic resonance (NMR) and others, such as flame ionisation detectors (FID) or sensor arrays (Chin and Sowndhararajan, 2020)

The Stable Isotope Ratio Analysis (SIRA) of the bioelements is among the most powerful techniques developed to assess the authenticity and traceability of agri-food products, including honey (Kelly., 2003). This methodology is based on the measurement of the ratios of stable isotopes of C, O, H, N and S ($^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{2}H/^{1}H$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$) of products and/or specific components. This technique provides information on the botanical and geographical origin of foodstuff, considered as important characteristics by both national and international consumers (Wang et al., 2021). Furthermore, several studies already demonstrated the ability of this mass spectrometry technique to geographically differentiate the honey to guarantee its authenticity (Schellenberg et al., 2010; Wu et al., 2015).

In this work, we evaluated the influence of the botanical composition (monofloral and polyfloral) of honeys coming from the same region on their stable isotope ratios. Moreover, we tested the ability of the SIRA to discriminate between honeys produced in the internal border regions between Argentina and Uruguay (namely, Concordia and Salto), particularly famous for citrus honeys, and honeys sampled in the two different Uruguayan coastal regions (Canelones and Maldonado). The melissopalynological analysis was performed on 82 honey samples for the classification as monofloral or polyfloral according to the different species involved. The isotopic parameters ($\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$, $\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$, $\delta^{18}O_{protein}$) were therefore determined.

2. Materials and methods

2.1. Sampling

Eighty-two honey samples [60 honeys from the internal region of Concordia (Entre Ríos, Argentina)-Salto (Uruguay) and 22 samples from coastal areas of Uruguay (Maldonado and Canelones)] were considered (see Figure S1 and Table S1). The samples were stored at room temperature. The botanical origin and the classification as monofloral or polyfloral of all the samples was determined by melissopalynological analysis before proceeding to the isotopic analysis.

2.2. Melissopalynological analysis

The melissopalynological analysis followed the method of Louveaux et al. (Louveaux et al., 1970). In brief, 10 g of each honey sample was diluted in 20 ml of distilled water and then clarified by centrifugation at 1200 ×g for 5 min. The supernatant was discarded, and the pellet was suspended in 3 ml glacial acetic acid, centrifuged as above and the acetic acid decanted. The obtained samples were then subjected to the acetolysis method (Erdtman, 1960) with acetic anhydride and sulfuric acid (9,1) at 70 °C until the pollen grains turned brown. The pollen grains were then harvested by centrifugation as above and divided into two portions. The first portion was preserved in glycerin jelly for light microscopy (LM) and the other in absolute ethanol for scanning electron microscopy (SEM). Pollen grains in glycerin jelly were transferred to glass slides for permanent slide preparation, covered by a cover slip and sealed with paraffin ready for LM analysis. The pollen grain samples in absolute ethanol were freeze dried by the critical point drying method, placed on the stubs with double sided tape and coated with gold by Emitech K550 UK, prior to SEM analysis using a JEOL JSM-6610LV (Japan) instrument at 5–10 kV, depending on the sample.

In the LM analysis, at least 300 pollen grains per sample were counted, identified and then compared with the pollen source catalogues of flowers in the study area. Morphological characteristics of the pollen used in their identification included the symmetry, shape, polarity, apertural pattern, exine and ornamentation. The results are expressed as the frequency class, using the criteria recommended by Louveaux et al. (Louveaux et al., 1970) and have been graphed in Fig. 1a and Fig. 1b.

2.3. Sample preparation for stable isotope analysis

About 0.7 mg of honey and 1.2 mg of extracted protein were weighed for $\delta^{13}C_{honey}$ comparison values to those of the $\delta^{13}C_{protein}$. The methodology that has been applied is described in the official method AOAC 998.12 (White, 1992; White and Winters, 1989). The $\delta^{34}S$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{18}O$ were also determined in extracted proteins (weight 1.2 mg) following the official method AOAC 998.12 and the guidelines reported in Bontempo et al. (Bontempo et al., 2017).

2.4. Stable Isotope analysis

The $^{13}C/^{12}C$ of bulk and extracted honey proteins and $^{15}N/^{14}N$ and $^{34}S/^{32}S$ ratios of extracted honey proteins were measured using an isotope mass spectrometer (IsoPrime, Isoprime Limited, Germany) after total combustion in an elemental analyser for EA (VARIO CUBE,

a

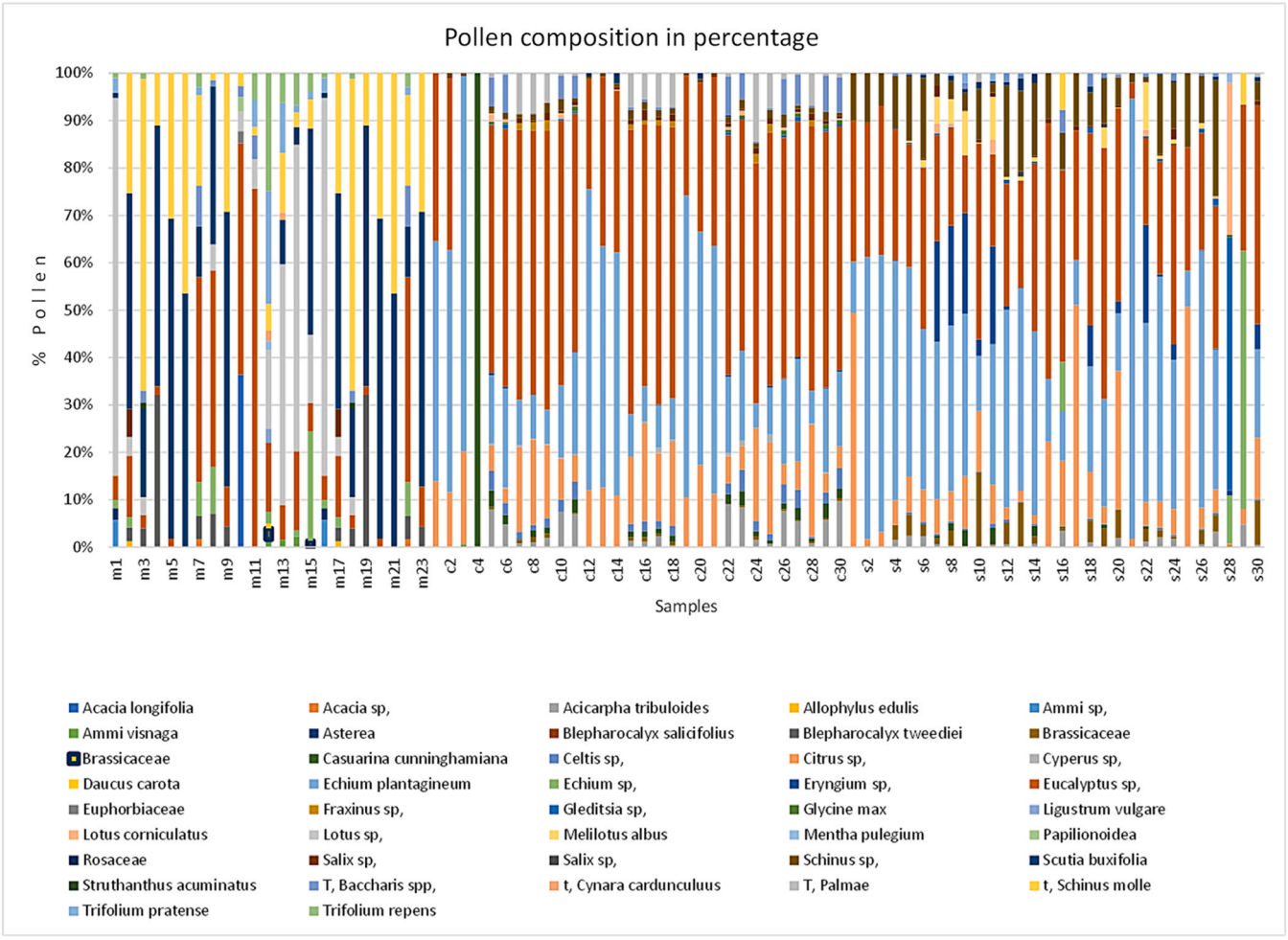


Fig. 1a. Melissoplinological analysis. The results are expressed as the frequency class, using the criteria recommended by Louveaux et al. (Louveaux et al., 1970).

Isoprime Limited, Germany). The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios of extracted honey proteins were measured using an IRMS (Finnigan DELTA XP, Thermo Fisher Scientific, Bremen, Germany) coupled with a pyrolyser (Finningan DELTA TC/EA, high temperature conversion EA, Thermo Fisher Scientific, Bremen, Germany). The isotope ratios were expressed in δ against V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$, Air for $\delta^{15}N$, Canyon Diablo Troilite (V-CDT) for $\delta^{34}S$ and V-SMOW (Vienna-Standard Mean Ocean Water) for $\delta^{18}O$ and $\delta^{2}H$ according to:

$$\delta_{ref}\left({}^{i}E/{}^{j}E, sample\right) = \left[\frac{R\left({}^{i}E/{}^{j}E, sample\right)}{R\left({}^{i}E/{}^{j}E, ref\right)}\right] - 1 \quad (1)$$

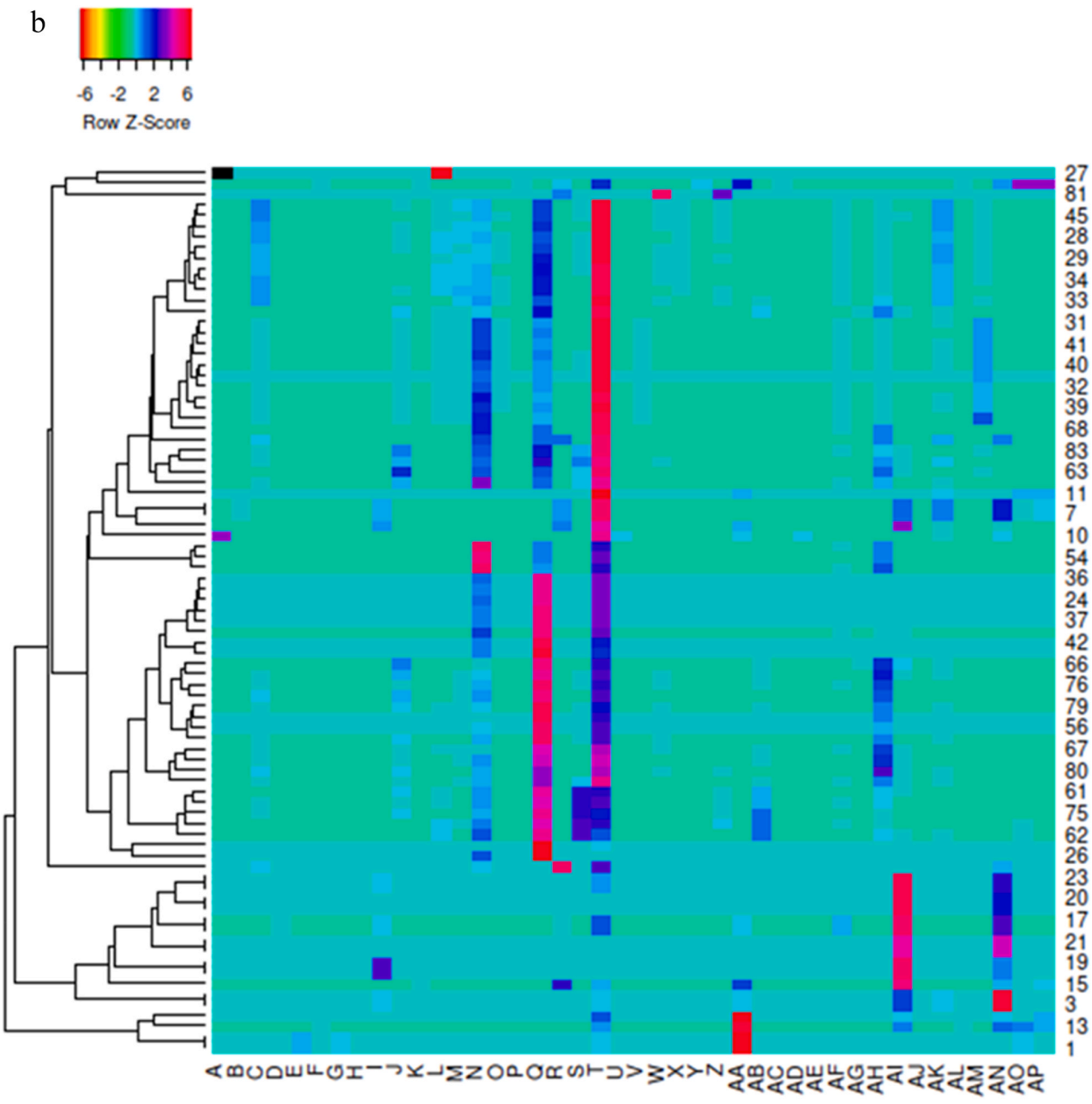
where i is the mass number of the heavier isotope of the element E, R_{sample} is the isotope ratio measured for the sample and $R_{standard}$ is the isotope ratio of the international standard. Sample analyses were carried out in duplicate.

The isotopic values were calculated against working in-house standards, which were themselves calibrated against international reference materials: fuel oil NBS-22 with $\delta^{13}C = -30.03‰$, sucrose IAEA-CH-6 with $\delta^{13}C = -10.45‰$ (IAEA-International Atomic Energy Agency, Vienna, Austria) and L-glutamic acid USGS 40 with $\delta^{13}C = -26.39‰$ and $\delta^{15}N = -4.52‰$ (U.S. Geological Survey, Reston, VA, USA) for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ and potassium nitrate IAEA-NO3 ($\delta^{15}N = +4.7‰$) from IAEA for $^{15}N/^{14}N$. Barium sulphate IAEA-SO-5 ($\delta^{34}S = +0.5‰$) and NBS 127 ($\delta^{34}S = +20.3‰$) from IAEA were used to obtain $^{34}S/^{32}S$ values.

Before carrying out the analysis of $\delta^{2}H$, the sample was left to equilibrate with the laboratory air for two days, subsequently it was placed inside a vacuum dryer with P_2O_5 for 48 hours and finally placed in a zero-blank autosampler to comply with the principle of identical treatment (Wassenaar et al., 2015). Keratins CBS (Caribou Hoof Standard, $\delta^{2}H = -157 \pm 2‰$) and KHS (Kudu Horn Standard, $\delta^{2}H = -35 \pm 1‰$) from U.S. Geological Survey were used to obtain $^{2}H/^{1}H$ values through the creation of a linear equation and by adopting a comparative equilibration procedure (Wassenaar et al., 2015). We used these two keratinous standards because of the absence of any international organic reference material with a similar matrix to our samples. The uncertainty of the method (calculated as two standard deviations when analyzing the same sample at least ten times under reproducible conditions) was 0.3‰ for $\delta^{13}C_{honey}$, 0.5‰ for $\delta^{13}C_{protein}$, 0.8‰ for $\delta^{34}S_{protein}$ and $\delta^{15}N_{protein}$, 2‰ for $\delta^{2}H_{protein}$ and 0.8‰ for $\delta^{18}O_{protein}$.

2.5. Multivariate statistical analysis

The analyses were performed with Statistica software (v. 7.0, Statsoft, Tulsa, USA) and XLSTAT statistical and data analysis solution (Addinsoft, Long Island, NY, USA, 2019 https://www.xlstat.com). Statistically significant differences were found using a Tukey HSD test. In all the cases, the cutoff value was set at $p<0.05$, which is associated with a significant difference between groups of values. Preliminary exploration of the stable isotope ratio data matrix was conducted by principal component analysis (PCA) and Discriminant Analysis (DA). PCA



Name	*Acacia longifolia*	*Acacia sp,*	*Acicarpha tribuloides*	*Allophylus edulis*	*Ammi sp,*	*Ammi visnaga*	*Asterea*
Code	A	B	C	D	E	F	G
Name	*Blepharocalyx salicifolius*	*Blepharocalyx tweedie*	*Brassicaceae*	*Brassicaceae*	*Casuarina cunninghamiana*	*Celtis sp,*	*Citrus sp,*
Code	H	I	J	K	L	M	N
Name	*Cyperus sp,*	*Daucus carota*	*Echium plantagineum*	*Echium sp,*	*Eryngium sp,*	*Eucalyptus sp,*	*Euphorbiaceae*
Code	O	P	Q	R	S	T	U
Name	*Fraxinus sp,*	*Gleditsia sp,*	*Glycine max*	*Ligustrum vulgare*	*Lotus corniculatus*	*Lotus sp,*	*Melilotus albus*
Code	V	WW	X	Y	Z	AA	AB
Name	*Mentha pulegium*	*Papilionoidea*	*Rosaceae*	*Salix sp,*	*Salix sp,*	*Schinus sp,*	*Scutia buxifolia*
Code	AC	AD	AE	AF	AG	AH	AI
Name	*Struthanthus acuminatus*	*T, Baccharis spp,*	*t, Cynara cardunculuus*	*T, Palmae*	*t, Schinus molle*	*Trifolium pratense*	*Trifolium repens*
Code	AJ	AK	AL	AM	AN	AO	AP

Fig. 1b. Heatmap of the botanical composition of honey samples.

identifies orthogonal directions, or principal components (PCs), the first describing the largest variance, the second describing the second-largest variance, and so on. Patterns in a data matrix can be emphasised by projecting objects and variables into the space of few significant F axes with minimal loss of information. Discriminant analysis is a statistical-mathematical discipline developed to separate objects and observations into distinct classes (clustering) and to allocate new observations into one of the previously defined classes (classification). In Table 2 S

the classification performance was quantified by the percentage of data samples correctly assigned to the predefined classes.

3. Results and discussion

3.1. Melissopalinological analysis of honey samples

The melissoplainological analysis allowed us to determine whether the honey samples having different geographical origin could be classified as monofloral or polyfloral. Regarding the coastal regions between Argentina and Uruguay, eight samples from Salto and twenty from Concordia were classified as monofloral from citrus, while twenty-two and ten, respectively, were classified as polyfloral (Fig. 1a). Moreover, eight samples from Uruguayan coastal region of Maldonado were classified as monofloral from *Scutia buxifolia*, two as monofloral from *Schinus mole* and six as polyfloral. Finally, four samples from the Canelones region were classified as monofloral by *Lotus sp.* and three as polyfloral.

Moreover, using the heatmap tool (Babicki et al., 2016) from the melissopalynological data (Fig. 1b), the honey samples could be classified into seven different groups: 1) monofloral *Citrus sp.* and 2) polyfloral from the Salto/Concordia production area; 3) monofloral *Lotus sp.* and 4) polyfloral from the Canelones production area; 5) monofloral from *Scutia buxifolia*; 6) monofloral from *Schinus molle*; 7) polyfloral from the Maldonado area. Heatmapper is a freely available web server that allows users to interactively view their own data in the form of heat maps through an easy-to-use graphical interface.

3.2. Stable isotope ratio analysis

Table S1 shows the isotopic values ($\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$, $\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$, $\delta^{18}O_{protein}$) obtained for honey samples. The different stable isotope ratio ranges of variability for the seven groups are shown in Fig. 2. No statistically significant differences ($p>0.05$) between the monofloral and polyfloral groups of the three sampling sites were highlighted. Except $\delta^{15}N_{protein}$ ($p<0.05$) in the comparison between monofloral honeys from *Schinus molle* and polyfloral honeys, none of the isotopic parameters investigated presented significant differences between the monofloral and polyfloral groups.

These results partially agreed with a previous work by Bontempo et al., according to which the botanical origin influenced not only the $\delta^{15}N_{protein}$ (as found in the present study) but also the $\delta^{13}C_{protein}$ (Bontempo et al., 2017). The other isotopic parameters ($\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$) had not been investigated by Bontempo et al. in order to establish their dependence on the botanical origin (Bontempo et al., 2017). Dinca et al., Bontempo et al. and Magdas et al. confirmed, as already reported by Giraudon et al., that the most significant botanical discrimination isotopic parameter is the $(D/H)_I$ of the ethanol (SNIF NMR analysis) obtained after fermentation and distillation of honey (Magdas et al., 2021; Giraudon et al. 2000; Bontempo et al., 2017; Dinca et al. 2015). On the contrary, the parameters $\delta^{13}C_{honey}$ (Bontempo et al., 2017; Dinca et al. 2015) and $\delta^{18}O_{protein}$, $\delta^{2}H_{protein}$ and $\delta^{13}C_{protein}$ (Magdas et al., 2021) appear to be more correlated to the meteorological conditions of the different sampling periods rather than discrimination markers that contribute to the classification on a botanical basis. Based on the results obtained in this study, $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$, $\delta^{18}O_{protein}$ do not seem to be affected by the botanical origin of the plant species involved in honey production.

3.2.1. Carbon isotopes

The $\delta^{13}C$ values range from −26.9‰ to −24.9‰ (-25.7‰ on average) in the bulk honey and from −26.8‰ to −24.4‰ (-25.7‰ on average) in the extracted proteins. This is consistent with plant species characterised by a C3 photosynthetic cycle, whose $\delta^{13}C$ values normally range from −30‰ to −23‰ (Smith and Epstein, 1971). Similar variability ranges were reported for Uruguayan honeys by Berriel et al. (Berriel, 2018) (-25.9‰ on average in bulk honey and −25.7‰ on average in extracted proteins).

Compared to literature data (ranging from average values of −27.4‰ for honey samples from Latvia (Labsvards et al., 2021) and Slovenia (Kropf et al. 2010) up to values of −22.48‰ in South African honey (Roßmann et al., 1992) and −22.34‰ in Romanian honey (Dinca et al. 2015)), the Uruguayan samples generally show high values typical of plants grown in sunny areas and therefore similar to those reported, for example, for the northeast Anatolia region of Turkey by Cengiz, Tosun, & Topal (from −26.1‰ to −25.2‰ of bulk and from −26.2‰ to −25.1‰ of the protein fractions) (Cengiz et al., 2018). The $\delta^{13}C$ values in fact depend on the amount of sun exposure to plants and air humidity; therefore, an increase of sunny days and less precipitation will result in higher $\delta^{13}C$ values (Schellenberg et al., 2010).

As reported in Fig. 3, the $\delta^{13}C$ of bulk honey is significantly different in the three sampling areas with higher values in the coastal site of Maldonado (average −25.3‰) with respect to the Salto/Concordia area (average −25.8‰). The $\delta^{13}C_{protein}$ allowed to distinguish the Maldonado site (average −25.1‰) from the Salto/Concordia one (average −25.9‰), while there is a partial overlap with the coastal site of Canelones.

3.2.2. Hydrogen and oxygen isotopes

As reported in the literature, the $\delta^{18}O$ and $\delta^{2}H$ depend on many variables, such as latitude, altitude and proximity to the sea (Bowen et al., 2007). The isotopic values of hydrogen and oxygen are closely related to precipitation and meteoric water (Araguàs-Araguàs et al., 2000; Bowen et al., 2007). This correlation is still valid, although not that strict, in the plants, where the only source of hydrogen is water, while oxygen can also derive from O_2 and CO_2 (Barbour, 2007).

Schellenberg et al. reported a $\delta^{2}H_{protein}$ variability range of honey from different European regions from −121‰ to −73‰ (Schellenberg et al., 2010), while Shuai et al. reported values between −116.4‰ and −103.5‰ for Chinese honeys, having $\delta^{18}O_{protein}$ between +5.5‰ and +19.0‰ (Shuai et al. 2023). When classifying Chinese honeys (including rape honey, acacia honey, vitex honey, and jujube honey), Wu et al. found that the $\delta^{2}H_{bulk}$ of rape honey ranged from −90‰ to −60‰ (Wu et al. 2015).

The values of the samples included in this study fall within these natural variability ranges, presenting $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$ values between −134‰ and −79‰ and between +11‰ and +21‰, respectively.

The $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$ of honey samples coming from citrus-growing areas (Salto and Concordia) do not show significant differences compared to those of the other areas of Uruguay (Fig. 3). In the absence of direct measurements of the groundwater used for irrigation, the values reported in the Water Isotope database created by Putman and Bowen (Putman and Bowen, 2019) leading to the development of useful global and regional scale models of precipitation isotopes, were used. The data available in the http://wateriso.utah.edu (accessed on 1 September 2023) database are monthly weighted average precipitation values for sites all over the world.

According to the Water Isotope database, the two considered areas (Salto+Concordia vs Coastal regions), located at similar latitudes (31°S vs 34°S), are characterised by similar meteoric water $\delta^{18}O$ (-3.7‰ vs −4.1‰) and $\delta^{2}H$ (-18‰ vs −21‰) values.

Indeed, the differences between the two groups for both $\delta^{18}O$ and $\delta^{2}H$ are close to analytical uncertainty and therefore these parameters do not guarantee a clear discrimination between them.

In this case, as also highlighted in the box plots of Fig. 3, the two isotopic parameters do not seem to contribute to the geographical discrimination of the three sites of honey production.

3.2.3. Nitrogen and sulphur isotopes

As reported in Section 3.2, the $\delta^{15}N$ seems to confirm its dependence on the botanical origin of honey. In particular, the monofloral samples from *Schinus molle* present significantly lower values than those

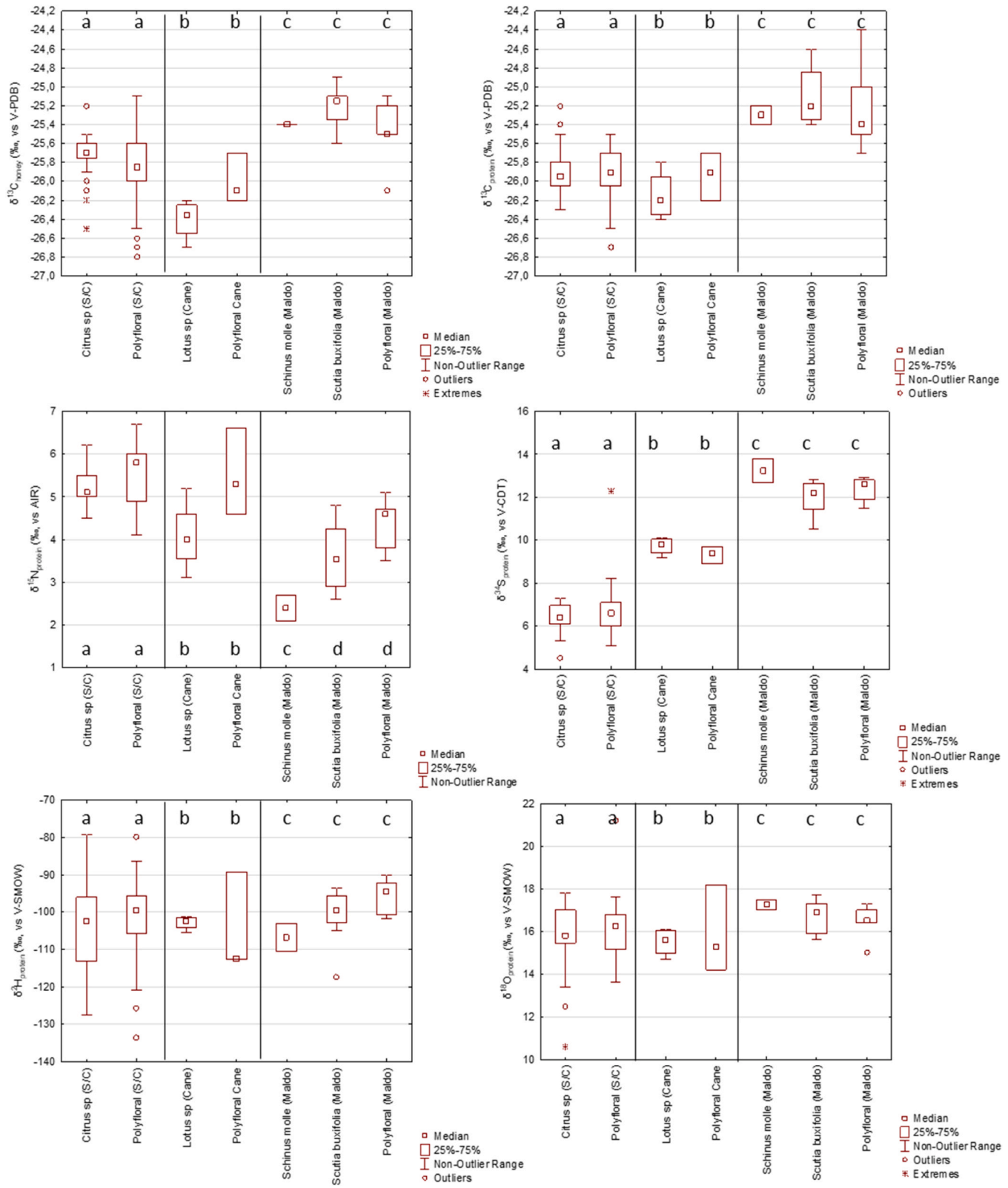


Fig. 2. Parallel box-plot diagrams representing the isotopic variation of $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$, $\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$, $\delta^{18}O_{protein}$ for the seven honeys group (monofloral *Citrus sp.* and polyfloral from the Salto/Concordia production area; monofloral *Lotus sp.* and polyfloral from the Canelones production area; monofloral from *Scutia buxifolia*; monofloral from *Schinus molle*; polyfloral from the Maldonado area). Significantly different values (Tukey's HSD, $p<0.05$) between mono vs polyfloral groups of any geographical origin are indicated with different letters.

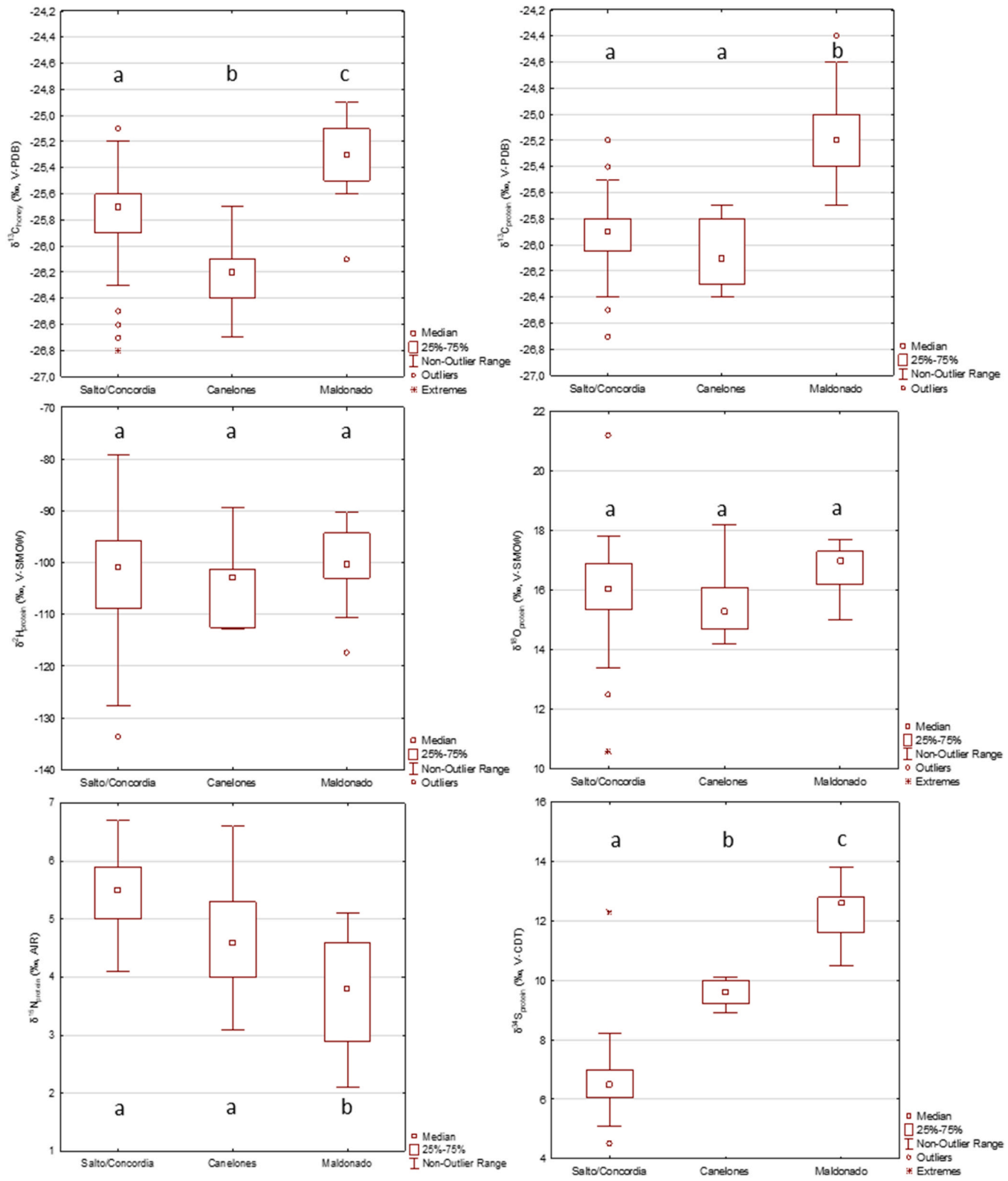


Fig. 3. Parallel box-plot diagrams representing the isotopic variation of $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$, $\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$, $\delta^{18}O_{protein}$ for the three honey groups (honey from Salto/Concordia, Canelones and Maldonado production areas). Significantly different values (Tukey's HSD, $p<0.05$) among groups are indicated with different letters.

measured in the other honeys from the Maldonado area (both monofloral and polyfloral). This result agrees with a previous study by Bontempo et al., which reported the correlation of this parameter with the botanical origin of honey (Bontempo et al., 2017). For this reason, the $\delta^{15}N_{protein}$ was not considered as a possible geographical tracer useful to discriminate between Salto and Concordia honey and the coastal areas products.

The main factor affecting the $\delta^{15}N$ in cultivated plants is the

fertilisation process used. Synthetic fertilisers, produced from atmospheric nitrogen via the Haber process, have $\delta^{15}N$ values between −4‰ and +4‰, while organic fertilisers are characterised by values between +0.6‰ and +36.7‰ (Bateman and Kelly, 2007; Vitòria et al., 2004).

Regarding the sampling areas for which the $\delta^{15}N$ was not statistically different between monofloral and polyflorar honey (likely due to similar fertilisation practices), the mean $\delta^{15}N$, regardeless of the botanical origin, can be considered in the comparison among sites. For instance, the mean $\delta^{15}N$ values of honey from the Concordia and Salto regions showed a higher average value (+ 5.4‰) than the other sampling areas of Uruguay (+ 3.9‰) (Fig. 3d). This is likely due to the use of organic fertilisers in these areas, characterized by a prevalent cultivation of citrus fruits (United Nations Conference on Trade and Development., 2003).

Plantation management technologies, including the adaptation of fertilisation to nutritional needs, aim to obtain high quality products, as this is a decisive factor for export. The criteria for fertilisation in Uruguay are variable, and there is little experimental information available for the adaptation of fertilisation programmes (Burna, 2014). To explain the lower values found in the samples from other areas of Uruguay, we can consider the possible use of both synthetic fertilisers and organic ones deriving from nitrogen-fixing plants (Ma et al., 2022; Yoneyama et al., 2001).

The $\delta^{34}S_{protein}$ of this study ranged between +4.5‰ and +13.8‰, not dissimilar to those reported by Schellenberg et al. for European honey (between +2.0‰ and +11.1‰) (Schellenberg et al., 2010).

The $\delta^{34}S_{protein}$ showed significant differences ($p<0.01$) between the three areas of sampling, with the lower values (average +6.6‰) for Salto/Concordia and higher ones (average +12.3‰) for Maldonado, while the Canelones area showed intermediate values (average +9.6‰) (Fig. 3).

Sulphur isotopes in plants are strongly influenced by many different factors, such as the abundance of sulphides in soil, plants aerobic and anaerobic growth, local bedrocks (Rubenstein and Hobson, 2004), active microbial process in the soil, fertilisation procedures, active deposition (Environmental Tracers in Subsurface Hydrology., n.d.). Therefore, the $\delta^{34}S$ values of honey protein fraction reflects the geological region of plants growing area (Förstel, 2007; Schmidt et al., 2005). As reported by Schellenberg et al. (Schellenberg et al., 2010), the $\delta^{34}S_{protein}$ is one of the most geographically discriminating parameters for honey, as it is not affected by annually changing climatic conditions or by the botanical origin of honey.

All the honey samples coming from the coastal areas of Canelones and Maldonado are distributed among the quartiles of the respective groups, while two samples coming from the sampling area of Sato/Concordia are outliers. According to its melissopalynological analysis (see Section 3.3), this is a non-citrus honey (polyfloral honey).

By observing the distance from the sea of the three sampling sites, it is possible to observe that it ranges from 500 km for the Salto/Concordia areas to 50 km for Canelones, while Maldonado is in front of the sea.

As reported by Trofimov et al. (Trofimov, 1949) and Thode et al. (Thode et al., 1949) sulphur isotopic composition in regions close to the sea is influenced by the so-called 'sea spray (or sea-salt sulphate) effect'. Sea water sulphate contains sulphur with $\delta^{34}S$ values close to +22‰. The precipitation of sea water aerosol in coastal regions is overlaying the geological source, as this kind of sulphur is better available for the plants growing in this area. This effect can result in higher $\delta^{34}S$ values in the plant products, including nectar and pollen.

The results obtained and represented in Fig. 3 show that this effect is evident on the analysed honey samples and that the $\delta^{34}S$ can be thus considered more efficient in discriminating Uruguayan coastal honeys compared to those from Salto and Concordia.

3.3. Evaluation of the multivariate analysis of the stable isotope data

The multivariate statistical analysis carried out on the stable isotope data matrix consisted in a preliminary PCA exploration of the whole dataset. The results of the PCA are summarised in Fig. 4, which displays the objects and the variables simultaneously projected in the space of the first two PCs explaining as a whole 63.5% of the total variance. In Fig. 4, samples are labelled with a numeric combination identifying the number of the sample itself and the group it belongs to (e.g., sample 12.1 represents sample n.12, belonging to group 1).

As reported in Section 3.1, using the heatmap tool from the melissopalynological data the honey samples could be classified into seven different groups. To perform the principal component analysis (PCA) and discriminant analysis (DA), the classification was reduced to 3 groups: 1: Salto - Concordia production area (monofloral *Citrus sp.* and polyfloral), 2: Canelones production area (monofloral *Lotus sp.* and polyfloral), and 3: Maldonado area (monofloral from *Scutia buxifolia*, monofloral from *Schinus molle* and polyfloral).

A projection of the variable loadings on the significant F axes allows the observed separation of honey categories in terms of variations in the stable isotope ratios to be explained. Separation of group 1, including the samples from Concordia/Salto area, from groups 2 and 3, corresponding to samples from the Maldonado and Canelones coastal areas near the Atlantic Ocean, particularly occurs on F1 and $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{34}S_{protein}$ seem to be the discriminant variables responsible for such separation. The partial overlap of the groups at the intersection of the two F axes makes it difficult to perform a definitive statement. In conclusion, and in our experimental conditions, the geographical origin appeared to have a strong influence on sample distribution aligned to $\delta^{34}S_{protein}$ and $\delta^{13}C_{protein}$.

When the results of the discriminant analysis were considered, it was found that the prediction of the groups was effective. The percentage of correct prediction is reported in Table S2: groups 2 and 3 present 100% correct prediction, while group 1 obtained 96.7%. In this group only two of the total 60 samples were not correctly classified (the samples 52.1 and 35.1, the first being classified in group 3 and the second one being classified in group 2). This is also evident in the PCA, where these samples are clustered together with those of group 2 and 3. Both samples are found in the initial classification of groups in group 2 (Salto / Concordia polyfloral). The centroids diagrams showed that each one of the samples integrated the groups 1, 2 and 3 (Fig. 5).

4. Conclusions

In this work, the analysis of the $\delta^{13}C_{honey}$ and of $\delta^{13}C_{protein}$, $\delta^{15}N_{protein}$, $\delta^{34}S_{protein}$, $\delta^{2}H_{protein}$, $\delta^{18}O_{protein}$ was applied in the study of monofloral and polyfloral honey samples from both the renowned area of citrus production located on the borderline between Argentina and Uruguay (Salto/Concordia) and from other coastal uruguayan sites (Maldonado and Canelones). With the exception of $\delta^{15}N_{protein}$, the other stable isotope ratios investigated were not significantly different ($p>0.05$) between mono- and polyfloral honey samples coming from the same geographic area.

The $\delta^{13}C_{honey}$ and, above all, the $\delta^{34}S_{protein}$, which is strongly influenced by the 'sea spray effect' and therefore by the distance from the sea, demonstrated an excellent ability to discriminate among the productions of the three sampling sites (Salto/Concordia, Maldonado and Canelones).

The first two factors of the PCA overall explain 63.5% of the total variance. Based on the LDA, a correct prediction of 96.7% for group 1 (Concordia/Salto samples), and 100% for group 2 and 3 was achieved (coastal samples from Canelones and Maldonado areas, respectively).

The results obtained allowed to add value to citrus honey, helped in the characterization of the product and are of fundamental importance for a country like Uruguay, which is among the top 20 world exporters of honey (1% of world trade). The methodology applied in this work can be useful to ensure the quality and safety of Uruguayan honey. For regulatory purposes, the creation of an isotope database that maps national production in detail would be desirable.

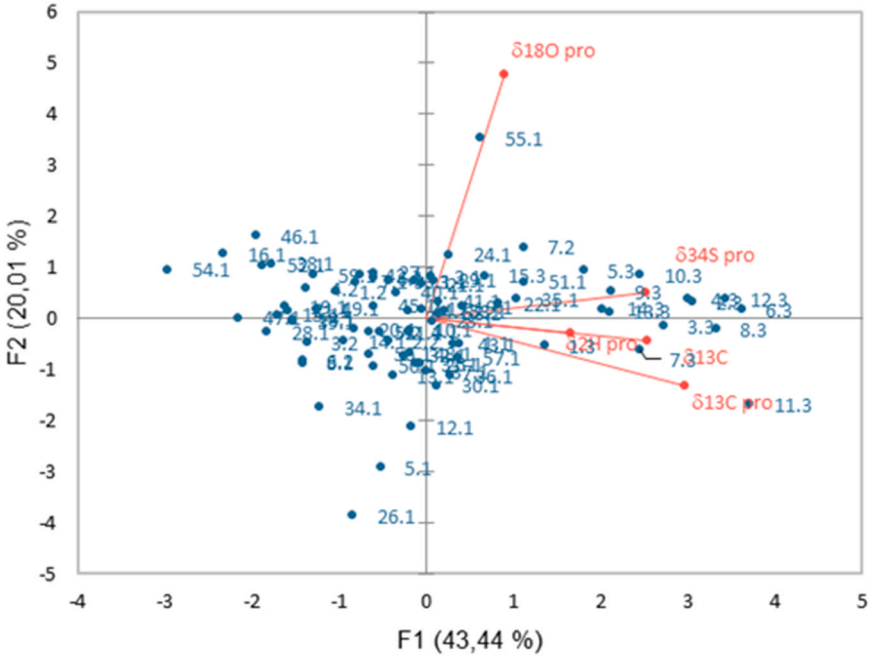


Fig. 4. Principal component analysis (PCA) score plot. Group 1: honeys from Concordia (Argentina) and Salto (Uruguay). Group 2: honeys from Canelones. Group 3: Maldonado; samples are labelled with a numeric combination identifying the number of the sample itself and the group it belongs to (e.g., sample 12.1 represents sample n.12, belonging to group 1).

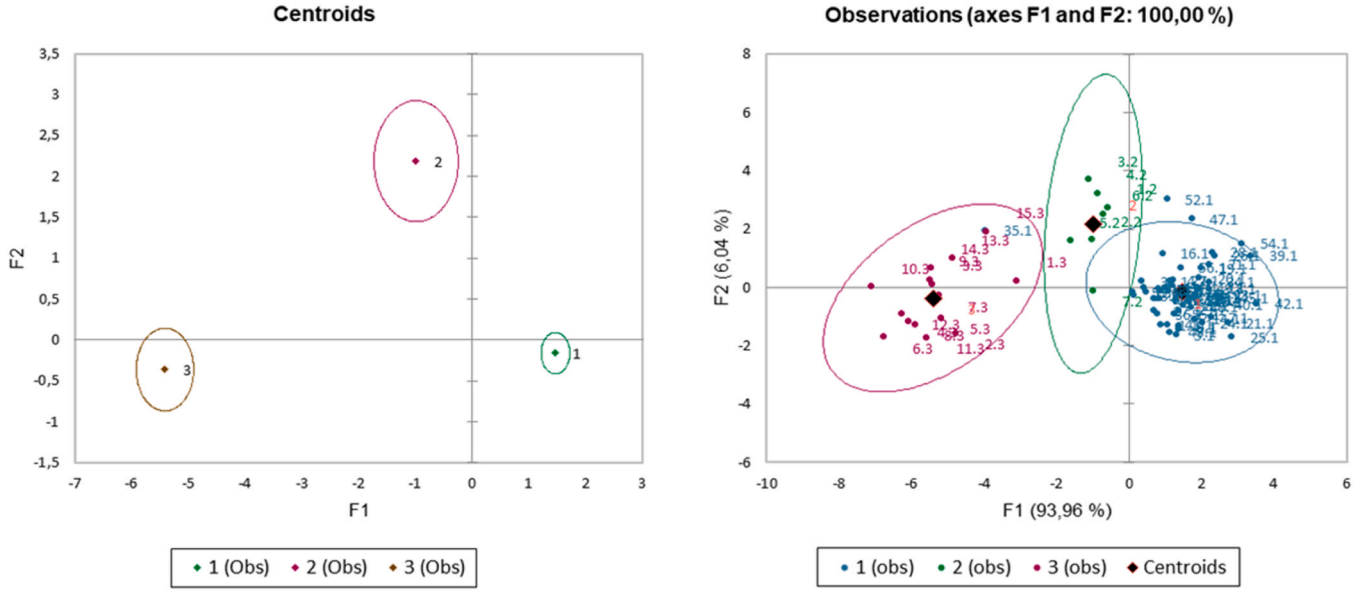


Fig. 5. Centroids from Discriminant Analysis (DA) and Discriminant function score plot for three observation honey groups: Group 1: honeys from Concordia (Argentina) and Salto (Uruguay). Group 2: honeys from Canelones. Group 3: Maldonado; samples are labelled with a numeric combination identifying the number of the sample itself and the group it belongs to (e.g., sample 12.1 represents sample n.12, belonging to group 1).

CRediT authorship contribution statement

Matteo Perini: Writing – review & editing, Writing – original draft, Visualization, Supervision, Conceptualization. **Ana Bonini:** Writing – review & editing, Writing – original draft, Conceptualization. **Gabriela Tamaño:** Formal analysis, Data curation. **Eduardo Dellacassa:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Silvia Pianezze:** Validation, Investigation, Formal analysis, Data curation. **Laura Fariña:** Formal analysis, Data curation. **Eduardo Boido:** Formal analysis, Data curation.

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

The data that has been used is confidential.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106132.

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