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Differences in the adulteration degree and antimicrobial activity of chilean *ulmo* honey versus multifloral honey revealed by stable isotope analysis

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ABSTRACT

Honey, valued for its nutritional and antimicrobial benefits, has experienced an increased production in recent decades. However, this rise has been accompanied by concerns of adulteration, often involving the fraudulent addition of sugars. Our study sought to compare the physicochemical and isotopic properties of various honeys available to Chilean consumers, assessing the extent of adulteration. Samples included honey produced from bees that fed on multiple flowers and those fed by *ulmo* flowers – an endemic species of South America that produces a high-quality, high-cost honey – and analyzed for antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The analysis of ash content (%), moisture (%), pH and total phenolic content (mg GAE/100 g honey) found little obvious differences among honeys regardless of the feeding flowers (multifloral vs *ulmo*), type of purchase market (formal vs informal) or origin of the honeys (Central vs Southern Chile). However, the use of stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of honey provided a powerful means to identify the degree of adulteration prior to the point of sale. Multifloral honeys purchased at informal markets were all adulterated, and *ulmo* honeys included both the least and most adulterated honeys. Regarding their antimicrobial activity, most multifloral honeys were less effective than *ulmo* honeys. Notably, while multifloral honey activity was independent of adulteration, the antimicrobial activity of *ulmo* honey was negatively affected by adulteration.

1. Introduction

Honey is a natural substance mostly produced by honey bees (*Apis mellifera*), the most studied and managed pollinators worldwide, able to increase yield in crops by 96% as well as wild plants (Klein et al., 2007). The sweetness and viscosity of honey arises from its high content of carbohydrates (up to 82%), with water representing only ~17% of its composition; as well as vitamins (B2, B3, B5, B6, B9 and C) and trace nutrients (K, Ca, P, Mg, Fe, Zn, etc.) (Da Silva et al., 2016; De-Melo et al., 2018). Honey collection by humankind started before the honey bee's domestication >8000 years ago (Crane, 1983, p. 360), being recognised as a healthy natural food acclaimed for its antimicrobial properties (Bogdanov et al., 2008). Honey production reached its peak in 2018 with >1,800,000 tonnes produced, with a rise of ~14% worldwide in the 2011–2018 period (Fig. 1). The worldwide rise in honey production contrasts with the decline of wild bee populations in the last 50 years, often driven by habitat loss and fragmentation (Winfree et al., 2009), parasite infections (Sammataro, et al., 2000), exposure to pesticides (Henry et al., 2012; Whitehorn et al., 2012), lack of flowers to pollinate (Goulson et al., 2015), recent climate change (Le Conte & Navajas, 2008), or interactions between these factors (Sánchez-Bayo & Wyckhuys, 2019).

Honey has become an increasingly scarce luxury, and therefore, a coveted substance, encouraging some unscrupulous farmers and other actors to adulterate honey by adding other sugars, i.e. fructose to impede crystallisation, syrups or compounds to change its flavour or viscosity,

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etc.; to increase production and reduce costs (Fakhlaei et al., 2020). This practice is not new, since already in ancient times honey was blended with plant syrups such as maple, birch or sorghum and sold to customers as pure honey, or crystallised and mixed with flour or other fillers until being liquefied to hide its adulteration (Bogdanov et al., 2008). In modern times, the most common adulterant is corn syrup, a clear, almost-flavourless substance which is difficult to differentiate from pure honey (Bogdanov et al., 2008). Honey adulteration can be direct with the addition of a substance directly to honey, or indirect when bees are fed with adulterating substances. According to current regulations, no other substances or additives should be incorporated into honey apart from other forms of honey (FAO, 2001). This reflects a need to counter food fraud where the customer overpays for a mislabelled product, as well as the risk of consuming a lower quality or even dangerous product given that adulterated honey can include substitutes that include dangerous cocktail of chemicals such as antibiotics, colourings or hydroxymethyl furfural, an organic compound potentially carcinogenic and genotoxic formed by the dehydration of reducing sugars (Abraham et al., 2011). Honey has been identified as one of the most easily and recurrently adulterated foods both in the United States and European Union (Fakhlaei et al., 2020; Guler et al., 2014).

Most (~95%) of Chilean honey production is exported in bulk without added value, which has grown ~28% in the 2011–2020 period from 8700 to 12,030 tonnes/year (FAO, 2022), representing the 25th largest honey producer in the world with <1% of the global trade (FAO, 2022). To improve its competitive value on the international market, Chilean honeys have been promoted based on their particular biological origins (Montenegro et al., 2008). Among them, the scented, creamy and whitish honey produced by bees collecting nectar from the flowers of the endemic plant *ulmo* (*Eucryphia cordifolia*), has been promoted for its vitamin and balsamic richness; biocide properties, which might be due to the abundant benzaldehyde and benzene derivatives; its potential as a direct topical application against free-radical scavengers; or as an inducer of apoptotic cell death due to increase in lactate dehydrogenase (Acevedo et al., 2017).

Historically, the authenticity of honey has been determined through High Resolution Liquid Chromatography (HPLC) coupled to different detectors (Andrade et al., 1997; Cabanero et al., 2006). Although widely used, this technique is often time consuming, and highly trained personnel are needed for its processing and analysis, and it is not suitable to detect sophisticated adulterations or determine low concentrations of sweetener additives (Chen et al., 2019; Fakhlaei et al., 2020). However, the development of new analytical methods based on the isotopic measurement of carbon stable isotope ratios using isotope ratio mass spectrometry (IRMS) resulted in the approach being recognised as one of the most effective techniques to analyse the addition of adulterants in different foods, due to its simplicity, speed of analysis, reduced pretreatment of the samples and great versatility, especially in honey (Chen et al., 2019; Tosun & Keles, 2021). The isotopic method is based on the carbon isotopic differences of $^{13}C/^{12}C$ shown between plants from which the honey is produced and those used to produce the adulterants (Elflein and Raezke, 2008; Fakhlaei et al., 2020; Guler et al., 2014). Thus, C3 plants have carbon isotopic values ranging from −32 to −21 ‰, while C4 plants have enriched values between −19 and −12 ‰, which makes additives based on common sweeteners such as high fructose syrup (Padovan et al., 2003), corn syrups (Guler et al., 2014; Sivakesava & Irudayaraj, 2001a), or other derivatives of sugar cane (Fakhlaei et al., 2020; Sivakesava & Irudayaraj, 2001b), easily recognizable isotopically in adulterated honey even at low concentrations.

The first goal of this study was to analyse physiochemical and isotopic characteristics of different multifloral and *ulmo* honeys produced in Chile, differentiating those collected from formal vs informal markets. A second goal was to analyse the potential antimicrobial activity of these honeys against three bacterial strains: *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

2. Materials and methods

2.1. Commercial honey samples

The honeys used in this study included samples labelled as being from either a multifloral (n = 16) or monofloral (*ulmo*) (n = 8) origin honey (Table 1). All honeys were randomly purchased from either formal, i.e. supermarkets (n = 12) or informal points of sale, i.e. small local markets that sell honey in containers without any brand label associated with the product (n = 12, Table 1). Only half of the honey samples (n = 12) included information regarding their geographical origin, which included both Central and Southern Chile (Table 1). *Ulmo* honey was ~100% more expensive (US$ 12–18/kg) than multifloral honey (US$ 6–10/kg).

2.2. Physicochemical properties and total phenolic content

Ash content (%), moisture (%) and pH were measured according to the Association of Official Agricultural Chemists methods (AOAC, 1999), nº 920.181, 969.38, 994.16 and 957.16, respectively. Total phenolic content (TPC), expressed as mg gallic acid equivalents (GAE)/100 g honey, was analyzed following the Folin-Ciocalteu assay (Bridi et al., 2014). Briefly, 0.2 g of sample was diluted in 4 mL of deionized water, then 0.5 mL of this solution was vortexed with 2 mL of Folin-Ciocalteu (1:10 v/v) and 1 mL of Na_2CO_3 solution, and later this mixture kept in darkness for 1 h at room temperature before absorbance

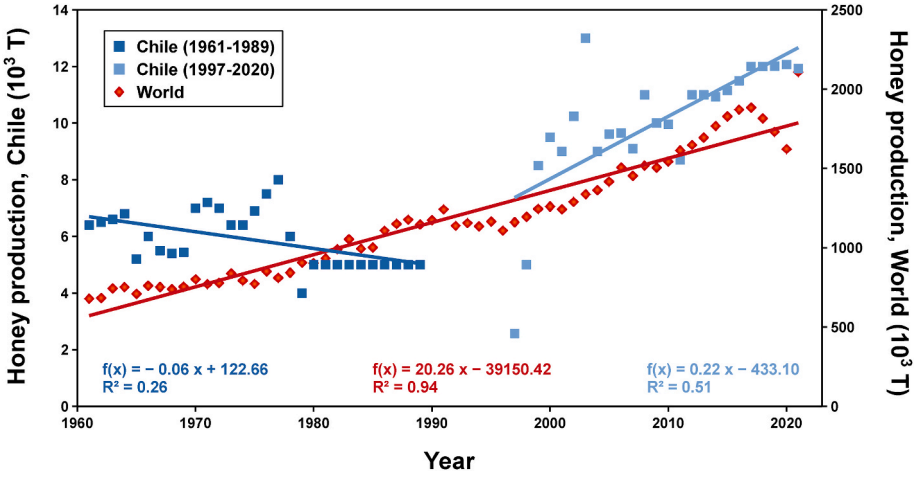

Fig. 1. Chilean vs World honey production (tonnes year^{-1}) over the period 1960–2021. Source: FAO, 2022.

Table 1
Physicochemical properties, isotopic values and adulteration values for analyzed honey samples. In bold, adulterated honeys (>−1‰ difference in $\delta^{13}C$ honey vs protein and/or > 5% calculated adulteration). Moist.: moisture; Adulter.: adulteration; n.e.: non extracted protein fraction; *: Calculation based on average protein isotopic value depending on the type of honey analyzed.

Sample	Type	Market	Origin (City)	Origin (Lat, Long)	Ash (%)	Moist. (%)	pH	TPC (mg GAE/ 100 g)	$\delta^{13}C$ honey ± SD (‰)	$\delta^{13}C$ protein ±SD (‰)	$\delta^{15}N$ protein ±SD (‰)	$\delta^{13}C$ honey - $\delta^{13}C$ protein (‰)	Adulter. (%)
M1	Multifloral	Formal	Codehua	−36.62, −72.08	0.3	13.4	4.5	65	−26.6 ± 0.2	−27.4 ± 0.1	2.3 ± 0.2	−0.81	4.6
M2	Multifloral	Formal	Rancagua	−34.17, −70.74	0.4	17.5	5.0	53	−27.0 ± 0.1	−27.5 ± 0.1	4.6 ± 0.1	−0.42	2.4
M3	Multifloral	Formal	San Fernando	−34.59, −70.99	0.2	14.1	5.2	59	−26.9 ± 0.1	−27.5 ± 0.1	2.3 ± 0.1	−0.59	3.3
M4	Multifloral	Formal	Casablanca	−33.32, −71.41	0.1	16.0	5.0	72	−26.8 ± 0.2	−27.5 ± 0.1	3.1 ± 0.2	−0.68	3.8
M5	Multifloral	Formal	Osorno	−40.57, −73.14	0.4	15.9	4.8	65	−26.6 ± 0.2	−27.4 ± 0.1	3.9 ± 0.1	−0.87	4.9
M6	Multifloral	Formal	Villa Alemana	−33.05, −71.35	0.2	14.0	5.1	71	−26.4 ± 0.1	n.e*	n.e.	–	5.0*
M7	Multifloral	Formal	San Fernando	−34.59, −70.99	0.1	15.3	5.0	61	−26.3 ± 0.1	−27.0 ± 0.1	3.2 ± 0.1	−0.72	4.2
M8	Multifloral	Formal	Rengo	−34.41, −70.86	0.4	15.0	4.7	68	−26.5 ± 0.1	−27.3 ± 0.2	4.2 ± 0.1	−0.85	4.8
M9	Multifloral	Informal	Unknown	Unknown	0.5	18.2	4.8	59	−26.7 ± 0.1	−27.8 ± 0.2	3.3 ± 0.1	**−1.16**	**6.4**
M10	Multifloral	Informal	Unknown	Unknown	0.3	17.4	5.2	44	−25.1 ± 0.1	−27.1 ± 0.2	2.2 ± 0.2	**−1.95**	**11.2**
M11	Multifloral	Informal	Unknown	Unknown	0.2	17.9	4.8	52	−24.6 ± 0.2	−27.0 ± 0.2	3.0 ± 0.1	**−2.34**	**13.6**
M12	Multifloral	Informal	Unknown	Unknown	0.1	16.6	4.9	60	−24.3 ± 0.1	n.e*	n.e.	-	**17.2***
M13	Multifloral	Informal	Unknown	Unknown	0.5	15.9	5.3	72	−25.3 ± 0.1	n.e*	n.e.	-	**11.4***
M14	Multifloral	Informal	Unknown	Unknown	0.4	17.1	5.2	49	−24.4 ± 0.2	n.e*	n.e.	-	**16.4***
M15	Multifloral	Informal	Unknown	Unknown	0.6	14.4	4.9	55	−26.1 ± 0.1	−27.3 ± 0.1	4.1 ± 0.1	**−1.22**	**6.9**
M16	Multifloral	Informal	Unknown	Unknown	0.4	15.1	5.3	64	−26.2 ± 0.1	−27.3 ± 0.1	2.9 ± 0.1	**−1.11**	**6.3**
U1	Ulmo	Formal	Ancud	−41.87, −73.83	0.3	13.6	5.1	77	−27.3 ± 0.2	−27.4 ± 0.2	0.3 ± 0.1	−0.09	0.5
U2	Ulmo	Formal	Futrono	−40.13, −72.40	0.2	14.0	4.9	81	−27.3 ± 0.2	−26.9 ± 0.2	−1.5 ± 0.2	0.36	2.1
U3	Ulmo	Formal	Purranque	−40.91, −73.16	0.4	14.8	5.5	65	−27.5 ± 0.1	−27.3 ± 0.1	−2.9 ± 0.2	0.22	1.3
U4	Ulmo	Formal	Los Lagos	−39.85, −72.83	0.2	15.4	4.8	70	−26.2 ± 0.1	−26.2 ± 0.1	−3.8 ± 0.1	0.02	0.1
U5	Ulmo	Informal	Unknown	Unknown	0.3	14.7	5.0	67	−20.5 ± 0.1	n.e*	n.e.	-	**35.8***
U6	Ulmo	Informal	Unknown	Unknown	0.2	13.9	4.8	49	−26.4 ± 0.2	−25.8 ± 0.1	4.2 ± 0.1	0.55	3.4
U7	Ulmo	Informal	Unknown	Unknown	0.2	14.8	5.6	75	−20.7 ± 0.2	n.e*	n.e.	-	**34.5***
U8	Ulmo	Informal	Unknown	Unknown	0.1	15.0	4.5	57	−22.1 ± 0.2	−25.6 ± 0.2	5.0 ± 0.2	**−3.48**	**21.9**

quantification at 760 nm using an Agilent 8453 UV–visible spectrophotometer (Palo Alto, CA, USA).

2.3. Protein extraction

Proteins were extracted according to the AOAC official method 998.12 (1999), with small changes. Briefly, ~10–15 g of sample was transferred into a clear 50 mL centrifuge tube with 4.0 mL Milli-Q water and the mixture vortexed until full dissolution. Then, a mixture of 2.0 mL of 10% Na_2WO_4 (Merck, Darmstadt, Germany) and 0.3 M H_2SO_4 (Merck, Germany) was added to the tube and later heated at 80 °C on a water bath for approximately 10 min to induce flocculation, adding inoculums of 1 mL of H_2SO_4 until no further flocculation was observed. Flocculated proteins were then concentrated by centrifugation (8 min at 3000 rpm) and rinsed 5 times with Milli-Q water. The resulting pellet was freeze dried for 18 h in a Labconco FreeZone Plus Cascade benchtop freeze dryer prior storage in a desiccator before isotopic analysis by Elemental Analyzer Isotope Ratio Mass Spectrometry (EA/IRMS).

2.4. $\delta^{13}C$ and $\delta^{15}N$ analysis of honey and proteins

Elemental percentages for carbon and nitrogen and stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were measured using a Pyrocube elemental analyzer linked to a visION continuous-flow isotope ratio mass spectrometer (Elementar, Langenselbold, Germany) at the Universidad de Antofagasta Stable Isotope Facility (UASIF), Chile. Stable isotope ratios were expressed using δ notation and are reported as per thousand (‰) relative to Vienna Pee Dee Belemnite for carbon and air for nitrogen. Several international standards were used in each batch to provide a multipoint calibration using the ionOS software package v4.1.005 (Elementar, Langenselbold, Germany). Glutamic acid and sulfanilamide (in house standard, internal precision), as well as certified reference materials USGS40, USGS41a and IAEA CH-6, were used for carbon and nitrogen calibration values. Repeated analysis of standards showed analytical errors (±1 SD) of ±0.04‰ for $\delta^{13}C$, ±0.06‰ for $\delta^{15}N$. Two types of calibration standards were used: (a) sulfonamide (Elementar) and (b) an in-house standard (*Oncorhynchus mykiss* dorsal muscle) to

correct for instrumental drift. The percentages of elemental composition for carbon and nitrogen were also analyzed following a similar statistics framework as that followed for $\delta^{13}C$ and $\delta^{15}N$. Noteworthy, $\delta^{15}N$ could only be measured in proteins. It was extremely difficult to precisely quantify $\delta^{15}N$ values in honey samples due to their relatively low concentrations of nitrogen (Schellenberg, et al., 2010; Kroft et al., 2010). Consequently, this work focuses exclusively on the $\delta^{15}N$ protein fraction.

Honey adulteration was identified when the difference between $\delta^{13}C$ of honey and the $\delta^{13}C$ of its protein fraction exceeded −1‰. The honey adulteration degree (%) was calculated following equation (1) (Chen et al., 2019), where values > 5 % are considered as being adulterated:

$$Adulteration(\%) = \frac{\left[\delta^{13}C_{(protein)} - \delta^{13}C_{(Honey)}\right]}{\left[\delta^{13}C_{(protein)} - \delta^{13}C_{(Sweetener)}\right]} x100 \qquad \text{equation 1}$$

This methodology is widely used by several organisations such as the FAO or the International Atomic Energy Agency (IAEA), which recommend the use of isotopic tools to provide reliable information about potential food adulteration (Kukurova et al., 2004; Padovan et al., 2003). When the $\delta^{13}C$ of the protein fraction was not extractable, the average value of the fractions extracted for each flower source was considered to calculate the adulteration degree.

Additionally, possible deviations in the analysis of percentage of adulteration were established. To do so, tests were carried out at different adulteration percentages of high fructose corn syrup (HFCS) or a sugar cane derivative (SCD), an artificial sweetener commonly used in Chilean honeys. Tests with HFCS and SCD were conducted by adding these sweeteners in a range of 0–25% to a previously unadulterated honey sample previously analyzed by the internal standard stable carbon isotope ratio analysis (ISCIRA) method (Table S1).

2.5. Antimicrobial assays

2.5.1. Bacterial strains

The antibacterial properties of 23 different honeys (all but M8) were tested against four bacterial isolates differing in their cell wall properties and antimicrobial resistance mechanisms, being able to induce enterocolitis, alongside other infection manifestations (Chuang et al., 2017; Wei et al., 2015): Two reference strains, *Escherichia coli* TOP10 (Gram-negative) and *Staphylococcus aureus* ATCC25923 (Gram--positive); and two multidrug-resistant strains of *Pseudomonas aeruginosa* (Gram-negative), one to erythromycin and the other one to multiple antibiotics. These strains were maintained in 20% glycerol suspension at −80 °C. Twenty-four hour cultures in Luria Broth (Sigma-aldrich, MO, USA) at 37 °C were used as an inoculum for experimental assay.

2.5.2. Spectrophotometric assay

We estimated the minimum inhibitory concentration (MIC) through broth dilution techniques. The minimal concentration of honey where bacterial growth was not visibly detected was identified as the MIC (Osés, et al., 2016). To accomplish this, five dilutions (v/v; 50%, 25%, 12.5%, 10% and 5%) of each honey (not gamma irradiated) were prepared aseptically in Miller Hilton broth (Sigma-aldrich, Missouri, United States). The assay was carried out using sterile 96 well flat-bottomed polystyrene plates (Thermo Fisher), with ten μL of bacterial culture (0.01 optical density) added to 190 μL of different honey dilution concentrations, with five replicates per dilution. Control wells containing each dilution (sterility control) were used to correct the optical density measurement and positive control for each strain. The plates were incubated for 24 h at 37 °C.

2.6. Statistical analysis

All statistical analyses were performed in Jamovi (Version 2.3) (The jamovi project, 2023), on open-source statistical application based on R. Normality of data was examined using Shapiro-Wilk tests, and Welch's or Fisher's one-way ANOVA were used assuming equal or unequal variances, respectively, to compare parameters from different honey types depending on the flower source, purchase source and origin. Homogeneity of variance was tested through both Levene's and Bartlett's tests, while *Post-Hoc* comparisons were conducted using Tukey or Games-Howell tests assuming equal or unequal variances, respectively. Statistical significance was determined using an alpha level of 0.05.

3. Results & discussion

3.1. Physicochemical properties of the honeys tested

Overall, there was little evidence for differences ($p > 0.05$) in the physicochemical properties of the honeys tested (n = 24), with a single significant ($p < 0.05$) exception (Fig. 2). Comparing honeys derived from bees that fed on multifloral (n = 16) or *ulmo* flowers (n = 8), mean ash content, 0.32 vs 0.24%, respectively ($p > 0.05$, Fig. 2A), pH, 4.98 vs 5.03, respectively ($p > 0.05$, Fig. 2D), and total phenolic content, 60.6 vs 67.6 mg GAE/100 g honey, respectively ($p > 0.05$, Fig. 2J), were similar; as opposed to mean moisture, 15.9 vs 14.5%, respectively, that significantly differed ($p < 0.05$, Fig. 2G). Comparing honeys according to their purchase location, i.e. formal (n = 12) vs informal markets (n = 12), no significant differences ($p > 0.05$) were observed in ash content (0.26 vs 0.31%, Fig. 2B), moisture (15.2 vs 15.6%, Fig. 2E), ph (4.91 vs 5.04, Fig. 2G) and total phenolic content (64.3 vs 62.3 mg GAE/100 g honey, Fig. 2H), respectively. Regarding the geographical origin of the honeys, no significant differences ($p > 0.05$) were observed between honeys of Central (n = 7) vs Southern Chile (n = 5) in ash content (0.24 vs 0.3%, Fig. 2C), pH (4.9 vs 5.0, Fig. 2F) moisture (15.0 vs 14.8%, Fig. 2I), and total phenolic content (64.1 vs 71.6 mg GAE/100 g honey, Fig. 2L), respectively. The lack of statistical differences in almost all the physicochemical parameters tested contrasts with previous reports that reported significant differences, mostly associated with high adulteration (Bodor et al., 2020; Gemeda et al., 2020). Thus, our findings would at first glance indicate a low prevalence of adulteration with artificial sweeteners mimicking the detectable organoleptic and physicochemical properties of both multifloral and *ulmo* honeys (Fakhlaei et al., 2020; Machado et al., 2022). However, the significant differences in multifloral vs monofloral honeys may not only be attributable to adulteration by sweeteners, but to differences in the biological activities of the originally collected floral sources, geographical provenance, seasonal effects, storage conditions, honey aging, bee colony health, and appropriate beekeeping methodologies contribute to these differences (Abdulkhaliq & Swaileh, 2017; Scripcă and Amariei, 2021). Therefore, it is necessary to emphasize the need of employing advanced methodologies to accurately assess low-scale adulteration in commercial honeys.

3.2. Honey stable isotopic values

Previous studies have shown that honey stable isotope values commonly vary, driven by differences in flower type and/or the climatic and geographical conditions where the flowers grow, although these factors may not explain all the apparent variability (Dinca et al., 2015; Schellenberg et al., 2010). In this study, the $\delta^{13}C$ honey values obtained from the multifloral honeys exhibited a similar variability in informal markets (−26.7 ± 0.1 to −24.3 ± 0.1 ‰, n = 8, Table 1) compared to formal markets (−27.0 ± 0.1 to −26.3 ± 0.1 ‰, n = 8, Table 1), but the difference in means was not significant ($p > 0.05$). Conversely, *ulmo* honeys from informal markets (−22.1 ± 0.2 to −17.6 ± 0.1 ‰, n = 4, Table 1) were significantly $\delta^{13}C$ honey enriched ($p < 0.05$) compared to *ulmo* honey sourced from formal markets (−27.5 ± 0.1 to −26.2 ± 0.1 ‰, n = 4, Table 1) (Fig. 3A).

Ulmo honey purchased at formal markets (−25.8 ± 0.1 to −25.6 ± 0.2 ‰, n = 2, Table 1) showed a significantly ($p < 0.05$) lower $\delta^{13}C$ protein than those purchased at informal markets (−27.4 ± 0.2 to −26.2 ± 0.1 ‰, n = 4, Table 1). Multifloral honeys had similar $\delta^{13}C$ protein

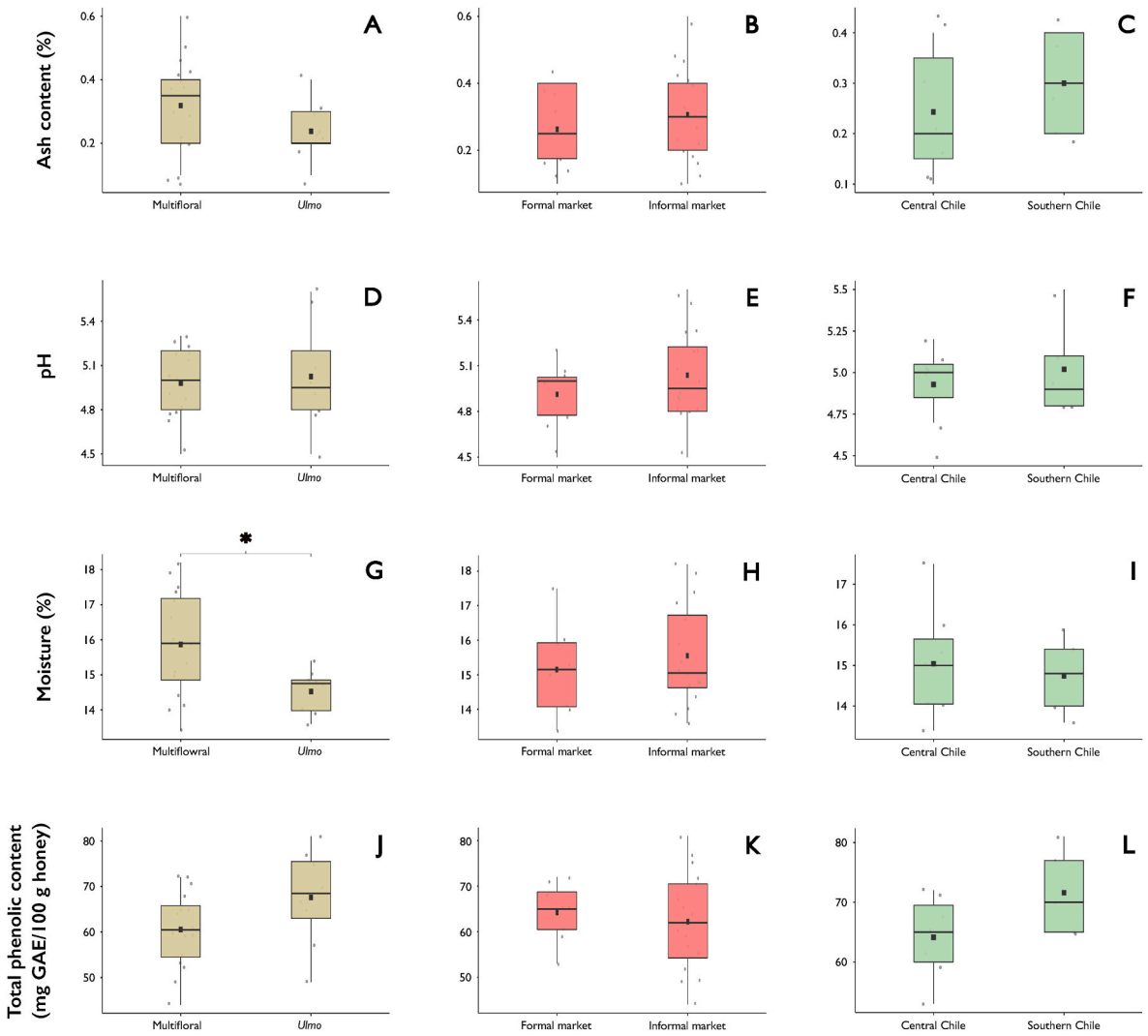

Fig. 2. Physico-chemical properties of the honeys used, based on the flower source (multifloral vs *ulmo*, panels A, D, G and J), commercial availability (formal vs informal market, panels B, E, H and K) or origin of the honey (Central vs Southern Chile, panels C, F, I and L). *p < 0.05.

values (p > 0.05) from both formal (−27.5 ± 0.1 to −27.0 ± 0.1 ‰, n = 7, Table 1) and informal (−27.8 ± 0.2 to −27.0 ± 0.1 ‰, n = 5, Table 1) markets (Fig. 3C). *Ulmo* honeys from informal markets had higher $\delta^{13}C$ protein values (p < 0.05) than multifloral honeys purchased at these markets (Fig. 3C).

These isotopic differences likely reflects the effects of isotopic adulteration highlighted by the contrast between the $\delta^{13}C$ values in the honey fraction (including the isotopically enriched sweeteners used in cases of fraud) and the $\delta^{13}C$ values in the protein fraction which are unaffected by adulteration. Protein $\delta^{13}C$ values showed some differences between market types, albeit greater in *ulmo* rather than multifloral honeys, likely due to its monofloral origin. However, $\delta^{13}C$ values did not vary between the two geographical origins (p > 0.05; Fig. 3B–D), despite the climatic variability between the dryer and warmer region of Central Chile and the wetter and colder region of Southern Chile (Aceituno et al., 2021).

Multifloral $\delta^{15}N$ protein values were similar (p > 0.05) in honey purchased from formal (2.3 ± 0.2–4.6 ± 0.1 ‰, n = 7, Table 1) and informal (2.2 ± 0.2–4.1 ± 0.1 ‰, n = 5, Table 1) markets (Fig. 3E), and in honey originating from Central (2.3 ± 0.2–4.6 ± 0.1 ‰, n = 6, Table 1) and Southern Chile (3.9 ± 0.1 ‰, n = 1, Table 1) (Fig. 3E–F). Conversely, honey sold as *ulmo* showed marked differences (p < 0.05) between purchase markets, with notably $\delta^{15}N$ depleted values (−3.8 ± 0.1–0.3 ± 0.1 ‰, n = 4, Table 1) in formal markets compared to informal markets (4.2 ± 0.1–5.0 ± 0.2 ‰, n = 2, Table 1) (Fig. 3E). Variation in honey nitrogen stable isotope values reflects baseline nutrient status of the soils (e.g. relative use of different fertilizers) which are then reflected in the flowers (Schellenberg et al., 2010). *Ulmo* honeys, due to their monofloral nature, showed a larger range of $\delta^{15}N$ protein values, reflecting the specific cultivation locations that often vary greatly in soil conditions and fertiliser usage (Bustos et al., 2008), where soils in the south of Chile are increasingly nutrient poor and plants show lower (and often negative) $\delta^{15}N$ values relative to those in the more nutrient-enriched Central region (Barrientos et al., 2020; Boeckx et al., 2005).

3.3. Honey adulteration

The ISCIRA, based on the official AOAC method (978.71), considers adulteration by sweeteners such as cane or corn sugar syrups when the isotopic difference between honey and its protein fraction surpasses 1 ‰ (Tosun., 2013). Following this threshold, of the 24 honey samples tested, 45 % (11 out of 24) were adulterated. Adulteration was higher (50 %) of the multifloral honeys (8 out of 16) compared to the *ulmo* honeys (38 %; 3 out of 8) (Fig. 4). Multifloral honeys purchased at formal markets showed isotopic differences that ranged between −0.9 and −0.4 ‰, indicating that they were all unadulterated. Conversely, all multifloral honeys purchased from informal markets were adulterated, with

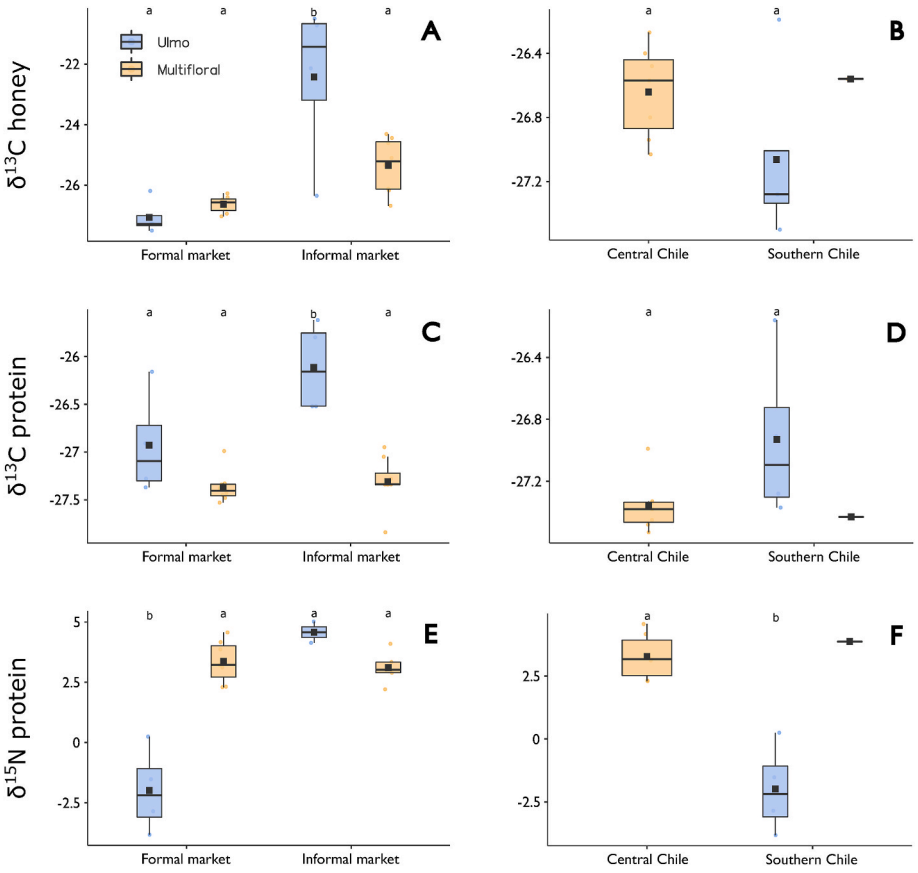

Fig. 3. Variation in stable isotope values ($\delta^{13}C$ honey, $\delta^{13}C$ protein and $\delta_{15}N$ protein) in the different honeys tested, based on the flower source (blue boxes, *ulmo*; yellow boxes, multifloral), commercial availability (formal vs informal market, panels A, C, and E) or the geographical origin of the honey (Central vs South Chile, panels B, D, and F). Small letters indicate the existence of significant statistical difference in mean values per group ($p < 0.05$).

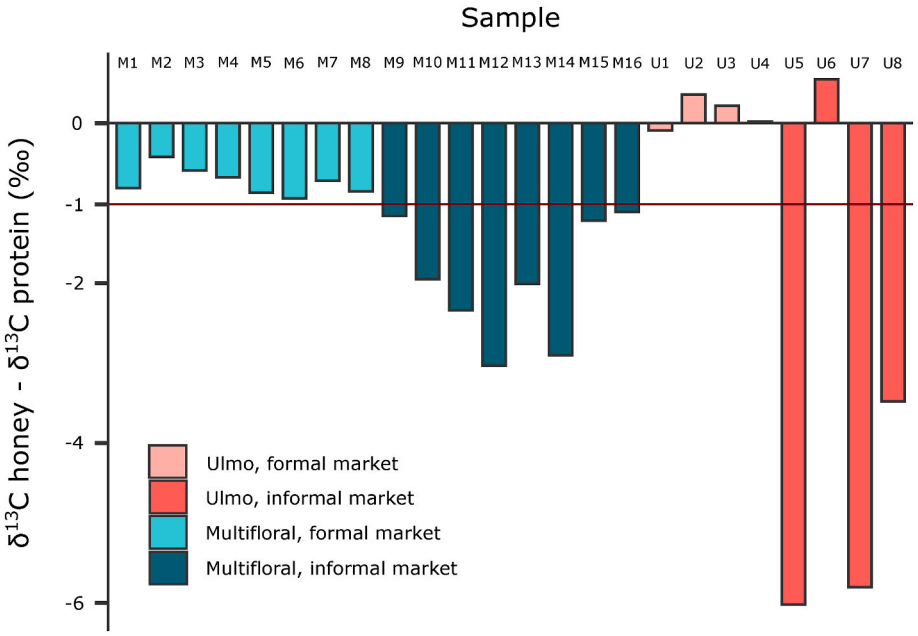

Fig. 4. Isotopic differences in per mil ($\delta^{13}C$ of honey - $\delta^{13}C$ of protein) of the honeys tested. Differences >1‰ are considered to be adulterated. Samples U1-4 are *ulmo* honeys from formal markets; U5-8 are *ulmo* honeys from informal markets, M1-8 are multifloral honeys from formal markets and M9-16 are multifloral honeys from formal markets.

isotopic differences >1‰ (Fig. 4). Adulteration of multifloral honeys therefore reached values as high as 17 % (Table 1), highlighting the likelihood that sale of these products involves the procurement of adulterated honey and fraud (Simsek et al., 2012; Padovan et al., 2003; Elflein and Raezke, 2008).

In the case of *ulmo* honey, those samples purchased at formal markets exhibited relatively consistent $\delta^{13}C$ values (Fig. 3), and the isotopic difference with their protein fraction remained low, from −0.1 to 0.4 ‰, indicating a lack of adulteration (Fig. 4). However, in samples from informal markets, extraction of the protein fraction was only possible in 2 out of the 4 samples: the unadulterated sample U6 showed an isotopic difference in $\delta^{13}C$ of 0.6 ‰ (3.4% adulteration) (Table 1) while the highly adulterated sample U8, showed a difference in $\delta^{13}C$ of −3.5 ‰ (22 % adulteration) (Table 1). The remaining two samples (U5 and U7) where the protein fraction was unextractable, showed adulteration percentages of 36 % and 35 %, respectively (Table 1). Despite finding a lower proportion of adulterated *ulmo* honeys (75 %) than multifloral honeys (100 %) in the informal market, their adulteration degree was significantly ($p < 0.05$) higher, from 22 to 36 % vs 6–17 %, respectively (Table 1). It is hypothesised that this difference may arise due to the intrinsic economic values of these honeys, since the price of *ulmo* honey can be double that of multifloral honey.

The high rates of adulteration in multifloral honeys from informal markets might be attributable to both a constant search for greater economic profits by the informal sellers, and to a lack of stricter policies by local authorities to pursue illegal practices. Implementing stronger regulatory measures, such as introducing quality seals, and improving the detection of adulteration through advanced technologies like isotopic analysis, could effectively address this issue and enhance consumer trust in the authenticity of honey products.

We also conducted experiments to explore whether sweeteners other than HFCS could have been used in the adulterated honey samples, as often more than one sweetener is added (Cabanero et al., 2006), a practice that varies between countries. In Chile, sugar cane derivatives (SCD) are utilised across various industries due to their low cost, sweetening capabilities and easy access (Holland et al., 2001). Adulteration tests ranging from 0% to 25% were performed on non-adulterated honey samples to evaluate the reliability of the SCIRA analysis, to examine the effect of considering other potential sweetening additives such as SCD as well as HFCS. The result obtained using both HFCS and SCD using SCIRA, showed no significant ($p > 0.05$) differences between estimated and recorded percentages (Table S1). The average $\delta^{13}C$ for SCD was −12.5 ‰ and HFCS was −9.7‰: this difference between both sweeteners did not represent a major difference in the adulteration calculation, considering the low levels of adulteration shown here, mostly <20% (Table 1), and the absence of differences in physicochemical properties (Fig. 2). However, further studies incorporating chromatographic profiles are required to fully examine the issue of the use of alternative sweeteners during honey adulteration.

3.4. The physicochemical and isotopic properties of adulterated vs unadulterated honeys

Following the identification of different honey samples as adulterated or not, we examined whether this was reflected in their physiochemical composition (ash content, pH, moisture and TPC). However,

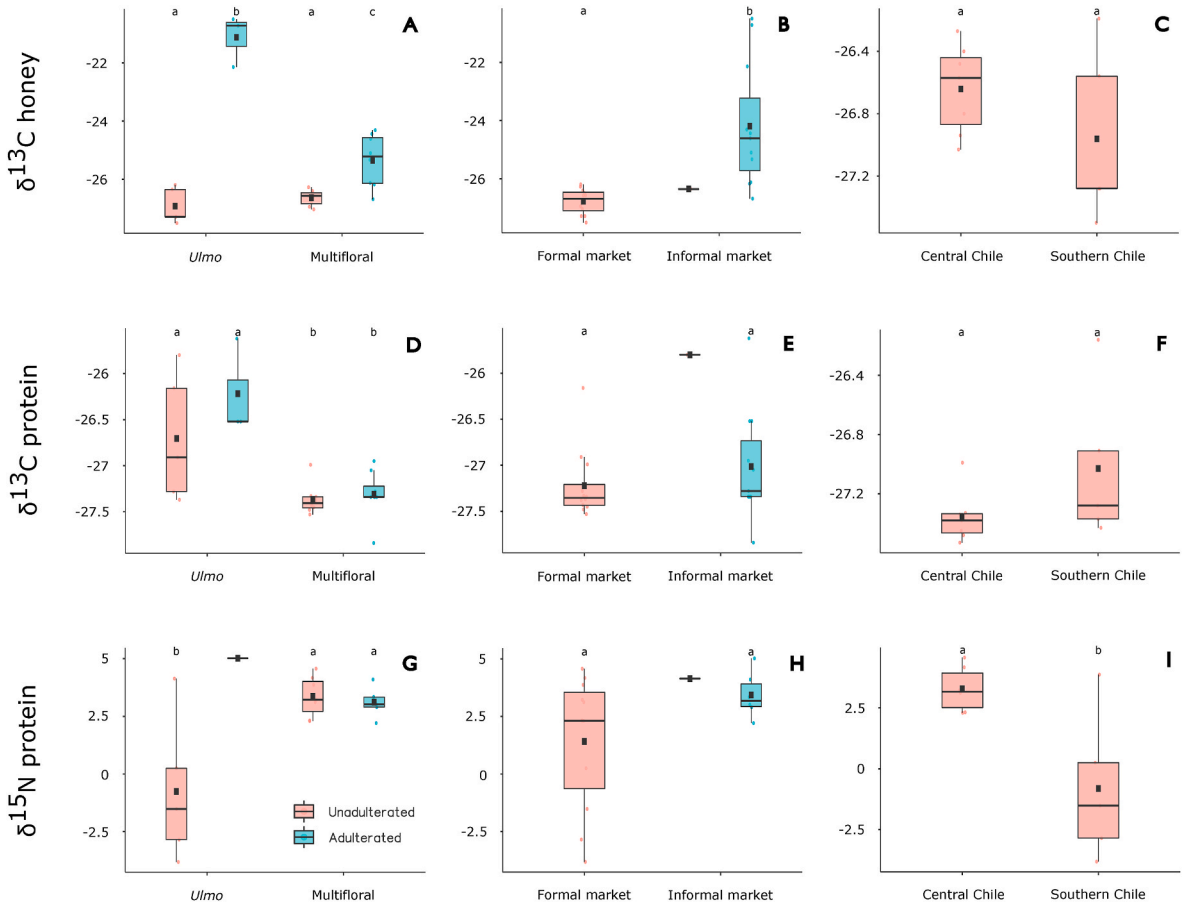

Fig. 5. Variation in stable isotope values ($\delta^{13}C$ of honeys, $\delta^{13}C$ of proteins and $\delta^{15}N$ of proteins) of the honeys tested, based on adulteration (Adulterated: pink boxes; Unadulterated: cyan boxes), the flower source (multifloral vs *ulmo*, panels A, D and G), commercial availability (formal vs informal market, panels B, E and H) or origin of the honey (Central vs Southern Chile, panels C, F and I). U1-4: *ulmo* honeys from formal markets; U5-8: *ulmo* honeys from informal markets; M1-8: multifloral honeys from formal markets; M9-16: multifloral honeys from informal markets. Small letters indicate significance ($p < 0.05$).

there were no significant ($p > 0.05$) differences associated with adulteration (Fig. S1) among the honeys, independent of the flower source, purchase market or geographical origin, except when comparing unadulterated *ulmo* honey with adulterated multifloral honey (Fig. S1). This highlights a worrying lack of utility in some classical measures used to quantify adulteration in honey (Cabanero et al., 2006; Fakhlaei et al., 2020).

When we re-examined the isotopic variation showed between the different honey samples based on their adulteration status, significant ($p < 0.05$) differences became clear associated with the flower source, purchase market and origin (Fig. 5). Thus, adulterated *ulmo* honeys showed significantly ($p < 0.05$) higher $\delta^{13}C$ honey values than that seen in adulterated multifloral honey, a difference not observed between unadulterated honeys of both types (Fig. 5A). Indeed, adulterated honeys at informal markets showed significantly higher values than those purchased from formal markets (Fig. 5B). *Ulmo* honeys showed significantly higher protein $\delta^{13}C$ values than multifloral honeys, independently of adulteration state (Fig. 5D), a pattern paralleled in unadulterated honeys purchased at informal markets (Fig. 5E). There was no evidence that honey $\delta^{13}C$ values were affected by geographical origin ($p > 0.05$; Fig. 5C–F), despite the climatic contrast between these two regions (Aceituno et al., 2021). Unadulterated *ulmo* honey showed significantly ($p < 0.05$) lower $\delta^{15}N$ values compared to adulterated *ulmo* honey. There was a similar pattern in unadulterated *ulmo* honey where samples from Southern Chile were very ^{15}N depleted relative to those from Central Chile (Fig. 5I). However, this difference was not apparent ($p > 0.05$) in multifloral honeys (Fig. 5G) or between market types (Fig. 5H). These differences in $\delta^{15}N$ values could eventually point to suspicious practices with fertilisers and other nitrogenous compounds depending on the origin of the honeys (Schellenberg et al., 2010) or just the biogeochemistry of both regions, as there is often low N availability in soils of the Southern South American (Haberzettl et al., 2005). Therefore further analysis should be undertaken to corroborate these findings due to the economic, social and/or legal consequences it may have.

When integrating simultaneously the stable isotope values of the honeys tested, 3 main clusters arose: cluster 1 with high $\delta^{13}C$ protein and low $\delta^{13}C$ honey and $\delta^{15}N$ protein values, corresponding to the unadulterated *ulmo* honeys U2, U3 and U4 (Fig. 6); cluster 2 with low (−24 to −28 ‰, except U8 with −22.1‰) $\delta^{13}C$ honey, low (−27.8 to −27 ‰, except U6 and U8 with −25.8 and −25.6‰, respectively) $\delta^{13}C$ protein and high (>2 ‰, except U1 with 0.3‰) $\delta^{15}N$ protein values, corresponding to the unadulterated *ulmo* honeys U1 and U6 and multifloral M2-M5 and M7-M8, and the adulterated *ulmo* honey U8 and the multifloral M9-M11 and M15-M16 (Fig. 6); and cluster 3 with no detectable values of $\delta^{13}C$ and $\delta^{15}N$ protein, corresponding to the adulterated *ulmo* honeys U5 and U7 and the multifloral M6, M12, M13 and M14, all but M6 adulterated (Fig. 6).

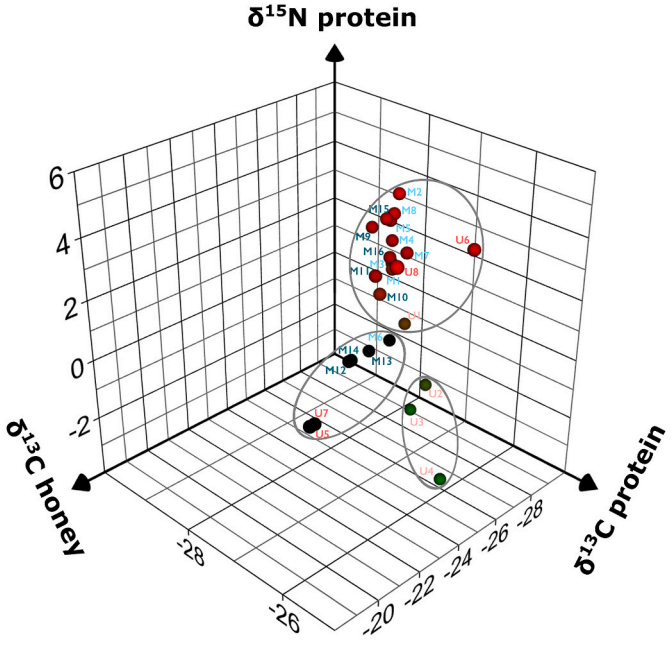

Fig. 6. 3D plot of the honeys tested based on stable isotope values ($\delta^{13}C$ of honeys, $\delta^{13}C$ of proteins and $\delta^{15}N$ of proteins).

Although honey $\delta^{13}C$ values higher than −23.5 ‰ may be considered indicative of adulteration (Guler et al., 2014), it is apparent that the informal market has been able to elude these new methodological approaches, mixing sweeteners with others of artificial origin to achieve isotopic values similar to those of pure honey (Fakhlaei et al., 2020; Mehryar et al., 2013). Determining the value of $\delta^{13}C$ in a commercial sample may not be sufficient by itself to detect adulteration, which was the driver of the modification of the SCIRA method to the ISCIRA method, being the current recommended method for detection of adulteration honey by the AOAC (Kropf et al., 2010). Thus, the carbon isotopic analysis ($\delta^{13}C$) of extracted proteins compared to an original honey sample may be a powerful tool to detect honey adulteration, since no significant differences (<1 ‰ difference) may arise if both the honey and the sweetener values come from same sources (Chen et al., 2019; Tosun, 2013).

3.5. Antimicrobial activity of adulterated vs unadulterated honey

Only 13% of the honey samples showed evidence of antibacterial efficacy at concentrations <12.5% (specifically, 10% [v/v]), according to broth dilution techniques (Fig. 7). Notably, for all the bacterial strains tested, *ulmo* honey exhibited a significant ($p < 0.05$; Fig. 7) greater antimicrobial activity compared to multifloral honey, confirming previous observations (Mandal & Mandal, 2011; Muñoz et al., 2023; Sherlock et al., 2010). Unadulterated *ulmo* honeys showed significantly greater ($p < 0.05$) antimicrobial activity compared to adulterated *ulmo* or multifloral honeys. This difference was not apparent ($p > 0.05$) between unadulterated and adulterated multifloral honeys (Fig. 7). The MIC values obtained from *ulmo* honeys from formal markets were higher (10% v/v) than those previously reported for *E. coli* and *P. aeruginosa* (3.1%–6.3% v/v) using the same spectrophotometric assay (Sherlock et al., 2010). Other studies with other honey types found comparable outcomes, with MICs ranging from 10% to 50%, depending on the specific strains and the efficacy of the honey type employed (Al-Nahari, et al., 2015; Almasaudi et al., 2017; Mullai & Menon, 2007).

Several factors determine the antimicrobial capacity of honeys, including the pH range, which in our study unlikely had an influence on variation in antimicrobial activity; the presence and concentration of glucose oxidase, which releases reactive oxygen species such as H_2O_2 (Albaridi, 2019); the protein content, which appeared to have no influence on antibacterial activity (Muñoz et al., 2023); as well as other factors, such as the osmotic pressure, the water activity, the viscosity of honey or the presence of bioactive compounds, i.e. methylglyoxal, defensin-1, flavonoids, phenolic acids or lysozyme (Feknous & Boumendjel, 2022). Moreover, lactic acid bacteria living in symbiosis with the intestinal microbiota of bees may produce antibacterial metabolites like organic acids, H_2O_2, CO_2, reutirin, diacetyl, bacteriocins, etc., further contributing to its antimicrobial activity (Feknous & Boumendjel, 2022).

However, variation in the stable isotope values in the different honeys did reveal interesting patterns concerning their antimicrobial activity (Fig. 7E–F). Thus, in the case of adulterated honeys, MICs significantly ($p < 0.05$) decreased when the isotopic values (both in $\delta^{13}C$ honey and $\delta^{15}N$ protein) arose (Fig. 7E–F), while in the case of unadulterated honeys the opposite trend was observed, with MICs significantly ($p < 0.05$) increasing as the isotopic values were enhanced (Fig. 7E–F).

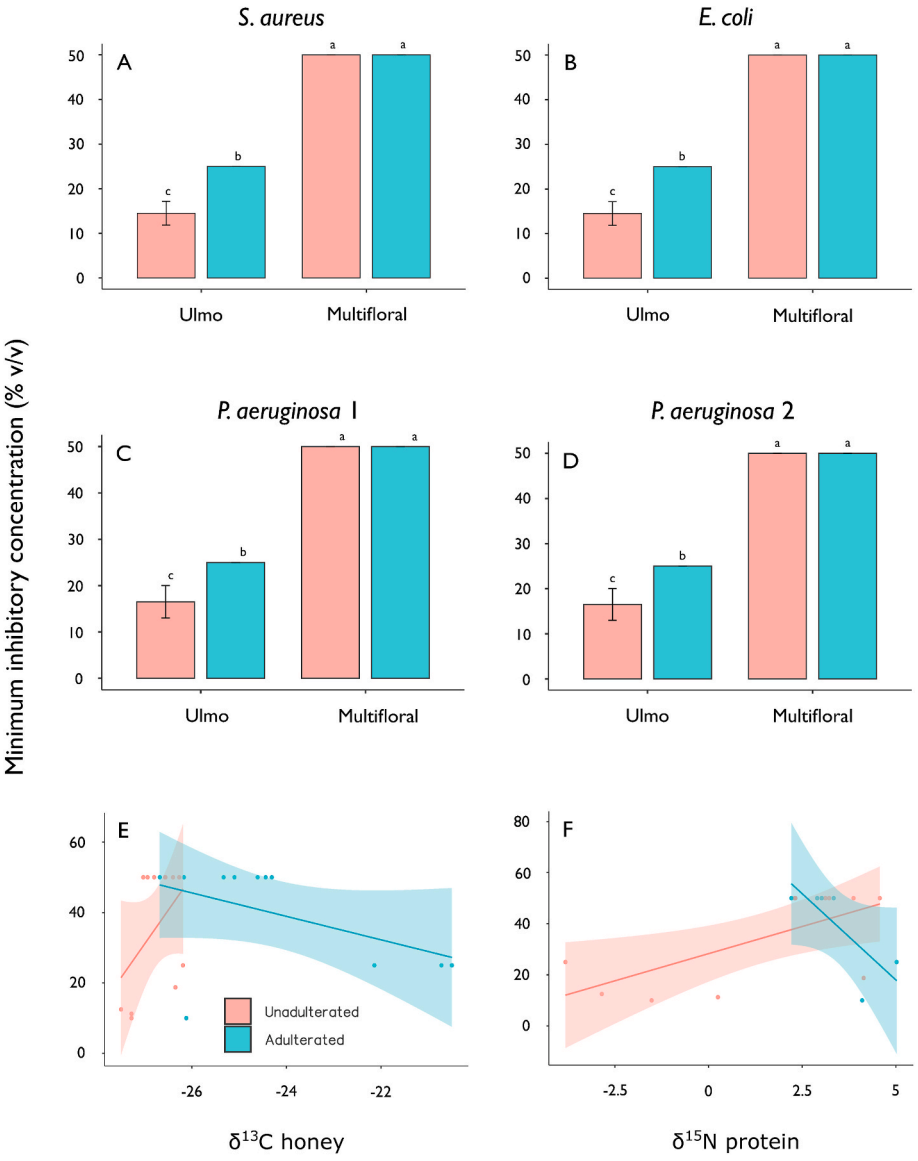

Fig. 7. Antimicrobial activities, expressed as minimum inhibitory concentrations (% v/v), of the *ulmo* and multifloral honeys to *S. aureus* (Panel A), *E. coli* (Panel B), *P. aeruginosa* (1- erythromycin resistant, Panel C) and *P. aeruginosa* (2- multi resistant, Panel D). Panels E–F: Average MIC of the four species vs the isotopic signatures of all the honeys tested (δ^{13}C honey and δ^{15}N protein).

4. Conclusions

Physicochemical properties of honeys did not differ independently of the flower source, market type or geographical origin of the honeys. Significant lower δ^{13}C values (pointing to adulteration) were observed in multifloral and Central Chile honeys vs *ulmo* and Southern Chile honeys. The least (n = 5) and the most (n = 3) adulterated honeys were *ulmo* honeys, the latter being associated with the informal market. In the case of multifloral honeys, adulteration differed depending on the point of sale: all samples of multifloral honey purchased from informal markets were adulterated, while those at formal markets were all unadulterated. The physiochemical characteristics of adulterated and unadulterated honeys were generally similar, highlighting an apparent inability of this approach to identify or quantify rates of the fraudulent adulteration of honey, as opposed to the honey δ^{13}C and honey protein δ^{13}C and δ^{15}N values approach used here. Most *ulmo* honey samples had a higher antimicrobial activity than multifloral honeys. Independently of market origin (formal vs informal) or adulteration, all multifloral honeys had the same antimicrobial activity. The antimicrobial activity of high-financial value *ulmo* honey was reduced by adulteration, highlighting that the effects of fraudulent adulteration of honey on consumers include both financial and medical impacts.

CRediT authorship contribution statement

Pablo Pérez: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paris Lavín:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chris Harrod:** Writing – review & editing. **Pedro Echeveste:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization.

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.foodcont.2024.110590.

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