# Relationship among the minor constituents, antibacterial activity and geographical origin of honey: A multifactor perspective

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## ARTICLE INFO

## ABSTRACT

Some minor constituents of honey samples were determined through a fluorometric-chemical characterization method and related multifactorially with their antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and with their geographical origin. Rotated principal component analysis identified five significant components in honey: three related to antibacterial activity and linked to phenolic compounds; Maillard products; proteins; the concentration of $H_2O_2$ at 3 and 24 h of incubation; and a tyrosine-containing entity. On the other hand, five constituents (phenolic compounds were the most relevant) allowed the classification of honey samples by geographical origin with 87% certainty. The results showed that phenolic compounds and Maillard products are related to the sustained production of $H_2O_2$ over time, which in turn boosts the antibacterial activity of honey. Native flora could promote this capability. The results showed the effect of geographic origin on the content of the analyzed minor constituents of honey, particularly phenolic compounds.

## 1. Introduction

Honey is produced by bees, which collect and store nectar as raw material from flowers, then enrich the nectar with compounds that they themselves produce metabolically. As a result, honey is essentially a concentrated aqueous solution of sugar but also contains a wide range of minor constituents that can reflect the surrounding flora near the hive and the metabolic state of the bees that compose it, as well as determining the nutritional and antibacterial properties of the honey. Among these minor constituents are those originating from plants, such as proteins, amino acids (where proline represents 50–80% of the amino acid content), phenolic compounds and vitamins. There are also those from bee origin, such as the major royal jelly proteins, the antimicrobial peptide defensin-1 and the enzymes glucose oxidase (GOX), glucosidase, diastase and catalase (Bogdanov, 2014; Di Girolamo, D'Amato, & Righetti, 2012; Missio da Silva et al., 2016).

It has been reported that the amount of protein from plant origin in honey is lower than that of protein from honey bee origin; the latter corresponds to enzymes and peptides that bees add to the collected nectar and pollen to transform them into honey (Di Girolamo et al., 2012). Proline in honey accumulates from different floral sources. However, bees prefer the nectar of plants rich in this amino acid

(Carter, Shafir, Yehonatan, Palmer, & Thornburg, 2006). On the other hand, the phenolic content in honey depends particularly on nectar origin, as well as seasonal and environmental factors. The presence and amount of some individual phenolic compounds may be useful markers in terms of the botanical and geographical origin of honey (Socha et al., 2011). Moreover, phenolic compounds from plant nectar have been proposed to be important factors determining the antibacterial activity of honey, where these phytochemical components could participate in redox reactions involving hydrogen peroxide, which has antiseptic properties (Brudzynski & Lannigan, 2012; Brudzynski & Miotto, 2011a,b; Brudzynski, 2006; Brudzynski, Abubaker, & Miotto, 2012). On the other hand, among the minor constituents of bee origin in honey, the peptide defensin-1 and GOX stand out; both have been identified as important antibacterial factors. Defensin-1, an antimicrobial peptide (AMP) effective against Gram-positive bacteria (Casteels-Josson, Zhang, Capaci, Casteels, & Tempst, 1994), is expressed in the head of workers bees by the hypopharyngeal glands (Valachova, Bucekova, & Majtan, 2016) and secreted into royal jelly and honey to protect the larvae (Kwakman et al., 2010). Among its 51 amino acids (5.5 kDa), defensin-1 has a residue of tryptophan at position 47 (Casteels-Josson et al., 1994). GOX is an 85-kDa enzyme that is also expressed in the hypopharyngeal glands of processor and forager bees and secreted into the

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nectar during the preparation of honey (Bucekova et al., 2014). This enzyme catalyzes the oxidation of β-ᴅ-glucose to gluconic acid with the generation of hydrogen peroxide under aerobic conditions in diluted honey, which is generally thought to be a key component in the antibacterial action of honey. Hence, the generation of hydrogen peroxide can be mediated enzymatically and probably chemically by the presence of phytochemicals such as phenolic compounds (Brudzynski et al., 2012; Bucekova, Buriova, Pekarik, Majtan, & Majtan, 2018). On the other hand, the presence of hymenoptaecin in honey samples has recently been reported (Erban, Shcherbachenko, Talacko, & Harant, 2019). This antimicrobial peptide of 10.5 kDa contains five tyrosine residues in its structure and principally inhibits the growth of Gram-negative bacteria (Casteels, Ampel, Jacobs, & Tempst, 1993).

Therefore, the combination of different minor constituents might contribute substantially to the antibacterial activity of honey. In fact, this property is recognized as being dependent on multiple factors in nature (Kwakman & Zaat, 2012). An example is the relationship observed between the generation of hydrogen peroxide and the presence of polyphenols or Maillard products in honey (Brudzynski & Miotto, 2011b; Brudzynski et al., 2012). However, there are no available reports that account for these multidependent connections as a part of the characterization of honey samples. Such an assessment would reveal the networks of components linked to the antibacterial activity of honey. Thus, assuming that a multifactor relationship exists among the antibacterial activity of honey and its minor constituents, which are linked to the surrounding flora near the hive and/or to the metabolism of the bees, it is expected that multifactorial analysis will make these relationships evident.

In this work, we characterized some minor constituents of honey samples, aiming to establish multifactorial relationships among these constituents, the antibacterial activity of honey against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the geographical origin of the honey samples. Since several of the minor constituents of honey are intrinsic fluorophores, whole honey (WH) collected from 31 beehives located in different geographical areas in central Chile and its peptide fraction (HPF; 3–10 kDa) were first characterized through total fluorescence spectroscopy (TFS) and parallel factor analysis (PARAFAC) to identify relevant fluorophores. In addition, the total protein content, proline content and concentration of hydrogen peroxide at 3 and 24 h of incubation were determined in WH. Then, multiple-variable regression and principal component analysis (PCA) with an orthogonal varimax rotation was employed to establish multifactorial relationships between the minor constituents and antibacterial activity of honey. Linear discriminant analysis (LDA) was used to classify the honey samples according to their geographical origin based on their profile of minor constituents.

## 2. Experimental

### 2.1. Honey samples

Honey samples were collected in November 2015 from nine apiaries (31 colonies) located in the vicinity of the towns of Chimbarongo (8 colonies from 2 apiaries; 34°40′1.36″S; 71° 0′28.39″W); Codegua (4 colonies from 2 apiaries, 33°59′55.24″S; 70°38′50.39″W); Peumo (8 colonies from 2 apiaries; 34°23′0.71″S; 71°10′58.96″W) and Rengo (11 colonies from 3 apiaries; 34°28′41.38″S; 70°44′36.21″W) in the O'Higgins region of Chile. The samples were placed in clean 250 mL containers, sealed and labeled with the sampling site information. Then, they were transported to the laboratory and stored at room temperature until analysis. In addition to the collection of the samples, the prevailing nectar source of honey was identified by the beekeepers based on the availability of flora for nectar foraging, the location of the apiary and the organoleptic characteristics of the honey (Table 1).

**Table 1**
Geographical origin and prevailing nectar source of the honey samples analyzed.

| Honey sample | Geographical origin in O'Higgins region of Chile | Prevailing nectar source |
|---|---|---|
| C1 | Chimbarongo | Multifloral |
| C2 | Chimbarongo | Multifloral |
| C3 | Chimbarongo | Multifloral |
| C4 | Chimbarongo | Multifloral |
| C5 | Chimbarongo | Multifloral |
| C6 | Chimbarongo | Multifloral |
| C7 | Chimbarongo | Multifloral |
| C8 | Chimbarongo | Multifloral |
| D1 | Codegua | Multifloral |
| D2 | Codegua | Multifloral |
| D3 | Codegua | Multifloral |
| D4 | Codegua | Multifloral |
| P1 | Peumo | *Cryptocarya alba* (peumo)* |
| P2 | Peumo | *Cryptocarya alba* |
| P3 | Peumo | *Cryptocarya alba* |
| P4 | Peumo | *Cryptocarya alba* |
| P5 | Peumo | *Cryptocarya alba* |
| P6 | Peumo | *Cryptocarya alba* |
| P7 | Peumo | *Cryptocarya alba* |
| P8 | Peumo | *Cryptocarya alba* |
| R1 | Rengo | *Quillaja saponaria* (quillay)* |
| R2 | Rengo | *Quillaja saponaria* |
| R3 | Rengo | *Quillaja saponaria* |
| R4 | Rengo | *Quillaja saponaria* |
| R5 | Rengo | *Quillaja saponaria* |
| R6 | Rengo | *Quillaja saponaria* |
| R7 | Rengo | *Quillaja saponaria* |
| R8 | Rengo | *Quillaja saponaria* |
| R9 | Rengo | *Quillaja saponaria* |
| R10 | Rengo | *Quillaja saponaria* |
| R11 | Rengo | *Quillaja saponaria* |

\* Common name.

### 2.2. Fluorimetric characterization of minor constituents in WH by PARAFAC

Honey solutions at 10 or 20% w/v were prepared with ultrapure water and filtered with filter paper (pore size 4 μm). A Varian Cary-Eclipse luminescence spectrometer (Mulgrave, Australia) equipped with a xenon flash lamp was used to obtain total fluorescent spectra. A Starna (Essex, England) quartz cell with a 3 mL inner volume and a $10 \times 10$ mm light path was used. The EEMs were recorded in the $\lambda_{exc}$ range of 220–450 nm every 10 nm and $\lambda_{em}$ range of 250–600 nm every 2 nm. The widths of the excitation and emission slits were 10 nm. The EEMs were saved in ASCII format and transferred to a computer for subsequent manipulation. The EEMs were pretreated to eliminate Rayleigh and Raman scattering peaks using an algorithm previously reported (Zepp, Sheldon, & Moran, 2004), arranged into a three-way mathematical array and then analyzed through PARAFAC with an algorithm available on the web (http://www.models.kvl.dk/algorithms). Nonnegativity constraints were applied in the excitation, emission and score modes. The number of factors needed to describe the systematic variation in the three-way array was evaluated through the standard deviation of the residual and the core consistency test (Bro & Kiers, 2003), which provide a measure of the variability of the experimental data reflected by the model. All the algorithms were implemented using the graphical interface of the MVC2 toolbox (Olivieri, Wu, & Yu, 2009), which is available on the Web (www.iquir-conicet.gov.ar/descargas/mvc2.rar). The computation and algorithms were implemented in MATLAB v.7.6 (Mathworks, Natick, MA).

## 2.3. Fluorimetric characterization of minor constituents in HPF by PARAFAC

To obtain HPF, 25 mL of honey solution at 20% w/v was prepared in 0.1 M HCl and filtered by using filter paper (pore size 4 μm). Then, 20 mL of this solution was placed in two 10-kDa molecular weight cutoff Amicon Ultra-15 tubes (Millipore, Bedford, MA, USA) and centrifuged at 4000 $g$ for 45 min at room temperature. The < 0-kDa filtrates were collected, transferred to a 3-kDa molecular weight cutoff Amicon Ultra-15 tube and recentrifuged at 4000 $g$ for 80 min at room temperature. The > 3-kDa retentates were subsequently washed in the filter tube with 5 mL of water, recovered (approximately 0.3 mL), merged and finally completed to 1 mL (equivalent to 4 g of honey per mL). This solution was diluted ten times in water before the total fluorescence spectra determination. The Varian Cary-Eclipse luminescence spectrometer was also used to obtain the spectra in Starna (Essex, England) quartz cells with an 800 μL inner volume and 5 × 5 mm light path. The TFSs were recorded in the $\lambda_{exc}$ range of 220–350 nm every 5 nm and in the $\lambda_{em}$ range of 250–450 nm every 2 nm. The widths of the excitation and emission slits were 5 and 10 nm, respectively. The EEMs were pretreated to eliminate Rayleigh and Raman scattering (Zepp et al., 2004) and analyzed through PARAFAC as previously detailed for whole honey in Section 2.2.

## 2.4. Chemical characterization of minor constituents in WH

### 2.4.1. Determination of protein content

The protein content in honey was determined by the Bradford method (Azeredo, Da, Azeredo, de Souza, & Dutra, 2003). Bovine serum albumin standard solution (10–100 mg/0.1 mL) in 0.15 M NaCl was prepared to build a calibration curve. Then, 0.1 mL of the bovine serum albumin standard solution or 20% w/v honey solution and 5 mL of 0.1 mg mL$^{-1}$ Coomassie Brilliant Blue G-250 (99%, Sigma-Aldrich, St. Louis, MO, USA) were added to a 10 mL test tube. The Coomassie Brilliant Blue was prepared in 5% v/v ethanol (95%) and 10% v/v $H_3PO_4$ (85%). After 2 min incubation at room temperature, the absorption at 595 nm was measured in an Agilent Cary 8454 spectrophotometer. The absorbance values were corrected by measuring a blank made by replacing the standard or sample with water.

### 2.4.2. Determination of proline

The proline concentration in honey was determined quantitatively using a modification of a previously described method (Ough, 1969). A solution of proline (99%, Sigma-Aldrich, St. Louis, MO, USA) of 1 g L$^{-1}$ was prepared in water and subsequently diluted to concentrations ranging from 10 to 90 mg L$^{-1}$ to build a calibration curve. Solutions of honey at 10% w/v were prepared in water and filtered with filter paper. Then, 0.5 mL of the standard solution of proline or honey was placed in a 15 mL screw cap test tube. Then, 0.25 mL of formic acid (98%, Merck) and 0.5 mL of a 3% w/v ninhydrin (99%, Sigma-Aldrich, St. Louis, MO, USA) solution prepared in ethylene glycol monomethyl ether (99.5% Merck) were added to the tube. After closing the caps, the tubes were mixed and heated in a boiling water bath for 15 min, ensuring that the water level exceeded the level of the solution inside the tubes, and then cooled in a water bath for 5 min. After cooling, 5 mL of a mixture of 2-propanol-water (1:1 v/v) was added, mixed and left for 35 min. Then, the absorbance was measured at 513 nm in a Cary 8454 spectrophotometer (Agilent Technologies). The absorbance values were corrected by measuring a blank made with a similar mix of reagents (diluted honey) without heating.

### 2.4.3. Determination of $H_2O_2$ concentration

The hydrogen peroxide concentration in honey was determined quantitatively using a modification of a previously described method (Kwakman et al., 2010). A 435 mg L$^{-1}$ solution of $H_2O_2$ (30%, Merck) was prepared in water and subsequently diluted at concentrations ranging from 2.2 to 17 mg L$^{-1}$ in 0.01 M phosphate buffer, pH 6.5, to build a calibration curve. A solution of honey at 20% w/v was prepared in 0.01 M phosphate buffer and pH 6.5, filtered with filter paper and left at room temperature for 3 and 24 h. Then, 0.5 mL of the standard solution of $H_2O_2$ or solution of honey was placed in a 15 mL screw cap test tube, to which was added 1.5 mL of 0.01 M phosphate buffer (pH 6.5); 0.5 mL of 0.05 mg mL$^{-1}$ horseradish peroxidase type IV (150 U/mg, Sigma-Aldrich, St. Louis, MO, USA) and 0.25 mL of 0.4 mg mL$^{-1}$ o-dianisidine (95%, Sigma-Aldrich, St. Louis, MO, USA). O-dianisidine was freshly prepared as a 1 mg mL$^{-1}$ stock in water, and peroxidase was diluted from a 1 mg mL$^{-1}$ stock solution in 0.01 M phosphate buffer (pH 6.5) stored at −20 °C. After 10 min of incubation at room temperature, the absorbance was measured at 440 nm in a Cary 8454 spectrophotometer. The absorbance values were corrected by measuring a blank made with a similar mix of reagents, where o-dianisidine was replaced with phosphate buffer.

## 2.5. Determination of the antibacterial activity of WH

*S. aureus* (clinically isolated strain resistant to methicillin) and *P. aeruginosa* ATCC®27853TM were grown in Luria-Bertani (LB) broth at 37 °C and monitored by measuring the culture optical density (OD) at λ 620 nm. The honey was diluted in the LB. Samples diluted to 1:32 v/v (3.1% v/v) were assayed against *S. aureus*, and samples diluted to 1:8 v/v (12.5% v/v) were assayed against *P. aeruginosa*. A total of 100 μL of each dilution in three replicates was applied to 96-well microtiter plates containing an inoculum of 100 μL of bacterial culture at a concentration of 5x10$^5$ CFU/mL. The control contained 100 μL of bacterial culture and 100 μL of LB. The OD was determined at 620 nm before (T$_0$) and after incubation for 24 h in the dark at 37 °C (T$_{24}$), and the adjusted OD was obtained by subtraction. The adjusted OD of each control well was assigned a value of 100% growth.

## 2.6. Statistical and multifactorial analysis

To assess the impact of the geographical origin of honey, as a single categorical factor, on the content of different minor constituents, the data were statistically analyzed using one-way ANOVA and the multiple-range Tukey's test for parametric data or the Kruskal-Wallis test to compare the medians for nonparametric data (p values lower than 0.05 were considered significant). This analysis was performed using Statgraphic XV software (StatPoint Inc., Rockville, Maryland, USA). The multivariate characterization of the samples with regard to minor constituents and antibacterial activity was performed by multiple-variable regression and PCA using Statgraphic XV software and Unscrambler software (CAMO PROCESS AS, Oslo, Norway), respectively. PCA was performed on the correlation matrix using the total cross-validation method to select the number of relevant components; then, an orthogonal transformation (normalized varimax) was applied. To interpret the respective factors, only loads whose absolute value was equal to or greater than 0.500 in each PC were considered in order to retain the least number of factors with the highest weights (Škrbić, Đurišić-MLadenović, & Cvejanov, 2005). The discrimination by geographical origin was performed by LDA with forward selection of parameters using Statgraphics Centurion XV software (StatPoint Inc., Rockville, Maryland, USA).

## 3. Results and discussion

### 3.1. Fluorimetric characterization of minor constituents in WH by PARAFAC

Fig. 1 shows the measured excitation-emission spectra (EEMs) for selected honey samples from the four different geographical areas (Table 1). In general, the emission patterns of the samples are similar, with several bands over the emission range of 325–550 nm and
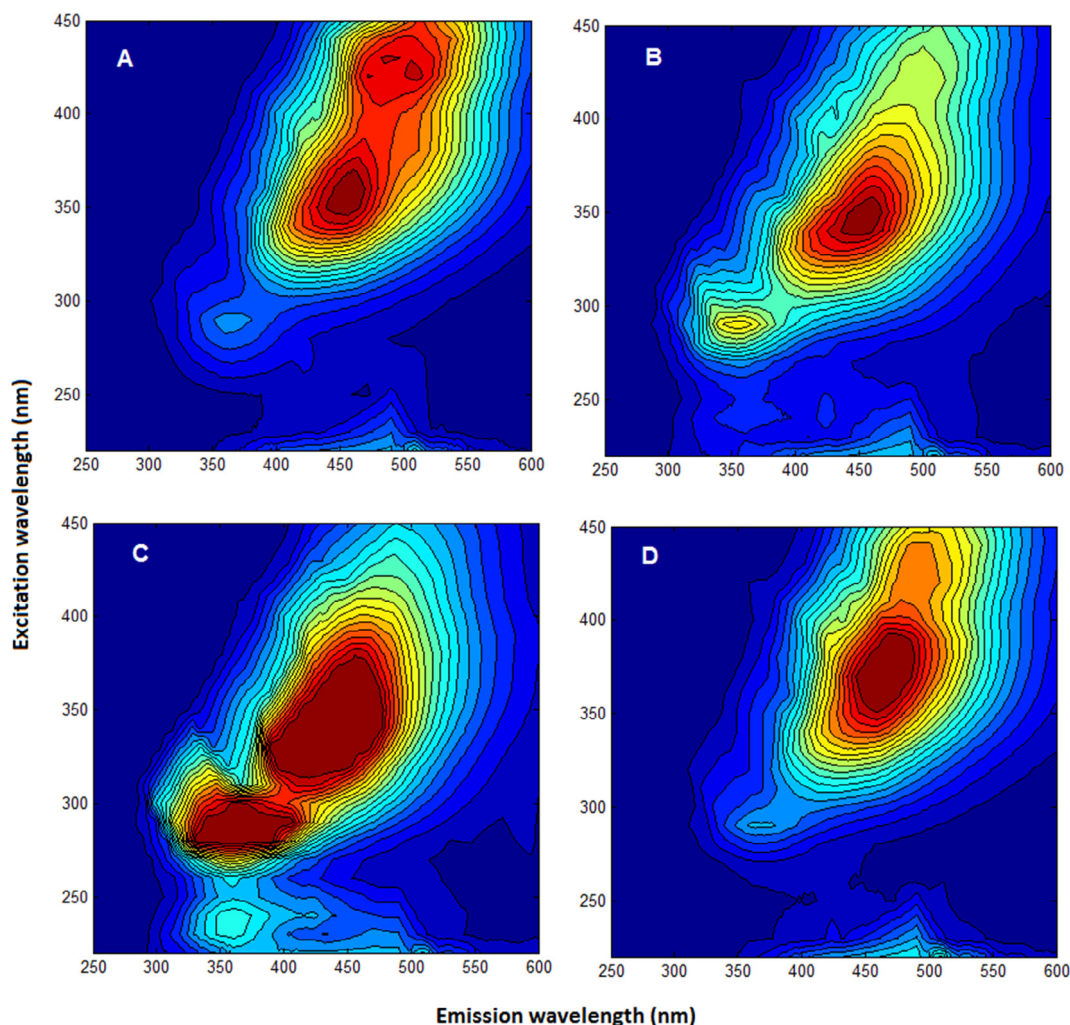
**Fig. 1.** EEM spectra after removing Rayleigh scattering of selected samples from (A) Chimbarongo (sample C1); (B) Codegua (sample D1); (C) Peumo (sample *P*3) and (D) Rengo (sample R1).

excitation range of 250–450 nm. This result indicates, a priori, the presence of the same minor fluorescent constituents in the samples. However, the intensities of these bands are variable depending on the geographical and/or botanical origin of the honey. Then, to obtain the number and type of fluorophores present in WH and their relative content, the EEMs of all samples arranged in a three-way mathematical array were analyzed through PARAFAC. PARAFAC is one of the most widely used tools for processing this kind of data because its intrinsic model is based on the properties of fluorescence signals (Lenhardt, Bro, Zekovic, Dramicanin, & Dramicanin, 2015). This analysis decomposes the three-way array of EEM data into trilinear components according to the number of fluorophores detected in the samples. When data are accurately modeled, the obtained parameters of the model can be further used to calculate the relative concentration of fluorophores in samples (Christensen, Miquel Becker, & Frederiksen, 2005).

The PARAFAC analysis extracted four relevant components. The average emission and excitation profiles of these components are observed in Fig. 2a and b, respectively. The first component (80% spectral variance explained, excitation maximum at 360 nm and emission maximum at 460 nm) was related to products of the Maillard reaction. These products showed maximum excitation and emission at 365 nm and 440 nm, respectively (Matiacevich & Buera, 2006). Maillard reaction products corresponding to multicomponent polymers consisting of protein–polyphenol-oligosaccharides (melanoidins) have been isolated from honey (Brudzynski & Miotto, 2011a). The second component

(14% spectral variance explained, excitation maximum at 440 nm and emission at 512 nm) was associated with riboflavin (vitamin B2) since the pure compound shows an excitation/emission pair of 450/525 nm (Yang et al., 2015). The third component (4% spectral variance explained, excitation maximum at 325 nm and emission at 410 nm) was related to the phenolic compounds present in honey, possibly hydroxycinnamic acids and/or flavonoids, which have excitation and emission maxima in the range of 260–340 nm and 400–426 nm, respectively (Rodríguez, Malovana, Perez, Borges, & Garcia Montelongo, 2001; Sergiel, Pohl, Biesaga, & Mironczyk, 2014). The fourth and last component (1% spectral variance explained, excitation maximum at 280 nm and emission at 350 nm) may be related to tryptophan and/or phenolic compounds (possibly hydroxybenzoic acids) present in honey since they have excitation and emission maxima in the range of 260–315 nm and 360–420 nm, respectively (Lenhardt et al., 2015; Rodríguez et al., 2001). Therefore, this component of WH was referred to as tryptophan-phenolic compounds (Trp-Phenolic). Table S1 shows the relative contents of the four relevant fluorophores in each WH sample obtained from the concentration mode of the PARAFAC model, where the first sample "C1" represents the unit value. Table 2 shows the mean relative content, standard deviation and comparison of these fluorophores for every geographical area. The concentration of the component associated with Maillard reaction products was higher in the Peumo and Codegua groups than in the other two groups. The components related to phenolic compounds and Trp-Phenolic were
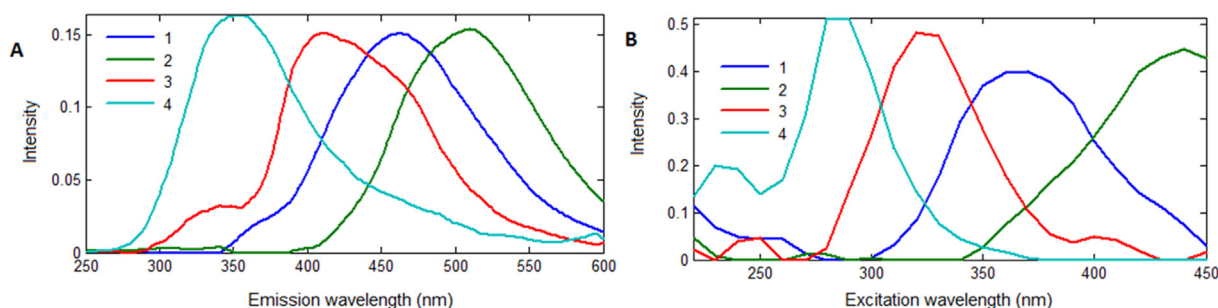
**Fig. 2.** Mean emission (a) and excitation (b) profiles of four components of WH from the PARAFAC model of EEMs with nonnegativity constrained. Components. 1: Maillard reaction products; 2: Riboflavin; 3: Phenolic compounds; 4: Trp-Phenolic.

**Table 2**
Mean relative content of minor fluorescent constituents in WH and HPF obtained from the concentration mode of the PARAFAC model. Mean relative concentration of minor constituents in WH obtained in the chemical characterization. Antibacterial activity of WH against *S. aureus* (ABSa) and *P. aeruginosa* (ABPa). The standard deviation is shown in brackets ().

| | Geographical area | | | |
|---|---|---|---|---|
| | Chimbarongo (n = 8) | Codegua (n = 4) | Peumo (n = 8) | Rengo (n = 11) |
| **Fluorescent constituent in WH** | | | | |
| Maillard products | 0.9 (0.4)[a] | 3 (1)[a] | 2.7 (0.4)[a] | 1.5 (0.7)[a] |
| Riboflavin | 0.9 (0.4)[a] | 1.0 (0.4)[a] | 0.8 (0.3)[a] | 1.3 (0.8)[a] |
| Phenolic compounds | 0.7 (0.4)[a] | 4 (1)[a] | 6.4 (0.4)[a] | 1 (1)[a] |
| Triptophan-Phenolic | 0.6 (0.4)[a] | 11 (7)[a] | 16 (4)[a] | 1 (1)[a] |
| **Fluorescent constituents in HPF** | | | | |
| AGEs | 1.8 (0.7)[a] | 2.1 (0.6)[a] | 7 (2)[a] | 4 (2)[a] |
| Triptophan-Phenolic | 1.9 (0.9)[a] | 1.8 (0.8)[a] | 8 (4)[a] | 4 (2)[a] |
| Unknown | 0.8 (0.2)[a] | 0.8 (0.1)[a] | 0.6 (0.3)[a] | 0.7 (0.1)[a] |
| Tyrosine | 1.3 (0.4)[a] | 3 (1)[a] | 2.4 (0.9)[a] | 2 (1)[a] |
| **Chemical characterization of WH** | | | | |
| Total protein (mg/kg) | 1040 (3 4 2)[a] | 716 (2 9 6)[a] | 634 (1 0 8)[a] | 1008 (2 3 7)[a] |
| Proline (mg/kg) | 297 (61)[a] | 512 (82)[b] | 532 (1 0 0)[a] | 485 (1 1 7)[a] |
| $H_2O_2$ 3 h (ug/g) | 16 (16)[a] | 1 (1)[a] | 23 (12)[a] | 4 (4)[a] |
| $H_2O_2$ 24 h (ug/g) | 2 (2)[a] | 0.6 (0)[a] | 40 (19)[a] | 1.0 (0.5)[a] |
| **Antibacterial activity of WH** | | | | |
| AB (% inhibition *S. aureus*) | 47 (42)[a] | 39 (18)[a] | 69 (39)[a] | 37 (45)[a] |
| AB (% inhibition *P. aeruginosa*) | 3 (8)[a] | 0[a] | 35 (43)[a] | 29 (36)[a] |

[a] Different letters indicate significant differences between groups of samples (p < 0.05).

higher in the Peumo group than in the other three and two groups, respectively. On the other hand, no differences were observed for the component associated with riboflavin among the groups.

### 3.2. Fluorimetric characterization of minor constituents in HPF by PARAFAC

For this analysis, honey samples were size-fractionated by ultrafiltration with 3- and 10-kDa molecular weight cut-off membranes. In this way, only the minor constituents of honey in this size range (named the honey peptide fraction, HPF) were isolated for subsequent analysis by TFS/PARAFAC. Fig. 3a to d show the measured EEM spectra of the HPFs of samples C1, D1, P3 and R1 at dilutions equivalent to 0.1 g of honey per mL, respectively. In the samples, three emission bands were observed: a first strong emission band at λem 325–425 nm, separated into two excitation bands at λexc 210–240 nm and 250–300 nm; a

second strong emission band at λem 380–450 nm and λexc 300–350 nm; and a weaker emission band at λem 275–325 nm, separated into two excitation bands at λexc 210–240 nm and 250–300 nm. As for WH, to obtain the number and type of fluorophores present in HPF and their relative contents, the EEMs, arranged in a three-way mathematical array, were analyzed through PARAFAC.

Four relevant components were obtained in the analysis. The average emission and excitation profiles of the four components are observed in Fig. 4a and b, respectively. The first component (emission maximum at 425 nm), which dominated the emission with an explained spectral variance of approximately 89%, was associated with fluorescent advanced glycation end products (AGEs). Proteins and peptides exposed to glucose over a long period (such as in honey) undergo several physicochemical changes due to the Maillard reaction. This process starts with the initial nonenzymatic condensation of the *N*-terminus and the residues of lysine and arginine in proteins and peptides with glucose (glycation) to form stable derivatives (Amadori products); later, the reaction diverges toward the fluorescent AGEs, which have a maximum fluorescence excitation/emission intensity at approximately 370/440 nm (Matiacevich & Buera, 2006; Nakamura, Nakazawa, & Ienaga, 1997; Séro et al., 2013). Therefore, it is expected that HPF would contain this kind of compound as a product of the Maillard reaction. The second component in HPF (8% spectral variance explained, excitation maximum at 280 nm and emission at 355), similar to that observed in WH, may be related to tryptophan present in peptides and/or to phenolic compounds included in complexes with peptides given the 3–10 kDa molecular weight cut-off. This kind of complex in honey has been previously described (Brudzynski, Sjaarda, & Maldonado-Alvarez, 2013). Because tryptophan is an amino acid occurring infrequently in the proteomes of commonly studied organisms (Russell et al., 2011) and defensin-1 contains this amino acid in its structure and falls within the selected molecular weight cut-off, defensin-1 may be related to the second component in HPF. Undoubtedly, more studies are necessary in this direction. Therefore, according to the available evidence, the second component in HPF was referred to as tryptophan-phenolic compounds (Trp-Phenolic). The third component (2% spectral variance explained) was not related to any particular compound, and its presence was relatively homogeneous in the samples. Finally, the fourth component (0.3% spectral variance explained, emission maximum at 305 nm) was associated with the fluorescence of tyrosine belonging to some peptide present in HPF. Table S1 shows the relative contents of the four relevant fluorophores in the HPF of every sample; these values were obtained from the concentration mode of the PARAFAC model, where the first sample "C1" represents the unit value. Table 2 shows the mean relative content, standard deviation and comparison of these fluorophores for every geographical origin. The concentration of the component associated with Trp-Phenolic was higher in Peumo than in the other three groups, while the concentration of the component associated with AGEs was higher in Peumo and Rengo than in the other two groups. No differences were observed for the component associated with tyrosine.
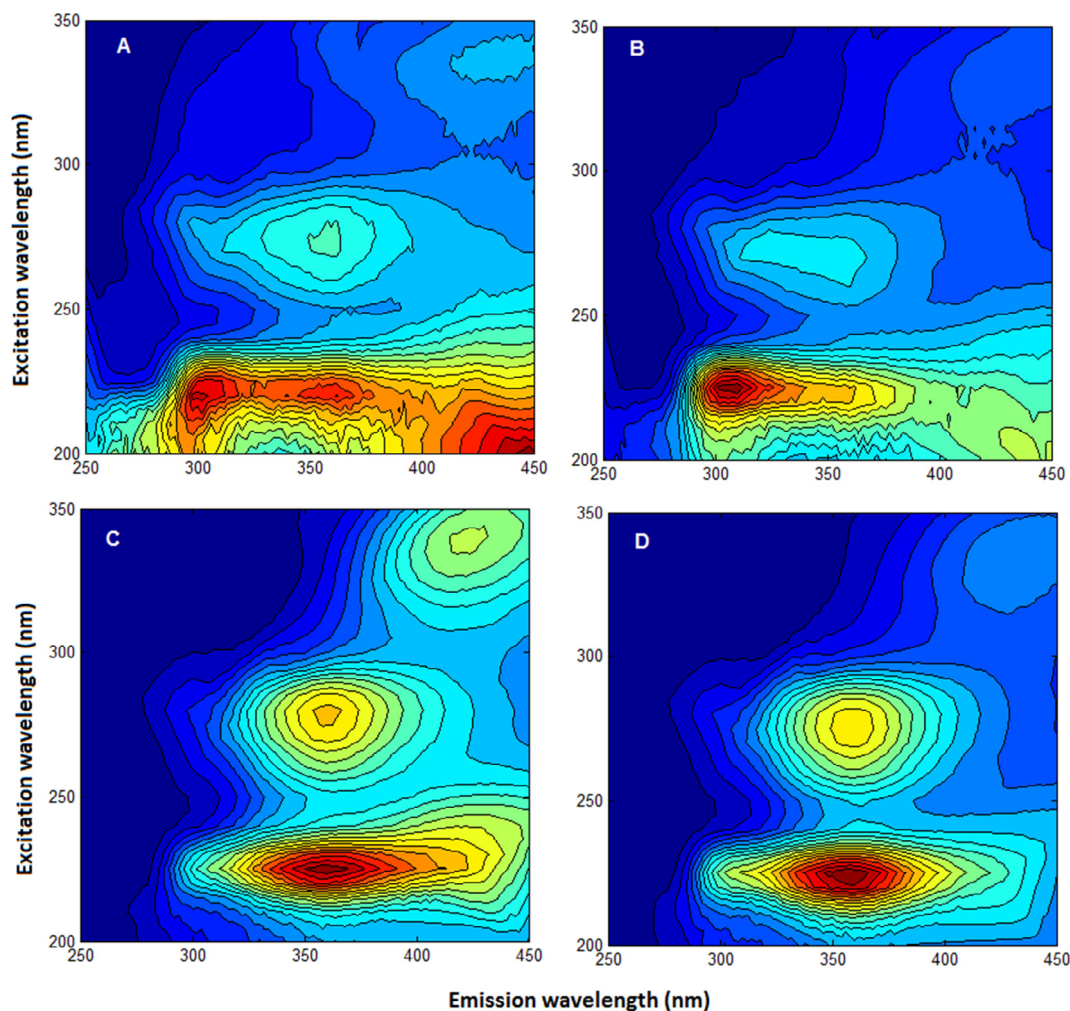
**Fig. 3.** EEM spectra after removing Rayleigh scattering of the HPF of selected samples from (A) Chimbarongo (C1), (B) Codegua (D1), (C) Peumo (*P*3) and (D) Rengo (R1) at dilutions equivalent to 0.1 g of honey per mL.

### 3.3. Chemical characterization of minor constituents in whole honey

Table S2 shows the concentrations of the minor constituents in WH determined for every sample through chemical characterization. Table 2 shows the mean relative content, standard deviation and comparison of these constituents for every geographical area. The total protein content of the analyzed honey ranged from 250 to 1,410 mg/kg (0.25 to 1.41%), which agrees with previously reported values of proteins in honeys of different floral origins produced by *Apis mellifera* (0.20–2.2%) (Azeredo et al., 2003). The content of proline ranged from 224 to 694 mg/kg, which meets the quality standard (minimum 180 mg/kg for a honey to be considered genuine, authentic and mature

(Bogdanov, 2014)). The protein content was higher in Chimbarongo and Rengo than in the other two groups, and the Peumo group showed the lowest content. The proline content was lower in Chimbarongo than in the other three groups.

On the other hand, the concentration of hydrogen peroxide evaluated at 3 h of incubation was between 0.6 µg/g (the limit of detection of the method) and 49 µg/g, and most of the samples presented quantifiable values. For comparison, for 4 h of incubation, Bucekova et al. (2014) reported values between 1 and 20 µg/g for honey samples from Slovakia, and Li et al. (2017) reported values between 0.2 and 7.2 µg/g for honey samples from China for 30 min of incubation. However, after 24 h of incubation, most of the samples showed a decrease in hydrogen
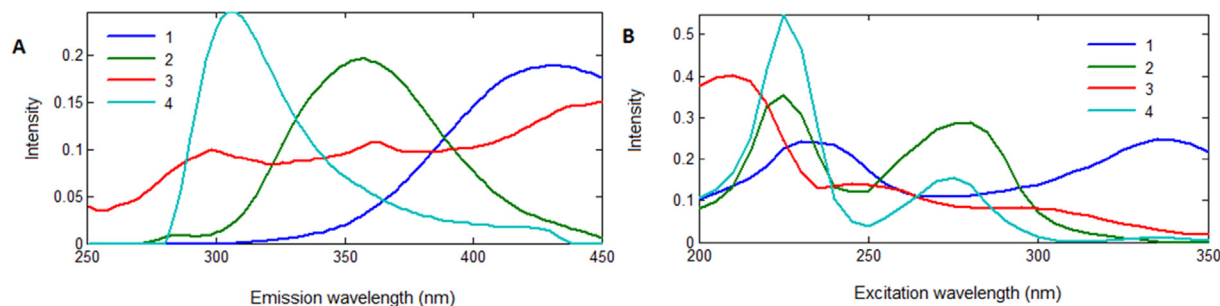


**Fig. 4.** Mean emission (a) and excitation (b) profiles of four components of HPF from the PARAFAC model of EEMs with nonnegativity constrained. Components. 1: AGEs; 2: Trp-Phenolic; 3: Unknown 4: Tyrosine.

peroxide concentrations, with values lower than 2 µg/g (the limit of quantification of the method) (Table S2). The prolonged incubation of honeys (30 min to 24 h) has revealed that typically, the hydrogen peroxide concentrations initially increased before decreasing to 0 at 24 h (Bang, Buntting, & Molan, 2003). The presence of catalase or a drop in GOX activity during prolonged incubation time could cause the decrease in hydrogen peroxide over time in most of the samples. The exceptions were the Peumo samples, which showed an approximately twofold increase in hydrogen peroxide concentration from 3 to 24 h of incubation (Table S2). Consequently, the concentration of hydrogen peroxide evaluated at 3 h of incubation was higher in Peumo and Chimbarongo than in the other two groups, while that measured at 24 h of incubation was higher in Peumo than in the other three groups.

### 3.4. Antibacterial activity of honey samples

Honey is a strong therapeutic agent, which has antibacterial and antibiofilm activity against a broad spectrum of bacteria. In this study, the antibacterial activity of honey samples against the two most clinically relevant bacteria frequently isolated from wounds, representatives of different gram, *S. aureus* (Gram-positive) and *P. aeruginosa* (Gram-negative), was determined. Both are species usually used for this purpose (Bucekova et al., 2018 and 2019). The inhibition percentages of the 31 honey samples against these bacteria are shown in Table S2. Considering the minimum inhibitory concentration values reported for these two tested bacterial strains (Bucekova et al., 2018 and 2019), dilutions of 1:32 and 1:8 of honey were used for *S. aureus* and *P. aeruginosa*, respectively. The evaluated samples were differentially effective against these tested bacterial strains. Almost all samples of honey inhibited the growth of *S. aureus* to different percentages at the dilutions considered; the samples from the Peumo area had the highest values, although these values were not significantly different from those of samples from the other three sectors (Table 2). While only 10 of the 31 honey samples showed the capacity to inhibit the growth of *P. aeruginosa* with different efficacies at the dilution considered, nine of them belonged to the Peumo and Rengo areas. Moreover, three honey samples from these areas (samples *P*2, *P*6 and R4), whose prevailing nectar source is *C. alba* (peumo) or *Q. saponaria* (quillay) (Table 1), were the only ones that showed a 100% inhibition in the growth of the two tested bacteria. Recently, the essential oil of the Chilean plant *C. alba* has been demonstrated to have antifungal activity against *N. ceranae*, which could be due to synergism among its different components (Bravo et al., 2017). Thus, compounds of this endemic plant with antiseptic properties could be transferred to honey, contributing to its antibacterial activity. This possibility deserves further study.

### 3.5. Multifactorial analysis of the minor constituents and antibacterial activity of honey

Multiple-variable regression and PCA with orthogonal varimax rotation were applied to the minor constituent data and the antibacterial activity to identify their multifactorial association. The variance–covariance matrix of the variables and the Pearson product moment correlations between each pair of variables are summarized in Tables S3 and S4, respectively; where p-values below 0.05 indicate statistically significant nonzero correlations at the 95.0% confidence level. On the other hand, the loading-rotated values and the contribution of explained variance (%) of each component are shown in Table S5. Only loadings above 0.500 are considered in the corresponding component to retain the least number of constituents with the highest weights (Škrbić et al., 2005). Five principal components showed eigenvalues higher or equal to 1.0 (Figure S1) and accounted for 85.6% of the total variance in the data. Loading-rotated plot and score plot of these five components are shown in Figures S2, S3 and S4. The first component (PC1), which explains 40% of the total variance, shows the association between phenolic compounds, Trp-Phenolic, Maillard products and

proteins in WH and hydrogen peroxide evaluated at 24 h of incubation. Therefore, these minor constituents in WH represent a specific chemically related group (significant correlations were observed between the constituents, as shown in Table S4) and/or incorporated into high-molecular-weight complexes (melanoidins), as suggested by Brudzynski and Miotto (2011a,b); these constituents are also associated with the sustained production of $H_2O_2$. Phenolic compounds originating from plant nectar have already been indicated to be important elements for the antibacterial activity of honey. It has been proposed that these phytochemical constituents emerged as active intermediates to promote the oxidative action of $H_2O_2$ on bacterial DNA (Brudzynski & Lannigan, 2012; Brudzynski et al., 2012) and/or act in synergy via pro-oxidative action by generating elevated levels of $H_2O_2$ in honey, which mediate the inhibition of bacterial growth as a result of oxidative stress (Bucekova et al., 2018). Our results point in the same direction: phenolic compounds and melanoidins mediate the sustained chemical production of hydrogen peroxide over time and consequently promote the antibacterial activity of honey. This relationship was clearly observed for samples from the Peumo zone, which showed the highest content of phenolic compounds and concentration of $H_2O_2$ at 24 h of incubation. The high content and/or the characteristic chemical profile of polyphenols contributed by the native flora in these samples would mediate the continuous generation of hydrogen peroxide. In this sense, the Chilean plant *C. alba* could have a significant effect on this capacity. Recently, a high content of polyphenols (1200 mg/kg) has been reported in honeys whose predominant botanical origin is this native species (Mejías et al., 2019). Thus, as suggested by Bucekova et al. (2018), it would be important to determine the critical concentration limit of total polyphenols in honey required for antibacterial activity, but it would also be necessary to know their chemical profile. On the other hand, in PC1, the protein content showed a significant inverse correlation with phenolic compounds (Table S4) but also with Trp-Phenolic in WH and HPF, probably as a reflection of the formation of high-molecular-weight insoluble protein–polyphenol complexes. Brudzynski et al. (2013) observed a decrease in protein content in honey associated with the formation of insoluble complexes with polyphenols, which accelerated under oxidizing conditions and high temperatures. Consistently, in our case, the group of samples that presented the highest content of phenolic compounds (the Peumo group) also showed the lowest protein content. Thus, PC1 was attributed to the "chemical profile supplying antibacterial activity" in honey.

The second component (PC2) accounts for 15% of the total variance and includes three of the four fluorophores observed in HPF (the unknown compound, AGEs and Trp-Phenolic). Significant correlations were observed among these fluorophores (Table S4). As described above for WH, the AGEs and Trp-Phenolic in HPF could correspond to a specific chemically related group and/or be incorporated into medium-molecular-weight complexes (3–10 kDa) comparable to melanoidins in WH. Therefore, PC2 was ascribed to a "medium-weight AGEs" in honey.

The third component (PC3) explains 12% of the total variance and includes the hydrogen peroxide evaluated at 3 and 24 h of incubation and the antibacterial activity against *S. aureus* and *P. aeruginosa*. However, the hydrogen peroxide concentration was related only to the antibacterial activity against *S. aureus* (Table S4), indicating that this strain is more sensitive than *P. aeruginosa* to hydrogen peroxide. Thus, PC3 was attributed to the "antibacterial effectiveness linked to $H_2O_2$" of honey, which was found to be related to both its initial and sustained hydrogen peroxide production capacity; the latter is stimulated by the presence of phenolic compounds and Maillard products, as previously discussed.

The fourth component (PC4), which explains 10% of the total variance, includes riboflavin, proline, and Maillard products in WH. The contents of these constituents in the honey samples were more homogeneous than those of PC1, PC2 and PC3. A significant correlation was observed between proline and several minor constituents, but particularly between proline and Maillard products ($r = 0.6874$, $p < 0.05$).

This fact may be associated with the capacity of proline in honey to trigger the Maillard reaction by the previous formation of Amadori compounds (Iglesias et al., 2006). Therefore, PC4 was ascribed to a "shared chemical profile" of honey.

Finally, the fifth component (PC5) explains only 8.6% of the total variance and includes the tyrosine in HPF and the antibacterial activity against *P. aeruginosa*. Although no significant correlation was observed between these variables, when considering only those samples that presented antibacterial activity against *P. aeruginosa*, the correlation between them was significant ($r = 0.6527$, $p < 0.05$). This result suggests that a chemical entity containing tyrosine in HPF would have activity toward this Gram-negative bacterium. On the other hand, tyrosine in HPF showed significant positive and negative correlations with Trp-Phenolic in HPF and protein in WH, respectively (Table S4), which indicates that this amino acid is also part of the medium-weight fluorescent AGEs defined by PC2 but has a smaller contribution. Thus, PC5 was attributed to the "antibacterial effectiveness linked to a tyrosine-containing entity" of honey.

### 3.6. Classification of honey samples based on minor constituents

LDA was used to classify samples according to geographical origin and was initially based on all the minor constituents. According to the results of forward selection (F to enter = 4), phenolic compounds, riboflavin, the hydrogen peroxide evaluated at 24 h of incubation, AGEs in HPF and Maillard products were determined to be statistically significant for discrimination ($p < 0.05$). Based on these constituents, LDA correctly classified 100% of the samples from Chimbarongo and Peumo, 75% of the samples from Codegua and 73% of the samples from Rengo (in total, 87% of the samples were correctly classified). As mentioned above, except for the factor associated with riboflavin, significant differences in the concentrations of these minor constituents in honey among groups were observed, with phenolic compounds exhibiting the largest differences (Table 2). Samples from Peumo and Codegua showed the highest concentrations of phenolic compounds and Maillard products ($p < 0.05$). Similarly, Lenhardt et al. (2015) reported that components related to fluorescent phenolic compounds and Maillard reaction products exhibited the largest difference among classes of honey of different botanical origins and contributed the most to the differentiation of such honeys. Sergiel et al. (2014) reported the possibility of distinguishing honey samples according to their different botanical origins by analyzing their fluorescent phenolic fractions. Therefore, some shared constituents and others are complementary in the differentiation of honey, where the content of phenolic compounds plays a relevant role. Thus, the geographical origin and consequently the surrounding flora can affect the content of the analyzed minor constituents in honey, particularly phenolic compounds, which can thus be used as factors of differentiation with respect to origin.

### 4. Conclusions

Total fluorescence spectroscopy provided information on eight relevant fluorophores as minor constituents in whole honey and the honey peptide fraction (3–10 kDa). This information complemented the contents of protein and proline, the concentration of $H_2O_2$ at 3 and 24 h of incubation and the antibacterial activity against *S. aureus* and *P. aeruginosa* of honey. Multivariate analysis of these data demonstrated that phenolic compounds and Maillard products are related to the sustained chemical production of hydrogen peroxide over time and consequently boost the antibacterial activity of honey, principally against *S. aureus*. This capability could be stimulated by the native Chilean plant *C. alba*. Moreover, the antibacterial activity against *P. aeruginosa* showed a relationship with an entity containing tyrosine present in the honey peptide fraction. On the other hand, the geographical origin and consequently the surrounding flora affect the content of the analyzed minor constituents in honey, particularly

phenolic compounds, which can thus be used as factors of differentiation with respect to origin.

### CRediT authorship contribution statement

**Gonzalo Cebrero:** Investigation, Validation. **Oscar Sanhueza:** Investigation, Validation. **Matías Pezoa:** Validation. **María E. Báez:** Methodology, Writing - review & editing. **Jessica Martínez:** Methodology, Writing - review & editing. **Mauricio Báez:** Methodology, Writing - review & editing. **Edwar Fuentes:** . : Conceptualization, Investigation, Writing - original draft.

### 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.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126296.

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