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Natural attributes of Chilean honeys modified by the presence of neonicotinoids residues

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Abstract Honeys in Chile are produced from native and endemic plant species that, due to phenolic compounds, present beneficial biological attributes. However, certain undesirable pollutants can exist in honeys from beehives located near agricultural crops or commercial industries. Neonicotinoids are a widely used pesticide group in farming, despite acute, negative effects to bee health. Indeed, neonicotinoids are associated with colony collapse disorder, one of the main causes for increased death rates in bee populations. Declining bee health in Chile may consequently be related to neonicotinoids exposure. To assess this threat, honey samples collected from different regions in Chile were analyzed to quantify phenolic contents, antioxidant activity, and the presence of four neonicotinoids (i.e., thiamethoxam, thiacloprid, acetamiprid, and imidacloprid). Pesticide-free honey samples were also fortified with three concentrations of the four neonicotinoids to evaluate changes in the chemical properties of honey. Total phenol contents decreased and antioxidant activity increased in relation to the assessed fortification concentrations. Since the agricultural use of neonicotinoids has been related to those negatives damages for bee health, in Chile, beehives should be strategically located to prevent the contamination of honeys with neonicotinoid pesticides.

Keywords Neonicotinoids · Honey · Antioxidant activity · Pesticides

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Introduction

Between 70% and 90% of all angiosperm species are pollinated by animals. These animal pollinators fulfill an ecosystem service that is widely used by mankind for productive purposes (Fontaine et al. 2006). Approximately 35% of farmed crops worldwide, and fruits in particular, are aided by animal pollination (Klatt et al. 2014). Bees are one of the most important

animal pollinators for both farmed crops and wild plants (Blacquière et al. 2012). Unfortunately, beehive colonies have been in decline since the 1990s across the United States and Europe (USDA 2018). This phenomenon, termed colony collapse disorder, is characterized by the partial or total loss of adult worker bees from a hive (vanEngelsdorp et al. 2009).

Different causative factors for colony collapse disorder have been proposed, including diseases and parasites linked to pesticide exposure in the environment (Mogren and Lundgren 2016; Sanchez-Bayo and Goka 2014; vanEngelsdorp et al. 2017). Neonicotinoids are one particularly concerning group of pesticides given that sub-lethal doses can cause nervous system disorders in bees, including disorientation, memory loss, behavioral changes, and communication issues (Aliouane et al. 2009; El Hassani et al. 2008; Williamson and Wright 2013). Neonicotinoids are additionally associated with immunodeficiency (Lu et al. 2014; Sanchez-Bayo and Goka 2014; vanEngelsdorp et al. 2009), which has also been related to colony collapse disorder.

While ample evidence exists for the negative consequences of pesticides on bee health (Panseri et al. 2014), the use of these compounds remains widely permitted in the agricultural industry. However, in 2013, the European Food Safety Authority (EFSA) issued a statement identifying clothianidin, imidacloprid, and thiamethoxam, three widely employed neonicotinoids, as risks to bee health. This conclusion was based on research detailing acute and chronic impacts to hive survival and development (EFSA 2013a, b, c). The use of these compounds is now restricted across the European Union, even for the treatment of seeds (EFSA Journal 2013; 3066; 3067; 3068). Other groups of insecticides, such as organophosphates, organochlorides, carbamates, and pyrethroids, might also negatively affect bees (Arena and Sgolastra 2014; Al Naggar et al. 2015; Kasiotis et al. 2014). As such, current guidelines for good agricultural practices include norms, standards, and regulations for the use of these compounds in farming (FAO COAG/2003/6).

Honeys in Chile are produced from native and endemic plant species that contain biologically relevant phenolic compounds. Nevertheless, information is lacking regarding the presence of pesticide residues in national apiculture products. This point is particularly important to address considering that 96% of honey exports are to countries in the European Union. Therefore, the aim of this study was to analyze the potential relationship between four neonicotinoids (i.e., acetamiprid, imidacloprid, thiacloprid, and thiamethoxam) and changes in the total phenolic contents and antioxidant activity of honey samples collected from hives located in proximity to agricultural areas across two regions in Chile.

Materials and methods

Honey samples

Ten honey samples (labeled A thru J) were harvested from beehives located between the Coquimbo (29°54′S) and Libertador Bernardo O'Higgins (34°22′S) Regions of Chile during 2015–2016 austral spring–summer. The samples were transported to the laboratory and stored at − 5 °C until analyses.

Determinations of botanical origin through mellisopalynological analysis

Quantitative analyses and counts of botanical elements were conducted according to Loveaux et al. (1978). Qualitative analyses were performed on acetolyzed slides containing bee pollen (20 g) (Montenegro et al. 2008a, b). 20 g of each honey sample was diluted with warm distilled water (20 mL, 40 °C). The solution was transferred to an appropriate tube and centrifuged at 3500 rpm for 10 min. The supernatant was discarded, and the pollen residue was re-suspended in distilled water (100 µL). An aliquot of this suspension (20 µL) was placed on a slide, and Calberla's solution (10 µL; either basic fuchsine or diamond) was subsequently added. The slide was then gently dried. Finally, melted glycerinated gelatin (15 µL) was added to the mixture. The pollen grain residues of each honey sample were identified using an optical microscope (400 × and 1000 × magnifications).

Preparation of phenolic extracts from honey samples

Honey samples (50 g) were mixed with distilled water (100 mL) acidified with HCl (pH 2). The mixture was placed in a volumetric flask, and water was added until

reaching a final volume of 250 mL. The extract was then filtered. Phenolic compounds were separated by column chromatography using the Amberlite XAD-2 resin (height = 250 mm; diameter = 20 mm; and drop speed = 2 mL/min). The column was washed with acid water (100 mL, pH 2), followed by neutral distilled water (200 mL). Finally, phenolic compounds were eluted with methanol p.a EMSURE® Merck (300 mL), and phenolic extracts were collected and concentrated in vacuo to dryness at 45 °C. The resulting residue was suspended in distilled water (5 mL). The suspension was placed in a decantation funnel, and diethyl ether (5 mL) was added. The organic phase was collected and washed twice with ether (5 mL). The solution was concentrated to dryness in vacuo at 45 °C. The obtained residue was re-suspended in high-performance liquid chromatography-grade methanol (2 mL), filtered (0.45 µm pore size), and stored at − 20 °C until analysis.

Determination of phenolic compounds through colorimetric assays

The procedures described by Singleton and Rossi (1965) and Buratti et al. (2007) were used, with minor modifications. Briefly, honey extracts (200 µL) were mixed with the Folin–Ciocalteu reagent (50 µL) and, subsequently, 20% Na_2CO_3 (150 µL). Finally, distilled water was added to a final volume of 1.00 mL. Absorbance was measured at 765 nm after 30 min in a UV–visible spectrophotometer (UV-1700; Shimadzu, Sao Paulo, Brazil). Gallic acid was used as a standard to derive the calibration curve (0–150 µg/mL). Results for phenolic contents were expressed as the mg equivalent of gallic acid/g of sample. This same procedure was implemented to determine phenolic contents in honey samples fortified by distinct pesticide concentrations (see section entitled "Sample fortification").

Determinationy of antioxidant activity through ferric reducing/antioxidant power (FRAP) assays

FRAP assays were performed according to Bertoncelj et al. (2007). Briefly, the FRAP reagent was prepared fresh (i.e., prior to each assay) by mixing 20 mM $FeCl_3$ (2.5 mL) with 2,4,6-tripyridyls-triazine (2.5 mL, containing 10 mM 2,4,6-tripyridyls-triazine/40 mM HCl). The reagent was completed by adding 0.3 M acetate buffer (25.0 mL, pH 3.6).

To measure antioxidant capacity, samples of each honey extract (0.2 mL) were mixed with the FRAP reagent (1.8 mL). Absorbance was read at 593 nm after 10 min. $FeSO_4 \cdot 7H_2O$ was used as a standard to derive the calibration curve (50–1000 mM). Antioxidant capacity values were expressed as mM equivalents of Fe^{+2}/g of sample. This same procedure was employed for honey samples fortified by distinct pesticide concentrations (see section entitled "Sample fortification"). FRAP assays of fortified honey samples also included a solution of the four tested neonicotinoids (0.15 ppm concentration each) to rule out positive reactions with colorimetric assays. No reactions to the pesticide solution were observed for either the phenolic or FRAP colorimetric assays.

Detection of pesticides in honey samples

Pesticides were extracted from honey samples following the methodology proposed by Barganska et al. (2013), with certain modifications. In summary, honey or beeswax samples (2 g) were mixed with 1% acetic acid (5 mL) in an acetonitrile and salt mixture included in the DisQuE Dispersive Solid Phase Extraction Kit (No. 176001903; Waters Corp., Milford, MA, USA) for use with QuEChERS methods. The salt mixture (tube 1) for the extraction process included $MgSO_4$ (4 g), NaCl (1 g), trisodium citrate dehydrate (1 g), and disodium hydrogencitrate sesquihydrate (0.5 g). Triphenyl phosphate (50 µL at 100 µg/mL) was then added as an internal standard. The samples were shaken vigorously for 1 min and centrifuged at 4400 rpm for 5 min. Samples were cleaned-up by transferring the obtained supernatant (4 mL) to a dispersive solid phase extraction tube (tube 2), which was then shaken for 45 s. The tube was then centrifuged at 5000 rpm for 2 min. The resulting supernatant was used for chromatographic analyses (see below). Tube 2 for the cleanup process contained 150 mg of $MgSO_4$ (150 mg), the primary-secondary amine (25 mg), and the primary-second amine sorbent C_{18} (25 mg).

Determination of neonicotinoid contents through ultra-performance liquid chromatography (UPLC)–tandem mass spectrometry (MS/MS)

Honey extracts were analyzed by UPLC–MS/MS using a XEVO Triple Quadrupole Tandem Mass Spectrometer (ACQUITY UPLC H-Class System; Waters Corp., Milford, MA, USA). Separation was facilitated by using an Acquity-BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters Corp.). Chromatographic analyses used a mobile phase gradient containing an aqueous solution of 10 mM ammonium acetate and 10 mM ammonium acetate in methanol. The oven temperature was 30 °C, and the injection volume was 10 µL. The MS/MS parameters were as follows: Ionization mode = positive; Scan type = MRM; Dwell-time = 20 ms; Ion spray voltage = 5500 V; and Source = 300 °C. Collision energy and transition (m/z) in the MRM were adjusted according to respective standards for each neonicotinoid (Table 1).

Sample fortification

To evaluate the effects of neonicotinoids on antioxidant properties, pesticide-free honey samples were fortified with increasing doses of acetamiprid, imidacloprid, thiacloprid, and thiamethoxa. Honey samples were verified pesticide-free using UPLC–MS/MS, as previously described, prior to fortification assays. Duplicate honey samples were used for each of the four separately assessed pesticides and at the following three concentrations: low, 0.001 ppm; medium, 0.003 ppm; and high, 0.006 ppm (Table 2). Honey samples (5 g) were fortified with aliquots of each pesticide, as taken from 0.5 ppm stock solutions diluted to each desired concentration. The fortification of each sample was corroborated by gravimetric evaluation. Once fortified, the samples were stored for 2 weeks in amber flasks at 25 °C.

Table 2 Final fortification low, medium, and high doses of each neonicotinoid detected in respective honey samples

Fortification compound	Final fortification dose (ppm)		
	Low	Medium	High
Acetamiprid	0.0018	0.0035	0.0064
	0.0016	0.0033	0.0070
Imidacloprid	0.0011	0.0034	0.0068
	0.0010	0.0032	0.0068
Thiacloprid	0.0004	0.0035	0.0067
	0.0004	0.0036	0.0068
Thiamethoxam	0.0015	0.0033	0.0068
	0.0015	0.0033	0.0069

Statistical analysis

Statistically analyses were performed in triplicate for each sample. An initial exploratory analysis of the data was conducted using Box-plots to determine appropriate statistical methodology (ANOVA, Linear Regression, and Tukey's Test). Statistical analyses were conducted in the 2016 R v3.11 software.

Table 1 Specific instrumental conditions obtained after analysis of standard 0.5 ppm solutions of each neonicotinoid compound

Compound[a]	Formula/mass	Sample	Parent m/z	Cone voltage	Daughters	Collision energy	Ion mode
Acetamiprid	223.67	1	255.02	34	127.96	20	ES+
		2	255.02	34	55.91	14	ES+
Imidacloprid	255.69	1	255.96	36	209.10	16	ES+
		2	255.96	36	175.01	22	ES+
Thiacloprid	252.72	1	252.92	58	125.92	30	ES+
		2	252.92	58	98.92	46	ES+
Thiamethoxam	291.72	1	291.99	32	211.02	20	ES+
		2	291.99	32	180.96	26	ES+

[a]Solutions were prepared gravimetrically in acetonitrile

Results

Determination of botanical origin

Mellisopalynological analysis was used to identify the primary plant origin of honeys collected between the Coquimbo (29°54′S) and Libertador Bernardo O'Higgins (34°22′S) Regions. The predominant species found in each analyzed sample are shown in Table 3. Unifloral and multifloral honeys of both native and non-native botanical origins were identified.

Chemical analysis of honey samples

The evaluated honey samples presented a lineal correlation (R = 0.961) between total phenol contents and antioxidant activity (Fig. 1). This result suggests that antioxidant ability depends on the phenolic composition of the samples. Furthermore, higher phenol contents were found in samples D, E, and H, the predominant botanical origins of which were *Cryptocarya alba* (50% and 28%) and *Medicago sativa* (42%), respectively.

Analysis of neonicotinoids content

All honey samples were subjected to UPLC–MS/MS analysis. Thiacloprid was not detected in any of the honey samples. In contrast, acetamiprid was found in samples B, F, H, and J, with significant differences found in paired comparisons (Fig. 2a) Imidacloprid was detected in honey samples A, B, C, D, and F (Fig. 2b), while thiamethoxam was found in samples A, B, C, D, F, G, and J (Fig. 2c). Two honey samples did not present any traces of the assessed pesticides—sample E, a unifloral honey of native botanical species from the O'Higgins Region, and sample I, a multifloral honey of non-native botanical species from the O'Higgins Region (Table 3). Honey E was selected for adding pesticides and as a control sample for fortification assays.

Changes in the chemical properties of honeys due to fortification with pesticides

All of the fortified honey samples presented a significant decrease in total available phenols as compared to the non-fortified control (Fig. 3a–d). Furthermore, as the concentration of thiamethoxam increased, total phenol contents decreased, with significant differences found between the three pesticide concentrations (Fig. 3d). Similarly, honey samples fortified with a high thiacloprid concentration presented significantly reduced phenol contents as compared to the low and medium thiacloprid concentrations (Fig. 3c).

In turn, antioxidant activity was significantly increased in all of the fortified honey samples as compared to the control (Fig. 3a–d). Two particular tendencies were in relation to pesticide concentration. First, samples fortified with acetamiprid and thiacloprid showed no significant differences between medium and high concentrations of either pesticide (Fig. 3a, c). Second, honey samples fortified with imidacloprid and thiamethoxam presented no differences between low and medium concentrations of either pesticide (Fig. 3b, d).

Table 3 Predominant botanical origins and classification of the assessed honey samples

Sample	Region	Predominant botanical origin	Classification	Origin
A	Coquimbo	*Escallonia pulverulenta* (26%)	Multifloral	Non-native
B	O'Higgins	*Escallonia rubra* (34%)	Multifloral	Non-native
C	O'Higgins	*Galega officinalis* (36%)	Multifloral	Non-native
D	Coquimbo	*Cryptocarya alba* (50%)	Unifloral	Native
E	Coquimbo	*C. alba* (28%)	Multifloral	Native
F	Coquimbo	*Quillaja saponaria* (85%)	Unifloral	Native
G	Coquimbo	*Azara sp.* (43%)	Multifloral	Native
H	Coquimbo	*Medicago sativa* (17%)	Multifloral	Native
I	Coquimbo	*M. sativa* (42%)	Multifloral	Non-native
J	Coquimbo	*G. officinalis* (54%)	Unifloral	Non-native

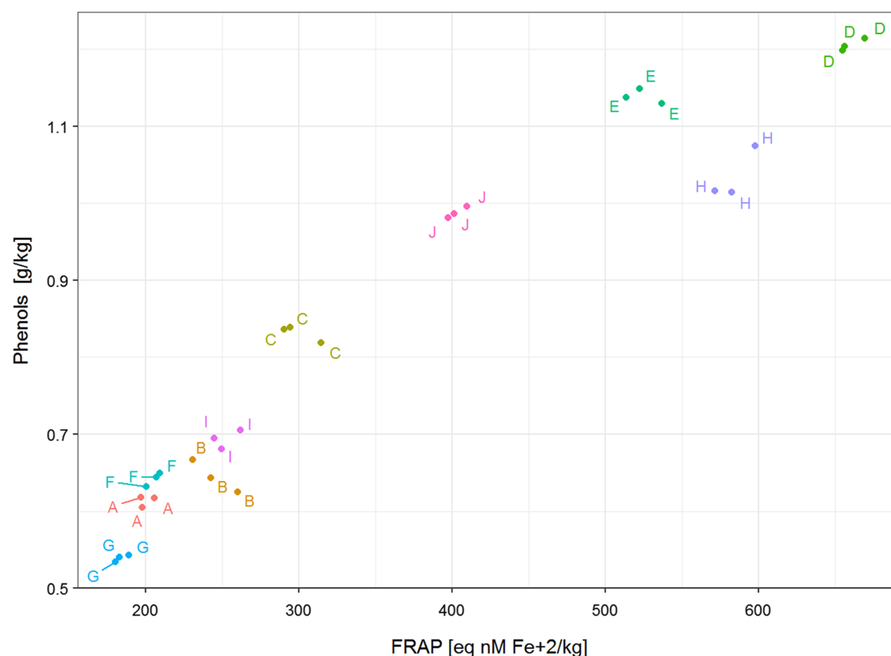


Fig. 1 Correlation between total phenol contents and antioxidant activity in the evaluated honey samples

Discussion

Chile produces approximately 10,000 tons of honey each year, accounting for 0.8% of worldwide production. This industry is spread across a large part of Chile's territory. Since the per capita consumption of honey within Chile is less than the global average, honey exportation is a promising commercial alternative (Montenegro et al. 2008a, b). Floral origin, geographical location, and environmental factors all influence the chemical composition of honeys (Przybyłowski and Wilczyńska 2001; Grembecka and Szefer 2013) Total polyphenolic content and antioxidant/antiradical activity are normally associated with the physiological and bioactive roles of the originating substances (Kroyer and Hegedus 2001). Therefore, the observed differences between total phenol contents and antioxidant activity (Fig. 1) in the assessed honey samples might, in part, be due to variations in the predominant botanical species of origin between the Coquimbo and O'Higgins Regions (Table 3).

The agricultural industry is present across nearly all of Chile, and animal pollinators, such as bees, are frequently used to improve farming yields (Klatt et al. 2014). Pesticides are commonly used in modern agriculture practices (Elgueta et al. 2017), with benefits being pest control and protected crop production. Nevertheless, the excessive use of these compounds can result in a number of negative consequences (Ecobichon 2001; Al Naggar et al. 2015).

Various reports have detected pesticides in honeys originating from geographical areas with nearby farming activities (Barganska et al. 2013; Kasiotis et al. 2014; Kujawski and Namieśnik 2011; Panseri et al. 2014), as found in the present study (Fig. 2). Interestingly, thiacloprid residues were not found in any of the currently analyzed honey samples, and two honeys showed no traces for any of the four assessed pesticides (samples E and I, Table 3). The *Codex Alimentarius* (2001) establishes maximum allowances for human consumption, but little information exists in Chile regarding human health risks associated with pesticide exposure and consumption through food products (Elgueta et al. 2017). Nevertheless, an environmental presence of these neonicotinoids is linked to negative impacts on bee behavior and overall hive health, with additional connections to colony collapse disorder (Aliouane et al. 2009; Williamson and Wright 2013; Sanchez-Bayo and Goka 2014; vanEngelsdorp et al. 2009). Furthermore, chronic pesticide exposure can be damaging for human health as a result of oxidative stress (Sharma et al. 2013). In the same way, the obtained results in this study are

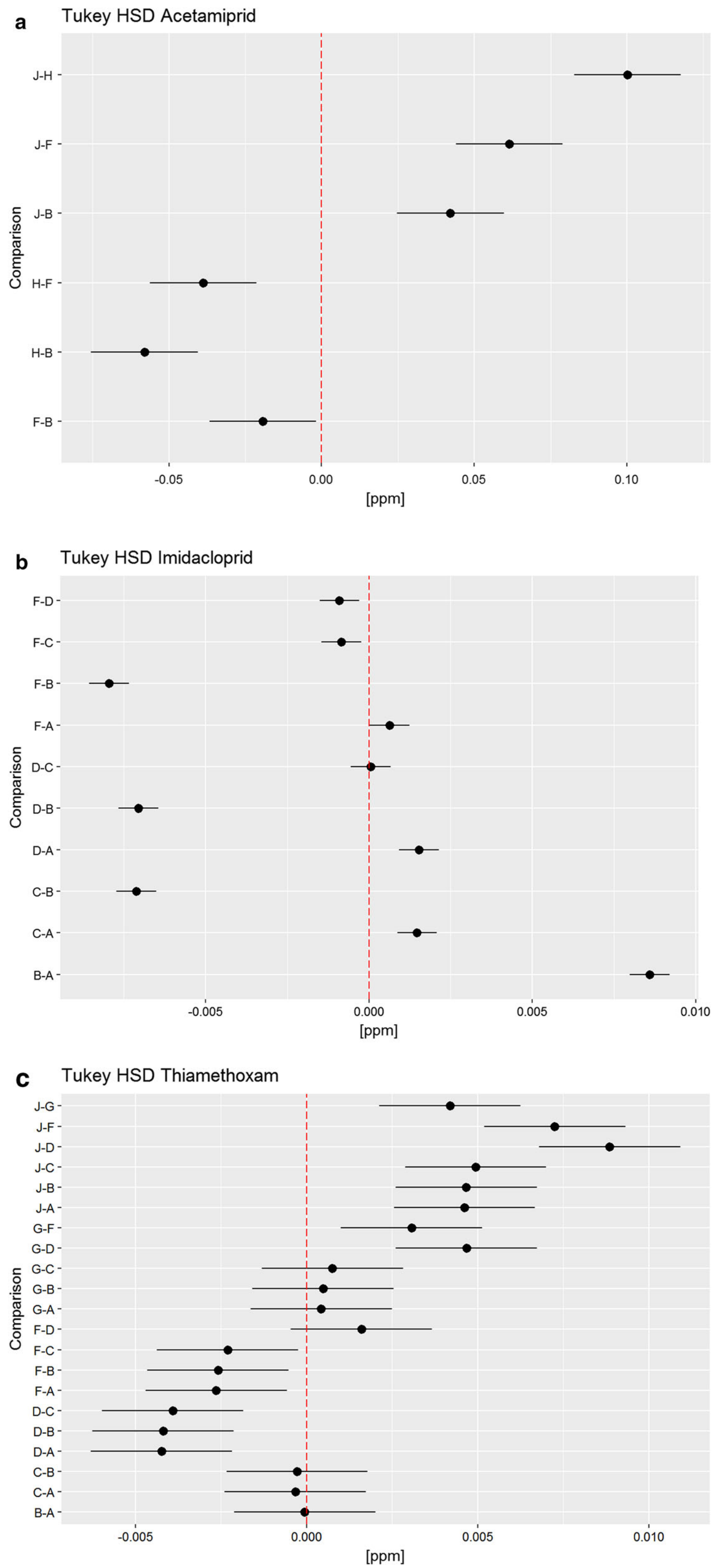
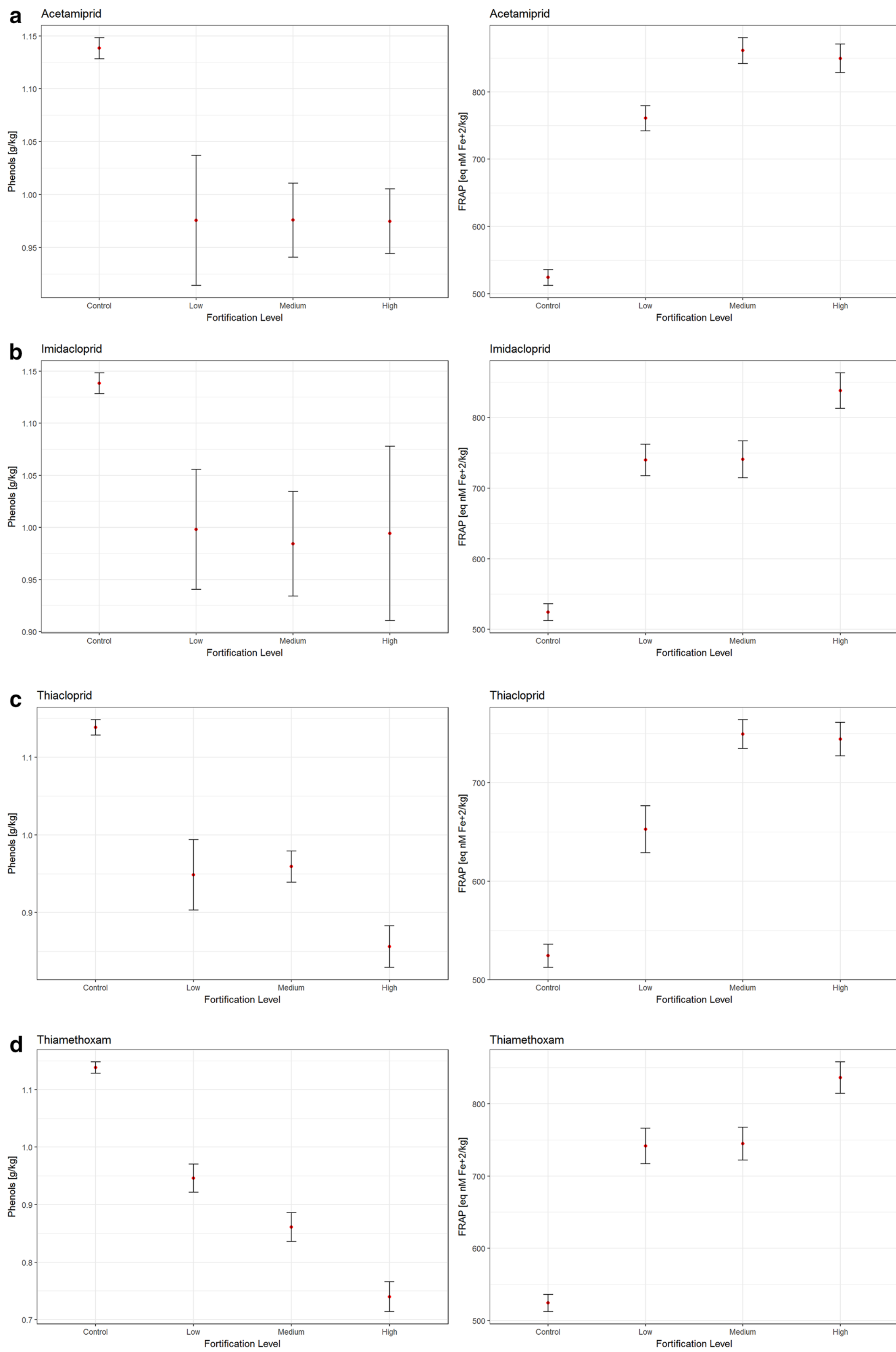


Fig. 2 Detection of pesticides in different analyzed honey samples. Comparisons are shown between samples positive for **a** acetamiprid, **b** imidacloprid, **c** thiamethoxam. Significant differences between sample pairs were established at $p < 0.05$


a
Acetamiprid
Phenols [g/kg]
1.15
1.10
1.05
1.00
0.95
Control
Low
Medium
High
Fortification Level
Acetamiprid
FRAP [eq nM Fe+2/kg]
800
700
600
500
Control
Low
Medium
High
Fortification Level
b
Imidacloprid
Phenols [g/kg]
1.15
1.10
1.05
1.00
0.95
0.90
Control
Low
Medium
High
Fortification Level
Imidacloprid
FRAP [eq nM Fe+2/kg]
800
700
600
500
Control
Low
Medium
High
Fortification Level
c
Thiacloprid
Phenols [g/kg]
1.1
1.0
0.9
Control
Low
Medium
High
Fortification Level
Thiacloprid
FRAP [eq nM Fe+2/kg]
700
600
500
Control
Low
Medium
High
Fortification Level
d
Thiamethoxam
Phenols [g/kg]
1.1
1.0
0.9
0.8
0.7
Control
Low
Medium
High
Fortification Level
Thiamethoxam
FRAP [eq nM Fe+2/kg]
800
700
600
500
Control
Low
Medium
High
Fortification Level

◀**Fig. 3** Changes in total phenol contents and antioxidant ability in relation to low, medium, and high concentrations of **a** acetamiprid, **b** imidacloprid, **c** thiacloprid, **d** thiamethoxam

according with other findings where the presence of at least one neonicotinoid has been reported in 75% of honey samples from different regions of the world. In those researches, it has been possible to classify samples depending on its geographical origin and neonicotinoid content. Thiacloprid is mainly detected in samples from Europe meanwhile in South America, imidacloprid predominates. We did not detect thiacloprid along the analyzed samples. Also, we found a similar trend in terms of percentage of positive samples with neonicotinoid presence (Mitchell et al. 2017).

To establish the effects of the four neonicotinoid pesticides on the chemical properties of honey, samples were fortified with increasing concentrations of each compound. Total phenol contents significantly decreased in fortified honey samples as compared to the control (Fig. 3). This would mean decreased honey quality since phenolic and polyphenolic contents act as antioxidants and anti-carcinogens, in addition to presenting cardioprotective actions (Rice-Evans et al. 1996). In contrast to the decrease in phenol contents, antiradical activity was significantly increased in fortified honey samples as compared to the control (Fig. 3). This is a contradictory finding as antioxidant ability can be predicted by the availability of phenolic compounds and flavonoids, which donate hydrogen radicals that can neutralize free radicals (Buratti et al. 2007). The presence of pesticides may trigger changes to the chemical properties of honey and, consequently, in the bioactive properties of honey. These changes may alter product quality.

The present study is the first to assess possible interactions between neonicotinoids and components in honey. Due to the composition and traits of the organic matrix of honey, pesticide-honey interactions likely vary in relation to differences in pH and humidity, among other external factors. Besides, the neonicotinoids have different ratio of water solubility providing different chemical interaction with natural compounds available in honey content. Thiacloprid shows the higher solubility (4.1 g L^{-1}) in comparison with value described for imidacloprid, 0.61 g L^{-1}. (Wirtz et al. 2018). This last fact may be the explanation of the different behavior of antioxidant properties of fortified samples. On the other hand, the widespread presence of pesticides detected in honeys across distinct geographical regions should serve as an environmental alert, particularly when considering the possible negative consequences for hive health.

Compliance with ethical standards

Conflict of interest No potential conflicts of interest were reported by the authors.

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