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Analysis and evaluation of (neuro)peptides in honey bees exposed to pesticides in field conditions☆

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Abstract

During the last years, declines in honey bee colonies are being registered worldwide. Cholinergic pesticides and their extensive use have been correlated to the decline of pollinators and there is evidence that pesticides act as neuroendocrine disruptors affecting the metabolism of neuropeptides. However, there is a big absence of studies with quantitative results correlating the effect of pesticide exposure with changes on neuropeptides insects, and most of them are conducted under laboratory conditions, typically with individual active ingredients. In this study, we present an analytical workflow to evaluate pesticide effects on honey bees through the analysis of (neuro) (Please remove the space "(neuro)peptides)peptides. The workflow consists of a rapid extraction method and liquid chromatography with triple quadrupole for preselected neuropeptides. For non-target analysis, high resolution mass spectrometry, multivariate analysis and automatic identification of discriminated peptides using a specific software and protein sequence databases. The analytical method was applied to the analysis of target and non-target (neuro)peptides in honey bees with low and high content of a wide range of pesticides to which have been exposed in field conditions. Our findings show that the identification frequency of target neuropeptides decreases significantly in honey bees with high concentration of pesticides (pesticide concentrations ≥ 500 μg kg^{-1}) in comparison with the honey bees with low content of pesticides (pesticide concentrations ≤ 20 μg kg^{-1}). Moreover, the principal component analysis in non-target search shows a clear distinction between peptide concentration in honey bees with high level of pesticides and honey bees with low level. The use of high resolution mass spectrometry has allowed the identification of 25 non-redundant peptides responsible for discrimination between the two groups, derived from 18 precursor proteins.

It has been shown a correlation between pesticide exposure and a decrease of some neuropeptides and other head peptides in honey bees.

Keywords: Neuropeptides; Honey bees; Pesticides; High resolution mass spectrometry; Field conditions

1 Introduction

Conservation of pollinator abundance and its role as ecosystem services, contribute decisively in moderating any negative impacts their deficit may provoke in agriculture, food production and environmental sustainability. The European honey bee (Apis mellifera L.) is the most commonly managed bee in the world. During recent years, declines in bee colonies are being registered as much in Europe as in other parts of the world (vanEngelsdorp and Meixner, 2010). Various factors have been identified causing the reduction in bee colonies, including parasites, pathogens and pesticide stressor along with other factors such as loss or fragmentation of habitat, invasive species and/or climate change (Sánchez-Bayo et al., 2016; vanEngelsdorp and Meixner, 2010). However, there is a big concern about the possible role that pesticides, particularly neonicotinoids insecticides and organophosphate miticides, may play in honey bee health (Cicero et al., 2017; Fairbrother et al., 2014; Palmer et al., 2013). Honey bees living and foraging near agricultural fields are exposed to pesticides as neonicotinoids (Cicero et al., 2017; Hakme et al., 2017; Palmer et al., 2013) and the extensive use of some of these pesticides has been correlated to the decline of bees and other pollinators (Samson-Robert et al., 2014). In addition, honey bees are also exposed to acaricides, used against Varroa in the hives, that can act whether alone or in combination with fungicides showing synergic effects (García et al., 2017; Gómez-Ramos et al., 2016; Sánchez-Bayo et al., 2016).

Neurotoxic insecticides have special importance at sublethal levels in honey bees, producing behavioral changes that interfere with foraging behavior, homing success, navigation performance and social communication (Stanley et al., 2016; Tison et al., 2016). Some studies demonstrated that pesticides and other environmental contaminants act as neuroendocrine disruptors capable or acting as agonist/antagonist or modulators of the metabolism of neuropeptides (Waye and Trudeau, 2011). Neuropeptides are 3–100 amino acid residues long, that are produced from precursor proteins by a series of enzymatic processing steps (Lee, 2016). Neuropeptides are key regulators in the majority of physiological and behavioral processes of any animal species, including insects (Boerjan et al., 2010). Some of these substances are involved in food intake of solitary insects such as Drosophila melanogaster (Melcher and Pankratz, 2005) and the German cockroach Blattella germanica (Pascual et al., 2008) and modulate odor perception and locomotor activity in Drosophila melanogaster (Kahsai et al., 2010; Winther et al., 2006). Regarding honey bees, several neuropeptides showed differences in brain abundance in association with nectar or pollen foraging (Brockmann et al., 2009). More recent studies have revealed that the suppression of ovary activation in worker honey bee is probably mediated through steroid and neuropeptide hormone signaling (Cardoen et al., 2012) and neuropeptides appear to have some functions in the honey bee brain that are specifically related to the age-related division of labor (Han et al., 2015; Pratavieira et al., 2014).

Because of the importance of neuropeptides in regulating neural communication and physiological modulation in organisms acting as neurotransmitters, neuromodulators and neurohormones, efforts have been undertaken in recent decades to identify them in a variety of insects, included in Apis mellifera, which is the best documented specie among the social insects (Audsley and Weaver, 2006; Boerjan et al., 2010; Han et al., 2015; Nässel and Winther, 2010).

Although there are several methods for the analysis of neuropeptides, also known as neuropeptidomics, mass spectrometry (MS), with its qualitative and quantitative capabilities, is ideally suited to the task (Lee, 2016; Yin et al., 2011). MS enables fast, sensitive, accurate and high-throughput analyses of neuropeptides without a priori knowledge of the peptide's identity, resulting in the identification of previously unknown neuropeptides (Lee, 2016). Two types of ionization are commonly used in the analysis of neuropeptides, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), each of them having its own advantages.

Direct tissue analysis by MALDI- based MS is usually performed by a simple sequence of steps, whereas ESI-MS can be coupled more easily with separation methods (Lee, 2016). Liquid chromatography (HPLC)- MS has proved to be particularly useful for the identification and quantification of neuropeptides, primarily due to its capability to unambiguously characterize peptides in complex biological samples (Yin et al., 2011). LC and nano-scale LC coupled to high resolution MS, using quadrupole time-of-flight (Q-TOF) or Orbitrap, have been used in some recent studies for the analysis of neuropeptides in animal-brain tissue (Yin et al., 2011), including honey bees (Han et al., 2015). Brain extract is a very complex matrix and in this context, high resolution is decisive in the discrimination of very similar compounds. Neuropeptides are typically identified with both MS and MS/MS fragmentation data, normally using neuropeptide prohormone databases to facilitate neuropeptide identification (Lee, 2016). MS has been used to characterize hundreds of putative signaling peptides in a range of animals (Yin et al., 2011). In honey bees, 158 neuropeptides derived from 22 precursor proteins have been identified in the brain using MS/MS techniques (Han et al., 2015). In addition, several MS-based measurement approaches have been developed and enable relative quantitation of peptide levels in biological samples, including correlating peptide levels to specific conditions or behaviors (Han et al., 2015; Lee et al., 2016; Yin et al., 2011). LC-MS/MS techniques, using relative quantitation, have been used to investigate connections between social behavior and bioactivities of neuropeptides (Han et al., 2015) including the regulation of foraging activity in honey bees (Brockmann et al., 2009; Schoofs et al., 2017) and labor division (Han et al., 2015). However, the physiological and behavioral functions of most neuropeptides in honey bees remain largely unknown (Han et al., 2015; Schoofs et al., 2017). Study of neuropeptide function is a challenging task, as it is known that more than one neuropeptide can be involved in the regulation of a physiological activity and multifunctionality is common for brain peptides (Nässel, 2002).

In this work, a new analytical method using a rapid and simple extraction method and LC with triple quadrupole (LC-QqQ-MS/MS) and high resolution MS (LC-QTOF-MS/MS) has been successfully applied for the target and not-target analysis of (neuro)peptides in honey bees with low and high content of pesticides to which bees have been exposed in field conditions. Neuropeptide differences, in concentration and detection frequency, were compared between the group of honey bees with low level content of pesticides and the group of honey bees with a high level of content. To our knowledge, this is the first work that studies the effects of pesticides in honey bees in relation with the presence and concentration of neuropeptides. This is of great importance for better understanding the neuronal basis of pesticide exposure of honey bees in the field.

2 Material and methods

2.1 Chemical reagents

A set of 12 neuropeptides were selected for the validation study. The neuropeptides were chosen as representatives of different neuropeptides families identified in Apis sp. (Brockmann et al., 2009; Han et al., 2015). The neuropeptides included in this study were supplied by Phoenix Pharmaceuticals Inc. (Burlingame, CA) at analytical grade (purity >95%). Individual standard stock solutions of the compounds were prepared in methanol 1% formic acid, at concentration of 200 mg L^{-1} and stored at −20 °C. Working standard solutions, at different concentrations, were prepared by appropriate dilution of the stock solutions with the mobile phases in a proportion of methanol/water (2:8 v/v). HPLC-grade methanol and formic acid (purity 98%) were supplied by Sigma-Aldrich (Steinheim, Germany). LC-MS grade water was obtained from Fisher Scientific (Geel, Belgium). Sodium chloride was purchased from J.T.Baker (Deventer, The Netherlands). Anhydrous magnesium sulfate was supplied by Panreac (Barcelona, Spain).

2.2 Sample collection and classification

Bee samples were collected from July to September of 2016 by beekeeper collaborators of 60 different apiaries distributed at diverse locations in Spain. Samples containing high level of pesticides were collected in apiaries close to areas of high intensive agricultural production with conventional practices of pesticide applications. Samples with low level of pesticides were collected in apiaries near fields with low agricultural production or intermediate areas near agricultural fields with conventional use of pesticides. Each collected sample was composed of approximately 500 adult forager honey bees (Apis mellifera iberica) from at least six colonies randomly selected in each apiary.

Before neuropeptide analysis, honey bees were analyzed for the characterization of the pesticide residue load. These results are part of another study that includes a multiresidue pesticide analysis of bee samples using a modified QuEChERs extraction. In this study, a total of 260 pesticides were analyzed using LC-MS/MS and GC-MS/MS. More information is available in Supplementary Information (Table S1). 30 bee samples were classified in two groups, (a) high level of pesticides (HLP) ($\sum$ pesticide concentrations ≥ 500 μg kg^{-1}) and (b) low level of pesticides (LLP) ($\sum$ pesticide concentrations ≤ 20 μg kg^{-1}).

2.3 Method validation

Method validation and performance were tested by assessing recovery, precision, linearity, matrix effects, limit of identification (LOI) and limit of quantitation (LOQ). Spiked honey bee head extracts were used for validation and five replicates were used for the recovery and precision check. The recovery studies were determined in bee head extracts fortified at a concentration level of 10 μg L^{-1}. Within laboratory-repeatability (RSD) was tested for 10 μg L^{-1} level. Due to the complexity of finding blanks, the samples were previously analyzed and the presence of the target compounds considered. LOI and LOQ were determined as the minimum detectable amount of the analyte in which the qualifier selected reaction monitoring (SRM2) transition had a signal-to-noise ratio 3:1 and 10:1 respectively. Linearity of the calibration curves was evaluated using matrix-matched calibration solutions prepared by spiking the extracts with the working solution at eight concentration levels, 0.05-1-5-10-20-50-75-100 μg L^{-1}.

Matrix effects were assessed by comparison of the slopes of the calibration curves of matrix-matched standards with the slopes of calibration curves in solvent. Matrix effects were calculated with the equation:

$$ME\,(\%) = \left(\left(\frac{\text{slope of calibration curve standard in matrix}}{\text{slope of calibration curve in solvent}} \right) - 1 \right) X\,100$$

For quantitative analysis of neuropeptides in bee heads, matrix matched calibration was used. The quantification was done using the closer concentration to the estimated concentration for each neuropeptide in the real samples.

2.4 Extraction procedure

For the neuropeptide extraction, 20 bee heads (0.2 g approx.) per sample were dissected and weighed in a 15 mL PTFE centrifuge tube, after which 0.5 mL of ultrapure water 1% formic acid were added. After homogenization, 2 ml of methanol 1% formic acid were added and shaken in an automatic axial extractor (AGYTAX®, Cirta Lab. S.L., Spain) for 7 min. Afterwards 0.1 g magnesium sulfate anhydrous and 0.05 sodium chloride were added and the samples were shaken in the automatic axial extractor for 7 min. The extract was then centrifuged (3500 rpm)

for 5 min and an aliquot of cleaned extract (1.5 mL) was transferred into a screw-cap vial. Finally, for the analysis, 300 µL of supernatant were transferred into a vial, evaporated until almost dryness with a gentle nitrogen stream and reconstituted with 60 µL of mobile phase methanol/water, 2:8. With this procedure the final matrix concentration is approximately 0.5 g mL^{-1}.

2.5 LC-QqQ-MS/MS analysis

For the method validation and the target analysis an Agilent 1290 UPLC system (Agilent Technologies, Santa Clara, CA) coupled to a triple quadrupole mass spectrometer was used. The UPLC was equipped with a reversed- phase C8 analytical column of 2.1 mm × 100 mm and 1.8 µm particle size (Agilent Technologies, Santa Clara, CA). Gradient LC elution was performed with 0.1% formic acid, 5 mM ammonium formate and 5% ultrapure water in methanol as mobile phase A and 0.1% formic acid, 5 mM ammonium formate and 5% methanol in ultrapure water as mobile phase B. The mobile phase composition is as follows: 20% A (2 min), 13 min linear gradient to 100% A (2 min), 2.5 min post-run time back to the initial conditions. The flow rate was 0.3 mL min^{-1} and the injection volume 10 µL. The UPLC system was connected to an Agilent 6490 triple quadrupole-tandem mass spectrometer from Agilent Technologies (Santa Clara, CA) equipped with an electrospray ionization source (ESI) operating in positive ionization mode and using DMRM (dynamic multi-reaction monitoring) software features. Parameters for the ESI source are gas temperature 120 °C and flow rate 13 L min^{-1}. Capillary voltage, 3000 V and nozzle voltage, 400; nebulizer, 45 psi; sheath gas temperature, 370 °C; sheath gas flow, 10 L min^{-1}. The iFunnel parameters were: high pressure RF 150 V and low-pressure RF 60 V. Nitrogen was served as the nebulizer and collision gas. Mass Hunter Data Acquisition; Qualitative Analysis and Quantitative Analysis software (Agilent Technologies, Palo Alto, CA, v.B.06 and v.B.05) was used for method development and data acquisition.

2.5.1 Optimization of LC-QqQ-MS/MS parameters

Due to its sensitivity and selectivity for trace analysis in complex matrix, LC-ESI-QqQ-MS/MS was the selected technique for the method validation and the target analysis of neuropeptides in bee head extracts. To obtain maximum sensitivity for identification and quantitation, collision energy was optimized for each analyte. The best sensitivity in multiple reaction monitoring was achieved through the acquisition of single reaction monitoring (SRM) transitions under DMRM conditions and with a time window of 60 s. The mass spectrometer operated in SRM mode with a resolution set to Unit for Q1 and Q3. The values of the optimized parameter and the SRM transitions selected in the analytical method are shown in Table 1. The most intense SRM transition was selected for quantitation purposes (SRM1) and one or two more transitions were chosen as qualifier transitions (SRM2 and SRM3).

2.6 LC-QTOF-MS/MS analysis

A liquid chromatography-electrospray ionization-quadrupole time-of-flight-tandem mass spectrometry (LC-ESI-QTOF-MS/MS) system, working in positive ionization mode, was used for the non-target analysis of peptides in the bee head extracts. The analytes were separated using a UPLC system consisting of vacuum degasser, autosampler, binary pump and an isopump (Agilent 1290 Series, Agilent Technologies), equipped with a reversed-phase C8 analytical column of 2.1 mm × 100 mm and 1.8 µm particle size (Agilent Technologies, Santa Clara, CA). Gradient LC elution was performed with 0.1% formic acid, 5 mM ammonium formate and 5% ultrapure water in methanol as mobile phase A and 0.1% formic acid, 5 mM ammonium formate and 5% methanol in water as mobile phase B. The mobile phase composition is as follows: 20% A (2 min), 13 min linear gradient to 100% A (2 min), 2.5 min post-run time back to the initial

conditions. The flow rate was 0.3 mL min^{-1} and the injection volume 10 µL. The UPLC system was connected to a Q-TOF-MS/MS (Agilent 6550 Series Accurate Mass QTOF-MS, Agilent Technologies, Santa Clara, CA). The instrument was operated in the 4 GHz high-resolution mode. Ions were generated by ESI using a Dual Spray Agilent Jet Stream ion source. Parameters for the ESI source are the super-heated nitrogen sheath gas temperature 350 °C and flow rate 14 L min^{-1}. ESI conditions were the following: capillary, 4000 V and nozzle voltage, 400; nebulizer, 30 psi; drying gas, 14 L min^{-1}; gas temperature, 160 °C; octapoleRFPeak, 750 V; fragmentor (in-source CID fragmentation), 360 V. The mass axis was calibrated using the mixture provided by the manufacturer over the m/z 100–3200 range. A sprayer with a reference solution was used as continuous calibration in positive ion using the following reference masses: 121.0509 and 922.0098 m/z (resolution: 38,000 ± 500, at 922.0098 m/z). Reference masses were pump by an isopump at a flow of 1 mL min−1 and it increase linearly to 2 mL min −1. Data were acquired in Full scan and MS/MS mode. MS and MS/MS spectra were acquired over the 100–3200m/z range at scan rate of 3 and 5 spectra/s respectively. MS/MS data were acquired in Target MS/MS (data-independent) mode. An inclusion list with the selected ions was included with a delta retention time of 0.25 min and isolation window of 1.3 m/z. Spectra were obtained using 10 and 20eV collision energy (CE).

2.6.1 Data processing and statistical analysis for differential profiling

Acquired MS data in LC-QTOF-MS were processed with Agilent MassHunter Qualitative Analysis (B.07.00). Automatic screening in full-scan mode was performed using the Molecular Feature Extraction (MFE) of Qualitative Analysis software tool using 10000 counts absolute height threshold, 500 counts chromatographic peak height threshold. This selection was decided upon to avoid non-relevant contributions or background compounds. This data process allows one to obtain a compound list with the molecular weight, charge state, retention time and intensity of all the matrix components detected.

Those features were exported as CEF files to the Mass Profiler Professional software (MPP). 13.1.1 (Agilent Technologies, Santa Clara, CA) for the statistical data analysis using unpaired t-test with Benjamini-Hocherberg multiple testing correction. Only compounds with an absolute height equal to or greater than 10000 counts and including charge state> 1 were considered for the study. Compounds were aligned with 15 ppm of mass and 0.2 min retention time window to compensate for minor variations, ensuring that identical compounds in different samples were accurately compared. To reduce false-negatives rates, these data were used for a recursive analysis in which the list of ions were targeted for re-extraction against the raw data. The aligned data was filtered, considering only features that were present in 80% of bee samples in at least one sample group. Only entities with p-values >0.01 and fold-change >2 were retained. Principal component analysis (PCA) was used to extract features of interest.

2.6.2 Tentative peptide identification in non-target analysis

Features that were considered characteristic were fragmented in a second injection in LC-QTOF-MS/MS for obtaining MS/MS high resolution spectral information. Automatic identification was performed using PEAKS Studio software (version 8.0, Bioinformatics Solutions, Waterloo, Canada). With this software, LC-QTOF-MS/MS data of selected compounds were used to tentative identify the peptides by comparing the obtained fragmentation pattern to theoretical fragmentation patterns of a database containing 29379 protein sequences of Apis mellifera. This database was sourced from the NCBI (National Center for Biotechnology Information). Selected parameters for automated identification were 10 ppm of parent mas error tolerance, 0.01 Da of fragment mass error tolerance, enzyme none, 3 maximum variable post translational modifications

(PTM) per peptide. Allowed variable modifications were C-terminal amidation (0.98), pyroglutamination from Q (−17.03) and deamidation (NQ, 0.98). A peptide score −10 log P ≥ 20.0 was established with a false discovery rate (FDR) ≤0.1%.

3 Results and discussion

3.1 Method procedure and validation

Sample preparation prior MS analysis is a critical step in neuropeptide analysis, due to the complexity of the biological matrix, containing salts, lipids and numerous protein degradation products. In this study, to simplify the sample processing the entire bee heads have been used, avoiding the time consuming brain tissue dissection step (Boerjan et al., 2010; Palmer et al., 2013). The sample extracts could be more complex, but also ensures that all neuropeptides are present in the sample, since in some cases the peptides are concentrated and localized into a small region within the tissue. A simple and fast procedure was used to extract the neuropeptides (see Fig. S1, in Supporting Information). Further clean-up steps (centrifugal filters for proteins of 3 k, protein-lipid removal filter cartridges and dispersive solid phase (d-SPE) extraction using zirconium dioxide-based sorbent) were tested to evaluate improvements of analytical performance, but all of them resulted in losses of some neuropeptides and in general the signal of the analytes was not improved, and was thus not applicable (more information is available in Supplementary Information, Tables S2 and S3). LC-ESI-QqQ-MS/MS was used for the validation and analysis of target peptides. This approach is very sensitive and selective, allowing the differentiation of the target neuropeptides in the complex biological samples. As can be observed in Table 1, neuropeptides are quite big multicharged molecules, and normally the precursor ions found with higher intensity in the mass spectra are the ones with charge +2 or +3. The physico- chemical properties of these molecules together with the complexity of the biological matrix, make this kind of analysis complex, and it is very difficult to obtain good analytical performance for all the compounds.

The analytical performance of the method was evaluated by using spiked bee head extracts. The results obtained are shown in Table 2. The method showed a linear response over the entire studied ranged, (Please change "ranged" to "range")with correlation coefficients higher than 0.991. The investigated compounds presented recoveries between 52 and 74%, except for NVASLARTYYLPQNA a (neuropeptide 8) (44%) and SVSSLARTGDLPVREQ (neuropeptide 9) (39%). Despite these low recoveries, the other validation data, such as repeatability and limit of identification (LOI) are good, and therefore a reliable determination of these compounds is feasible. The recovery values of the 5 replicates presented a relative standard deviation (RSD) less than 20% in all the cases, except for MVPVPVHHMADELLRNGPDTVI (neuropeptide 6) (32%). The LOI and LOQ, determined with the second transition, were within the range of 0.1–5 μg L^{-1} and 0.5–10 μg L^{-1}, respectively, except for NIDEIDRTAFDNFF (neuropeptide 12) (10 and 20 μg L^{-1}). The LOIs reported here are sufficient to characterize mean concentrations measured in real samples (see Table 3). Values of matrix effect (ME) are presented in the validation Table 2, a value of 0% indicates the absence of matrix effects, and negative and positive values are indicative of signal enhancement and suppression, respectively. Signal suppression is observed in all the analytes, seven neuropeptides showed intermediate matrix suppression (23–55%) and five compounds showed strong matrix suppression (60–84%), demonstrating the importance of using matrix-matched calibration to improve the accuracy of the quantification.

Table 1 Molecular formula, molecular mass, acquisition and chromatographic parameters obtained by LC-QqQ-MS/MS for a set of 12 neuropeptides described as relevant for honey bees in previous studies (Han et al., 2015).

	Neuropeptide	Molecular Formula	Molecular Mass	t_R (min)	Z	SRM1	CE1 (V)	SRM2	CE2 (V)	SRM3	CE3 (V)
1	RQYSFLA	$C_{40}H_{60}N_{12}O_{10}$	868.4555	6.3	+3	869.4/852.1	40	869.4/267.9	40		
2	GRDYSFGL amide	$C_{41}H_{60}N_{12}O_{12}$	912.4454	6.68	+2	457.2/783	20	457.2/755.1	20	457.2/550.9	30
3	GRQPYSFGL amide	$C_{47}H_{70}N_{14}O_{12}$	1022.5297	6.58	+2	512.3/893	20	512.3/865.2	30	512.3/503.3	15
4	ALMGFQGVR amide	$C_{43}H_{72}N_{14}O_{10}S$	976.5276	6.58	+2	489.3/793.3	20	489.3/662.2	20	489.3/157	20
5	IDLSRFYGHFNT	$C_{68}H_{96}N_{18}O_{19}$	1468.7098	8.35	+2	735.4/675.7	30	735.4/120	40		
6	MVPVPVHHMADELLRNGPDTVI	$C_{101}H_{174}N_{30}O_{31}S_2$	2439.2402	9.43	+3	814/737.4	15	814/770	20		
7	NVGSVAREHGLPY amide	$C_{61}H_{96}N_{20}O_{18}$	1396.7211	5.4	+2	699.3/232.8	40	699.3/560.6	30	699.3/278.1	40
8	NVASLARTYYLPQNA amide	$C_{70}H_{116}N_{22}O_{22}$	1616.8635	6.96	+2	809.4/428.1	20	809.4/1190.4	20	809.4/411	30
9	SVSSLARTGDLPVREQ	$C_{71}H_{123}N_{23}O_{26}$	1713.901	5.95	+3	572.3/764.7	20	572.3/159	20		
10	NVPIYQEPRF	$C_{59}H_{87}N_{15}O_{16}$	1261.6455	7.26	+2	631.8/525.2	20	631.8/1049.5	20	631.8/839.4	20
11	NLDEIDRVGWSGVF	$C_{72}H_{107}N_{19}O_{23}$	1605.7787	9.56	+2	803.9/199.9	30	803.9/1198.2	20	803.9/471.8	30
12	NIDEIDRTAFDNFF	$C_{77}H_{109}N_{19}O_{26}$	1715.7791	10.1	+2	858.9/776.3	20	858.9/199.8	40	858.9/120	20

Z- Charge state of selected precursor ion; **SRM-**Single Reaction Monitoring; **CE-**Collision Energy.

Table 2 Validation data for the analysis of target neuropeptides in honey bee head extracts.

	Neuropeptide	Linearity µg L^{-1}	ME (%)	Recovery N = 5 10 µg L^{-1}	RSD N = 5 (%)	LOI* µg L^{-1}	LOQ* µg L^{-1}
1	RQYSFLA	5–100	53	61	13	5	10
2	GRDYSFGLa	0.5–100	60	60	6	0.1	0.5
3	GRQPYSFGLa	0.5–100	68	52	4	0.5	1
4	ALMGFQGVRa	0.5–100	84	52	12	0.1	0.5
5	IDLSRFYGHFNT	1–100	23	53	12	1	5
6	MVPVPVHHMADELLRNGPDTVI	5–75	54	68	32	5	10
7	NVGSVAREHGLPYa	1–100	34	52	8	1	5
8	NVASLARTYYLPQNAa	0.5–100	55	44	8	0.1	0.5
9	SVSSLARTGDLPVREQ	0.5–100	67	39	16	0.5	1
10	NVPIYQEPRF	0.5–100	66	74	9	0.1	0.5
11	NLDEIDRVGWSGVF	1–100	50	56	7	1	5
12	NIDEIDRTAFDNFF	10–75	39	69	5	10	20

ME- Matrix effects; **RSD-** Relative standard deviation; **LOI-** Limit of identification; **LOQ-** Limit of quantification; **(*)** determined with SRM2.

Table 3 Occurrence and concentration of target neuropeptides detected in honey bee head samples. Pesticide load for each sample it is also included. Each sample represents 20 individuals.

Samples	Pesticide Load up (μg kg^{-1})	Gro	Neuropeptide concentration (μg kg^{-1})										
			2	3	4	5	6	7	8	9	10	11	12
A1	0	LLP	1.1		4.4	8.4	48.6		1.2	2.1	2.7	8.4	45.1
A2	1	LLP	1.6	1.7	5.7	13.4	43.5		1.5	2.8	2.3		34.6
A3	1	LLP	1.0		2.2	12.1	31.4		2.0	2.8	2.9		
A4	4	LLP											
A5	5	LLP			3.6	8.8	18.8		0.8	0.8	1.8	7.6	
A6	5	LLP			1.2	8.1	35.1						
A7	7	LLP					31.7						
A8	8	LLP	0.8				21.5						

A9	9	LLP	1.5		4.6	13.1	37.5		2.0	2.7	3.8	8.8	31.4
A10	10	LLP	1.9			9.4			1.6	3.6	3.3		
A11	10	LLP			0.8		34.0				1.6		
A12	12	LLP											
A13	17	LLP	1.9		13.1	12.3	86.8		1.8	2.5	5.5		22.7
A14	20	LLP											
A15	20	LLP			1.1					1.1			
A16	500	HLP											
A17	513	HLP											
A18	612	HLP											
A19	629	HLP											
A20	692	HLP											
A21	805	HLP											
A22	899	HLP											
A23	927	HLP											
A24	1114	HLP											
A25	1149	HLP											
A26	1315	HLP											
A27	1525	HLP			5.3	19.5	38.6				2.5		
A28	2142	HLP											
A29	3618	HLP			5.7	18.7	64.9				1.8		
A30	6580	HLP				6.9	17.3						
Neuropeptide mean Concentration			**1.4**	**1.7**	**4.3**	**11.9**	**39.2**	**< LOQ**	**1.6**	**2.3**	**2.8**	**8.3**	**33.5**
Identification Frequency (%)			**37**	**17**	**43**	**60**	**47**	**27**	**33**	**27**	**67**	**57**	**20**

LLP- low level of pesticides**; HLP-** high level of pesticides; **LOQ**-limit of quantification; **Neuropeptides: 1-** RQYSFLA; **2-** GRDYSFGLa; **3-** GRQPYSFGLa; **4-** ALMGFQGVRa; **5-** IDLSRFYGHFNT; **6-**MVPVPVHHMADELLRNGPDTVI; **7-**NVGSVAREHGLPYa; **8-**NVASLARTYYLPQNAa; **9-** SVSSLARTGDLPVREQ; **10-** NVPIYQEPRF; **11-**NLDEIDRVGWSGVF; **12-** NIDEIDRTAFDNFF.

3.2 Target analysis of real samples

The optimized and validated method was used to analyze 30 honey bee samples. Each extracted sample was composed of 20 bee heads, so a total of 600 individual honey bees were used for this study. To study the correlation of pesticide residue content in honey bees with neuropeptides, the samples were classified in two groups according to their pesticide concentration after being analyzed for a total of 260 pesticides. The pesticides analyzed belong to different families, including cholinergic pesticides such as neonicotinoids and organophosphate miticides (see the list of pesticides in Table S1, Supporting Information). The groups were organized as follow: low level of pesticide (LLP) group, samples with ∑pesticide concentrations ≤ 20 μg kg^{-1} and high level of pesticides (HLP) group, samples with ∑ pesticide concentrations ≥ 500 μg kg^{-1}. The two groups were chosen to be in the extremes; low and high pesticide level, to minimize the influence of external parameters other than pesticide content (e.g. honey bee labor, age, etc.). The first part of the work was the target analysis of the 12 neuropeptides used for the validation of the method. These neuropeptides were chosen as representatives of different neuropeptides families identified in honey bees (Apis sp.) in previous studies (Brockmann et al., 2009; Han et al., 2015). Occurrence and concentrations of the target neuropeptides measured in honey bee head samples, pesticide residue load in the samples and group classification are given in Table 3. Fig. S2 (Supporting Information) presents examples of identification of the target neuropeptides using LC-QqQ-MS/MS.

The total pesticide concentrations of LLP samples ranged from 0 to 20 μg kg^{-1} and in the case of HLP samples ranged from 500 to 6580 μg kg^{-1}. As can be observed in Table 3, neuropeptide 1, RQYSFLA, was the only target neuropeptide that was not identified in any of the samples. Moreover, the neuropeptide 7, NVGSVAREHGLPYa, was identified although below the LOQ in all samples. Taking into account all the samples, neuropeptide mean concentrations were between 1.4 μg kg^{-1} (GRDYSFGLa, neuropeptide 2) and 39.2 μg kg^{-1} (MVPVPVHHMADELLRNGPDTVI, neuropeptide 6). The highest concentrations were detected in LLP samples, for MVPVPVHHMADELLRNGPDTVI (neuropeptide 6) (86.8 μg kg^{-1}) and NIDEIDRTAFDNFF (neuropeptide 12) (45.1 μg kg^{-1}). We cannot compare these results with other studies because, to our knowledge, there are not studies that report neuropeptides concentrations in honey bees, the available studies use relative quantification of peptides for the comparison of multiple samples or conditions. Neuropeptide 10, NVPIYQEPRF, was the neuropeptide with higher identification frequency, being identified in 67% of the samples. This neuropeptide has been reported before to be an abundant neuropeptide in honey bee brain (Boerjan et al., 2010). The neuropeptides ALMGFQGVRa, IDLSRFYGHFNT, MVPVPVHHMADELLRNGPDTVI and NLDEIDRVGWSGVF (Neuropeptides 4, 5, 6 and 11) were identified in 43%, 60%, 47% and 57% of the samples, respectively. The other neuropeptides had low identification frequencies in general (between 17% and 37%). In Fig. 1 it is shown a graphic with the identification frequency of target neuropeptides in LLP and HLP. The results show interestingly that the identification frequency of all target neuropeptides is clearly higher in LLP samples than in HLP samples. In LLP samples the identification frequency was higher than 50% for most of the neuropeptides, however the identification frequency of the target neuropeptides in HLP samples it was lower than 30% in most of the cases, and neuropeptides 7 and 9, NVGSVAREHGLPYa and SVSSLARTGDLPVREQ, were not identified in any of the HLP samples.

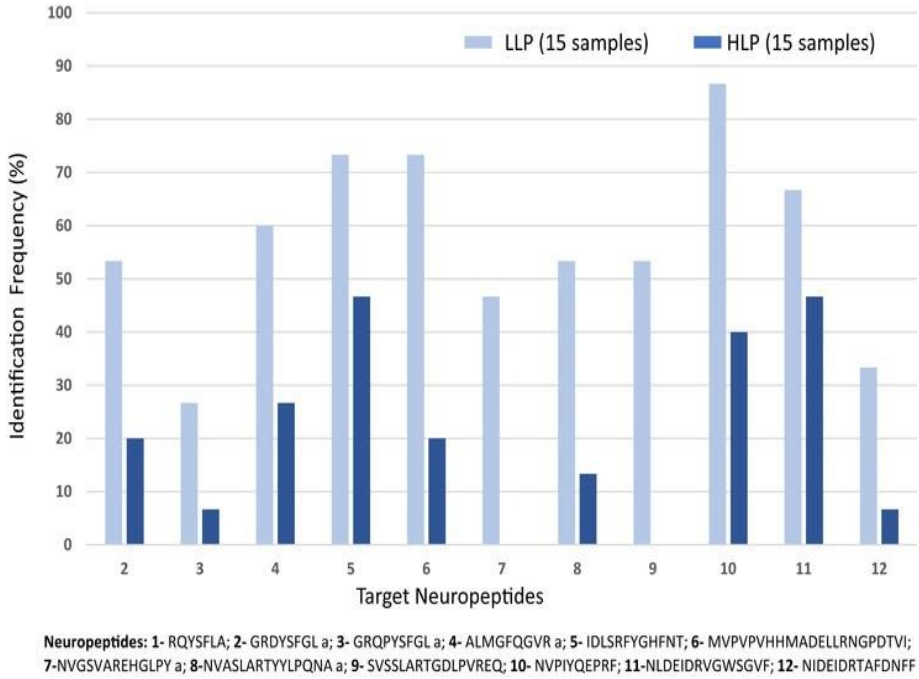

Fig. 1 Identification frequency (values > LOI) of target neuropeptides analyzed by LC-QqQ-MS/MS in LLP and HLP honey bee samples.

In the principal component analysis from Fig. S3, in Supporting Information, can be observed that LLP samples were grouped together and clearly distinct from HLP samples. These data suggest a correlation between pesticide level and a decrease in the number of target neuropeptides in honey bees. In other studies (Fairbrother et al., 2014; Palmer et al., 2013) it has been shown that cholinergic pesticides, such as the neonicotinoids imidacloprid and clothianidin, and the organophosphate miticide coumaphos oxon, are potent neuromodulators in the honey bee brain at the levels that are normally encounter in these insects. Besides, there is evidence that exposure to multiple cholinergic pesticides and in general all classes of pesticides that target neuronal function cause enhanced neurotoxicity (Palmer et al., 2013). However, the knowledge on the change of the neuropeptides during different honey bee situations, as is the case of exposure to pesticides, is still very limited.

3.3 Non-target analysis of peptides in bee head extracts

The bee head is mainly composed of the brain and associated ganglia, hypopharyngeal glands, mandibular glands, salivary glands, antennae, all contributing to neuronal, endocrine and/or exocrine functions (Scharlaken et al., 2007). Pesticides can induce diverse effects on honey bees making them more susceptible to diseases, cold, nutritional stress and/or affecting their learning activity (Blacquière et al., 2012). Some of these effects may be due to the fact that after xenobiotic exposure, the expression pattern of some proteins involved in pathogen susceptibility, neuronal chemical stress, neuronal protein misfolding, higher occurrence of apoptosis, damaged synapsis formation, brain degeneration etc., can change (Roat et al., 2014). In the second part of this work, a non-target analysis in bee head extracts has been conducted to discriminate peptide profile between honey bees containing high or low pesticide residues.

The proposed analytical strategy for non-target analysis combines the use of accurate mass data and statistical evaluation of sample constituents to select the most important peptides for further automatic identification using MS/MS spectral data information together with protein sequence databases and a specific proteomic based search software. Fig. 2 shows the applied

workflow for non-target strategy.

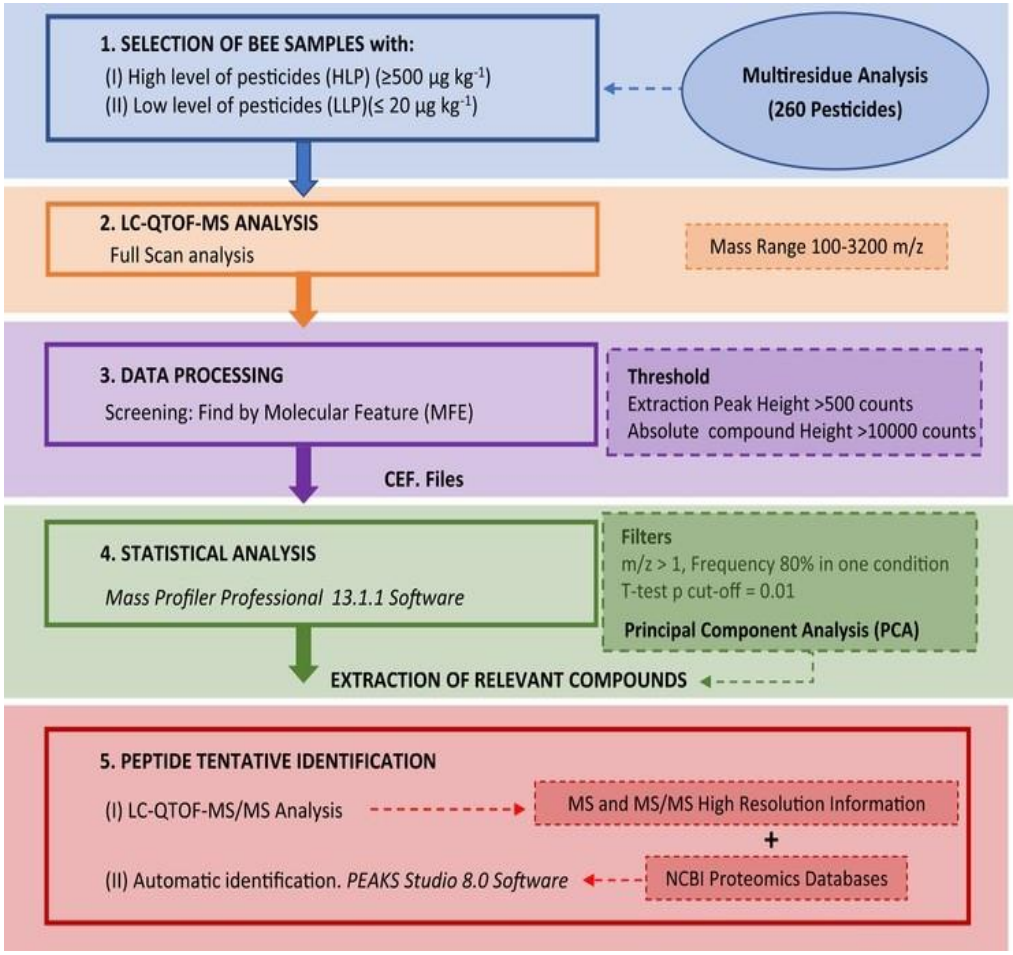

Fig. 2 Workflow for non-target analysis in LLP and HLP honey bee samples.

As in the case of target analysis, honey bee samples were classified in two groups according to their total load of pesticides HLP and LLP, containing each group 15 samples. All matrix components detected in full scan in the two studied groups were compared using multivariate data tools to facilitate the isolation of the compounds responsible for separation between groups. After the multivariate analysis, the compounds were reduced from 1959 to a group of 158 compounds of interest (Table TS2). The principal component analysis (PCA) in Fig. 3, shows a clear separation between bee samples with HLP and LLP. Only sample A14, with a low level of pesticides, was not totally separated, not conforming to the expected pattern in both, target and non-target analysis, and as a consequence is considered in the study as an outlier data. The profile plot for one individual peptide (Fig. 3) was used to explore the abundance of compounds across the samples. As an example, it is shown the profile plot of the peptide ISKTTVAPIERVKL with m/z 777.9871, in the 30 investigated samples. As can be observed, this peptide is present in all the bees with low level of pesticides, with intensity values in most of the cases above 3 e10^6. In the case of bees with high level of pesticides the peptide is absent or with intensity levels below 1 e10^6, much lower than in the LLP group.

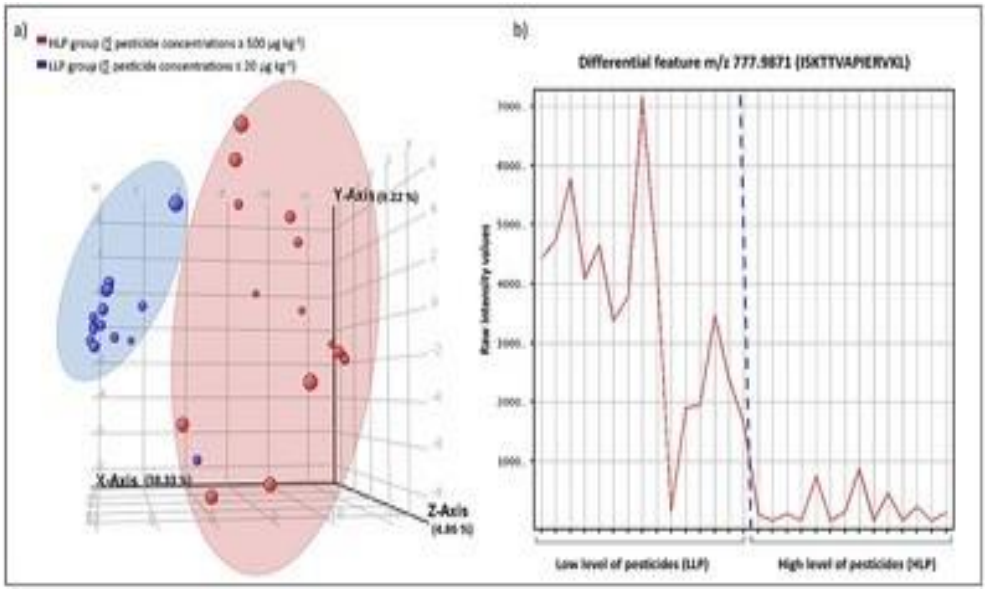

Fig. 3 a) Principal Component Analysis (PCA) in non-target analysis of two bee sample groups: Low Level of Pesticides (∑ pesticide concentrations ≤ 20 µg kg-1) (LLP) and High Level of Pesticides (∑ pesticide concentrations ≥ 500 µg kg-1) (HLP). b)Profile plot for one individual peptide across multiple samples.

The 158 compounds responsible for discrimination between the two groups were selected for further automatic identification using PEAKS Studio software. Fig. 4 illustrates an example of the tentative automatic identification of one of the selected compounds using the extracted MS/MS spectra and a composite database containing 29379 protein sequences of Apis mellifera deposited in the NCBI. By means of this approach, 25 nonredundant peptides derived from 18 precursor proteins were identified in the honey bee heads (Table 4). With the filters and conditions applied in this study, no neuropeptides were automatically identified. The precursor proteins of the identified peptides include among others, structural proteins, proteins involved in detoxification processes, energy house-keeping, stress responses, etc. It is reported that pesticide exposure can alter immune responses (Boncristiani et al., 2012). In a threating situation for the bee, as a bacterial infection, the activation of the immune system can downregulate the expression of some proteins in honey bee head as proteins involved in exocrine secretion, learning and memory formation, odor sensing and visual functioning. Activation of immune system also can influence the expression of structural proteins, proteins involved in signal transduction, energy house-keeping and stress response (Scharlaken et al., 2007). In the present study in general, the concentration level and the detection frequency of the identified peptides are lower in honey bees with high content of pesticides (HLP) (Fig. 5). For some peptides, mean concentration levels are similar in both studied groups, however the frequency of detection is significantly lower in the honey bees HLP. This is the case of the peptide PVKGLGEPIRFL derived from Glutathione s-transferase (GST) enzyme, related to detoxification and stress responses of xenobiotics (Gong and Diao, 2017). The two peptides derived from the enolase enzyme (DVTSQSDIDNFL and IILPVPAF) are also detected less frequently in the bee heads with HLP. This enzyme is involved in glycolysis and responsible for the majority of energy production in all organisms (Kikuchi et al., 2017). In other studies, the enzyme has been found downregulated in bee head after a bacterial infection (Scharlaken et al., 2007), however these results are opposite to another study where honey bee workers were exposed to sub-lethal doses of the pesticide fipronil, and enolase was found upregulated, relating this overexpression with the requirement of metabolic energy in the neurons of honey bees affected for the pesticide (Roat et al., 2014).

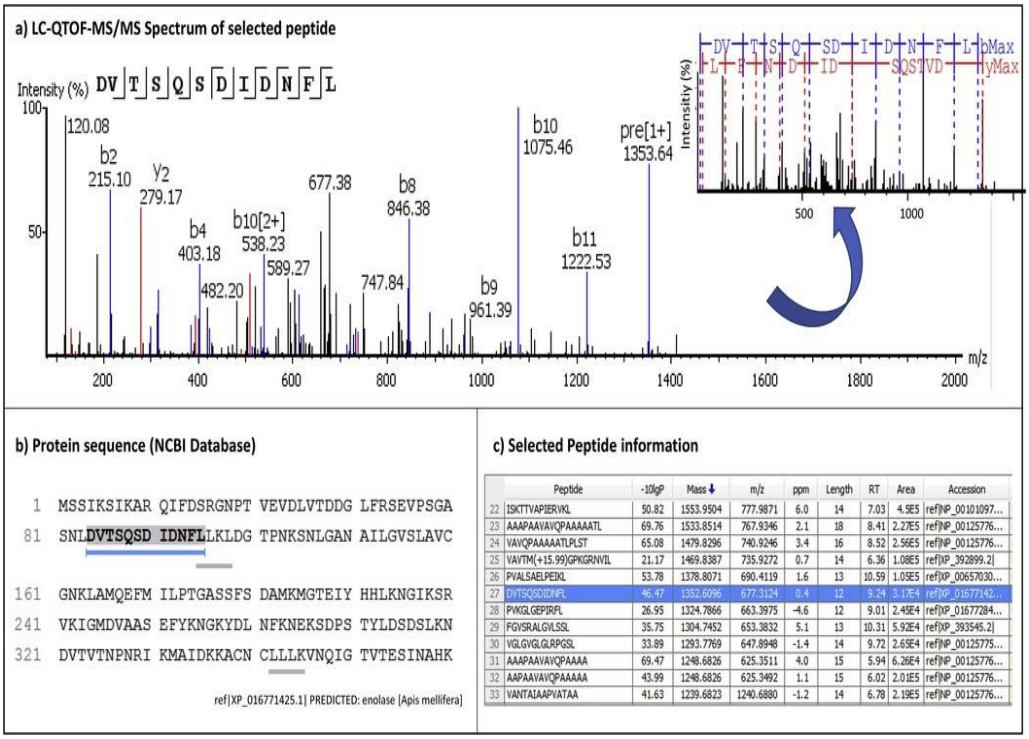

Fig. 4 Example of tentative automatic identification of peptides by comparing LC-QTOF-MS/MS spectra to theoretical fragmentation patterns of a protein sequence database with PEAKS Studio software.

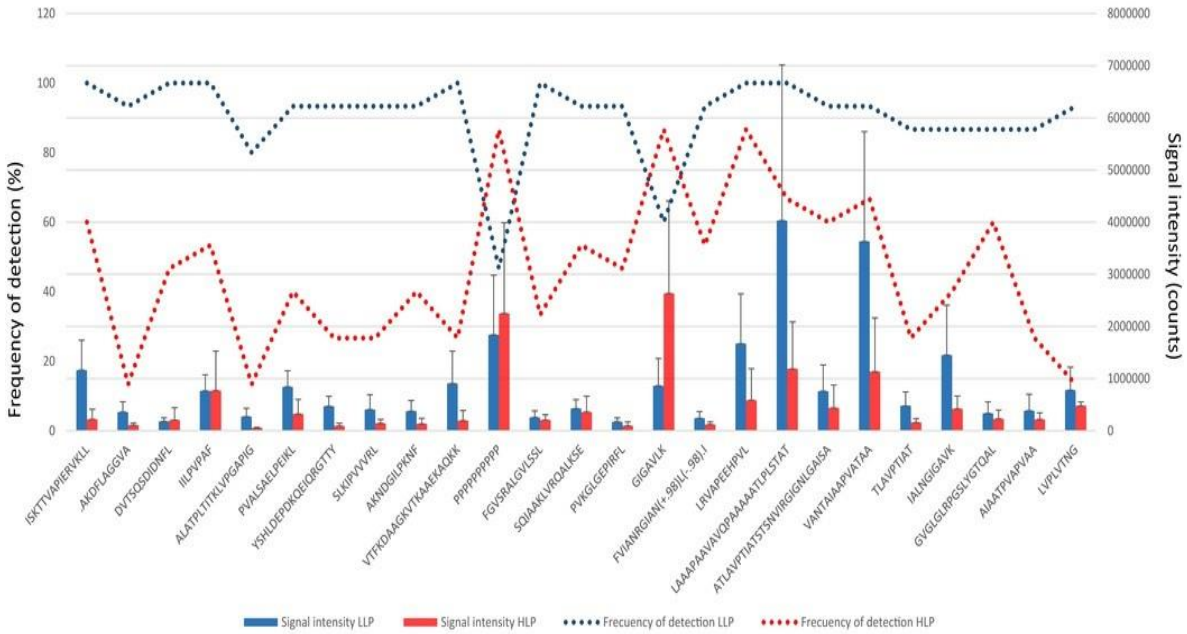

Fig. 5 Signal intensity and detection frequency of automatic identified peptides by LC-QTOF-MS/MS using a protein sequence database from NCBI in LLP and HLP honey bee samples.

Table 4 Automatic identified peptides in honey bee heads by LC-QTOF-MS/MS using a protein sequence database from NCBI.

Precursor Protein	Peptide	Mass	m/z	RT (min)	Mass error (ppm)	−10lgP	Length	Protein accesion
ADP/ATP translocase	ISKTTVAPIERVKL	1553.9504	777.9871	7.03	6	50.82	14	ref|NP_001010975.1|
	ISKTTVAPIERVKLL	1667.0345	834.5247	8.01	0.2	31.61	15	
	VAPLERVKL	1023.644	512.8311	6.76	3.6	25.32	9	
	AKDFLAGGVA	947.5076	948.5104	7.14	−4.7	22.82	10	
Predicted: Enolase	DVTSQSDIDNFL	1352.6096	677.3124	9.24	0.4	46.47	12	ref|XP_016771425.1|
	IILPVPAF	868.5422	869.5531	10.9	4.2	44.39	8	
Predicted: Uncharacterized protein LOC725838	LATPLTITKLVPGAPIG	1661.0127	831.5143	10.32	0.9	46.32	17	ref|XP_001121640.4|
	ALATPLTITKLVPGAPIG	1732.0498	867.033	10.5	1	20.73	18	
Predicted: 40S ribosomal protein S5	PVALSAELPEIKL	1378.8071	690.4119	10.59	1.6	53.78	13	ref|XP_006570300.1
Predicted:Probable aconitate hydratase mitochondrial isoform X2	YSHLDEPDKQEIQRGTTY	2179.0181	727.345	5.12	−2.2	52.37	18	ref|XP_006560252.1|
Predicted:Uncharacterized protein LOC551958	SLKIPVVVRL	1122.7488	562.3829	9.57	2.3	35.27	10	ref|XP_624343.2|
Predicted:40S ribosomal protein S21	AKNDGILPKNF	1215.6611	608.8398	7	3.3	42.39	11	ref|XP_006560961.1|
Predicted:Elongation factor 1-alpha isoform X1	VTFKDAAGKVTKAAEKAQKK	2118.2161	707.0831	6.24	5.4	41.87	20	ref|XP_006569957.1|
Predicted:Protein prickle-like isoform X3	PPPPPPPPPP	988.5381	989.5435	5.34	−2	28.53	10	ref|XP_006569671.1|
Predicted:Probable citrate synthase 2 mitochondrial	FGVSRALGVLSSL	1304.7452	653.3832	10.31	5.1	35.75	13	ref|XP_393545.2|
Predicted:ATP synthase subunit epsilon mitochondrial-like isoform X2	SQIAAKLVRQALKSE	1640.9573	821.4868	7.51	1	29.35	15	ref|XP_003249712.1|
Predicted:Glutathione S-transferase S1 isoform X1	PVKGLGEPIRFL.L	1324.7866	663.3975	9.01	1.6	25.77	7	ref|XP_016772844.1|
Melittin	GIGAVLK	656.4221	657.4304	6.06	1.6	25.77	7	prf||670043A

Predicted:Uncharacterized protein LOC552356 isoform X3	FVIANRGIAN(+.98)L(-.98)	1186.6823	594.3471	8.66	−2.2	25.61	11	ref|XP_006568984.1|
Actin related protein 1	LRVAPEEHPVL	1258.7034	630.3628	6.78	6.1	63.19	11	ref|NP_001172074.1|
Cuticular protein CPF2 isoform 1 precursor	AAVAVQPAAAAATLPLSTAT	1793.9886	898.0042	8.87	2.9	70.45	20	ref|NP_001257761.1|
	AAAPAAVAVQPAAAAATLPLSTAT	2104.1528	1053.087	9.3	3.4	70.44	24	
	AAAPAAVAVQPAAAAATL	1533.8514	767.9346	8.41	2.1	69.76	18	
	AAAPAAVAVQPAAAAATLPLST	1932.068	967.0447	9.23	3.6	69.75	22	
	AAAPAAVAVQPAAAA	1248.6826	625.3511	5.94	4	69.47	15	
	AAVAVQPAAAAATLPLST	1621.9038	811.9617	8.76	3.1	67.02	18	
	VAVQPAAAAATLPLST	1479.8296	740.9246	8.52	3.4	65.08	16	
	APAAVAVQPAAAAATLPLSTAT	1962.0785	982.0499	9.11	3.4	59.68	22	
	LAAAPAAVAVQPAAAAATLPLSTAT	2217.2368	1109.625	9.86	−0.4	56.39	25	
	AAAPAAVAVQPAAA	1177.6455	589.8309	5.86	1.5	55.89	14	
	AAAPAAVAVQPAAAAATLPLSTA	2003.1051	1002.561	9.41	0.8	53.08	23	
	AAAPAAVAVQPAAAAATLP	1630.9042	816.462	8.5	3.2	53.06	19	
	ATLAVPTIATSTSNVIRGIGNLGAISA	2567.4282	856.8177	11.73	1.2	44.62	27	
	VANTAIAAPVATAA	1239.6823	1240.688	6.78	−1.2	41.63	14	
	TLAVPTIAT	885.5171	886.5251	8.64	0.8	39.15	9	
	LAAAPAAVAVQPAAAAATLPLSTA	2116.1892	1059.1	9.89	−2.3	37.89	24	

	AAPAAVAVQPAAAAATLPLST	1861.0309	931.5234	9.15	0.8	31.16	21	
	IALNGIGAVK	954.5862	955.5968	7.37	3.5	29.34	10	
	NGIGAVKVL	869.5334	870.5417	7.7	1.2	28.12	9	
Cuticular protein 5	GVGLGLRPGSLYGTQAL	1657.9152	829.9669	9.02	2.5	49.14	17	ref|NP_001257750.1|
	AIAATPVAPVAA	1050.6073	526.3128	7.23	3.6	38.92	12	
	VGLGVGLGLRPGSL	1293.7769	647.8948	9.72	−1.4	33.89	14	
Cuticle protein 18.7-like	LVPLVTNG	811.4803	812.4913	7.51	4.5	37.74	8	ref|XP_006568331.1|

RT- Retention time.

4 Conclusions

We propose a new analytical workflow to evaluate pesticide effects on honey bees through the analysis of (neuro)peptides content in honey bee heads. The present study has demonstrated the high potential of the developed analytical method to identify and quantify target and non-target (neuro)peptides in honey bees. LC-QqQ-MS/MS is a very sensitive and selective technique to identify and quantify target neuropeptides. LC-QTOF-MS/MS analysis in combination with multivariate statistical analysis, protein sequence databases and proteomic based search engines have shown to be a very powerful tool for the non-target analysis of (neuro)peptides and discriminating the peptide profile between two sample groups.

The application of the developed method to the analysis of neuropeptides in honey bees exposed to a field concentration of pesticides, has shown that the frequency of detected target neuropeptides from honey bee exposed to high level of pesticides is affected. Moreover, non-target analysis approach showed a clear distinction between the head peptides found in the two groups; (Please change ";" to ",") honey bees with high level content of pesticides and honey bees with low levels.

All this data show a correlation between exposure to high pesticide levels and a decrease of some neuropeptides and head peptides in honey bees. To our knowledge, this is the first field study which correlates pesticide exposure with neuropeptides content in honey bees.

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CRediT authorship contribution statement

María del Mar Gómez Ramos: Validation, Investigation. María José Gómez Ramos: Supervision, Project administration, Funding acquisition M.D. Gil García: Methodology, Validation Visualization. M. Martínez Galera: Conceptualization, Visualization, Writing – original draft Amadeo R. Fernández-Alba: Conceptualization, Visualization, Supervision.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.12.091.

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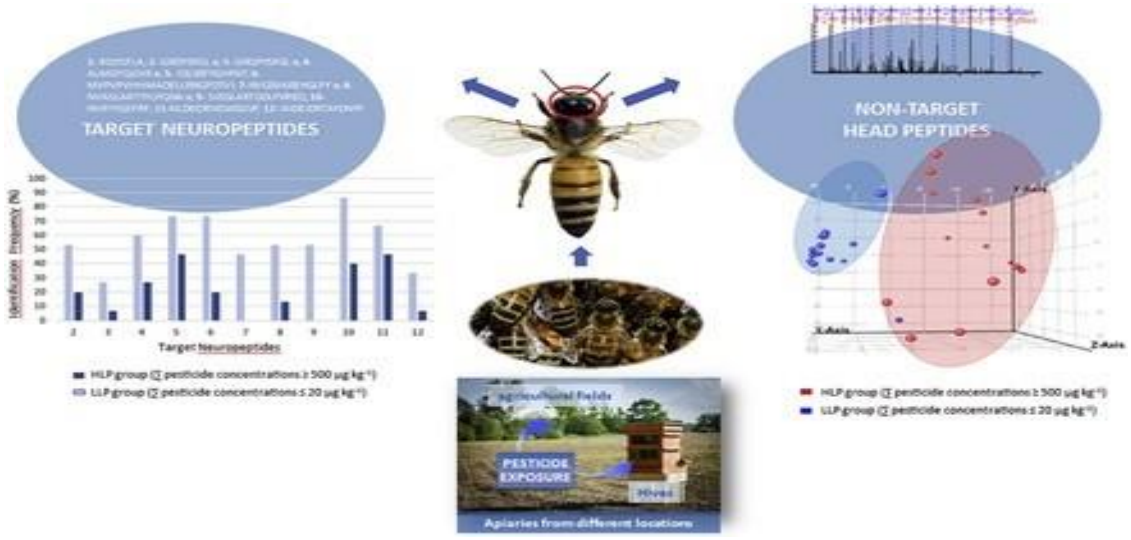
Graphical abstract



alt-text: Image 1

Highlights

- Evaluation of pesticide effects on honey bees through the analysis of (neuro)peptides.
- A workflow for the target and non-target analysis of (neuro)peptides in bee heads.
- Application to honey bees exposed to pesticides in field conditions.
- Head peptide differences between honey bees with HLP and honey bees with LLP.
- Identification of 25 non-redundant and discriminant head peptides.