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Validation of a QuECheRS method for analysis of neonicotinoids in small volumes of blood and assessment of exposure in Eurasian eagle owl (*Bubo bubo*) nestlings

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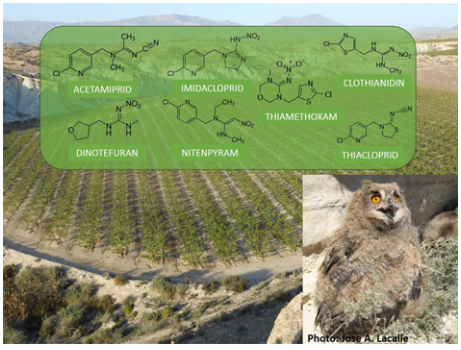
HIGHLIGHTS

- Exposure and effects of neonicotinoid pesticides are unknown in birds of prey.
- Two QuEChERS methods were compared to analyse small blood samples from birds.
- Acetate buffered QuEChERS was considered the most suitable method and was validated.
- Imidacloprid was the only neonicotinoid detected in eagle owls from Southeast Spain.
- More studies including toxicokinetics and toxicodynamics are recommended to assess the risk for birds.

GRAPHICAL ABSTRACT



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ABSTRACT

Neonicotinoid pesticides have gained great interest in the last years both for agricultural and domestic use. Since the information on their environmental distribution or the effects derived from exposure to ecosystems and biota is scarce, new analytical techniques are being developed for monitoring studies. In this sense, two extraction techniques based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methodology to detect the neonicotinoids authorised in Spain (acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, nitenpyram and thiamethoxam) were adapted and compared: a) using acetate buffer (AB); and b) using citrate buffer (CB). For detection and quantification, high performance liquid chromatography coupled with time of flight mass spectrometry (HPLC/TOF-MS) was used. The CB method provided a wide range of recoveries (68–134%) and accuracy (4–9%). The AB method provided good recoveries (59–76%, 59% corresponded to clothianidin) precision (4–11%) linearity (0.987–0.998%) and limit of quantification (2–10 ng/mL) for all the compounds. To test the effectiveness of the technique, we analysed 30 blood samples of free-ranging nestlings of Eurasian eagle owl (*Bubo bubo*). The only compound detected, in one nestling from a dry land farming area, was imidacloprid, with a concentration of 3.28 ng/mL. To our knowledge, this is the first study of neonicotinoid pesticides in free-ranging birds of prey using non-destructive samples, providing the first insight for biomonitoring studies. Further studies, including toxicokinetics and toxicodynamics, are recommended to assess the risk for these species.

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1. Introduction

Neonicotinoids were developed in the nineties as a safer and more effective class of pesticides. Their development was motivated by the increased insect resistance to former pesticides and the growing regulations for human and environmental safety (Simon-Delso et al., 2015; Tomizawa and Casida, 2005).

This group of compounds has a wide range of applications for agricultural, domestic and veterinary use. Due to their benefits, they are considered one of the most produced and distributed pesticides worldwide (Jeschke et al., 2011; Simon-Delso et al., 2015).

These compounds are derived from nicotine and bind to the nicotinic acetylcholine receptors in the postsynaptic neuron, leading to neurotoxic symptoms. The first toxicological studies suggested that they are apparently safe in vertebrates, based on their lower affinity to the receptors (Tomizawa and Casida, 2005). Nevertheless, recent studies have shown direct effects in non-target invertebrates (Vijver and Van den Brink, 2014) and direct and indirect effects in vertebrates (Barrios, 2007; Gibbons et al., 2015; Kreutzweiser et al., 2008). One of the most controversial examples may be the mortality of some pollinator organisms (Chagnon et al., 2015; Mommaerts et al., 2010; Pisa et al., 2015). This led to the implementation of regulations (i.e. the Commission Implementing Regulation (EU) No 485/2013 that bans the use of clothianidin, thiamethoxam and imidacloprid during blooming). In birds, early experimental studies already proved their toxicity (Mineau and Palmer, 2013). More recent studies showed adverse effects from biochemical, reproductive or immune alterations to mortality in Red-legged partridges (*Alectoris rufa*), Red avadavat (*Amandava amandava*) and chickens (*Gallus gallus domesticus*) (Balani et al., 2011; López-Antia et al., 2013; Pandey and Mohanty, 2015). Therefore, the risks of neonicotinoid pesticides may have been underestimated, especially by regulatory agencies (Mineau and Palmer, 2013). This highlights the need to investigate the distribution and effects in ecosystems and living beings through monitoring studies (Anderson et al., 2015; Millot et al., 2015). In this sense, birds of prey are considered especially suitable sentinels, as they are on top of food chains and therefore susceptible to accumulate and integrate contaminants over time (Furness, 1993; García-Fernández et al., 2008; Gómez-Ramírez et al., 2014; Sergio et al., 2005). However, sampling from wild animals need the minimization of stress and the improvement of techniques in order to use lesser size or volume of sample. Thus, non- or less-invasive sampling methods are more ethically acceptable, and nowadays they are being frequently used to biomonitoring studies. Samples such as blood (Espín et al., 2014; Eulaers et al., 2011; Gómez-Ramírez et al., 2011; Sonne et al., 2010), feathers (Espín et al., 2012; García-Fernández et al., 2013; Eulaers et al., 2011), or unhatched eggs (Gómez-Ramírez et al., 2012a; Martínez-López et al., 2007) are suitable for these purposes. In the case of blood, the sample amount is limited by the body weight, since maximum 1% or 2% of the body weight in any 14-day period can be extracted (McGuill and Rowan, 1989; Voss et al., 2010).

Currently there are many analytical techniques with a wide range of solvents for quantifying pesticides, including neonicotinoids. Most of them are used for vegetables and fruits (Appendix, Table A1), and less frequently, for animal or human samples (Appendix, Table A2). Since the development of the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methodology by Anastassiades et al. (2003), many techniques based on this method have been set up. This is mainly due to its characteristics, as described by Anastassiades et al. (2003): rapid, simple, inexpensive, effective, safe and potentially rugged, using minimal amounts of solvents, no special equipment or glassware, but providing high quality results for a wide range of pesticides. However, to our knowledge, most studies have used QuEChERS to analyse neonicotinoids in vegetables or honey, but only one analysed liver samples (Table 1). Modifications of this method using small samples have been successful in the analysis of other pesticides (Gómez-Ramírez et al., 2012b). This led us to choose a QuEChERS method for the analysis of neonicotinoids in birds' blood. Therefore, our first objective was the development of an analytical technique using small volumes of blood for the simultaneous detection of all the neonicotinoid pesticides authorised in Spain (AEMPS, 2016; MAGRAMA, 2015).

The study area, the Region of Murcia (Spain), comprises a heterogeneous landscape: mountains and hills with natural vegetation (scrubland and pine forest) and valleys with farming land (irrigation and dry farming). As a matter of fact, agriculture is the main use of land in the study area (León-Ortega, 2016). The Eurasian eagle owls (*Bubo bubo*) mainly breed in small cliffs in mountains and hilly areas dominated with scrubland, but near the edge of farming land with high prey availability (rabbits *Oryctolagus cuniculus*, partridges *Alectoris ruffa* and pigeons *Columba* spp.; León-Ortega et al., 2016). Thus, in our study area, the Eurasian eagle owl can be considered a suitable sentinel species for monitoring pesticides as it meets the requirements established by the National Research Council (1991), i.e. abundance of individuals, knowledge of the census, ease to capture and sampling, and to be exposed to environmental pollutants such as neonicotinoids, mainly through the ingestion of contaminated prey. Hence our second objective was to carry out a first insight of the exposure to neonicotinoids in this population, analysing blood samples from nestlings with the validated technique.

2. Material and methods

2.1. Biological samples

For the development of the analytical technique, blood samples were obtained from 10 chickens from the Laboratory Animal Section (Research Support Service, University of Murcia). The chickens were healthy and had never been exposed to chemicals. Blood

Table 1
Comparison of acetate buffered QuEChERS method validated in this work with other QuEChERS techniques that analyse neonicotinoids.

Matrix (sample amount)	Compounds	Recovery[a] (%)	Spiking levels (ng/mL)	LOQ[a] (ng/mL)	References
Birds blood (500 μL)	Acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, thiamethoxam	59–76	2, 10, 20, 50, 100	2–10	This work
Fruits and vegetables (15 g)	32 pesticides of different chemical classes including imidacloprid	103	50, 250, 1000	10	Lehotay et al., 2010
Potato, orange and cereal-based baby food (10 g)	52 pesticides of different chemical classes including acetamiprid, imidacloprid, thiacloprid	85–110	10, 100	5–10	Leandro et al., 2007
Soil from cocoa plantation (5 g)	Acetamiprid, imidacloprid, thiacloprid, clothianidin, thiamethoxam	64–98	8, 80	2–9	Dankyi et al., 2014
Northern bobwhite (*Colinus virginianus*) and scaled quail (*Callipepla squamata*) liver (10 g)	Clothianidin, imidacloprid, thiamethoxam	90–113	Not mentioned	3.61, 3.49, 3.42	Turaga et al., 2016

LOQ = limit of quantification.
[a] Range of values obtained from all neonicotinoid pesticides analysed.

collection was performed by puncturing the brachial vein with 23G needle and 5 mL syringe, using heparin as anticoagulant. Blood samples were pulled, homogenized and frozen at −40 °C until use.

In order to assess the exposure to neonicotinoids in a population of wild birds, blood samples were obtained from free-ranging Eurasian eagle owl nestlings born in the Southeast of the Region of Murcia (Fig. 1). To provide a representative sample of the main uses in the study area, the eagle owl nests were selected according to land uses in the proximities: irrigation farming (n = 10), dry land farming (n = 10) and non-agricultural use (n = 10). These nests were monitored by four surveys during the reproductive period (January–May), to take the samples when the chicks were large enough (mean age and weight of 35 days old and 1305 g respectively), between March and June (León-Ortega et al., 2014). Thirty samples from 30 different nests (the oldest nestling of each nest) were selected. The blood samples were obtained following the EURAPMON sampling and monitoring protocol for raptors (Espín et al., 2016), which coincides with the protocol for blood sampling in chickens.

2.2. Chemicals and standards

Neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) reference standards were obtained from Sigma-Aldrich® (USA). Acetonitrile was purchased from Lab-Scan® (Poland) and glacial acetic acid from Panreac® (Spain). Magnesium sulphate, sodium chloride, sodium citrate dibasic sesquihydrate, sodium citrate tribasic dihydrate, sodium acetate, PSA bonded silica (Supelclean PSA: polymerically bonded, ethylenediamine-*N*-propyl phase that contains both primary and secondary amines) and C18 (Discovery DSC-18: octadecylsilane 18% C) were purchased from Supelco® (USA). All the chemicals and standards were of residue quality (>99.9% purity). A stock solution containing all the neonicotinoids in acetonitrile was made at 2000 ng/mL. This mix was used to spike the chicken blood samples at different final concentrations (2, 10, 20, 50, and 100 ng/mL) to validate the technique.

2.3. Analytical procedure

For the choice of extraction method, two modifications of the QuEChERS method were compared: a) acetate buffered QuEChERS extraction (AB) based on Dankyi et al. (2014) and Lehotay et al. (2010) methods, similar to that published by the AOAC International (2011); and b) citrate buffered QuEChERS extraction (CB) based on Gómez-Ramírez et al. (2012b) and Payá et al. (2007) methods, similar to that published by the European Committee for Standardization (2008).

For the AB extraction, 500 μL of whole blood was mixed with 1500 μL of glacial acetic acid 1% in acetonitrile. The mix was homogenized with ultrasonic homogenizer (Labsonic®) for 1 min and shaken vigorously with a vortex for another minute. A combination of salts (1.2 g magnesium sulphate and 0.3 g sodium acetate) was then added and the tube was again vigorously shaken with vortex for 1 min. The tube was centrifuged at 5000*g* for 5 min, and frozen at −4 °C for 1 h. The tubes were again centrifuged under the same conditions as the previous step. The supernatant was then transferred to another tube and mixed with 180 mg magnesium sulphate, 30 mg PSA and 30 mg DSC-18. The tube was shaken similarly to the first step and centrifuged again in a microcentrifuge at 5000*g* for 5 min. The supernatant was transferred to a chromatography vial where it was evaporated under a gentle nitrogen stream up to 500 μL.

The CB extraction process is identical to the extraction technique QuEChERS in acetate buffer, but the solvent was acetonitrile without acetic acid; and a different mix of salts for the first extraction step were used (0.8 g of magnesium sulphate, 0.2 g sodium chloride, 0.1 g sodium citrate dibasic sesquihydrate and 0.2 g dibasic sodium citrate tribasic dehydrate).

2.4. Instrumental analysis

Detection and quantification of extracts was performed using high-performance liquid chromatography (HPLC, Agilent Series 1200, Agilent Technologies, Santa Clara, CA, USA) which include: autosampler, binary pump and control temperature module. The separation was performed on a Waters Sunfire C18 column of 250 mm × 4.6 and 25 μm particle size. Column was held at a constant temperature of 40 °C. The mobile phase was 0.1% formic acid in water/ammonium formiate 5 mM (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient program was 15% B from the start ramped to 75% B over the course of 20 min and held until 22 min, and reduced in $t = 22.1$ at 15% until the end (25 min). The electrospray interface

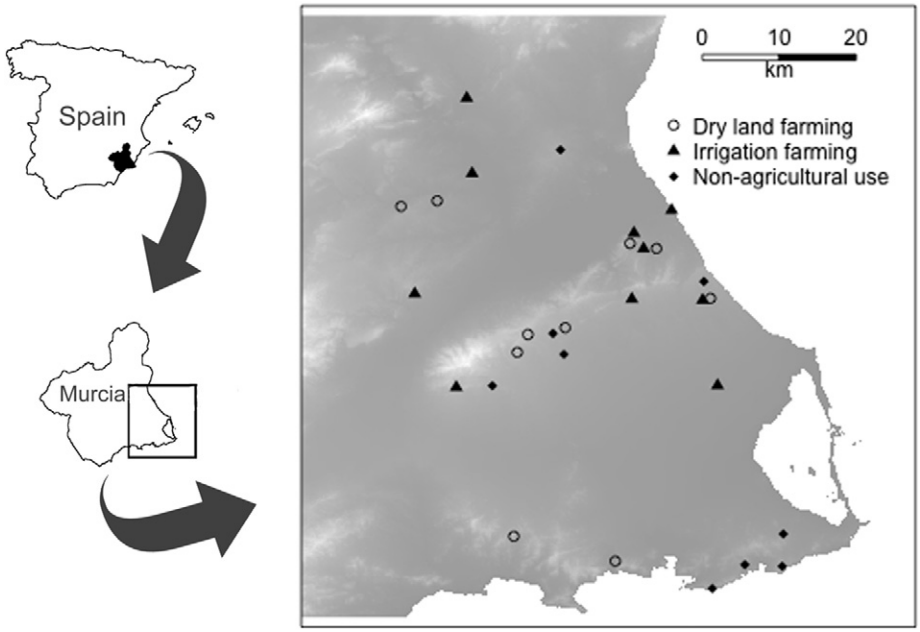

Fig. 1. Study area and location of the nests sampled in this study.

was set in positive ionization mode. The HPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 accurate mass TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in the positive ionization mode, using the following operation parameters: capillary voltage, 3000 V; nebulizer pressure, 60psig; drying gas flow rate, 12.0 L/min; gas temperature, 350 °C; skimmer voltage, 65 V; octapole RF 250 V; fragmentor voltage, 180 V. LC–MS accurate mass spectra were recorded across the *m/z* range of 100–1000. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduced the flow from the outlet of the analytical column together with a low flow (approximately 40 L/min) of a calibrating solution which contained the internal reference masses purine ($C_5H_4N_4$, at *m/z* 121.050873, in positive ion mode) and HP-0921 (Hexakis-(1H,1H,3H-tetrafluoropropoxy) phosphazine, $C_{18}H_{18}O_6N_3P_3F_{24}$, at *m/z* 922.009798 in positive ion mode). The instrument provided a typical resolution higher than 10,000 at *m/z* 118 and higher than 18,000 at *m/z* 1522. The full scan data were recorded with Agilent Mass Hunter Data Acquisition software (version B.06.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.06.00).

2.5. Selection and validation of extraction technique

The analytical method was validated following the "Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed from the Directorate-General for Health and Food Safety of the European Commission" (SANTE/11945/2015, 2016). Firstly, five replicates of spiked chicken blood with a mix of the 7 neonicotinoids at 50 ng/mL were extracted using the two different methods. The method was selected based on the best values of accuracy and repeatability. Afterwards, accuracy, precision, linearity and limit of quantification were calculated after the extraction by the selected method of five replicates of spiked chicken blood with a mix of the 7 neonicotinoids at different concentrations (2, 10, 20, 50, and 100 ng/mL). The unspiked chicken blood samples were analysed as blank in quintuplicate in order to ensure that they were free from neonicotinoids and to test for matrix effect.

Accuracy of the method was assessed by studying the recovery of neonicotinoids in chicken blood spiked with the mix of neonicotinoids. The extraction recoveries were calculated comparing peak heights obtained from extracted spiked samples with peak heights of the standard solutions. Linearity of an analytical method is defined as the ability to elicit test results directly proportional to the concentration of analytes in samples within a given range. The range and number of levels of fortification are highly related to the applicability of the method. In this case, linearity was calculated using a blank sample as 0 and five replicates of spiked blood samples with the mix of neonicotinoids at five levels (2, 10, 20, 50, and 100 ng/mL). Linear regression of data to a matrix-matched calibration curve was performed using the method of least squares. The acceptance criterion for linearity was a correlation coefficient $r \geq 0.9$. Precision of a method can be defined by repeatability and reproducibility tests. Repeatability proves the ability to provide similar results when the technique is repeated in the same sample, by the same operator. The acceptance criterion is based on the relative standard deviation (RSD) of five replicates of spiked blood samples. Reproducibility proves the ability to provide similar results when the technique is repeated in the same sample but by different operators or different laboratories. To validate reproducibility of our technique, chicken blood samples spiked at 100 ng/mL were analysed by different analysts on different days. Repeatability and reproducibility acceptance criterion was RSD ≤ 20%.

2.6. Statistical analysis

Statistical analysis of the data was performed using Microsoft Excel 2016 spreadsheet and SPSS (version 21), using paired *t*-test to compare both methods (recoveries and repeatability), with a level of significance of $\alpha = 0.05$. Linear regression of data to a matrix-matched calibration curve was performed using the method of least squares.

3. Results and discussion

3.1. Method choice and validation

Both extraction methods showed good recoveries and repeatability (Table 2). However, paired *t*-test showed significant differences (<0.05) between the methods in both parameters and the AB method was chosen based on the best values of repeatability and suitable recoveries. Although both methods offer good recoveries, method CB values are less acceptable since they exceed about 30% of the spiked levels applied for imidacloprid and nitenpyram. In addition, RSD for repeatability at 50 ng/mL was lower for AB (0.2–2%) than for CB (4.1–8.6%).

Validation parameters of the chosen method (AB), taking into account 5 spiking levels, are shown in Table 3. Representative chromatograms and mass spectra of spiked blood samples are shown in Figs. 2 and 3.

Recovery values for all the compounds were around 70%, except for clothianidin (59%). In multi residue methods, recoveries below 60% are also acceptable if linearity and precision values are good (SANTE/11945/2015, 2016), which is the case of clothianidin (Table 3). In regards to technical precision, repeatability and reproducibility, the values are below 12%, and therefore within the accepted range (<20%) (SANTE/11945/2015, 2016). Linearity above 0.987 for all compounds confirms excellent correlation of the data, and high quality of fit. The limit of quantification was 2 ng/mL for acetamiprid, imidacloprid and thiacloprid, and 10 ng/mL for clothianidin, dinotefuran, nitenpyram, and thiamethoxam. As indicated by SANTE/11945/2015 (2016), good values for limit of quantification in pesticides should be below or equal to Maximum Residues Levels (MRL). Based on this criterion, we can consider that the method AB shows good sensitivity since European legislation established the lowest MRL at 10 ng/g for these neonicotinoids (Commission Regulation (EU) 500/2013, 491/2014, 2015/846, 2015/1200, 2016/156).

If we compare with similar extraction techniques that analysed neonicotinoids using QuEChERS (Dankyi et al., 2014; Leandro et al., 2007; Lehotay et al., 2010; Turaga et al., 2016; Table 1), or other extraction methods (Luzardo et al., 2015; Seccia et al., 2008; Stivaktakis et al., 2016; Table 4), our recovery values were slightly lower. The main reason could be that we used a smaller sample amount (500 μL) compared to the other techniques. On the other hand, the concentrations of the spiking solutions were usually higher (200–50,000 ng/mL) than in our study (2–100 ng/mL). Finally, matrix effect should be considered, since different matrices have been used in the studies reviewed (urine, milk, vegetables, soil, etc). However, our limits of quantification

Table 2
Comparison between citrate and acetate buffered QuEChERS method for 7 neonicotinoid pesticides in spiked blood samples. Parameters were calculated as the average of 5 replicates at 50 ng/mL.

	Citrate buffered QuEChERS		Acetate buffered QuEChERS	
	Recovery (%)	Repeatability (RSD %)	Recovery (%)	Repeatability (RSD %)
Acetamiprid	77	5.4	69	1.1
Clothianidin	83	7.3	68	2.0
Dinotefuran	68	5.6	64	1.8
Imidacloprid	134	4.1	97	0.7
Nitenpyram	132	8.6	92	3.2
Thiacloprid	78	4.1	71	0.2
Thiamethoxam	91	6.2	75	0.9

RSD = relative standard deviation.

Table 3
Validation parameters for 7 neonicotinoid pesticides in spiked blood samples for acetate buffered QuEChERS extraction method analysed with HPLC-TOF/MS.

	Acetate buffered QuEChERS									
	Recovery[b] (%)						Repeatability[b] (RSD %)	Reproducibility[c] (RSD %)	Linearity[b] (r)	LOQ[a] (ng/mL)
	M[a]	2	10	20	50	100				
Acetamiprid	70	68	70	76	69	66	4.8	4.2	0.991	2
Clothianidin	59	ND	62	50	68	58	9.2	4.8	0.993	10
Dinotefuran	68	ND	87	62	92	62	4.4	4.6	0.987	10
Imidacloprid	68	67	54	63	97	59	6.5	6.9	0.995	2
Nitenpyram	76	ND	72	68	64	66	8.9	5.6	0.998	10
Thiacloprid	71	72	66	76	71	70	7.5	11.1	0.992	2
Thiamethoxam	69	ND	66	64	75	70	5.7	4.1	0.993	10

ND = not detected; r = regression coefficient; LOQ = limit of quantification.
[a] Average recoveries of the 5 spiking levels.
[b] Average of 5 replicates at 5 concentrations (2, 10, 20, 50 and 100 ng/mL).
[c] Average of 5 replicates at 100 ng/mL.

(2–10 ng/mL) are similar or lower than other techniques (3.42–40 ng/mL in animal samples; Tables 1 and 4). Therefore, the validated technique is accurate, sensitive, with excellent linearity, and good recovery values.

3.2. Applicability of the technique

A total of 30 samples of whole blood from Eurasian eagle owl nestlings from the Region of Murcia were analysed using the selected and validated technique. Imidacloprid was the only compound detected, in a single sample. The sample was obtained in dry land farming area, with a concentration of 3.28 ng/mL.

The use of imidacloprid, as well as clothianidin and thiamethoxam is restricted during blooming in the study area (Commission Implementing Regulation (EU) No 485/2013). For most of the plants and trees grown in the irrigation farming areas (peach, apricot, melon, watermelon, etc.), this blooming coincides with the sampling season of eagle owls. On the contrary, almond trees together with olive trees, which are grown in the dry land farming area, usually bloom earlier (almond trees) or later (olive trees) than the sampling of eagle owls. Therefore, the probability of finding residues would be higher in the dry land farming areas. In addition, imidacloprid is registered in the highest number of products available on the market in Spain, both for agricultural and veterinarian use (AEMPS, 2016; MAGRAMA, 2015). Moreover, it is also the most widely used neonicotinoid in the Region of Murcia (Sanz-Navarro, 2008). Also, this compound has greater persistence in the environment than the other neonicotinoids (Miranda et al., 2011).

A priori the results of this study suggest that there is practically no exposure to neonicotinoid pesticides in this Eurasian eagle owl population. This lack of exposure would lead to suspect the suitability of this species as sentinel in neonicotinoid biomonitoring studies. However, several hypotheses may explain the low frequency or nil detection of these compounds. In spite of the high frequency of use of neonicotinoids, as mentioned above for imidacloprid, these may not have been used in the study area or in the sampling season. Due to degradation in the soil or water, the bioavailability of neonicotinoids could be too low after their agricultural application. Neonicotinoids could also have been quickly metabolized and/or excreted from the owls prey. In mammals these compounds are rapidly absorbed, widely distributed and fastly metabolized and excreted mainly by urine (Kapoor et al., 2014; Marrs, 2012). Thus we can hypothesize that the potential for bioaccumulation in mammals is scarce or non-existent. However, similarly to other compounds (Ex.: Organohalogen - Walker, 1983), there could be differences in the metabolism between mammals and birds. In fact, although to our knowledge no toxicokinetic studies exist, López-Antia et al. (2015) found that after repeated oral doses of imidacloprid, this accumulated in the liver of Red-legged partridges. Thus, further analysis of neonicotinoids in rabbits, partridges and pigeons, the main prey of eagle owls in the study area, may be useful.

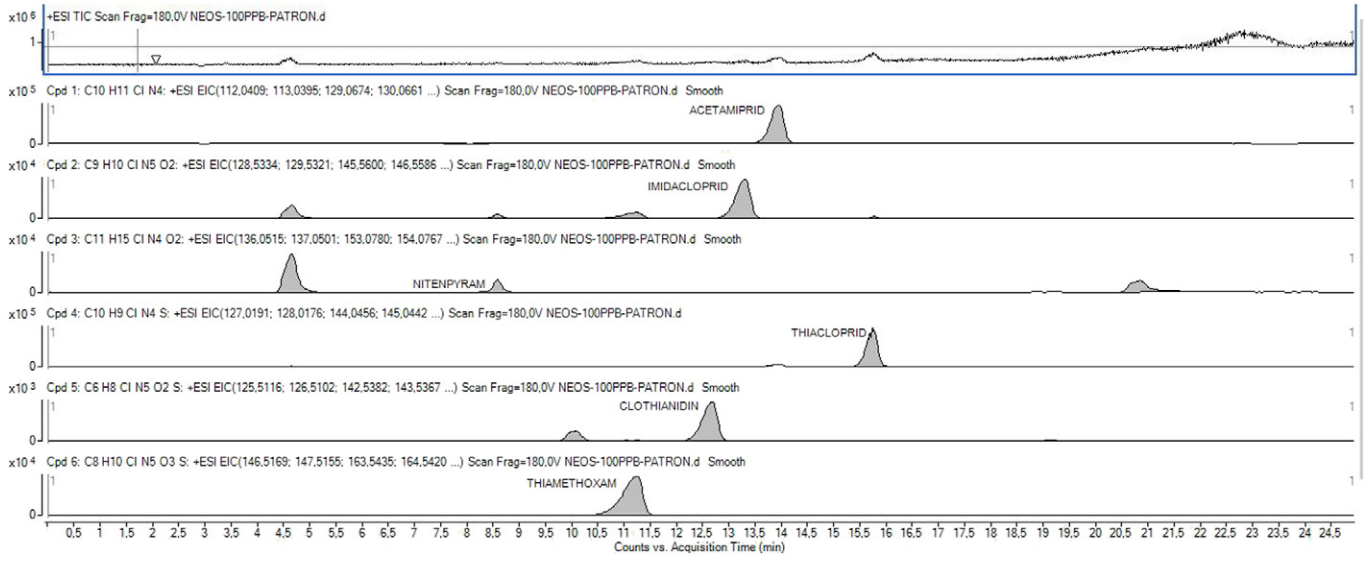

Fig. 2. HPLC-TOF/MS chromatograms of the neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, nitenpyram and thiamethoxam) in a multi-standard solution containing 100 ng/mL of each analyte.

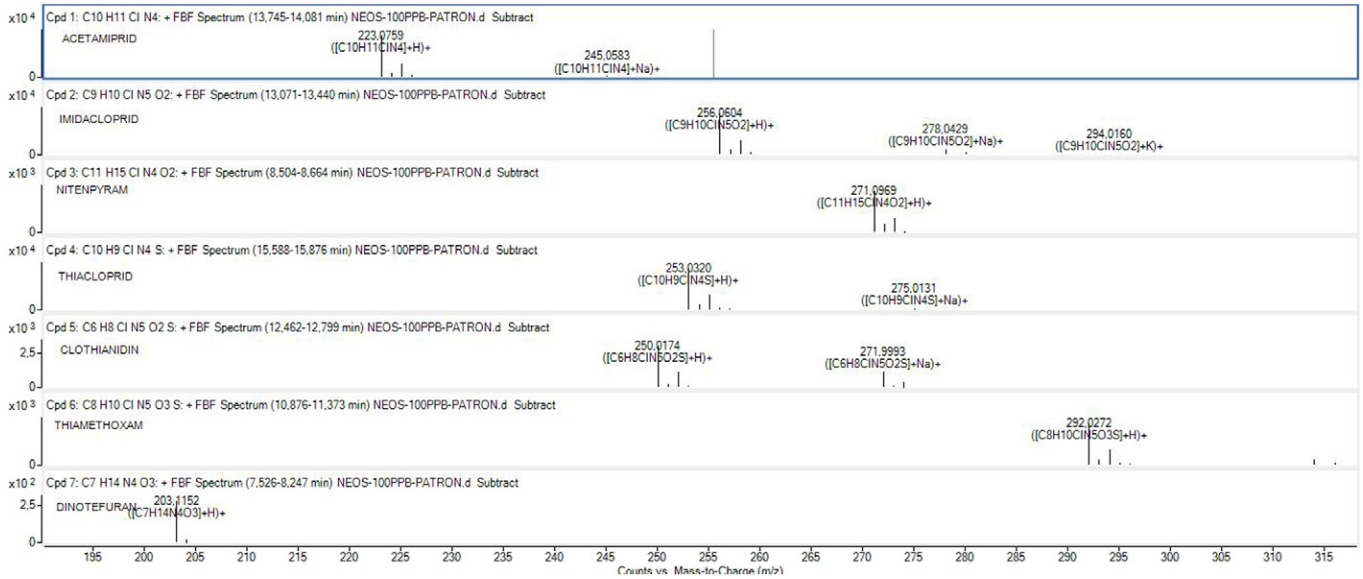

Fig. 3. HPLC-TOF/MS mass spectra of the neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, nitenpyram and thiamethoxam) in a multi-standard solution containing 100 ng/mL of each analyte.

Lastly, it is also possible that, in the owls sampled, neonicotinoids were distributed to other organs, metabolized or excreted previously to sample collection. Therefore, although neonicotinoids were not detected in blood, their metabolites could have been detected. It would be recommended to perform experimental studies to understand better the toxicokinetics of neonicotinoid pesticides in birds.

Considering these arguments, we still cannot confirm or rule out the suitability of eagle owl as a sentinel species. It will be necessary to deepen the knowledge and analytical methods for these compounds. It could also be interesting to analyse blood samples obtained from adults caught at different seasons of the year. The usefulness of other matrices such as feathers or liver, in the case of dead birds can also be investigated.

Due to the scarce information about toxic levels in birds, the toxicological interpretation of the levels of imidacloprid in the positive sample is difficult. To our knowledge, the effects of neonicotinoids on birds of prey have not been studied yet. In any case, long-term effects should not be ruled out, paying attention to biochemical, reproductive and immunological alterations.

4. Conclusions

Based on validation parameters, the QuEChERS method with acetate buffer is suitable for application in the analysis of neonicotinoid pesticides in small volumes of bird blood.

Table 4
Comparison of acetate buffered QuEChERS method validated in this work with different analytical techniques applied to animal fluid samples.

Matrix (sample amount)	Compounds	Extraction solvent (extraction method)	Recovery[a] (%)	Spiking levels (ng/mL)	LOQ[a] (ng/mL)	References
Chicken blood (500 μL)	Acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, thiamethoxam	Acetonitrile (QuEChERS)	59–76	2, 10, 20, 50, 100	2–10	This work
Bovine whole milk (5 g)	Acetamiprid, imidacloprid, thiacloprid, thiamethoxam	Dichloromethane (solid-liquid extraction)	85–99.7	10,50,100	10–40	Seccia et al., 2008
Human urine (5 mL)	Acetamiprid, imidacloprid, thiacloprid, thiamethoxam + 83 pesticides of different chemical classes	Dichloromethane (solid phase extraction with C-18 cartridges)	67–101	5, 10, 50	0.04–0.7	Cazorla-Reyes et al., 2011
Chicken muscle and liver (≤5 g)	Acetamiprid, clothianidin, imidacloprid, thiacloprid, thiamethoxam + 102 pesticides and some degradation products	Ethyl-acetate: cyclohexane (liquid-liquid extraction)	72–91	100, 1000	25	Taylor et al., 2013
Rats blood and urine (1 mL)	Imidacloprid, 6-chloro nicotinic acid (6-CNA) and 6-hydroxy nicotinic acid (6-HNA)	Acetonitrile (solid phase extraction in C-18 cartridges)	Not mentioned	Not mentioned	Not mentioned	Kapoor et al., 2014
Human blood (2 mL)	Imidacloprid + 18 pesticides of different chemical classes	Dichloromethane: ethyl-acetate: acetone (liquid-liquid extraction)	88	200	10	Luzardo et al., 2015
Rabbit serum (500 μL)	Imidacloprid and 6-chloronicotinic acid	Dichloromethane (liquid-liquid extraction)	88.4–89.5	500, 1000, 2500, 5000, 10,000, 25,000, 50,000	6–18	Stivaktakis et al., 2016

LOQ = limit of quantification.
[a] Range of values obtained from all neonicotinoid pesticides analysed.

Imidacloprid was the only compound detected in this initial evaluation of the exposure to neonicotinoid pesticides in Eurasian eagle owl. This could be explained because it is the most widely used neonicotinoid in the Region of Murcia, and also the most persistent in the environment.

With the available data, we still cannot confirm or rule out Eurasian eagle owl suitability as a sentinel species for neonicotinoid pesticides exposure. To shed light on exposure to neonicotinoids it is necessary: a) further knowledge about the bioavailability of neonicotinoids on birds at the top of the food chain, such as the Eurasian eagle owl; b) further research on the analytical method, including the main metabolites of each compound and other matrices such as feathers or liver; and c) experimental studies of exposure to better understand the kinetics of neonicotinoid pesticides on birds.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.03.246.

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