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Development of a QuEChERS method for simultaneous analysis of antibiotics in carcasses for supplementary feeding of endangered vultures

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HIGHLIGHTS

- There is a need to assess the exposure to pharmaceuticals in Cinereous vultures.
- A QuEChERS technique was validated to analyse antibiotics in animal tissues.
- The carrion disposed to Cinereous vultures was analysed.
- Oxytetracycline was the most common and abundant antibiotic in the carrion.
- These results can be used to assess the risk to antibiotics in the vultures.

GRAPHICAL ABSTRACT

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ABSTRACT

Antibiotics have been beneficial for human and animal health. However, an excessive use in livestock and a deficient management of the carcasses can lead to adverse effects in the scavengers that ingest them, especially in "supplementary feeding sites" (SFS). The aim of this study was to assess the potential risk of exposure to antibiotics for an endangered population of Cinereous vultures (*Aegypius monachus*) from southeastern Portugal. Hence, a multi-residue method based on QuEChERs was adapted and validated to analyse, in small volumes of tissues, the most frequent antibiotics used in livestock. The method was applied to 87 samples of liver, muscle and kidney from 7 goats and 25 sheep disposed in SFS. According to questionnaires to farmers, the animals had not been treated with antibiotics, but analyses showed residues in 29% of the samples. Antibiotics were more frequent in goats (42.9%) than in sheep (24.2%), and oxytetracycline and trimethoprim were the most common (both 13.8%). Oxytetracycline, the most common antibiotic for livestock in Portugal, showed the highest concentration (1452.68 ng g^{-1}). To our knowledge, this is the first study of presence of antibiotics in carrion from SFS. The concentrations of antibiotics in carrion do not seem to pose a risk of acute intoxication for adult Cinereous vultures. However, subtle and likely chronic exposure with unknown health consequences may occur,

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which requires more research. Moreover, the results of this first study can be used in future studies to assess the risk for avian scavengers.

1. Introduction

The development and use of pharmaceuticals has been greatly beneficial for human and animal health. However, due to a rapid increase of intensive farming, where animals are kept at high densities with frequent transmission of diseases, the use of veterinary drugs has increased. Manuring, treatment of animals and disposal of carcasses, offal, urine, faeces, and unused products can contaminate the environment with veterinary pharmaceuticals (Margalida et al., 2014). As a consequence, some negative effects have been evidenced in living beings, including humans (Jørgensen and Halling-Sørensen, 2000). Particularly, antibiotics (the most frequent veterinary drugs in livestock, including grazing animals; Sarmah et al., 2006) or their metabolites, are known to cause allergic reactions in human (dermatitis, cutaneous eruptions, anaphylaxis, and gastro-intestinal symptoms due to the ingestion of poultry products contaminated with β-lactams, Mund et al., 2017; Paige and et al., 1997), as well as disruption of the normal microbiome both in human and wildlife (Mund et al., 2017; Pitarch et al., 2017). Microbial resistance and predisposition to fungal infections (Blanco et al., 2017a, 2017b; Pitarch et al., 2017) are especially of increasing concern. In fact, antibiotic resistance is one of the biggest threats to global health, food security, and development for the World Health Organization (2017).

Vultures, as species at the top of food chains, can be considered especially at risk of exposure and accumulation of environmental contaminants such as heavy metals and pesticides (García-Fernández et al., 2005; Gómara et al., 2004). Their exposure to veterinary pharmaceuticals is of increasing concern, mainly after the "crisis of diclofenac", a non-steroidal anti-inflammatory drug (NSAID) which caused a population decline of Gyps vultures in Asia after feeding on medicated cows (Cuthbert et al., 2011; Taggart et al., 2009). However, other drugs could also cause death (i.e. barbiturics, Aldeguer et al., 2009; ketoprofen, Naidoo et al., 2010) or indirect effects on scavenger birds by affecting pathogens (i.e. antibiotics, Blanco et al., 2017a, 2017b; Pitarch et al., 2017), immune (i.e. corticosteroids; Höfle et al., 2007) or reproductive system (hormones, metabolites and endocrine disruptors; Höfle et al., 2007). Antibiotics like penicillin, tetracycline, oxytetracycline and sulfonamide drugs may cause deformities in embryos when they are administered to hens near or during the breeding season (Dumonceaux and Harrison, 1994). For these reasons, the risk of pharmaceuticals for scavenger birds is considered of especial interest for the "One Health" initiative (Margalida et al., 2014). This concern is especially relevant in the Iberian Peninsula, which holds 95% of the European population of scavengers (Margalida et al., 2010). Nevertheless, the literature regarding this issue and the results related to antibiotic residues in carrion and its effects on scavenger bird populations are still scarce (Blanco et al., 2016, 2017a, 2017b; Casas-Díaz et al., 2016; Pitarch et al. 2017).

The practice of supplementary feeding, concentrating carcasses at just a few sites called "vulture restaurants" or "supplementary feeding sites" (SFS), has been used as a strategy for the conservation of avian scavengers in southern Europe (Cortés-Avizanda et al., 2010; Moreno-Opo et al., 2010, 2015a, 2015b, 2016; Zuberogoitia et al., 2013). Because there are currently no regulations in this sense, the carcasses can come from medicated animals. Hence, the risk of exposure to residues of pharmaceuticals can be higher for scavengers that feed on them. In the case of Cinereous vultures (*Aegypius monachus*) from southern Portugal, carcasses of domestic ruminants have been their main feed, especially after the declines of rabbit populations. Also, as populations of wild ungulates increased, these have constituted an important part of the Cinereous vultures diet (Lourenço et al., 2013).

More recently, measures to protect this endangered species have been taken. These measures include the implementation of a network of SFS with carcasses of domestic ruminants (Lourenço et al., 2013), the creation of buffer zones to reduce human disturbance at breeding sites (Margalida et al. 2011) or the management of SFS (Moreno-Opo et al., 2010). In this sense, the Liga para a Protecção da Natureza provided carcasses for Cinereous vultures in a special protected area of 2000 Nature Network, within the project "LIFE Habitat Lince Abutre" (LIFE08 NAT/000227). The selection of carcasses from non-medicated animals was based on the responses to the questionnaires addressed to farmers. In order to confirm the lack of residues of antibiotics, samples of carrion would be analysed. For this reason, our first objective was to develop a method to analyse several antibiotics simultaneously in a single analysis. This method consists in a modification of QuEChERS (short name for Quick, Easy, Cheap, Effective, Rugged, and Safe). QuEChERS has been successfully used for a great number of compounds of different chemical groups, including quinolones, β-lactams and tetracyclines in different matrices of animal origin such as milk or meat (Lombardo-Agüí et al., 2012; Freitas et al., 2014). Based on the information provided by local breeders and published literature (Almeida et al., 2014), we focused on the antibiotics more frequently used in the species of livestock (cow, goat and sheep) that could be provided to the vultures in the study area. Hence, the validation of the method was carried out for tetracyclines (tetracycline, chlortetracycline, oxytetracycline), quinolones (enrofloxacin, ciprofloxacin, nalidixic acid), β-lactams (penicillin G, ampicillin), macrolides (erythromycin A) sulphonamides (sulfadiazine) and 2,4-Diaminopyrimidine (trimethoprim). Sample amount is usually scarce in biomonitoring studies for risk assessment in wildlife. The current method was developed with this purpose, as future studies using vulture samples could be performed. Hence, the main modifications of the existing QuEChERS method were made in this regard, reducing both sample and chemicals amount.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS), using triple quadrupole, is the most frequently used for residue analysis of antimicrobials in food. Other accurate mass full scan MS techniques as time-of-flight mass spectrometry (HPLC–TOFMS) have been recently applied (Freitas et al., 2014; Villar-Pulido et al., 2011). HPLC–TOFMS provides accurate masses for both parent and transition ions, and it also gives a full-scan product ion spectrum of the analytes. Thus, this technique is preferred for final confirmation of proposed analyte identities in complex matrices (Stolker et al., 2004). After the validation of the technique, the second objective was to confirm the lack of residues of antibiotics in the carrion disposed in SFS from Portugal.

2. Material and methods

2.1. Biological samples

Blank rabbit muscle (obtained from a local supermarket) was minced and homogenized to use subsamples to prepare spikes for the method validation. We used rabbit muscle to minimise background residues, as it was expected to be free from antibiotics and was confirmed with the blank analysis.

To assess the potential risk of exposure to antibiotics in a Cinereous vulture population from southeastern Portugal, samples from livestock carcasses were collected periodically from July 2013 to May 2014. Samples were obtained from 7 goats and 25 sheep (>9 months-old) and consisted of liver ($n = 30$: 7 from goats, 23 from sheep), muscle ($n = 29$; 7 from goats, 22 from sheep) and kidney ($n = 28$; 7 from goats, 21 from sheep). Although we intended to analyse liver, muscle and kidney of every individual, this was not always possible due to field conditions. About 20 g of each sample were obtained by necropsy at the moment of carcass disposal in the SFS. Samples were kept frozen until laboratory analysis (<12 months).

Animals were bred in different extensive farms ($n = 13$) in a special protected area of 2000 Nature Network: Moura-Mourão-Barrancos (Herdade da Contenda, southeastern Portugal, Fig. 1).

No specific regulation bans using veterinary antibiotics in protected areas (Olmeda et al., 2014). However, personnel of the Liga para a Protecção da Natureza of the project LIFE Habitat Lince Abutre (LIFE08 NAT/000227) used questionnaires to ask the farmers if the animals had been treated. Only non-treated or after withdrawal period animals were disposed in the SFS. However, there could be mistakes in the questionnaires and some treated animals would be disposed.

2.2. Chemicals and standards

Antibiotics reference standards of different chemical classes were purchased as follows: tetracycline, chlortetracycline, oxytetracycline, ciprofloxacin, enrofloxacin, nalidixic acid, penicillin G, ampicillin and trimethoprim were purchased by Dr. Ehrenstorfer® (Germany) and erythromycin A and sulfadiazine by Sigma-Aldrich® (USA). Methanol of residue quality (>99.9% purity) was obtained from Lab-Scan® (Poland). Magnesium sulfate, sodium chloride, sodium citrate dibasic sesquihydrate, sodium citrate tribasic dihydrate, 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).

Stock solutions of 1.0 mg mL^{-1} of the standard compounds were prepared by dissolving 10.0 mg of each reference standard compound in 10 mL of HPLC grade methanol. A standard mix containing all the antibiotics was made at 1000 ng mL^{-1} by mixing a portion of stock solution of each compound with an appropriate amount of methanol. This mix was used to spike the rabbit muscle samples for the method validation.

2.3. Instruments and conditions

Detection and quantification of the antibiotics were performed on a HPLC system consisting of vacuum degasser, autosampler and a binary pump (Agilent Series 1200, Agilent Technologies, Santa Clara, CA) equipped with a reversed phase rapid resolution Waters Sunfire C18 column of 150 mm × 4.6 and 5 μm particle size. The column was held at a constant temperature of 25 °C. The injection volume was 40 μL. The mobile phase (A) was water with formic acid 0.1% and (B) acetonitrile with formic acid 0.1% at a flow rate of 0.8 mL/min and a gradient where at t = 0 min, 10% B and at t = 19 min 100% B until the end (25 min). 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, 4000 V; nebulizer pressure, 60 psig; drying gas flow rate, 13.0 L min^{-1}; gas temperature, 350 °C; skimmer voltage, 65 V; octapole RF 250 V; fragmentor voltage, 160 V. HPLC–MS accurate mass spectra were recorded across the m/z range of 50–1000. 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 0.4 L min^{-1}) 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.02.00, Patch 3) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.02.00, Patch 3). Identification of the compounds was based on the

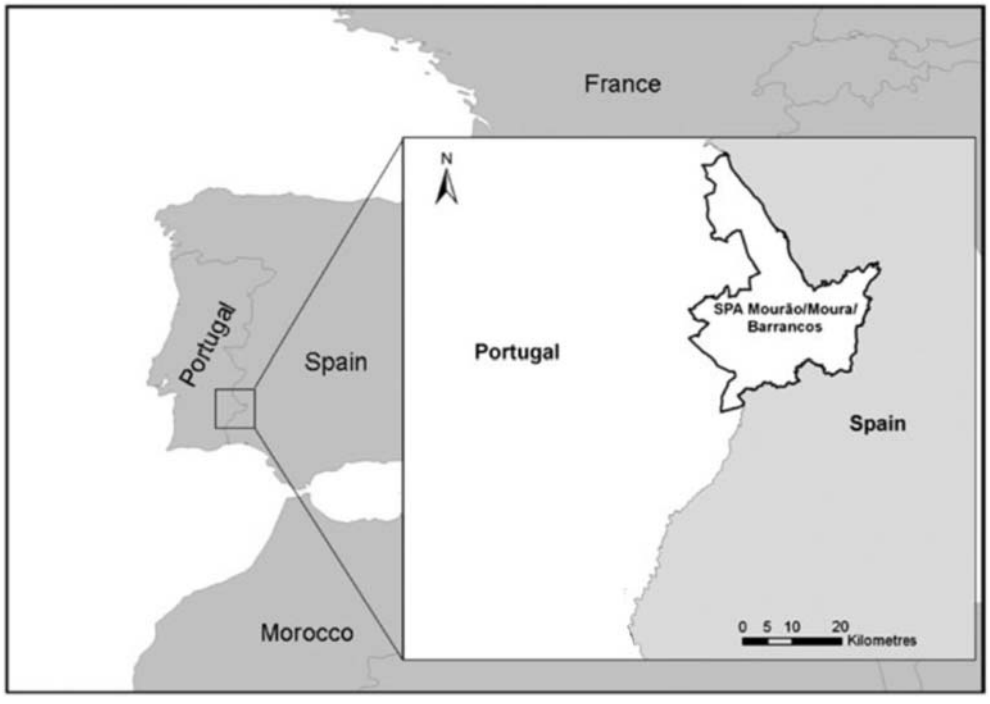

Fig. 1. Location of the carrion sampling area (Moura-Mourão-Barrancos (Herdade da Contenda, southeastern Portugal).

comparison of the retention times of the analyte and a standard compound (±0.5 min) and the difference of the theoretical exact mass and the measured accurate masses of the analyte (≤±5 ppm). A table with this information for each compound is provided as Supplementary Information (Table S1).

2.4. Sample preparation

We used a modification of the technique described by Anastassiades et al. (2003), a dispersive solid phase extraction (dSPE), commonly known as QuEChERS, based on the modification developed by Gómez-Ramírez et al. (2012). Tissues were minced a using a food hand blender (Taurus Robot 250, Taurus, Berlin, Germany). To avoid contamination, all the material used to prepare the samples, including the blender, was washed and rinsed with distilled water and acetone. About 2 g of sample were mixed with 2 mL of methanol. The mix was shaken vigorously with a vortex for about a minute and a combination of salts (1.3 g magnesium sulfate, 0.3 g sodium chloride, 0.2 g sodium citrate dibasic sesquihydrate and 0.3 g sodium citrate tribasic dehydrate) was then added, followed by 2 mL of methanol more. Tubes were again vigorously shaken with vortex for 5 min. This mix separates the liquid phase and stabilizes the analytes. The tubes were centrifuged at 5000*g* for 5 min with a centrifuge Sanyo® MSE MISTRAL 2000 R and frozen at −4 °C for 1 h. The total supernatant was transferred to another tube and mixed with 300 mg magnesium sulfate, 50 mg PSA and 50 mg DSC-18. The tube was shaken similarly to the first step and centrifuged again at 5000*g* for 5 min. The supernatant was evaporated until dryness under a gentle nitrogen stream and redisolved in 1 mL methanol for analysis.

2.5. Method validation

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).

Linearity of an analytical method is the ability to elicit test results that are 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 rabbit muscle samples spiked with the mix of antibiotics at five levels (10, 20, 50, 100 and 200 ng g^{-1}). Linear regression of data to a 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 is used to prove 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), which is calculated analysing five replicates of rabbit muscle samples spiked with the mix of antibiotics at five levels (10, 20, 50, 100 and 200 ng g^{-1}). This is calculated as follows: $RSD\ (\%) = (SD/Xm) \times 100$ where *SD* is the standard deviation of the whole series of measurements, and *Xm* is the mean.

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, 5 aliquots of the same sample of spiked rabbit muscle (200 ng g^{-1}) were analysed by different analysts and different days. The acceptance criterion for repeatability and reproducibility were RSD ≤ 20%.

Accuracy of the method was assessed by studying the recovery of antibiotics in rabbit muscle samples spiked with the mix of the compounds of interest. Rabbit muscle samples were analysed as blank in quintuplicate in order to ensure that they were free from the antibiotics analysed and to test matrix effect. Recoveries of antibiotics were tested using five replicates of spiked rabbit muscle at five levels (10, 20, 50, 100 and 200 ng g^{-1}). The extraction percent recoveries were determined by comparing peak heights obtained from extracted spiked samples with peak heights obtained in the working standard solutions. This was calculated as follows: $Recovery\ (\%) = (Cm/Cp) \times 100$, where *Cm* is the concentration of each compound in the sample and *Cp* is the concentration of each compound in the standard solution. The field samples were corrected for recoveries.

The limit of detection (LOD) was defined as 3× the signal to noise ratio for the analysed matrix.

2.6. Statistical analysis

Microsoft Excel 2015 was used to calculate the validation parameters of the technique as described in Section 2.5. Descriptive statistics for compounds detected in >3 samples were calculated using IBM© SPSS© Statistics version 21. For this, data below the LOD were assigned a value of f × LOD, where f is the proportion of samples above LOD (Voorspoels et al., 2002). As f was <50% for all the compounds, no statistical inference was calculated.

3. Results and discussion

3.1. Method validation

Method validation parameters are shown in Table 1. For all the compounds, a strong relationship between concentrations of antibiotics: area of chromatographic peak was found, as correlation coefficients were above 0.98 (Table 1). RSD of repeatability and reproducibility indicated good precision, because they were lower than the threshold set to accept the validation of the analytical method (20%). The only exception was oxytetracycline, although this was also close to the threshold (23.89%; Table 1). The over and under recovery seen in some compounds may be caused by matrix effects, which can enhance or suppress ionization. As shown in Table 1, recoveries were above 70%, except for nalidixic acid (41.48%). However, in multi-residue methods, recoveries below 60% are also acceptable if linearity and precision are good (SANTE/11945/2015, 2016), which is the case of nalidixic acid. In addition, some method validation guidelines such as the established by the U.S. Department of Agriculture (2017) expand this range to 50–150% for recoveries and 20% of RSD. Therefore, all the analytes, except tetracycline and oxytetracycline would meet the criteria (196.74 and 177.98%, respectively). Nevertheless, future modifications of the method may be performed to improve the recoveries. In addition, low LOD were achieved for most compounds (0.16–20 ng g^{-1}; Table 1). Hence, this method permits the quantification of antibiotics in meat at levels below the Maximum Residue Levels (MRL) in food set by the European Union legislation (Commission Regulation (EU) No 37/2010, see Table S2) and it can be used to analyse livestock samples for human consumption.

Since 2003, when QuEChERS method was first developed by Anastassiades et al., several studies have applied and modified it to analyse a great number of compounds of varied properties and structure (Gómez-Pérez et al., 2013; Gómez-Ramírez et al., 2012; Orlando and Simionato, 2013; Stubbings and Bigwood, 2009). In the case of antibiotics in animal products, the techniques are limited to few compounds of the same group, usually in more simple matrices such as milk (Arroyo-Manzanares et al., 2014; Fernandes et al., 2015; Freitas et al., 2014; Lombardo-Agüí et al., 2011; Orlando and Simionato, 2013; Pérez-Burgos et al., 2012). As already mentioned in the introduction of this manuscript, most of these studies use LC–MS/MS. When more accurate instruments such as UHPLC-MS-MS or HPLC-TOFMS are used, the methods are more efficient (Abdallah et al., 2014; Freitas et al., 2014). Nevertheless, our LOD (Table 1) are generally much lower than in the mentioned techniques (0.5–3 ng g^{-1} wet weight; Abdallah et al., 2014; Arroyo-Manzanares et al., 2014; Fernandes et al., 2015; Freitas

Table 1
Accuracy, linearity and precision of the antibiotics from spiked rabbit meat samples.

	Recovery[b] (%)						Repeatability[b] (RSD %)	Reproducibility[c] (RSD %)	Linearity[b] (r)	LOD[a] (ng g^{-1})
	M[a]	10	20	50	100	200				
Sulfadiazine	82.10	46.48	79.78	89.15	94.41	100.65	11.28	6.86	0.978	1.01
Nalidixic acid	41.48	44.19	46.57	48.21	36.60	31.82	15.50	13.53	0.979	0.16
Trimethoprim	134.89	69.99	105.85	164.53	147.09	187.00	14.00	11.40	0.995	0.30
Penicillin G	71.38	100.44	82.42	45.45	57.39	71.21	18.97	24.21	0.990	3.16
Ampicillin	95.90	120.03	118.72	63.74	148.64	28.34	18.40	22.17	0.989	5.17
Ciprofloxacin	105.89	68.19	133.99	115.31	112.72	99.22	16.93	11.36	0.998	0.26
Enrofloxacin	154.39	123.28	185.05	188.52	152.18	122.91	12.98	5.55	0.998	0.27
Chlortetracycline	80.58	76.50	63.88	100.00	71.52	90.97	10.77	3.73	0.999	3.00
Tetracycline	196.74	157.48	229.32	221.65	148.75	226.51	18.45	13.14	0.990	2.52
Oxytetracycline	177.98	214.23	168.79	185.59	158.34	162.94	23.89	23.95	0.990	3.66
Erythromycin A	144.02	<LOD	101.30	151.21	178.81	144.78	16.88	7.88	0.986	20.00

LOD = limit of detection.
[a] Average recoveries of the 5 spiking levels.
[b] Average of 5 replicates at 5 concentrations (10, 20, 50, 100 and 200 ng g^{-1}).
[c] Average of 5 replicates at 200 ng g^{-1}; r = regression coefficient.

et al., 2014; Lombardo-Agüí et al., 2011; Orlando and Simionato, 2013; Pérez-Burgos et al., 2012). Also compared to most QuEChERS methods that analyse antibiotics in food samples, including meat (Abdallah et al., 2014; Blasco et al., 2011; Lopes et al., 2012), the method developed here implies a reduction of the costs: about half of the solvent and at least a third of the salts are needed to detect antibiotics in only 2 g of sample. Moreover, the use of a smaller amount of methanol for the extraction also reduces time and nitrogen needed to evaporate the extract. Biomonitoring programmes often use samples from small animals (i.e. *Milvus milvus*, Gómez-Ramírez et al., 2014). Different contaminant types are usually studied simultaneously in the same individual's sample (Gómez-Ramírez et al., 2014). Thus, multi-residue techniques are preferred. In addition, reduction of costs in analytical processes is an aim for optimization, especially when the funding resources are limited (OECD, 2017). For these reasons, we think that this method can be useful for the assessment of antibiotics exposure in scavenger animals.

Due to the relevance for human health of detecting veterinary medicines in animal tissues and products, many other multi-residue methods different to QuEChERS have been developed. Compared to our method, some of the most recent methods showed similar recoveries but lower sensitivity (>0.5 ng g^{-1} wet weight; Jank et al., 2017; Moreno-González et al., 2017; Rizzetti et al., 2017).

3.2. Application to real samples

As mentioned above, we aimed to confirm the lack of antibiotics in the carcasses of grazing ungulates disposed in SFS. Concentrations in ng g^{-1} wet weight (ww) are shown in Tables 2 and 3. As shown in Table 2, antibiotics were more frequently detected in goats (42.9%) than in sheep (24.2%), and oxytetracycline and trimethoprim were the most common antibiotics in all the samples analysed (13.8%). In addition, the highest concentrations were also found for oxytetracycline (1452.68 ng g^{-1}).

Traces of antibiotics were found in 29% of the samples ($n = 25$). While nalidixic acid, ampicillin and chlortetracycline were not detected, penicillin G was detected in muscle of a sheep (34.87 ng g^{-1} ww) and a goat (174.24 ng g^{-1} ww). Sulfadiazine was also poorly detected, in three muscle samples, being higher in a goat (41.23 ng g^{-1} ww) than in two sheep (11.05 and 7.85 ng g^{-1} ww). Regarding quinolones, ciprofloxacin and enrofloxacin were found in muscle (4.89 and 65.5 ng g^{-1} ww, respectively) and liver (3 and 49.2 ng g^{-1} ww, respectively) of the same goat. This was the only animal with traces of these compounds.

These results seem to be related to the frequency of use of antibiotics in veterinary medicine. According to questionnaires and published

Table 2
Concentrations expressed as median; mean ± SD (range) in ng g^{-1} wet weight (frequency of detection) of antibiotics in each species. <LOD = below limit of detection.

	Total ($n = 87$)	Goat ($n = 21$)	Sheep ($n = 66$)
Sulfadiazine	ND; 0.73 ± 4.62 (ND-41.23) (3.4%)	41.23[a]	11.05; 7.85[b]
Nalidixic acid	<LOD	<LOD	<LOD
Trimethoprim	ND; 0.86 ± 1.99 (ND-7.11) (13.8%)	ND; 0.91 ± 2.11 (ND-6.47) (14.3%)	ND; 0.84 ± 1.97 (ND-7.11) (13.6%)
Penicillin G	174.24; 34.87[b]	174.24[a]	34.87[a]
Ampicillin	<LOD	<LOD	<LOD
Ciprofloxacin	3; 4.89[b]	3; 4.89[b]	<LOD
Enrofloxacin	4.89; 65.5[b]	4.89; 65.5[b]	<LOD
Chlortetracycline	<LOD	<LOD	<LOD
Tetracycline	ND; 0.43 ± 1.17 (ND-7.02) (4.6%)	ND; 0.9 ± 1.82 (ND-5.73) (14.3%)	7.02[a]
Oxytetracycline	ND; 34.23 ± 169.40 (ND-1452.68) (13.8%)	ND; 51.32 ± 132.22 (ND-491.32) (19%)	ND; 28.78 ± 180.18 (ND-1452.68) (12.1%)
Erythromycin A	<LOD	<LOD	<LOD
∑Antibiotics[c]	10.81; 127.94 ± 301.83 (4.45–1452.68) (28.7%)	62.91; 140.51 ± 177.55 (4.45–497.05) (42.9%)	8.03; 120.86 ± 358.89 (5.15–1452.68) (24.2%)

[a] Only 1 sample > LOD.
[b] Only 2 samples > LOD.
[c] Calculated only for samples > LOD.

Table 3
Concentrations expressed as median; mean ± SD (range) in ng g^{-1} wet weight (frequency of detection) of antibiotics in each tissue. <LOD = below limit of detection.

	Liver ($n = 30$)	Muscle ($n = 29$)	Kidney ($n = 28$)
Sulfadiazine	<LOD	ND; 0.73 ± 4.62 (ND-41.23) (10.3%)	<LOD
Nalidixic acid	<LOD	<LOD	<LOD
Trimethoprim	ND; 0.82 ± 1.97 (ND-6.47) (13.3%)	ND; 0.61 ± 1.63 (ND-5.51) (13.8%)	ND; 1.14 ± 2.36 (ND-7.11) (17.9%)
Penicillin G	<LOD	174.24; 34.87[b]	<LOD
Ampicillin	<LOD	<LOD	<LOD
Ciprofloxacin	3[a]	4.89[a]	<LOD
Enrofloxacin	49.2[a]	65.5[a]	<LOD
Chlortetracycline	<LOD	<LOD	<LOD
Tetracycline	7.02[a]	5.48[a]	4.45; 5.73[b]
Oxytetracycline	120.42; 330.55[b]	ND; 17.78 ± 55.09 (ND-232.68) (13.8%)	ND; 71.13 ± 286.12 (ND-1452.68) (21.4%)
Erythromycin A	<LOD	<LOD	<LOD
∑Antibiotics[c]	34.97; 89.56 ± 128.96 (5.15–335.71) (20%)	46.14; 80.50 ± 88.33 (5.48–232.68) (27.6%)	7.11; 183.37 ± 445.87 (4.45–1452.68) (39.3%)

[a] Only 1 sample > LOD.
[b] Only 2 samples > LOD.
[c] Calculated only for samples > LOD.

literature (Almeida et al., 2014), oxytetracycline is the most commonly used in livestock from the study area and in Portugal in all the livestock species, including goat and sheep.

To our knowledge, this is the first study of presence of antibiotics in carrion disposed in SFS. The analysis of different tissues, as well as pharmacokinetics and tissue distribution, should be considered for risk assessment as interspecific differences in food preferences have been noticed for different vulture species. For example, while Griffon vultures (*Gyps fulvus*) typically feed on viscera, Cinereous vultures tend to choose firmer tissues such as muscle and cartilage (del Hoyo et al., 1994; Moreno-Opo et al., 2015a). In fact, antibiotics were more frequently found in muscle and kidney than in liver (see Table 2 and Fig. 2). However, due to the low frequency of detection, these differences could not be tested statistically. On the other hand, as mentioned above, the information regarding dose-related toxic effects in birds of prey is scarce. Hence, the assessment of risk of acute intoxication of vultures fed with carcasses in SFS was based on the data found for other bird species. Considering that an adult Cinereous vulture ingests on

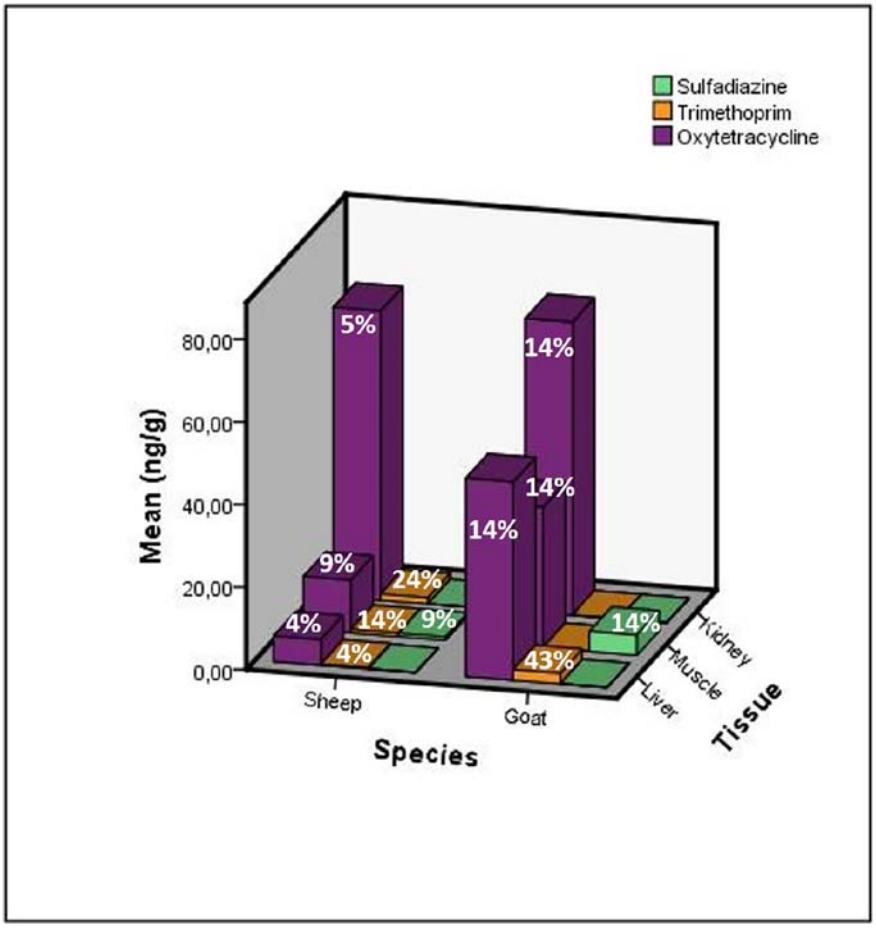

Fig. 2. Mean concentrations (ng g^{-1} wet weight) and frequency of detection of the most frequent antibiotics (sulfadiazine, trimethoprim and oxytetracycline) in each species and tissue.

average 575 g/day of carrion (Corbacho et al., 2007) and its average body weight is 10 kg, we calculated the doses of each antibiotic that could be ingested. This was calculated for every organ of every goat or sheep analysed: Exposed dose = highest concentration of each antibiotic in each organ x (575 g carrion/day)/body weight. According to this, the calculated doses were lower (in most cases >1000 times lower) than the recommended therapeutic doses for birds of different species (Marx, 2006). However, experimental studies or data from case reports would be needed to demonstrate the relation dose-toxic effect for this species. Nevertheless, the chronic, pulsed and chaotic exposure to antibiotics can caused other adverse effects: Antibiotic-resistant bacteria have been found in several wildlife species, including birds of prey and vultures (Porrero et al., 2013; Radhouani et al., 2014) and the use of antibiotics may be related to an increase of other pathogens in scavenger birds (Blanco et al., 2017a, 2017b; Höfle et al., 2007; Pitarch et al., 2017). Moreover, other factors may be also important to take into account, including the consumption of food other than livestock, which can differ between vulture species, especially regarding wild animals as ungulates and rabbits (Donázar et al., 2010; Margalida et al., 2009, 2012).

Also aforementioned, the Commission Regulation (EU) No 37/2010 established MRL for food from animal origin in the European Union, including the antibiotics analysed in this study. According to this, oxytetracycline levels were above MRL (100 ng g^{-1}) in the organs of 3 individuals (2 sheep and one goat). These goat and sheep were provided by the same farmer, although in different days. Nevertheless, it should be taken into account that MRL were set for the human, following the "precautionary principle". Hence, the comparison in our case would only indicate that withdrawal periods of the drugs were not always fulfilled before providing the carcasses in the SFS.

The lack of residues in most samples could be explained by two facts: the animals had not been treated with these drugs or, if they had, the time between the treatment and the death of the animals was longer that the withdrawal period (usually 20 days). In this case, antibiotics would have been eliminated from the organism. Hence, the risk for vultures would be negligible for the antibiotics analysed using the current method, except for the acquisition of bacterial resistance developed by medicated livestock.

On the other hand, as mentioned above, other pharmaceuticals such as NSAIDs may have been administered to the goats and sheep before they died. It is well-known that vultures are especially sensitive to most of these drugs (i.e. diclofenac, ketoprofen, flunixin; Naidoo and Swan, 2009; Naidoo et al., 2010; Zorrilla et al., 2015), being responsible for the population decline of several species in India (Taggart et al., 2009). The fact that residues of antibiotics have been found in carrion disposed for vulture feeding may be of concern, as this may imply that some farmers may have lied in their responses to questionnaires regarding the pharmacological treatment. This treatment may include other substances. This is especially important in the sense that NSAIDs as diclofenac, whose lethal toxicity to vultures is well-known, have been recently authorised for livestock in at least five EU countries (Spain, Italy, Estonia, Czech Republic and Latvia) and currently there is a request for a permit in Portugal (Margalida and Oliva-Vidal, 2017). There is currently no regulation on the disposal of medicated animals in SFS, not even at special protected areas of 2000 Nature Network (Olmeda et al., 2014). In the case of diclofenac, the veterinarian becomes the responsible for not treating animals that may be further ingested by scavenger birds (AEMPS, 2015). The fact that diclofenac is authorised for many uses and a large number of pigs and cattle are bred in the Iberian Peninsula, implies a high risk of exposure (Margalida et al., 2014). A mathematical model suggests that between 715 and 6389 Griffon vultures can die annually in Spain due to the ingestion of cattle treated with diclofenac (Green et al., 2016). As mentioned above, most of the vultures populations are found in the Iberian Peninsula. Hence, the impact of this product could seriously be a threat for these remaining large populations of European vultures (Margalida and Oliva-Vidal 2017). Therefore, to perform a proper risk evaluation of pharmaceuticals, and to reduce the risk, residues of NSAIDs should also be evaluated in the carrion disposed for the Cinereous vultures.

4. Conclusions

The purpose of the validation of a modified QuEChERS method has been fulfilled, since it allows the simultaneous quantification of low levels of 11 antibiotics of different chemical classes in a single analysis, using a small amount of tissue. Moreover, the validation of our method has reduced sample amount as well as solvents, reagents and the time required for the analyses. This implies a great advantage to reduce laboratory expenses and to optimize laboratory work. Overall, we think that this method can be useful to assess the risk for exposure to antibiotics in scavenger species.

To our knowledge, this is the first study of presence of antibiotics in carrion disposed for feeding endangered scavenger birds. Although, according to questionnaires, the carcasses provided in SFS were free from antibiotics, 29% of the samples presented residues. The information related to toxic doses in this species is missing, but based on therapeutic doses for other bird species, the concentrations of antibiotics in carrion do not seem to pose a risk of acute intoxication for adult Cinereous vultures. The impact on the health of Cinereous vultures from southeastern Portugal is still to be assessed, but based on the studies carried out in Spain, opportunistic mycosis may be occurring in these vultures due to chronic and pulsed exposure. Hence, the analysis of a higher number of carcasses, together with analysis of blood samples from vultures and study of oral cavity may be necessary to evaluate the risk for exposure to antibiotics.

Nevertheless, and based on the recent published literature, we consider that the disposal of medicated animals in SFS should be regulated to minimise the risk for scavenger birds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.01.060.

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