Identification and quantification of 12 pharmaceuticals in water collected from milking parlors: Food safety implications

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ABSTRACT

The introduction of drug residues into the food chain and their presence in drinking water has been recently investigated. The aim of this work was to monitor the presence of 19 active drugs in water samples collected from milking parlors of dairy farms located in Galicia (northwest Spain), one of the main Spanish milking areas. Overall, 65% of the samples tested positive for at least one of the compounds analyzed. A total of 12 drugs were measured, with concentrations ranging between 17 and 3,941 ng/L. Considering that a mixture of compounds may contribute to the overall effect of each compound and might increase or reduce its toxicity, it should be noted that 29% of the samples tested contained more than one pharmaceutical. To date, the effects of the continuous consumption of these mixtures of drugs in water or milk are unknown; however, antimicrobials may affect the human gut microbiota or have toxic effects in sensitive individuals.

Key words: residue, drinking water, dairy farms, antimicrobial

INTRODUCTION

Pharmaceuticals are important and essential elements in life because they are used in agriculture, human, and veterinary medicine. According to the European Federation of Animal Health (FEDESA, 1998), the annual consumption of antibiotics in the European Union (EU) is about 13,000 t, with about 65% used in human medicine, 29% in veterinary medicine, and 6% used as food additives. The use of these substances in animal production is a common practice, not only for therapeutic purposes but also for prophylactic treatments (Iglesias et al., 2014). Once consumed, pharmaceuticals

are metabolized and part of the initial dose is excreted unchanged, with the higher percent excreted in the form of metabolites; both the parent compound and metabolites are excreted via feces and urine in both human and animals (Jjemba, 2006). These substances can therefore reach the aquatic environment through wastewater treatment plants because the plants are not able to remove them completely (Kosma et al., 2014). Another way of introducing these active compounds is through the use of contaminated manure in agricultural soils as fertilizer, contaminating rivers, lakes, and drinking water sources (Watkinson et al., 2009; Rodil et al., 2012). Additionally, during the manufacture and production of drugs, the environment can be contaminated with pharmaceuticals due to breaks, leaks, and uncontrolled washes (Rodil et al., 2012). Depending on the physicochemical properties of drugs and the characteristics of the water and soil, these active substances reach the environment, particularly the water system, through water runoff and leaking, contaminating aquifers, rivers, lakes, seas (Ayscough et al., 2000) and even human drinking water (Watkinson et al., 2009; Iglesias et al., 2014).

Farms across the European Union produced approximately 147,904 million tonnes of cow milk in 2014 (Eurostat, 2014); in particular, Spain produced 6,679 million tonnes. Galicia, in northwest Spain, is an area with high milk production at 2.65 million tonnes (36.6% of the total produced in Spain; MAGRAMA, 2014) and has 14,415 livestock and 450,271 head of cattle (IGE, 2014). Water is very important on dairy farms, not only because it is used to feed animals but also for cleaning the facilities. Inadequate quality of the water used on dairy farms can lead to food safety concerns, because water contaminated with pharmaceuticals can come in contact with milk during equipment cleaning (Llena, 2011). In general, milk farms use water from public distribution, but the use of water from water wells is also common practice in Spain, particularly in Galicia. European legislation states that “water for cleaning food facilities shall be potable water” (Regulation

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853/2004; EU, 2004), because this water is in contact with food. In Spain, health criteria for water quality for human consumption are regulated by the Spanish standard Real Decreto 1120/2012 (BOE, 2012) and EU Directive 2015/1787 (EU, 2015).

If even small amounts of water carrying pharmaceuticals remain in the milk tank after cleaning, the new fresh milk would also be contaminated with these pharmaceuticals. The amount of water that remains in the tank is variable, depending on the size of the tank and the quality of the water system tubing; leaks due to broken valves can result in more water remaining compared with the residual left after cleaning. Milk could also contain residues of pharmaceuticals as a result of overdosing cattle treatment, incorrect separation of milk from medicated and unmediated animals, dry cow therapy anomalies, and, to a lesser extent, inadequate withdrawal period (MAGRAMA, 2005). Thus, methods for analysis of pharmaceuticals in milk exist to control and prevent food safety problems as much as possible. Maximum residue limits of pharmaceuticals allowed in foods of animal origin within the EU are provided in Regulation 37/2010 (EU, 2004). However, pharmaceuticals are not usually controlled in the water used for cleaning facilities, although legislation states that cleaning water used on farms should be of the same quality as human drinking water.

Even if the concentration of pharmaceuticals in the final milk is low and below the level set for antimicrobials in Regulation 37/2010 (EU, 2004), the unknown consumption of pharmaceuticals in this way may lead to interactions with knowingly consumed drugs or by increasing or reducing the effects of the prescribed drugs (Pomati et al., 2008). In the particular case of antimicrobials, continuous consumption of low levels of these active compounds can lead to the development of bacteria with resistance genes in consumers, which may result in synergistic or antagonistic effects and the need to increase or decrease the dose of the treatment (De Liguoro et al., 2009). Additionally, these substances can have harmful effects on the environment; for example, the synergistic activity of sulfamethoxazole and trimethoprim results in greater inhibition of the growth

of the alga *Selenastrum capricornutum* compared with the respective individual activities (Santos et al., 2010). Therefore, low levels of active compounds could have negative effects on sensitive consumers, such as infants, the elderly, and allergic individuals who are frequent consumers of milk.

In accordance with the above data, research into the presence of pharmaceuticals in water used to clean milking facilities is needed. The aim of this study was to collect water from dairy farms located in the province of Lugo, one of the main milking areas in Spain, and to investigate the presence of 19 pharmaceuticals by using solid-phase extraction and HPLC-tandem mass spectrometry (MS/MS).

MATERIALS AND METHODS

Study Area

The weather in the area is classified as continental oceanic with an average temperature of 13.6°C. The relief is very heterogeneous, constantly altering elevations and depressions, where the surface is broken by rivers or streams. The average rainfall is 678 mm/yr (Meteogalicia, 2015). Table 1 shows average climatological data (temperature, relative humidity, rain, light hours, and hydric balance) of the province of Lugo over a 17-wk period from February to June 2015. The region investigated was divided into areas (Figure 1): South (1 sample), Coast (6 samples), Mountain (7 samples), Flatland (15 samples), and Center (23 samples).

Chemicals, Reagents, and Stock Solutions

Methanol and acetonitrile (HPLC grade, $\geq 99.9\%$) were purchased from Scharlau Chemie (Barcelona, Spain), formic acid (purity, $>99\%$ for analysis) from Acros Organics (Geel, Belgium), and hydrochloric acid solution (0.1 N HCl) from Merck (Darmstadt, Germany). Nitrogen gas (purity, $>99.98\%$) was generated by an in-house nitrogen generator from Peak Scientific Instruments Ltd. (Chicago, IL), and purified water was prepared in-house with a Milli-Q water system

Table 1. Climatological data for Lugo (Spain) during the months of water sample collection

Sampling month	Average temperature (°C)	Average relative humidity (%)	Rain (L/m ²)	Sunlight hours (h)	Hydric balance (L/m)
February	5.55	83.74	4.78	10.12	3.67
March	7.77	68.34	1.29	10.40	-0.70
April	11.84	75.82	3.55	12.98	-0.81
May	14.33	72.80	1.17	14.01	-3.32
June	17.36	73.53	0.56	14.71	-4.65
Average	11.37	74.84	1.61	12.44	-1.16

from Millipore (Bedford, MA). The internal standards sulfadoxine- d_3 and robenidine- d_8 and the pharmaceuticals betamethasone, cortisone, decoquinat, enrofloxacin, flumethasone, griseofulvin, maduramycin, 6 α -methylprednisolone, monensin, narasin, salinomycin, sulfachloropyridazine, sulfadiazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, trimethoprim were purchased from Sigma-Aldrich (St. Louis, MO), all with purity >98%. The drugs were accurately weighed to prepare stock solutions of individual compounds at a concentration of 0.8 mg/mL in methanol. Stock solutions of the individual compounds were then mixed with 0.1% formic acid in methanol to obtain stock solutions of 10 μ g/mL; these solutions were further diluted with 0.1% formic acid in methanol to obtain standard mixtures of drugs at 75, 150, and 300 ng/mL. All standard solutions were stored in the dark at -18°C for a maximum of 6 mo.

The stock solutions of individual drugs at 0.8 mg/mL were mixed to obtain a working stock solution of 1

μ g/mL with 0.1% formic acid in methanol. The therapeutic and chemical properties of the selected drugs are presented in Table 2.

Equipment

A MS2 Minishaker vortex mixer from IKA (Staufen, Germany), a vacuum station manifold and Strata-X solid-phase extraction (SPE) cartridges (60 mg, 3 mL) from Phenomenex (Macclesfield, UK), and a Turbo Vap II evaporator from Zymark (Hopkinton, MA) were used for sample preparation and extraction.

Samples were analyzed on a liquid chromatography-MS/MS system consisting of an HPLC 1100 separation module from Agilent Technologies (Waldbronn, Germany), equipped with a vacuum degasser and an autosampler, and an API 4000 mass spectrometer from Applied Biosystems/MDS Sciex (Toronto, Canada) equipped with a TurbolonSpray source. The Software Analyst 1.6 from Applied Biosystems was used to control the system. The chromatographic analyses were performed

Figure 1. Map of the study area. Color version available online.

Table 2. Therapeutic class and limit of quantification (LOQ) of selected veterinary drugs

Analyte	Therapeutic class	LOQ (ng/L)	Formula
Betamethasone	Glucocorticosteroid	12.5	C ₂₂ H ₂₉ FO ₅
Cortisone	Corticosteroid	12.5	C ₂₁ H ₂₈ O ₅
Decoquinate	Coccidiostats	12.5	C ₂₄ H ₃₅ NO ₅
Enrofloxacin	Antimicrobial	25.0	C ₁₉ H ₂₂ FN ₃ O ₃
Flumethasone	Corticosteroid	25.0	C ₂₂ H ₂₈ F ₂ O ₅
Griseofulvin	Antifungal	25.0	C ₁₇ H ₁₇ ClO ₆
Maduramycin	Coccidiostats	12.5	C ₄₇ H ₈₃ NO ₁₇
6 α -Methylprednisolone	Corticosteroid	25.0	C ₂₂ H ₃₀ O ₅
Monensin	Coccidiostats	12.5	C ₃₆ H ₆₂ O ₁₁
Narasin	Coccidiostats	12.5	C ₄₃ H ₇₂ O ₁₁
Salinomycin	Coccidiostats	12.5	C ₄₂ H ₇₀ O ₁₁
Sulfachloropyridazine	Antimicrobial	12.5	C ₁₀ H ₉ ClN ₄ O ₂ S
Sulfadiazine	Antimicrobial	25.0	C ₁₀ H ₁₀ N ₄ O ₂ S
Sulfamethazine	Antimicrobial	25.0	C ₁₂ H ₁₄ N ₄ O ₂ S
Sulfamethizole	Antimicrobial	25.0	C ₉ H ₁₀ N ₄ O ₂ S ₂
Sulfamethoxypyridazine	Antimicrobial	12.5	C ₁₁ H ₁₂ N ₄ O ₃ S
Sulfapyridine	Antimicrobial	25.0	C ₁₁ H ₁₁ N ₃ O ₂ S
Sulfaquinoxaline	Antimicrobial	25.0	C ₉ H ₉ N ₃ O ₂ S ₂
Trimethoprim	Antimicrobial	12.5	C ₁₄ H ₁₈ N ₄ O ₃

by injecting 10 μ L of extract into a Synergi 2.5 μ m Polar-RP 100A column (50 $\times$ 2.0 mm) connected to a Polar-RP security-guard cartridge (4.0 $\times$ 2.0 mm), both obtained from Phenomenex.

Water Samples

Water samples collected for this research were clean-ing water collected in the milking parlors of 52 milking farms from the province of Lugo (Figure 1). The samples were from private water wells (52% of the samples), from distribution systems (17%), from both sources as the farm has access to both water systems (4%), and unknown origin (27%). Water samples were collected in 1-L polyethylene plastic vessels for pharmaceutical detection and in 150-mL polyethylene plastic containers for microbial analyses. Within 4 h after collection, water samples were transported cooled to the laboratory and stored at 4°C until sample preparation for analysis. Because European legislation establishes that the quality of the water used in milking facilities should be potable (i.e., of the same quality as drinking water), hereafter, we refer to samples as water or drinking water.

Sample Preparation

To remove suspended solids, water samples were filtered (800 mL) under vacuum using a 0.47- μ m glass microfiber filter from Filter-Lab (La Rioja, Spain). The samples were acidified with 0.1 N HCl to a pH of 3. After treatment, the samples were stored at -18°C until analysis.

Extraction Procedure

For quantification, 4 control samples were acidified, fortified, and extracted following the same analytical procedure as the experimental samples. These control samples include a blank (tap water) and 3 fortified samples (tap water enriched with drugs) at 75, 150, and 300 ng/L, respectively. Before extraction, experimental and control samples were spiked with 10 μ L of the internal standard (sulfadoxine-d₃ and robenidine-d₈) solution (1 μ g/mL) and then shaken and transferred into the conditioned cartridges at a flow rate of 1 mL/min.

The extraction procedure was conducted based on Iglesias et al. (2012). Briefly, cartridges were conditioned with 4 mL of methanol and 4 mL of Milli-Q water. After loading the whole volume of sample (0.8 L), the cartridges were dried under vacuum for 30 min. Sample vessels were rinsed with 4 mL of methanol, which was transferred to the SPE cartridges and left to soak for 5 min; the eluent was collected in a 10-mL conical graduated Pyrex glass tube (16.5 $\times$ 110 mm). Elution was completed with the addition of 4 mL of methanol, and the final mixture of eluents was evaporated to dryness under a stream of air at 45°C. The dried extracts were reconstituted with 200 μ L of 0.1% formic acid in methanol, vortexed, and transferred into a 300- μ L glass insert, fused into an amber 2-mL screw-top standard vial (12 $\times$ 32 mm) and stored at -18°C until analysis by HPLC-MS/MS.

HPLC-MS/MS Method

The HPLC method was conducted following Iglesias et al. (2012). Analytes were separated using a gradient

Table 3. Retention time (t_R) and precursor and product ions used to identify the selected drugs

Analyte	t_R (min)	Precursor (m/z)	Product 1 (m/z)	Product 2 (m/z)
Betamethasone	15.3	393	355	373
Cortisone	14.8	361	163	121
Decoquinatone	20.1	418	372	204
Enrofloxacin	13.1	360	342	316
Flumethasone	15.7	411	391	253
Griseofulvin	17.2	353	285	165
Maduramycin	22.3	935	629	647
6 α -Methylprednisolone	15.2	375	339	161
Monensin	21.7	693	461	479
Narasin	22.5	787	431	531
Salinomycin	21.8	773	431	531
Sulfachloropyridazine	13.9	285	156	92
Sulfadiazine	10.9	251	156	92
Sulfamethazine	12.4	279	186	92
Sulfamethizole	13.0	271	156	92
Sulfamethoxypyridazine	12.9	281	156	92
Sulfapyridine	11.3	250	156	92
Sulfaquinoxaline	15.3	301	156	92
Trimethoprim	11.9	291	230	123

mixture of 2 components and a flow rate of 0.15 mL/min; component A was 0.1% formic acid in acetonitrile and component B was 0.1% formic acid in water. Mass-spectrometry measurements were performed using positive electrospray (ESI⁺), and pharmaceuticals were identified using 2 multiple reaction monitoring transitions and their retention times. The retention times and precursor and product ions used are summarized in Table 3.

Microbiological Determinations

The microbiological quality of water samples was investigated for the presence of total aerobic mesophilic and total coliform microorganisms. To determine total aerobic mesophilic counts, 1 mL of water was poured onto 15 mL of plate count agar, which was prepared following the manufacturer's instructions (Biolife, Milan, Italy) and subsequently incubated at 30°C for 72 h. All colonies were counted to obtain the total aerobic mesophilic count. Coliform determinations were made by aseptically filtering 100 mL of water through a 45- μ m filter (Millipore). Then, filters were placed on a Petri plate and overlaid with 15 mL of violet red bile lactose agar, prepared following the manufacturer's instructions (Merck), and subsequently incubated at 24°C for 30 h. After incubation, pink to red colonies were considered to be coliforms and taken as a positive result.

Statistical Analysis

The results were analyzed using the PAWS Statistics 18 SPSS Software Inc. (Chicago, IL) to identify statisti-

cally significant trends in the drug concentration. The effects of weather conditions (Table 1), sampling date, and localization were tested using one-way ANOVA with significant differences at a 95.0% confidence level (P -values < 0.05).

RESULTS AND DISCUSSION

Presence of Pharmaceuticals in Water Samples

Out of the 19 pharmaceuticals analyzed, 12 were detected in water used for cleaning milking parlors, which should be of potable water quality. Betamethasone, decoquinatone, enrofloxacin, monensin, salinomycin, sulfachloropyridazine, sulfadiazine, sulfamethazine, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, and trimethoprim were detected in water samples. These pharmaceuticals belong to 3 of most widely used therapeutic groups: antimicrobials, coccidiostats, and corticosteroids. Contamination of the samples investigated may be caused by contamination of the water source, such as rivers, lakes, and water wells via the discharge of incompletely clean final effluent from wastewater treatment plants (Chang et al., 2007; Kosma et al., 2014) or due to the application of manure over culture lands as fertilizer (Díaz-Cruz and Barceló, 2006; Baquero et al., 2008), which is common practice in the area. Additionally, the adsorption of the pharmaceuticals present in the environment to soil particles delays biodegradation and explains the long-term survival of drugs in the environment (Baquero et al., 2008); specifically, sulfadiazine and trimethoprim can persist for months or years depending on the sediment and they are highly persistent (Kasprzyk-Hordern et al., 2008).

The pharmaceuticals cortisone, flumethasone, griseofulvin, maduramycin, 6 α -methylprednisolone, narasin, and sulfamethizole were not detected. There might be several reasons for this, including chemical properties of the compounds, their minimal use in veterinary or human medicine, or their presence at concentrations below the limit of detection of the method used.

Antimicrobials were the most frequently detected group: sulfonamides and quinolones were measured in 57% of the samples (Figure 2). The high rate of detection of antimicrobials is coincident with previous studies of surface water (Kasprzyk-Hordern et al., 2008; Patrolecco et al., 2013). The frequency of detection is probably related to their high use in both human and veterinary medicine (FEDESA, 1999) and consequently their high rates of excretion and low rates of removal in wastewater treatment plants (Petrović et al., 2003).

Overall, the drugs most frequently detected were sulfadiazine and sulfachloropyridazine, which were present in 27 and 22% of the samples, respectively (Table

4). The concentrations of all drugs detected ranged between 17 and 3,941 ng/L, with the highest concentration being measured for sulfadiazine. The next highest compounds after sulfadiazine were sulfachloropyridazine (3,826 ng/L) and sulfamethoxypyridazine (905 ng/L). The relation between maximum concentration and frequency was observed for antimicrobials, in particular for the group of sulfonamides, where 47% of the samples analyzed were positive for at least one sulfonamide (Table 4 and Figure 2). This is not the first time that the presence of residue of active substances such as sulfonamides has been reported in drinking water systems (Valcárcel et al., 2011; Sun et al., 2015). After sulfonamides, trimethoprim was the most frequently detected drug; it was present in 17% of the samples, whereas trimethoprim was found in only 2% of samples of finished drinking water from Canada (Kleywegt et al., 2011). The simultaneous presence of trimethoprim and sulfonamides was confirmed in 5 samples, probably because these drugs are often administered together

Figure 2. Detection of the veterinary drugs assessed. Color version available online.

Table 4. Maximum, minimum, average concentrations, and number of detections of the veterinary drugs detected

Analyte	Concentration (ng/L)			No. of detections
	Maximum	Minimum	Average	
Betamethasone	34	34	34	2
Decoquinat	732	17	111	8
Enrofloxacin	28	28	28	1
Monensin	56	31	44	2
Salinomycin	23	23	23	1
Sulfachloropyridazine	3,826	17	716	11
Sulfadiazine	3,941	164	1,109	14
Sulfamethazine	180	29	80	3
Sulfamethoxypyridazine	905	92	184	9
Sulfapyridine	400	400	400	1
Sulfaquinoxaline	337	104	110	4
Trimethoprim	552	23	137	9

(Arnold et al., 2014). Trimethoprim was also measured in drinking water from Spain (Valcárcel et al., 2011), the United States (Benotti et al., 2009), China (Sun et al., 2015), and Canada (Kleywegt et al., 2011).

To date, no data indicate clear human health risks associated with the continuous consumption of low levels of pharmaceuticals (ng/L) via drinking water or other type of food because ecotoxicity tests are usually performed using therapeutic doses and short periods of time. However, many investigations have probed toxic effects of pharmaceuticals on large and small terrestrial and aquatic organisms and microorganisms (Wollenberger et al., 2000; Madden et al., 2009; Liu et al., 2011; Mišik et al., 2014; Bártiková et al., 2016; Kovacs et al., 2016). For example, lethal and sublethal effects on embryos and larvae of zebrafish exposed to sulfadiazine, sulfamethoxazole, or sulfamethazine have been observed. Concentrations between 1 and 1,000 µg/L caused toxic effects on spontaneous movements, heartbeats, and hatching of embryos, and resulted in malformations (pericardial edema, yolk sac edema, hemagglutinations, tail deformation, and swim bladder defects) in embryos and larvae (Lin et al., 2013). Ando et al. (2007) investigated the effect of antimicrobials on the growth of 8 cyanobacteria and demonstrated that 7 antimicrobial agents, including sulfadimethoxine and trimethoprim, inhibited the growth of cyanobacteria even at low concentrations. For humans, the continuous ingestion of milk contaminated with low level of antimicrobial via water could lead to the development of resistant bacterial strains as has been shown for animals (Kemper, 2008).

The second most frequently detected group was coccidiostats, present in 20% of samples; they are also the second most used drug group in veterinary medicine (FEDESA, 1999). Decoquinat was measured in 16% of samples at concentrations ranging between 17 and 732

ng/L. As this drug is only used in veterinary medicine, it can be considered an indicator of farming contamination (Iglesias et al., 2013). It is notable that of the 8 samples (15%) with measurable levels of decoquinat, 5 were sourced from farms using well water. These results could support the idea of lixiviation or runoff of pharmaceuticals after using manure contaminated with decoquinat as fertilizer on agricultural soils. The average concentration of decoquinat (111 ng/L) was higher than that reported in surface water collected from the same area (23 ng/L; Iglesias et al., 2012), probably due to the degradation effect of decoquinat. Decoquinat is toxic in fetal rats (at 300 mg/kg of BW per day) with minor developmental variations resulting (retarded skeletal ossification; Boxall et al., 2011). In general, toxicity of coccidiostats for animals is well documented; however, scientific literature does not contain toxicity information for long-term effects at low levels of coccidiostats (ng/L; Hansen et al., 2009). It has also been demonstrated that ionophores might have synergistic effects when combined with other biologically active compounds. The case of cattle death is remarkable because, out of 988, a total of 562 cattle died (57%) in an 8-wk period due to the consumption of monensin-containing feed contaminated with macrolide antibiotic residues (Basaraba et al., 1999). It has also been shown that narasin combined with nicarbazine is 5 time more toxic than the predicted additive effect (Hansen et al., 2009).

Out of 52 drinking water samples analyzed in the current study, 33 (63.5%) were positive for the presence of one or more drugs. Considering that the mixture of compounds may contribute to the overall effect of the mixture and might increase the toxicity of individual compounds (Arnold et al., 2014), it is important to note that 29% of the samples contained more than one pharmaceutical; 6 samples were positive for 2 drugs,

4 samples for 3 drugs, 3 samples were for 4 drugs, 1 sample for 5 drugs, and 1 sample was positive for 6 drugs. One sample contained decoquinatone, monensin, sulfachloropyridazine, sulfadiazine, sulfamethazine, and trimethoprim and another sample contained measured levels of sulfachloropyridazine, sulfadiazine, sulfamethoxypyridazine, sulfaquinoxaline, and trimethoprim. Pomati et al. (2008) reported the effect of a mixture of 13 drugs at concentrations in the nanograms per liter range and demonstrated that the mixture could inhibit the proliferation of human embryonic cells by affecting their physiology and morphology. Even if sulfonamides and trimethoprim are often administered simultaneously, adverse reactions to this combination have been reported in humans (Leoung et al., 2001). In the last decade, other research has investigated the adverse effect of pharmaceutical mixtures at the nanograms per liter level (Zou et al., 2012; Vasquez et al., 2014; Wang et al., 2014). Even if effects of mixtures of drugs measured in water collected from milking parlors has not been reported yet, it is likely that these combinations of active substances lead to toxic effects, as has been

reported for other groups of drugs (Pomati et al., 2008; Arnold et al., 2014).

When data were analyzed by sampling area, the Coast area (Figure 2) had the fewest contaminated samples; none of the samples analyzed contained residues of the pharmaceuticals at the limit of quantitation of the method used (Table 2). On the other hand, more than 70% of the samples from the Flatland, Mountain, Center, and South areas were contaminated with drugs. In these areas, the antimicrobials were the most frequently detected—found in more than 50% of the samples of each area. We noted that the number of contaminated samples by area correlated with the number of farms and total number of cattle by area; the Coast and South areas had fewer farms and cattle, approximately 10% of the total number of cattle (Figure 1).

The Flatland and Mountain areas had the higher number of samples positive for more than one pharmaceutical; 60 and 57% of the samples had more than one pharmaceutical in these areas, respectively. In fact, the samples with 5 and 6 pharmaceuticals were collected in farms located in the Flatland area. The Central area

Table 5. Statistical analysis (*P*-values) of the presence and concentration of each drug detected against sampling area, average sunlight, rainfall, temperature, water source, and sampling month

Drug	Sampling area	Average sunlight (h)	Average rainfall (L/m ²)	Average temperature (°C)	Water source	Sampling month
Betamethasone						
Presence	0.675	0.199	0.199	0.199	0.587	0.199
Concentration	0.675	0.199	0.199	0.199	0.587	0.199
Decoquinatone						
Presence	0.633	0.147	0.147	0.147	0.846	0.147
Concentration	0.943	0.176	0.176	0.176	0.938	0.176
Enrofloxacin						
Presence	0.117	0.199	0.199	0.199	0.587	0.199
Concentration	0.117	0.199	0.199	0.199	0.587	0.199
Monensin						
Presence	0.898	0.295	0.294	0.295	0.434	0.294
Concentration	0.864	0.278	0.278	0.278	0.454	0.278
Sulfachloropyridazine						
Presence	0.007	0.002	0.002	0.002	0.917	0.002
Concentration	0.226	0.003	0.003	0.003	0.797	0.003
Sulfadiazine						
Presence	0.227	0.164	0.164	0.164	0.243	0.164
Concentration	0.230	0.227	0.227	0.227	0.432	0.227
Sulfamethazine						
Presence	0.159	0.000	0.000	0.000	0.329	0.000
Concentration	0.440	0.036	0.036	0.036	0.474	0.036
Sulfamethoxypyridazine						
Presence	0.801	0.187	0.187	0.187	0.251	0.187
Concentration	0.347	0.088	0.088	0.088	0.820	0.088
Sulfapyridine						
Presence	0.675	0.199	0.199	0.199	0.587	0.199
Concentration	0.675	0.199	0.199	0.199	0.587	0.199
Sulfaquinoxaline						
Presence	0.675	0.844	0.573	0.573	0.587	0.573
Concentration	0.675	0.573	0.573	0.573	0.587	0.573
Trimethoprim						
Presence	0.375	0.606	0.606	0.606	0.674	0.606
Concentration	0.142	0.358	0.358	0.358	0.779	0.358

had the lowest risk samples: 31% of the samples did not contain any pharmaceuticals and 60% only one pharmaceutical. These results are interesting because the Central area had greater numbers of cattle and farms and also the highest number of farms with well water.

In the statistical analysis, the presence and concentration of each drug was evaluated against sampling area; average sunshine, rainfall, and temperature; water source, and sampling month. Table 5 compiles the *P*-values obtained in each case. Significant differences were found for the presence and concentration of sulfachloropyridazine and sulfamethazine with respect to weather conditions and sampling months (Table 5). Sulfamethazine was only detected during February and sulfachloropyridazine was only detected during February and March; these were the months with the lowest recorded average temperature and hours of sunshine. Additionally, higher rainfall was registered during these 2 mo. These results could indicate that sulfamethazine and sulfachloropyridazine were more degraded during April, May, and June by the higher temperatures, more sunshine, and lower rainfall (Table 1).

Microbiological Results

According to Regulation 853/2004 (EU, 2004), the water used to clean milk facilities must be potable. The Spanish standard Real Decreto 1120/2012 (BOE, 2012) and the World Health Organization (WHO, 2008) state that the number of coliform bacteria in 100 mL must be zero and the maximum bacterial count at 22°C must be 100 cfu/mL. Microbiological analyses of the 52 water samples collected from milking parlors in the current study showed 13 samples to be positive for the presence of coliforms (in a total volume of 100 mL), and 10 of these samples came from farms with well water (out of 27 wells analyzed). None of the water samples from farms with distribution systems were positive for coliforms. Higher contamination of well water with coliforms was observed in a study conducted on water quality of private water wells from a farming region in Ohio (United States), where 82% (*n* = 180) of the samples were contaminated with coliform bacteria (Won et al., 2013). It is also remarkable that the area in our study with the highest number of total coliforms detected was the Center region (46%), which also had the greatest number of farms and cattle (Figure 1). These results could indicate a possible relation between ground water contamination and dairy farm contamination but this finding should be investigated in more detail. Regarding mesophilic bacteria, 10% of samples contained more than 100 cfu/mL; again, such samples are not suitable for use as drinking water for humans and consequently cannot be used in the food industry.

We found no relation between mesophilic bacteria and sample origin or sampling area.

CONCLUSIONS

Analysis of water samples from milking parlors collected from farms in Galicia (Spain) revealed the presence of pharmaceuticals in 65% of samples, with the concentration of pharmaceuticals in positive samples ranging between 17 and 3,941 ng/L. Antimicrobials were present in 57% of the samples, with sulfadiazine and sulfachloropyridazine being the most commonly detected drugs. Coccidiostats were the second most frequently detected group, being present in 20% of the samples. These 2 groups are the most frequent classes used in veterinary medicine and antimicrobials are the most frequency prescribed drug in human medicine. Considering that a mixture of compounds might increase the toxicity over the additive effect of individual compounds, 29% of samples contained more than one pharmaceutical. Our results confirm that pharmaceuticals penetrate the human food supply via milking parlors. To date, the effects of continuous consumption of these mixtures of drugs are unknown; however, antimicrobials may affect the human microbiota or have toxic effects in sensitive people. No regulations exist for the presence of pharmaceuticals in drinking water and the long-term effects of low-dose pharmaceuticals need to be investigated. On the other hand, microbiological analyses demonstrated that the water used in most cases is drinkable, and even suitable for human consumption; however, there is evidence of fecal contamination, because 26% of the samples were positive for total coliforms.

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