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Determination of antibiotics and other veterinary drugs in the solid phase of pig manure

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ABSTRACT

The presence of residues of veterinary pharmaceuticals in farm wastewaters such as pig slurry represents a problem that needs to be tackled to avoid further contamination of environmental waters and the development of resistant bacteria. For their monitoring and control, it is necessary the existence of reliable analytical tools. The present paper describes for the first time the development and optimization by statistical experimental design of a specifically designed analytical method for the analysis of 21 veterinary drugs, including 18 antibiotics of several families (β-lactams, tetracyclines, fluoroquinolones, sulfonamides, macrolides, among others), 1 antiparasitic, 1 analgesic and 1 hormone, in a complex environmental matrix such as the fresh solid phase of pig slurry. The resulting method, consisted of an ultrasound assisted extraction (UAE) combined with *in-situ* dispersive solid phase extraction (d-SPE) from a 0.3 g of freeze-dried sample aliquot followed by a preconcentration step by compact solid phase extraction (c-SPE) and subsequent instrumental analysis by ultra-high-performance-liquid-chromatography (UHPLC) coupled to mass spectrometry in tandem (MS/MS) by a triple quadrupole, was successfully validated as a very sensitive (method limit of quantification in the low ng g^{-1}) and reliable method (relative recoveries around 100% and method repeatability featured by a general relative standard deviation below 20%). Provided raw data was intended to be processed by matrix-matched quantification approach. The resulting methodology was applied to the characterization of several pig manures from different Spanish farms sampled across breeding season between 2018 and 2019. Sample precedence showed to have a high impact in the positives, its frequency and concentration.

1. Introduction

The consumption of pork meat is growing annually worldwide. Consequently, the number of pig farms is gradually increasing. The European Union (EU) is the world's second biggest producer of pork, only surpassed by China. In particular, Germany, Spain and France are leading the European ranking, and between them they represent half of the EU's total production.

In industrial intensive swine farming, pigs are usually raised in stables. In order to comply with health and hygiene controls, pigsties are regularly cleaned by using high volumes of water pressure. The resulting piggery wastewater (PWW) mainly contains pig urine and feces, in addition to other components such as rests of feed and straw bed. PWW is made up to 98% of water and high concentrations of inorganic nutrients and organic matter (Makara and Kowalski, 2015).

For many years, PWW has been commonly discharged as fertilizer directly onto the agricultural land surrounding the farms. It was the simplest and most inexpensive way to dispose this kind of wastewater (Khatri and Tyagi, 2015). However, underlying aquifers were becoming contaminated by nutrient leaching. Thus, in 1991 the EU issued the Directive 91/676/EEC in order to protect the waters against pollution caused by nitrates from agricultural sources, and limited the discharge of total nitrogen to 170 kg ha^{-1} y^{-1}.

At the same time, since 2006 the use of antibiotics as growth promoters in livestock is only authorized under prescription in EU, and its use has been severely restricted in the US since 2016. However, little or no control is enforced in many other countries around the world. Furthermore, as it happens for other types of drugs, many antibiotics are poorly absorbed in the digestive system and are scarcely metabolized. Therefore, they are mainly excreted and incorporated into the manure (Ezzariai et al., 2018) (Spielmeyer, 2018). Stunning antimicrobial loads of up to 100 mg L^{-1} have been reported in the literature revision published by (Van Epps and Blaney, 2016). In fact, it is very difficult to find residues-free manure (Ezzariai et al., 2018) (Spielmeyer, 2018). Consequently, when PPW is discharged, antibiotics are probably released into the environment too, which enhances the risk of developing antibiotic resistance in pathogenic bacteria, as consistently reported in the last years (Peeples, 2015).

Highest amounts of nitrogen are found in the liquid phase pig manure. In addition, transportation costs drastically decrease when the liquid phase is removed from the pig manure. Hence, latest manage-

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ment approaches tend to separate both phases. Liquid phase is usually further treated as sewage and solid phase manure is commonly processed to be used as fertilizer in agriculture.

To date, only a few methods intended to determine pharmaceuticals in pig slurry (Patyra et al., 2020) (López-Serna et al., 2019) (Bourdat-Deschamps et al., 2014), and only one tailor-made it for its solid phase (Marti et al., 2020). The latter used of pressurized liquid extraction as technique to extract the analytes from the matrix, which is expensive and not often available in analytical laboratories. In addition, its efficiency to extract the analytes is often depleted by its associated matrix effect, as it also shows a high efficiency extracting the matrix (Pérez-Lemus et al., 2019). It is paramount to assess the feasibility of a wider range of techniques, including the most commonly present in routine-analysis laboratories, which would enlarge analytical capacity for this type of analysis. This study aimed at developing and optimizing a creative analytical method consisting of Ultrasound-assisted-extraction (UAE) with *in-situ* dispersive solid phase extraction (d-SPE) followed by compact-solid-phase-extraction (c-SPE) and Ultra-High-Performance-Liquid-Chromatography (UHPLC) coupled to Mass spectrometry in tandem (MS/MS) for 21 veterinary drugs in the solid phase of pig slurry, using statistical experimental design. To the authors' knowledge, this is the first time statistical design has been specifically tailor-made to find the optimum conditions to analyze veterinary drugs of different chemical properties and medical indications in swine manure solid-phase.

2. Materials and methods

2.1. Standards and reagents

Twenty-one veterinary pharmaceuticals, including 18 antibiotics (5 sulfonamides, 4 fluoroquinolones, 3 tetracyclines, 2 β-lactams, 1 macrolide, 1 pleuromutilins and 2 others), as well as 1 anti-parasitic, 1 hormone and 1 analgesic were selected according to their high prescription in veterinary farms in Spain, particularly in Castilla y León, region where the farm providing with samples for this study was located (AEMPS, 2011). Drug standards of high purity grade (>95%) were obtained from Sigma-Aldrich, except for tylosin and florfenicol which were purchased from LGC Standards (Supplementary data 1). All of them were acquired as neutral non-solvated molecules, except for amoxicillin (trihydrated) and, penicillin G, doxycycline, oxytetracycline, tetracycline, tiamulin, and tylosin, which were obtained as salts. An isotopically labelled internal standard, enrofloxacin-d_5, was supplied by Sigma-Aldrich. Stock solutions for each compound (1 g L^{-1}) were prepared in methanol (MeOH), except for amoxicillin and danofloxacin (dissolved in a (1:1) H_2O/MeOH mixture) and ciprofloxacin (dissolved in (1:1) H_2O/MeOH in presence of 0.2% (v/v) hydrochloric acid (HCl)). Mixture and work solutions were prepared in MeOH and stored at −80 °C in darkness until further use.

LCMS-grade MeOH and formic acid (FA) were supplied by Scharlau. Ultrapure water was obtained by a Milli-Q Advantage A10 water purification system from Merck Millipore. HCl (37%) was acquired from Sigma-Aldrich. Oasis® HLB cartridges (60 mg, 3 mL) were provided by Waters Chromatography. Powdered activated carbon (AC), alumina (Al_2O_3) and Na_2EDTA were purchased from Panreac, while octadecylsilane (ODS) was purchased from Supelco. Al_2O_3 was activated before use by heating at 110 C for 12 h.

2.2. Method development and optimization

2.2.1. Solid phase pig slurry analytical methodology

Swine manure from a pig farm at Segovia (Spain), which was freshly collected and transported at 4 C and darkness to our laboratory within the day, was used to develop and validate the methodology further discussed in Sections 3.1 and 3.2.1.

2.2.1.1. Sample pretreatment The solid-phase pig-slurry analytical method was designed as follows: 1) Within a 24-h time span since its collection, aliquots of 100 mL of fresh homogenized swine manure were centrifuged in a Thermo Sorvall Legend RT + Refrigerated Benchtop Centrifuge at 14,000 rpm (30,000 × *g*) for 10 min. The resulting decanted suspended solids constituted the target matrix, the so-called solid phase of the pig slurry. This is also the usual procedure to obtain environmental solid matrixes such as sewage sludge (Pérez-Lemus et al., 2020) (López-Serna et al., 2018). Thus, the remaining supernatant was discarded. The amount of analytes remaining in the solid phase interstitial water was considered negligible. 2) The wet solid phase was, then, freeze-dried and stored in darkness at −20 C if not immediately analyzed. 3) An aliquot of ca. 300 mg was accurately weighed into a 50-mL centrifuge tube, and a volume of 100 μL of a 0.5 mg L^{-1} solution of the isotopically labelled internal standard (enrofloxacin-d_5) was added. 4) The mixture was thoroughly vortex-stirred for approximately 1 min and, then, was let it settle in darkness overnight to allow solvent evaporation and internal standard fixation. The loss of analytes during about 12 h in darkness of settling was considered negligible. This spiking procedure is common practice in solid samples (Jelić et al., 2009) (Gago-Ferrero et al., 2011). 5) An amount of 0.3 g of previously activated alumina (Al_2O_3) was added to the sample for *in situ* cleanup (dispersive solid phase extraction (d-SPE)). 6) A volume of 10 mL of 10:90 MeOH/H_2O was added as extraction solvent and the mixture was, then, vigorously vortex-stirred to obtain a homogenous suspension. 7) Then, the centrifuge tube underwent Ultrasound Assisted Extraction (UAE) for 15 min at room temperature in a JP Selecta Univeba ultrasound bath of 50 W and 60 Hz. 8) Subsequently, the suspension was centrifuged for 10 min at 14,000 rpm (30,000 × *g*). 9) Six millilitres of the resulting supernatant were collected with a glass pipette, filtered through 0.7 μm-pored glass fibre and transferred to a 100-mL volumetric flask. 10) Steps 6–9 were repeated once more, adding 15 mL of extraction solvent and collecting 9 mL of the resulting supernatant, this time. Extracts from both UAE cycles were combined. As in sewage sludge (Pérez-Lemus et al., 2019), two *in situ* d-SPE-UAE cycles were considered enough to quantitatively extract the target analytes without augmenting unnecessarily the matrix effect by excessively co-extracting matrix components. 11) Then, Na_2EDTA was added to reach a concentration of 0.1% and, the solution was diluted up to 100 mL with ultrapure water. This diluted extract was concentrated and cleaned up by compact solid phase extraction (c-SPE) as described elsewhere (López-Serna et al., 2019). In brief, Oasis® HLB cartridges with 60 mg of stationary phase (Waters Chromatography) were conditioned with MeOH and H_2O. Afterwards, samples were extracted and, then, eluted with 6 mL of ACN. Finally, the organic solvent was evaporated to dryness and, the extracts reconstituted in 1 mL of 0.1% FA in a 95:5 H_2O/MeOH mixture.

2.2.1.2. LC–MS/MS analysis Analytes in the extracts were determined by ultra-high performance liquid chromatography (UHPLC) – tandem mass spectrometry (MS/MS). Chromatographic separation was carried out by a Sciex Exion UHPLC equipped with a Phenomenex reversed-phase column Kinetex EVO C18 (2.1 mm × 50 mm, particle size 1.7 μm). The mobile phase consisted of H_2O and MeOH containing 0.1% FA as modifier working under gradient conditions (from 5% MeOH, held for 1 min, to 95% MeOH in 2 min, held for 3 min). The flow rate was 0.5 mL min^{-1} and the injection volume was 10 μL. The column was kept at 40 °C and samples were preserved in the autosampler at 15 C. Mass detection was performed by the triple quadrupole Sciex 6500+ QqQ in selected reaction monitoring (SRM) mode. LC and MS instruments were interphased by electrospray ionization (ESI) in positive mode. The full list of SRMs is given in Supplementary data 2. Some of the ESI conditions were set as follows: capillary voltage, 5.5 kV; source temperature, 400 °C. N_2 was used for the curtain gas, ion source gas 1 and 2, and collision gas at a flow rate of 20, 45 and 9 L min^{-1}, respectively. To achieve the optimum mass sensitivity/selectivity ratio, unit resolution was set at both first (Q1) and third (Q3) quadrupole. Instrument control and data acquisition were performed by Analyst® software. Data processing was carried out by SciexOS software.The optimized method based on UAE – SPE – LC-MS/MS was applied to the analysis of swine manure from pig farms in Segovia and

Almeria (both in Spain) at different times of the year and pig raising seasons, and the results are presented in Section 3.2.2.

2.2.2. Experimental design

Previous studies such as (López-Serna et al., 2019) (Liu et al., 2017) (Östman et al., 2017) (Golet et al., 2002) (Guo et al., 2017) (López Zavala and Reynoso-Cuevas, 2015) (Goulas et al., 2017) (Montesdeoca-Esponda et al., 2012) (Silva et al., 2011) (Lara-Martín et al., 2014) (Kantiani et al., 2010) (Liu et al., 2011) were reviewed. Based on them, influencing parameters were identified and then narrowed down into the most significant ones. Their subsequent optimization was divided into two steps. Firstly, variables such as sample size, type of cleanup adsorbent and extraction technique were tested, in triplicate, in a classical one-factor-at-a-time approach. The analytical method described in section 2.2.1 was used by default. The experimental design consisted of a replicated fractional factorial 2^{k-1}, with two central points. Thus, a set of 20 experiments was randomly performed to optimize extractant pH, organic solvent content in the extractant, amount of cleanup agent, and amount of complexing agent. Afterwards, the software Statgraphics Centurion XVII was used to process the acquired experimental data and find out the potential influence of some operation parameters. During the whole optimization, samples were spiked at 1000 ng g^{-1} of each target compound by adding 150 μL of a mix stock solution at 2 mg L^{-1} in MeOH during the step 3) of section 2.2.1.1, and both individual (IS/N) and total (TS/N) signal to noise ratios were used as response variables. TS/N was calculated as the sum of the IS/N of each target analyte in the resulting chromatogram.

3. Results and discussion

3.1. Pretreatment method development and optimization

3.1.1. Preliminary experiments

Some preliminary experiments were carried out in triplicate in a one-factor-at-a-time approach to optimize the *sample size*, *type of cleanup agent* and *extraction technique*. These qualitative discrete variables were not tested within the experimental design described in section 3.1.2 as they included more than two levels per factor, which would complicate the design. The method protocol described in section 2.2.1 was used as starting point and only the tested parameter was modified in each case. Once a parameter was optimized, the method protocol was updated accordingly. Samples were spiked at 1000 ng g^{-1} of each target compound during the step 3) of section 2.2.1.1. This level was in the top range of expected concentration in real sample for the target analytes. Hence, optimization at a real scenario could be carried out comfortably. As the method includes the simultaneous analysis of several compounds with quite different physico-chemical characteristics, the performance conditions needed to be compromised. In this case, the total method sensitivity, characterized by the TS/N was selected as criterion to achieve a multicomponent method. Fig. 1 shows the accumulated percentage of the IS/N for all the tested levels in each parameter.

3.1.1.1. Sample size Three amounts of freeze-dried solid phase pig manure, i.e., 0.1, 0.3 and 0.5 g were tested. Due to matrix effect, sensitivity was not expected to constantly improve with sample size. The breakthrough sample mass was then investigated. In general, the smaller the sample size the more ecologically sustainable is the analysis and easy to handle and safer for the analyst (López-Serna et al., 2018). Fig. 1A shows 0.3 g provided the best S/N for most of the compounds, and a TS/N of (176 ± 145) x 10^6, versus (108 ± 57) x 10^6 for 0.1 g and (130 ± 89) x 10^6 for 0.5 g. It was particularly well functioning for sulfathiazole with an IS/N of (66 ± 1) x 10^3 (versus 10 × 10^3 and 2 × 10^3 for 0.1 g and 0.5 g, respectively). In contrast, trimethoprim showed poor sensitivity at this sample size with an IS/N of (95 ± 1) x 10^3 (versus 125 × 10^3 and 387 × 10^3 for 0.1 g and 0.5 g, respectively).

3.1.1.2. Type of cleanup agent After reviewing the literature (Pérez-Lemus et al., 2020) (Perestrelo et al., 2019) (Wang et al., 2020), three adsorbent substances intended to be used as cleanup agent during the *in situ* d-SPE step, i.e., activated Al_2O_3, ODS and AC, were tested. Alumina, both in-house packed or in commercial cartridges, has been traditionally used for the isolation and cleanup of analytes from biological matrices such as animal tissues and vegetable material (Telling et al., 1977) (Cheng et al., 2009) (Zhu et al., 2018), but rarely from non-biological environmental matrices. A blank sample without cleanup agent was also run and subsequent c-SPE step could not be completed as cartridges became blocked due by the matrix content in the extract. Hence, the d-SPE cleanup agents turned out to be essential to decrease the matrix effect in a solid matrix as complex as the present one. However, their efficiency needed to be limited to the removal of matrix components and not to the analytes. This balance between effectiveness and selectivity was assessed.The results confirmed AC had an extraordinary adsorption capacity, even visually observed as the extracts were transparent and colourless when it was in use. However, the overall low extraction recovery showed by most of the analytes when in use, was attributed to the adsorption of the analytes onto it too. In Fig. 1B, relative S/Ns, when the different tested cleanup agents, is depicted. Despite ODS seemed to be more suitable for some drugs, such as penicillin G and the tetracyclines, Al_2O_3 turned out to be slightly more efficient adsorbent for most of the compounds, with a TS/N of (496 ± 332) x 10^6 versus (427 ± 286) x 10^6 for ODS and (9 ± 6) x 10^6 for AC. Thus, alumina was selected as the optimized cleanup agent from that point on.

3.1.1.3. Extraction technique Ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE) were proposed as solid-liquid extraction techniques as they were reported to be cheap, environmentally friendly and effective in extracting the analytes of interests from environmental matrices (Pérez-Lemus et al., 2020).TS/N seemed to be higher for UAE (26 ± 21) x 10^3 for UAE vs. (19 ± 10) x 10^3 for MAE) as well as for most of the IS/N ratios (Fig. 1C). Nonetheless, penicillin G was the most notable exception with an IS/N of 5 ± 4 (versus 11 ± 4 for MAE). In addition, the simplicity in the technical use and maintenance led to the selection of UAE as the preferred option in the final optimized method.

3.1.2. Experimental design

With the method updated after the preliminary assays, the influence of the remaining four factors was assessed under a replicated fractional factorial design at two levels (2^{k-1}), i.e., *extractant* pH *(Low level: 3; High level: 8)*, *organic solvent content in the extractant (Low level: 10% MeOH; High level: 35% MeOH)*, *amount of alumina for the cleanup (Low level: 33% of the sample size; High level: 100% of the sample size)*, and *amount of complexing agent (Low level: 0.05 g of EDTA; High level: 0.1 g of EDTA)*, including two central points. These factors and levels were selected after reviewing related literature (Pérez-Lemus et al., 2020) (Li et al., 2018) (Pan and Chu, 2017) (Li et al., 2017) (Wang et al., 2019) (Conde-Cid et al., 2018) (Gros et al., 2019) (Qian et al., 2016). The principles of the so-called green chemistry were also taken into consideration by proposing lower percentages of methanol during the UAE extraction, in comparison to most of the reported UAE analysis so far, where organic solvents usually made more than 50% of the extractant (Li et al., 2015) (Widyasari-Mehta et al., 2016) (Hu et al., 2010). The resulting 20 experiments, whose analytical conditions are gathered in Table 1, were randomly performed. Again, aliquots from the same freeze-dried solid phase pig slurry were spiked at 1000 ng g^{-1}. TS/N values, obtained under each experiment, were introduced in the Statgraphics Centurion XVII software as response variable. After mathematically fit the data to a second order polynomial model through the least squares method, only one factor, the amount of Al_2O_3 cleanup agent, turned out to have a significant effect (R^2 = 57%; $p < 0.05$). Hence, as it could be expected, a positive relationship between the added amount of Al_2O_3 and the TS/N value was observed. That meant that the response variable increased when increasing the

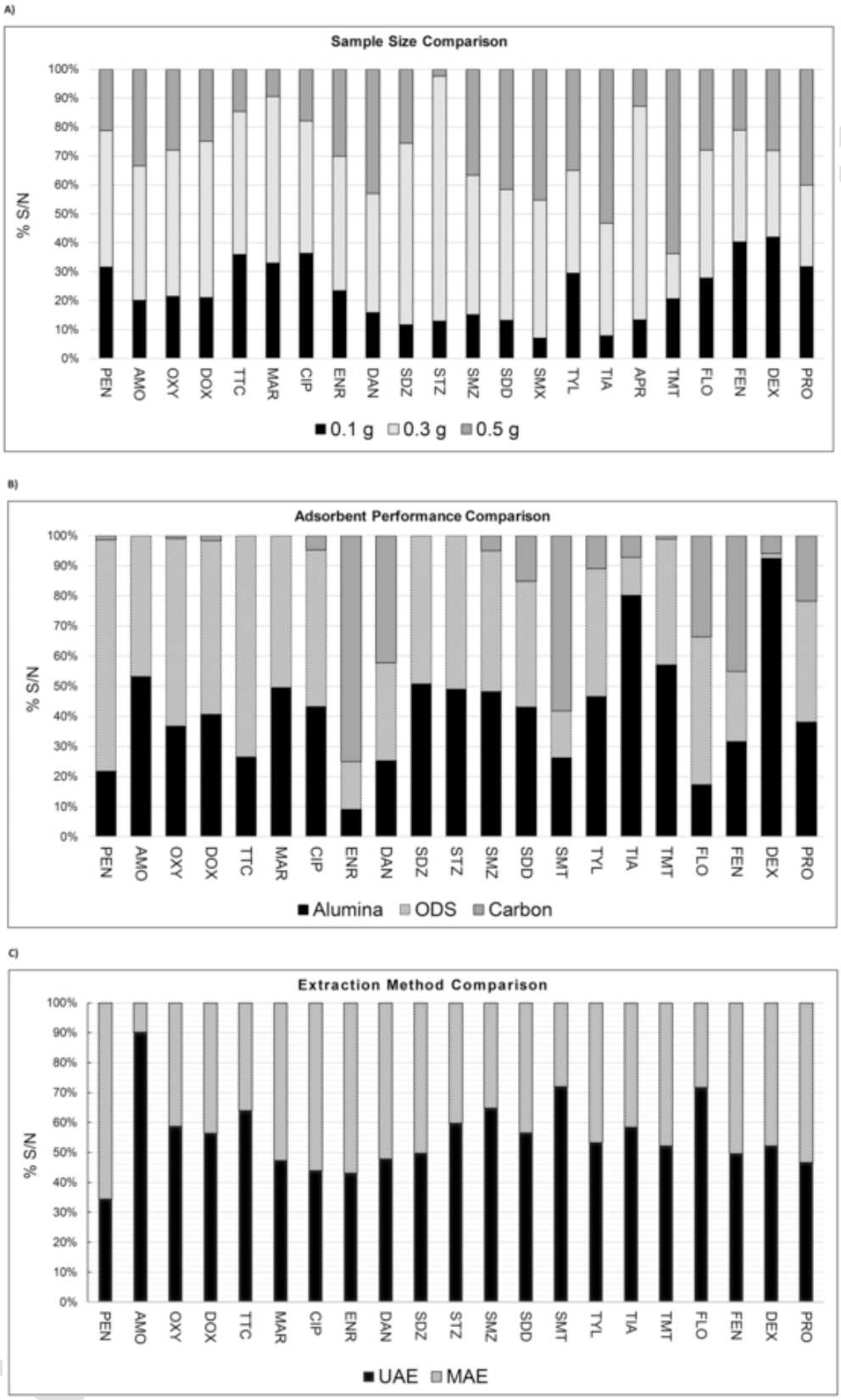

Fig. 1. Preliminary experiments.

alumina amount. Al_2O_3 may have decreased the amount of co-extractives in the extract by preferentially adsorbing the matrix components over the analytes, and, therefore, the related ion suppression in the electrospray interface, increasing the subsequent TS/N ratio. In addition, IS/N were also statistically processed for each drug. Individual significant effects are shown in Supplementary data 3. The amount of

Table 1
Replicated fractional factorial 2^{k-1}, with two central points, experimental design.

Experiment	pH	%MeOH	% Al_2O_3	Mass EDTA (g)
1	3	10	33	0.05
2				
3	3	10	100	0.1
4				
5	3	35	33	0.1
6				
7	3	35	100	0.05
8				
9	8	10	33	0.1
10				
11	8	10	100	0.05
12				
13	8	35	33	0.05
14				
15	8	35	100	0.1
16				
17	5.5	22.5	66	0.05
18				
19	5.5	22.5	66	0.1
20				

Al_2O_3 turned out to be a significant factor ($p < 0.05$) for the analysis of 9 drugs, with a positive effect in all cases, except for amoxicillin. For the other 11 analytes, the presence of alumina did not show any significant effects (either positive or negative) in their IS/N. This translated into a negligible or balanced adsorption of both matrix and analyte components onto the alumina. It is worth pointing out to the fact that 8 of those drugs with positive effect presented some of the highest lipophilia among all, and had a log P above 1 in all cases. In contrast, this parameter is below 1 for amoxicillin (Supplementary data 1). Therefore, the addition of 0.3 g of alumina (high level) was included in the optimized method. The TS/N response variable was not able to detect the significance of the percentage of MeOH in the extractant solution. However, three drugs, in particular tetracycline, oxytetracycline and ciprofloxacin, showed a negative significant effect when the IS/Ns were taken into consideration. This meant their IS/Ns decreased when the %MeOH increased. This is compatible with their high polarity, especially in the cyclines (Supplementary data 1). Therefore, only 10% of MeOH was decided to be contained in the extractant solvent of the final method, to foster the extraction of these particular compounds. This came to show that actually low organic contained extractans might be preferred when it comes to desorb microcontaminants from solid matrixes by solid-liquid extraction. On the other hand, as no individual or joint effect was significantly detected for the pH and the amount of complexing agent, in the optimized method pH was not adjusted in the extracting solvent and the EDTA was added in high concentration (0.1 g, equivalent to 0.1% in dilution).

3.2. Method validation and application

3.2.1. Method validation

Five validation parameters, i.e., accuracy, matrix effect, precision, sensitivity and linear dynamic range were determined for all 21 target analytes in the developed analytical method for solid phase of swine manure. With this purpose, matrix-matched calibration curves were built from 3 to 2000 ng g^{-1}, spiking identical samples at 8 different concentrations. Spiking concentrations at 170 (low level) and 1300 (high level) ng g^{-1} were prepared in triplicate. In addition, non-spiked samples (blanks) were also analyzed in duplicate. All samples were spiked with Enrofloxacin-d5 at 170 ng g^{-1}, intended to correct the signal of the fluoroquinolone antibiotics, for which an internal standard calibration curve was built. With the combination of both quantification approaches, any matrix effect or any analyte degradation along the method procedure would be compensated. The results, which are discussed below, are shown in Table 2.

1) *Accuracy:* Relative recoveries, calculated after comparing the spiked concentrations to the obtained with the quantification approach, after applying the optimized analytical method, are shown in Table 2. They turned out to be between 92 and 106% for all compounds (n = 3).

Table 2
Validation parameters.

Veterinary drug	Accuracy	Precision	Matrix effect	Sensitivity	Dynamic range		
	Relative recovery (%)	RSD (%)	Signal suppression (%)	MLQ (ng g^{-1})	R^2	Linear range (ng g^{-1}) [a]	Equation
Amoxicillin	94	7	27	136	0.995	MLQ – 2000	y = 314 x – 22,305
Penicillin G	101	2	25	6.8	0.998	MLQ – 1333	y = 1628 x – 23,232
Oxytetracycline	92	2	38	0.4	0.993	MLQ – 2000	y = 1074 x + 124,563
Tetracycline	101	28	33	20	0.999	MLQ – 2000	y = 1389 x + 108,267
Doxycycline	104	19	37	1.3	0.999	MLQ – 2000	y = 1089 × 209,791
Marbofloxacin	101	19	95	3.3	0.994	MLQ – 2000	y = 0.0109 x - 1.471
Ciprofloxacin	106	30	96	1.3	0.992	MLQ – 2000	y = 0.0074 x - 2.288
Enrofloxacin	96	15	96	3.9	0.997	MLQ – 2000	y = 0.0155 x - 1.387
Danofloxacin	100	18	94	4.7	0.993	MLQ – 2000	y = 0.0099 x - 0.737
Sulfadiazine	96	2	74	0.06	0.991	MLQ – 1333	y = 48,307 x + 1,927,222
Sulfathiazole	105	2	65	0.1	0.992	MLQ – 2000	y = 67,947 x + 3,713,376
Sulfamethizole	101	2	73	0.11	0.999	MLQ – 2000	y = 73,778 x + 630,974
Sulfadimidine	101	5	74	0.04	0.999	MLQ – 2000	y = 96,187 x + 1,856,201
Sulfamethoxazole	98	2	84	0.07	0.999	MLQ – 2000	y = 26,731 x – 249,646
Tiamulin	92	9	81	0.08	0.995	MLQ – 2000	y = 49,581 x + 1,044,982
Tylosin	97	19	69	0.18	0.992	MLQ – 1333	y = 2638 x + 433,605
Trimethoprim	97	4	63	0.02	0.994	MLQ – 1333	y = 116,798 x + 5,376,861
Florfenicol	100	1	−16	0.3	0.999	MLQ – 2000	y = 4797 x + 89,605
Fenbendazole	96	14	99	5.6	0.998	MLQ – 2000	y = 2382 x – 108,796
Dexamethasone	104	6	86	0.3	0.998	MLQ – 2000	y = 4294 x + 62,214
Progesterone	101	2	98	0.4	0.998	MLQ – 1333	y = 1628 x – 23,232

[a] MLQ: Method limit of quantification.

2) *Matrix effect*: Although it was corrected by the implemented quantification method, it was considered relevant to determine how much a matrix as complex as the solid phase of pig slurry affected the analysis. For this, areas obtained after applying the optimized analytical method to spiked samples corrected by blanks and samples without matrix, were compared. % signal suppression by the matrix is shown in Table 2. In all cases, effect was positive between 25 and 99%, except for florfenicol which showed a matrix signal enhancement of 16%.
3) *Precision*: As shown in Table 2, thirteen of the compounds showed a relative standard deviation (%RSD, n = 3) below 10%. All the rest presented a repeatability below 20% with the only exception of tetracycline (28%) and ciprofloxacin (30%).
4) *Sensitivity*: Method limits of quantification (MLQ), defined as the concentrations that provided signal-to-noise ratios of 10, are displayed in Table 2 and were observed to be below 0.1 ng g^{-1} for 6 analytes (sulfadiazine, sulfathiazole, sulfadimidine, sulfamethoxazole, tiamulin, trimethoprim), below 1 ng g^{-1} for another 6 analytes (oxytetracycline, sulfamethizole, tylosin, florfenicol, dexamethasone, progesterone), and below 10 ng g^{-1} for the rest, except for amoxicillin and tetracycline.
5) *Dynamic range*: Table 2 gathers the linearity parameters, i.e., concentration range providing a linear equation with an $R^2 > 0.99$. At least 3 and up to 5 orders of magnitude of linearity range were observed for most of the compounds.

All these indicators were considered to be highly satisfactory and, then, the method successfully validated as sensitive, efficient, reliable and capable for its purpose.

3.2.2. Method application

Once the analytical method was proved to be valid for its purpose, it was applied to the analysis of four pig slurries from two different farms (Almeria and Segovia, Spain) sampled at different times along the period 2018–2019. Each farm used a different pigsty cleaning procedure which contributed, along with the breeding season, to the differences in the composition of the pig manures. Grab samples were taken from the collecting tank. Average concentrations in nanograms per gram of freeze-dried solid phase pig slurry are shown in Table 3 β-Lactams, some fluoroquinolones such as marbofloxacin and danofloxacin, some sulfonamides such as sulfathiazole and sulfamethizole and the hormone progesterone, were not detected in any of the samples. In contrast, all three monitored tetracyclines, the antibiotics trimethoprim and florfenicol, and the parasitic fenbendazole were ubiquitous. The rest of compounds were detected in some of the samples depending on their location or time of the sampling. Thus, enrofloxacin and sulfamethoxazole were only found in the manure solid phase from Segovia pig farm. In general, more veterinary drugs and at higher concentrations were observed in samples from Segovia. Thus, pig slurries 1 and 2 showed quantifiable levels in 12 and 11 drugs accounting for a total load of 31.3 and 36.0 ng g^{-1} versus 7 and 9 pharmaceuticals summing 2.0 and 3.4 ng g^{-1} in samples 3 and 4, respectively. Differences within farms along the time could be attributed to variations in the medication routines depending on the moment of the breeding season (piglets growing, sow farrows, pig fattening, epidemic breakouts, etc.). Sample characterization has shown a very polarized picture. Hence, some compounds were not detected whatsoever and, in contrast, others were quantified at very high concentrations, above or at the very top limit of the linearity range, which led to sample dilution for reliable quantification. Oxytetracycline and doxycycline accumulated at such high concentration (≫ 10 times above top limit of the linearity range) that their concentration could only be estimated. Only other study concerning the presence of pharmaceuticals in the solid phase of pig slurries was found (Marti et al., 2020) and similar concentrations were reported. Hence, they also found tens of micrograms per gram of tetracyclines, ubiquitously, in Spanish pig farms. Likewise, the fluoroquinolones ciprofloxacin and enrofloxacin were determined to be present at units of ng g^{-1} in most of samples too, but danofloxacin absent. However, those Catalonian pig farms seemed not to use fenbendazole as parasitic, as they were not reported to be present in their solid phase samples, despite its high analytical response factor. The high occurrence of the tetracyclines in the solid phase, despite their low lipophilia

Table 3
Method application.

		Therapeutic class	Therapeutic subclass	Pig slurry 1	Pig slurry 2	Pig slurry 3	Pig slurry 4
				Nov-18	Feb-19	Jan-19	May-19
				Segovia		Almeria	
1	Amoxicillin	Antibiotic	β-lactams	< MLD	< MLD	< MLD	< MLD
2	Penicillin G			< MLD	< MLD	< MLD	< MLD
3	Oxytetracycline		Tetracyclines	>40,000	>40,000	>40,000	>40,000
4	Tetracycline			24,112 ± 4149	30,445 ± 6158	< MLD	581 ± 21
5	Doxycycline			>40,000	>40,000	>40,000	>40,000
6	Marbofloxacin		Fluroquinolones	< MLD	< MLD	< MLD	< MLD
7	Ciprofloxacin			2364 ± 86	< MLD	< MLD	< MLD
8	Enrofloxacin			2225 ± 193	1681 ± 124	< MLD	< MLD
9	Danofloxacin			< MLD	< MLD	< MLD	< MLD
10	Sulfadiazine		Sulfonamides	174 ± 11	< MLD	1668 ± 121	< MLD
11	Sulfathiazole			< MLD	< MLD	< MLD	< MLD
12	Sulfamethizole			< MLD	< MLD	< MLD	< MLD
13	Sulfadimidine			19 ± 1	11 ± 11	1 ± 0	10 ± 0
14	Sulfamethoxazole			70 ± 5	7 ± 7	< MLD	< MLD
15	Tiamulin		Pleuromutilins	2028 ± 14	3474 ± 110	< MLD	218 ± 7
16	Tylosin		Macrolide	< MLD	< MLD	< MLD	2147 ± 10
17	Trimethoprim		Others	< MLQ	< MLQ	< MLQ	< MLQ
18	Florfenicol			< MLQ	< MLQ	< MLQ	< MLQ
19	Fenbendazole	Anti-parasitics		349 ± 12	359 ± 21	292 ± 9	460 ± 79
20	Dexamethasone	Analgesic/Anti-inflammatory		< MLD	7 ± 7	< MLD	< MLD
21	Progesterone	Hormones		< MLD	< MLD	< MLD	< MLD

MLD: Method limit of detection, defined as the concentration providing signal-to-noise ratios of 3.

(Supplementary data 1) show their high use in pig raising and/or their low degradability. In fact, tetracyclines were pointed out as one of the most commonly used antibiotic class in Europe and North America (Lekagul et al., 2019) and the most in Spain (Casal et al., 2007). In addition, the majority of first-generation tetracyclines are not metabolized (though 5% of tetracycline is metabolized to a less active metabolite). Instead, they are most often eliminated by renal excretion (Vojtová and Urbánek, 2009). Once the pig slurry is produced, oxytetracycline is completely degraded after 18 days, but around a 7% of tetracycline might still remain after 20 days of incubation, according to (Chang et al., 2014). Nonetheless, in our particular study, samples were collected immediately after they were produced and solid phase was separated and frozen the same day they were sampled. Thus, the latter kind of degradation was considered negligible in the present work. Either way, the use of this kind of biosolids as soil amendment should be carefully controlled in order to avoid the contamination of aquifers and/or the development of antibiotic resistance. In contrast, the absence of progesterone, in spite of its high log P (Supplementary data 1), may show either it was not included in the therapeutic routines during the sampling periods, or it degrades at a high rate. This latter fact has been reported in the literature. Hence, up to 80% of the administered progesterone is immediately metabolized and will not be excreted as the original drug by the animal unless it was transdermal administered (De Ziegler and Fanchin, 2000).

4. Conclusions

The development of a tailor-made method for the analysis of 21 veterinary drugs in the very complex matrix of the fresh solid phase pig slurry has been conducted. The resulting methodology based on UAE with *in situ* d-SPE followed by c-SPE and UHPLC-MS/MS was intra-laboratory validated and subsequently applied to real samples. The main conclusions drawn were as follows:

1. After some preliminary assays, aliquots of 0.3 g of freeze-dried sample, activated Al_2O_3 and UAE were selected as the best performing sample size, *in situ* d-SPE cleanup agent and extraction technique, respectively.
2. A fractional factorial design showed that the pH in the extraction mixture and the EDTA amount during the SPE process had not a significant influence in the overall method sensitivity. In contrast, the amount of activated Al_2O_3, and in a lower extent the MeOH percentage in the extraction mixture, resulted to be significant factors; MeOH and Al_2O_3 amounts were set to 10% and 0.3 g, respectively, in order to achieve the best results. The use of TS/N ratio as response variable showed to be a valid compromise solution as it detected the most predominant significant factor.
3. Despite the complexity of the target matrix, a simplified sample pretreatment was achieved by successfully implementing *in situ* d-SPE during the UAE. The resulting analytical method could be considered both reliable and sensitive, with general relative recoveries between 92 and 106%, repeatability expressed as %RSD below 20%, MLQ under 10 ng g^{-1} and a linearity range of up to 5 orders of magnitude, which validated it as a method suitable for its purpose. This came to dismantle the conventionally accepted need of high percentages of organic solvent in the solid-liquid extractants, and showed that more environmentally sustainable options are also possible.
4. The direct application of the methodology to the analysis of real samples from different farms and sampled in different periods along the year showed big variations in the list of detected analytes as well as their concentrations. Nonetheless, the antibiotics tetracyclines were ubiquitous at stratospheric concentrations ranging the tens of micrograms per gram.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.130039.

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