



Determination of veterinary drugs in microalgae biomass from photobioreactors fed with piggery wastewater

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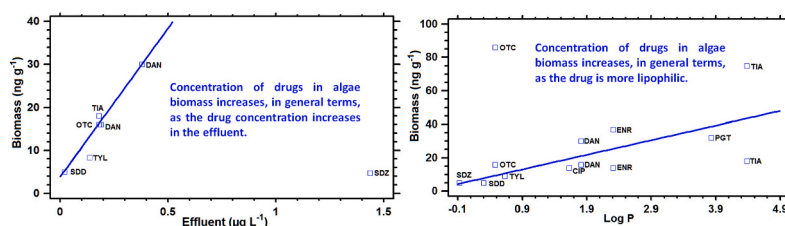
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HIGHLIGHTS

- Some tetracyclines and quinolones cannot be detected by the QuEChERS methodology.
- A solid-liquid extraction is preferable for the analysis of pharmaceuticals.
- Pharmaceutical concentrations in algae biomass are relatively low.
- Lipophilic pharmaceuticals tend to be accumulated in algae biomass.
- The concentrations in biomass are generally proportional to effluent concentrations.

GRAPHICAL ABSTRACT



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ABSTRACT

Concentration data of veterinary drugs in microalgae biomass collected from photobioreactors fed with piggery wastewaters are presented for the first time in this work. To this aim, a QuEChERS methodology and an ultrasound-assisted solid-liquid extraction have been assessed as sample preparation procedures with the purpose of determining 20 veterinary drugs, mainly antibiotics of different physico-chemical properties in addition to dexamethasone, fenbendazole and progesterone. Some critical operation parameters of the QuEChERS procedure were optimized by an experimental design but tetracycline, oxytetracycline, doxycycline, marbofloxacin and ciprofloxacin were not detected by the QuEChERS sample preparation. The use of a longer and thorough approach, a solid-liquid extraction with water/methanol in presence of primary secondary amine as a clean-up agent followed by solid-phase extraction on Oasis HLB cartridges, is recommended to monitor all intended analytes. The determination in extracts is carried out by ultra-high performance liquid chromatography–tandem mass spectrometry in selected reaction monitoring mode. Limits of detection about 0.2–42 ng per g of lyophilized microalgae sample, and repeatabilities about 6–46% ($n = 5$, RSDs) are reached. The solid-liquid extraction method was applied to microalgae biomass samples collected from a photobioreactor. Nine drugs were detected in the samples at relatively low concentration and a proportional relationship between the found concentrations and the octanol/water partition coefficients of the drugs has been outlined. Moreover, a linear ratio between the concentrations measured in biomass and effluent has been observed for most of the drugs.

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1. Introduction

The animal husbandry entails the yield of urines and feces which constitute most of the manure together with rests of beddings and livestock feed among other minor components. Antibiotics are the main type of drug applied in industrial husbandry to treat or prevent animal diseases and they are consumed in largest amounts in the commercial swine production compared with other livestock (Zhang et al., 2015). These antibiotics are poorly absorbed in the digestive tract of the animals and their metabolization rate is relatively low (Sarmah et al., 2006), most of them being excreted. In fact, antibiotics are frequently detected in manure samples (Spielmeyer, 2018) reaching concentrations up to 700 mg kg⁻¹ (Widyasari-Mehta et al., 2016). Manure is usually stored in lagoons or applied to soils as organic fertilizer, it is also used as substrate in biogas plants in which case the digestate becomes a residue. In any case, manure is a hazardous waste for the environment not only for the content of antibiotics but also for its content of organic matter, inorganic nitrogen and phosphorus, and occasionally metals too (Wei et al., 2019) (Albero et al., 2018) (Wang et al., 2016). Thus, these compounds may enter water bodies and the food chain. Antibiotics are particularly relevant because of their potential toxicity to microorganisms living in the environment and the appearance of bacterial resistance genes.

Algae and their consortia with bacteria act as a detoxification device of the aquatic environment (Torres et al., 2008). Thus, nowadays, different configurations of photobioreactors entailing the growing of microalgae biomass are being assayed to purify farm and urban wastewaters and, particularly, to remove hazardous microcontaminants (Tolboom et al., 2019) (Larsen et al., 2019). The removal capacity of microcontaminants is commonly assessed by the determination of the residues in influents and effluents of the reactors (Villar-Navarro et al., 2018) (López-Serna et al., 2019a) (López-Serna et al., 2019b) (Mata-moros et al., 2015). The analysis of pharmaceutical and veterinary drugs in microalgae biomass has not been described yet, and it would be interesting to have reliable analytical methods in order to know the accumulation of residues in biomass and to understand the removal mechanisms involved in the photobioreactors. Furthermore, the biomass is intended to be used as biofertilizer, animal feed or for the production of value-added products such as carbohydrates and proteins (Lorenzo et al., 2019) (Visca et al., 2017), for which it is recommendable to determine the concentrations of drugs, mainly antibiotics, in the biomass.

As regards the determination of organic microcontaminants in algae (seaweed, plankton and microalgae), the extraction of organochlorine compounds, polybrominated diphenyl ethers and petroleum hydrocarbons with an *n*-hexane/acetone mixture and other low-polarity solvents has been described (Qiu et al., 2017) (Phaneuf et al., 1999) (Siriwong et al., 2008) (Moreno et al., 2007) (Bettinetti et al., 2012) (Kovacic et al., 2018) (Carvalho et al., 1999). Also, some pharmaceuticals bioaccumulated in algae have been determined by subjecting the sample to hydrolysis with NaOH (Ali et al., 2018) or after treatment with dichloromethane/methanol to achieve the cell lysis (Kiki et al., 2020). Pharmaceuticals, and mainly antibiotics, are compounds with a relatively high polarity and, consequently, their extraction from solid samples is often performed by shaking with water-rich solvent mixtures, as in the case of the sample preparation procedures commonly applied to the analysis of veterinary drugs in sludge, soil and manure samples (Li et al., 2017) (Iranzo et al., 2018) (Van den Meersche et al., 2016) (Ho et al., 2012) (Huang et al., 2013). These polar extraction solvents should be appropriated for algae biomass, too.

On the other hand, the QuEChERS (Quick Easy Cheap Effective Rugged Safe) sample preparation was initially developed to analyze pesticides at trace levels in fruits and vegetables with high content of moisture. Nowadays, the application scope of this technique has been widespread regarding both the physico-chemical properties of the target analytes and the nature of the samples which can be of different

consistency and complexity. The simplicity of the procedure, its quickness as well as the minimized use of reagents and solvents have favored its spreading and popularity. Parameters such as the extraction solvent, solvent/water ratio and clean-up adsorbent have been selected in each particular case to improve the performance of the extraction procedure (Perestrelo et al., 2019) (Santana-Mayor et al., 2019) (Lopes et al., 2012) (Wang et al., 2019).

Hence two sample preparation procedures have been assayed for the multiresidue determination of 20 piggeries-used veterinary drugs, mainly antibiotics, in microalgae biomass (and their associated bacteria consortium) grown in photobioreactors fed with swine manure. A conventional QuEChERS methodology has been evaluated after devising an experimental design to test the influence of operation parameters such as pH, primary secondary amine (PSA) amount and ethylenediaminetetraacetic acid (EDTA) presence among others. The analytical characteristics of this method have been compared with those achieved by a Solid-liquid extraction (SLE) assisted by ultrasound technology followed by solid-phase extraction (SPE), modifying a procedure for the analysis of veterinary drugs in the solid phase of pig manure (Argüeso et al., 2021). Analytes in the extracts were determined by ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry in tandem (MS/MS) by using a triple quadrupole system. The proposed SLE method was applied to determine, for the first time, the concentrations of veterinary drugs in microalgae biomass from photobioreactors designed for the treatment of piggery wastewaters. The potential adsorption of the drugs contained in the liquid phase of the photobioreactor on the biomass is discussed.

2. Experimental

2.1. Material and reagents

Twenty veterinary drug standards of high purity grade (>95%) were purchased from LGC Standards (Barcelona, Spain) and Sigma-Aldrich (Tres Cantos, Madrid, Spain). They included antibiotics of different chemical families, in addition to dexamethasone, progesterone and fenbendazole (Table SM1 in supplementary material). Individual stock solutions (1 g L⁻¹) were prepared in methanol (MeOH), except for fluoroquinolones which were dissolved in a H₂O/MeOH mixture (1:1, v/v) containing 0.2% (v/v) hydrochloric acid (HCl). Mixture stock solutions and work solutions were subsequently prepared in MeOH and stored at -80 °C until they were employed.

Ultrapure water (pH 6) was generated by a Milli-Q Advantage Ultrapure Water purification system from Merck Millipore (Billerica, MA, USA). MeOH, acetonitrile (ACN) and formic acid (FA) of analytical grade were supplied by Sigma-Aldrich. HCl (37%), NaOH and EDTA disodium salt dehydrate were obtained from Sigma-Aldrich. EDTA was dissolved in H₂O at a concentration of 1 g per 100 mL. PSA, alumina and PTFE disposable syringe filter units, 0.70, 0.45 and 0.22 μm pore-size, were purchased from Scharlab (Barcelona, Spain). Reagent grade anhydrous MgSO₄ and NaCl were obtained from Panreac (Barcelona, Spain). Oasis HLB cartridges (60 mg, 3 mL) for SPE were acquired from Waters (Milford, MA, USA).

A vortex mixer was obtained from Thermo Scientific (Waltham, MA, USA), a PK120 centrifuge from ALC (Winchester, VA, USA), an Univeba ultrasonic bath from Selecta (Barcelona, Spain), an N-Evap 11,250 evaporation system from Organomation (Berlin, MA, USA) and nitrogen gas (99.999% minimum purity) from Carbueros Metálicos (Barcelona, Spain).

2.2. Spiking of microalgae samples

Fresh microalgae samples were collected from open pond photobioreactors fed with swine manure diluted at 5% with H₂O, they were operated in the experimental station "Las Palmerillas" (El Ejido, Almería, Spain). *Chlorella* sp. and *Scenedesmus* sp. were the most abundant

microalgae, their cell percentage was about 80% with respect the total microalgae population. Samples were isolated by centrifugation, lyophilized (moisture 78%) and kept under $-5\text{ }^{\circ}\text{C}$ until use. Then, an amount of 0.25 or 0.30 g of powdered lyophilized algae was spiked with a volume (50–500 μL) of drug solution in MeOH at appropriated concentration to carry out matrix-standard calibrations and other assays. Spiked samples were allowed to stand at room temperature and darkness for 12 h to evaporate the organic solvent and to embed the analytes in sample. Blanks of microalgae samples were also analyzed.

2.3. QuEChERS sample preparation

Operation parameters such as pH, EDTA presence, ACN/ H_2O ratio and amounts of PSA and MgSO_4 added in the dispersive-SPE step, were evaluated for their influence in the performance of the method. The final proposed method was the following: Lyophilized microalgae sample (0.25 g), placed into a centrifuge tube coated with aluminum foil to avoid exposure to light, was mixed with 4 mL of H_2O (pH 6), 5 mL of ACN, and 0.5 mL of 1% (w/v) EDTA. The tube was closed and shaken for 1 min using a vortex mixer. Then, the ionic strength was increased by the addition of 1 g of NaCl and 2 g of anhydrous MgSO_4 (dehydrating); the mixture was shaken again in the mixer for another 1 min, and after that the phases were separated by centrifugation at 5000 rpm and $4\text{ }^{\circ}\text{C}$ for 8 min. A volume of 3.2 mL of the organic upper layer (ACN) was collected and poured into a microtube containing 200 mg of MgSO_4 and 70 mg of activated PSA (PSA was activated by heating at $120\text{ }^{\circ}\text{C}$ for 24 h and cooled in a desiccator). Once more the mixture was shaken in the vortex for 1 min and the extract in ACN was isolated from the solid phase by centrifugation (5000 rpm, 8 min, $4\text{ }^{\circ}\text{C}$). A volume of 3 mL was transferred to a vial and evaporated at room temperature under a nitrogen stream. Then, the dry residue was dissolved, with the help of a vortex mixer, in 1 mL of $\text{H}_2\text{O}/\text{MeOH}$ (95:5, v/v) containing 0.1% (v/v) of FA. Finally, the extract was filtered through a 0.22 μm pore-size PTFE filter before its injection in the UHPLC system.

2.4. Solid-liquid extraction followed by solid-phase extraction

Lyophilized microalgae sample (0.30 g) was mixed with 0.30 g of activated PSA and 10 mL of 90:10 (v/v) $\text{H}_2\text{O}/\text{MeOH}$ extractant; the suitable adsorbent for dispersive-SPE clean-up was selected after comparing the use of PSA and alumina. The mixture was first homogenized in a vortex for 1 min and then, the drugs were extracted by ultrasonic shaking (50 w, 60 Hz) for 15 min at room temperature. Afterwards, the liquid phase was separated by centrifugation at 10,000 rpm and $4\text{ }^{\circ}\text{C}$ for 8 min and a volume of 6 mL was collected. A 15 mL volume of the $\text{H}_2\text{O}/\text{MeOH}$ mixture was added to the sample and the above-described procedure was repeated, but 9 mL of supernatant were collected after centrifugation. The two collected portions were combined and filtered through 0.7 μm pore-size PTFE. Subsequently, 5 mL of 1% EDTA were added and the resulting extract was made up to 100 mL with ultrapure H_2O .

An Oasis HLB cartridge was conditioned by successive elution of 3 mL of MeOH and 3 mL of H_2O . Next, the diluted extract (100 mL) was eluted through the cartridge at about 5 mL min^{-1} using a suction system. Then, 3 mL of $\text{H}_2\text{O}/\text{MeOH}$ (95:5, v/v) mixture were poured into the cartridge, eluted and discarded to clean-up the extract. Afterwards, the cartridge was dried under air flow for about 10 min and the extract was eluted with 2 aliquots of ACN (3 mL each one). The resulting organic solution was evaporated under a gentle nitrogen stream and reconstituted in 1 mL of 0.1% (v/v) FA in a mixture $\text{H}_2\text{O}/\text{MeOH}$ (95:5, v/v) with the help of a vortex. Finally, the extract was filtered through a 0.22 μm pore-size PTFE for its subsequent chromatographic analysis.

2.5. Analysis of drugs in effluent from photobioreactors

The analysis of the content of veterinary drugs in the purified

effluent from the bioreactor was based on a previously published procedure (López-Serna et al., 2019a). Briefly, a volume of 100 mL of filtered sample (0.45 μm) was spiked with 5 mL of 1% EDTA and the mixture was subjected to SPE on Oasis HLB cartridges, evaporation and re-dissolution as stated in the above section 2.4.

2.6. Determination by UHPLC-MS/MS

The chromatographic separation was carried out using an Exion LC AD equipment from AB Sciex (Framingham, MA, USA) and a reversed-phase column EVO C18 (50 \times 2.1 i.d. mm, 1.7 μm particle size) from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 0.1% (v/v) FA in H_2O (A) and 0.1% (v/v) formic acid in MeOH (B). The elution started at 5% of B during 1 min and, then, was linearly increased to 95% B in 2 min and kept isocratic for 3 min. Re-equilibration time was 4 min. The column was thermostated at $40\text{ }^{\circ}\text{C}$, the mobile phase flow rate was 0.5 mL min^{-1} and the injection volume was 10 μL .

MS/MS detection was performed by the triple quadrupole 6500+ from AB Sciex. Analyses were done in positive electrospray ionization, working on selected reaction monitoring (SRM) mode. Nitrogen was used as nebulizing gas (45 units), drying gas (45 units, $450\text{ }^{\circ}\text{C}$) and curtain gas (35 units). For each compound, SRM transitions between the precursor ion and the two most abundant fragment ions were monitored. The list of transitions and particular instrumental conditions to monitor them is shown as supplementary material (Table SM2). Data acquisition and peak integration were done via the Analyst and OS softwares (AB Sciex), respectively. Peak areas were used for quantification.

3. Results and discussion

3.1. QuEChERS. Operation parameters

Five operation parameters of the QuEChERS procedure were tested by a two-level half fractional factorial design with three central points. The factors (and levels) were: pH of H_2O (3, 8), ACN/ H_2O solvent ratio (0.5, 2, v/v), EDTA concentration in the aqueous phase (0, 0.11%, v/v), amounts of PSA (70, 200 mg) and MgSO_4 (200, 400 mg). The signal-to-noise (s/n) ratio of each drug was chosen as response variable. Tetracycline, oxytetracycline, doxycycline, marbofloxacin and ciprofloxacin were not detected. Tables SM3 and SM4 show the experimental domain and results, respectively.

The ACN/ H_2O ratio and PSA amount were found to be statistically significant ($p < 0.05$) although their influence was different depending on the compound. The MgSO_4 mass was not a significant factor and an acidic pH was preferable for the extraction. The EDTA concentration in H_2O only significantly ($p < 0.05$) increased the s/n ratio of florfenicol, but it did not affect the sulfonamides and tetracyclines for which the EDTA addition is sometimes advised (López-Serna et al., 2011) (Feng et al., 2016) (Conde-Cid et al., 2018) (Pan and Chu, 2017) (López-Serna et al., 2019a). The selected extraction parameters were: addition of H_2O at pH 6, EDTA (0.11%), 200 mg of MgSO_4 , 70 mg of PSA, and use of a ACN/ H_2O ratio of 1.25 (4 mL H_2O , 5 mL ACN). A discussion more detailed of the results can be found in the supplementary material.

3.2. QuEChERS methodology. Analytical characteristics. Influence of the matrix

A matrix-standard calibration was done by spiking aliquots of microalgae sample with increasing amounts of drugs to obtain concentrations ranging from 1.6 to 4000 ng g^{-1} . At the same time a blank was also analyzed to apply a standard-addition calibration method in order to calculate the concentration of the compounds identified in the real microalgae sample used in the assays. These results (Table SM5) were: trimethoprim 72 ng g^{-1} , danofloxacin 291 ng g^{-1} and tiamulin 243 ng g^{-1} . Table 1 shows the linearity ranges of the matrix-standard calibration graphs drawn against the total (own plus added) concentration in

Table 1

Analytical parameters for the matrix-standard calibration obtained by the QuEChERS sample preparation. Relative and absolute recoveries, and precisions (expressed as relative standard deviation, RSD) obtained after spiking microalgae samples at 80 and 2400 ng g⁻¹ (n = 5).

Drug	Matrix-standard calibration data						80 ng g ⁻¹			2400 ng g ⁻¹		
	R ²	Slope	Intercept	Linearity range (ng g ⁻¹)	LOD (ng g ⁻¹)	LOQ (ng g ⁻¹)	Relative recoveries (in %)	Absolute recoveries (in %)	RSD (in %)	Relative recoveries (in %)	Absolute recoveries (in %)	RSD (in %)
Penicillin G	0.99	438,193	-66961941	80–2960	7	16	104	25	7	97	22	22
Enrofloxacin	0.96	1874993	-325782359	200–4000	38	94	-	-	-	77	1	36
Danofloxacin ^a	0.96	1240879	-96,478,997	371–3251	47	155	133	1	13	99	3	26
Sulfadiazine	0.94	22,066,749	170,219,842	16–4000	6	15	116	-	22	93	-	32
Sulfathiazole	0.98	14,587,839	-665,472,475	16–4000	5	16	121	8	33	92	6	23
Sulfamethizole	0.97	4,716,646	-319,116,735	16–4000	5	16	151	3	26	84	2	25
Sulfadimidine	0.99	47,815,068	785,005,034	16–4000	4	13	136	19	25	90	9	8
Sulfamethoxazole	0.97	16,311,000	-910,222,157	16–4000	4	12	166	7	31	116	5	18
Tylosin	0.99	2,624,339	-109,260,406	16–2960	6	15	156	9	3	124	11	14
Tiamulin ^a	0.97	127,979,586	7,746,277,059	259–4243	3	11	81	10	18	103	12	9
Trimethoprim ^a	0.99	102,342,265	2,759,417,432	74–4072	11	36	76	24	9	104	25	3
Florfenicol	0.990	8,104,739	-29,518,886	16–4000	6	16	118	54	8	90	43	4
Fenbendazole	0.97	43,622,517	3,673,481,884	16–4000	4	12	120	9	21	119	7	25
Dexamethasone	0.98	5,430,204	96,298,341	1.6–2960	0.3	1.3	132	16	8	104	11	8
Progesterone	0.97	1,582,439	14,134,652	80–2960	9	27	100	2	27	102	2	14

–: without data.

^a Compounds detected in the sample.

sample. Linearity ranges covered, in general, concentrations from 16 to 3000–4000 ng g⁻¹; low concentration standards did not adjust to the linear fittings in some cases. Limits of detection (LODs) and limits of quantification (LOQs) were established as three and ten times the s/n ratio; danofloxacin and enrofloxacin showed the highest LOD, about 40 ng g⁻¹. Table 1 also lists the relative and absolute recoveries achieved at two spiked concentrations, 80 and 2400 ng g⁻¹. Relative recoveries were calculated against the matrix-matched calibration while absolute recoveries were calculated from a conventional calibration done with standards dissolved in mobile phase. At 2400 ng g⁻¹ recoveries were close to 100% (they comprise 77–124% values) as it was expected but at 80 ng g⁻¹, near the LOQs, many recoveries were rather far from 100%. Absolute recoveries were mostly lower than 20%. Precision, expressed as relative standard deviation varied from 3 to 36% (n = 5).

An external-standard calibration was also performed by spiking the ACN/H₂O mixture without microalgae sample to test the influence of the matrix in the measured peak areas. The microalgae matrix decreased

perceptibly the responses of most of the analytes in comparison with those measured in absence of matrix. This fact can be outlined from the graphical representation of the peak areas obtained in the matrix-standard calibration against those obtained in the external-standard calibration. As the calibration concentrations were similar, linear fitting slopes were expected to be around 1 in absence of matrix effect (Table SM6, Fig. SM1). For all compounds, except tylosin, a signal reduction was observed, which is coherent with a possible ion-suppression phenomenon in the electrospray interface, and perhaps with a saturation of the ACN phase with co-extracted matrix compounds.

3.3. Solid-liquid extraction methodology. Analytical characteristics

The SLE procedure assayed to analyze drug residues in algae biomass was developed to monitor them in the solid phase of pig manure and it entailed the use of alumina as clean-up agent, which was added to the H₂O/MeOH mixture in the first extraction (Argüeso et al., 2021). Thus,

Table 2

Signal-to-noise ratios (mean and 95% confidence interval, n = 7) obtained with different extraction procedures at 1000 ng g⁻¹ for each drug: SLE in presence of alumina or PSA (with and without the addition of EDTA) as adsorbents, and QuEChERS.

	SLE PSA		SLE PSA and EDTA		SLE Alumina		QuEChERS	
	s/n ratio mean	Confidence interval	s/n ratio mean	Confidence interval	s/n ratio mean	Confidence interval	s/n ratio mean	Confidence interval
Penicillin G	39.4	8.6	45.2	4.0	51.7	17.3	108.5	8.1
Doxycycline	81.5	21.8	180.3	39.6	99.1	11.2	-	-
Tetracycline	20.8	10.2	38.2	17.9	26.5	6.6	-	-
Oxytetracycline	21.3	9.0	22.1	8.1	51.8	10	-	-
Marbofloxacin	170.9	19.3	60.0	117.3	97.8	81.0	-	-
Ciprofloxacin	218.0	32.9	49.1	65.3	176.1	49.5	-	-
Enrofloxacin	406.5	38.6	1100.8	208.8	400.6	140.8	25.3	2.9
Danofloxacin	141.1	147.7	1539.6	487.6	334.6	168.5	12.0	5.1
Sulfadiazine	432.7	43.2	462.6	141.0	466.3	90.8	556.8	79.8
Sulfathiazole	422.8	55.7	515.8	59.2	441.0	35.1	519.7	95.5
Sulfamethizole	161.2	31.7	137.3	26.1	97.0	12.5	224.5	25.7
Sulfadimidine	34.5	26.7	32.6	5.3	19.6	2.0	91.9	9.3
Sulfamethoxazole	29.7	3.2	25.5	7.8	24.7	3.5	85.7	7.7
Tylosin	39.3	14.7	145.2	43.6	74.8	22.8	172.7	29.0
Tiamulin	1059.1	610.8	1278.0	495.8	990.6	182.8	1827.7	337.0
Trimethoprim	1549.0	211.6	1502.7	184.8	1630.9	159.7	1360.4	135.0
Florfenicol	17.3	5.5	21.2	6.9	16.9	5.1	22.5	3.5
Fenbendazole	22.8	6.6	18.5	3.6	77.6	27.4	67.6	16.2
Dexamethasone	42.9	9.7	51.2	10.6	40.3	5.1	52.8	9.8
Progesterone	63.6	7.7	17.9	3.8	38.6	8.0	24.5	17.1

–: not detected.

besides alumina the use of PSA, in presence or absence of EDTA (0.1%, w/v), was initially considered with the purpose of knowing which of them improved the responses of the analytes. Table 2 (Fig. SM2) shows the s/n ratio means and their 95% confidence intervals ($n = 7$) obtained after spiking at a concentration of 1000 ng g^{-1} . Adopting the criterion that two means are different if their confidence intervals are not overlapping it can be assumed that the s/n ratios keep constant for 10 compounds when the extraction conditions were changed; the use of alumina as adsorbent was the best option for only 2 compounds while the addition of PSA without EDTA was favorable for 4 compounds and with EDTA for other 4 compounds. Finally, PSA, without adding EDTA, was chosen to perform a dispersive SPE as clean-up mode in the SLE. In addition, the s/n ratio results of the QuEChERS methodology are included in Table 2, too. This latter approach supplied higher s/n ratios than the SLE method for compounds such as penicillin, sulfamethizole, sulfamethoxazole and tiamulin. The chromatograms obtained by both sample preparation procedures were similar, which was foreseeable according to the selectivity of the detection system (Fig. SM3).

Table 3 shows the analytical characteristics of a matrix-standard calibration done by the SLE method after spiking samples with concentrations ranging between 5 and 3344 ng g^{-1} . A concentration of 271 ng g^{-1} of progesterone was determined in this different biomass sample by an earlier standard-addition calibration (Table SM7). SLE procedure was able to analyze all intended compounds, in particular the cyclines tetracycline, oxytetracycline and doxycycline and the quinolones ciprofloxacin and marbofloxacin, which were not monitored by the QuEChERS method. Regarding the LODs, they ranged between 0.2 and 42 ng g^{-1} for the SLE method and were similar or slightly better with respect to the QuEChERS procedure for many compounds whenever the comparison was feasible. In particular, danofloxacin, enrofloxacin, sulfadimidine and trimethoprim had LODs about 10-50-fold lower. Consequently, the linear dynamic range for SLE method was lengthened towards lower concentrations. The exception was dexamethasone whose LOD by the QuEChERS method was ten-fold lower. The relative recoveries achieved by the SLE procedure differed more from the expected value, 100%, in comparison with those from QuEChERS procedure. This agreed with the somewhat worse precision observed in the SLE (RSD 6–46%, $n = 5$). Absolute recoveries were, in general, somewhat higher than those calculated for the QuEChERS approach, they were now higher than 10% except for 4 compounds. There are no similar methods in the bibliography for the analysis of pharmaceuticals in algae samples

in order to compare the efficiency of the SLE method.

Furthermore, a positive significant statistical correlation ($r = 0.767$, $p = 0.0014$) has been found between the absolute recoveries of the SLE and QuEChERS (2400 ng g^{-1}) assays considering the analytes detected by both procedures (Fig. SM4). And the same for SLE and QuEChERS (80 ng g^{-1}) recoveries ($r = 0.730$, $p = 0.0046$). The higher the absolute recovery for SLE method, the higher the absolute recovery for QuEChERS methodology. An unequivocal explanation of this behavior has not been found because the recoveries depend on the ion suppression in the electrospray ionization and the capacity of extraction of both sample preparations. However, assuming that the influence of the matrix in the ion suppression is similar for both extraction types it could be ascertained that the physico-chemical properties of these analytes are the main factor influencing the capacity of extraction. At this respect, a statistical correlation with the octanol/water partition coefficients has not been established.

3.4. Analysis of drugs in biomass and effluent from a photobioreactor

The SLE method with PSA as adsorbent was applied to the determination of drugs in two samples of algae biomass collected in the months of October and November 2018 from a photobioreactor fed with pig manure. The photobioreactor was operated under recirculation and the hydraulic retention time was 3 days. Some characteristics and operation details of the photobioreactor can be found in the supplementary material. Photobioreactor effluent containing the biomass was sampled and the biomass was separated from the liquid effluent by centrifugation, and kept lyophilized until analysis. Liquid effluent was also analyzed to determine the same drugs. In both cases quantification was performed by a standard-addition calibration. Table 4 shows the drugs and concentrations found in biomass and effluent. Table SM8 lists the detection and quantification limits estimated for the effluent samples.

Nine drugs were detected in biomass in relatively low concentrations, close to the LOQs of the analytical method. Oxytetracycline, enrofloxacin, danofloxacin and tiamulin were detected in the two assays while ciprofloxacin, sulfadiazine, sulfadimidine and tylosin were present in only one of the assays. The oxytetracycline concentration was the highest found, 86 ng g^{-1} (ng per lyophilized g), roughly equivalent to 19 ng of drug per fresh g. However, it should be pointed out here that some major concentrations, about $240\text{--}290 \text{ ng g}^{-1}$, were found for

Table 3

Analytical parameters for the matrix-standard calibration achieved after solid-liquid extraction. Relative and absolute recoveries, and precisions (expressed as relative standard deviation, RSD), obtained after spiking algae samples at 1000 ng g^{-1} ($n = 5$).

	R ²	Slope	Intercept	Linearity range (ng g ⁻¹)	LOD (ng g ⁻¹)	LOQ (ng g ⁻¹)	Relative recoveries (in %)	Absolute recoveries (in %)	RSD (in %)
Penicillin G	0.96	274	32,317	25–3344	8	25	142	37	18
Doxycycline	0.996	3054	-270,302	85–2281	42	85	109	4	14
Tetracycline	0.95	6950	98,098	29–2833	9	22	96	15	46
Oxytetracycline	0.97	5137	-43597	16–2833	9	16	127	38	14
Marbofloxacin	0.92	3398	54,216	85–3344	32	85	131	31	26
Ciprofloxacin	0.998	2127	145,062	12–3344	3	9	141	21	35
Enrofloxacin	0.98	19,029	62,901	5–3344	0.8	3	128	17	24
Danofloxacin	0.996	9369	-10069	14–2281	4	14	115	4	27
Sulfadiazine	0.98	24,949	-326,811	5–3344	1	4	110	40	22
Sulfathiazole	0.96	1508	341,917	32–3344	11	32	126	16	9
Sulfamethizole	0.98	18,820	-1143,622	12–3267	4	12	66	11	22
Sulfadimidine	0.998	70,715	-613,522	5–3344	0.5	2	116	15	22
Sulfamethoxazole	0.97	3088	138,602	12–3444	4	12	135	8	22
Tylosin	0.97	6433	-9356,505	8–3344	3	7	111	15	6
Tiamulin	0.96	32,738	-1464,561	5–3344	2	5	112	3	14
Trimethoprim	0.98	99,412	8,165,886	5–3344	0.2	5	105	51	13
Florfenicol	0.996	8231	-239,689	16–3344	5	16	95	36	9
Fenbendazole	0.97	2604	-149,266	70–3344	6	19	85	10	18
Dexamethasone	0.994	2583	80,721	11–3344	3	11	97	10	9
Progesterone ^a	0.97	211	437	283–3615	17	32	103	2	17

^a Compound detected in the sample.

Table 4

Concentrations of veterinary drugs found in effluents ($\mu\text{g L}^{-1}$) and algae biomass (ng per g of lyophilized biomass) grown in those photobioreactors fed with piggery wastewater. The biomass concentration standard deviation estimated from the standard-addition calibration data is shown in parentheses.

	Assay 1		Assay 2	
	Effluent	Biomass	Effluent	Biomass
Penicillin G	-	-	-	-
Doxycycline	-	-	-	-
Tetracycline	-	-	-	-
Oxytetracycline	-	86 (54)	0.18	16 (106)
Marbofloxacin	-	-	-	-
Ciprofloxacin	-	14 (40)	-	-
Enrofloxacin	-	37 (39)	-	14 (80)
Danofloxacin	0.19	16 (13)	0.38	30 (26)
Sulfadiazine	0.63	-	1.44	<5
Sulfathiazole	-	-	-	-
Sulfamethizole	-	-	-	-
Sulfadimidine	0.03	-	0.02	<5
Sulfamethoxazole	0.04	-	-	-
Tylosin	0.05	-	0.13	9 (34)
Tiamulin	0.18	18 (25)	-	75 (63)
Trimethoprim	-	-	-	-
Florfenicol	0.13	-	-	-
Fenbendazole	0.05	-	-	-
Dexamethasone	-	-	-	-
Progesterone	-	-	-	<32

-: not detected.

progesterone, tiamulin and danofloxacin in the biomass samples used to develop an analytical method, in addition to trimethoprim (72 ng g^{-1}). In six sampling points drugs were detected simultaneously in the biomass and in the effluent that contained it. There are no previously published comparable data about biomass drug concentrations to discuss these results.

Tetracyclines, sulfonamides and fluoroquinolones are commonly found in manure worldwide (Wohde et al., 2016), and tetracyclines, penicillins, sulfonamides and macrolides are the most sold antimicrobial classes for food-producing animals in the European Union in 2018. In Spain, in this year, 1750 tonnes of antibiotic drugs were sold for this purpose, of which about 540 tonnes were penicillins, 490 tonnes were tetracyclines, 74 were sulfonamides, 44 were fluoroquinolones, other 44 were amphenicols, 39 were for pleuromutilins and 13 were of trimethoprim. According to the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC, 2020) about 12% of these tonnes were destined for the pig production. The medication is mainly administrated in the suckling and post-weaning periods although there is no uniformity of behavior among the producers (Lekagul et al., 2019). The use of antibiotics for growth promotion and disease prevention without diagnosis is completely restricted in European Union and other countries but they are still allowed to these aims in exporting countries such as China and Brazil (Cheng et al., 2020). Thus, it seems logical to find antibiotics of these families in the analysis of manure or photobioreactor samples; penicillins are relatively labile in aqueous medium which implies that they are not frequently detected. On the other hand, fenbendazole is an authorized anthelmintic already detected before in pig manure while progesterone is a natural hormone that, in principle, is not used in piggeries as veterinary drug (Argüeso et al., 2021).

Fig. 1 depicts the concentration in algae biomass against a) the effluent concentration and b) the octanol/water partition coefficient (log P) of the drug. The LOQ value was used when the drug was found in a concentration lower than its LOQ. Excluding the sulfadiazine and oxytetracycline points marked in Fig. 1, in the first and second graph respectively, a quasi linear fitting can be observed in the two representations. In fact, there was a positive significant statistical correlation between the biomass and effluent concentrations ($r = 0.978$, $p = 0.0007$) and the biomass concentration against the drug log P value ($r = 0.696$, $p = 0.012$). These findings suggest that the accumulation of drugs

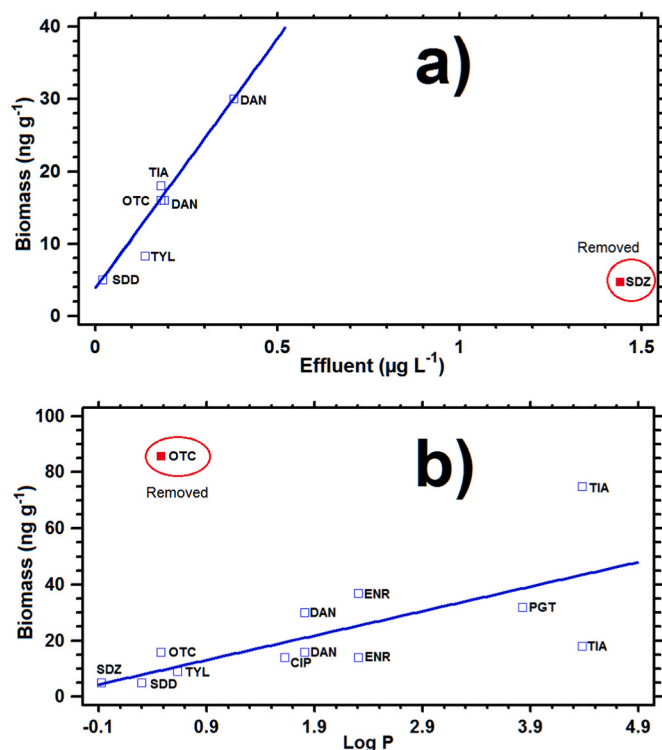


Fig. 1. Representation of the concentrations found in biomass against a) effluent concentrations and b) logarithm of partition coefficient. Removed data for the statistical correlations are circled. See Table SM1 for abbreviations.

in the algae biomass increases, in general terms, as the drug concentration increases in the effluent as it would be expected. In the same way, it is inferred that lipophilic drugs tend to be retained for the microalgae. Furthermore, sulfadiazine, sulfadimidine, sulfamethoxazole and tylosin were observed exclusively in the effluent of some assays which could be consistent with their log P values lower than 1 (Table SM1) and, thus, a higher affinity for the aqueous phase. In opposition, florfenicol and fenbendazole have log P values higher than 1 (1.2 and 2.4, respectively) and they were detected only in the aqueous phase, too. On the other hand, ciprofloxacin, enrofloxacin, tiamulin and progesterone were detected exclusively in the biomass on other assays which agrees with their high log P values (1.6, 2.3, 4.4 and 3.8, respectively).

The adsorption mechanism by itself is not able to explain all the experimental data but it has proven to be a relevant phenomenon in the process of purification of piggery wastewaters according to the relationships established with log P. The adsorption onto algae biomass has been proposed as a relevant way of removing some antibiotics from an aqueous medium after laboratory-scale assays; thus, tetracycline and ciprofloxacin tend to be adsorbed unlike sulfonamides which are slightly removed by this mechanism (de Godos et al., 2012) (Hom-Diaz et al., 2017) (Kiki et al., 2020) (Zambrano et al., 2021). Obviously, several other mechanisms such as photodegradation, chemical degradation, biodegradation and bioaccumulation are involved in the removal of drugs from farm wastewaters by using photobioreactors with microalgae and their influence should also be taken into account.

4. Conclusions

The amount of PSA added as clean-up adsorbent was the main operation parameter affecting the performance of the QuEChERS sample preparation procedure to determine drugs in microalgae biomass. The amount of MgSO_4 added as dehydrating agent was not a relevant factor, and the presence of EDTA only influenced the analysis of florfenicol. The influence of the matrix in the QuEChERS procedure turned out to be

prominent and a satisfactory explanation of the narrow scope of this procedure has not been found.

SLE assisted by ultrasound followed by SPE is required to analyze cyclines (tetracycline, oxytetracycline, doxycycline) in lyophilized microalgae samples and to include a major number of fluoroquinolones (ciprofloxacin, marbofloxacin) in the multiresidue method. The use of PSA as adsorbent increased the number of drugs with high chromatographic responses. SLE procedure provided better LODs for some drugs. A positive correlation has been observed for the absolute recoveries calculated by both extraction procedures.

The concentration of drugs in microalgae biomass is relatively low and in most cases it is directly related to the drug concentration in the liquid phase of the effluent. The log P value seems to explain the distribution of some drugs between the liquid phase of the effluent and the biomass in suspension. The adsorption mechanism would have an important role in the removal process of drugs from piggery wastewaters.

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.2021.132076>.

Credit author statement

Rebeca López-Serna: Methodology, Validation, Data curation, Writing – original draft, Silvia Bolado: Funding acquisition, Project administration, Rubén Irusta: Investigation, Resources, Juan J. Jiménez: Conceptualization, Supervision, Formal analysis, Writing – review & editing

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