# Determination of contaminants of emerging concern in raw pig manure as a whole. Difference with the analysis of solid and liquid phases separately.

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The content of veterinary drugs in manure is usually estimated by the amount of residues determined in its solid or liquid phase, individually, which previously requires a separation step. As alternative, a multiresidue method for the analysis of 48 veterinary drugs and other contaminants of emerging concern (CECs) in swine raw manure as a whole has been developped and in-house validated in this work. The impact of several experimental factors during the ultrasound assisted extraction was assessed. Hence, the use of alumina seemed to especially decrease the matrix effect and improved the overall recovery of drugs, mainly those with high octanol-water partition coefficient. CECs in the extracts were analyzed by ultra-high performance liquid chromatography coupled to mass spectrometry in tandem. A standard addition-matrix matched calibration was used for quantification. The method application to two related samples (raw manure and farm centrifuged raw manure) from a facility revealed that the concentrations of CECs determined in the raw manure by the comprehensive methodology were higher than those calculated by adding the concentrations measured in the solid and liquid phases, separately. This was attributed to the loss of CECs adsorbed to fine particles in suspension during the sample preparation procedure of the liquid-phase. Furthermore, the decrease of residues in the raw manure when this is centrifuged in the farm to yield compost is shown.

## 1. Introduction

Swine raw manure (RM) generated in pig farms is an aqueous slurry made of pig urine and droppings, food scraps and pigpen-floor straw, resulting after the farm routine washing pigpen protocols. Most manures have up to 90% of water, 2-4% of dry matter, 1-3% organic matter, 0.2-0.4% total nitrogen, 0.07-0.10% total phosphorus and 0.09-0.14% potassium, among other components[1].

Pig manure solid phase (MSP) and manure liquid phase (MLP) are routinely spread as fertilizer. However, the intensification of pig industries and the high polluting power of the generated slurry are producing numerous environmental problems. The use of drugs in pigs as a veterinary resource implies another limitation when using and storing this slurry. After their administration to animals, it is estimated that between 30-90% of the drugs are excreted in their original form, unchanged, or as an active metabolite of the parent species. In fact, concentrations of antibiotics up to 200 mg $Kg^{-1}$ or mg $L^{-1}$ have been reported in swine manure[2], although antibiotic concentrations in this kind of manure are more commonly between 1 and 10 mg $Kg^{-1}$ or mg $L^{-1}$. Hence, if untreated slurry is spread onto farming land as fertilizer, antibiotics could accumulate in the soil and/or be transferred to the underlying groundwater. Eventually, they could enter in the food chain through crops and/or drinking water. Additionally, it could entail important issues related with the development of resistant bacteria[3], among other environmental problems.

Antibiotics, other pharmaceuticals and, in general, any micropollutants which are not currently included in the systematic monitoring programs are known as contaminants of emerging concern[4]. They are biologically active substances in many occasions, designed to be resistant to degradation, and thus, scientific community has shown concern about their toxicity on untargeted individuals[5].

There are a wide variety of sample preparation methods for the determination of veterinary drugs both in MSP and MLP samples. The extraction of analytes of interest in wastewaters is usually performed by solid phase extraction (SPE)[6,7,8] whereas for solid samples a solid-liquid extraction (SLE), with a wide variety of solvents and additives, is carried out first in most revised publications. This SLE can normally be assisted by ultrasounds[9,10], pressurization of the liquid[11] or microwaves[12,13]. After extraction, a clean-up stage of the resulting supernatant liquid is carried out by SPE extraction[14]. Alternative extraction methodologies such as QuEChERS approach[15] have been assayed with the objective of improving the simplicity, sensitivity and selectivity of the analysis[16]. There are no published analytical methods, in our knowledge, to determine the drug content in RM collected from piggeries, without previous separation of the MSP and MLP, although a method involving a solvent extraction with acetonitrile and ethyl acetate has been published for the determination of four

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antibiotics in dairy manure[17]. When the overall content in the slurry must be estimated both phases can be separated and analyzed individually[18]. In addition, the analytical methods are focused in the veterinary drugs used to keep the animal health, only in a work the analysis of a family of CECs (perfluorinated compounds) in poultry manure[19] has been considered.

This work deals with the direct analysis of CECs in the whole pig RM, without any previous sample handling or phase separation, which dramatically decreases the need of disposable resources and the produced residues in the laboratory, while shortening analysis time by around half, if the total amounts of CECs in RM are determined. The proposed method has been in-house validated and includes an ultrasound assisted extraction (UAE) of the RM sample, in combination with dispersive-SPE as clean-up, followed by SPE to enhance the clean-up and concentrate the extract. Some operating parameters of the sample preparation are optimized by an statistical experimental design. Analytes in extracts are determined by ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry in tandem (MS/MS) by a triple quadrupole analyzer (QqQ). A RM sample collected from a farm and a RM sample arising from the low speed centrifugation in the farm of the first sample were analyzed. Furthermore, MSP and MLP have been separated in both RM samples and also analyzed individually to compare the concentration results. Theses assays revealed that the CEC concentrations determined in the direct analysis of the pig RM sample as a whole differed notably from those expected according to the concentrations measured in MSP and MLP.

## 2. Experimental

### 2.1. Standards and reagents

Fifty-seven compounds, mainly human and veterinary drugs such as antibiotics, antiparasitics, hormones, analgesics, lipid regulators and other CECs were initially chosen for their determination in manure samples. The selected veterinary drugs were authorized in piggeries from the European Union while the rest of CECs were microcontaminants usually found in the environmental samples of the region. Standards were purchased of high purity grade, >95% (Supplementary data 1†), and individual stock solutions were done at a concentration range 800-1000 mg $L^{-1}$ in methanol, except for danofloxacin and ciprofloxacin which were dissolved in (1:1) water/methanol. Mixture and work solutions were also prepared in methanol. Solutions were stored at -30 ˚C in darkness until further use.

LC-MS grade methanol 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 2M was acquired from Sigma-Aldrich. Oasis® HLB cartridges (60 mg, 3 mL) were provided by Waters Chromatography. Alumina ($Al_2O_3$) and $Na_2EDTA$ (disodium salt of ethylenediaminetetraacetic acid) were purchased from Panreac, while octadecylsilane (ODS) was purchased from Supelco. $Al_2O_3$ was activated before use by heating at 110 ºC for 24 h.

### 2.2. Collection and treatment of samples

Two raw swine manure samples from a pig farm at Segovia (Castile and Leon, Spain) were freshly collected and transported to our laboratory where it were stored at -30ºC until their treatment. For each RM sample MSP and MLP were separated in the laboratory by centrifugation at 10000 rpm for 10 min. One RM sample was the crude manure yielded in the farm. Its amount of solids in suspension, which constitute the MSP, was 7.4% (w/w) and the density of MLP (92.6%, w/w) was 1.02 g $mL^{-1}$. The second one was the previous RM which was subjected to centrifugation in farm (2000 rpm) to produce compost. The MSP of the centrifuged manure constituted now 4.8% (w/w) and the density of its MLP (95.2%, w/w) was 1.01 g $mL^{-1}$.

### 2.3. Sample preparation for raw swine manure analysis

An usual procedure to analyze CECs in wastewaters and sludge matrixes[14,16] was modified and optimized, by using environmentally friendly solvents such as methanol and water. The proposed procedure consists of: 1) The RM slurry is shaken manually to homogenize it and take an aliquot whose pH is set to 3 with HCl 0.5 M. 2) An amount of 1 g is placed in a 50 mL polyethylene Falcon tube. 3) An amount of 0.1 g of activated alumina ($Al_2O_3$) is added for in situ clean-up of the sample. 4) Afterwards, two extraction steps are performed. For the first extraction, 10 mL of (10:90, v/v) water/methanol mixture and 1 mL of EDTA (5%, w/v) solution are added. 5) The mixture is shaken for 1 minute on a Vortex. 6) Sample undergoes UAE for 15 min in a Sonorex Digitex Bandelin Ultrasonic bath at 25ºC. 7) The suspension is centrifuged for 10 minutes at 10000 rpm. 8) A volume of 6 mL of the resulting supernatant liquid is collected, filtered through 0.70 and 0.45 µm pore-size PTFE filters and transferred into a 100 mL flask. 9) For the second extraction, steps 4-8 are repeated but without adding EDTA, adding 15 mL of water/methanol mixture and collecting 9 mL of the resulting supernatant. 10) The extracts from both UAE cycles are combined. Then, 2 mL of 5% EDTA solution are added and the solution is diluted up to 100 mL with water.

This diluted extract was concentrated and cleaned-up by SPE as described elsewhere[20]. 11) Oasis HLB cartridges are conditioned with 3 mL of methanol and 3 mL of $H_2O$. 12) Afterwards, samples are eluted and the cartridges are washed with 3 mL of methanol/water (5:95, v/v). 13) Cartridges are dried with air for approximately 20 minutes and then analytes are eluted with 6 mL of acetonitrile on a vial. 14) The organic solvent is evaporated under $N_2$ in a water bath at a maximum temperature of 30ºC. 15) The extracts are redissolved in 3x0.5 mL of methanol to sweep along the extract from the upper walls of the vial, and evaporated to dryness once more. 16)

Finally, the extracts are reconstituted in 1 mL of 0.1% FA in a 95:5 (v/v) water/methanol mixture and filtered through 0.22 µm pore-size PTFE filter. A scheme of the procedure is shown in Supplementary data 2†.

#### 2.4. Liquid and solid phase sample preparation

CECs in the MSP were determined by a previously described procedure[9] that it is basically similar to that described for RM samples. Briefly, 0.3 g of lyophilized MSP (78-80% moisture) were extracted in presence of alumina with a water/methanol mixture by UAE, then extract was diluted with water and subjected to SPE. The MLP was filtered through 0.70 and 0.45 µm pore-size PTFE filters, successively, 5 mL of MLP were diluted to 100 mL and a SPE procedure similar to that described in the steps 11-16 of section 2.3 was applied.

#### 2.5. UHPLC–MS/MS analysis

Analytes in the extracts were determined by ultra-high performance liquid chromatography coupled to tandem mass spectrometry. Chromatographic separation was carried out in a Sciex Exion UHPLC equipped with a reversed-phase column Kinetex EVO-C18 (2.1 mm × 50 mm, particle size 1.7 µm) from Phenomenex. The mobile phase consisted of 0.1% (v/v) FA in a water/methanol mixture under gradient conditions to perform the separation. The initial methanol percentage was 5% held for 1 minute, after which it was increased linearly up to 95% in 2 minutes, held for 3 minutes. Afterwards, the percentage of methanol was reduced to 5% to reequilibrate the system during 4 minutes. Injection volume was 10 µL and the flow rate was 0.5 mL $min^{-1}$ (at 40ºC). A Sciex 6500+ (QqQ) mass spectrometer equipped with an electrospray ionization (ESI) interface was used as detector in selected reaction monitoring (SRM) mode. Some of the ESI conditions were set as follows: capillary voltage, 5.5 kV; source temperature, 400ºC. $N_2$ was used for curtain gas, ion source gas, and collision gas at a flow rate of 20, 45 and 9 L $min^{-1}$, respectively. To achieve the optimum mass sensitivity/selectivity ratio, the unit resolution was set to the first and third quadrupole. Supplementary data 3† shows the SRMs monitored and other instrumental parameters. Instrument control and data acquisition were performed by the Analyst® software. Peak area integration and data processing was carried out by the SciexOS software.

## 3. Results and discussion

### 3.1. Analytical method development

#### 3.1.1. Selection of clean-up adsorbent

Two adsorbents and their mixture were evaluated as dispersive-SPE clean-up agent in the initial UAE: alumina (0.2 g), ODS (0.2 g) and a ODS-alumina mixture (0.1 g + 0.1 g). Out of the 57 available compounds, amoxicillin, nalidixic acid, bisphenol A, 1,4-benzoquinone, 17-β-estradiol, 4-nonylphenol, sulfapyridine, triclosan and 4-tert-octylphenol were not detected in any extract chromatograms, suggesting that the sample preparation is not useful for these 9 compounds. Peak area mean values (n=7) and most suitable adsorbents are shown in Supplementary data 4† for the 48 analyzed CECs. Significant differences among the peak area means were established by an analysis of variance (ANOVA). Alumina provided the highest responses for 22 compounds while ODS was preferable for 7 compounds; the addition of ODS or alumina, indifferently, was the best alternative for 15 compounds. The use of alumina seems specially advisable for the analysis of CECs with high partition coefficients as shown in a dispersion diagram (Fig. 1). The mix of alumina and ODS did not turn out to be a good choice for any of the CECs. Alumina was the best option for a higher number of analytes, thus, it was selected for the next assays. As regards this selection it is assumed that the best clean-up adsorbent supplies the highest peak areas because the adsorbent minimize the ion suppression phenomenon, favored by the co-extracted matrix, in the ESI interface.

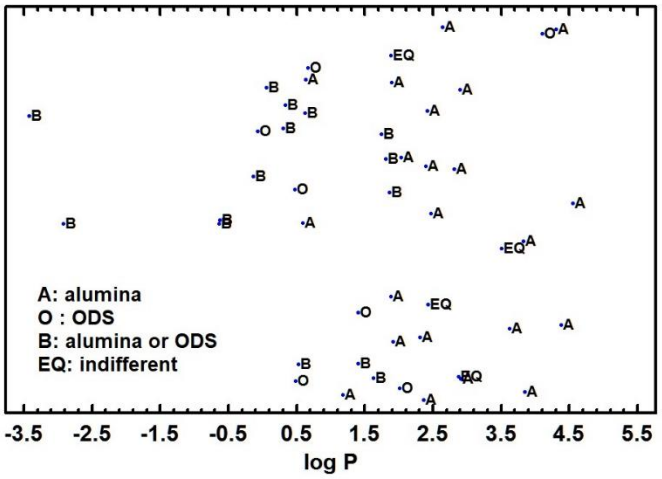


Fig. 1: Representation of the clean-up adsorbent most suitable for the analysis of each CEC in raw manure against the log P value of the CEC.

#### 3.1.2. Optimization of sample preparation parameters

After consulting scientific publications related to similar researches[20-22] four potentially-influential experimental parameters were identified and optimized by a $2^{k-1}$ replicated fractional factorial design with three central points. The sample preparation parameters (and their levels in parenthesis) are: extraction pH (3-8), methanol content in the UAE (10-20 %), alumina amount (0.1-0.3 g) and EDTA content in the UAE (0-0.4%, w/v). The experiments and results are given in Supplementary data 5 and 6†. Data were processed with the Statgraphics Centurion XVII software.

The percentage of methanol only significantly ($p<0.05$) affected the iohexol analysis whose peak area decreased when methanol amount increased. This behavior is understandable due to the very polar nature of iohexol (partition coefficient, log P= -2.921). Therefore, a 10% of methanol was selected as suitable. The pH value turned out to be significant for 21 compounds. For 13 of these compounds the effect was

Table 1: Validation parameters, relative and absolute recoveries (Rec.) and their relative standard deviations (RSDs) for two concentration levels (n=5).

| Compound | $R^2$ | Linear range (ng $g^{-1}$) | Interception | Slope | LOD (ng $g^{-1}$) | LOQ (ng $g^{-1}$) | High concentration level (n=5) | | | | Low concentration level (n=5) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Relative Rec (%) | RSD (%) | Absolute Rec. (%) | RSD (%) | Relative Rec. (%) | RSD (%) | Absolute Rec. (%) | RSD (%) |
| Penicillin G | 0.986 | 7-5137 | 75871 | 1748 | 2 | 7 | 106 | 9 | 24 | 9 | 95 | 13 | 25 | 10 |
| Oxytetracycline | 0.978 | 29-4891 | 104864 | 56889 | 0.3 | 1 | 114 | 10 | 45 | 9 | 86 | 11 | 76 | 4 |
| Doxycycline | 0.994 | 1977-6776 | 1838970 | 61050 | 2 | 6 | 113 | 8 | 30 | 8 | 103 | 10 | 28 | 10 |
| Tetracycline | 0.966 | 8-5127 | -448809 | 54081 | 0.1 | 0.3 | 127 | 6 | 67 | 6 | 97 | 15 | 59 | 12 |
| Marbofloxacin | 0.970 | 137-7791 | 967994 | 71235 | 0.2 | 0.7 | 113 | 9 | 32 | 9 | 88 | 6 | 25 | 6 |
| Enrofloxacin | 0.996 | 135-7945 | 3569959 | 85821 | 2 | 5 | 143 | 10 | 10 | 10 | 93 | 11 | 7 | 10 |
| Danofloxacin | 0.997 | 57-5770 | 1207025 | 101813 | 1 | 3 | 138 | 10 | 11 | 10 | 99 | 16 | 4 | 35 |
| Sulfadiazine | 0.989 | 25-7757 | 60007 | 21524 | 0.2 | 0.7 | 114 | 3 | 10 | 3 | 93 | 6 | 3 | 15 |
| Sulfathiazole | 0.988 | 9-7663 | 9988199 | 88125 | 0.06 | 0.2 | 126 | 5 | 18 | 5 | 86 | 14 | 15 | 12 |
| Sulfamethizole | 0.958 | 9-7663 | -5192907 | 88524 | 0.07 | 0.2 | 140 | 7 | 14 | 7 | 180 | 7 | 14 | 9 |
| Sulfadimidine | 0.972 | 10-7819 | 11389432 | 61681 | 0.1 | 0.3 | 129 | 4 | 10 | 4 | 99 | 7 | 9 | 5 |
| Sulfomethoxazol | 0.999 | 9-7663 | 684288 | 90178 | 0.1 | 0.3 | 115 | 6 | 16 | 7 | 90 | 8 | 9 | 12 |
| Tylosin | 0.954 | 8-6651 | 2518615 | 16131 | 0.07 | 0.2 | 119 | 8 | 39 | 8 | 122 | 16 | 72 | 8 |
| Tiamulin | 0.960 | 92-1612 | 8634495 | 205632 | 1 | 2 | 84 | 6 | 14 | 6 | 109 | 15 | 20 | 14 |
| Apramycin | 0.963 | 9-5547 | 5268656 | 34413 | 0.07 | 0.2 | 155 | 7 | 33 | 7 | 86 | 22 | 30 | 13 |
| Trimethoprim | 0.986 | 319-8051 | 19845456 | 172870 | 0.7 | 2 | 137 | 2 | 14 | 2 | 105 | 11 | 12 | 10 |
| Florphenicol | 0.995 | 374-8170 | -249821 | 12147 | 3 | 10 | 114 | 8 | 25 | 9 | 92 | 13 | 18 | 16 |
| Fenbendazole | 0.973 | 52-5765 | 252251 | 22887 | 1 | 4 | 119 | 5 | 1 | 5 | 113 | 9 | 0.6 | 17 |
| Dexamethasone | 0.981 | 60-7713 | 169795 | 12879 | 3 | 9 | 131 | 7 | 20 | 7 | 117 | 4 | 14 | 6 |
| Progesterone | 0.978 | 10-5781 | 2057775 | 12303 | 0.3 | 1.0 | 138 | 5 | 3 | 5 | 105 | 16 | 4 | 9 |
| Methylparaben | 0.995 | 81-7891 | -382714 | 9852 | 3 | 9 | 129 | 4 | 20 | 4 | 106 | 6 | 16 | 6 |
| Acetaminophen | 0.998 | 109-7841 | 472919 | 18411 | 2 | 6 | 117 | 4 | 3 | 5 | 71 | 9 | 1 | 35 |
| Carbamazepine | 0.983 | 24-390 | 15475939 | 1195846 | 0.05 | 0.2 | -- | -- | 3 | 24 | 105 | 5 | 17 | 5 |
| Propanolol | 0.994 | 213-7060 | 4381222 | 79534 | 0.7 | 2 | 149 | 5 | 12 | 5 | 99 | 9 | 8 | 10 |
| Metronidazole | 0.997 | 10-7741 | 3784698 | 39816 | 0.6 | 2 | 122 | 8 | 6 | 8 | 55 | 31 | 2 | 43 |
| Naproxen | 0.995 | 113-7923 | 852552 | 33468 | 3 | 9 | 124 | 4 | 6 | 4 | 102 | 3 | 1 | 19 |
| Clarithromycin | 0.967 | 10-1905 | 29390998 | 202183 | 0.1 | 0.2 | -- | -- | 33 | 6 | 89 | 15 | 70 | 8 |
| Erythromycin | 0.998 | 9-7663 | -50308 | 6186 | 1 | 3 | 103 | 10 | 26 | 10 | 92 | 11 | 18 | 14 |
| Acetylsalicylic acid | 0.946 | 1907-7741 | 3845 | 10 | 193 | 642 | -- | -- | 0.3 | 2 | -- | -- | 4 | 2 |
| Norfloxacin | 0.974 | 9-7663 | -2398499 | 32116 | 1 | 4 | 99 | 6 | 18 | 7 | 92 | 6 | 4 | 26 |
| Atorvastatin | 0.946 | 4-3612 | -2127320 | 79405 | 0.1 | 0.5 | 108 | 7 | 15 | 7 | 100 | 14 | 21 | 19 |
| Atenolol | 0.996 | 9-7663 | 10313 | 510 | 3 | 9 | 109 | 27 | 0.1 | 23 | 91 | 12 | 0.3 | 2 |
| Caffeine | 0.989 | 51-7743 | 1379606 | 114427 | 0.1 | 0.4 | 137 | 5 | 24 | 5 | 95 | 13 | 11 | 20 |
| Atrazine | 0.979 | 10-1918 | 24473790 | 242998 | 0.1 | 0.4 | -- | -- | 9 | 2 | 98 | 4 | 13 | 3 |
| Iohexol | 0.997 | 9-7624 | 46695 | 575 | 1 | 4 | 107 | 13 | 2 | 13 | 62 | 29 | 1 | 24 |
| DEET | 0.976 | 84-1963 | 10499385 | 277515 | 1 | 2 | -- | -- | 12 | 3 | 98 | 6 | 10 | 10 |
| Ciprofloxacin | 0.992 | 10-7780 | -7051285 | 105627 | 0.4 | 1 | 119 | 12 | 9 | 13 | 98 | 14 | 1 | 32 |
| 17-α-ethinylestradiol | 0.992 | 7704-11532 | -1398791 | 342 | 229 | 764 | -- | -- | 6 | 28 | -- | -- | 5 | 40 |
| Crotamiton | 0.998 | 9-365 | -1001671 | 289112 | 0.1 | 0.3 | -- | -- | 11 | 7 | 91 | 10 | 16 | 12 |
| Estrone | 0.939 | 195-7741 | 1295075 | 1597 | 32 | 106 | 107 | 10 | 6 | 8 | -- | -- | 28 | 31 |
| Ethylparaben | 0.986 | 10-7819 | 213756 | 1690 | 2 | 6 | 126 | 7 | 10 | 8 | 75 | 18 | 3 | 38 |

--: no data

Table 2: Validation parameters, relative and absolute recoveries (Rec.) and their relative standard deviations (RSDs) for two concentration levels (n=5). Continued.

| Compound | $R^2$ | Linear range (ng $g^{-1}$) | Interception | Slope | LOD (ng $g^{-1}$) | LOQ (ng $g^{-1}$) | High concentration level (n=5) | | | | Low concentration level (n=5) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Relative Rec (%) | RSD (%) | Absolute Rec. (%) | RSD (%) | Relative Rec. (%) | RSD (%) | Absolute Rec. (%) | RSD (%) |
| Propylparaben | 0.994 | 10-7819 | 157525 | 1509 | 1 | 3 | 128 | 4 | 6 | 4 | 103 | 8 | 7 | 5 |
| Diclofenac | 0.991 | 51-7321 | 3449 | 1807 | 4 | 13 | 99 | 7 | 3 | 8 | 95 | 13 | 1 | 30 |
| Ibuprofen | 0.991 | 193-7663 | 49061 | 166 | 14 | 46 | 131 | 4 | 9 | 4 | -- | -- | 6 | 51 |
| Salicylic acid | 0.908 | 1907-7741 | 187478283 | 26715 | 572 | 1907 | -- | -- | 8 | 4 | -- | -- | 85 | 9 |
| Clofibric acid | 0.988 | 192-7768 | 1043703 | 19007 | 1 | 4 | 131 | 4 | 14 | 4 | 74 | 6 | 7 | 7 |
| 4-hydroxibenzoic acid | 0.976 | 16001-23647 | -20779652 | 24317 | 25 | 84 | 100 | 6 | 10 | 7 | 92 | 9 | 9 | 10 |
| Gemfibrozil | 0.998 | 9-7663 | -134709 | 5250 | 1 | 3 | 100 | 6 | 5 | 6 | 120 | 20 | 4 | 30 |

--: no data

negative (peak area increased for decreasing pH values) while for the other 8 the effect was positive (peak area increased at high pH values). A pH value of 3 was selected because the number of compounds analyzed under optimum conditions was higher. The amount of alumina had a significant effect for 12 analytes, all of which presented a negative effect except for sulfamethoxazole, dexamethasone, naproxen and clofibric acid, in which cases the effect was positive. Therefore, the addition of 0.1 g of alumina was considered preferable. On the other hand, for those CECS whose optimum pH value was 3, mainly fluoroquinolones which are majorly ionized at this pH, the optimum alumina amount resulted to be 0.1 g (Supplementary data 7†).

The percentage of EDTA affected to 7 CECs, with a positive effect in all cases. So, EDTA was added in the initial extraction at a concentration of 0.4% (w/v). Although most of the methods published for antibiotic analysis include the addition of EDTA before SPE, it should be noted that the presence of EDTA during a SLE step can also improve the analytical response of some compounds, even if they are not antibiotics, as it happens for salicylic acid, atorvastatin and 4-hidroxibenzoic acid in this work. These last also possess functional groups able to form complexes with $Ca^{2+}$ and $Mg^{2+}$ ions incorporated in the MSP and MLP, preventing the extraction of the analytes.

### 3.2. Validation of the method for raw manure samples

As it is not possible to obtain the RM matrix free of CECs residues the detection and quantification of the own compounds of the RM used to validate the method is necessary. Hence, a standard addition, and matrix-matched, calibration was applied. To this aim, aliquots of sample were spiked with increasing CECs amounts and subjected to analysis; two aliquots of sample without spiking (blanks) were included in the calibration, too. Results can be consulted in Supplementary data 8†. Tables 1 and 2 show the parameters of a matrix-matched calibration in which peak areas were plotted against the total concentration of each CEC in sample: sum of the own and spiked concentrations. The concentration levels of the calibrations graphs can be seen in Supplementary data 9†. The coefficients of determination ($R^2$) varied between 0.960 and 0.998 after the sample preparation, except for 6 CECs whose $R^2$ was lower. A linear fitting was considered as good if the $R^2$ value was higher than 0.99, and it was considered as acceptable if the $R^2$ value was only higher than 0.95. For two analytes (atorvastatin and estrone) the regression was on the limit of the acceptability. It must be pointed out that the variation of peak area against the concentration was markedly parabolic towards high concentrations for five analytes: carbamezapine, atrazine, DEET, crotamiton and clarithromycin, for which the upper linear range reached up to 2000 ng $g^{-1}$ only. The observation of the parabolic plots is attributed to the saturation of the mass spectrometric detector. Limits of detection (LODs) and quantification (LOQs) of the method were calculated as three and ten times, respectively, the signal-to-noise ratio. This ratio was recorded by the integration software in the chromatograms of extracts of samples spiked with CEC low concentrations. As it can be seen in Tables 1 and 2, LODs vary widely since CECs with different functional groups and physicochemical properties have been included in the multiresidue method. They ranged between 0.05 and 4 ng $g^{-1}$ except for 6 compounds detected in relatively high concentrations. LOQs ranged between 0.016 and 13 ng $g^{-1}$ for most of the

Table 3: Influence of the matrix, slopes of the calibrations without manure matrix and slope ratios (manure matrix/blank). Concentrations measured in a typical blank, expressed as raw manure concentration.

| Compound | Blank Slope | Slope Ratio | Blank concentration (ng $g^{-1}$) | | Compound | Blank Slope | Slope Ratio | Blank concentration (ng $g^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Penicillin G | 16451 | 0.11 | ** | | Metronidazole | -- | -- | ** |
| Oxytetracycline | 713560 | 0.20 | ** | | Naproxen | 1422815 | 0.06 | ** |
| Doxycycline | 9186 | 0.66 | 15 | | Clarithromycin | 913538 | 0.55 | ** |
| Tetracycline | 643291 | 0.21 | ** | | Erythromycin | 373143 | 0.04 | ** |
| Marbofloxacin | 614471 | 0.29 | ** | | Acetylsalicylic acid | -- | -- | ** |
| Enrofloxacin | 384858 | 0.22 | 2 | | Norfloxacin | -- | -- | ** |
| Danofloxacin | 280926 | 0.36 | ** | | Atorvastatin | 550046 | 0.36 | ** |
| Sulfadiazine | 77913 | 0.28 | ** | | Atenolol | 104810 | 0.01 | ** |
| Sulfathiazole | 313696 | 0.28 | ** | | Caffeine | 821899 | 0.35 | ** |
| Sulfamethizole | 247445 | 0.36 | ** | | Atrazine | 5290924 | 0.11 | 1 |
| Sulfadimidine | 313696 | 0.20 | ** | | Iohexol | 56357 | 0.03 | ** |
| Sulfomethoxazol | 363632 | 0.25 | ** | | DEET | 3053522 | 0.23 | ** |
| Tylosin | 45629 | 0.35 | ** | | Ciprofloxacin | 1422469 | 0.19 | ** |
| Tiamulin | 856945 | 0.24 | 0.03 | | 17-$\alpha$-ethinylestradiol | -- | -- | ** |
| Apramycin | 144066 | 0.60 | ** | | Crotamiton | 1039048 | 0.28 | 0.1 |
| Trimethoprim | 2177022 | 0.20 | ** | | Estrone | 30518 | 0.05 | ** |
| Florphenicol | 76382 | 0.40 | ** | | Ethylparaben | 29690 | 0.06 | ** |
| Fenbendazole | 2013541 | 0.03 | 0.29 | | Propylparaben | 27761 | 0.05 | ** |
| Dexamethasone | 229354 | 0.14 | ** | | Diclofenac | 61749 | 0.03 | ** |
| Progesterone | 143628 | 0.21 | ** | | Ibuprofen | -- | -- | ** |
| Methylparaben | 83771 | 0.29 | ** | | Salicylic acid | 182169 | 0.15 | ** |
| Acetaminophen | 773818 | 0.06 | ** | | Clofibric acid | 205213 | 0.09 | ** |
| Carbamazepine | 6369885 | 0.47 | 1 | | 4-hydroxibenzoic acid | -- | -- | ** |
| Propanolol | 1645610 | 0.12 | ** | | Gemfibrozil | 110900 | 0.05 | ** |

--: no data

**: not detected.

compounds in agreement with LODs. The lowest LODs corresponded to carbamazepine, apramycin, sulfathiazole, sulfamethizole and tylosin (<0.1 ng $g^{-1}$) while the worst were found for acetylsalicylic acid and 17-$\alpha$-ethinylestradiol (>100 ng $g^{-1}$). On the other hand, it must be noted that other approach is sometimes applied to validate an analytical method when the target analytes are commonly found in the sample. It is based on the subtraction of the background signal and consequently the peak areas would be plotted exclusively against the added concentration; thus, the presence of a concentration gap between the LOQ and the lowest calibration level is not observed. This approach does not deal with real concentrations in samples and for this reason it has not been considered in this work.

Tables 1 and 2 shows the repeatabilities and recoveries achieved at two concentration levels (n=5), too. The concentrations of the CECs were different but they were close to 2800 ng $g^{-1}$ for the high level and 190 ng $g^{-1}$ for the low level. Relative recoveries, obtained from the matrix-matched calibration, were about 100% as it was expected because the calibration was carried out with extracts of spiked samples although some extreme values, up to 140 and 55%, were calculated, too. The precision, expressed as relative standard deviation (RSD), was almost always lower than 20%. Absolute recoveries, obtained from a conventional external-standard calibration (CEC calibration standards dissolved in initial mobile phase), were generally lower than 20%. These low recoveries are usual owing to the loss of analyte during the sample preparation and the ion suppression phenomenon that occurs commonly in the ESI interface. Relatively high absolute

recoveries have been achieved for tetracycline, oxytetracycline and tylosin, in addition to apramycin, salicylic acid and clarithromycin, these last in the low concentration assays. RSDs were similar to those obtained for the relative recoveries in the assays at high concentration but at low concentration RSDs were now somewhat worse, for some compounds, with respect to RSD data achieved for absolute recoveries. Some chromatograms of extracts can be seen in Supplementary data 10-12†).

The matrix effect in the quantification is due mainly to the suppression of signal in the ionization source of the mass spectrometer, and to a lesser extent, to the matrix influence on the sample preparation. The combined effect on both stages can be estimated by the absolute recoveries but also comparing the slopes of calibration graphs built with and without matrix. The sample preparation procedure was also applied without adding the manure matrix. The extraction solvent was directly spiked with increasing CEC amounts. The peak areas of these blanks were integrated and plotted against the concentration to calculate the corresponding slope. As the loss of analytes in the sample preparation operation is similar in the analysis with and without manure the difference between the slope values was attributed to the presence of manure matrix. Table 3 shows the blank slopes and the slope ratios (manure matrix slope divided by blank slope). As expected from absolute recovery data the slope ratios were lower than 1, which indicated that the presence of matrix decreased the signal. The matrix decreased notably the signal of CECs such as fenbendazole, acetaminophen, naproxen, erythromycin, atenolol, iohexol, estrone, diclofenac, clofibric acid and parabens, for which the ratio was lower than 0.1.

Finally, selectivity of the method was tested by the injection of solvent blanks and it resulted to be satisfactory. In this regard, instrumental carryover was assessed. It was calculated by dividing the peak area registered for each CEC in a matrix-free solvent solution by the peak area for the same CEC in the extract of manure spiked at the highest concentration level (about 7000-8000 ng $g^{-1}$), injected immediately before. The blanks contained less than 2% of the preceding signal in the worst cases. Hence, carryover was deemed negligible. Concentrations in a typical blank can be seen in Table 3 to assess the certainty of the low measured concentrations, expressed as ng of CEC per g of raw manure. Also, the material cleaning protocols were verified by the injection of the corresponding blanks.

### 3.3. Application of the method

#### 3.3.1. Concentration of CECs in raw manure

Table 4 lists the concentration mean values (n=2, Supplementary data 13†) found in the farm centrifuged and uncentrifuged RM samples as well as in their corresponding MSP and MLP. These concentrations were determined by applying a standard addition-matrix matched calibration to each type of matrix. Twenty-seven CECs were found in RM in concentrations lower than 100 ng $g^{-1}$ and five CECs in concentrations higher than 1000 ng $g^{-1}$. Seventeen CECs were expected compounds or veterinary drugs authorized in piggeries placed in the European Union[23], mostly antibiotics (doxycycline and marbofloxacin in the highest concentration) in addition to analgesics/anti-inflammatories (acetylsalicylic acid, salicylic acid and acetaminophen) and hormone derivatives. The other ten compounds were drugs prescribed for human health such as carbamazepine, propranolol, naproxen, clarithromycin, atorvastatin, crotamiton and clofibric acid, besides caffeine, DEET (insecticide), methylparaben, and 4-hydroxibenzoic acid. This last compound, detected at high concentration (14000-16000 ng $g^{-1}$), is a degradation product of parabens but it is a natural product, too. As regards the origin of these ten compounds in RM samples it is supposed that they arise mainly from cleaning waters of the facilities but their incorporation as micropollutants to animal feed, bed straw or, even, animal tissues cannot be discarded.

The percentage of removal of CECs in the farm during the separation of solid residues by centrifugation at low speed (2000 rpm) to obtain compost is shown in Table 4, too. Acetaminophen, progesterone, florphenicol, naproxen, acetylsalicylic acid and clofibric acid were not detected in the centrifuged RM while salicylic acid, methylparaben, clarithromycin and caffeine had high removal percentages (higher than 60%). Thus, it is deduced that these CECs tend to be adsorbed on the particles of relatively high size removed in the centrifugation. On the contrary, the concentrations of DEET and doxycycline were higher in the centrifuged RM (removal percentages about -70/-80 %) which suggest that they have a higher affinity for the liquid phase (including the particles in suspension) of the uncentrifuged manure than for the isolated particles. An explanation of the partitioning between the two phases seems not possible according to the log P values, ionization state (Supplementary data 14†) or capacity to form complexes with metals according to the functional groups present in their chemical structures.

A significant correlation between the concentrations determined in both types of RM, uncentrifuged and centrifuged, was found ($r=0.891$, $p<0.0001$). Alike, the centrifuged RM concentrations were positively correlated with the concentrations present in their MSP ($r=0.756$, $p=0.0004$) and MLP ($r=0.510$, $p=0.04$). However, the concentrations in the uncentrifuged RM were correlated with their MSP concentrations ($r=0.685$, $p=0.003$) but there was not significant relationship with those measured in its MLP.

#### 3.3.2. Distribution of CECs in liquid and solid phase

Table 4 shows the percentage of each CEC in the MLP and MSP with respect to the CEC total content determined directly in the RM. The distribution percentages in each phase are very variable depending on the compound and, even, the RM sample. It is noteworthy that the sum of percentages in MLP and MSP is far from 100%. A negative significant correlation was found between the percentages of CECs in MLP and

Table 4: Concentrations (ng $g^{-1}$) of CECs in the uncentrifuged and centrifuged RM samples (RM), solid phase (MSP) and liquid phase (MLP) of each RM sample (n=2). Removal of CECs during the centrifugation in farm. Concentrations calculated for each RM sample (C-RM) from the MSP and MLP analysis, and difference with respect to the concentration measured directly in the RM. Distribution (in percentage) of CECs between the solid and liquid phase, referred to the RM concentration.

| Compound | Concentration in uncentrifuged manure | | | | Concentration in centrifuged manure | | | | Removal in farm (%) | Difference (%) (C-RM – RM) / RM | | Uncentrifuged manure | | Centrifuged manure | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RM | MSP | MLP | C-RM | RM | MSP | MLP | C-RM | | Uncentrifuged | Centrifuged | % in MLP | % in MSP | % in MLP | % in MSP |
| Oxytetracycline | 28 | 29 | 23 | 24 | 21 | 105 | 5 | 9 | 25 | -14 | -57 | 77 | 8 | 20 | 24 |
| Doxycycline | 1091 | 75 | 26 | 29 | 1969 | 392 | 10 | 28 | -80 | -97 | -99 | 2 | 0.5 | 0.5 | 1 |
| Marbofloxacin | 156 | 151 | 77 | 83 | 128 | 461 | 28 | 49 | 18 | -47 | -62 | 46 | 7 | 21 | 17 |
| Enrofloxacin | 24 | 31 | 1 | 3 | 26 | 22 | 0.7 | 2 | -8 | -88 | -92 | 4 | 10 | 3 | 4 |
| Danofloxacin | 83 | 95 | 1 | 8 | 75 | 28 | 0.5 | 2 | 10 | -90 | -97 | 1 | 8 | 0.7 | 1.8 |
| Sulfadiazine | 74 | 19 | 12 | 12 | 49 | 17 | 14 | 14 | 34 | -84 | -71 | 15 | 2 | 27 | 2 |
| Tiamulin | 57 | 3 | 0.4 | 0.5 | 48 | 13 | 0.4 | 1 | 16 | -99 | -98 | 0.6 | 0.4 | 0.8 | 1 |
| Trimethoprim | 30 | 7 | 0.9 | 1 | 35 | 8 | 0.1 | 0.5 | -16 | -95 | -99 | 3 | 2 | 0.3 | 1 |
| Florphenicol | 77 | 51 | 11 | 14 | nd (<3) | nd (<0.1) | nd (<0.3) | -- | 100 | -82 | -- | 13 | 5 | -- | -- |
| Progesterone | 37 | 77 | 70 | 70 | nd (<0.3) | nd (<0.2) | 11 | 10 | 100 | 89 | 100 | 173 | 15 | -- | -- |
| Methylparaben* | 79 | 8 | nd (<0.2) | 0.6 | 20 | 8 | 0.6 | 0.9 | 75 | -99 | -96 | 0 | 0.8 | 3 | 2 |
| Acetaminophen | 66 | 29 | 7 | 9 | nd (<2) | nd (<1) | nd (<0.2) | -- | 100 | -86 | -- | 10 | 3 | -- | -- |
| Carbamazepine* | 9 | 2 | 0.4 | 0.5 | 8 | 6 | 0.4 | 0.6 | 7 | -94 | -93 | 4 | 2 | 4 | 3 |
| Propanolol* | 27 | 25 | 3 | 4 | 31 | 54 | 1 | 4 | -14 | -85 | -87 | 9 | 7 | 4 | 9 |
| Naproxen* | 72 | 91 | 19 | 24 | nd (<3) | nd (<2) | nd (<0.5) | -- | 100 | -67 | -- | 25 | 9 | -- | -- |
| Clarithromycin* | 34 | 5 | 0.4 | 0.7 | 0.4 | nd (<0.1) | 0.5 | 0.4 | 99 | -98 | 0 | 0.9 | 1 | 128 | 0 |
| Acetylsalicylic acid | >7741 | 192 | 27 | 39 | nd (<193) | nd (<41) | nd (<0.3) | -- | 100 | -99 | -- | <0.3 | <0.2 | -- | -- |
| Atorvastatin* | nd (<0.1) | nd (<2) | nd (<0.05) | -- | nd (<0.1) | 2 | 0.2 | 0.3 | -- | -- | 100 | -- | -- | -- | -- |
| Caffeine* | 10 | 4 | 2 | 2 | 2 | 7 | 1 | 1 | 85 | -80 | -50 | 19 | 3 | 69 | 22 |
| DEET* | 4 | 4 | 0.8 | 1 | 6 | nd (<1) | 0.9 | 0.8 | -72 | -75 | -87 | 20 | 8 | 13 | 0 |
| Ciprofloxacin | 12 | 5 | 0.3 | 0.6 | 10 | 50 | 1 | 3 | 17 | -95 | -70 | 2 | 3 | 10 | 25 |
| 17-α-ethinylestradiol | 1953 | 694 | 211 | 246 | 1856 | 292 | 36 | 48 | 5 | -87 | -97 | 10 | 3 | 2 | 0.8 |
| Crotamiton* | 52 | 4 | 0.7 | 0.9 | 29 | 4 | 0.3 | 0.4 | 44 | -98 | -99 | 1 | 0.6 | 0.9 | 0.6 |
| Estrone | <764 | 110 | 23 | 29 | <764 | 222 | 9.2 | 19.4 | -- | -- | -- | >5 | >2 | >2 | >2.8 |
| Salicylic acid | 39575 | 620 | 8 | 53 | 15605 | 610 | 25 | 53 | 61 | -99 | -99 | 0.02 | 0.1 | 0.2 | 0.2 |
| Clofibric acid* | 20 | 5 | 2 | 2 | nd (<1) | nd (<0.8) | nd (<0.1) | -- | 100 | -90 | -- | 10 | 2 | -- | -- |
| 4-hydroxibenzoic acid* | 14783 | 692 | 8 | 58 | 15906 | 463 | 19 | 40 | -8 | -99 | -99 | 0.05 | 0.3 | 0.1 | 0.1 |

nd: not detected. Detection limit is shown in parentheses. --: without data.

*: non-veterinary drug or compound not related to animal hormones.

log P (r=-0.659, p=0.006) for the centrifuged manure. As the MLP is the main constituent of the manure, this correlation suggests that the centrifuged manure is impoverished in lipophilic CECs, which would tend to be adsorbed by the high density particles in the uncentrifuged RM and, subsequently, be removed by the low speed centrifugation in the farm. However, there is not an apparent relation with the removal percentages of the CECs as it was stated above.

The concentration of CECs in RM could be determined as the sum of the MSP and MLP concentrations, taking into account the percentage of each phase in the RM. Eq. 1 resumes the calculation of the RM concentration ($C_{RM}$). The MSP moisture to convert the concentration determined on lyophilized MSP ($C_{MSP}$) in a concentration per fresh g, and the inverse of the experimental density of the MLP (V/w) to convert the measured MLP concentration (ng $L^{-1}$) in ng per g, must be

$$C_{RM} = C_{MSP} \times \left[1 - \frac{\%_{moisture}}{100}\right] \times \frac{\%_{w_{MSP}/w_{RM}}}{100} + C_{MLP} \times (V/w)_{MLP} \times \frac{\%_{w_{MLP}/w_{RM}}}{100} \quad (Eq.1)$$

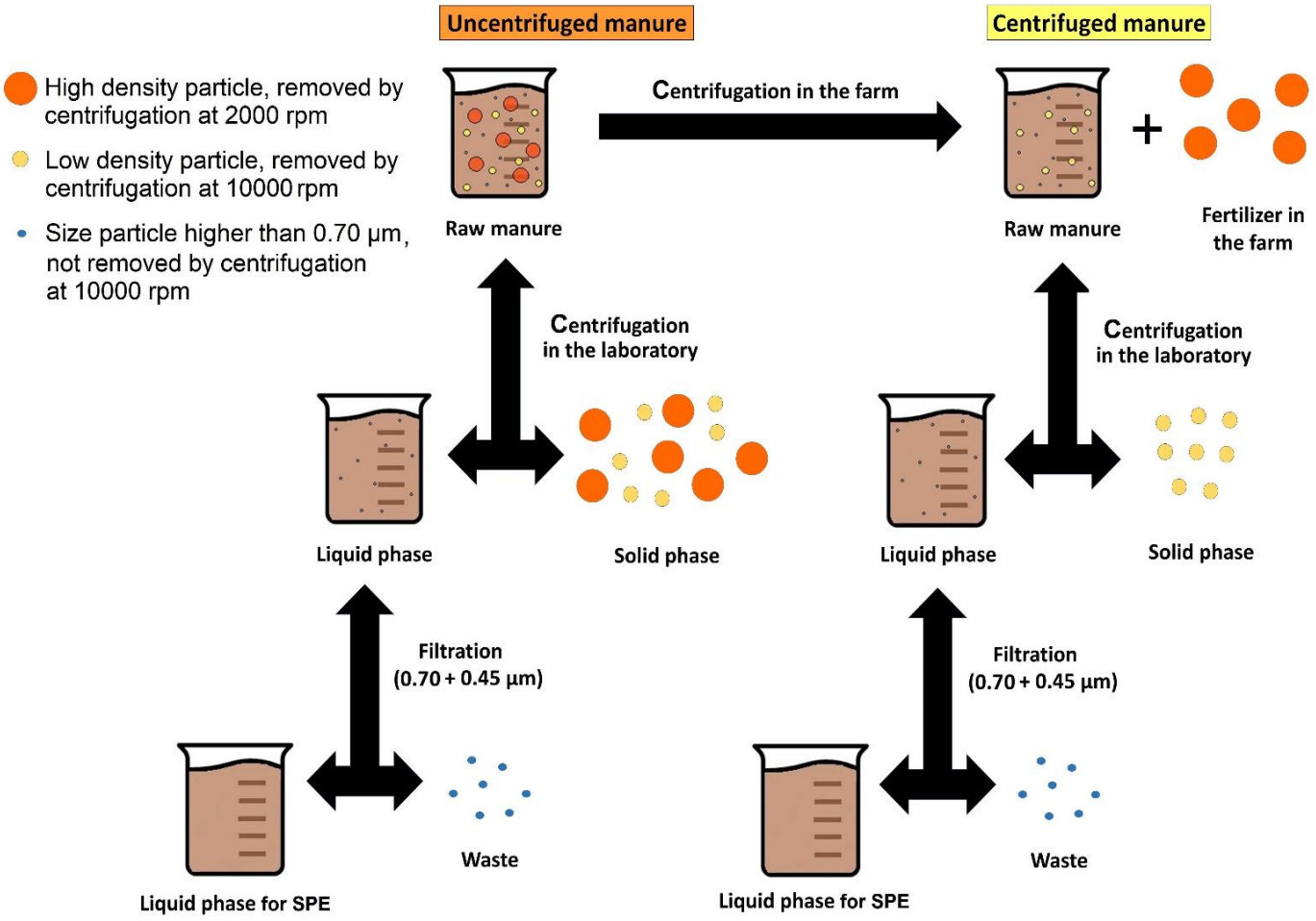


Fig. 2: Scheme of the separation of manure phases and solid particles contained in the raw manure.

considered. Table 4 gives the calculated concentrations in both manures and the concentration differences according to the method of determination of the CEC content in the raw sample. The difference is expressed as a percentage referred to the RM concentration (without previous phase separation). For most compounds, the concentration measured directly in the raw manure is much higher than that obtained by the sum of MLP and MSP concentrations, so the percentages obtained are negative and lower to -70%. Only for oxytetracycline, marblofloxacin and naproxen the difference percentage was inferior, between -14% and -67%. Progesterone was the only CEC whose concentration in RM was lower when this was determined by the procedure proposed in this manuscript, without separation of the phases (positive percentages, 89 and 100%). It must be noted that the reliability of the percentage data in the centrifuged manure is limited for progesterone, clarithromycin and atorvastatin because they were not detected in the RM or the concentrations measured were very low.

Assuming the homogeneity of the RM samples and the representativeness of the separated MSP and MLP, the explanation of the notable concentration difference for the two samples analyzed in this work could lie in the filtration step previous to the SPE for the analysis of the MLP. Fig. 2 outlines the separation of the three types of solid material considered in this work: material of high density separated in farm that constitutes the compost used as fertilizer, the material of lower density separated in laboratory that constitutes the MSP and the fine material kept in suspension after the laboratory centrifugation with a particle size higher than 0.70 µm. This fine material must be removed from the MLP sample to prevent the obstruction of the SPE cartridges and, consequently, is a RM aliquot lost when the determination of the CEC total content is performed by the sum of contents of each phase. This interpretation entails that the amount of CECs linked to the removed and suspended fine material in the MLP, likely by adsorption, is considerably high (high difference percentages were observed, except for progesterone) while the content of CECs really solubilized in the MLP should be relatively scarce. This explanation seems coherent with the worse correlations observed between the concentrations measured in the RM and those in the MLP with respect to the RM and MSP concentrations. It can be deduced that the CEC amounts not included in the MLP analysis are the reason of the minor correlation. Thus, the analysis of the MSP could provide a better profile of distribution of CECs in the RM than the analysis of the MLP. As well, this idea is asserted by the fact that the RM concentrations in the centrifuged manure are correlated with the concentrations in the MSP of the uncentrifuged manure ($r=0.806$, $p=0.0002$) but there was no correlation between the centrifuged RM and uncentrifuged MLP concentrations.

The sum of CEC percentages in MSP and MLP does not reach the 100% for this reason. The difference should be ascribed to those CEC amounts linked to the non-analyzed fine material in suspension. Apparently anomalous results were obtained here for the those compounds determined in concentrations close to the LOQs, as already happened earlier, but in general terms the percentage of CECs in the MSP was lower than 10%. The percentage of CECs in the MLP was similar, or higher, to that in MSP for many compounds, about 70% of them.

## 4. Conclusions

An analytical method to determine CECs in raw manure as a whole, without phase separation, has been developed and in-house validated. The repeatability (RSD) is in general lower than 20% (n=5), the linearity of the calibration graphs is about 3-4 magnitude decades and the limits of detection ranged mostly between 0.05 and 4 ng $g^{-1}$.

The use of alumina as a cleaning agent (dispersive solid phase extraction) in the initial solid-liquid extraction is preferable to the use of octadecylsilane or (1:1) octadecylsilane-alumina mixture to obtain more intense peak areas from a greater number of CECs. According to an experimental design, the pH-value and the amounts of alumina and AEDT are the most influential operating parameters in the extraction with water-methanol. Low alumina amounts are suitable for the analysis of fluoroquinolones.

More than twenty CECs have been detected in raw manure samples, in a wide concentration range. Besides veterinary drugs, the finding of CECs such as caffeine, DEET and carbamezapine among other should be ascribed to their occurrence as environmental micropollutants. Lipophilic CECs tend to be removed, in different proportion, when the raw manure is centrifuged in the farm to yield compost. Doxycycline and DEET tend to remain in the raw manure.

The previous filtration step in the solid phase extraction to prevent the blockage of the cartridges in the analysis of the liquid phase of the manure seems to remove a notable amount of CEC linked to the small diameter particles in suspension, so that the determination of the amount of CEC contained in the liquid phase could be underestimated. The determination of the total amount of CECs in raw manure as a whole seems an advisable option compared to the analysis of the phases separately.

## Author Contributions

Cristina Portela Monge: Investigation, Validation, Data curation, Writing, Original draft

Silvia Bolado: Funding acquisition, Project administration

Rebeca López-Serna: Methodology, Investigation, Data curation, Resources

Juan José Jiménez: Conceptualization, Methodology, Formal Analysis, Supervision, Writing, Review & editing

## Conflicts of interest

There are no conflicts to declare.

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