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Performance critical comparison of offline SPE, online SPE, and direct injection for the determination of CECs in complex liquid environmental matrices

Miguel Ángel de la Serna Calleja [a,b], Silvia Bolado [b,c], Juan José Jiménez [a,b], Rebeca López-Serna [a,b,*]

[a] Department of Analytical Chemistry, Faculty of Sciences, University of Valladolid, Campus Miguel Delibes, Paseo de Belén 7, 47011 Valladolid, Spain
[b] Institute of Sustainable Processes, Dr. Mergelina s/n, 47011 Valladolid, Spain
[c] Department of Chemical Engineering and Environmental Technology, School of Industrial Engineering, University of Valladolid, Dr. Mergelina, s/n, 47011 Valladolid, Spain

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ABSTRACT

Sample preparation for the analysis of organic micropollutants in wastewater samples is commonly carried out by solid-phase extraction (SPE) procedures, which involve different manual laboratory operations. This conventional approach requires several hours of counter labour and entail the use of a lot of disposable material, and the subsequent contaminated non-recyclable plastic-residue production. In contrast, by coupling and automatizing the pre-treatment to the instrumental analysis most of that burden erases, sample size gets miniaturized and, thus, storage becomes freed-up. Even lab counters get cleared off from sample pre-treatment apparatus. However, method performance could get alter as a trade-off. This paper presents the results from a study in which methodology, including SPE online-coupled to UHPLC-MS/MS chromatography, was developed for multi-residue (58) determination of veterinary and pharmaceutical drugs in urban and piggery wastewater (influent and effluent to wastewater treatment plants (WWTPs)). Similarly, the direct injection (DI) of large volumes (hundreds of μL) of same matrix samples into the chromatographic system was optimized too. The performance of both automated methods was statistically compared with the classical off-line SPE. As dealing with trace analysis, suitable injection volumes for the alternative approaches were selected on the premise of low limits of quantification (MLQs). Under the selected conditions, validation parameters such as linearity range, method quantification limits, peak shape and carry over were determined. Usually more than 50 % of the analytes showed MLQs below 50 ng/L, for all matrices and methodologies, especially for DI. Real wastewater samples from a local urban WWTP and farm were analysed with all three tested methodologies. Determined concentrations and removal rates were statistically compared and turned out being quite similar. However, analysis under offline SPE and DI approaches provided a larger amount of information as they reached lower MLQs. Offline-SPE provided the worst precision among all.

1. Introduction

Contaminants of emerging concern (CECs) are anthropogenic substances which are being continuously generated and released into the environment in huge quantities. This is beginning to cause serious health problems to living beings, including humans, as they rapidly spread through the aquatic compartments, where they are already ubiquitous, and move up in the food chain [1–3].

Nowadays, the most studied class of CECs is the pharmaceuticals and personal care products (PPCPs) which is made of ~3000 different substances (painkillers, lipid regulators, hormones among others) [4]. They are consumed by humans or animals (provided in farms) and then partially excreted and directly discharged into nature or collected into urban or livestock sewage systems [5–9]. Current conventional wastewater treatment plants (WWTPs) are not usually provided with equipment or appropriate processes to remove many of these types of

* Corresponding author at: Department of Analytical Chemistry, Faculty of Sciences, University of Valladolid, Campus Miguel Delibes, Paseo de Belén 7, 47011 Valladolid, Spain.
E-mail address: rebeca.lopez@uva.es (R. López-Serna).

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pollutants [10,11]. Thus, they may remain in the effluents at a high extent and eventually be discharged into the receiving natural waters as CEC cocktail-mixtures, where aquatic flora and fauna are directly impacted [12,13]. Once in the aquatic environment and food chain, CECs end up finding their way to humans either through drinking water or food intake [3,14,15].

Some CECs have been reported to be endocrine disruptors [16,17]. Pharmaceuticals have been designed to exert a biological response even at low concentrations. And acute or chronic toxicity has been confirmed or highly suspected for many others [18,19].

Current CEC concentrations in both urban and livestock wastewaters vary compound to compound and depend on the area population density. Nonetheless, they could reach up to thousands of tens of ng/L [7,20,21].

This concerning situation has increased the demand for the development of reliable methods for the analysis of CECs in environmental matrices. They are required to be highly multicomponent, including very diverse compounds, to have considerable low limits of detection and quantification, as CECs are present at trace levels, and to have high throughput and be cost effective, so that CECs could be extensively monitored. In addition, these analytical methods should contain their environmental impact so that they do not become part of the problem.

With the development of the fast chromatography techniques coupled to tandem mass spectrometry, throughput limitations are mainly bottle-necked in the time-consuming sample pretreatments. For liquid samples such as natural or waste waters, they usually include solid phase extraction (SPE) in lab counter manifolds followed by cartridge elution, extract evaporation and reconstitution steps, which typically extend over two full working days. In addition, they entail analyst chemical exposure, human error introduction and large quantities of solvents and disposable material consumption.

In this work, a fast and completely instrumental sample pretreatment, based on online solid-phase-extraction (online SPE), has been developed for the analysis of 58 CECs, including PPCPs of 14 different classes, in both urban and livestock wastewaters. Additionally, non-pretreated direct sample introduction (DI) has been also considered. Both scenarios have been compared with a conventional offline SPE methodology. In all cases, sample pretreatment (if any) has been followed (or coupled) to the same ultra-high-liquid-chromatography (UHPLC) with triple-quadrupole (QqQ)-mass spectrometry detection routine, as it is known to provide the best versatility, selectivity and sensitivity [22–24]. These three analytical approaches have been statistically compared, and pros and limitations have been arisen. Large injection volumes were widely assayed during the '90s [25] for pesticides in environmental waters. However, CECs have been barely ever tested under these methodologies, despite the new developments in the mass spectrometry sector, especially in terms of limits of detection. Hence, just a few studies have been published regarding the analysis of PPCPs in aqueous matrixes by online SPE [26,27], and just one was found to report the performance of the direct injection with no pretreatment [28]. All of them included <20 analytes belonging to a reduced number of therapeutical classes. To the authors' knowledge, this study represents the first technical comparison of all three approaches, especially in a matrix as complex as the pig slurry.

2. Materials and methods

2.1. Chemicals

All pharmaceutical standards were of high purity grade (>95 %) and are listed in the **Supplementary data 1**.

Both individual stock standard and isotopically labelled internal standard solutions were prepared on a weight basis at 1 g/L in methanol (MeOH), except for amoxicillin which was prepared in a MeOH/H_2O (1/1) solution, and danofloxacin, enrofloxacin, marbofloxacin, ciprofloxacin, ofloxacin, and norfloxacin which were prepared in a 0.2 % HCl MeOH/H2O (1:1) to improve their solubility.

Mixture stock solutions at 20, 2, 0.5, 0.05, 0.005 and 0.0005 mg L^{-1} were prepared in MeOH by appropriate dilution of the individual stock solutions. Similarly, a separate mixture of 12 isotopically labelled internal standards at 0.5 mg L^{-1} was also prepared in MeOH. All individual and working stock solutions were immediately stored in darkness at −80 °C to avoid degradation [29,30]. LCMS-grade MeOH and acetonitrile (ACN) as well as 98 % pure formic acid (FA) were supplied by Scharlau. Ultrapure deionized water was in-house obtained by a Milli-Q Advantage A10 water purification system from Merck Millipore. Nitrogen for drying, 99.995 % of purity, was from Air Liquide (Madrid, Spain).

2.2. Analytical methodology

2.2.1. Sample collection and conservation

Both influent and effluent urban wastewater were collected in November 2020 from the municipal WWTP in Aldeamayor de San Martín (Spain) constituted by an aerobic reactor (18 × 6 × 4.5 m), a circular secondary decanter with an average retention time of 0.43 h and a biological sludge reactor, among other conventional treatments. It serves >5000 inhabitants. Additionally, two batches of raw piggery wastewater were collected in April 2021 from a farm located in Cantalejo, Spain, and then they were diluted at 5 % v/v, and stored at 4 °C. The resulting water was treated by an indoor open photobioreactor located at the Institute of Sustainable Processes in Valladolid, Spain, which was inoculated with microalgae culture (with >95 % of *Chlorella* sp.) working at a retention time of 5 days. In addition, tests were also run in deionized water to assess the impact of each methodological proposal in the absence of the matrix effect.

Wastewater samples were collected in 2.5-L glass containers and immediately transported to the laboratory at 4 °C in darkness, where they were centrifuged in a Thermo Sorvall Legend RT + Refrigerated Benchtop Centrifuge at 14,000 rpm (30,000×*g*) for 10 min, and the supernatant was filtered through 0.7 μm glassfiber membranes and then through 0.45 um nylon membranes from Filterlab (Barcelona, Spain), and stored at −20 °C and darkness in amber polyethylene terephthalate (PET) bottles until analysis.

2.2.2. Conventional Offline methodology proposal

The selected conventional method is described [31] and has been properly adapted for the analysis of 58 PPCPs both in urban and pig slurry. The selection of those target substances was based on their high concentration levels and ubiquity in aquatic environments as well as their variable degradability in conventional wastewater treatments [32]. Additionally, many of the selected human PPCPs are highly used in daily life and/or have a recognized toxicity. High consumption of the selected veterinary drugs has also been reported by the Spanish Agency of Medicines and Medical Devices (AEMPS), according to their sales in Spain [33]. In brief, it consisted of **1)** 100 mL of 0.45-μm-filtered samples were spiked at 1,000 ng L^{-1} of internal standards. **2)** Subsequently, they underwent conventional SPE clean-up onto Oasis® HLB cartridges (60 mg, 3 cc) by Waters Chromatography (Barcelona, Spain), previously activated and conditioned with 3 mL MeOH and deionized water, respectively. After extraction (assisted by vacuum in a manifold) was carried out, cartridges were rinsed with 3 mL of a 5 % MeOH solution, and then dried for 20–30 min. Cartridges were stored at −20 °C in darkness if not immediately eluted. For the elution, 2 × 3 mL of ACN were used, with no vacuum assistance. Extracts were then dried under a N_2 stream using an Organomation *N*-EVAP 11,250 evaporator (Berlin, MA, USA) and stored at −20 °C in darkness until the instrumental analysis by LC-MS/MS. Then, the extracts were reconstituted with 1.0 mL of 0.1 % FA in MeOH/water mixture (5:95, v/v), as it constituted the mix of mobile phases at initial conditions in the LC gradient used during the chromatographic gradient described below. **3)** Then, a filtration through a polytetrafluoroethylene (PTFE) 0.22-μm syringe filter took

Table 1
Median (μ) and percentiles (P25% and P75%) for MLQ and dynamic range under each methodological approach (Offline SPE, DI and Online SPE) in urban wastewater and piggery wastewater.

	URBAN					
EFF	**OFF SPE**					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	13	4	41	39,555	99,024	99,588
	ID					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	23	10	67	19,982	99,908	99,888
	ON SPE					
	MLQ (ng/L)			Dynamic range (ng/L)		
	μ	P25%	P75%	μ	P90%	P95%
	103	35	142	79,519	99,917	99,989
INF	**OFF SPE**					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	23	8	120	49,937	99,973	99,993
	ID					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	37	14	296	79,745	99,970	99,992
	ON SPE					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	112	15	386	79,990	99,985	99,991
	PIGGERY					
EFF	**OFF SPE**					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	4	0.3	19	19,991	39,981	39,996
	ID					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	18	5	131	19,991	39,959	39,980
	ON SPE					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	36	6	235	19,997	39,989	39,996
INF	**OFF SPE**					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	P25%	**P75%**	**μ**	**P90%**	**P95%**
	5	1	33	19,990	39,996	39,998
	ID					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	28	12	227	19,997	39,964	39,971
	ON SPE					
	MLQ (ng/L)			**Dynamic range (ng/L)**		
	μ	**P25%**	**P75%**	**μ**	**P90%**	**P95%**
	34	5	122	26,269	39,986	39,998

MLQ: Method limit of quantification, defined as the concentration providing signal-to-noise ratios of 10.

Table 2
Chromatographic peak shape in terms of peak width for A) Urban and B) Piggery wastewater. Comparison of automatic vs conventional methodologies.

2A)			
EFF	**OFF SPE**		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.13	0.33	0.35
	ID		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.14	0.40	0.45
	ON SPE		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.52	0.99	1.14
INF	**OFF SPE**		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.13	0.38	0.39
	ID		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.12	0.42	0.47
	ON SPE		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.56	0.89	1.08
2B)			
EFF	**OFF SPE**		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.13	0.34	0.48
	ID		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.12	0.46	0.56
	ON SPE		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.58	0.99	1.14
INF	**OFF SPE**		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.14	0.32	0.34
	ID		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.13	0.37	0.50
	ON SPE		
	Width at 5 % (min)		
	μ	**P90%**	**P95%**
	0.51	1.01	1.05

μ=median, P%=percentile.

place. The filtrate was collected in an amber 1.5-mL vial before instrumental injection. **4)** An instrumental LC-MS/MS analysis was performed using an UHPLC Sciex Exion system connected to a Sciex 6500 + triple-quadrupole mass spectrometer from Sciex (Washington, DC, USA) equipped with an electrospray ionization (ESI) source operated in both positive and negative mode in the same run. Chromatographic separation was achieved by a Phenomenex (Washington, DC, USA) reversed-phase column Kinetex EVO C18 (2.1 mm × 50 mm, particle size 1.7 μm), which was temperature-controlled at 40 °C along the entire chromatogram. The gradient run at 500 μL min^{-1} with 0.1 % FA (v/v) in water and 0.1 % FA in MeOH as mobile phases starting with 5 % of the organic phase for 1 min and then increasing to 95 % in 2 min, held at 95 % for 3 min, and finally returning to the initial conditions, which were kept for 4 min (system rebalancing). The total run time for each injection was 10 min. Injection volume was set at 10 μL.

Mass spectrometry acquisition was performed in selected-reaction monitoring (SRM) mode, recording the transitions between the precursor ion and the two most abundant product ions for each target analyte, thus achieving four identification points per compound (2002/657/EC) [14]. The specific details of the UHPLC-MS/MS conditions are shown in the **Supplementary data 2**. In addition, ESI operational settings were: capillary voltage, 4500 V; capillary temperature, 400 °C; both gas 1 and 2, 45 psi. Data acquisition and evaluation were performed by SciexOS software.

2.2.3. *Fast Online SPE proposal*

For this methodological version, the protocol described in **section 2.2.2** was followed during step 1. However, step 2 was skipped. In contrast, 2 mL of the resulting solution from step 1 were directly filtered as described in step 3. Subsequently, the filtrate underwent instrumental online SPE coupled to the LC-MS/MS method described above. For this purpose, a Strata-X 25 μm 20 × 2.0 mm online reusable mini-cartridge by Phenomenex was used. This sorbent was selected for its similarities with the one used in the Offline SPE approach described above.

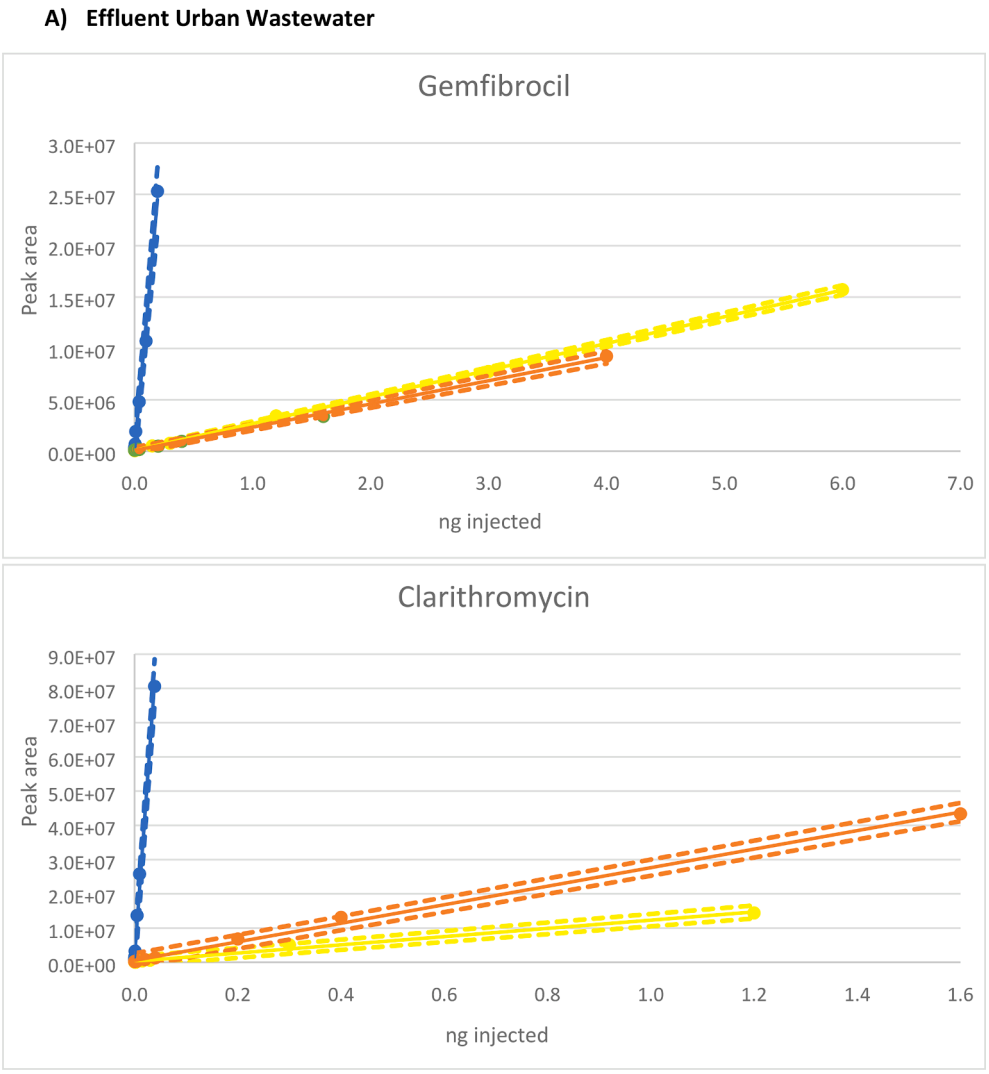

Fig. 1. Representation of peak area to amount injected (ng) for SPE-Offline, DI and SPE-Online for Gemfibrozil and Clarithromycin in Urban Wastewater samples. The regression bands are depicted in dashed lines.

Untreated water samples were conveyed to the cartridge by a 5 % MeOH solvent at a flow rate of 1 mL min^{-1} for 1 min in the Sciex Exion system. In these conditions, extracts were fully seeded in the cartridge and rinsed. Subsequently, UHPLC gradient described above would elute backwards the cartridge towards the analytical column. Six injection volumes, i.e., 25, 50, 100, 200, 300 and 400 μL, were tested.

2.2.4. Fast direct injection (DI) proposal

The protocol for the DI methodological option was identical to the one described in **section 2.2.2**, except for step 2 which was skipped. In contrast, 2 mL of the resulting solution from step 1 were directly filtered as described in step 3 and continued with the protocol (step 4) without any sample pre-treatment at all. Six injection volumes, i.e., 25, 50, 100, 200, 300 and 400 μL, were tested.

3. Results and discussion

3.1. Injection volume optimization in online SPE and DI methodologies

A 15-level-calibration curve from 0.5 ng/L to 100 μg/L was built in each target matrix, included deionised water. For that, aliquots from suitable stock solutions were added to the samples during the step 1 of the protocol. Two additional blanks per matrix were built with no standard fortification.

In order to select the optimum injection volume for the fast methodologies (Online SPE and DI) method limit of quantification (MLQ) was selected as the most suitable criterion as trace level sensitivity was intended. Hence, six injection volumes, i.e., 25, 50, 100, 200, 300 and 400 μL were tested in deionized water and a MLQ below 50 ng/L was set as the threshold for a methodology to be considered valid for the purpose intended. **Supplementary data 3** shows the number of analytes showing a MLQ below 50 ng/L under both automatic analytical procedures for each injection volume. A slightly higher number of compounds were detected under Online SPE than DI for all injection volumes, ranging between 45 and 47 analytes by Online SPE, and 42–45 by DI. However, DI methodology provided a bigger number of quantifiable analytes. Injection volumes of 25 and 50 μL rendered insufficient number of analytes with MLQ < 50 ng/L in Online SPE. Best performance in terms of number of detected analytes and analytes with good sensitivity was also achieved at injection volumes of 100 μL and larger for DI too. Therefore, the two smallest tested injection volumes, 25 and 50 μL, were discarded and were no longer tested on the target matrices, as described below.

Aliquots of 100, 200, 300 and 400 μL of inlet and outlet of both urban

B) Influent Urban Wastewater

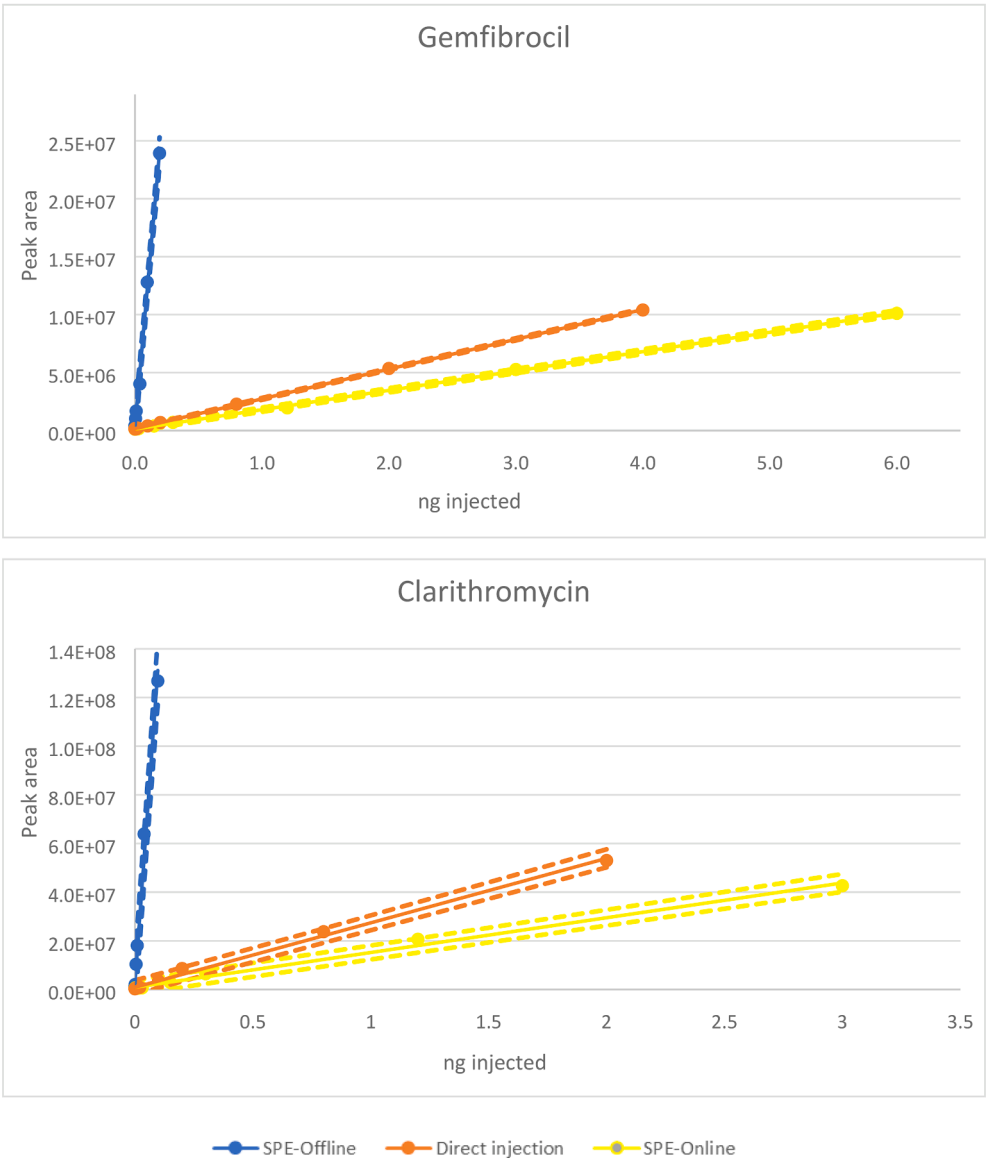

Fig. 1. (*continued*).

and livestock wastewaters were, then, injected under the fast methodologies in order to optimize the injection volume in each case. **Supplementary data 4 and 5** show the results obtained for urban and piggery wastewater, respectively.

As expected, the number of detected analytes dropped when analyzing target matrices in comparison to deionized water. Hence, the number fell between 40 and 41 and 39 to 42 in effluent and influent urban wastewater, respectively, and from 38 to 41 in piggery wastewater. In addition, fewer analytes with MLQ below 50 ng/L were also registered in the presence of matrix. In contrast to the performance observed in deionized water, online SPE did not clearly provide a higher number of detected analytes in any of the tested wastewaters. However, again, DI delivered more compounds with MLQ < 50 ng/L, especially in urban wastewater. Performance between matrices did not follow a consistent trend for all cases, but piggery wastewater usually provided a higher number of detected analytes quantifiable under 50 ng/L.

The injection volume showed a bigger impact on the number of analytes with good MLQ than on the number of detected compounds. Hence, based on the number of compounds with MLQ < 50 ng/L, the injection volumes which provided the best results were 400 and 200 μL for urban effluent and influent wastewater, respectively, by DI, and 300 μL for both urban wastewaters by online SPE. Regarding piggery wastewater, 300 and 100 μL showed to be the best options in effluent and influent urban wastewater, respectively, by DI, and again 300 μL for both effluent and influent livestock wastewaters. It should be noticed that in the case of several injection volumes providing the same number of good quantifiable compounds, the number of detected compounds was used to settle.

3.2. *Method validation for the optimized fast analytical approaches*

Six validation parameters, i.e., absolute recovery, matrix effect, relative recovery (bias), precision, sensitivity and dynamic range were determined for 44 of the 58 total initial target analytes. The remaining 14 did not show sufficient response factor under the instrumental conditions and, thus, they were ruled out of all the proposed methodologies. These were amoxicillin, acetaminophen, sulfapyridine, nalidixic acid, acetylsalicylic acid, estradiol, ethynylestradiol, estrone, salicylic acid, bisphenol A, triclosan, octylphenol, nonylphenol and 4-hydroxibenzoic acid. In addition, carryover was assessed to check the absence of

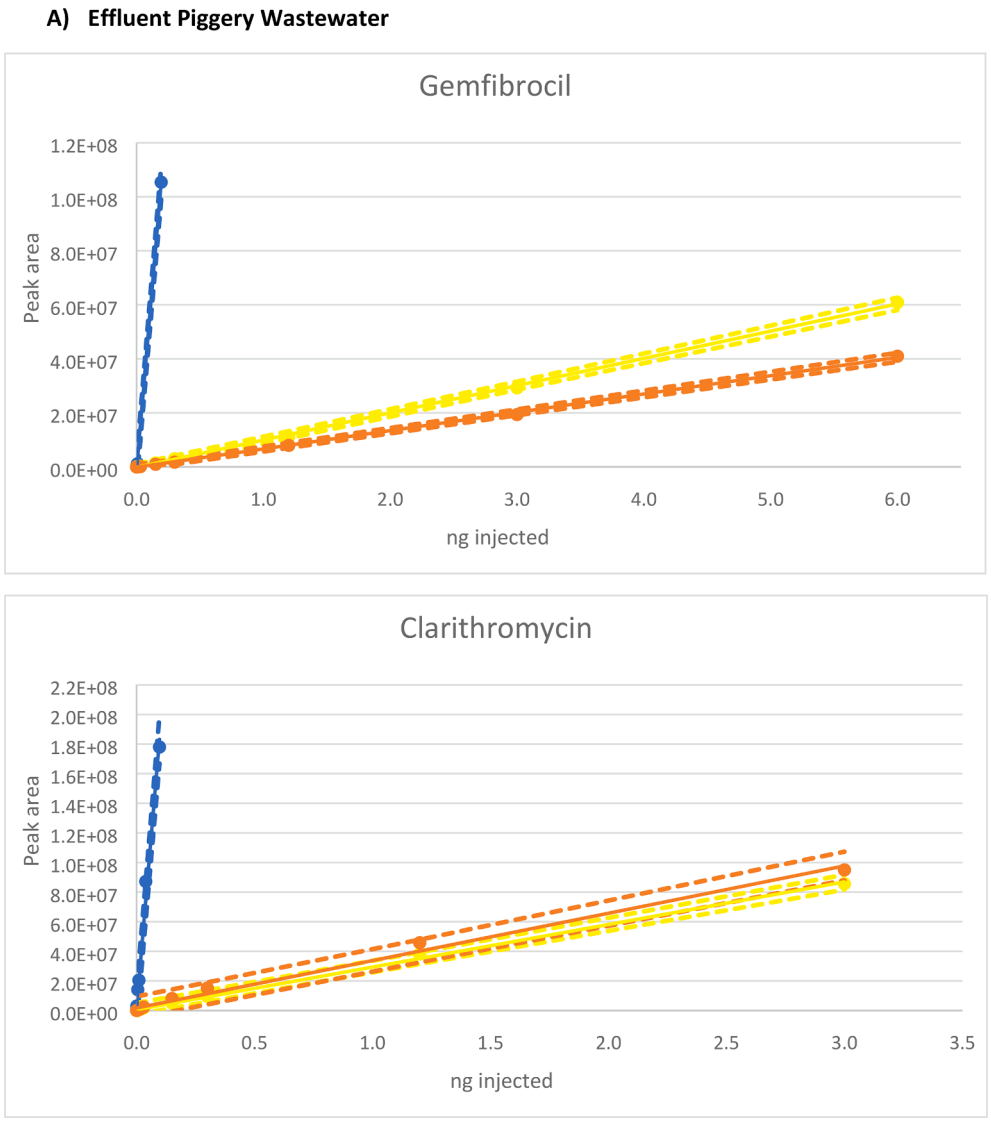

Fig. 2. Representation of peak area to amount injected (ng) SPE-Offline, DI and SPE-Online for Gemfibrozil and Clarithromycin in Piggery Wastewater samples.

contamination among samples during the instrumental analysis.

3.2.1. Trueness

Three parameters have been studied to evaluate the veracity of the automatic methods: absolute recovery, matrix effect and relative recovery.

3.2.1.1. Absolute recovery. They were calculated by comparing the signal (peak area) registered for each analyte in the spiked matrices after the subtraction of the average signal (peak area) in the blanks, versus the signal (peak area) registered by direct injection of standard solutions in absence of matrix.

Supplementary data 6**A** shows average percentages (n = 15) calculated for all the spiked concentration levels within the linear dynamic range. Associated %RSD is provided too. The overall absolute recovery average (excluding the outliers) for each wastewater and analytical approach are shown at the bottom of the table. They were very similar under both fast methodologies, averaging from 33 % to 50 % for piggery wastewater and from 40 % to 44 % for urban wastewater. Oxytetracycline, tetracycline, sulfadiazine, apramycin, 1,4 benzoquinone, caffeine and iohexol provided recoveries over 200 %, so they were not included in the table.

3.2.1.2. Matrix effect. It was calculated by dividing the peak area obtained for each matrix under each analytical method (corrected by the average area in the blank) by the peak area registered in the deionized water for the same injection volume. Values for all levels within the dynamic range were averaged (n = 15) and are shown in Supplementary data 6**B** as percentages. Percentages above 100 % show signal enhancement, percentages below 100 % show signal suppression. The overall average for all the analytes was determined for the three analytical protocols in each matrix and is shown at the bottom of the table. They were very similar under both automatic methods, and above 100 %, i.e., signal enhancement, in all cases, except for the effluent of piggery wastewater. In contrast, the conventional methodology (Offline SPE) showed the lowest matrix effect among the three methods with an overall average below 100 %, which is associated to signal suppression (except for the effluent of piggery wastewater). Danofloxacin and 1,4 benzoquinone showed a poor response in the matrix samples, hence, matrix effect was not able to be calculated reliably.

3.2.1.3. Relative recovery (bias). They were calculated by dividing the registered peak area for each analyte by the peak area predicted by the regression line associated to each matrix under each method procedure. Values for all levels within the dynamic range were averaged (n = 15)

B) Influent Piggery Wastewater

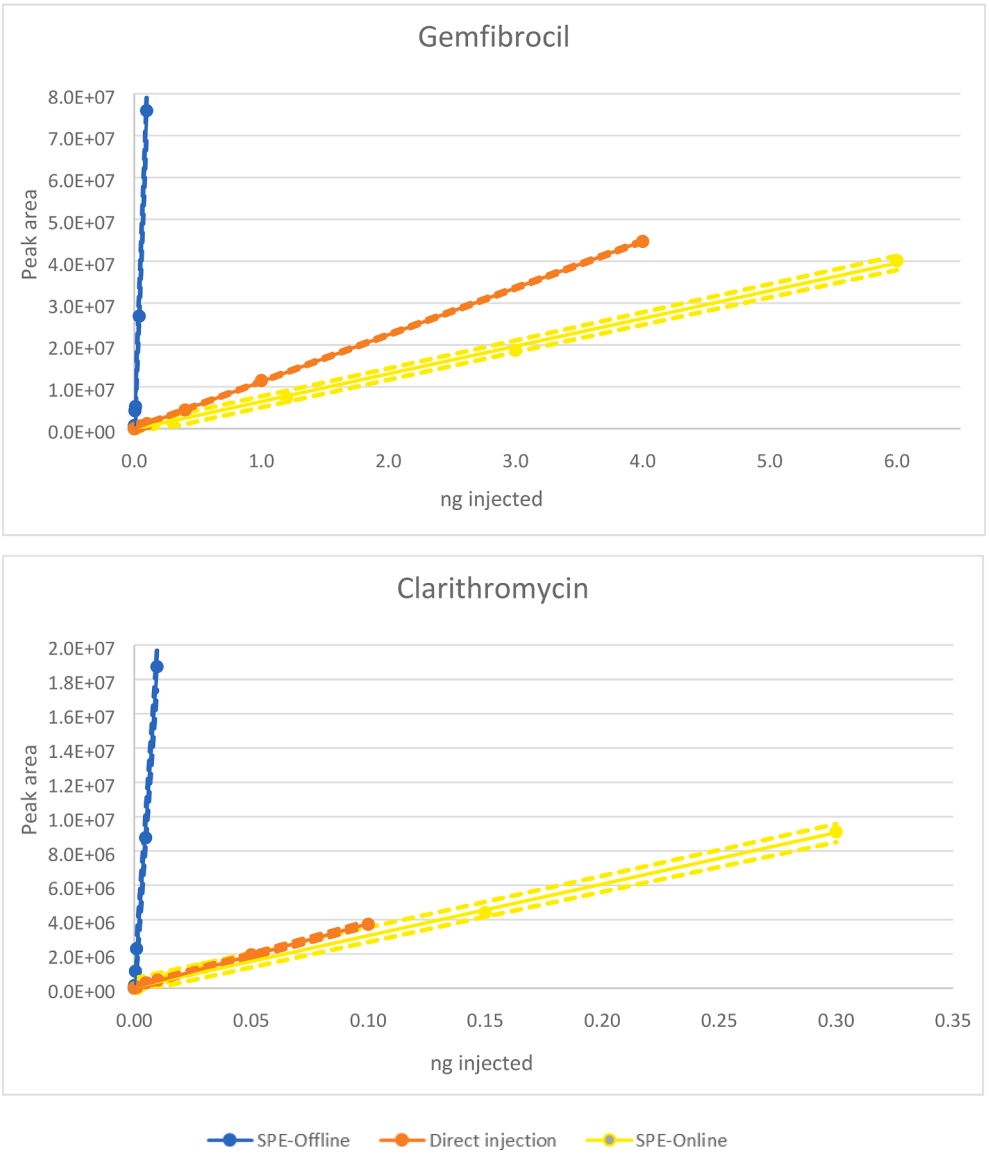

Fig. 2. (*continued*).

and are shown in Supplementary data 6**C** as percentages. Relative recoveries between 90 and 110 % were considered acceptable. Trueness associated to analytes under methodologies providing relative recoveries between 75 and 90 % was deemed to be unreliable.

A higher percentage of analytes with acceptable trueness (90–110 %) was provided when they were analyzed by DI or Offline SPE (similar trueness was achieved between them). The Online SPE was clearly the worst of the studied analyzing method in all the matrices.

The analytes 1,4 benzoquinone and caffeine showed poor calibration curves, hence low relative recoveries (<70 %) were determined for them. Thus, systematic error by underestimation of their concentrations was expected for these compounds.

3.2.2. *Precision*

It was calculated by determining the %RSD of the peak area at two concentration levels (0.05 and 40 μg L^{-1}) in triplicate (n = 3). It was determined for each analysis method and matrix. They are shown in Supplementary data 6**D**. The overall average for all the analytes was also determined for the three analytical methodologies in each matrix and is shown at the bottom of the table.

Both fast methods turned out to be more precise than the conventional one, especially at the most concentrated level with %RSD under 10 % in most cases, both in influent and effluent of either urban and agricultural wastewater. This could probably be attributed to a decrease in the human error introduction associated with the decrease of the manual sample manipulation.

The %RSD for oxytetracycline, tetracycline, sulfadiazine, apramycin, 1,4 benzoquinone and iohexol could not be properly determined as fewer of three replicates were quantified, probably because the tested concentrations were out or in the limit of their dynamic range.

3.2.3. *Sensitivity and dynamic range*

Method limit of quantification (MLQ), defined as the concentration providing a signal-to-noise ratio of 10, represents the minimal concentration of analyte that can be quantified by a method.

Dynamic ranges, extending from the MLQs to the maximum concentration within the linear range, were also studied. They represent the range of concentration within each analyte that can be quantified by each method.

Supplementary data 6**E** shows the determined values for each of

Table 3
Concentration (±standard deviation) of target analytes in urban and piggery wastewater determined by the validated fast and conventional methods, expressed in ng/L, and elimination rates in monitored A) Urban and B) Piggery wastewaters.

A)

Analyte	URBAN								
	EFF			INF			Removal (%)		
	SPE-OFF	DI	SPE-ON	SPE-OFF	DI	SPE-ON	SPE-OFF	DI	SPE-ON
Doxycycline	8	284 ± 28	42	447	480 ± 223	1	98	41	<0
Enrofloxacin	1	116 ± 3	6	9	**41**	63	89	<0	90
Danofloxacin	0.2	56 ± 1	68	0.2	547 ± 203	172	0	90	60
Sulfamethoxazole	16.78 ± 0.03	**3**	68	41.3 ± 2.3	52.4 ± 0.2	6	59	94	<0
Tylosin	1	10.0 ± 0.1	59	2	53 ± 2	31	50	81	<0
Tiamulin	**2**	10.6 ± 0.1	16.1 ± 0.7	2.97 ± 0.01	45 ± 3	59 ± 3	33	76	73
Trimethoprim	16.7 ± 0.1	9.30 ± 0.1	17.96 ± 0.04	2.805 ± 0.002	**0.1**	**24**	<0	<0	25
Fenbendazol	**1**	42.2 ± 0.5	101.5 ± 0.9	**1**	70 ± 1	148 ± 5	0	40	32
Progesterone	**1**	**6**	**95**	20.8 ± 0.1	100 ± 8	119.4 ± 0.1	95	94	20
Methylparaben	651 ± 169	739 ± 212	620 ± 498	2,655 ± 44	2,652 ± 254	2,319 ± 50	75	72	73
Carbamazepine	12.0 ± 0.1	9.64 ± 0.01	7.36 ± 0.03	18.8 ± 0.2	16.93 ± 0.01	8.62 ± 0.04	37	41	22
Propranolol	19.60 ± 0.04	24.30 ± 0.02	49.4 ± 0.7	26.68 ± 0.02	32.33 ± 0.03	56.2 ± 0.6	26	25	13
Ofloxacin	164 ± 100	77.55 ± 0.02	177.6 ± 0.5	492 ± 163	473 ± 3	475 ± 7	67	84	63
Naproxen	3,104 ± 20	3,067 ± 23	2,873 ± 48	4,022 ± 77	3,926 ± 89	3,526 ± 200	23	22	19
Clarithromycin	19.65 ± 0.03	15.42 ± 0.01	25 ± 121	37.8 ± 0.1	32.67 ± 0.06	65.4 ± 0.3	47	55	62
Erythromycin	206	406.0 ± 0.5	**n.d.**	4	969 ± 17	**n.d.**	<0	58	–
Norfloxacin	4	56.5 ± 0.3	52	141	**1**	1	97	<0	<0
Atorvastatin	51 ± 92	70.72 ± 0.04	75.9 ± 0.1	73 ± 54	80.0 ± 0.1	94.09 ± 0.04	30	11	19
Atenolol	298.9 ± 0.03	329.8 ± 0.2	279 ± 1	434 ± 353	510.2 ± 0.9	462 ± 6	31	35	40
Atrazine	8.28 ± 0.01	10.41 ± 0.06	9.09 ± 0.01	13 ± 0.1	11.801 ± 0.003	10.08 ± 0.01	38	17	10
DEET	156 ± 1	125.3 ± 0.3	129.0 ± 0.1	232 ± 1	266.7 ± 0.3	257 ± 1	33	53	50
Ciprofloxacin	101 ± 542	143 ± 6	306.1 ± 0.3	598 ± 77	494 ± 8	602 ± 46	83	71	49
Crotamiton	41 ± 2	18.60 ± 0.04	14.13 ± 0.03	27.0 ± 0.1	23.24 ± 0.07	**0.1**	<0	17	<0
Ethylparaben	80.2 ± 0.1	111.6 ± 0.2	**n.d.**	335 ± 3	304.9 ± 0.4	385	76	63	–
Propylparaben	319 ± 176	370 ± 304	349 ± 23	433 ± 1	569 ± 2	484 ± 2	26	35	28
Diclofenac	14	424 ± 1	423 ± 3	3	524 ± 1	551 ± 4	<0	19	23
Ibuprofen	219	9,925 ± 367	9,860 ± 1136	341	16,904 ± 726	19,337 ± 1178	36	41	49
Clofibric acid	22 ± 11	22 ± 8	32	34.2 ± 0.2	27.12 ± 0.01	22	35	19	<0
Gemfibrocil	103 ± 58	94 ± 5	116 ± 3	295.3 ± 0.3	286 ± 1	235 ± 5	65	67	51
# detected	**26**	**29**	**26**	**28**	**26**	**26**			
# quantified	**18**	**27**	**19**	**20**	**26**	**18**			

Underlined values correspond to < MLQ. Estimated concentrations were calculated by (MLQ-MLD)/2
Bold and underlined values correspond to < MLD. Estimated concentrations were calculated by MLD/2

3B).

Analyte	PIGGERY								
	EFF			INF			Removal (%)		
	SPE-OFF	DI	SPE-ON	SPE-OFF	DI	SPE-ON	SPE-OFF	DI	SPE-ON
Doxycycline	n.d.	132 ± 99	n.d.	n.d.	346 ± 25	n.d.	–	62	–
Marbofloxacin	n.d.	n.d.	3.0 ± 0.7	n.d.	n.d.	42.53 ± 0.03	–	–	93
Enrofloxacin	n.d.	28	n.d.	n.d.	138 ± 19	n.d.	–	93	–
Danofloxacin	n.d.	84 ± 12	88 ± 16	n.d.	102 ± 2	123 ± 18	–	17	28
Sulfadiazine	0.1	21	n.d.	230 ± 5	165 ± 8	n.d.	99	96	–
Sulfadimidine	0.4	n.d.	n.d.	0.2	n.d.	n.d.	–	–	–
Tiamulin	0.2	3.33 ± 0.01	0.972 ± 0.004	0.5	7.483 ± 0.001	1.813 ± 0.002	40	61	64
Fenbendazol	0.4	13.48 ± 0.01	**35**	9.696 ± 0.002	19.97 ± 0.07	22	99	37	–
Progesterone	n.d.	45.5 ± 0.2	n.d.	n.d.	50.2 ± 0.6	n.d.	–	9	–
Carbamazepine	0.1	2.688 ± 0.001	n.d.	0.5	4.4107 ± 0.0002	n.d.	80	30	–
Acetaminophen	1	n.d.	1	4.48 ± 0.01	n.d.	201 ± 4	99	–	99
Ofloxacin	n.d.	39.1 ± 0.6	49 ± 3	n.d.	90 ± 12	87 ± 9	–	58	43
Naproxen	n.d.	n.d.	282 ± 75	n.d.	n.d.	351 ± 5	–	–	20
Caffeine	49 ± 100	56.89 ± 0.04	1	142 ± 8	179 ± 32	1	66	68	–
Atrazine	n.d.	3	n.d.	n.d.	55 ± 6	n.d.	–	97	–
DEET	29.81 ± 0.03	44.21 ± 0.09	44.8 ± 0.3	85 ± 4	118 ± 49	103 ± 9	65	63	57
Ciprofloxacin	n.d.	n.d.	132 ± 9	n.d.	n.d.	182 ± 116	–	–	27
Crotamiton	n.d.	3	n.d.	n.d.	51 ± 4	n.d.	–	97	–
Clofibric acid	2.4 ± 0.2	n.d.	n.d.	74 ± 13	n.d.	n.d.	98	–	–
Gemfibrocil	10	n.d.	n.d.	7 ± 2	n.d.	n.d.	99	–	–
# detected	**10**	**13**	**9**	**10**	**13**	**10**			
# quantified	**3**	**9**	**7**	**7**	**13**	**8**			

n.d.: non detected.

these parameters under the assessed conditions. A higher number of analytes showed a MLQ below 50 ng/L for this trace analysis in piggery than in urban wastewater, varying from 30 to 38 or 16 to 27 analytes, depending on the methodological approach, respectively.

For an improved method comparison, median (μ) and percentiles (P25% and P75%) of both parameters were also calculated and are shown in Table 1. Offline SPE showed the lowest MLQ μ, P25% and P75% of all methodologies, closely followed by DI in all cases. Median

Table 4
ANOVA F factors and the conclusion of the analytical study.

	URBAN		PIGGERY	
	INF	EFF	INF	EFF
F calculated	0.54	0.56	2.20	3.75
F critical	3.10	3.10	3.13	3.13
Conclusion	Comparable	Comparable	Comparable	NOT comparable

values were below 30 ng/L both in Offline SPE and DI, in most of the cases. In contrast, Online SPE never rendered MLQs median below that. Thus, Online SPE turned out to be the less sensitive methodology. Additionally, effluents both from urban and piggery wastewater showed dynamic ranges from lower concentrations.

In contrast, no substantial differences were observed in the amplitude of the dynamic range under any of the studied analytical approaches or wastewater matrices, which varied between 4 and 5 orders of magnitude.

Tetracycline, 1,4 benzoquinone and caffeine showed a poor lineal regression with $R^2 < 0.99$ in the whole tested concentration range.

3.2.4. Carryover

It was calculated by dividing the peak area registered for each analyte in a matrix- and analyte-free aqueous solution by the peak area for the same analyte in the highest calibration curve level (100 μg/L) of each matrix under study, injected immediately before. The resulting values are shown in percentage (%) in **Supplementary data 6F**.

Except for very minor exceptions, carryover maintained under 5 %, including Online SPE, for which a reusable preconcentration column was used. Thus, carryover was not considered a remarkable issue for any of the matrixes under any of the assessed analytical protocols.

The standard for 100 μg/L in doxycycline, tetracycline, 1,4 benzoquinone, caffeine and iohexol provided, in these particular assays, a rather wide peak (probably saturated) so it was not considered for the determination of their carry over.

3.3. Performance comparison for the three methodological proposals. Statistical assessment

The two automatic methods (DI and Online SPE) were validated and statistically compared to the manual conventional method (SPE-Offline) to find out if they implied an improved alternative.

Conventional manual methodologies, such as Offline SPE are highly time consuming and are affected to high uncertainty as shown in this study in **section 3.2.2**. Gross and systematic errors are also less likely in an automatized process. In contrast, both fully automatic analytical methods proposed here involved direct instrumental injection of very low sample volumes, below 500 μL in all cases, in an UHPLC-MS/MS without any sample manual pre-treatment. For the Online SPE approach the analyte solid phase extraction was performed directly by the chromatograph.

The overall analysis time was therefore decreased dramatically from over a working day with the conventional routine to just a few minutes, basically the time required to run the chromatographic gradient described in step 4 of **section 2.2.2**, that is 10 min. That would allow to increase drastically the analytical throughput and many more samples could be processed per day. Even out-of-hour time could be used as analytical time as these automatized analytical procedures could be run unattended, and even remotely controlled.

Additionally, the fact that no counter lab work is required in the proposed alternative methodologies led to a realm where barely no one-use-only material was required. Hence, most of the plastic disposable items and solvents were disregarded in the fast alternative methodological proposals, which resonates with an improved individual analyst and global planet health protection demanded by the principles of the so-called green chemistry, especially required to be complied in environmental analysis in order not to be part of the problem. Even the extraction column was reusable with no wear sign after around 2,000 analysis, in the Online SPE approach.

The fact that only a few hundreds of μL of sample is required to carry out the analysis under the automatic methods proposed here versus the hundreds of mL with the conventional ones (>250 times larger), tackles the recurring storage problem many environmental analytical labs struggle with nowadays. Furthermore, the need for devices such as SPE manifolds and N_2-assisted solvent evaporators are no longer needed which helps save lab space and costs.

On the other hand, conventional manual methodologies like the one described here are still currently the most commonly applied as they required lower-tech instrumentation which are more readily available in all kind of the analytical laboratories around the world. As explained in **section 3.2**, the conventional manual method showed good validation outcome, especially excelling in regard to sensitivity, which is paramount in trace analysis, as it is the case. The number of analytes with MLQ < 50 ng/L of the DI method is slightly under the one achieved by the conventional manual method, as it is shown in the **Supplementary data 6E,** especially in urban wastewater (20 to 27 ng/L in influent and 28 to 31 ng/L in effluent, respectively). The SPE-Online represents the worst scenario although it provided similar MLQs than DI in the piggery inlet wastewater, which was the most complex matrix. In comparison to the DI, the effectiveness in the matrix effect decrease provided by the cleanup during the automatic SPE was probably compensating for the eventual analyte losses taking place there. Nonetheless, validation parameters related to the method effectiveness and trueness such as analytical recoveries rendered worse outcome with the conventional methodology than under the automatic approaches, as shown in **Supplementary data6A** and **6C**, respectively.

Thus, the DI approach entails a good compromise as it gathers the advantages from both sides. It is an automatic fast protocol simplified to the extreme of not including any pretreatment whatsoever, relying most of the selectivity to the mass spectrometry detection. Despite the complexity of the matrixes involved, urban and livestock wastewater, validation parameters, including sensitivity with the MLQs, remained very close to the ones observed for the conventional time-consuming manual Offline SPE approach, but incorporating all the advantages of the simple, fast and automatized methodology a DI without any sample pretreatment has. It did not require even the use of reusable online preconcentration columns, making it the most cost-effective and environmentally friendly of all the proposed methodologies. Nonetheless, it should not be dismissed a possible analytical column lifespan shortening and a tentative LC-MS/MS instrument extra wearing due to the lack of prior sample cleanup as the raw sample is conveyed directly into the chromatographic system.

Online SPE, on the contrary, lost the competition versus DI. It gathered all the benefits fully automatized and miniaturized technology entails but with high trade-offs, as it did not pass the validation test satisfactorily in most of the parameters, including the critical sensitivity, with MLQ values significantly worse (higher) than the ones achieved in the DI and Offline analysis specially in urban wastewater. Hence, for example, in the effluent the MLQ average of SPE-Offline was near to the DI and both much lower than the SPE-Online. In particular, 91 to 106 to 316 ng/L respectively. The chromatography was also deficient in this case, rendering poor peak shapes for many analytes as is shown in Table 2. In fact, a statistical study was conducted to determine if the type of analytical methodology and environmental matrix (influent or effluent) significantly affects the peak width at 5 % height. The influence on these two factors in urban wastewater and piggery wastewater was analyzed by MANOVA (multifactorial ANOVA) studies.

Hence, peak area in urban wastewater seemed only to be affected by the method of analysis ($p = 0.0020$). The type of matrix did not show a significant effect ($p > 0.05$). Hence, peak areas measured by DI were significantly lower. Regarding the peak width at 5 % height, it showed to be affected by both factors: the method of analysis ($p = 0.0001$) and the

sample matrix ($p = 0.0090$). Peak widths were significantly higher in the Online SPE chromatograms, and they were very similar for the other two methodological approaches, that is DI and Offline SPE. In addition, they were higher in the influent than in the effluent. The fact that the MLQ in DI were better than the ones in SPE-Online, although the peak area of the DI are significantly lower, might be due to the fact that the SPE-Online peaks were wider, and the MLQ is directly related by the peak height (no area), which is higher in the DI than the SPE-Online ones.

For piggery wastewater the peak area was only affected by the analytical method ($p = 0.0001$). The sample matrix did not seem to affect ($p > 0.05$). Again, peak areas measured by DI were significantly lower. Unlike urban wastewater, the width at 5 % height was affected only by the analytical protocol ($p = 0.0001$) and did not seem to be impacted by the matrix ($p > 0.05$). Similarly, to the urban wastewater results, the width was significantly higher in the SPE-Online method.

Figs. 1 and 2 show the calibration curve comparison among the three tested methodologies. The regression bands ($p = 0.05$) have been also depicted for each of them. The two automatic methods offered similar slopes in many cases. Nonetheless, DI provided better results than Online SPE in six out of eight scenarios.

With all the results at sight, DI was clearly the best behaving automatic method out of the tested here, both in urban and piggery wastewater. And comparing to Offline SPE, it provided close results in peak shape, number of analytes with MLQ < 50 ng/L, and even better ones in the recoveries. As conclusion, DI rendered similar performance to Offline SPE, but it incorporated all the advantage of a fully automatized, fast, analyst- and environmental-friendly methodology, thus it was proposed as a valid and convenient alternative to the conventional approaches.

3.4. Application of the validated methodologies. Results comparison

The three assessed methodologies, with the optimized injection volume, were used for the quantification of the analytes for which they were validated in target urban and piggery wastewater. Urban wastewater was grabbed sampled, in duplicate, from the WWTP located in Aldeamayor de San Martín, Spain, the same as indicated in **section 2.2.1** in March 2021. Raw piggery wastewater was provided by a farm located in Cantalejo, Spain, the same as indicated in **section 2.2.1,** also treated at the Institute of Sustainable Processes in Valladolid, Spain in April 2021. Calibration curves, including 15 levels and 2 blanks, were matrix-matched built from 0.5 ng/L to 100 μg/L.

As shown in Table 3**,** DI methodology provided the highest number of detected (>MLD) and quantified (>MLQ) analytes both in the urban and in the piggery wastewater samples, confirming the good sensitivity this procedure was rendering, probably due to the analyte loss DI approach was saving by skipping the SPE step. The two SPE methods provided a similar number of detected and quantified analytes. More differences between the two were obtained in the piggery wastewater as the one including Online SPE was able to determine a higher number of analytes.

Quantified concentrations and elimination percentages by the three methods were very similar in both studied wastewaters. The concentrations determined by the three analytical methods, in urban and piggery wastewater, both in influent and effluent streams, were examined by an ANOVA test to know if they are comparable. The calculated F values, shown in the Table 4, pointed that all three methodologies provided comparable concentrations, that is F calculated lower than F critical, except for the Online SPE in the effluent of piggery wastewater, probably due to the few analytes detected under that method.

4. Conclusions

Three different methodological approaches have been proposed for the determination of 58 CECs in urban and livestock wastewater. In all cases, sample pretreatment (if any) has been followed (or coupled) to the same UHPLC-MS/MS instrumental analysis. The performance of the conventional offline SPE method has been statistically compared to both fast methodologies online SPE and DI, and pros and limitations have been arisen. The main conclusions drawn were as follows:

- The best validation results were obtained for the conventional method, Offline SPE, and the fully automatized alternative direct injection of high volumes approach, DI.
- The total analysis time and some validation parameters, such as the systematic (trueness) and random (precision) errors, were worse with the conventional procedure. This made the DI approach a very promising methodology as it also entailed a much lower overall cost and one-time-use-only material, so it was environmentally friendlier. It entailed an analytical move towards miniaturization. Hence, massive sample storage (>250x) and lab counter space saving was also achieved.
- A possible disadvantage for the DI methodology could revolve about the fact that it does not include any cleanup steps prior the instrumental analysis to protect the instrument, thus column lifespan could prematurely get shortened.
- MLQ median values were below 30 ng/L both in Offline SPE and DI, in most of the cases. In contrast, Online SPE never rendered MLQ medians below that. Thus, Online SPE and DI turned out being the less powerful methodological approach of the three in terms of sensitivity, which is critical in trace analysis.
- Concentrations determined with all three methodologies were significantly the same. Nonetheless, Offline and DI offered a larger amount of information as they were able to provide signals above MLD and MLQ for a bigger number of analytes.

CRediT authorship contribution statement

Miguel Ángel de la Serna Calleja: Investigation, Validation, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Silvia Bolado:** Resources, Funding acquisition, Project administration. **Juan José Jiménez:** Conceptualization, Supervision, Formal analysis, Writing – review & editing. **Rebeca López-Serna:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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