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Pressurised liquid extraction and ultra-high performance liquid chromatography-tandem mass spectrometry to determine endogenous and synthetic glucocorticoids in sewage sludge

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ABSTRACT

Glucocorticoids are a type of steroid hormones classified as endocrine disrupting chemicals (EDCs) and they are widely used by human and animal medicine. In this paper, we describe the development and validation of an analytical method for the determination of 9 selected glucocorticoids (betamethasone, cortisol (hydrocortisone), cortisone, dexamethasone, flumethasone, methylprednisolone, prednisolone, prednisone and triamcinolone acetonide) in sewage sludge by pressurised liquid extraction (PLE) and ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). PLE with on-cell clean-up followed by solid-phase extraction (SPE) clean-up (Oasis HLB) has been applied to extract the analytes and clean up the matrix. Recoveries of the method were from 8% for prednisone and cortisone to 73% for triamcinolone acetonide. The developed method achieves limits of detection (LODs) between 1 and 5 μg/Kg dry weight (d.w.) in sludge and the repeatability expressed as relative standard deviation (%RSD, 50 μg/Kg (d.w.), $n=3$) is less than 8%. The PLE/SPE/UHPLC-MS/MS method was successfully applied to determine these pollutants in sludge samples from two sewage treatment plants located in the Tarragona area. Cortisone was found at levels below LOQ, cortisol between 5.2 and 6.1 μg/Kg (d.w.) and prednisolone between $<$LOQ and 6.0 μg/Kg (d.w.).

1. Introduction

A lot of pharmaceuticals are discharged into the environment without any concern for the world's population. However, in recent years it has become a crucial issue since the danger to human health from chronic exposure to some of these compounds has been proven [1]. Pharmaceuticals used in human and animal medicine are a very heterogeneous class of emerging organic pollutants and they have been determined in several environmental matrices such as sewage [2,3], ground water [4] or marine sediments [5].

The main intrusion route of these compounds into aquatic systems is the excretion of free or conjugate form of pharmaceuticals by either humans or animals. The pharmaceuticals arrive via this route to sewage treatment plants (STPs) and several studies show that conventional treatments used are not fully effective in eliminating them [6]. The fate of these pharmaceuticals during sewage treatment is partial adsorption onto activated sludge. This can result in the transfer of these pollutants to rivers and oceans directly via effluent sewages or in the contamination of agricultural soils as a result of using contaminated sludge from STPs as manure.

Synthetic glucocorticoids are a large group of drugs intended for human or veterinary use with a wide range of therapeutic applications, such as combating asthma, skin allergies or inducing labour. Glucocorticoids are a sub-group of steroids with significant physiological functions and, therefore, their ecotoxicological risk is apparent. Studies in fish have shown that long-term treatment with cortisol causes adverse effects to locomotion, inhibits aggressive behaviour, influences the immune response and may also affect the sexual behaviour of fish [7,8]. Unfortunately, they have a significant effect as growth promoters and, therefore, are banned in the EU for this use. Maximum residue levels (MRLs) were established in 1990 in order to eliminate bad farming practices (EU no. 37/2010) [9]. As a result, numerous studies have been published lately related to their determination in biological matrices such as plasma, liver, eggs and milk [10–16]. However, only a few articles refer to their determination in environmental matrices. To our knowledge, there are published papers that report methods to determine some glucocorticoids in surface waters [17–20], sewage [17,19,21–25], sewage sludge [17,25], sediments [26] and soils [26,27]. To date, however, evidence of the presence of glucocorticoids has only been reported in river water [17,18] and sewage [17,19,21–23]. Sewage

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sludge is a possible destination for these steroids, due to their removal in sewage treatment by activated sludge. However, in-depth information about their occurrence and fate in sludge is not available.

Various analytical extraction techniques have been used to extract different types of steroids from solid samples such as soil or sludge. Conventional Soxhlet extraction and ultrasonic solvent extraction (USE) [17] are the most commonly used techniques because they do not involve expensive equipment, but they often require large volumes of solvent and time. As a result, in recent years, extraction techniques such as microwave-assisted extraction (MAE) [28] or pressurised liquid extraction (PLE) [27] have been widely accepted, due to reduced time and solvent consumption as well as the possibility of the automation of extraction and hence improved reproducibility.

To determine glucocorticoids, liquid chromatography combined with tandem mass spectrometry [21], fluorescence [29] or UV–vis [30] is the most frequently used detection system. In addition, gas chromatography [31] or capillary electrophoresis [32] has also been used as a separation technique. In recent years, ultra-high performance liquid chromatography (UHPLC) has been established as a day-to-day technique because of its capacity to provide faster analysis and better efficiency in the separation of compounds. These benefits combined with the specificity, sensitivity and selectivity that tandem mass spectrometry (MS/MS) provides make UHPLC-MS/MS the most suitable technique for determining these emerging organic pollutants in environmental matrices.

In the present paper, we develop an analytical method for the simultaneous determination of nine corticosteroids, endogenous (cortisol and cortisone) and synthetic, in sewage sludge by using PLE followed by UHPLC-MS/MS analysis. Several parameters that affect the extraction procedure have been evaluated and, moreover, different clean-up strategies (on-cell clean-up and solid-phase extraction) have been evaluated in order to prevent ion suppression in mass spectrometry when the sludge extracts are analysed.

2. Experimental

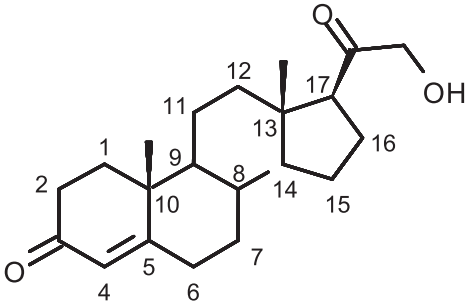
2.1. Reagents and standards

All the standards: betamethasone, cortisol (hydrocortisone), cortisone, dexamethasone, dichlorisone acetate, flumethasone, methylprednisolone, prednisolone, prednisone and triamcinolone acetonide were purchased from Sigma-Aldrich (St. Louis, USA). The chemical structure of glucocorticoids is presented in Fig. 1. Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and stored at −20 °C. Fresh stock solutions of 100 mg/L of each compound in methanol were prepared every month and stored at 4 °C. A mixture of all compounds in water/acetonitrile (4:1) at a concentration of 1 mg/L was prepared weekly and working solutions were prepared daily by diluting these solutions with water/acetonitrile (4:1) or acetone.

Ultrapure water was obtained with an ultrapure water purification system from Veolia waters (Sant Cugat del Vallés, Spain). Acetone, acetonitrile (ACN), methanol (MeOH), n-hexane and ethyl acetate (EtOAc) were of HPLC grade from SDS (Peypin, France) and nitrogen gas was from Carburos Metálicos (Tarragona, Spain). Formic acid from Merck (Darmstadt, Germany) was used to adjust the pH of the mobile phase and the diatomaceous earth used in PLE was purchased from Sigma-Aldrich.

2.2. Instrumentation and equipment

The chromatographic system was an Agilent 1200 series (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). It was equipped with an automatic injector, a degasser, a binary pump, and a column oven. The chromatographic column was Zorbax Eclipse XDB-C_{18} (50 × 4.6 mm, 1.8 μm) (Agilent Technologies, Waldbronn, Germany). The freeze-drying system was supplied by Labconco (MO, USA) and PLE was carried out with



		1-2	6	9	11	16	17	16-17
Betamethasone	BMS	=		F	OH	CH_3	OH	
Cortisol (Hydrocortisone)	HCOR				OH		OH	
Cortisone	COR				=O		OH	
Dexamethasone	DMS	=		F	OH	CH_3	OH	
Flumethasone	FMS	=	F	F	OH	CH_3	OH	
Methylprednisolone	MPNL	=	CH_3		OH		OH	
Prednisolone	PNL	=			OH		OH	
Prednisone	PNS	=			=O		OH	
Triamcinolone acetonide	TACA	=		F	OH			O-C(CH_3)$_2$ O

Fig. 1. Chemical structure of glucocorticoids.

an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA). A 200 mg Bond Elut Plexa (Varian, Agilent Technologies) connected to a manifold (Teknokroma, San Cugat del Vallés, Spain) with a pump as a vacuum source was used for the SPE procedure.

2.3. Sampling and sample preparation

The sewage sludge samples were collected from two domestic sewage treatment plants (STPs) provided with activated sludge biological treatment. Both plants are located in the area of Tarragona and each one treats sewage from a population of more than 100,000 inhabitants. STPs receive urban wastewaters and some industrial discharges. The average flow rates are 30,000 m^3/day (STP_1) and 16,000 m^3/day (STP_2) and the biological oxygen demand (BOD_5) is 400 mg/L for both treatment plants. The sewage sludge samples corresponded to a mixture of primary and secondary sewage, which was anaerobically digested and then dehydrated using press filters. Sludge was frozen before being lyophilised and then was crushed in a mortar and pestle and sieved (125 μm) to obtain particles with the same diameter.

Spiked samples were prepared by adding the stock mixture of standards to acetone (the required volume to wet and cover the sludge). The solvent was slowly evaporated at room temperature inside an extractor hood with frequent homogenisation of the sample throughout the two days prior to extraction.

2.4. Pressurised liquid extraction

One gram of freeze-dried sample was placed in a 11 mL stainless steel extraction cell and mixed with 1 g of diatomaceous earth. The PLE method included two steps: the first step was defatting of the sample with n-hexane. The extraction was performed at 40 °C and 1,500 psi with a preheating period of 5 min, two cycles of 1 min, flush volume of 100% of cell volume and nitrogen purge of 360 s. This extract was rejected. The second step was the extraction of analytes with a methanol:acetone (80:20) mixture at 40 °C and 1,500 psi. Operational conditions were as follows: preheating period of 5 min, one cycle of 10 min, flush volume of 30% of cell volume and nitrogen purge of 120 s. The methanol:acetone extract was evaporated to dryness under a flow of N_2 and the residue was redissolved with 25 mL of water prior to further clean-up with an SPE procedure.

2.5. SPE clean-up

To decrease the ion suppression resulting from a high matrix effect, an SPE procedure was applied after the PLE of the sludge. SPE was carried out using 200 mg Bond Elut Plexa cartridges. These were preconditioned with 5 mL of MeOH followed by 5 mL of ultrapure water. The PLE extract, redissolved in 25 mL of water, was loaded into the cartridge which was slightly dried under vacuum and washed with 5 mL of ultrapure water. Before the elution step, the sorbent was further dried under vacuum and then the analytes were eluted from the cartridge with 10 mL of MeOH. The eluate was concentrated under a flow of nitrogen gas to dryness and the residue was redissolved in 1 mL of water:-acetonitrile (4:1) and filtered with a 0.22 μm nylon filter before UHPLC–MS/MS analysis.

2.6. UHPLC-MS/MS analysis

The UHPLC-MS/MS method was developed in a previous paper [19]. Briefly, chromatographic separation of analytes was performed with a gradient elution. Solvent A was water:acetonitrile (78:22 v:v) with formic acid (0.1%) and solvent B was methanol:acetonitrile (78:22 v:v) with formic acid (0.1%). The gradient started at 0.8% of solvent B, then increased to 5% at 5 min, 15% of B at 11.5 min, 50% at 12 min and then remained constant for 1.5 min more. Finally, the gradient was increased to 99.9% at 14 min and remained constant for 0.5 min before returning to initial conditions over 0.5 min. All the compounds eluted within 11.3 min. The chromatographic system operated at 50 °C to reduce the back pressure and decrease the retention factor of analytes and, in addition, the flow rate was 1 mL/min to reduce the time per run. Injection volume was 50 μL.

Table 1
UHPLC-MS/MS acquisition parameters in MRM mode.

Compound	tr (min)	CV (V)	Parent ion (m/z)	Daughter ion (m/z)		
Prednisone	3.7	100	403	**327 (10)**	299 (15)	285 (30)
Prednisolone	3.8	100	405	**329 (10)**	280 (30)	295 (30)
Cortisol	4.1	100	407	**331 (10)**	297 (35)	282 (35)
Cortisone	4.2	100	405	**329 (10)**	301 (15)	311 (30)
Methylprednisolone	7.3	110	419	**343 (10)**	294 (35)	309 (35)
Betamethasone	7.7	110	437	**361 (15)**	307 (35)	292 (35)
Flumethasone	7.9	110	455	**379 (15)**	305 (35)	325 (30)
Dexamethasone	8.2	110	437	**361 (10)**	307 (35)	292 (35)
Triamcinolone acetonide	11.3	110	479	**413 (15)**	337 (20)	375 (10)

CV is cone voltage.
Collision Energies are in brackets (eV).
The most intense daughter ion for each compound is in bold.

ESI interface and MS/MS conditions were individually optimised for each compound with flow injection of a standard solution of each compound. The average conditions selected for the optimum performance of the ESI in the negative mode were nebuliser pressure 40 psi, drying gas (N_2) flow rate 12 L/min, drying gas temperature 350 °C, and capillary voltage 2,000 V. Cone voltage and collision energies for each compound are described in Table 1. Chemical structure of each product ion is proposed in a previous paper [19].

3. Results and discussion

3.1. UHPLC-MS/MS

The UHPLC-MS/MS method was developed in a previous paper in which the studied glucocorticoids were determined in water samples from rivers and sewage [19]. The chromatographic method uses gradient elution with a ternary mobile phase (water, acetonitrile and methanol). The use of methanol in the ternary mobile phase allows a slight chromatographic separation of prednisone/prednisolone and cortisone/cortisol couples (set of polar compounds) and separation between betamethasone and flumethasone compounds. In addition, the acetonitrile allows the chromatographic separation of the epimers betamethasone and dexamethasone.

Moreover, the use of methanol provides good analyte desolvation in the electrospray interface and we can therefore work at an elevated flow rate (1 mL/min). This flow rate reduces the run time (the compound with the highest retention elutes in 11.3 min) without losses in column efficiency because they are composed of sub −2 μm particles. The run time is not as short as would normally be expected for a UHPLC analysis due to the necessary separation of epimers which cannot be differentiated by MS/MS.

A comprehensive study of the optimisation of several parameters of the tandem mass spectrometer was also carried out in the previous paper [19]. It was observed that the negative ionisation mode provided more intense MRM transitions than positive ionisation mode. Only triamcinolone acetonide had less

intense MRM transitions in negative mode because its fragmentation pathways were slightly different from other glucocorticoids under study. Assigned fragments for all analytes and their cone voltages and collision energies are described in Table 1.

3.2. Pressurised liquid extraction

To achieve a fast and efficient extraction of the target compounds from a solid matrix using a PLE system, several operational parameters must be optimised. The solvent, temperature, time and number of cycles should therefore be tested. Pressure, flush volume and purge time can also be optimised, but it is well known that these parameters do not have a significant effect on the extraction efficiency.

Literature reports a methanol/acetone mixture [27] or pure ethyl acetate [17] as the most suitable solvents for the extraction of glucocorticoids from environmental solid samples. Thus, water, methanol, acetone, ethyl acetate and n-hexane were tested in the first instance, as pure solvents or as mixtures between them. Other initial conditions were as follows: 60 °C, 5 min to preheat cell, 5 min of static time, 1,500 psi of pressure, 1 cycle, 30% flush volume and 120 s of purge time. Lyophilised sludge samples were spiked at high concentration (1 mg/kg (d.w.)) for each compound to ensure that there is no possibility of the matrix interferences affecting the accurate quantification of analytes. For optimisation purposes, extracts with water-miscible solvents (15–20 ml approx.) were brought up to 50 mL of final volume with ultrapure water to prevent ion suppression and achieve conditions similar to the mobile phase prior to their injection into the UHPLC system. The extracts containing ethyl acetate and/or hexane were evaporated to dryness under nitrogen flow and the residues were redissolved with about 15 mL of methanol and then brought up to 50 mL with ultrapure water.

An important aspect to be considered is how the sludge was spiked (procedure is further described in Section 2.3). We observed experimentally that the recoveries of these compounds are highly dependent on the contact time between the matrix and analytes before extraction process because when this time was shorter (some hours) the recoveries were close to 100%. Consequently, we carried out a batch of experiments with the extraction conditions stated above and acetone as extraction solvent to determine the time necessary to obtain constant recoveries along the time. The experiments led us to wait two days prior to extraction.

Results showed (see Tables 2 and 3) that the methanol:acetone mixtures were the most efficient for extracting the target analytes from sludge, rather than pure methanol or acetone. Water or ethyl acetate was also capable of extracting the glucocorticoids from sludge, but always with a lower efficiency than methanol/acetone mixtures. N-hexane could not extract any compound due to its low polarity.

Table 2
PLE recoveries (%) obtained with different pure solvents.

Compound	Water	Methanol	Acetone	Ethyl acetate
Prednisone	8	10	6	2
Prednisolone	14	13	12	7
Cortisol	7	5	4	2
Cortisone	13	24	12	11
Methylprednisolone	17	21	22	11
Betamethasone	24	12	24	7
Flumethasone	10	8	14	6
Dexamethasone	14	12	17	11
Triamcinolone acetonide	33	20	31	24

Sludge was spiked at 1 mg/Kg (d.w.) and %RSD($n=3$) $<10\%$.
Extraction conditions are described in Section 3.2.

In methanol:acetone mixtures, recoveries were between 13% and 30% for more polar compounds (prednisone, prednisolone, cortisol and cortisone), between 25% and 49% for medium-polar compounds (methylprednisolone, betamethasone, dexamethasone and flumethasone) and between 62% and 74% for the non-polar compound (triamcinolone acetonide). It is notorious that the two compounds with a ketone group at position C11 (prednisone and cortisone), rather than an alcohol group like the other glucocorticoids, showed much lower recoveries than the other compounds. The results show that a mixture rich in methanol (80% in volume) is able to enhance the efficiency of extraction of triamcinolone acetonide from 60% in rich acetone mixtures (80%) to 74% in rich methanol mixtures (80%). For the rest of the compounds, no significant differences in their extraction were found. Methanol:acetone (80:20) was therefore chosen for the PLE process.

Regarding the extraction temperature, a range from room temperature (~25 °C) up to 100 °C was studied with a mixture of methanol:acetone (80:20). The other extraction conditions were the same as described previously. A temperature of 40 °C provided the best recoveries for all analytes (between 16% and 39% for more polar analytes, between 34% and 56% for medium-polar and 90% for the non-polar). These recoveries were better than at room temperature. The recoveries decreased when temperature was increased to 60 °C or 80 °C and were below 10% for the majority of compounds when the temperature was 100 °C. This fact is probably due to a thermal decomposition of analytes and may indicate that, at 40 °C, the thermal decomposition of compounds is very low.

Static extraction time and number of cycles were also studied in order to enhance the efficiency of extraction. Static times of 3, 5, 10 and 20 min were studied. A static time of 10 minutes resulted in a slight improvement of the extraction process (recoveries were between 19% and 43% for more polar, 41% and 65% for medium-polar and 90% for non-polar). A time of 20 min did not result in a significant increase in the extraction efficiency. Regarding the number of cycles, 1, 2 and 3 cycles with a static extraction time of 10 min were tested. Two cycles of extraction only improved recoveries by 2% for some compounds and then, increase in the number of cycles was discarded. Therefore, one cycle and a static time of 10 min were chosen as optimal parameters for the PLE extraction.

In addition, the fact that n-hexane does not extract the compounds from sludge was exploited in order to carry out an on-cell matrix clean-up. Thus, we extracted the insoluble-water interfering substances such as fat and oil before the extraction of analytes with the methanol:acetone mixture. A much cleaner extract was obtained and differences in the recovery of analytes were not observed when this on-cell clean-up was applied.

Finally, it was decided to concentrate the PLE extract in order to minimise the dilution of the sample and therefore improve the method detection limits. Unfortunately, the final extract was extremely dark and turbid when evaporated residues were redissolved with small volumes of water:acetonitrile (4:1) and filtered (0.22 μm). Therefore, to improve the cleaning of the sample, reduce the matrix effect and minimise the final volume of dilution, a SPE clean-up was applied prior to analysis by UHPLC-MS/MS.

3.3. Solid-phase extraction clean-up

Our prior knowledge of the determination of glucocorticoids in sewage and river waters led us to choose a polymeric sorbent for SPE which is capable of retaining both polar and non-polar

Table 3
PLE recoveries (%) obtained with different solvent mixtures.

Compound	MeOH/Acetone (20:80)	MeOH/Acetone (50:50)	MeOH/Acetone (80:20)	MeOH/EtOAc (50:50)	Acetone/EtOAc (50:50)	MeOH/Acetone/EtOAc (35:35:30)
Prednisone	13	13	15	12	5	9
Prednisolone	24	27	28	20	8	17
Cortisol	25	27	30	7	3	6
Cortisone	13	13	13	27	12	20
Methylprednisolone	25	26	28	37	14	23
Betamethasone	47	49	48	29	12	21
Flumethasone	34	34	31	18	7	12
Dexamethasone	32	33	34	22	9	16
Triamcinolone acetonide	62	68	74	42	23	36

Sludge was spiked at 1 mg/Kg (d.w.) and %RSD($n=3$) $<10\%$.
Extraction conditions are described in Section 3.2.

Table 4
SPE recoveries (%) obtained with different washing steps.

Compound	water	water/MeOH (9:1 v/v)	n-hexane
Prednisone	76	73	85
Prednisolone	77	72	87
Cortisol	108	83	101
Cortisone	100	82	100
Methylprednisolone	81	82	89
Betamethasone	91	86	88
Flumethasone	81	74	74
Dexamethasone	83	74	88
Triamcinolone acetonide	96	82	77

Sludge extracts (~25 mL) were spiked at 50 μg/L.
SPE procedure was described in Section 2.6 %RSD (50 μg/L, $n=3$) $<8\%$.

compounds. Bond Elut Plexa (hydroxylated surface and polystyrene-divinylbenzene core with a macroporous structure) was selected as the most suitable for this purpose based on the excellent results obtained in a previous study [19]. This sorbent was applied for the SPE of glucocorticoids in sewage and it provided recoveries above 90% for all compounds in this matrix. Subsequently, a similar strategy was applied to the sludge extracts. The PLE extracts were then evaporated under a stream of nitrogen and were subsequently redissolved with about 25 mL of ultrapure water to load into the cartridge. Other considerations of the SPE procedure are extensively described in Section 2.5.

In order to minimise the sample interferences without involving significant losses in the recovery of the analytes, different washing stages were tested in the SPE clean-up step, including 5 mL of ultrapure water, 5 mL of water:methanol (90:10, v-v) and 2 mL of n-hexane. Sludge extracts were spiked at 50 μg/L for each compound after the PLE extraction and evaporation process and were used for the SPE experiments. Recoveries were calculated by comparing the signal obtained by the SPE extracts with a sludge extract subjected to the same SPE process, spiked after the elution step. Moreover, the signal obtained was compared with a standard in order to evaluate the matrix effect. The recoveries associated with the SPE with the different washes are shown in Table 4.

The recoveries obtained when ultrapure water was used in the washing step were between 80% and 100% depending on the compound. These results are similar to those obtained for sewage and river waters [19] and indicate that the matrix did not decrease analyte–sorbent interaction. This indicated that the properties of the sorbent were adequate for these analytes in this matrix. Regarding the washing step experiments, washing with water:methanol gave the worst results of the three washes (recoveries between 70% and 80%) due to the analytes being partially eluted in this step. Hexane wash did not affect the recovery of polar and medium-polar compounds (when these are compared with water wash results), but the recovery of most non-polar compounds, such as triamcinolone acetonide, decreased from 95% when water was used to 77% with hexane. In addition, results showed that all of the different washes involved a matrix effect below 20% for all compounds. A wash step with ultrapure water was therefore selected based on these results (recoveries and clean-up) as well as for the sake of simplicity.

3.4. Method validation

The method validation involved the evaluation of the linear range, LOD, LOQ, repeatability and reproducibility between days (expressed as % Relative Standard Deviation) and recoveries for glucocorticoids under the optimised sample extraction procedure (PLE and SPE). All these parameters were calculated with a sludge sample which did not contain the glucocorticoids and are shown in Table 5.

To calculate the recoveries, a calibration curve obtained from a set of *posteriori* (after PLE and SPE) fortifications (between 1 and 100 μg/Kg (d.w.)), except for triamcinolone acetonide (between 5 and 100 μg/Kg (d.w.)), of blank sludge samples was used. Then, three blank sludge samples were spiked at 50 μg/Kg (d.w.), analysed by the entire method and recoveries were calculated by the calibration curves already mentioned.

Recoveries of the analytes had a higher variation range depending on their polarity, as shown in the results. Thus, the group of high-polar compounds showed poor recoveries in a range from 8% to 20%. For the medium-polar compounds that range increased from 28% to 43% and for the low-polar compounds their recovery increased up to 73%.

Although the matrix effect had been reduced significantly, it was decided to use a matrix matched calibration curve (with sludge samples spiked before PLE) for the quantification of analytes in order to obtain more accurate results and also correct the recoveries. However, it would be possible to use an external calibration curve for a quick and easy quantification of the glucocorticoids with the developed method because the ion suppression was less than 20%. We also discarded the use of an internal standard to correct ion suppression due to its low commercial availability and high price. Dichlorisone acetate, a compound of the same family, was proposed in some papers for this purpose [12]. However, this option does not have a total guarantee that a compound of the same family as the target analytes was not present in samples. A major discussion of this issue was included in a previous paper [19].

Linear range, limit of quantification (LOQ) and detection (LOD) were obtained experimentally by spiking sludge at different levels

Table 5
Validation values for PLE/SPE/UHPLC–MS/MS method.

Compound	Recovery[a] (%)	Repeatability[b] (%RSD, $n=3$)	Reproducibility[c] (%RSD, $n=3$)	LOD (μg/Kg (d.w.))	LOQ (μg/Kg (d.w.))
Prednisone	8	1	2	0.5	1.0
Prednisolone	18	1	3	0.5	1.0
Cortisol	20	4	6	0.5	1.0
Cortisone	8	2	3	0.5	1.0
Methylprednisolone	43	6	9	0.5	1.0
Betamethasone	43	6	9	0.5	1.0
Flumethasone	28	1	4	0.5	1.0
Dexamethasone	28	3	6	0.5	1.0
Triamcinolone acetonide	73	8	10	1.0	5.0

[a] Recoveries calculated by a 50 μg/Kg (d.w.) spiked sludge.
[b] Intra-day repeatability calculated as %RSD by a 50 μg/Kg (d.w.) spiked sludge.
[c] Day-to-day reproducibility calculated as %RSD by a 50 μg/Kg (d.w.) spiked sludge.

prior to the extraction procedure. These parameters were equal for all glucocorticoids except triamcinolone acetonide, for which the parameters were higher than the others. This is due to their lower signal response, as previously discussed. The linear range started at LOQ (defined as the lowest calibration point) and went up to 100 μg/Kg (d.w.) with good linearity for all compounds ($R^2 > 0.997$). The analyte concentrations for LODs corresponded to a signal/noise ratio equal to 3 times. Thus, LOQ was 1 μg/Kg (d.w.) and LOD was 0.5 μg/Kg (d.w.) for all glucocorticoids except triamcinolone acetonide (for which LOQ was 5 μg/Kg (d.w.) and LOD was 1 μg/Kg (d.w.)).

Repeatability (intra-day) and reproducibility (day-to-day) were obtained with three replicates of a sample spiked at 50 μg/Kg (d.w.). Repeatability (%RSD, $n=3$) was always less than 8% and was commonly found to be between 1% and 4%. Reproducibility between days was always less than 10% (%RSD, $n=3$). An MRM chromatogram obtained from a spiked sludge at 10 μg/Kg (d.w.) is shown in Fig. 2.

In general, recoveries were not very high, but linearity was good and the method limits of quantification and detection were reasonably good in comparison with previous studies for this type of matrix and analytes. Previously, Liu et al. [17] developed a method to determine five glucocorticoids in sludge with an ultrasonic solvent extraction with ethyl acetate. The LODs (0.8–2.06 μg/Kg (d.w.)) and LOQs (1.95–6.86 μg/Kg (d.w.)) achieved with this method were slightly higher than in our study. Moreover, Fan et al. [25] described a method to determine seven glucocorticoids in sludge based on ultrasonic solvent extraction with methanol:acetone (50:50) followed by three SPE clean-up stages (HLB followed by an NH_2-SPE and finally, with a florisil (or silica) cartridge). The LODs achieved with this method (0.02–0.2 μg/Kg (d.w.)) are slightly better than those from our study but the LOQs were not reported.

In addition, two other papers determined the glucocorticoids in environmental solid samples such as soils [26] and sediments [26,27]. In these two papers, a PLE method for the extraction of glucocorticoids from samples was applied. Gineys et al. [27] reported recovery values similar to those found in our study but only two glucocorticoids were determined and the method was only applied to spiked soil samples (PLE extraction with methanol: acetone (50:50) at 80 °C). Pérez-Carrera et al. [26] in a study into the determination of 32 human and veterinary residues detected the presence of prednisolone (below LOQ) in one sediment sample.

4. Application to environmental samples

The PLE/SPE/UHPLC-MS/MS method was applied to determine glucocorticoids in eight sludge samples from the two STPs over a period of seven months and only three samples contained low residues of glucocorticoids (Table 6). The presence of these compounds in the different samples was corroborated by their retention time and two qualifier ion ratios, as can be seen in Table 6 under requirements of Commission Decision 2002/657/EC [33].

Thus, hydrocortisone was determined to be more frequent, between 5.2 and 6.1 μg/Kg (d.w.) in three samples taken from the two urban sewage treatment plants. Prednisolone and cortisone were also determined in few samples but their concentrations were lower than that of hydrocortisone. Prednisolone only appeared in one sample at 6 μg/Kg (d.w.) and in another sample, prednisolone and cortisone were found below LOQ. The other glucocorticoids were not detected in any of the sludges analysed. The presence of hydrocortisone in the sludge was expected since hydrocortisone has a natural origin and its concentration levels may therefore be expected to be higher than the synthetic glucocorticoids. However, the presence of prednisolone should be highlighted due to its synthetic origin (exogenous). Prednisolone is usually detected in influent sewage at a level lower than hydrocortisone or cortisone. However, the concentration of prednisolone (together with prednisone) in influents is the most elevated of the synthetic glucocorticoids [19].

These results demonstrate that activated sludge is one destination of glucocorticoids. Despite this, their elimination from the sewage is not fully complete as shown in previous studies [19]. In addition, glucocorticoids remain in the sludge and if used as manure, it may lead to new forms of contamination of the environment.

To our knowledge, the presence of glucocorticoids in sludge has only been studied in two papers. Liu et al. [17] developed a method to determine prednisone, prednisolone, cortisone, cortisol and dexamethasone, but no residue of glucocorticoids was found. Nevertheless, Fan et al. [25] determined prednisone, prednisolone, corticosterone, cortisone, cortisol, methylprednisolone and dexamethasone in sludge and found five of them within the range of 0.05–0.31 μg/Kg (d.w.).

5. Conclusions

A sensitive analytical method was developed to determine nine glucocorticoids simultaneously in sludge samples with pressurised liquid extraction as the extraction technique. An on-cell clean-up with n-hexane was applied to defat the sludge before the PLE with methanol:acetone (80:20). An SPE clean-up with a polar/non-polar sorbent was applied in order to minimise the matrix interference and reduce the ion suppression. The two

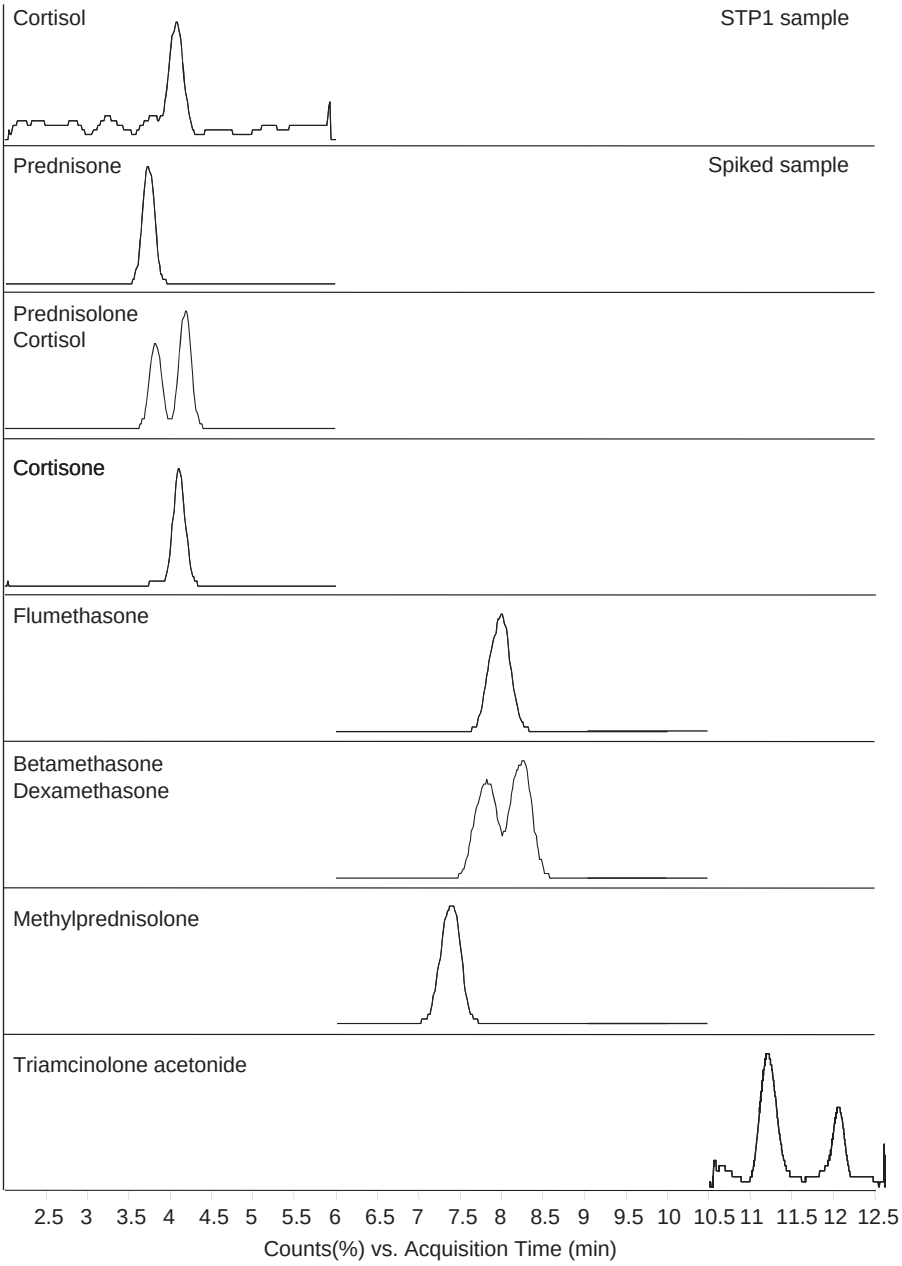


Fig. 2. Quantification of MRM trace of cortisol detected in one sample from STP1 and MRM chromatogram of a spiked sludge (10 μg/Kg (d.w.)).

Table 6
Concentrations (μg/Kg (d.w.)) and qualifier ion ratios (Q/q) in sewage sludge samples.

Compound	S1 (STP_1)	S2 (STP_2)	S3 (STP_2)	Q/q_1 (%)	Q/q_2 (%)
Prednisolone	n.d.	< LOQ	6.0 (22,11)	20	9
Cortisol	5.2 (17,15)	6.1 (18,16)	5.6 (19,16)	16	15
Cortisone	n.d.	< LOQ	n.d.	21	8

Qualifier ion ratios (%) in samples are in brackets.

clean-up stages ensured that the extracts did not have significant matrix effects (< 20%). Recoveries were from 8% to 73% depending on the compound.

Purified extracts were analysed by UHPLC-MS/MS and the method showed LOD around 0.5 μg/Kg (d.w.) for the target analytes. Cortisone (< LOQ), cortisol (5.2–6.1 μg/Kg (d.w.)) and prednisolone (< LOQ – 6.0 μg/Kg (d.w.)) were detected in some sludge samples.

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