Comparison of extraction techniques for isolation of steroid oestrogens in environmentally relevant concentrations from sediment

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ABSTRACT
The comparison of four extraction techniques for isolation of five native and one labelled steroid oestrogens from sediment was described. The three conventional extraction techniques Soxhlet warm extraction (SWE), accelerated solvent extraction (ASE), microwave-assisted extraction (MAE) and a promising technique QuEChERS were tested for isolation of low environmentally relevant oestrogen concentrations using different extraction conditions. The least expensive and time-consuming method QuEChERS provided the best extraction recoveries (53–84%) from all techniques. MAE achieved the highest recovery from conventional techniques for less polar oestrogens using dichloromethane:acetone 3:1 mixture as an extraction solvent (50–71%), but for extraction of the whole group of oestrogens including more polar estriol acetone or methanol must be used. ASE provided higher extraction recoveries using dichloromethane at 60°C (53–74%) for less polar oestrogens. However, the repeatability of results was unsatisfactory and recoveries using other extraction conditions were lower than for MAE. The most time-consuming SWE achieved the worst extraction recoveries and for isolation of low oestrogen concentrations from sediments, it is completely unsuitable.

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1. Introduction
Over the past 10 years, a wide diversity of compounds has been found to possess endocrine-disrupting properties [1,2]. Steroid oestrogens are biologically active compounds that are synthesised from cholesterol and contain a cyclopentane – o-perhydrophenanthrene ring. Natural oestrogens such as estrone (E1), 17β-estradiol (βE2) and estriol (E3) are secreted by the adrenal cortex, testis, ovary and placenta in humans and animals. The synthetic oestrogen 17α-ethynylestradiol (EE2), found in contraceptive pills, is considered the most potent oestrogenic chemical [3–5]. The main sources of natural...
and synthetic steroid oestrogens are associated with human or animal excrement, which enter into the water from sewage treatment plants after incomplete removal. Steroid oestrogens are largely excreted from the human or animal body as sulphate and glucuronide conjugate through urine. After entering into the aquatic environment, these compounds are de-conjugated by bacteria to the free oestrogens form. Agricultural run-off, septic systems and animal manure are considered to be other significant sources of steroid oestrogens. Steroid oestrogens such as E1, E2 and EE2 have shown potencies and a combination effect on fish which is associated with an increase in plasma vitallogenin, a biomarker for endocrine disruption. This effect was observed at the very low and environmentally relevant concentrations of about 1 ng/L (E2) and lower than 0.1 ng/L (EE2). In the water ecosystem, steroid oestrogens tend to accumulate in solid matrices such as sediments due to their lipophilicity expressed by their higher octanol–water partition coefficient (log $K_{ow}$) values in the range 2.8–4.2. These values predicted that E1, E2 and EE2 would have a higher tendency to sorption on the top layer of sediment compared to E3 that is less likely to be associated with solids. Sorption of oestrogens is influenced by the structure and composition of sediment and the total organic carbon content (TOC) plays an important role. A relationship between the concentration of oestrogens in sediment and TOC and also the relationship between log $K_{ow}$ and the organic carbon–water partition coefficient (log $K_{oc}$) were observed. This indicates that sediment with a higher TOC is more potent to retain oestrogens from the aquatic environment depending on the oestrogen’s $K_{ow}$ value. To gain an understanding of steroid behaviour in the water-sediment system, a rigorous extraction technique followed by sensitive and selective analysis is necessary.

Many different conventional (Soxhlet warm extraction [SWE], ultrasonic-assisted extraction) and more advanced conventional extraction techniques (accelerated solvent extraction [ASE], microwave-assisted extraction [MAE]) or the newest, promising method QuEChERS can be used for isolation of steroid oestrogens from solid matrices. Modified Soxhlet extraction known as SWE is based on evaporation of solvents from a glass-heated vessel, condensation and the flow down of an extraction solvent to the extraction chamber with the sample. The extraction chamber is heated by an upper heating element. This technique is used or tested for isolation of a wide range of chemical contaminants from soils and sediments such as organochloride pesticides or polychlorinated biphenyls, polycyclic aromatic hydrocarbons and explosives. Limited information about the use of this method for extraction of steroid oestrogens from soils and sediments is available. Matejicek et al. published a study focusing on the possibility of using SWE for extraction of oestrogens from spiked sediment (20 ng/g of E1, 17α-estradiol [αE2], βE2, E3, EE2 and their sulphate, glucuronide and acetate conjugates). Methanol/water (75:25) was used as an extraction solvent and extraction was performed at 130°C for 60 min. The main disadvantages of this technique are that it is time consuming and a large volume of extraction solvent must be used for extraction. The conventional extraction technique based on ultrasound was also often used. Various extraction conditions with different solvents and extraction volumes were performed using an ultrasonic probe or bath according to the initial sediment weight. One extraction step usually takes several dozen minutes or less time with repeating the extraction and collecting the extracts.
ASE is widely used for the isolation of various chemical compounds from soils and sediments. The method utilises a temperature higher than the boiling point of the solvent in order to achieve the best extraction efficiency. The solvent remains in the liquid phase due to the high pressure. The high temperature improves mass transfer and increases the solvent’s capacity to solubilise analytes. More information and comparisons of extraction methods are known concerning the use of ASE for isolation of oestrogens from soils. Beck et al. presented results focused on optimisation of ASE for extracting E1, αE2, βE2 and E3 from spiked (2 ng/g) soils. Thirteen extraction solvents were tested in a temperature range, the highest recovery being achieved with acetone at 60°C, and also acetonitrile, ethylacetate and dichloromethane:acetone achieved high extraction efficiency [20]. Salvia et al. compared five extraction solvents for isolation of steroid compounds, antibiotics and other human contaminants using spiked (50 ng/g) soil at 80°C. The high recoveries for E1, αE2, βE2 and EE2 were achieved using methanol, a methanol:acetone mixture (50:50) and acetone. However, for E3, acetone provided the highest extraction recoveries in comparison with the previous two solvents [21]. Less information and comparisons are known for extraction of oestrogens from sediments. Dichloromethane [22], acetone:methanol mixture (1:1) [23] and water:acetone:methanol mixture (1:2:1) [24] were used for extraction of E1, E2, EE2 and E3 from sediment in various temperature ranges from 40 to 120°C using usually two or three 5-min static extraction cycles.

The MAE technique makes use of a temperature controlled with microwave power for extraction of a diverse array of solid matrices in a short time. This technique is very useful for extraction and isolation of many different organic compounds from solid matrices. Studies on isolation of steroid oestrogens from sediments have also been published. Methanol or methanol:water mixtures are predominantly recommended in many studies due to the high ability to heat these solvents. A small amount of sediment sample (1–5 g) was usually placed into the polytetrafluoroethylene (PTFE) cartridges and a moderate volume of extraction solvent (5–25 mL) depending on sediment weight was used for extraction. Extraction experiments were usually performed at 90–130°C. An extraction time of 5–15 min was recommended [17,25,26].

The method QuEChERS, also known as dispersive solid phase extraction (SPE), is an extraction and cleanup technique originally developed for recovering the residues of polar pesticides in fruit and vegetables. Nowadays, this technique is developed for applications in the environmental analysis [27]. This technique involves shaking a homogenised sample with acetonitrile and salt that facilitates solvent partitioning and improves recovery of polar analytes (MgSO₄, anhydrous), reduces the amount of polar interferences (NaCl) or acts as buffers. Another different reagent can be used for extract purification (PSA – primary secondary amine, C18 chromatographic sorbent, GCB – graphitised carbon black) after extraction. Information on the application of the QuEChERS method for isolation of oestrogens from soils and sediments is limited. However, some studies tested modified or buffered QuEChERS coupled with SPE or using GCB as a purification step [28,29]. This technique provides satisfactory results in these studies and can represent a very promising tool for effective, rapid and inexpensive extraction of oestrogens from soils and sediments.

Our study was focused on comparison of three conventional extraction techniques: SWE, ASE and MAE for isolation of five native and one labelled steroid oestrogens (E1,
αE2, βE2, EE2, E3 and EE2d4) from sediment using different extraction conditions (solvent, extraction time and temperature). Also the promising extraction technique QuEChERS, primarily used for extraction of polar organic compounds (pesticides) from different kinds of food, was used in two variants (1) original QuEChERS and (2) buffered QuEChERS for this comparison. Only very low environmentally relevant oestrogen concentrations (1 ng/g) were spiked to sediment for evaluating the extraction recoveries and selecting the best of them for this purpose.

2. Experimental

2.1. Chemicals

Oestrogen standards E1 (min. 99%), αE2 (min. 98%), βE2 (min. 98%), E3 (min. 97%) and EE2 (min. 98%) were purchased from Sigma Aldrich (Germany). Deuterated oestrogen standards 17α-ethynylestradiol 2,4,16,16-d4 (EE2d4, min. 98.3%), 17β-estradiol 2,4,16,16-d4 (βE2d4, min. 98%) and 16α-hydroxy-17β estradiol-2,4-d2 (E3d2, min. 98.9%) came from CND isotopes (Canada). Formic acid (98%), ammonium hydroxide (NH4OH, 28–30%), acetic acid (≥99.0%), dansylchloride (min. 99.0%), sodium bicarbonate (99.5–100.5%), sodium chloride anhydrous (NaCl ≥ 99%), magnesium sulphate anhydrous (MgSO4 ≥ 97%), sodium hydrogencitrate sesquihydrate (99%) and sodium citrate tribasic dihydrate (99.5%) were purchased from Sigma Aldrich (Germany). Solvents used in this work came from Sigma Aldrich (Spain) – methyl tert-butyl ether (MTBE, min. 98%, Chromasolv), from Biosolve (France) – methanol for LC–MS (MeOH, 99.95%), acetonitrile for LC–MS (ACN, 99.95%) and from Chromservis (Czech Republic) – acetone (99.8%), dichloromethane – chromapur (DCM, 99.9%), n-hexane (95%). PSA-bonded silica was purchased from Supelco (USA).

2.2. Preparation of sediment samples before all extraction experiments

Standard stock solutions of all oestrogens (100 µg/mL) were prepared separately dissolving the E1, αE2, βE2, E3, EE2, deuterated 17α-ethynylestradiol EE2d4 as surrogate standard, βE2d4 and E3d2 as internal standards (ISs) in ACN and stored at 4°C in a refrigerator. Standard spike solutions of each oestrogen and surrogate EE2d4 (100 ng/mL of each analyte in ACN), ISs βE2d4 and E3d2 (10 ng/mL) were prepared by diluting standard stock solutions. Sediment used for all extraction experiments was collected from the Brno area (Holasky reservoir). The top layer of sediment (20 cm) was sampled to a dark glass vessel rinsed by MeOH and transported to the laboratory. Freeze-dried sediment was homogenised by mortar and pestle, passed through a 2-mm sieve and stored at −20°C. The TOC of tested sediment was 1.40%, percentage of dry mass = 65.79%, loss of ignition = 3.91% in dry mass, total N = 0.16% in dry mass, nonpolar extractable compounds <30.0 mg/kg in dry mass and pH (H2O) = 8.60.

Five grams of sediment sample was spiked by standard spike solution and standard spike surrogate solution (1 ng/g of each oestrogen) before extraction. The sample was carefully mixed by spatula and allowed to stand for 2 h before extraction. This equilibration time was recommended as sufficient time for the analytes to partition onto the solid matrix but not sufficient time for possible degradation [30]. A sample of used sediment
was also extracted without any spike of oestrogens to determine any possible primary contamination in the sampling area.

2.3. **Conventional extraction techniques**

2.3.1. **Soxhlet warm extraction (SWE)**

Automatic extractor B-811 (Büchi, Switzerland) was used for SWE. Five grams of spiked sediment was placed into the extraction chamber and 100 mL of extraction solvent (DCM, acetone, MeOH and ACN) were used. The total extraction time was 60 min (40 min extraction, 20 min dropping). The obtained extract (approximately 80 mL) was transferred to a round-bottomed flask and evaporated by a rotary evaporator to approximately 5 mL, subsequently evaporated to dryness by means of a gentle stream of nitrogen and finally redissolved in 40 mL of 5% acetone (pH = 3, adjusted with acetic acid) prior to SPE. Three parallel samples of sediment were extracted.

2.3.2. **Accelerated solvent extraction (ASE)**

Extraction experiments were performed using an automatic extractor ASE 150 (Thermo Scientific Dionex, USA). Five grams of spiked sediment were filled into an 11 mL stainless extraction cell, the bottom and the top of the cell were covered by filter paper. Extraction was started by a 5-min preheated step without solvent, followed two 5-min static extraction cycles (flush 40%). At the end of the extraction, a 3-min nitrogen purge was used. DCM, acetone, MeOH and ACN as extraction solvents were tested with different extraction temperatures (40, 60 and 80°C). The extract (approximately 20 mL) was evaporated to dryness by means of a gentle stream of nitrogen and redissolved in 40 mL of 5% acetone (pH = 3, adjusted with acetic acid) prior to SPE. Three parallel samples of sediment samples were extracted.

2.3.3. **Microwave-assisted extraction (MAE)**

An automatic extractor Multiwave 3000 (Anton Paar, Austria) was used for this extraction experiment. Five grams of spiked sample was placed into a PTFE cartridge, after which extraction was performed by 25 mL of solvent (DCM:acetone 3:1, acetone, MeOH and ACN were tested). In order to improve the ability to heat of DCM in MAE, acetone were added to this solvent. Two different time and temperature regimens, based on procedures recommended by Labadie et al. [25] and Matejicek et al. [17] with some modifications, were used (A) 5 min static extraction at 90°C and (B) 10 min static extraction at 110°C (ramp 5 min). These extraction conditions were selected for comparison of extraction efficiency using mild extraction (A) and extraction with higher temperature and longer extraction time (B). In the case of DCM:acetone, only the (A) extraction conditions were used because higher extraction temperature and long extraction time caused the total evaporation of the solvent from the PTFE cartridges. Extracts (approximately 23 mL) were filtered through a glass fibre filter (1.2 µm) to vials (60 mL). Sediment samples in PTFE vessels were then rinsed two times with 5 mL of tested solvent. All filtrates were combined and evaporated to dryness by means of a gentle
stream of nitrogen and then redissolved in 40 mL of 5% acetone (pH = 3, adjusted with acetic acid) prior to SPE. Three sediment samples were extracted.

### 2.3.4. SPE for conventional extraction techniques

Extracts from all extraction experiments (SWE, ASE and MAE) were subjected to preconcentration by SPE [31]. Oasis HLB (500 mg, 12 cm³, Waters-Ireland) was conditioned by 7.5 mL MTBE, 7.5 mL MeOH and 7.5 mL MiliQ water, with 40 mL of sample subsequently loaded into the cartridge. Organic interferents were removed by 7.5 mL 40% MeOH and after equilibration by 7.5 mL of MiliQ water, the inorganic interferents were removed by 7.5 mL 10% MeOH with 2% NH₄OH. Finally, 12 mL of MTBE:MeOH (90:10) mixture was used for elution. This volume was evaporated to dryness by means of a gentle stream of nitrogen and redissolved in 1 mL of DCM:hexane (50:50) mixture.

Two different SPE cleanups were used for purification of extracts. An SPE cartridge with florisil sorbent (Strata-Florisil, 500 mg, 3 cm³, Phenomenex-USA) was used in the first step. The sample (1 mL from previous step) was applied to the cartridge after conditioning by 5 mL of acetone:hexane (75:25) and activation by 5 mL of DCM:hexane (50:50). The cartridge was washed by 10 mL of DCM:hexane (50:50) and oestrogens were eluted by 8 mL of acetone:hexane (75:25). Solvents were evaporated to dryness by means of a gentle stream of nitrogen and redissolved in 1 mL of MeOH.

The second cleanup step was performed on aminopropyl cartridge (Sep-Pak Vac, 500 mg, 6 cm³, Waters-Ireland). The sample (1 mL from florisil cleanup) was applied to aminopropyl cartridge after washing by 5 mL of MeOH. Finally, 10 mL of MeOH was used for elution. The purified extract was evaporated to minimal volume, transferred to a minivial and evaporated to dryness by means of a gentle stream of nitrogen. Deuterated βE2 as an IS (in the case of MAE, also E3d2) was added before derivatisation. The solution was evaporated to dryness again.

### 2.4. QuEChERS

For the original QuEChERS technique, 15 mL of ACN and 10 mL of MiliQ water were added to 5 g of spiked sediment. The sediment with the mixture of solvents was first manually shaken for 1 min and subsequently extracted in an ultrasonic bath for 15 min. Four grams of MgSO₄ and 1 g of NaCl were then added to the extract and extraction (manually shaking + ultrasound) was repeated once again. Centrifugation (3000 rpm, 5 min) was used and 10 mL of extract was evaporated by means of a gentle stream of nitrogen at 40°C to dryness. Prior to derivatisation, the extract was transferred to minivial, then deuterated ISs E2d4 and E3d2 were added and the content was evaporated to dryness again. In the case of buffered QuEChERS without purification, the sodium citrate (1 g) and sodium hydrogencitrate (0.5 g) were added to MgSO₄ and NaCl, and the following preparation was the same as the original QuEChERS.

The purification step for both QuEChERS techniques was also tested. PSA (250 mg) and MgSO₄ (1.5 g) were applied to 10 mL of extract and third manually and ultrasonic extraction were performed followed by centrifugation. Five millilitres of
purified extract was prepared for derivatisation as well as extracts without purification.

Three parallel samples of sediment were extracted for each QuEChERS variant.

### 2.5. Derivatisation

In order to increase the LC-MS signal of oestrogens, dansylchloride was used as a derivatisation agent. Dry and purified extracts from all extraction techniques were finally dissolved in 20 µL of acetone and 50 µL of sodium bicarbonate (100 mmol/L in water, pH = 10.5 adjusted by sodium hydroxide); 50 µL of dansylchloride (1 mg/mL in acetone) was added and thoroughly stirred. The solution was incubated for 3 min at 60°C followed by cooling to laboratory temperature. Finally, the derivatised extract was evaporated to dryness by means of a gentle stream of nitrogen and redissolved in 1 mL of 40% MeOH. The scheme of oestrogen derivatisation is displayed in Supplementary material (Figure S1).

### 2.6. HPLC–MS/MS conditions

An Agilent 1260 Infinity chromatographic system (Agilent, USA) equipped with a vacuum degasser, quaternary pump and autosampler and column thermostat was connected online to a ESI/QqQ mass spectrometer Agilent TripleQuad 6460 (Agilent, USA). The chromatographic/mass spectrometric system was controlled by Mass Hunter software.

An analytical column Poroshell 120 EC-C18 (2.1 mm × 150 mm, 2.7 µm particle size, Agilent, USA) and guard column Poroshell 120 EC-C18 (2.1 mm × 5 mm, 2.7 µm particle size, Agilent, USA) were used for analytical separation. The column temperature was set to 30°C. A mobile phase consisted of 7 mmol/L formic acid in water (solvent A) and ACN (solvent B). The mobile phase gradient was as follows: 0–10 min from 50% B to 100% B, held 100% B to 11 min, with a subsequent equilibration step 50% B to 15 min. The flow rate was 0.35 mL/min; 10 µL of sample was injected.

The parameters of the ion source were set as follows: gas temperature 200°C, gas flow 10 L/min, nebuliser 50 psi, sheath gas temperature 350°C, sheath gas flow 10 L/min, capillary voltage 3500 V and nozzle voltage 2000 V. The precursor and product ions, collision energies and fragmentor voltage used for multiple reaction monitoring can be found in Table 1. Validation parameters of analytical method and chromatographic record of oestrogen standards are described in Supplementary material (Table S1, Figure 2).

### Table 1. Mass spectrometer conditions.

| Compound name | Precursor ion (m/z) | Product ions (m/z) | Collision energy (V) | Fragmentor voltage (V) |
|---------------|---------------------|--------------------|----------------------|------------------------|
| E1            | 504                 | 171; 156           | 38                   | 140                    |
| E3            | 522                 | 171; 156           | 42                   | 140                    |
| αE2           | 506                 | 171; 156           | 42                   | 140                    |
| βE2           | 506                 | 171; 156           | 42                   | 140                    |
| EE2           | 530                 | 171; 156           | 40                   | 140                    |
| βEE2d4        | 510                 | 171; 156           | 42                   | 140                    |
| EE2d4         | 534                 | 171; 156           | 40                   | 140                    |
| E3d2          | 524                 | 171; 156           | 42                   | 140                    |

E1: Estrone; E3: estriol; αE2: 17α-estradiol; βE2: 17β-estradiol; EE2: 17α-ethynylestradiol; βEE2d4: deuterated 17β-estradiol; EE2d4: deuterated 17α-ethynylestradiol; E3d2: deuterated 16α-hydroxy-17β estradiol.
2.7. Average extraction recovery calculation

Extraction recovery was calculated using the equation:

\[ R_E(\%) = \frac{[A_s] - [A_0]}{5 \text{ ng}} \times 100 \]

where \( R_E \) is the average extraction recovery, \([A_s]\) the average calculated concentration (normalised to the IS) of analyte \( A \) in the extracts of the spiked samples \((n = 3)\), \([A_0]\) the calculated concentration of analyte \( A \) in their corresponding unspiked sample and 5 ng is the spike oestrogen concentration for 5 g of sediment. Statistical significance of obtained \( R_E \) was evaluated using ANOVA and results are described in Supplementary material (Table S2). Statistical differences among extraction solvents and extraction conditions for all tested extractions were evaluated at a significance level \( p < 0.05 \).

3. Results and discussion

3.1. Soxhlet warm extraction (SWE)

In our study, SWE was used for extraction of oestrogens from sediment spiked by 1 ng of E1, αE2, βE2, E3 and EE2 per gram of sediment, the oestrogen amount typically occurring in environmental matrices. Deuterated EE2d4 was used as a possible surrogate standard. Four extraction solvents (DCM, MeOH, acetone and ACN) often used and recommended for extraction of organic contaminants from solid environmental matrices were used for comparison. Extraction recoveries \((R_E)\) obtained for four different extraction solvents are given in Figure 1. The results show that SWE did not provide high values of \( R_E \) in the case of very low spike concentrations of steroid oestrogens in comparison with the study of Matejicek et al. where \( R_E \) reached more than 70% for all tested oestrogens but the spiked concentration was twenty times higher than in our experiment [17]. Acetone provided the highest \( R_E \) of the four extraction solvents 26–37%, with relative standard deviation (RSD) = 5–13%; E3 – 160%, RSD = 23%. The

![Figure 1. Soxhlet warm extraction – extraction recoveries. Whiskers represent standard deviations.](image-url)
other three solvents provided the $R_E$ to 27% with RSD from 2% to 17% for E1, αE2, βE2, E3, EE2 and EE2d4. Estriol was extracted with $R_E$ 93% (RSD = 4%) using MeOH, 38% (RSD = 10%) using ACN and in the case of DCM extraction, the E3 was totally lost. SWE provided very low $R_E$, probably caused by the long extraction time with high extraction temperature and also losses of analytes during treatment of a large volume of extract.

3.2. Accelerated solvent extraction (ASE)

Four extraction solvents which provided the high recovery for extraction of oestrogens from soils and sediments according to published studies and three temperature conditions were chosen in our study for comparison of ASE with other extraction procedures. Results of ASE extraction are given in Figure 2. In the case of such concentration, the $R_E$ were lower than the $R_E$ reached in previous studies, namely Petrovic et al. (94–104% for E1, E2, EE2 and E3) [23] or Gorga et al. (81–114% for E1, E2, EE2 and E3) [24] for sediments spiked by a more than ten times higher amount of oestrogens per gram of sediment. Comparable data are not available in the literature. Beck et al. presented using different solvents for extraction of oestrogens from soil spiked by 2 ng/g of soil. $R_E$ values were in the range of 89–103% for acetone, 75–93% for ACN, 71–100% for ethylacetate and 77–84% for mixture of acetone:DCM, respectively [20]. However, any comparison with sediments is problematic due to the quite different composition of soil and sediment matrices. In our study, the $R_E$ were reached using DCM at 60°C (53–74%) for E1, αE2, βE2, EE2, EE2d4 and 240% for E3 with RSD from 8% to 31%. In the case of acetone, the highest $R_E$ was achieved at 80°C, from 29% for EE2 to 42% for βE2 and 172% for E3 with RSD from 8% to 39%; however, differences between $R_E$ obtained at 60°C

![Figure 2. Accelerated solvent extraction – extraction recoveries using (a) DCM, (b) acetone, (c) MeOH and (d) ACN at different extraction temperatures. Whiskers represent standard deviations.](image-url)
C and 80°C are statistically insignificant for all tested compounds. $R_E$ of oestrogens using MeOH or ACN ranged from 12% to 35% (RSD = 1–22%) at all temperature conditions, with the exception of E3 which was in the range of 87–172% (RSD = 8–15%). $R_E$ values of E1, EE2 and EE2d4 were lower than αE2 and βE2 in almost all combinations of extraction solvents and temperatures. On other hand, the $R_E$ of E3 exceeded 100% in most cases. The reason for the overestimation of E3 is most likely the use of the usually recommended βE2d4 as an IS for quantification of the oestrogen set. The IS βE2d4 is often recommended for the analysis of mixtures of oestrogens in various matrices, but due to the different physico-chemical properties of E3, which is reflected in the different chromatographic behaviour, the matrix effect is significantly different from other oestrogens in these complex environmental components such as sediment.

Extraction temperature plays an important role in oestrogen isolation depending on the selected solvents. The use of DCM provides the significant improving the $R_E$ at 60°C for EE2. The same observation was described by Beck et al. using acetone for isolation of oestrogens from soils where the reduction of $R_E$ at temperatures higher than 60°C was explained by destabilisation or destruction of compounds or by increasing the formation of non-extractable complexes [20]. However, the same trend was observed only for DCM in our study. Different results (recoveries in the range of 85–96%) were published by Zhang et al., who used DCM for extraction of E1, E2 and EE2 from sediment at 100°C [22]. However, the spiked concentration was one hundred times higher than those in our study. Using acetone and MeOH in our study, the $R_E$ gently increased with increasing temperature from 40 to 80°C for most tested compounds. Extractions at low temperature (40°C) probably caused ineffective desorption and dissolution of target compounds and the obtained $R_E$ were lower. In the case of ACN, the $R_E$ of E3 and EE2 were also significantly higher at 60 and 80°C compared with 40°C.

### 3.3. Microwave assisted extraction (MAE)

Four extraction solvents (DCM:acetone 3:1, acetone, MeOH and ACN) were tested in our study, although only MeOH for the higher ability to heat in MAE or a mixture of MeOH with water were used in published studies. These solvents were tested in two extraction conditions with different extraction times and temperatures (A: 90°C, 5 min, ramp 5 min, B: 110°C, 10 min ramp 5 min) that were modified using the studies of Labadie et al. and Matejicek et al. [17,25]. The results in Figure 3 show that the best $R_E$ values were achieved using DCM:acetone 3:1 at the extraction condition A. $R_E$ were above 50% (50–71%, RSD = 2–9%) for E1, αE2, βE2, EE2 and EE2d4. In the case of estriol, an $R_E$ of 109% (RSD = 14%) was obtained. Acetone also provided $R_E$ over 50% (51–60%, RSD < 8%) using extraction condition B. An extraction recovery of 115% with RSD = 12% was achieved for estriol. Slightly lower $R_E$ values were obtained for E1 using acetone in regime A. MeOH as the most commonly used extraction solvent provided similar results in both the A and B extraction conditions. $R_E$ in the range 47–55% for E1, αE2, βE2, EE2, EE2d4 and 145–161% for E3 were obtained. The repeatability of results using MeOH was a low RSD = 13–37% for all tested oestrogens. Labadie et al. obtained $R_E$ in the range of 65–130% (RSD 6–24%) for E1, E2 and EE2 using two sediments with different TOC content and two extraction regimes (90°C, 5 min; 110°C, 15 min). However, the spike concentration was ten times higher than in our experiments.
Liu et al. and Matejicek et al. tested MeOH for MAE with an oestrogen spike several times higher than 1 ng/g using different extraction conditions. Matejicek et al. used 100°C and 10 min for extraction while Liu et al. optimised extraction condition 110°C and 15 min. Although MeOH is the most common extraction solvent for extraction of oestrogens from sediment, the results in our study with a significantly lower spike concentration of oestrogens were not satisfactory in comparison with other tested solvents due to problems with repeatability. ACN values were between 40% and 55% (RSD = 19–27%) for E1, αE2, βE2, EE2 and EE2d4 using regime A and between 46% and 58% (RSD = 4–15%) using regime B. The R_E of E3 decreased rapidly with the increasing extraction time and temperature from 134% to 26%.

3.4. Evaluation of E3 overestimation

The R_E of E3 using tested extraction techniques with SPE preconcentration and cleanup were very overestimated in comparison with other oestrogens, most likely due to the use of the IS E2d4 for oestrogen analysis. This assumption was tested using deuterated E3d2 as an IS. MAE, a better extraction technique compared with SWE and ASE, was used for this purpose. The E3 R_E obtained using E2d4 and E3d2 were compared and are shown in Figure 3 (E3/E2d4 vs. E3/E3d2). The results obtained using E3d2 confirmed this assumption. Calculated recoveries were significantly lower (6–59%, RSD = 5–18%) compared to those using IS E2d4 (26–161%, RSD = 5–22%) in all combinations of extraction solvents. The calculation of E3 final concentrations with the deuterated IS E3d2 provided reliable results, because this compound has a very similar structure and properties, is eluted from the chromatographic column in almost the same retention time and the same background of the matrix as E3. Compared to results calculated using IS E2d4 which is eluted at different retention time and different background of matrix caused the overestimation of E3. For final calculation of other oestrogens (E1, αE2, βE2, EE2 and EE2d4), the use of E2d4 provided reliable results because these oestrogens were eluted at similar retention times and with a similar matrix composition. The results demonstrated that E3d2 is a preferable IS for calculating the final concentration of E3. For other tested oestrogens, E2d4 can be reliably used.
QuEChERS

Two types of the very promising extraction technique QuEChERS for isolation of oestrogens from sediment were tested (1) original QuEChERS and (2) buffered QuEChERS, both with and without PSA purification. The results in Figure 4 show that buffered QuEChERS provided $R_E$ from 46% to 59% (RSD = 4–25%), for less polar oestrogens (E1, αE2, βE2, EE2 and EE2d4) without purification and original QuEChERS from 40% to 54% (RSD < 13%). However, the PSA purification step plays an important role and caused a significant increase in $R_E$ for αE2. The original QuEChERS with PSA purification provided $R_E$ 53–65% with RSD = 5–18% for less polar oestrogens. On the other hand, the PSA purification step did not cause an improvement in $R_E$ for buffered QuEChERS and the obtained results were in the range of 47–58% with RSD = 9–16%. Comparison of our results can be made using the results for soil samples. For example, Salvia et al. presented results of ACN-based extraction followed by two SPE cleanup steps (SAX and StrataX) used for isolation of steroid oestrogens (E1, αE2, βE2, EE2 and E3), veterinary and human drugs from soil. Three concentration levels 1.5, 50 and 500 ng/g were tested. $R_E$ were below 50% (RSD = 11–20%) for all oestrogens for the spiked amount of 1.5 ng/g each and higher $R_E$ were achieved with a spiked amount of 500 ng/g (80–104%, RSD = 4–20%) [29]. Berlioz-Barbier et al. also used the acetate-buffered QuEChERS for isolation of emerging pollutants from sediments including E1. The cleanup step using PSA/GCB was performed and $R_E$ 79%, RSD = 8% was achieved with a spike of 3 ng/g of steroid [28].

Two different ISs (E2d4 and E3d2) were used for calculation of final concentration of E3 as in our previous MAE experiments. The overestimation of the calculated concentration of E3 using E2d4 as an IS was also observed. Estriol $R_E$ determined using E2d4 was 133% (RSD = 17%) for original QuEChERS and 141% (RSD = 14%) for buffered QuEChERS without purification. $R_E$ calculated using E3d2 as the IS were 64% (RSD = 1%) for original QuEChERS and 68% (RSD = 29%) for the buffered variant. However, experiments with a PSA purification step showed smaller differences between E2d4 and E3d2 that were used for calculation of E3 concentration than previous experiments without purification. Estriol $R_E$ calculated using E2d4 as the IS
were 105% (RSD = 17%) and 120% (RSD = 33%) for original and buffered QuEChERS, respectively. Estriol $R_E$ calculated using E3d2 as the IS were 84% (RSD = 11%) for original QuEChERS and 93% (RSD = 24%) for buffered QuEChERS. These results confirmed that the different matrix composition in different retention time for E2d4 and E3d2 causes an overestimation of E3 concentration using the IS E2d4 during calculation. However, with increasing purification efficiency, the differences between the total concentrations of E3 calculated using both ISs (E2d4 and E3d2) are lower. Nonetheless, E3d2 should be prioritised as the most appropriate IS for calculation of E3 concentration.

3.6. Statistical evaluation (ANOVA)

Original QuEChERS method with PSA cleanup was found to be the best extraction method from all tested methods with statistical significance ($p < 0.05$). Results obtained using Original QuEChERS methods with PSA cleanup were compared with the results obtained for each extraction method at the best extraction conditions (extraction solvent, temperature and time) and also with other variants of QuEChERS method.

Significant differences in $R_E$ were found between the Original QuEChERS method with PSA cleanup and SWE using acetone as extraction solvent ($p < 0.0240$) for less polar oestrogens.

No significant differences in $R_E$ (with exception of E3) were observed between the Original QuEChERS method with PSA cleanup and ASE (DCM, 60°C). However, the repeatability was better compare to ASE method.

Significant higher $R_E$ of E3/E3d2 ($p < 0.0218$) were found for Original QuEChERS method with PSA cleanup comparing to MAE (DCM:acetone or acetone and MeOH in regime B).

The Original QuEChERS method with PSA clean up was also compared with other QuEChERS variants. Significantly higher $R_E$ were achieved for αE2 compared to Original QuEChERS without PSA cleanup ($p = 0.0294$) and for βE2 compared to Buffered QuEChERS with PSA cleanup ($p = 0.0476$).

Complete results of ANOVA evaluation are inserted in Supplementary material (Table S2).

3.7. Overall comparison of extraction techniques

Four extraction techniques and four different extraction solvents were tested for extraction of oestrogen from sediment. The results showed that the extraction solvent plays an important role and it is not possible to determine which solvent provides the best $R_E$ for all types of extraction techniques. Acetone provided the highest $R_E$ using SWE, but this technique is not suitable for extraction of low polar oestrogens E1, αE2, βE2, EE2 and EE2d4 due to low $R_E$ (11–37%) and the very time-consuming extraction. DCM provided the best $R_E$ using ASE at three extraction temperatures. Maximum values of $R_E$ were 53–74% at 60°C, but the repeatability of results was worse in comparison with other extraction techniques. ASE is a very time-consuming extraction technique using our conditions, because only one sample can be extracted at a time. In the case of MAE, the
DCM:acetone mixture (90°C, 5 min) provided the best $R_E$ and satisfactory repeatability for less polar oestrogens (50–71%, RSD < 9%) compared to all conventional extraction techniques. MeOH as an extraction solvent, often used and recommended for extraction of oestrogens by MAE, provided lower repeatability in both extraction conditions in comparison with DCM:acetone mixture. MAE is less time consuming than the SWE and ASE techniques, as up to 16 samples can be extracted in one extraction run. However, different ACN-based QuEChERS procedures that are frequently used due to this technique’s speed, simplicity and low financial demands provided a satisfactory $R_E$. The best $R_E$ values of less polar oestrogens (53–65%) were achieved using original QuEChERS with PSA cleanup.

Evaluation of $R_E$ for E3 was more complicated. As for SWE and ASE extraction, the results showed that the IS E2d4 used for calculation of E3 concentration led to overestimation due to the effect of the different matrix on the ionisation of E3 and E2d4 eluted at quite different retention times. IS E2d4 is eluted and ionised with more complex matrix at retention time $t_R = 9$ min. For this reason, the suppression of MS/MS signal is different in comparison with E3 which eluted at $t_R = 6$ min. These differences cause the overestimation during the calculation of E3 final concentration. In order to eliminate these differences, it was necessary to use E3d2 as the IS with properties similar to E3. The suitability of E3d2 as the IS was tested using MAE and QuEChERS extraction techniques and the results were compared with those obtained using E2d4. MAE did not provide the highest $R_E$ using a less polar DCM:acetone mixture due to the higher polarity of E3 in comparison with other oestrogens. In the case of MAE, the more polar extraction solvent (acetone or MeOH) is necessary for extraction of whole group of oestrogens including E3. The QuEChERS method that uses ACN as the extraction solvent provided $R_E > 64\%$ for all four extraction variants using E3d2 as the IS. If PSA was used for cleanup, the $R_E$ of E3 exceeded 80%.

Choosing a suitable extraction technique is very important for studying the behaviour of oestrogens in the water–sediment system, such as sorption, degradation or transformation processes. Our study was focused on comparison of extraction techniques at environmentally relevant oestrogen concentrations. This comparison not only described the extraction process more realistically but also showed it to be more complicated due to the unrecoverable fraction of analyte that was probably irreversibly sorbed on the surface of sediment particles. In the case of low oestrogen concentrations, the higher portion of these oestrogens is sorbed in comparison with a higher oestrogen concentration [32]. For this reason, lower extraction recoveries at the concentration level used can be tolerated. The acceptable recoveries depending on the analyte level were presented by Gonzalez et al. [33]. For our case, the recovery in the range of 40–120% is acceptable. This criterion was met using the QuEChERS method and MAE in many extraction conditions.

4. Conclusion

Due to the limited amount of information on comparing the methods for extracting oestrogens from sediment, this paper has focused on comparison of extraction recoveries of five native and one labelled oestrogens from sediment using three conventional...
extraction techniques and the promising QuEChERS method. The comparison was carried out on the level of environmentally relevant concentrations.

MAE provides the best extraction recoveries and satisfactory repeatability among conventional extraction techniques. SWE and ASE provided low extraction recoveries or unsatisfactory repeatability in the case of all extraction conditions. These two techniques are also more time consuming compared to MAE. The QuEChERS extraction technique also provides high extraction recoveries using the original QuEChERS variant and purification of extract by PSA sorbent. The main advantages of this QuEChERS variant are its lower price and its less time-consuming nature, while maintaining the satisfactory recoveries and repeatability of conventional extraction techniques. The original QuEChERS with PSA cleanup could be further optimised according to the specific properties of a matrix and used for appropriate fate studies.

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