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Solid-phase extraction of estrogen hormones onto chemically modified carbon cryogel

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Abstract: This study introduces a novel solid-phase extraction (SPE) method utilizing pristine and chemically treated carbon cryogel (CC) as an adsorbent for the isolation and enrichment of estrogen hormones (estrone, 17 β -estradiol, and 17 α -ethinylestradiol) from water samples. High recovery values (82–95 %) were obtained after optimizing the SPE technique, which included adsorbent mass and chemical treatment, sample volume and pH, and elution solvent type and volume. The developed analytical method, based on SPE coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS), proves to be selective, efficient, and cost-effective for the determination of selected estrogens. The utilization of self-made cartridges with chemically modified CC produced results comparable to those obtained with commercial cartridges while employing significantly less material. Furthermore, the selectivity of the employed materials contributed to minor matrix effects. The optimized method was successfully applied to analyze estrogen hormones in groundwater, surface water, and wastewater samples, with the results highlighting the importance of monitoring these contaminants in the aquatic environment.

Keywords: estrogens; SPE; surface water; groundwater; waste water; liquid chromatography–tandem mass spectrometry.

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INTRODUCTION

Estrogen hormones are acknowledged as endocrine-disrupting compounds (EDCs) capable of disrupting the endocrine systems of both humans and animals, leading to adverse health effects.¹ Wastewater is the main route by which these hormones get into the environment. Existing wastewater treatment plants (WWTPs) struggle to entirely eliminate hormones, contributing to their pervasive presence in environmental water.² The presence of these compounds in the aquatic environment can affect fish sexual development and reproduction.^{3,4} Long-term estrogen exposure also has negative effects, such as bioaccumulation in aquatic species, which can eventually reach people through the food chain.³ Therefore, the removal of these substances from wastewater and their monitoring in the aquatic environment is becoming increasingly important.

Estrogens are generally present in the aquatic environment at very low concentrations (ng dm^{-3}), so their detection requires an efficient isolation and pre-concentration method before analysis. This step is of crucial importance for the outcome of further analysis, especially when dealing with complex matrix samples where the components of interest are present at trace concentrations. Solid-phase extraction (SPE) is commonly used to enrich ambient water samples prior to analysis.⁴⁻⁸ The choice of the adsorbent is essential in the application of the SPE technique since it affects parameters such as affinity, selectivity and extraction capacity.⁹

Due to their well-developed specific surface area, wide porosity range, and consequently high adsorption capacity, numerous carbon-based materials have been used as efficient adsorbents for the removal or extraction of different environmental pollutants from water.¹⁰⁻¹² Additionally, the surface of carbon materials can be easily tailored or modified by various treatments in order to improve the adsorption features of the examined materials for specific water pollutants.¹³⁻¹⁵ Due to their easily controllable mesoporosity, carbon cryogel (CC) has become an attractive material for adsorption purposes.¹⁰ By selecting the right precursor material and managing the synthesis settings, it is possible to customize the pore structure of CC.¹⁶ The predominantly mesoporous structure of CC provides a fast transfer of adsorbate through the pore network, so this material has been used as an adsorbent for different organic and inorganic solutes from the liquid phase.^{10,14,16}

Previously, it was shown that modified and unmodified CC have high efficiency in the removal of estrone (E1), 17β -estradiol (E2) and 17α -ethinylestradiol (EE2) from water, showing higher Langmuir adsorption capacities for all three hormones in comparison with carbonized and activated hydrothermal carbons, multi-walled carbon nanotubes, and activated carbon cloths.^{14,15} The results also demonstrated that the matrix component of surface water, groundwater and

wastewater samples did not significantly affect the adsorption capacity of CC towards E1, E2 and EE2.¹⁴

The objectives of this study were to assess the possibility of using self-made cartridges packed with pristine and chemically modified CC as SPE adsorbents and to develop a new, reliable, efficient and cost-effective method for the determination of E1, E2 and EE2 from environmental water samples. Also, hormone recoveries obtained using the most efficient CC adsorbent were compared with those obtained by commercially available cartridges. To the best of our knowledge, CC material has not been used as an adsorbent for hormone extraction from water so far. The instrumental method used for hormone detection was liquid chromatography–tandem mass spectrometry (LC–MS/MS) with electrospray ionization. The optimized and validated method was applied to the analysis of real water samples.

EXPERIMENTAL

Material preparation

The CC was manufactured at the Vinča Institute of Nuclear Sciences, and a detailed synthesis procedure is described in the literature.¹⁰ Briefly, a solution of resorcinol (R) and formaldehyde (F) with sodium carbonate as a basic catalyst was poured into a glass tube, sealed and gelled for 7 days. Wet RF gel was washed in *t*-butanol, pre-frozen (–30 °C) and freeze-dried for 24 h under a vacuum (0.4 mbar). The obtained RF cryogel was finally carbonized to 800 °C under a nitrogen atmosphere with a heating rate of 5 °C min^{–1} and cooled to room temperature. The resulting material was squashed into powder and stored closed in a PVC box.

Chemical modification of CC was carried out by heating a suspension of the material in HNO₃ or KOH water solution, and modified materials were labeled as CC/HNO₃ and CC/KOH, respectively. Applied treatment conditions during the chemical modification process are described in a previous paper.¹⁴ Chemical modification with the mentioned agents leads to the formation and/or alternation of oxygen functional groups.^{17,18} An increased amount of oxygen groups may enhance the adsorption efficiency of tested estrogens since these hormones possess hydroxyl groups, which may form hydrogen bonds on the adsorption surface.^{15,19}

Solid-phase extraction

In order to obtain high recoveries of the SPE method, the following parameters were optimized: the mass of the adsorbent, the volume and initial pH value of the water samples, and the type and volume of the organic solvent for hormone elution. In addition, the possibility of improving the method through CC modification was investigated.

Initially, spiked water samples were prepared by spiking deionized water with a mixed hormone stock solution (5 mg dm^{–3} of each hormone in methanol) to a concentration of 2.5 µg dm^{–3} per hormone. The SPE cartridges (3 cm³ volume) were made by packaging a selected amount of CC between two Teflon frits. The cartridges were conditioned by passing 5 cm³ of a chosen organic solvent followed by 5 cm³ of pH-adjusted deionized water. After conditioning, spiked water samples of the required volume and pH value were passed through the cartridges. Then, the cartridges were dried under vacuum for 10 min and eluted with a chosen organic solvent until the optimal eluent volume was achieved. The eluents were collected in

glass tubes, evaporated to dryness under N₂, and reconstituted in 1 cm³ of the mobile phase. After reconstitution, all samples were vortexed and filtered through the polyvinylidene difluoride (PVDF) 0.45 µm filters into glass vials.

The optimal parameters of the extraction procedure were selected based on the highest recovery values obtained. The optimization process involved varying the adsorbent mass, using 20, 50 and 100 mg of material. Subsequently, the optimal volume of water samples was selected based on recoveries obtained by performing the extraction from 25, 50, 100 and 200 cm³. To find an optimal pH value of water samples, extraction of selected hormones was carried out by using 20 mg of adsorbent and 200 cm³ of water sample with an initial pH adjusted to 5, 6, 7, 8, 9, 10 and 11. An optimal organic elution solvent was selected according to SPE recoveries gained using methanol (MeOH), acetonitrile (ACN), ethyl acetate (EtOAc), a 1:1 volume ratio mixture of dichloromethane and methanol (DCM/MeOH) and a 1:1 volume ratio mixture of ethyl acetate and methanol (EtOAc/MeOH). Further optimization steps involved determining the appropriate eluent volume, with elution performed using 5, 10 and 15 cm³ of the optimal organic solvent. In the final optimization step, three types of materials (CC, CC/HNO₃ and CC/KOH) were evaluated as potential adsorbents, maintaining the parameters optimized in previous steps. Additionally, recovery values gained using cartridges packed with the most efficient CC material were compared with the recoveries gained using commercially available cartridges: Supelclean Envi Carb, Supelclean Envi-18, Supelclean LC-SCX, Supelclean LC-18 (Sigma–Aldrich) and Oasis HLB (Waters, USA).

LC–MS/MS analysis

The concentrations of the hormones in the final extracts were measured by liquid chromatography coupled with tandem mass spectrometry. The separation of tested hormones was performed using a Surveyor LC system (Thermo Fisher Scientific, USA). The analytical column used for reverse-phase separation was an Agilent Zorbax Eclipse XDB-C18 column (75 mm×4.6 mm×3.5 µm). According to literary sources, a mixture of water, MeOH, and/or ACN is often utilized during LC–MS analysis of E1, E2 and EE2, with NH₄OH as a mobile phase modifier in negative mode and formic acid in positive mode.^{7,20,21} During the optimization of the LC–MS method, we obtained the most stable and intense signals in positive mode, with the optimal mobile phase composition of 25 % formic acid (0.1 % water solution) and 75 % methanol. The method was isocratic, with a constant flow rate of 0.3 cm³ min⁻¹.

For detection and quantification of the hormones, LCQ Advantage (Thermo Fisher Scientific, USA) mass spectrometer with an electrospray ion source and quadrupole ion trap mass analyzer was used. The measurements were conducted in positive ionization mode, with optimal source parameters set at: source voltage, 4.5 kV; sheath gas, 23 au; auxiliary gas, 5 au; capillary temperature, 350 °C. The selected reaction monitoring mode (SRM) was used for quantification purposes. Table S-I of the Supplementary material to this paper lists the selected precursor ion, the optimal collision energy, the most abundant fragment ion, and its isolation width for each hormone, whereas Fig. S-2 of the Supplementary material shows an example of a SRM chromatogram.

Real water samples analysis

The applicability of the optimized SPE method was tested by analyzing selected hormones in groundwater, surface water and wastewater samples. Two groundwater, four surface water, and four wastewater samples were collected. Groundwater samples GW1 and GW2 were collected from observation wells close to the river Danube in the vicinity of Kovin. Surface water samples were collected from the rivers Danube (locations Novi Sad and Kladovo,

labeled SW1 and SW2, respectively), the Velika Morava (location 1 km from the confluence with the Danube, labeled SW3) and the Pek (location 8 km from the confluence with the Danube, labeled SW4). Two wastewater samples were taken from Belgrade (WW1, sampled at discharge into the Danube near the confluence of the Sava into the Danube, and WW2, sampled at discharge near Belgrade Fair), while two samples were from the entrance and exit of the wastewater treatment plant (WWTP) Arandelovac (WW3 and WW4). Water samples were stored in 1 dm³ plastic bottles and stored in a freezer. Prior to the SPE procedure, the water samples were filtered through 1–3 μm glass fiber filters (Whatman GmbH, Dassel, Germany).

RESULTS AND DISCUSSION

Optimization of solid-phase extraction

The initial step in optimizing the SPE method for hormone analysis from water samples was to determine an adequate adsorbent mass. The influence of the adsorbent mass on the extraction efficiency is demonstrated in Fig. 1a. Results showed that recoveries of all three investigated hormones increased as the mass of CC decreased. It can be assumed that the increase in adsorbent mass leads to a decrease in the homogeneity of close-packed material, preventing satisfactory contact between the adsorbent particles and solution. The highest recovery values, ranging from 65 to 70 %, were obtained for the adsorbent mass of 20 mg. Therefore, 20 mg of adsorbent was chosen for further experiments.

Choosing the appropriate volume of the water sample is an important step in the SPE optimization process. Low sample loading volumes are advantageous when considering potential matrix effects and extraction times, yet extraction efficacy and pre-concentration factors generally increase as sample volume is increased.²² Fig. 1b illustrates the SPE method's recoveries for all tested hormones. Recoveries increase with sample volume, yet volumes above 200 cm³ weren't tested due to analysis time constraints. Thus, 200 cm³ was chosen as optimal. The effect of the initial pH value of the water sample was also investigated by varying the initial pH values in the range from 5 to 11. Recoveries obtained for all selected hormones (Fig. 1c) in the tested pH range were high and acceptable (72–85 %). As pH did not have a significant influence on extraction efficiency, the neutral pH value was selected for further experiments. Additionally, at a neutral pH value, the highest recoveries for EE2 were achieved.

Fig. 1d shows the effects of different eluents on extraction efficiency. The experiment was done under the following conditions: material mass was 20 mg, sample volume was 200 cm³ and initial pH value was 7. The elution conditions were chosen based on the optimization of the parameters in the previous steps. The tested elution solvents were MeOH, ACN, EtOAc, DCM/MeOH and EtOAc/MeOH. All tested solvents used individually yielded low recoveries, with ACN providing the lowest recovery. When using DCM/MeOH and EtOAc/MeOH mixtures, high and acceptable recoveries for all tested hormones were obtained.

Even though the DCM/MeOH mixture produced slightly higher recoveries than the EtOAc/MeOH mixture, the latter was chosen as a suitable elution solvent due to its lower toxicity when compared to DCM.^{23,24}

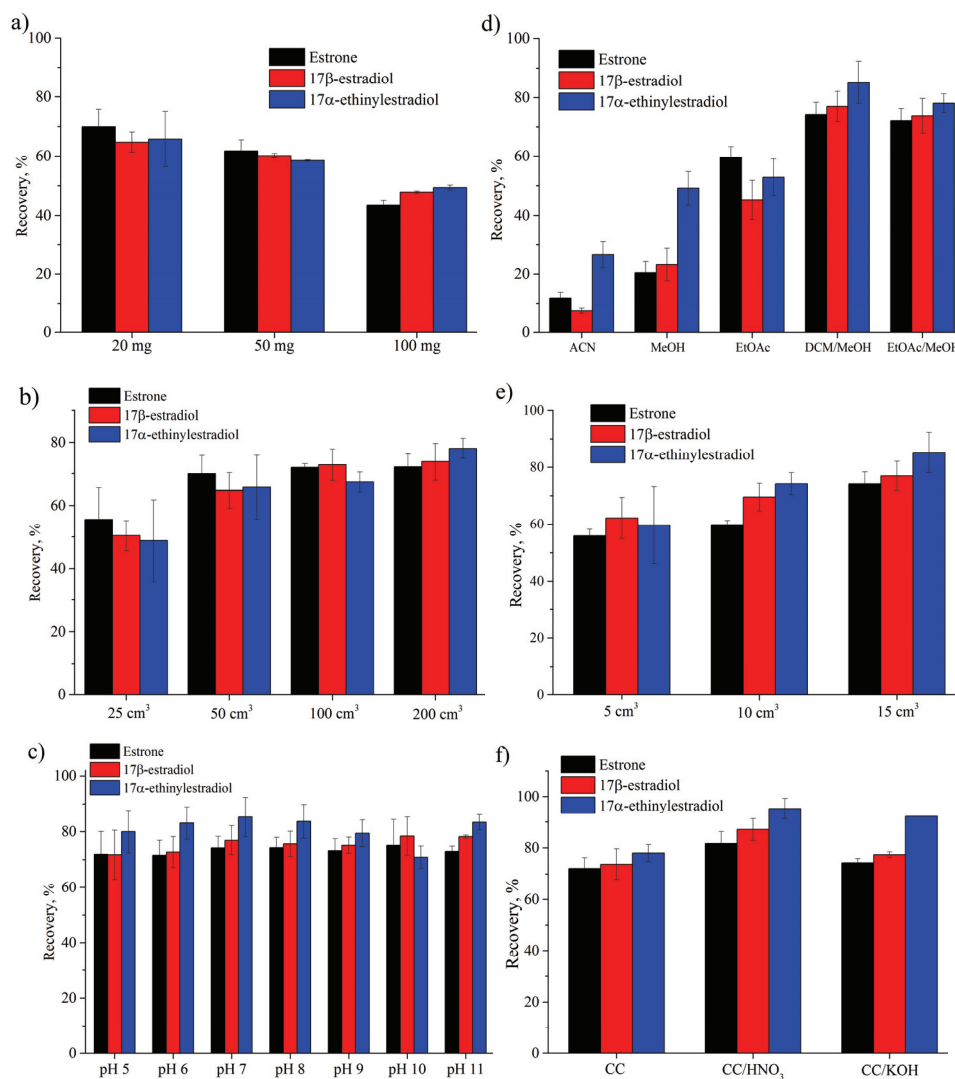


Fig. 1. Recoveries of selected hormones obtained using different a) adsorbent masses, b) sample volumes, c) initial pH values of the sample, d) elution solvents, e) eluent volumes and f) unmodified and chemically modified CCs.

In the next step, the eluent volume was optimized. As shown in Fig. 1e, the recoveries of the tested hormones consistently improved with the increase in elu-

ent volume. A 15 cm³ eluent volume proved to be adequate, providing satisfactory recoveries for all observed hormones (74–85%). Therefore, this volume was selected as the optimal choice for subsequent analyses.

The final step in SPE optimization was the selection of the material modification method that would provide the highest efficiency of CC for the extraction of tested hormones. The obtained results (Fig. 1f) show that after the chemical modification of CC, modest variations in recovery values were found. However, utilizing CC/HNO₃ as an adsorbent produced better results for all three hormones with high recoveries (82–95 %), hence CC/HNO₃ was chosen for real water sample analysis. In our previous study, we found that modifying the material with HNO₃ resulted in an increase of carboxyl functional groups.¹⁴ In the present study, it is observed that functionalization leads to a slight increase in the recovery values of the tested hormones, possibly due to the prevalence of hydrogen bonds in the mechanism of adsorption, which is a consequence of the increase in oxygen surface groups.²⁵ The final optimized analysis procedure was as follows: the 3 cm³ cartridge, packed with 20 mg CC/HNO₃, was conditioned with 5 cm³ of EtOAc/MeOH (1:1) mixture followed by 5 cm³ of deionized water; 200 cm³ of the water sample, with the initial pH adjusted to 7, was passed through the preconditioned cartridge; the cartridge was dried under vacuum for 10 min, and analytes were eluted with EtOAc/MeOH mixture (1:1) until 15 cm³ of extract was collected in a glass test tube; the extract was evaporated to dryness, reconstituted with 1 cm³ of mobile phase, and the final extract was filtered through a PVDF filter (0.45 μm) into the glass vial and analyzed.

Method validation

In order to determine the applicability of the developed analytical method for the extraction of observed hormones from real water samples, the linearity, repeatability, matrix effect, limit of detection (*LOD*) and limit of quantification (*LOQ*) were estimated. Details related to method validation are given in Supplementary material. The calibration curves based on six calibration levels ranging from 5 to 500 μg dm⁻³ showed good linearity, with determination coefficients (*R*²) of 0.9981 for E1, 0.9909 for E2 and 0.9971 for EE2. The calibration curves are presented in Fig. S-3 of the Supplementary material. The relative standard deviation (*RSD*) was in the range of 3.2–5.9 %, indicating good repeatability. For all observed hormones, the calculated values of *LOD* and *LOQ* were in the range of 2.63–6.36 and 8.77–21.19 ng dm⁻³, respectively. The recoveries, *RSD*, *LOD*, *LOQ* and *R*² values are given in Table S-II of the Supplementary material. The *LOD* values of our method are lower than the corresponding values obtained in some studies^{20–27} and comparable to some recently published studies.^{1,7} Glineur *et al.*²⁸ developed an analytical method in which lower *LOD* and *LOQ* were

achieved, but that method requires more time because an additional sample purification technique is necessary.

The existence of certain co-extracted substances, including natural organic matter and other contaminants, in environmental samples has the potential to impact the signal intensity of LC–MS/MS through either suppression or enhancement of the signal. Hence, assessing the matrix effect becomes crucial for a comprehensive understanding of the analytical results. The results presented in Table S-II show that the matrix effect has no significant influence on the determination in this case. In all studied matrices, the deviation of the results was less than 20 %, indicating the strong selectivity of CC/HNO₃ for the investigated hormones. However, in order to improve accuracy, the standard addition method was used to analyze real water samples.

By comparing recoveries gained using commercially obtained cartridges and cartridges packed with CC/HNO₃ (Fig. 2), it was demonstrated that CC/HNO₃ could be successfully used as a solid-phase adsorbent for the analysis of tested hormones in water samples. According to the results presented in Fig. 2, it is evident that recoveries obtained using cartridges packed with CC/HNO₃ were comparable to the recoveries gained by commercial cartridges. The performance of the proposed method also was on par with a few previously documented methods,^{7,29} which utilized expensive commercial cartridges. Notably, our cartridges utilized a significantly smaller amount of material, 20 mg as opposed to 200–500 mg in the referenced studies, underscoring the cost-effectiveness of this approach.

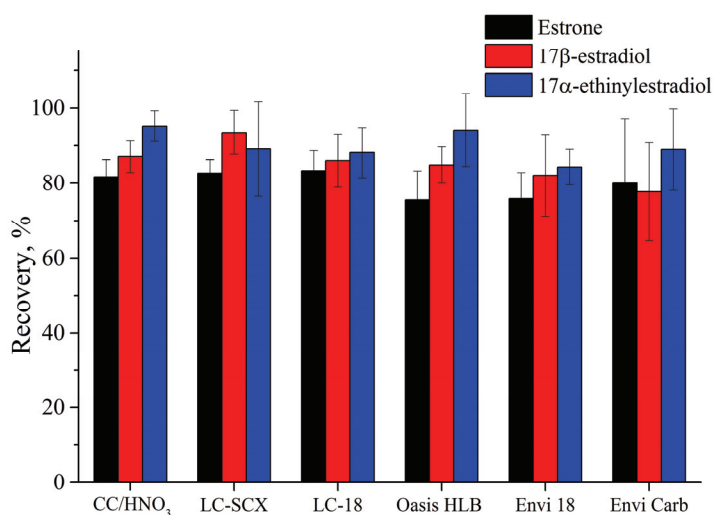


Fig. 2. Recoveries of selected hormones obtained using commercial cartridges and CC/HNO₃.

Real water samples

In Table I, detected concentrations of examined hormones in groundwater, surface water, and wastewater samples are presented. At least one hormone was found in eight, out of the ten samples that were tested, indicating a considerable prevalence of these compounds in the aquatic environment.

TABLE I. Hormone concentrations (ng dm⁻³) detected in ground, surface and wastewater samples

Sample	Location	Hormone		
		E1	E2	EE2
Groundwater				
GW1	Kovin 1	< LOQ ^a	< LOQ	< LOQ
GW2	Kovin 2	– ^b	< LOQ	–
Surface water				
SW1	Danube, Novi Sad	36.2	10.1	–
SW2	Danube, Kladovo	–	–	–
SW3	Velika Morava	–	8.8	–
SW4	Pek	22.8	10.2	9.6
Wastewater				
WW1	Belgrade, Confluence	99.3	–	–
WW2	Belgrade, Belgrade Fair	71.3	–	–
WW3	WWTP Arandelovac, influent	163.5	101.8	91.0
WW4	WWTP Arandelovac, effluent	–	–	–

^a(< LOQ) detected, but below LOQ; ^b(–) not detected

In GW samples, only trace amounts of the studied hormones were detected, below LOQ levels, which is in accordance with results obtained in some previous studies.^{30,31} Higher concentrations were detected in GW samples in Poland (≤ 43 ng dm⁻³ for estrone and ≤ 48 ng dm⁻³ for 17 β -estradiol).³² None of the tested hormones were found in the SW2 sample collected from the Danube, Kladovo, while in the remaining SW samples, hormone concentrations ranged from 8.8 to 36.2 ng dm⁻³, which was comparable with hormone concentrations obtained in previous studies in Italy,³³ Poland,³⁴ China,²⁹ Indonesia³⁵ and Malaysia (river Pahang).³⁶

Higher concentrations, up to 820 ng dm⁻³ were recorded in the Bacanga River in Brazil.¹ This river is positioned in an area affected by urbanization, burning, deforestation, water contamination, and siltation, which may explain the high concentration of the selected compounds detected in the water samples of that river.¹ Zhang *et al.* detected observed hormones in river water in Switzerland at concentrations up to 3.7 ng dm⁻³,³⁷ which was lower than the results obtained in the present study. Rocha *et al.* determined estrone, 17 β -estradiol, and 17 α -ethinylestradiol in a river estuary in Portugal at concentrations ≤ 16 , ≤ 18 and ≤ 11 ng dm⁻³, respectively.³⁸ As anticipated, wastewater samples contained the high-

est quantities of the hormones. Concentrations of E1, E2 and EE2 in the present work were up to 163.5, 101.8, and 91.0, respectively. The relatively high concentrations of E1 can be explained by the conversion of E2 and EE2 into E1 before it can be transformed further.³⁹ The highest concentrations of hormones were detected in sample WW3, from the inlet of WWTP Arandelovac, while there was no detectible amount left in the sample from the exit of the same WWTP. Concentrations of E1 in sample WW3 were comparable with obtained E1 concentrations of wastewater influents in France and Slovenia.^{40,41} The concentration of E2 in the WW3 sample was also comparable with the E2 concentration of influent wastewater in Malaysia,⁴² while higher levels of E1 and EE2 were recorded in wastewater influents in Brazil.³⁹

Taking into account the obtained results, it can be concluded that the developed method was efficiently applied for the selective determination of trace estrogens in complex environmental water samples.

CONCLUSION

In the present study, pristine and chemically modified CC was used as a new solid-phase extraction adsorbent for the determination of estrogen hormones in environmental water samples. An efficient SPE method was developed by optimizing the adsorbent mass (20 mg), volume (200 cm³) and initial pH value (pH 7) of the water sample, the type and volume of elution solvent (15 cm³ of EtOAc/MeOH 1:1 mixture), and by selecting HNO₃ treated CC as an adsorbent. The optimized SPE method provided high recovery values for all tested hormones (82–95 %), comparable with the recoveries obtained using commercially available cartridges. Notably, our method used significantly less material than is customary, demonstrating the cost-effectiveness of this approach. The developed SPE/LC–MS/MS method was successfully applied to the analysis of ground, surface and wastewater samples, whereby the matrix effect of examined water samples did not have a significant impact on method accuracy. The highest concentrations of tested hormones were found in wastewater samples. However, the fact that the tested hormones were detected and quantified in most of the tested samples indicates the significant presence of these pollutants in the aquatic environment, which requires further monitoring.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/12844>, or from the corresponding author on request.

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ИЗВОД
ЕКСТРАКЦИЈА ЕСТРОГЕНИХ ХОРМОНА НА ХЕМИЈСКИ МОДИФИКОВАНОМ
УГЉЕНИЧНОМ КРИОГЕЛУ

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У овом раду је представљена нова метода екстракције на чврстој фази, коришћењем немодификованог и хемијски модификованог угљеничног криогела (енгл. carbon cryogel, CC) као адсорбента за изоловање и предконцентрисање естрогених хормона (естрона, 17β -естрадиола и 17α -етинилестрадиола) из узорака воде. Након оптимизације методе, која је обухватила оптимизацију масе и хемијског третмана адсорбента, запремине и рН-вредности узорка и типа и запремине растварача за елуирање, добијене су високе вредности приноса (82–95 %). Развијена аналитичка метода, базирана на SPE екстракцији и течной хроматографији–тандем масеној спектрометрији, показала се селективном, ефикасном и економичном за одређивање одабраних естрогених хормона. Коришћењем кертрица са модификованим CC постигнути су резултати који су били упоредиви са резултатима добијеним при употреби комерцијалних кертрица, уз коришћење знатно мање масе материјала. Поред тога, селективност одабраног материјала је допринела малом ефекту матрице. Оптимизована метода је успешно примењена за анализу естрогених хормона у подземним, површинским и отпадним водама, при чему резултати указују на важност праћења ових загађујућих материја у воденој средини.

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