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Determination of endocrine disrupting compounds using temperature-dependent inclusion chromatography
II. Fast screening of free steroids and related low-molecular-mass compounds fraction in the environmental samples derived from surface waters, treated and untreated sewage waters as well as activated sludge material

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ABSTRACT

In the present work solid-phase extraction protocol based on C18 tubes and organic water washing solvents as well as isocratic HPLC procedure focused on quantification of free steroids and related low-molecular-mass endocrine disrupting compounds (EDCs), characterized by different polarity varied from estetrol to progesterone, were studied. Described separation method involves temperature-dependent inclusion chromatography with mobile phase modified with β-cyclodextrin. Using such analytical approach the environmental samples derived from Baltic Sea, selected lakes and rivers of the Middle Pomerania in northern part of Poland as well as untreated and treated sewage water from municipal sewage treatment plant near Koszalin were analyzed. Moreover, some preliminary data concerning estriol, testosterone and equilin biodegradation involving activated sludge material were reported. Cluster and principal components analysis of the acquired data sets confirms a high separation and quantification throughput of the solid-phase extraction and isocratic HPLC protocols presented. The method can be useful for simple and rapid classification of the environmental samples characterized by different sources of EDCs loading. The results of this work extend the utility of temperature-dependent inclusion chromatography as an inexpensive, efficient and accurate analytical tool appropriate for characterisation and quantification of complex environmental samples.

1. Introduction

Hormonal systems are essential elements in the life of all living organisms. Many biogenic or synthetic chemicals that are present in our natural environment may affect hormone receptors, modulate hormone actions as well as significantly change their transport within multi-cellular organisms [1]. The endocrine disrupting phenomenon was brought to scientific community during the 1980s when deformities in fish were observed across the European rivers [2]. Presently, the endocrine modulation is mainly related to the potentially dangerous consequences to human and wildlife, by reason of the presence of natural and anthropogenic endocrine disrupting compounds (EDCs) in the aquatic environment [3–5]. It is noteworthy that endocrine disrupters are not defined by their chemical nature but by their biological effect [6,7]. Therefore, many different classes of common pollutants including: pesticides, polycyclic aromatic hydrocarbons, plasticizers, polychlorinated biphenyls, dioxins as well as natural steroids such as phytoestrogens are collectively referred to as EDCs [6,8,9].

One of the important group of chemicals considered as endocrine disrupters are steroids [6,7,10]. This group of compounds is extensively used in modern medical science particularly in treatment of infertility, certain cancers, menstrual and menopausal hormonal disorders as well as commonly used for birth-control. The important issue is that many drugs e.g. birth-control pharma-

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ceutical formulations are composed of estrogens and progestogens that show high physiological activity even at very low concentrations [1,3,11,12]. They are excreted through urine mainly as water-soluble conjugates, then discharged into the environment via sewage treatment plants. However, it has been reported that less active conjugated forms can be effectively deconjugated during, e.g. wastewater treatment and can generate the more potent parent compounds [13,14]. It is very important that both groups of steroids should be stable under typical environmental conditions and their concentration ranges few factors from ng to μg levels per litre, depending on type of water [2,10,12,15]. Therefore, the monitoring of steroids-like compounds in aquatic environment is of great importance and numerous studies have recently been conducted to develop analytical procedures suitable for quantification of a wide range of steroids in water samples [6,7,10,16,17]. Nevertheless, due to low level of the components of interest that are usually present in unstable biological matrix with different organic substance loads, the simultaneous measurement of the multiple form of steroids and related chemicals in complex environmental samples is still a real analytical challenge. Results of our studies that were presented in the first part of this paper indicate that isocratic HPLC procedure, where retention of analytes is driven by temperature and a "host-guest" interaction is capable for efficient separation of complex samples containing a wide range of polar and non-polar substances. This approach, which was combined with optimised solid-phase extraction method, was successfully applied for quantification of low quantities of steroid hormones that were present in complex biological samples coming from fetal cord blood [18,19].

The aim of this proposal is to utilise a temperature-dependent inclusion chromatography with UV–vis diode-array detection following a selective solid-phase extraction in order to isolate, separate and quantify a wide range of low-molecular-mass compounds, from surface water samples. Recorded chromatographic profiles containing mostly unknown substances should reflect the samples quality according to EDCs loading. Particularly, we focus our studies on profiling EDCs related chemicals that can be present in treated and untreated sewage waters as well as surface water samples, which were collected from the lakes and rivers located in the area of Middle Pomerania in the northern part of Poland (Fig. 1). Moreover, some preliminary studies concerning biodegradation of selected steroids under activated sludge conditions were reported.

2. Experimental

2.1. Chemicals and reagents

Analytical standards of steroids and low-molecular-mass compounds were obtained from Steraloids (London, UK) including estetrol, 20α-hydroxyprogesterone, cortisol, cortisone, ethynylestradiol, norgestrel isomers, medroxyprogesterone, mestranol, norethindrone and diethylstilbesterol. Equilin, *d*-equilenin, 17β-estradiol, 17α-hydroxyprogesterone, bisphenol A, 4-*tert*-butylphenol, dimethyl phthalate, dibutyl phthalate, dioctyl phthalate, 17β-estradiol 3-sulfate sodium salt, β-estradiol 3,17-disulfate dipotassium salt, β-estradiol 17-(β-D-glucuronide) sodium salt and 7,8-dimethoxyflavone (internal standard) were product of Sigma (St. Louis, MO, USA) whilst estriol, estrone, 17α-estradiol were obtained from Aldrich (Milwaukee, WI, USA). Testosterones (testosterone, methyltestosterone) were purchased from Polfa (Jelenia Góra, Poland), tetrahydrocortisol, tetrahydrocortisone were products of Koch-Light Labs. (Colnbrook, UK) and progesterone as well as β-cyclodextrin were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (38%, analytical purity) and solid sodium hydroxide were products of POCh (Gliwice, Poland) and PPH Standard (Lublin, Poland), respectively.

Organic liquids including methanol, ethanol and acetonitrile were obtained from Merck and used as received without further purification (LiChrosolv; HPLC grade). Double distilled water was used for binary mobile phase preparation.

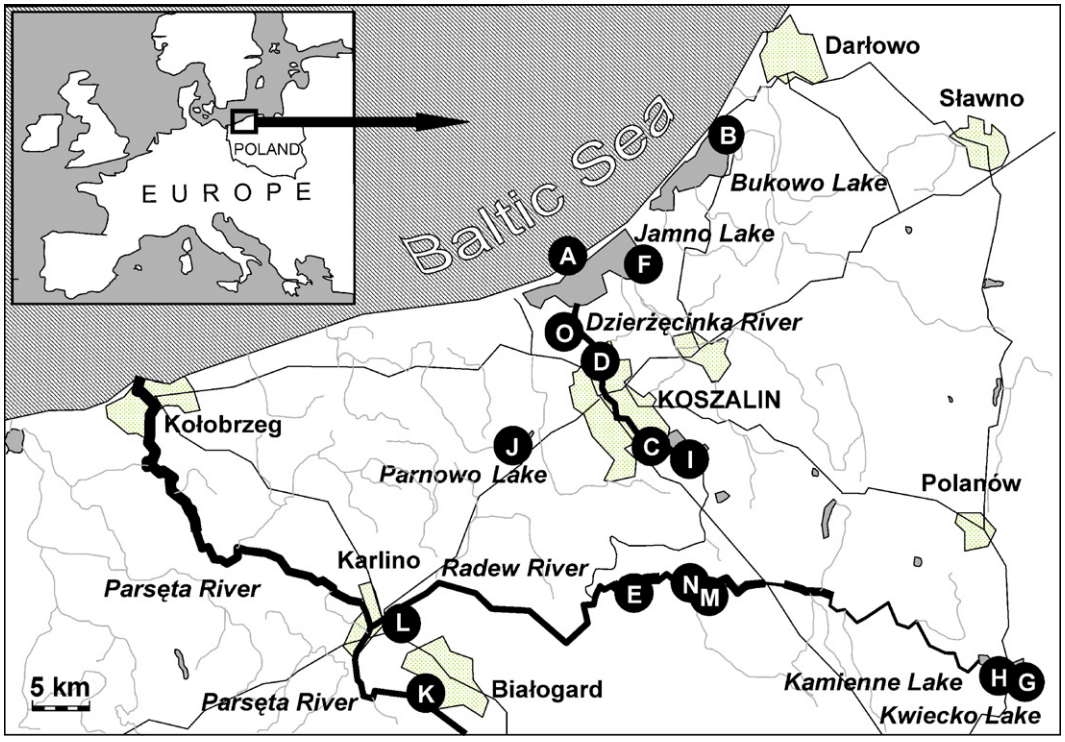

Fig. 1. Map of area of interest including sampling locations. Sample area labels correspond to codes listed in Table 1.

Table 1
Surface water sampling locations data.

Area Name	Coordinates: latitude/longitude	Sample area code
Baltic Sea Coast	N54 17.067 E16 07.887	A
Bukowo Lake	N54 22.622 E16 18.835	B
Dzierżęcinka above Koszalin	N54 10.075 E16 13.433	C
Dzierżęcinka under Koszalin	N54 12.824 E16 09.982	D
Hajka Lake	N54 04.251 E16 12.823	E
Jamno Lake	N54 17.062 E16 12.567	F
Kamienne Lake	N54 01.695 E16 44.053	G
Kwiecko Lake	N54 01.570 E16 42.064	H
Lubiatowo Lake	N54 09.627 E16 17.200	I
Parnowo Lake	N54 09.939 E16 03.363	J
Parsęta River	N54 00.226 E15 57.304	K
Radew River	N54 02.720 E15 52.980	L
Rosnowo Lake	N54 03.989 E16 17.971	M
"Old Radew" Channel	N54 04.704 E16 17.375	N
"Jamno" Wastewater Treatment Plant	N54 14.071 E16 08.998	O

2.2. High-performance liquid chromatography

Chromatographic experiments were performed using 25 cm column (Supelcosil LC-18, I.D. = 4.6 mm, 5 μm). Mobile phase was composed of 35%, v/v, acetonitrile/water with addition of β-cyclodextrin at level of 10 mM. Mobile phase flow rate was set at 1 mL/min. Column temperature was set at 47 °C and was controlled by foam insulated water jacket connected to circulating thermostat (Nestlab RTE7; product of Thermo Electron Corporation, Newington, USA). HPLC system consisting of isocratic pump (LC-10ADvp), injector (Rheodyne 7725i, Rohner Park, CA, USA) with 20 μL loop, an SPD-M20A photodiode-array detection (UV–vis-DAD) system and computer system for data acquisition with software LC Solution (version. 1.21 SP1; 2002–2005) was product of Shimadzu (Suzhou New District, Jiangsu, China). Stock solution of chromatographic standards were prepared in methanol at concentration of 1 mg/mL. Appropriate injecting solutions at concentration ranging from 1 to 50 μg/mL were prepared in mobile phase without macrocyclic additives.

2.3. Solid-phase extraction

Steroids and related substances fraction ranging with polarity from estetrol to progesterone extracted from surface water,

Table 2
Basic characteristic of water ecosystems, which have been chosen for sampling.

Water ecosystem	Area characteristics
(1) Baltic Sea Coast	A brackish inland sea recognized as the largest body of brackish water in the world, which is a result of abundant freshwater runoff from the surrounding land. The sampling station was located at inshore point of the southern Baltic Sea, far from the gulfs and estuaries. Sampling area was free of the organic sediments deposit.
(2) Shallow, physically unstable lakes with high intensity of water/sediment interactions	(a) Lake Lubiatowo, eutrophic, receiving mostly in 1970–80s high agricultural loads from institute farms in Bonin, and contemporary from its catchment used as forest and extensive agriculture areas. (b) Coastal hypertrophic lake Jamno, located in the same catchment below Lubiatowo lake and below Koszalin, a 110-thousand city expected as important source of EDCs in its sewage. (c) Coastal eutrophic lake Bukowo, serving as a reference system for the above lake, without large cities or intensive agriculture in its catchment.
(3) Mesotrophic floodplain of the river Parsęta	Typical lowland river with many meander and slow midstream, receiving main stream of pollutants from local agriculture areas and eutrophic floodplain of the river Dzierżęcinka (27 km length river, passing across Koszalin City).
(4) Mesotrophic/eutrophic floodplain of the river Radew with moderate anthropogenic impact, but with diversified functions of water bodies and relatively deep lakes with epilimnetic waters cut off from sediments:	(a) Physically unstable (as a result of a pumping-storage electric plant operation) lakes Kwiecko and Kamienne, fed by calcareous groundwater, with large reedbelts in its littoral, undergoing fungal decomposition in spring. (b) Stable and sharply stratified lake Rosnowo (a dam reservoir), with distinct zones of sedimentation, primary production and decomposition. (c) Stable, but weakly stratified and warm in summer lake Hajka (a dam reservoir), receiving only surface water from the above located lakes (Rosnowo, "Old Radew" Channel, Kamienne, Kwiecko). (d) Deep groundwater from the floodplain, interacting with surface waters due to high hydraulic conductivity of the sandy bedrock, being a source of drinking water in Koszalin. (e) The "Old Radew", an ancient river channel between Hajka and Rosnowo lakes, now cut off and intensively fed by groundwater passing through forest and agricultural soils in the floodplain.
(5) Stable, stratified and highly eutrophic lake Parnowo,	Groundwater-fed, impacted by intensive agriculture and animal farms.
(6) The "Jamno" municipal wastewater treatment plant	Municipal wastewater treatment plant modernised in year 2000, presently receiving around 90% of the sewage from the Koszalin City. The plant is equipped with two technological line of mechanical and biological treatment extended with denitrification chamber to ensure high reduction of nitrogen.

treated and untreated sewage waters and activated sludge samples were purified and concentrated using Supelclean LC-18 solid-phase extraction (SPE) tubes (6 mL, 0.5 g) and 12-ports vacuum manifold obtained from Supelco (Bellefonte, PA, USA). Extraction procedure was optimized for efficient purification and high recovery of components of interest and internal standard substance (7,8-dimethoxyflavone) using modified SPE protocol described previously [18,19]. Surface water, treated and untreated sewage waters as well as activated sludge samples in volumes of 1000, 100 and 1 mL, respectively, were spiked with 1 μg of internal standard substance (100 μL of I.S. solution at concentration of 10 μg/mL in acetonitrile/water, 35:65, v/v). SPE tubes were conditioned with 5 × 1 mL of 100% methanol and 5 × 1 mL of methanol/water (1:99, v/v) mixture. Water samples were passed through solid-phase extraction cartridge and purified with 5 × 1 mL of washing solvent composed of methanol/water (30:70, v/v) solution. The analytes fraction was eluted with 4 × 0.5 mL of 100% methanol and dried under vacuum at room temperature using vacuum centrifugal evaporator Savant SPD121P Speed Vac. System equipped with Refrigerated Vapor Traps RVT4104 and VLP80 oil vacuum pump, which were products of Thermo, Milford, MA, USA. Extracts were reconstituted in 100 μL of acetonitrile/water (35:65, v/v) liquid and quantified using HPLC–DAD machine.

2.4. Environmental samples collection and sampling area characterization

Surface water samples were collected from the lakes and rivers located in the area of Middle Pomerania in northern part of Poland (Fig. 1, Tables 1 and 2). Treated and untreated sewage waters as well as activated sludge samples were collected from "Jamno" wastewater treatment plant near Koszalin City. Samples collection was conducted within 2007/2008 period, and processed immediately with SPE protocol. Collected SPE extracts were stored at temperature of −20 °C until HPLC quantification.

Numerous lakes and rivers, characterised by different sources of EDCs loading (and different amounts and composition expected) are present within a short (up to 50 km) distance from Koszalin, Poland. These waters are located within the same geological and climatic region what enables comparisons, but differ also in their physical stability, temperature regime, trophy and overall microbial activities, what are expected to act as factors affecting metabolism

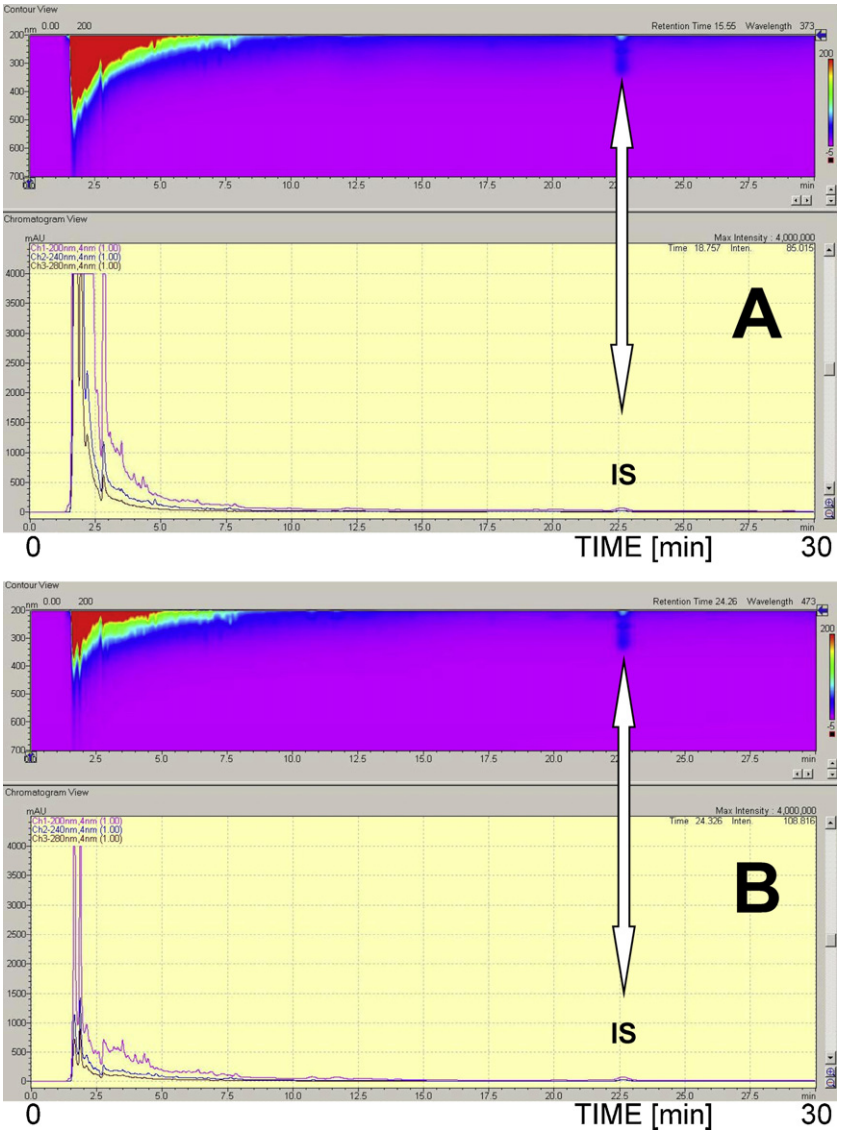

Fig. 2. Comparison of chromatographic profiles of EDCs fraction extracted from Lubiatowo Lake using SPE protocols without (A) and with (B) washing solvent composed of methanol/water (30:70, v/v).

of EDCs and their persistence in ecosystems. Some characteristic water ecosystems, located in the same geographical region but differing in terms of their limnological functions and EDCs sources have been chosen for sampling are presented in Table 2.

2.5. Steroids biodegradation experiment

Steroids degradation including estriol, testosterone and equilin was studied using a method adapted from Ternes et al. [20,21]. Particularly, 10 mL of activated sludge from "Jamno" wastewater treatment plant was mixed with 90 mL of tap water. Three volumes, each of 20 mL, were transferred to separate Erlenmeyer 100 mL flasks. Simultaneously, additional three samples consisting of 20 mL of tap water (instead activated sludge) were prepared as the reference samples. Each sample in the Erlenmeyer flask was spiked with 200 μg of steroid (200 μL at concentration of estriol, testosterone and equiline of 1 mg/mL in methanol). The samples were mixed at room temperature using magnetic stirrers and oxygenated using membrane aquarium pump through the whole experiment period. For steroids quantification 1 ml of sample were taken in following time intervals: 1, 5, 30 and 60 min as well as 6, 24, 48 and 72 h. To inhibit microbial degradation of the target analytes each 1 mL sample was mixed with 20 μL of 0.5% hydrochloride acid. Then low pH value of the sample (pH 3) was adjusted to the neutral using 20 μL of 0.5% sodium hydroxide. Until SPE purification the samples were stored in glass containers with PTFE caps in refrigerator at sub-ambient temperature.

During experiment the concentration of oxygen in the samples was measured using the oxygen sensor COG-2 (Elmetron, Zabrze, Poland) coupled with acquisition data unit CO-411. Temperature and pH parameter were measured via Handylab pH 11 unit connected to pH/temperature electrode BlueLine 24pH (Schott Instruments, Mainz, Germany).

2.6. Data analysis

Each water sample was characterized by 104 variables including selected physicochemical parameters like pH, water and air temperature and oxygen concentration as well as relative intensities of chromatographic peaks using an internal standard method involving 7,8-dimethoxyflavone as the quantitative marker. Due to strong differences in the background matrix profiles and number of different peaks observed on the chromatographic profiles recorded from the environmental samples, each chromatogram was split and char-

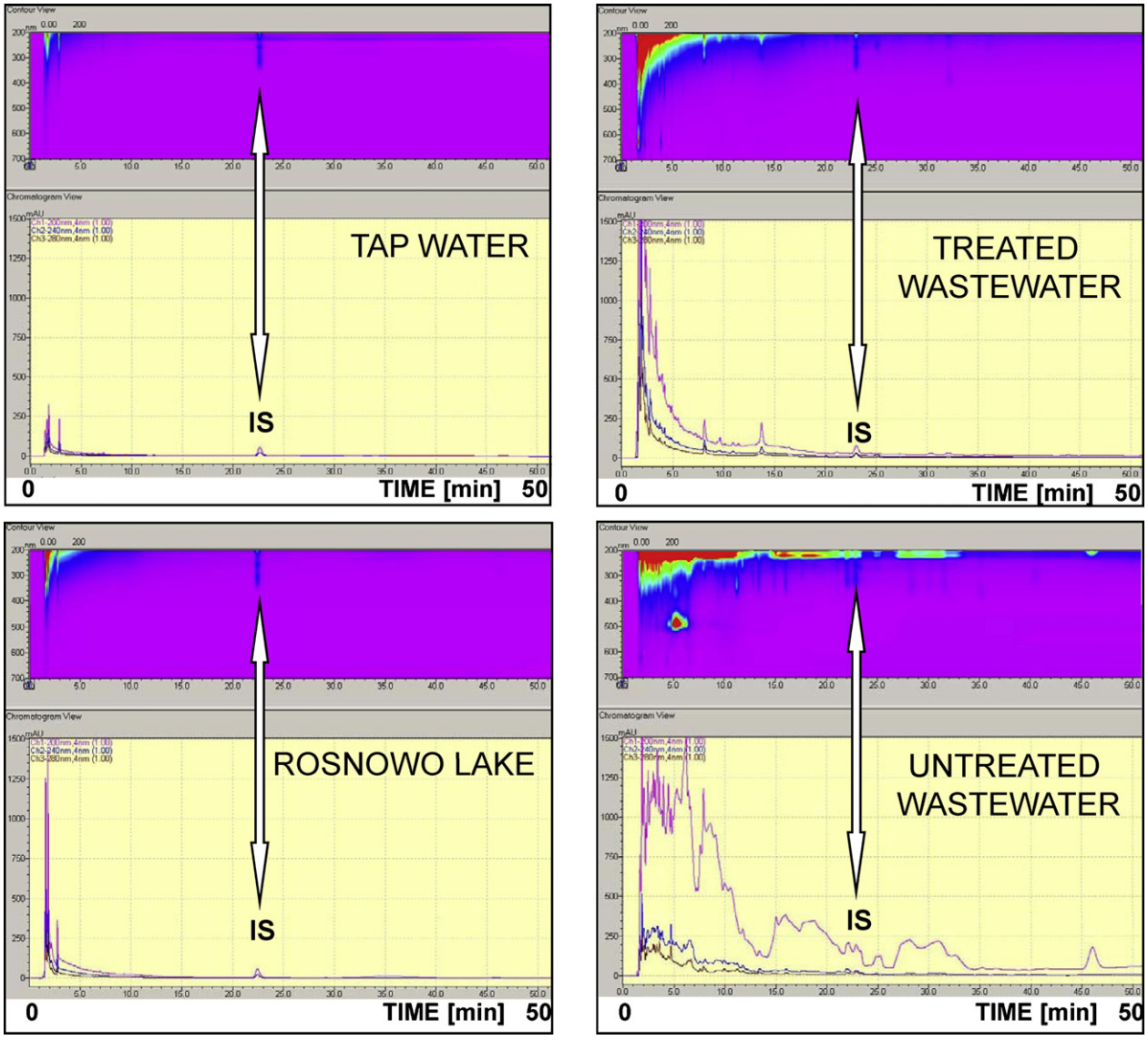

Fig. 3. Typical chromatograms (UV–vis-DAD) of EDC fraction extracted from waters with different organic substances load.

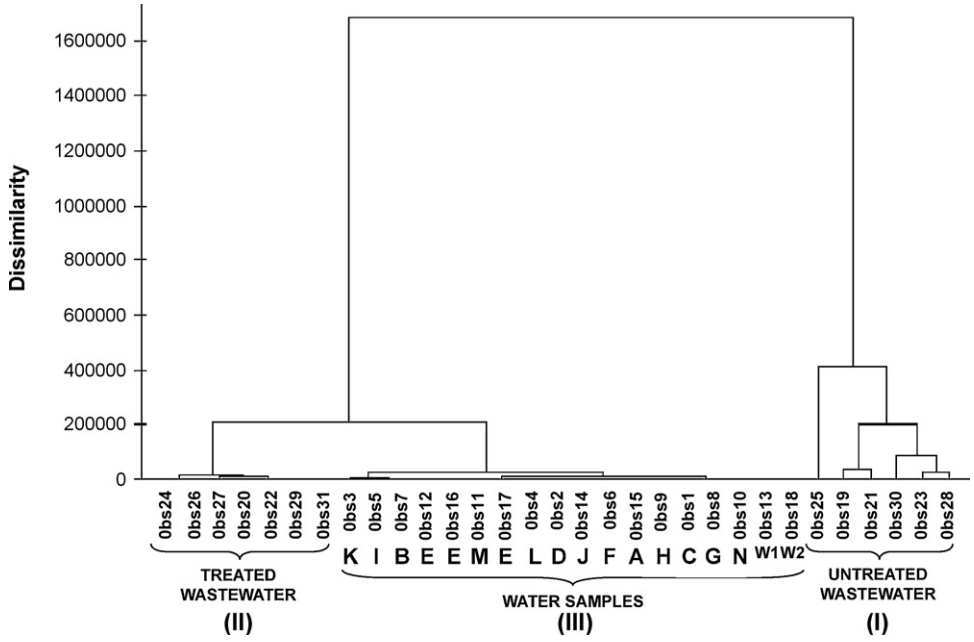

Fig. 4. Results of cluster analysis involving data matrix composed of water samples (observations; rows) versus chromatographic peak groups and selected physicochemical parameters (variables; columns). Dendrogram of agglomerative hierarchical cluster analysis involves Ward's method as the aggregation criterion and represents the clustering of the water samples according to the chromatographic and physicochemical parameters measured. Samples (observations) labels correspond to codes listed in Table 1; in addition W1 denotes distilled water and W2 tap water samples.

acterized by 33 peaks clusters. Particularly, within four time periods ranging from 0 to 5 (with 0.5 min step), 5 to 10 (with 1 min step), 10 to 20 (with 2 min step) and 20 to 72 min (with 4 min step) individual peaks groups were selected. They were recorded at three analytical wavelengths of 200, 240 and 280 nm, resulting in 99 clusters in total, for each sample. Under such conditions each specified cluster characterizes peaks eluted within time period covering two base peak distance, approximately. Within each cluster the relative intensities of individual peaks observed were summarized.

The experimental data were inspected with the agglomerative hierarchical clustering and principal components multivariate statistical procedures using XLSTAT-Pro/3DPlot (version 2008.2.01) provided by Addinsoft, Paris, France.

3. Results and discussion

In our previous studies we optimized SPE procedure toward simultaneous purification, extraction and concentration of steroid hormones from biological samples involving C18 tubes and methanol/water eluents [18,19]. It has been found that such separation protocol is capable for efficient fractionation of steroids and related low-molecular-mass compounds ranging from polar estetrol to relatively non-polar progesterone. Within this polarity range number of unknown and identified steroids relating substances can be captured [19]. In present work we tested this procedure for the environmental samples (Fig. 1, Tables 1 and 2) by extracting of large volume of surface water up to 1000 mL. Under such condition, samples concentration ratio was set at 1:10000. This allows a low ng/L sensitivity of steroids and related substances (Table 3), quantified by UV-DAD in the environmental extracts separated under temperature-dependent inclusion chromatography conditions, which is comparable with LC and GC protocols involving MS detectors and reported in literature [22–24]. Chromatograms presented on Fig. 2 clearly demonstrate that high level of background matrix peaks eluted within the first minutes of chromatographic run can be significantly reduced using washing solvent composed of 30%, v/v, methanol in water. It has been found that with such SPE protocol, average recovery rate exceed 90% (Table 4). Chromatograms presented within Fig. 3 show typical UV-DAD profiles of the environmental samples characterized by different loading of organic substances, particularly tap water, surface water from the lake as well as treated and untreated wastewater collected from the municipal wastewater treatment plant. Noteworthy, chromatographic separation conducted at elevated temperature (47 °C) provides high separation throughput of the extract components and baseline separation of the internal standard from all the peaks observed.

Using this SPE protocol and separation of extracts via temperature-dependent inclusion chromatography a fraction of low-molecular-mass substances was extracted from environmen-

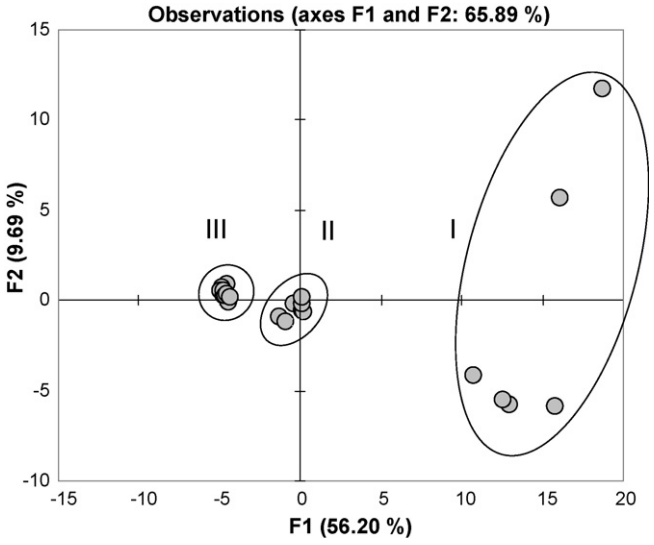

Fig. 5. Principal component plot showing relationships between all objects investigated in respect to 1 and 2 factor scores (samples within I, II and III areas corresponds to clusters presented in Fig. 4.).

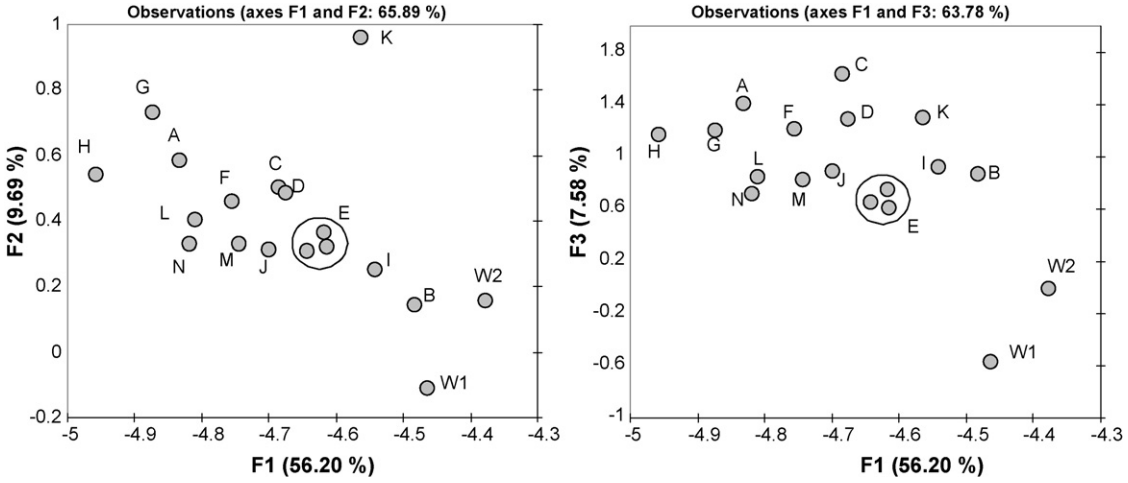

Fig. 6. Principal component plot showing relationships between water samples located within cluster III (according to Fig. 5) in respect to 1 and 2 (left) as well 1 and 3 (right) factor scores.

tal samples including Baltic Sea, lakes and rivers surface water as well as treated and untreated wastewater (Fig. 1, Table 1). As the reference samples, distilled and tap water from our laboratory was also proceed. The results of cluster analysis (CA) presented in the dendrogram form (Fig. 4) suggest that samples collected can be effectively fingerprinted and characterized by the extraction and quantification protocol described. Analytical protocol is capable to distinguish significant differences between environmental samples that are not readily identifiable using univariate measurements of single low-molecular-mass substance. As can be seen, the chromatographic patterns of UV detected fractions may capture key information about sample source. Particularly, two main associations consisting of untreated wastewater cluster (I) and remaining samples cluster, which clearly split into treated wastewater (II) and surface water samples (III) groups, can be recognized. To obtain more quantitative data concerning water samples clustering a principal components analysis (PCA) was performed. The main advantage of PCA is to reduce the dimensions of the large data matrix to fewer uncorrelated variables. Both CA and PCA chemometric methods are exploratory data analysis tools for solving classification problems of large data sets, allowing data reduction and determination of latent information from the raw data set. For these reasons, CA and PCA are widely used in chemistry, separation sciences and environmental studies [17,25–28]. In the case of the present PCA studies, a raw data matrix consisted of 3224 experimental points made up of 31 water samples (objects), which were characterized by 104 parameters (variables including chromatographic and physicochemical data) was investigated. As the result of PCA computation the sequence of 10 eigenvalues greater than 1 were obtained. Therefore, instead of the Kaiser criterion, in which only factors with eigenvalues greater than 1 are retained, the factorial scree test (cattell test) was used to graphically determine the optimal number of factors to retain. According to this criterion, the first two factors were selected and these explain over 65.8% of total variability. Particularly, factors 1 and 2 account for 56.2% and 9.7% of the variance. Graph presented in Fig. 5 show calculated score plot for the water samples investigated. It is clearly seen that objects form three associations, particularly considering F1 component (*X*-axis) which carry most of the data set variability. The groups specified by PCA procedure correspond to those previously visualized by the CA dendrogram. PCA investigation revealed that mechanical and biological units of the municipal wastewater treatment plant can reduce and, which is more important, to unify the organic pollution load of untreated sewage water. However, due to significant separation, which is observed between clusters II and III presented on Fig. 5, the EDCs fraction in the resulting treated wastewater may still have significant impact on the natural environment.

Plots presented on Fig. 6 prove that chromatographic patterns of UV detected analytes, contains the key information that may reveal differences between water samples located within cluster no. III. This can be easily observed considering the first three PC factors, which explain over 73.4% of total variability (plot F1 versus F2 and F1 versus F3 on Fig. 6). As can be seen, distilled and tap water samples (W1 and W2) are clearly separated from the Baltic Sea (A), Kwiecko (H) and Kamienne (G) lakes, which are physically unstable as a result of the wave's action or pumping-storage electric plant operation. Interestingly, lack of the separation between samples collected from Dzierżęcinka River (above and under Koszalin; C and D, respectively) indicate low contribution of the Koszalin City to organic pollutants load of this river. Moreover, the object association located within the circle confirms the method robustness, since the samples for those objects were acquired from the same area (Hajka Lake; E).

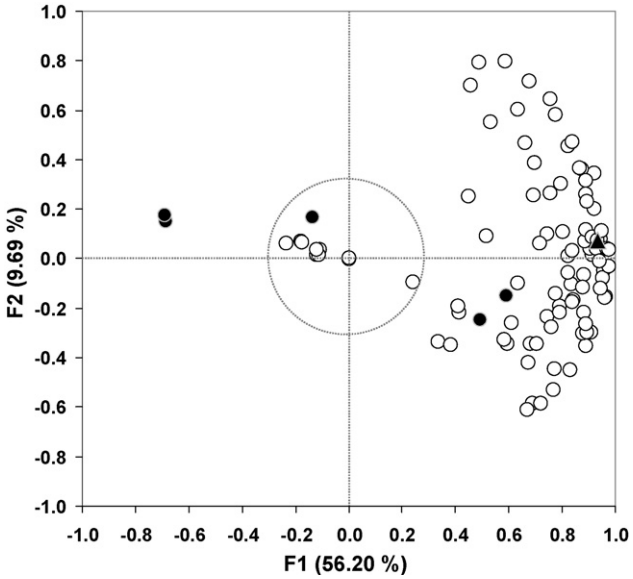

Fig. 7. Projection of all variables considered in the principal component loadings space.

Fig. 7 shows the loading plot, which reflects similarities and dissimilarities among quantitative variables considered. Generally, variables far from zero in the horizontal direction are responsible for the environmental samples grouping described above (Figs. 5 and 6). Data presented on Fig. 7 clearly shows the prevalent influence of physicochemical variables including oxygen concentration and water or air temperature (black dots on the left and right side of the plot) as well as majorities of the chromatographic peaks recorded (empty dots, right side of the plot) for the environmental samples classification. Influence of pH parameter (black dot

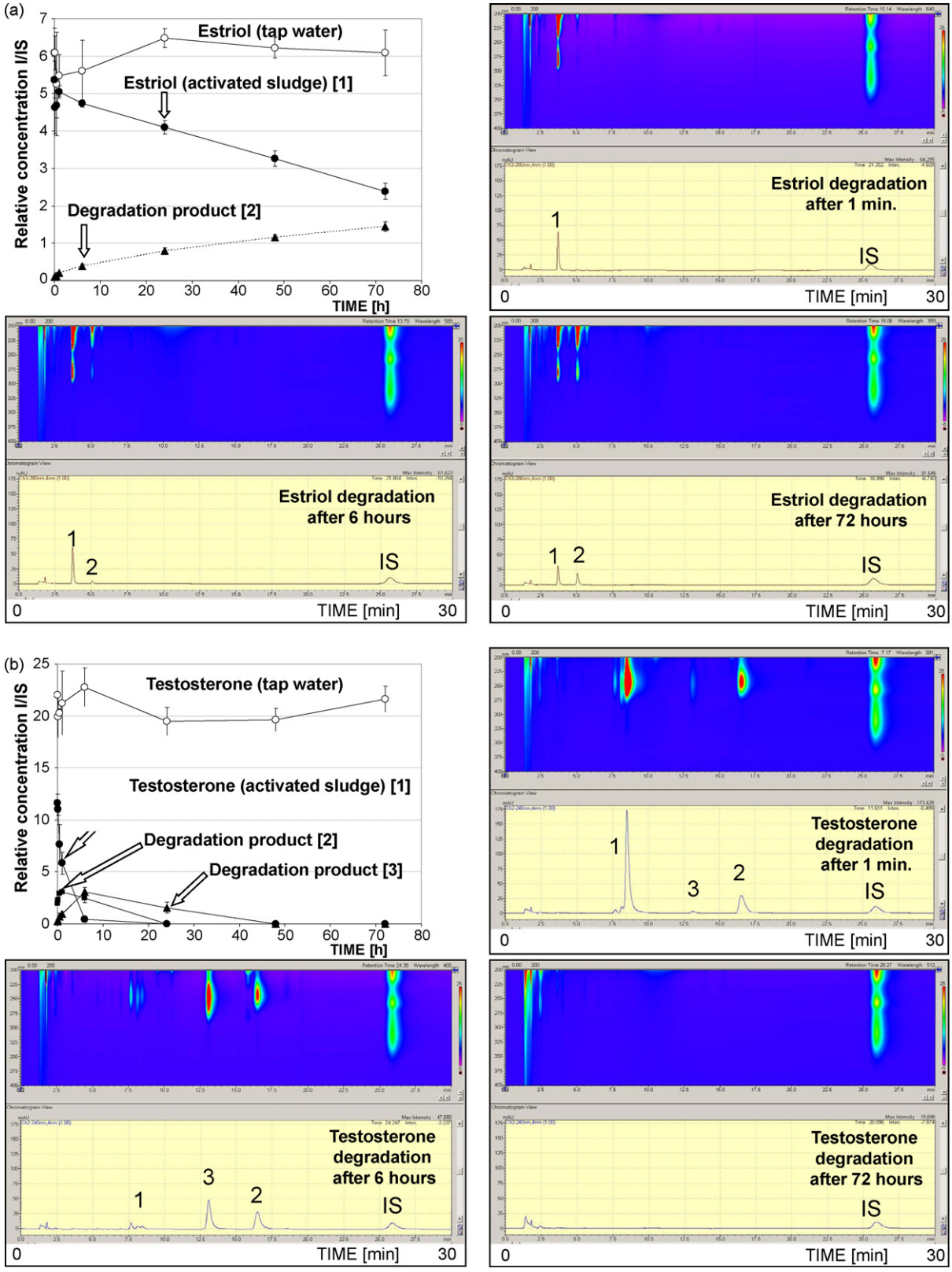

Fig. 8. Biological degradation of estriol (A), testosterone (B) and equilin (C) under activated sludge conditions.

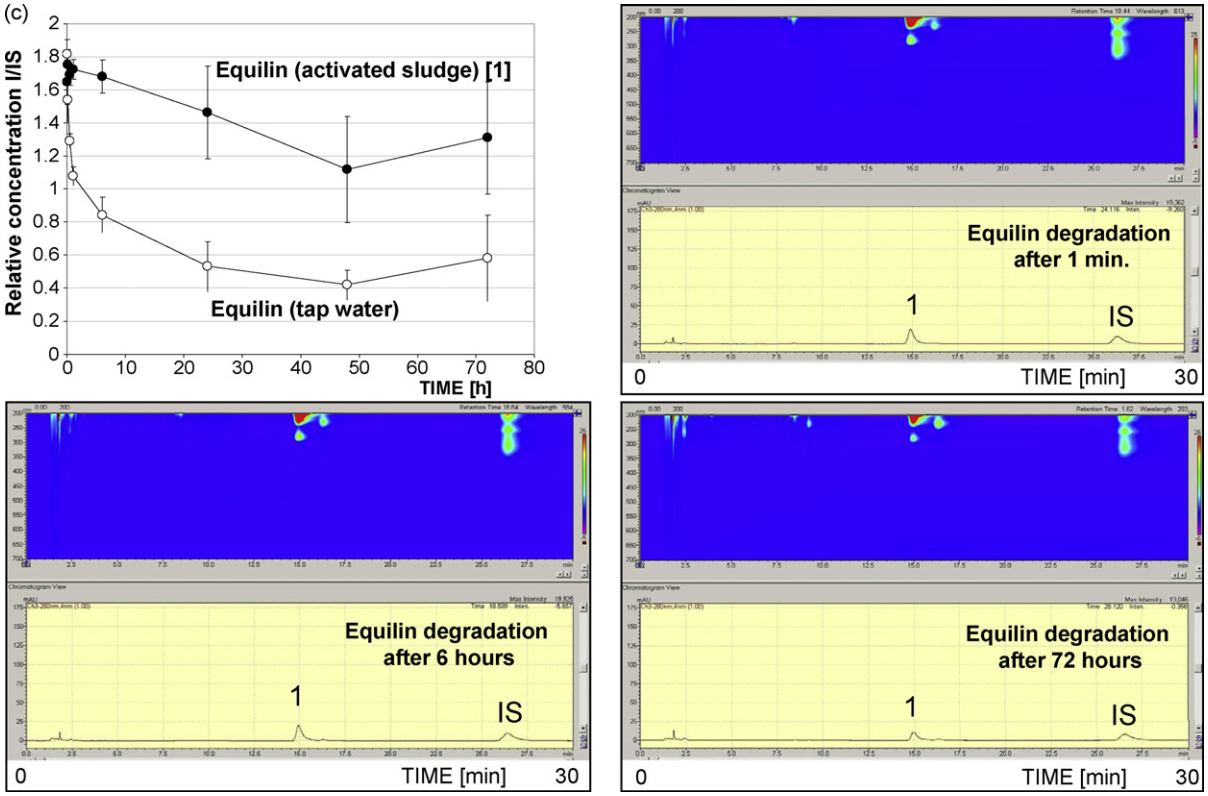

Fig. 8. (*Continued*).

Table 3
Detection limits for investigated analytes measured for mobile phase composed of 35%, v/v, acetonitrile/water with addition of β-cyclodextrin (injection loop: 20 μL).

Analyte	Analytical wavelength (nm)	Detection limit (ng/1000 mL)	Standard deviation
Estetrol	200	0.37	±0.02
	280	3.6	±0.2
Estriol	200	0.22	±0.02
	280	1.9	±0.1
Cortisol	240	0.37	±0.03
Cortisone	240	0.49	±0.03
Tetrahydrocortisol	200	1.75	±0.04
Tetrahydrocortisone	200	3.8	±0.3
Dimethyl phthalate	200	0.23	±0.01
	280	2.8	±0.1
Bisphenol A	200	0.28	±0.02
	280	1.81	±0.08
17β-Estradiol	200	0.51	±0.03
	280	4.7	±0.2
Testosterone	240	0.33	±0.02
Norethindrone	240	0.44	±0.02
17α-Estradiol	200	0.47	±0.02
	280	4.5	±0.2
d-Equilenin	230	0.24	±0.02
	280	3.8	±0.3
Methyltestosterone	240	0.56	±0.04
Equilin	200	0.86	±0.08
	280	7.4	±0.6
Ethynylestradiol	200	1.64	±0.08
	280	25.6	±0.7
7,8-Dimethoxyflavone*	200	0.95	±0.06
	260	0.59	±0.04
	312	0.74	±0.05

Table 3 (*Continued*)

Analyte	Analytical wavelength (nm)	Detection limit (ng/1000 mL)	Standard deviation
Estrone	200	0.79	±0.05
	280	9.6	±0.6
17α-Hydroxyprogesterone	240	1.07	±0.06
4-*tert*-Butylphenol	200	0.52	±0.01
	280	2.29	±0.09
Toluene	207	1.2	±0.2
	261	24.3	±4.1
Norgestrel	240	1.41	±0.08
Diethylstilbesterol	200	0.52	±0.04
	240	1.0	±0.1
20α-Hydroxyprogesterone	240	2.1	±0.2
Medroxyprogesterone	240	1.57	±0.08
Progesterone	240	2.3	±0.2

* Internal standard.

located within circle in the picture center) seems to be negligible, probably due to narrow pH values range recorded. Within all samples investigated, bisphenol A has been found at quantitative level of 17.8, 23.7, 36.3 ng/L (average contents in surface water, treated and untreated sewage waters, respectively). According to loading plot pattern, this variable (black triangle located on the right side of the plot) can be considered as important factor for environmental samples clustering. The level of remaining chemicals listed in Tables 3 and 4 was below of ng/L, which may indicate low contribution of organic pollutants generated by the humans to composition of EDCs fraction present in water ecosystems located in the area of Middle Pomerania in northern part of Poland.

Data presented on Fig. 8 A–C, summarize the results of the aerobic batch experiments involving activated sludge obtained from the municipal sewage treatment plant near Koszalin. As can be seen, the method is capable to separate sludge matrix peaks and trace steroids of interest as well as number of their degradation products.

Table 4
Recovery values of the analytes and internal standard at concentration corresponding to 100 ng/L of water sample, for the extraction protocol proposed (number of samples = 5).

Analyte	Recovery (%)	Standard deviation
Estetrol*	95.4	±5.8
Estriol	94.6	±3.7
Cortisol	94.3	±4.5
Cortisone	95.0	±4.1
Tetrahydrocortisol	96.3	±6.2
Tetrahydrocortisone	87.5	±9.8
Dimethyl phthalate	82.3	±9.5
Bisphenol A	96.5	±5.2
17β-Estradiol	99.5	±2.9
Testosterone	94.3	±5.5
Norethindrone	96.7	±6.6
17α-Estradiol	93.6	±7.7
d-Equilenin	89.7	±3.5
Methyltestosterone	93.6	±7.3
Equilin	89.7	±6.8
Ethynylestradiol	96.4	±8.9
7,8-Dimethoxyflavone**	95.4	±5.0
Estrone	96.6	±9.4
17α-Hydroxyprogesterone	95.7	±5.4
4-*tert*-Butylphenol	30.2	±6.2
Norgestrel	95.6	±4.8
Diethylstilbesterol	77.8	±1.9
20α-Hydroxyprogesterone	92.8	±5.7
Medroxyprogesterone	89.7	±5.0
Progesterone	90.2	±8.8

* Recovery data according to Ref. [18].
** Internal standard.

4. Conclusions

Presented results of cluster and principal components analysis confirms a high separation and quantification throughput of the solid-phase extraction and isocratic HPLC protocols studied. Based on the chromatographic profiles collected via UV–vis-DAD, number of environmental samples derived from surface water including Baltic Sea, selected lakes and rivers of the Middle Pomerania in northern part of Poland, were effectively fingerprinted and characterized. Moreover it has been demonstrated that this method can be directly applied for steroids biodegradation batch experiments involving complex biological matrix like activated sludge material. In case of characterization of the environmental samples organic load, the results of our experiment indicate that separation protocol using temperature-dependent inclusion chromatography with UV–vis-DAD may be simple and non-expensive alternative for fingerprinting protocols based on LC-MS machines.

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