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# Temperature-controlled micro-TLC: A versatile green chemistry and fast analytical tool for separation and preliminary screening of steroids fraction from biological and environmental samples

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ABSTRACT

This paper is a continuation of our previous research focusing on development of micro-TLC methodology under temperature-controlled conditions. The main goal of present paper is to demonstrate separation and detection capability of micro-TLC technique involving simple analytical protocols without multi-steps sample pre-purification. One of the advantages of planar chromatography over its column counterpart is that each TLC run can be performed using non-previously used stationary phase. Therefore, it is possible to fractionate or separate complex samples characterized by heavy biological matrix loading. In present studies components of interest, mainly steroids, were isolated from biological samples like fish bile using single pre-treatment steps involving direct organic liquid extraction and/or deproteinization by freeze-drying method. Low-molecular mass compounds with polarity ranging from estetrol to progesterone derived from the environmental samples (lake water, untreated and treated sewage waters) were concentrated using optimized solid-phase extraction (SPE). Specific bands patterns for samples derived from surface water of the Middle Pomerania in northern part of Poland can be easily observed on obtained micro-TLC chromatograms. This approach can be useful as simple and non-expensive complementary method for fast control and screening of treated sewage water discharged by the municipal wastewater treatment plants. Moreover, our experimental results show the potential of micro-TLC as an efficient tool for retention measurements of a wide range of steroids under reversed-phase (RP) chromatographic conditions. These data can be used for further optimalization of SPE or HPLC systems working under RP conditions. Furthermore, we also demonstrated that micro-TLC based analytical approach can be applied as an effective method for the internal standard (IS) substance search. Generally, described methodology can be applied for fast fractionation or screening of the whole range of target substances as well as chemo-taxonomic studies and fingerprinting of complex mixtures, which are present in biological or environmental samples. Due to low consumption of eluent (usually 0.3–1 mL/run) mainly composed of water–alcohol binary mixtures, this method can be considered as environmentally friendly and green chemistry focused analytical tool, supplementary to analytical protocols involving column chromatography or planar micro-fluidic devices.



## 1. Introduction

The growing demand for development of environmentally friendly analytical protocols usually results with miniaturization of the existing and well established methods. In case of liquid chromatography (both column and planar techniques), modern micro-separation systems may work with low volumes of mobile phases, typically less than 1 mL. In analytical practice thin-layer chromatography (TLC) is still commonly applied as the method for fast qualitative and quantitative analysis as well as screening of target substances from complex biological and environmental samples [1–3]. It is noteworthy to say that modern high-performance planar chromatography is an excellent illustration of how an older technique may evolve due to the development of new sensitive detectors and acquisition data methods in the last decade [4–6]. Most recently, number of new detection methods like direct analysis in real time (DART) involving mass spectrometry (MS) techniques were introduced [7,8]. Such MS-based sophisticated analytical tools including matrix-assisted laser desorption/ionization mass spectrometry (TLC–MALDI-MS) or electron impact ionization mass spectrometry (TLC–EI-MS) were successfully applied for analysis of complex biological samples

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allowing the use of planar chromatography in metabolomic studies [9]. To extend the separation power of the classical TLC plate a high-performance thin-layer chromatography (HPTLC) and/or two-dimensional developing mode can be easily selected. In practice, typical separation power of non-forced flow rate HPTLC systems lies between 10 and 20 spots per plate measured in one direction. However, working under 2D-TLC mode the number of spots separated can be significantly increased even by one factor more [10,11]. Our previous experimental data have revealed that 2D developing protocol involving micro-HPTLC plate is capable of separating more than 240 spots consisting of low-molecular mass compounds like steroids or herbs extracts [12,13].

In analytical practice, a modern high-performance thin-layer chromatography (HPTLC) involving reversed phase (RP) plate is particularly suitable for the method miniaturization. It is noteworthy that using HPTLC plates, the mobile phase developing distance can be reduced to less than 50 mm, which is well documented in the literature [14–16]. Generally, this conclusion is based on the observation that minimum values of the plate height ($H$) can be achieved if the solvent migration distance along the HPTLC plate ranges from 30 to 40 mm [11,14]. Under such conditions total analysis time can be dramatically reduced in comparison to chromatographic separations performed on typical 10 or 20 cm long TLC plates. Moreover, it allows the miniaturized planar chromatographic devices to work with μL volumes of the mobile phase to complete the whole analytical run, and therefore this technique can be classified as micro-TLC [4,10,13,17]. The next advantage of planar chromatography over its column counterpart is that each TLC run can be performed using non-previously used stationary phase. In case of column chromatography including GC and HPLC such samples must be accurately pre-purified and usually diluted. Particularly, all substances that are strongly adsorbed by stationary phase must be carefully removed to provide columns and detectors long life. Using micro-TLC it is possible to perform inexpensive fractionation or separation of complex samples characterized by heavy biological or environmental matrix loading [18].

This work is a continuation of our previous research focusing on development of micro-TLC methodology for fast fractionation and analysis of low-molecular mass compounds, mostly from complex samples [12,13,15,17–19]. The main goal of this paper is to demonstrate the separation and detection capability of micro-TLC technique towards steroids compounds that can be present in highly loaded organic matrix extracts. Target components from environmental or biological samples including extracts from surface water ecosystems or treated sewage water as well as steroids fraction of fresh and hydrolyzed fish bile were analyzed. The samples were isolated using lyophilization, simple direct organic liquid extraction or optimized solid-phase extraction techniques. Described methodology can be applied for fast and non-expensive screening or fingerprinting of steroids main fraction from complex mixtures, which are present in biological or environmental samples. Moreover, micro-TLC method can be used as fast retention data screening tool for selection of internal standard substances for HPLC protocols or optimization of SPE procedure for target substances.

## 2. Experimental

### 2.1. Materials and reagents

Analytical standards of testosterone and testosterone enanthate were obtained from Polfa (Jelenia Góra, Poland). Steroids standards including estetrol and 20α-hydroxyprogesterone were purchased from Steraloids (London, England). Progesterone was obtained from Merck (Darmstadt, Germany). Equilin and 17α-hydroxyprogesterone were products of Sigma (St. Louis, MO, USA). Estriol, 17α-estradiol, 17β-estradiol and estrone were obtained from Aldrich Chemical Co (Milwaukee, WI, USA). Methanol (LiChrosolv 99.8% for liquid chromatography) was obtained from Merck (Darmstadt, Germany). Phosphomolybdic acid was purchased from Chempur (Piekary Śląskie, Poland) and methyl red was a product of POCH SA (Gliwice, Poland). Double-distilled tap water was used for mobile phase preparation.

### 2.2. Micro-TLC chromatography

Separation experiments were performed on glass-based HPTLC RP18W and RP18W$F_{254}$S plates that were products of Merck (Darmstadt, Germany). Before sample application, the factory-prepared plates (100 × 100 mm) were cut to a working size of 50 × 50 mm. In each case, a sample starting line was placed 5 mm from the plate bottom edge, allowing a maximum eluent front migration distance of 45 mm. Micro-planar separations were performed using a home-made temperature-controlled removable horizontal micro-TLC chamber unit described previously [13]. Particularly, a chromium-coated brass unit was working inside a foam insulated metal oven connected to an external liquid circulating thermostat (Ultra-Low Refrigerated Circulator FP51-SL, Julabo, Seelbach, Germany) filled with ethanol as a circulating liquid. The system provided a constant TLC plate temperature, which was set at given temperature with an accuracy of ±0.02 °C.

To obtain chromatograms, the following chamber working protocol was applied: a micro-TLC plate with samples spotted on the starting line was positioned horizontally inside a chamber module with the stationary phase layer placed up side down. Afterwards, the chamber module was transferred into a temperature-regulated oven cavity and sealed using a 1 mm thin glass cover. Then, the movable cover of the oven was slid so as to reach the position above the TLC chamber module and the temperature equilibration step was performed for 15 min. The chromatographic process was started after injecting a given eluent in a volume from 300 μL to 1 mL through an injection pipe into a mobile phase application bar. Finally, the TLC plate was removed from the chamber module immediately after the mobile phase front reached the plate edge located opposite to the application bar.

Chromatographic separations were performed under unsaturated or saturated chamber conditions using various methanol:water composed binary mobile phases. Spots patterns on the developed plates were acquired by direct scanning under visible light conditions and/or after application (by dipping method) of a visualization reagent consisted of 10% phosphomolybdic acid (PMA) in methanol. Under such conditions blue-gray colored spots were generated after the plates were dipped in the PMA reagent and heated (gravity convection oven BMT Ecocell, Conbest; Krakow, Poland) for given time and temperature, depending on the target compounds mixture. Visualization protocol conditions for steroids investigated were optimized previously [20]. Time and temperature data for given analysis are enclosed within appropriate figures captions.

Detailed analytical condition concerning testosterone quantification presented within Fig. 1: temperature 20 °C; mobile phase composition – methanol:water (8:2, v/v); unsaturated chamber, stationary phase HPTLC RP18W$F_{254}$S; samples application – manual using Hamilton-type syringe; detection *via* fluorescence quenching ($\lambda_{EX}$ = 254 nm, $\lambda_{EM}$ = visible light).

Retention screening of estrogens and progestogens (Fig. 2): stationary phase HPTLC RP18W; mobile phase methanol:water; temperature 20 °C (saturated chamber conditions); steroids mass 1 μg/spot; samples application – manual using Hamilton-type syringe; spots visualization: 10% PMA (120 °C, 10 min).

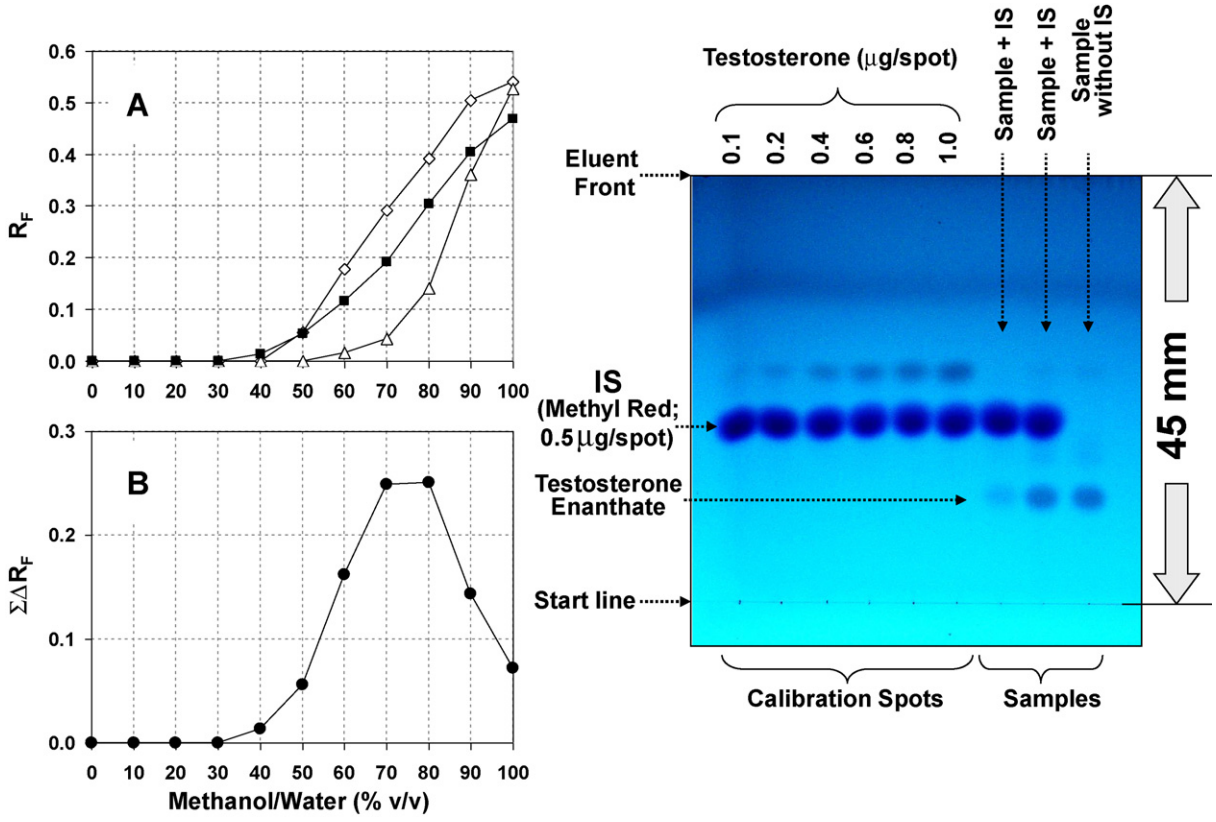


**Fig. 1.** Selection of the internal standard substance for quantification of testosterone impurity in the testosterone enanthate sample. Graph A illustrate chromatographic retention profiles of testosterone (diamonds), testosterone enanthate (triangles) and methyl red (squares, IS). Optimization graph (B) was constructed as the sum of $\Delta hR_F$ parameter for the adjacent spots.

Fingerprinting of SPE extracts derived from surface water ecosystems presented in Fig. 5. Samples volume – 5 µL, sample application – 4 mm band using spray-on technique, separation temperature +30 °C, stationary phase – HPTLC RP18W$F_{254}$S, mobile phase composition – 80% (v/v) methanol:water (unsaturated chamber); fluorescence detection ($\lambda_{EX}$ = 366 nm; $\lambda_{EM}$ = visible light) using 5.0 Mega pixel digital camera Olympus Camedia 5050 Zoom equipped with a 43 mm UV filter (shutter speed 4 s, $F$ = 8.0);

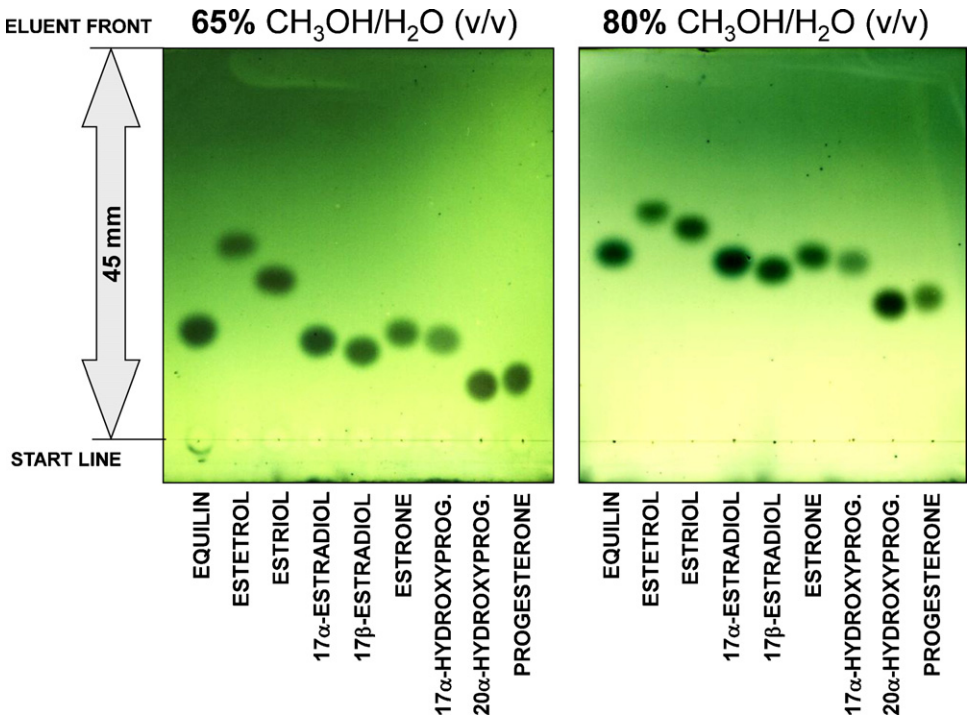


**Fig. 2.** Retention screening of estrogens and progestogens under reversed-phase chromatographic conditions.

phosphomolybdic acid derivatization conditions: 10% PMA, 80 °C, 20 min. Chromatogram acquisition method (PMA plate) – direct digital scan under visible light conditions using Plustek OpticPro S12 USB office scanner.

Screening of treated sewage water discharged by the municipal wastewater treatment plant "Jamno" (Fig. 6): sample application – 5 mm bands; chromatogram acquisition method (PMA plate) – direct digital scan under visible light conditions using Plustek OpticPro S28 scanner. Remaining experimental conditions are similar to those specified for Fig. 5.

Micro-TLC separation of main components of sea trout bile (Fig. 7). Bile samples volume – 6 μL; sample application – 5 mm bands; separation temperature: +50 °C (unsaturated chamber); stationary phase – HPTLC RP18WF$_{254}$S; mobile phase composition – 70% (v/v) methanol:water; spots detection: direct visible light scan (A), fluorescence quenching mode $\lambda_{EX}$ = 254 and $\lambda_{EM}$ = visible light (B), fluorescence $\lambda_{EX}$ = 366 nm and $\lambda_{EM}$ = visible light (C) as well as visible light scan after phosphomolybdic acid staining (D) using 10% PMA (60 °C, 25 min.). Chromatogram acquisition methods are similar to those specified within Fig. 5, except of digital camera shutter speed that was set at level of 1 and 10 s for 254 and 366 nm UV light exposure, respectively.

### 2.3. Micro-TLC plates application protocol

Micro-TLC plates were spotted with given liquid samples manually using Hamilton-type glass syringe or *via* Linomat 5 semi-automatic application instrument (Camag, Switzerland), controlled through the Planar Chromatography Manager (winCATS software, 1999–2008, version 1.4.4.6337). Using the spray-on technique narrow 4 or 5 mm long bands were formed along start line, which was located 5 mm from the bottom edge of micro-TLC plate.

### 2.4. Solid-phase extraction

SPE of target compounds ranging with polarity from estetrol to progesterone extracted from surface water, treated and untreated sewage waters 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) [21]. Particularly, SPE tubes were conditioned with 5 × 1 mL of 100% methanol and 5 × 1 mL of methanol:water (1:99, v/v) mixture. Water and sewage 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 solvent composed of acetonitrile/water (35:65, v/v).

### 2.5. Micro-chromatograms digitalization

Picture acquisition was performed using a Plustek OpticPro S12 or S28 USB scanners (Plustek, Taipei, Taiwan) with an 8-bit per RGB channel color deep mode, 600 DPI resolution, and saved as TIFF files without compression with the help of image-acquisition software: Image Folio v. 4.2.0 (1991–2000, NewSoft Technology Corporation).

Fluorescence visualization for $\lambda_{EX}$ = 254 and 366 nm was performed using a Cobrabid UV lamp (Warszawa, Poland). For that purpose the TLC plate was placed on the black background 23 cm from the light source (the angle between lamp/plate/digital camera lens was 15°, approximately). The chromatographic pattern observed under visible light was acquired using an Olympus Camedia 5050 Zoom, 5.0 Mega pixel digital camera (Olympus Optical Co. Ltd., Japan) equipped with a 43 mm UV filter (Marumi, Japan). The camera lens was positioned 26.5 cm above the TLC plate center and digital shots were taken by using the following camera settings: focusing mode – manual, shutter speed 1 and 10 s for $\lambda_{EX}$ = 254 and 366 nm, respectively, aperture F8.0, ISO sensitivity 64, recording mode RAW, image resolution 2560 × 1920. All Olympus RAW files (16 bits per RGB channel color deep mode) were transformed into an 8-bit TIFF file.

After data acquisition an appropriate TLC plate area was cropped from the original frame size, and subsequently auto-balance or gray scale conversion filters were applied using Scion Image for Windows software (ver. Alpha 4.0.3.2; Scion Corporation; www.scioncorp.com). Selected cross-sections of the chromatographic lanes were extracted from the TIFF digital pictures with the help of ImageJ software (ver. 1.42q Wayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij).

### 2.6. Preparation of biological and environmental samples

#### 2.6.1. Surface water ecosystems

Extraction procedure was performed according to an analytical protocol reported in our previous study concerning determination of endocrine disrupting compounds using temperature-dependent inclusion chromatography [21]. Surface and sewage waters samples were collected in the volumes of 1000 and 100 mL, respectively. SPE protocol was briefly described in Section 2.4.

#### 2.6.2. Preparation of fish bile

Bile was collected from the sea trouts (*Salmo trutta* m. *trutta*) that were available commercially. A liquid content of fish gallbladder was directly transferred to the glass tube and sealed with PTFE lid. Collected bile samples were stored at temperature of −20 °C, until sample processing. Unfrozen bile samples (50 μL, approximately) were lyophilized. Deproteinization step involved addition of 1 mL of methanol to 10 mg of dry bile residue. Low-molecular mass compounds fraction was extracted from the sample by sonication (ultrasonic bath, 15 min.). Solid particles, which were still present in the samples after methanol addition and sonication, were separated from the liquid by centrifugation (5800 rpm, 15 min). Clear bile solution was used for chromatographic separation.

Freeze-drying procedure was performed using Refrigerated Vapor Traps RVT4104 (Thermo, Milford, MA, USA). Deep freeze (below −100 °C) bile samples in glass containers were dried under vacuum at room temperature using vacuum centrifugal evaporator Savant SPD121P Speed Vac connected to VLP80 oil vacuum pump, which were products of Thermo, Milford, MA, USA.

#### 2.6.3. Acid and base hydrolysis of fish bile samples

Depending on hydrolysis type, lyophilized (according to the protocol described above) bile samples, were mixed with 50 μL of hydrochloric acid (at concentration of 35–38%, approximately) or 50 μL of 10 M sodium hydroxide. Both acid and base-type hydrolyses were performed in water bath (90 °C for 60 min.). After that, samples were adjusted to pH ∼ 8 using appropriate volumes of HCl or NaOH as well as $NaHCO_3$ (10% solution). Hydrolyzed samples were dried under vacuum conditions in 60 °C and subsequently: extracted with methanol (accordingly to the protocol described above, methanol volume was based on the initial mass of dry bile residue after lyophilization) and centrifuged. Resulting hydrolyzed bile solutions were used for further micro-chromatographic analysis.

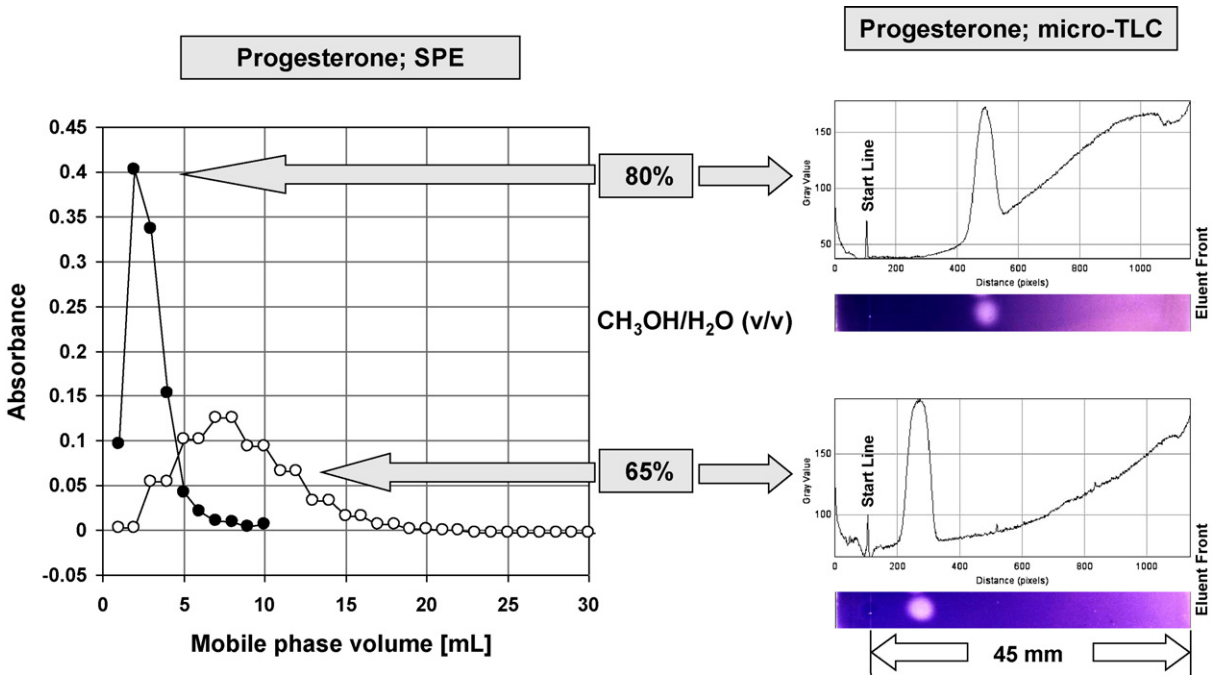


**Fig. 3.** Elution profiles of progesterone from C-18 solid-phase extraction (SPE) tube and corresponding micro-TLC chromatograms obtained using RP18W plates. In both separation systems studied the retention data were obtained using eluents composed of 65 and 80% (v/v) methanol in water.

## 3. Results and discussion

In general, separation efficiency of planar chromatography is usually considered to be poorer than performed by its column counterpart. However, in particular cases conventional, non-forced flow HPTLC (high-performance thin-layer liquid chromatography) can provide more effective and robust separation system than column chromatography. Especially, with regard to peak distribution across chromatogram of mixtures composed of low-retarded analytes. It should be noted that from its nature, isocratic planar chromatography has a great capability to separate mixtures composed of low-retarded components of interest. This problem can be simply explained taking into account nonlinear relationship that exists between column ($k$) and planar ($R_F$) retention parameters *via* equation formed as $\log k = R_M$, in which $R_M$ values can be derived from planar chromatogram using relationship $R_M = \log(1/R_F - 1)$. One of the interesting consequences of such nonlinear interdependence between $k$ and $R_F$ retention parameters is the high capability of planar systems to separate complex mixtures composed of relatively weakly retarded analytes ($k < 10$). This can be easily demonstrated through, *e.g.* graphical representation of $\log k = R_M$ equation [22]. Particularly, we demonstrated that the isocratic separation of four weakly adsorbed natural estrogens including estetrol, estriol, 17β-estradiol, and estrone at different temperatures can be more

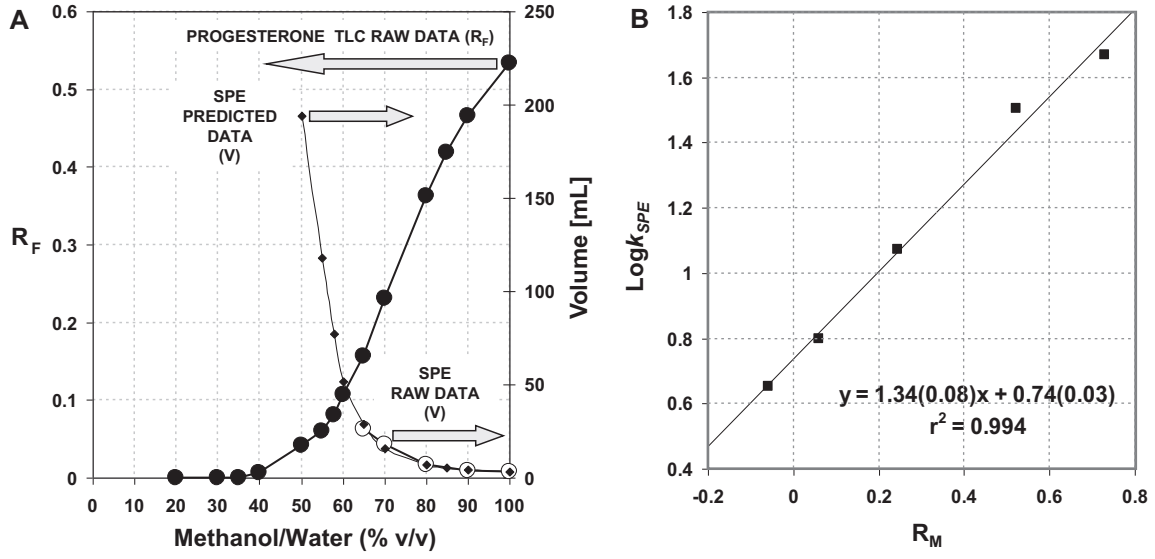


**Fig. 4.** Estimation of progesterone elution volumes from octadecylsilica type SPE tubes involving micro-TLC data. Circles and black dots on graph A correspond to the real elution data points obtained from SPE and micro-TLC experiments, according to protocol presented in Fig. 3. Diamond marked points (graph A) represent a back-calculated SPE elution volumes of progesterone, using the micro-TLC data *via* equation formed as $\log k_{SPE} = aRM + b$. Appropriate regression line and computed correlation coefficients are plotted within graph B.

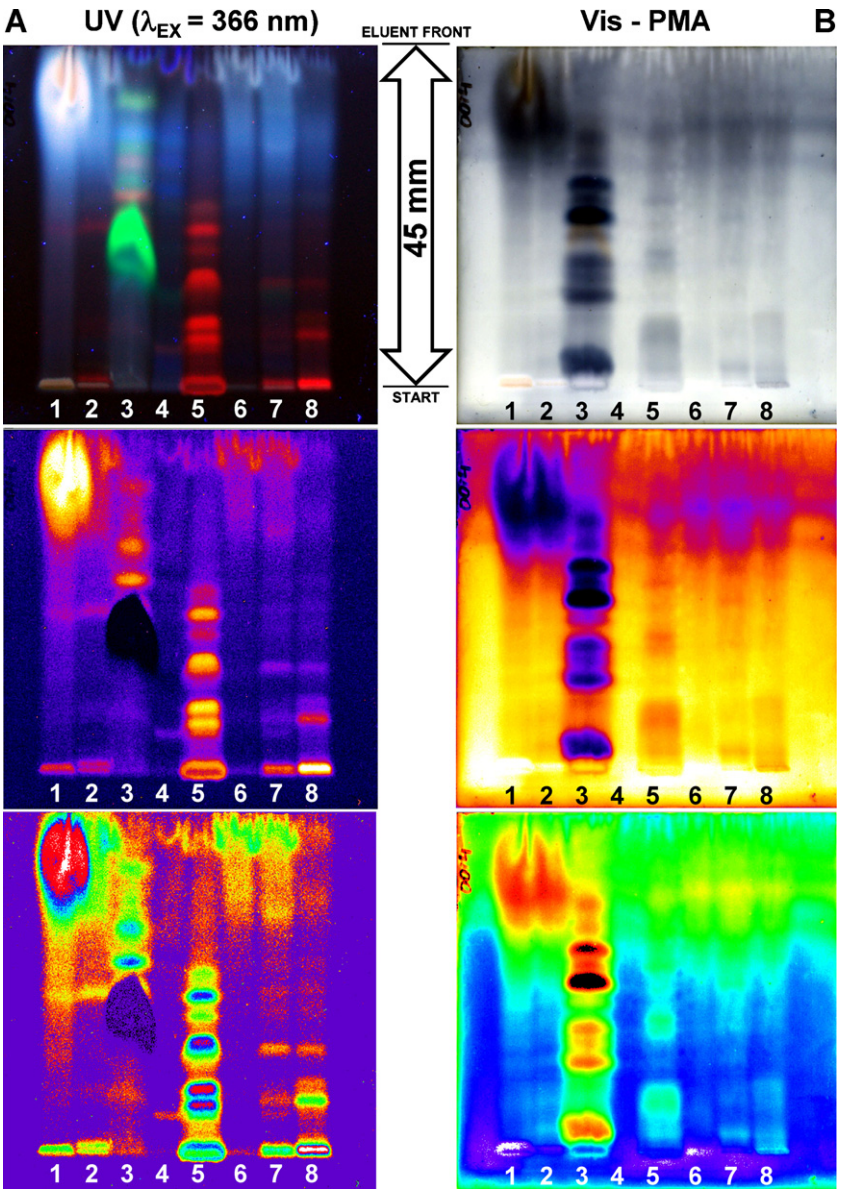


**Fig. 5.** Fingerprinting of SPE extracts derived from surface water ecosystems. Low-molecular mass substances with polarity ranging from estetrol to progesterone obtained from environmental samples were visualized using fluorescence detection (plate A) and PMA reagent (plate B). Middle and bottom located pictures within column A and B correspond to artificial color representation of the top located original scans, involving "Fire 2" and "Rainbow" filters (Scion Image freeware). Samples I.D.: 1 – Lubiatowo Lake (SPE protocol without cleaning solvent step); 2 – Lubiatowo Lake (full SPE procedure); 3 – untreated sewage water; 4 – treated sewage water (municipal wastewater treatment plant "Jamno"); 5 – Baltic Sea; 6 – Kamienne Lake; 7 – Dzierżęcinka River (under Koszalin City); 8 – Jamno Lake.

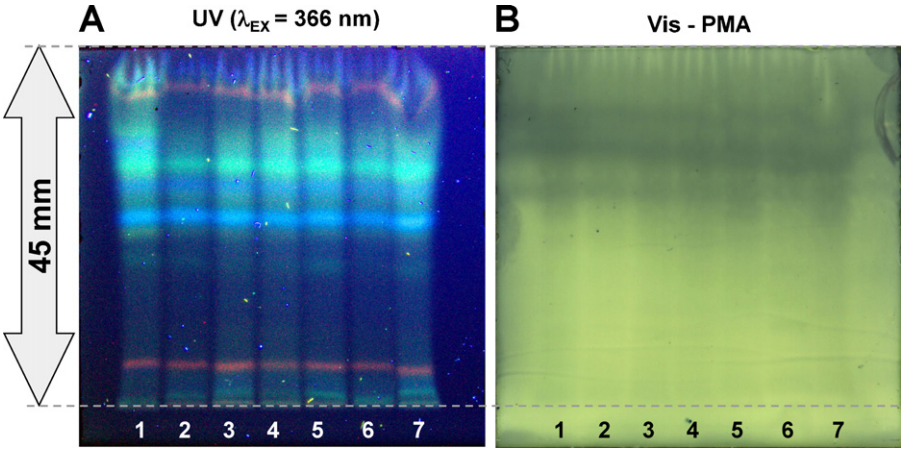


**Fig. 6.** Screening of treated sewage water discharged by the municipal wastewater treatment plant "Jamno" (7-days period with 24 h steps).

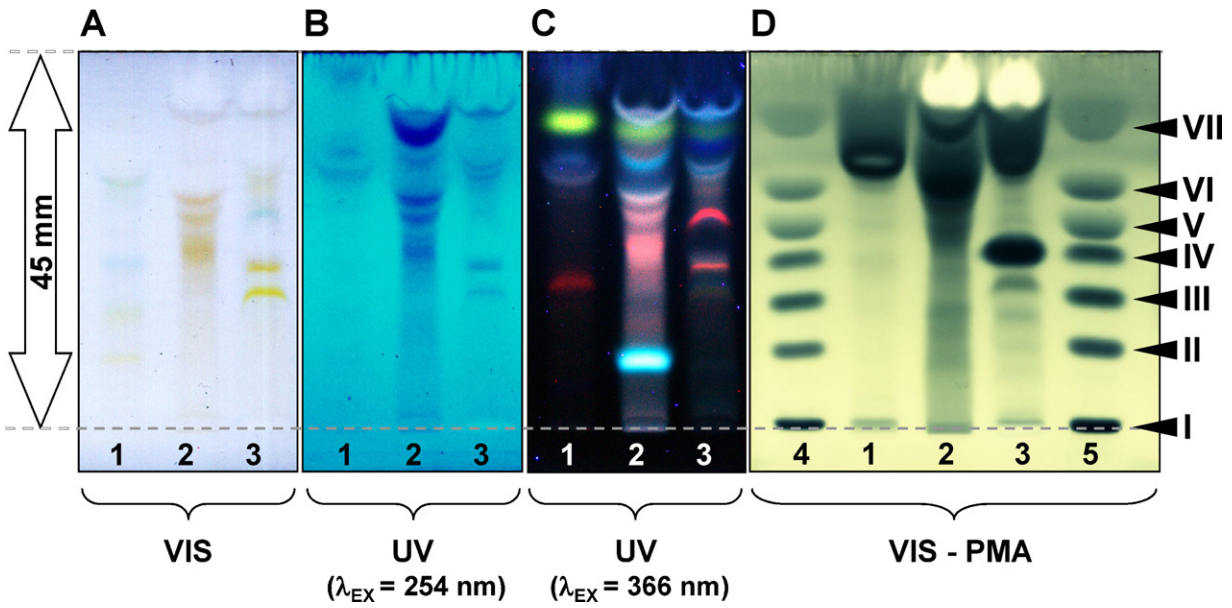


**Fig. 7.** Micro-TLC separation of main components of sea trout bile detected under different UV–Vis light exposure and visualization reagent conditions. Direct visible light scan (A), fluorescence quenching mode (B), fluorescence (C), phosphomolybdic acid staining (D). Lane no. 1 corresponds to the sample consisted of deproteinized bile methanolic solution, lanes nos. 2 and 3 reflect separation of bile samples after acid and base type hydrolysis, respectively. Lanes nos. 4 and 5 correspond to cholesterol (I) and litocholic (II), deoxycholic (III), cholic (IV), glycodeoxycholic (V), glycocholic (VI) acids as well as taurodeoxycholic acid sodium salt (VII) chromatographic standards mixture, separated at quantity of 1 and 2 μg/spot, respectively.

effective under HPTLC conditions in comparison to HPLC separation. Noteworthy, under proper experimental conditions, in which the heat evolved during solvent adsorption and mobile phase "distillation process" is minimized, micro-planar chromatographic systems can be very suitable for separation at elevated and sub-ambient temperatures [6,12]. This is mainly because of the low flow rate and small amount of the mobile phase, usually less than 0.5 mL that is necessary to perform the separation process. Depending on the plate size, solvent viscosity and temperature, the results of HPTLC separation can be obtained within short period of time, even less than 5 min [13].

### 3.1. Retention profiling study and optimization of solid-phase extraction

Low consumption of the eluent and short separation time makes the micro-TLC technique a suitable tool for fast retention screening of target steroids across the whole range of binary mobile phases compositions. Such analytical approach can be applied as an effective method for the internal standard (IS) substance search. Previously, we reported the micro-TLC protocol for determination of testosterone residue containing testosterone derivatives in complex samples involving estriol as the IS marker [12]. Due to the potential problem with overlapping impurity peaks, in this study we demonstrate fast selection of the internal standard substance, which is different than steroid like estriol. Retention profiles of testosterones that are presented in Fig. 1A allow fast selection of the optimal separation conditions (Fig. 1B), together with selected non-steroidal low-molecular mass marker (methyl red) proposed instead of estriol. Based on the simple optimization criterion expressed as the sum of $\Delta R_F$ values calculated for the adjacent spots, the best separation conditions for testosterone, testosterone enanthate and methyl red can be easily selected, within whole range of binary methanol:water mobile phase investigated. As can be seen from the micro-plate scan presented in Fig. 1 the internal standard spot is well separated from the components of interest and the remaining background interfering spots. Presented protocol is capable to develop up to nine independent samples simultaneously, including *e.g.* six calibration lanes and three investigated sample lanes. The separation process can be completed within 18 min at temperature of 20 °C using 300 μL of the eluent.

Water tolerable reversed phase HPTLC system involving *e.g.* Merck RP-18W wettable plates allows fast retention screening of whole range of weakly adsorbed natural steroids with polarity ranging from estetrol to progesterone (Fig. 2). Under such conditions micro-TLC retention data can be linearized by plotting $R_M$ values of steroids against a reciprocal form of the organic modifier molar fraction ($1/X_s$) [18]. Using this approach, the values of the retention parameter of steroids investigated could be easily back-calculated for a wide range of mobile phase compositions, using few initially selected experimental data points. Based on the elution profiles of progesterone from C-18 solid-phase extraction (SPE) tube and corresponding micro-TLC chromatograms obtained using RP-18W plates (Fig. 3), an accurate solid-phase extraction conditions can be estimated (Fig. 4), beyond the SPE breakthrough volumes that can be measured experimentally. Particularly, the shape of predicted SPE elution curve for progesterone allows to design of high-recovery purification and extraction protocols for wide range of more polar steroids with polarity up to estetrol, from biological and environmental samples [21,22].

### 3.2. Preliminary screening of environmental samples towards EDCs like chemicals

One of the advantages of planar chromatography over its column counterpart is that each TLC run can be performed using non-previously used stationary phase. Therefore, it is possible to fractionate or separate complex samples characterized by heavy matrix loading. In case of column chromatography such samples must be carefully pre-purified. Particularly, all substances that are strongly adsorbed or chemisorbed by stationary phase must be carefully removed to provide column long life. In our previous work concerning determination of endocrine disrupting compounds (EDCs) using temperature-dependent inclusion chromatography we collected number of samples derived from Baltic Sea, selected lakes and rivers of the Middle Pomerania in northern

part of Poland [21]. Samples were pre-purified and concentrated using SPE protocol capable of extracting low-molecular mass compounds within polarities ranging from estetrol to progesterone. In this study we tested if micro-TLC separation is capable of generating specific profiles of environmental extracts using different detection modes, including spots fluorescence or derivatization with visualization reagent (phosphomolybdic acid, PMA). According to chromatograms presented in Fig. 5, such samples can be fingerprinted using micro-TLC analysis. Particularly, SPE extracts of samples derived from following locations were chromatographed:

a) Baltic Sea – recognized as the largest body of brackish water in the world.
b) Shallow, physically unstable lakes with high intensity of water/sediment interactions including eutrophic lake Lubiatowo and coastal hypertrophic lake Jamno, located in the same catchment and below Koszalin, a 110-thousand city.
c) Lake Kamienne – physically unstable as a result of a pumping-storage electric plant operation.
d) River Dzierżęcinka, 27 km length river, passing through Koszalin City.

Similarly, to HPLC UV–Vis chromatographic profiles of solid-phase extracts obtained previously from environmental samples, micro-TLC separation involving fluorescence detection (left located scans, Fig. 5A) or PMA staining detection (Fig. 5B) is capable of efficient fingerprinting and characterization of environmental samples. It is clearly demonstrated that fraction of very polar substances, migrating close to the eluent front can be efficiently removed from the sample using SPE protocol with optimized concentration of methanol in washing solvent (bands nos. 1 and 2, Fig. 5A and B). It should be noted that presently, direct optical detection based on fluorescence is frequently employed in miniaturized systems focusing on bioanalysis [24]. Fluorescence scans and digitally post-processed pictures of the investigated microplate revealed the number of red-fluorescing bands that exist mainly in the Baltic Sea (band no. 5) as well as Dzierżęcinka and Jamno samples (bands nos. 7 and 8, respectively). These bands correspond to chlorophyll decomposition products, which may reflect the total algae activity in the given surface water ecosystems. PMA derivatization of the lanes (Fig. 5B) clearly distinguishes the samples characterized by high organic carbon load including EDCs compounds (*e.g.* lane no. 3, which corresponds to untreated sewage water sample). This approach can be useful as simple and non-expensive complementary method for fast control and screening of treated sewage water discharged by the municipal wastewater treatment plants. To demonstrate this we analyzed the SPE extracts of treated sewage water samples derived from "Jamno" Wastewater Treatment Plant near Koszalin City. It is easy to see that within seven days period, treated water samples discharged by this sewage water processing system were characterized by the equal chromatographic profiles (Fig. 6A), importantly not affected by high carbon load impurities (Fig. 6B) with polarity ranging from estetrol to progesterone.

### 3.3. Fractionation of fresh and hydrolyzed fish bile

Fish bile contains at least three chemical classes of reproductive pheromones including bile acids, sex steroids and prostaglandins, which can be present at high level in such material [25]. Among them bile acids and prostaglandins are difficult for direct determination using common spectrophotometric detectors, because they are transparent to UV–Vis light. Due to crucial role of these substances in fish reproduction, the fast and non-expensive quantification of bile components is presently a great interest of fish industry. This is because number of bile acids can be released and

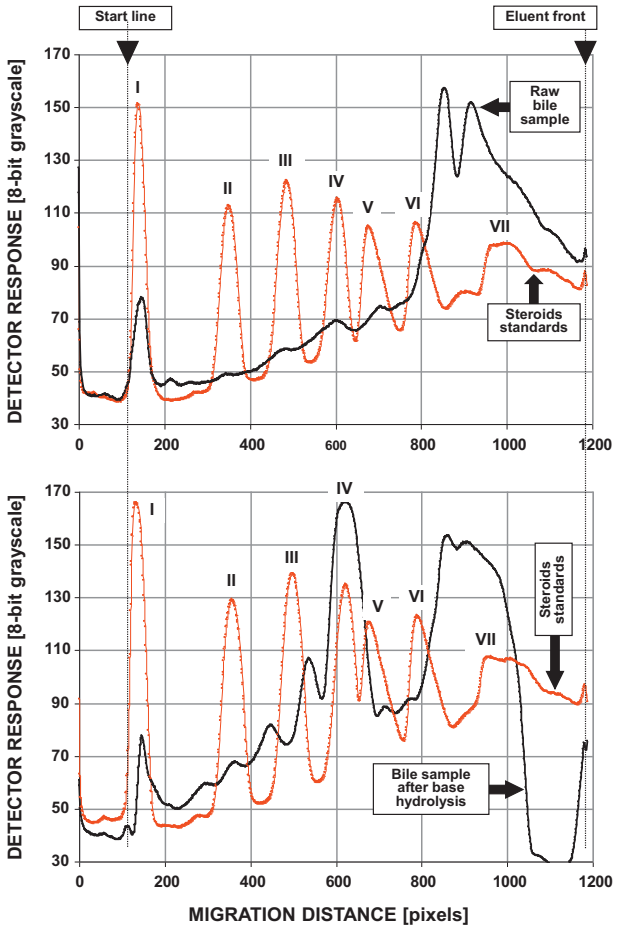


**Fig. 8.** Selected densitometric profiles of untreated and base hydrolyzed bile samples derived from PMA microchromatograms presented in Fig. 7D. Top graph combines lane 1 (raw bile) and lane 4 (steroids standards 1 μg/spot), while the bottom graph overlaps lane 3 (bile after base hydrolysis) and lane 5 (steroids standards 2 μg/spot). Steroids I.D.: cholesterol (I), litocholic (II), deoxycholic (III), cholic (IV), glycodeoxycholic (V), glycocholic (VI) acids and taurodeoxycholic acid sodium salt (VII).

detected by many fishes and consequently work as the efficient pheromones [26,27]. In analytical practice preparation of fish bile samples for qualitative and quantitative analysis of steroids fraction is fairly complex, especially considering high level of protein matrix as well as the presence of wide range of steroids as highly polar conjugated derivatives [28,29]. Therefore, numbers of pre-treatment protocols for quantification of steroids in raw or hydrolyzed bile were invented [28–30]. In this work we demonstrated that micro-TLC can be effectively applied for fast fractionation of bile steroids without additional purification step of the bile samples processed with base and acid hydrolysis.

For micro-TLC analysis of fish bile we used simple deproteinization step based on freeze-drying procedure. Such fast pre-treatment involving low volume samples (10–100 μL level) can be easily performed using common laboratory equipment including freezer and vacuum evaporator or any small chamber connected to the vacuum. Concurrently, the fish bile sample was also hydrolyzed at low and high pH conditions. In all cases, methanol was used as the final extraction medium for low-molecular mass fraction in fish bile investigated. According to chromatograms presented in Fig. 7A–D, number of UV–Vis transparent substances including bile acids can be detected *via* fluorescence and particularly after staining with phosphomolybdic acid. It is clearly demonstrated that acid hydrolysis may strongly decompose bile acid fraction. However, number of resulting spots under visible and

UV-light can be generated and used as potential markers for future sex-specific studies or intra- and inter-species comparisons. As can be seen from densitograms presented in Fig. 8, micro-TLC method is capable of detecting quantitatively a wide range of non-conjugated bile acids in the fresh bile sample, without extensive sample pre-treatment. Moreover, it is possible to distinguish the differences of steroids levels within free bile acids fraction occurring after base hydrolysis, which reflects the total level of these steroids in the bile sample. Particularly, the high level of cholic acid was determined in the bile from sea trout investigated. Such a high content of cholic acid was also observed in the bile samples that were originated from different fish species [29].

## 4. Conclusions

Despite the high-resolution metabolomic approach involving mass spectrometry detection, which is presently commonly applied for characterization of biological systems, number of complex problems can be solved using relatively low resolution analytical methods. Previously, we demonstrated that focusing on selected fraction of metabolites (mainly non-transparent for UV light steroids and related low-molecular mass compounds) in complex biological material like cord blood, significant differences between clinical groups (*e.g.* labour and non-in-labour) can be distinguished. This was performed using simple separation method involving isocratic HPLC system driven by host–guest interactions and multivariate statistic processing of raw data in sample-feature matrix [23,31]. The main advantage of such approach is that confident quantitative data can be acquired using manual peak matching on the analyzed chromatograms. It is noteworthy to say that manual peak matching is currently considered as the best approach for the processing of small data sets (approximately 20–50 peaks per chromatogram), particularly, in case of irregular shaped base line and peaks overlapping [32]. From that point of view described micro-TLC technique can be promising and non-trivial technique for preliminary screening and classification of high level metabolites fraction in complex biological samples. The main advantages of isocratic non-forced flow rate planar chromatography result from its simplicity, easy operation and inexpensive equipment needed. Contrary to column chromatography and due to planar chromatography method principles, all sample components including substances that are strongly retarded or chemisorbed by stationary phase on the start line, can be visualized after plate development. The results of our experiments indicate that in particular cases fractionation and/or separation as well as characterization of complex biological materials and the environmental samples *via* analytical protocol involving temperature-controlled planar micro-chromatography is efficient, simple, fast and non-expensive. The total analysis cost involving $5 \times 5$ cm micro-plate that is capable of separating simultaneously up to nine samples, does not exceed one Euro. Such separation approach can be an alternative for fingerprinting protocols based on HPLC machines equipped with UV–Vis detectors or simple separation systems involving planar micro-fluidic devices. Particularly, it has been demonstrated that using micro-TLC separation, a fast screening of complex materials like surface and sewage waters or fish bile samples towards steroids search, can be successfully performed. Our investigations has revealed that specific bands patterns for samples derived from surface water of the Middle Pomerania in northern part of Poland, can be easily observed on obtained micro-TLC chromatograms. Moreover, our experimental data show the potential of micro-TLC as an efficient tool for retention measurements of wide range of steroids under reversed-phase chromatographic conditions. These data can be used for further SPE and HPLC method optimalization. Furthermore, micro-TLC based analytical approach can be applied as an effective method for the internal standard (IS) substance search. Due to low consumption of eluents (usually 0.3–1 mL/run) mainly composed of water–alcohol binary mixtures, this method can be considered as environmentally friendly and green chemistry focused analytical tool.

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