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***Professor Lamparczyk regrettably deceased in November 16, 2012.**

Krótki przegląd zastosowań cyklodekstryn dotyczący analiz farmaceutycznych, biomedycznych oraz środowiskowych

Streszczenie: *Przedstawiona praca jest ostatnim projektem naukowym nad jakim pracował Profesor Henryk Lamparczyk, który przedwcześnie zmarł 16 listopada 2012 roku w wieku 65 lat. Przeglądowa praca o charakterze dydaktyczno-poglądowym dotyczy zastosowania cyklodekstryn w bioanalityce i została napisana w oparciu o prace opublikowane przez Profesora i współpracowników. Niniejsza wersja bazuje na artykule pt. "Natural cyclodextrins: development in theory, chromatography and pharmacy, A. Chmielewska, H. Lamparczyk;" opublikowanej w materiałach Supramolecular Chemistry and Advanced Materials. Wojciech Macyk & Konrad Szaciłowski, Editors, Kraków Jagielonian University 2007, pages 131-136 i jest rozszerzona o wiadomości przedstawione w najnowszych publikacjach dotyczących zastosowania cyklodekstryn w HPLC, głównie do oznaczeń sterydów i analiz przesiewowych próbek środowiskowych.*

Słowa kluczowe: *Cyklodekstryny, chromatografia gazowa, wysokosprawna chromatografia cieczowa, chromatografia cienkowarstwowa, zależność struktura-retencja, sterydy, próbki biologiczne i środowiskowe, efekty temperaturowe, kompleksy inkluzyjne.*

Short review of cyclodextrins applications in separation science focusing on pharmaceutical, biomedical and environmental analisys

Abstract: *This is the last research project of Professor Henryk Lamparczyk that prematurely passed away at age 65. In his intention it was to prepare a demonstrative review paper concerning application of cyclodextrins in bioanalysis, based on his and co-worker contributions to the field of supramolecular chemistry. This version of paper is based on the manuscript entitled "Natural cyclodextrins: development in theory, chromatography and pharmacy, A. Chmielewska, H. Lamparczyk;" that was published in Supramolecular Chemistry and Advanced Materials. Wojciech Macyk & Konrad Szaciłowski, Editors, Kraków Jagielonian University 2007, pages 131-136 and now is extended for the latest papers concerning HPLC application of cyclodextrins for steroids analysis and environmental samples screening. The main tools applied in described investigations were various chromatographic techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Topics such as structure-retention relationships, equilibrium constants between cyclodextrins (CDs) and guest molecules, thermodynamics and CDs separations were discussed. Additionally few examples of practical applications of CDs in pharmacy as well as biomedical and environmental analysis are given.*

Key words: *Cyclodextrins, gas chromatography, high-performance liquid chromatography, thin-layer chromatography, structure-retention relationships, steroids, biological and environmental samples, temperature effects, inclusion complexes.*

1. Introduction

Natural cyclodextrins (CDs) were discovered and described by Villiers in 1891. The natural CDs are produced from starch by the action of cyclodextrin glucosyltransferase, an enzyme present in several organisms, *Bacillus macerans* being the earliest source.

Cyclodextrins are toroidal-sharped cyclic oligomers of α-1,4-D-glucopyranose unit. The three most commonly employed natural cyclodextrins contain six seven and eight glucopyranose units and are denoted as α-CD, β-CD and γ-CD respectively. Native cyclodextrin consisting of nine units is called δ-CD. The dimensions of the cyclodextrin cavity increase with the number of glucose units in the ring, while the height of the cavity is the same for all three types. Internal dimensions of cavities are 0.47-0.53, 6.0-6.5 and 7.5-8.3 in α-CD, β-CD and γ-CD respectively. The ability of a cyclodextrin to form an inclusion complex with guest molecule is a function of two key factors. The first is steric and depends on relative size of the cyclodextrin to the size of guest molecule. If the guest is the wrong size, it will not to fit properly into the cyclodextrin cavity. The second critical factor is the thermodynamic interactions between the different components of the system CD-guest-solvent. For a complex to form, there must be a favourable net energetic driving force that pulls the guest into cyclodextrin. The aqueous solubilities of α- and γ-CD at 25°C are 0.121 and 0.168 M respectively, whereas that of β-CD is roughly a factor less, *i.e.* 0.0163 M. The interior of CD cavities is relatively hydrophobic. It is formed by a circular configuration of hydrogen atoms and glucoside oxygen atoms, while all the hydroxyl groups are outside of the molecule. Therefore CD's and their inclusion complexes, even with nonpolar organic compounds are quite soluble in water. The CD complexation process is highly stereoselective.

2. Discussion on results included in our former works

2.1. Structure retention study

In supramolecular chemistry the binding forces are determined by electrostatic interactions, molecular geometry, and hydrogen bonds. Polycyclic aromatic hydrocarbons (PAH) are good model compounds to study these effects using chromatographic techniques.

The electrostatic interactions (van der Waals) my be devided into three groups: the orientation interactions (Keesom), the inductive interactions (Debey) and the dispersive interations (London). When the solutes are nonpolar, like PAH, and their ionization potentials are nearly identical, the operating interactions should be inductive and dispersive. Under these circumstances the molecular polarizability (α) of the solute appears to be the most important factor governing the retention order [1,2]. The second factor should be geometry of the solute molecules The molecular geometry was expressed by the shape parameter (η). Mention above parameters were used to study the relationships between chromatographic retention data and structures of selected PAH [3,4] using GC technique with β-CD bonded to stationary phase and cyclodextrin modified mobile phase in the case of HPLC. In both systems this idea was fully confirmed by regression equations and moreover by the elution order of solutes. Slightly different attempt was applied in structure-retention study of eight isomeric dimethylnaphtalenes [5]. On the basis of GC and HPLC retention data stability constants of the inclusion complexes with β- and γ-CDs were calculated. All complexes under investigation were of moderate stability, except the complex of 1,8-dimethylnaphthalene with β-CD. Remarkable 1,8-dimethylnaphthalene is the most compact molecule among investigated compounds.

2.2. Temperature effect and thermodynamics

Methanol and acetonitrile are commonly used as organic components of the mobile phases for a number of prepurification and separation techniques, including liquid or solid phase extraction and capillary electrophoresis as well as planar and column chromatography. Particularly, temperature-dependent inclusion chromatography requires the presence of macrocyclic additives in mobile phases, especially those that are not retarded by the stationary-phase components [6]. As it was mentioned above, the solubility of native cyclodextrins in binary organic/water mixtures is very complex and usually non-linear. For most of the isocratic separations that involve cyclodextrin additives, the concentration of water should be as high as possible to allow good solubility of such macrocycles in the mobile phases. Therefore, a concentration of organic liquids that is close to the mole fraction of Xs = 0.16 is often used. Our experimental data confirmed that under elevated temperature conditions β-cyclodextrin is almost three-times more soluble in acetonitrile/water than a methanol/water mixture [7]. At sub-zero temperature the change in solubility between both phases is more evident. Under such conditions β-cyclodextrin is almost 5-times more soluble in acetonitrile/water than in a methanol/water mixture (1.6 and 7.9 mM, respectively).

Therefore, the effective application of β-cyclodextrin as a modifier of mobile phases based on methanol/water mixtures at sub-ambient temperature may be strongly limited. It is noteworthy that the HPBCD derivative is at least two factors more soluble than native β-CD under all of the conditions investigated.

In order to demonstrate experimentally the temperature effect not any sophisticated equipment is required [8]. It is well known phenolphthalein in alkaline medium is purple coloured (reference solution). The colour diminishes when phenolphthalein is incorporated into the cavity of β or γ-CD (thermochromic solution). In iced water the thermochromic solution due to high stability constant of the inclusion complex is colourless, whereas the reference solution is purple coloured. When the temperature was raised to 40ºC the thermochromic solution became pale purple. In 70ºC water bath the colour intensity of both solutions is identical. After moving the coloured thermochromic solution to iced water the colour will disappear. This can be explained because the complexation processes occurring in solution is reversible and hysteresis phenomena is not observed.

In the next work the equilibrium constants of β-CD-phenolphthalein complex in wide range of temperatures (10 to 70°C) and β-CD/phenolphthalein ratios (from 0.8:1 to 427:1) was studied spectroscopically [9]. Additionally, the competitive effect of tetrahydrofuran, solvent commonly used in HPLC and TLC was also examined. The stoichiometric proportion and binding constants (K_{11}) were calculated using Scott's equation. The stoichiometric ratio of investigated complex was 1:1, the K_{11} values ranged from 0.26×10^4 M^{-1} (70°C) to 7.44×10^4 M^{-1} (10°C). Strong competition at the binding site between phenolphthalein and tetrahydrofuran was observed. On the basis of this observation it can be now easily explained, that relatively high concentration of CD is needed, when tetrahydrofuran-water binary system is used in chromatography. The formation of inclusion complexes between dyes and cyclodextrins has proven to be an excellent model system for studying the nature of noncovalent binding forces in water based solutions. This phenomenon was widely applied to indirect post-column detection of macrocycles in chromatographic methods as well as measurement of association constant of low molecular mass compounds. Recently, we explored the host-guest complex formation between selected bile acids (dehydrocholic, cholic, deoxycholic, taurodeoxycholic, glycodeoxycholic, glycocholic and chenodeoxycholic acid) and cyclodextrins (β-cyclodextrin and its hydroxypropyl derivative) at sub-ambient and elevated temperature, using as a probe the phenolphthalein-cyclodextrin inclusion complex detected via UV-Vis spectrophotometry [10]. In order to explore the general trends in the complexation ability of the bile acids by macrocycles investigated, the quantitative data set containing ΔAU values was analyzed by principal component analysis (PCA). It has been found that within investigated bile acids group the strongest interaction with cyclodextrins was observed for chenodeoxycholic acid at subambient temperature. Such behaviour can be explained by lack of keto or hydroxyl groups linked to C12 carbon atom in the steroid structure. The results of chemometric investigation indicate that under particular conditions an effective inclusion complex formation may improve chromatographic separation of bile acids, especially those that are difficult to separate using unmodified with macrocycles mobile phases.

Generally, in classical chromatography solute retention is inversely related to temperature and is known as van't Hoff plot which is linear. Nevertheless any reversible process which alters the enthalpy or entropy of adsorption in principle give rise to nonlinear dependency. For example presence of multiple types of retention mechanisms or multiple types of binding sites leads to non-linearity of the van't Hoff plots.

In the subsequent work [11] the influence of CD modified mobile phases on thermodynamic parameters and multiple separation was studied. Investigated compounds; estriol, 17β-estradiol, 17α-estradiol, d-equilenin, equilin and estrone, belong to the group of female sexual hormones. However estriol, estrone and 17β-estradiol are human hormones, while d-equilenin and equilin are horse hormones both groups were applied in human medication. When unmodified mobile phase was used the van't Hoff plots are linear for all steroids and the retention times are very long. Modification of mobile phase with β-CD produce deviation from the linearity, particularly in low temperature region, moreover the retention time is shorter. At subambient temperature region the selectivity of the chromatographic systems is greatly improved even for very complex multiple separations. Similar results were obtained when prednisone, cortisone, testosterone, 17α-methyltestosterone and 17α-hydroxyprogesterone were used as a model compounds [12]. The best separation in the shortest retention time of the last solute was achieved when phase modified with 16 mM β-CD at 5°C was applied.

We observed that in liquid chromatographic systems modified by macrocycles a decrease of retention is followed by an increase of solute complexation by inclusion modifier of the mobile phase [6,12,13]. This behaviour can be explained by assuming that the retention of cyclodextrin is considerably lower than the retention of solutes chromatographed and the predominating mechanism for retention is the formation of guest-cyclodextrin complexes in the mobile phase. Our experimental data indicated that at the wide range of temperatures investigated the retention of β-cyclodextrin is significantly lower than the retention of the steroids chromatographed using an unmodified mobile phase [6]. The results seem to indicate that retention of inclusion complexes can be varied between two lines formed by the van't Hoff plot of the cyclodextrin and van't Hoff plot of the uncomplexed steroid. Additionally, in the low temperature region the inclusion modifier

action is more efficient due to high values of the binding constant of the complexes created and large differences in retention of β-cyclodextrin and uncomplexed solutes.

In our further study we explored the capability of native α-, β- and γ-cyclodextrin as well as their hydroxypropyl derivatives for host-guest interaction in different temperatures with 7,8-dimethoxyflavone, selected steroids (estetrol, estriol, estradiol, estrone, testosterone, cortisone, hydrocortisone, progesterone and 17α-hydroxyprogesterone) and polycyclic aromatic hydrocarbons (toluene, naphthalene, 1,8-dimethylnaphthalene, 1-acenaphthenol, acenaphthylene and acenaphthene) under reversed-phase liquid-chromatography conditions [14]. The study has revealed that native cyclodextrins interact more efficiently with the analytes investigated than do their hydroxypropyl counterparts. In the low-temperature region, enormously high ratios were observed for polycyclic aromatic hydrocarbons, particularly 1,8-dimethylnaphthalene, acenaphthene and acenaphthylene chromatographed on a β-cyclodextrin-modified mobile phase. In such a case, the retention times of the polycyclic aromatic hydrocarbons were strongly reduced and were close to the hold-up time of the high-performance liquid chromatography (HPLC) system. Experimental data revealed that mobile phases modified with native β-cyclodextrin and γ-cyclodextrin have ability for chiral separation of acenaphthenol optical isomers. At subambient temperature the peak of the mixture of enantiomers was broadened and split (Rs = 0.02 and 0.73 for γ-cyclodextrin and β-cyclodextrin modified eluents, respectively). Baseline separation of acenaphthenol enantiomers (Rs = 1.62; α = 1.14) under the same temperature and mobile-phase conditions was observed by applying a 15-cm column filled with Develosil ODS-UG-5 stationary phase. Circular dichroism spectra derived from the extracted fractions of pure enantiomers indicated that the (-) isomer was eluted first. Within the steroids group investigated, strong interaction was observed for estradiol and testosterone. The results of cluster analysis indicate that β-cyclodextrin as well as γ-cyclodextrin and its hydroxypropyl derivative can be most effective mobile-phase additives under reversed phase HPLC conditions for 3D-shape-recognition-driven separation, performed at subambient and elevated temperatures, respectively. It is noteworthy that irrespective of the temperature and macrocyclic modifier type, no significant interaction was observed for 7,8-dimethoxyflavone. Taking into account the 3D structures of the analytes it is clear that 7,8-dimethoxyflavone is less compact than steroids having similar molecular mass. Therefore, such compounds cannot enter the internal cavity of the macrocycles and form stable host-guest complex.

The results of our study clearly show the key role of temperature in chromatographic separation, which is driven by native cyclodextrins and their hydroxypropyl derivatives used as the mobile phase additives. For a given concentration of macrocycles in the eluent, most effective host-guest complexation of analytes performed at subambient and elevated temperature was observed for β-cyclodextrin as well as γ-cyclodextrin and its hydroxypropyl derivative, respectively. Such macrocycles should be considered as the first choice mobile-phase additives for separation of low molecular mass compounds *via* temperature-dependent inclusion chromatography.

2.3. Chromatographic properties of cyclodextrins and macrocyclic antibiotics

In particular cases conventional planar chromatography can actually provide more effective and robust systems than column chromatography. This is the case of retention measurements of CDs and other macrocycles. Although, HPLC technique were formerly used this technique has a number of disadvantages for such purposes.

Since CDs show almost no UV absorption, universal detectors of low sensitivity such as refractive index or light scattering detector must be used. This requires overload injections which resulting enormous adsorption effect, strongly influencing the retention measurements. Furthermore, the mobile phase composition which can be investigated is strictly restricted because under extreme conditions the retention time is too long or the solute does not elute from the column. Considering these disadvantages, TLC seems to be the method of choice.

It is well know that the aqueous solubilities of α- and γ-CDs are ten time higher than β-CD. The solubility behaviour in binary mixtures commonly used as mobile phases in chromatography is even more complicated. Moreover, in real chromatographic systems the influence of stationary phases cannot be neglected. Therefore, a detailed knowledge of macrocycles behaviour in chromatographic systems is of great importance because it supports the theoretical basis of chiral separations and relates to other industrial applications of CDs.

In the first two works [15,16] of this cycle the retention behaviour of α-, β- and γ-CDs has been examined using RP-18W [15] and polyamide [16] plates as stationary phases. Six binnary solvent mixtures (acetonirile-water, methanol-acetonitrile, methanol-water, ethanol-water, propanol-water, and tetrahydrofuran-water) in wide rage concentrations 0-100% v/v were used as mobile phases. The retention behaviour of CDs in both systems is difficult to rationalise. Generally, the separation of CDs on polyamide phase is worse than on RP-18W [16]. On RP-18W phase [15] the most hydrophobic β-CD migrates last when methanol-water and ethanol-water are used as mobile phases. In propanol-water the elution behaviour of all the cyclodextrin considered is similar, whereas in tetrahydrofuran-water mixtures the CDs again behave

differently. At very low concentrations of tetrahydrofuran β-CD migrates furthest whereas at high tetrahydrofuran concentration its migration is lowest. It is noteworthy that in contrast with reversed-phase chromatography, the order of elution of CDs on polyamide plates corresponds to the order of their molecular weights [16]. Hence, the shortest migration distance was observed for γ-CD and the longest for α-CD. This contradicts commonly accepted idea that the retention of structurally similar compounds is determined by their solubility in mobile phase, *i.e.* retention decreases as the solubility increases. According to this concept the elution order should be α-, γ- and β-CD, this is not observed, therefore it can be concluded that the solubilities of CDs cannot be directly related to retention data.

In the subsequent works temperature controlled chromatographic chambers were applied. Problem of chambers construction is discussed in details in work [17].

Thermostated TLC was used to study the retention behaviour of α-, β- and γ-CDs on RP-18W reversible stationary phase [18]. As mobile phases, a homologous series of n-alcohols from ethanol to butanol, and their mixtures with water were investigated. Chromatographic experiments were performed either at constant 30°C temperature and over wide range of binary mixtures (0-100%, v/v), for ethanol and propanol, as well as at fixed mobile phase composition and different temperatures from 5°C to 60°C. Using isoelution binary mobile phases, the effect of temperature on retention of CDs was examined. Thermodynamic parameters such as the change of ethalpy (ΔH°) and the change of entropy (ΔS°) were estimated. In each case the sign of the calculated parameters is negative. Nonlinear van't Hoff plots were observed when propanol or butanol was used as a component of binary mobile phase. Most recently, we demonstrated that native β-cyclodextrin and its methyl, dimethyl as well as trimethyl derivatives can be effectively separated under isocratic temperature controlled micro-TLC conditions. Fast separation was performed using simple binary mobile phase consisted of 50% (v/v) acetonitrile:water and RP18W HPTLC plates chromatographed inside horizontal micro-chamber [19].

In other work [20] the mobile phase system was restricted to methanol-water from 0 to 100% v/v binary compositions but two additional solutes were added *i.e*. rifamycyn and rifampicin. Both solutes belongs to the group of macrocyclic antibiotics. They are frequently used in human medicine, particularly in case of tuberculosis. It was observed that temperature changes produce significant differences in migration of the investigated compounds. Generally, the plots of the rate mobility factor (R_M) versus the reciprocal of the absolute temperature are linear. From these plots thermodynamic parameters were calculated. In each case the sign of (ΔH°) and the (ΔS°) is negative. However, the magnitudes of (ΔH°) and the (ΔS°) indicate significant thermodynamic differences between two groups of solutes, one of which includes α- and γ-CDs and the other which includes macrocyclic antibiotics and β-CD. From the practical point of view, when the macrocycles are choosing as a candidate for mobile phase additives, theirs retardation factor (R_F) should be equal unity. The most adsorbed solute is rifampicin for which the R_F value never reaches unity. On the other hand, rifampicin might be the best candidate for physical immobilization on the support and therefore serve as stationary phase modifier.

2.4. Examples of application in pharmacy

Living organisms are largely constructed from chiral compounds. Therefore, in such a highly chiral environment it should not be surprising that some drugs, which possess an asymmetric centre, exhibit a high degree of stereoselectivity in their interactions with various enzymatic systems and also with receptor. Moreover, most of the enantiomers should be considered as a separate active substances.

Norgestrel [(±)13β-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one] belongs to the group of synthetic progestagenes. It is frequently used as a component of various oral contraceptive pills. Enantiomers of this compound have different intrinsic pharmacological activity. Currently norgestrel is manufactured as separate enantiomer named levonorgestrel. Therefore, method suitable for checking optical purity is very important. In the proposed method [21] mobile phase modified with β-CD and temperature controlled HPLC were used. The base line separation of two enantiomers was achieved at 0°C using acetonitrile-water (25:75 v/v) modified by the addition of 14 mM β-CD. On a molecular level, enantioselectivity in this temperature can probably be interpreted as being due to slower rotation of the guest and host molecules and hence a steric fit is possible.

Semi-natural mixtures of menthol, α- and β-pinene, borneol,, camphene, cineol and menthone have choleretic, spasmolytic, and bacteriostatic properties. Disolved in oliva oil they are used to treat cholesterol stones in the gall bladder and the bile ducts as well as in the treatment of patients with uretic stones. The enzymatic mechanism of terpene action suggest that the enantiomeric composition of the drug might be an important factor determining its therapeutic properties. Despite this fact in commercially available preparations the enantiomeric composition is not standardised and controlled. They are usually described as a mixture of six terpenes in which even the α- and β- pinenes are not discriminated. The enantiomeric composition of five commercially available drugs *i.e*. Terpichol, Terpinex, Rowachol, Rowatinex and Uroterp, were studied using a GC system with α-CD [22]. It was found that, depending on the manufacturer, drugs

possessing similar chemical compositions differ considerably from one to another regarding the content of enantiomers.

In biomedical analysis solution of two problems is substantial. Firstly, the compounds of interest should be separated from biological matrix and secondly, the detection of usually small amount of active substance should be as high as possible. In solution of these problems CDs might be very useful.

The fluorescence spectra of anethole and eugenol, compounds commonly present in essential oils, dissolved in methanol-water binary systems with the addition of α- and β-CD were studied [23]. In both cases, anethole and eugenol, detection limits were improved after addition of cyclodextrins. This phenomenon can be applied for improvement of both direct fluorescence and HPLC assays.

Estrogens are of clinical and analytical interest for many reasons. Biochemical studies have demonstrated that there are characteristic changes in the concentration of estradiol in both plasma and urine during the menstrual cycle. Most of these studies indicated that defined changes of urinary estrogens might be used as chemical indices to locate the start and finish of fertile period. The level of estrogens should be also controlled in menopausal and postmenopausal woman specially when hormone replacement therapy is applied. During pregnancy, estrogens urinary concentration in patients with gestosis is lower than those in normal pregnant subjects.

Hence, a simple procedure for simultaneous determination of 17β-estradiol, estriol and estrone in human urine was elaborated [24]. After acid hydrolysis of the sulphate and glucoronide conjugates, estrogens were extracted into diethyl ether and after concentration applied directly to an HPLC column. Using acetonitrile-water (25:75 v/v) modified by the addition β-CD as mobile phase, the separation of estrogens was achieved within 20 minutes. Estrogens were detected using spectrofluorimetric detector (λ_{exc} = 280 nm, λ_{em} = 312nm). In this case β-CD plays double role, improving separation and detection. Described method was fully validated and applied practically for measurements of estrogens level in nonpregnant and pregnant women.

2.5. Application of temperature-dependent inclusion chromatography for metabolomic investigation involving biological and environmental samples

Clinical and metabolomic investigations of complex human fluids require cost-effective methodologies that can rapidly assess the whole steroid hormone pattern of individual samples. We optimised the solid-phase extraction protocol for the extraction of a range of steroids of varied polarity from estetrol to progesterone from human plasma [25]. In this paper, we also improved the separation methodology of our previous work using isocratic elution with a mobile phase composed of 35% acetonitrile and 12 mM of β-cyclodextrin at 29ºC. Under these conditions most of the fluid components including estetrol were detected in the first 10 min with progesterone appearing at 43 minutes. We applied these pre-purification and separation analytical protocols for separation of cord blood extracts [26]. Human male and female fetal cord blood samples were purified, selectively extracted and separated to examine a fraction of steroids ranging from polar estetrol to relatively non-polar progesterone. Resulting UV diode array chromatographic patterns revealed the presence of 27 peaks. Observed chromatographic patterns of UV detected steroids were analyzed using principal components analysis, which revealed differences between male/female and labour/not-in-labour clusters. Quantitative analysis of nine identified steroids including: estetrol, 17β-estradiol, estrone, estriol, cortisol, cortisone, progesterone, 20α-hydroxyprogesterone and 17α-hydroxyprogesterone were not significantly different between males and females. Significant differences between male and female fetuses were related to as yet unidentified compounds. Four peaks were significantly different with labour which corresponded with cortisol, cortisone and two unidentified compounds. This protocol may distinguish significant differences between clinical groups that are not readily identifiable using univariate measurements of single steroids or different low-molecular mass biomarkers. Moreover, we have provided new evidence that despite the absence of testosterone there are number of steroids and low-molecular mass compounds that differ between male and female fetuses.

In our last works we optimised the separation of battery of key UV non-transparent low-molecular mass compounds having possible endocrine disrupting compounds (EDCs) activity or which may be used as the endocrine effect biomarkers [27,28]. Simple optimization strategy was based on strong temperature effect in presence of cyclodextrin in HPLC mobile phase. Particularly, the effect of temperature involving native β-cyclodextrin and its hydroxypropyl derivative to improve separation of number of natural (d-equilenin, equilin, estetrol, estriol, estrone, 17β-estradiol, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, cortisol, cortisone, progesterone, testosterone, tetrahydrocortisol and tetrahydrocortisone) and artificial steroids (ethynylestradiol, norgestrel isomers, medroxyprogesterone, mestranol, methyltestosterone, norethindrone, 17α-estradiol) as well as non-steroidal compounds (diethylstilbesterol, bisphenol A, 4-tert-butylphenol, dimethyl phthalate, dibutyl phthalate and dioctyl phthalate) was investigated. It has been found that successful isocratic separation of 27 chemicals can be achieved using acetonitrile/water eluents modified with β-cyclodextrin or hydroxypropyl-β-cyclodextrin at concentration of 10 mM and temperature of 47ºC. Separation protocol may be easily adapted for rapid

separation and quantification of wide range of given steroids and related EDCs in environmental samples, particularly those that are characterised by unstable biological matrix and components of interest load.

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 [28]. 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. Using this approach, two main associations consisting of untreated wastewater cluster and remaining samples cluster, which clearly split into treated wastewater and surface water samples groups, can be recognized. 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, the EDCs fraction in the resulting treated wastewater may still have significant impact on the natural environment. Our investigations concerning of EDCs compounds level presented in Dzierżęcinka river (passing through Koszalin City) indicate low contribution of the Koszalin to organic pollutants load of this river. 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. Such approach may be simple and non-expensive alternative for fingerprinting protocols based on LC-MS machines.

3. Conclusion

As it can be seen from this compilation, which is a tiny fraction comparing to enormous amount of worldwide published works, supramolecular chemistry or chemistry beyond the molecule is a fascinating branch of science. It offers solution to many problems connected with life sciences and technical applications. Separation protocols based on isocratic temperature-dependent inclusion chromatography provides the opportunity for steroids and related low-molecular mass compounds of differing polarities to be robustly profiled in biological and environmental samples and provides a great deal of information on a physiological situation and ecosystems without the need to perform more complex and expensive assays.

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