Paweł K. ZARZYCKI, Michał J. BARAN, Filip B. HARASIMIUK, Magdalena M. ŚLĄCZKA
POLITECHNIKA KOSZALIŃSKA, WYDZIAŁ BUDOWNICTWA I INŻYNIERII ŚRODOWISKA,
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Spectrophotometric study of interaction between selected bile acids and cyclodextrins

Dr hab. Paweł K. ZARZYCKI

Paweł K. Zarzycki is directing the Section of Toxicology and Bioanalytics at the Koszalin University of Technology, where he has been appointed as Associate Professor. Since obtaining his doctoral degree from Medical University of Gdańsk in 1993, he has focused his research interest on thermochromic properties of inclusion complexes, temperature effect in liquid chromatography, miniaturization of TLC systems as well as biomedical analysis driven by HPLC involving cyclodextrins and multivariate data proceeding.

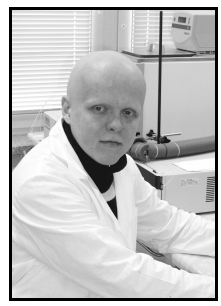
e-mail: pkzarz@wp.pl

Dr Michał J. BARAN

Dr Michał J. Baran was graduated from the Agricultural University of Szczecin and his Ph.D. project was based on the agricultural science involving animal biology and reproduction. His research interest is focusing on genetic, cell biology and endocrinology. Particularly, he studied the methods for cythogenetic analysis of molucs genotype. His latest research includes measurement of endocrine disrupting compounds in water ecosystems via TLC, HPLC and microchip-electrophoresis methods.

e-mail: michal.baran@wbiis.tu.koszalin.pl

Mgr inż. Filip B. HARASIMIUK



Filip B. Harasimiuk is a Ph.D. student at the Koszalin University of Technology. He is graduated from Agricultural University of Szczecin. His research interest is centered on fast and efficient separation of fullerenes. Moreover, his latest studies involve micro-TLC chromatography and detection of selected steroids including bile acids using supramolecular chemistry methods.

e-mail: filiphk@wbiis.tu.koszalin.pl

Mgr Magdalena M. ŚLĄCZKA

Magdalena M. Ślączka is a Ph.D. student at the Koszalin University of Technology. She is graduated from Nicolaus Copernicus University in Toruń and got a Master's Degree in Environmental Chemistry. Since 2002 she worked in the number of environmental testing laboratories across UK and USA, focusing on GC analyses of organic pollutants of soil, water and air samples. Presently, her research involves LC separation and quantification of low-molecular mass biomarkers and endocrine modulators present in surface water ecosystems.

e-mail: m030177@yahoo.com

Abstract

The main goal of present work was to explore 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. 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.

Keywords: bile acids, steroids, cyclodextrins, UV-Vis spectrophotometry, planar chromatography, micro-TLC, temperature, supramolecular chemistry, host-guest interactions, inclusion complexes, multivariate statistics, chemometrics, principal component analysis.

Badanie spektrofotometryczne oddziaływania wybranych kwasów żółciowych z cyklodekstrynami w różnych temperaturach

Streszczenie

W pracy przedstawiono wyniki badań nad oddziaływaniem wybranych kwasów żółciowych (kwas dehydrocholowy, cholowy, deoksycholowy, taurodeoksycholowy, glikodeoksycholowy, glikocholowy oraz chenodeoksycholowy; rys. 1) z substancjami makrocyklicznymi (β-cyklodekstryna i jej hydroksypropylowa pochodna) w różnych temperaturach (0 oraz 30°C), wykorzystując jako detektor kompleks inkluzyjny makrocykli z fenoloftaleiną (rys. 2 i 3). Z punktu widzenia chemii analitycznej i diagnostyki medycznej kwasy żółciowe są grupą związków trudnych w detekcji i analizie ilościowej. Zastosowanie kompleksów supramolekularnych typu gość-gospodarz, w których skład wchodzi substancja barwna np. fenoloftaleina, umożliwia zastosowanie spektroftometrii do detekcji kwasów żółciowych oraz badań ich oddziaływania ze związkami makrocyklicznymi. Jest to istotne z punktu widzenia zastosowań praktycznych np. analizy kwasów żółciowych w materiałach biologicznych rozdzielanych metodami chromatograficznymi. W prezentowanej pracy dane eksperymentalne (ΔAU) uzyskane za pomocą spektrofotometrii UV-Vis (rys. 4) były analizowane metodą PCA (Principal Component Analysis; rys. 5). Wyniki analizy wskazują na kluczową rolę temperatury oraz podstawnika przy węglu C12 szkieletu sterydów na siłę oddziaływania badanych kwasów żółciowych z cyklodekstrynami (rys. 6). Zaobserwowano, iż w warunkach mikro-chromatografii planarnej jest bardzo trudno rozdzielić parę kwasów chenodeoksycholowy/ deoksycholowy (rys. 7). Uzyskane wyniki wskazują na możliwość poprawy rozdzielania chromatograficznego wybranych par kwasów żółciowych przy zastosowaniu faz ruchomych modyfikowanych cyklodekstrynami (rys. 8).

Słowa kluczowe: kwasy żółciowe, sterydy, cyklodekstryny, spektrofotometria UV-Vis, chromatografia planarna, mikro-TLC, temperatura, chemia supramolekularna, oddziaływanie typu gość-gospodarz, kompleksy inkluzyjne, statystyka wielowariancyjna, chemometria, analiza czynników głównych.

1. Introduction

Bile acids (BA) are well-known metabolites of cholesterol. This class of compounds can form micellar structures with various organic substances [1, 2]. As size, charge and shape of structures formed are strongly dependent on the physicochemical properties of bile steroids, these supramolecular complexes have been studied extensively [3, 4]. In living organisms bile steroids play a main role in the cholesterol balance and fat digestion or absorption. Therefore, determination of bile acids and their metabolites in biological samples is becoming increasingly important for the diagnosis of several diseases and disorders [5, 6]. Because of low absorption of bile acids in the UV-Vis region, these steroids are very difficult to quantify using common high-performance liquid chromatography or capillary electrophoresis machines equipped with spectrophotometric or fluorimetric detectors without target components derivatization. On the another hand, due to number of sensitive and non-expensive visualization reagents, planar chromatography seems to be very attractive method for bile acids quantification [7, 8]. It is noteworthy, that using such approach separation and quantification can be directly performed in biological fluids without earlier sample pre-purification.

Cyclodextrins (CD) are a group of toroidal-shaped oligosaccharides that contribute to several guest associated phenomena in solution [9, 10]. The three most commonly employed natural cyclodextrins contain six, seven and eight glucopyranose units in macrocyclic rings and are denoted as α-, β - and γ- cyclodextrin, respectively. The interior of CD cavities is relatively hydrophobic because all the hydroxyl groups are on the outside of the molecule. Moreover, the C2–OH and C3–OH groups form a ‘‘belt’’ of hydrogen bonds, making the molecule more rigid. The CD complexation processes are highly selective and can be considered as the method of choice for resolution of various isomers: structural, geometrical, diastereomeric and enantiomeric [10, 11]. Particularly in chromatography, cyclodextrins are commonly used as chiral selectors and for improving separation of other stereoisomers [12]. The primary factors in CD-guest complexes are Van der Waals forces, hydrophobic interactions and bonding. However, other factors such as shape of the guest molecules may also be important [13]. Cyclodextrins are extensively used in the pharmaceutical, cosmetics and food industries in order to improve solubility, dissolution rate, stability, and the bioavailability of drugs as well as to enhance the fluorescence intensity of certain compounds [14-16].

The main goal of present work is to explore the host-guest complex formation between selected bile acids (dehydrocholic, cholic, deoxycholic, taurodeoxycholic, glycodeoxycholic, glycocholic and chenodeoxycholic acid) and two macrocycles (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.

2. Experimental

2.1. Materials and reagents

Bile acids (Fig. 1) were purchased from Sigma (St. Louis, MO, USA).

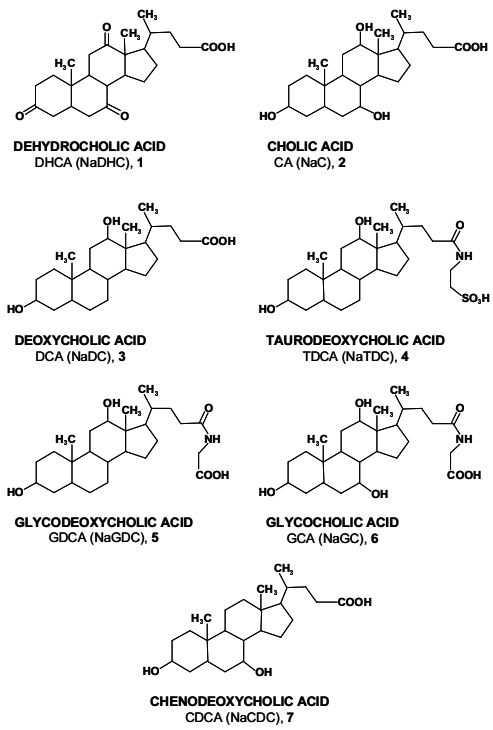


Fig. 1. Chemical structures and abbreviations of bile acids investigated. Steroids abbreviations, which are placed in the parenthesis correspond to sodium salt of given bile acid

Rys. 1. Wzory chemiczne oraz literowe skróty badanych kwasów żółciowych. Skróty sterydów umieszczone w nawiasach dotyczą soli sodowej odpowiedniego kwasu żółciowego

Macrocycles (β-cyclodextrin and its hydroxypropyl derivative) were products of Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland), respectively. Phenolphthalein, sodium carbonate and phosphomolybdic acid (PMA) were obtained from Chempur (Piekary Śląskie, Poland). Methanol (LiChrosolv 99.8% for liquid chromatography) was obtained from Merck (Darmstadt, Germany). Double-distilled tap water was used for solutions and mobile-phase preparation.

2.2. UV measurements

The absorption spectra were recorded using a Specord M40 (Carl Zeiss, Jena, Germany) UV-Vis two beam spectrophotometer. All measurements were carried out using standard 1 cm thick quartz cells under elevated (30°C) and sub-ambient (0°C) temperature conditions. Samples constant temperature was maintained using home-made anti-frosting thermostating module (Fig. 2) connected to constant temperature bath through Pump NPD 14/2 Totton (Southampton, UK). Such equipment provided fast and accurate sample equilibration inside standard UV-Vis cuvette, for given sub-ambient and elevated temperature (Fig. 3).

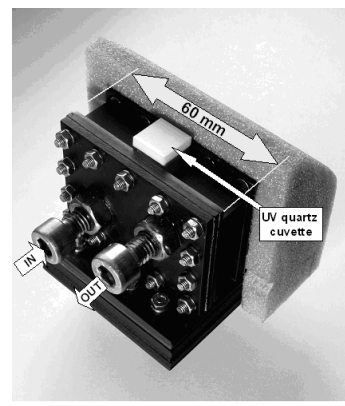


Fig. 2. Perspective view of temperature controlled module of UV-Vis cuvette that was used for spectrophotometric experiments

Rys. 2. Widok ogólny termostatującego modułu kuwety UV-Vis, zastosowanego w pomiarach spektrofotometrycznych

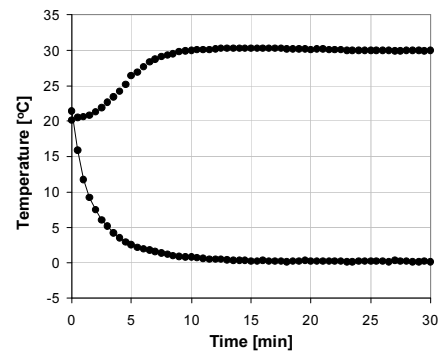


Fig. 3. Temperature equilibration rate of UV-Vis quartz cuvette filled with 4 mL of water, measured from room temperature to the thermostating module temperature of 0 and 30◦C

Rys. 3. Profile szybkości zmian temperatury kuwety kwarcowej wypełnionej 4 mL wody, mierzonej od temperatury pokojowej do uzyskania temperatury modułu termostatującego 0 oraz 30◦C

Alkaline solution of phenolphthalein at a concentration of 30 μM was prepared in aqueous sodium carbonate 0.02 M (pH = 10.4). The samples were modified by the addition of β-cyclodextrin or hydroxypropyl-β-cyclodextrin at 1 mM concentration. Bile acids were added to phenolphthalein-macrocycle solution at concentration of 1 or 10 mM. Appropriate alkaline solutions without phenolphtalein were used as the references. All of the solutions were prepared freshly in the day of use.

2.3. Micro-chromatography and chromatograms digitalization

Separation experiment was performed on glass-based HPTLC RP18W plates that were product 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 [17]. Chromatographic separations were carried out under unsaturated chamber conditions using 8:2 (v/v) methanol:water mobile phase. Spots patterns were acquired by direct scanning under visible light conditions 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 at 60°C for 25 min.

Micro-TLC plates were spotted with steroid mixture (at concentration of 1 mg/mL methanol) using 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 5 mm long bands (containing analytes mass of 1 μg of each steroid) were formed along start line, which was located 5 mm from the bottom edge of TLC plate.

Picture acquisition was performed using a Plustek OpticPro S12 USB scanner (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).

2.4. UV-Vis and micro-TLC data analysis

Data from spectrophotometric measurements were inspected with principal components multivariate statistical procedure using XLSTAT-Pro/3DPlot (version 2008.2.01), provided by Addinsoft (Paris, France). Selected cross-sections of the chromatographic lanes (for bile acids micro-TLC separation) were extracted from digital pictures with help of Scion Image freeware (Scion Corp., Frederick, MD; Version 4.0.3.2; http://www.scioncorp.com/).

3. Results and discussion

The formation of inclusion complexes between small organic molecules and cyclodextrins has proven to be an excellent model system for studying the nature of noncovalent binding forces in water based solutions [18]. It has been found, that the addition of cyclodextrin (CD) to solution containing phenolphthalein (PP) or azo-dyes results in a decrease of its absorbance in UV-Vis region [19]. 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 like *n*-alkohols with α- and β-cyclodextrins [20-23]. The principle of spectrophotometric measurement of host-guest interaction involving cyclodextrin-phenolphthalein complex is demonstrated in Fig. 4. As can be seen an alkaline solution of phenolphthalein absorb visible light (close to λ = 554 nm) and this absorption band is markedly lowered in the presence of cyclodextrin. If target compound (*e.g.* bile acid) can competitively interact with macrocycle internal cavity, this may be simply observed as the increase of phenolphthalein absorbance. In our previous work we reported that CD-PP supramolecular complex involving β-cyclodextrin as the host molecule is strongly temperature dependent [24]. Decreasing of temperature induce a decrease in UV-visible absorbance of phenolphthalein. Under favorable conditions such termochromic effect is very significant (0.1 AU per 10°C) [25].

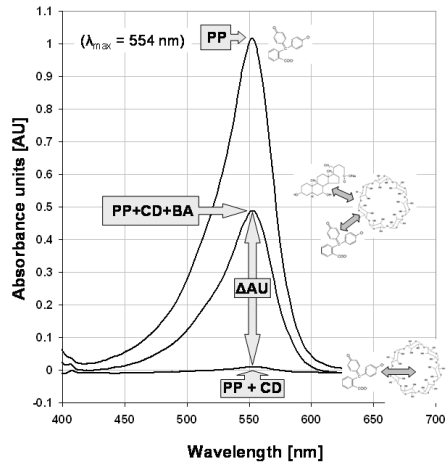


Fig. 4. Principle of spectrophotometric measurement of host-guest interaction involving phenolphthalein-β-cyclodextrin complex formation; PP - phenolphthalein, CD - β-cyclodextrin, BA - bile acid

Rys. 4. Zasada pomiaru spektrofotometrycznego oddziaływania typu gość-gospodarz przy pomocy kompleksu fenoloftaleina-β-cyklodekstryna; PP - fenoloftaleina, CD - β-cyklodekstryna, BA - kwas żółciowy

In the present study we measured the host-guest complex formation occurring between selected bile acids (in form of sodium salt) including dehydrocholic, cholic, deoxycholic, taurodeoxycholic, glycodeoxycholic, glycocholic and chenodeoxycholic acid (Fig. 1) and two macrocycles (β-cyclodextrin and its hydroxypropyl derivative) at sub-ambient and elevated temperature (0 and 30°C, respectively). From spectrophotometric data appropriate ΔAU values for bile acids studied were derived, according to the method presented in Fig. 4. Spectrophotometric experiment was performed under constant macrocycles concentration (1 mM) and for two bile acids concentrations (1 and 10 mM). Therefore, resulting raw data matrix was composed of seven target analytes (bile acids) against eight variables (experimental conditions).

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). This chemometric method is common exploratory data analysis tool for solving classification problems, allowing data reduction and determination of latent information from the raw data set [26]. In the case of our studies, a raw data matrix consisting of 56 experimental points made up of 7 objects (bile steroids) characterized by 8 variables (experimental conditions) was investigated. For the initial data matrix the first 2 factors explained over 97% of the total variability were analysed. The graph presented in Fig. 5 shows the PC score plot for the objects investigated. It is clearly seen that chenodeoxycholic acid (point No 7) is well separated from remaining bile acids, which form one cluster. This compound is characterized by high ΔAU values observed under all experimental conditions studied. Particularly, the highest ΔAU value for this compound was observed at temperature of 0°C in presence of β-cyclodextrin as the inclusion agent (Fig. 6). Such strong interaction can be explained by shape-recognition, taking into account that only this particular bile acid structure does not contain the keto or hydroxyl groups linked to C12 carbon atom. Therefore, strong host-guest interaction is preferred, similarly to steroids like 17β-estradiol, testosterone or 20α-hydroxyprogesterone, which has been previously reported based on liquid chromatography experiment [12]. Interestingly, under planar chromatographic conditions chenodeoxycholic acid is difficult to separate, particularly from deoxycholic acid (Fig. 7). Similarly to our spectrophotometric and chromatographic data obtained for polycyclic aromatic hydrocarbons [27,28], the results of the present work strongly suggest that the use of cyclodextrin as mobile phase additive, may

significantly improve separation between those bile acids. In order to find more quantitative data concerning interaction of C12 substituted BA with cyclodextrins, chenodeoxycholic acid data were excluded from further investigations. Hence, the chemometric calculations were based on a 48-point data matrix consisting of 6 objects versus 8 variables. Under such conditions, the first 2 factors were characterized by eigenvalues greater than 1 and these explain more than 80% of the total variability. Data presented in Fig. 8 suggest that there is significant difference in BA-CDs interaction, which may help to improve bile acids separation involving chromatographic mobile phases modified with cyclodextrins. Especially, for steroids pairs No 2 and 5 as well as No 1 and 6, according to micro-TLC chromatographic profile presented in Fig. 7.

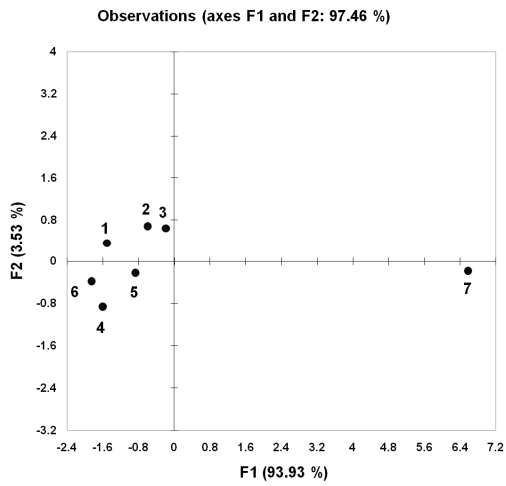


Fig. 5. Principal component plot showing relationships between all objects (steroids) investigated in respect to 1 and 2 factor scores (dots numbers corresponds to bile acids numbers presented in Fig. 3)

Rys. 5. Grupowanie wszystkich obiektów badań (sterydy) w przestrzeni dwuwymiarowej uwzględniającej dwa pierwsze czynniki główne F1 oraz F2 (numeracja punktów na wykresie odpowiada numerom kwasów żółciowych przedstawionych na rys. 3)

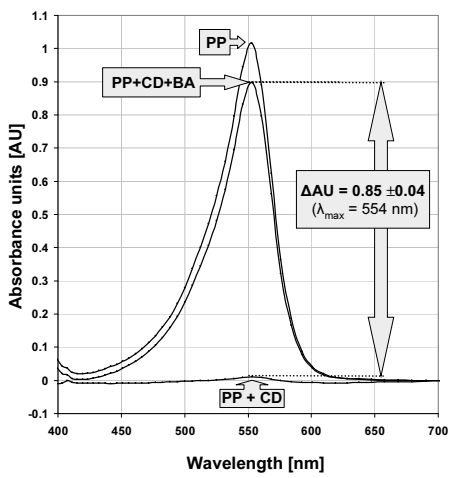


Fig. 6. Change of absorbance (**ΔAU**) of phenolphthalein-β-cyclodextrin complex after chenodeoxycholic acid addition (as sodium salt; NaCDC; No 7), measured at subambient temperature (0°C). Experimental conditions: β-cyclodextrin, phenolphthalein and NaCDC concentrations: 1 mM, 30 μM and 10 mM, respectively (prepared in water containing 0.02 M sodium carbonate; pH = 10.4)

Rys. 6. Zmiana absorbancji (**ΔAU**) kompleksu fenoloftaleina-β-cyklodekstryna po dodaniu kwasu chenodeoksycholowego (w postaci soli sodowej; NaCDC; No 7) mierzona w obniżonej temperaturze (0°C). Warunki prowadzenia eksperymentu: stężenie β-cyklodekstryny, fenoloftaleiny oraz NaCDC odpowiednio 1 mM, 30 μM oraz 10 mM (przygotowane jako wodny roztwór zawierający 0.02 M węglanu sodu; pH = 10.4)

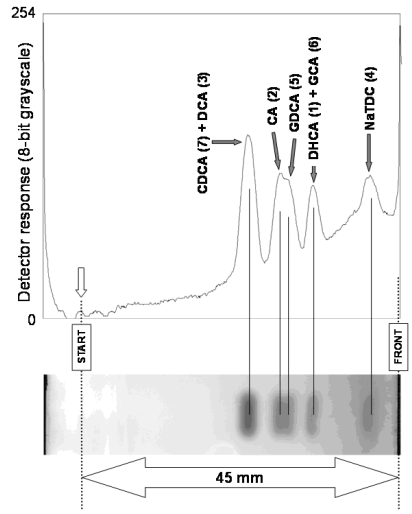


Fig. 7. Separation of bile acids using temperature-controlled planar micro-chromatography (micro-TLC). Analytical conditions: sample application - 5 mm band using spray-on technique (mass of individual steroids: 1 μg); separation temperature +50°C; stationary phase - HPTLC RP18W; mobile phase composition - 80% (v/v) methanol/water; spots visualization: developed chromatogram dipped in 10% (w/v) phosphomolybdic acid (PMA) in methanol and heated for 25 min at 60°C; chromatogram acquisition method - direct digital scan under visible light conditions using Plustek OpticPro S12 USB office scanner. Densitogram corresponding to original chromatogram was derived via Scion Image software

Rys. 7. Rozdzielenie kwasów żółciowych za pomocą mikrochromatografii planarneej (mikro-TLC) w warunkach kontrolowanej temperatury. Warunki wykonania chromatogramu: sposób naniesienia próbki - pasmo długości 5 mm techniką natryskową (masa poszczególnych sterydów: 1 μg); temperatura procesu rozwijania +50°C; faza stacjonarna - HPTLC RP18W; skład fazy ruchomej - 80% (v/v) metanol/woda; detekcja plamek: płytka z rozwiniętym chromatogramem zanurzona w 10% (w/v) metanolowym roztworze kwasu fosforomolibdenowego (PMA) i wygrzewana przez 25 min. w temperaturze 60°C; rejestracja chromatogramu: obraz na płytce zeskanowano przy użyciu skanera biurowego Plustek OpticPro S12 USB. Densytogram odpowiadający rozwiniętemu i wywołanemu chromatogramowi uzyskano za pomocą programu Scion Image

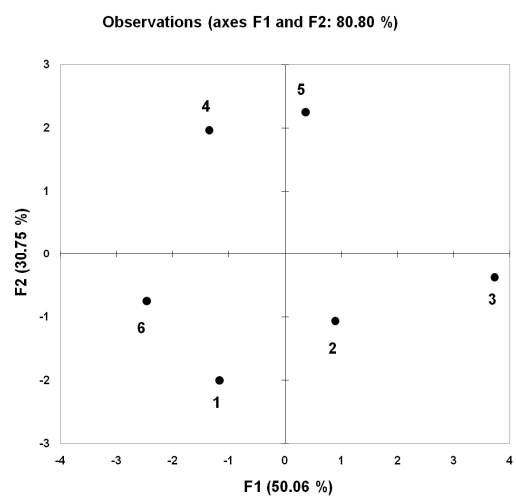


Fig. 8. Principal component plot showing relationships between bile acids except chenodeoxycholic acid in respect to 1 and 2 factor scores (dots numbers corresponds to steroids numbers presented in Fig. 3)

Rys. 8. Grupowanie kwasów żółciowych z wyłączeniem kwasu chenodeoxycholowego w przestrzeni dwuwymiarowej uwzględniającej dwa pierwsze czynniki główne F1 oraz F2 (numeracja punktów na wykresie odpowiada numerom sterydów przedstawionych na rys. 3)

4. Conclusions

The formation of inclusion complexes between phenolphthalein and cyclodextrins has proven to be an excellent model for studying of bile acids interaction with macrocycles in water based

solutions *via* UV-Vis spectrophotometry. 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. Spectrophotometric data were explored using principal component analysis. 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.

5. References

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INFORMACJE

IX Sympozjum pt. Niepewność Pomiarów

W dniach 15 – 19 lutego w Świnoujściu odbyło się IX Sympozjum pt. Niepewność Pomiarów, zorganizowane przez Zakład Metrologii Katedry Sterowania i Pomiarów Wydziału Elektrycznego Zachodniopomorskiego Uniwersytetu Technologicznego w Szczecinie.

Podczas otwarcia Sympozjum uczestników powitał prodziekan Wydziału Elektrycznego ZUT, prof. Ryszard Pałka, a wiceprezes Głównego Urzędu Miar, Włodzimierz Popiołek, odczytał list od pani Prezes GUM, Janiny M. Popowskiej. Pani Prezes, która objęła Sympozjum swoim patronatem, podkreśliła w liście, że ceni rolę Sympozjum jako forum wymiany poglądów między pracownikami nauki a przedstawicielami administracji miar i życzyła uczestnikom Sympozjum owocnych obrad. Pan Wiceprezes W. Popiołek przedstawił też referat nt. kierunków prac, mających na celu doskonalenie administracji miar w Polsce.

W Sympozjum wzięło udział 41 osób, w tym 12 pracowników administracji miar. Obrady przeprowadzono w sześciu sesjach pod przewodnictwem profesorów wyższych uczelni oraz pracowników GUM.

Idea wymiany doświadczeń znalazła pełne odbicie w przebiegu Sympozjum i, co istotne, okazało się, że rozróżnienie między pracownikami administracji miar a pracownikami nauki ma tylko formalny charakter. Przedstawiciele obu grup zaprezentowali zarówno referaty szczegółowe, dotyczące pomiarów konkretnych wielkości lub wzorcowania konkretnej aparatury, jak i referaty o ogólniejszym charakterze, w szczególności dotyczące ogólnej problematyki niepewności pomiaru, – w tym referat zwracający uwagę na problem oceny niepewności w przypadku stosowania miary logarytmicznej i referat uwypuklający znaczenie problematyki niepewności w procesach produkcyjnych. Ponadto wygłoszono referaty omawiające technikę obliczeń niepewności lub przedziału rozszerzenia, w tym technikę opartą o symulację Monte Carlo, referaty dotyczące estymacji wartości mezurandu i rozkładu prawdopodobieństwa błędu pomiaru, wreszcie referat podejmujący zagadnienie opisu niedokładności pomiaru za pomocą teorii reprezentacji w zbiorach rozmytych.

Przyjęte szeroko zasady oceny niedokładności pomiaru ukształtowały się przede wszystkim w środowiskach fizyków, elektryków, elektroników i mechaników, natomiast nie znajdują pełnego zastosowania w analizie chemicznej. Ten istotny problem był również dyskutowany.

Podczas trwania Sympozjum odbyła się też sesja poświęcona dyskusji nad formułą Sympozjum „Niepewność Pomiarów" na następne lata, a w szczególności jego roli jako forum kontaktów środowiska metrologów uczelnianych z pracownikami administracji miar. Liczni dyskutanci z obu stron opowiedzieli się za kontynuacją Sympozjum w dotychczasowej formie.

Nawiązując do referatu, wygłoszonego przez wiceprezesa GUM, p. W. Popiołka podczas sesji otwarcia IX Sympozjum, środowisko metrologów uczelnianych wyraziło też żywe zainteresowanie problematyką doskonalenia administracji miar w Polsce.

Materiały Sympozjum w formie prezentacji dostarczonych przez Autorów są zamieszczone na stronie internetowej PAK: www.pak.info.pl/Konferencje PAK.

Opracowanie: Prof. dr hab. inż. Stefan KUBISA