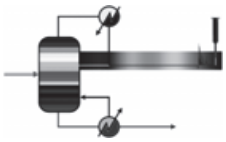

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Szybka metoda frakcjonowania lipidów i substancji niepolarnych w piórach ptaków z wykorzystaniem termostatowanej mikrochromatografii planarnej (micro-TLC)

Fast method for fractionation of lipids and related non-polar substances from birds' feathers using thermostated micro-TLC

Streszczenie: *Pióra, ze względu na powszechną obecność ptaków w różnych typach środowisk, mogą znaleźć zastosowane jako materiał do badań biomonitoringowych. Wymaga to opracowania ekstrakcji niepolarnych związków zawartych w upierzeniu oraz metody ich skutecznego rozdzielania z wykorzystaniem technik chromatograficznych. Zasadniczym problemem analitycznym jest obecność w piórach substancji o charakterze wosków, które bardzo silnie oddziaływują z chromatograficzną fazą stacjonarną na bazie krzemionki lub oktadecylsilanu. Adsorbenty te powszechnie stosowane jako fazy stacjonarne w technikach cienkowarstwowych i kolumnowych. Głównym problemem jest to, iż naniesienie na kolumnę chromatograficzną nieodpowiednio oczyszczonych ekstraktów próbek biologicznych zawierających woski jako zanieczyszczenia, zazwyczaj prowadzi do jej zniszczenia. Zaletą chromatografii planarnej jest w tym przypadku to, że dana płytka jest wykorzystywana do rozdzielania tylko raz. Celem niniejszej pracy było opracowanie szybkiej procedury analitycznej z wykorzystaniem prostej jednoetapowej ekstrakcji cieczą oraz rozdzielania uzyskanych ekstraktów substancji niepolarnych obecnych na powierzchni piór ptaków, za pomocą mikropłytek chromatograficznych. Uzyskane wyniki wskazują na przydatność metodologii ekstrakcji cieczą oraz detekcji fluorescencyjnej w badaniach chemotaksonomicznych oraz biomonitoringu.*

Słowa kluczowe: mikro-TLC, chromatografia planarna, ptaki, pióra, myszołów, kormoran, pójdźka

Abstract: *Planar chromatography (TLC) is known as an effective tool for separation of complex biosamples highly loaded with interfering substances. The main goal of this research communication is to demonstrate a capability of micro-TLC methodology followed by simple one-step liquid extraction as fast fractionation tool for wide range of non-polar, low-molecular mass substances extracted from birds' feathers samples. The main analytical problem concerning birds' feathers is the presence of interfering matrix, mainly composed of waxes-like impurities. Such substances can be strongly adsorbed on the stationary phase. Proposed simple extraction protocol and fast micro-TLC separation procedure is based on dichloromethane: methanol mixtures. We used this methodology for fractionation of non-polar compounds present in feathers of buzzard, cormorant and little owl. Described methodology can be applied for fast fractionation or screening of whole range of target substances as well as chemo-taxonomic studies and fingerprinting of complex mixtures, which are present in raw biological samples. Particularly, the mi-*

cro-TLC separation combined with PMA staining and UV light detection seems to be an effective tool for fast and low-cost characterization of non-polar compounds from birds' feathers or uropygial gland secretion products.

Key words: *micro-TLC, planar chromatography, phosphomolybdic acid, fluorescence, densitometry, birds, feathers, Buzzard, Cormorant, Little owl*

1. Introduction

Modern monitoring of environmental pollutions (biomonitoring) requires to use of non-destructive, intravital techniques for samples collection. A lot of birds during the extra active life cycle can accumulate number of chemicals, especially living in various habitats as well as during short-distance or transcontinental migrations. Number of heavy metals and organic contaminations were found on the surface and inside of the feathers structure [1-4]. Chromatographic techniques including GC-MS and HPLC, are often used for qualitative and quantitative analysis of the feathers adsorbed chemicals [3-5]. However, there is still on demand to design new, fast, simple and low cost analytical techniques for environment screening of wide range of components from complex biological samples. From analytical point of view planar chromatography fractionation has a great potential as the initial examination tool of such complex raw samples. Particularly, taking into account fast and efficient multidimensional separation and sensitive detection of UV-Vis transparent chemicals via common visualization agents including iodine or phosphomolybdic acid (PMA). Moreover, the advantage of planar chromatography over column technique is simple fluorescence or fluorescence quenching detection of TLC spots for efficient fingerprinting and characterization of complex environmental samples.

The main goal of this research communication is to demonstrate a capability of reversed-phase micro-TLC methodology as fast fractionation tool for wide range of non-polar, low-molecular mass substances extracted from birds' feathers complex samples. Particularly, in presence of highly loaded organic matrix, mainly composed of waxes relating impurities strongly adsorbed on octadecylsilane stationary phase.

2. Experimental

Materials and reagents

Methanol (LiChrosolv 99.8% for liquid chromatography) and dichloromethane (99.8% GR for analysis, stabilized with about 50 ppm 2-methyl-2--butene) were obtained from Merck, Darmstadt, Germany. Phosphomolybdic acid was purchased from Chempur, Piekary Śląskie, Poland. Double-distilled tap water was used for mobile-phase preparation.

Birds samples collection

The objects investigated were found dead and no birds were killed for the purpose of this research. Particularly, the causes of death included traffic accidents or natural reasons. Carcasses of birds were collected from the Middle Pomerania area in northern part of Poland with the permission of the General Directorate for Environmental Protection.

Extraction of feathers' compounds

The flight feathers (*Phalacrocorax carbo* – Great Cormorant, *Athene noctua* – Little Owl and *Buteo buteo* – Common Buzzard; figure 1), after cutting of the subcutaneous part of the shaft, were fragmented into small pieces (1-3 mm). The material of interest (400 mg, approximately) was extracted three times using 6 mL of solvent consisted of dichloromethane:methanol (2:1, v/v) binary mixture, followed by sonication (3 × 90 min). The feathers extracts were centrifuged at 3500 rpm for 15 min. and combined supernatants for individual objects were evaporated under vacuum conditions at room temperature. The residue was reconstituted in freshly prepared extraction solvent. The resulting concentration of the feathers extract substances was set at level of 5 mg/mL.

Rys. 1. Przykładowa lotka myszołowa (*Buteo buteo*) użyta w pracach doświadczalnych
Fig. 1. Typical secondary flight feather of buzzard (Buteo buteo) used for the experiment

***Micro*-TLC procedure**

Chromatographic separations were performed on glass-based HPTLC plates RP18 F254s purchased from Merck (Darmstadt, Germany). Given volumes of the feather extract were sprayed on the plate start line using semi-automatic sampler Linomat 5 (Camag, Muttenz, Switzerland). The plates were developed in home-made unsaturated horizontal micro-chamber module [6] at elevated temperature (30°C), using binary dichloromethane:methanol mobile phase (2:1; v/v). After plate development and mobile phase evaporation from the plate surface the resulting bands were photographed under UV light (254 and 366 nm, UV lamp, Cobrabid, Warszawa, Poland) and using SLR digital camera Nikon F80 plus Nikkor AF 35-70 mm/3.3-4.5 lens with manual control of exposure (Nikon, Bangkok, Thailand). Components of interest were also visualized by 10% (v/v) solution of phosphomolybdic acid (POCh, Gliwice, Poland) in methanol, followed by heating at 80°C for 20 minutes. After visualization process the TLC plates were scaned at visible light

conditions using the Plustek OptiPro S28 desktop scanner (Plustek, Taipei, Taiwan) with 8 bit per RGB channel colour deep mode and 600 dpi resolution.

Digital images were saved as NEF and TIFF files for the Nikon RAW system and the Plustek scanner, respectively. The ImageJ freeware (ver. 1.42q, Wayne Rasband, National Institutes of Menthal Health, Bethesda, MD, USA) was used to digital pictures processing.

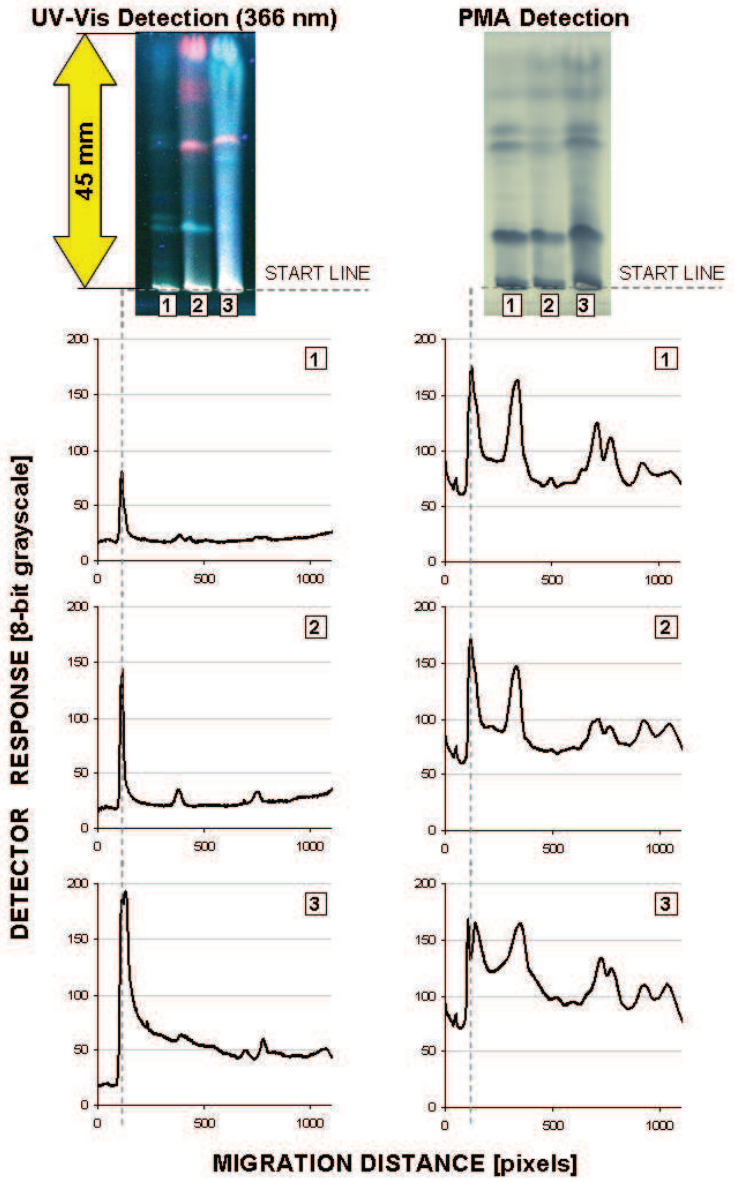
3. Results and discussion

The main advantages of isocratic non-forced flow rate planar chromatography result from its simplicity, easy operation and the inexpensive equipment needed. The advantage of modern planar chromatography is that such method can easily combine pre-purification and quantification steps within one analytical protocol. Particularly using micro-TLC technique number of complex samples consisting of bile acids, lipids, fullerenes as well as cyanobacterias and herbs dyes can be separated and quantificated without pre-purification steps [6, 7].

The birds' feathers contain a number of low molecular mass compounds like waxes and lipids synthesized and secreted by uropygial gland [8, 9]. Such substances are essential for proper function of plumage (body's heat, water insulation, flight ability) and birds protective or signalling coloration. On the other hand birds plumage can contain various of low molecular mass compounds adsorbed on the feathers during birds lives, especially in the polluted natural environment. This conclusion is particularly important from biomonitoring researches point of view that look for samples containing long-life organic chemicals and biomarkers including e.g. endocrine disrupting compounds (EDCs). However, the waxes fraction, which is present in the organic feathers extract, generate number of serious analytical problems. Particularly, such substances are extremely strongly adsorbed on both silica and octadecylsilane stationary phases. This can course the column destruction, if non-properly pre-purified material is injected. In case of TLC such problem doesn't exist because the plate is used just once for given chromatographic run. It noteworthy to say that micro-TLC based separation system is much more efficient in terms of separation power in comparison to e.g. micro-chip or micro-fluidic devices, which are recently developed [10-12]. Planar chropmatography is also less sensitive for sample overloading [13].

Designed analytical protocol involves simple liquid extraction of low-molecular mass, non-polar substances using dichloromethane: methanol mixture, direct extract application on TLC RP18 micro-plates, chromatographic separation using binary mobile phase consisting of dichloromethane: methanol (developing distance 45 mm) and detection under UV light and after PMA derivatization (figure 2). Such micro-TLC separation and quantification protocol can be successfully applied for fast screening of lipids and related non-polar substances from birds' feathers or uropygial gland secretion products. Particularly, micro-TLC separation combined with PMA staining for UV-Vis transparent peaks visualization seems to be effective tools

for fast and low-cost characterization of non-polar compounds present in birds' plumage. Presented on figure 2 fluorescence densitometric profiles have revealed that this technique can be useful for birds species chemotaxonometric studies. Particularly, that acquired spots patterns were similar to those obtained from less concentrated samples analyzed previously [13].



Rys. 2. Wynikowy obraz rozdziału chromatograficznego ekstraktów z piór pójdźki (1), myszołowa (2) i kormorana (3)

Fig. 2. Micro-chromatograms and related densitograms of extracts derived from Little Owl (1), Buzzard (2), and Cormorant (3) feathers. Biological samples - feathers mass - 400 mg; extraction solvent – dichloromethane/methanol; chromatographed samples volume - 10 µL; extract mass applied on the start line - 50 µg/spot, approximately. Chromatographic conditions: stationary phase HPTLC RP18 F254s; mobile phase - 15:85 (v/v) dichloromethane/methanol; unsaturated chamber; developing temperature 30°C; developing time 8 min.; Detection: fluorescence 366 nm and visible light as well as visualization using 10% phosphomolybdic acid (PMA)

4. Conclusions

The results of our experiments indicate that in particular cases fractionation and/or separation as well as characterization of complex raw biological materials via analytical protocol involving temperature-controlled planar micro-chromatography is efficient, simple, fast and non-expensive. Such separation approach can be an alternative for fingerprinting protocols based on HPLC machines equipped with UV-Vis detectors, especially in case of samples with high level of interfering waxes like substances. Particularly, it has been demonstrated that using micro-TLC separation followed by simple liquid extraction without additional pre-purification steps, a fast screening of bird's feathers samples can be successfully performed. PMA staining as well as fluorescence of target peaks seems to be an effective detection approach for fast and low-cost characterization of low-molecular mass metabolites and non-polar compounds present in birds' plumage.

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