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Application of micro-thin-layer chromatography as a simple fractionation tool for fast screening of raw extracts derived from complex biological, pharmaceutical and environmental samples

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ABSTRACT

The main goal of present paper is to demonstrate the separation and detection capability of micro-TLC technique involving simple one step liquid extraction protocols of complex materials without multi-steps sample pre-purification. In the present studies target components (cyanobacteria pigments, lipids and fullerenes) were isolated from heavy loading complex matrices including spirulina dried cells, birds' feathers and fatty oils as well as soot samples derived from biomass fuel and fossils-fired home heating systems. In each case isocratic separation protocol involving less that 1 mL of one component or binary mixture mobile phases can be completed within time of 5–8 min. Sensitive detection of components of interest was performed *via* fluorescence or staining techniques using iodine or phosphomolybdic acid. 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 or environmental samples.

1. Introduction

In analytical practice thin-layer chromatography (TLC) is still commonly applied method for fast qualitative and quantitative analysis as well as screening of low-molecular mass compounds from complex biological and environmental samples [1–3]. This is mainly due to inexpensive equipment needed and parallel sample processing. In many cases a single use of TLC plate allows fast screening of raw materials without time-consuming sample pre-purification [4,5]. Moreover, the advantage of planar chromatographic approach is simple detection of separated bands under visible light as well as using sensitive visualization reagents for a variety of ultraviolet–visible (UV/vis) transparent bioactive analytes [6,7]. The resulting spots pattern on developed TLC plate can be easily digitalized using simple office scanners or digital cameras working with dynamic signal range up to 16 bits per RGB channel. 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 [5,7,8]. Our 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 [8,9].

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 [8,9]. This is mainly because of the low flow rate and small amount of the mobile phase that is necessarily to perform the separation process. Moreover, there is no Joule's heat evolved due to electric current flow as in *e.g.* classical planar electrophoresis systems, therefore, micro-TLC units, new planar chromatographic and electro-chromatographic chambers as well as micro-fluidic paper-based devices are still constructed [9–15]. Depends 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 [9]. Recently, a number of new detection methods like direct analysis in real time (DART) involving mass spectrometry (MS) techniques were introduced [16,17]. 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 [18,19].

This work is a continuation of our previous research focusing on development of micro-TLC methodology [8,9,20–22]. The main goal of present paper is to demonstrate the separation and detection capability of micro-TLC technique towards highly loaded organic matrix extracts involving simple analytical protocols without multi-steps sample pre-purification. Components of interest from biological and environmental samples including dry spirulina cells, birds' feathers and fatty oils as well as soot dust materials were isolated using direct organic liquid extraction. Described methodology can be applied for fast and non-expensive fractionation of target substances including cyanobacteria pigments, lipids and fullerenes as well as screening and fingerprinting of complex mixtures, which are present in raw biological or environmental samples.

2. Experimental

2.1. 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. Acetone (99.9% HPLC grade) and toluene (99.5% A.C.S. Reagent) were received from Sigma–Aldrich, Steinheim, Germany. *n*-Hexane 95% was a product of Fluka Chemie AG, Buchs, Switzerland and tetrahydrofuran (HPLC 99.9%) was purchased from Aldrich Chemical Co. Inc., Milwaukee, WI, USA. Pure analytical standards of fullerenes (C60 > 99% and C70 > 98%) were obtained from TCI (Tokyo Kasei Kogyo Co., Ltd., Japan). Phosphomolybdic acid was purchased from Chempur, Piekary Śląskie, Poland and iodine (cryst., ACS, pure P.A.) 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 $RP18F_{254}S$, RP18W, and $RP18WF_{254}S$ plates that were products of Merck (Darmstadt, Germany). Before sample application, the factory-prepared plates (100 mm × 100 mm) were cut to a working size of 50 mm × 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 (Fig. 1.), described previously [9]. 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 thermostating 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 250 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.

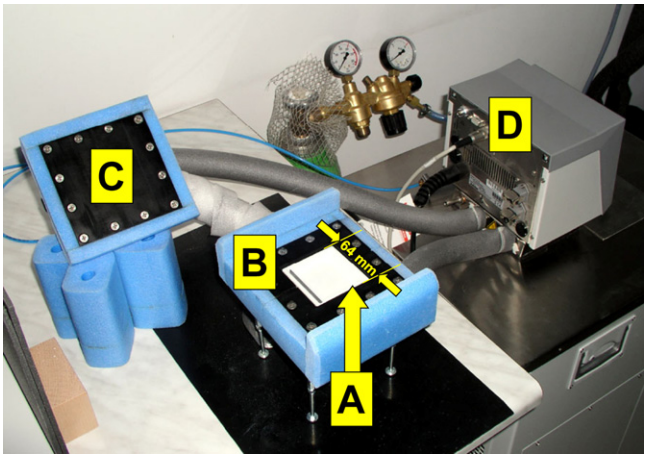

Fig. 1. Perspective view of temperature controlled micro-planar chromatography device: horizontal chamber unit (A) working inside temperature controlled metal oven (B) equipped with movable cover (C) and connected to external liquid circulating thermostat (D).

Chromatographic separations were performed under unsaturated chamber conditions using 3:7 (v/v) acetone:*n*-hexane, 15:85 (v/v) dichloromethane:methanol and pure *n*-hexane for spirulina, feathers and soot extracts, respectively. Spots patterns on the plated developed were acquired by direct scanning under visible light conditions and 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 80 °C for 20 min. Additional spots, corresponding to visible light transparent substances, on the plates with spirulina samples were detected by iodine vapor exposure (room temperature; 30 min).

2.3. Chromatograms digitalization

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).

Fluorescence visualization for λ_{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 using the following camera settings: focusing mode—manual, shutter speed 1 or 10 s for λ_{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 using Adobe Photoshop Software (San Jose, CA, USA).

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. Selected cross-sections of the chromatographic lanes were extracted from the digital pictures with the help of ImageJ software (ver. 1.43q Wayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij).