APARATURA
BADAWCZA I DYDAKTYCZNA

Application of a scanning densitometer for environmental and biomedical research

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ABSTRACT
The article focuses on the possibility of using densitometry. It also presents the results of studies using a densitometer as a detector in planar chromatography for the determination of environmental pollutants, pro- and carcinogenic compounds found in food as well as biomarkers of exposure to tobacco smoke determined in urine, serum and amniotic fluid.

Zastosowanie densytometru skaningowego w badaniach środowiskowych i biomedycznych

Słowa kluczowe: densytometr, badania środowiskowe, analiza metabolitów

STRESZCZENIE
W artykule zwrócono uwagę na możliwości zastosowania densytometrii. Przedstawiono również rezultaty badań z zastosowaniem densytometru jako detektora w chromatografii planarnej do oznaczania związków zanieczyszczających środowisko naturalne, związków kancerogennych i prokancerogenów występujących w żywności oraz biomarkerów narażenia na dym tytoniowy oznaczanych w moczu, surowicy i płynie owodniowym.

A scanning densitometer is dedicated to measurements of optic density of transparent and non-transparent materials. It can be used in various branches.

In medicine computerized densitometers to measure bone mineral density, acting on the basis of X-ray to diagnose and monitor treatment of osteoporosis and body composition analysis with fat and muscle tissue determination. Moreover densitometer may enable measurement of the concentration of bacterial cells in the fermentation process, the identification of microorganisms using appropriate reagents and the measurement of the resistance of bacteria to antibiotics [1].

Densitometer allows measurement of light transmission, reflectance and fluorescence. Thanks to this it is widely used as a detector in reading as well as TLC chromatograms, and electrophoretic gels. This types of instruments are usually equipped with two or three automatically switched light sources: tungsten lamp, deuteron lamp and mercury lamp. Detection is carried out using highly sensitive photomultipliers provides a wide spectral range. Software of densitometer allows to optimize the detection conditions determined compounds, qualitative and quantitative analysis with the creation of the calibration charts [2].

In the printing industry densitometry is also a traditional method of measurement, evaluation and characterization of surface colour at different stages of production [3]. Different instruments equipped with software running in the Windows environment for acquisitions and recording of densitograms and obtained spectra are used.

Densitometers allow scanning of the chromatographic plates depending on the needs in two modes "linear" and "zigzag". Selection of scanning options affects the quality of received chromatogram and sensitivity of detection (Fig. 1).

In addition to the use of a densitometer for the determination of polycyclic aromatic hydrocarbons and their oxygen, sulphur and nitrogen derivatives in the environment, as well as the device was used for the analysis of selected metabolites of xenobiotics in the biological material [4-9].

Some of the results obtained in a research to clarify the role of the interaction of the analyte in the creation of the band of thin layer chromatography was also performed using a UV-Vis densitometer [10]. Other such studies focused on the development of mathematical models of physical and chemical processes related to the separation of organic compounds, and deepening knowledge of the rules describing transport of mass in the mobile phase and in adsorbent grains. This research, mainly related to the mass transfer kinetics and thermodynamics of adsorption at liquid chromatography using TLC technique (called Thin Layer Chromatography) and OPLC (called Overpressured Layer Chromatography) [11, 12].

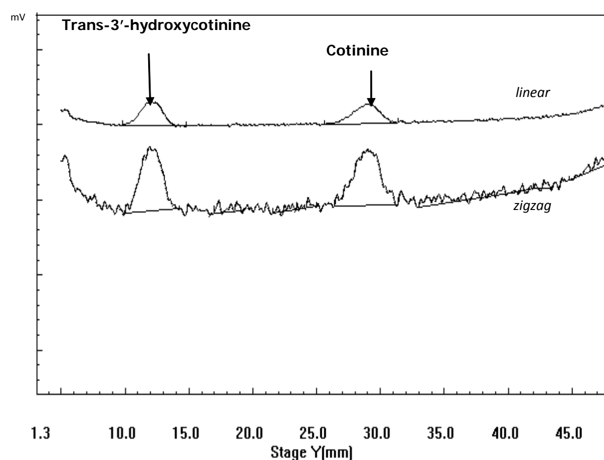

Figure 1 Comparison of the sensitivity determinations major metabolites of nicotine obtained through the plates by scanning densitometry by zigzag and linear at λ =260 nm

Rysunek 1 Porównanie czułości oznaczeń densytometrycznych głównych metabolitów nikotyny uzyskanych jako wynik skanowania płytek HPTLC Nano-Sil techniką zigzag oraz linear, λ =260 nm

Another applications of a densitometer were aimed at identify and determine the quantitative selected ingredients of Polish ethanol extract of propolis (EEP). For this purpose TLC analysis with densitometry using RP C18 TLC plates (Macherey Nagel) were performed.

The mobile phase was a mixture of toluene + dioxane + methanol (80+10+10, v/v/v), and the chromatograms were visualized with a mixture of chloroform + methanol + formic acid (44+3.5+25; v/v/v). After drying the plates, colour of spots and peak positions on densitograms obtained using the fluorescent lamp allows to eliminate or to confirm the presence of certain components of the EEP tested. Unfortunately TLC technique with scanning densitometry could only confirm the presence of certain EEP ingredients (Fig. 2) previously identified by HPLC (High Performance Liquid Chromatography).

What deserves special attention is usage of a tungsten lamp that allows the detection and quantification in fluorescence mode of procancerogenic nitrogen compounds such as aminoa-

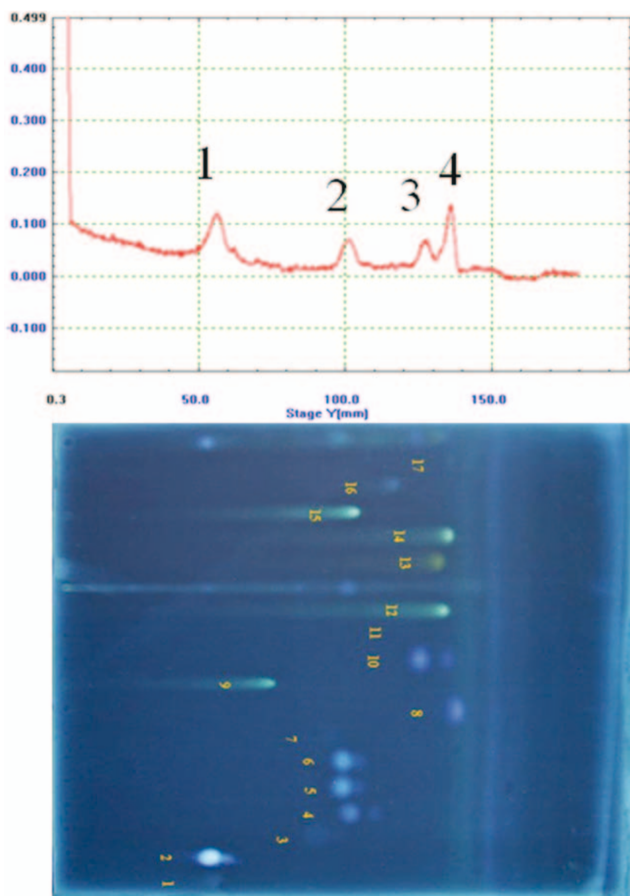

Figure 2 Chromatographic plate of propolis ingredients under UV light l=366 nm and its densitogram obtained in system RPC18 / chloroform+methanol+formic acid (44+3.5+2.5; v/v/v): 1. protocatechuic acid; 2. caffeic acid; 3. p-coumaric acid; 4. ferrulic acid; 5. isoferrulic acid; 6. m-coumaric acid; 7. o-coumaric acid; 8. salicylic acid; 9. quercitin; 10. 3.4-dimetoxycinnamic acid; 11. cinnamic acid; 12. kaempferol; 13. chrysin; 14. galangin; 15. 4'-methoxy-3,5,7trihydroxyflavone; 16. caffeic acid phenylesther; 17. artepillin - C

Rysunek 2 Płytka chromatograficzna z rozdzielonymi składnikami propolisu obserwowana w świetle lampy UV light l=366 nm i densytogram składników EEP uzyskany w układzie RP18 / chloroform+metanol+kwas mrówkowy (44+3,5+2,5; v/v/v): 1. kwas protokatechowy; 2. kwas kawowy; 3. kwas p-kumarowy; 4. kwas ferulowy; 5. kwas izoferulowy; 6. kwas m-kumarowy; 7. kwas o-kumarowy; 8. kwas salicylowy; 9. kwercetyna; 10. kwas 3,4- dimetoksycynamonowy; 11. kwas cynamonowy; 12. kamferol; 13. chryzyna; 14. galangina; 15. 4'-methoksy-3,5,7trihydroksyflawon; 16. ester fenyloetylowy kwasu kawowego; 17. artepilina - C

zaarenes at relatively low concentrations in sewage sludge and in the air, as well as in thermally processed food [13].

In the similar manner 7-ketocholesterol and 7-hydroxycholesterol as products of cholesterol biotransformation were determined in the meat samples [14]. These derivatives are formed by free radical or enzymatic transformation of cholesterol. These compounds can be isolated from serum samples of patients with hypercholesterolemia and determined by TLC with densitometry and a fluorescent lamp in the free form, and after derivatisation using Liebermann-Burchard reagent [15, 16].

The use of fluorescence detection in planar chromatography with densitometry can often increase the limits of detection and quantification of xenobiotics, but it is necessary to carry out optimization of the detection conditions, the best for each of the determined compounds separately, i.e., method of visualization, wavelength, beam width, time of determination, scanning mode (Fig. 3), etc.

Majority of carcinogenic polycyclic aromatic hydrocarbons isolated from the aqueous matrix and separated on HPTLC plates coated with silica gel impregnated with caffeine were determined at a wavelength of 365 nm [17].

Analysis of procancerogenic compounds with sufficient sensitivity (and even in some cases with more sensitivity than using a UV-Vis detector) and good reproducibility was performed using a fluorescent lamp [18].

In several studies using Dragendorff's reagent, visualization of nicotine and its N-oxide was carried out, and orange-red spots on a yellow background were observed. This enables the detection and determination of these metabolites of nicotine in biological and environmental samples using visible light. It was possible to increase almost 5 times the detection limits of nicotine in the analysed material compared to the limit of detection obtained for nicotine without spraying with Dragendorff's reagent (Fig. 3) [19].

Visualization of nicotine and its metabolites on the chromatographic plates by spraying reagents may only be used for the qualitative confirmation of their presence in the test samples, mainly because the resulting colour spots are nondurable. The main metabolites of nicotine such cotinine and trans-3'-hydroxycotinine cannot be detected in this way. However, they can be detected and determined by planar chromatography with densitometry not only in extracts isolated from a variety of biological materials under the conditions described previously [20-22], but also in environmental samples after the transformation of nicotine under the influence of environmental factors, e.g. in air [23].

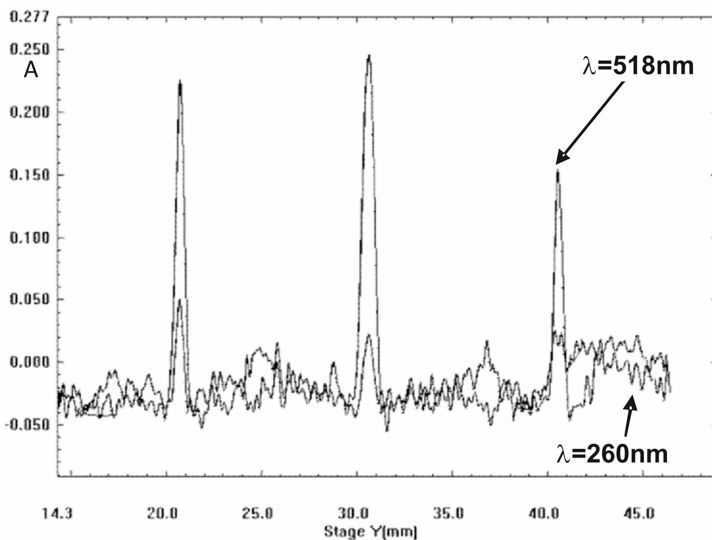

Figure 3 Nicotine identified on RP18 plates before (lower peaks) and after spraying with Dragendorff's reagent (higher peaks)

Rysunek 3 Nikotyna identyfikowana na płytkach RP18 przed (niższe piki) i po spryskaniu odczynnikiem Dragendorffa (wyższe piki)

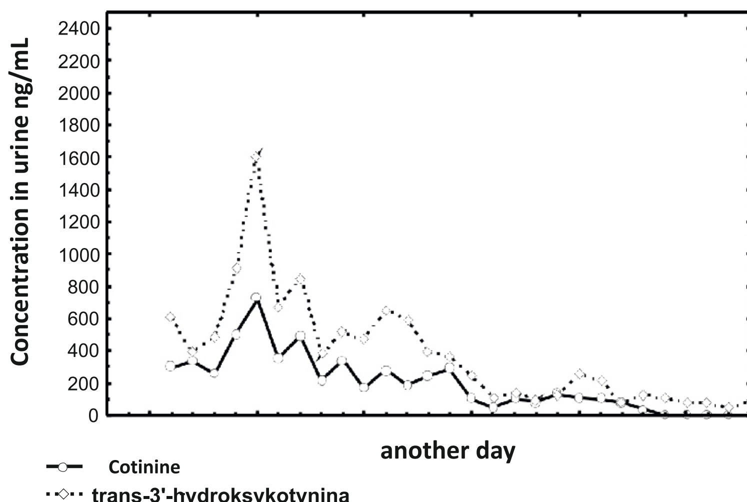

Figure 4 Monitoring the major metabolite of nicotine concentrations in urine of people quitting smoke: cotinine and trans-3'-hydroxycotinine determined by TLC with densitometry

Rysunek 4 Monitorowanie stężeń głównych metabolitów nikotyny w moczu osoby rzucającej palenie: kotyniny i trans-3'-hydroksykotyniny oznaczonych przy wykorzystaniu techniki TLC i densytometrii

Defined smokers often attempt to immediate or gradual cessation with/without nicotine replacement therapy (NRT). A detailed analysis of the major metabolites of nicotine excreted with the urine during stopping smoking was performed by means of planar chromatography with densitometry in the chromatographic system described previously [19]. An example of such monitoring is shown in Figure 4.

A similar effect of separation of the major nicotine metabolites to the previously mentioned can be achieved using Overpressured Layer Chromatography (OPTLC) – the fact that the obtained results are achieved in a slightly shorter period of time [19]. Comparison of chromatographic separation selectivity of major nicotine metabolites (cotinine and trans-3'-hydroxycotinine) by OPLC and TLC techniques in the same chromatographic system reveals that slightly better results can be obtained by the OPLC technique (Fig. 5).

However, the choice of the technique for routine determinations depends not only on selectivity, but also the overall analysis time. In this case, using this chromatographic system under study, a small shorten of separation time of the major nicotine metabolites does not compensate for time consuming procedure of preparing plates. Furthermore, this technique is limited by the use of only certain types of chromatographic plates [19].

The observed results obtained by TLC with scanning densitometry suggest that the predominant metabolite in urine of people during stopping smoking can be trans-3'-hydroxycotinine, as evidenced by the molar ratio of the major metabolites in urine, as well as the time after which the major nicotine metabolites are eliminated with urine after smoking cessation.

Studies using a densitometer, in which the molar ratios of the main nicotine metabolites or its percentages in serum, urine and amniotic fluid were carried out in earlier described chromatographic system, enabled assessment of exposure to tobacco smoke in pregnant women and their offspring from the region of Upper Silesia, [21] and can be applied in metabolomics (Fig. 6).

The synthesis of new stationary phases create other additional features in the biochemical and environmental analysis [24]. The results obtained in the pilot study on the use of aluminium plates coated with silica gel bound with C30 alkyl chains for the separation of the metabolites of nicotine, indicate the possibility of improved retention parameters in the same chromatographic system [19].

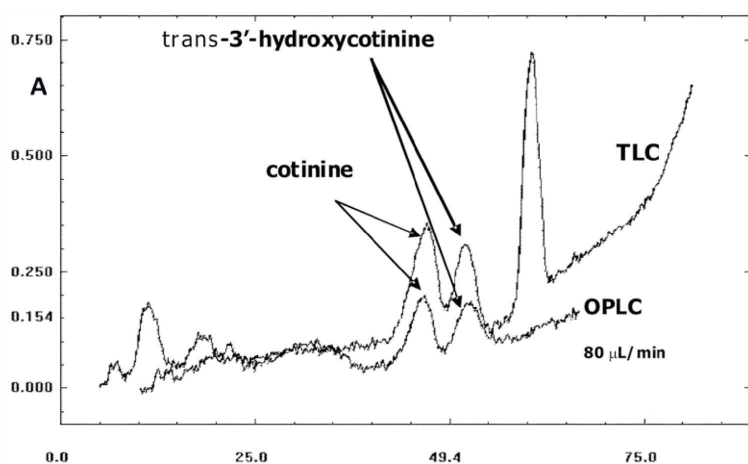

Figure 5 Comparison of the effects of the main metabolites of nicotine separation in the chromatographic system of RP-18 / acetonitrile+water (88+12, v/v) + sodium octane sulfonate (50 mg/100 ml of the mobile phase) separated by techniques planar chromatography: OPLC and TLC

Rysunek 5 Porównanie efektów separacji głównych metabolitów nikotyny rozdzielanych w układzie chromatograficznym RP-18/ acetonitryl+woda (88+12, v/v) + oktanosulfonian sodu (50 mg/100 ml fazy ruchomej) technikami chromatografii planarnej TLC i OPLC

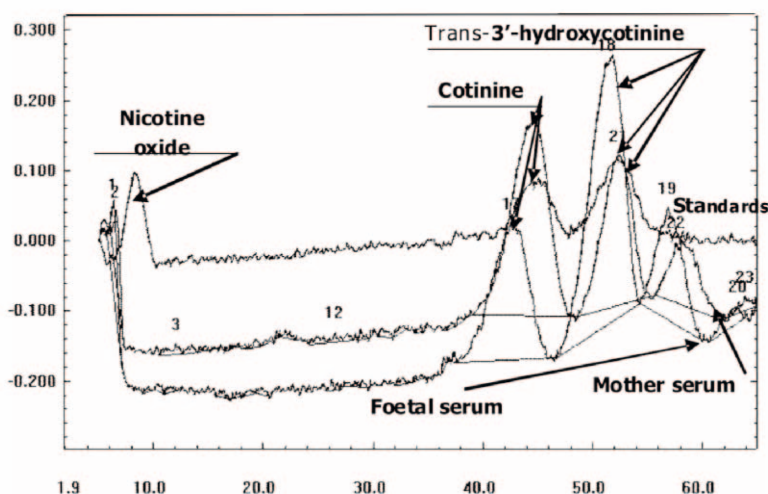

Figure 6 Thin layer chromatograms of nicotine and its main metabolites determined in the serum of mothers and their newborn children; TLC-RP-18 / acetonitrile+water (88+12, v/v) + sodium octane sulfonate (50 mg/100 ml of the mobile phase), scanning „zigzag" mode, at l=260 nm)

Rysunek 6 Chromatogramy cienkowarstwowe nikotyny i jej głównych metabolitów oznaczonych w surowicy krwi matek i ich nowo narodzonych dzieci; TLC-RP18/ acetonitryl+woda (88+12, v/v) + oktanosulfonian sodu (50 mg/100 ml fazy ruchomej, skanowanie techniką „zigzag"przy l=260nm)

Densitometer used in planar chromatography allows not only qualitatively and quantitatively determination of separated substances but also offers opportunities to obtain spectra of these compounds on chromatographic plates (Fig. 7).

A scanning densitometer CS 9310 Shimadzu (Japan) equipped with a deuterium and a tungsten lamp, as well as a fluorescence lamp described in this paper, is one of the most used appliances in the Department of Chemistry, Faculty of Medicine, Division of Medicine with Dentistry in Zabrze, Silesian Medical University in Katowice.

The device has been repaid many times, and the results of its use led to the writing of several doctoral dissertations and three postdoctoral work. Furthermore, densitometry was applied in several ministerial projects, statutory works and several collaborations between the Units of Silesian Medical University, and beyond SUM (Rzeszow University of Technology, University of Silesia, University of Humanities and Technology in Bielsko-Biala), as well as work carried out under cooperation with the University of Tübingien (Germany) [24].

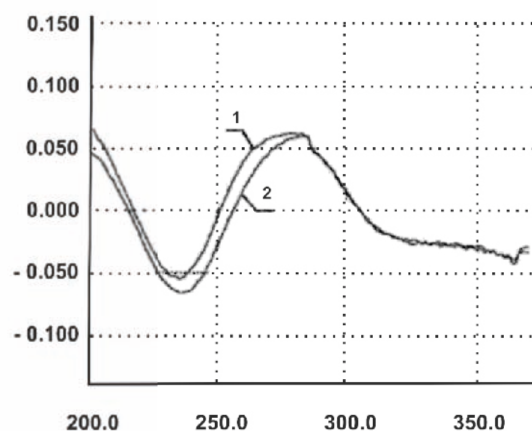

Figure 7 UV spectra standard of cotinine – (1) and cotinine identified in smoker's urine – (2) on RP18 TLC plate after background substraction coming from stationary phase

Rysunek 7 Widmo UV wzorca kotyniny – (1) i kotyniny zidentyfikowanej w moczu palacza – (2) na płytce RP18 TLC po odjęciu tła pochodzącego z fazy stacjonarnej

An important advantage of the application of the almost already obsolete device is its reliability, and above all, low running costs, not very complicated operation, the possibility of multiple repetition of determinations, ever under the same conditions for samples and reference substances.

A disadvantage of the detector densitometry is relatively low sensitivity, which can be of importance in the determination of the compounds present in the samples with low concentrations in the samples, the collection of larger volumes is difficult or even impossible.

As long as the computers dedicated to densitometer software are accessible, there is a chance of using this reliable apparatus to scan thin layer chromatograms. The problem of the work out of the data becomes even more complex in the age of frequently updated web browsers because they require corresponding quality computers. This extends the time of achieving of the results, because the data transfer cannot be carried out on-line.

At present densitometer is used to assess exposure to tobacco smoke of medical students [25] and patients suffering from multiple sclerosis. The more that the urine samples, in which are determined the major nicotine metabolites can be harvested non-invasively and in any volume.

Due to the large scientific role of described above, albeit not very modern and even obsolete densitometer the question can be raised: what to do with such indestructible devices that can fulfil didactical needs rather than be recommended as scientific apparatus?

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