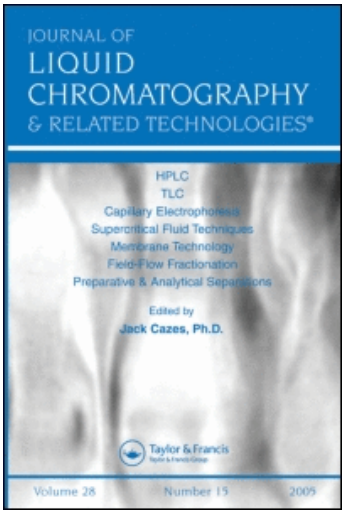

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Improved Detection of Ergosterol, Stigmasterol, and Selected Steroids on Silica Coated TLC Plates using Phosphomolybdic Acid Staining

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Abstract: In this work, improved detection of selected steroids on silica coated TLC plates using post-run derivatisation with phosphomolybdic acid (PMA) was investigated. Particularly, spot visualization of ergosterol, stigmasterol, dihydrocholesterol, 4-cholesten-3-one, cholecalciferol, and cholesterol acetate was studied. It has been found that the quantitative effect of PMA dyeing is strongly time and temperature dependent. Contrary to the data reported in literature, our experimental work revealed that the best conditions for robust detection of analytes investigated can be expected if the silica coated chromatographic plates are heated within a relatively low temperature region, between 40 and 80°C and for more than 20 minutes.

Keywords: Thin-layer chromatography, Spots visualization, Densitometry, Temperature, Phosphomolybdic acid (PMA), Steroids

INTRODUCTION

Thin-layer chromatography (TLC) allows fast identification of a number of different class of compounds, including steroids by post-run application of simple visualization reagents. From a practical point of view, quantitative determination of analytes is also possible; however, it depends strongly on

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the derivatisation reagent and procedure, the TLC plate data acquisition technique, as well as the chromatographer's manual skills. For example, phosphomolybdic acid (PMA) can give intensely blue mixed oxides with a number of substances, particularly for those that are UV-VIS light transparent. Such a coloring process may be considered as a quantitative method and possible color reaction mechanism was described in literature.[1] Blue colored derivatisation products, for which analytical wavelength λ_{max} is usually in the range 600–900 nm, can be viewed by light and electron microscopy, photographed, scanned, or measured by spectroscopic techniques.

Recently, the PMA reagent is frequently referred to as the most commonly employed stain for thin-layer chromatographic detection of lipids, saponins, prostaglandins, terpenes, aminophospholipids, and common sterols, including bile acids and cholesterol esters.[2–7] Visualization protocols based on the color reaction of analytes with phosphomolybdic acid are frequently applied for purity confirmation and substance identification protocols as described in the European Pharmacopoeia monographs.[8]

Our previous work was focused on the optimization of TLC detection by phosphomolybdic acid staining for robust quantification of UV-transparent steroids like cholesterol and bile acids.[9] In this study, the detection ability of PMA for different classes of steroids important from the medicine and pharmacy point of view including ergosterol, stigmasterol, cholesterol acetate, dihydrocholesterol, 4-cholesten-3-one, and cholecalciferol was investigated.

EXPERIMENTAL

Chemicals

Ergosterol, stigmasterol, dihydrocholesterol, 4-cholesten-3-one, and cholecalciferol were products of Sigma (St Louis, MO, USA). Cholesterol acetate was obtained from The British Drug Houses LTD. Mobile phase components: methanol (LiChrosolv) and dichloromethan (LiChrosolv stabilized with 2-methyl-2-buten), as well as phosphomolybdic acid hydrate were the products of Merck (Darmstadt, Germany).

Steroids TLC Separation and Visualization

Steroids standards stock solutions were prepared in methanol at a concentration of 1 mg mL^{-1}. In each case, 1 μg (1 μL of stock solution) was transferred onto the plate starting line using a Hamilton type micropipette. Chromatography was performed on 50 × 100 mm Kieselgel 60WF254S glass TLC plates (Merck, Darmstadt, Germany). The chromatographic plates were developed in a horizontal chamber (Chromdes, Lublin, Polska) using methanol-dichloromethane mobile phase (5:95%, v/v). All runs were

performed under saturated mobile phase vapor conditions at constant temperature, 30°C. The separation temperature was controlled by an external thermostatic air circulating oven (ST2/B/40 Pol-Eko-Aparatura, Wodzisław Sl., Poland). Steroid spots were visualized by spraying the plates twice with 10% phosphomolibdic acid in methanol (the second portion of PMA reagent solution was applied on the plate surface after 5 minutes). After solvent evaporation, the plates were heated in the gravity convection oven (Ecocell, BMT, Brno, Czech Republic) at temperatures of 40, 60, 80, 100, 120, and 140°C, and times ranging from 2 to 40 minutes (2, 5, 10, 20, or 40 minutes). In each case, the TLC plates were cooled at room temperature and under low humidity conditions (glass cabinet with blue silica gel) for 15 minutes and then immediately scanned using a digital office scanner (Plustek OptiPro S12 scanner).

Chromatograms Digitalization and Image Data Procedure

The TLC plate raw images were recorded with 300 DPI optical resolution and 8 bits per RGB channel color deep mode, and then saved as the TIFF files without compression. Chromatographic spots quantification was performed with Scion Image freeware (Scion Corporation; ver. 4.0.3.2; http://www.scioncorp.com/). Appropriate time, temperature contour maps were generated with the help of Statistica V.6.0 (StatSoft) software, using the distance weighted least squares algorithm.

RESULTS AND DISCUSSION

According to the data presented in the literature, typical conditions for analytes visualization with PMA reagent involve fast heating of TLC plates (usually for less than 10 minutes) at a temperature more than 100°C.[2–8] Unfortunately, robust spot detection under such conditions cannot be obtained, because within the first minutes of the plate heating, the temperature changes very quickly and the average surface temperature is substantially different from the desired destination temperature.[9] Such unsteady, nonlinear, and uncontrolled conditions can be a significant source of the poor precision in quantitative thin-layer chromatography performed on the glass based plates. Moreover, it has been found that if the PMA detection is performed for a short time and under high temperature conditions, the signal/noise ratio and spots signal stability are usually low.[9] This may result in poor repeatability of TLC spots quantitative measurements.

To explore this problem, the PMA detection of selected steroids using a wide range of temperatures from 40 to 140°C and different heating times from 2 to 40 minutes has been studied. Selection of the component of interest was due to the steroids high UV transparency and their potential

application as the environmental biomarkers for endocrine disrupting phenomenon; particularly, detection of ergosterol, stigmasterol, cholesterol acetate, dihydrocholesterol, 4-cholesten-3-one, and cholecalciferol was investigated.

Graphs in Figure 1 show signal intensity maps calculated from the raw data points measured on the silica coated TLC plates. On these maps, the dark areas correspond to the maximum values of the spot signal expected.

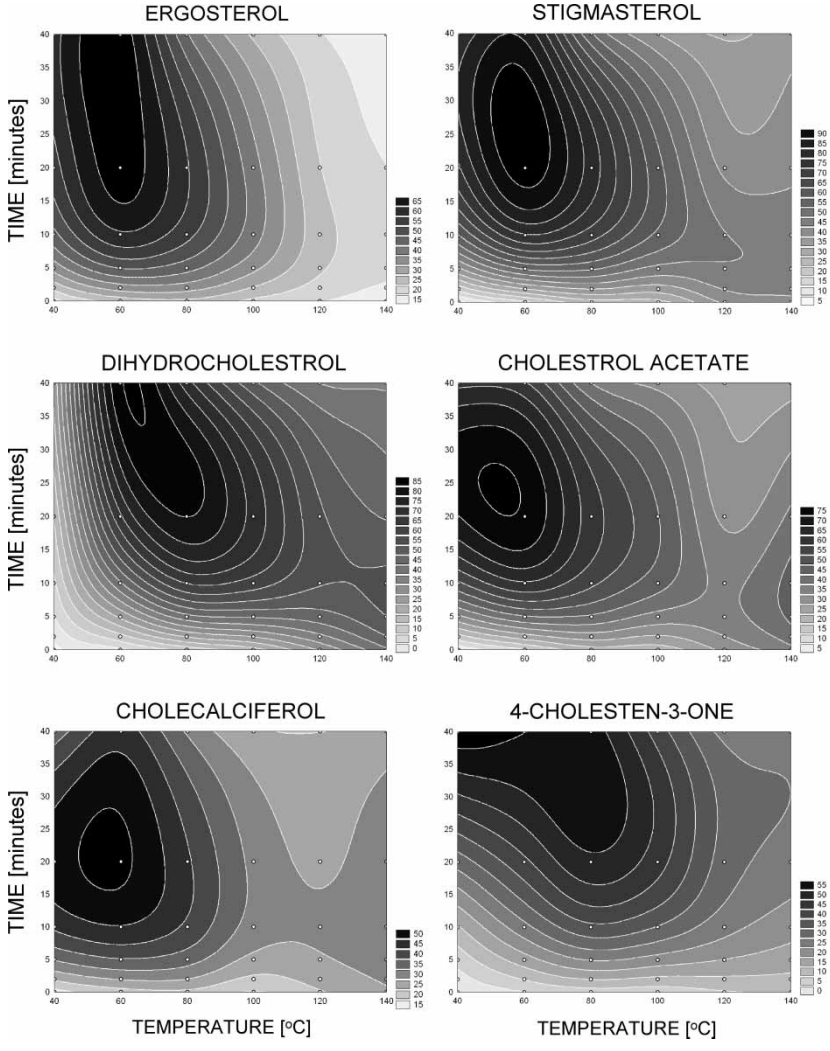

Figure 1. Signal intensity contour maps generated for the steroids studied. Two-dimensional profiles were calculated from the raw data obtained for analytes visualized on silica gel coated glass based TLC plates.

As can be seen, the effect of PMA dyeing is strongly time and temperature dependent. Similar, to cholesterol and bile acids,[9] for each steroid the investigated maximum signal intensity is located in the region of low temperature and long heating time. According to the data obtained, robust detection of the components of interest may be expected if the plates are heated in a relatively low temperature region, between 40 and 80°C and for more than 20 minutes. Under such conditions, sensitive detection of steroids can be expected, especially for stigmasterol, dihydrocholesterol, and cholesterol acetate.

CONCLUSIONS

To minimize uncertainty and maximize reproducibility of quantitative phosphomolybdic acid staining on the thin-layer chromatographic plates, the temperature and time of heating optimization is necessary. Contrary to the data presented in literature, a robust quantification of steroids studied can be obtained if post-run derivatisation is performed under a relatively low temperature and for longer heating times.

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