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CONTRIBUTION TO THE TLC SEPARATION OF URSOLIC AND OLEANOLIC ACID MIXTURE

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The aim of the study was to develop a rapid, simple, effective and reproducible TLC method for separation of a naturally occurring mixture of ursolic and oleanolic acids. Because of the similarity of chemical structures, *in situ* derivatisation by iodine was necessary to separate these triterpenic acids. Separation was achieved on silica gel plates. After derivatisation, a chromatographic plate was developed with the mobile phase consisting of light petrol, ethyl acetate and acetone (8.2:1.8:0.1, v/v/v) following visualisation by spraying with sulphuric acid in diethylether (25%, v/v) and heating to 120°C for 5 min. The method used enabled chromatographical differentiation of ursolic and oleanolic acid mixtures in all tested mutual ratios.

Keywords: *oleanolic acid – ursolic acid – TLC – separation*

INTRODUCTION

Oleanolic and ursolic acids belong to the pentacyclic triterpenes and are common constituents of many medicinal herbs, plants [1] and also vegetable oils [2]. These terpenes may exist in the form of free acids or as triterpenoid saponin aglycones in many plant species. A lot of them are used as medicinal plants in traditional medicine [3]. They possess a broad spectrum of important pharmacological effects. There are numerous data on their anti-inflammatory, hepatoprotective, anti-tumour, anti-HIV, antimicrobial, antifungal, antiulcer, gastroprotective, hypoglycaemic, antihyperlipidemic and cardiotonic properties in the literature [4–7].

Separation of oleanolic and ursolic acids by TLC is difficult because of the closely related physicochemical properties of both structural isomers (Fig. 1). Several methods have been described for the isomeric pair separation: HPLC [8–9], HPTLC [9–10], capillary zone electrophoresis [11] and GC-MS [12]. All these exploited sophisticated

equipment. An excellent, very simple and effective TLC method of ursolic and oleanolic acids separation after iodine derivatisation on HPTLC plates has been described by Wojciak-Kosior [13]. However, relatively high costs of analysis including the price of HPTLC plates was the reason to apply this method to the better available and economically more acceptable silica gel plates.

EXPERIMENTAL

Standard and sample preparation

All solvents and reagents were *pro analysis* grade from Mikrochem (Bratislava, Slovak Republic). Triterpenic acid standards were purchased from Sigma (St. Louis, MO, USA).

Stock solutions of ursolic acid (UA) and oleanolic acid (OA), were prepared by dissolving 0.003 g of UA in 5 mL of methanol (final concentration: 0.6 mg/mL), and 0.005 g of OA in 5 mL of methanol (final concentration: 1 mg/mL).

Chromatographic conditions

TLC plates coated with silica gel (Kieselgel 60 F_{254} Fertigplatten and DC-Alufolien Kieselgel 60 F_{254}, Merck, Darmstadt, Germany; Silufol UV 254 nm, Kavalier Votice, Czech Republic) were used in experiments. The plates were pre-washed with methanol and dried in a stream of a hot air before use. Standard solutions of ursolic and oleanolic acids were mixed in different ratios: 1+9; 2+8; 3+7; 4+6; 5+5; 6+4; 7+3; 8+2 and 9+1. Three μL of each mixture were spotted on a TLC plate.

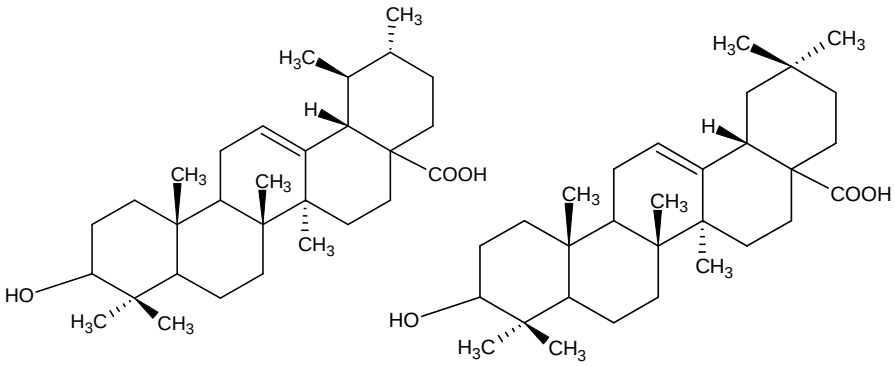
Prechromatographic derivatisation

The plates were developed in a horizontal chamber with 1% iodine solution in chloroform until spotted samples were covered by iodine solution. The plates were placed in darkness for 10 minutes. When the derivatisation reaction was finished, the plates were dried in a stream of warm air to remove the excess of iodine.

Chromatographic procedure and detection

The plates were developed once with a mixture of light petrol:ethyl acetate:acetone (8.2:1.8:0.1, v/v/v) as a mobile phase. After drying in a stream of warm air the plates were sprayed with H_2SO_4/diethyl ether reagent, dried for 10 minutes and then heated to 120°C for 5 minutes.

The visualised spots were documented by a digital camera in daylight (Fig. 2, Fig. 4) and in UV 366 nm (Fig. 3) respectively.



Ursolic acid **Oleanolic acid**

Figure 1. Structure of ursolic and oleanolic acids

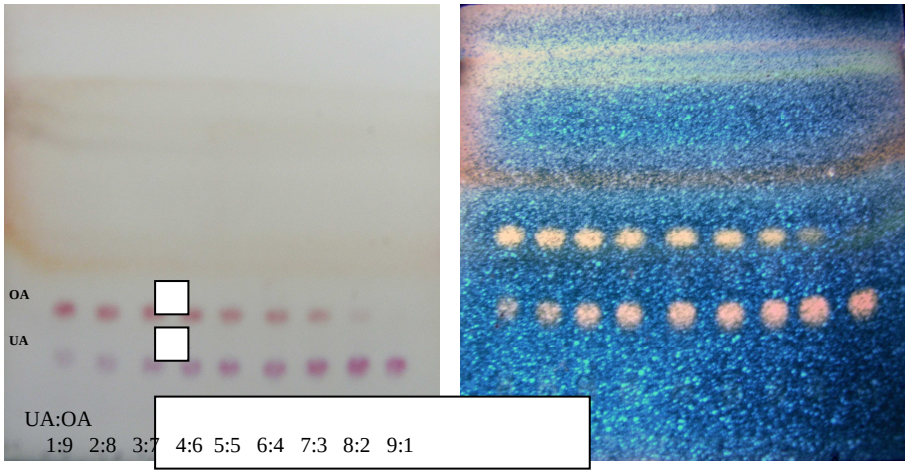


Figure 2 Figure 3

Figure 2. Daylight visualisation of ursolic and oleanolic acid mixture separation after single development of the TLC plate. Upper spot: oleanolic acid, lower spot: ursolic acid.
Figure 3. UV 366 nm visualisation of ursolic and oleanolic acid mixture separation after double development of the TLC plate. Upper spot: oleanolic acid, lower spot: ursolic acid.

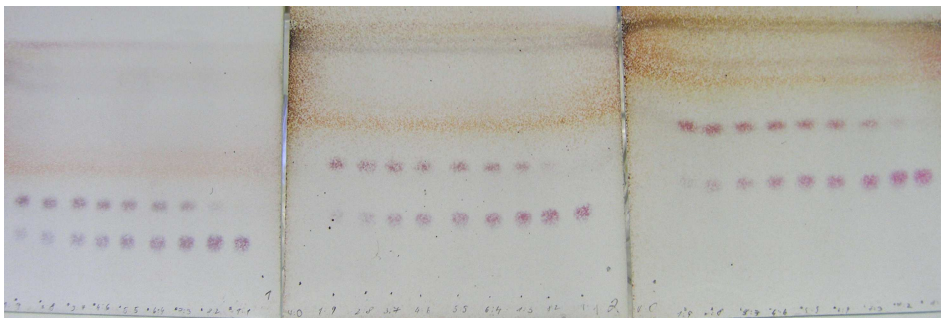


Figure 4. Daylight visualisation of ursolic and oleanolic acid mixture separation after single (left), double (middle) and triple (right) development of the TLC plate. Upper spot: oleanolic acid, lower spot: ursolic acid.

RESULTS AND DISCUSSION

Standard methods of separation and chromatographic carriers commonly used for TLC separation did not allow a separation of the triterpenic acids mentioned above. Our experiments on silica gel TLC plates using various organic solvents mixtures did not bring any satisfactory results. Distinguishable differences in Rf values were described in literature using HPTLC plates [9, 10] or C_{18} RP-HPTLC ones developed in different elution systems [9], but their application for analysing complex plant material with different ratios between both acids is questionable due to their very similar Rf values. Because of high cost of HPTLC plates, we tried to apply a method that includes derivatisation with iodine on routinely available silica TLC plates.

Stock solutions of oleanolic and ursolic acids were prepared by dissolving appropriate mass of each compound in 5 mL of methanol (final concentration: 0.6 mg UA/mL and 1 mg OA/mL). After preparation of mixtures of different mutual ratios, samples were spotted on TLC plate and developed. Due to lower sensitivity of oleanolic acid to detection by sulphuric acid (data not shown), stock solution of this one needed to be slightly more concentrated than stock solution of UA. Usage of silica gel plates of different producers did not influence quality of separation (data not shown). Excellent separation was achieved on all types of TLC plates even after the first development (ursolic acid Rf = 0.15; oleanolic acid Rf = 0.31). According to Wojciak-Kosior [13] oleanolic acid reacts more readily with iodine, so the reaction was carried out at room temperature, whereas ursolic acid requires a higher temperature (above 40°C). The second development had positive influence on the quality of separation and increased the difference between Rf values of UA and OA. Additional (third) TLC plate development did not show any significant influence on Rf values (for details see Fig. 4).

This method shows sufficient effectiveness of studied separation process even in low concentrations of tested acids. Positive visualisation of ursolic acid at daylight after detection with sulphuric acid reagent was achieved after spreading 0.18 µg of UA in mixture UA:OA 1:9. Probably lower detection sensitivity of OA does not allow a visualisation at higher dilutions levels (UA:OA 9:1 and 8:2 – data not shown). The lowest concentration for a visualisation of OA was 0.3 µg in a UA:OA ratio 9:1.

As a conclusion, we can confirm that the method used is simple, rapid, reproducible and effective in TLC separation of ursolic and oleanolic acids or their combinations with different mutual ratios.

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PRÍSPEVOK K TLC SEPARÁCII ZMESI URSOLOVEJ A OLEÁNOLOVEJ KYSELINY

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Cieľom práce bolo vyvinúť rýchlu, jednoduchú, účinnú a reprodukovateľnú metódu na separáciu ursolovej a oleanolovej kyseliny pomocou tenkovrstvovej chromatografie. Vzhľadom na štruktúrnu podobnosť oboch látok bolo nevyhnutné použiť ich derivatizáciu jódom *in situ.* Separácia sa podarila na platniach silikagélu. Po derivatizácii sa platne vyvíjali v sústave: petroléter:etylacetát:acetón v pomere (8,2:1,8:0,1 v/v/v) s následnou vizualizáciou škvŕn detekciou kyselinou sírovou v dietyléteri (25%, v/v) a následným zahriatím pri 120 °C počas 5 minút. Metóda umožňuje chromatografické odlíšenie zmesí kyseliny ursolovej a oleanolovej vo všetkých vzájomných skúšaných pomeroch.

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