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Two-dimensional thin-layer chromatography with adsorbent gradient as a method of chromatographic fingerprinting of furanocoumarins for distinguishing selected varieties and forms of *Heracleum* spp.

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ABSTRACT

There are a lot of taxonomic classifications of the genus *Heracleum*, and many authors indicate they need revision. Morphological identification is difficult to perform, as there are only few characteristic differences between each *Heracleum* species, varieties and forms. Furanocoumarins are characteristic compounds for the *Apiaceae* family, and they can be found in the whole genus in large quantities. Despite this fact, it is difficult to use the furanocoumarin profiles of plants, for their discrimination, as furanocoumarins are difficult to separate, due to their similar chemical structures and physicochemical properties. In this paper, a new, simple method is proposed for the discrimination of selected species, varieties and forms of the genus *Heracleum*. Thin-layer chromatography (TLC) with an adsorbent gradient (unmodified silica gel + octadecylsilica wettable with water) enables complete separation of the structural analogues. The proposed method gives the possibility to distinguish selected species, varieties and forms of the *Heracleum* genus, as they produce distinctive furanocoumarin fingerprints. The method is characterised by high specificity, precision, reproducibility and stability values. It is for the first time that graft TLC is used for constructing fingerprints of herbs. The complete separation of ten structural analogues, by combining gradient TLC with the unidimensional multiple development technique, has not been reported yet.

1. Introduction

The genus *Heracleum* includes biennial and perennial plants characteristic for the northern hemisphere. There are almost 125 *Heracleum* species in the world [1]. Plants from this genus are little used in modern herbalism, but have been employed in the treatment of laryngitis and bronchitis. The roots and leaves of *Heracleum sphondylium* are rarely used as aphrodisiac, digestive, mild expectorant and sedative drugs. A tincture made from aerial parts of the plant has also been used to relieve general debility. Different *Heracleum* species were reported to be used in rheumatic diseases, lumbago, gastralgia, fractures, contusions and strains, in the treatment of hypertension, epilepsy, paralysis, dysentery and diarrhea [1]. Modern studies reveal the essential oil of some *Heracleum* species exhibits antibacterial and anticandidal activity [2,3]. The genus *Heracleum* is also a popular source of furanocoumarins (e.g. bergapten, byakangelicol, phellopterin, xanthotoxin, isopimpinellin, imperatorin, etc.) which exhibit biological activity of wide spectrum [4–6]. They are important drugs in vitiligo and psoriaris therapy [7,8]. Their ability to covalently modify nucleic acids is used in the process called "extracorporeal photopheresis", that is medically necessary for either of the following clinical indications: erythrodermic variants of cutaneous T-cell lymphoma (e.g. mycosis fungioides, Sezary's syndrome) or chronic graft-versus-host disease, refractory to standard immunosuppressive therapy [9,10].

The taxonomic classification of the whole genus *Heracleum* is very complicated. For example Gawłowska mentions two main species, present in Polish flora, *H. sphondylium* and *Heracleum sibiricum*, that are divided in different varieties and forms [11]. Ochsmann indicates the taxonomic classification of the mentioned genus needs revision, as all existing treatments do not take into account the high variation of the characters used [12].

The amount of particular furanocoumarins depends on the enzymes active in plants' secondary metabolism. Plants with similar enzyme profile contain comparable amount of secondary

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metabolites, that are products of chemical reactions induced by these enzymes. Thus furanocoumarins' content, in different species, varieties and forms may contribute to their better distinction, and better understanding of the taxonomy of the whole genus *Heracleum*.

Chromatographic fingerprinting is one of the most popular methods in the herbal medicine studies. It has been widely introduced and accepted by WHO (World Health Organisation), FDA (US Food and Drug Administration), EMEA (European Medicines Agency), German Commission E, British Herbal Medicine Association, Indian Drug Manufacturer's Association [13]. According to its definition chromatographic fingerprint is a chromatogram that represents the chemical characteristics of a herb [14]. In the majority of published methods, chromatographic fingerprints of herbs have been constructed by a single chromatogram [15–20]. These methods are usually focused on qualitative and quantitative determination of individual or several known compounds, but are inadequate in case of more complex mixtures' quality assessment, or in case of plants containing substances, that are structural analogues. Some authors recommend using a combination of analytical methods with different separation principles, to produce multiple chromatographic fingerprints [21–26]. These methods are complicated, demand sophisticated equipment, and are usually time-consuming.

In recent years, HPTLC with digital scanning is becoming more and more popular in chemical fingerprints construction. It is due to the ability of this method to produce picture-like images, that can be stored as documentation, and easily compared with other images [27,28]. HPTLC plates can be also evaluated with the use of different derivatization reagents, at any time. TLC is the method of choice when many samples must be compared, when flexibility is important, and when rapid quantitative data are needed at low cost per sample [27]. Unfortunately structural analogues are difficult to separate in one chromatographic run, and this is the case in furanocoumarins' investigations. They have similar chemical structures and physicochemical properties and their separation and isolation demand multistep procedures [29–32].

Multidimensional chromatography is a popular method for separation of multicomponent mixtures. Planar chromatography gives the possibility to conduct two-dimensional separations with the use of the same stationary phase with different eluent systems [33–37] or by using stationary phase gradient [38–41]. The highest resolution in separation is achieved by combining two comprehensive (characterised by different selectivity) systems. These are usually normal phase systems with adsorption separation mechanism, and reversed phase systems with partition separation mechanism [36]. The use of RP-18W and silica gel dual phase plates is complicated because solvent used in the first direction (normally MeOH, MeCN, tetrahydrofuran in water) modifies stationary phase (strong adsorption of water on silica gel). It leads to inadequate reproducibility, as the mobile phase usually cannot be quantitatively removed. In such case the use of two single plates is recommended [42]. It can be realized with the use of the method called "graft TLC". In this method partially separated compounds, in the first run, are transferred to the another adsorbent and chromatographed in the second dimension [32,38].

The aim of this study was to verify as to what degree graft TLC can be applied for constructing chromatographic fingerprints of furanocoumarins for distinguishing selected varieties and forms of *Heracleum* species.

Already presented chromatographic systems cannot be applied for constructing fingerprints, as they do not provide sufficient coumarin resolution. The combination of TLC with adsorbent gradient and unidimensional multiple development technique is used for the first time to separate ten closely related coumarins, and then applied for constructing fingerprints. The new systems are intended also to shorten the analysis time, which was quite long in case of already presented graft TLC systems [32].

2. Experimental

2.1. Apparatus and reagents

Xanthotoxin and umbeliferone were obtained from Fluka (Buchs, Switzerland), and the others were isolated and purified in the Department of Pharmaceutical Botany. The process of isolation and purification of the coumarins was described elsewhere [43]. The used standards are listed in Table 1.

Binary mixtures of solvents: methanol (MeOH) in water, and ethyl acetate (AcOEt) in *n*-heptane (H) were used as mobile phases. All solvents were of analytical grade from. E. Merck (Darmstadt, Germany).

TLC was performed on 10 cm × 10 cm and 10 cm × 20 cm glass-backed RP-18W F_{254S} HPTLC plates, and silica gel TLC 60 F_{254S} plates, all purchased from Merck

Solutions (1.6 mg/ml) of all test substances and plant extracts were applied to chromatographic plates, as spots, by means of a Camag automatic TLC sampler (Camag, Muttenz, Switzerland), and developed in horizontal DS chambers (Chromdes, Lublin, Poland). The locations of the spots were determined under UV light (λ = 254 and 366 nm). Results were visualised with the use of Camag TLC Reprostar 3 with computer program Videostore, and scanned with densitometer Camag TLC scanner3 with computer program CATS 4 (Camag) or with diode-array spectrophotometer working in the range 191–612 nm (J&M Aalen, Stuttgart, Germany).

2.2. Standard substance solutions and plant extracts

Bergapten, byakangelicol, heraclenin, phellopterin, isopimpinellin, xanthotoxin, umbelliferone, herniarin, scoparone and aesculetin were dissolved in methanol to prepare solutions at a concentration of 1.6 mg/mL each.

Extracts were obtained from fruits and roots of different varieties and forms of *H. sphondylium* and *H. sibiricum*. The list of investigated plant material and their collected location are present in journal supplement. All fruits were collected in August 2006, and roots in September 2006. The plant material was dried at room temperature. All samples were authenticated by Bogucka-Kocka (Pharmaceutical Botany Department, Medical University of Lublin, Poland, where voucher specimens were deposited) according to morphological characteristics.

Accurately weighed (approximately 15 g) powder of analysed fruits was extracted in Soxhlet apparatus with use of petroleum ether, for about 15 h. Obtained extracts were concentrated in a vacuum evaporator, at temperature of 40 °C. By keeping in the refrigerator, for 10 days, the crystalline coumarin fraction was obtained. It was separated from the liquid residue, which was again placed in the refrigerator for second cristallisation. Both obtained crystalline fractions were combined. About 25 mg of this combined coumarin fraction was dissolved in a calibrated flask and filled with methanol to 25 mL.

The chromatography is strongly affected by lipids present in the extract, so this way of treating coumarin containing extracts enables their purification from fats. The liquid residue, after the second precipitation, was checked for the presence of furanocoumarins. HPLC analysis showed only the trace amounts of coumarins. This did not influence the results of further experiments.