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Applications of Thin Layer Chromatography in the analysis and isolation of coumarins derived from medicinal plants

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ABSTRACT

Because of the common occurrence in plants and broad spectrum of their activity, coumarins are one of the most promising groups of natural compounds to investigate. TLC is very quick and simple method to analyze the composition of secondary metabolites in plants. It can be successfully applied as a screening procedure to verify a presence of coumarin compounds in a sample. In simple way it can be also used for evaluation of some biological activity of this compounds. Current knowledge of application of TLC to analytical separation of coumarin compounds is presented in this review.

Keywords: coumarins, chromatography, thin layer chromatography

INTRODUCTION

Structure and physical properties of coumarins

Coumarins are one of the most common secondary metabolites occurring in plants, especially widespread in families like *Apiaceae, Rutaceae, Rubiaceae, Hippocastanaceae, Solanaceae, Moraceae, Leguminosae, Compositae, Oleaceae, Gramineae*. They can be found both in the free form or in the glycoside form. They occur in a lot of different part of plants like fruits, underground organs, crust, leaves or stems at the concentration about 0.5-2% sometimes even up to 5-6% [11, 33]. Coumarins all are benzo--α-pyrone derivatives. Structurally the simple coumarin nucleus is phenylopropanoid with C6 benzene ring linked to C3 aliphatic chain [50].

Biosynthesis of coumarin in plants starts when phenylopropane passes through the schickimic acid cycle lead to trans-cinnamic acid. After that trans-cinnamic acid passes to o-coumaric acid, its glycoside and coumarin. In the similar way p-coumaric passes to umbeliferone. Furano- and pyranocoumarins are formed when the active isopren C5 join umbeliferon and this product cyclizes [27,50].

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Structurally, coumarins are divided into four basic groups:

- Sipmple coumarins which have basic benzo-á-pyrone structure;
- Isocoumarins which have 3,4-benzo-2-pyrone structure;
- Furanocoumarins:
  - psolarene type: contains furan ring condensed with benzo-α-pyrone structure in position 6,7;
  - angelicyn type: contains furan ring condensed with benzo-α-pyrone structure in position 7,8;
- Pyranocoumarins:
- Xanthyletine type: contains pyran ring condensed with benzo-α-pyrone structure in position 6,7;
- Seseline type: contains pyran ring condensed with benzo-α-pyrone structure in position 7,8.

Most of coumarins are substituted in position C-5, C-6 and C-8 with hydroxyl, metoxyl groups or aliphatic chains. In this positions glycoside bond can be also created. Coumarins glycosides when drying, undergo enzymolysis process and convert to aglycones. Solubility of coumarins depends of its structure. Glycosides are more likely soluble in water than free molecules. Generally they are soluble in nonpolar and medium polar solvents such as dichloromethane, chloroform or diethyl ether. Coumarins easy sublimate especially under reduced pressure. Coumarins solutions reveal strong fluorescence in UV light, which is helpful in identification of them [27].

Medical significance of coumarins

It is well known that coumarins are biologically active compounds. Their activity is closely related to its structure. Below the main activities are described:

Coumarins as an anti-tumoral agent

A lot of different cytostatic properties and cytotoxic activity have been observed along studies of coumarins. The mechanism of their action is not yet fully understood. Probably, they inhibit cell proliferation by interfering with mitotic spindle microtubule function. Their broad range of effects on the tumors shown by various in vitro and in vivo experiments and clinical studies are discussed. Low toxicity against normal cells and selectively high for neoplastic cells makes this group of compounds hopeful to investigate as a chemotherapeutic agents [38,58].

Coumarins as a neuroprotective agent in central nervous system (CNS) disorders

Coumarins are important as inhibitors of key enzymes like MAO- A, MAO – B, COX – 2, GABA – T, acting at different levels in the biosynthesis and metabolism of lipids, antioxidant and many others. This is the object of study in treatment of various diseases like Parkinson disease or Alzheimer disease [10]. Luszczki et al. [34], proved that imperatorin and osthol, two natural coumarin compounds, exerted the clear-cut antielectroshock action in mice. The direct comparison of the anticonvulsant profiles of these two compounds with valproate, the classical antiepileptic drug, revealed that imperatorin and osthol possess similar dose ranges protecting the animals against maximal electroshock-induced seizures. This research showed that imperatorin and osthol are very promising compounds, which may be used in the future to create a new formula of antiepileptic drugs.

Coumarins as an anti-fungal, anti-viral and anti-microbial agents

Coumarins are produced in plants as defense substances, when they are wounded or attacked by another organism. Selected coumarins are known with bacteriostatic and antifungal activities [7,57]. There are also reports that coumarins shows activity as a nonnucleoside reverse transcriptase inhibitors of human immunodeficiency virus (HIV) [29].

Coumarins UV absorption and phototherapy

Because of photosensibilized properties, furanocoumarins are applied in therapy of albinism and psoriasis. Some of metoxy- and hydroxycoumarins are used as additives in sun blocking creams because of their ability of absorbing UV light [7,50].

Other

There is a lot of other activities of coumarins, one of the major is a property to blocking Ca-channel in heart muscle and circular vessels by pyranocoumarins present in *Ammi visnaga L.* fructus as visnadin, dihydrosamidin and samidin [7,11]. Aesculin isolated from *Aesculus hippocastanum* bark, flowers and fruits is widely used as component of oral drugs, ointments and suppositories in therapy of hemorrhoids. Ointments and oral drugs with aesculin are also used in therapy of varicose veins of lower limbs.

The anticoagulant effect of some coumarins is related to blocking by them hepatitic synthesis of certain blood clotting factors and thus the impairment of this process. This is particularly the protrombin and factors VII, IX and X of the blood coagulation cascade. In creation of these proteins vitamin K is necessary. Some coumarins because of close structural relationship to vitamin K replace it in synthesis cycle. Structural similarity is not related to functional similarity and does not occur in normal synthesis of these proteins in blood coagulation cascade. Coumarins therefore act as the so-called anti-metabolites of vitamin K. Most known coumarin used of its anti-coagulant effects are warfarin and dicoumarol [32].

Coumarins are used in colorimetry and in construction of dye lasers widely used in a lot of fields, among other things in medicine. Some of antipyretic, analgesic, antiphlogistic, anti-inflammatory, antioxidant, spasmolitic, choleretic, cholagogic activities of coumarins can also be found in literature [7,11,38,50]. Because of the wide use, coumarins are very promising group of compounds to investigate.

ANALYSIS OF COUMARINS – SAMPLE PREPARATION

Sample pretreatment is typically one of most time-consuming steps of analytical process, particularly when solid samples are involved. Depending on the physicochemical properties of target compounds, the most efficient solvent for extraction should be chosen. Because of very differentiated chemical structure and various hydrophobic and hydrophilic properties of coumarins, many solvents with increasing polarity and solution strength are used to extract them in classical extraction method like, maceration, percolation, extraction on cooling water bath or Soxhlet extraction. The individual solvent strength and selectivity values are used to formulate extraction strategy [36]. Głowniak et al. [17] proved that application of mixed binary or ternary solvent from N, A or B group gives significant (up to 30%) increase of extraction efficiency of coumarins. For group A, dichloromethane, chloroform, trichloroethylene, for group N oil ether, petrol, tetrachloromethane and for group B diisopropyl ether were used. The fruits of *Angelica archangelica* and *Libanotis intermedia Rupr.* were exhaustively extracted with this solvent system. Further analysis of obtained extracts was made by TLC using silica gel as a stationary phase and mixture of benzene and ethyl acetate (85:15), as a mobile phase. For fruits of *Angelica archangelica* three coumarins (impera-

torin, bergapten, xantotoxin), and for fruits of *Libanotis intermedia Rupr.* one dominating coumarin compound (edultin) was identified.

Next to the traditional methods of extraction, some modern methods like: ultrasound-assisted solvent extraction (USAE), microwave-assisted solvent extraction (MASE), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) and others are applied in extraction of coumarins.. The comparison of some classical and modern method of extraction of furanocoumarins was showed by Komaszewska et al. [28] In this research the classical methods, like maceration and extraction on water bath under reflux with more modern methods like maceration with shaking, USAE and USAE after homogenization were compared. As a solvent for extraction petroleum ether was used. The concentration of furanocoumarins in particular extracts was determined by densitometry detection. The highest yield of furanocoumarins was obtained by homogenization followed by 100% sonnification under reflux at the temperature of boiling solvent.

Accelerated solvent extraction as an efficient method of extraction of coumarins from *Heracleum leskowii* fruits was examined by Skalicka-Woźniak and Głowniak [43]. Effects of most important parameters in ASE, temperature and solvent, on the yield of extraction were evaluated. Dichloromethane and methanol were chosen as the most suitable solvents for extraction of umbeliferone, xanthotoxin, angelicin, isopimpinellin, bergapten and isoimperatorin. It has been proved that increase of temperature, affects higher yield of extraction when petroleum ether and dichloromethane were used as a solvents.

The most modern method that can be applied to isolate coumarins from plant material is supercritical fluid extraction (SFE). The supercritical fluid is characterized by high diffusion coefficient, low density and viscosity, and lack of surface tension and gases. Because of its properties the fluid penetrates the matrix of plant material with high efficiency, dissolving the substances in this matrix. The main advantages of this method are: high process speed, the lack of organic solvent used, low risk of oxidation or thermal degradation of extracted substances and possibility of "on-line" and "off-line" extraction", coupling with qualitative and quantitative chromatographic methods. The SFE method was successfully applied for the separation of furanocoumarins from *Angelica archangelica* [13].

There is a method of isolation of coumarins from plant material that is based on its lactone type structure. The alcohol or water – alcohol solution of KOH destroys the lactone ring in coumarins and coumarin acids arise. After acidation these acids cyclize to coumarins again, and then they are extracted with oil ether. This method is successful but it can destroy the epoxide structures and ester bonds in side chains of coumarins [53]. For isolation of coumarins the sublimation and fractionating distillation in high vacuum and crystallization from organic solvents can be also successfully used [2,51]. Sample preparation stage is first and very important stage in analysis of plant material and should be done properly to give good results in further stages of analysis.

ANALYSIS OF COUMARINS – THIN LAYER CHROMATOGRAPHY (TLC)

Thin-layer chromatography is a simple chromatographic technique that can be successfully used in analysis of wide variety of classes compounds. Solvent consumption is relatively low and sample can be analyzed without earlier purification like it is necessary in other chromatographic techniques (HPLC). Crude extracts can be successfully analyzed in this method. Because of simplicity it can be used in preliminary experiments that may provide basic information on the mixture to be separated and its behavior in the chromatographic system in further analysis with another method like HPLC [19, 21, 41]. TLC has the highest sample throughput compared to other chromatographic techniques. Up to 30 individual samples can be separated in the same time on one plate [40]. The stationary phase in TLC is used only once, there is no problem with "memory effect", thus it increases repeatability of this method. The plates can be also easily storage after use. [50]

Stationary and mobile phases in analysis of coumarins

First step in TLC analysis is choosing of a proper combination of stationary and mobile phases.

Polarity relationship between mobile and stationary phase is most important. One can choose between normal phase system (NP) in which sorbent is more polar than mobile phase, or reversed phase system (RP) in which sorbent is less polar than mobile phase. In the most cases in the literature the normal phase system and silica gel as a stationary phase is first choice in the TLC analysis of coumarins.

The next very important in TLC is selection of mobile phase. Nyiredy et al. [35,36] created an optimization model assist in the selection of the optimal eluent systems for planar chromatographic techniques called "PRISMA". Basic concept of this system is first to optimize the solvent strength and subsequently the selectivity in a triangular mixture solvent diagram, which is a graphic representation of the solvent strength and the proportions of the solvents. The PRISMA optimization system consists of three parts. In the first part basic parameters like the stationary phase and the solvent are selected. In the second part the optimal combination of the selected solvents is chosen using the PRISMA model. The third part includes selection of suitable method and transfer of the optimized mobile phase to chromatographic techniques [22].

Härmala et al. [21] have studied the retention behavior of some closely related coumarins in TLC according to PRISMA model and coupling it with further HPLC analysis. They presented that the mobile phase composition has a similar effect on the elution in both techniques and normal-phase TLC can be used as a HPLC preassay method in the PRISMA system. Głowniak and Bieganowska [14] studied retention behavior of coumarins and furanocoumarins in both, normal and reversed-phase system TLC and HPLC. In this paper linear relationships between experimentally obtained retentions and content of organic modifier in mobile phase are shown.

Chromatogram Development

There are a lot of possibilities to choose a method of chromatogram development like 2D development, one-dimensional multiple development (UMD), gradient development and others. Choice of the most suitable type seems to be very important step of the analysis. Isocratic linear development is the first choice in analytical and preparative chromatography of coumarins but other methods can also give very good results.

There are some modern methods in which the eluent system can migrate through the stationary phase under additional influence of forced flow. The forced flow can be achieved by application of a pump system for overpressured layer chromatography (OPLC), by centrifugal force for rotation planar chromatography (RPC), or by electric field for high-speed thin layer chromatography (HSTLC) [44]. The retention behavior of fifteen closely related coumarins in normal phase OPLC was investigated by Vuorela et al.[47] and compared to HPLC and TLC techniques. OPLC was also successfully used for separation of six main coumarins in *Peucedanum palustrae* [48]. Clean separations of coumarins and furanocoumarins was obtained with OPLC on silica gel, with the mixture of ethyl acetate and chloroform 60:40 was used as a mobile phase [12]. The forced flow techniques are called the bridge between the planar chromatography and the column chromatography and they are one of the most promising techniques to improve in the future.

Analytical TLC of coumarin compounds

TLC is a very quick and popular method for the identification of compounds presented in plant extracts, by measuring the retention parameters or UV spectra taken directly from the layer by use of densitometry [52]. It is also used as good method for fraction monitoring in the process of isolation of coumarins from plant material [3,25].

The normal phase and silica layer are the first choice of separation system for coumarins. Although there is some research in which the reversed phase or polar bonded stationary phase was used. Reversed-phased system on the RP 18 layer was used for lipophilicity assessment of coumarins. The RP 18 plates as a stationary phase and three solvent systems were used as mobile phase: methanol-water, acetonitrile-water and tetrahydrofuran-water binary mixtures, with a varying content of organic modifier. Based on retention parameters at several compositions of the three different binary solvent systems composed of organic modifier and water the lipophilicity parameters were calculated for twelve different coumarins [9]. The polar bonded phases such as CN-Silica and Diol-Silica were successfully applied for 2D analysis of coumarins by Waskmundzka – Hajnos et.al. [53]. Multiphase plates, connected with C18 strips and silica layers, were used with aqueous and nonaqueous eluents. Such layers were successfully applied for the separation of selected coumarins.

Very interesting application of modified 2D separation technique called the Graft TLC for analysis of coumarins has been researched by Cieśla et al.[4, 6] This research shows that Graft TLC method can be successfully used for both qualitative and quantitative analysis of closely related coumarins, which is very difficult in one-dimensional analysis. The basic condition for obtaining good results is a choice of two systems with different selectivity for satisfactory resolution of spots. Grafted Sillica to RP 18 and Grafted CN-silica to silica layers have been successfully used for separation. 2D TLC was also proposed as a method that enables obtaining a comprehensive profile of a plant, and it is ideally suited to construct fingerprints of closely related plant species. The graft-TLC images of *Heracleum* species, varieties and forms show distinctive fingerprints [5].

Automated Multiple Development (AMD) with decreasing solvent gradient was used by Sieniawska et al.[42] for analysis of coumarin fraction from *Angelica archangelica.* Five-step multiple development with n-hexane and dichloromethane (1:1) with gradient of ethyl acetate (0.5% to 2.5%) as a mobile phase were used. Developed plates were further analyzed with densitometer. Identification of five furanocoumarins (imperatorin, isoimperatorin, isopimpinelin, bergapten and phellopterin) was confirmed by comparison of R_f values and UV spectra with adequate standards. It has been proved that multiple developments with gradient elution can be used as a routine screening procedure to verify a presence of coumarin compounds in a sample.

TLC coupled with fluorometric method was successfully applied to for finding the proper system for separation of determine the partition coefficient of target coumarins in HSCCC solvent system instead of HPLC method [22]. Some examples of using TLC for analytical separation are placed in Table 1.

Preparative Thin Layer Chromatography (PTLC) of coumarins

As mentioned earlier, normal phase system and silica gel is the first choice for analysis of coumarins but also for

Table 1. TLC systems in analysis of coumarins

Adsorbent	Mobile Phase	Source	Remarks	Compounds	References
Silica TLC	Benzene : Ethyl acetale (7:3)	*Vaccinum vitis – idaea L.* *Vaccinum myrtillus L.* *Vaccinum uliginosum L.* *Oxycoccus quadripetalus Gilib.* *Calluna vulgaris L. Hull.* *Ledum palustre L.*	Scopoletin determination	scopoletin	[52]
Silica TLC	Hexane : Ethyl acetate : Ethanol	*Angelicae gigantis*	Coumarins (decursin and decursinol) as the marker compounds for TLC with DART-MS system	decursin, decursinol	[24]
CN – Silica TLC Diol – Silica TLC	Binary eluents with polar modifier	*Heracleum sphondylium* *Heracleum sibiricum,* *Archangelica officinalis*	2D TLC Analysis of coumarins	isopimpinellin, methoxsalen, umbeliferone, phellopterin.	[53]
Silica TLC	Binary eluents with polar modifier	*Heracleum sphondylium L.* *Pastinaca sativa L.* *Sium sisarium L.* *Libanotis intermedia Rubr.*	Stepwise gradient elution TLC	xanthotoxin, bergapten, imperatorin	[13]
Silica TLC	Petroleum ether: Ethyl acetate	*Ferula szowitsiana*	Fraction monitoring from preparative column	senecioylprangol, 3'-senecioyloxymarmesin, 3'-hydroxyprantschimgin and 2"-senecioyloxymarmesin	[26]
Silica TLC	Various mobile phases	*Peucedanum palustrae*	OPLC, PRISMA system	Coumarin isomers	[46]
RP 18 TLC	Binary solvents	*Seseli annuum*	lipophilicity assessment	Some natural and synthetic coumarins	[9]
Silica TLC	Dichloromethane: n hexane: Ethyl acetate	*Angelica archangelica L.*	5 step Multiple Development HPTLC	imperatorin, isoimperatorin, isopimpinelin, bergapten, phellopterin	[42]
Sillica TLC	Chloroform : Ethyl acetate	*Ipomoea batatas L.*	Determine the partition coefficient of target compounds in HSCCC	6,7-dimethoxycoumarin, 5-hydroxymethyl-2-furfural	[22]
Sillica TLC	Petroleum ether : Ethyl acetate : Methanol	*Psoralea coryfolia fruits*	TLC antioxidant bioautographic assay of isolated compounds by HSCCC	psoralen, isopsoralen, psoralidin, corylifol, bavachinin	[59]
Sillica TLC	Hexane: Ethyl acetate	*Peucedanum ostruthium*	TLC anti-acetylocholinoesterase bioauthographic assay of isolated compounds by CPC	ostruthin, imperatorin, ostruthol, oxypeucedanin hydrate	[45]

preparative scale TLC. The thickness of the adsorbent layer is greater, typically around 0.5 – 2.0 mm (for analytical purposes it is usually around 0.1 – 0.25 mm).

Preparative Thin Layer Chromatography in combination with other chromatographic methods like preparative column chromatography and HPLC was used by Głowniak et al. [16] and Bartnik et al. [1] for isolation and purification of coumarins from *Peucedanum tauricum* leaves and fruits. In this research the fractions have been obtained from LC separation, those richest in coumarin compounds were chromatographed by preparative TLC on plates precoated with 0.5mm layers of silica gel. Plates were developed twice with cyclohexane – ethyl acetate 80:20 (v:v), then dried and re-chromatographed with more selective mobile phases. The preparative TLC in reversed phase system was used for purification of coumarin sediment before RPHPLC analysis. For this purpose 0.5mm RP-2 layers as a stationary phase and methanol : water 9:1 (v:v) as a mobile phase were used. As a result oxypeucedanin hydrate and officinalin isobutyrate was first time isolated from *Peucedanum tauricum* aerial parts, other isolated coumarins were: peucedanin, bergapten and isoimperatorin.

The retention behavior of coumarins from *Libanotis doloichostyla* on silica and Florisil layers has been researched by Zgórka [60]. Both silica and Florisil layers as a stationary phase and ternary mobile phases, consisting of a hexane-dichloromethane mixture (1:1) and polar modifiers of ester, ketone or ether type in different concentration were used. The results of this experiment enabled the isolation of ten coumarins (umbeliferone, scopoletin, osthol, ostruthin, xanthotoxin, bergapten, imperatorin, isoimperatorin, edultin. (+)*cis*-khellactone) from *Libanotis dolichostyla* fruits using preparative TLC analysis both on silica and Florisil layers.

PTLC was used as a method of isolation coumarin compound from *Ferula persica* roots by Ahmad-Reda Shahverdi et al. [39] Preparative Thin Layer Chromatography was carried out on sillica gel using petroleum ether and ethyl acetate (2:1) as the mobile phase. The fractions were visualised under UV light at 254nm. The isolated coumarin was identified as a umbeliprenin and it was further investigated for antibacterial properties

Some other examples of using PTLC of coumarin compounds are placed in Table 2.

Methods of detection

One of the properties of coumarins is that they reveal a strong fluorescence in the UV light (365nm). It is very helpful in detection on the chromatogram without using any of the chromogenic agents. Sometimes it is possible to identify the structural class of coumarins by the color, which they display under UV light. For example purple fluorescence generally signifies 7-alloksycoumarins whereas blue fluorescence is usually characteristic for 7- hydroxy- and 5,7 – deoxygenated coumarins. The fluorescence can be more intense or its color can be changed after spraying the chromatogram with ammonia [55]. In the case of UV spectrophotometric analysis, coumarins are identified by a presence of absorptions bands at certain specified wavelengths.

Table 2 TLC for the isolation of coumarins from the plant material

Adsorbent	Mobile Phase	Source	Compounds	References
Sillica TLC RP2 TLC	Binary and ternary eluents Methanol : Water	*Peucedanum tauricum* leaves	isoimperatorin, bergapten	[1]
Sillica TLC	Trichloromethane : Metanol : Water	*Peucedanum ostruthium*	isoimperatorin, oxypeucedanin hydrate	[23]
Sillica TLC	Heptane : Dichloromethane : Ethyl acetate	*Peucedanum verticilare*	pteryxin, epoxypteryxin	[30]
Sillica TLC RP2 TLC	Binary and ternary eluents Methanol : Water	*Peucedanum tauricum* fruits	isoimperatorin, bergapten	[16]
Silica TLC	Binary eluents	*Ferulago capilaris,* *Ferulago brachyloba*	senecioylprangol, 3'-senecioyloxymarmesin, 3'-hydroxyprantschimgin 2"-senecioyloxymarmesin	[26]
Silica TLC	Petrol : Ethyl aceteate	*Ferula szowistana*	szowitsiacoumarin A, szowitsiacoumarin B, 2-epihelmanticine, auraptene, umbelliprenin, galbanic acid, methyl galbanate, farnesiferol B, farnesiferol C, persicasulfide A, β-sitosterol, stigmasterol	[25]
Silica TLC Florisil TLC	Ternary eluents	*Archangelica officinalis* fruits *Heracleum sosnowskyi* fruits	furanocoumarins	[48]
Sillica TLC	Petrol: Trichloromethane	*Polygala paniculata*	herniarin, aurapten	[20]
Silica TLC Florisil TLC	Ternary eluents	*Libanotis dolichostyla*	umbeliferone, scopoletin, osthol, ostruthin, xanthotoxin, bergapten, imperatorin, isoimperatorin, edultin, (+)cis-khellactone	[60]
Sillica TLC	Petroleum ether : Ethyl acetate	*Ferula persica*	umbelliprenin,	[39]

There are some chemical methods for an identification of coumarins including conventional azo-addition with diazo component, lacto probe and some other color reactions. These reactions can be used jointly with TLC techniques but it has to be remembered that the reaction is not specific for coumarins [**33**].

CONCLUSION

TLC always will be an attractive technique used for separation of natural products, because of its simplicity and no cost. The main application field of TLC is quick fingerprint analysis of herbal mixtures. There are a lot of possible modifications of this method and there is still a place for new upgrades that will significant rise its the role. Faster, cheaper and more accurate analysis that always will be a primary goal for usage of separation techniques including planar chromatography. We have to benefit from all the advantages that the different techniques of chromatography can give us and create the method that will be best for our research. Coupling of liquid chromatography to mass spectrometry is one of the best methods of analysis in phytochemistry, maybe coupling planar chromatography to mass spectrometry is that what we are looking for. Applying modern methods and modifications of Thin Layer Chromatography technique for analytical separation and isolation of coumarins will let us more efficient research this very potent group of compounds. Future looks very promising at this field.

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