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Review

Two-dimensional thin-layer chromatography in the analysis of secondary plant metabolites

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ABSTRACT

Drugs, derived from medicinal plants, have been enjoying a renaissance in the last years. It is due to a great pharmacological potential of herbal drugs, as many natural compounds have been found to exhibit biological activity of wide spectrum. The introduction of whole plants, plant extracts, or isolated natural compounds has led to the need to create the analytical methods suitable for their analysis. The identification of isolated substances is relatively an easy task, but the analysis of plant extracts causes a lot of problems, as they are usually very complex mixtures. Chromatographic methods are one of the most popular techniques applied in the analysis of natural mixtures. Unfortunately the separation power of traditional, one-dimensional techniques, is usually inadequate for separation of more complex samples. In such a case the use of multidimensional chromatography is advised. Planar chromatography gives the possibility of performing two-dimensional separations with the use of one adsorbent with two different eluents or by using bilayer plates or graft thin-layer chromatography (TLC) technique; combinations of different multidimensional techniques are also possible. In this paper, multidimensional planar chromatographic methods, commonly applied in the analysis of natural compounds, were reviewed. A detailed information is given on the methodology of performing two-dimensional separations on one adsorbent, on bilayer plates, with the use of graft TLC and hyphenated methods. General aspects of multidimensionality in liquid chromatography are also described. Finally a reader will find a description of variable two-dimensional methods applied in the analysis of compounds, most commonly encountered in plant extracts. This paper is aimed to draw attention to the potential of two-dimensional planar chromatography in the field of phytochemistry. It may be useful for those who are interested in achieving successful separations of multicomponent mixtures by means of two-dimensional TLC.

Contents

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; 3D, three-dimensional; ACN, acetonitrile; AcOEt, ethyl acetate; BMD, bivariant multiple development; CCD, charged couple-device; DEA, diethylamine; EtOH, ethanol; GC, gas chromatography; GMD, gradient multiple development; HPLC, high-performance liquid chromatography; HPLC-MS, high-performance liquid chromatography–mass spectrometry; HPTLC, high-performance thin-layer chromatography; ICH, International Conference on Harmonisation; IMD, incremental multiple development; iPrOH, isopropanol; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MD-PC, multidimensional planar chromatography; MeOH, methanol; MGD, multiple gradient development; NP-OPLC, normal-phase over-pressured liquid chromatography; NP-TLC, normal-phase thin-layer chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; RP-TLC, reversed-phase thin-layer chromatography; T, toluene; UMD, unidimensional multiple development.

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1. Introduction

The amount of medicinal plants and herbal medicinal products used worldwide has risen dramatically in the last decades. The advent of new herbal medicines caused the need for finding quality assurance tools for ensuring identity, purity and quality of botanical materials [1]. Scientists focused on chemotaxonomical research also indicate the need for finding new methods in the analysis plant extracts.

Unfortunately plant extracts and herbal medicinal products are usually highly complex samples, and in most cases it is difficult to identify all compounds by means of common approaches (e.g. one-dimensional liquid chromatography). However, there are a lot of methods, in which traditional techniques are used in the analysis of complex samples. These methods are usually focused only on some components, which identity is known, regarding them as "marker compounds" [2]. Contrary to synthetic drugs and isolated natural products the entire plant extracts should be regarded as active components, so the aforementioned methods may sometimes appear insufficient in the quality control of highly complex natural samples.

Multidimensional chromatography offers many advantageous features in the analysis of medicinal plants, which enables solving lots of analytical problems encountered in the separation of multicomponent mixtures [3].

1.1. Multidimensionality in liquid chromatography

In multidimensional separation a sample is first subjected to separation via one method, and then the separated compounds are further resolved by at least one additional independent method [4]. Theoretically the sample can be subjected to unlimited number of separation methods. In practice, the constructional constrains and difficulties in finding three or more methods characterized by orthogonal mechanisms, caused the majority of multidimensional separations are two-dimensional [5].

According to Giddings multidimensional chromatography includes two conditions [6]:

(1) the separation mechanisms of the applied steps must be orthogonal,
(2) resolution gained in the first dimension may not be lost in any subsequent dimension.

Multidimensional chromatography can be easily realized by use of gas chromatography (GC) [7]. The realization of multidimensionality in liquid chromatography is somewhat difficult. It is normally caused by mobile phase components which are difficult for switching from one system to another. Multidimensional LC–LC can be realized with the use of ion-exchange column as the first and reversed-phase column in the second direction, with the use of buffered mobile phase in the first dimension. Good results are also achieved when cyanopropyl column packing is used as the first dimension adsorbent, followed by a C8 or C18 adsorbent in another direction. In both cases aqueous mobile phase is used, but what is important it should be weaker in the first column [8].

There are several classifications of two-dimensional separations. Generally 2D chromatographic methods can be divided into two main groups: 2D-in-time and 2D-in-space [5]. In the first case, the analyzed compounds are subjected to a 1D separation (normally on a LC column) and then individual fractions from the first column undergo the analysis in a second 1D separation. 2D-in-time techniques can be further divided into off-line and on-line 2D separations. In off-line methods partly separated compounds are collected from the first dimension, and then separated in the second direction. On-line techniques involve switching the fractions, resolved in the first dimension, directly to the second dimension, while the first-dimensional separation continues simultaneously. Two subgroups of chromatographic methods can be distinguished from among on-line techniques: "heart-cutting two-dimensional liquid chromatography" and "comprehensive two-dimensional chromatography" [9]. In the first of the aforementioned methods, only a desired segment of the first column effluent is subjected to further analysis. In "comprehensive 2D-chromatography" all sample components, separated in the first direction, are analyzed in the second direction. Comprehensive two-dimensional chromatography is more powerful than heart-cutting one because it provides greater peak capacity.

In case of methods that are two-dimensional in-space, a separation is performed over a two-dimensional planar surface, e.g. a chromatographic plate (planar chromatography) or gel (gel electrophoresis).

From among 2D-in-space techniques, two-dimensional thin-layer chromatography (TLC) is one of the most widely used methods due to its numerous advantages:

(a) in the second geometric direction unlimited number of secondary "columns" can be applied; multidimensional TLC is the only real multidimensional separation method in which, after the first separation in the first direction, all compounds can be passed to a second direction [3],
(b) it is one of the most simple methods that can be performed without sophisticated equipment that is needed in case of two-dimensional LC–LC methods [10],
(c) the plate is used once only, there is no problem of strongly adsorbed constituents; complicated clean-up procedures do not have to be performed [11],
(d) there are almost no limits as far as mobile phase components are concerned, because they can be evaporated from the layer after development in the first direction [12],
(e) multiple detection enables the analysis of wide spectrum of compounds [12] and
(f) visual results can be easily presented [1].

Two-dimensional chromatography, as any other technique, also has notable disadvantages. One of them is the possibility

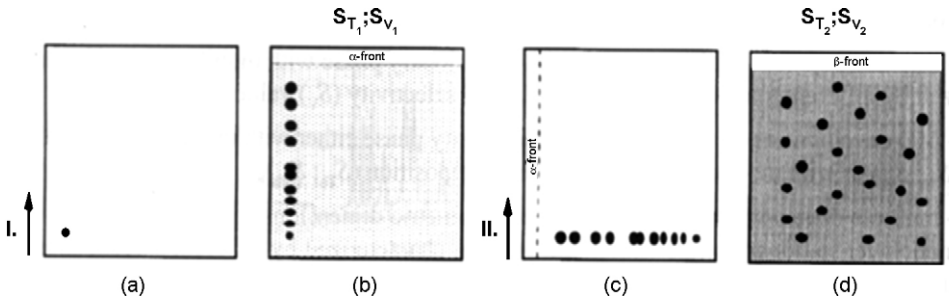

Fig. 1. Schematic representation of comprehensive two-dimensional chromatography on one adsorbent: spotting a sample at the corner of a chromatographic plate; development of the plate in the first direction with the first eluent; drying of the plate; development of the plate in the second direction perpendicular to the first one with the second eluent. From ref. [3] with permission from Springer.

of artifact formation during the chromatography process, so all two-dimensional separations should be validated with respect to possible formation of artifacts [1]. However, the greatest shortcoming of 2D-TLC, when compared to other planar techniques, is its limitation to one sample per plate. As a consequence, standards have to be applied individually for each dimension [11]. An interesting solution of this problem was presented by Medič-Sarič et al. [13].

Another downside of this technique is that very polar and non-volatile solvents should be avoided as components of the mobile phase, as they are difficult to be removed from the adsorbent, e.g. DMSO (dimethyl sulfoxide), acetic acid, trimethylamine, as well as ion-pair reagents and non-volatile buffer components. If the plates are not carefully dried, after first development, "ghost peaks" formation may also appear [14].

Multiple developments ultimately extend the analysis time, which makes some two-dimensional separations time-consuming.

Nyiredy divides multidimensional planar chromatography into the following groups [15,16]:

(1) comprehensive 2D planar chromatography – development on the same adsorbent with eluents characterized by different eluent strength and selectivity; or separation on a bilayer plate with the application of the same eluent or two different mobile phases,
(2) targeted or selective 2D planar chromatography – after the first development only chosen spots are subjected to subsequent analysis (similarly to a heart-cutting multidimensional column chromatography),
(3) modulated 2D planar chromatography – first the monolayer is developed with eluent of decreasing strength, and in the second direction the plate is redeveloped several times with solvent mixture of different selectivity, at constant eluent strength,
(4) coupled-layer planar chromatography, also called "graft-TLC" – in this method partially separated compounds, in the first direction, are transferred to another adsorbent, with the use strong eluent (usually MeOH), and then redeveloped in the second direction,
(5) combination of MD-PC methods – a combination of at least two of the methods mentioned above, and
(6) automated coupling of two chromatographic techniques – on-line NP-OPLC in the first direction coupled with RP-TLC or HPLC, and RP-HPLC or GC in the first direction followed by NP-TLC, UMD in the first direction followed by IMD or BMD orthogonally.

2. Multidimensional thin-layer chromatography methods commonly applied in medicinal plant analysis

As far as the analysis of botanicals and herbal drugs is considered, only several of the planar chromatographic methods, distinguished by Nyiredy, have been often applied. These are: comprehensive 2D planar chromatography realized on mono- and bilayers, coupled-layer chromatography, combination of MD-PC techniques and hyphenated methods.

2.1. Comprehensive two-dimensional chromatography on one adsorbent

In this method the analyzed sample is spotted at the corner of the stationary phase and developed in the first dimension. Then it is carefully dried, rotated at 90° and redeveloped in the second direction (see Fig. 1) [17,18].

In its most simple version, the plate is developed with the same eluent system in both directions. In such case the increase in resolution, when compared to one-dimensional chromatography, is due to the fact that the migration distance is increased by a distance of $\sqrt{2}$ [19]. After application of this technique, one will observe the spots lying on the diagonal of the plate. Two-dimensional separation, on monolayer, with the use of the same mobile phase is one of the most important prevalidation tests for checking the stability of the analyte during chromatographic process. All separated zones should be located on the diagonal, any deviation indicates a decomposition product or chemisorption [20,21]. The spot capacity of such two-dimensional separations is only slightly higher when compared to one-dimensional technique [22–24].

The use of a single sorbent layer and two solvent systems of different selectivity is the most popular method of performing two-dimensional separations in medicinal plant research. Ideally, if two truly orthogonal systems are applied in both directions, the spots should be spread over the whole plate, and it should be possible to obtain spot capacity between 100 and 250 [22]. However, it is clear that current theory overestimates the potential of 2D-TLC, as such spot capacity has never been reported.

The limited amount of successful two-dimensional separations on monolayers is due to the fact that finding two orthogonal systems is not a trivial task [10]. Different methods have been proposed for finding chromatographic systems characterized by different selectivity. For instance Gonnord et al. [25] proposed a computer-aided technique for finding optimum mobile phases. Two equations – the sum of the squared distances between the spots (D_A) and the inverse of the sum of the squared distances between spots (D_B) were proposed for judging the quality of two-dimensional separations. The modification of these methods was proposed by Steinbrunner et al. [26]. Nurok proposed several strategies for optimizing the mobile phase in planar chromatography, one of which was the planar response function (PRF) [27].

All of the aforementioned methods have been rarely used for the optimization of two-dimensional separations, except for computer assisted optimization which allows the identification of best systems by screening a large set of these systems, based on data collected using 1D systems. Its usefulness was proven in case of the analysis of steroids [28].

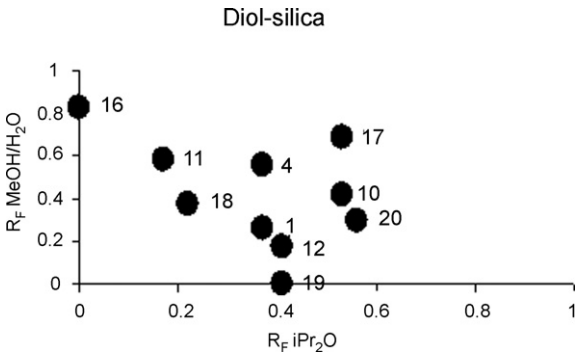

Fig. 2. Correlation of R_F parameters for coumarin standards chromatographed on diol-silica in systems: I direction: 10% MeOH/H_2O/1% HCOOH (v/v) and II direction 100% isopropanol, double developed. 1 = Isopimpinellin, 4 = umbelliferone, 10 = coumarin, 11 = aesculetin, 12 = bergapten, 16 = aesculin, 17 = 3,4-dihydrocoumarin, 18 = dimethylfraxetin, 19 = phellopterin, 20 = 6-methylcoumarin. From ref. [45] with permission from Preston Publications.

The most widely applied method, for selection of optimum mobile phase, was the use of correlation coefficients between R_F and R_M values obtained after one-dimensional development, in two different solvent systems, first reported by de Spiegeleer et al. [29] (see Fig. 2). Although being the simplest approach, this method appeared to be useful in the analysis of multicomponent mixtures of pesticides [30–32], coumarins [33] or polyphenolic compounds [34]. Some latest reports state the lowest correlation coefficients technique should be applied hand in hand with the visual comparison of real chromatograms, obtained with the use of the analyzed systems [35].

Silica gel is the adsorbent that has been used most often in two-dimensional separations. Unfortunately, in almost all cases the two solvent systems used, were not truly orthogonal in properties.

Two-dimensional separations on silica gel are especially not recommended for analysis of mixtures containing structural analogs, and substances that can be oxidized on polar adsorbents (e.g. carotenoids). Despite the disadvantages several successful separations of medicinal plant constituents have been achieved. These were usually mixtures containing substances spanning a wide polarity range.

Except for silica gel there have been several papers reporting the use of alumina [36–38] cellulose [39,40] and alkyl-bonded phases [41–43] used as adsorbents in two-dimensional separations. Also in these methods the separation space remained usually unused.

Despite the difficulties, the application of these adsorbents, in two-dimensional separations should not be diminished. In all cases 2D-TLC always produced greater spot capacity when compared to one-dimensional methods. These techniques may be applied in the analysis of less complex samples, however, not fully resolved with the use of traditional chromatographic approach.

The greatest selectivity differences are obtained by combining normal-phase (NP) and reversed-phase (RP) chromatography [3]. Surface-modified adsorbents (especially polar stationary phases bonded to silica matrix) of moderate polarity, can be used both for NP and RP separations [44]. These sorbents are compatible with mixtures of organic solvents, applied in NP systems, as well as with aqueous eluents (containing also buffers, amines, etc.) used in RP systems. Except for CN-silica, operated in NP and RP systems, aminosiloxane-bonded layers can also be used as an ion-exchange system with acidic mobile phases [45].

A cyanopropylsilanized silica gel has been used most often in two-dimensional separations of natural mixtures, however, the use of diol-silica layers has also been reported [46,47]. A rational approach for method development for 2D-TLC of multicomponent mixtures on CN-silica was proposed [32].

One of the greatest disadvantages, when two-dimensional separations are applied on one adsorbent, is that eluent systems used in the first dimension may modify the stationary phase. Good results are obtained only if the applied solvent is completely removed from the adsorbent, or the modification is reproducible. The drying step is also critical, as some components may be lost, especially volatile ones. When new method is developed it should be checked, whether both the adsorbent's modification, and drying step procedure do not cause any analytical problems. Thus, it is advised to use aqueous mobile phases in the second direction, to avoid lengthy drying steps. If mobile phase additives, such as formic or acetic acid (e.g. phenolic acid analysis), ammonia, amines (alkaloids analysis) have to be used, to obtain good results, such mobile phases should rather be applied in the second direction. As far as alkaloids are concerned, ammonia can be used instead of diethylamine, in the first direction, as it is easier to be quantitatively removed from the layer.

2.2. Two-dimensional chromatography on bilayer plates

Great selectivity differences can be achieved when the analysis is performed on so called bilayer plates, on which two different sorbents are coated side by side. In this technique the analyzed sample is spotted to a narrow strip of the first adsorbent, then chromatographed in the first dimension. After drying step the plate is rotated at 90° and redeveloped in the second direction (first the sample molecules interact with the active sites of the first adsorbent, used to coat the strip, and then are developed on the second adsorbent) [48]. The most popular, commercially available layers consist of a narrow strip of silica gel (or octadecylsilica gel) adjacent to a strip of C18 (or silica) [49]. These plates (Multi K SC5 and CS5) are available, for example, from Whatman (Kent, UK).

Other combinations of adsorbents have also been reported: silica gel + alumina, cellulose + silica gel, poliamide + silica, poliamide + cellulose [50–52].

Before bilayer plates were used for two-dimensional separations, they would be applied for improving the chromatographic performance in one-dimensional chromatography [53]. The narrow strip of adsorbent was used as a preconcentrating zone.

The effect of preconcentration is still used in two-dimensional separations – the obtained zones are symmetric and well separated, after development in the second direction. It was proven to ease the densitometric estimation of two-dimensional chromatograms [54].

Bilayer plates can also be used for removing the interfering substances from plant extracts, which usually contain constituents spanning a wide polarity range. The sample can be spotted to a narrow strip without any clean-up procedure. Strongly adsorbed constituents remain on the narrow strip of adsorbent, and the other substances, present in the extract, are separated on the adjacent adsorbent.

If a silica gel is used as the narrow zone, it can be used for removing very polar compounds, as they are strongly adsorbed on silica gel. It can be applied in case of extracts containing large amounts of carbohydrates, tannins or polar glucosides.

A narrow zone of octadecylsilica wettable with water can be used for removing apolar constituents (e.g. lipids,waxes) from the sample. Such a procedure can be used, for example, for the analysis of extracts obtained from fruits, as they often contain lipophilic excipients (e.g. the analysis of furanocoumarins in fruits of different *Apiaceae* plant species).

Two-dimensional separations on bilayer plates can be achieved in two modes: with the use of the same eluent and with two different eluents. The first one is the opposite of the classical 2D-TLC on the same monolayer stationary phase, with two different eluents, and is rarely performed [3]. The second one has been used for

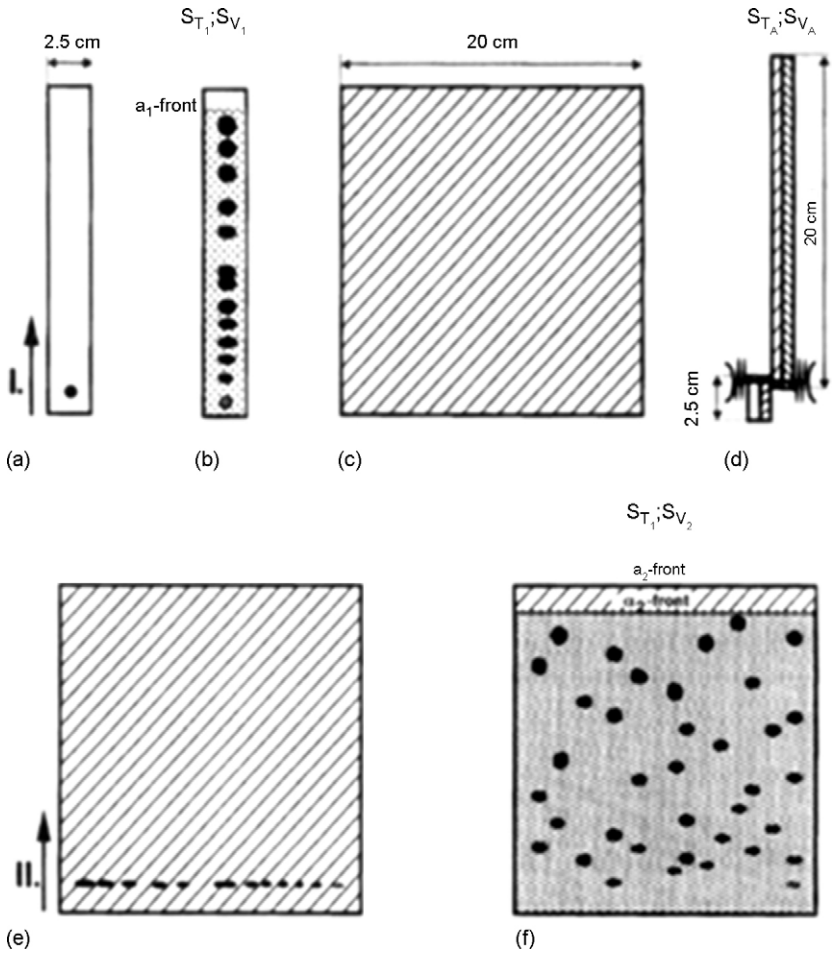

Fig. 3. Schematic representation of coupled-layer planar chromatography (graft-TLC): sample application to the first plate – 2 × 10 cm; development of the plate in the first direction; (c and d) clamping the second plate with the narrow strip and transferring partly separated compounds from the first layer; drying of the plate; development of the second plate in the second direction perpendicular to the first one with the second eluent. From ref. [3] with permission from Springer.

solving some analytical problems encountered in the analysis of plant extracts.

Multi K SC5 and CS5 plates have dominated the analysis of multicomponent mixtures, performed with the use of bilayer plates.

On silica gel the constituents are separated according to differences in polarity, while on octadecyl silica the differences in substituents' position play an important role (structural analogs can be separated).

Due to different mechanisms of interaction, multiphase plates enable obtaining a complete resolution of very complex samples. Such plates can be useful when the aim of the procedure is to divide the constituents into subgroups of different polarity, as it was in case of the coumarins' analysis [46].

A major problem of analysis performed on bilayer plates is the difficulty in obtaining reproducible results. It is caused by modification of stationary phase by eluent constituents, used in the first dimension. The same problem can be encountered in case of monolayer plates, but is easier to overcome. Multiphase plates usually consist of a narrow octadecyl silica gel, so the analysis in the first dimension is performed on this adsorbent. Eluents used in reversed-phase systems contain water that is strongly adsorbed on silica gel and causes deactivation of the layer. Unless it is completely removed, it is difficult to obtain reproducible results. The analysis time is also quite long, as drying step is a time-consuming procedure, when water is used as the mobile phase constituent. However, reproducible results, with the use of commercially available bilayer plates, have been reported [31,49].

If there are difficulties in operating multiphase plates, the use of two separate plates coated with different stationary phases is suggested [3]. It can be realized with the use of the technique called graft-TLC.

2.3. Coupled-layer planar chromatography – graft-TLC

In this chromatographic procedure partly separated compounds, in the first run, are transferred to another adsorbent and chromatographed in the second dimension (see Fig. 3) [55]. The analyzed compounds are transferred with the use of solvent with high eluent strength, without the usual scrapping of bands, extraction and re-spotting.

When compared to analysis on mono- and bilayers, more mixtures can be analyzed at the same time. It is possible because several samples can be developed, in the first direction, on the first adsorbent (up to five in case of 10 cm × 10 cm plates). After drying, step plate used in the first run is cut into narrow strips (usually 2 cm × 10 cm) in the direction parallel to the direction of the performed development. Then individual strips are clamped to another plates and compounds are transferred. The procedure of performing reproducible graft-TLC analysis was well described by Tuzimski [56].

It is important that both plates are turned face to face and pressed (grafted) together, the layers should overlap. It can be done by putting both precoated plates between glass plates and joining them with the use of clamps. A device that eases the transfer of

partly separated mixtures from one stationary phase to another was also described earlier [47]. The transfer of analyzed compound is performed in vertical glass chamber, as the joined plates are difficult to be developed in horizontal chambers.

The most important issue in this method is to check whether the analyzed compounds are quantitatively transferred from the first adsorbent to another. It is difficult to perform in case of strongly adsorbed constituents. It is of crucial importance to choose a proper adsorbent for the first direction. It should be a rule of thumb to perform the first development on adsorbent on which the analyzed substances are not strongly adsorbed. Polar substances (e.g. phenolic acids, flavonoid glycosides, polar alkaloids, etc.) are advised to be chromatographed first in reversed-phase systems, for example, on RP-18W plates, or CN-silica developed with aqueous eluent. Apolar compounds (furanocoumarins, steroids, lipids, etc.) should be developed first on polar adsorbents, mainly silica.

Usually silica gel and octadecyl silica are applied in graft-TLC, as they produce greatest selectivity differences, but other combinations of adsorbents have also been reported: CN-silica + silica, DIOL-silica + RP-18W plates, etc. [41].

The application of narrow strip of the first adsorbent may also play the same role as the preconcentrating zone in case of bilayer plates. The samples are not only separated on the first adsorbent, but also concentrated, and as-such developed in the second direction. The concentration is also performed during the transfer, as the strong mobile phase used in this procedure, introduce the analyzed substances, to another adsorbent, as very thin bands.

As far as the solvent used for transferring substances is concerned, MeOH is usually applied. Some authors used also aqueous MeOH [57], or simply used eluent, applied in the second direction [47], as the transferring liquid. If the analyzed compounds are strongly adsorbed on the first adsorbent, what was observed in case of alkaloids [58], first developed on silica, the addition of organic acids, to transferring solvent, is advised.

In order to obtain reproducible results some technical aspects should also be considered. First of all the cut strips of the first adsorbent should have smooth edges, any irregularities may lead to deformation of zones during the transfer to the second adsorbent layer. When the clamped plates are placed in the vertical chamber, the external glass plates should not be immersed in transferring solvent, because flow of MeOH may also cause zone deformations.

Graft-TLC has been successfully applied in the analysis of the following natural compounds: quinolizidine alkaloids in the herb and in-vitro cultures of *Genista* species [47]; a mixture of 11 phenolic acids [57]; saponins from *Ginseng* preparation [59]; furanocoumarins in fruits of different species of the *Apiaceae* family [35,60]; isoquinoline alkaloids in the herb of *Fumaria officinalis*, *Glaucium flavum* and *Chelidonium majus* [61], polyphenolic compounds in extracts from *Polygonum* and *Verbascum* spp. [62].

2.4. Combination of MD-PC methods, hyphenated techniques

In this method at least two different modes of development are combined in both directions. The most popular is the application of multidevelopment one-dimensional techniques with two-dimensional procedures. As far the combination of unidimensional multiple development [35,54] and multiple gradient development (MGD) [63] with two-dimensional techniques has been described.

UMD procedure has been used in both directions in two-dimensional separations performed on one-adsorbent, on bilayer plates and along in graft-TLC procedure. UMD consists of the repeated development of the same plate, with the mobile phase of constant composition, for the same distance [24]. By use of UMD, differences between R_F values are increased for compounds in the lower R_F range and reduced for those in the upper range. Therefore mobile phase of low eluent strength should be used. Other aspects of unidimensional multiple development were well described by Poole et al. [14]. An interesting example of combination of MD-PC procedures is the use of graft-TLC and unidimensional multiple development technique for separation of closely related coumarins [35,54,60]. The combination of graft-TLC with UMD enabled complete separation of all investigated compounds.

When UMD is used in any of the two perpendicular directions, several limitations and downsides should be taken into account. The addition of non-volatile compounds into the mobile phase may cause "ghost peaks formation", the whole procedure is also time consuming. If there is no great improvement in separation, it should be considered if such procedure pays off.

Matysik [63] proposed an interesting method of joining multiple gradient development technique with two-dimensional separation on one adsorbent. In GMD technique each stage of rechromatography is performed with a mobile phase of different composition, while the development distance remains constant, or may be also varied. Different aspects of multiple gradient development were described in several papers [64–66]. The author managed to completely separate antraquinone aglycones and glycosides on silica gel plate.

Cisowski and co-workers proposed also a method of applying MGD with graft-TLC. This method turned out to be the most selective and efficient in case of phenolic acids analysis [57].

Tuzimski [67] proposed a new procedure, characterized as [PC × (^{n}PC + PC + PC + PC)], for the separation of complex mixtures by a combination of different modes of multidimensional chromatography. In this technique, after the development in the first direction, lines were scrapped in the direction perpendicular to the first development, that analyzed compounds were divided into five fractions. Then a certain portion of adsorbent was removed, that the mobile phase reached only the first fraction. The whole procedure was repeated five times, in each step only one particular fraction was developed. This method is not as time consuming as similar technique – graft-TLC, and is relatively cheap. Thus it can be applied in routine analysis of multicomponent mixtures, after preliminary clean-up procedures.

The real potential of combined MD-PC methods has not been used yet. There are several possibilities of performing such separations. One of them is the combination of BMD technique with two-dimensional separations. In BMD development distance is increased and the eluent strength reduced for consecutive steps. The analyzed substances are separated according to differences in their polarity. Such separations can be performed not only on one adsorbent, e.g. CN-silica, but also with the use of graft-TLC. For example, BMD can be first performed on silica gel, then the analyzed substances are transferred to RP-18W plate and developed in the second direction. The combination of different unidimensional methods can be also performed, e.g. UMD in the first direction, and BMD or IMD in another one, etc. Nyiredy [3] proposed a method in which in the first direction components are developed with solvent mixture of decreasing strength, at constant selectivity, the substances are separated according to polarity differences. In perpendicular direction, selectivity of the mobile phase is varied, while solvent strength remains constant. Methods in which both stationary phases (bilayer plates, coupled layers) and mobile phase composition ensure the criteria of MD-PC are regarded as double MD-PC.

Hyphenated techniques, in which TLC is combined with other complementary techniques, are another means of realizing multidimensional separations. TLC can be used as the first or second dimension method. In the first case scrapping of the adsorbent is needed, and such procedure rarely provides reproducible results, thus it is performed not very often. TLC is usually used as the second

dimension with GC, HPLC, supercritical fluid chromatography, capillary electrophoresis, counter-current chromatography, etc. [10].

Hyphenated techniques have been rarely applied in the analysis of medicinal plants, the most common is the combination of HPLC and TLC, for this reason it is the only method described in this paper.

Similarly to LC–LC techniques, hyphenated HPLC–TLC methods can be divided into two groups: on-line and off-line ones. In case of on-line methods the use of more sophisticated equipment is needed. The main problem is to apply the column effluent onto the layer. This is normally achieved with the use of spray-jet applicator. In case of columns operated at higher flow rates, a splitter to the spray-jet applicator should be used to allow the proper transfer of effluent from the column [68]. The use of spray-jet mode has several limitations, the inability to use mobile phase additives (buffers, ion-pair reagents), being the most serious.

In case of natural compound analysis, the practice of using mobile phase additives is frequent, as in many cases acids, amines or buffers significantly improve chromatographic system efficiency. This is why the off-line HPLC–TLC procedure is more widely applied in the analysis of plant derived products. In this method the analyzed substances are first chromatographed in reversed-phase system, on RP-18 column. The fractions of the column effluent are collected, evaporated and after dissolution applied on the chromatographic plates. The particular fractions are then developed in normal-phase system, usually on silica gel.

The off-line combination of RP-HPLC and NP-TLC was used for example for the analysis of coumarins [69] and flavonoids [70].

2.5. Future aspects of multidimensional planar chromatography

As it has been already stated the real potential of multidimensional thin-layer chromatography has not been used yet. Several methods are waiting to be applied in the analysis of highly complex samples, as those mentioned in Section 2.4.

Guiochon and Siouffi [71] gave the theoretical basis for the enhanced separation of multicomponent samples with the use of 3D-TLC separation. Due to the technical obstacles this idea has not been used in practice. To solve the problems that may be encountered in performing three-dimensional analysis a new stationary phase was proposed – Empore silica. According to Poole and Poole [72], the separating power of this adsorbent, when mobile phase flow is due to capillary flow, is only about 60% of that of traditional TLC. Thus it has been proposed to use overpressure to overcome this obstacle. Technical, as well as theoretical aspects of the use of overpressured 3D-TLC are summarized in the work of Botz et al. [73].

In theory, three-dimensional planar chromatography is able to generate the spot capacity of n^3, where n is the number of completely separated zones, in one direction. When combined with other multidimensional techniques, e.g. multiple developments in one direction it can provide, in the future, useful solutions for qualitative analysis of medicinal plants and other complex samples.

Another means of realizing multidimensional separations was proposed by Botz et al. [74,75]. The authors introduced long-distance OPLC for complete resolution of investigated compounds. In this method several stationary phases are applied, placed on top of each other. Special technical solutions should be used to allow the continuous mobile phase flow.

New methods coupling TLC with other methodologies may be introduced, in the field of phytochemical analysis, for example, new interfaces in hyphenated techniques, that will enable the use of mobile phase additives, e.g. acids, amines, etc.

One of the main reasons two-dimensional TLC is normally applied only for qualitative purposes is the lack of proper instrumentation for performing quantitative analysis. Although some solutions have been proposed, 2D-TLC is only occasionally applied in quantitative analysis. The situation may change after the introduction of CCD cameras, which can be applied in quantitative evaluation of 2D chromatograms. Petrović et al. [76] performed video-densitometric quantitation of the 2D chromatograms, of selected pesticides, and evaluated the results with the use of Camag Videoscan software, and found it suitable for routine quantitative analysis.

As far as quantitation is considered one important question remains still unanswered: does quantitative analysis after two-dimensional separations improve the detection limit. Some papers indicate that LOD and LOQ values are smaller, in case of 2D-TLC, due to suppression of background and matrix interference and as a result of better separation [54,77]. However, some authors stand on the position that quantitative results obtained in two-dimensional chromatography are characterized by greater LOD and LOQ values, in comparison to 1D-TLC. This may be attributed to greater diffusion during two-dimensional development [49]. More data is needed before giving the final response to this inquiry.

3. Natural compounds analyzed with the use of multidimensional planar techniques

3.1. Phenolic acids

Phenolic acids are compounds nearly ubiquitous in the plant kingdom, and play an important role in plant physiology, e.g. stimulating plant growth. Phenolic acids also exhibit biological activity of wide spectrum. These compounds were proven to have fungistatic, bacteriostatic, choleretic, sedative and anticonvulsant activity [78].

Separation of phenolic acids is quite difficult task due to several problems that can be encountered in their analysis. First of all there are many isomeric forms that are difficult to resolve, second – they are polar, ionizable compounds, and usually appear on chromatograms as tailing spots, especially on polar adsorbents (e.g. cellulose).

Two-dimensional separations have been widely applied in the analysis of these polyphenol compounds. The method used most

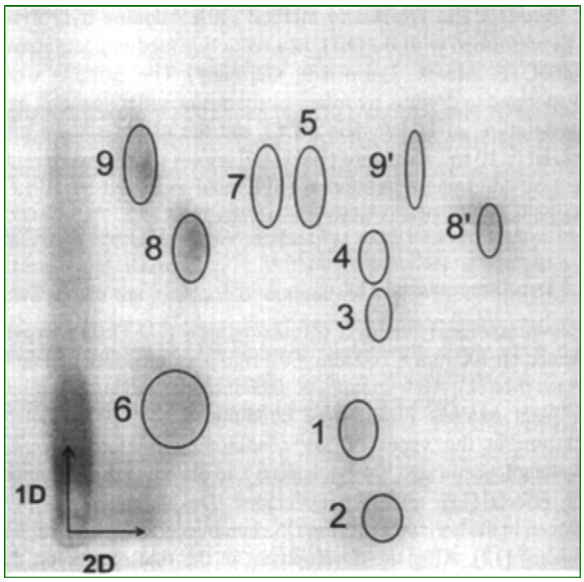

Fig. 4. Two-dimensional separation of phenolic acids from the foliage of *Peucedanum tauricum*, on cellulose. I direction eluent: T/MeOH/CH_3COOH/ACN (75.5:10:5:7.5, v/v/v/v/v), II direction eluent: HCOONa/HCOOH/H_2O (10:1:200, v/v/v). 1 = Protocatechuic acid, 2 = chlorogenic acid, 3 = genistic acid, 4 = *p*-hydroxybenzoic acid, 5 = vanillic acid, 6 = caffeic acid, 7 = syringic acid, 8 = *p*-coumaric acid, 8′ = *cis*-*p*-coumaric acid, 9 = ferulic acid, 9′ = *cis*-ferulic acid. From ref. [84] with permission from Akademia Kiado.

Table 1
Examples of solvent systems used in the analysis of phenolic acids.

No.	Mobile phases		Adsorbent	References
	First direction	Second direction		
1	MeOH/ACN/benzene/CH_3COOH (10:5:80:5, v/v/v/v)	HCOONa/HCOOH/H_2O (10:1:200, v/v/v)	Cellulose	[79]
2	H_2O/benzene/CH_3COOH (15:80:5, v/v/v)	HCOONa/HCOOH/H_2O (10:1:200, v/vv)	Cellulose	[82]
3	H_2O/toluene/CH_3COOH (15:80:5, v/v/v)	HCOONa/HCOOH/H_2O (10:1:200, v/vv)	Cellulose	[83]
4	Benzene/MeOH/absolute CH_3COOH (45:8:4, v/v/v)	HCOONa/HCOOH/H_2O (10:1:200, v/vv)	Cellulose	[84]
5	Benzene/CH_3COOH/H_2O (6:7:3, v/v/v)	CH_3COOH/H_2O (3:17, v/vv)	Cellulose	[85]
6	Toluene/Dx/ HCOOH (7:2:1, v/v/v)	Multiple gradient development: diisopropyl ether/HCOOH (80:20, v/v) – 2 cm, diisopropyl ether/HCOOH/cyclohexane (78:2:20, v/v/v) – 9 cm (2×)	Silica	[57]
7	$CHCl_3$/MeOH/HCOOH (44:3.5:2.5, v/v/v)	*n*-Hexane/AcOEt/CH_3COOH (31:14:5, v/v/v)	Silica	[13]
8	30% iPrOH/*n*-hexane (v/v)	100% AcOEt	Silica	[86]
9	10% AcOEt/85% toluene/5% MeOH (v/v)	10% MeOH/AcOEt (v/v)	Silica	[86]
10	MeOH/H_2O (40:60, v/v)	Multiple gradient development: diisopropyl ether/HCOOH (80:20, v/v) – 2 cm, diisopropyl ether/HCOOH/cyclohexane (78:2:20, v/v) – 9 cm (2×)	Graft-TLC: RP-18W/silica	[57]

often, according to literature, is a technique proposed by Smolarz and Waksmundzka-Hajnos [79]. The analyzed compounds were chromatographed on cellulose layers applying non-aqueous I direction eluent – adsorption mode and aqueous eluent and in the II direction – partition mode (for details see Table 1). The authors managed to completely separate 14 phenolic acids and their isomeric forms. Data found in the literature indicate the proposed method has been widely applied for identification of phenolic acids in plant material, e.g. in the leaves, roots and fruits of *Peucedanum verticillare* [78], in the petrioles of *Rheum* spp. [80], in the leaves of *Lavathera trimestris* [81], in the leaves of *Polygonum amphibium* [79].

Unfortunately the aforementioned system is characterized by poor efficiency, almost all bands are tailing, especially in the first direction. Despite this fact this system is advantageous when distinguishing *cis* and *trans* isomers of cinnamic acid derivatives is important (see Fig. 4).

Several modifications of the aforementioned method were proposed [82–85]. However, no further improvement has been observed when compared with the original methodology, all of the systems can be found in Table 1.

Silica gel is also a popular adsorbent in two-dimensional analysis of the polyphenol compounds. The great advantage of chromatographic systems, where the addition of organic acid was applied, is its good efficiency. However, only few phenolic acids can be completely separated, with the use of one-dimensional chromatography, on silica. As it was observed in most of the presented papers, cinammic acid derivatives are difficult to resolve, as they usually migrate as one band. Cisowski and co-workers proposed the use of multiple gradient development on silica [57]. More compounds were separated after the application of the gradient program, however, the resolution of the phenolic acid mixture was far from ideal. To improve the separation selectivity of individual phenolic compounds the authors performed 2D-TLC on silica. The development was performed with non-aqueous eluent in the first direction and, after drying, with the mobile phase gradient (for details see Table 1). The analyzed substances appeared as sharp zones, but some acids still remained unresolved (see Fig. 5). It should be noticed that performing the aforementioned two-dimensional analysis is a quite time-consuming procedure (four developments, and three drying steps).

Another disadvantage of all chromatographic systems already mentioned is that they can be only applied after the isolation of phenolic acids' fraction from plant material. They are not useful in case of more complex mixtures containing also other polyphenol compounds, e.g. flavonoids.

There are several methods in which phenolic acids are analyzed together with flavonoids, as these two groups of natural compounds are normally found in plant extracts. Medič-Šarič et al. proposed an interesting method of quantification of phenolic acids present in propolis [13]. The authors were able to establish the amount of caffeic and ferulic acid in the analyzed extracts.

Hawrył and Soczewiński analyzed the mixture of flavonoids and three phenolic acids on silica gel in normal-phase systems [86]. The optimal systems enable complete resolution of the analyzed phenolic acids and flavonoids, and can be successfully applied for identification purposes in case of plant extracts containing natural mixtures of polyphenolic compounds.

RP-18 plates have been rarely used in the analysis of phenolic acids. It can be caused by their specific behavior on this kind of adsorbent. As it was already stated polar ionizable compounds may appear as tailing spots, as they occur in solution as ionized and unionized forms. Cisowski and co-workers chromatographed a mixture of 11 phenolic acids on RP-18W plate in the first direction [57], then partly separated substances were transferred to silica

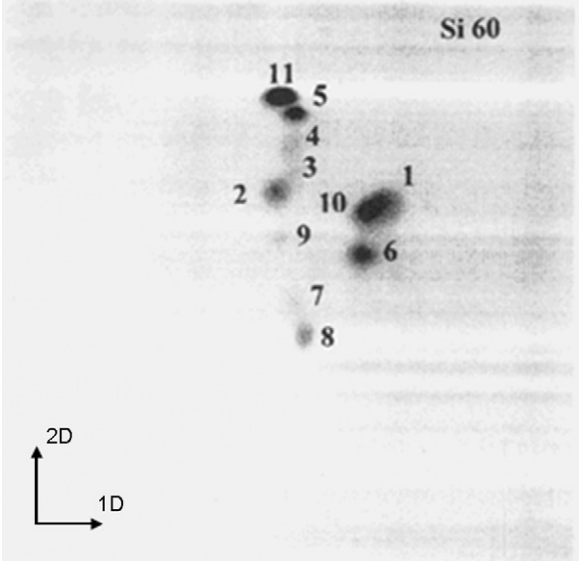

Fig. 5. Two-dimensional separation of phenolic acids standards on the silica layer. I direction eluent: T/Dx/HCOOH (7:2:1, v/v/v), II direction eluent: mobile phase gradient (for details see ref. [56]). 1 = 3,5-Dihydroxybenzoic acid, 2 = vanillic acid, 3 = *p*-coumaric acid, 4 = *p*-hydroxybenzoic acid, 5 = genistic acid, 6 = caffeic acid, 7 = syringic acid, 8 = sinapic acid, 9 = ferulic acid, 10 = protocatechuic acid, 11 = 2,4-dihydroxybenzoic acid. From ref. [56] with permission from Akademia Kiado.

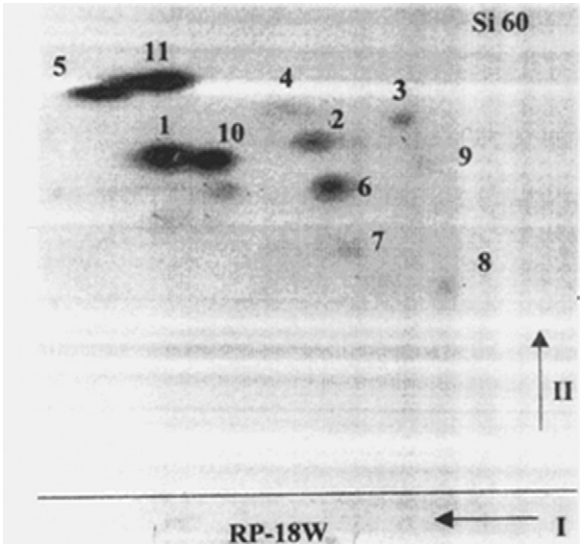

Fig. 6. Two-dimensional separation of phenolic acids standards with the use of graft-TLC procedure. I direction: RP-18W plate developed with MeOH/H_2O (40:60, v/v/v), II direction: silica developed with the mobile phase gradient, the same as in Fig. 5. The standards were transferred from the first to the second layer with 70% aqueous MeOH. Symbols are as in Fig. 5. From ref. [56] with permission from Akademia Kiado.

gel plate with the use of 70% aqueous MeOH. Phenolic acids were redeveloped with the use of mobile phase gradient (see Table 1). Complete resolution of 11 phenolic acids was obtained. Unfortunately the use of reversed-phase system, in the first direction, causes band tailing of acids containing two hyroxyl groups. Artifacts are also formed during the process of chromatography, in proposed graft-TLC technique (see Fig. 6).

Coupled layer planar chromatography technique was also proposed by Hawrył and Waksmundzka-Hajnos. The authors analyzed phenolic compounds present in extracts from *Polygonum* and *Verbascum* species [62]. First the plant extracts were developed on the RP-18W plate, with mixture of water and acetone, and after the transfer with methanol, redeveloped on silica or diol layers with the use of eluent comprising of AcOEt with MeOH, or propan-2-ol. This method can be useful for analyzing highly complex samples containing compounds spanning a wide polarity range, and with similar chemical structure.

It can be easily noticed that in case of graft-TLC technique phenolic acids should be first developed in revered-phase systems. It is of crucial importance, as strong adsorption of very polar constituents may lead to incomplete transfer of the substances from polar adsorbent.

Hawrył et al. [87] used also CN-silica plates for separations of natural mixtures of polyphenols present in extract from *Sambucus nigra* flowers. Several solvent mixtures were used for finding optimal mobile phases. Some of them are given in Table 2, where examples of solvent systems used for two-dimensional chromatography of polyphenolic compounds, are presented.

To sum up, when the analysis of phenolic acids is performed, before choosing the right two-dimensional method, it is important to answer the question of its aim, whether it is identification of isomers, or phenolic acids present in mixtures containing other polyphenols, etc. The answer will lead to a proper choice of one of the chromatographic systems mentioned above.

3.2. Coumarins

Coumarins are benzo-α-pyrone derivatives, and may be divided into three main groups: simple coumarins, furanocoumarins and pyranocoumarins. These substances have versatile biological activity and have been proven to exhibit pharmacological activity as: calcium channel blockers, anticoagulants, cytostatics, antifungal drugs, etc. [88]. Furanocoumarins are important drugs in vitiligo and psoriaris therapy. They were also applied in the process called "extracorporeal photopheresis" that is used for treating chronic graft-versus-host disease or erythrodermic variants of cutaneous T-cell lymphoma [89,90].

Each subgroup of coumarins have different polarity, thus they are easy for group separation. Unfortunately in particular groups these substances have comparable polarity and similar chemical structures. Thus the separation of closely related coumarins demands multistep procedures [91–93].

Several adsorbents have been applied for the chromatographic analysis of coumarins, e.g. silica, C18 layers, alumina, poliamide, Florisil, etc. Unfortunately one-dimensional chromatography is inadequate in the analysis of closely related coumarins (structural analogues), due to their similar chemical structure and physicochemical properties.

In case when complete separation is needed, the use of multidimensional chromatography should be considered.

Silica gel is the most popular and least expensive adsorbent, thus it has been widely used in different chromatographic methods. However, in case of two-dimensional separations of coumarins, it has been rarely applied as it is difficult to select solvent systems which are complementary in selectivity. Härmälä et al. [33] proposed very interesting method for the separation of 16 coumarins from the genus *Angelica* with the use of silica gel as an adsorbent. The application of two-dimensional overpressured layer chromatography enabled complete resolution of the analyzed substances. The authors described a very useful procedure of choosing complementary systems that can be applied in the analysis of complex mixtures, and not only coumarins. It turned out that the systems, I direction – 100% $CHCl_3$ and II direction – AcOEt/*n*-hexane (30:70, v/v) provided excellent separation of all coumarins, although having only the fourth poorest correlation value. The other solvents mixtures used in two-dimensional separations can be found in Table 2.

Table 2
Examples of solvent systems used in the analysis of coumarins.

No.	Mobile phases		Adsorbent	References
	First direction	Second direction		
1	100% $CHCl_3$	AcOEt/*n*-hexane (30:70, v/v)	Silica	[33]
2	100% $CHCl_3$	Methyl *tert*-buthyl ether/*n*-hexane (60:40, v/v)	Silica	[33]
3	Methyl *tert*-buthyl ether/*n*-hexane (60:40, v/v)	Water in dichloromethane	Silica	[33]
4	100% $CHCl_3$	Methyl ethyl ketone/*n*-hexane (24:76, v/v)	Silica	[33]
5	100% diisopropyl ether (double developed)	10% MeOH/H_2O/ 1% HCOOH (v/v)	Diol-silica	[46]
6	35% AcOEt/*n*-heptane (v/v) (triple developed)	30% ACN/H_2O (v/v) (double developed)	CN-silica	[46]
7	55% MeOH/H_2O (v/v)	35% AcOEt/*n*-heptane (v/v) (triple developed)	Multiphase plate RP-18W/silica	[46]
8	35% AcOEt/*n*-heptane (v/v) (triple developed)	55% MeOH/H_2O (v/v)	Graft-TLC: silica/RP-18W	[35,54,60]
9	30% ACN/H_2O (v/v) (double developed)	35% AcOEt/*n*-heptane (v/v) (triple developed)	Graft-TLC: CN-silica/silica	[35,54]
10	100% $CHCl_3$	Hexane/pentane/AcOEt (35:35:30, v/v/v)	Silica	[124]

Due to the possibility of the application of normal- and reversed-phase systems, polar bonded phases have been an often choice for two-dimensional separations. In case of coumarins, the use of diol- and cyanopropyl-silica have been reported.

Waksmundzka-Hajnos et al. reported the use of diol-silica for the separation of 10 coumarin standards [46]. First the compounds were chromatographed with the use of 100% diisopropyl ether (double development), then in the perpendicular direction: 10% MeOH/H_2O (v/v) containing 1% HCOOH. The use of the first direction eluent caused the separation of analyzed substances into three main groups. Thus it is useful for group separation of natural mixtures of coumarins. Chromatography in reversed-phase system enabled the complete resolution of all tested standards. The disadvantage of the applied reversed-phase system is fact, that it has low efficiency, most substances, especially those containing hydroxyl groups are tailing. Diol-silica is similar in its properties to deactivated silica, thus the application of aqueous eluent may be responsible for tailing, which was only slightly reduced after the addition of formic acid.

Better results were obtained after the application of CN-silica. In this case, coumarin standards were first chromatographed with the use of normal-phase, then in reversed-phase system (see Table 2). The plate was triple developed in the first direction to improve separation of strongly retained polar coumarins. Double development, in the second direction was aimed to improve the resolution of apolar constituents, with low R_F values in this system. The aforementioned system seems to be suitable for the analysis of even more complex mixtures. However, some coumarins remain unresolved in this system (see Fig. 7). Despite this disadvantage it is useful for identification of particular compounds present in coumarin containing extracts, as it was shown for *Heracleum spondylium*, *Heracleum sibiricum* and *Archangelica officinalis* fruit extracts.

The authors investigated also the use of multiphase plates for the identification purposes. Coumarins were first chromatographed on a RP-18W strip with 55% MeOH/H_2O (v/v), then in perpendicular direction it was triple developed with: 35% AcOEt/*n*-heptane (v/v). The use of reversed-phase system caused the separation of investigated coumarins into two groups: coumarins containing hydroxyl group, and furanocoumarins. The separation, according to the differences in polarity, is even greater than that observed on diol-silica. This system was then applied for separation of furanocoumarin fraction from fruits of *H. sibiricum*, seven compounds were identified in the extract.

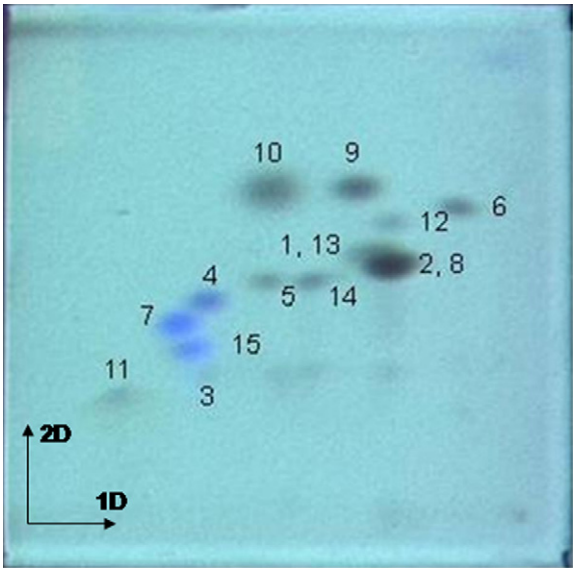

Fig. 7. Videoscan of two-dimensional separation of coumarin standards on CN-silica plate. I direction: 30% (v/v) ACN in H_2O, triple developed; II direction: 35% (v/v) AcOEt in *n*-heptane, double developed. 1 = Isopimpinellin, 2 = byacangelicol, 3 = fraxidin, 4 = umbelliferone, 5 = xanthotoxin, 6 = imperatorin, 7 = scopoletin, 8 = byacangelicin, 9 = angelicin, 10 = coumarin, 11 = aesculetin, 12 = bergapten, 13 = heraclenin, 14 = xanthotoxol, 15 = isoscopoletin. From ref. [45] with permission from Preston Publications.

The use of graft thin-layer chromatography of coumarins was also reported [35,54,60]. The authors applied two combinations of adsorbents: silica + RP-18W, and CN-silica + silica gel, eluents used can be found in Table 2. Both systems enabled complete resolution of investigated standards, and both were suitable for identification of coumarins in plant extracts. However, the first one seems to have more advantages when compared to the second one. First of all it produces greater selectivity differences, thus furanocoumarins are better resolved. Second, it does not demand as many separation steps, as the system CN-silica + silica.

The graft-TLC system silica + RP-18W was successfully applied for construction of chromatographic fingerprints of different plants from the *Heracleum* genus (see Fig. 8).

Two-dimensional chromatography has been also applied for quantitative analysis of furanocoumarins in plant extracts [54]. In order to obtain reproducible results, all investigated compounds should be completely separated. Graft-TLC with the use of adsorbents silica + RP-18W was proven to be the most suitable for quantitative analysis. Resolution of compounds was insufficient in case of 2D-TLC on one adsorbent (CN-silica), standards had to be divided into two separate groups for accurate estimation of peaks' surface area.

Quantitative analysis is difficult to perform after two-dimensional chromatographic run, as densitometers are not adjusted to scan two-dimensional chromatograms. This problem may be overcome if small steps between scans are used. In the proposed method the authors scanned the plate with the slit of dimension 5 mm × 0.2 mm, operated at λ = 366 nm, obtaining 36 tracks that were not overlapping. This wavelength was chosen to get rid of intensive baseline noise, observed at lower wavelengths. Peak areas were measured with the use of the method called "peak approximation".

A hyphenated, HPLC-TLC procedure for separation of couamrins, has been proposed by Hawrył et al. [69]. A mixture of 12 coumarins from *A. officinalis* was completely separated as a result of the different selectivities of the two combined chromatographic techniques, RP-HPLC and NP-TLC. First the analyzed compounds were separated by means of RP-HPLC. The optimal eluent: 60% MeOH in water was chosen with the use of DryLab program. All HPLC fractions were collected, evaporated and finally developed in normal-phase system, on silica gel, with the use of solvent mixture: 40% AcOEt (v/v) in dichloromethane/heptane (1:1). All fractions were completely separated. The combination of these methods gave successful results, although both methods, if used separately, failed to give good resolution (see Fig. 9). This procedure may be useful for micropreparative separation of coumarins. It is a lengthy procedure, but the time devoted to performing the analysis pays off by excellent separation results.

3.3. Flavonoids

Similar as phenolic acids, flavonoids are natural substances present in almost all plant species, thus they have been widely investigated. They are benzo-γ-pyrone derivatives, and are present in plants as glycosides and aglycones. The most frequently encountered aglycones can be divided into the following groups: flavones, flavonols, anthocyanidins, isoflavones, flavanones, dihydroflavonols, biflavonoids, chalkones and aurones [94].

Flavonoids are usually used in medicine as antioxidant, antimutagenic, diuretic, antibacterial, and antiviral drugs. They are

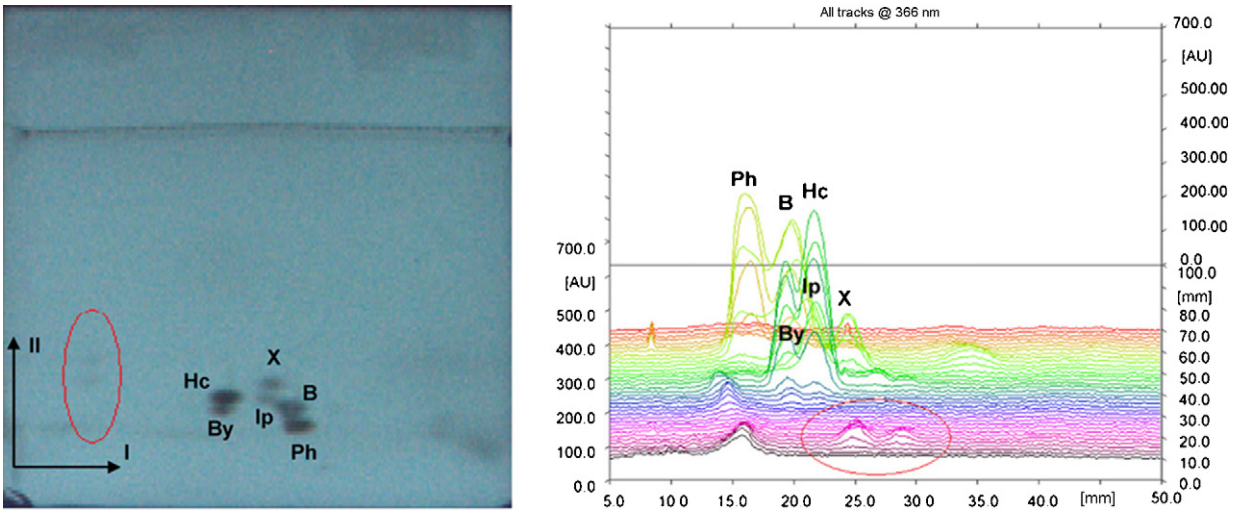

Fig. 8. Videoscan and densitogram of a RP-18W plate, on which *H. sibiricum* sample was developed in the second direction, after the transfer, with MeOH, from a silica plate. The silica plate was triple developed with 35% (v/v) AcOEt in *n*-heptane, and in case of a RP-18W plate, 55% (v/v) MeOH in water was applied. B = Bergapten, By = byacangelicol, Hc = heraclenin, Ip = isopimpinellin, Ph = phellopterin, X = xanthotoxin. From ref. [59] with permission from Elsevier.

probably responsible for reducing the risk of cardiovascular disease and stroke [95]. Due to their great number in plant kingdom (more than 4000 identified substances), their pharmacological potential is enormous and is still to be discovered.

Flavonoids are frequently encountered in plant extracts as complex mixtures, containing not only flavonoid aglycones and glucosides, but also other phenolic constituents (phenolic acids, tannins, etc.). The chromatographic behavior of flavonoids is very often similar because of their similar molecular structures. What is more, their separation is sometimes more difficult, due to the fact, that some flavonoids contain unsubstituted hydoxyl groups, and possess slightly acidic properties.

Polar adsobents (silica, cellulose, poliamide) were the first choice in case of two-dimensional chromatography of flavonoids.

Hawrył et al. [86] investigated behavior of the selected deriavtives of two flavonoid aglycones: flavones and flavanones. The authors observed that systems containing a polar modifier in a non-polar diluent (*n*-hexane) are selective only in relation to some groups of flavonoids.

Two pairs of solvent systems, characterized by the lowest correlation coefficients, were applied in the separation of polyphenol fraction present in *Betula* leaves. The applied solvent systems can be found in Table 3. The separated compounds lie on a straight line, so the applied systems are not truly orthogonal. Also the efficiency of the applied systems seems to be poor, most of the spots are diffused (see Fig. 10). Despite the disadvantages these systems can be used for preliminary identification of flavones and flavanones derivatives in plant extracts.

Silica gel was also applied in the work of Medič-Šarič et al. [13], who performed quantitative analysis of selected flavonoids in propolis samples. The remarks on the quantitative analysis of polyphenolic compounds can be found in the section devoted to phenolic acids. The authors write that they managed to perform quantitative analysis for seven flavonoids.

Detailed description of the quantitative analysis of some phenolic compounds in propolis can be found in work by Prosek et al. [96]. The authors described a computer program which enables scanning and evaluation with a TLC scanner.

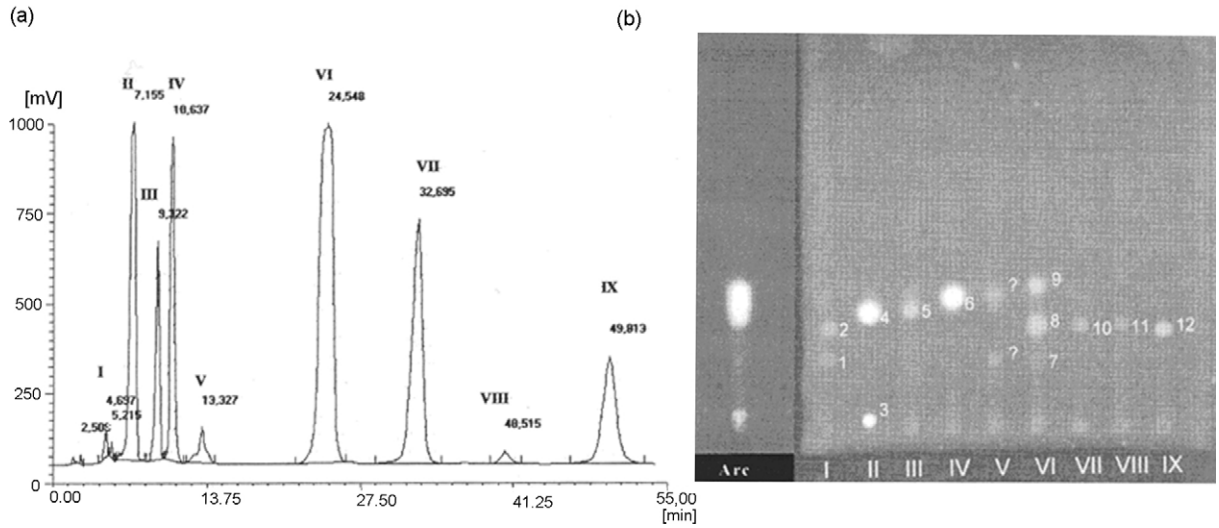

Fig. 9. (a) HPLC chromatogram of coumarins fraction from *A. officinalis* obtained by using 60% (v/v) methanol in water. Roman numbers are numbers of fractions collected during HPLC separation; (b) silica plate with the collected fractions (eluent: 40%, v/v, AcOEt in *n*-heptane/dichloromethane (1:1). 1 = Xanthotoxol, 2 = umbelliferone, 3 = archangelicin, 4 = xanthotoxin, 5 = isopimpinellin, 6 = bergapten, 7 = pimpinellin, 8 = imperatorin, 9 = phellopterin, 10 = osthol, 11 = umbelliprenin, 12 = isoimperatorin, ? = unidentified compounds, Arc = *Archangelica off.* From ref. [68] with permission from Elsevier.

Table 3
Examples of solvent systems used in the analysis of flavonoids.

No.	Mobile phases		Adsorbent	References
	First direction	Second direction		
1	30% iPrOH/*n*-hexane (v/v)	100% AcOEt	Silica	[86]
2	10% AcOEt/85% Tol/5% MeOH (v/v)	10% MeOH/AcOEt (v/v)	Silica	[86]
3	$CHCl_3$/MeOH/HCOOH (44:3.5:2.5, v/v/v)	*n*-hexane/AcOEt/glacial CH_3COOH (31:14:5, v/v/v)	Silica	[13]
4	AcOEt/HCOOH/H_2O (8:1:1, v/v/v)	Cyclohexane/acetone/HCl conc. (40:55:5, v/v/v)	Silica	[125]
5	Butanol/CH_3COOH/H_2O (50:10:40, v/v/v)	Cyclohexane/AcOEt/CH_3COOH (60:38:2, v/v/v)	Silica	[125]
6	AcOEt/HCOOH/MeOH/H_2O (100:5:4:8, v/v/v)	Tol/HCOOEt/HCOOH (5:4:1, v/v/v)	Silica	[126]
7	AcOEt/H_2O/HCOOH (6:1:1, v/v/v)	Ethylene diammonium sulfate/H_2O (1:1, v/v)	Silica	[127]
8	60% acetone/*n*-hexane (v/v)	50% MeOH/H_2O (v/v)	CN-silica	[87]
9	40% propan-2-ol/*n*-hexane (v/v)	50% THF/H_2O (v/v)	CN-silica	[87]
10	40% propan-2-ol/*n*-hexane (v/v)	50% Dx/H_2O (v/v)	CN-silica	[87]
11	Tol/CH_2Cl_2/MeOH/MEK/butanol (60:50:42:1, v/v/v/v)	H_2O/MeOH/MEK/butanol (800:240:80:1, v/v/v/v)	Polyamide	[128]
12	H_2O/butanol/acetone/Dx (70:15:10:5, v/v/v/v)	Benzene/MeOH/butanone/H_2O (55:25:23:2, v/v/v/v)	Polyamide	[129]
13	Tol/MeOH/MEK/butanol (30:20:15:0.3, v/v/v/v)	H_2O/*n*-butanol/acetone/Dx (110:15:10:5, v/v/v/v)	Polyamide	[130]
14	H_2O/*n*-butanol/acetone/Dx (70:15:10:5, v/v/v/v)	Benzen/MEK/MeOH/H_2O (55:23:20:2, v/v/v/v)	Polyamide	[131]
15	H_2O/*n*-butanol/acetone/Dx (70:15:10:5, v/v/v/v)	1,2-Dichloroethane/MeOH/butanone/H_2O (55:20:22:3, v/v/v/v)	Polyamide	[132]
16	Benzene/MEK/MeOH/butanol (40:20:15:0.2, v/v/v/v)	H_2O/butanol/acetone/Dx (18:3:2:1, v/v/v/v)	Polyamide	[98]
17	$CHCl_3$/MeOH/MEK/acetylacetone (15:2:2:1, v/v)	H_2O/MeOH/MEK/acetylacetone (6:3:2:1, v/v/v/v)	Polyamide	[97]
18	6% CH_3COOH/H_2O (v/v)	2-Butanol/CH_3COOH/H_2O (14:1:5, v/v/v)	Cellulose	[133]
19	Butanol/acetone/H_2O (4 + 1 + 5, v/v/v)	Acetone/HCl/H_2O (15:3:82, v/v/v)	Cellulose	[134]
20	n-butanol/CH_3COOH/H_2O (4:1:5, v/v/v)	CH_3COOH/H_2O (15:85, v/v)	Cellulose	[99]
21	3% HCOOH/H_2O (v/v)	Butanol/acetone/H_2O (4:1:5, v/v/v)	Cellulose	[135]
22	AcOEt/$CHCl_3$/2-propanol/H_2O (6:6:2:1, v/v/v/v)	MeOH/H_2O/HCOOH (35:15:3, v/v/v)	Graft-TLC: silica + RP-18W	[102]

In all investigated chromatographic systems, organic acids were used to suppress the ionization of slightly acidic compounds. In all cases the pseudo-reversed phase system was applied in the first direction, followed by normal-phase system in the perpendicular direction.

Polyamide was an often choice in two-dimensional separations of flavonoids in 1980s [97,98]. Because of its polarity, polyamide is normally used for separation of polar compounds, and this is the case in flavonoids (mainly flavonoid glycosides) analysis. Mixtures of polar solvents were used as mobile phases. Usually the mixtures of *n*-butanol and water were applied in the perpendicular directions.

Cellulose is normally applied in the analysis of hydrophilic and very polar constituents. Several systems, with this adsorbent, in the analysis of benzo-γ-pyrone derivatives, have been reported. Cellulose is always developed with aqueous eluents, which usually contain formic or acetic acid additives. The reported chromatographic systems have better efficiency, when compared with those on polyamide. They are especially beneficial in case of the analysis of very polar compounds, e.g. flavonoid glucosides, as it was well shown in case of phenylethanoid glucosides from *Plantago lanceolata* [99], flavonols and anthocyanins in subgenus *Rosa* [100], or various proanthocyanidins [101].

The greatest selectivity differences are obtained when normal- and reversed-phase systems are used in orthogonal directions. Soczewiński et al. [34] investigated several systems, on CN-silica, for the complete resolution of selected flavonoids. The best systems

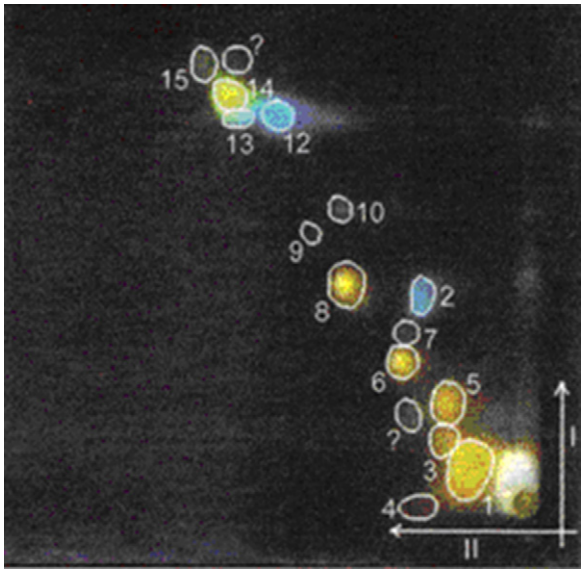

Fig. 10. Two-dimensional separation of compounds from *Betula* leaves on silica. I direction eluent: AcOEt/T/MeOH (10:85:5, v/v), II direction eluent: 10% (v/v) MeOH in AcOEt. 1 = Rutin, 2 = chlorogenic acid, 3 = naringin, 4 = hesperidin, 5 = hyperoside, 6 = astragalin, 7 = quercitrin, 8 = myricetin, 9 = caffeic acid, 10 = ferulic acid, 12 = quercetin, 13 = acaetin, 14 = kaempferol, 15 = naringenin, ? = unidentified compounds. From ref. [87] with permission from Akademia Kiado.

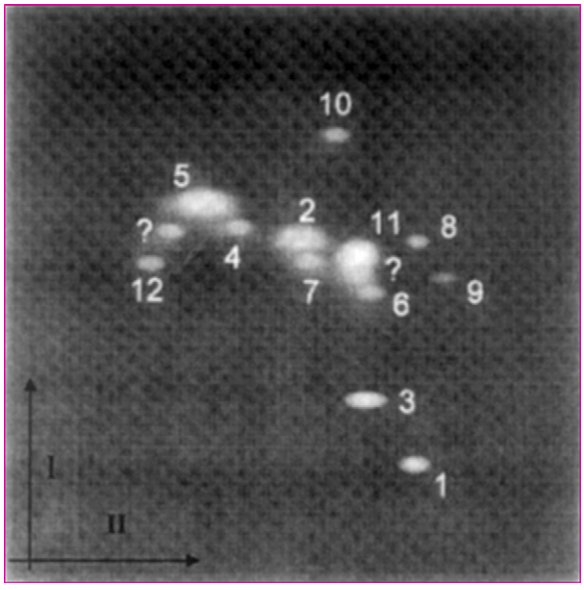

Fig. 11. 2D-TLC separation of an extract from *Flos Sambuci* on CN-silica, with 60% (v/v) acetone in *n*-hexane as the mobile phase for the first development and 50% methanol in water as the mobile phase for the second development. 1 = Myricetin, 2 = naringenin, 3 = luteolin, 4 = apigenin, 5 = acacetin, 6 = hyperoside, 7 = quercetin, 8 = naringin, 9 = rutin, 10 = hesperetin, 11 = quercitrin, 12 = astragalin, ? = unidentified compound. From ref. [88] with permission from Akademia Kiado.

Table 4
Examples of solvent systems used in the analysis of alkaloids.

No.	Mobile phases		Adsorbent	References
	First direction	Second direction		
1	MeOH/CCl_4, (50:50, v/v)	Acetone/CH_3COOH/H_2O, (72:8:20, v/v/v)	Silica	[104]
2	Diisopropyl ether/MeOH (85:15, v/v)	Acetone/CH_3COOH (90:10, v/v)	Silica	[105]
3	MeOH/$CHCl_3$/ NH_3 (85:15:0.7, v/v/v/v)	Hydrated diethyl ether/acetone/DEA (85:8:7, v/v/v)	Silica	[106]
4	MeOH/H_2O (80:20, v/v) + 1% NH_3	MeOH/acetone/diisopropyl ether/DEA (15:15:69:1, v/v/v)	Silica	[58]
5	AcOEt/MeOH/conc. NH_3 (85:10:5, v/v/v)	MeOH/conc. NH_3 (99:1, v/v)	Silica	[77]
6	MeOH/conc. NH_3 (99:1, v/v)	Tol/DEA/MeOH/AcOEt (60:10:10:20, v/v/v)	Silica	[77]
7	AcOEt/MeOH/conc. NH_3 (85:10:5, v/v/v)	Acetone/H_2O/conc. NH_3 (20:20:1, v/v/v)	Silica	[77]
8	60% MeOH/H_2O/2% NH_3 (v/v)	10% MeOH/iPr_2O/2% NH_3 (v/v)	CN-silica	[114]
9	10% MeOH/iPr_2O/2% NH_3 (v/v)	70% MeOH in water/0.1 M DEA (v/v)	CN-silica	[114]
10	MeOH/Me_2CO/DEA (50:48:2, v/v/v)	MeOH/aqueous phosphate buffer (pH 3.4) (1:3, v/v) containing 0.001 M HDEHP	Multi-K SC5 silica + RP-18	[115]
11	AcOEt/MeOH/H_2O/CH_3COOH	AcOEt/MeOH/H_2O/HCOOH (100:2.7:5:3, v/v/v/v)	Silica	[136]
12	$CHCl_3$/MeOH/25% NH_3 (85:15:1, v/v/v)	ACN/H_2O/36 % HCl (30:100:7, v/v/v)	Diol	[47]
13	$CHCl_3$/MeOH/25% NH_3 (85:15:1, v/v/v)	ACN/H_2O/36 % HCl (30:100:7, v/v/v)	Graft-TLC: diol + RP-18W	[47]
14	10% MeOH/iPr_2O/2% NH_3 (v/v) (double developed)	80% MeOH/acetate buffer (pH 3.5)/0.05 ml DEA (v/v) (double developed)	Graft-TLC: CN-silica + RP-18	[61]
15	10% MeOH/iPr_2O/2% NH_3 (v/v) (double developed)	5% Me_2CO/diisopropyl ether/2% NH_3 (v/v) (double developed)	Graft-TLC: CN-silica + silica	[61]

were chosen by correlating R_M values obtained in normal- and reversed-phase systems.

Hawrył et al. [87] managed to completely separate the extract of *Flos Sambuci* by use of the systems, most orthogonal in properties. Twelve polyphenolic compounds were identified in the extract (see Fig. 11). The applied eluents can be found in Table 3.

The applied chromatographic systems are characterised not only by high selectivity but also they are very efficient, all zones are narrow, and well shaped, no tailing is observed. This is a great advantage over two-dimensional separations on silica gel.

Krauze-Baranowska et al. [102] proposed graft-TLC method for the resolution of flavonol truxinic esters and flavonoids from *Pseudotsuga menziesii*. The application of graft-TLC procedure (silica + RP-18) led to complete separation of flavonoid complex occurring in *P. menziesii* (for the eleunts used see Table 3). The authors managed to resolve the mixture containing: hyperoside, astragalin, *trans*-tiliroside, *trans*-ditiliroside, daglesioside I, II, III, IV, kaempferol, quercetin and isorhamnetin.

Hawrył and Soczewiński [70] proposed a method of combining RP-HPLC and NP-TLC for the fractionation of 10 flavonoids. First the flavonoid separation was carried out by means of isocratic RP-HPLC (50% MeOH in water as an eluent), then compound fractions not separated on the HPLC column were developed with the use of three-step gradient on silica gel (5%, 10% and 15% MeOH/AcOEt/0.1% HCOOH, for a distance of 3 cm for each gradient step).

The method should be used for micropreparative purposes, rather than for identification. The investigated flavonoids can be easily identified by means of 2D-TLC on CN-silica, and this procedure is not as complicated as the hyphenation of HPLC and TLC.

Chromatographic systems for the analysis of flavonoids can be chosen on the basis of the polarity of investigated compounds. If the polarity of substances, being investigated, is known, silica or cellulose may be used for the analysis of polar fractions, in case of a mixture containing substances of different polarity, CN-silica is a good choice. Chromatographic systems employing this adsorbent may be also beneficial in case of the analysis of samples containing flavonoids of unknown polarity.

3.4. Alkaloids

Heterogeneous nitrogenous substances have been grouped under this heading. The alkaloids have been divided into several groups, according to their chemical structure and biosynthetic precursors.

Alkaloids are widely used as pharmaceuticals, and belong to a group of the most potent drugs (lots of them have poisonous properties). Their pharmacological activity is enormous, they are used in hundreds of illnesses, and health problems, just to mention: analgesic, antihypertensive, antibacterial, anticancer, spasmolytic, antitussive, expectorant, stimulant properties, and many others.

The chromatographic separation of alkaloids is not a trivial task, as they are normally found in complex mixtures; what is more they are heterocyclic bases and occur in solution as ionized and unionized forms. The greatest problems associated to alkaloid analysis are normally caused by the effect of silanols. The effect of chromatographic conditions on the separation of selected alkaloids is well covered by the works of Petruczynik et al. [58,103].

The attempts to use two-dimensional chromatography in alkaloids resolution were made soon after the introduction of thin-layer chromatography; several chromatographic systems, mainly on silica, were used in the analysis [104,105] (for details see Table 4).

The use of ammonia or amines as the eluent additives is often performed in chromatographic separations of heterocyclic bases, as they compete with the analytes for adsorbent silanol sites. Chromatographic procedures with the use of ammonia and diethylamine were reported in the early papers of Viala et al. [106] and Agurell [107].

Most modern, chromatographic systems, using silica as a stationary phase, incorporate non-aqueous mobile phases as eluents, as it has been reported for separation of the following alkaloid groups: tryptophane [108], carbazole [109], indole [110], ergoline [111] and benzophenantridine derivatives [112]. However, the use of silica with aqueous buffered mobile phases in so called pseudo-reversed-phase systems for the analysis of isoquinoline alkaloids was reported by Gołkiewicz et al. [113].

The pseudo-reversed system, was used as the first direction in 2D separation of isoquinoline alkaloids. Petruczynik et al. managed to completely separate a mixture of nine alkaloids, with the use of MeOH/H_2O (80:20, v/v) + 1% NH_3 in the first direction and MeOH/acetone/diisopropyl ether/DEA (15:15:69:1, v/v) applied orthogonally [58]. The system was successfully applied in the identification of alkaloids present in *F. officinalis* herb extract. The applied systems resulted in good efficiency and peak symmetry. The second direction eluent, when applied in one-dimensional chromatography gives also good results, seven alkaloids were resolved. It may replace 2D system in the analysis of less complex alkaloid mixtures.

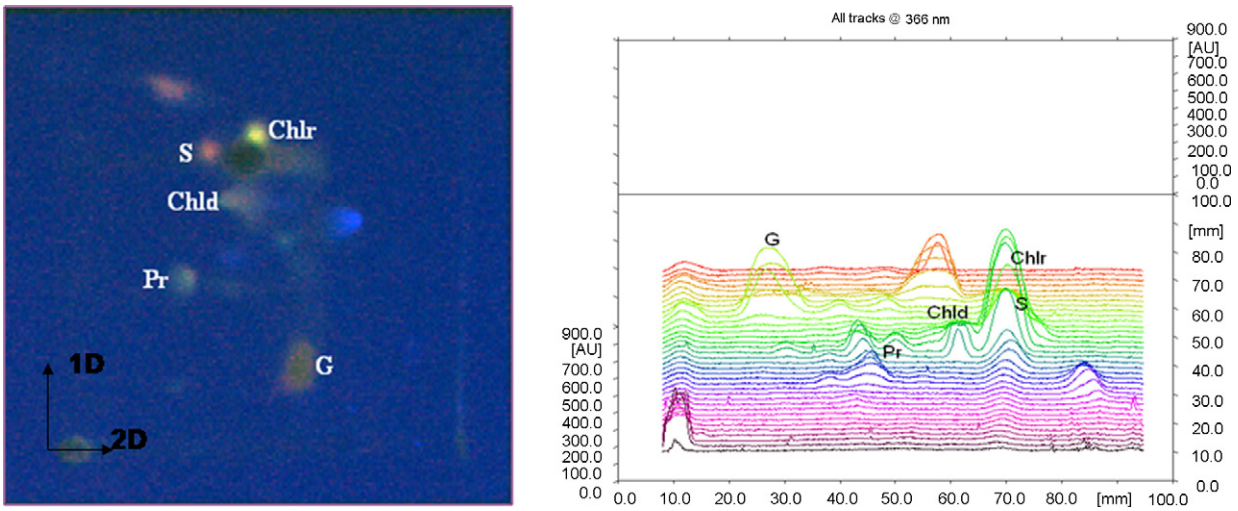

Fig. 12. Videoscan and densitogram of the CN-silica plate on which *Glaucium flavum* extract was chromatographed. Eluent systems: I direction – 10% MeOH in diisopropyl ether/2% NH_3, II direction – 70% (v/v) MeOH in water/0.1 M of DEA. G = Glaucine, Pr = protopine, Chld = chelidonine, S = sanguinarine, Chlr = chelerithrine. From ref. [121] with permission from Preston Publications.

Nováková [77] described a procedure of opiates detection by 2D HPTLC on silica. The author chromatographed the analyzed sample along with standards on the same chromatoplate. Six opiates were identified with the use of the technique, eluents applied can be found in Table 4.

The author indicates the use of HPTLC plates and two-dimensional procedure resulted in the improvement of the detection limit, when compared to traditional one-dimensional approach.

Petruczynik et al. [114] investigated the influence of different mobile phase additives on the retention, system selectivity and efficiency in case of the alkaloid analysis on cyanopropyl silica layers. Both aqueous and non-aqueous eluents were checked in the research. The most selective and efficient systems were applied for the separation of alkaloid fraction from *Chelidonium officinalis*, *F. officinalis* and *G. flavum* extracts by 2D-TLC. Reversed-phase system was applied in the first direction, followed by normal-phase one, applied orthogonally in the analysis of the two first extracts. A disadvantage of the applied system is rather poor shape of the obtained spots (see Fig. 12). A modified eluent system was used for the separation of *G. flavum* herb extract (see Table 4).

Bilayer plates have also been used in the analysis of alkaloids. Gadzikowska et al. [115] performed two-dimensional chromatography on Multi-K SC5 TLC plates containing a 3 cm × 20 cm strip of silica and a 17 cm × 20 cm layer of C18. The tropane alkaloids fraction was first developed in normal-phase system, then it was redeveloped on octadecyl-silica. The authors had to apply methanol (as in the graft-TLC procedure) to transfer the alkaloids to the C18 layer, then the silica strip was cut off and plate developed in the second direction. The cutting off the adsorbent ensured that resolution is maximized and the separation time is minimized. It was proved by Poole et al. [14] that most favorable separation conditions are achieved by minimizing the distance between the solvent entry position and the sample application position. The applied system can be used for qualitative analysis of natural mixtures containing tropane alkaloids.

Two-dimensional thin-layer chromatography with adsorbent gradient was proposed by Łuczkiewicz et al. [47] in the analysis of quinolizidine alkaloids. Graft-TLC procedure was used: quinolizidine alkaloids were first chromatographed in normal-phase system on diol-silica and after the transfer on RP-18W plate. The transfer was made by the mobile phase used in the second direction (ACN/H_2O/HCl, 30:100:7, v/v), what is rather unusual in graft-TLC. Ammonia was chosen as the mobile phase additive in the first direction because diethylamine led to problems in detecting the compounds (the use of Dragedorff's reagent gave intense orange background due to incomplete removal of DEA). Hydrochloric acid was used as acidity modifier in order to convert the compounds into their hydrochlorides, which migrate in the polar mobile phase. Although the chromatographic system applied in the first direction is characterized by rather poor efficiency (tailing bands), the use of graft-TLC enabled complete separation of the alkaloids. The optimized system was eventually applied to separate alkaloid fractions from plant and in-vitro cultures of *Genista* spp. (see Fig. 13).

An interesting method of joining CN-silica plates with other adsorbents for the analysis of isoquinoline alkaloids was presented by Petruczynik et al. [61]. Cyanopropyl-silica was used as the first direction adsorbent in normal-phase system, with the addition of ammonia. After the transfer with methanol the alkaloids were re-chromatographed on RP-18W plate or on silica (normal phase

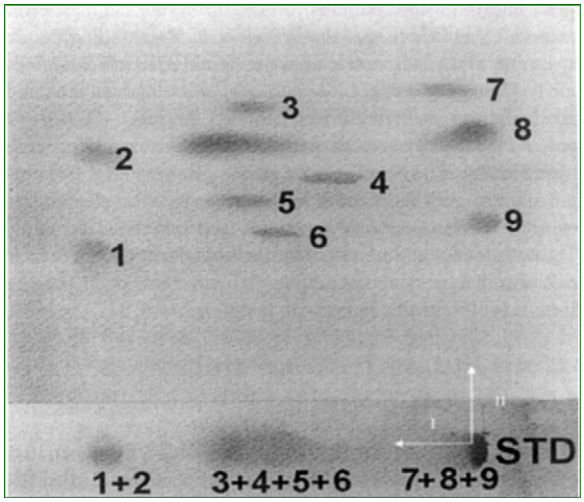

Fig. 13. 2D-TLC with an adsorbent gradient of quinolizidine alkaloid standards. Systems: I direction – diol-silica/$CHCl_3$/MeOH/ NH_3 (85:15:1, v/v/v), II direction – RP-18W/ACN/H_2O/36% HCl (30:100:7, v/v/v). The second mobile phase was used for transferring the alkaloids from diol-silica to RP-18W plate. 1 = Lupanine, 2 = methylcitisine, 3 = cytisine, 4 = hydroxylupanine, 5 = sophocarpine, 6 = lusitanine, 7 = retamine, 8 = sparteine, 9 = isosparteine. From ref. [46] with permission from Akademia Kiado.

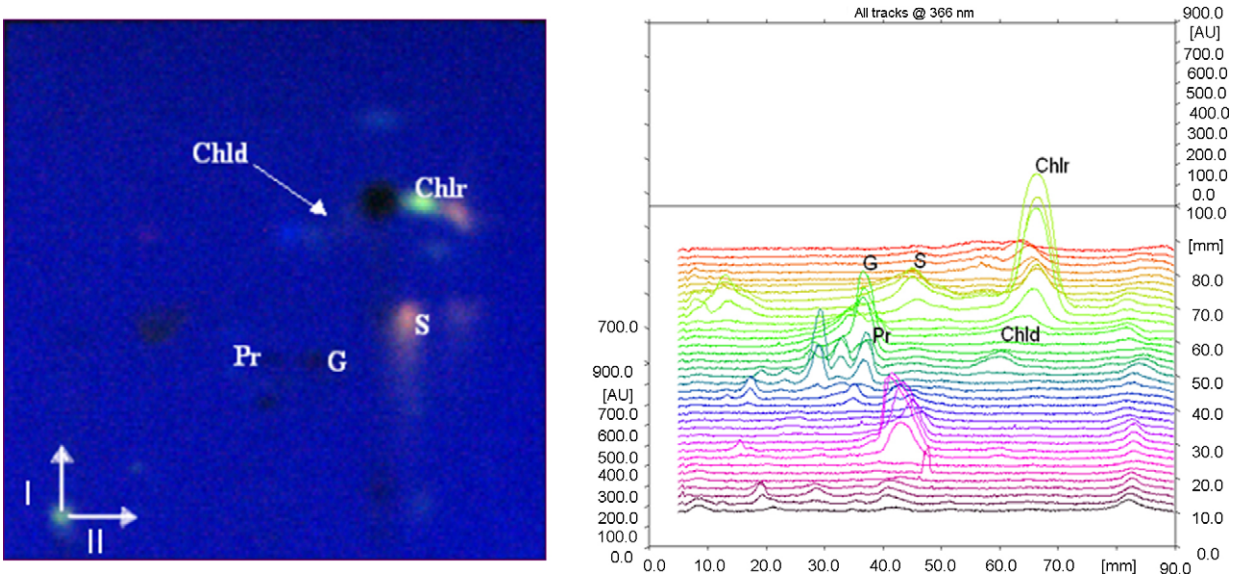

Fig. 14. Videoscan and densitogram of the plate on which *Glaucium flavum* extract was chromatographed. Systems: I direction – CN-silica 10% MeOH/diisopropyl ether/2% NH_3 (v/v), II direction – RP-18W 80% ACN/H_2O/2% NH_3 (v/v). G = Glaucine, Pr = protopine, Chld = chelidonine, S = sanguinarine, Chlr = chelerithrine. From ref. [60] with permission from Preston Publications.

system). When compared to two-dimensional separations on CN-silica, the applied systems are much more orthogonal in properties, especially the one with octadecylsilica adsorbent. Despite the fact, that obtained spots have poor shape, these systems turned out to be useful in the analysis of alkaloid fractions present in some species of the *Papaveraceae* family (see Fig. 14).

Two-dimensional analysis of alkaloids is not a simple task. It is of crucial importance to carefully choose the mobile phase additives, influencing chromatographic systems efficiency (ammonia, DEA, acids, ion-pair reagents, etc.). Depending on the alkaloids properties, as well as the aim of the analysis, a proper procedure of performing two-dimensional analysis should be chosen.

3.5. Triterpenoids

Triterpenoids can be divided into at least four major classes: true triterpenes, steroids, saponins and cardiac glycosides [116]. Saponins, ecdysteroids, triterpene and cardiac glycosides have been separated by means of 2D-TLC so far.

Saponins are widely distributed in plant species, and according to the nature of the aglycone can be classified into steroidal and triterpene groups. Saponins have many health benefits. Studies have illustrated the beneficial effects on blood cholesterol levels, cancer, bone health and stimulation of the immune system [116].

Their determination is a difficult task, as they are present in plant extracts in complex mixtures, and due to the fact that they are constituents spanning a wide polarity range. Thin-layer chromatography is often applied in the analysis of these compounds, as they do not have suitable chromophore for UV detection, thus are difficult for HPLC analysis [117].

The potential of two-dimensional separation has been occasionally applied in their analysis.

Kapusta et al. [118] proposed a method of saponins separation from aerial parts of *Medicago tranculata*. The authors used two-dimensional system for the resolution of investigated saponins. First the sample was developed on silica with the use of AcOEt/CH_3COOH/H_2O (7:2:2), and re-chromatographed with $CHCl_3$/MeOH/H_2O (63:32:5). Complete resolution of saponins was obtained, thus the aforementioned system is useful in the analysis of complex saponin mixtures.

Glensk et al. [59] developed a method of resolution of saponins from a *Ginseng* preparation. The authors decided to check whether graft-TLC procedure is useful in the analysis of ginsenosides. The pharmaceutical formulation was first developed on RP-18 plate with MeOH/H_2O (70:30, v/v). After the transfer with methanol, to a silica plate, the investigated sample was re-developed in perpendicular direction with a mixture: $CHCl_3$/MeOH/H_2O (70:30:4, v/v/v). The application of adsorbent gradient caused the spots were well resolved when compared to two-dimensional chromatography on one adsorbent (see Fig. 15). This method could be useful for routine analysis of crude plant extracts and pharmaceutical formulations rich in saponins.

There has been a report of using graft-TLC technique for analysis of saponins in *Silene vulgaris* [119]. In this case the analyzed extract was also first developed on RP-18W plate, and then on silica HPTLC

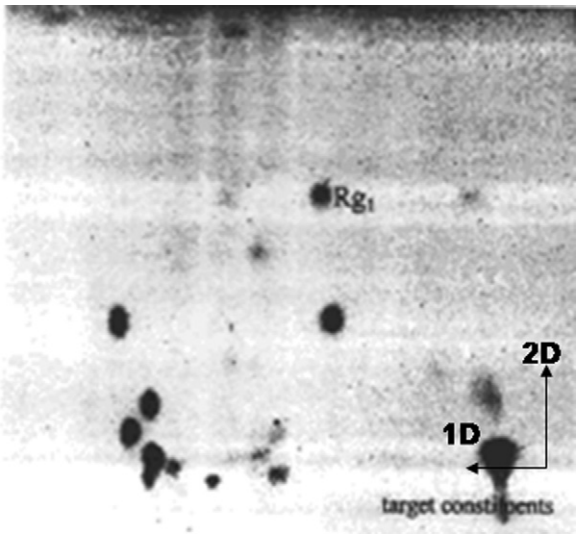

Fig. 15. 2D-TLC of a *Ginseng* preparation with an adsorbent gradient. Systems: I direction – RP-18W MeOH/H_2O (70:30, v/v), II direction – silica/ $CHCl_3$/MeOH/H_2O (70:30:4, v/v/v). After the first development the saponins were transferred to the second plate with MeOH. Rg_1 = Ginsenoside Rg_1. From ref. [58] with permission from Akademia Kiado.

Table 5
Examples of solvent systems used in the analysis of saponins.

No.	Mobile phases		Adsorbent	References
	First direction	Second direction		
1	AcOEt/CH_3COOH/H_2O (7:2:2, v/v/v)	$CHCl_3$/MeOH/H_2O (63:32:5, v/v/v)	Silica	[118]
2	$CHCl_3$/MeOH/H_2O (70:30:4, v/v/v)	1-Butanol/AcOEt/H_2O (4:1:1, v/v/v)	Silica	[59]
3	MeOH/H_2O (70:30, v/v)	$CHCl_3$/MeOH/H_2O (70:30:4, v/v/v)	Graft-TLC: RP-18 + silica	[59]
4	HCOOH/MeOH (3:7, v/v)	$CHCl_3$/MeOH/HCOOH/H_2O (10:4:1:1, v/v/v/v)	Graft-TLC: RP-18 + silica	[59]
5	$CHCl_3$/MeOH/H_2O (65:35:1, v/v/v)	$CHCl_3$/MeOH/acetone (2:1:2, v/v/v)	Silica	[137]
6	$CHCl_3$/MeOH/H_2O (21:11:4, v/v/v)	1-Butanol/AcOEt/H_2O (4:1:1, v/v/v)	Silica	[138]
7	$CHCl_3$/MeOH/H_2O (14:16:1, v/v/v)	$CHCl_3$/MeOH/H_2O (7:3:1, v/v/v) + CH_3COOH (9:1, v/v)	Silica	[139]

plate. The application of 1% aqueous HCOOH/MeOH (3:7, v/v) in the first direction and $CHCl_3$/MeOH/HCOOH/H_2O (10:4:1:1, v/v/v/v) orthogonally, resulted in separation the extract into 18 components compared to nine obtained in conventional one-dimensional TLC.

As it is seen in the examples, when graft-TLC technique is applied in the analysis of saponins, they should be first chromatographed in reversed-phase system, as they are easier to transfer to another adsorbent.

Other two-dimensional chromatographic systems, as well as those, mentioned above can be found in Table 5.

Mobile phases at different pH values were applied in orthogonal dimensions for separation of several groups of triterpene plant glycosides: neutral bidesmoside glycosides, bidesmoside glycosides with additional free carboxylic groups in the aglycone, monodesmoside glycosides, acidic glycosides with glucuronic acid and sulfate residues, etc. [120]. The use of neutral eluent ($CHCl_3$/MeOH/H_2O, 100:30:5, v/v) in the first direction and alkaline ($CHCl_3$/MeOH/25% NH_3, 100:30:6 or 100:40:10, v/v) in the perpendicular one can divide triterpenoid derivatives into groups of mono- and bidesmosidic glycosides. The application of neutral and acidic ($CHCl_3$/MeOH/H_2O, 100:30:5, v/v/v, addition of HCOOH (3–5%)) mobile phases enabled the separation of glycosides with uronic acids from neutral glycosides. All separations were performed on Silufol TLC plates.

Among steroids only ecdysteroids have been analyzed most often, with the use of two-dimensional thin-layer chromatography. These substances occur frequently in plant extracts as polar (glycosides) and non-polar (acetates) compounds.

Bathori et al. [121] chromatographed ecdysteroids, in normal-phase systems, in the first direction, e.g. toluene/acetone/96% EtOH/25% NH_3 (100:140:32:9, v/v/v/v), and reversed-phase ones orthogonally, e.g. $CHCl_3$/MeOH/benzene (25:5:3, v/v/v). The authors proved the usefulness of these systems in the separation of 16 different ecdysteroids from *Silene italica* subsp. *nemoralis*. The applied systems gave satisfactory separation even in the presence of substantial impurities and for mixtures containing many ecdysteroids.

Cardiac glycosides are natural compound used in the treatment of heart muscle failure. Chemically they are glycosides with a steroid moiety. Clarke and Cobb [122] separated a mixture of 32 cardenolides on silica gel. First the substances were chromatographed with a mixture: AcOEt/CH_2Cl_2/MeOH/H_2O (120:72:7:4, v/v/v/v), and in the second: CH_2Cl_2/MeOH (9:1).

3.6. Antraquinones

Antraquinones are the most widely distributed quinone derivatives in plant kingdom. As far as their medicinal use is considered, they have been usually used as laxatives. The latest studies show the potential use of antraquinones as antioxidants, antibacterial and antifungal drugs. Like many other groups of natural compounds antraquinones are isolated as groups of similar structures, their complete separation is a difficult task.

Different chromatographic methods were applied in the analysis of these quinone derivatives. Two-dimensional chromatography has been rarely used in the analysis of this group of natural compounds, however, there are interesting methods of their analysis, worth mentioning.

Reynolds [123] used two-dimensional chromatographic separations in chemotaxonomic research. On the basis of two-dimensional fingerprint the author proved that some, plants originally grouped under the name *Aloe turkanensis*, are taxonomically distinct as a new species *Aloe scabrifolia*. The plant extracts were chromatographed on silica, with the use of: propanol/iPrO/H_2O (7:5:1) and $CHCl_3$/EtOH/ H_2O orthogonally.

Matysik [63] proposed a new two-dimensional approach for a complete resolution of sennoside A and B monoantraquinones. The author used multiple gradient development technique in one direction, and then developed the plate in the perpendicular direction.

4. Conclusions

Two-dimensional chromatography is a popular method for solving some analytical problems encountered in the analysis of complex samples of natural origin. Its potential to produce greater spot capacity, when compared to one-dimensional chromatography, can be used as one of the first laboratory methods for examining botanical material. It can be followed by HPLC, that enables obtaining more reliable quantitative data, or HPLC-MS that gives the possibility to confirm the structures of the separated compounds.

A very important task is the evaluation of the data after two-dimensional separation. Some 2D-TLC methods have been introduced, however, most of them are not properly validated, thus they still may not compete with data obtained after one-dimensional separation, or those produced by HPLC.

There is a growing trend of combining different chromatographic techniques in orthogonal dimensions. The use of different unidimensional methods with two-dimensional techniques belongs to one of the most popular ones. If such problems, as time consuming procedures or insufficient reproducibility are overcome, such techniques may be of value in the analysis of samples containing substances with different polarities or structural analogs.

Two-dimensional thin-layer chromatography is becoming very popular in phytochemical analysis. New multidimensional planar chromatography methods may be a proper solution in everyday laboratory work. For example, they can be applied in chemotaxonomical research for (1) screening plant extracts that are only partly resolved after one-dimensional separation, (2) checking stability of a sample during chromatography, (3) resolving complex samples that may lead to column contamination and (4) checking the presence of adulterants in highly complex samples, etc.

In our opinion the future will bring new two-dimensional chromatography methods for separation of real botanical samples. If properly validated, these methods may be applied in routine phytochemical analysis, along with HPLC-MS, HPLC-NMR HPLC-HPLC, or other methods.

Definitions

BMD: repeated development, in the same direction, during which both the development distance and mobile phase composition change; the development distance is increased and the eluent strength of the mobile phase reduced for consecutive development steps.
GMD: repeated development, in the same direction, during which the development distances of consecutive development steps are identical, while the mobile phase strength is increased.
IMD: repeated development, in the same direction, performed by increasing the development distance using the same mobile phase composition.
UMD: repeated development of the plate, in the same direction, over the same development distance, with a mobile phase of constant composition, with careful drying between development steps.
MD-PC: multidimensional planar chromatography, a method in which different modes of unidimensional techniques are combined with two-dimensional methods in order to obtain greater spot capacity when compared to one dimensional separations.

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