16 Application of TLC in the Isolation and Analysis of Flavonoids

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16.1 INTRODUCTION

16.1.1 Chemistry, Biochemistry, and Medicinal Significance of the Flavonoids

Flavonoids constitute one of the largest and recently very popular group of phytochemicals. They are virtually ubiquitous in green plants and as such are likely to be

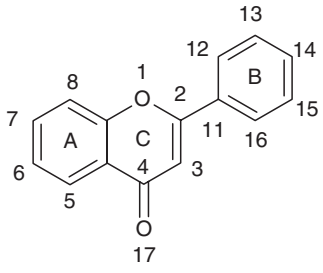


FIGURE 16.1 Basic flavonoid nucleus. Subdivision to different groups is primarily based on the presence (or absence) of a carbonyl substituent on the position C4, the presence (or absence) of a double bond between carbon atoms 2 and 3, and a phenyl substitution at the positions 2 or 3 of the pyrone ring.

encountered in any work involving plant extracts. The term flavonoid is a collective term for plant pigments, mostly derived from benzo-γ-pyrone, which is synonymous with chromone.[1] In plants, flavonoid aglycones (flavonoids without attached sugars) occur in a variety of structures, all containing fifteen carbon atoms arranged in a C_6–C_3–C_6 configuration (flavonoid nucleus is depicted in Figure 16.1).

Until now, more than 4000 flavonoids have been identified and this number is constantly growing because of the great structural diversity arising from the various hydroxylation, metoxylation, glycosylation, and acylation patterns. Most frequently encountered groups of flavonoid aglycones include flavones, flavonols, anthocyanidins, isoflavones, flavanones, dihydroflavonols, biflavonoids, calchones, and aurones. Flavonoid aglycones possess the chemical properties of phenolics, and thus they are slightly acidic. Those possessing a number of unsubstituted hydroxyl groups, or sugar moieties, are polar substances and soluble in polar organic solvents. The presence of sugar makes flavonoid more water soluble, while less polar aglycones like isoflavones, flavanones, and highly methoxylated flavones and flavonols tend to be more soluble in ether or chloroform.[2] Flavonoids have important roles in plant physiology and are components of the diet of numerous herbivores and omnivores, including humans. This group of compounds exhibit an extraordinary array of biochemical and pharmacological activities in mammalian systems, such as anti-inflammatory, antioxidant, immunomodulatory, hepatoprotective, antimicrobic, and antiviral.[3,4] With the increased popularity and use of herbal medicines containing flavonoids, the question of composition determination and standardization arises. It is almost impossible to compare any kind of biological activity without the chemical characterization of the plant extract. For this reason it is important that scientists working in this field are aware of the importance of the content analysis and for that purpose use the appropriate analytical tools.

16.1.2 Brief Overview of Use of TLC in the Analysis of Flavonoids in Plants

Since the early 1960s, TLC has been used in flavonoid analysis. Paper chromatography (PC) was the preferred method in the past and valuable descriptions of the

use of PC in flavonoid identification and isolation are given in Markham's *Techniques of flavonoid identification*.[2] To identify or isolate individual compounds, two-dimensional PC was used. Thin layer chromatography slowly replaced the paper chromatography, as new stationary phases (such as microcrystalline cellulose, polyamide, and silica) were developed. Numerous TLC protocols that can be used in flavonoid separations (including the analysis of very well known flavonoid drugs such as *Arnicae flos*, *Calendulae flos*, *Crataegi flos* and *folium*, *Betulae folium*, *Rubi ideaei folium*, *Equiseti herba*, and many others) are listed in the TLC atlas of plant drug analysis.[5] A good review on the use of TLC in this field is given in Sherma's review article as well.[6]

TLC was the method of choice for herbal analysis before instrumental chromatographic techniques such as gas chromatography (GC) or high performance liquid chromatography (HPLC) were established. Although GC and HPLC are nowadays considered to be the leading techniques (as described in Ref. 7 as well as in the review article of de Rijke et al.[8]), TLC is still the basic tool for the identification of natural compounds given in various pharmacopoeias. It is often used to provide the first characteristic fingerprints of herbs.[9] In addition, TLC technique is constantly improving.[10,11] Modern high-performance TLC (HPTLC) is an efficient instrumental technique and optimized quantitative HPTLC using a densitometric evaluation can produce results that are analogous to those obtained with GC and HPLC.

In this chapter we will focus on the use of TLC in flavonoid analysis during the past decade, especially on the use of modern instrumental techniques, as well as new methods like rotation planar chromatography (RPC) and overpressured-layer chromatography (OPLC).

16.2 TLC TECHNIQUES

16.2.1 Basic Principles for Flavonoid Separations

Nowadays TLC analysis of flavonoids is performed mostly on silica gel as stationary phase, using adsorption chromatography as the mode of separation. For normal-phase separations a variety of mobile phases is available, when compared with the reversed-phase chromatography, in which only a few water-miscible organics can be used (in this case methanol is the typical organic phase). As PC was the first form of layer chromatography, identification of flavonoids (prior the silica gel became the most popular stationary phase) was performed on cellulose as well. In Markham's book, recommendations for different solvent and sorbent type (cellulose, polyamide or silica) combinations can be found, depending on the particular flavonoid group (Table 16.1).[2]

Flavonoid aglycones and glycosides separations listed in the TLC atlas of plant drug analysis were performed on silica gel TLC plates, containing fluorescence indicator. According to the authors, ethyl acetate–formic acid–glacial acetic acid–water (100:11:11:26, v/v) or formic acid–water–ethyl acetate mixed in different proportions (with or without ethylmethyl ketone) are suitable mobile phases for the TLC screening of polar flavonoid glycosides. The good start for investigation of less polar flavonoid aglycones would be a mobile phase composed of toluene–ethyl

TABLE 16.1
Recommended Solvent/Adsorbent Combinations for TLC Identification of Different Flavonoid Types

	Mobile Phase		
Adsorbent Type	**Celullose**	**Polyamide**	**Silicagel**
Flavonoid group			
Flavonoid glycosides	• *t*-Butanol–acetic acid–water (3:1:1, v/v)[a] • *n*-Butanol–acetic acid–water (4:1:5, v/v)[a]	• water–methanol–ethylmethylketone–acetylacetone (13:3:3:1, v/v)	• Ethylacetate–pyridine–water–methanol (80:20:10:5, v/v), especially flavone C-glycosides
Polar flavonoid aglycones (e.g., flavones, flavonols)	• *t*-Butanol–acetic acid–water (3:1:1, v/v)[a] • Chloroform–acetic acid–water (30:15:2, v/v)[b]	• Methanol–acetic acid–water (18:1:1, v/v)	• Toluene–pyridine–formic acid (36:9:5, v/v)
Nonpolar flavonoid aglycones (e.g., dihydroflavonoids, isoflavones, methylated flavones)	• 10–30% acetic acid		• Chloroform–methanol (15:1 to 3:1, v/v)

[a] Mobile phase is mixed thoroughly in a separating funnel and upper phase is used.
[b] Mobile phase is mixed thoroughly in a separating funnel and excess of water is discarded.

formiate–formic acid (50:40:10, v/v) or toluene–dioxan–glacial acetic acid (90:25:4, v/v).[5] Combination of hydrophobic organic solvents such as *n*-hexane or chloroform with more polar ones (ethyl acetate or methanol), with addition of acetic or formic acid (e.g., *n*-hexane–ethyl acetate–acetic acid, 31:14:5, v/v, or chloroform–methanol–formic acid, 44:3.5:2.5, v/v), might be also used.[12]

Detection of flavonoids is usually performed under the UV light at 254 nm (all flavonoids cause fluorescence quenching) or at 366 nm (depending on the structural type, flavonoids show dark yellow, green, or blue fluorescence). Fluorescence can be enhanced using various spray reagents, which will lower the detection limit. The most popular ones are natural product—polyethylene glycol reagent (NP/PEG; the plate is first sprayed with 1% methanolic diphenylboric acid-β-ethylamino ester, followed by 5% ethanolic polyethylene glycol-4000) and 1% ethanolic or methanolic aluminum-chloride ($AlCl_3$) solution. Other derivatization reagents such as diazotized sulphanilic acid (compounds with free phenolic hydroxyl groups will show as yellow, orange or red spots), vanilin-5% HCl (red or purplish-red spots will be produced by cathechins and proanthocyanidins immediately after spraying and warming the plates, and by flavanones and dihydroflavonols more slowly), and fast blue salt B (phenolic compounds will form blue or blue-violet azo-dyes with

this reagent) can be used as well. Detailed descriptions for preparation of spraying reagents are given in Markham's *Techniques of flavonoid identification* and the TLC atlas of plant drug analysis.[2,5]

16.2.2 Sample Preparation

Medicinal plant extracts are known to be very complicated samples, which contain a variety of different compounds. Therefore, matrix effect cannot be ignored when the analysis of such sample is needed. Isolation of flavonoids from the sample matrix is thus the most important step prior to any analysis scheme.

The ultimate goal of a sample preparation is the preparation of a sample extract enriched in all compounds of interest and free of interfering matrix impurities. Usually it includes a number of steps from extraction of crude sample, purification, and preconcentration of obtained extract. Sample handling strategies for determination of biophenols (including flavonoids) in food and plants are reviewed in the paper of Tura and Robards,[13] and later in Robard's article.[14] Contaminating non-flavonoid substances such as carbohydrates, proteins, lipids, pigments, etc., do interfere with later chromatographic analysis so the development of modern purification techniques significantly facilitates identification of phenolics in different samples.[11,15] Sample preparation depends on the nature of both analyte and sample, particularly on analyte's physicochemical properties such as solubility and lipophilicity (partitioning between hydrophobic and hydrophilic phases). Liquid–liquid extraction (LLE), which is based on the partition of analyte usually between two immiscible phases (solvents), is a simple method that can be used for purifying herbal extracts. An illustrative example of LLE is removal of chlorophylls, xanthophylls, and other hydrophobic contaminants (various fats and terpenes) from aqueous extracts by extraction with hydrophobic solvents (e.g., chloroform or hexane).[2] In such way LLE was used in the work of Mbing et al.[16] First described LLE step was purification of aqueous extract of *Ouratea nigroviolacea* Gilg ex Engl. with hexane (to remove the hydrophobic components) and such extract was later partitioned with ethyl acetate. This method was used to purify the extract and facilitate the isolation of two biflavonoids—ouratine A and B. Solid-phase extraction (SPE) appeared as an alternative to LLE and it is now certainly the most widely used clean-up method, which is also applied for fractionation and concentration of analytes from herbal extracts. Nevertheless, its use in flavonoid analysis is relatively new. A brief overview of use of SPE in polyphenols analysis is given in Ref.7 and in different review articles.[8,13,14] Considering the physicochemical properties of flavonoids, in most cases reversed phase C18-bonded silica is the sorbent of preference and the sample solution and used elution solvents are usually slightly acidified to prevent ionization of the flavonoids, which would reduce their retention. Under the aqueous conditions, the flavonoids are retained on the column, while the most of the water soluble contaminants are not. After rinsing the cartridge with water to remove the hydrophilic impurities, flavonoids can be eluted with an appropriate volatile solvent such as methanol. Although TLC is known to be an analytical technique that does not require a complicated sample pretreatment, there are more and more examples in the literature in which SPE is used to diminish the matrix

effect and in such a way facilitate quantification. In the work of Nowak and Tuzimski[17] SPE was used for preliminary cleaning of rose leaves extracts to remove the interfering matrix components that affected the baseline resolution and caused difficulties during identification and quantification of flavonoids of interest quercetin and kaempferol. Pretreated aqueous extracts were passed through octadecyl SPE microcolumns. After application of the samples, the microcolumn was washed with water and adsorbed aglycones were desorbed with pure methanol and subjected to further TLC analysis. Chromatography was performed on silica gel and cellulose HPTLC plates and developed in a horizontal chamber. For quantitative determination silica plates were used with 1,4-dioxane–toluene–85% acetic acid ($6 + 24 + 1$, v/v) as mobile phase. Densitograms were recorded at 373 and 364.8 nm, respectively. Direct application of wine samples to the chromatographic plate in the work of Lambri et al.[18] led to the spot broadening during TLC migration and to nonuniform migration of polar compounds. Such results implied that SPE would be a necessary step in analysis of anthocyanins. Consequently, samples were submitted to purification on a reversed-phase SPE cartridge previously activated by elution of methanol and water. Applied wine samples were first washed with water and then eluted with methanol–formic acid (90:10, v/v). Collected purified samples were applied to the RP-18 WF_{254s} HPTLC plates. The best separation of anthocyanins was achieved by methanol–water–trifluoroacetic acid (55:45:1, v/v). Qualitative and quantitative analysis of obtained chromatograms was performed at 520 nm using scanning densitometry. To improve the separation of flavonoid glycosides (eriocitrin, hesperidin, luteolin-7-*O*-rutinoside and diosmin) and rosmarinic acid from *Mentha x piperita* L., aqueous extracts were applied to an SPE octadecyl microcolumn. Obtained methanolic eluates were subsequently subjected to the TLC analysis on unmodified silica gel layers and on silica gel chemically modified with polar (HPTLC NH_2, HPTLC CN) and nonpolar groups (HPTLC RP-18W). The mobile phase acetone–acetic acid ($85 + 15$, v/v) enabled successful separation on the aminopropyl adsorbent, while optimal mobile phase for octadecyl layer was water–methanol ($60 + 40$, v/v).[19] SPE was also used to separate the glycerine from the glycerinic extracts of *Ribes nigrum* L., *Sorbus domestica* L., and *Tilia tomentosa* Moench, so rutin could be quantitatively determined by using silica gel 60 F_{254} plates scanned at 260 and 440 nm, respectively, after spraying the plateswith 2-aminoethyldiphenylborinate.[20]

16.2.3 Separation of Flavonoid Aglycones and Glycosides on Silica Gel and Reversed Phase TLC

Analysis of medicinal plants is traditionally one of the oldest fields of application for TLC. In the last decade, regulation of the herbal industry and the demands for analytical methods that can ensure the quality and safety of herbal medicines have been constantly growing. Identification can be considered as the main application of TLC technique. Additionally, HPTLC fingerprints are often used to establish the optimal conditions of extraction, to standardize extracts and to detect changes or degradation of the plant material during the extract formulation. Methods can also be optimized to identify and quantify a specific target (marker) substance, or a class of

substances.[21] In modern approaches in the field of medicinal plants, TLC analysis (including the analysis of flavonoids) are reviewed in the recent article of Gocan and Cimpan,[22] and in two comprehensive papers published in 2006.[8,23]

During the last decade, the attention has been headed to the development of chromatographic fingerprint profiling methods to determine the quality of complex herbal extracts. The HPTLC fingerprint on silica plates combining digital scanning profiling was developed to identify flavonoids puerarin, daidzin, genistin, and daidzein in order to distinguish *Pueraria lobata* (Willd.) Ohwi and *P. thomsonii* Benth. samples (chromatograms showing aglycone fingerprints of *P. lobata* and *P. thomsonoii* roots are shown in Figure 16.2).

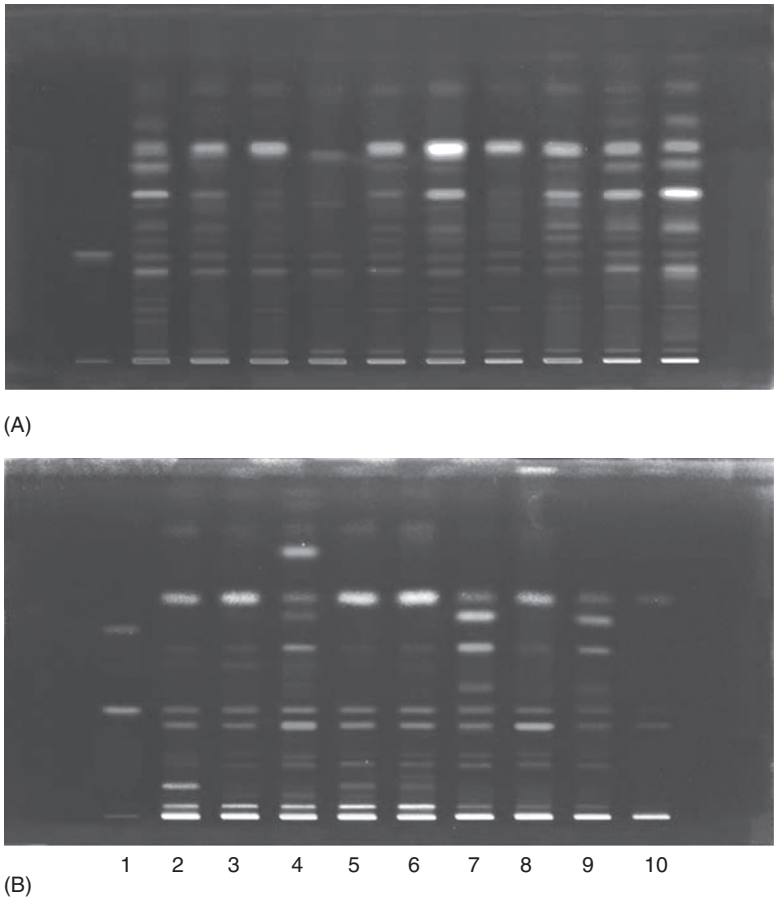


FIGURE 16.2 (A) HPTLC fluorescence images under the excitation wavelength 366 nm of aglycone fraction of root of *Pueraria lobatae* (RPL) and chemical reference substances. (Track 1: daidzein; tracks 2–11: RPL samples). (B) HPTLC fluorescence images under the excitation wavelength 366 nm of aglycone fraction of root of *Pueraria thomsonii* (RPT) and chemical reference substances (Track 1: daidzein; tracks 2–10: RPT samples). (From Chen, S.B. et al., *J. Chromatogr. A*, 1121, 114, 2006. With permission.)

Flavonoid aglycones were identified using toluene–butyl acetate–formic acid (60:30:5, v/v) as the mobile phase, while the separation of glycosides was performed with chloroform–methanol–ethyl acetate–water (20:40:22:10, v/v, lower phase was used).[24] The quality of two samples (Indian and German mother tincture) of *Calendula officinalis* L. prepared from marigold flowers was evaluated in the work of Lalla et al.[25] Two biomarkers, rutin and quercitin, were used for microfingerprinting analysis. Chromatography was performed on silica gel HPTLC plates and for rutin and quercitin identification different mobile phases were used—*n*-butanol–glacial acetic acid–water ($3.6 + 0.5 + 0.5$, v/v) and toluene–chloroform–acetone–glacial acetic acid ($2 + 2 + 2 + 0.5$, v/v), respectively. Quantification was performed by scanning densitometry and developed method was validated for both standards. The results enabled clear differentiation between the samples of Indian and German mother tincture. Fingerprint analysis was also performed by Matysik et al.[26] in order to optimize the extraction procedure of *Calendula officinalis flos*. Different solvents were tested and the highest amount of marker flavonoids (rutin, isoquercitrin, narcissin, and isorhamnetin-3-glucoside) was found in the methanolic extract. HPTLC separation of listed flavonoids was performed on silica gel plates containing fluorescent indicator with the mobile phase consisting of toluene–ethyl acetate–formic acid–water (1:9:2.5:2, v/v). NP/PEG reagent was used for the location of the spots under the UV light (366 nm). The spectrum of constituents of different Hawthorn extracts was compared with three different TLC fingerprint chromatograms in different polarity ranges and by means of quantitative determinations of different groups of constituents (procyanidins, flavonoids, total vitexin, total polyphenol) in the paper of Vierling et al.[27] Lederer et al.[28] compared HPTLC fingerprint analysis of Chinese star anise (*Illicium verum* Hook. F.) with HPLC/MS/MS technique to evaluate the reliability of the planar chromatographic method. HPTLC was shown to be an useful tool for the rapid identification of Chinese star anise in the quality control of commercial batches using rutin, hyperoside, chlorogenic acid, and caffeic acid as standards. Furthermore, TLC identification of pinostrobin, pinocembrin, galangin, chrysin, kaempferol, benzyl ferulate, and phenethyl caffeate was used to establish the identity of propolis samples from different regions of Turkey.[29] A combination of planar chromatographic and electrophoretic procedures allowed Adelmann et al.[30] to establish the chemical correlation between an almost homogeneous flora source, poplar buds, and proplis sample based on the content of a particular bioactive flavanone (pinocembrin). In the paper of Prashanth Kumar et al.[31] a simple, specific and sensitive method for estimation of the rutin content of three therapeutically important Indian plants (*Tephrosia purpurea* (Linn.) Pers., *Leptadenia reticulata* W. & A., and *Ruta graveolens* L.) was reported. Chromatographic analysis was performed on silica gel 60 F_{254} plates with ethyl acetate–*n*–butanol–formic acid–water ($5 + 3 + 1 + 1$, v/v) as mobile phase and the plates were scanned at 366 nm. The method was validated by determination of precision, accuracy, and repeatability. A new, simple and selective HPTLC method for the separation and determination of kaempferol and quercetin in an extract of *Ginkgo biloba* leaves was reported by Jamshidi et al.[32] Glass-backed silica gel 60 F_{254} HPTLC plates were used and developed in toluene–acetone–methanol–formic acid ($46 + 8 + 5 + 1$, v/v). Evaluation was performed densitometrically at 254 nm.

Selectivity, repeatability, and accuracy of the method were determined and method was shown to be suitable for standardization of Ginkgo preparations. A rapid and sensitive HPTLC method was developed for determination of apigenin in liquid products of *Matricaria chamomilla* L. Samples were applied directly to silica gel 60 F_{254} HPTLC plates which were developed with toluene–methanol ($10 + 2$, v/v) as mobile phase. Densitometric evaluation of apigenin was performed at 343 nm.[33] HPTLC and HPLC methods were established and compared for separation and quantitative determination of puerarin, 3′-methoxypuerarin, daidzin, and daidzein, the main isoflavonoid components of several kudzu (*Pueraria lobata* (Willd.) Ohwi) samples. Fluorescence-quenching zones were quantitative evaluated by UV absorption scanning densitometry at 254 nm after developing HPTLC silica gel F_{254} plates with chloroform–methanol–ethyl acetate–water ($16.2 + 18.8 + 52 + 3$, v/v) as mobile phase. The results obtained from the use of validated HPTLC and HPLC methods showed that either can be successfully applied for determination of the amounts of different isoflavone components in kudzu root samples.[34] Likewise, Janeczko et al.[35] used HPTLC silica gel F_{254} plates developed with chloroform–methanol–water ($23 + 8 + 1$, v/v) to determine isoflavone glycosides (genistin and daidzin) in different cultivars of soy (*Glycine max* L.). Another comparison of HPTLC and HPLC methods is given in the paper of Pereira et al.[36] According to the authors, HPTLC method was shown to be suitable for routine fingerprint analysis for quality and authenticity validations of plant material, extracts, and preparations containing *Passiflora* L. species. The objective of the work of Bilušić Vundać et al.[37] was to determine the presence and quantity of flavonoids (hyperoside, isoquercitrin, luteolin, luteolin-7-*O*-glucoside, rutin, vitexin, quercetin, and quercitrin), and phenolic acids (caffeic and chlorogenic acid) in seven plants belonging to Croatian *Stachys* taxa by the use of HPTLC method performed on silica gel 60 F_{254} plates with ethyl acetate–acetic acid–formic acid–water ($100 + 11 + 11 + 26$, v/v), as mobile phase. After preliminary screening, the amount of each compound was determined using image (chromatogram) analysis technique, following the spraying of the plates with the natural products reagent—polyethylene glycol. A representative chromatogram of rutin analysis in some *Stachys* L. taxa samples is shown in Figure 16.3.

Similar investigation of certain phenolics was performed by Maleš et al.[38] in some Croatian *Hypericum* L. taxa.

Reversed-phase thin-layer chromatography is a seldom used technique for the analysis of flavonoids. Examples of such separations are given in previously described papers by Lambri et al. and Fecka et al.[18,19]

16.2.4 Use of Mathematical Methods for the Optimization of Chromatographic Conditions for Flavonoid Separations

Method development is the central element of any applied analytical technique and TLC is not an exception in this respect. Finding a suitable developing solvent for a certain separation problem is one of the most important steps in TLC analysis. When it comes to the separation of flavonoids, two approaches can be found in the

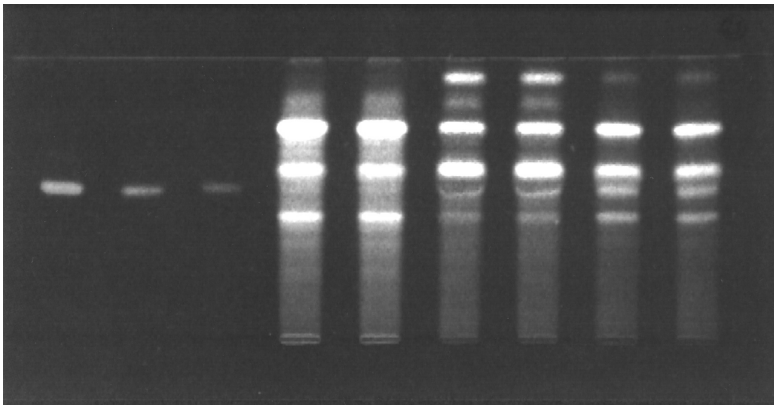


FIGURE 16.3 Analysis of rutin in some *Stachys* taxa (Tracks 1–3: rutin in different concentrations; samples 4L – *Stachys salviifolia* leaves, 4C – *Stachys salviifolia* flowers, 6L – *Stachys recta* subsp. s*ubcrenata* leaves, 6C – *Stachys recta* subsp. s*ubcrenata* flowers, 7L – *Stachys palustris* leaves, 7C – *Stachys palustris* flowers). (From Bilušić Vundać, V., unpublished data, 2006. With permission.)

literature—PRISMA model (more empirical version of solvent triangle approach proposed by Snyder) developed by Nyiredy and the method of numerical taxonomy and information theory developed by Massart (optimization methods are briefly described in the book *Planar chromatography—a retrospective view for the third millennium*).[39] The example given in the paper of Reich and George illustrates the application of the PRISMA model to find conditions that enable comparison of essential oils by HPTLC.[40] Guided by the PRISMA model, ten oils (among which *Citronella* oil, rosemary oil and peppermint oil that all contain flavonoids) were simultaneously developed on silica gel HPTLC plates in the neat solvents that have different solvent strength and according to the Snyder's classification, belong to the different groups: diethyl ether and diisopropyl ether, 2-propanol, tetrahydrofurane–hexane $(1 + 5,\ v/v)$, dichloromethane, 2-chloropropane, acetone–hexane $(1 + 5,\ v/v)$, toluene, and chloroform. It should be noted that the optimization of separation conditions was accomplished in approximately two working days. In the work of Medić-Šarić and other authors, the efficiency of different TLC systems is compared using numerical methods.[12,41–43] Parameters such as information content can be used to compare the quality of chromatographic systems and discriminating power is used as a measure of their effectiveness. Flavonoids and phenolic acids in various extracts (different plant, wine and propolis extracts) were analyzed on silica plates developed with various mobile phases and the methods were optimized using the given procedure of numerical taxonomy. An interesting example of computer-aided optimization of stepwise gradient TLC of plant extracts performed by Matysik and Soczewiński[44] showed that this technique may noticeably increase separation efficiency.

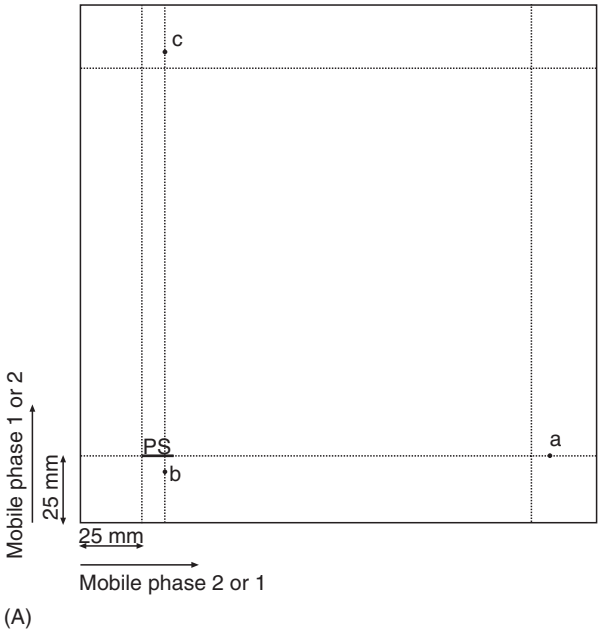
16.2.5 Multidimensional Planar Chromatography (Unidimensional Multiple Development and Two-Dimensional Development)

Multidimensional methods used to improve the separation capacity in planar chromatography include unidimensional multiple development and two-dimensional development. For difficult separation problems the application of multidimensional (MD) TLC is necessary, because the power of one-dimensional chromatography is often inadequate for complete resolution of the components present in complex samples.[39]

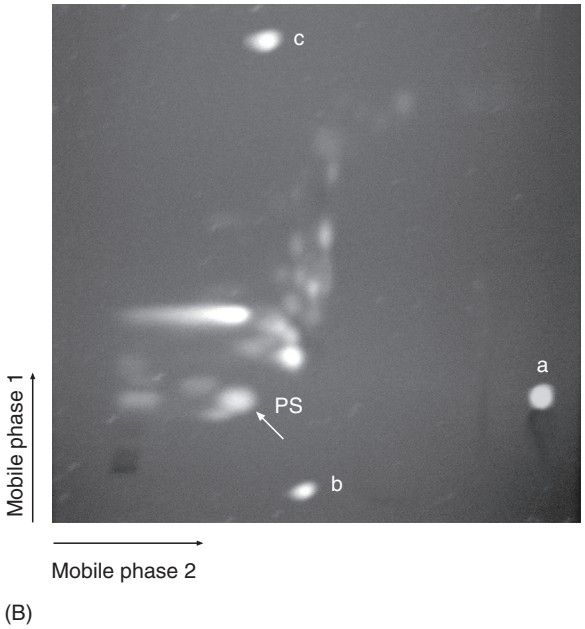
The automated multiple development (AMD) system is considered to be a ''focusing planar chromatography'' which is characterized by the successive and progressive development of a chromatographic plate, with a drying step in between each development. The most important characteristic that allows the successful application of AMD with high-performance thin-layer chromatography in complex mixtures analysis is the possibility of carrying out the separation process using a gradient development mode. Multi-development produces a band reconcentration effect, which means that bands could migrate over a certain distance without appreciable band broadening. Gradient development and band reconcentration effect allow homogenous spreading over a single chromatogram of many compounds which might belong to very different polarity ranges.[39] In the paper of Menziani et al. a basic approach to an optimized AMD-HPTLC experimental procedure and its ability to separate several classes of natural phenolic compounds is presented.[45] To test the proposed method, *Chamomilla recutita* (L.) Rauschert extracts were chosen as well known natural sources of phenolics from a wide chemical polarity range. Among other phenolic substances, 6 flavonoid aglycones and 4 glycosides were used as standard compounds. Chromatography was performed on silica gel 60 HPTLC plates without fluorescence indicator. The AMD gradient optimization was carried out on mixtures of selected standards. General stepwise gradient conditions were gradually modified and the final optimized gradient composition started from methanol–dichloromethane–water–formic acid (70.5:25:4.5:1, v/v) and proceeded through 15 steps ending with pure dichloromethane. The flavonoids were detected after derivatization with 4% aqueous aluminium sulphate solution. After 10 min exposure to UV light, they exhibited yellow fluorescence that was stable for weeks. Fluorescence was detected densitometrically, using an excitation wavelength of 360 nm and by measuring the emission through a cut-off filter (400 nm). Ten plant extracts (among which were three flavonoid drugs—*Carduus marianus* L., *Baptisia tinctoria* L., and *Lycopus europaeus* L.) were analyzed using AMD-TLC. The separation was performed on silica plates with fluorescence indicator and four AMD gradients were tested. Optimal separation was achieved in 25 steps with mobile phase composed of methanol, ethyl acetate, toluene, 1,2-dichloroethane, and 25% ammonia solution and anhydrous formic acid as modifiers. The chromatograms were scanned at 254 nm and quantitatively evaluated. The optimal AMD separation was also compared with the isocratic chromatography described for listed plant extracts in Homeopathic Pharmacopeia and it was shown that AMD-TLC technique can be used to achieve better simultaneous separations of plant extracts than it is in the case of official TLC methods.[46] The separating power of two chromatographic methods—isocratic TLC and

two-dimensional AMD—for the separation of extracts of four related plants—*Artemisia abrotanum* L., *A. absinthium* L., *A. vulgaris* L., and *A. cina* Berg., that contain flavonoids is discussed in the paper of Olah et al.[47] Silica gel 60 F_{254} HPTLC plates were used for isocratic and AMD separations. Isocratic TLC was performed with ethyl acetate–formic acid–water (80:10:10, v/v) as mobile phase, while AMD was performed in two steps (two-dimensionally) by the use of 16 steps two gradient systems (gradient I was composed of methanol, dichloromethane, hexane, and 25% ammonia; and gradient II of methanol, diethyl ether, hexane, and 25% ammonia). After development, spots were visualized under UV light at 254 nm in reflection mode. The number of compounds separated was greater by use of AMD than by isocratic TLC. Two- and three-step gradient elution for separation of flavonoid glycosides and multiple development for separation of aglycones from *Vaccinium myrtillus*, L. and *V. vitis-idaea*, L. was performed by Smolarz et al.[48] Silica gel HPTLC plates were developed with mixtures of toluene, hexane, ethyl acetate, and methanol. Stepwise gradient was used for hyperin, isoquercitrin, and avicularin, while multiple gradient elution for quercetin. Qualitative and quantitative evaluation of *Passiflora coerulea* L. flavonoids (isoorientin, orientin, and vitexin) was published in the paper of Pastene et al.[49] Fingerprint analysis of plant extracts was obtained by using the optimized multiple development with a mixture of ethyl acetate–formic acid–water as mobile phase. Soczewinski et al.[50] used two-step gradient development to separate nine flavonoid glucosides and seven aglycones. Chromatography was performed on silica gel HPTLC plates. In the first step, more polar glycosides were separated using an eluent with high solvent strength (ethyl acetate–formic acid–water, $85 + 15 + 0.5$, v/v). After solvent evaporation, the aglycones were separated in a second step in the same direction with another eluent (dichloromethane–ethyl acetate–formic acid, $85 + 15 + 0.5$, v/v).

Two-dimensional TLC (2D-TLC) when compared with the AMD is still seldom used as a qualitative or quantitative technique in spite of its enormous potential. Still, examples in literature prove the usefulness of this method. In the work of Hawryl et al.[51] 2D-TLC identification of twelve flavonoids and three phenolic acids from *Betula* sp. leaves was performed using normal-phase systems. Optimization of mobile phases was conducted and complete separation of the investigated compounds was achieved by use of two optimum nonaqueous mobile phases on silica plate. Similarly, 2D-TLC on cyanopropyl-bonded silica was performed by the same author in order to separate eight flavonoids and three phenolic acids in *Sambuci flos*. As the first dimension a normal-phase separation was used for which seven binary eluents were tested, and the second one (a reversed-phase separation) was studied by using three binary eluents.[52] From among the 21 combinations, the three best ones that all contained *n*-hexane in the first, and water in the second dimension were used. Two-dimensional TLC was shown to be a suitable method for quantitative determination of flavonoids and phenolic acids in Croatian propolis samples.[53] Analysis was performed on silica gel 60 F_{254} plates according to the scheme depicted in the Figure 16.4A. The mobile phases used were *n*-hexane–ethyl acetate–glacial acetic acid ($31 + 14 + 5$, v/v) and chloroform–methanol–formic acid ($44 + 3.5 + 2.5$, v/v). Quantitative evaluation was based on the image (chromatogram) analysis after spraying the plates with 1% ethanolic solution of aluminium



(A)



(B)

FIGURE 16.4 (A) Two-dimensional chromatography; scheme for application of standard and sample: PS, propolis sample; a, standard solution (m_{maximum}); b, standard solution (m_{minimun}); c, standard solution (m_{medium}); (B) Two-dimensional chromatogram of kaempferol in propolis sample from Slavonia ($m_{\text{minimun}} = 0.83$ μg, $m_{\text{medium}} = 1.67$ μg, $m_{\text{maximum}} = 2.50$ μg and $m_{\text{PS}} = 2.82$ μg). (From Medić-Šarić et al., *J. Planar Chromatogr.*, 17, 459, 2004. With permission.)

chloride (a representative chromatogram of kaempferol analysis in one propolis sample is shown in Figure 16.4B).

16.2.6 Quantitative Evaluation

Quantitative analysis is carried out in situ by measurement of herbal extract sample and standard zones on layers with a slit-scanning densitometer or video or CCD camera (image processing). Slit-scanning instruments have a number of advantages and were used in most of the representative densitometry papers cited in this chapter.[36,48] However, in some articles quantification based on image analysis is used.[37,53] Desitometry is usually performed at the wavelength of maximum absorption for UV/VIS absorption scanning and the maximum excitation/emission wavelengths for fluorescence scanning.[17,20] Notable examples of quantification and validation of the method are described in the papers of Jamshidi et al.,[25] Prashanth Kumar et al.,[31] Lalla et al.,[32] and Fang et al.[34]

16.3 MODERN TLC TECHNIQUES IN THE SEPARATION OF FLAVONOIDS

Forced flow planar chromatography (FFPC) is considered to be a modern (improved) planar chromatography technique, in which the solvent system (except the capillary force) moves across the layer under the influence of additional force. Forced flow can be achieved either by the application of external pressure (e.g., vacuum, hydrostatic pressure) like in case of OPLC, while centrifugal force is used in rotation planar chomatography (RPC) and electric field in electro-planar chromatography. This state of the art of planar chromatography is summarized in the book *Planar chromatography—a retrospective view for the third millennium*[39] and latter paper of Nyiredy.[10] Forced flow methods are also proposed by Nyiredy[11] as one of the possible planar techniques for the separation of plant constituents in complex mixtures.

16.3.1 Overpressured-Layer Chromatography

Forced-flow development by OPLC involves pumping of the mobile phase through a layer that is sandwiched between a rigid plate and a flexible membrane under pressure. OPLC, together with AMD, is used in the work of Galand et al. for the identification of certain flavones, flavonols, flavanones, isoflavones, and anthocyanins in extracts obtained from bitter orange tree (*Citrus aurantium* L. subsp. *amara* L.), common horse chestnut (*Aesculus hippocastanum* L.), and high mallow (*Malva sylvestris* L.).[54] For OPLC separation, silica gel 60 F_{254} plates were used. A gradient formed from ethyl acetate and chloroform mixed in various proportions was used as mobile phase. Separated flavonoids were detected under UV and visible light at 365 and 530 nm, respectively, after spraying the dry plates with NP/PEG reagent. In this paper, OPLC and AMD (together with densitometric evaluation of the plates) were shown to be appropriate and valuable techniques for identification of flavonoids in herbal extracts. OPLC was also used in the work of Ligor and Buszewski in which

both TLC and OPLC methods were developed for the determination of anthocyanins in multicolored *Coleus*, *Prunus cerasifera* Ehrh., and *Rhus hirta* leaves.[55]

16.3.2 Rotation Planar Chromatography

RPC is the oldest described FFPC method and it is a technique that can be used for both preparative (using the planar column) and analytical work. An example of use of this method for isolation and identification of flavonoids (quercetin, quercitrin and rutin) from oak bark (*Quercus robur* L.) is given in the paper of Vovk et al.[56] Oak bark is recognized as a rich source of flavonoid and similar phenolic compounds. The main aim of this work was to study RPC as a tool for preparative (extraction of plant material and fractionation of the obtained extract) and also for analytical separation and detection of some characteristic compounds. Fractionation was performed and obtained fractions were screened by conventional TLC, which was performed on silica gel 60, 20×20 cm TLC plates, with *n*-hexane–ethyl acetate–formic acid (20:19:1, v/v) as mobile phase. Analytical RPC was performed on 20×20 cm cellulose TLC plates. The separation was performed in the ultramicrorotation planar chromatographic (U-RPC) chamber with water as the developing solvent. Fluorescence of flavonoids was detected under the UV light at 366 nm. The advantages of described technique in the preparative separations of complex mixtures are easy and rapid filling of the planar column and possibility to use adsorbent material of small particle size. The possibilities to use commercially available TLC plates, the adjustable volume of vapor phase, and the optimization of developing solvent are beneficial characteristics of the method in analytical separations.[56]

16.4 USE OF TLC IN THE ISOLATION OF FLAVONOIDS FROM PLANT EXTRACTS

To isolate individual flavonoids for further study, PC was used in two-dimensional or one-dimensional mode. After achieving satisfactory separation, spots of interest were cut out, cut into small strips and flavonoids were extracted with a suitable solvent.[2] Today, paper is replaced with different sorbents and preparative layer chromatography (PLC) is carried out on thicker layers with application of larger weights and volumes of samples in order to separate and recover 10–1000 mg of compound for further structure elucidation. Silica gel is a layer of preference and many examples of flavonoid isolation could be found in recently published papers. A representative example is the paper of Pieroni et al.[57] The presence of flavonoid structures (luteolin-4′-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, apigenin, and luteolin) from the leaves of *Phillyrea latifolia* L. was revealed by the preparative TLC performed on conventional thick silica gel and reversed-phase layers, followed by UV and MS analysis. Preparative TLC was used in the work of Ponce et al.[58] as well, and isolation and full characterization of four flavonoids from *Anthemis triumfetti* (L.) DC. were performed. For further reading, papers published by Pavlović et al.[59] and Kim et al. are recommended.[60]

16.4.1 Future Trends

Nowadays the main application field of TLC technique is quick fingerprint analysis of herbal mixtures. The exclusive feature of chromatographic image of HPTLC coupled with digital scanning profile is more and more attractive to the herbal analysts to construct the chromatographic fingerprints of herbal extracts. With the introduction of automated multi development, gradient elutions and over-pressured layer chromatography, planar chromatography may play more and more important role in the analysis of complex mixtures such as herbal extracts. With the coupling of TLC with different mass spectrometric methods (TLC-MS), a more reliable identification in extremely complex matrices could be achieved.

Validation of results to meet good laboratory practice (GLP)/good manufacturing practice (GMP) standards is of a great importance today as well. Complete validation of a TLC/HPTLC would be necessary if the method is proposed for the quality assurance of commercially available herbal product.

Special attention should be paid to the development of PLC methods, as a very useful tool for the separation and isolation of flavonoids from different plants as well.

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