

Plant Analysis 2008 – Planar Chromatography

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Abstract

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 For 70 years, planar chromatography has been closely connected with the analysis of plants. Also today in its most advanced form “high performance thin-layer chromatography” (HPTLC), the technique plays an important role in identification of medicinal plants and dietary supplements. Detection of adulteration and quantitative determination of marker substances are other widely used applications. The advancement of instrumentation and methodological concepts has created a basis for reliable qualitative and

quantitative results in HPTLC. Remarkable progress has been made in the manageability of the entire planar chromatographic process, particularly in the control of chromatogram development and the utilization of images to obtain and compare information about separated samples. Integration of biological detection systems as well as hyphenation to mass spectrometry has widened the applicability of planar chromatography as an analytical technique that is both orthogonal and complementary to HPLC.

Introduction

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 Seventy years ago in 1938 thin-layer chromatography (TLC) was invented by Izmailov and Shraiber. Named “drop-chromatography” this form of circular planar chromatography was a rapid way to analyze galenic formulations of plant extracts. About thirty years later Egon Stahl’s “Thin-layer Chromatography – a Laboratory Handbook” was published. A significant portion of the book was dedicated to the analysis of natural products and provided the scientific basis for TLC not only to become a universally accepted and widely applicable analytical technique, but also a standard method in the pharmacopoeias for quality control of medicinal plants and synthetic drugs. Today in 2008 an overwhelming number of TLC analyses are still performed in the same traditional way. Yes, there are pre-coated plates (or sheets) and little convenience items like spotting guides and disposable pipettes but the focus has always been and remains on simplicity, low cost and rapid results. Many papers are published each year demonstrating what great results can be achieved with limited budgets, simple tools, and lots of patience while practicing. Many papers about plant analysis simply refer to the fact

that TLC has been used to verify certain results, with little or no detail on the methodology. Comparatively rare is the recent literature on the analytical aspects of the technique.

This review of planar chromatography is focused on high performance thin-layer chromatography (HPTLC) – the TLC of the 21st Century – and what it can offer to plant analysis.

Planar Chromatography Today
**▼
 What is HPTLC?**

The term HPTLC was originally used to describe thin-layer chromatography performed on pre-coated plates with small particles having an average size of 5 μm and narrow size distribution as opposed to TLC material of 15 μm average particle size and broader size distribution. HPTLC plates offer a significantly higher efficiency and shorter developing times. The improvement over conventional TLC plates is comparable to that achieved by switching from a 5 μm packing in an HPLC column to a shorter column with 3 μm packing [1]. The differentiation of TLC and HPTLC based on plates is also the approach taken by the United States Pharmacopeia (USP) and the Euro-

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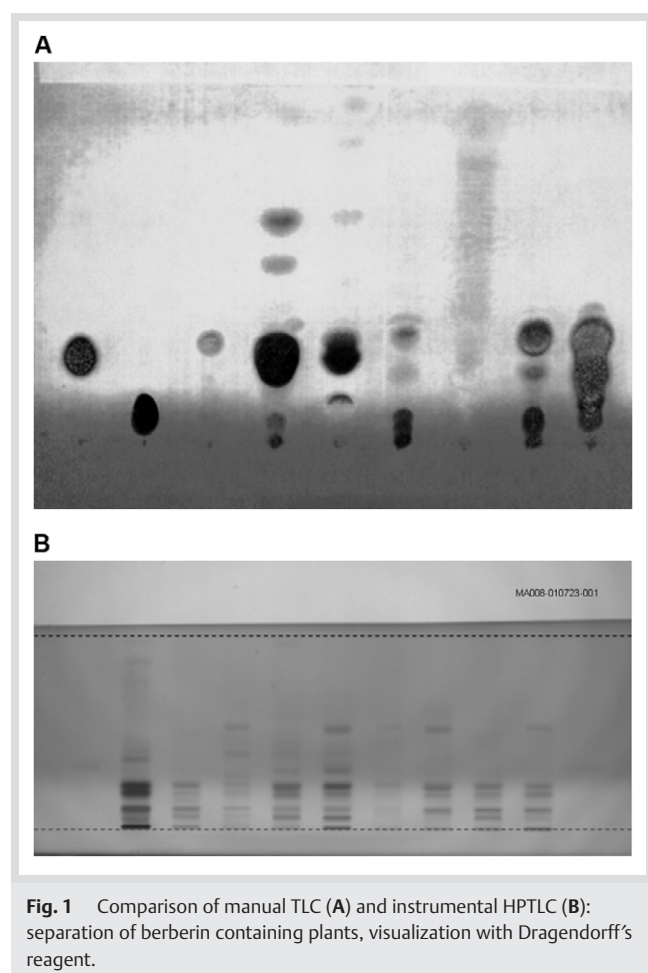
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pean Pharmacopoeia (EP) in their respective general chapters [2], [3]. The decision about whether, and to what degree, instruments are used for the analysis is left to the analyst. While "TLC" is commonly used for mostly manually performed analyses on TLC plates, "HPTLC" implies the use of instruments for all steps of the chromatographic process on HPTLC plates (● Fig. 1). Instruments for HPTLC have been dramatically improved over the last decade and driven up the performance and reliability of planar chromatography to the level of any other state-of-the-art analytical method [4].

HPTLC today is more than just plates and instruments. It is also a concept, including a scientific basis [5], standardized methodology [6], and validated methods [7].

Why HPTLC for plant analysis?

Since its inception planar chromatography was always indispensable in plant analysis, be it as a rapid tool for comparison of column fractions, optimization of solvent mixtures for preparative column chromatography, identification of plant material, screening for certain biological activity or content of markers and others. It is not necessary to repeat all the general advantages inherent to the planar chromatographic process at this point [8], but the level of sophistication available today in HPTLC has raised the analytical quality of results significantly. Accepting the principal equivalence in quality, HPTLC can be viewed as an orthogonal technique to HPLC enabling a look at an analytical problem from a different angle for complementary information.

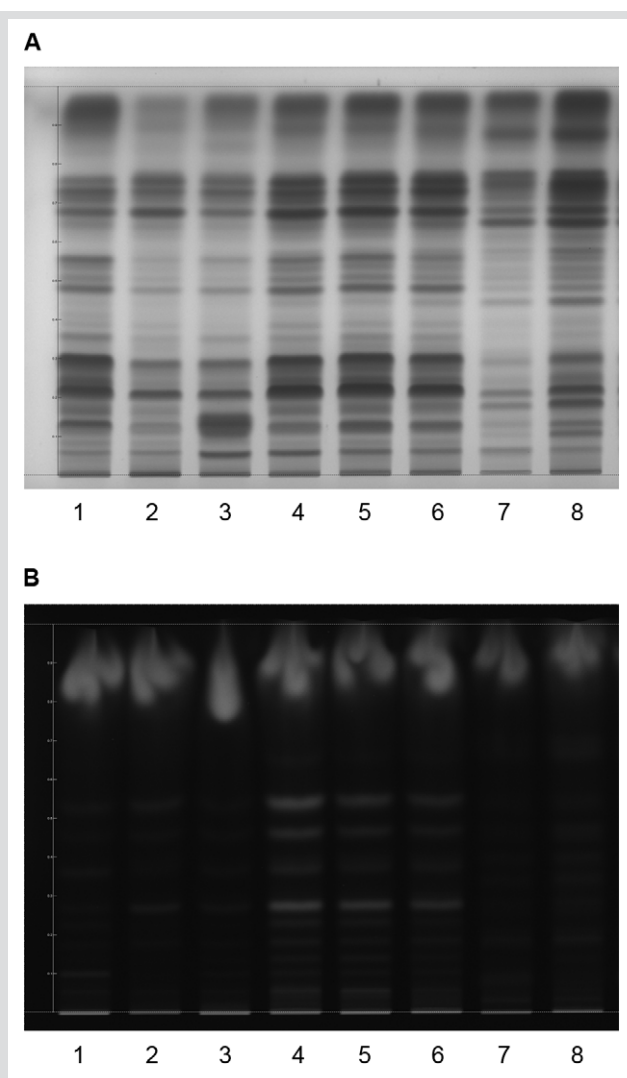


1) Most HPLC separations are performed in reversed phase (RP) mode using partition chromatography, while with HPTLC separations, normal phase silica gel is most frequently employed, using adsorption chromatography, which benefits from the ability to separate substances according to the type, number and position of functional groups.

There are also other modern stationary phases including reversed, amino-, diol-, and cyano-bonded phases available for HPTLC. This makes setting up two orthogonal separations possible (● Fig. 2).

For difficult separation problems the stationary phases can be easily modified by impregnation to utilize very specific interactions [9]. An interesting example is the separation of alpha- and beta-sarone achieved on silica gel impregnated with caffeine [10].

2) While in HPLC elution of a compound is the requirement for detection, in HPTLC it is a compound's migration to a certain point in the chromatogram. In addition, substances permanently adsorbed or un-retained will remain on the developed plate and



can be detected. While HPLC offers the convenience of hyphenation and full automation, HPTLC works as an off-line technique with individual steps. Of particular advantage is the possibility to evaluate multiple samples developed in parallel on a plate, and subsequently on the same plate, re-evaluate in different ways with or without chemical derivatization. Even tests for biological activity can be performed on the plate [11], [12].

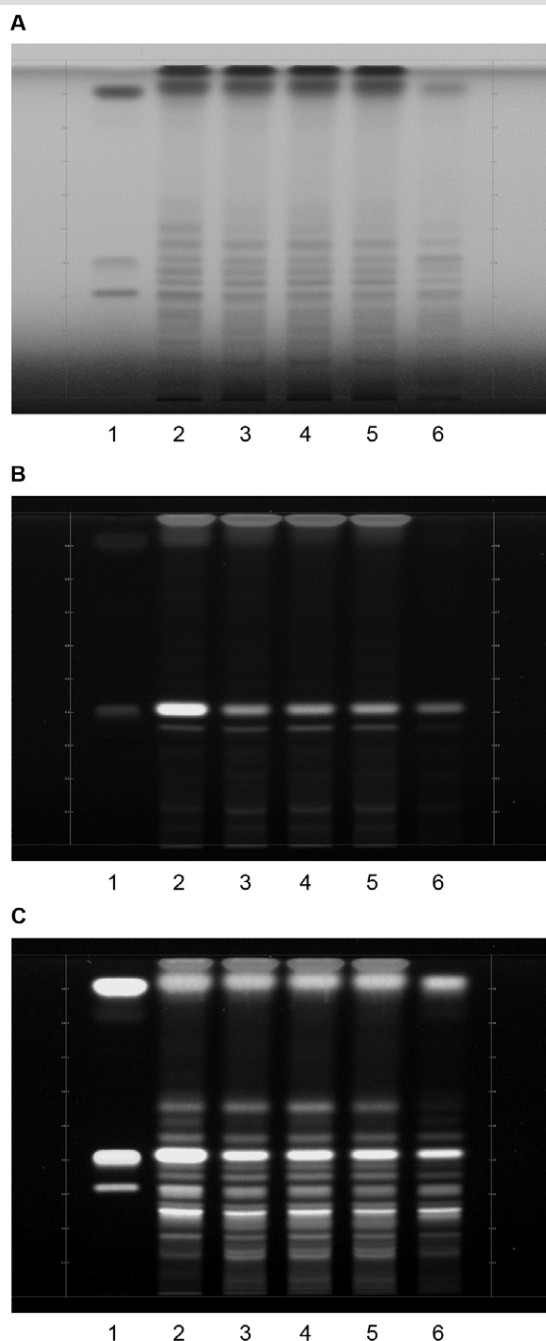


Fig. 3 HPTLC fingerprints of *Ginkgo* (1: reference substances, 2–5: *Ginkgo* leaf, different samples, 6: *Ginkgo* tincture); **A** UV 254 nm, **B** UV 366 nm, **C** UV 366 nm after derivatization with Natural Products reagent; for details on the method see [14]. Minor differences are seen in the fingerprint of the leaf samples representing natural variability. The tincture represents the starting material in a balanced way. Chlorophyll (red zone under UV 366 nm close to the solvent front) is not present in the tincture.

3) Certainly the most striking advantage of HPTLC and also one of the fastest growing areas of application is the possibility of presenting chromatograms of multiple samples as an image, which can either be visually evaluated or further processed by dedicated software. Images of the same plate can be obtained with different illumination modes, prior to and/or after derivatization (● **Fig. 3**). The power of using images to illustrate issues of similarity and difference, to set specification for rapid pass/fail tests, or to communicate results is unsurpassed. Thus the quality and reproducibility of the imaging process have become a central element of HPTLC and it is evident that TLC of the kind shown in ● **Fig. 1** (left side) will not be suitable.

What are the limitations?

Naturally there are also limitations to planar chromatography. The off-line principle has some drawbacks in that it requires manual interaction. Although there are computer controlled instruments available for each step, full automation is not yet a feasible option. With respect to the reproducibility of the work, critical manual steps may be difficult to standardize. Some of the parameters affecting the chromatographic result (such as humidity, composition of the gas phase in the chamber) are difficult to change, but they should at least be kept as constant as possible.

The planar chromatographic system is open. The plate is exposed to environmental and climatic factors (temperature, light, fumes) and mechanical stress. Volatile and sensitive samples require special care. In order to achieve reproducible results, the plate must be treated with extra caution.

The quality of data generated by planar chromatography is closely related to the level of instrumentation and the rigor of the employed methodology. Qualitative and quantitative HPTLC data can be as reliable as data obtained with other chromatographic techniques as long as the methods have been properly optimized and validated. The separation power of HPTLC is lower than that of HPLC. Particularly for complex samples like botanicals it is often difficult to achieve sufficient resolution for all components. This fact plus the wider linear working range of HPLC are the main reasons why quantitative determinations are less frequently performed by HPTLC.

Applications

The following sections are essentially focusing on a few examples that illustrate principal aspects. Recently, two books, “High Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants” [15] and “Thin Layer Chromatography in Phytochemistry” [16], have been published. Both cover in depth practical issues and theory as well as applications. While the former places emphasis on various aspects of quality control, the latter covers separation of individual substance classes.

Identification of plants

HPTLC is very well suited for the identification of plants based on fingerprints [17]. This is not only true for fundamental and applied research but also for regulatory purposes:

- The pharmacopoeias of Europe, the United States and of China acknowledged this fact in recent revisions of their general chapters [3], [2], [18]. More detailed and specific guidance is provided concerning suitable methodology. The Chinese Pharmacopoeia has created an atlas of HPTLC chromato-

grams. Several authenticated samples of the same plant obtained in different Chinese provinces are shown side by side. The USP is in the process of publishing a “Dietary Supplements Compendium” for 2008–2009 which will now include HPTLC chromatograms as well. The European Pharmacopoeia recently adopted an extensive program to create monographs for Traditional Chinese Medicinal (TCM) plants used in Europe. Identification by HPTLC will be an important element of the elaboration process.

- In 2007 the FDA released current Good Manufacturing Practices (cGMP) for dietary supplements [19]. By 2010 the entire US botanical industry has to be in compliance with this regulation. Two elements of the document are of great importance: (1) all incoming material must be tested for identity and (2) suitable, scientifically appropriate methods have to be employed for that purpose. HPTLC will be a very valuable tool for this industry.

From an analytical point of view two important objectives need to be addressed for proper identification of plants by HPTLC. There must be clarity about the employed methodology to ensure that results from official or other published methods can be reproduced by other laboratories. This requires a certain degree of validation, which in principle is not difficult to achieve [20]. More difficult to deal with is the relationship of the botanical identity and chemical composition of a plant, which may be quite variable.

When developing an HPTLC method for identification, specificity is of great importance. Typically, a known marker compound, which might later on serve as target for quantitative measurements, is selected for convenience. Differences in plant species may, however, be found in the content of other, known or unknown substances. Proper plant identification can be best ensured if either multiple species of the same genus or any known adulterant are analyzed on the same plate (● Fig. 4).

In traditional medicine it is not unusual to use several species of a genus interchangeably. Examples are willow bark (several *Salix* species) and hawthorn leaf and flower (several hawthorn species). HPTLC fingerprints can of course also be created in a way that they are less specific and emphasize the similarities of the samples.

Detection of adulteration and quantitation of individual substances

Adulteration, more specifically falsification of plant material is a widespread problem, not just from a regulatory but also from an economic point of view. Discovering a simple mix-up of two similar species on the basis of specific fingerprints is generally no problem. The monographs of the “American Herbal Pharmacopoeia” [21] as well as the series “Chinese Drug Monographs and Analysis” [22] include not only TLC/HPTLC fingerprints of the monographed species but also those of common adulterants. A greater analytical challenge arises if two species are mixed.

- If the adulterant has a specific, known marker that can be detected in a mixture of both plants, the analytical task becomes a limit test. A good example is the detection of aristolochic acids in TCM. An HPTLC method was developed for the investigation of a possible mix-up between *Stephania tetrandra* (Han fangji) and *Aristolochia fangji* (Guang fangji) [23], [24]. The method is very sensitive and allows detection of aristolochic acid I at the 1 ppm level. Recently it was proposed to include it as part of a General Test 2.8.21 for herbal drugs into the European Pharmacopoeia [25].

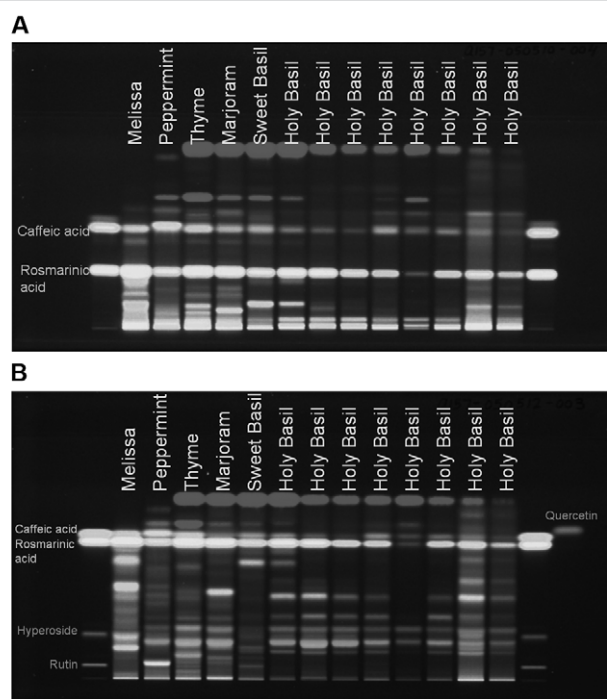


Fig. 4 Fingerprints of various Lamiaceae: Identification of Holy basil (*Ocimum sanctum*). Because the plant contains significant amounts of rosmarinic acid, a method for the determination of that marker may be chosen (A). By itself the resulting fingerprint is not bad, but if it is compared to those of sweet basil and some other Lamiaceae, specificity is not sufficient. In HPTLC the same set of samples can just as conveniently be chromatographed also with other methods, i. e., one that targets flavonoids (B). This method is highly specific and allows distinguishing all investigated species with certainty.

- If the fingerprints of the involved species are complex or very similar, and a majority of constituents is unknown, the detection of mixtures at low levels requires another approach. ● Fig. 5 shows how easily semi-quantitative investigations can be performed in HPTLC. A series of mixtures can be compared either visually or densitometrically to establish detection limits for adulterants. 3% or, respectively, 1% of the toxic Germander species (*Teucrium chamaedrys* and *T. canadense*) are detectable in mixtures with Skullcap (*Scutellaria lateriflora*).
- Another example of a plant with great potential for adulteration is black cohosh, *Cimicifuga racemosa*, economically a very important medicinal plant from North America. The plant not only shows a considerable natural variability in the HPTLC fingerprint, but it is also easily confused with another American *Cimicifuga* species (*C. americana*) and often substituted by either of three Chinese medicinal plants (*C. foetida*, *C. heracleifolia*, *C. dahurica*). Taking advantage of convenient post chromatographic derivatization combined with evaluation of images captured of the plate, HPTLC enables detecting the presence of 5% or more of either adulterant [26] in mixtures with black cohosh. The determination is based on fingerprints and one specific zone (marker) for each adulterant. Even without knowing the chemical identity of the markers and its absolute amount in the corresponding plant, reliable quantitation is achieved, based on a calibration derived from mixtures that were prepared from representative batches of each species.

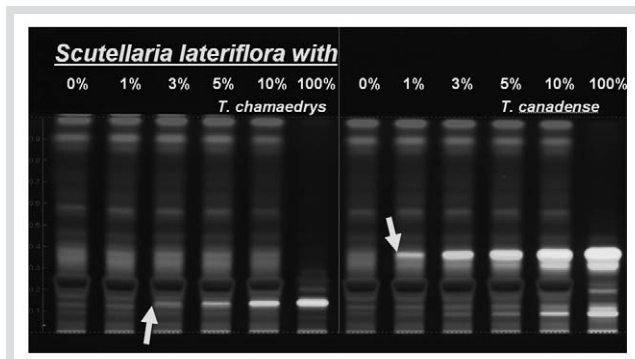


Fig. 5 Investigation of mixtures containing Skullcap and Germander. For details see text.

Although planar chromatography is primarily regarded as a qualitative technique it can also offer precise and accurate quantitative data [27]. By HPTLC it is not difficult to determine the presence of chemical substances added to plant derived products. In dietary supplements, examples of HPTLC assays range from those for painkillers and antidiabetic drugs [28] or sildenafil [29], [30], to caffeine [31], and glucosamine [32]. For quality control of raw material or finished products, numerous assays for marker compounds have been developed and validated. In 2007 a total of about 50 papers were published on the subject [33], [34]. HPTLC as a quantitative tool is of particular advantage if the analyte does not have a chromophore and would require a special detector in HPLC. An example is the determination of artemisinin in *Artemisia annua* [35]. Based on specific derivatization of the target compound with a modified anisaldehyde reagent and a highly selective measurement of fluorescence (excitation at 520 nm, measurement > 540 nm), detection/quantitation is possible over a wide concentration range from 20 ng absolute (0.05% in the dried plant material) up to a theoretical amount of 1300 ng (3.25%). The method can be applied to screen the artemisinin content of 9 samples in an hour.

Instrumentation

The overall quality and performance of instruments continue to improve. A comprehensive discussion of the functionality and features of today's equipment for instrumental TLC can be found in [36]. Due to the off-line character of planar chromatography, separate devices are used for sample application, chromatogram development as well as derivatization, documentation and evaluation of the plate. Each device by itself can make the final result better and more reproducible, but only if operating parameters for all instruments are properly selected and compatible with each other, the full power of HPTLC can be utilized. It is a wrong assumption that just because a modern densitometer is used, "hand-spotted" plates, developed in a simple glass container, will produce good quantitative results. It is also wrong to say that good HPTLC must always include a "horizontal developing chamber" and/or a scanner. HPTLC is a concept! Modern instrumentation that is properly employed helps to implement it.

Sample application

Sample application is the first step of TLC and its quality sets the limits for the final results:

- The dimension of the applied zone (band or spot) limits the achievable resolution.
- The application position of all zones on the plate relative to one another determines the quality of identification by migration distance/RF.
- The application volume affects the quality of quantitative results.

Unlike in pharmaceutical analysis where most samples come as clear solutions in methanol or acetonitrile, plant analysis often has to deal with samples of unknown composition. HPTLC can accept sample solvents of all kind and usually, as long as the application device (needle or capillary) is not clogged, requires only minimum clean-up. In many respects sample application using the spray-on technique is preferred over contact spotting. Instrument parameters have to be selected that ensure sharp, homogeneous bands. If the application position was dried properly, e.g., the sample solvent was completely evaporated, the chief requirements for clearly structured chromatograms are met. All instruments available today can ensure proper positioning and control of the applied sample volume.

Chromatogram development

The geometry of a developing chamber and the composition of the gas phase at the beginning and during chromatography affect the chromatogram almost as strongly as the composition of the mobile phase [37]. Yet most publications do not even mention how, for example, a "saturated chamber" was saturated. This is largely due to the assumption that everybody knows what chamber saturation is and how it is achieved. Whether one or more filter paper was used to line the chamber, or whether the time before introduction of the plate was 15 or 20 min may be considered a minor detail. But it is a fact that the status of the gas phase in a saturated chamber opened under a running fume hood is at best unpredictable. In this light the availability of a fully automatic developing chamber can be regarded as a major breakthrough [38]. The actual chamber is always closed or just opened mechanically as much as required to introduce the plate through a narrow slit. The saturation is precisely time controlled and never disturbed so that a highly homogenous gas phase is obtained. As a result the solvent front and the same substances on multiple tracks will form straight lines parallel to the lower edge of the plate and RF values are reproducible from plate to plate with variation of < 0.02 units [39]. Accurate measurement of the solvent front position is important, because the developing distance influences the resolution [40]. In the automatic chamber the migration distance of the front is measured by a CCD element in 100 μm steps so that a solvent front overrunning a pencil mark as it is often seen in classical TLC is no longer a threat to reproducibility.

During the drying of the plate that follows chromatography, zones of the separated substances are moving towards the layer surface [41]. Reproducibility of quantitative determination across the plate can be significantly improved if the drying process is controlled [42].

Relative humidity is a powerful factor that can affect the result in planar chromatography to a large extent. Aside of changes in overall migration distances due to changing activity of the stationary phase, humidity also may affect the selectivity of separation as seen in **Fig. 6**. During sample application and manual handling, the plate is usually exposed to ambient atmosphere and its humidity. Therefore an activation of the plate as it was recommended in the days of self-made plates is not the solution.

In order to achieve results corresponding to a constant relative humidity/activity of the stationary phase, the plate after sample application must be either manually conditioned in a conditioning tray or an automatic humidity control unit should be utilized.

Documentation

The documentation step in planar chromatography is unique. It aims at converting the visual impression of the chromatogram into an image and storing it in a durable way. Principally two approaches can be taken: (1) scanning the plate with a flat bed scanner or (2) using a camera to capture an image. Visual evaluation of planar chromatograms is primarily performed in daylight, that is if the analyzed substances are colored or were changed to colored derivatives during a post-chromatographic derivatization step. Invisible substances can be visualized with a suitable UV lamp if they absorb UV 254 nm or can be excited by UV 366 nm to fluoresce. Plates containing a so-called fluorescence indicator F_{254} emit, depending on the type, green or blue fluorescence when exposed to UV light at a wavelength of 254 nm. Any substance absorbing that wavelength becomes visible as a dark zone on such plates. Under UV 366 nm certain substances or their derivatives become visible as colored fluorescent zones on a dark background.

Flat bed scanners are simpler and less costly and by design more suitable to capture images from flat objects, but due to the fact that illumination under UV 254 and 366 nm can be achieved more conveniently in a "light box", camera based documentation systems are more versatile. In the context of HPTLC documentation has to be taken to a much higher level. Images of chromatograms on HPTLC plates are not just photographs but electronic files that contain qualitative and quantitative information. In order to obtain such information reproducibly and accurately the capturing process must be under complete control. It cannot be left to the skills and creativity of the analyst to produce a "nice" image. Neither can the analyst rely on the automatic programs of high-end professional cameras which are designed to create "picture-perfect" impressions. One would expect the image of an HPTLC chromatogram to as close as possible

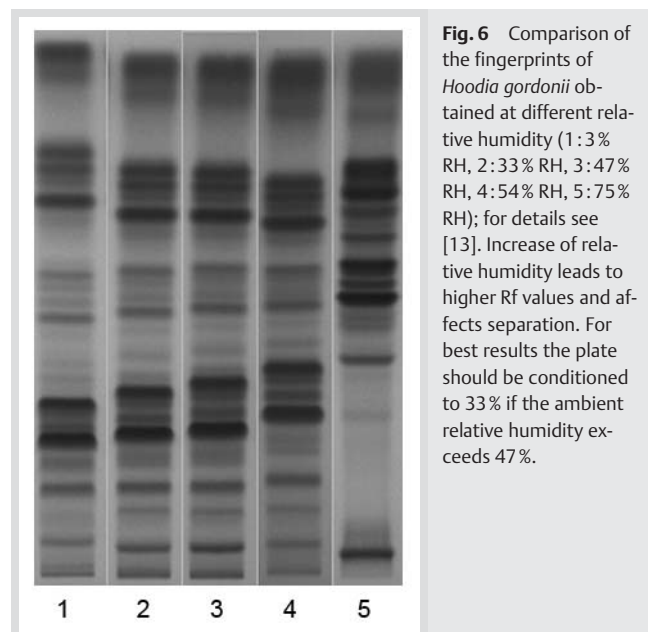


Fig. 6 Comparison of the fingerprints of *Hoodia gordonii* obtained at different relative humidity (1: 3% RH, 2: 33% RH, 3: 47% RH, 4: 54% RH, 5: 75% RH); for details see [13]. Increase of relative humidity leads to higher R_f values and affects separation. For best results the plate should be conditioned to 33% if the ambient relative humidity exceeds 47%.

to what is seen on the plate. Whether the blue sky on a photograph was as blue in reality is probably not important, but an orange fluorescence under a UV lamp is usually not expected to be green on the image, even though nobody would be able to define "orange" in simple words.

The most advanced documentation systems for TLC/HPTLC plates feature optimized illumination under white light and UV 254/366 nm and a powerful high-resolution CCD digital camera with outstanding linearity. During qualification each system is adjusted to achieve absolutely homogeneous intensity levels across the entire plate. Minor differences in the light sources are corrected as well as all errors resulting from the fact that a square or rectangular object is pictured through a lens. All capture parameters are applied according to defined rules always in the same way. This way "true" images which are independent of personal taste of the operator can reproducibly be obtained. To further improve images for quantitative evaluation an image generated of the neat plate, prior to sample application can be "subtracted" from images of the developed plate. Together with an up to 12-fold noise reduction this provides the basis for an unsurpassed image quality and the ability to detect separated compounds at very low levels [43].

◉ **Fig. 7** illustrates the possibilities of such systems for the detection of adulteration of *Cimicifuga racemosa* as discussed before. In a blind test the analyst has to determine which of four samples compared to a reference sample of *C. racemosa* is adulterated with 5% *C. americana*. This adulteration can be detected by looking at the developed, but not derivatized plate under UV 254 nm. A fluorescence quenching zone at $R_f = 0.30$ is specific for *C. americana*. Image **B** in ◉ **Fig. 7** was electronically amplified but the zone in question is barely detectable. Image **A** shows significantly less noise and better contrast. It was obtained by subtracting an image of the neat plate from Image **B**.

Detection and evaluation

Today evaluation of planar chromatograms by scanning densitometry can be regarded as a standard technique [44]. In addition to absorption measurements densitometers generally can also measure fluorescence, which makes detection very flexible particularly in combination with optional chemical derivatization. Modern instruments utilize wavelengths from 190 to 800 nm in single or multi-wavelength scans and can also record spectra [45]. Results obtained this way can be compared to those from modern photo diode array detectors in HPLC [46], [47], [48], [49]. Also images of the plate can be quantitatively evaluated. By so-called video densitometry the individual chromatogram tracks are converted into analogue chromatogram curves [50]. The quality of results is quite comparable to that of scanning densitometry [51]. Video densitometry lacks the spectral selectivity of a scanning densitometer but it can take advantage of multiple images obtained from different illumination modes. For semi-quantitative evaluation analogue curves of tracks from the same or different images can be superimposed (◉ **Fig. 8**). With suitable software, qualitative evaluation by visual comparison of HPTLC fingerprints is possible. A fingerprint is the slice of an image representing an individual chromatogram track. This technique allows using information from multiple images of the same or different plates. With the help of dedicated pattern recognition software the information contained in such image slices might aid in identification of plants [52].

The possibility of detecting biological activity of sample components separated on the TLC/HPTLC plate by *in situ* tests has al-

ready been mentioned before. If such tests are well standardized they can be used in routine quality control or in effect directed analysis. A commercially available test is BioLuminex [53], which is based on the luminescent bacteria *Vibrio fischeri*. When the developed HPTLC plate is briefly immersed into a suspension of bacteria and then observed in a dark room, biologically active compounds can be seen as dark zones on a fluorescing background. This effect can be monitored and documented under controlled conditions with the BioLuminizer [54]. The test has been successfully used to detect adulteration of medicinal plants [55].

Great progress has been made in hyphenating planar chromatography with mass spectrometry (MS). A comparison of various techniques, interfaces, and performance criteria has been published by Morlock and Schwack [56]. Today it is possible to identify by MS substances that have been separated by HPTLC. Substances can either be detected by conventional techniques (e.g., densitometry) and then be individually transferred into the MS,

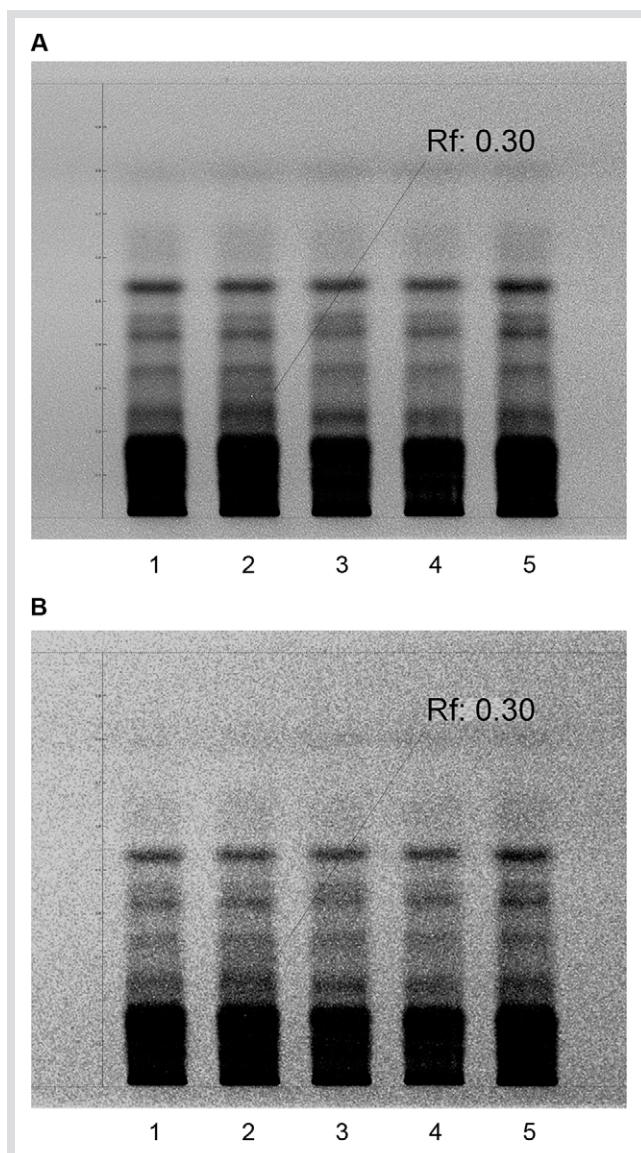


Fig. 7 Effect of image correction (**A**) for the detection of 5% *Cimicifuga americana* in *Cimicifuga racemosa*; **B**: without correction (1: *C. racemosa* reference, 2–5: Test samples), for details see text.

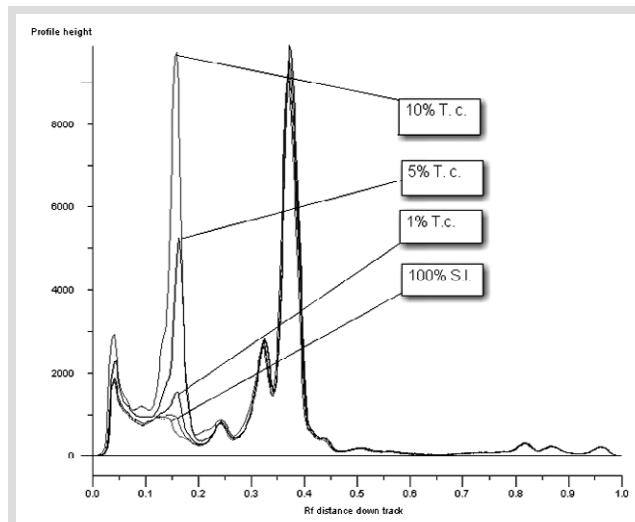


Fig. 8 Adulteration of *Scutellaria lateriflora* with *Teucrium chamaedrys*. Video-densitometric evaluation allows detection of 1% adulteration (see also **Fig. 5**).

or the entire track can be scanned. Van Berkel et al. demonstrated the suitability of the latter approach for the analysis of dietary supplements [57]. With the commercial availability of suitable interfaces it can be expected that HPTLC-MS will become a standard analytical tool in the near future.

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