PARAMETERS OF HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY (PART 1)

TLC is a simple, quick, and flexible way to analyze samples, and there is no right or wrong way of doing it. However, if efficient, standardized, and reproducible results are desired, HPTLC is the right choice. HPTLC implies the control of all parameters of the process.

SAMPLE PREPARATION

Sample preparation is at the beginning of the HPTLC process and can significantly influence the result. For practical reasons, it should be kept as simple as possible. Three main steps are included:

- The sample homogenization, comprising drying, grinding, freezing, lyophilizing and/or milling step(s);
- The choice of solvents;
- The extraction method.

Choosing a suitable extraction solvent involves several considerations:

- It should be non-hazardous (health, explosion, flammability), stable, and not reactive with the sample;
- It should be inexpensive and easily removable from the plate.

Polar solvents are water, ethanol, and methanol, medium polarity solvents are ethyl acetate, dichloromethane, and ethers, non-polar solvents are toluene, and cyclohexane.
Some extraction methods are:

- Maceration and percolation;
- Reflux/Soxhlet;
- Steam distillation (for essential oils / terpenes);
- Supercritical fluid extraction;
- Shaking;
- Extraction in the ultrasonic bath (sonication);
- Warm water-bath (boiling);
- Liquid-liquid extraction;
- Microwave-assisted extraction;
- Accelerated solvent extraction.

For samples with unknown properties, or as a convenient starting point, CAMAG proposed a simple extraction scheme covering different solvent polarities: 0.5 g of the powdered sample (amount can be adjusted) are extracted by sonication for 10 minutes with 5.0 mL of the following solvents:

Neat solvents and their mixtures	Acidic mixtures	Basic mixtures
Heptane/Toluene	Methanol – acetic acid 9:1 (V/V)	Methanol – ammonia 25% 8:2 (V/V)
Butyl methyl ether	Water – acetic acid 9:1 (V/V)	Water – ammonia 25% 8:2 (V/V)
Dichloromethane		
Ethyl acetate		
Acetone		
Acetonitrile		
Ethanol		
Methanol		
Water		
Ethanol – water 7:3 (V/V)		
Methanol – water 8:2 (V/V)		

The 15 test solutions can be analyzed in parallel with a mobile phase from the literature or a universal solvent system.

APPLICATION

During sample application, the following basic requirements must be met. The position of the sample must be precise to ensure a simultaneous start of the chromatography for all samples on the plate. This is prerequisite for the comparability of R_F values of separated zones. For that, a standardized plate layout is adopted. Precise application volumes are required for qualitative and quantitative analysis. A common problem is sample overloading, which decreases the separation power and causes tailing of zones. Furthermore, it is also clear that damages to the layer during sample application can affect the movement of the mobile phase.

CONTACT OR SPRAY-ON APPLICATION?

During contact application, the sample solution is transferred onto the plate with a (fixed volume) capillary or a syringe. The volume and volatility of the solvent affect the size of the applied spot. The strength of the solvent determines the distribution of the sample components in the applied spot (circular chromatography during application). Compact spots improve the chromatography resolution (see example below, where a test dye solution dissolved either in methanol or in toluene was applied by contact application, Figure 1A).

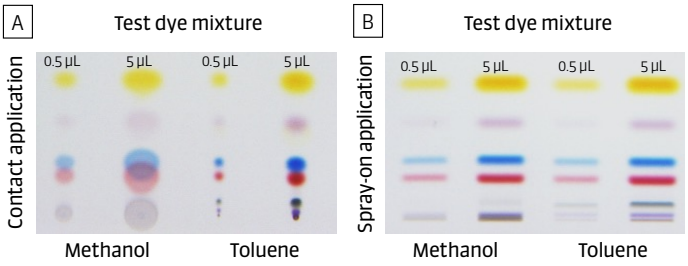

Figure 1: Contact application as spots (A) and spray-on application as narrow bands (B)

Spray-on application vaporizes the sample solution with a gas stream as it is dispensed from a syringe. Before it reaches the layer, a significant part of the solution's solvent is evaporated, and mainly the sample is transferred to the plate. That way, sharp lines are applied for improved separation during chromatography, regardless of the strength of the solvent (in Figure 1B). The spray-on technique can concentrate large sample volumes into narrow bands.

BAND OR SPOT APPLICATION?

During visual evaluation, the resolution seems to improve with the length of the separated zones, but that is just an optical illusion. Nevertheless, evaluation of the homogenous center part of a band improves the signal to noise ratio during the generation of peak profiles.

Most pharmacopoeias prefer band application in HPTLC due to better suitability for visual evaluation.

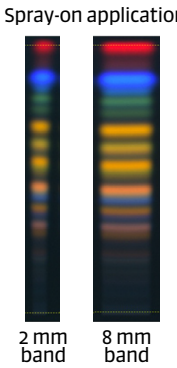

Figure 2: Comparison of 2 mm and 8 mm band length

PLATE LAYOUT

The standard format of the HPTLC plate is 20 x 10 cm. A precise application position is essential for proper identification of separated zones. For the application of samples with respect to plate layout, certain parameters (see Figure 3) need to be considered.

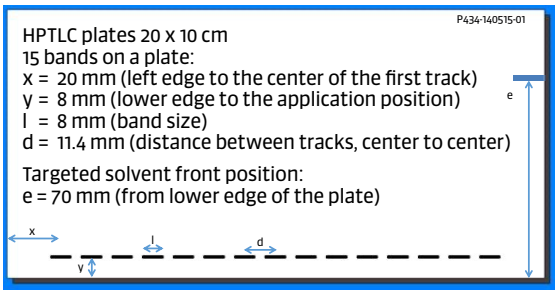

Figure 3: Plate layout proposed by the HPTLC Association and adopted by The United States Pharmacopoeia (USP), European Pharmacopoeia (Ph. Eur.), and British Pharmacopoeia (BP).

INSTRUMENTATION

CAMAG® HPTLC PRO MODULE APPLICATION

The degree of automation and productivity are key factors for the HPTLC laboratory. The Module APPLICATION is part of the CAMAG® HPTLC PRO SYSTEM, a fully automated sample analysis and evaluation system using HPTLC plates (20 x 10 cm), which is best suited for routine quality control of analytes extracted from complex matrices and provides reproducible and reliable results.

The Module APPLICATION plays a crucial role within the HPTLC PRO SYSTEM as it largely determines the quantification accuracy. Designed for highly precise application of samples as bands onto HPTLC glass plates, it supports the autonomous application of up to 75 samples on up to five HPTLC plates. To avoid cross contamination, the Module APPLICATION employs an S/N marked syringe generating a highly effective separation bubble between the rinsing solvent and the sample solution. To ensure highly precise application of samples as narrow bands, the Module APPLICATION laser-controls the needle distance and dosage speed, which vary depending on the sample solvent used.

The HPTLC PRO SYSTEM and the individual modules are controlled by *visionCATS*, a state-of-the-art software platform for workflow organization and data evaluation management.

Figure 4: CAMAG® HPTLC PRO Module APPLICATION

CAMAG® AUTOMATIC TLC SAMPLER 4 (ATS 4)

The ATS 4 is a highly advanced, versatile and powerful system, enabling the fully automated application of 66 samples from vials or 96 samples from well plates using either the spray-on technique or spot application by contact transfer. Any x- and y- position on the TLC plate can be selected for application. Samples can also be applied as rectangles, a feature that is very useful for large volumes of samples containing the analyte in very low concentration. Prior to chromatography, the rectangles are focused to narrow bands

with a solvent of high strength. Designed for routine use and high sample throughput, the automated ATS 4 is suited for qualitative and quantitative analyses as well as for preparative separations, while offering great flexibility by supporting any plate format up to 20 x 20 cm. The ATS 4 is controlled by *visionCATS* HPTLC Software.

Figure 5: CAMAG® Automatic TLC Sampler 4 (ATS 4)

CAMAG® LINOMAT 5

A widely used sample applicator is the semi-automatic Linomat 5, a device that introduced all advantages of the spray-on technique to HPTLC. Precise volume dosage and exact positioning combined with flexibility and convenient handling are among the features of the instrument. The user only loads the sample manually into a syringe and selects the y-position of the application; the instrument manages all other parameters of the application process. Like every other software-controlled HPTLC instrument from CAMAG, the Linomat 5 is controlled by *visionCATS* HPTLC Software.

Figure 6: CAMAG® Linomat 5

DEVELOPMENT

Development is one of the core elements of HPTLC. During development, the analyst needs to be aware of several parameters, which will affect the chromatography and the reproducibility of the results. The following paragraphs will discuss the influence of the separation distance and humidity in the chromatography, and the different chamber configurations.

SEPARATION DISTANCE

In (HP)TLC, the velocity of the mobile phase depends on capillary forces and is not constant as in HPLC or GC. With increasing separation distance, the velocity of the mobile phase decreases because of increasing resistance caused by the silica gel layer, which affects resolution.

With a decreasing velocity of the mobile phase diffusion increases. That is the reason why zones get broader in the upper part of the chromatogram. Even though their distance still increases, resolution/separation of zones does not improve beyond 6 cm separation distance (see Figure 7). This is why many compendia adopted 6 cm as optimum separation distance.

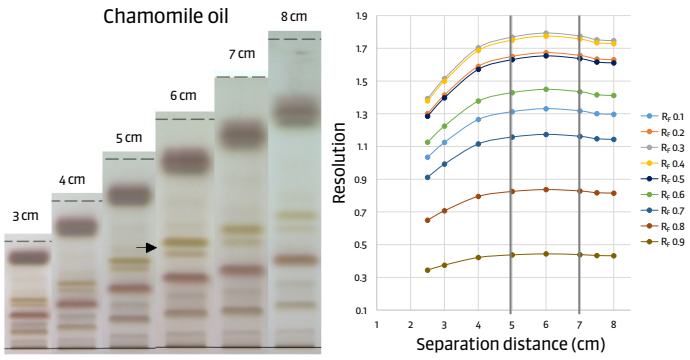

Figure 7: Separation of chamomile oil on HPTLC silica gel 60 plates. Mobile phase: toluene – ethyl acetate 19:1 (V/V), derivatization by dipping in 10% sulfuric acid in methanol

INFLUENCE OF THE RELATIVE HUMIDITY

Silica gel has a hydrophilic nature. Silanol groups on its surface interact strongly with water vapor adsorbed from the environment. This leads to deactivation of the layer, reducing the analyte's interaction with the stationary phase and increasing R_F values.

Exposing silica gel to high temperatures (e.g., 120°C) or low relative humidity removes adsorbed water (activation). The silanol groups become more active, and the R_F values of the analytes will be lower.

Because HPTLC is performed in an open system, results are always affected by the relative humidity of the environment. To ensure reproducible results, it is recommended to adjust the activity of the plate prior to development by exposing the plate for a certain time to a defined relative humidity established over a saturated salt solution.

Saturated salt solution	% relative humidity
NaCl	75
NaBr	58
KSCN	47
$MgCl_2$	33
$CaCl_2$	31
KCOOH	20
(Molecular sieve)	0-5

HPTLC CHAMBER CONFIGURATIONS

Chamber configuration and the corresponding gas phase will affect the chromatographic result. In standardized HPTLC, a saturated Twin Trough Chamber (TTC) is generally utilized, but also other modes of operation are possible:

- **Unsaturated TTC:** The development starts by introducing the plate immediately after the developing solvent is poured into one of the troughs of the TTC. The other trough remains empty. During development, components of the mobile phase may evaporate from the plate. At the same time, the developing solvent is evaporating into the chamber. Equilibrium between liquid and gas phase (saturation) is not achieved; the chamber remains "unsaturated".
- **Saturated TTC:** Both troughs of the TTC are charged with developing solvent. The rear trough is fitted with filter paper (saturation pad). With the lid closed (e.g., for 20 minutes), equilibrium of the developing solvent and its vapor in the chamber is achieved before the plate is introduced for development. Unless the developing solvent is a neat solvent, the component(s) with the higher vapor pressure will have a higher proportion in the gas phase.
- **Pre-conditioning in TTC:** The developing or another conditioning solvent is poured into the rear trough of the TTC, which may contain a filter paper (saturation pad), and the chamber is saturated (e.g., for 20 minutes) with the lid closed. Then, the plate is inserted into the front trough and, thus, exposed to the gas phase. After a certain time (e.g., 10 minutes) of pre-conditioning, the developing solvent is introduced into the front trough, and chromatography starts.

Special features of the unsaturated TTC are:

- Sharper zones;
- Higher R_F values;
- Sometimes better separation of critical pairs;
- Longer developing time;
- Chromatography susceptible to variation in temperature, humidity and chamber geometry;
- Results less reproducible.

Special features of saturated TTC and pre-conditioning are:

- Broader zones;
- Lower R_F values;
- Shorter developing time;
- Chromatography is less affected by variation in temperature, humidity and chamber geometry;
- Reproducible results;
- Pre-conditioning further decreases the R_F values.

Practical tip: The level of developing solvent must always be below the application position on the plate. In HPTLC, the level is 5 mm with an application position of 8 mm.

POST-CHROMATOGRAPHIC DRYING OF THE PLATE

Once the mobile phase reached the targeted solvent front position, the plate should be immediately removed from the chamber and dried to stop chromatography and suppress any diffusion of separated components. During the drying, the plate is kept in a vertical position.

INSTRUMENTATION

CAMAG® HPTLC PRO MODULE DEVELOPMENT

Designed to develop 20 x 10 cm HPTLC glass plates, the Module DEVELOPMENT obtains highly reproducible analytical results and offers high flexibility regarding the choice of the solvents and configuration of the separation process. Its unique chamber geometry allows for the full control of the gas phase prior to and during chromatography. Prior to development, the stationary phase can be pre-dried, activated, and pre-conditioned. During development, the gas phase can be actively circulated to achieve the best possible separation of the analytes. After development, the mobile phase is drained off the chamber and a running blower removes the residual mobile phase from the stationary phase. Combined with the Modules PLATE STORAGE and APPLICATION, the Module DEVELOPMENT allows for the sequential execution of several analysis files, enabling the autonomous development of up to five different HPTLC glass plates with up to three different developing solvents.

Figure 8: CAMAG® HPTLC PRO Module DEVELOPMENT

CAMAG® AUTOMATIC DEVELOPING CHAMBER ADC 2

The Automatic Developing Chamber 2 (ADC 2) is an instrument for reproducible development, performing the development step fully automated, and independent of environmental effects. The activity and pre-conditioning of the layer, chamber saturation, developing distance and final drying can be preset and automatically monitored by the ADC 2. The fully automated ADC 2 employs a conventional 20 x 10 cm Twin Trough Chamber for development, allowing to retain the chromatographic conditions of already existing analytical procedures while excluding environmental and operational effects. The option Humidity Control allows reproducible chromatography at defined activity of the layer. This feature is essential in method development when the influence of relative humidity shall be investigated.

Figure 9: CAMAG® Automatic Developing Chamber ADC 2

CAMAG® AMD 2 SYSTEM AUTOMATED MULTIPLE DEVELOPMENT

The separation of complex samples is a challenging task for every chromatographic system, particularly when the sample components span a wide polarity range. The software-controlled AMD 2 is designed to solve difficult separation problems that cannot be handled by isocratic HPTLC. The AMD procedure offers an excellent solution as it allows stepwise gradient elution over increasing separation distances. As a result, acids, bases, neutral, hydrophilic, and lipophilic substances can be separated in a single AMD run. This makes AMD suitable for a variety of applications. The technique is frequently used in lipid analysis and in routine analysis of drinking water. Pigment formulations with a complex composition, resins as well as additives of mineral oil products are other typical applications of AMD analysis.

Figure 10: CAMAG® AMD 2 System Automated Multiple Development