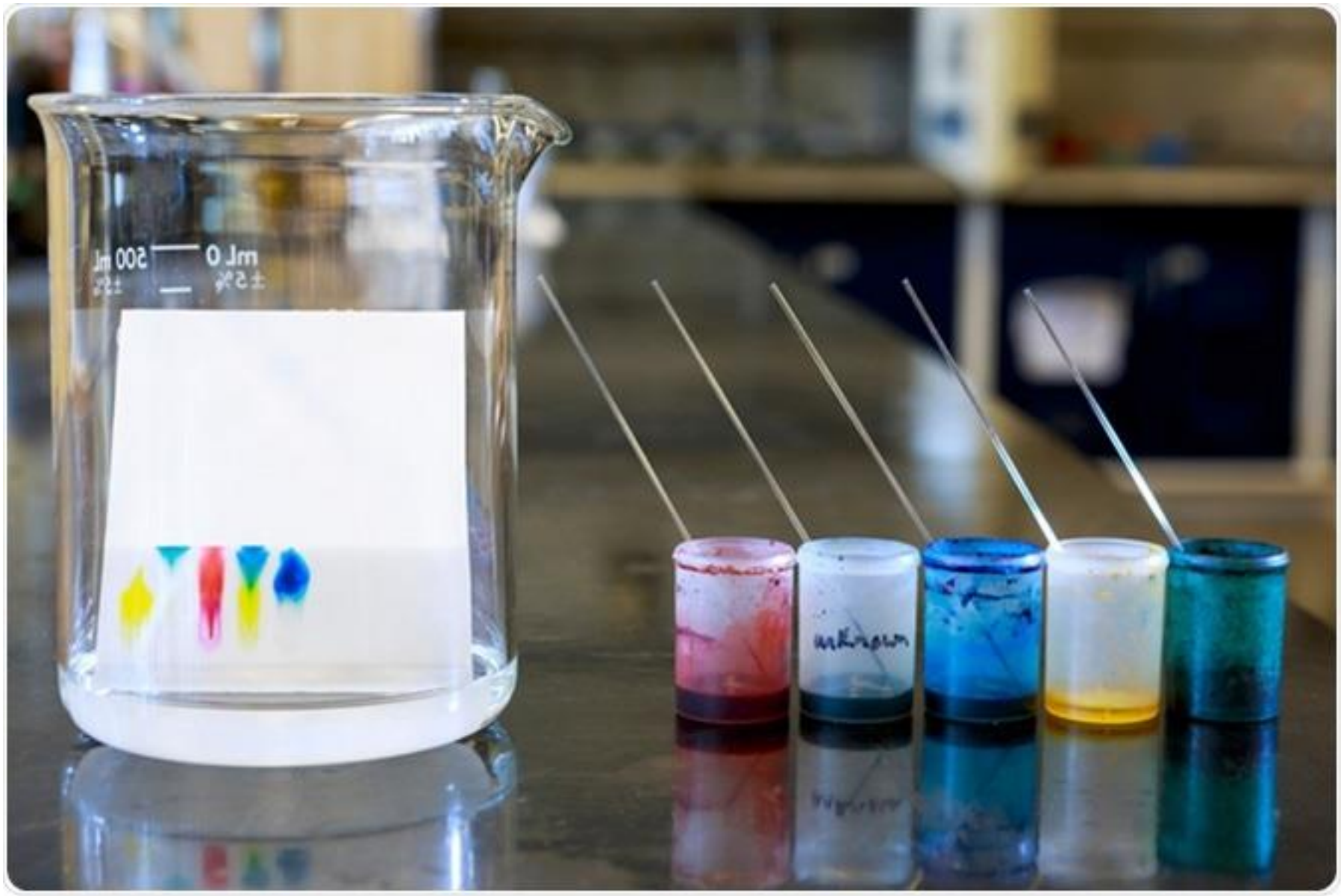
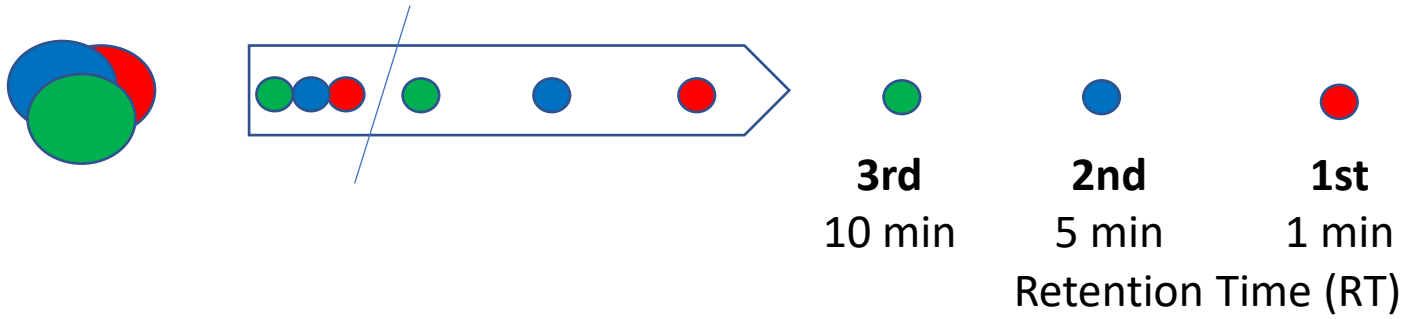
Planar Chromatography (and column)



Identifying Components of a Mixture

Chromatography is a process by which compounds within a mixture are separated. Compounds are be separated by properties such as size, and how the compounds interact with the mobile and stationary phases of chromatography.



The sample is mixed into the **mobile phase**, usually a liquid or a gas, which is then passed over the stationary phase, usually a solid or a liquid. If a compound (compound A) within a mixture has a low affinity for the **stationary phase**, then it will not interact much with the stationary phase. However, if another compound (compound B) within a mixture has a high affinity for the stationary phase, then it will bind to the stationary phase. This results in compound A moving through the mobile phase more quickly than compound B, and thus compounds A and B can be separated from the mixture.

Different types of chromatography

~~(Will be / were dealt with at some other time)~~

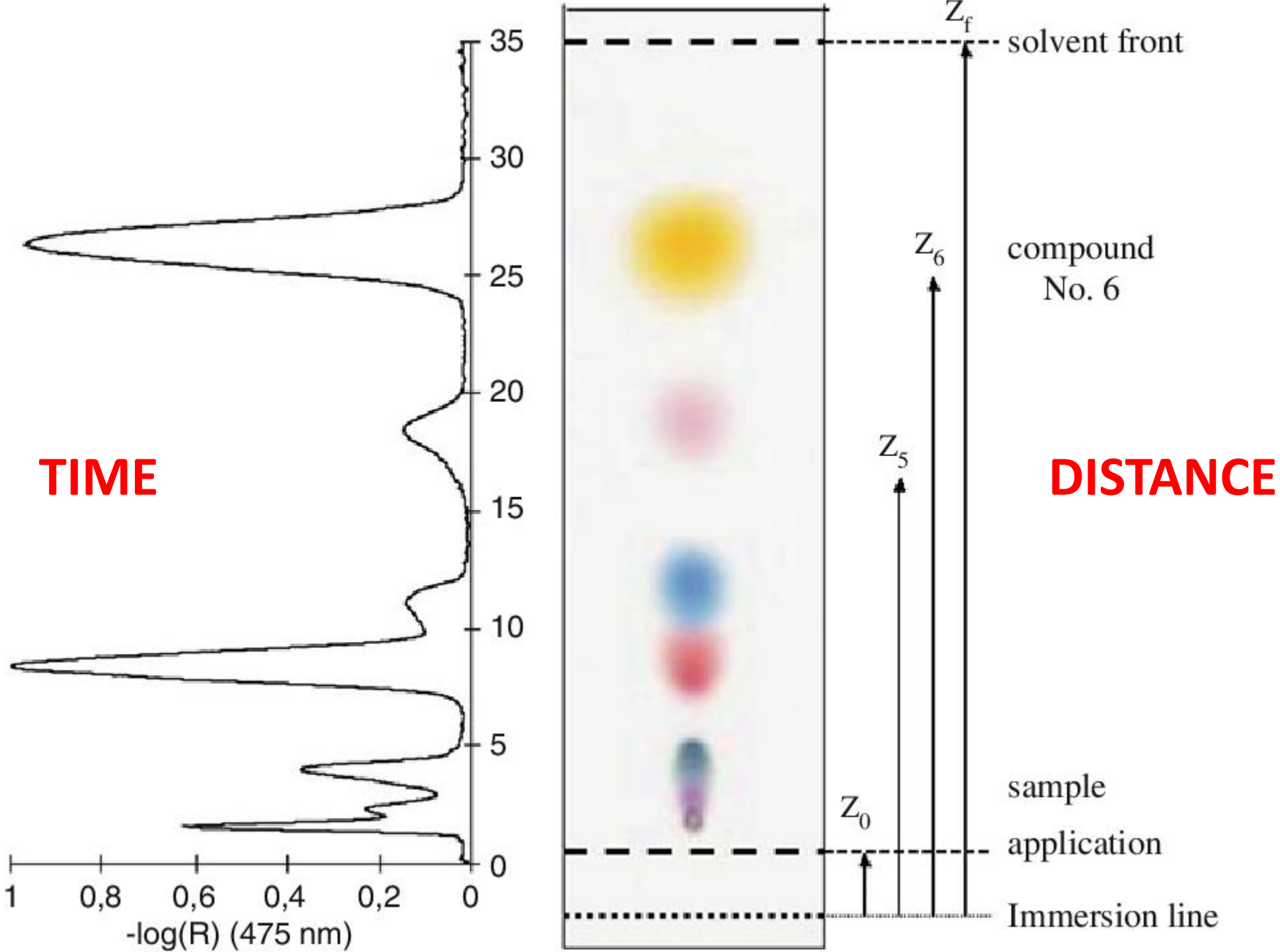
Chromatography technique can be explained / **classified** into three fundamental ways:

- Based on the shape of chromatographic bed.
  - **Planar chromatography (TLC and paper chromatpography)**
  - ~~**Column chromatography**~~
- Based on the physical nature of the stationary and mobile phases.
  - ~~**Gas chromatography (GC)**~~
  - ~~**Liquid chromatography (**~~**LC,** ~~**HPLC, UHPLC)**~~
  - ~~**Supercritical fluid chromatography (SFC)**~~
- Based on the mechanism of the separation.
  - ~~**Ion- exchange**~~
  - ~~**Affinity**~~
  - ~~**Size exclusion**~~
  - ~~**Hydrophobic interaction**~~

Planar Chromatography: What is it?

Definition: **Planar chromatography** is a liquid chromatography in which the stationary phase is arranged in the form of a planar or flat bed and the mobile phase moves by capillary action.

Planar chromatography is a form of **liquid** chromatography: A liquid mobile phase dissolves the substances of a mixture and transports them through a **flat (planar) stationary phase.** Different affinities of substances to the two phases afford separation as they cause different retardation with respect to the velocity of the mobile phase. In column chromatography, this affects the **time** of elution (retention time) while in planar chromatography the **position** of substance relative to the front of the mobile phase on the plate is changed. Because planar chromatography takes place in a developing chamber, also a gas phase is established, which can influence the separation.


TIME
35
30
25
20
15
10
5
0
1
0,8
0,6
0,4
0,2
0
-log(R) (475 nm)
Z_f
solvent front
Z_6
compound
No. 6
Z_5
DISTANCE
Z_0
sample
application
Immersion line

Planar Chromatography: Separation principles

Like other chromatographic techniques, thin-layer chromatography (TLC) depends on a separation principle. The separation relies on the **relative affinity** (**coefficient of partition o coefficient of distribution**) of compounds towards both the phases. Besides, **adsorption** also play an important role.

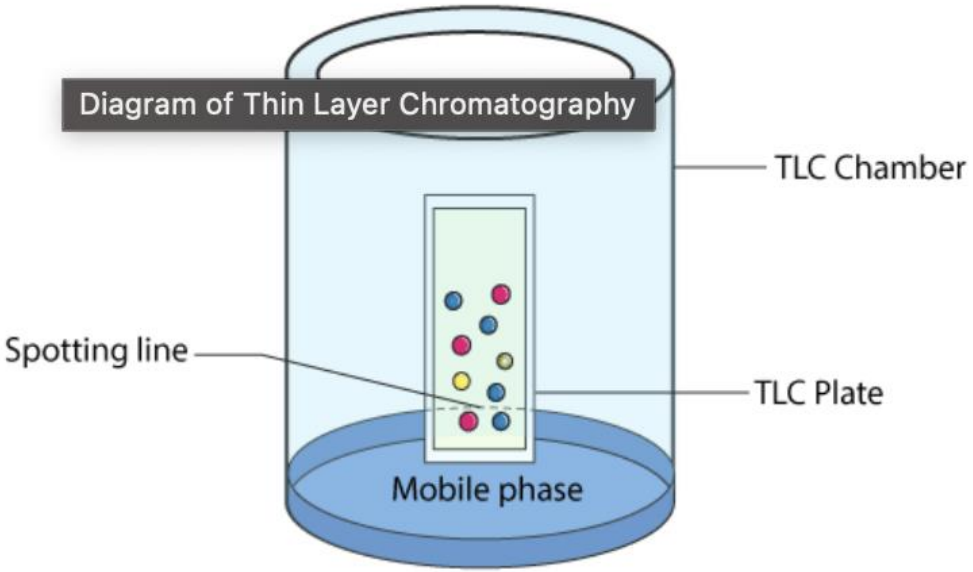
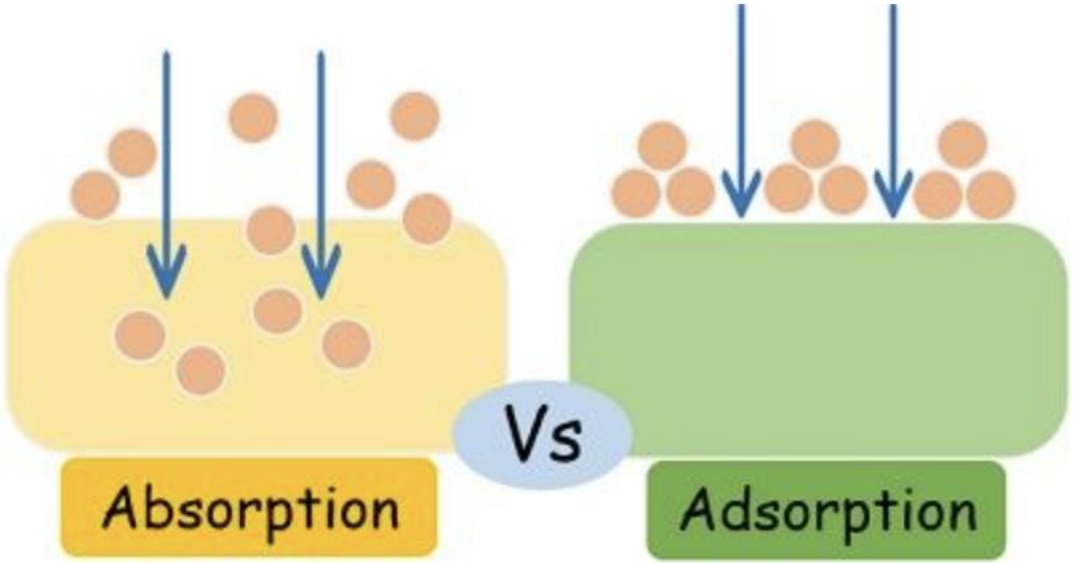
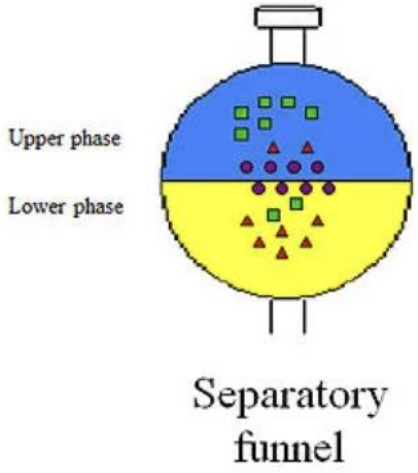


Diagram of Thin Layer Chromatography



Planar Chromatography: Separation principles

Distribution coefficient is the ratio of the concentration of both ionized and un-ionized species of a compound in a mixture of two immiscible phases. We can denote this phenomenon as “D”. Here, one of the two immiscible phases is essentially water or an aqueous solution. The other phase is usually a hydrophobic phase which is immiscible with water (or any other aqueous phase we use here).



$K_D > 1$

$K_D = 1$

$K_D < 1$

$$K_D = \frac{[A]_{upper}}{[A]_{lower}}$$

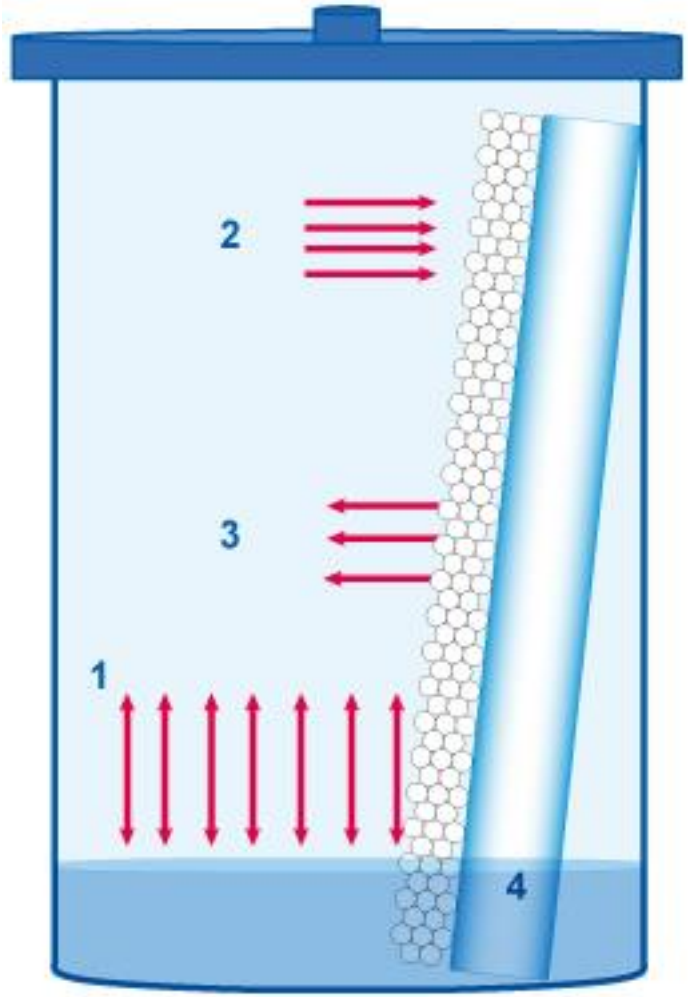
ORGANIC

AQUEOUS

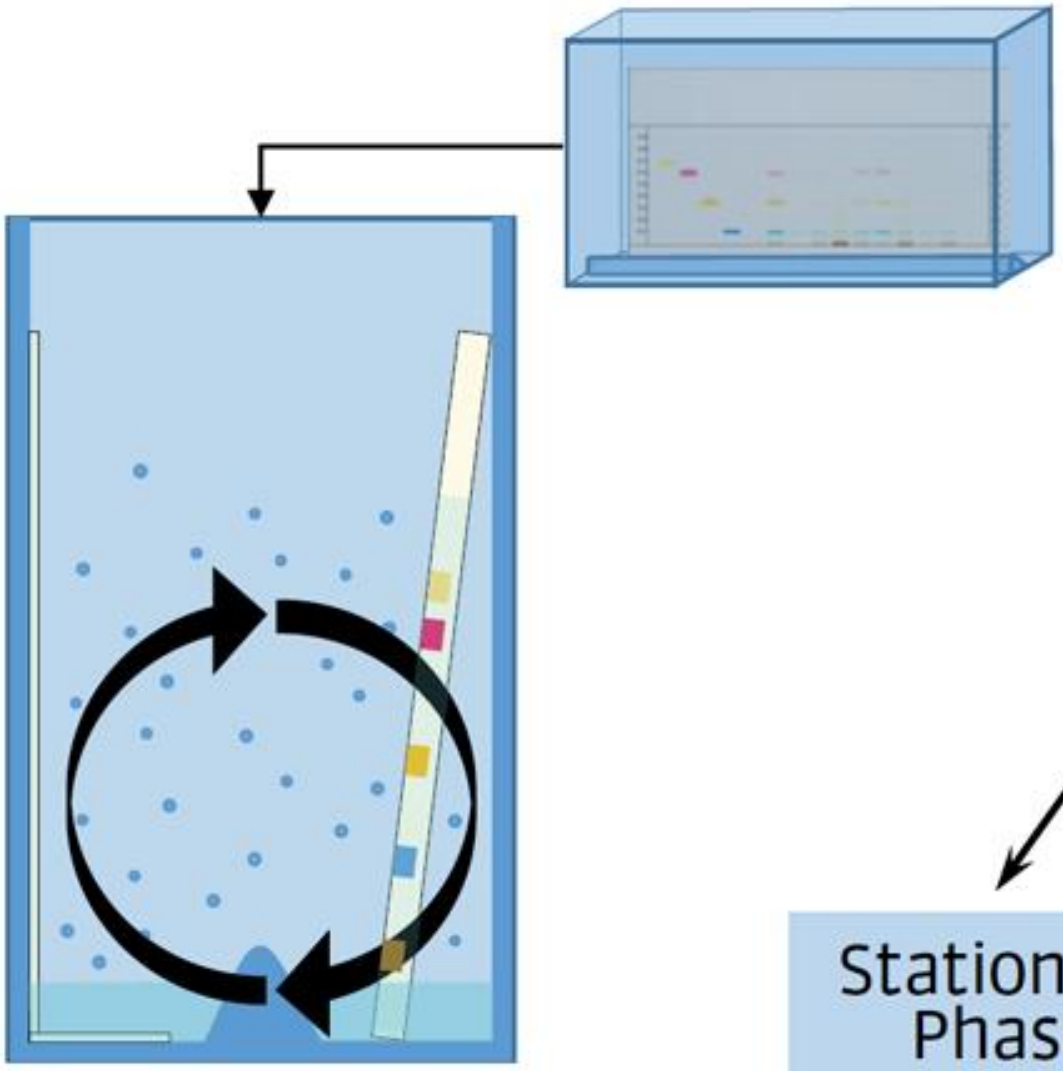
Note: **The key difference between partition coefficient and distribution coefficient** is that the term partition coefficient refers to the concentration of un-ionized chemical species of a compound whereas the term distribution coefficient refers to the concentration of both ionized and un-ionized chemical species of a compound.

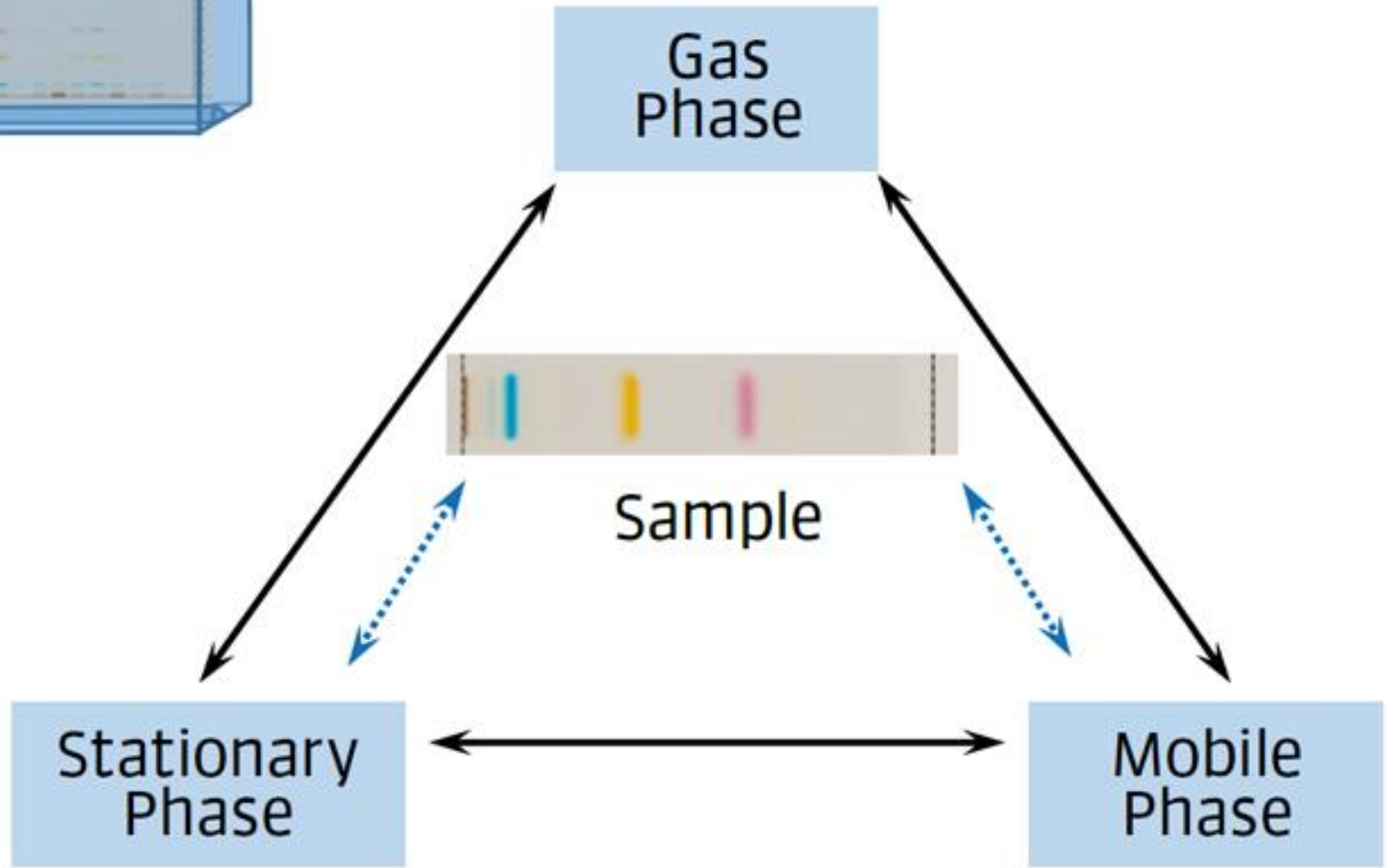
Planar Chromatography: Effect of gas phase

If the head space of the chamber is not saturated with solvent, the solvent that is rising up the plate will evaporate in an attempt to saturate the air. This will lead to high Rf values and poor resolution. You want your chamber air thoroughly impregnated with solvent vapor in planar chromatography because this keeps the stationary phase from drying out before the process is finished. The solvent evaporating from the paper saturates the chamber's air so it doesn't wick solvent off the stationary phase as quickly.



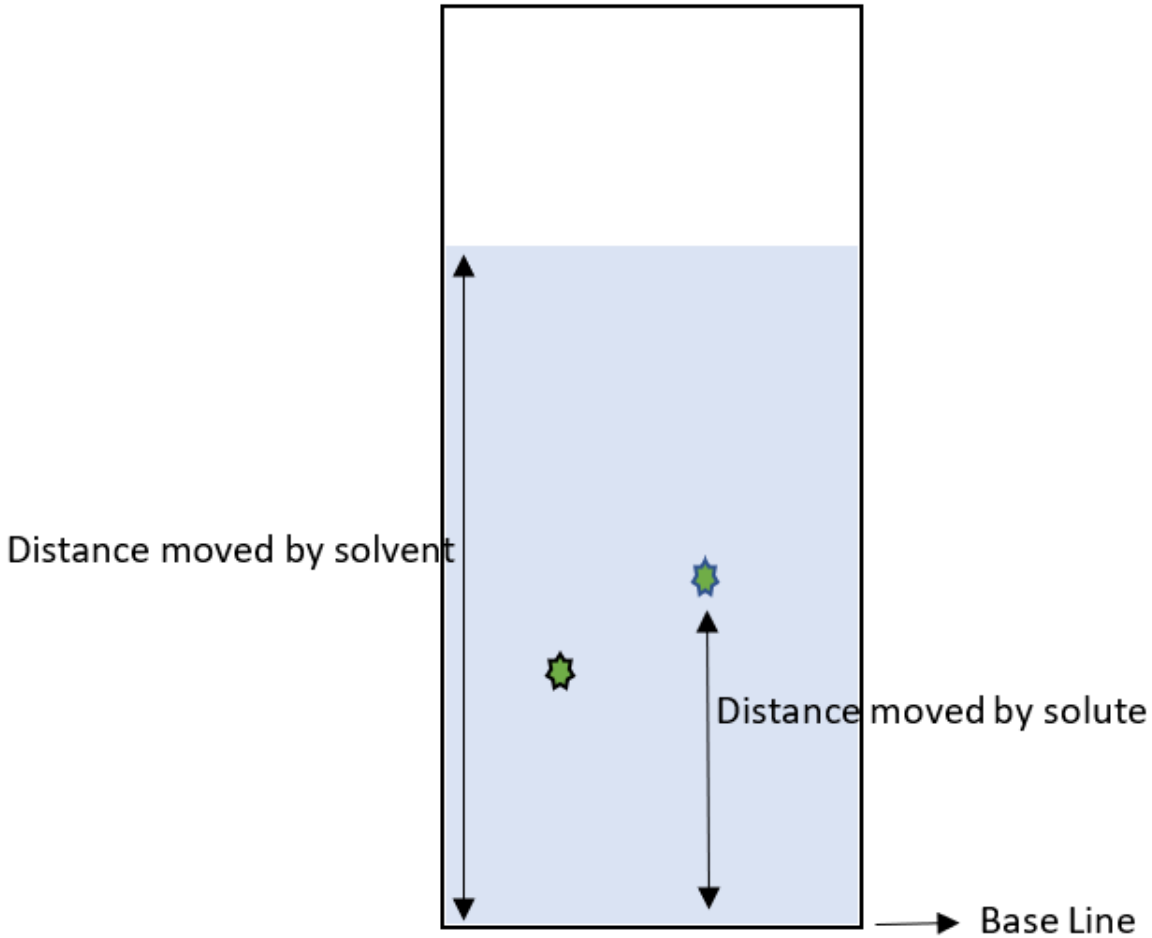
Planar Chromatography





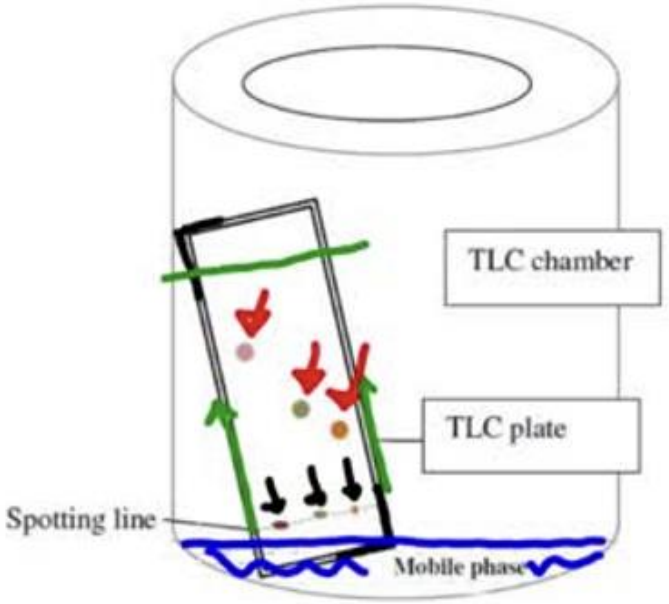
Retention Factor (Rf)

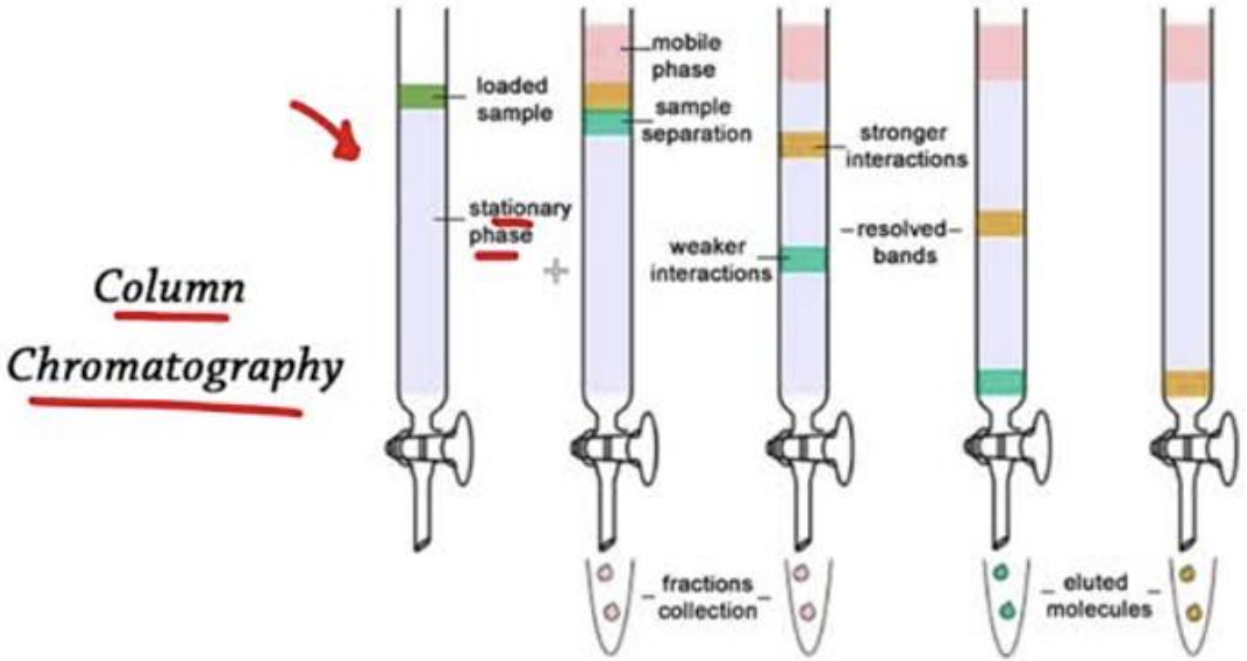
The **retention factor (RF)** is a measure of how far a compound travels up a TLC /paper chromatography plate relative to the solvent front. **It is calculated by dividing the distance the compound has traveled by the distance the solvent front has traveled**. The RF value is specific to a particular solvent system and TLC plate, and it can be used to identify and compare compounds in a mixture based on their chromatographic behavior.



The RF value is used to **help identify unknown compounds** by comparing their RF values to those of known compounds under the same conditions. It is also used to optimize solvent systems for separating mixtures of compounds.

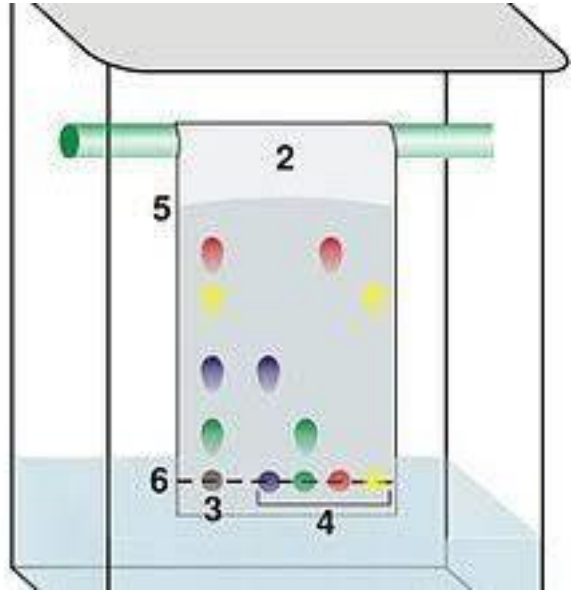
Planar Chromatography vs Column Chromatography





Planar Chromatography (vs column): Advantages

- Several samples may be analyzed at the same time.
- The analytes are separated by this method within a short period.
- A complex sample mixture can be easily separated as it is sensitive.
- Planar chromatography requires fewer sample volumes for analysis.
- This can be automated in the form of high-performance thin-layer chromatography HPTLC).
- Compared with other separation techniques it is an economical method.
- The use and setup of the planer chromatography are simple.
- This technique requires very few tools to carry out the process.
- In this method, organic as well as inorganic molecules can be possible to identify

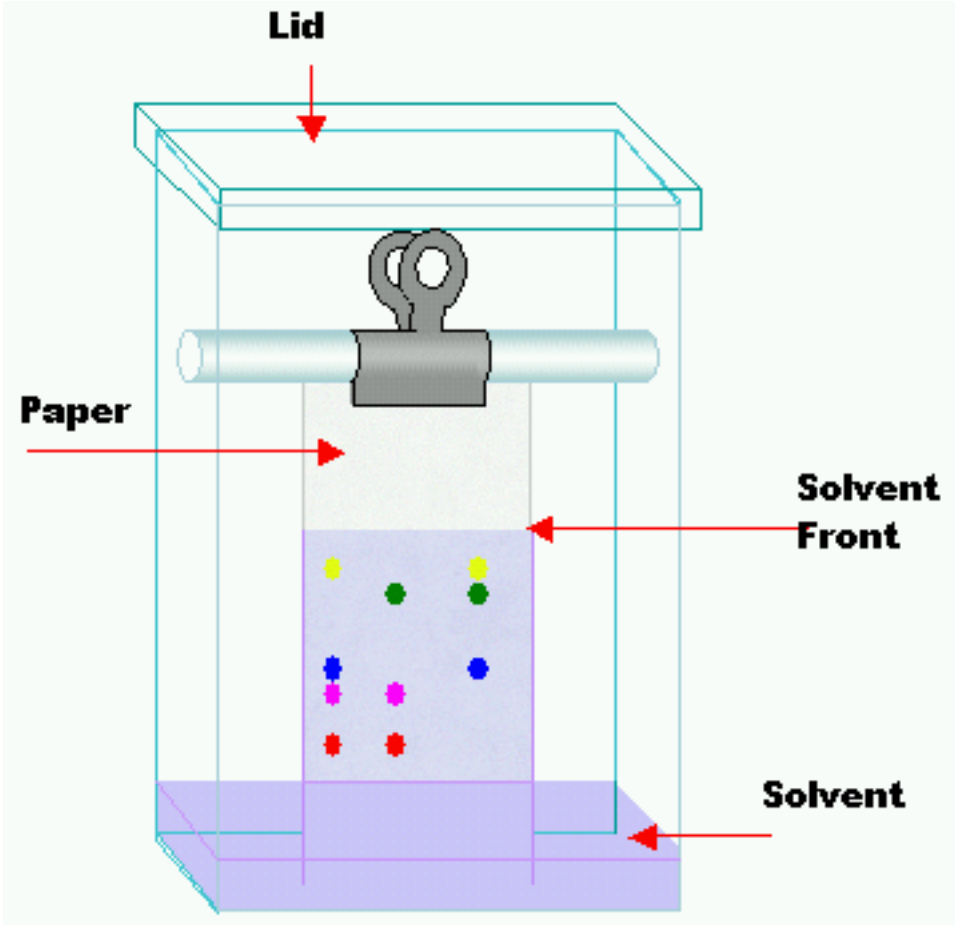


Planar Chromatography (vs column): Disdvantages

- The use of the planar chromatography method cannot separate **volatile** components.
- Since it is an open system, **humidity and temperature** can affect the results.
- Another disadvantage of planar chromatography is that the molecules **must be soluble**.
- Planar chromatography isolates only **small sample volumes**.
- It has **less accuracy** when compared to HPLC (although HPTLC is rapidly shortening the breach among techniques based on precision and accuracy).

Planar Chromatography: Types

There are two forms of planar chromatography: **Paper Chromatography**



and **Thin-Layer Chromatography (TLC).**

Planar Chromatography: Types

Advantages of each method

Paper Chromatography	Thin-layer Chromatography
• Cheap • Little preparation • More efficient for polar and water-soluble compounds • Easy to handle and store	• Faster • Detects smaller amounts • Better separation of less polar compounds • Corrosive material can be used • A wide range of stationary phases is available

Thin Layer Chromatography (TLC): Advantages vs paper chromatography

TLC has some advantages over paper chromatography. For example:

- The mobile phase moves more quickly through the stationary phase
- The mobile phase moves more evenly through the stationary phase
- There is a range of absorbencies for the stationary phase
- TLC tends to produce more useful chromatograms than paper chromatography, which show greater separation of the components in the mixture - and are therefore easier to analyze

Paper Chromatography

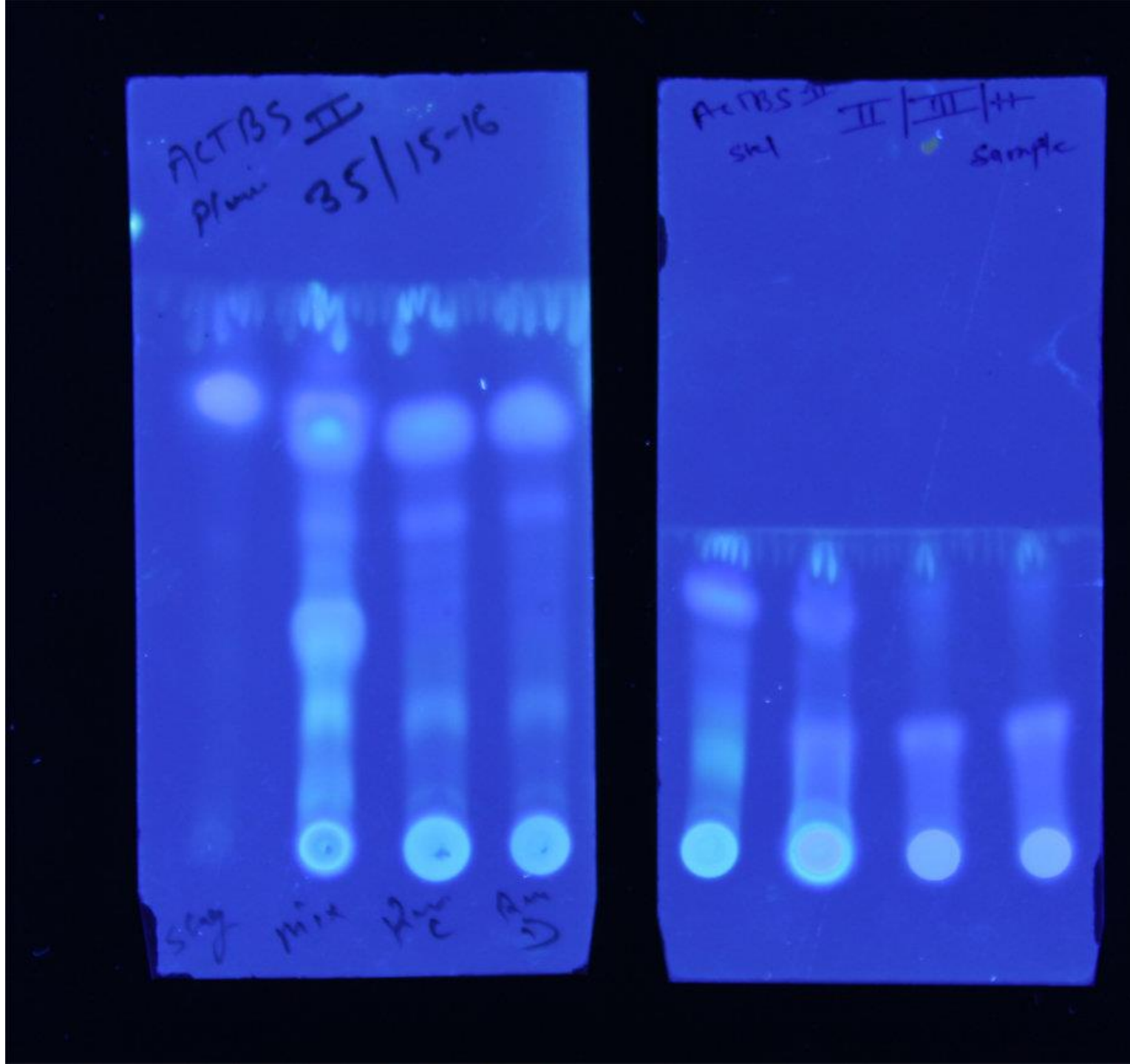
Paper chromatography is a type of chromatography used to separate and identify different components of a mixture. It involves placing a small amount of a mixture on a piece of **filter paper** and placing the paper in a container with a solvent, which then moves up the paper by capillary action.

As the solvent moves up the paper, it carries the different components of the mixture with it. Each component will have a different affinity for the paper and the solvent, causing them to separate into distinct bands along the paper. The distance each component travels up the paper is determined by its affinity for the paper and the solvent, as well as the composition of the mixture.

Once the bands have formed, they can be visualized by exposing the paper to a developing agent, such as a stain or a UV light. The distance each band traveled can be measured and compared to a standard to identify the components of the mixture.

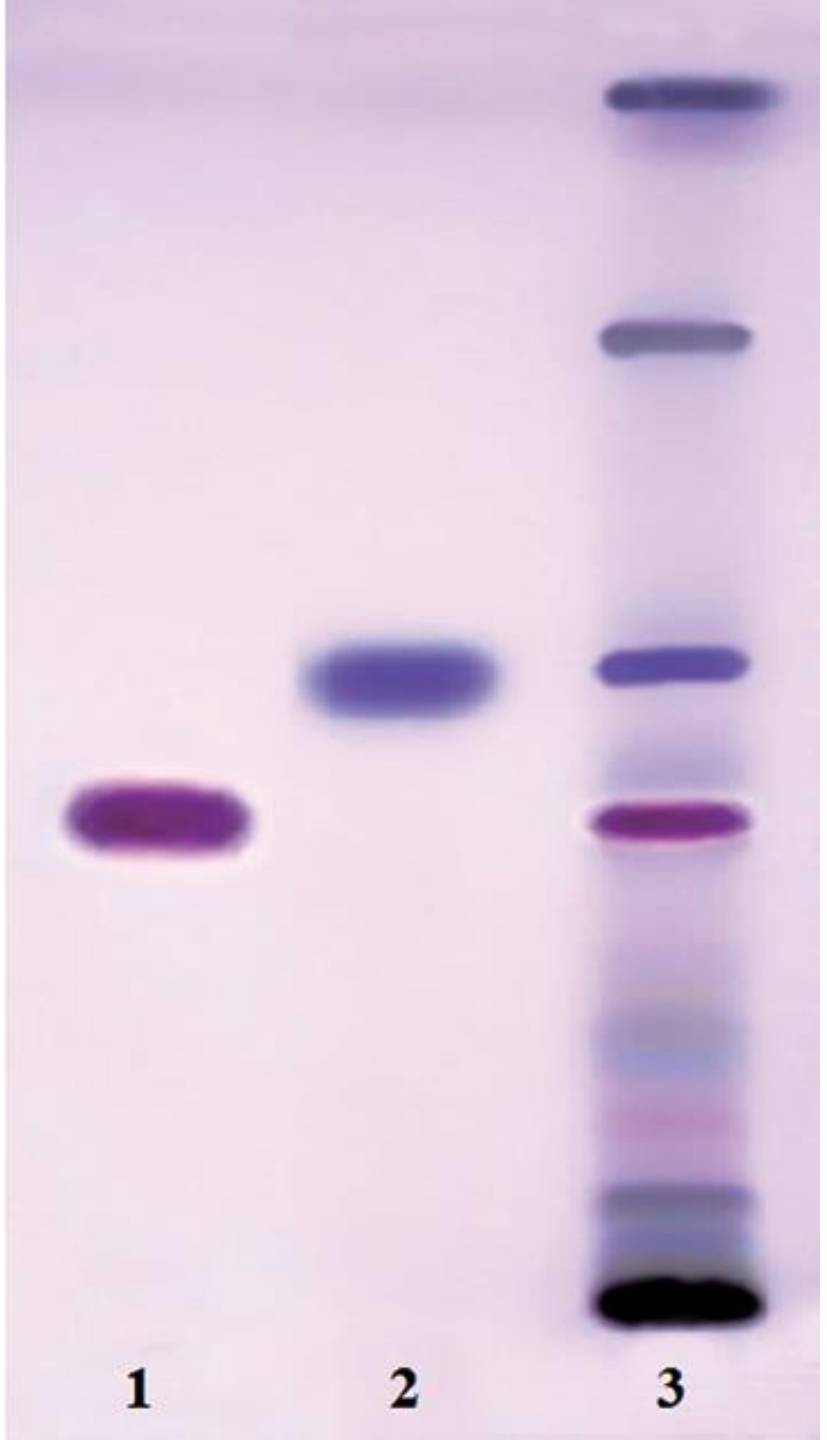
Thin Layer Chromatography

Thin layer chromatography (TLC) is similar to paper chromatography but instead of paper, the stationary phase is a thin layer of an inert substance (eg silica) supported on a flat, unreactive surface (eg a glass plate). It is generally treated with a fluorescent compound that allows for separated compounds identification under UV Light



TLC: Derivatization

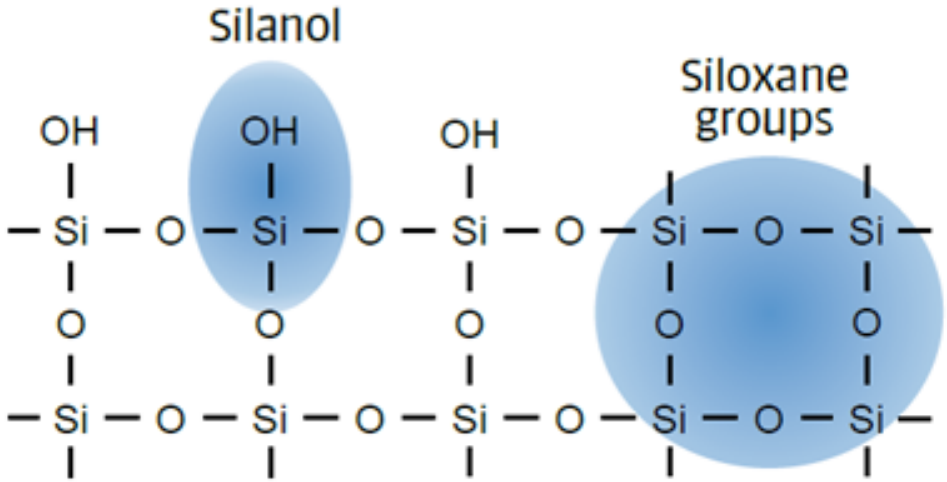
What is Derivatization? In TLC**, derivatization** is used to enable the detection of separated compounds that are colorless and cannot be visualized with UV radiation or fluorescence. A suitable reagent is applied to the TLC plate, which reacts with the sample compounds and transforms them into detectable derivatives.

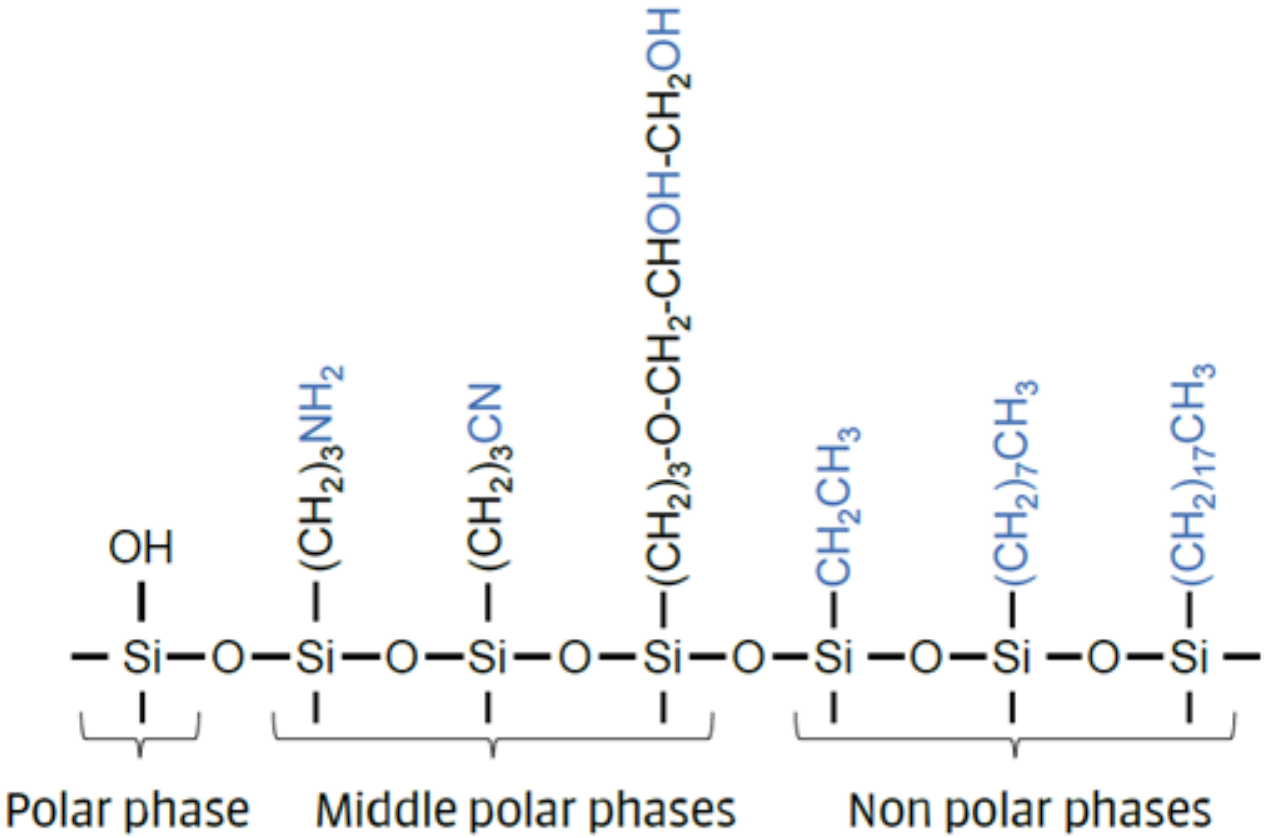


TLC: Plates

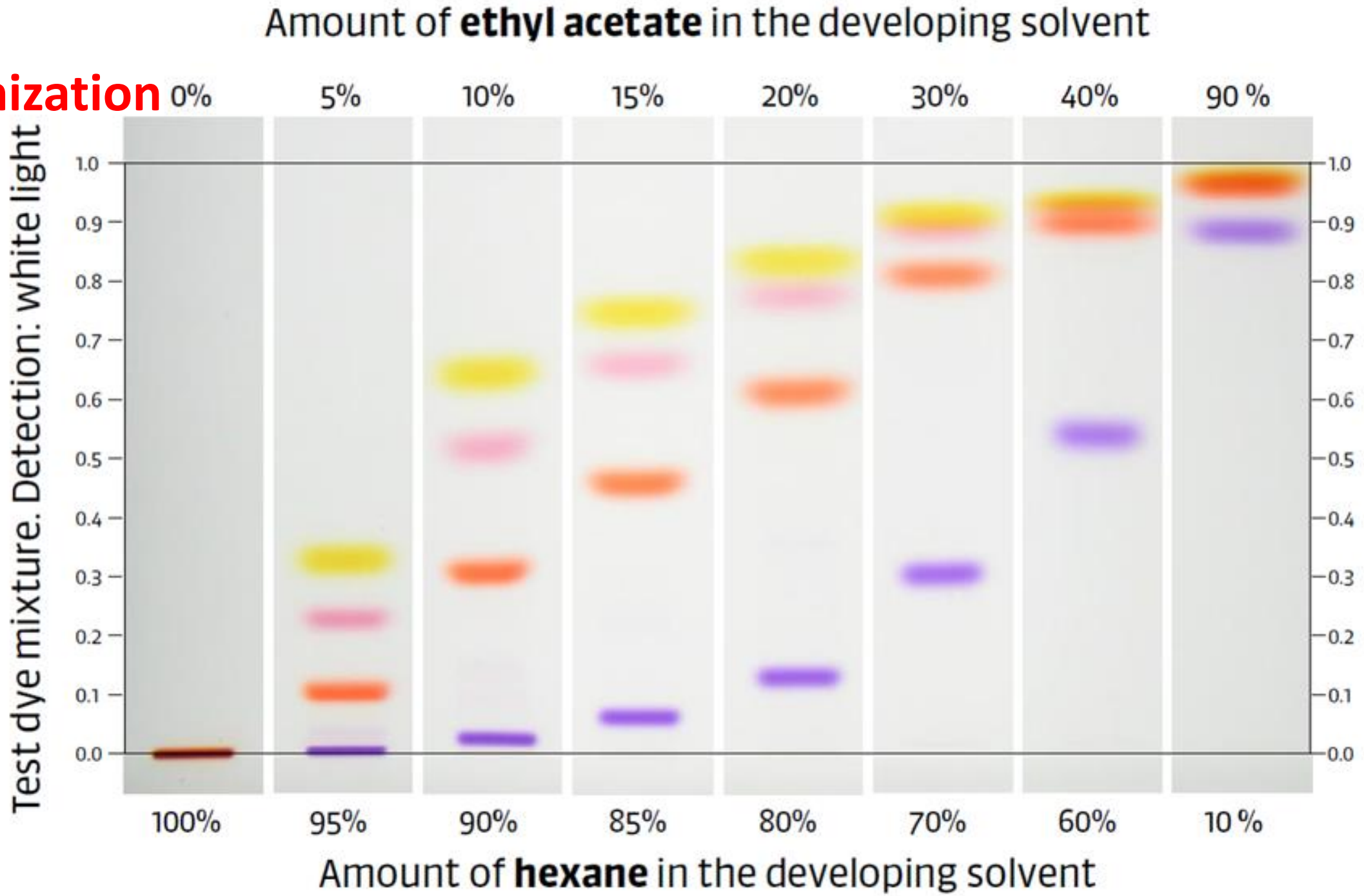
Name (Merck)	Functionality	Polarity	Principal use as
RP 2 (C2)	Dimethyl	Non-polar	RP (partition)
RP 8 (C8)	Octyl	Non-polar	RP (partition)
RP 18 (C18)	Octadecyl	Non-polar	RP (partition)
Silica gel (SiOH)	Silanol	Polar	NP (adsorption/partition)
Amino (NH2)	3-aminopropyl	Middle polar	RP/NP (partition/adsorption)
Cyano (CN)	3-cyanopropyl	Middle polar	RP/NP (partition/adsorption)

TLC: Plates


Silanol
Siloxane groups
OH
Si
O


OH
(CH2)3NH2
(CH2)3CN
(CH2)3-O-CH2-CHOH-CH2OH
CH2CH3
(CH2)7CH3
(CH2)17CH3
Si
O
Polar phase
Middle polar phases
Non polar phases

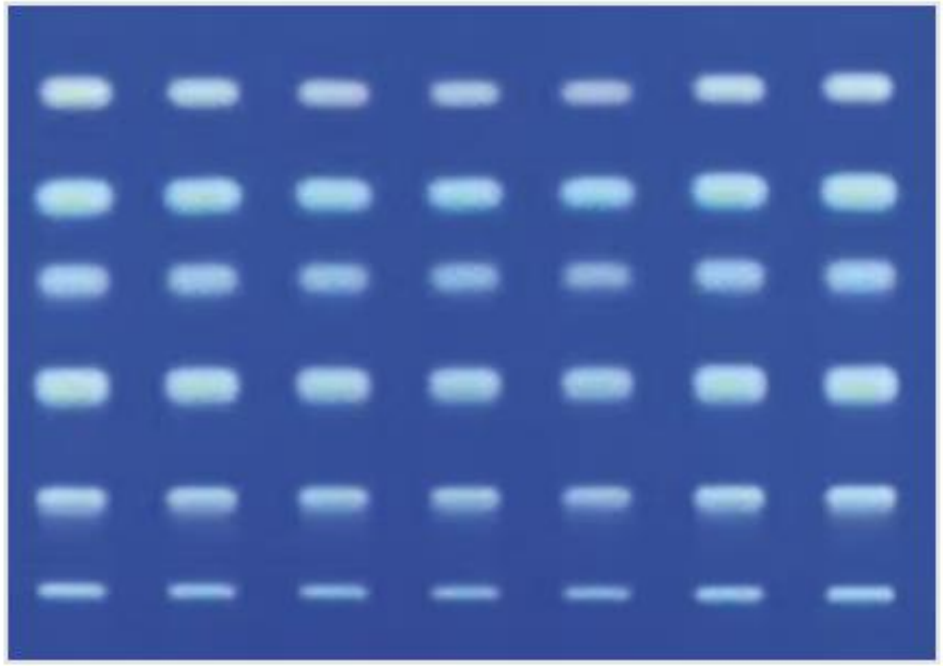
TLC optimization



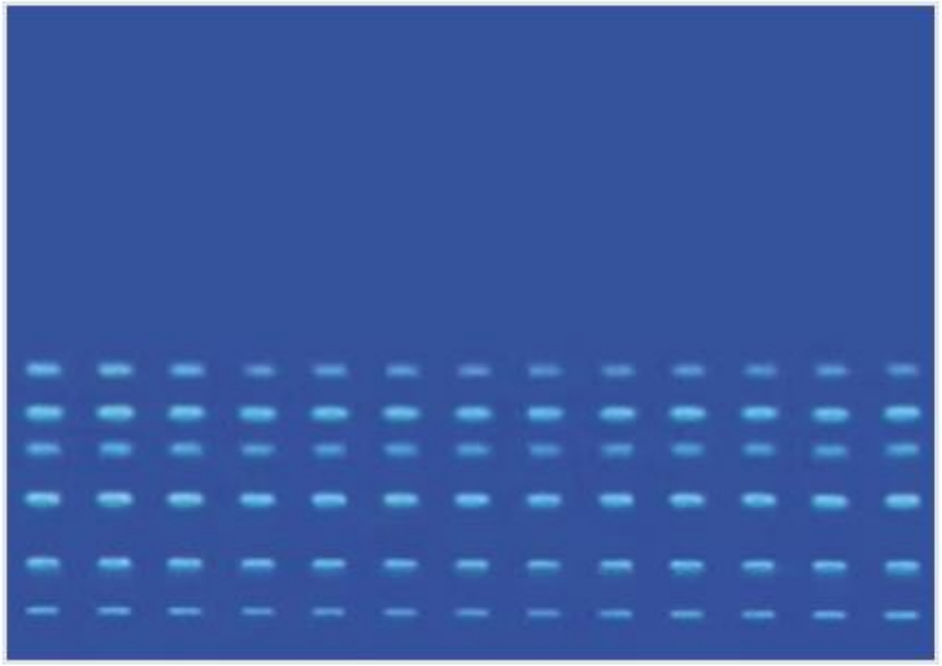
High Performance Thin Layer Chromatography (HPTLC)

HPTLC (high-performance thin layer chromatography) is a **sophisticated form of TLC**, which provides superior separation efficiency. The HPTLC concept includes validated methods for qualitative and quantitative analysis, and fulfills all quality requirements for use in fully regulated environments

TLC



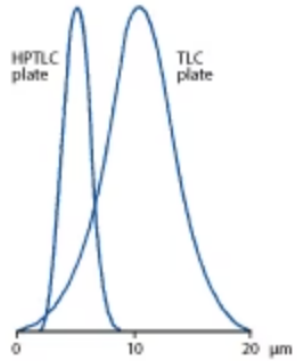
HPTLC



HPTLC vs TLC (1)

The process steps of HPTLC are **identical** to classical TLC. The main difference between them is in the **characteristics of the separation plate and the automatization in HPTLC**. HPTLC plates are based on optimized silica gel 60 with a significantly smaller particle size than used for classical TLC. This allows a higher packing density and a smoother surface. Hence, sample diffusion is reduced, resulting in compact bands or spots. Furthermore, the smaller particle size and thinner layer significantly increase detection sensitivity and analysis speed.

Features of HPTLC versus classical TLC	HPTLC	Classical TLC	
Mean particle size	5 - 6 µm	10 - 12 µm	
Particle size distribution	4 - 8 µm	5 - 20 µm	
Layer thickness	200 µm (100 µm)	250 µm	
Plate height	12 µm	30 µm	
Typical migration distance	3 - 6 cm	10 - 15 cm	
Typical separation time	3 - 20 min	20 - 200 min	
Number of samples per plate	< 36 (72)	< 10	
Sample volume	0.1 - 0.5 µl	1 - 5 µl	
Detection limits: absorption	100 - 500 pg	1 - 5 ng	
Detection limits: fluorescence	5 - 10 pg	50 - 100 pg	



HPTLC vs TLC (2)

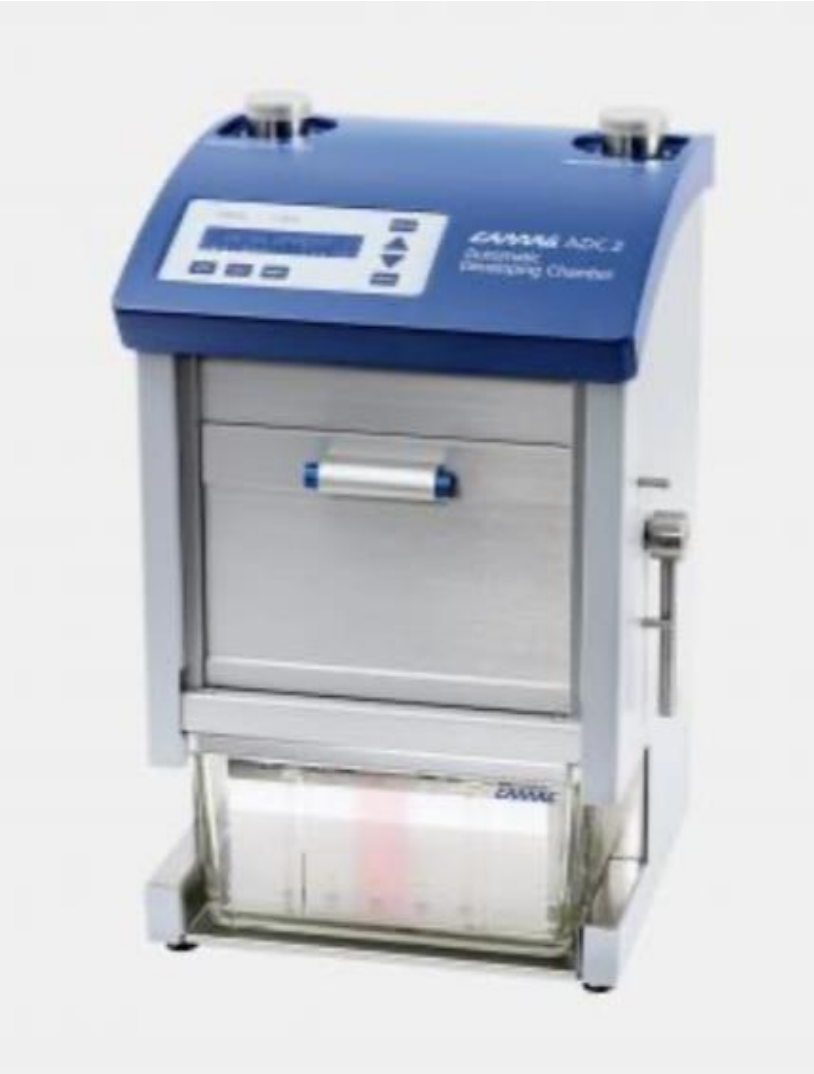
	A. TLC	B. HPTLC
Mobile phase	Ethyl acetate/methanol/propianic acid (20/10/3)	
Detection	UV 366	
Sample volume	4 µm	0.3 µm
Migration distance	10 cm	5 cm
Analysis time	42 min	13 min 45 sec
-	-	-

Automatization in HPTLC

1. Application:

The sample extracts are applied as bands onto the plate with a software-controlled applicator. Precision of the applied volume, exact positioning and compactness of application zone determine the quality of the final result.

Automatization in HPTLC (2)



2. Development:

Automatic development chambers allow to make the development step more reproducible and less dependent on human interaction. Chamber configuration, activation, developing distance, and final drying can be preset. The progress of the development step is monitored.

Automatization in HPTLC (3)

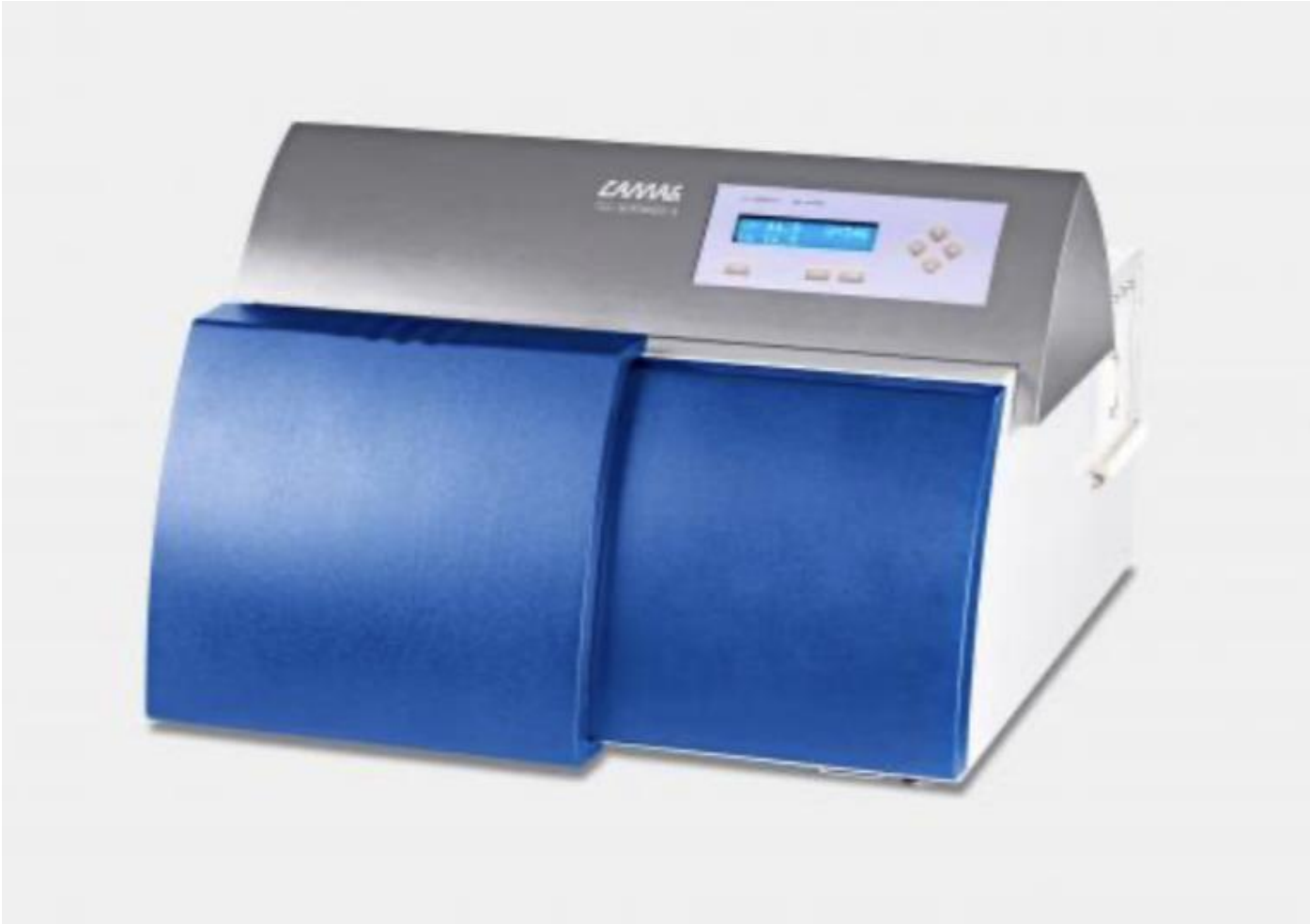
3. Derivatization:

Substances without chromophores or fluorophores can be visualized or made detectable through derivatization. The required reagents are transferred onto the chromatogram

Automatization in HPTLC (4)

4. Detection:

HPTLC chromatograms are captured images of the plates under white or ultraviolet light. For electronic image acquisition a digital camera captures visible polychromatic light. The obtained data can be edited, archived and evaluated. For quantitation densitograms or image profiles are used



Automatization in HPTLC (5)

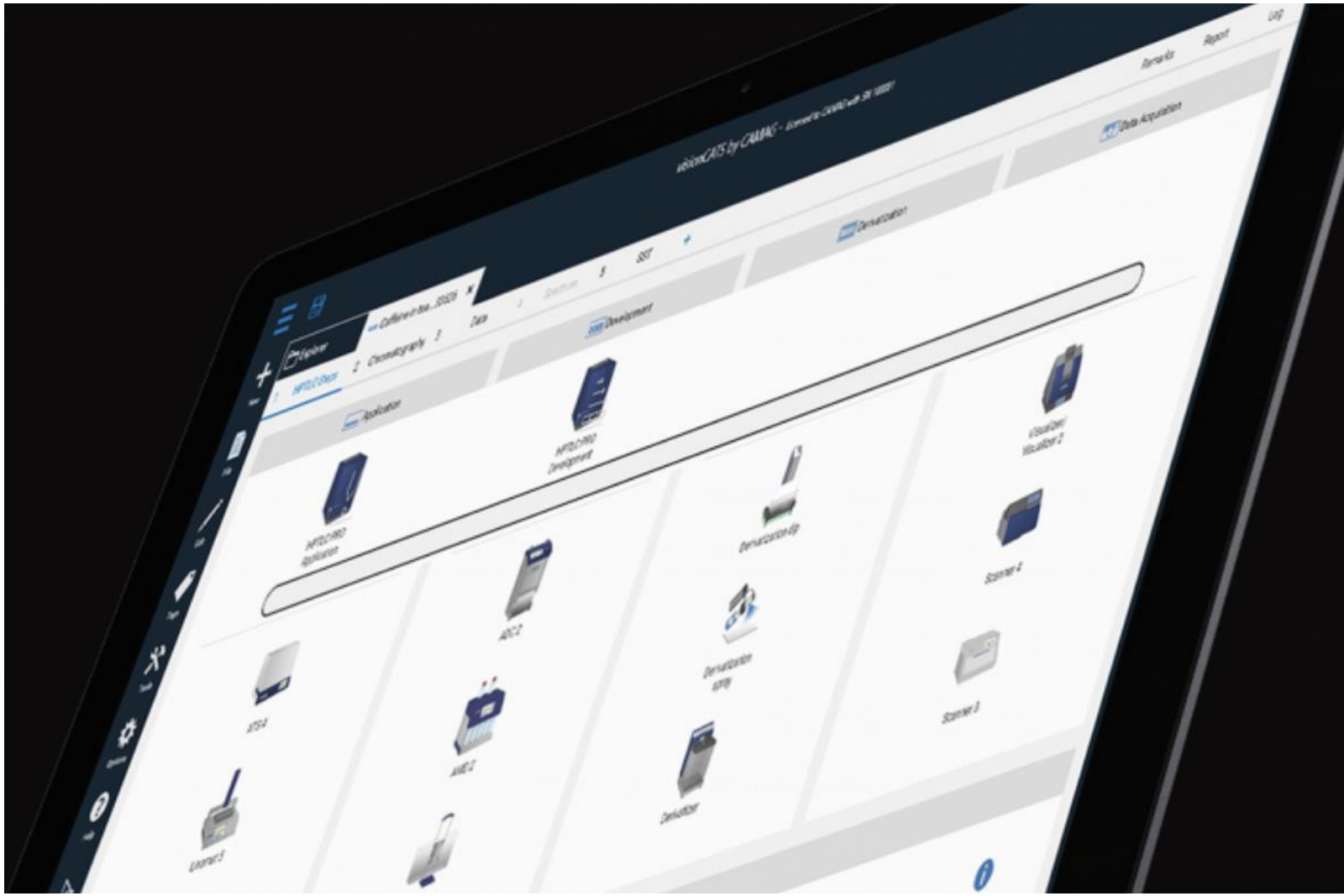
5. MS Interface:

TLC-MS coupling allows for verification of the chemical structure of analytes by Mass Spectrometry. Analytes can be directly eluted to an MS or the eluate can be collected for further analysis offline.

Automatization in HPTLC (6)

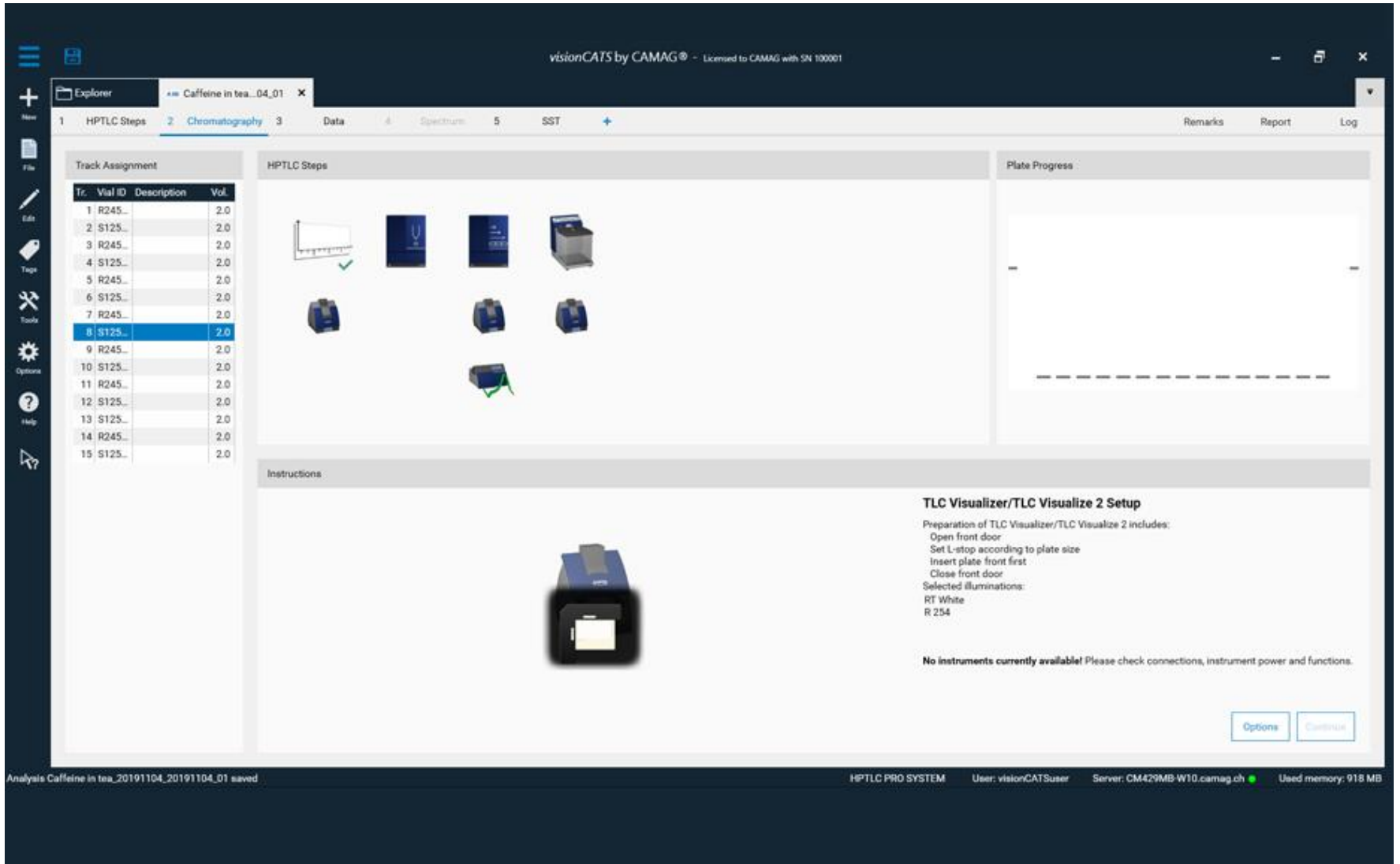
6. Software integration:

HPTLC may be integrated with a controlling sofware. For example: The HPTLC Software visionCATS organizes the workflow of the HPTLC analysis, controls the CAMAG instruments, and manages data evaluation and automated spraying.



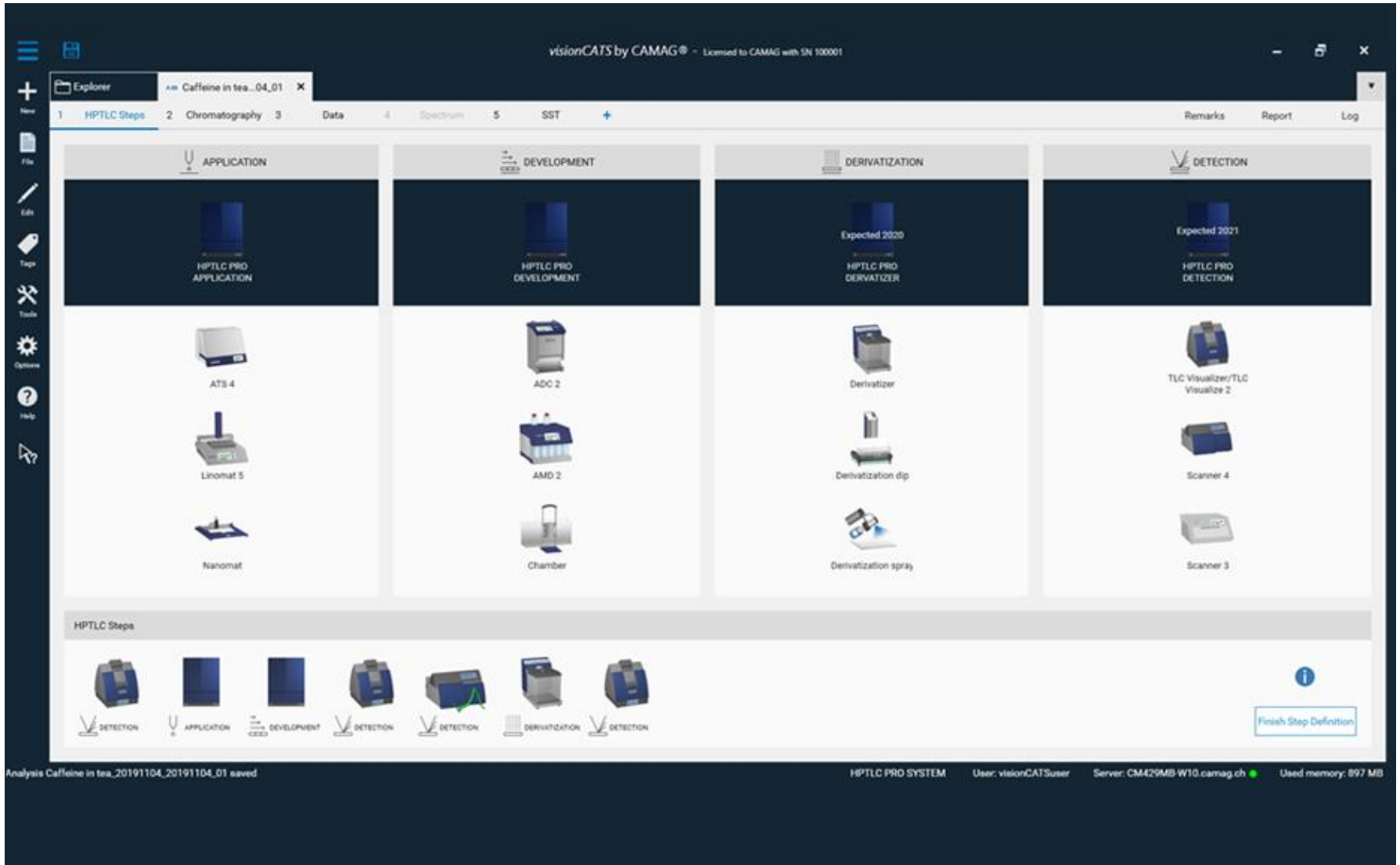
HPTLC: Software integration

2) It allows for method creation with simple and straight forward steps



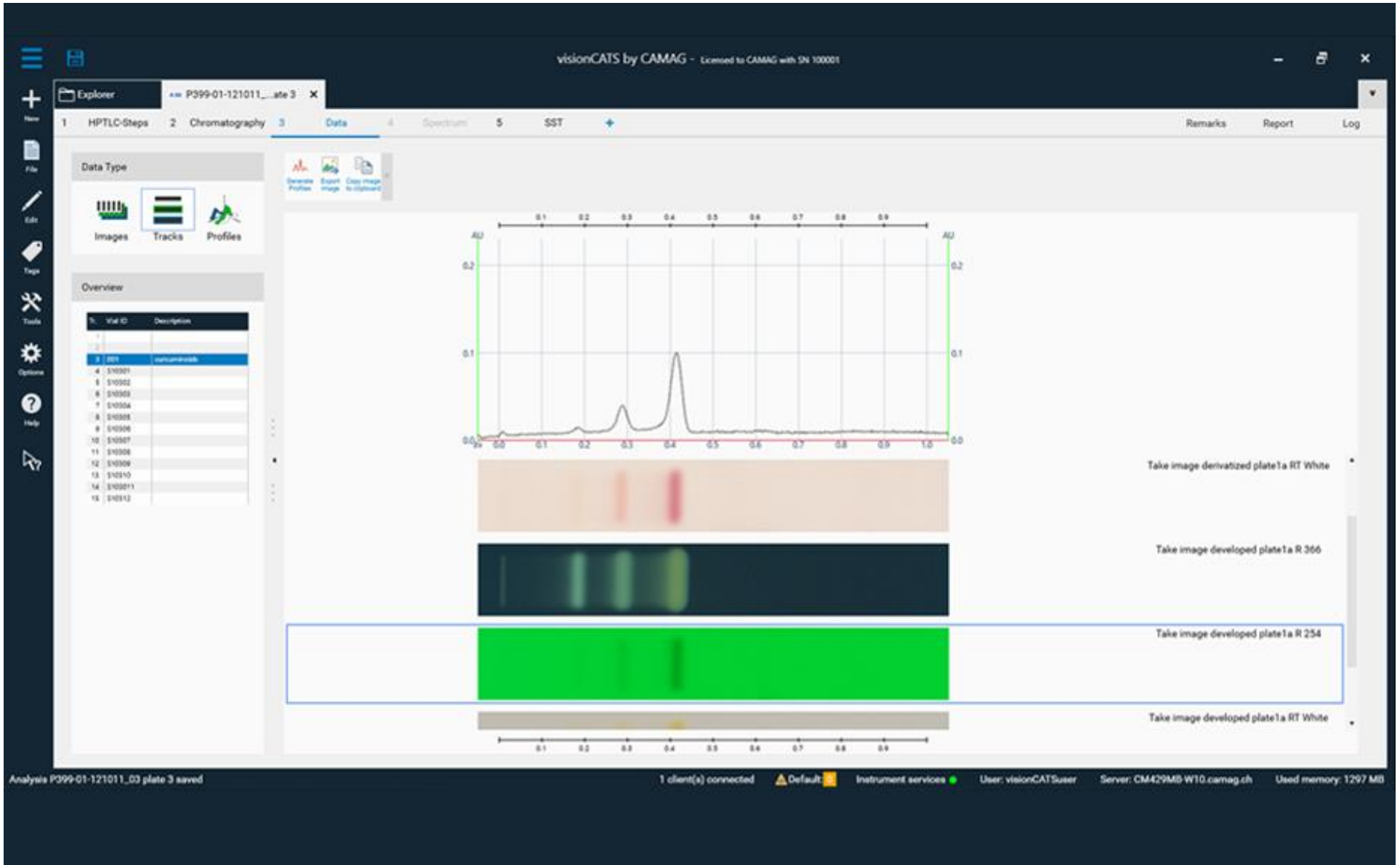
HPTLC: Software integration

1) It can control several HPTLC systems at the same time



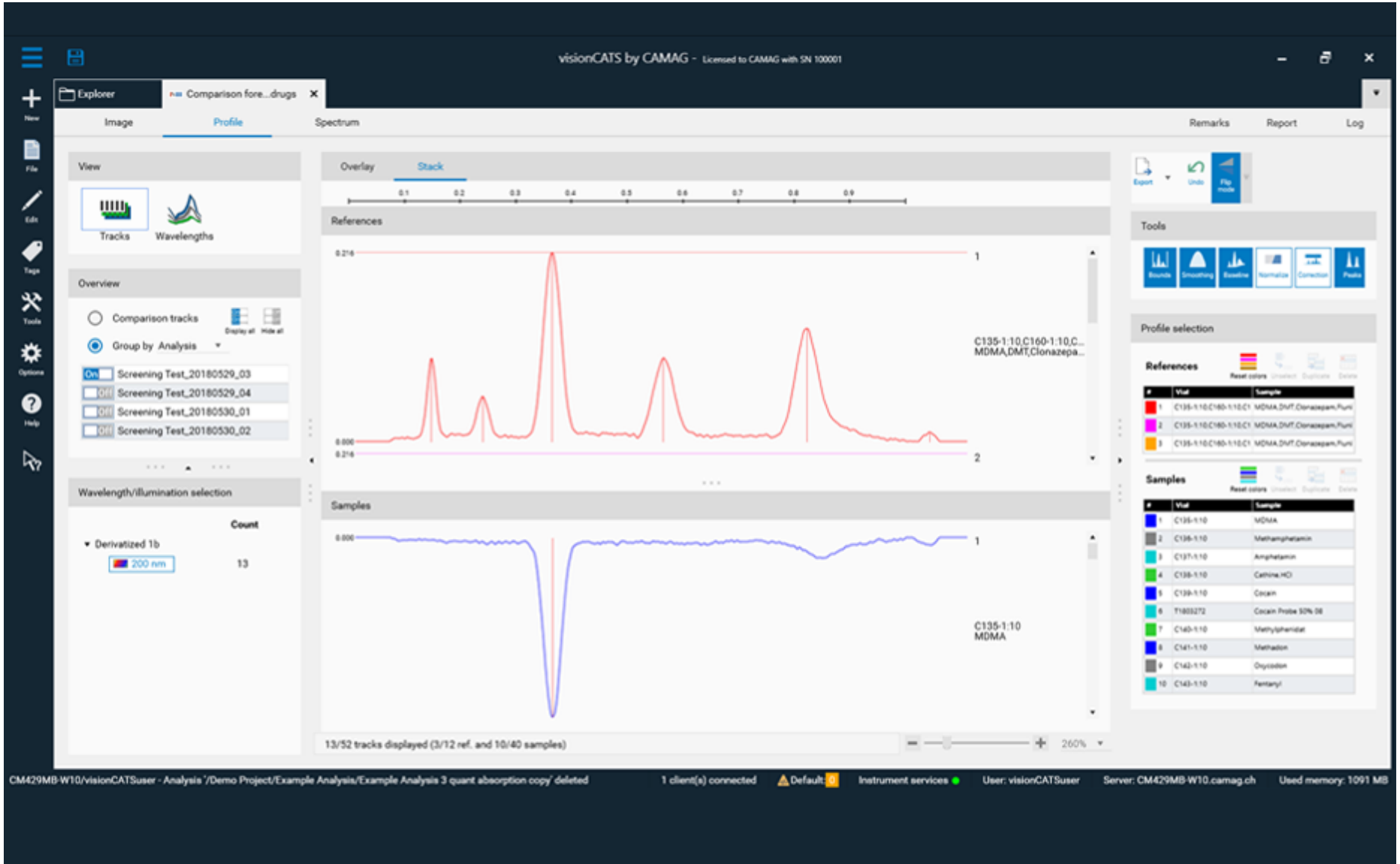
HPTLC: Software integration

3) Derivatization / visualization options made easy



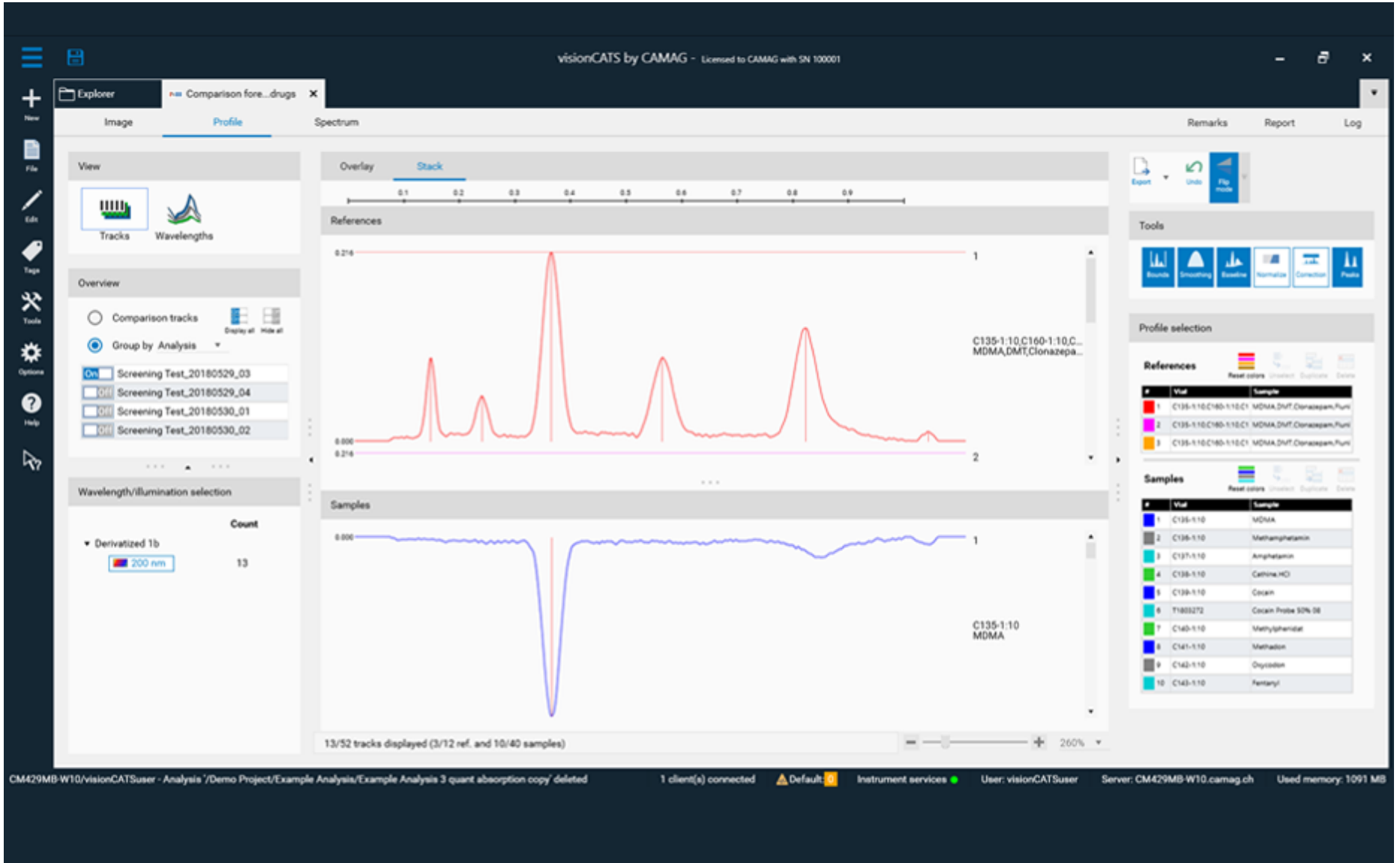
HPTLC: Software integration

4) Possibility of comparing data developed in different solvent systems



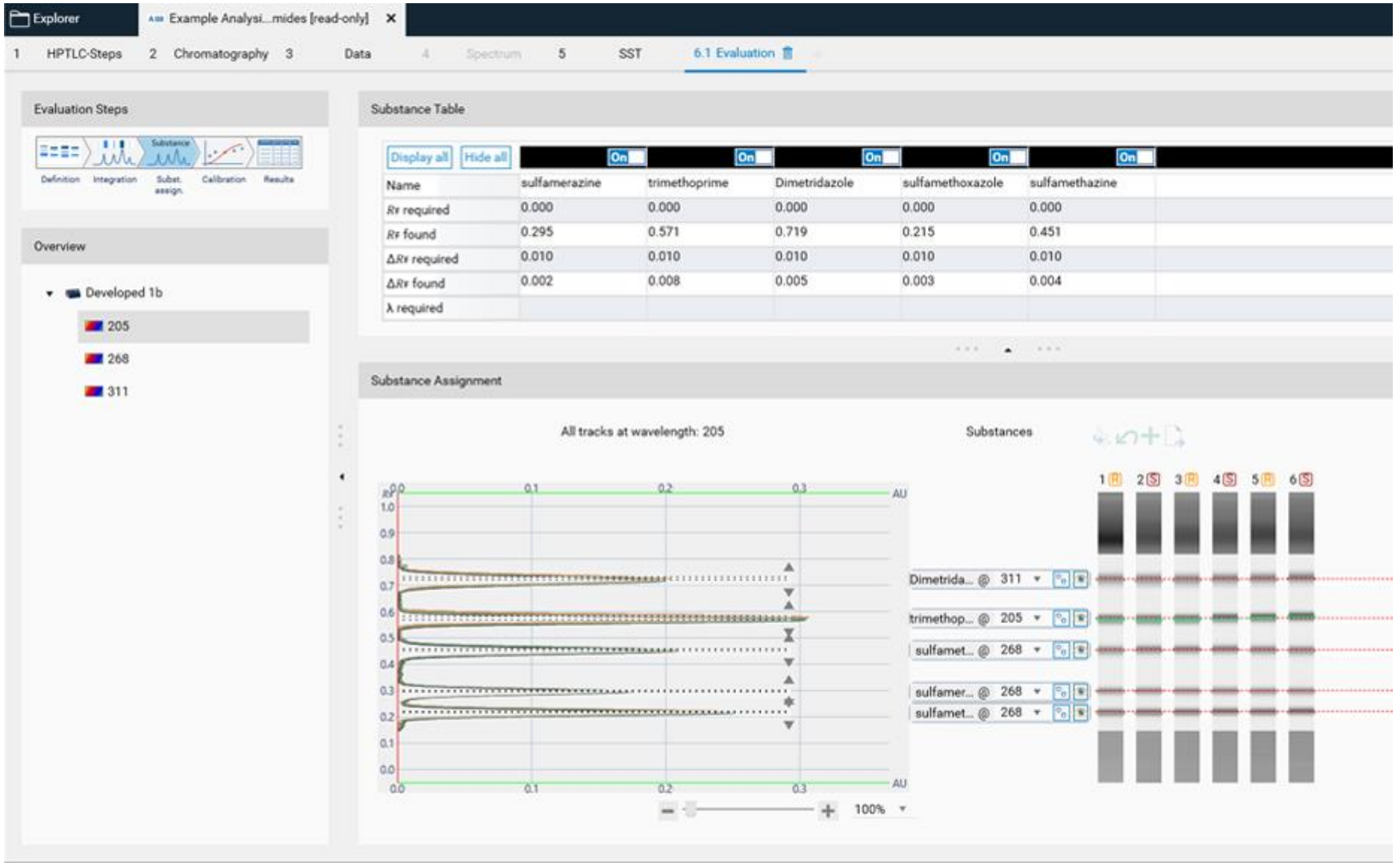
HPTLC: Software integration

5) Possibility of comparing data developed in different solvent systems



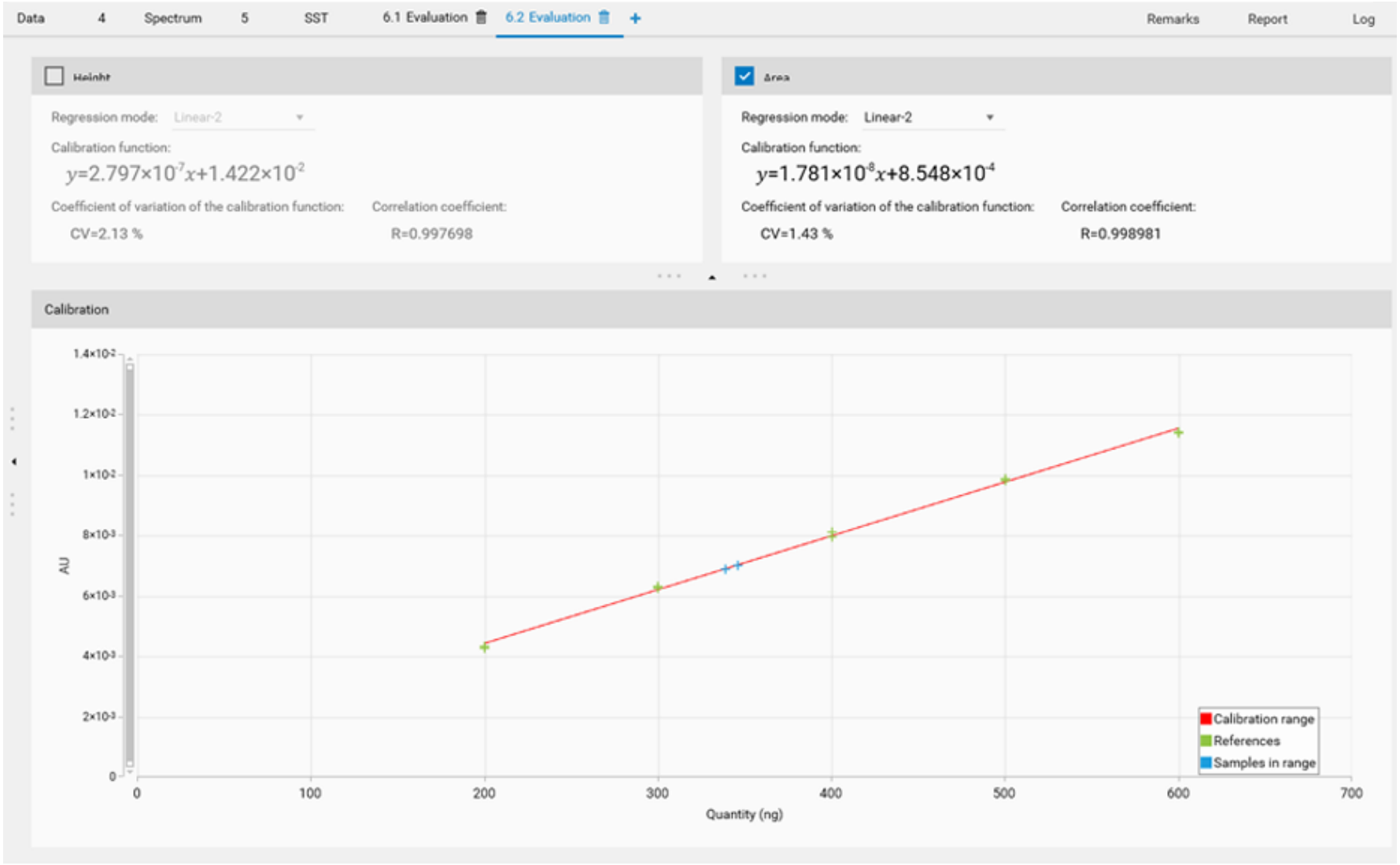
HPTLC: Software integration

6) Equivalence between Rf and RT



HPTLC: Software integration

7) Quantification made possible



HPTLC: Advantages

- Faster analysis, only 3 to 20 minutes for optimal separation
- 5 to 10 times better detection sensitivity than classical TLC
- Highly reproducible, sharp bands for quantitative analysis
- Easy coupling with bioassays, thus particularly beneficial for effect-directed analysis
- Defined zones can be absorbed by mass spectrometry (MS) after evaluation, hence no need to record every run including matrix and background