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PLANAR CHROMATOGRAPHY

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Planar Chromatography in the Year of Chemistry 2003

stimulates and makes chromatography comprehendible –

as example for the discrimination of true saffron and adulterants

Important Date:

International Symposium for High Performance Thin-Layer Chromatography, Lyon, October 15–18, 2003

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IN THIS ISSUE

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Column: Know CAMAG

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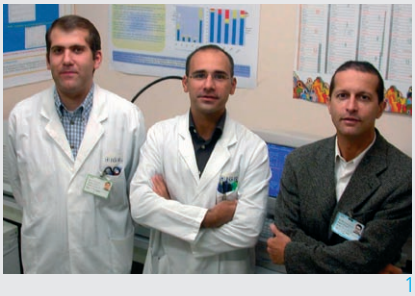
Rapid analysis of vinca alkaloids in infusion bags



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◀ *L. Mercier, Dr. A. Paci, Dr. P. Bourget (Head of the Department), from left to right*

For 3 years Dr. Paci* and his team have been working in the leading French center for cancer research and treatment, the Institute Gustave Roussy. For the Department of Clinical Pharmacy, which ensures the production of about 35.000 medical preparations each year, planar chromatography is an integral part of the pharmaceutical quality assurance program. They prefer HPTLC as a relatively simple and inexpensive assay method, which does neither require the use of radio labeled drugs, complex derivatization nor expensive equipment. HPLC and GC are considered as efficient, but time-consuming methods for the analysis of numerous samples. With several other HPTLC methods for a total of 24 cytotoxic agents already developed, the HPTLC platform has become a major quality management tool according to ISO 9001 for the production unit.

Scope

The last CBS featured an excellent paper on Vinca-alkaloids by Prof. Creche et al., but from the point of view of biotechnological process control and optimization. In this paper Dr. Paci and his team describe planar chromatography as a powerful analytical method for the assay of chemotherapeutic infusion bags containing vinca alkaloids. They determine four anti tumor vinca alkaloids, i.e. vindesine, vinblastine, vinorelbine and vincristine, used in infusion bags as antineoplastic compounds in a chemotherapeutic regimen.

The group prefers planar chromatography, because of its rapidness, cost-effectiveness and efficiency. They apply up to 60 samples on one plate. Chromatography of all samples takes only 15 min (10 min for pre-conditioning and 5 min for chromatography). All in all, including application and evaluation, the analysis of 20 samples requires 30 min, i.e. 1.5 min per sample compared with more than 10 min using a typical HPLC method. There is no need for a conditioning step as with HPLC. HPTLC analysis is by a factor of 6 more rapid and also less expensive.

Sample preparation

Batch samples are diluted in an aqueous methanolic solution (1:1).

Chromatogram layer

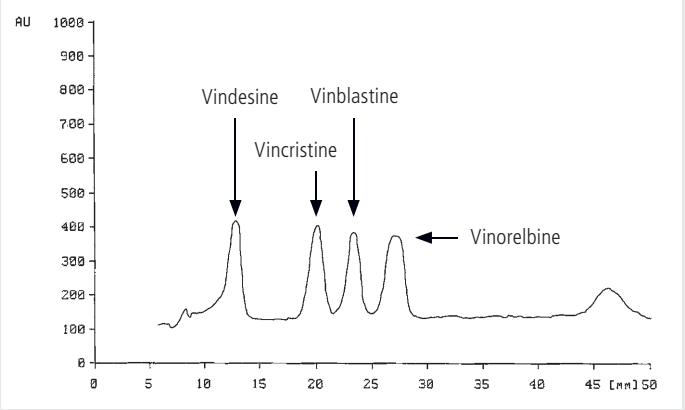
HPTLC plates silica gel 60 F_{254} (Merck), 20 x 10 cm

Sample application

Bandwise with Automatic TLC Sampler, 60 tracks (30 on each side), band length 3 mm, application volume 50–300 nL, distance from the lower edge 8 mm, distance from the side 15 mm, track distance 5,5 mm

Chromatography

In the Horizontal Developing Chamber 20 x 10 cm from both opposing sides with each 5 mL dichloromethane – methanol 93:7[1] with pre-conditioning for 10 min, developing distance ca. 50 mm, developing time 5 min



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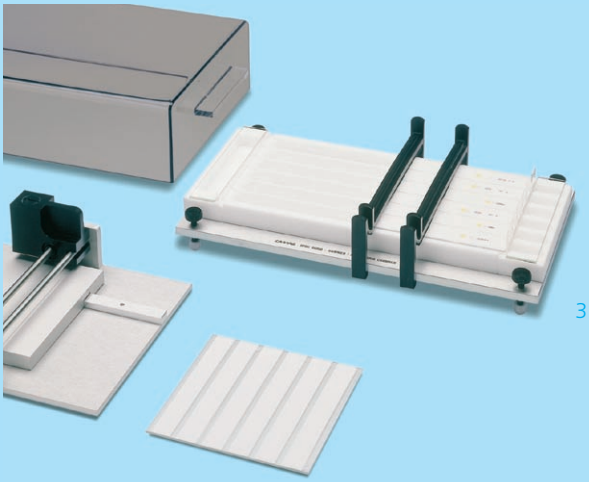
▲ *Densitogram of the four vinca-alkaloids*

[1] This mobile phase was optimized in the HPTLC Vario Chamber in which up to 6 mobile phases can be tested simultaneously.



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HPTLC Vario System

In this application the HPTLC Vario System was used by Dr. Paci and his team for optimization of the mobile phase, i.e. for efficient method development.

In the HPTLC Vario Chamber six solvents can be tested side by side on a 10 x 10 cm plate in one run, making selection during method development very convenient. Another advantage is the low consumption of solvent (1 mL each). 90% of solvent are saved compared to a flat bottom chamber.

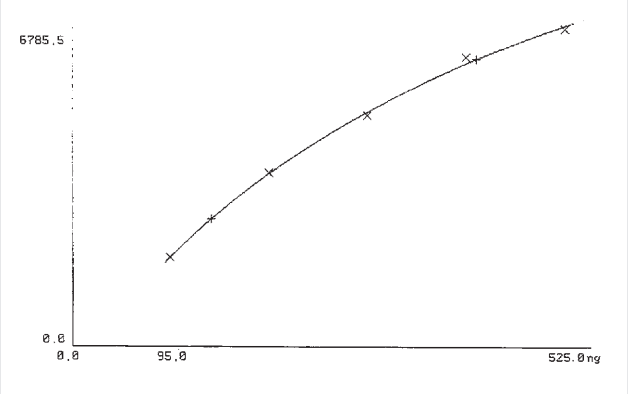
Further variations of developing conditions can be freely combined:

- Sandwich as well as tank configuration can be simulated side by side, making results directly comparable.
- Six different conditions of pre-equilibration, including relative humidity, can be tested simultaneously.

Concerning the optimization of the separation system the HPTLC Vario Chamber is unsurpassed in flexibility and from an economic point of view.

Densitometric evaluation

TLC Scanner 3 with CATS Software, absorption measurement at 274 nm, Michaelis-Menten regression of peak area



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▲ *Michaelis-Menten regression of vinorelbine*

Quality control at		Vincristine	Vinorelbine	Vindesine	Vinblastine
low concentration level	mean	73.4 (75)	152.0 (150)	150.6 (150)	150.9 (150)
	± RSD	2.58	7.70	3.56	4.36
medium concentration level	mean	181.1 (175)	251.2 (250)	251.5 (250)	245.5 (250)
	± RSD	6.72	11.05	8.68	5.88
high concentration level	mean	231.3 (225)	443.7 (450)	452.8 (450)	444.9 (450)
	± RSD	11.43	8.44	14.38	9.34

▲ *Accuracy for three different concentrations (target value) (n = 6)*

Coefficient of variation of	Quality control at	Vincristine	Vinorelbine	Vindesine	Vinblastine
Repeatability CV_r (%)	low concentration level	2.1	1.4	2.7	2.3
	medium concentration level	1.8	2.0	2.6	1.3
	high concentration level	0.7	2.2	3.5	2.7
Intermediate precision CV_i (%)	low concentration level	3.5	5.1	2.4	2.9
	medium concentration level	3.7	4.4	3.5	2.4
	high concentration level	4.9	1.9	3.4	2.1

▲ *Repeatability (CV_r) and intermediate precision (CV_i) (n = 6)*

Discussion und Results

The analytical method allows the quantitation of the four vinca alkaloids with good confidence according to the intended purpose. The method is accurate and precise. Repeatability is better than 3.5% and intermediate precision better than 5.1%. Setting the acceptance range as ± 10% of the theoretical concentration, 97,8% of the tested solutions were found to be conform. The resulting non-conformity rate of 2,2% can be accepted for routine analysis. Out of range analyses always appeared to be due to incorrect homogenization of the manufactured batch before sampling.

More information is available from A. Paci (apaci@igr.fr) or from CAMAG.

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Improved separation of benzodiazepines by AMD

▲ *Dr. R. Werner, Chr. Arndt und Dr. U. Demme (from left to right)*

Dr. Demme*, Dr. Werner and Christina Arndt work at the toxicological-chemical laboratory of the Institute of Forensics at the Hospital of the Friedrich Schiller University in Jena. For more than 20 years the laboratory deals with systematic toxicological analysis, that is the chemical-analytical search for an unknown toxin in biological material. The oldest method used in this field is thin-layer chromatography. Today the lab employs also ion trap GC-MS and HPLC, which in the last 10 years has been replaced to a large degree by HPTLC. HPTLC in combination with spectra recording is not only used for detecting unknown compounds (in this field the high resolution power of two-dimensional development is utilized), but also for quantitative determination of pharmaceuticals in therapeutic drug monitoring.

*Dr. Ulrich Demme, Dr. Rolf Werner, Christina Arndt, Hospital of the Friedrich-Schiller-University of Jena, Institute for Forensics, Fürstengraben 23, D-07743 Jena, Germany

Introduction

A group of pharmaceuticals of high toxicological relevance are the benzodiazepines, because these substances are often used in therapy and have a high potential of abuse (for instance as addition to drugs of abuse). In Germany 22 benzodiazepines are available. In the case of suspected abuse of drugs or pharmaceuticals or in cases of unspecified poisoning these substances must be tested for. Part of the investigation are also clotiazepam and two active metabolites (amino flunitrazepam and norflunitrazepam). An analytical method was needed which had to be able to separate as many of the 25 compounds as possible in one run. For this purpose 8 mobile phases (from the literature) and 13 gradients have been tested.

The combination of chromatographic data with spectra recording enables a good identification. Separation of the 25 benzodiazepines was improved by AMD. This is a result of better discrimination of the migration distances and the smaller peak width as well as an improved removal of the biological matrix. With the best separation system only 2 structurally closely related pairs out of the 25 benzodiazepines remain unresolved. Separation power is comparable to that of HPLC; if two-dimensional separation is performed it is even higher. Another advantage is the possibility of serial analyses, up to 20 analyses can be performed on one plate. The method is used as a cost efficient and rapid screening tool.

Sample preparation

For this article, sample preparation was not further investigated. Serum can be extracted with 1-chlorobutane from weakly alkaline solution (pH ~ 9, phase ratios ≥ 3:1).

Chromatogram layer

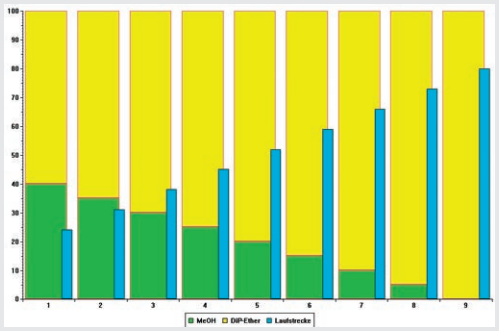
HPTLC plates LiChrospher® silica gel 60 F_{254} (Merck), 20 × 10 cm

Sample application

Bandwise, 25 tracks, band length 2 mm, application volume 1–4 µL (200 ng benzodiazepine each), distance from lower edge 10 mm, distance from the side 10 mm, track distance 7 mm

Chromatography

In AMD2 system methanol, diisopropyl ether, toluene, dichloromethane and t-butyl methyl ether were tested as solvents as well as ammonia and formic acid as polar modifier. A 9 step gradient based on methanol and diisopropyl ether showed the best separation power (migration distance 80 mm).



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▲ *AMD 2 gradient on basis of methanol and diisopropyl ether*

Densitometric evaluation

TLC Scanner with CATS software, absorption measurement at 230 and 320 nm followed by spectra recording from 200 to 330 nm

Results and discussion

The following table include the migration distances of the 25 benzodiazepines as well as the measured halve base widths (average of three experiments) and the maxima of the absorption spectra (*= main maximum).

$$DP = 1 - \frac{2 \times M}{N\,(N-1)}$$

No	Benzodiazepine	Y (mm)	$B_{1/2}$ (mm)	λ_{max} (nm)	Matches
1	Alprazolam	27,7	1,3	219	
2	Amino-FNZ	42,7	1,5	239*, >330	M1
3	Bromazepam	45,3	1,7	234*, 325	M2, M3
4	Brotizolam	30,5	1,2	245*, 310	M4
5	Chlordiazepoxid	37,5	1,2	261*, 310	
6	Clobazam	51,5	1,6	228*, 291	M5, M6, M7
7	Clonazepam	51,3	1,5	258, 315*	M5, M8, M9
8	Clotiazepam	57,8	1,4	247*, 324	M10, M11
9	Diazepam	57,1	1,5	230*, 317	M10, M12
10	Dikaliumchlorazepat	53,9	1,5	228*, 318	M13, M14, M15
11	Flunitrazepam	53,1	1,4	258, 314*	M13, M16, M17, M18
12	Flurazepam	31,9	2,8	228*, 314	M4
13	Loprazolam	15,0	1,1	>330	
14	Lorazepam	44,4	2,7	225*, 322	M1,M2, M19
15	Lormetazepam	48,1	2,1	227*, 319	M20
16	Medazepam	58,3	1,5	249	M11, M12, M21
17	Midazolam	36,1	1,3	229	
18	Nitrazepam	52,2	1,4	267, 313*	M6, M8, M16, M22
19	Nordazepam	53,8	1,7	227*, 318	M14, M17, M23
20	Nor-Flunitrazepam	52,5	1,5	257, 314*	M7, M9, M15, M18, M22, M23
21	Oxazepam	44,2	2,5	228*, 317	M3, M19
22	Prazepam	61,3	1,7	232*, 315	
23	Temazepam	49,1	2,0	229*, 317	M20
24	Tetrazepam	59,6	1,4	228*, 310	M21
25	Triazolam	25,8	1,3	218	

▲ *Migration distance Y, halve peak width $B_{1/2}$ and maxima of the UV spectra λ_{max} of 25 benzodiazepines, separated by the AMD gradient mentioned above (green: distinguishable, pink: not distinguishable)*

As a criterion of overlap and therefore mutual disturbance of the detection, aside of the migration distance also the halve peak widths were looked at. If the distance of two peak maxima is larger than the sum of the corresponding halve base widths ($\Delta Y_{1\text{-}2} > (B^1_{½} + B^2_{½})/2$ two peaks are regarded as separated. When applying this criterion 23 overlaps were observed (matches M1 to M23). That means for the separation of these 25 benzodiazepines (N = 25) the discriminating power (DP) is 0.923.

Next the substance pairs of the matches were separated with the same gradient (each match on a starting point). With the help of migration distance and UV spectra 11 of the 23 matches could be separated, that means the substances can be clearly distinguished. This way DP was increased to 0.96. With a single step isocratic development of the remaining 12 matches with dichloromethane – methanol 9:1 only two overlaps remained, i.e. nitrazepam and nor-flunitrazepam as well as lorazepam and oxazepam. With other isocratic developing solvents more matches occurred. Lorazepam and oxazepam did not separate clearly in any of the investigated solvent systems.

With the combination of two separation systems as mentioned above in a two-dimensional development the discriminating power could be increased to 0.993.

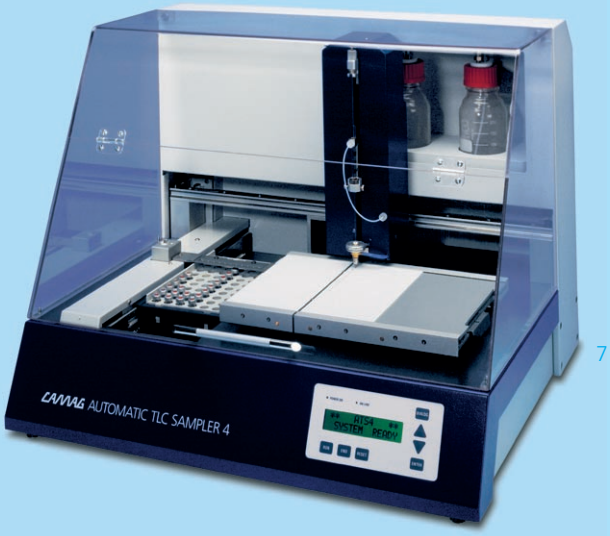
Match	Compound A	Compound B	Y_A (mm)	Y_B (mm)
M4	Brotizolam	Flurazepam	28,0	22,0
M5	Clobazam	Clonazepam	53,3	46,7
M8	Clonazepam	Nitrazepam	46,7	52,3
M9	Clonazepam	Nor-Flunitrazepam	46,7	52,9
M11	Clotiazepam	Medazepam	50,0	55,6
M13	Dikaliumchlorazepat	Flunitrazepam	46,3	56,9
M14	Dikaliumchlorazepat	Nordazepam	46,3	51,0
M16	Flunitrazepam	Nitrazepam	56,9	52,3
M17	Flunitrazepam	Nordazepam	56,9	51,0
M18	Flunitrazepam	Nor-Flunitrazepam	56,9	52,9
M19	Lorazepam	Oxazepam	39,2 ($B_{1/2}$= 2,3)	41,9 ($B_{1/2}$= 3,1)
M22	Nitrazepam	Nor-Flunitrazepam	52,3	52,9

▲ *Migration distance Y of single step isocratic development of the remaining 12 matches with dichloromethane - methanol 9:1 (green: distinguishable, pink: not distinguishable)*

The HPLC spectrum library by Pragst et al. [1] contains 23 benzodiazepines (no.10 and 20 are missing). When following relative retention times and assuming an average peak width of 0,25 min, 5 matches (DP = 0,98) would occur. The results illustrate that the use of AMD can improve the separation of benzodiazepines even in comparison to the for these substances most suitable mobile phase dichloromethane – methanol 9:1.

Further information is available from the authors (Ulrich.Demme@med.uni-jena.de) on request.

[1] F. Pragst, M. Herzler, S. Herre, B.-T. Erxleben, M. Rothe: UV Spectra of Toxic Compounds, Verlag Dr. Dieter Helm, Heppenheim, Germany, 2001.



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CAMAG Automatic TLC Sampler (ATS 4)

In several articles of this issue the ATS 4 is mentioned for fully automatic application of substances onto the plate (see pages 3, 12 and 14). The ATS 4 is easy to operate. It applies substances under a cover to protect the object from environmental factors. Samples can be applied as bands using the spray-on technique or as spots by contact transfer. Sample application in form of rectangles allows the application of large volumes.


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Now available with heated spray nozzle

Especially in the field of trace analysis the employment of ATS 4 with heated spray nozzle improves sample application because often large volumes have to be applied to reach the detection limit. Moreover, for aqueous extracts due to the low vapor pressure and the high elution power on silica gel layers, a low application rate is needed. For example: application of 50 µL of an aqueous extract requires 15 min in normal application, but heating the spray nozzle to 60° C reduces the necessary time for application to only 7 min. A nozzle temperature between 30 and 60° C can be selected.

Change of CEO

Since January 2003 CAMAG under new leadership


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▲ *Hans Reichenbach, CEO 1994 to 2002*


10

▲ *Peter Jänchen, CEO*

After 38 years of wholehearted engagement for CAMAG Mr. Reichenbach has retired from the management in July of 2003. Despite of this step he will be available as advisor. He also continues to serve on the board and will run for re-election as board member for the next three-year period in 2004.

His position as CEO was already taken over by Mr. Peter Jänchen on January 1st of 2003. He will continue the successful company policy. Also in the future CAMAG will profile planar chromatography as modern analytical method with technically and qualitatively upscale products.

Many of you already know Peter Jänchen from his 10 years of work as manager of research and development. In that function he was introduced in CBS 71. Together with his development team he gave an innovative image to instrumental planar chromatography.

We thank you all for trusting CAMAG and are grateful for your continuing support of planar chromatography as an especially creative and visual chromatographic method. It´s worth it!

Parameters of Planar Chromatography

The articles in this series are dedicated to the important steps of planar chromatography and their parameters which influence the chromatographic result. Hints for optimization are given to help the reader to use planar chromatography most efficiently.

Collecting these pages is recommended.

Handling of plates

Planar chromatography uses the off-line principle and although there is modern instrumentation for each individual step, no complete automation is available. This requires manual handling of the plate between the steps. Also the preparation of the plate for use, which is not necessarily a part of the TLC process, can involve several manual operations. The chromatographic plate is a very sensitive object and can be easily damaged or contaminated. It must always be dealt with using special care. This chapter shall provide practical tips for improving the plate performance and avoiding common problems.

1. Storage, pre-cleaning

Impurities on the plate do not only accumulate from the lab atmosphere but also from packing material such as shrink-wrapping foil. When plates are stored in the original Styrofoam box, the plate on the top of a stack should be kept face down. While for most qualitative analyses plates are typically used "out of the box" without any pre-treatment, it is important to consider a standardized cleaning procedure if the analytical method has to be validated (stability test, quantitation) and reproducible results are required. To remove impurities from the layer a plate pre-washing or pre-development is recommended. During pre-washing, the plate is immersed into a large volume of isopropanol and soaked for several minutes. During this time impurities diffuse into the washing liquid. After drying the plate in a clean bench the procedure may be repeated with fresh solvent. Much easier to perform is pre-development. The plate is developed with a strong solvent to the upper edge. In the case of silica gel plates methanol is often recommended for use, but may not be the most sufficient cleaning agent if the contaminants are rather non-polar. Figure 1 shows the effect of pre-washing on plates, which are contaminated with impurities from the packaging material. It is not a good practice to pre-develop with a portion of the actual mobile phase. In this case secondary fronts may be established, which could collect impurities and are difficult to remove during the actual chromatography.

The cleaned plate is dried (and activated) in an oven at 120° C for 20 to 30 min. In a dust and fume free environment (for example a large empty desiccator) the active plate is cooled down to room temperature and equilibrated with the relative humidity of the laboratory atmosphere.

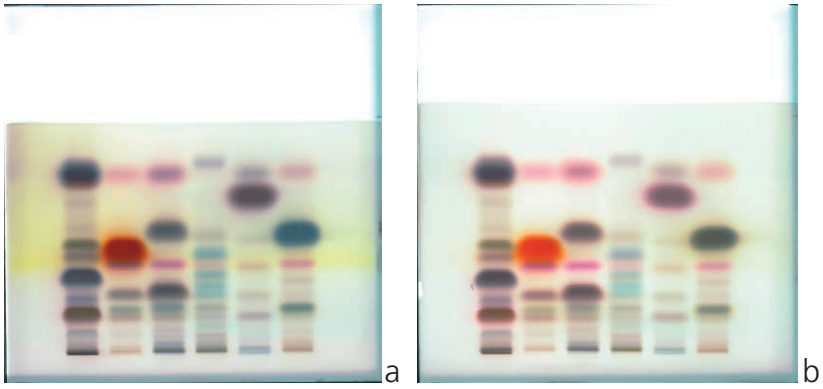

Figure 1: Effect of pre-washing on plate background (a) no pre-washing, (b) pre-washing with methanol
Mobile phase: toluene, ethyl acetate (95:5), derivatization by dipping in anisaldehyde reagent, followed by heating at 100° C for 3 min.
Left to right: chamomile, thyme, lavender, bitter orange, anise, spruce needle oils.

2. Activation

Upon storage and handling silica gel will interact with the surrounding laboratory atmosphere, thus adsorbing water vapor. This affects the activity of the plate, which is inversely proportional to the relative humidity, and the R_F value of the analyte. However, because water adsorbed on the surface of the stationary phase can also influence selectivity of a separation, this statement cannot be generalized. As seen in Figure 2 it is possible that humidity/activity changes affect different compound to different extents. While the position of β-sitosterol (red dotted line) remains constant, the zones above and below (alkylamides) have lower R_F values when humidity decreases.

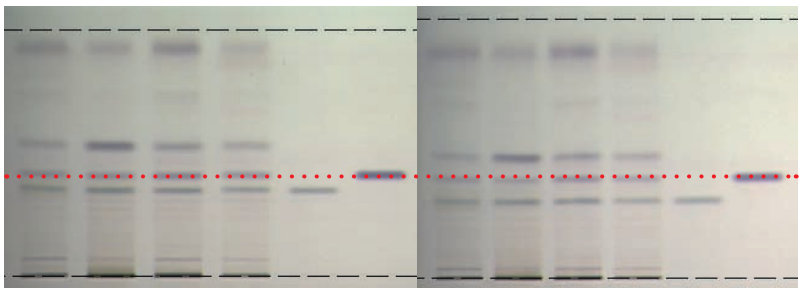

Figure 2: Effects of relative humidity on the fingerprint (alkylamides) of Echinacea purpurea. Mobile phase: toluene – ethyl acetate – cyclohexane – formic acid 24:6:3:0.9, derivatization with anisaldehyde.
Left: 45% rel. hum., right: 32% rel. hum.
Tracks 1-4: sample, track 5: dodecatetraenic acid isobutylamide, track 6: β-sitosterol

Heating a silica gel plate to 120° C in an oven can maximize activity. At that temperature adsorbed water is completely removed from the surface. High activity is not necessarily desirable, because it can cause tailing and even chemical reactions. Technically it is rather difficult to maintain a specific activity of the silica gel for chromatography. During transport and during sample application the stationary phase is again in contact with the relative humidity of the surroundings. A common approach, covering most of the plate with a counter plate during sample application is not a reproducible solution. It is more useful to equilibrate the active plate with the humidity of the surroundings by cooling it down to room temperature in a dust/fume free environment. In any case it is advisable to record the actual relative humidity and the temperature during chromatogram development. To adjust the activity of the plate conditioning prior to development over sulfuric acid or a saturated salt solution for an extended period of time is possible (Table). One disadvantage of saturated salt solution is their ability to "move" move up the wall and out of the container.

mass % H_2SO_4	% rel. humidity	Saturated salt solution	% rel. humidity
10	96	$Pb(NO_3)_2$	98
20	88	KBr	84
30	75	$NaNO_2$	66
40	56	$NaHSO_4 \cdot H_2O$	52
50	35	KF	31
60	16	HCOOK	21
70	3	$ZnCl_2 \cdot 1.5\ H_2O$	10

Table: Solutions for establishing defined relative humidity.

3. Labeling

Prior to chromatography the TLC plate should be labeled (see Inlay CBS 89 for details). Labeling is performed with a soft pencil according to Fig. 3. Touching the active layer on the plate must be avoided. The use of GLP coded plates is a convenient alternative.

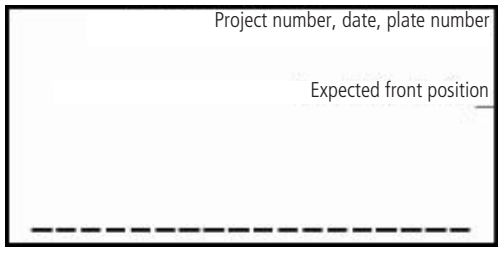

Figure 3 Labeling of an HPTLC plate

4. Impregnation

The chromatographic performance of a TLC plate can be significantly altered by impregnation. Impregnation is mostly performed by dipping the plate in a 5–10% solution of low-volatile impregnation reagent, which is dissolved in a highly volatile solvent. Drying the plate in an oven at 100–120° C for 30 min evaporates the solvent. The table on the next page gives an overview. Figure 4 illustrates the effect of impregnation in the case of separation of ginkgolides.

Formation of complexes

Impregnation with	Concentration of impregn. solution	Fields of application
EDTA	10%	Cephalosporins, tetracyclines, metal ions, phospholipids, phenols
Boric acid or borate	5%	Ascorbic acid derivatives, sugar, phosphatidylinositols, urethan derivatives, mono-/di-/triglycerides, stearic, lipids
Transition metal salts	5–20%	Amino acids, aromatic amines, sulfonamide, anilines, quinolines, phenol derivatives
Iron(III)salts	5–20%	Phenolic acids
Silver nitrate	3–20%	Interaction of Ag^+ with π-elektrons of double/triple bounds. Fatty acids, diglyceride/triglyceride, phospholipids, glycolipids, steroids

Formation of charge transfer complexes

Caffeine	4%	Polycyclic aromatic hydrocarbons (PAH)

Ion-pairing

Quaternary ammonium salts	0.05M	Sulfa drugs, penicillins

Adjustment of pH value

Inorganic acids	0.1–0.5N	Phenols, acids, aromatic amines
Potassium/sodium hydroxide	0.1–0.5N	Alkaloids, amines, basic compounds
Buffer salts		Curcumin derivatives, sugars, heavy metals, phloroglucinols

Change the analyte's solubility in the "liquid" stationary phase (partition chromatography), modification of partition coefficient

Formamide		Local anesthetics, alkaloids, digitalis glycoside, nitrophenols
Ammonium sulfate		Lipids, phospholipids
Sodium nitrite		Phenols
Sodium bisulfite-citrate-buffer		Sugars
Sodium sulfate	0.1M	Sugars
Sodium acetate	4–10%	Terpene lactones
Lithium/sodium/potassium salts		Metal ions, aromatic amines
Ammonium thiocyanate		Metal ions
Butylamine		Metal ions

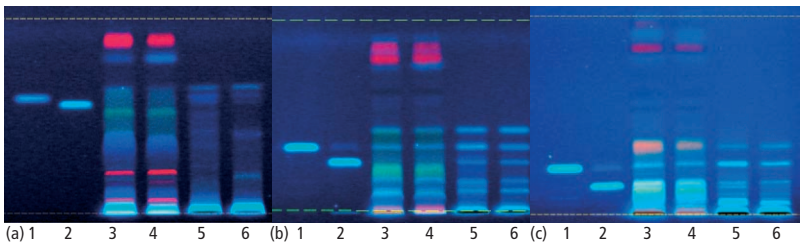

Figure 4: Effects of plate impregnation on the separation of terpene lactones from Ginkgo biloba (a) no impregnation, (b) impregnation in a 4% solution of sodium acetate for 2 s, (c) impregnation in a 10% solution of sodium acetate for 20 s. Mobile phase: toluene – ethyl acetate – acetone – methanol 20:10:10:1.2, derivatization with acetic anhydride. Track 1: ginkgolide A, track 2: ginkgolide B, track 3, 4: ginkgo leaves, track 5, 6: extracts.

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Identification of true Saffron


11

▲ *Participants of a HPTLC workshop at the Institute for Didactic of Chemistry at the University Erlangen-Nuremberg: Ms. Elke Hahn-Deinstrop (left), team leader Dr. Kathrin Sommer (right), 3 participants*

The "Year of Chemistry" should bring the abstract and often suspicious term **"Chemistry"** closer to the broad public, inform citizens and spark their interest. Ms. Hahn-Deinstrop*, who you may already know as an author (Thin Layer Chromatography, Practice and Avoidance of Mistakes, ISBN 3-527-29839-8), works for Heumann Pharma GmbH at Feucht. She performed the following experiment with an advanced chemistry class of a highschool and at the Institute for Didactic of Chemistry, University of Erlangen-Nuremberg, with young teachers, thus very effectively demonstrating to students and becoming teachers the principle of chromatography.

Here planar chromatography shows its strongest side – it is a very visual tool to make chromatography comprehendible.

The method was developed together with Dr. Angelika Koch, Frohme-Apotheke Hamburg. It can be easily repeated because saffron is readily available in stores and pharmacies.

Introduction

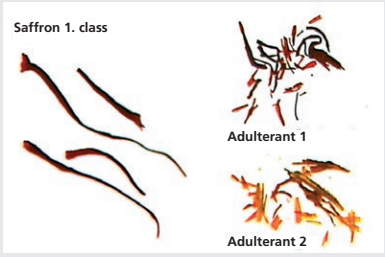
In a vacation hotel at the Red Sea the guests received a thank you note as far-well gift in their room together with an attached small bag containing about 10 g of "Saffron" and a recipe for a chicken dish. This generosity was suspicious, because saffron is the most expensive spice in the world. For example, in March of this year 100 mg of whole or ground saffron cost 1.95 Euro. The amount of spice given for free would have been equivalent to about 195 Euro! Therefore, a rapid and cost efficient method for identification of powdered saffron was needed. The German Arzneimittelkodex (DAC) 1999, the Homeopathic Pharmacopoeia (HAB) 2000 and also the European Pharmacopoeia (Ph.Eur.) 4 include monographs for saffron. Planar chromatography is listed as an identification test.

The TLC method of Ph.Eur. monograph "Crocus for homeopathic preparations" was adapted for use with HPTLC plates. The developing time was reduced by 50% and 75% of the volume of developing solvent were saved. For the school experiment even a plate format of 5 × 5 cm, 1 mL developing solvent and a run time of only 8 min were sufficient for 5 samples.

Saffron consists of the dried stigmas of *Crocus sativus L.* (Iridaceae). It has a characteristic smell similar to Iodoform. The principal constituents are water soluble pigments (crocin), bitter principles (picocrocin) and essential oil components (safranal, which is formed by thermal decomposition of picrocrocin). Several samples from Egypt, Germany, Spain, Iran and Denmark have been included in the investigation. As reference, saffron 1. class obtained from a pharmacy was used as well as the so-called "Wild Saffron" of Safflower (*Flores Carthami*).


12

▲ *Blooming saffron, Crocus sativus L. (Iridaceae)*



13

▲ *Saffron filaments 1. class and two adulterants*

Sample preparation

100 mg drug are grounded and wetted with 0.2 mL water. After 3 min 5 mL methanol are added. The mixture is left in the dark for 20 min and then filtered through glass wool. The clear filtrate is used for chromatography.

As reference solution according EuAB (only for description of R_F value of main component) 5 mg naphthol yellow S are dissolved in 5 mL methanol and 5 mg sudan red G in 5 mL dichloromethane. Both solution are mixed 1:1.

Chromatogram layer

Instead of the prescribed TLC plate silica gel 60 F_{254} several other HPTLC plates were evaluated (Silica gel 60 F_{254}, LiChrospher® silica gel 60 F_{254}, RP-18 F_{254} and DIOL F_{254}). The HPTLC plate silica gel 60 F_{254} worked best. The plates were pre-washed with 2-propanol and activated at 100° C for 15 min.

Sample application

Sample solution 2 µl and standard solution 1 µL, 15 tracks, band length 8 mm, distance from lower edge 8 mm, distance from the side 10 mm, distance between tracks 12 mm

Chromatography

In the CAMAG Horizontal Developing Chamber 20 x 10 cm with ethyl acetate – 2-propanol – water 13:5:2 with chamber saturation, developing distance 6.5 cm, developing time 24 min. Alternatively a CAMAG Twin Trough Chamber can be employed.

Postchromatographic derivatisation

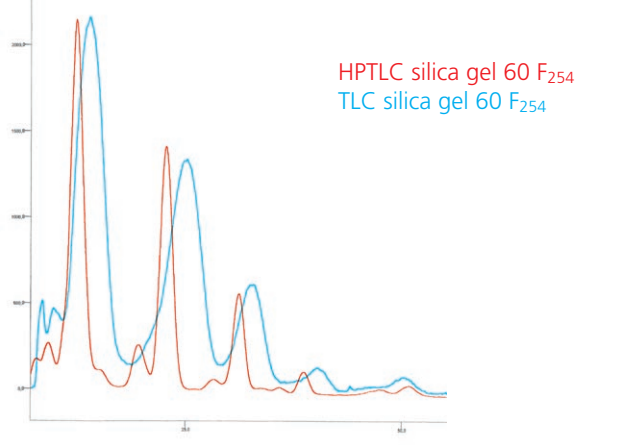
Spraying with anisaldehyde-sulfuric acid reagent, followed by heating for 5–10 min at 110° C.

Visual evaluation

In white light (transmission prior to, reflectance after derivatization) and under UV 254 nm

Results and discussion

To assess the different separation power of the utilized pre-coated plates always one track per plate was scanned at 430 nm and the tracks of different plates were compared by overlay. The separation of zones is most efficient on HPTLC plates silica gel 60 F_{254}.



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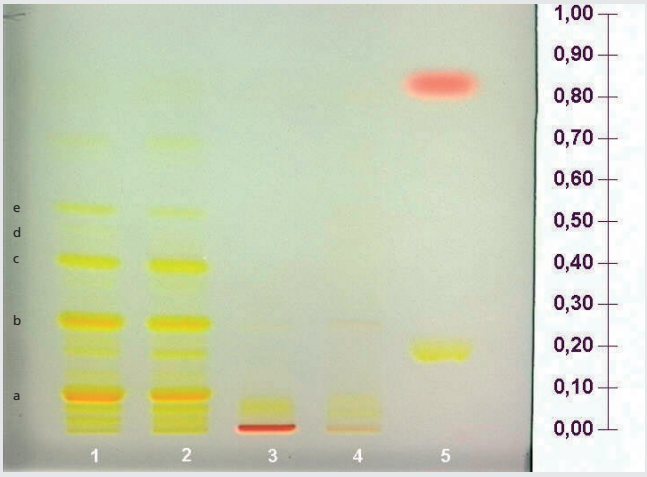
▲ *Comparison of separation power of different silica gel 60 plates*

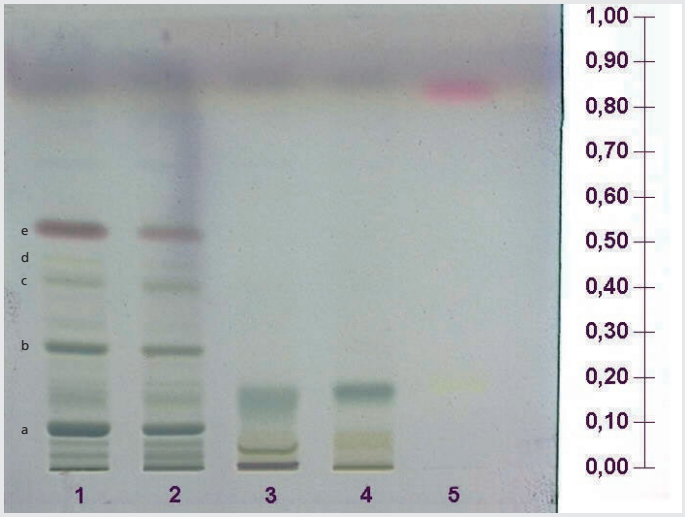
In white light (transmission) prior to derivatization up to 8 yellow zones are visible, of which 3 have a significantly higher concentration (zones a-c).



15

▲ *Image in white light prior derivatization (transmission); track 1: saffron 1. class (reference), track 2: saffron purchased in Germany, track 3: saffron special, Bazar Hurghada, track 4: Safflower (wild saffron), track 5: reference solutions (naphthol yellow S and sudan red G)*

According to the literature [1] the lower strongly yellow zone (crocin, hR_F-value 7–9) should have the highest intensity. Under UV 254 nm an additional fluorescence quenching zone is seen at hR_F 48–50 (zone e). After derivatization with anisaldehyde-sulfuric acid reagent, zones a-d turn blue-gray and zone e changes rather quickly from Bordeaux to red-gray.



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▲ *Image in white light after derivatization (reflectance); track assignment see image before*

Based on the reference chromatograms the authenticy of the Egyptian saffron samples could be disproved. It seems that the "hotel samples" are safflower. Maybe in the common name "wild saffron" for the drug the "wild" was omitted. The commercial samples from Germany, Denmark, and Spain comply with the reference drug.

Currently saffron bulbs have been planted. Following the harvest in autumn it shall be checked on the fresh samples, whether the additional yellow zones (between application position and crocin) found for all samples are degradation products of the trade samples.

Further details can be obtained from the authors on request: (elke.hahn_deinstrop@arcor.de, Koch@FrohmeApotheke.de).

* Elke Hahn-Deinstrop, Kleingeschaidter Str. 23, D-90542 Eckental and Dr. Angelika Koch, Frohme-Apotheke, Frohme Str. 14, D-22457 Hamburg, Germany

[1] Hagers Handbuch der Pharmazeutischen Praxis, 5. Auflage, Folgeband 2, Drogen A–K, S. 442, Springer-Verlag Berlin-Heidelberg, 1998


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Horizontal Developing Chamber

The Horizontal Developing Chamber (HDC) is not only used in this application, but also for the rapid analysis of vinca alkaloids (see pages 2–4). For the alkaloid separation the HDC is very time saving. It allows development of a plate from both opposite sides toward the middle, thus doubling the number of samples per plate compared to conventional developing techniques. 60 tracks are simultaneously developed in 5 min!

Only 5 mL solvent per side are required for chromatography on 20 × 10 cm plates. Thus, compared to other chamber types the HDC consumes less solvents, e.g. 75% less compared to a flat bottom chamber.

Easy handling is combined with a high degree of flexibility for conditioning in the tank configuration or for chromatography in the sandwich configuration. The HDC is unsurpassed from an economic point of view, in flexibility and reproducibility of the result in routine work, especially for mass analysis. Test it!

Stability tests of Vitex agnus castus (Chaste Tree) extracts

18 ◀ *Franziska Wahli*

In cooperation with the University of Basel and under supervision of Prof. Beat Meier and Prof. Willy Schaffner Ms. Franziska Wahli has worked on her diploma thesis in Pharmaceutical Biology at the CAMAG Laboratory.

In the analysis of medicinal plants planar chromatography (TLC/HPTLC) is primarily used for identity tests, but the resulting fingerprints can also provide information about the quality of the drug. In the following it is shown that HPTLC fingerprints are suitable as well to detect changes in extracts, which have been induced during stress tests. The method is sufficiently selective to identify such changes.

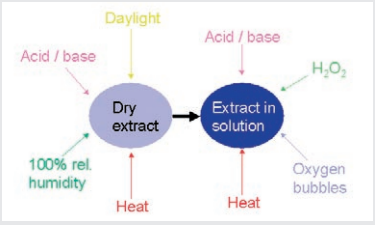
Planar chromatography allows reliable evaluation of stability of plant extracts. The results aid decisions about shelve life and storage conditions for the respective product.

Sample preparation

1 g Chaste Tree extract is dissolved in 10 mL methanol, sonicated for 5 min and filtered. Standards are dissolved in methanol (0.1 mg/mL).

Stress tests

The extract was exposed in dry and dissolved form to various stress conditions.



19 ◀ *Applied stress conditions*

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 × 10 cm

Sample application

Bandwise with Automatic TLC Sampler 4, 12 tracks, band length 8 mm, application volume 2 µL, distance from lower edge of plate 8 mm, distance from left edge 20 mm, distance between tracks automatic

Chromatography

In Horizontal Developing Chamber, separation distance 52 mm (60 mm from lower edge)

- Flavonoides with 5 mL tetrahydrofuran – toluene – formic acid – water 16:8:2:1
- Diterpenes with 5 mL toluene – ethyl acetate 9:2
- Iridoides with 5 mL ethyl acetate – methanol – water 77:15:8, chamber saturation

Derivatization

With Chromatogram Immersion Device

- Flavonoides: dipping of warm plate into Natural products reagent (0.5% in ethyl acetate) followed by polyethylene glycol 400 solution (5% in dichloromethane).
- Diterpenes: dipping into 10% methanolic sulfuric acid, heating to 105° C.
- Iridoides: dipping into 4-dimethylaminobenzaldehyde-reagent (1% in 1N methanolic HCl).

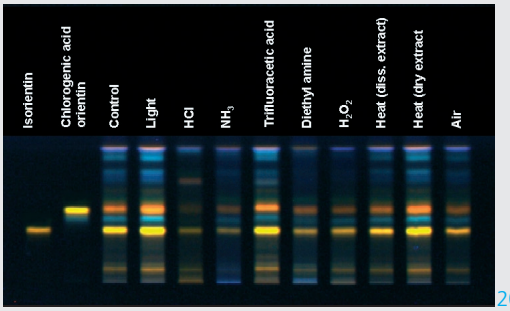
Documentation and densitometric evaluation

With Video Documentation System (VideoStore) under UV 366 nm (flavonoides), white light (lipophilic components) and white light with sharp cut filter 560 nm (iridoidea). Evaluation with Video Densitometry (VideoScan).

Results and discussion

Drastic stress conditions have been chosen to make sure that changes in the extract would occur. The stability of the different investigated substance classes strongly varies. The stress tests yielded information about storage conditions and shelve life. Diterpenes are less stable, flavonoides and iridoides rather stable. The diterpene rotundifuran, which is contributing to the activity of the extract, is very light sensitive and degrades within a few days. The substance is also more sensitive to acids and hydrogen peroxide. Concentrated hydrochloric acid and hydrogen peroxide change all constituents of the investigated groups. Only the lipophilic flavonoid casticin is stable in all stress tests.

Please contact the CAMAG Laboratory for further information (labnet@camag.com)



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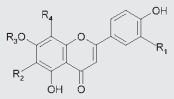
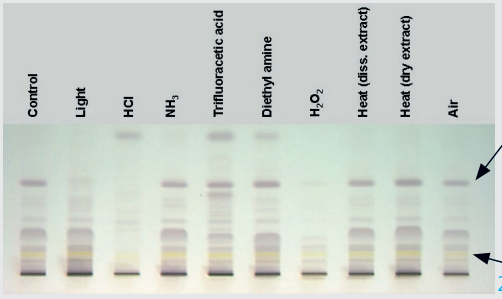
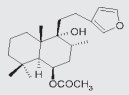


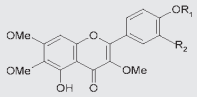
Isoorientin:
R_1= OH
R_2 = Glucose
$R_{3,4}$ = H
Orientin:
R_1 = OH
$R_{2,3}$ = H
R_4 = Glucose

▲ *Results of stress tests on flavonoides (UV 366 nm)*



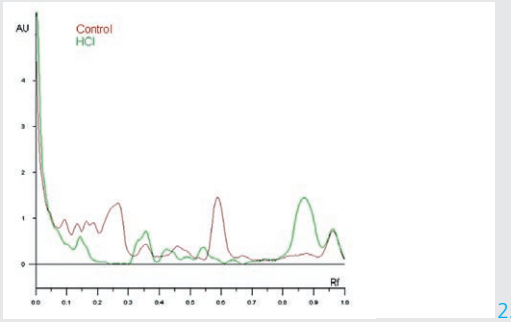


Rotundifuran



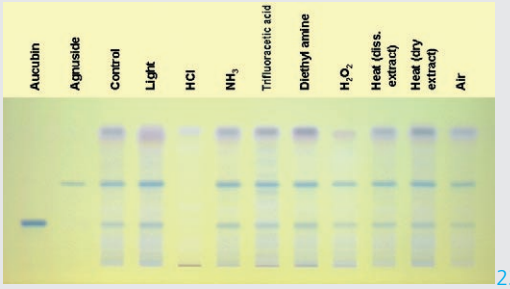
Casticin:
$R_1 = CH_3$, R_2 = OH

21

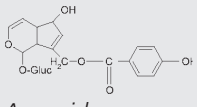


22

▲ *Results of stress tests on diterpenens: chromatogram under white light and densitogram of superimposed tracks 1 (control) and 3 (exposure to HCl)*



Aucubin



23 *Agnuside*

▲ *Results of stress tests on iridoides under white light with cut-off filter 560 nm*

Analysis of flavone glycosides from Hawthorn and Passion flower

◀ *Valeria Widmer*

24

Ms. Valeria Widmer has worked on her Diploma thesis in Pharmaceutical Biology at the CAMAG Laboratory in cooperation with the University of Basel. The work was supervised by Prof. Beat Meier and Prof. Willy Schaffner.

25

▲ *Hawthorn*

26

▲ *Passion flower*

Extracts of Hawthorn (*Crataegus monogyna Jacq.* and *Crataegus laevigata Poiret*) and Passion flower (*Passiflora incarnata L.*) are constituents of many Herbal Medicinal Products. Ensuring of quality of herbal drug, extract, and commercial product are a major prerequisite. TLC fingerprint analysis is stipulated by the European Pharmacopoeia for identification of both drugs. However, the description of the chromatograms is not sufficient to discriminate the two drugs with certainty.

The principal part of the work was devoted to the development and optimization of separation systems based on HPTLC layers, which yielded reproducible results. Aside of clear identification also the quality of the drug can be assessed. All flavonoides have a similar structure in common, but are different in the number and type of substituents and glycosidic bonds. In the green parts of Passion flower only flavonoids with a C-glycosidic bond are present (flavone-C-glycosides), whereas in Hawthorn besides flavone-C-glycosides also O-glycosides have been identified.

The following two independent and reproducible methods should be preferred over the singular method for identification as stipulated by the European Pharmacopoeia. The detection of chrysine, a controversial constituent of Passiflora is possible by HPTLC.

Sample preparation

Various amounts of drugs are extracted at 65° C or in the ultrasonic bath at room temperature with methanol (about 1 g/10 mL). Standards are dissolved in methanol (about 0.1–0.6 mg/mL).

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck) 10 x 10 cm und 20 x 10 cm (for Hawthorn and Passions flower)

HPTLC plates silica gel RP-18 W, 10 x 10 cm (for chrysine)

Sample application

Bandwise with Automatic TLC Sampler 4, band length 8 mm, 8 (10 x 10 cm plate) or 15 tracks (20 x 10 cm plate), application volume 2 µL, distance from lower edge of plate 8 mm, distance from left edge 20 mm, distances between tracks automatic

Chromatography

In saturated Twin Trough Chamber, developing distance 52 mm (60 mm from lower edge)

- Hawthorn extract (silica gel 60 F_{254}) with ethyl acetate – methanol – water – formic acid 50:2:3:6
- Passion flower extract (silica gel 60 F_{254}) with tetrahydrofuran – toluene – formic acid – water 16:8:2:1
- Chrysine (RP-18 W) with tetrahydrofuran – methanol – water – formic acid 4:6:14:1

Derivatization

The warmed plate is dipped into Natural products reagent (0.5% in ethyl acetate) with the Chromatogram Immersion Device. Following drying the fluorescence on the plate is stabilized by dipping into a polyethylene glycol 400 solution (5% in dichloromethane).

Documentation

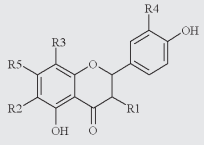
With Video Documentation System (VideoStore) under UV 366 nm

Results and discussion

Compared to the methods of the European Pharmacopoeia, the optimized or new methods provide better separation and significantly higher reproducibility from plate to plate with respect to sequence, intensity and color of the zones. By spiking experiments it was proven that chrysine is clearly detectable with the proposed method on HPTLC silica gel RP-18 W plates. However, none of the investigated drugs contained chrysine. The high flexibility of planar chromatography with respect of possible combinations of stationary and mobile phases particularly benefited the results of this work.

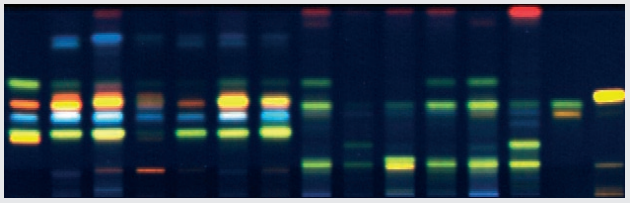
	Compound	R_1	R_2	R_3	R_4	R_5
O-Glycoside	Hyperoside	O-gal	H	H	OH	OH
	Rutin	O-rut	H	H	OH	OH
C-Glycoside	Vitexin	H	H	glc	H	OH
	Isovitexin	H	glc	H	H	OH
	Vitexin-2″-O-rhamnoside	H	H	glc-rha	H	OH
	Isovitexin-2″-O-glucoside	H	soph	H	H	OH
	Orientin	H	H	glc	OH	OH
	Isoorientin	H	glc	H	OH	OH
	Isoorientin-2″-O-glucoside	H	soph	H	OH	OH
Aglycons	Chrysine	H	H	H	H	OH

glc = β-D-glucosyl
rut = rutinose (α-rhamnosyl-1–6-β-D-glucosyl)
gal = β-D-galactosyl
soph = 2-(β-D-Glucopyranosyl)-D-glucopyranose
rha = α-L-rhamnosyl

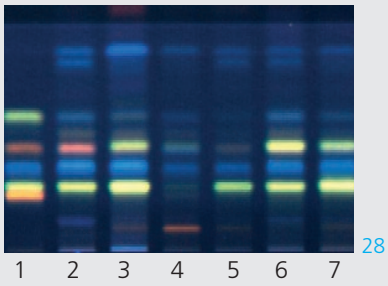


▲ *Structures of flavonoides in Hawthorn and Passion flower*

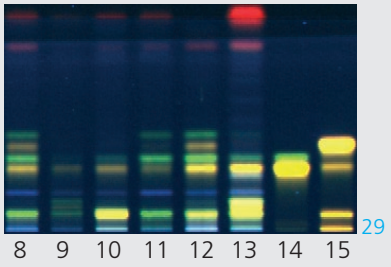
Please contact the CAMAG Laboratory for further information (labnet@camag.com)



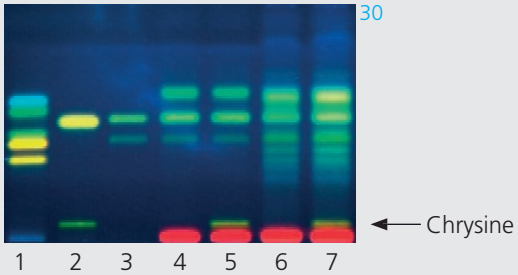
▲ *Method of Ph. Eur. 3 for Hawthorn and Passion flower; mobile phase: ethyl acetate – ethyl methyl ketone – formic acid – water 5:3:1:1*
Track 1: Rutin, vitexinrhamnoside, chlorogenic acid, hyperoside and vitexin (bottom to top); Tracks 2–7: Drugs C. monogyna, C. monogyna, C. laevigata, C. laevigata, C. herba, C. dry extract; Tracks 8–13: Drugs P. incarnata, P. incarnata, P. incarnata, P. incarnata, P. dry extract, P. caerulea; Track 14: Isoorientin and Isovitexin; Track 15: Isoorientin glucoside, Isoorientin and Orientin



▲ *Optimized method for Hawthorn*
Track 1–7 see image above



▲ *Optimized method for Passions flower*
Track 8–15 see image above



▲ *Detection of chrysine (spiked) in Passion flower*
Track 1: Rutin, Hyperoside, vitexin rhamnoside, vitexin, chlorogenic acid; track 2: chrysine, orientin; Track 3: Isovitexin, isoorientin; Track 4: P. caerulea; Track 5: P. caerulea spiked with chrysine; Track 6: P. caerulea; Track 7: P. caerulea spiked with standard chrysine

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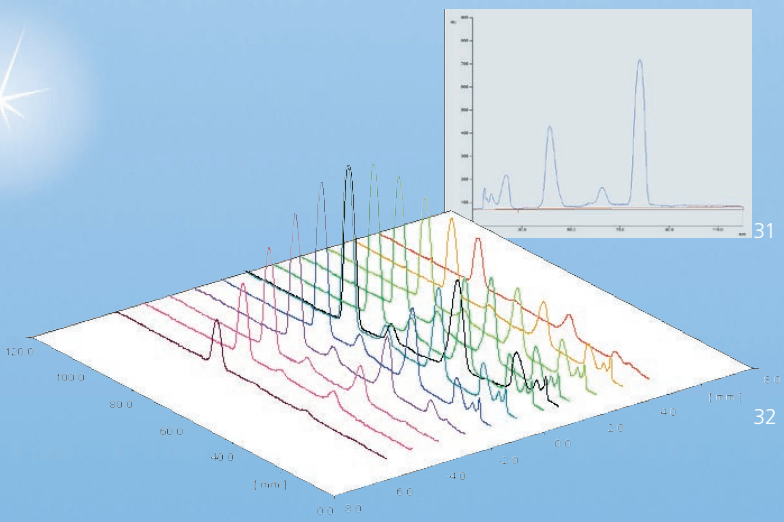
With respect to instrument control, collection of data, evaluation and documentation almost nothing remains to be desired.



▲ *Analog curves of track optimization, 3D view*

winCATS – adapted to customer's needs

The development team is continuously implementing into winCATS features, which are requested by our customers. For example now the AMD 2 and the video camera Hitachi HV-C20 can be controlled. Quantitative evaluation is complemented by Michaelis-Menten regression and other calculation routines. We intensively work on the option 21 CFR Part 11 "compliance ready". It will be available in 2003.

▲ *Video camera: live image and parameters view*

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