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Detection, Identification, and Documentation

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I. INTRODUCTION

For detection and identification of chromatogram zones, in situ techniques are generally employed. As in an analytical disk (1), the information stored in the chromatogram can be used for various detection and identification methods, even successively, because the processes of chromatographic development and detection or identification are independent in both time and space. Detection in HPTLC takes place in the absence of the mobile phase and therefore offers much greater choices than any other chromatographic technique. This means that

1. Multiple subsequent detection of the same chromatogram is possible. In addition to recording, e.g., an absorbance or fluorescence scan using visible or ultraviolet (UV) light, a Fourier transform infrared (FTIR) or Raman spectrum can be recorded, and these methods can be followed by a suitable microchemical reaction or mass spectrometry (MS) to provide additional information.
2. Detection can be repeated with different parameters, e.g., portions of the chromatogram can be selectively evaluated.
3. Postchromatographic derivatization can easily be performed on the plate. A great variety of selective or specific reagents can be used to ease detection and identification.

Absorbance or fluorescence spectrometry and microchemical detection are commonly employed in TLC (Fig. 1). Bioactivity-based reactions, i.e., microbiological and biochemical detection methods, have gained interest for toxicologically relevant substances. In situ FTIR spectroscopy has become a practical method for detection and identification, and Raman spectroscopy has gained importance with the introduction of lasers as the light source. Furthermore, the combination of TLC with in situ MS (see Chap. 9) can be employed. Detection and identification of radioactively labeled substances by autoradiographic, fluorographic, spark chamber, or scanning techniques are discussed in a special chapter of this Handbook (see Chap. 12). The combination of TLC with flame ionization detection (Chap. 13) is used only for special purposes (2).

With all of these methods, accurate documentation is necessary to provide reliable and reproducible results. Factors of influence must be documented in detail. Nowadays, protocols and image documentation of the plate involve the use of computers, but drawing, sketching, tracing, photocopying, or photographing can also be used to obtain images of the plate.

II. DETECTION

For detection, physical, microchemical, microbiological, and biochemical methods are available in HPTLC. Physical detection methods mainly include either absorbance or fluorescence mea-

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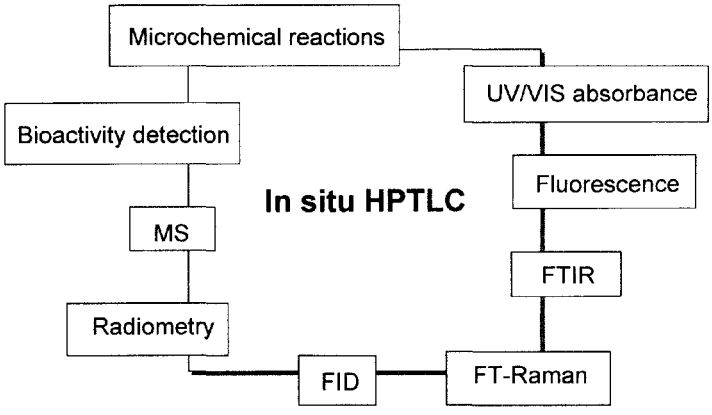

Figure 1 In situ detection and identification methods.

surement. Other physical methods are based on the difference in solubility, iodine vaporization, the addition of pH indicators, or the detection of radioactively labeled substances. Microchemical detection methods can be carried out either before chromatography (prechromatographic derivatization) or afterward (postchromatographic derivatization). Therefore, a variety of universal reagents or group characterizing reagents are available (3). Microbiological or biochemical detection methods take account of the biological and/or physiological activity of the separated components independently of their physical or chemical properties.

Detection is performed on a dry plate. After development, the plate is dried with either air or nitrogen gas, e.g., by means of a plate heater, an oven, or a hair drier, to remove the residual mobile phase. The zones are now ready for various detection methods or detection sequences, if more than one detection method is successively used.

A. Physical Detection Methods

Physical detection methods are nearly nondestructive. They mainly include the photometric measurement of either absorbance or fluorescence, i.e., the emission of electromagnetic radiation. Suitable detectors may be the eye (visual detection) or more sensitive sensors, such as a photomultiplier for TLC scanners or a charge-coupled device for image processing systems (photometric detection). Other physical methods are based on the difference of solubility, iodine vaporization, the addition of pH indicators, or the detection of radioactively labeled substances.

1. Visual Detection

Colored substances may be viewed in daylight. Owing to the fact that such compounds absorb a particular portion of polychromatic light in the visible range, the remaining reflected radiation can be detected by the eye as the visible color of the substance zone.

Colorless substances that can be excited to produce fluorescence or phosphorescence by mostly longwave (366 nm) UV radiation can be irradiated under a UV lamp. The emitted longer wavelength visible radiation (above 400 nm) can be viewed as red, yellow, orange, green, blue, or violet zones against the dark layer background.

Colorless, nonluminescent substances that show self-absorption in the shortwave UV region can be visualized under a UV lamp (254 nm) by using HPTLC plates with a fluorescent indicator. On layers containing a fluorescent indicator, the emission is reduced in regions where UV-active substances absorb the UV light with which they are irradiated. Such substances appear as dark zones on a fluorescent background. This effect is wrongly referred to as fluorescence quenching; it should be described as *phosphorescence inhibition* because the decay of emission of radiation lasts longer than 10^{-8} s after exciting radiation is cut off. As fluorescence indicators (correctly, phosphorescence indicators), inorganic substances are mainly used, for instance, acid-resistant

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alkaline earth metal tungstates (4) that emit blue light, e.g., for reversed phases, or manganese-activated zinc silicates (5) that emit yellow-green light, e.g., for silica gel plates.

Various UV lamps are commercially available. The plates are best viewed in a darkened room or corner. UV lamps can be equipped with a stand that shields off extraneous light on three sides (Fig. 2). Objects up to 2 mm thick can be pushed through under the back screen. For inspection without a dark room, UV viewing cabinets (Fig. 3) are recommended. UV lamps incorporate longwave (366 nm) or shortwave (254 nm) UV light, or both. Usually the supply voltage is converted to a high-frequency current (25–30 kHz) on which the tubes operate. This ensures instantaneous illumination at the selected wavelength as well as the absence of the flickering that is observed with 50/60 Hz systems.

2. Photometric Detection by TLC Scanners

Photodetectors are more sensitive sensors than the human eye. Generally, photomultipliers are employed; these have replaced photocells in TLC scanners. Photomultipliers depend on the external photoeffect and are evacuated photocells that incorporate an amplifier. The photocurrent is

Figure 2 CAMAG dual wavelength (254/366 nm) UV lamp with stand. (Photograph courtesy of CAMAG.)

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Figure 3 CAMAG UV cabinet for inspection without a dark room. (Photograph courtesy of CAMAG.)

amplified by a factor of 10^6-10^8 by using secondary electrodes (dynodes). Various types of photomultipliers, e.g., "side on" or "head on," can be employed. Photodetectors that depend on the internal photoeffect, such as photoelements and photodiodes, are also used in TLC. Photodiodes are used, e.g., for gel electrophoresis as an additional detector for transmission measurements. Photoelements such as the charge-coupled device (CCD element) are used for detection with video technology.

A diffraction grating is usually employed as the monochromator. Grating monochromators have an approximately linear wavelength scale, which can easily be automated, a constant and non-wavelength-dependent dispersion, and a higher light transmission (above 270 nm) than prism monochromators.

As light sources, continuous and spectral line sources are installed. In the UV region, hydrogen, deuterium, or high-pressure xenon lamps, and in the visible range incandescent tungsten lamps or halogen lamps are employed as continuous sources to record absorbance scans or spectra. Fluorescent substances are commonly excited with a mercury vapor lamp, a spectral line source that radiates more powerful major bands than a xenon lamp. Furthermore, lasers are being discussed for use. However, they should be tunable to afford a wider choice of usable wavelengths.

Densitometric measurements of planar chromatograms are made by reflectance, in either absorbance or fluorescence modes. Transmission measurement was used at the very beginning of densitometry in planar chromatography, and today it is still used for the evaluation of gel electrophoresis.

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a. Transmission. Densitometry of TLC plates started with the measurement of transmission in the 1960s analogously to the photometry of solutions using the Lambert–Beer law. However, an important prerequisite of that law is that the measured TLC zone does not scatter the measured light. This is not fulfilled, because the sorbent layer scatters the light to a great extent, and that is why there is often not a linear relationship between extinction and amount of substance per zone. Moreover, the adsorbent and its support (glass plate) absorb UV light, which means that transmission measurement beyond 325 nm is not possible. There are still more reasons, such as the bad signal-to-noise ratio, why transmission measurement is not reasonable in TLC. Nowadays, transmission measurement is used only for gel electrophoresis. A photodiode mounted below the object is used as detector, and a higher step resolution, e.g., 25 μm, is necessary.

b. Reflectance. The diffuse reflection of the measured light on the sorbent layer is used for reflectance measurements. Therefore, the measuring photomultiplier is aligned at an angle of 30° to the normal. The physical facts of diffuse reflection, i.e., what happens in the sorbent layer concerning reflection, refraction, and diffraction, are best described by the Kubelka–Munk equation. Reflectance can be measured in either absorption or fluorescence modes.

Absorbance measurement. Substances that absorb light in the UV or visible range can be measured by absorption. Generally, they are determined at the maximal absorption wavelength. The principle of direct absorbance measurement functions as follows. The reflected light energy is detected by the photomultiplier, i.e., photons strike the photomultiplier cathode and are intensified by the dynodes. As the chromatogram is scanned, the voltage differences produced at the detector are plotted as a function of position of measurement to yield an absorption scan. If the plate background is scanned, the full light intensity is reflected and generates the 100% signal because there are no substances that absorb the light. Thus the signal has to be inverted to get the familiar representation of the baseline. If a chromatographic zone is scanned, it absorbs a proportion of the light irradiating it and emits a lower light intensity than the plate background. This negative peak has to be inverted to generate the usual analytical peak.

The same principle is used by indirect absorbance measurement, i.e., substances that absorb between 250 and 300 nm are detected on HPTLC plates with fluorescence indicator, also called UV indicator. HPTLC plates with fluorescence indicator enable visual evaluation and ease of positioning the plate in the TLC scanner. Because fluorescence indicators absorb in the same wavelength range (between 250 and 300 nm), the stimulation of luminescence is diminished by the substance because it absorbs energy in the excitation range of the indicator. When a deuterium lamp is used, the radiation energy is low. Consequently, unlike the total radiation, the fluorescence radiation makes up only a small signal. Thus, results of indirect absorbance measurement between 250 and 300 nm on HPTLC plates with fluorescence indicator are changed by only a small extent when the deuterium lamp is used.

A further principle of indirect absorbance measurement is fluorescence quenching (correctly, phosphorescence inhibition). When the deuterium lamp is replaced with a mercury vapor lamp, the radiation intensity is much greater. The short-wavelength emission line of 254 nm excites the fluorescence indicator much more intensely. Before the detector, a cutoff filter is inserted in the light beam to absorb the excitation wavelength of 254 nm. The plate background, i.e., the fluorescence of the indicator, is set to 100% emission. Substances that absorb in the wavelength range around 254 nm reduce the emission of the fluorescence indicator and generate negative peaks that have to be inverted. By scanning the chromatogram, the inverted voltage differences produced at the detector are illustrated as a function of measurement position, thus producing the fluorescence quenching scan. Indirect absorbance measurement is generally not as sensitive as direct measurement. The detection limits of absorbance measurement are 0.01–0.2 μg of substance per chromatogram zone in the most favorable cases.

Fluorescence measurement. In fluorescence measurement, substances are irradiated at a definite wavelength to generate the fluorescent light that is measured. For irradiation, a high-pressure mercury vapor lamp is used. It provides wavelengths of high energy that are listed in Table 1. Fluorescent substances emit the absorbed light energy instantaneously as radiation, usually of a longer wavelength than the incident light. To measure just that fluorescent light of longer wavelength, a special filter is positioned before the detector to block out the excitation wavelength.

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Table 1 Emission Lines and Their Relative Intensities for the High-Pressure Mercury Vapor Lamp (St 48)

Wavelength (nm)	Relative intensity
238; 240	3
248	8
254	55
265	25
270	5
275	4
280	10
289	7
297	18
302	3
313	69
334	7
366	100
405; 408	43
436	81
546	108
577; 580	66

Either a cutoff or monochromatic filter can be used. A cutoff filter blocks out the light beyond a definite wavelength, e.g., a K 400 filter blocks wavelengths beyond 400 nm. A monochromatic filter passes the light at a definite wavelength, e.g., an M 460 passes light at wavelengths of 460 nm.

Fluorescence measurement functions as follows: In a scan of the substance-free background, no signal is measured because the excitation wavelength is blocked out by the filter. If a fluorescent zone is scanned, it emits light of longer wavelength that passes through the filter and thus generates a signal at the detector, i.e., a peak. The most luminescent substance is set to 100% emission.

Fluorescence measurements have the following advantages compared to fluorescence quenching or absorption measurements:

Increased selectivity. An increased selectivity is caused by two factors: (a) matrix that is not fluorescent is not measured and (b) both excitation and emission wavelengths can be selected. The ideal combination of these wavelengths eases detection and quantitation. For example, with a 600 nm filter, an orange fluorescent substance can be detected and easily quantified even when a blue fluorescent substance is overlapping that zone. In this case, bad resolution is compensated for by good detection selectivity.

Increased sensitivity. Compared to absorption measurements, fluorescence measurements are more sensitive by a factor of 10–1000. Normally, substances on the plate can be detected in the picogram and lower nanogram range.

Signal is independent from zone shape. The distribution of the substance within the zone has no influence on the signal if the scanning slit passes over the whole zone. Thus, for fluorescence measurements a slit length longer than the zone diameter or length is selected.

Increased linearity. Between fluorescence intensity and substance concentration there exists a linear relationship over a wide concentration range due to the applicability of the Lambert–Beer law (Chap. 10).

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For these reasons, for inherently fluorescent substances, fluorescence measurement should be preferred, and for nonfluorescent substances, derivatization reactions to render them fluorescent should always be considered for optimal detection and quantification.

3. Photometric Detection by Video Technology

Photodetectors depending on the internal photoeffect, such as the charge-coupled device (CCD element), are used for detection by video technology and are incorporated in video cameras or digital cameras. As a video camera, a 3-CCD color camera, a 1-CCD color camera, or a 1-CCD monochrome (black-and-white) camera can be employed. All cameras are equipped with the long-time integration feature because the standard exposure time of 20 ms is often not sufficient to detect weakly fluorescent substances. Images recorded with a video camera are digitized, creating a color or gray scale image of the chromatogram. This is performed by a video documentation system (Fig. 4), which is composed of

A high resolution, highly sensitive CCD camera, capable of long-time integration

Special hardware, normally called a frame grabber, that digitizes the analog video signal (supplied by the CCD camera) into the digital PC memory

A lighting module with direct UV light of 254 or 366 nm wavelength, direct or transmitted white light, or all combined, and a cabinet cover to shield off extraneous light, with a camera stand

Figure 4 CAMAG Reprostar 3 with cabinet cover, camera bellows, camera support, and 3-CCD camera with zoom objective. (Photograph courtesy of CAMAG.)

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A computer with monitor, printer (with good color and graphics capabilities), and special software for chromatogram documentation

Using professional software for chromatogram evaluation, tracks are marked on the chromatogram image and converted to analog curves by considering the average gray scale level of the pixels in each line of the selected track. Integration of the analog curves and their quantitative evaluation are easy to handle and very fast because all tracks are simultaneously integrated and evaluated in response to a mouse click. As an additional new feature, tracks of different plates can be compared with each other by superimposing the analog curves. This kind of profile comparison of several tracks on different chromatograms is used, e.g., for pattern recognition in drug analysis.

Strong points of chromatogram evaluation with video technology are speed of evaluation, low cost (only additional software for evaluation is needed), and time-independent evaluation, i.e., electronically saved chromatograms can be evaluated at any time. However, only the visible part of the spectrum is used (similarly to the human eye). Thus, only visible substances, fluorescent substances excited at a wavelength of 254 or 366 nm (offered by the lighting module), or substances that absorb around 254 nm on fluorescent plates (fluorescence quenching) can be detected. Therefore, a spectral range comparable to that offered by TLC scanners is not available, and spectral selectivity and recording of spectra are not possible. Sensitivity, accuracy, and precision may become comparable to those of TLC scanners, but only in certain cases, e.g., when the absorbance of the substance is at or near the excitation maximum of the fluorescence indicator (254 nm). In general, sensitivity, accuracy, and precision are not quite as good as those achieved by densitometry with TLC scanners, but in most cases they are sufficient for the analytical task. That is why, especially with its rapidity and ease of handling, video technology is used for detection and evaluation of the chromatograms. However, the strong points of densitometry with TLC scanners are spectral selectivity, use of the entire UV range down to 190 nm, recording of spectra, and high accuracy and precision.

4. Other Physical Detection Methods

Another physical method is based on lipophilicity. Lipophilic substances on hydrophilic adsorbents such as silica gel or aluminum oxide can be viewed and marked by spraying or dipping the plate in water (using special plates that can be wetted with water). Transparent layers result that show the lipophilic substances as dry white zones that can be recognized best by holding the TLC plate against the light. With this kind of solubility detection, compounds such as herbicides, hydrocarbons, sapogenins, phosphoinositides, and triterpene derivatives can be detected.

In the same way, aqueous dye solutions such as methylene blue or patent fast blue are employed instead of water. Lipophilic substances such as anion-active detergents appear pale on a transparent blue background. This phenomenon is the reverse on reversed phases, in which case the lipophilic part of the detergent is aligned with the RP chains whereas the hydrophilic part is colored by the dye, and therefore deeply colored blue zones appear on a pale background. Using lipophilic dye solutions for the detection of lipophilic substances on a hydrophilic phase yields dark zones on a pale background.

The fact that substance zones dipped in or sprayed with fluorescent solutions lead to increased fluorescence can be exploited as well. For example, spraying lipophilic substances with a dichlorofluorescein solution produces yellow-green fluorescent zones on a purple background.

Moreover, the reversible reaction of iodine vaporization can be employed as a universal detection method for lipophilic substances such as indoles, amino acids, steroids, or lipids. The solvent-free chromatogram can be treated with iodine vapor or dipped in or sprayed with an iodine solution. Iodine dissolves in or forms weak charge transfer complexes with most organic substances, leading to first slightly yellow then dark brown zones on a pale yellow or tan background. To stabilize the iodine on the chromatogram plate, the plate can be immersed in or sprayed with a dilute starch solution to produce blue iodine inclusion compounds that are stable for a long time.

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For detection of acidic or basic substances, pH indicators can be employed. Dipping or spraying the chromatogram with an indicator solution whose pH is adjusted to be close to the endpoint of the basic or acidic substances results in a change in their color.

B. Microchemical Detection Methods

In addition to physical methods of detection, chemical derivatization methods can be employed to yield or complement results. Derivatization to colored, fluorescent, or UV-absorbing compounds can be carried out pre- or postchromatographically. Prechromatographic derivatizations, during sample preparation or on the starting zone of the HPTLC layer, are employed primarily to improve selectivity of the separation by changing of substance properties and to stabilize labile substances that would degenerate during chromatography. The main purpose of postchromatographic derivatizations, however, is to visualize substances, i.e., to render them detectable, and to improve detection limits and the linearity of the calibration function.

1. Prechromatographic Derivatization

In contrast to derivatization during sample preparation, in which case samples and standard solutions have to be treated individually one after the other, derivatizations performed as in situ reactions at the starting zone or in the concentration (preadsorbent) zone of the TLC plate offer the following advantages:

1. Derivatization reagents can be automatically applied on the layer, and derivatization is performed simultaneously on all tracks. In contrast to derivatization of each single sample and standard solution, prechromatographic derivatization on HPTLC plates is rapid and easy to handle.
2. If substances are not stable, prechromatographic derivatization can produce stable zones for analysis.
3. Reactivity of substances, e.g., toward the stationary phase, can be reduced.
4. Linearity of calibration curves can be improved.
5. Sensitivity of detection can be increased by adding a chromophore or fluorophore to the analyte molecule.
6. Chromatographic selectivity can be improved by specific chemical derivatization.

In practice, the derivatization reagent is applied as a band first. Then the sample or standard solution is applied onto the same starting zone. This method of application is called overspraying. The solvent in which the sample or standard solution is dissolved should not cause the reagent to spread outward. If necessary, the reagent solution can be applied once more so that it is present in excess or a second reagent solution, necessary for the proper reaction, can be applied. Finally, the starting zone should be covered with a glass strip before being placed on a hotplate or in an oven if heat is necessary to accelerate the reaction. The derivatization reagent can also be applied as a vapor. The layer, except for the applied substance zones, is covered with a glass plate and placed in a chamber over the vapor of the reagent that produces the derivatization reaction. Examples of prechromatographic in situ reactions are compiled in Table 2. For quantification, derivatization products have to be proportional to the quantity present on the layer, and derivatization reagents in excess should not interfere with the subsequent chromatographic separation. If necessary, a prechromatographic run can be used to separate the excess derivatization reagents.

2. Postchromatographic Derivatization

Colorless, nonluminescent substances that cannot be detected by UV absorbance, fluorescence quenching, or prechromatographic derivatization have to be derivatized after chromatography to render them detectable. The primary purposes of postchromatographic derivatizations are to

Visualize substances

Increase selectivity of detection

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Table 2 Examples for Prechromatographic Derivatizations In Situ

Reaction	Compound class	Derivatization reagent	Ref.
Acid hydrolysis	Cardenolide glycosides	37% Hydrochloric acid	6
Alkaline hydrolysis	*n*-Hexadecyl esters	Methanolic sodium hydroxide solution	7
Enzymatic hydrolysis	Digitalis glycosides	Luizyme solution	8
Oxidation	Geraniol	20% Chromic acid in glacial acetic acid	9
Reduction	Alkaloids	Sodium borohydride solution	10
Chlorination	Acetanilides	Chlorine vapor	11
Bromination	Capsaicinoids	Bromine vapor	12
Iodination	Phenolic steroids	Iodine vapor	13
Nitration	Phenols	Nitrous vapor	14
Diazotization	Estriol	Saturated ethanolic Fast Dark Blue R salt solution	15
Hydrazone formation	Aldehydes and ketones	2 N 2,4-Dinitrophenylhydrazine in acetic acid	16
Esterifications	Aflatoxins	Trifluoroacetic acid	17
Etherifications	Carboxylic acids and organophosphoric acids	Ethereal diazomethane solution	18
Dansylation	Fatty acids	Dansyl semicadaveride solution; *N,N'*-dicyclohexylcarbodiimide solution	19

Improve sensitivity of detection

Improve the linearity of the calibration function

The substance concentration needed for derivatization is reagent-dependent because not every derivatization reagent can detect the substance at the same sensitivity level. Therefore, a separation can seem to be a good one when the outer part of the substance zones is not detected because substance concentration is too low for reaction with the derivatization reagent. This effect can confuse the results. With increasing R_f values diffusion increases, thus leading to a lower zone concentration and less detection sensitivity. That is why visualization of substances in the chromatogram is reduced by increasing R_f values.

Postchromatographic reactions can be performed as universal reactions or with functional group selectivity. Universal reagents react with a wide variety of compound types:

Hydrochloric acid vapor reacts with organic substances to form colored products and finally dark brown carbon (20).

Phosphomolybdic acid causes blue-black zones to appear against a yellow background (21).

Anisaldehyde–sulfuric acid reacts with natural products and leads to differently colored zones, whereby zone identification is possible (22).

Antimony(III) or (V) chloride produces zones of different characteristic colors on a white background (23).

Ammonium hydrogen carbonate vapor reacts with many organic compounds and leads to fluorescent products when heated (15).

Zirconium salts form mainly yellow green to blue fluorescent zones (24).

Sequences of microchemical detection (3), i.e., the consecutive application of different derivatization reagents onto the plate, can be used for complex mixtures. An intermediate drying or heating step is employed, and the chromatogram documented and/or evaluated after each derivatization step.

Examples for functional group–specific reagents are listed in Table 3.

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Table 3 Functional Group-Specific Postchromatographic Derivatizations

Functional group	Derivatization reagent	Reference
Acetylene compounds	Dicobalt octacarbonyl	25
Aldehydes	2,4-Dinitrophenylhydrazine	26
Alcohols	Lead(IV) acetate dichlorofluorescein	26
Amines	Ninhydrin	26
Carboxylic groups	2,6-Dichlorophenyl indophenyl (Tillmans' reagent)	26
Halogen derivatives	Ammoniacal silver nitrate (Dedonder's/Tollens' or Zaffaroni's reagent)	27
Ketones	2,4-Dinitrophenylhydrazine	26
Nitro derivatives	Benzocyanide benzyltrimethylammonium hydroxide	28
Peroxides	1-Naphthol/N^4-ethyl-N^4-(2-methyl-sulfonamidoethyl)-2-methyl-1,4-phenylenediamine	26
Phenols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	26
Thiols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	26

In practice, the derivatization reagent is added by exposure to vapor, by immersion, or by spraying after evaporation of the mobile phase. The procedures for chromatogram immersion or exposure to vapor are preferable because of their precision and repeatability. The derivatization reagent may also be added to the mobile phase if the reagent is evenly spread over the layer and if it elutes with the solvent front. For example, acids have been added to the mobile phase for detection of quinine alkaloids (29), and fluorescamine has been added for detection of biogenic amines (30).

a. Immersion. The procedure of dipping or immersing the chromatogram plate into a derivatization reagent solution offers three advantages:

1. Contamination with toxic derivatization reagents is reduced in comparison with spraying or evaporation. When the immersion device is not in use, it is always covered with a stainless steel lid.
2. Consumption of derivatization reagents is low if reagents are used repeatedly.
3. The layer is homogeneously coated with the reagent. This results in a better baseline structure, consequently a lower detection limit, and better reproducibility compared with spraying methods.

A chromatogram immersion device is available from CAMAG (see Chap. 5). The derivatization reagent is poured into a dip tank that holds either 20 × 20 cm or 20 × 10 cm plates. Then the dried chromatogram is automatically immersed and withdrawn at a uniform speed. The advantage of using an immersion device instead of manual dipping is the uniform speed of immersion and withdrawal of the plate. Thus, irregularities of manual dipping, such as tide marks that can interfere with densitometric evaluation, are avoided. The vertical speed (selectable between 30 and 50 mm/s) as well as the immersion time (selectable between 1 and 8 s) can be set as required. After immersion, the chromatogram is removed slowly to allow excess reagent to drain back into the dip tank and the back of the plate is cleaned off. Then the plate is dried with either air or nitrogen gas, or it is heated with a plate heater to start the derivatization reaction.

Generally, dipping solutions are about 80% less concentrated than corresponding spray solutions, and if necessary they can be modified during preparation. For instance, water is often replaced with an alcohol or another lipophilic solvent because on the one hand water can dissolve the silica gel layer and on the other hand it cannot penetrate reversed phases. But, of course, dipping solutions must not dissolve the substances or their reaction products out of the stationary phase. If the dipping solution is too polar, it can penetrate the layer, thus leading to more intense zones at the back of the layer than at its surface. In this case, the dipping solution has to be

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rendered less polar. A chromatogram immersion device can also be employed to impregnate adsorbent layers with a detection reagent prior to sample application. This preimpregnation method has been used successfully with silver nitrate and with phosphomolybdic acid.

b. Exposure to Vapor. The most homogeneous way to cover the chromatogram with a derivatization reagent is by exposing it to vapor. For instance, iodine can be sprayed onto the chromatogram as a 1% alcoholic solution, but it is simpler to place the plate in a closed standard developing chamber that contains a few iodine crystals at the bottom and is saturated with iodine vapor. Twin-trough chambers or special conditioning chambers can be used for this purpose as well. Surprisingly good quantitative results can be obtained using another HPTLC plate, which can be exposed to iodine vapor for several days and then used as a source of vapor for the investigated plate for a time ranging from a few minutes to a couple of hours (31). To stabilize the iodine on the chromatogram plate, the plate can be immersed into a dilute starch solution to produce blue iodine inclusion compounds that are stable for a long time. Iodine vapor allows nonspecific, and in most cases nondestructive, detection of many lipophilic substances such as indoles, amino acids, steroids, and lipids. Besides iodine, bromine, chlorine, formaldehyde, ammonia, diethylamine, ammonium hydrogen carbonate, acids, or sulfur dioxide can be applied as vapors.

c. Spraying. For spraying the chromatogram plate with derivatization reagents, electropneumatic sprayers (Fig. 5) or simple glass sprayers with a rubber bulb pump are mainly used. Alternatively, a computer-controlled instrument, the Chromajet (Desaga), can be employed to spray on defined amounts of derivatization reagent. Derivatization reagents are atomized into a fine aerosol spray with particles in the range of 0.3–10 μm. Glass sprayers can also be operated using a compressed air or an inert gas. Usually the derivatization reagent solution is sprayed onto the layer at a pressure of 0.6–0.8 bar. Spraying should be performed in a spray cabinet, which ensures the complete removal of excess spray from the sprayer and of spray particles that have rebounded from the chromatogram plate. The spray jet is not deflected before it reaches the chromatogram, an effect that often occurs in a normal laboratory fume hood. Spraying is carried out manually from a distance of 20–30 cm. It is performed two-dimensionally (first horizontal then vertical lines) in a meandering pattern, returning outside the chromatogram. The very first spray should be directed beside the TLC plate until a very fine aerosol spray is supplied. However, sprayers are operated manually and can never be used very uniformly. That means that the resulting chromatogram visualization differs from individual to individual and from spraying to spraying. Reproducibility is not as good when a chromatogram immersion device or evaporation application is used. However, spraying cannot be circumvented when two derivatization reagents

Figure 5 CAMAG TLC sprayer. (Photograph courtesy of CAMAG.)

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have to be applied successively without intermediate drying. Aerosol sprayers using fluorogenated hydrocarbons, etc. as the propellant gas, quite common in former years, should be avoided for environmental considerations. After each use, the sprayer should be cleaned by spraying with a suitable solvent to prevent clogging. Some derivatization reagents, like manganese heptaoxide– and perchloric acid–containing reagents, sodium azides, and iodine azide solutions can cause explosions in the exhaust ducts of the spray cabinet and should never be sprayed. Plates should preferably be immersed into such reagents.

d. Heating. After immersion into, spraying with, or vapor application of the derivatization reagent, heating is often necessary to produce the required color or fluorescence of the substance zones, i.e., to start and complete the derivatization reaction. Instead of a plate heater, which is commonly used, an IR source, a microwave apparatus, or an oven can be employed. A plate heater (Fig. 6) can usually be regulated over a temperature range of 25–200°C. The temperature is uniformly maintained over the entire surface of the heating plate. However, chromatogram plates should be positioned in the center of the heating plate. Programmed and actual temperatures are digitally displayed. Temperature and heating time depend on the derivatization reagent and sorbent layer used.

3. Stabilization or Intensification

Generally, chromatograms should be protected from light and oxygen during storage. After derivatization, it should be determined that the chromatogram will be sufficiently stable until it is evaluated. Various stabilization treatments can be employed if a reduction in the fluorescence or color intensity is observed (3). For colored substances, for example, cadmium or copper salts can be added if a ninhydrin reagent has been used, a sodium nitrite solution can be sprayed to stabilize

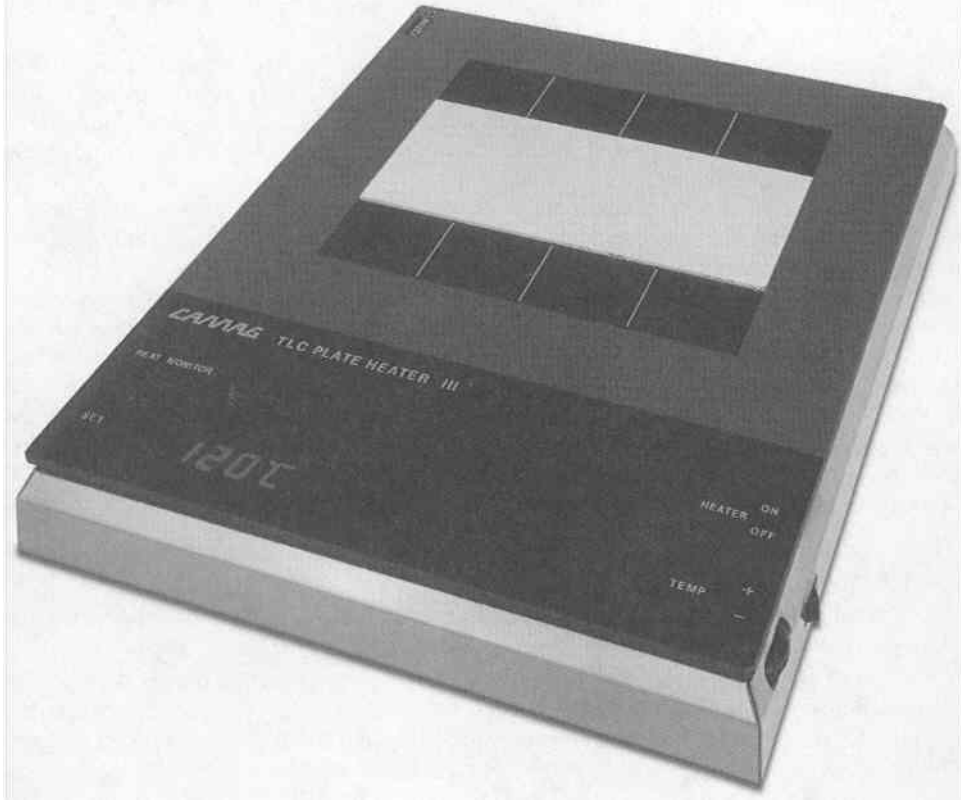

Figure 6 CAMAG TLC plate heater. (Photograph courtesy of CAMAG.)

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the van Urk reaction with lysergic acid derivatives, or an ammonia solution or ammonia vapor can be employed to stabilize the reaction of tryptamine with 2,6-dibromoquinone-4-chloroimide. For fluorescent zones, the chromatogram plate is treated with a viscous lipophilic or hydrophilic agent. These agents evidently influence the rotation of the molecules and keep out the ambient air to help eliminate quenching. As lipophilic stabilization agents, particularly liquid paraffin, but also silicone, kerosene, isooctane, or dodecane are used at low concentrations. Often, more concentrated solutions additionally yield an intensification of the fluorescence. As hydrophobic stabilization agents, for example, polyethylene, triethylamine, triethanolamine, or Triton X-100 are used.

C. Bioactivity-Based Detection Methods

Microbiological and biochemical methods of detection do not exploit chemical or physical properties but the biochemical or biological–physiological activity of substances. Bioactivity-based reactions are employed mostly for the detection and determination of environmental or toxic compounds such as pesticides (insecticides, fungicides, herbicides), antibiotics, alkaloids, mycotoxins, cytotoxins, hot or bitter substances, and saponins. Such compounds have in common that they stimulate or inhibit an appropriate enzyme or test organism during incubation. Either enzymes or test organisms can be applied directly onto the sorbent layer (bioautographic detections) or the sorbent layer itself is placed on the test organism medium (reprint methods). For biochemical detection, enzymes are used. Appropriate test organisms for microbiological detection can be mold spores, yeast cells, cell organelles, or bacteria in a nutrient medium.

For instance, saponins are detected by blood cells. After chromatography, a blood–gelatin suspension is directly applied onto the layer. Then active agents diffuse from the layer to the blood–gelatin suspension and stimulate or inhibit the test organism during incubation. Saponins cause hemolysis of blood cells, so they are visible as transparent, nearly colorless zones on a turbid red blood–gelatin background.

Antibiotics in environmental samples can be detected by the bacterium *Bacillus subtilis*. The plate is dipped in the bacterial solution. After incubation, the plate is sprayed with MTT–tetrazolium salt reagent, which, after incubation, gives a blue-violet background. Antibiotics inhibit the growth of the bacteria and cause bright zones of inhibition on the colored background (Merck Chrom Biodip®, bioautography test kit).

The principle of enzymatic reactions is the formation of an enzyme–substrate reaction (32). The developed chromatogram is dipped in an enzymatic solution, e.g., a solution of cholinesterase, and incubated for a short period. Then it is dipped into a substrate solution, e.g., 1-naphthyl acetate/Fast blue salt B. In presence of the active enzyme, 1-naphthyl acetate is hydrolyzed to 1-naphthol and acetic acid. Further, 1-naphthol is coupled with Fast blue salt B to form a violet-blue azo dye. This enzyme–substrate reaction is inhibited by pesticides, such as organophosphates, organochlorines, carbamates, or pentachlorophenol, which inhibit the enzyme cholinesterase. Consequently, such substances cause bright zones of inhibition on a violet-blue background (33,34). Also, other enzyme test systems, such as (chymo)trypsin, elastase, urease, amylase, aminolevulinic acid dehydratase, vegetable peroxidase, or catalase can be applied.

Advantages of bioactivity-based detection are

1. High specificity and reduced interference of the matrix, leading to a reduced need for sample cleanup.
2. More sensitive detection limits, i.e., typical detection limits are found to be in the subnanogram and even in the lower picogram range.
3. Coupling of chromatography with bioactivity, allowing identification of toxic compounds, degradation products, or metabolites, not just the summation of damaging effects in a specified test system as in biomonitoring tests. Separated fractions are stored in the chromatogram and can easily be used for bioactivity-based reactions.

Coupling of bioactivity detection with TLC enables the assignment of physically detected substances to a specific activity, which means that toxic active substances can be identified, not just

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detected. Thus, in a sample, further unknown toxic compounds that affect the test system can be detected, leading to a complete toxicity profile of the sample related to the test system.

III. IDENTIFICATION

In planar chromatography, a substance is identified by comparison with an authentic standard cochromatographed on the same plate. Parameters that are compared are the hR_f value, the analog curve (absorption at a definite wavelength), the color of the zone if the zone is visible or inherently fluorescent, the spectrum, and/or the reaction with a derivatization reagent.

Unknown substances can be detected and identified by a special feature, called a multiwavelength scan (CAMAG TLC Scanner3), with which the plate is automatically scanned at up to 30 different wavelengths. The analog curves at the different wavelengths are overlaid in one graphic (Fig. 7), and the spectral and chromatographic properties are compared to a series of identification standards. Thus, unknown constituents of a sample can be detected and identified over a wide wavelength range.

For fingerprint identification, all samples on one plate are compared to one another at the same time. If necessary, analog curves of samples on different plates are overlaid by a special feature of the CAMAG VideoScan. Thus, for example, in plant analysis the chemical constituent profile is linked to the botanical identity of a plant.

Generally, UV/vis spectra are recorded because they can easily be measured with a conventional TLC scanner. The spectrum can then be compared with a standard cochromatographed on the same plate or with a spectral library. However, if possible, more information is given by recording an FTIR, Raman, or mass spectrum. In former times, zones of interest were recovered by extraction from the adsorbent and were then characterized by FTIR-, Raman, or mass spectrometry. Nowadays, there seems little need to go through time-consuming recovery procedures. FTIR or Raman spectra can be directly recorded on the plate using appropriate instrumentation. Recording of in situ mass spectra is described in detail in Chapter 9, and the detection and identification of radioactively labeled substances in Chapter 12. For identification of very complex mixtures, coupling of separation methods (especially HPTLC with HPLC) is used to cope with difficult separations and to get rid of interfering matrix.

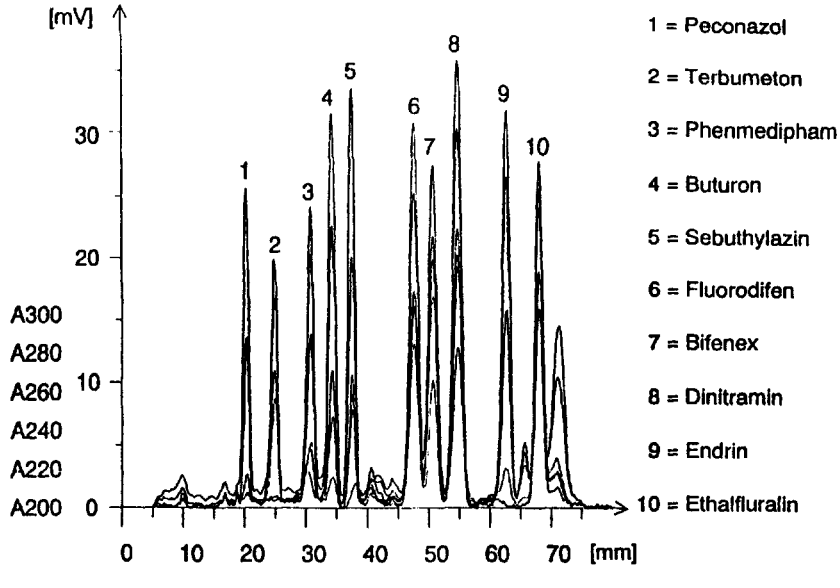

Figure 7 Multiwavelength scan of pesticides recorded at six different wavelengths that are superimposed to get a spectral scan of the track.

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A. Ultraviolet/Visible Spectra

Spectral data can be processed after the chromatographic run to identify individual fractions by comparison with spectra of authentic standards cochromatographed on the same plate or stored in a spectral library (Fig. 8). Spectra can also be recorded to check identity by superimposing the spectra of all fractions within the same R_f window. Moreover, the spectral data allow the determination of the optimum wavelength(s) for quantitative scanning and the checking of the purity of fractions by superimposing the spectra from different positions within a spot.

If substances are well separated and possess different chromophores, their UV/vis spectra can be used for recognition and identification, as shown in Fig. 9. However, in the case of unknown mixtures, it is necessary to employ other identification methods such as direct in situ FTIR measurement, because for example, phenazone scarcely differs from other pyrazolone derivatives such as propylphenazone, and caffeine does not differ from purine derivatives such as theophylline or theobromine. The situation is similar for designer drugs of the 3,4-methylenedioxybenzene series. They can be well separated by chromatography, but they cannot be distinguished at all by means of their UV spectra (Fig. 10a). However, this is possible after derivatization with a definite reagent solution (Fig. 10b).

Ultraviolet/visible (UV/vis) spectra can easily be recorded by a conventional TLC scanner that is described in detail in Chapter 5. The spectrum is automatically corrected by measuring the spectra of the lamp or light, the plate background, and any solvent traces thereon, i.e., a substance-free area of the layer:

$$\lambda_{\text{corrected}} = \lambda_{\text{substance on HPTLC plate}} - \lambda_{\text{lamp}} - \lambda_{\text{plate background}}$$

HPTLC spectra usually correspond to the spectra of the same substances in solution. However, either bathochromic or hypsochromic shifts can be caused by interaction of the substance molecules with adsorbents (e.g., silanol, amino, or polyamide groups) and with any solvent traces still on the plate (e.g., if acids or bases have been used in the solvent mixture). Thus, HPTLC spectra are compared to authentic standards chromatographed on the same plate or are searched for in a self-made spectral library.

HPTLC spectra are dependent upon the amount of substance, especially in the range of the detection limit. At low amounts, the bondings between adsorbent and substance influence the reflection, whereas at high amounts only the substance itself contributes to the reflection. This means that spectra have to be compared at similar concentration levels.

For spectral comparison, the correlation or difference of spectra can be displayed as well as the overlaying of the spectral shape. Spectra of unknown substances can be searched for in libraries. As search criteria, the following are used:

The characterization number (wavelength maxima, the number of wavelength maxima, and similar features of the spectra)

The position [migration distance, R_f value, hR_fc value (corrected R_f value)]

The correlation (statistical comparison of two spectra)

A list shows the best matching spectra. Up to 1200 self-recorded spectra can be saved in one library file. Besides comparing spectra of unknown substances with those in a library file, spectra of two different library files can be compared. In this way, application-specific library files can be compiled.

B. FTIR Spectra

Direct in situ HPTLC-FTIR measurement is carried out by diffuse reflectance using a diffuse reflection infrared Fourier transform (DRIFT) spectroscopic unit (Fig. 11) (35). It is necessary to take into account the fact that at wavelengths at which the absorption is great and the refractive index is high, the incident radiation is almost 100% normally reflected at the surface so there is scarcely any diffuse reflection. However, only this part of the reflection contains the spectral information concerning the sample, in contrast to the normal (Fresnel) reflection. This means that reflectance minima, and not the expected reflectance maxima, are obtained at wavelengths of

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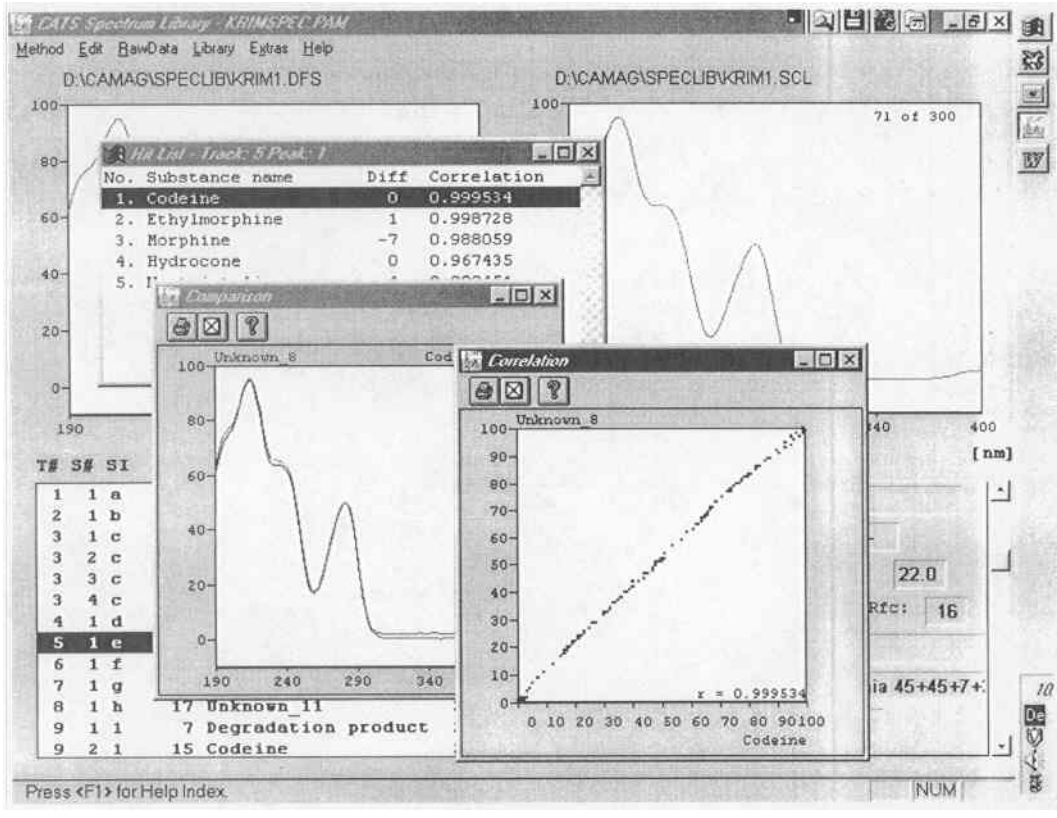

Figure 8 Spectrum library search; codeine was found to be the best hit for the unknown. (Photograph courtesy of CAMAG.)

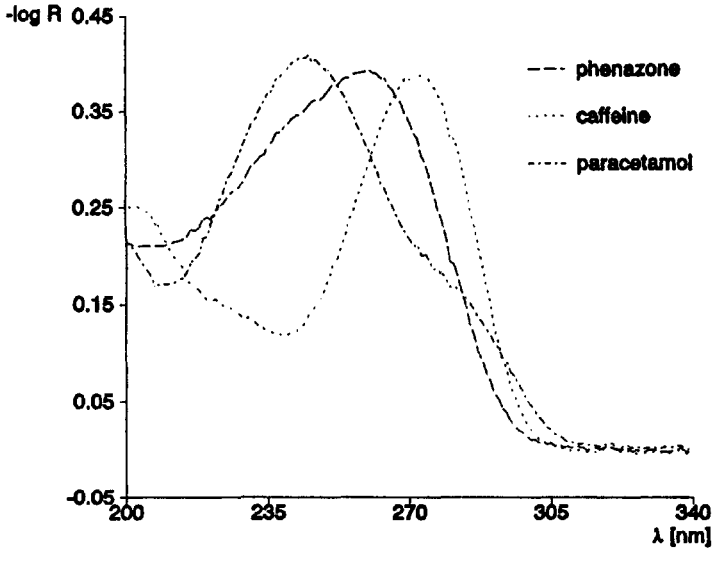

Figure 9 HPTLC UV spectra of phenazone (---), caffeine (· · ·), and paracetamol (— · —).

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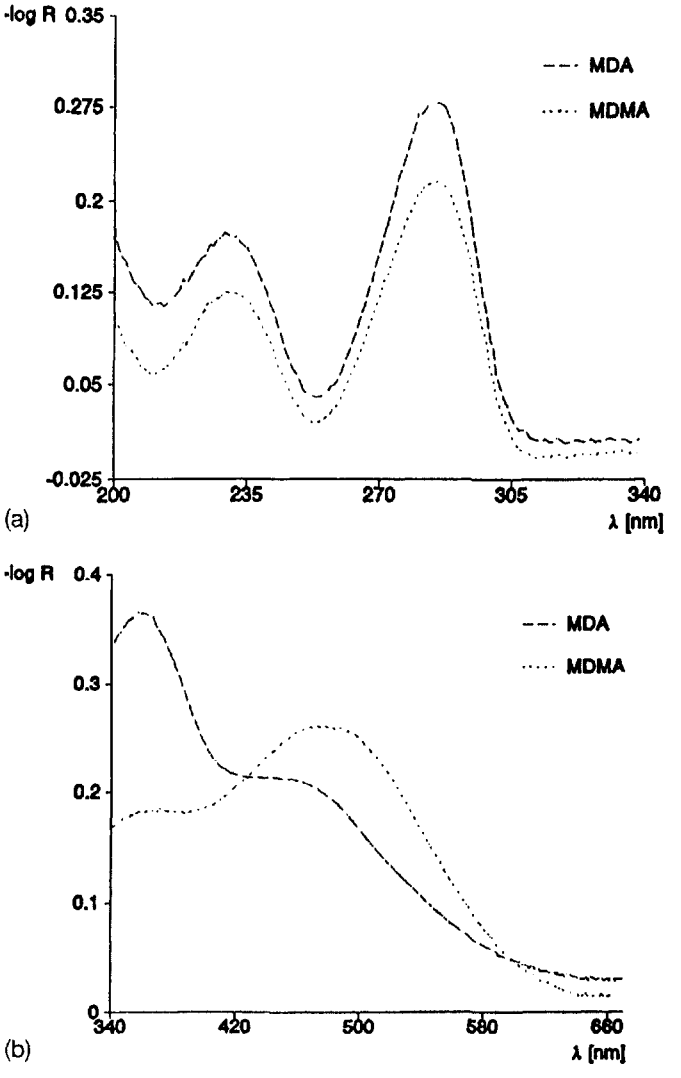

Figure 10 HPTLC UV spectra of MDMA (---) and MDA (· · ·) (a) before and (b) after dipping in an *o*-(benzenesulfonamido)-*p*-benzoquinone solution.

strong absorption. With silica gel, the absorption maxima, also known as residual radiation bands, dominate considerably in the 1300 to 1000 cm^{-1} region, so the diffuse reflectance of interest is negligibly small. Therefore, measurements in this region are not possible on this sorbent. In contrast, it is possible to make measurements up to a wave number of 1000 cm^{-1} on cellulose stationary phase. In spite of the limited wavelength range, it is still possible to carry out in situ measurements on silica gel to characterize and identify substances that have been separated by HPTLC if use is made of an HPTLC-FTIR reference library with automatic comparison of band position, width, and intensity and if this is supplemented by comparison of the sample spectrum with the best matching spectra.

The Fourier transformed interferograms provide IR spectra that can be recorded and converted at will of the library search into normalized reflectance spectra (reflectance units R) (Figs. 12A and 12B), into quasi-absorbance units that are not proportional to concentration (−log R) (Fig. 12C), or into Kubelka–Munk units that are proportional to concentration (Fig. 12D). The sub-

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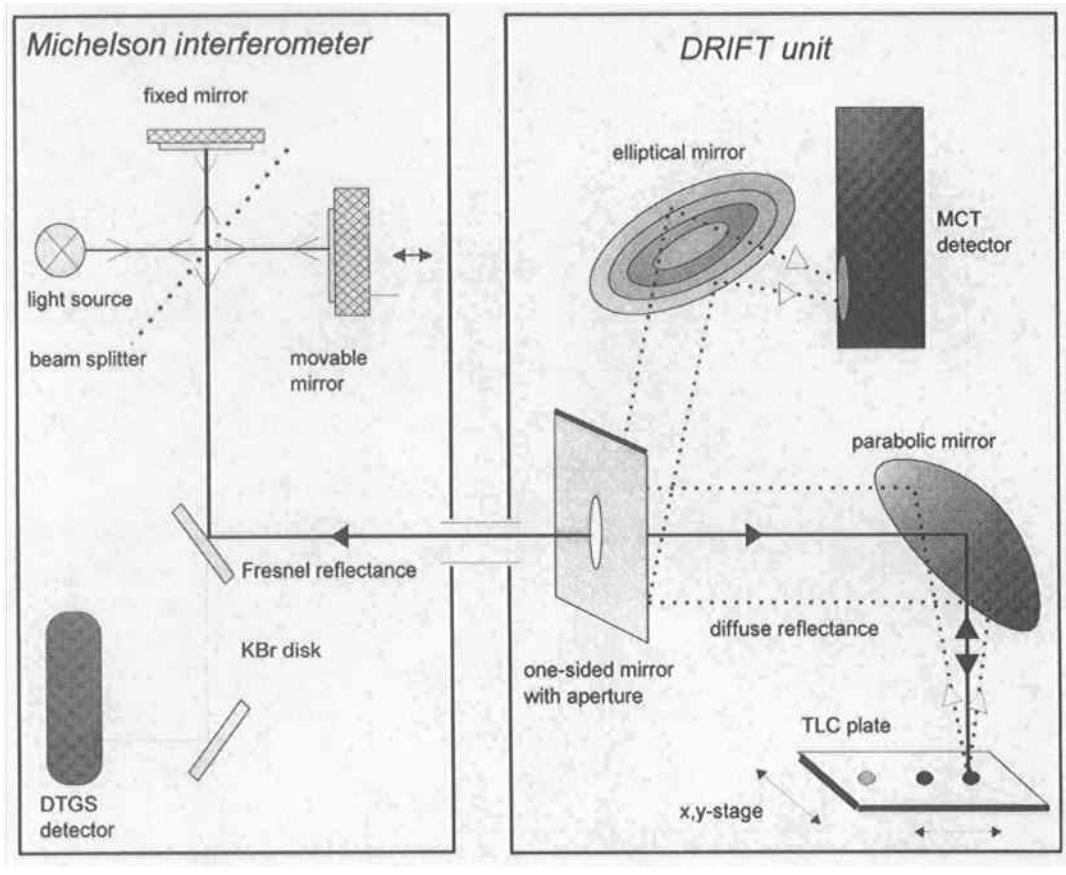

Figure 11 Schematic overview of the Bruker HPTLC-DRIFT unit for on-line measurement.

stances can be localized on the TLC plate by using either spectral windows chosen at will (Fig. 12E) or the Gram–Schmidt technique (Fig. 12F). The first of these two methods can be used to increase selectivity (e.g., the spectral window can be chosen so that it detects only compounds with carbonyl groups), whereas the second is universally applicable and independent of wave number.

The large quantity of data generated by HPTLC-FTIR coupling can be printed out as a three-dimensional plot of a spectral series, with the wave numbers on the x-axis, the distances on the z-axix, and the absorptions on the y-axis. However, because the whole picture can then become very complex, a two-dimensional contour plot is better suited for the recognition of band overlaps and small quantities of impurities.

The HPTLC-FTIR method is particularly suitable for identification and quantification of substance mixtures. Depending on the specific IR absorbance of the substance and the distance run in the chromatogram, the limits of identification, the validated detection limits, and the limits of quantification lie between 15 ng and 2.5 μg.

The power of this coupling method is confirmed by examples from various fields of analysis such as drug identification (36), forensic chemistry (37), environmental analysis (38), and quality control of essential oils (39). The most recent developments include the design of a silica gel sorbent containing 50% magnesium tungstate, which considerably enhances the interpretable wavelength range (40). This allowed an efficient HPTLC-UV/FTIR coupling procedure for the separation and rapid identification of flurazepam hydrochloride and its related substances in bulk

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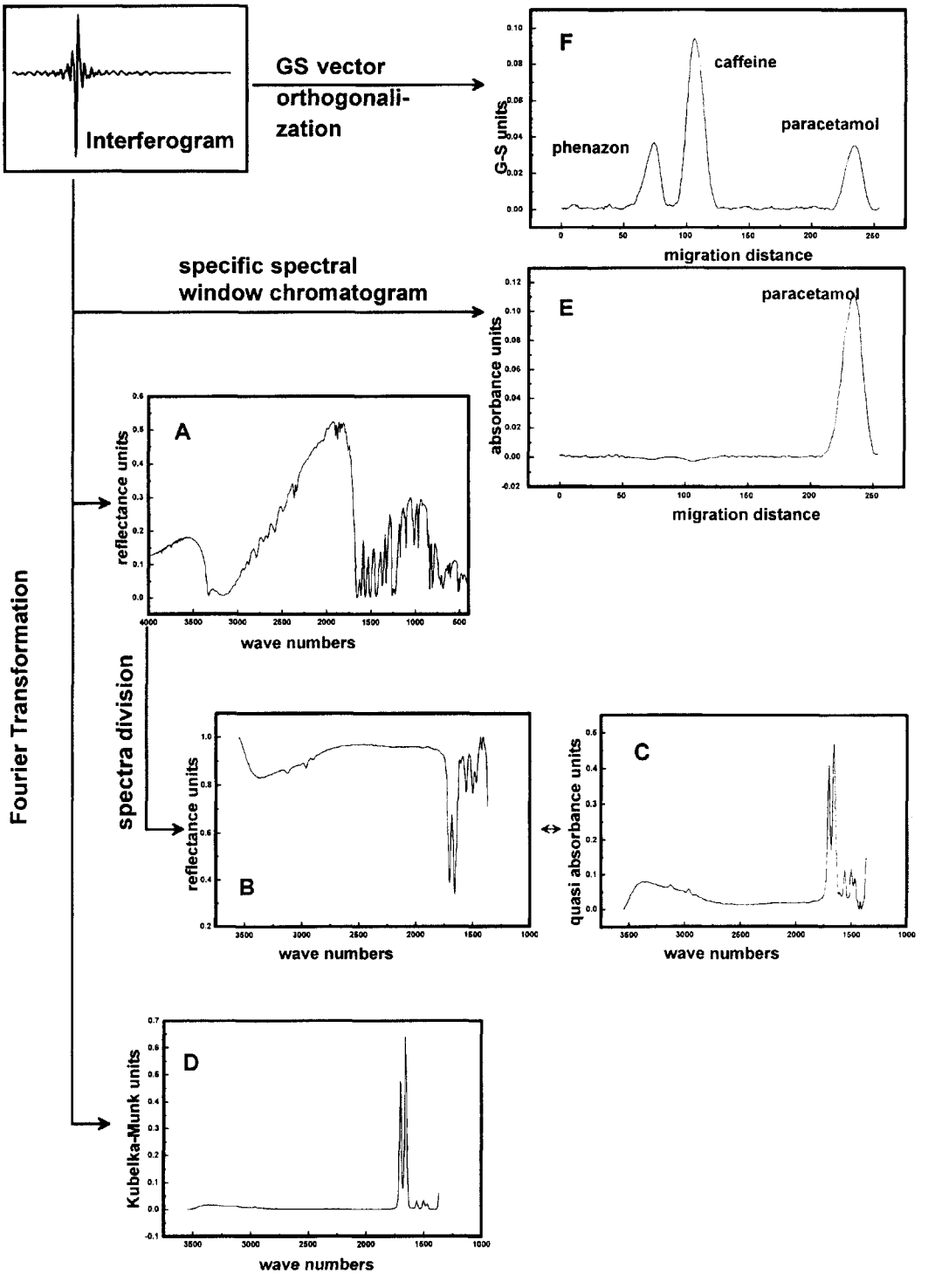

Figure 12 Schematic overview of data presentation possibilities.

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powder and capsules (41). Compared to the related compound test of the Pharmacopoeia, this procedure shows several advantages, e.g., baseline separation of the known impurities and detection of the substances as peaks in the UV region (Fig. 13) as Gram–Schmidt or window chromatograms (Fig. 14). Furthermore, unambiguous identification is obtained by postchromatographic extraction of the DRIFT spectra and comparison with reference spectra in the library. Quantification of the related compounds was carried out densitometrically.

C. Raman Spectra

With the use of argon ion, HeNe, or YAG lasers as monochromatic light sources and the improvement of detection methods by the employment of more sensitive CCD detectors instead of photomultiplier tubes, Raman spectroscopy has gained in importance. This identification technique serves primarily for the investigation of apolar atomic groups and of symmetrical groups of atoms that are infrared-inactive. It is also possible to assign vibrations from FTIR spectroscopy with the aid of Raman spectra. However, little progress has been made with quantitative evaluation.

For in situ identification in TLC especially, the surface-enhanced Raman scattering (SERS) technique is used in the subnanogram range. After development and drying of the chromatogram, the plate is dipped in or sprayed with a colloidal silver suspension (42). The silver colloids (about 15 nm particle size) are prepared by reduction of silver nitrate with sodium citrate. With the use of this technique, the investigated substances experience an intensity enhancement of about 10^6 due to the metal microstructure on the surface of the chromatogram, thus leading to greater electron–photon coupling at the atomically rough metal surface and simultaneous charge transfer to orbitals of the adsorbates. Consequently, one of the advantages of the SERS technique is the

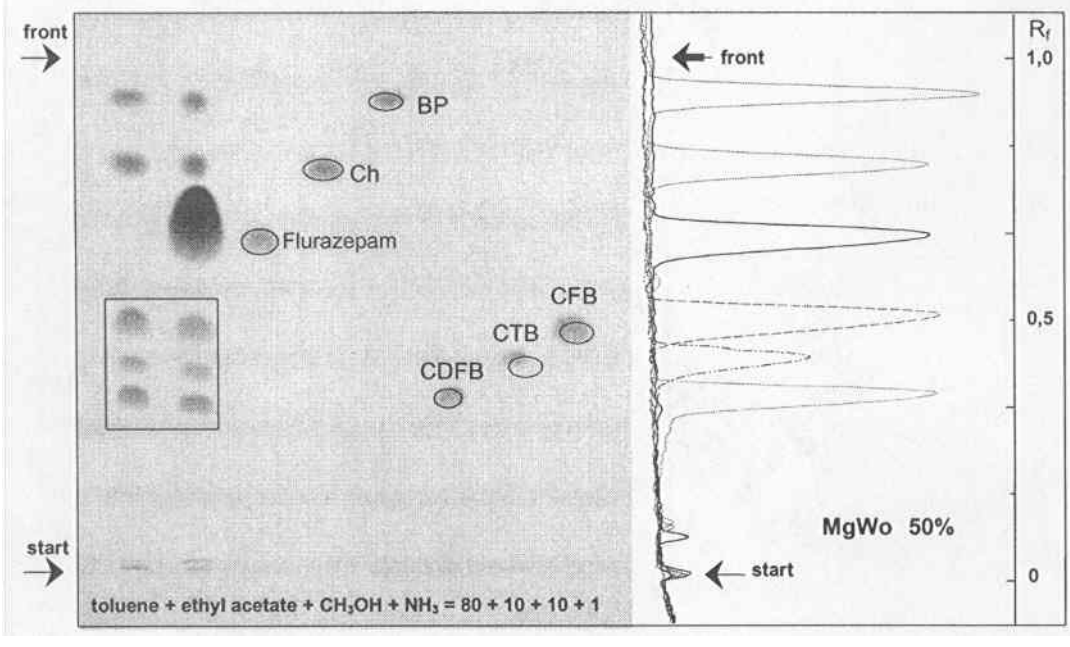

Figure 13 Separation and detection of Flurazepam and its impurities, Ch, 3-Amino-6-chloro-1-[2-diethylamino)-ethyl]-4-(2-fluorophenyl)-chinolin-2-one hydrochloride; BP, 5-chloro-2-[2-(diethylamino)ethylamino]-2′-fluorobenzophenone hydrochloride; CDFB, 7-chloro-1,3-dihydro-1-[(2-ethylamino)-ethyl]-5-(2-fluorophenyl)-2*H*-1,4-benzodiazepin-2-one hydrochloride; CTB, 7-chloro-1-[(2-ethylamino)-ethyl]-5-(2-fluorophenyl)-2*H*-1,4-benzodiazepin-2-one hydrochloride; CFB, 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

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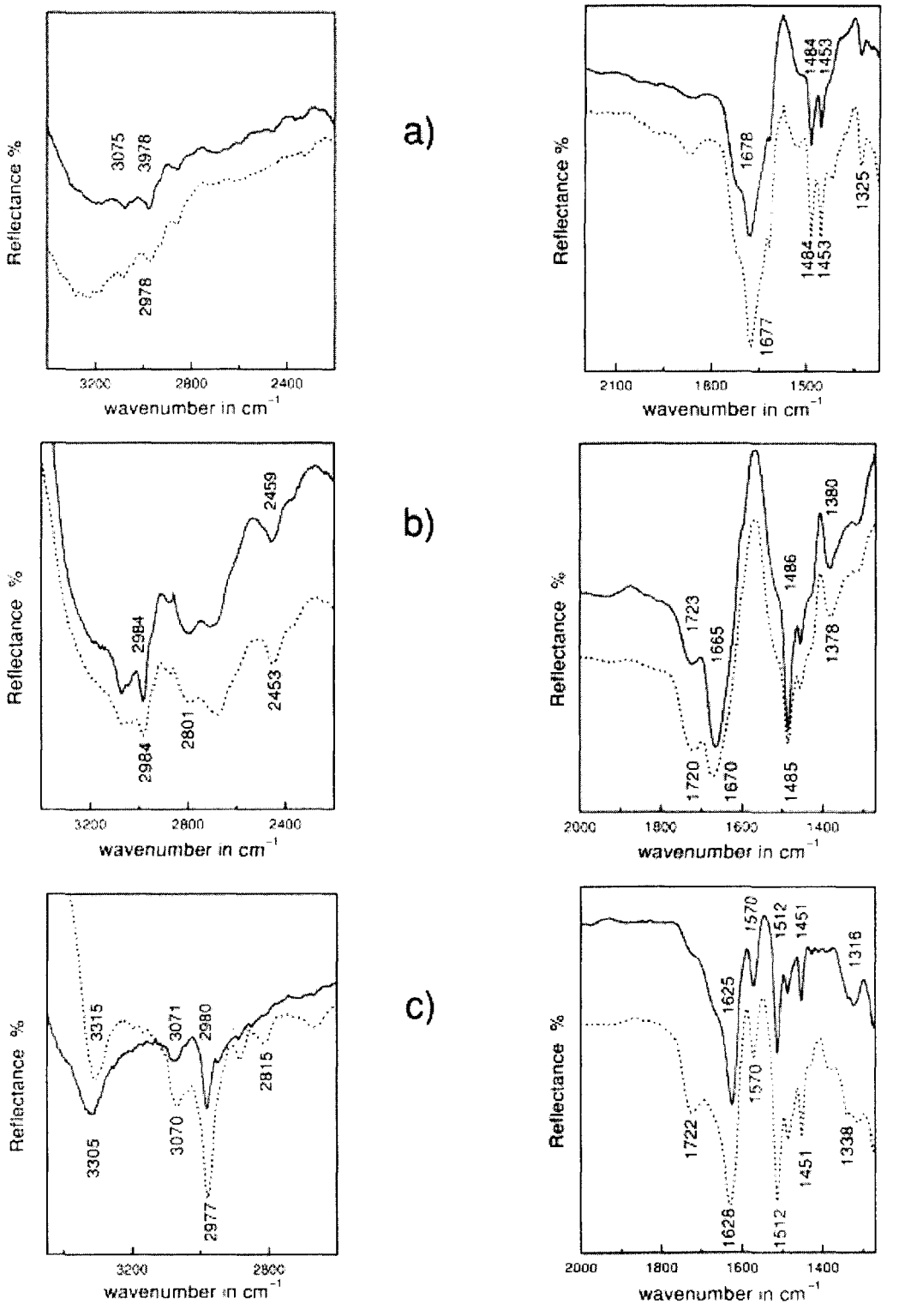

Figure 14 DRIFT spectra of degradation products (solid curves) in capsule after stress treatment [hit qualities (a) CDFB (652), (b) CTB (711), (c) BP (839)] and reference (dotted curves).

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high enhancement factor, permitting in situ analysis of TLC zones even down to picogram amounts.

To avoid diffusion effects at the zones of interest when the plate is dipped in or sprayed with an aqueous colloidal silver suspension, the silver molecules can be evaporated onto the HPTLC plate (43). Plates (10 × 10 cm) are placed in an evaporation device (Fig. 15) in which silver (about 600 mg) is evaporated at high temperature under high vacuum. Figure 16 shows the intensity enhancement by evaporation with silver molecules very clearly.

Highly Raman-active compounds such as optical brighteners (Fig. 17) (M. Moss, M. Zeller, personal communication, 1995) can also be detected without surface-enhanced scattering on specially modified silica gel plates. The identification limit is about 25 ng for these substances and about 100 ng for dyes.

D. Mass Spectra

For this in situ identification method, FAB (fast atom bombardment), liquid SIMS (secondary ion MS), or laser desorption is generally employed as the ionization technique (44,45). The analytes are sputtered directly from the TLC foil (Fig. 18) (46), or the TLC plate is placed on a movable table. However, the amount of substance needed for recording reliable mass spectra still lies in the submicrogram range. More details are supplied in Chapter 9.

E. Coupling of Separation Methods

Coupling of TLC with gas chromatography, supercritical fluid extraction, or the thermal separation technique (TAS) has been employed for special analytical tasks. Coupling of HPLC with either rotation planar chromatography (RPC) or overpressured layer chromatography (OPLC) (47) and the coupling of different stationary phases, known as long-distance OPLC (48), have also been demonstrated.

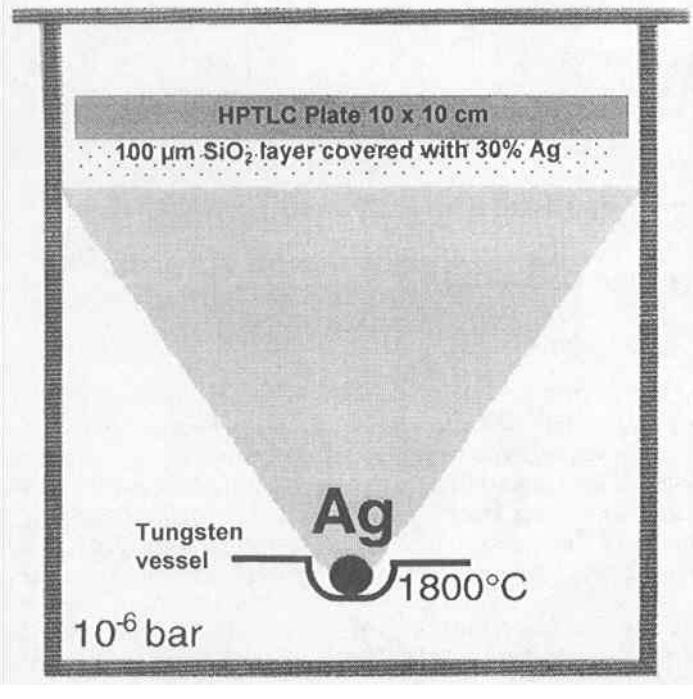

Figure 15 Evaporation device for SERS-Raman spectroscopy.

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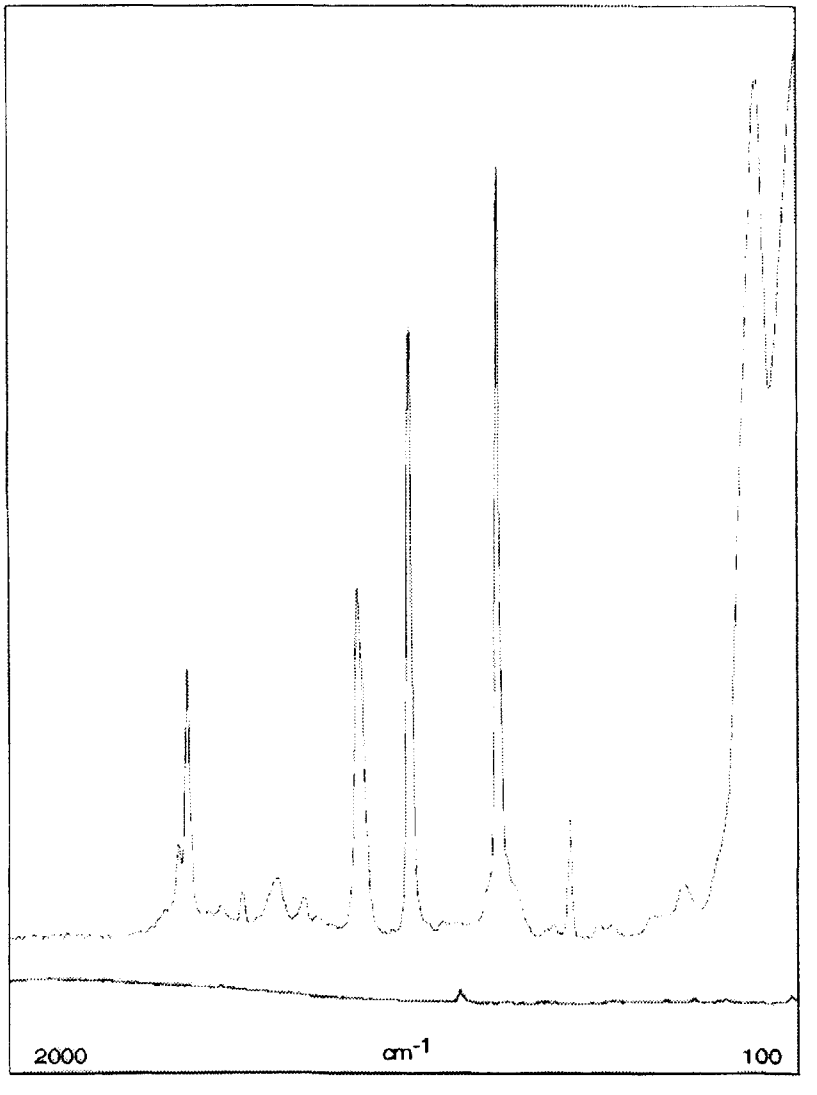

Figure 16 Intensity enhancement by evaporation with silver molecules. Raman spectra of 300 ng phthalic acid before (lower) and after (upper) evaporation.

Of major interest is the coupling of HPLC with automated multiple development (AMD) because of the immense increase in separation power it achieves. It seems to afford a low-price and rapid way to cope with difficult separations and to get rid of interfering matrix components of complex mixtures. HPLC separations are primarily carried out by bonded phase partition chromatography, whereas TLC separations on silica gel take place according to the principles of adsorption chromatography. Coupling of these two highly efficient separation methods greatly increases the information content of analyses (Fig. 19) (K. Burger, personal communication, 1994). In practice, a complex mixture is first separated on a microbore system, thereby providing a low flow rate of about 60 μL/min. This low flow rate enables a connection without a splitter. Selected HPLC fractions are automatically transferred onto the HPTLC plate by using a special application device (CAMAG DuoChrom) that can cope with an application flow rate of about 60 μL/min and can be heated if desired. Thereafter, planar chromatography is continued as usual.

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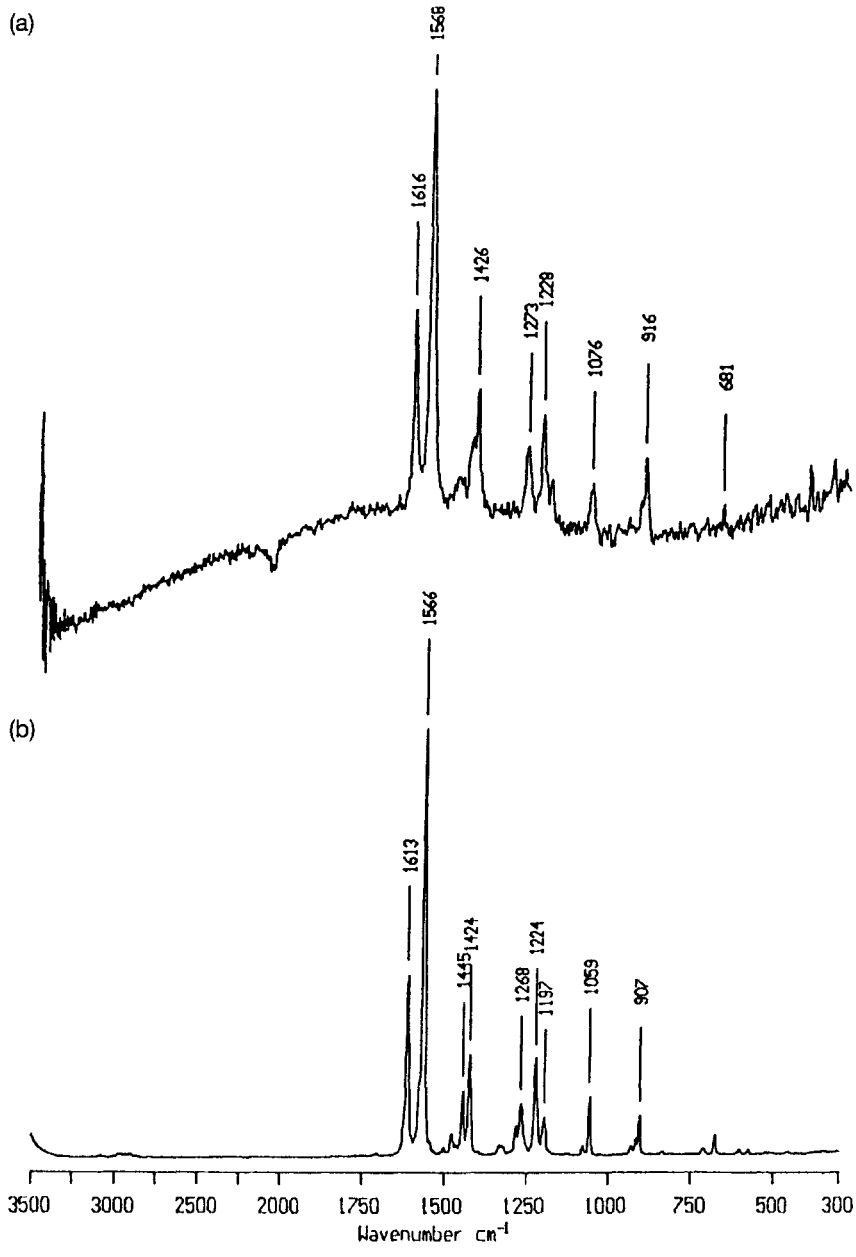

Figure 17 In situ Raman spectra of 100 ng of an optical brightener (a) and its reference substance (b).

IV. DOCUMENTATION

Planar chromatography is an open system, in contrast with high-performance liquid chromatography or gas chromatography. Thus, it can more easily be affected by the environment, and possible factors of influence have to be monitored more consciously and documented in detail (49). Accurate documentation seems to be the basis for reproducible planar chromatographic results.

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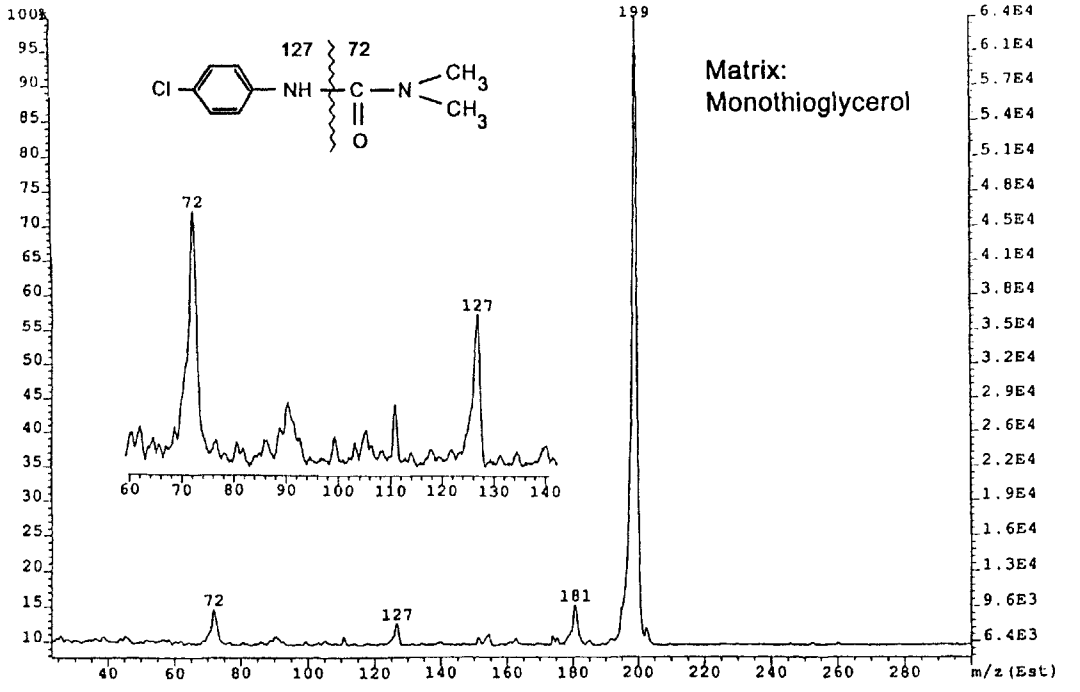

Figure 18 In situ positive-ion FAB-MS/MS analysis of the phenylurea herbicide Monuron.

A. Documentation of the Method

Good laboratory practice (GLP), good manufacturing practice (GMP), and standard operating procedures (SOP) and procedures such as accreditation, auditing, and certification involve nothing more or less than determining a range of parameters and demonstrating their reliability by means of statistical methods. Thus, it is necessary to ensure the quality of the working instructions and to document the chromatographic conditions for reproducible and reliable results. Some important items of documentation are compiled in Table 4.

These items can easily be documented with a computer. Nowadays, software [e.g., winCATS, (CAMAG)] is specially designed to manage, monitor, and control all constituent steps of the planar chromatographic procedure. From sample application to TLC plate development to classical densitometry and image documentation, all necessary parameters are documented in one data file. The software manages and supervises all software-driven instruments. For nonsoftware-driven equipment, e.g., development in a glass tank or pre- or postchromatographic derivatization, the user can manually enter all related parameters, and the software will archive and report them in compliance with GMP/GLP. That means that one type of software, one data file, and one protocol are sufficient for the entire TLC procedure inclusive of the devices used.

B. Image Documentation

For documentation of the size, shape, and color of the individual zones, the chromatographic result can be reproduced graphically or stored as a whole (manual documentation), or it can be recorded as a photocopy, photograph, or electronic image (electronic documentation).

1. Manual Documentation

In former times, the original chromatograms were stored, i.e., the plate itself was the document. Storage of chromatograms was more convenient if TLC foils had been employed or if the adsorbent layer was fixed and removed from the plate as a whole. The latter was achieved by smoothly

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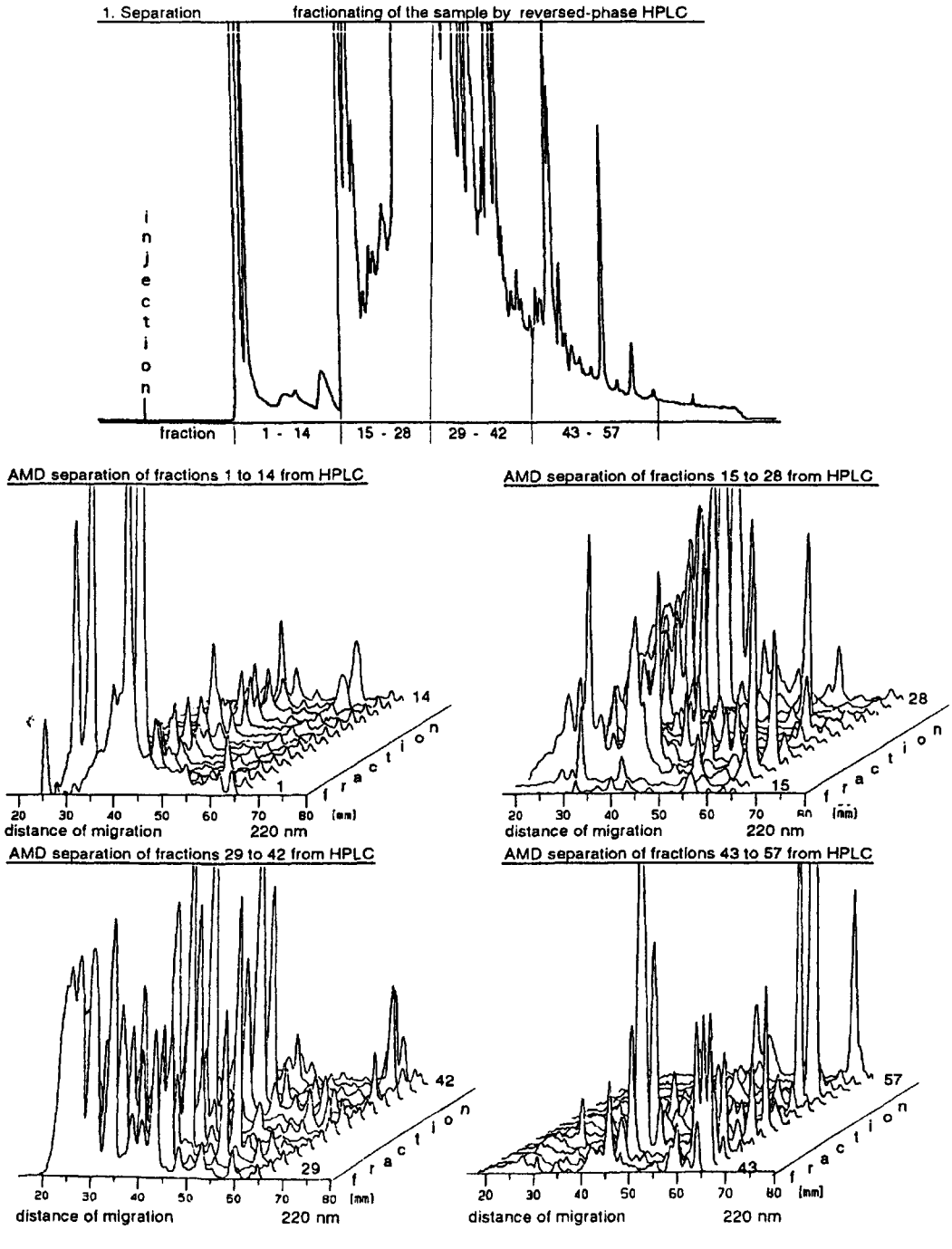

Figure 19 On-line HPLC/HPTLC (AMD) analysis of wastewater.

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Table 4 Important Items for Method Documentation

Sample preparation	Reference substance: name, amount, dilution factor, manufacturer, batch, purity. Solvents: manufacturer, batch, purity, stabilizer. Sample preparation or cleanup procedure.
Stationary phase	Type of plate, plate size, indicator, layer thickness, manufacturer, batch, description of pretreatment, impregnation, or conditioning.
Mobile phase	Composition of mobile phase, equilibration of vapor phase. Solvents: manufacturer, batch, purity, stabilizer.
Application	Application device, spot or spray technique, application scheme, application volume and other application parameters, drying mode after application.
Development	Technique of development, developing chamber system, volume of mobile phase, migration distance and time, temperature, humidity, drying mode after development.
Derivatization	Pre- or postchromatographic derivatization, preparation of derivatization reagents (manufacturer, batch, purity, stabilizer, etc.), detailed derivatization technique (spraying, evaporation, immersion). Reagents for stabilization or intensification of zones. Heating mode, temperature, and heating time of the plate.
Evaluation	Detection mode, principle of measurement, software version, scanner type, parameters of measurement, integration, quantification or spectroscopic identification.
Documentation	Date, time, user name, identification number, parameters of image acquisition, comments.

pressing cellophane tape or clear contact paper on top of the layer so that the adhesive came into uniform contact with the layer. Then the tape and the attached layer were carefully peeled away and fastened into a notebook. Treatment of the chromatogram with collodium (50) or plastic dispersions based on polyacrylic ester, polyvinyl chloride, or polyvinyl propionate (E. Merck, company literature about Neatan, 1975) was used also. These kinds of storage methods often entail degradation, fading of the zones, as well as changing of the color or blurring of the contours.

Furthermore, TLC separations can be reproduced by drawing, sketching, or tracing. For example, transparent paper can be placed on top of a glass-covered chromatogram, and the zones can be traced directly and colored with crayons or pens or marked in accordance with a color key system to reproduce the impression of color. However, these methods are tedious, time-consuming, and subjective.

2. Electronic Documentation

Direct copying on Ozalid or Ultrarapid blueprint paper (51) and contact printing (52) have been replaced by photocopying, photographing, or electronic image processing. Such phototechniques allow rapid retakes to produce the best possible result. Instant photography, photocopying, and electronic image processing even provide for immediate reproduction and decision making regarding acceptance or retake under different conditions.

a. Photocopying. Photocopying is the simplest way to record visible chromatogram zones. Relatively good reproductions can be achieved in black and white or even in color. Intense zones can be duplicated better than light ones.

b. Photographing. Chromatograms can be photographed in black and white or true color under visible or UV light with appropriate filters. Aside from electronic image processing, color photography is probably the best method for documenting chromatograms. When a long exposure time is necessary, especially for photographing fluorescent zones or for using filter combinations, handheld lights and cameras are undesirable and do not provide exact documentation. Therefore, commercial camera stands and suitable lighting units that can be combined with a large variety

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of conventional and instant cameras, for example, a Polaroid multipurpose reflex camera or a standard 35 mm camera, should be used. Lighting units feature direct shortwave UV light (254 nm), direct longwave UV light (366 nm), and direct and/or transmitted white light (400–750 nm). Additionally, transmitted midrange UV light (302 nm) is offered. The tubes operate with high-frequency (25–30) current; this ensures optimum light efficiency and eliminates synchronization problems with electronic cameras. The cabinet cover ensures complete exclusion of ambient light, so image capturing under all kinds of light is feasible in an undarkened room.

Ultraviolet photography. For UV photography, the entire chromatogram has to be illuminated uniformly by the UV light source. This is more difficult than with brighter, more intense white light sources. Illumination strikes the chromatogram at a proper angle from two sides in the reflected mode. In addition to completely excluding ambient light by using a cabinet cover, the excitation wavelength has to be cut off with a filter (barrier filter) placed before the camera lens. Further, UV tubes have to be covered with a special filter (bandpass filter) that permits only UV light to pass through and illuminate the zones, because otherwise a "wash-out" effect due to the excessive contamination of white light emitted from the UV tubes is observed. The effectiveness, in other words the transparency, of the blue bandpass filter can be reduced with increasing duration of irradiation, especially in the shortwave UV range. The resulting slight blue coloration of photos can be avoided by using a yellow or pale orange filter. In the transmittance mode, the frosted glass that is used as support for the HPTLC plate is replaced by a bandpass filter that allows through the midrange UV light (302 nm) emitted by tubes in the base of the instrument. This mode is used mainly for electrophoresis gels.

The above-mentioned barrier filter is used to absorb or remove unwanted UV radiation to prevent it from being recorded on the film because it is much brighter than fluorescence and causes the film to be overexposed. Thus, the more residual UV radiation is absorbed, the darker the background will become on the photograph. A correctly chosen barrier filter (Table 5) (53) will transmit only the visible wavelength of the fluorescent zone. Generally, a Wratten 2 E filter, which blocks all UV radiation but also cuts into the visible range, is recommended for recording yellow-green fluorescent zones at an excitation wavelength of 365 nm. For blue to indigo fluorescent zones, a Wratten 2 A or 2 B filter can be recommended. If all fluorescent zones should be recorded on the film, a Wratten 2 C filter can be used, but the residual UV irradiation between 385 and 400 nm will pass the filter and cause a grayish appearance on black-and-white film or a brownish background on color film. Wratten 3, 4, and 8 filters produce a very dark black background but cut off almost all of the visible blue spectrum. Consequently, violet and blue fluorescent zones are lost when these filters are used.

After the proper choice of the UV barrier filter, contrast and rendition can be enhanced by controlling the exposure time. The exposure time is primarily dependent on the intensity of the fluorescence and has to be optimized for each chromatogram. Experience has shown that operating with a range of exposure times, i.e., an aperture of f/8 with exposures of 15, 30, 60, 120, and 240 s, always leads to one optimal exposure time. In certain situations, substances can be adversely affected by UV light and fade rapidly under prolonged exposure (photobleaching). The exposure time for photographing zones of fluorescence quenching at a wavelength of 254 nm often applies for several recordings using the same conditions. A special glass filter (GG 435) placed in front of the camera lens often improves rendition (3). Color-correction filters are used in UV photography to lessen the amount of yellowness created by Wratten barrier filters (53). For example, a G (green) color correction filter, which absorbs red and blue, or an R (red) filter, which absorbs blue and green, can be used. Moreover, contrast filters for black rendition (mostly Wratten filters Blue 47 or Red 25) are employed for black-and-white UV photography to darken the zones against a bright fluorescent background. Corresponding contrast filters for white rendition (e.g., Wratten filter Green 58) brighten specific fluorescent colors (e.g., green) and make them appear white against a dark background (53).

CAUTION: All radiation below 350 nm is considered to be dangerous. Therefore, protective gear must be worn to protect the eyes and skin.

White light photography. In white light photography, a frosted glass plate serves to support the HPTLC plate as well as to diffuse the light. In normal cases, the zones are more visible in

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Table 5 Wratten Barrier Filters for UV Photography

Wratten gelatin filter number	Absorption of UV radiation (at and below)
2 C Pale yellow	385
2 B Pale yellow	390
2 A Pale yellow	405
2 E Pale yellow	415
3 Yellow	440
4 Yellow	450
8 Yellow	465

the transmission mode, with illuminating white light tubes at the instrument base, than in the reflection mode. Color-corrected white light is recommended rather than cool or warm white illumination for obtaining better color rendition. Most color films are designed to perform best at 5500 K. Therefore, when using warm light UV tubes of about 4000 K for illumination, a color temperature filter (Table 6) (53) is usually employed for color correction. Usually a Wratten gelatin filter is positioned between the camera lens and the UV barrier filter. Moreover, color correction filters are used to accentuate the color and control the contrast. Photographing through a filter of a complementary color (e.g., a yellow filter for a blue zone) makes the zone appear darker. The blue zone will appear lighter when photographed through a blue filter.

c. Electronic Image Processing. Video documentation systems for acquiring, printing, and archiving images of planar chromatograms have largely replaced instant photography systems. Their salient advantages are low cost per image, previewing and immediate optimization of the images on the screen, full compatibility with GMP requirements, high user-friendliness, and rapid data storage on the PC, all of these leading to durable results. The chromatograms are photographed in direct and/or transmitted light, depending on their quality. Even multiple detections of the chromatogram, i.e., several images of the same plate (visualization under white light, fluorescence quenching at UV 254, fluorescence at 366 nm), can be easily documented. The appropriate configuration, which includes the electronic settings for the CCD camera and frame grabber for a special illumination mode, has to be chosen. After the optimum contrast, contour, sharpness, illumination, etc., have been determined, images are captured, i.e., a digital "snapshot" is taken to create a colored or gray-scale image of the entire chromatogram. Single tracks or fractions of the chromogram can be edited very comfortably, and annotations can be made. Raw data and all parameters of their acquisition are stored in a secure file format that cannot be manipulated. The images can be exported in various open image formats. An image database makes it possible to manage many images along with their (computer-generated) ID, date and time of capture, infor-

Table 6 Illumination Filter Correction for Color Film (5500 K)

Illumination source	Blue filter number	Increase in exposure stops
3200 K	80 A	~1
3400 K	80 B	~1⅔
3800 K	80 C	~1
4200 K	80 D	~⅓

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Figure 20 CAMAG Reprostar 3 with cabinet cover and mounted digital camera. (Photograph courtesy of CAMAG.)

mation about the user, and special notes. Display and print formats can be selected. Images from the database can be selected at will for comparison, and all entries are searchable.

Photo Scanners and digital cameras are less expensive electronic image processing systems than CCD cameras. If photo scanners are used for image documentation, only visible wavelength zones (those illuminated in direct white light) can be documented. With a high-resolution digital camera (Fig. 20), the image quality is comparable to that of pictures taken with a conventional or instant camera. However, digital cameras have relatively low data transfer rates and are slower than image documentation systems that use a CCD camera. The software supplied with the digital camera or photo scanner is usually suitable for simple applications but is unfortunately not GMP/GLP-compliant so far because of the open file format. If this problem is solved in the near future, then high-resolution digital cameras will probably replace the more expensive video cameras.

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