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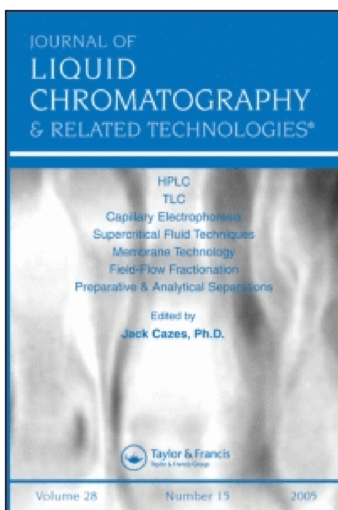

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NEW APPROACHES IN TLC-DENSITOMETRY

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NEW APPROACHES IN TLC-DENSITOMETRY

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ABSTRACT

The use of a TLC scanner can be regarded as a key step in high performance thin layer chromatography (HPTLC). Densitometric measurements transform the substance distribution on a TLC plate into digital computer data. Systems that allow quantitative measurements have been available for many years for either fluorescence or ultraviolet absorption measurements, while lately the reflection analysis mode for both types is the most common application. New scanning approaches are designed to aid the analyst who has common demands for TLC-densitometry without using special data, such as scanned images. Two examples that have been developed lately in the laboratories of the authors are described in this paper. These approaches were developed on the basis of current needs for analysts who employ TLC as a

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tool in research, as well as in routine analysis. One approach is aimed to support analysts in economically disadvantaged areas, where cost intensive apparatus is unsuitable but trace analysis by simple means is required. The other system, allows the spectral determination of chromatographic spots on TLC plates covering the ultraviolet and visible range, thus, revealing highly desired information for the analyst.

INTRODUCTION

Thin-layer chromatography (TLC) is one of the simplest chromatographic methods and can be used for identification, as well as quantification of chemical substances. Analytes are commonly identified by their retention factor (R_f) values, while the simplest way for quantification is based on visual inspection. The latter is limited to those cases where analytes can be visualized (e.g., by color or fluorescence) and where no data documentation (numerical values) is required.

Therefore, the use of a TLC scanner can be regarded as a key step in high performance thin layer chromatography (HPTLC). Densitometric measurements transform the substance distribution on a TLC plate into digital computer data. However, quantitative determinations in TLC require densitometric measurements of the spots on the TLC plate. Systems that allow such measurements have been available for many years for either fluorescence or ultraviolet (UV) absorption measurements, while lately the reflection analysis mode for both types is the most common application.

Very few new approaches in densitometry have been made over the past decade. However, the opposite is true regarding progress in other scientific fields, especially microelectronics and related issues. New optical sensors, such as the charged coupled device (CCD) modules, which have a sufficient sensitivity in both the UV and visible (VIS) range and are widely used in other applications (e.g., video cameras, computer scanners), as well as sufficiently powerful personal computers to perform image analysis are available today. The impact of these developments is illustrated by numerous publications in this field,[1–25] as well as specific conferences on image-evaluation in TLC. It can generally be expected, that video and scanner technologies available nowadays, can achieve results comparable to conventional TLC scanners with regard to precision performance for many applications. However, measurements by video imaging with a CCD-camera are cost intensive, and the possibility of obtaining spectral data of the substances is limited. Systems for the acquisition of spectral data have been described for TLC[25] and require interferometers in addition to the video system. However, such systems cover the spectral range of 400 nm–1000 nm only, which would limit their application, while spectral data acquisition down to the

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range of 200 nm is a highly desired feature for substance identification, as well as for quantification.

Other approaches have been made with the use of computer scanners where the principle is the same used in video-imaging. One example of the application of commercially available computer scanners has been shown to work satisfactorily, and the simple algorithms used have been published.[26] A number of commercial software applications are also available, such as the sorbfil software package.[27] However, it must be noted, that the image evaluation software used for video-acquisition does not differ technically from those designed for computer scanners, since both deal with the same type of data images. An interesting example that shows the advantages of the scanner technology, was the determination of lactose in animal feed after TLC separation.[23]

Simple modifications can make office scanners suitable for fluorescence measurements to overcome the limitations of working in the VIS range. It has been reported, that replacing the light source with a commercially available mercury tube and measuring the emission selectively by inserting a cut-off filter causes the excitation of fluorescent spots.[29] Remarkable results have been achieved for the detection of aflatoxins (excitation 366 nm, emission 435 nm), allowing the determination of their fluorescent spots on TLC plates at the lowering level.[28]

The approaches described below are the result of constant progress in the above fields, and they are designed to aid the analyst who has common demands for TLC densitometry without using special data such as scanned images. One approach is aimed to support analysts in economically disadvantaged areas, where cost intensive apparatus is unsuitable, but trace analysis by simple means is required. The other approach allows the spectral determination of chromatographic spots on TLC plates covering the UV and VIS range, thus, revealing highly desired information for the analyst.

SEMICONDUCTOR BASED DETECTION CELL

A different type of a low cost approach based on a simple detection cell[29] has been described, and the system has recently been further developed. The main improvements were achieved by the use of new components that were previously not available, such as a single-band filter (436 nm) rather than a cut-off filter, and the use of a different type of UV-diode with less sensitivity in the lower UV range (<400 nm) and a further modified electronic circuit. Since the circuit is under constant development, the current versions are available on the Internet for downloading.[30]

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These modifications increased the sensitivity significantly, mainly by reducing the previously observed background noise and, therefore, allowing the determination of e.g., aflatoxin spots at lower levels than described before. As can be seen in Fig. 1, the densitogram obtained is sufficiently free of background noise even at a level of 0.5 ng of aflatoxin B1 per spot on silica gel 60 TLC plates. When signals were integrated, the correlation coefficient was found to be 0.998, which shows the linearity for measurements between 0.5 ng–5 ng per spot.

An amount of 0.5 ng per spot corresponds approximately to visual detection (untrained eye) under UV light. This reflects a contamination level several times below what is regulated by current legislation, in combination with an adequate sample clean-up method.[31]

Since such a basic prototype is generally not appropriate for most analysts, even though easy to re-build, additional efforts were made to increase the comfort of data acquisition. Two main features were identified to be essential for an easy and successful application: a) supply of simple and adequate software to record the signals on a computer (preferably handheld devices) and b) assurance that no

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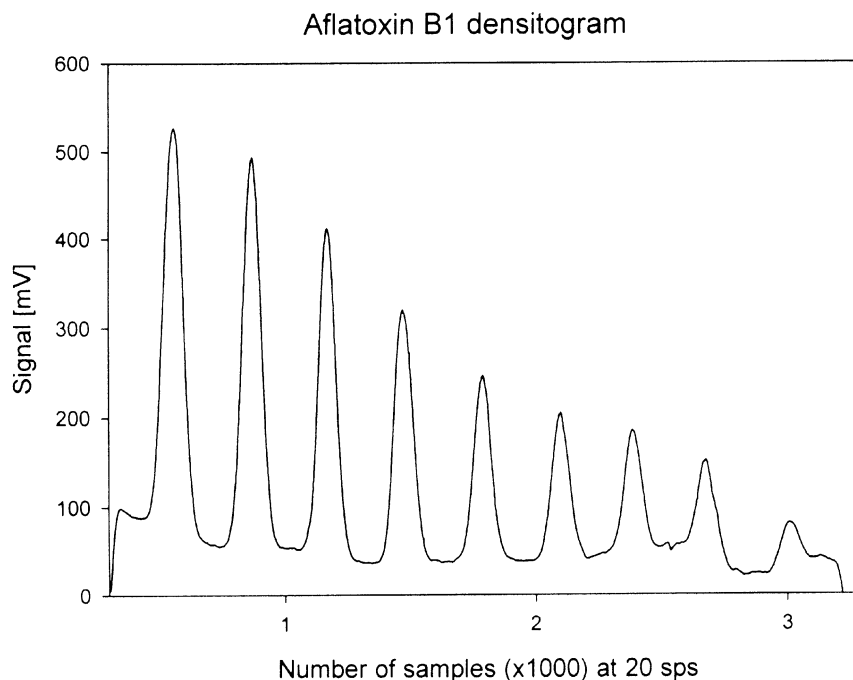

Figure 1. Vertical fluorescence densitogram of aflatoxin spots ranging from 4.5 ng to 0.5 ng in 0.5 ng steps obtained with a further developed detection cell system.

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mechanical movement is required of the analyst during scanning. The latter can be achieved by the use of a simple musical-box engine, which has proven to be sufficiently stable in movement (signal determination is performed by height) and does not require electrical supply or electronic control circuitry. A software based on WindowsCE® was developed for facilitated data acquisition, which allows the evaluation of the recorded scan on a mobile pocket-size PC. While this software can be downloaded from the Internet,[30] additional efforts are currently being made to modify a simple Gameboy® for data acquisition of densitometric scans. Similar applications of such devices (modified Gameboys®) have been described in the past.[32–34]

A simple and almost general derivatization principle for a broad number of different compounds, especially for simple fluorescence measurements, was described about 30 years ago.[35] This procedure resulted in fluorescent derivatives for many different substances with an excitation wavelength around 360 nm–380 nm and an emission of >400 nm. Substances could be quantified in the ng range. This principle was further investigated and modified, showing that visual fluorescence observation down to 1 ng for several different substances could be achieved.[36–38] Similar approaches for the visualization of sugars on amine modified TLC plates have been described[39,40] when TLC plates are heated for a short period. These methods should be applicable to a wide range of substances when the derivatization is modified accordingly. Currently, research is performed in our laboratories to further simplify this derivatization procedure and to evaluate its applicability for trace analysis of mycotoxins and other classes of food contaminants in combination with the simple detection cell.

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SLIT SCANNING DENSITOMETER

Another novel approach is based on a slit scanning densitometer, which works reliably by showing a small resolution on HPTLC plates and being able to scan a track simultaneously at different wavelengths within less than one minute. Such a system allows the spectral data acquisition with high sensitivity, resolution, and speed.

EXPERIMENTAL

Apparatus

An item was designed for direct spectrophotometry of HPTLC plates that consists of a diode array spectrophotometer (J&M Aalen, Germany) working in a range of 197 nm to 612 nm with an average optical resolution of 0.8 nm.[40]

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A home-made reflection attachment of 50 identical optical fibers, with a diameter of 100 μm each, transports light of different wavelengths from a deuterium lamp to the HPTLC plate and back to the diode array detector. In this detecting mode, the light source and detector are both placed 450 μm above the surface of the HPTLC plate. Commonly used scanners need an angle of 45° between the light emitting device and the bulky detector. Due to the Lambert cosine law $I = I_0 \cos \vartheta$, this angle (ϑ) is responsible for a reflected light intensity reduction (I) of nearly 30%.[41] The new light fiber array overcomes this limitation. For dense light intensities, the light emitting and the light detecting fibers are arranged parallel to each other, because the Lambert cosine law only predicts an optimum response in this arrangement. The HPTLC plate is placed horizontally on a mechanical stage, which can be moved by using two motors from the Micropack company (Stuttgart, Germany). The linear slide system works with constant velocity during reflection measurements. The whole device does not need any lenses, filters, or slit-width adjustments.

The constructed optical fiber arrangement is shown in Fig. 2. In the double row interface, a set of 25 fibers arranged in a separate line, transports light on to the plate.[42] The detecting channel is formed as a line as well, fixed close to the illuminating row. This double row array shows a length of nearly 3.5 mm and a resolution on a plate of better than 145 μm.[43] The size of the scanning slit should be as small as possible, in order to achieve a concentration distribution within the

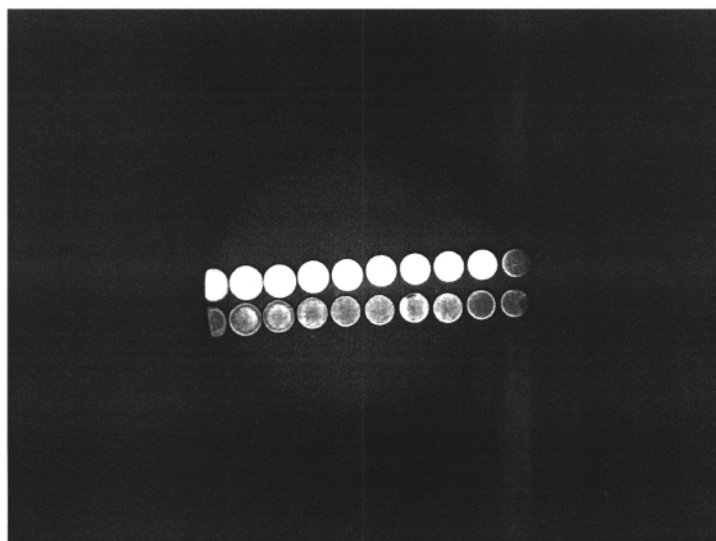

Figure 2. Close-up image of the double row interface.

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area of the substance spot on the HPTLC plate that is approximately constant. Each illuminating fiber forms a light spot on the HPTLC plate, the size of which depends on the numerical aperture of the fiber used. In the same way, the reading fibers can only register spots of identical size. The measurement input of this fiber array is a function of all overlapping areas of the illuminating and detecting fiber spots. The result is a small scanning slit, which is mainly dependent on the distance of the fiber array above the plate. Checking the distance of the fiber arrangement over the HPTLC plate is the only adjustment needed.[40]

Reagents and Measurements

HPTLC plates (10 × 10 cm) with the stationary phase silica gel K60 F254 were used. The plates as well as sodium hydroxide, sodium hydrogen carbonate, methanol, and ethyl acetate were obtained from Merck (Darmstadt, Germany).

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Sample Preparation

One mL of urine sample was made alkaline with 0.2 sodium hydroxide (0.5 mol L^{-1}) and adjusted to pH 9 by the addition of sodium hydrogen carbonate. Three mL of ethyl acetate was used for extraction. After centrifugation, the organic layer was separated and evaporated to dryness with help of an argon stream at 40°C. The residue was reconstituted in 50 μL methanol–ethyl acetate (1 : 1).

Sample Application and Chromatography

An aliquot of 5 μL was spotted in a band (7 mm) at a distance of 10 mm from the plate side using a Camag Linomat IV device. The plate was developed in a saturated chamber to a distance of 50 mm using ethyl acetate–methanol–ammonia (25%) (85 : 10 : 5) as the mobile phase. For the scan of the whole track, 450 spectra in the range of 197 nm to 612 nm were measured over a distance of 45 mm. Each spectrum was measured within 0.5 sec.

FORENSIC APPLICATIONS

HPTLC is a common technique in forensic sciences for detecting drugs and potential harmful compounds over a large polarity range in body fluids like blood and urine. Many standardized solvent systems have been developed for this purpose.[42] The local anaesthetic lidocaine and diphenhydramine, a drug against

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travel sickness, are both difficult to detect, because they show light absorptions only in the wavelength range below 230 nm. It is hardly possible to register UV spectra of both compounds using HPLC because of mobile phase interference. The HPTLC technique offers the measurement of UV spectra without any interference of the mobile phase. The new fiber array scanner allows detection and quantification of compounds with restricted spectral absorption in the wavelength range below 230 nm.

While the scanner measures an HPTLC track, light from a deuterium lamp is transported to the plate surface. This light can be either absorbed or scattered inside the layer. The scattered light is reflected as remission light I_{rem} (λ) from the plate surface. The diode-array equipment detects this light, which carries the relevant information of how much light is being absorbed either by the sample or the stationary phase. A set of remission values $R(\lambda)$ at different wavelengths is calculated using the remission light I_{rem} (λ) corrected by a reference according to Eq. (1). The remission light I_{ref} (λ) should be taken from a clean plate surface. The reference values $R(\lambda)$ are dependent only on the absorption of the separated compounds.

$$R(\lambda) = \frac{I_{rem(\lambda)}}{I_{ref}(\lambda)} \tag{1}$$

The open-ended selection of an appropriate reference I_{ref} (λ) offer HPTLC a powerful way to individual analyte adoptions and base line corrections.[44] Usually the reference spectrum is chosen at the computer terminal after the track measurement is finished. This makes different reference selections easily available to find the evaluation optimum. If a sample spot absorbs more light than the chosen reference, remission values $R(\lambda) < 1$ are observed. For transformation into positive absorption values, it was found best to use the negative logarithm according to Eq. (2).

$$A(I_{rem}, \lambda) = \ln R(\lambda) \tag{2}$$

All parts of the track that absorb more light than the chosen reference spectrum are shown as colored. Fluorescent spots can easily be seen if Eq. (3) is used for spectral calculations.

$$F(I_{rem}, \lambda) = -\ln R(\lambda) \tag{3}$$

Using Eq. (3) all parts of the track that emit more light than the chosen reference are shown as colored. The scanner detects fluorescence without the use of any special arrangements.[44] The scan data include the fluorescent information as well as the information about absorptions.

The contour plot of a urine sample evaluated using formula (2) in combination with a reference spectrum measured at 30 mm is shown in Fig. 3.

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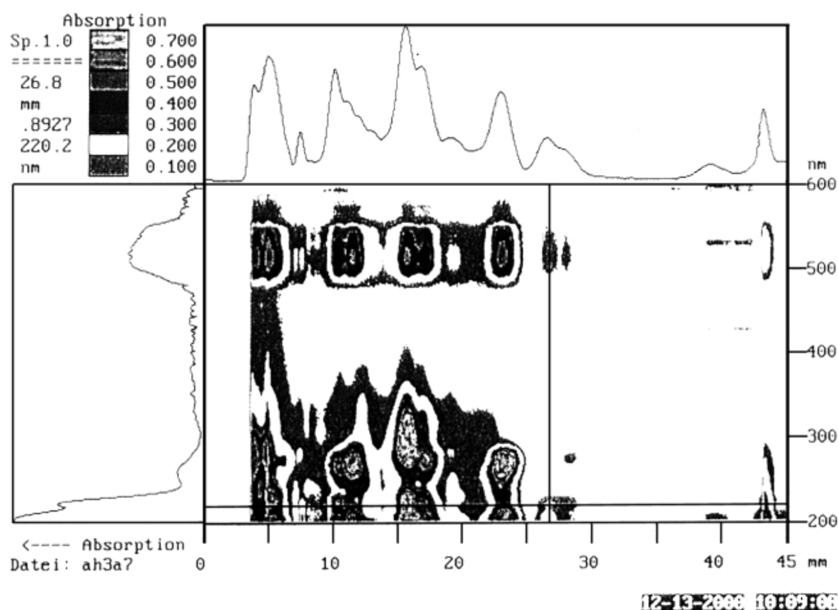

Figure 3. Contour plot of a urine sample with diphenhydramine spot at 26.8 mm. The spectrum of diphenhydramine is shown on the left and the densitogram at 220.2 nm on top.

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The point of application at 4 mm and the front signal at 43 mm can be clearly seen. Over the distance from 5 mm to 30 mm, several spots are visible. All these separated compounds show light absorptions only in a range from 200 nm to 400 nm. Absorption signals are also seen around 530 nm. These signals are caused by the fluorescent dye fixed in the stationary phase of the HPTLC plate. This dye absorbs light around 245 nm and emits fluorescent light between 500 nm and 550 nm. The reference spectrum at 30 mm shows high fluorescent light intensities between 500 nm and 550 nm. If a separated compound covers the dye it prevents light absorption and makes light emission around 530 nm impossible. This loss of light intensity in comparison to the reference is responsible for the absorption signals above 500 nm. The marked spot at the separation distance of 26.8 mm shows a spectrum (left) with weak absorptions around 245 nm. This results in poor absorptions between 500 nm and 550 nm. The spot at 26.8 mm distance shows perceivable absorption values only below 220 nm. In the densitogram on top of Fig. 3 taken at 220.2 nm, the spot at 26.8 mm is seen only as a weak and insufficiently base-line separated peak. The spectrum (left) indicates diphenhydramine with a spectral fit of better than 99% compared with a

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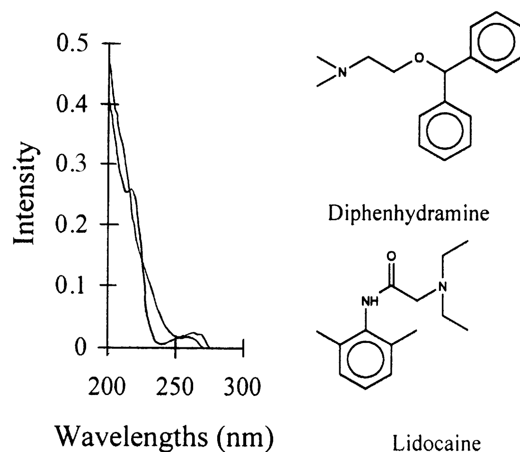

Figure 4. Structures and spectra of 250 ng of diphenhydramine (lower graph) and 250 ng of lidocaine (upper graph).

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reference spectrum taken from a spectral library.[45] Spectra and structures of diphenhydramine and lidocaine are given in Fig. 4.

CROSS-CORRELATION FUNCTION

For spectra identification, we found it best to use the cross-correlation function of two absorption spectra.[45] The multiplication of the sample spectrum $A(\lambda)$ and the reference spectrum $A_{\text{ref}}(\lambda)$ at n wavelengths can be described as:

$$\Im\Re = \begin{pmatrix} A_1 & * & A_{\text{ref}1} \\ A_2 & * & A_{\text{ref}2} \\ \vdots & * & \vdots \\ A_n & * & A_{\text{ref}n} \end{pmatrix} \tag{4}$$

It results in the cross-correlation vector $\Im\Re$. The auto-correlation of the sample vector is calculated by multiplication of each wavelength-dependent value of the sample spectrum by itself. For n values, the vector $\Im\Im$ is the result.

$$\Im\Im = \begin{pmatrix} A_1 & * & A_1 \\ A_2 & * & A_2 \\ \vdots & * & \vdots \\ A_n & * & A_n \end{pmatrix} \tag{5}$$

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The auto correlation vector of the reference spectrum $\Re\Re$ is obtained appropriately. To classify the rate of percentage identity $\aleph$ (the fit factor), all vectors have to be summed up over the desired wavelengths.

$$\aleph = \frac{\sum_1^n \Im\Re}{\sum_1^n \Re\Re}\sqrt{\frac{\sum_1^n \Re\Re}{\sum_1^n \Im\Im}} * 100\% \quad (6)$$

The value of $\aleph$ represents the percentage fit of the sample spectrum in comparison to the reference spectrum. A fit of 100% means full agreement of sample and reference. A fit of above 90% is usually regarded as sufficient for a definite compound identification.[45]

For peak identification, it is reasonable to use an averaged spectrum of seven single spectra. These seven spectra cover a space of 0.7 mm on the TLC plate. All peaks in TLC or HPTLC are broader than 0.7 mm, and this allows peak identification by spectra bundling.

The UV spectra of different amounts of diphenhydramine (125 ng, 250 ng, 375 ng and 500 ng) were compared with the diphenhydramine spectrum of a urine sample according to formula (6). For calculation of fit values, the spectral range from 200 nm to 300 nm was used. The calculation resulted in fit values between 99.3% and 99.7%. It is, thus, definitely possible to identify diphenhydramine in the urine sample. The identification of lidocaine in a similar urine sample[45] yielded fit values between 98.0% and 98.4% respectively.

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QUANTIFICATION OF DIPHENHYDRAMINE

An important advantage of the new scanner is the possibility to bundle different diode-array signals to one single densitogram.[44] This is a very effective way of reducing noise. Figure 5 shows the densitograms of 125 ng of diphenhydramine measured at 200 nm by use of a single diode (bottom) and the averaged signals of seven single diodes, active in the wavelengths range from 200 nm to 210 nm (densitogram second from the bottom). The noise can be further reduced using a five data-point moving average for smoothing (densitogram third from the bottom). The densitogram on the top consists of 450 vectors $\Im\Re$, summed up in the wavelength range between 200 nm and 400 nm, respectively. A single vector $\Im\Re$ is the result of the multiplication of a sample spectrum $A(\lambda)$ with a diphenhydramine library spectrum $A_{ref}(\lambda)$. This densitogram is very similar to the densitogram derived from the seven averaged diodes, but here the choice of wavelength range is redundant.

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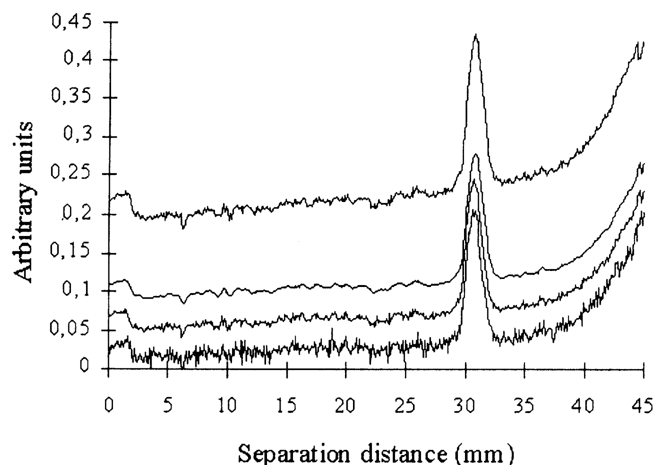

Figure 5. Densitogram of 125 ng of diphenhydramine measured at 200 nm by use of a single diode (bottom), averaged signals of seven single diodes, smoothed densitogram, and cross-correlation densitogram (top).

The cross-correlation of all track spectra with a target spectrum automatically results in the best densitogram for this target compound.

PEAK PURITY TEST AND QUANTIFICATION

A successful peak-purity check is recommended before quantification of the peak. Besides peak identification, the cross-correlation function makes peak purity evaluation easily available. Using the fit function (6), there is absolutely no reason for choosing individual wavelength combinations for common peak purity investigations. Peak identification and peak purity investigation can be done parameter-free and automatically.[45] If the cross-correlation function is applied to all measured spectra of an HPTLC track and if the diphenhydramine spectrum is used as reference, the fit-distribution of Fig. 6 is the result. Here the fit function and the track densitogram measured at 200 nm are drawn. The track densitogram is shown as a thick line and the fit function as a thin line.

The fit-function measured for all 450 spectra shows a nearly rectangular peak shape around 26.8 mm with a fit value of nearly 100%. The high fit value identifies the peak as diphenhydramine. A rectangular shape would indicate a pure peak but the fit function is not completely rectangular. The slightly curved fit distribution on the right side of the peak assumes a contaminated peak. The

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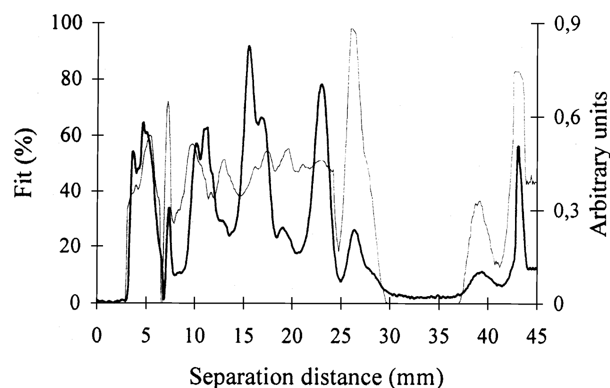

Figure 6. Fit distribution (thin line) and densitogram of a urine sample track (thick line).

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diphenhydramine peak is incompletely separated but, nevertheless, a quantification using the peak height is possible.

The calibration function of different diphenhydramine amounts in a range from 125 ng to 1000 ng shows linearity (Fig. 7). This result is observed either for peak area or peak height evaluation. Using the diode-array scanner in combination with formula (2) for calculation, linearity over more than two magnitudes can be observed.[44] Probably, the diode-array detector, instead of the use of a photomultiplier and the avoidance of a Lambert cosine term in the reflected light, is responsible for this result. Nevertheless, the use of different evaluation formulas affects linearity as well.[45] Further research has to be done in this field.

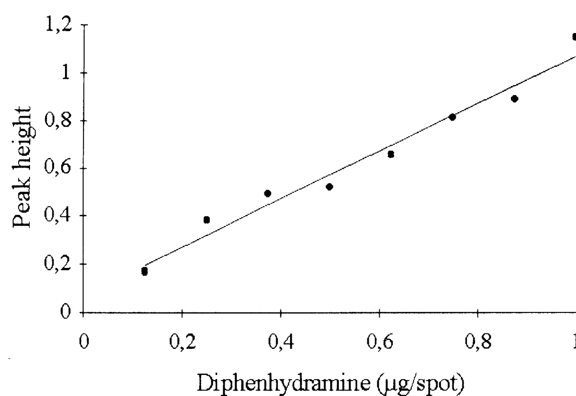

Figure 7. Calibration function of various diphenhydramine concentrations.

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CONCLUSION

The approaches described here, indicate that current developments in other fields of science prove to be helpful tools for solving current challenges in TLC. These new applications are complementary to other current systems such as video scanning, increasing the overall application of TLC to solve current problems in analytical chemistry.

The miniaturized semiconductor-based detection cell was found to be an inexpensive promising alternative to the currently used equipment for aflatoxin quantification by TLC. Due to low power consumption, this approach offers operation by means of batteries. In addition, the facilitated data acquisition using simple informatic tools makes this approach economically attractive for control of food or other commodities, even under very simple laboratory conditions.

The basic invention of the slit scanning densitometer is the use of some special fiber arrangements for high light detection, even below 200 nm, in combination with a diode array detector. The new system scans spectra in absorbance and fluorescence modes without the use of any mirrors or lenses. The simultaneous measurements at different wavelengths allow quick peak identification and peak purity investigation. In conclusion, the system is well suited, even to investigate complex samples such as urine, blood, and gastric contents. The system identifies target compounds automatically by use of UV spectra libraries. Therefore, this new fiber arrangement meets the demands of a modern HPTLC scanner as well.

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