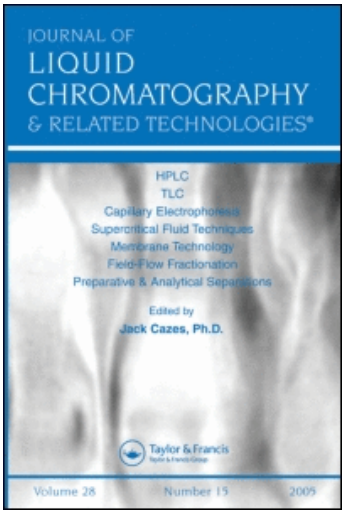

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A SIMPLE METHOD FOR QUANTIFYING TRIAZINE HERBICIDES USING THIN-LAYER CHROMATOGRAPHY AND A CCD CAMERA

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A SIMPLE METHOD FOR QUANTIFYING TRIAZINE HERBICIDES USING THIN-LAYER CHROMATOGRAPHY AND A CCD CAMERA

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□ *We present a video-densitometric quantification method for the triazine herbicides atraton, terbumeton, simazine, atrazine, and terbutylazine. Triazine herbicides were separated on silica gel using methyl-t-butyl ether, cyclohexane (1 + 1, v/v) as mobile phase. The quantification is based on a derivation reaction using chlorine and starch-iodine which forms red-brown triazine zones. Measurements were carried out using a 16 bit ST-1603ME CCD camera with 1.56 megapixel from Santa Barbara Instrument Group, Inc., Santa Barbara, USA. A white LED was used for illumination purposes. The range of linearity covers two magnitudes using the (1/R-1) expression data transformation. The signal-to-noise ratio increases directly linearly with the measurement time. The separation method is cheap, fast and reliable.*

Keywords derivatization, HPTLC, linear calibration range, quantification, TLC, triazine herbicides, video densitometry

INTRODUCTION

Modern TLC-scanners can measure in absorption, fluorescence, and also in transmittance. TLC-scanners cover the whole wavelength range from 200 up to 1000 nm. The disadvantage of TLC and HPTLC scanners is their high purchase price and maintenance costs. The high price of modern TLC-scanners makes image analysis in thin-layer chromatography (TLC) so interesting.[1] Most TLC-applications are designed to work in the wavelength range from 400 to 800 nm, using human eyes as detectors. Scanning equipment like CCD-cameras (charge coupling device-cameras) or flatbed-scanners working in the visible range are cheaply available and can be used for plate evaluation.[1] The term video-densitometer has also been introduced for such scanning devices.

This work is dedicated to Dr. R. E. Kaiser on the occasion of his 80th birthday.

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Historically, the first publication contributing to this subject was published in 1968. Hannig and Wirth described a new electronic densitometer based on the principle of the "flying spot." A light spot of a cathode tube dissects transparent gels. The transmission light is measured by use of a TV camera (Vidikon).[2] The principle of transmission scanners is still used to a large extent for evaluating electrophoresis gels.

The evaluation of two-dimensional electropherograms by use of a Kodak film was published 1979. The method was used for zone localisation. No peak quantification was described although the paper title is "Quantitative comparative analysis of complex two-dimensional electropherograms."[3]

The quantitative measurement of two-dimensional radioactive gels was published in 1979. A film scanner with a resolution of 8 bits rendered a silver-photo developed by the gel radioactivity into digital data. A non-linear calibration curve was published plotting radioactivity against scanner grey-level.[4]

The evaluation of a Coomassie blue stained protein gel by a video-camera picture was published in 1980. Linear calibration curves for stained proteins in the range from 1.5 to 6 μg per spot were published.[5] The evaluation of a stained TLC-plate for the quantification of the antibiotics benzylpenicillin and kanamycin B using ninhydrin for staining was described in 1980. The stained TLC-plate was digitised by a television scanning device (Optomax). Quantification was done by setting logarithm concentrations equal to measured zone areas.[6]

The first video-densitometric quantification directly from a TLC-plate was published in 1983. An RCA black-and-white camera with 280 columns and a 192 row array and 8 bit intensity resolution was used to evaluate charred cholesterol and ceramide. A curved calibration function in the range from 0.2 μg to 4 μg per spot was obtained.[7]

Prošek and Kaiser published, in 1984, similar results for ergot alkaloids in the range from 50 to 300 ng per HPTLC zone.[8] Video-densitometric evaluation of gel electrophoresis separations have been published by Manabe and Okuyama. The direct video-densitometric quantification by use of a black-and-white TV-camera resulted in a curved calibration function for a mass-range from 0.05 to 3.2 μg of bovine serum albumin per zone. This kind of quantification strongly depends on the available microcomputer, as the publication title states.[9]

In contrast to reflectance measurements, the video-densitometric evaluation of fluorescence zones results in linear calibration curves. Tetramethylrhodamine isothiocyanate shows a linear response in the range from 2.5 to 125 ng.[10] In general, measurements in reflectance using video-densitometric devices always result in a poor linear calibration range of less than one magnitude.[11]

The advantage of video-densitometric devices is their small size and low weight which make these camera systems portable.[12] The use of a CCD device provides ultraviolet response and a larger dynamic range than a photomultiplier.[13,14] The evaluation of 2D separations is possible which is not possible with slit-scanners. The principle of CCD-scanning is not restricted to cameras. A flat-bed scanner can also be used for plate measuring.[15] Commonly used flatbed-scanners illuminate the plate with white light and can scan coloured zones. Even fluorescence can be measured if the flat-bed scanner is equipped with a UV-lamp.[16] The cheapest CCD-technique for TLC evaluations is to use a hand-scanner.[17]

The disadvantage of a video-densitometer is that spectral information is not available. This makes spectral peak identification and spectral peak-purity tests impossible. Otherwise, most substances show no light absorption or fluorescence in the vis-range. To make separation more specific, we recommend a staining step which often makes spectral identification and peak-purity testing unnecessary.

What features should be taken into consideration when buying a CCD-camera? Quantitative video-densitometric measuring needs a detector, which can linearly digitalize light intensity measurements. Double-fold light intensity must result in doubled signal values, which can be checked by changing the measurement time. Therefore, double measurement time must result in doubled measured values. The digital resolution of commonly used cameras is 8 bit. A signal is rendered in $2^8 = 256$ different increments (grey levels), which is not sufficient for quantification purposes because at least 12 bit capacity is necessary for quantifying ($2^{12} = 4096$ increments). CCD-cameras with a resolution of 16 bits are much better because such cameras render $2^{16} = 65536$ grey scales. Relatively inexpensive cameras with suitable software that meet these requirements are available for astronomy observations. These cameras produce TIFF-pictures, because the TIF-Format (Tagged Image File Format) supports 16 bit data storage.

Although inexpensive flatbed scanners and cheap cameras are not linearly calibrated, it is nevertheless possible to quantify planar chromatography separations. For example the heavy metal complexes cobalt-dithizone and zinc-dithizone can be quantified but the working range covers only a single magnitude due to the non-linear detector.[17]

The group of triazine herbicides, which includes atrazine, causes underground water contamination. Atrazine is said to have a carryover, a generally undesirable property for herbicides. In 1952 H. Gysin and E. Knüsli synthesized a number of substituted triazines. A first publication appeared in 1955.[18] Triazine herbicides are colourless compounds detectable only in UV. Triazine herbicides react with chlorine and iodine-starch forming brown-red spots[19] when separated by thin-layer chromatography.[18,20–22]

This reaction can be used to quantify TLC-separated triazine herbicides using a CCD-camera.

The purpose of this work is to show that video-densitometric measurements provide a powerful tool for inexpensive quantitative thin layer chromatography. Linear calibration functions over two magnitudes and more can be obtained using a linear measuring device in combination with the correct remission theory.

THEORY

In planar chromatography, light is used for detecting separated sample spots by illuminating the TLC-plate from the top with light of known intensity. A clean illuminated plate will absorb a share of this illuminating light by the layer. The share of light, which is not absorbed but reflected by the surface, should be J_0. If this reflected light shows higher intensity than the reflected light (J) from a sample zone, a fraction of light must be absorbed by the sample (the analyte). The difference between these light intensities is absorbed by the analyte and defines the analyte absorption coefficient *a*:

$$I_{abs} = J_0 - J = aJ_0 \tag{1}$$

Increasing sample amounts will induce a decreasing light reflection (*J*). Therefore, a transformation algorithm is needed which turns decreasing light intensities into increasing signal values. Ideally, there should be a linear relationship between the transformed measurement data and the analyte amount.

With the abbreviation:

$$R = \frac{J}{J_0} \tag{2}$$

we see that theoretical considerations lead to the following equation for transformation purposes that show linearity between the transformed measurement data (TMD) and the absorption coefficient.[23]

$$TMD(k) = k\left(\frac{1}{R} - R\right) + (R - 1) = \frac{a}{(1 - a)} \tag{3}$$

k: backscattering factor ($k \geq 0$ and $k \leq 1$); a: absorption coefficient

The value of the so called backscattering factor k is in the range between 0 and 1 and depends on the scattering quality of the stationary phase. In TLC the Kubelka/Munk theory is often used for evaluation purposes. The Kubelka/Munk theory was first published in the year 1931

and is based on the assumption that half of the scattered flux is directed forward and half backwards.[23] The backscattering factor in the Kubelka/Munk–theory is $k = 1/2$ and the correct Kubelka/Munk–expression can be used to obtain linear calibration curves for high analyte concentrations. In trace analysis, it is mostly sufficient to use a k-factor $k = 1$ for obtaining linearity for calibration curves.

$$TMD(k = 1) = \frac{1}{R} - 1 = \frac{a}{1 - a} \tag{4}$$

For k = 0 no incident light is reflected to the plate top and the resulting expression can be used for fluorescence evaluation.[23]

EXPERIMENTAL

Preparation of Standards and Application on HPTLC-Plates

All the chemicals used were of analytical reagent grade. Terbumeton, simazine, atrazine, terbutylazine, atraton have a purity of ≥98% and where purchased from Ehrenstorfer, Augsburg. Potassium iodide was from Riedel-de Haën, Seelze, Germany and ethanol from Roth, Karlsruhe, Germany. Cyclohexane, starch (according to Zulkowsky), HCl, $KMnO_4$ and methyl-t-butyl ether were purchased from Merck, Germany as well as silica gel K60 Lichrospere® (with a fluorescent dye) used as the stationary phase.

Stock solutions were prepared by dissolving 4,000 mg of standard trazine herbizides in 25 mL of methanol. For calibration purposes, the stock solution was subsequently diluted with methanol in order to apply amounts of 1 to 20 µL.

Samples and standards were spotted dash-like (7 mm) on an HPTLC silica gel Licrospere® plate (10 × 10 cm, with fluorescent dye) using a DESAGA AS 30 device. The plates were developed in a vertical developing chamber without vapour saturation to a distance of 70 mm from the starting point, using methyl-t-butyl ether, cyclohexane (1 + 1, v/v) as the mobile phase.

Plate Staining

The plate was dried in a gentle stream of air for 5 minutes and placed in a chlorine containing chamber for 5 minutes. Chlorine was produced from 10 mL $KMnO_4$-solution (3 g $KMnO_4$ dissolved in 100 mL of water) and 10 mL HCl (25 mL 32% HCl dissolved in 50 mL of water). Five minutes after mixing, the chamber was filled with chlorine and the TLC-plate could be placed.

The staining reagent starch-iodine was found to be sufficiently sensitive. To produce the starch-iodine reagent, 800 mg of potassium iodide was dissolved in 20 mL of water. 800 mg starch (according to Zulkowsky) was dissolved in 20 mL of water. Both solutions were mixed and dissolved with 10 mL of ethanol. The mixture is stable for one day.

Red-brown zones are formed on a slightly dark background, if the chlorinated plate is dipped for 1 s in starch-iodine reagent. The colors are stable for days, if stored in the dark.

Apparatus

For direct video-densitometric evaluation, a ST-1603ME CCD camera with 1.56 megapixel from Santa Barbara Instrument Group, Inc., Santa Barbara, USA was used. The camera was mounted with a Kodak KAF-1603ME CCD pixel array containing 1530×1020 pixel. The array size is 13.8×9.2 mm with a pixel size of 9×9 microns. The camera uses a 16 bit A/D converter and a high speed USB interface. The camera was used in combination with a Schneider SKR KMP Xenoplan 28/2,0 – M30,5 lens. For plate evaluation the CCD-array was cooled to $-5°C$. The plate was measured in the dark using two LEDs emitting white light. The time of 6 seconds is necessary to measure the full 16 bit range.

RESULTS AND DISCUSSION

The HPTLC-plate is placed below the camera at a distance of 30 cm. This distance is adjusted so that 8.5 cm are detected by 1020 pixel providing a resolution of 83.3 μm per pixel. A single mm separation distance is measured by 12 diodes producing 12 data points.

Figure 1 shows the result of a video-densitometric evaluation of the five triazines atraton, terbumeton, simazine, atrazine and terbutylazine (50 ng each), separated on silica gel with the mobile phase methyl-t-butyl ether and cyclohexane (1 + 1, v/v). The plate was stained with iodine-starch reagent after treatment with chlorine. The application band width of different analyte amounts is 7 mm. Each application band was measured with 64 data points resulting in 64 densitograms. These 64 densitograms measured from each band-wise application were combined in a single densitogram. This data averaging improves the signal-to-noise ratio by a factor of 8 in comparison to the signal-to noise ratio of a densitogram registered by only a single pixel. It's important for all tracks to be evaluated with the same number of measurement data located in the centre of the application band. To achieve reliable evaluation, all tracks must also be evaluated using the same position within the track and the same evaluation widths.

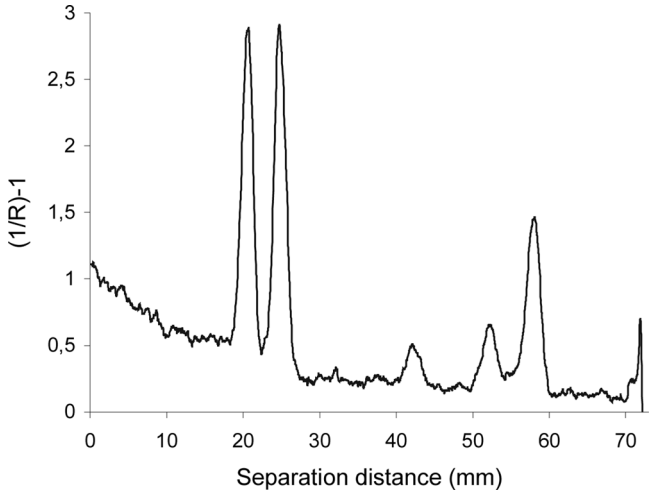

FIGURE 1 Plotted is the separation of atraton, terbumeton, simazine, atrazine and terbutylazine (50 ng each, from left to right) on silica gel evaluated with expression (4). As eluent methyl-t-butyl ether, cyclohexane (1 + 1, v/v) was used for a separation over a distance of 70 mm.

Figure 2 shows the brightest plate area in bits plotted against the measurement time. The plot emphasizes that the camera measures light intensities in a directly linear way. The response curve is not logarithmic as is the case in all commonly used cameras in our daily life.

A sample application was measured at six different time intervals (1 to 6 seconds) and evaluated using a single diode in the CCD-array. The

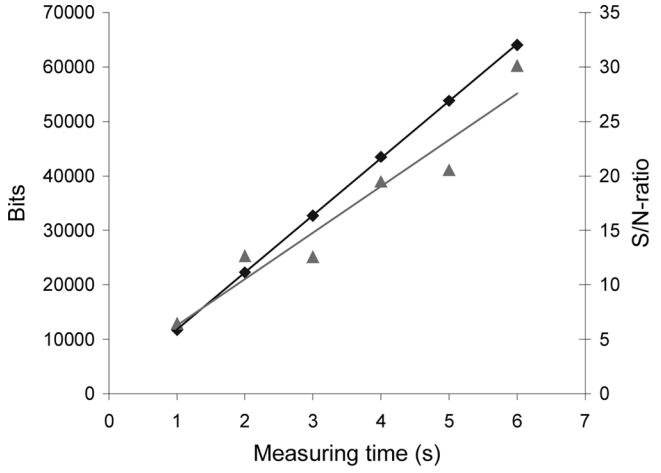

FIGURE 2 Plotted is the response curve of the ST-1603ME CCD-camera (bits against measuring time) and the obtained signal-to-noise ratios (S/N-ratios) plotted as triangles.

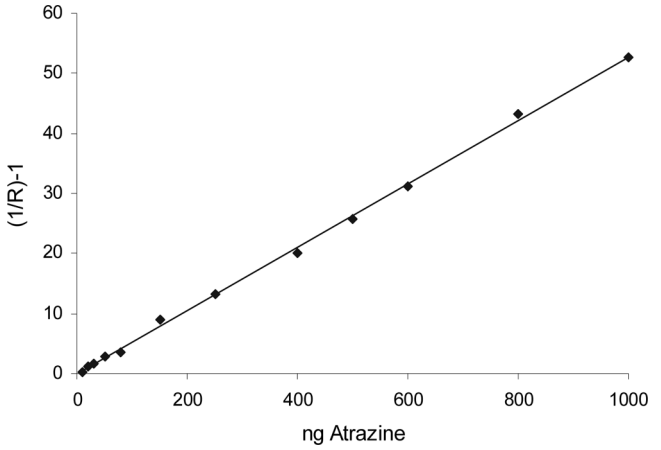

FIGURE 3 Plotted is the range of linearity for atrazine from 10 ng to 1000 ng.

signal-to-noise ratios of these six densitograms (using the atrazine-peak) where measured. The more the bit-range measurement increases, the more the linear signal-to-noise ratio increases as well. This is also plotted in Fig. 2 and shows that the signal-to-noise ration increases directly linear with the measuring time.

Different amounts of atrazine were separated, stained and measured using 6 seconds measuring time and 64 densitograms bundled. Peak areas were calculated by use of home-made integration software.[24] The result is plotted in Fig. 3. The densitograms can be used to quantify atrazine in a strictly linear calibration range of two magnitudes. To obtain linearity, the camera must show a linear response curve. Moreover it is absolutely necessary to use the correct transformation algorithm for calculating absorption values.

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