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IMAGE PROCESSING OF HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC PLATES

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SUMMARY

High-performance thin-layer chromatography (HPTLC) is often used to separate complex mixtures for qualitative and quantitative analysis. The use of a video camera and an image processing system allows images of the developed HPTLC plates to be enhanced for visual examination or enables quantitative information to be extracted, thus providing a fast and attractive alternative to conventional scanning densitometry.

In this paper, an image processing system consisting of a solid state camera, framestore and computer is described. This system enables high-resolution images of plates to be recorded and processed in a variety of ways. To correct for non-uniform illumination, images of developed plates are ratioed with that of a blank plate. Software associated with the system then allows the images to be enhanced, smoothed and automatic quantitative analysis to be carried out.

INTRODUCTION

High-performance thin-layer chromatography (HPTLC) has evolved from thin-layer chromatography (TLC) because of recent improvements in stationary phases, chromatographic development modes, and microprocessor controlled applicators and densitometers. These improvements have allowed complex mixtures to be separated and accurate quantitative analysis to be performed. Traditionally the plates are scanned by mechanically driven densitometers. This is usually the rate determining step in an analysis. The use of a video camera to record an image of the plate and a computerised image processing system for the analysis provides a fast, efficient, state-of-the-art alternative. At present, image processing hardware is more expensive than a modern scanning densitometer but, if present trends continue, this situation will reverse itself in the near future. In this paper we do not seek to describe a system that would necessarily be chosen to replace a scanning densitometer but to specify the image processing operations required to extract quantitative information from the HPTLC plates and to explore some of the advantages that such a system would have.

A number of papers[1–13] have been published highlighting the use and advantages of video camera–microcomputer systems. Most of this work has been quali-

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tative. However, Prosek *et al.*[14] have used an optical RAM device (128 × 256 pixels) to quantitatively analyse a mixture of sugars separated on HPTLC plates. Gianelli and co-workers[15,16] and Burns *et al.*[17] have described a video camera system and multidimensional data analysis technique which can be used to carry out quantitative analysis on chromatographic plates. They have shown that normal spectroscopic relationships such as Beer's law or, for reflectance work, the Kubelka–Monk equation hold well.

We have previously used a high-resolution image processing system for two-dimensional chromatographic image manipulation[18]. Digital filtering and the use of false colour allowed the images to be enhanced for visual interpretation. This system was also shown to have some applicability for quantitative analysis. In this paper we describe an improved camera system and a more refined technique for quantitative analysis.

The major problem associated with acquiring quantitative images from a relatively large area such as an HPTLC plate (10 cm × 10 cm) is to ensure uniform illumination. This is difficult to achieve in practice, and some means of correction must be employed. To do this an image of the developed HPTLC plate was ratioed against one of a blank plate. For ratioing to be effective it is important that the two images are captured under identical illumination and camera conditions. The Newvicon video camera, which we have previously used[18], was equipped with an automatic gain control. A charge-coupled device camera, on which we could disable this control, was substituted. Other possible benefits provided by this type of camera are slightly extended spectral response (300–1100 nm) and better long term stability.

EXPERIMENTAL

Image aquisition system

The image acquisition system (Fig. 1) consisted of a "light box" containing two 300-mm 8-W "cool white" fluorescent tubes and a video camera (high-resolution charge-coupled device, Pulnix TM-46K, supplied by Brian Reece Scientific Instruments, Newbury, U.K.), equipped with a 17.5–105 mm zoom lens. Appropriate Kodak Wratten filters were used to provide some measure of spectral selectivity. The output signal from the camera was digitised by a framegrabber and framestore (In-

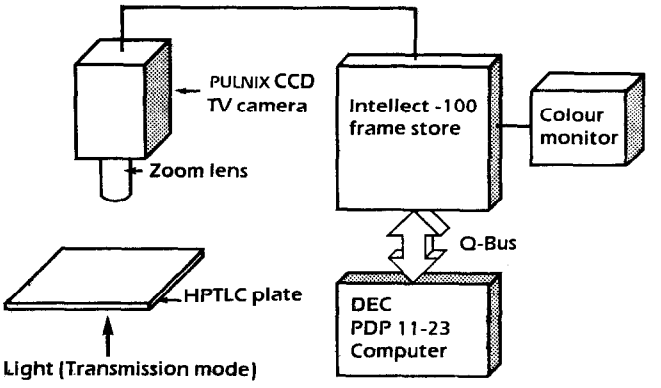


Fig. 1. Apparatus.

tellect 100, Quantel, Kenley, U.K.), which was interfaced to a computer (PDP 11/23, Digital Equipment Corporation, Maynard, MA, U.S.A.). Digital images (512 × 512 × 8 bit) could then be displayed in black and white or false colour on a high-resolution colour monitor (DEC, VR 241-A) and/or stored on a 10.5-Mbyte hard disc unit (DEC-RL02).

Scanning densitometer system

For comparison purposes, the HPTLC plates were also analysed using a scanning densitometer (Shimadzu CS930 supplied by V. A. Howe, London, U.K.). This was carried out in the reflectance mode at the appropriate wavelength.

Chromatographic procedure

All solvents and visualisation reagents were of analytical reagent grade (BDH, Poole, U.K.). Solvents were redistilled prior to use. Samples analysed were a five component dye mixture (TLC Test Dye Mixture, 15271, BDH) and an amine based antioxidant in gas oil. Toluene was used as the solvent for the dye mixture and chloroform–ethanol (90:10) was used for the gas oil. The chromatography was carried out on pre-cleaned reactivated (120°C) HPTLC silica gel 60F254 plates (Merck, BDH). Samples were applied as 1-μl aliquots using an HPTLC applicator (Linomatt IV, Camag, supplied by Baird and Tatlock, London, U.K.). The plates were developed in the appropriate solvent system in a linear development chamber (Camag). The solvent front was allowed to migrate 50 mm along the length of the plate. The plate was then dried in a stream of nitrogen. For the detection of the amine component a malonic acid visualisation spray was applied (0.2 g malonic acid and 0.1 g salicylaldehyde in 100 ml absolute ethanol).

Image capture and processing

For the analysis of the amine a deep blue filter (Wratten 47B) was placed in front of the lens. This filter has a band pass centred at 430 nm, which considerably enhances the contrast between the yellow of the amine derivative and the lighter plate background. The aperture of the lens was adjusted so that the dynamic range of the image did not exceed that of the framestore and then an image was captured. An image of a blank plate was also obtained under exactly the same lighting conditions. Transmitted light was used exclusively for these experiments. We now believe that this is not the best form of illumination and that reflected light, which would be less sensitive to variations in stationary phase thickness, will produce better quantitative results. However, a uniform reflected light system is more difficult to implement.

Initially, the framestore memory was divided into two so that a 512 × 256 pixel image of the developed plate could be stored in one half of the framestore and a 512 × 256 pixel image of the blank stored in the other. The following processing steps were then performed.

(1) A mean dark current luminance value was subtracted from both the developed plate and the blank images.

(2) To produce positive images the luminance values of both images were inverted (*i.e.* their value was subtracted from 256).

(3) To correct for non-uniform illumination the developed plate and blank images were ratioed pixel-by-pixel.

(4) All points in the ratioed image were rescaled such that the full dynamic range of the framestore (0–255 luminance levels) was utilised.

(5) Noise spikes, present in the image, were smoothed by running a 3 × 3 convolution filter over the entire image. This is the exact 2-dimensional equivalent of using a digital filter, of the Savitzky–Golay type, to smooth a noisy signal.

(6) The processed image was stored on disc.

For quantitative analysis, the processed image was transferred back into the frame store from disc. A threshold value, usually taken to be 1.5 standard deviations of the background plus the background mean, was calculated. The cursor was positioned over one lane of spots in the image and the luminance values of the pixels along this line were read until the threshold was encountered. A chain contouring routine was then invoked to track the threshold around the spot. The value of all pixels contained within this area were summed to produce an "integral" over the area of the spot. The cursor was then positioned over the next lane of spots and the operation was repeated until all the spots present were analysed.

The parameter p^2/a (p is the spot perimeter length, a is the spot area) was calculated for each spot. This is a shape parameter which may be used in a simple pattern recognition technique to characterise the spots. For a perfectly round spot $p^2/a = 4\pi$. For elongated spots, such as found with unresolved mixtures, this value increases. We have chosen the value of $p^2/a = 18$ as an indication that spots may be unresolved. These spots were labelled with the letter M and reserved for subsequent analysis.

RESULTS AND DISCUSSION

HPTLC was used to separate a five-component dye mixture, the resultant im-

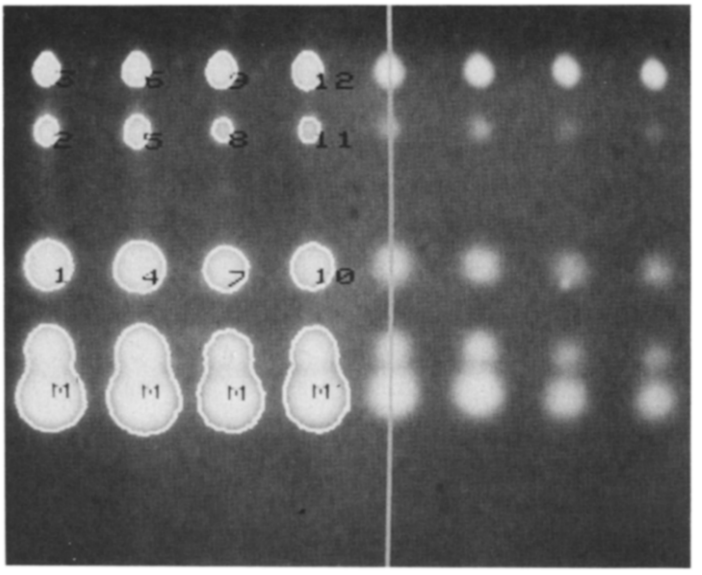


Fig. 2. Processed image of a series of chromatograms of differing dilutions of a five-component dye mixture. Chain contouring is in progress marking the extent of the spots.

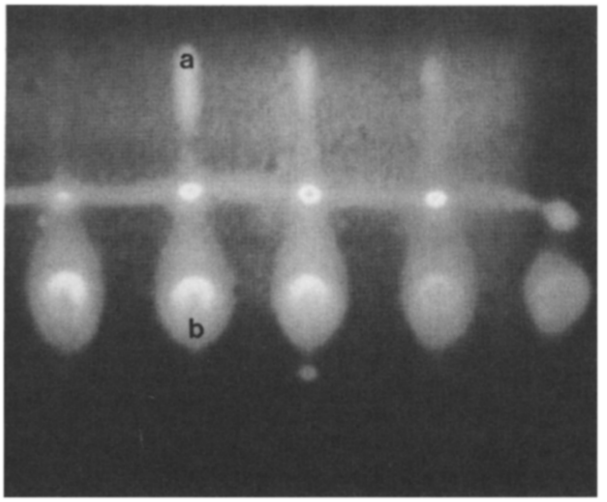


Fig. 3. Processed image of the separation of gas oil. The amine component (a) is separated from a complex solvent system (b). Visualisation of the amine was achieved by the use of a malonic acid spray.

age displayed on the monitor is shown in Fig. 2. This image has been ratioed, smoothed and is in the process of being analysed. Spots which have been integrated are automatically identified by a number. The thin white line around these spots indicate the extent of the spot defined by the threshold and the chain contouring algorithm. The vertical cursor is positioned over the next lane of spots to be integrated. The spots labelled with M are flagged as possible unresolved multicomponent mixtures, their p^2/a value being greater than 18. This test is obviously fairly crude but serves to indicate whether a spot is likely to be an unresolved mixture and also demonstrates the type of operation which may gainfully be employed on chromatographic images.

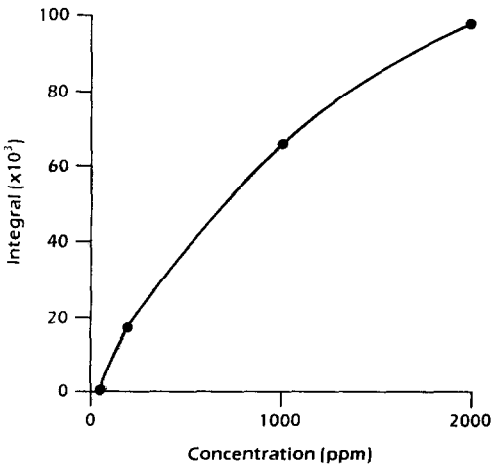
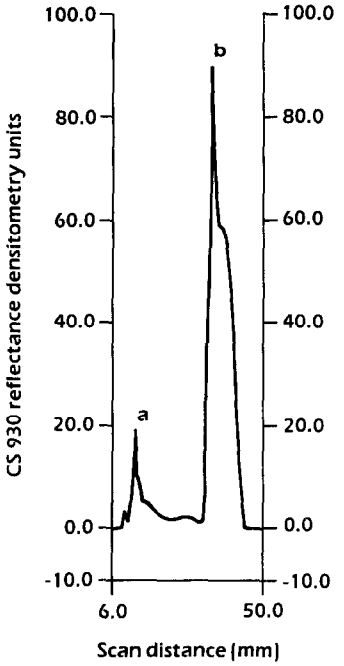


Fig. 4. Calibration of the amine component obtained by image processing.



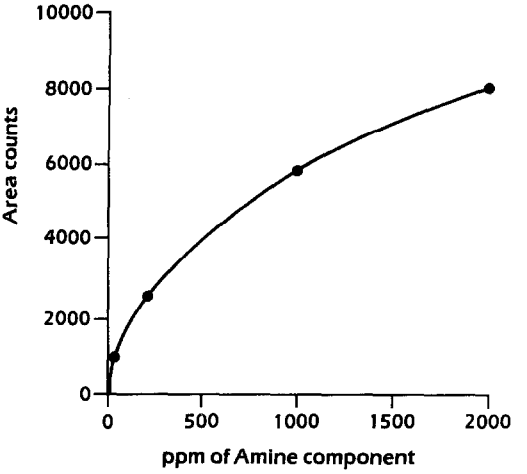


Fig. 5. Chromatogram of antioxidant mixture obtained by the scanning densitometer set at 395 nm.

Fig. 6. Calibration of the amine component obtained by scanning densitometry.

Fig. 3 shows the chromatogram obtained for the gas oil. The small spot labelled (a) is the amine of interest which has been visualised using the malonic acid reagent. Good contrast of this pale yellow spot has been achieved by the use of the blue Wratten filter. The calibration curve obtained by image processing is shown in Fig. 4. The trace measured by the scanning densitometer shown in Fig. 5 and the calibration curve obtained is shown in Fig. 6. Very similar calibration curves were obtained with both systems.

CONCLUSIONS

Image processing provides an attractive alternative to scanning densitometry for the analysis of HPTLC plates and could be equally well applied to other two-dimensional separation techniques. The advantages we perceive are: (1) speed; acquiring data from the plates is virtually instantaneous, storage and retrieval of images is also very fast. This speed advantage may be offset somewhat by processing time. With our system full analysis of a plate image, including ratioing of the developed image to that of the blank, takes about 10 min. (2) The data produced are in a format highly suitable for processing and analysis. This enables smoothing, resolution enhancement and pattern recognition to be easily employed. (3) There are no moving parts in the apparatus.

The present disadvantages are: (1) the relatively high cost of the apparatus. We estimate, at today's prices, an image processing system of sufficient sophistication

to replace a microprocessor-controlled densitometer would cost nearly twice as much. However, if present trends continue, we expect this situation to be reversed in the near future. (2) Solid state and tube cameras have poor response in the UV region. This is not a fundamental problem associated with detecting UV photons but is due to the absorbance of the glass used in the construction of the imaging optics. This problem could be avoided by the use of quartz optics and "optically thin" detectors. Conversely the high sensitivity of charge-coupled device cameras in the near infrared (700–1100 nm) often leads to poor contrast in the image. Unfortunately, most imaging optics and all Wratten type filters transmit well in this region. The use of a suitable infrared filter would further improve image quality. (3) A high-resolution image (512 × 512 pixels) contains 256K (= 262 144) data points. This demands a high-performance or purpose-designed computer system if computation is to be achieved in a reasonable time. Likewise, if there is a requirement to store a large number of images, for instance in setting up a HPTLC data base, a high-volume data storage facility must be available. At present, we are evaluating the use of an optical disc, which can store up to 2G bytes of information, as a solution to this problem.

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