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Reproducibility of densitometric and image analysing quantitative evaluation of thin-layer chromatograms[1]

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Abstract

This paper presents a comparison of quantification of thin-layer chromatographic plates with slit-scanning densitometry and with two image-analysing systems. Camag test dye mixture III was used as a sample. The influence of different measuring beam dimensions (slit width and slit length) on the quantitative evaluation of thin-layer chromatograms was studied in the reflectance as well as in the transmission mode. The slit length has a much higher influence on the reproducibility of the measured areas than the slit width. The results obtained with both CCD cameras show linear correlation between response area and concentration of Camag test dye mixture III in a higher concentration range than the densitometrically obtained results. The sensitivity of the used image-analysing systems was much lower than that of the slit-scanning densitometer. © 1997 Elsevier Science B.V.

Keywords: Image analysis; Densitometry; Thin-layer chromatography

1. Introduction

While a number of techniques have been used for quantification of thin-layer chromatographic (TLC) plates, the most common detection method is slit-scanning densitometry. However, image-analysing systems are an emerging detection technique in thin-layer chromatography (TLC). There are several advantages of charge-coupled device (CCD) imaging technology combined with planar chromatography for detection and quantification. The main advantages of image-analysing systems are: fast data acquisition, absence of moving parts and simple instrument design. Nevertheless image-analysing systems cannot compete with mechanical scanners in terms of cost, sensitivity, available wavelength measuring range and dynamic response range [1,2]. However, image-analysing systems provide more information concerning the conditions inside the layer than densitometers due to a bigger illumination field and larger number of scattered beams [3].

The principal sources of errors in scanning densitometry have been identified as the reproducibility of the: sample application, chromatographic conditions, positioning of the spot in the centre of the measuring beam and the measurement [4,5]. The main reason for the poor reproducibility in TLC is the positioning error [4]. This error can be divided into two parts, one attributable to unequal and inhomogeneous distribution of sample molecules on the stationary phase, and the other to irregular migration of the spot. The effect of the position of a

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spot on the absorbent layer on the intensity of the measured signal has already been studied by different multilayer models [6–11] and by photoacoustic spectroscopy [12,13]. The effect on reproducibility of the relative positions of the spots and scanning slit has also been described [14]. The influence of the measuring beam dimensions on the resolution and signal-to-noise ratio has already been studied with a Shimadzu CS-910 scanning densitometer [15–18].

This paper presents reproducibility of densitometric and image-analysing quantification of thin-layer chromatograms of Camag test dye mixture III. The effect of the measuring beam dimensions (the slit width and the slit length) on the measured signal and reproducibility in slit-scanning densitometry was studied. Obtained results were compared with those obtained by using a CCD camera, with the total plate illuminated.

2. Experimental

2.1. TLC

TLC was performed on 10×20 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with a 0.25 mm layer of Kieselgel 60 F_{254}. Test dye mixture III (Camag, Muttenz, Switzerland) was diluted with toluene (Kemika, Zagreb, Croatia) to obtain 20%, 40%, 60% and 80% solutions. Diluted and non-diluted solutions of test dye mixture III were applied to the layer in spots using 0.5 μl glass-capillaries and Nanomat III applicator (Camag). Fifteen spots were applied 15 mm from the bottom edge, 10 mm apart by using data-pair technique. The plates were developed twice in an unsaturated horizontal developing chamber (Camag) using toluene as a mobile phase, the migration distance being 7 cm. After the separation, the plates were dried in a stream of warm air for about 5 min.

2.2. Scanning and image processing

Evaluation of the developed HPTLC plates was performed densitometrically using the Camag TLC scanner II equipped with a built-in 12 bit ADC, and controlled by an external personal computer (PC) via an RS232 interface. The QTLC-pack (KIBK-IFC,1990) and the IMAGE-pack (KIBK-IFC,1990) software were used. The scanner was set to the reflectance or to the transmission mode; the monochromator bandwidth was 30 nm at λ=560 nm. The measurements were made using different combinations of the slit width and the slit length as can be seen from the figures.

Two different image-analysing systems were used for data acquisition. The first system (system 1) was constructed in our laboratory. It consists of commercially available parts, such as an illumination system (Desaga, Heidelberg, Germany), with a camera holder, CCD camera Chromachip IV Model JE-3622X (Javelin Electronics, Los Angeles, CA, USA) and a PC with programme Video blaster FS200, Application version 3.01 (Creative Technology, 1993–1994).

The second system (system 2) used for imaging and archiving the thin-layer chromatograms was the Camag Video Documentation System in conjunction with the Reprostar 3. The objects were captured by means of highly sensitive video camera - 3×1/2 in. (1 in.=2.54 cm) CCD camera, Model HV-C20 (Hitachi, Denshi, Japan). A special digitising board (frame grabber) assists rapid processing via the PC system. Image acquisition, processing and archiving are controlled via Video Store 2, a high-performance documentation software running under Windows 95. All images were obtained using attachment lens +2 Dpt for optimum adaption to the size of the object. The Camag Video Scan 1.16 program was used for the evaluation of thin-layer chromatograms.

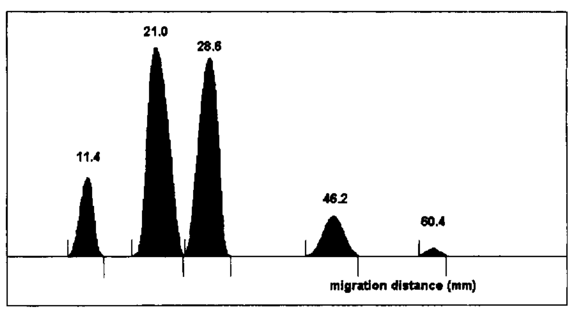

Fig. 1. Densitogram obtained for Camag test dye mixture III at λ=560 nm in the reflectance mode.

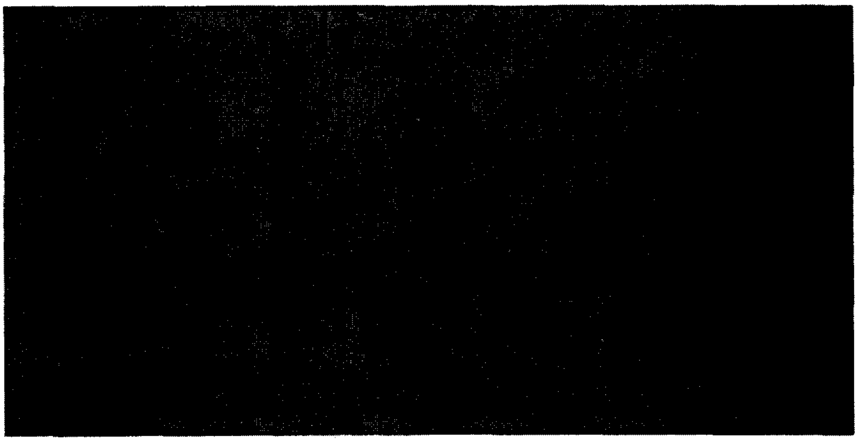

Fig. 2. CCD image showing the separation of dyes from Camag test dye mixture III.

3. Results and discussion

TLC plates were scanned with densitometry and with two image-analysing systems. Data acquisition was made by Camag TLC scanner II, Camag Video Documentation System in conjunction with the Reprostar 3 and with system 1. Fig. 1 illustrates the separation of dyes from the Camag test dye mixture III scanned with Camag TLC scanner II. The results obtained for four dyes presented as the yellow, the violet, the blue and the red spots in Fig. 2 showed similar variations. Therefore, only the results for the red spots with the diameters from 2.5 mm to 3.5 mm at migration distance 21.0 mm (Fig. 1) are presented in this paper.

The slit width and the slit length, which control the size of the measuring beam, were expected to have the greatest effect on the quantitative evaluation of thin-layer chromatograms. The measuring beam is a rectangle defined by the selected values for the slit width and the slit length. The slit width is adjustable from 0.025 mm to 1.2 mm and defines the dimension of the measuring beam in the direction of scanning.

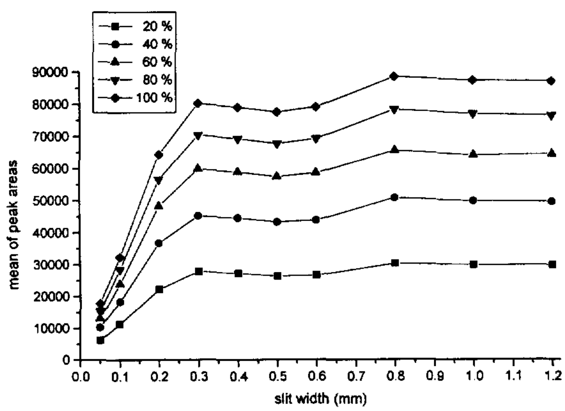

Fig. 3. Mean of peak areas ($n=3$) for the red spots as a function of slit width for different concentrations of Camag test dye mixture III measured in the reflectance mode (slit length=4 mm).

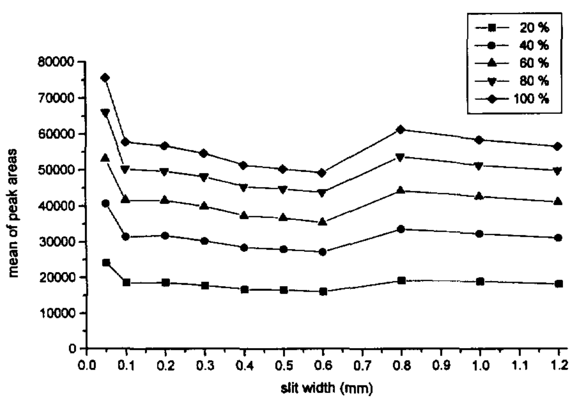

Fig. 4. Mean of peak areas ($n=3$) for the red spots as a function of slit width for different concentrations of Camag test dye mixture III measured in the transmission mode (slit length=4 mm).

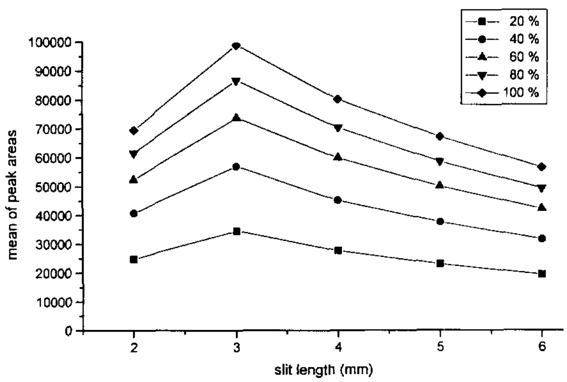

Fig. 5. Mean of peak areas ($n=3$) for the red spots as a function of slit length for different concentrations of Camag test dye mixture III measured in the reflectance mode (slit width=0.3 mm).

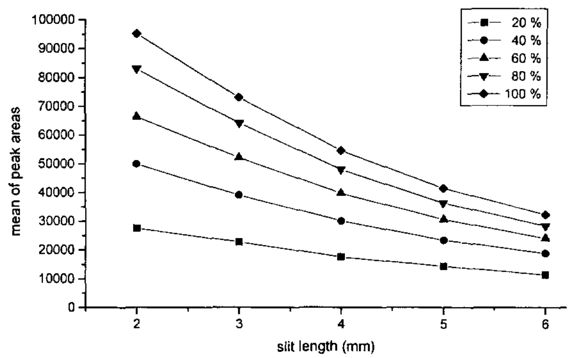

Fig. 6. Mean of peak areas ($n=3$) for the red spots as a function of slit length for different concentrations of Camag test dye mixture III measured in the transmission mode (slit width=0.3 mm).

The slit length is adjustable from 0.25 mm to 12 mm and defines the dimension of the measuring beam in the direction which is orthogonal to the direction of scanning. As can be seen from Figs. 3 and 4 for all concentrations the change of signal with the slit width is relatively small for the slit width values larger than 0.2 mm in both measuring modes. Slit length has much greater effect for all concentrations in the reflectance and in transmission mode, as shown in Figs. 5 and 6. For small slit-length values, larger integrated areas are obtained at all concentrations of Camag test dye mixture III. When the slit length was larger compared to the spot diameter, a large amount of light was transmitted or reflected from the blank area of the plate and the contribution from the subtracted (absorbed) light was small, while

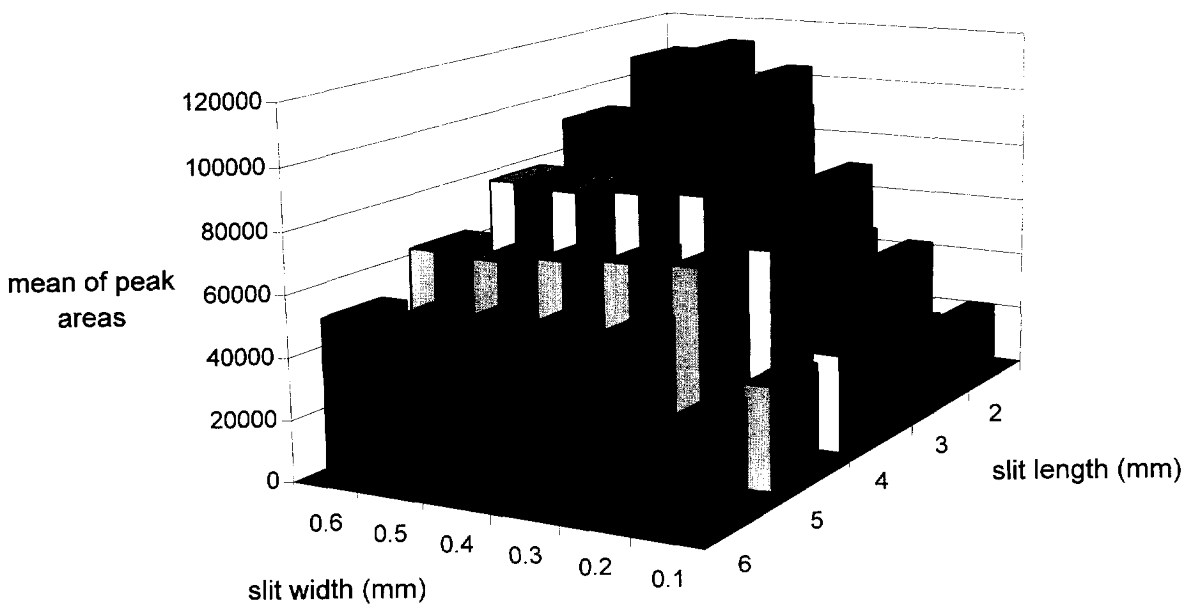

Fig. 7. Influence of the slit width and the slit length on the mean of peak areas ($n=3$) obtained for the red spots (conc.=100%) in the reflectance mode.

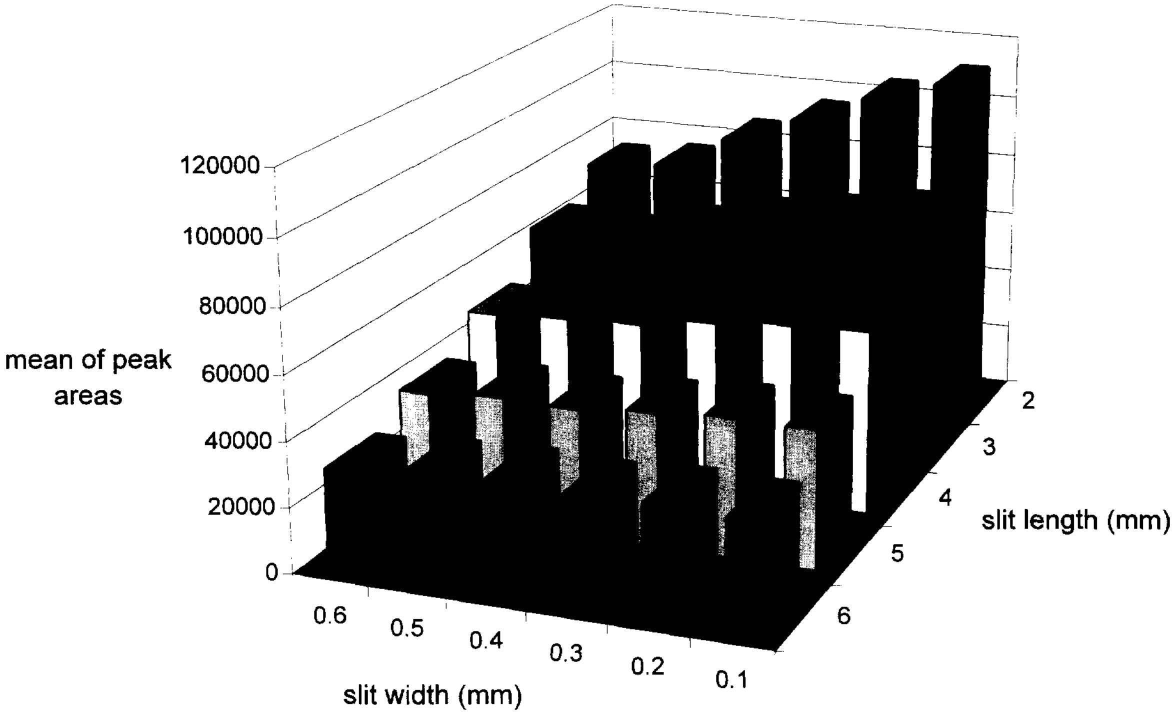

Fig. 8. Influence of the slit width and the slit length on the mean of peak areas ($n=3$) obtained for the red spots (conc.=100%) in the transmission mode.

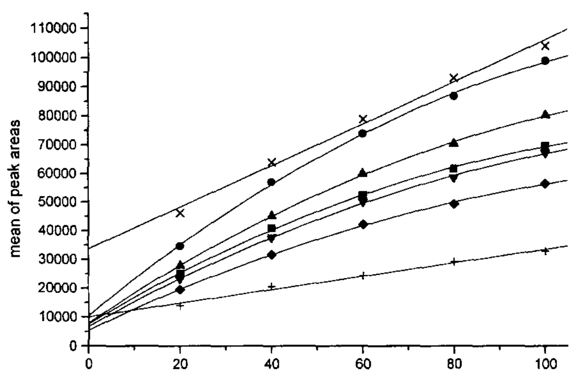

Fig. 9. Calibration curves for the red spots obtained in the reflectance mode using constant slit width (0.3 mm) and different slit length from 2 mm (■), 3 mm (●), 4 mm (▲), 5 mm (▼) and 6 mm (♦), system 1 (X measured values were divided with factor 1.5) and Camag Video Documentation System (+ measured values were multiplied with factor 100).

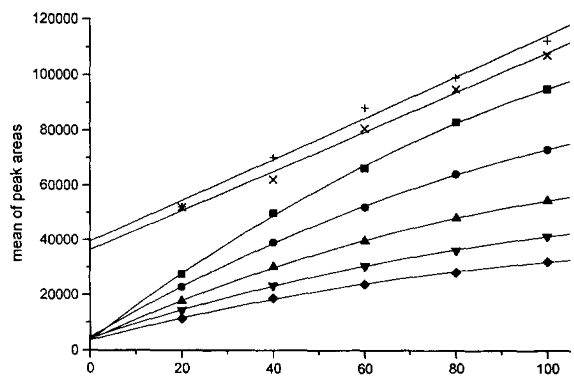

Fig. 10. Calibration curves for the red spots obtained in the transmission mode using a constant slit width (0.3 mm) and different slit length from 2 mm (■), 3 mm (●), 4 mm (▲), 5 mm (▼) and 6 mm (♦), system 1 (X measured values were divided with factor 1.5) and Camag Video Documentation System (+ measured values were multiplied with factor 200).

the signal was weak. Fig. 7, demonstrates that in the reflectance mode larger integrated areas are obtained for small slit length and large slit width. However, in the transmission mode integrated areas obtained for small slit length and small slit width are larger, as indicated in Fig. 8. From the results shown in Figs. 9 and 10 it can be concluded that the shape of calibration curve is independent of the slit-length value; only the absolute integrated area is changed in both measuring modes. The increase of the slit length from 2 mm to 6 mm caused a decrease of the slopes of the regression lines, which means lower sensitivity. The results obtained with both image-analysing systems showed linear correlation between response area and concentration of Camag test dye mixture III within the higher concentration range than the densitometrically obtained results. This was proved in the reflectance and in transmission mode, respectively. The results obtained in the reflectance mode (Fig. 9) showed that system 1 gave higher slopes of the regression lines than the densitometer when the slit width was 0.3 mm and the slit length was 2 mm, 5 mm or 6 mm. Furthermore, we can conclude that Camag Video Documentation System is much less sensitive compared to the densitometer in the reflectance mode. However, in the transmission mode (Fig. 10) the slopes of the regression lines obtained for measurements with both image-analysing systems are lower compared to the slopes obtained for densitometric measurements when the slit width was 0.3 mm and the slit length was 2 mm. Since the measured values obtained with Camag Video Documentation System were very low and the measured values obtained with system 1 were very high, normalization of data was necessary to obtain better presentation.

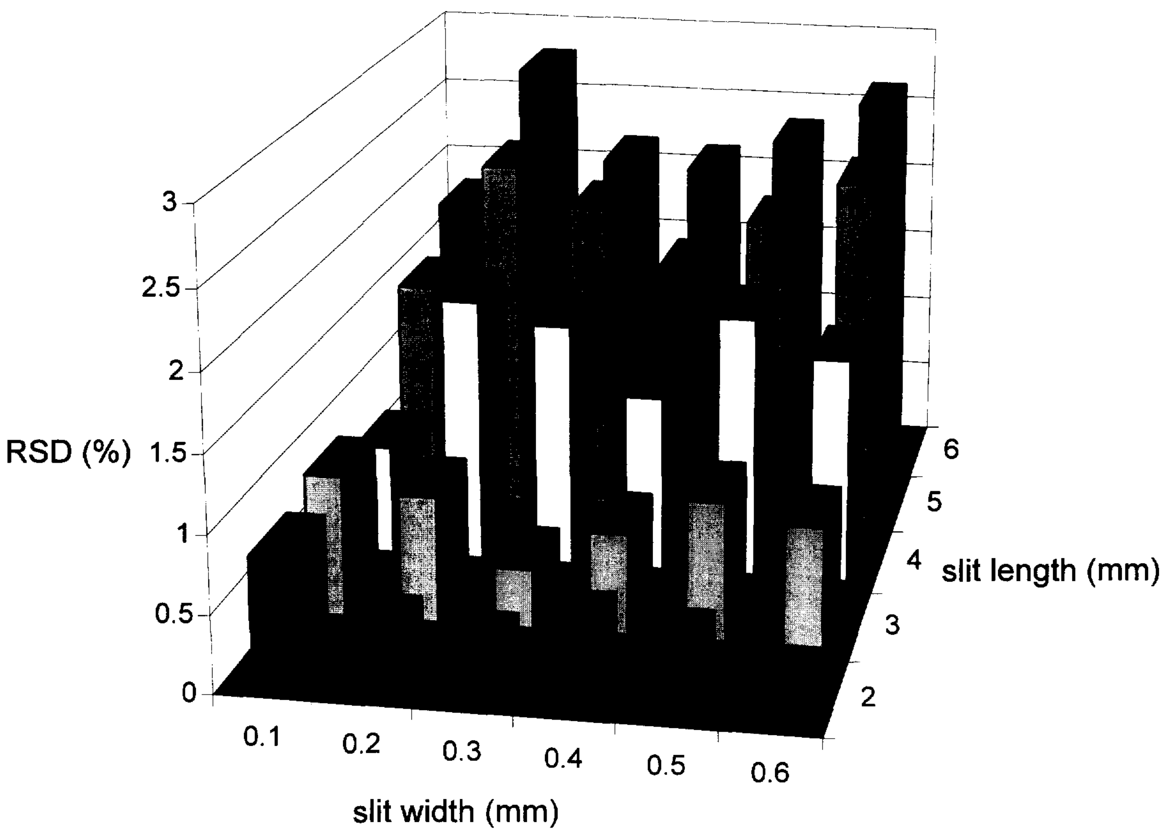

Fig. 11. Influence of the slit width and the slit length on the R.S.D. values ($n=3$) obtained for the red spots (conc. = 100%) in the reflectance mode.

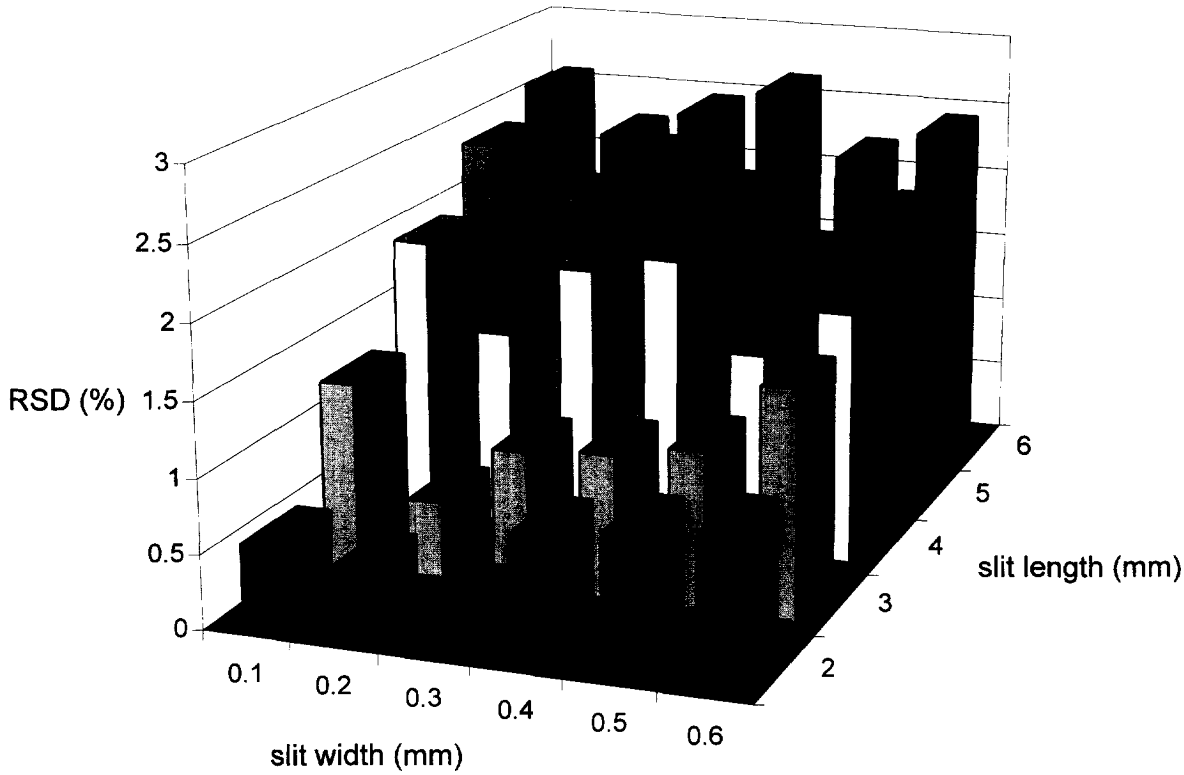

Fig. 12. Influence of the slit width and the slit length on the R.S.D. values ($n=3$) obtained for the red spots (conc. = 100%) in the transmission mode.

The slit length has a much higher effect on the reproducibility of the measured areas than the slit width. For small slit length values lower R.S.D. values were calculated for the reflectance, as well as for the transmission mode as indicated in Figs. 11 and 12. Finally, the results of the reproducibility obtained with system 1 showed lower R.S.D. values for the transmission mode than for the reflectance mode (Fig. 13). However, there is only a small difference between R.S.D. values for both measuring modes when Camag Video Documentation System was used.

4. Conclusions

According to our previous investigations [3,19] we assume that CCD cameras can also provide a lot of information from the inner part of the layer which results from the whole plate illumination. Therefore, the results obtained with both CCD cameras show linear correlation between response area and concentration of Camag test dye mixture III in a higher concentration range than the densitometrically obtained results. However, the sensitivity of the used image-analysing systems is much lower than that of the slit-scanning densitometer.

We can conclude with certainty that the reproducibility in slit-scanning densitometry is highly affected by the chosen slit length value in the reflectance as well as in the transmission mode. On the other hand, the reproducibility in image-processing is influenced by inhomogeneous illumination of the TLC plate. Nevertheless, we hope that this problem will be solved soon and that quantification of the TLC plates with image-analysing systems will soon be acceptable in routine work.

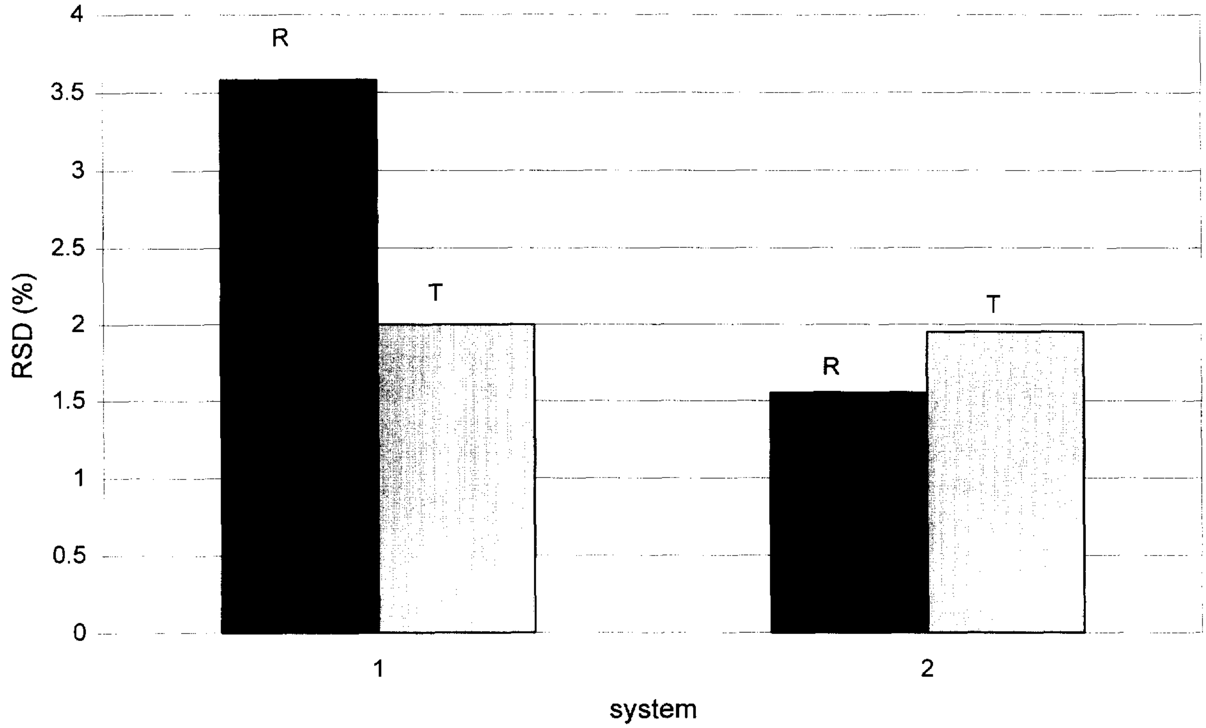

Fig. 13. R.S.D. of the areas ($n=3$) obtained for the red spots (conc.=100%) with system 1 and Camag Video Documentation System (system 2) in the reflectance (R) and in transmission (T) mode.

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