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Short communication

Quantitative evaluation of chromatograms from totally illuminated thin-layer chromatographic plates[1]

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Abstract

Saccharose in fermented and non-fermented soybean flour was determined by thin-layer chromatography. A comparison was made between quantification of TLC plates with CCD camera, with classical densitometry and by scanning with a small slit. In the last case the signal response of such a slit represents the signal from one pixel, which means that scanning with camera was simulated. All the measurements were made in reflectance and in transmission mode. The results obtained with a charge-coupled device camera have a linear correlation between response area and concentration of saccharose within a higher concentration range than the densitometrically obtained results. The linearity of calibration curves was better in transmission than in reflectance mode.

Keywords: Soybean; Detection, TLC; Densitometry; Saccharose

1. Introduction

Nowadays an increasing interest in thin-layer chromatography can be seen among the users of chromatography. Great technological progress in the field of digitized image acquisition and processing provides new possibilities for quantitative TLC.

The current situation offers two different approaches to the quantification in TLC. Slit-scanning densitometry is the most common method of detection. But an emerging detection technique in TLC is an image analysing system. In this case TLC plates are scanned with video cameras equipped with video chips, which have more than 400 000 detectors (pixels). Many analysts are unaware of the great difference between quantification with densitometer and quantification with camera, which are two completely different systems. Slit-scanning densitometers operate by observing a small portion of the light emanating from the chromatographic surface defined by the scanning slit. Thus most of the diffusely reflected light is lost or returned to a detector (photomultiplier) without giving any useful information about plate parameters. For quantitative work, the slit size is defined by the largest spot diameter on the plate and remains constant during the scanning process. This reduces the sensitivity of components represented by smaller spot diameters [1,2] and components which are deeper inside the sorbent. This problem is not present if a charge-coupled device (CCD) camera is used, where the whole plate is illuminated while the intensity of diffused light is much higher than in densitometric evaluation (where a relatively small part of the plate is illuminated).

Most of the densitometric measurements are made in the UV wavelength region, where only the reflect-

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ance mode can be used. However, our previous studies [3–6] have shown that the position of a compound in a layer highly affects the reflectance measurements because each compound has a different depth distribution in the TLC plate. This can be proved by using laser techniques [7]. In order to partly avoid this problem, chromatographic conditions must be well defined. We try to find out the position of the compound inside the layer and its influence on the results. Special models have been made to ascertain the difference between both data acquisition modes and meanwhile both detection techniques were used for the routine analysis.

We expected a great difference between scanning of the totally illuminated TLC plate using a CCD camera and slit-scanning densitometry. Therefore, we decided to study both systems in detail. This paper presents the comparison between two different approaches to the quantification of TLC plates where data acquisitions were made by a CCD camera and with the Camag TLC Scanner II, using saccharose and soybean flour as testing materials.

2. Experimental

2.1. Chemicals

All chemicals used, except H_3PO_4 (Kemika, Zagreb, Croatia) and 2-aminoethyl diphenylborinate (Fluka, Neu Ulm, Germany), were produced by Merck, Darmstadt, Germany. The solutions of samples and standards were prepared by dissolving in a mixture of methanol–deionized water (Milli-Q, Millipore, Bedford, MA, USA).

2.2. TLC

TLC was performed on 10×20 cm glass-backed Kieselgel 60 HPTLC plates (Merck). Standards (saccharose) and samples (fermented and non-fermented soybean flour) were applied to the plates by means of the Linomat IV applicator (Camag, Muttentz, Switzerland) equipped with a 100 μl syringe: the band length was 10 mm; the application volume was 5 μl; the application rate 4 μl/s. Ten bands per plate were applied 10 mm from the bottom edge, 15 mm apart. The plates were developed twice in an unsaturated glass twin through chamber (Camag) in a solvent system 3.0 mmol NST=2-aminoethyl diphenylborinate in 100 ml acetonitrile–deionized water–methanol (17:3:0.25, v/v), the migration distance being 8 cm. After separation, the plates were dried in a stream of warm air for ca. 5 min and then immersed for 10 s in DAP reagent [2% diphenylamine+2% aniline in methanol–H_3PO_4 (8:2, v/v)] by means of a Camag chromatogram immersion device II. Heating on a Camag TLC plate heater at 120°C for 10 min furnished coloured bands for the saccharose.

2.3. Scanning and image processing

Evaluation of the developed HPTLC plates was performed densitometricaly using the Camag TLC scanner II equipped with a built-in 12 bit ADC, and controlled by an external personal computer 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 reflectance or to transmission mode; the monochromator band width was 30 nm. The measurements were taken using the following combinations of slit width and slit length 0.8 mm:6 mm (for classical densitometry) and 1.2 mm:0.5 mm (for scanning with a small slit, Fig. 1) at λ=525 nm (wavelength of maximum absorbance).

Two different image analysing systems have been used for data acquisition. The first system (**system 1**) which is illustrated in Fig. 2 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

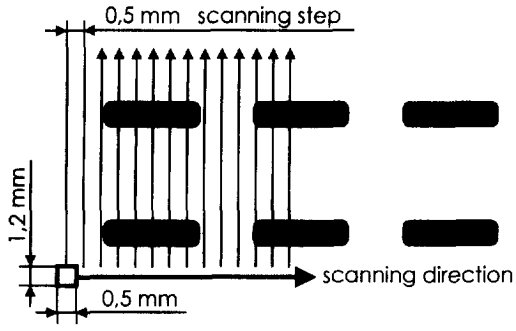

Fig. 1. Schematic presentation of scanning with a small slit.

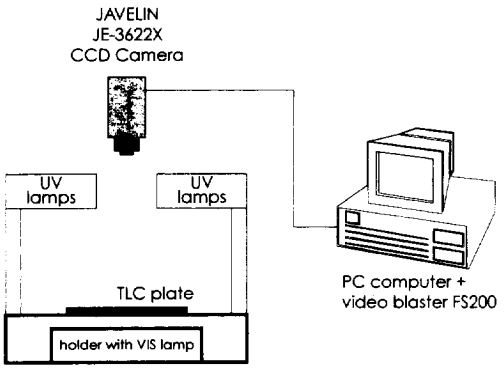

Fig. 2. **System 1** for data acquisition with CCD camera and PC.

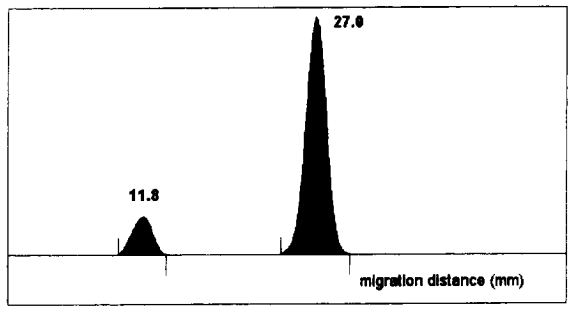

Fig. 3. TLC chromatogram showing the separation of saccharose (27.0) from the sample of soybean flour.

Electronics, Los Angeles, CA, USA) and a personal computer with programme Video blaster FS200, Application version 3.01 (Creative Technology, 1993–1994).

The second system used for imaging and archiving 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. CCD camera (1 in.=2.54 cm), Model HV-C20 (Hitachi, Denshi, Japan). A special digitizing board (frame grabber) assists rapid processing via a personal computer system. Image acquisition, processing and archiving are controlled via Video Store 2, a high-performance documentation software running under Windows 95. The Camag Video Scan program was used for the evaluation of thin-layer chromatograms.

3. Results and discussion

The TLC separation of saccharose from the sample of soybean flour is presented in Fig. 3. As already mentioned in the introduction, TLC plates were scanned by using classical densitometry, densitometric scanning with small slit, with **system 1** and with Camag Video Documentation System. Densitometry was carried out in two different modes. A large slit was used in the normal mode, while a small slit was used for camera simulation mode. In the latter the signal from such a slit represents the signal from one pixel. In this mode TLC plate was divided into about 15 000 pixels each with 16 bits of information. Lanes and spots were constructed after the data acquisition using collected data, like in image analysing system. When **system 1** was used, there were 228 096 pixels with 8 bits of information.

According to our results in the Vis part of the spectra (Figs. 4 and 5), the differences between the three different principles of measurements in reflectance and in transmission mode are not significant. Quantification is possible with all systems since there is a linear correlation between response area and concentration of saccharose within the concentration range 0.5 to 2.5 μg/5 μl. However, the slopes of the regression lines obtained for measurments with both image analysing systems are lower in comparison with the slopes obtained for densitometric measurements. Therefore, it can be concluded that both

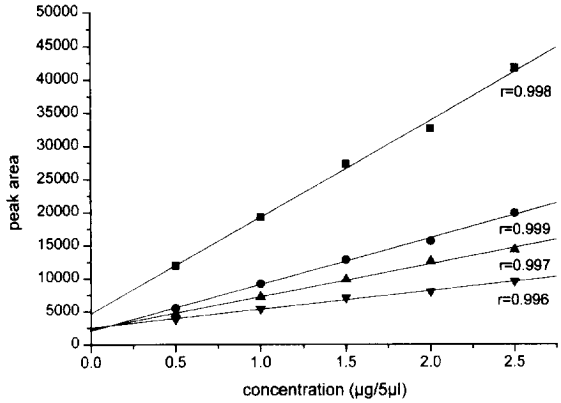

Fig. 4. Linear calibration curves for saccharose obtained with classical densitometry (■), scanning with a small slit (●), with **system 1** (▲) and with Camag Video Documentation System (▼-measured values are multiplied with factor 50) in reflectance mode.

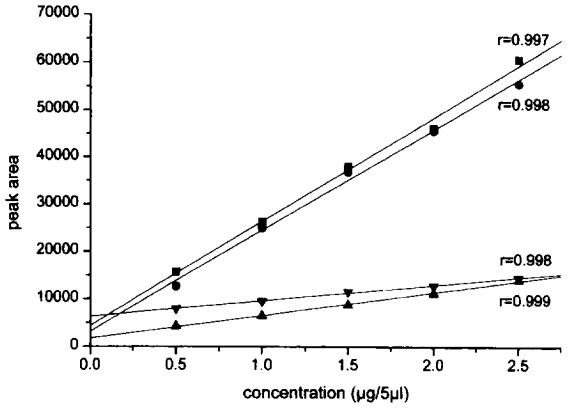

Fig. 5. Linear calibration curves for saccharose obtained with classical densitometry (■), scanning with a small slit (●), with **system 1** (▲) and with Camag Video Documentation System (▼-measured values are multiplied with factor 50) in transmission mode.

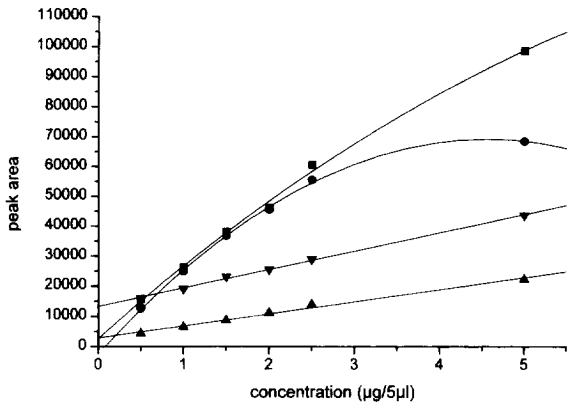

Fig. 7. Linear and polinominal calibration curves for saccharose obtained with classical densitometry (■), scanning with a small slit (●), with **system 1** (▲) and with Camag Video Documentation System (▼-measured values are multiplied with factor 100) in transmission mode.

image analysing systems are much less sensitive compared to the densitometer. The measured values obtained with Camag Video Documentation System were very low. That is why multiplication with factor was necessary to obtain better presentation.

Figs. 6 and 7 proved our expectations that image analysing systems would give a linear correlation between response area and concentration of saccharose within a higher concentration range than densitometry. Densitometric scanning by using a small slit has shown smaller dependence on the spot shape. In transmission with a small slit too much information caused the signal saturation, because the slit was very small, which means that it is very sensitive and quickly becomes non-linear. Nevertheless, this problem is not so big in reflectance mode where the amount of information is automatically smaller compared to transmission mode.

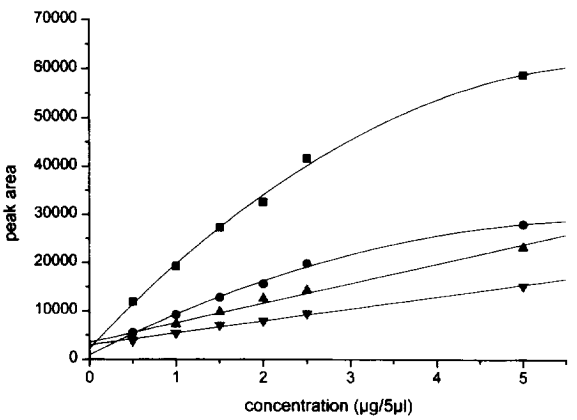

Fig. 6. Linear and polinominal calibration curves for saccharose obtained with classical densitometry (■), scanning with a small slit (●), with **system 1** (▲) and with Camag Video Documentation System (▼-measured values are multiplied with factor 50) in reflectance mode.

When **system 1** was used, it was not possible to prepare optimal scanning sensitivity, in spite of the fact that the sensitivity of our camera was very good. The disadvantage of **system 1** was the interface, which does not have enough memory and did not enable picture cumulation and thus automatical noise reduction. For that reason we had to use one picture only or sum up the pictures after each scan. This was not a significant disadvantage of **system 1** in comparison with commercially available systems in the visible part of the spectra.

From the results presented in Table 1 it can be concluded that in order to prove the difference between all systems, studies by using model substances and multilayer models should be made.

Analytical chemists try to maximize the information that can be obtained from TLC separations. Unfortunately, compared to densitometry, scanning with a small slit cannot be used in routine analysis because the analysis would take a considerable amount of time in comparison with densitometry.

Table 1
Concentrations of saccharose in the samples of soybean flour obtained with classical densitometry (D), scanning with a small slit (S) and with **system 1** (C) in reflectance and in transmission mode (Sample 1=fermented soybean flour, sample 2=non-fermented soybean flour)

	Reflectance		Transmission	
	$c_{\text{sample 1}}$ (μg/5 μl)	$c_{\text{sample 2}}$ (μg/5 μl)	$c_{\text{sample 1}}$ (μg/5 μl)	$c_{\text{sample 2}}$ (μg/5 μl)
D	1.623	1.423	1.649	1.545
S	1.645	1.459	1.793	1.731
C	1.893	1.478	1.955	1.634

However, the application of a CCD camera offers enhanced data acquisition and evaluation capability. Compared to densitometry, the main limitation of image processing is that no solution has been found for illuminating a TLC plate uniformly with monochromatic light.

Acknowledgments

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