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Journal of Chromatography A, 704 (1995) 173–178

JOURNAL OF CHROMATOGRAPHY A

# Validation of quantitative chromatographic analysis on laboratory-prepared thin layers☆

Mira Petrović*, Marija Kaštelan-Macan

*Laboratory of Analytical Chemistry, Faculty of Chemical Engineering and Technology, Marulićev trg 20, 41000 Zagreb, Croatia*

First received 13 October 1994; revised manuscript received 10 December 1994; accepted 5 January 1995

**Abstract**

Validation of the determination of amino acids on laboratory-prepared plates was performed on a mixed natural zeolite and microcrystalline cellulose sorbent. Chromatograms were recorded in the visible region using a Datacolor DC 3890 reflectance spectrometer, Camag Turner Fluorimeter 111 slit-scanning densitometer and Leco 2001 image analyser. The precision, detection limit and limit of quantification for each method were determined. A lower total R.S.D. was obtained by measuring colour remission with the Datacolor system (2.7% for high concentration and 3% for low concentration), with an instrumental R.S.D. of 0.1–0.2%. Densitometry and image analysis resulted in a lower precision, especially at low concentration (total R.S.D. > 10%), with a significant instrumental error.

## 1. Introduction

Commercial precoated TLC plates with performance and consistency acceptable for quantitative analysis are generally used by analytical chemists. However, in some instances, such as in the investigation of new or physically and chemically modified sorbents, laboratory-prepared layers are needed. Problems connected with quantitative analysis on such layers are numerous. The principal barrier is the noise component of the signal obtained by sophisticated scanning densitometers as a result of heterogeneity of the layer structure, low abrasion resistance and damages to the sorbent surface [1]. Application of precoated plates minimizes the error that is the result of the signal-to-noise ratio. However, the problem of diffuse and irregular spots is not restricted only to laboratory-made supports. On commercial precoated plates diffuse spots can appear when the analyte concentration is very low and large sample volumes are applied to the chromatographic plate. Irregular spots usually appear near the second solvent front. Additional problems occur when chromatograms are made visible by application of chromogenic reagents resulting in a coloured and non-uniform background. Therefore, the precision and accuracy of quantitative TLC analysis depend on the layer characteristics and the validation procedure should take these parameters into account.

The aim of this work was to validate the results of intra-laboratory studies dealing with

* Corresponding author.

☆ Presented at the *International Symposium on Chromatographic and Electrophoretic Techniques, Bled, Slovenia, 10–13 October 1994.*

0021-9673/95/$09.50 
*SSDI* 0021-9673(95)00018-6

quantitative analysis on laboratory-prepared plates. For this purpose the following chromatographic system was used: sorbent, natural zeolite mixed with microcrystalline cellulose; and test samples, α-alanine and glycine. This system was chosen as a model system for the determination of limiting factors in the quantitative analysis of diffuse and irregular spots obtained on laboratory-prepared plates.

## 2. Experimental

### 2.1. Chromatographic system

*Sorbent*

A mixture of microcrystalline cellulose (Merck) and natural tuff (1:1, w/w) was suspended in water, homogenized with an electric stirrer and spread on glass plates (20 × 20 cm) with a Camag applicator. The natural tuff, originating from the vicinity of Donje Jasenje, Croatia, consisted mainly of clinoptilolite, a mineral of the zeolite group, with minor amounts of feldspar, illite, sepiolite and calcite. The tuff was previously sieved and the fraction with particle size <40 μm was used for the layer preparation. The thickness of the wet layer was 300 μm. All experiments were performed on layers dried at room temperature.

*Sample*

A mixed stock standard solution of α-alanine and glycine was prepared by dissolving accurate amounts of powdered amino acids (Merck) in ethanol–$H_2O$ (1:1, v/v). This solution was suitably diluted to give a mass concentration of each compound in the range 5–500 μm/ml.

A 10-μl Hamilton syringe was used for sample spotting. Samples were applied either as a narrow band 15 mm long or as spots with an approximate diameter of 5 mm.

*Developing system*

Phenol (saturated with water)–ethanol–acetic acid–water (12:4:1:4, v/v) was used.

*Detection reagent*

Spots were detected by spraying with ninhydrin solution (1% in 1-butanol) and heating at 80°C for 20 min.

### 2.2. Apparatus and conditions for determination

The following were used:

1. Datacolor DC 3890 reflectance spectrometer (Datacolor, Switzerland) equipped with an IBM-PC XT/AT computer; monitoring range, 400–700 nm with 10-nm intervals; screen diameter, 18 mm; monitoring wavelength (remission minimum), alanine λ = 510 nm and glycine λ = 500 nm.
2. Leco 2001 image analyser (Leco, Germany) equipped with a 486XT computer and high-resolution CCD camera with zoom; calibration, 0.1609 μm per pixel; determination of spot area in manual mode.
3. Camag Turner fluorimeter 111 slit-scanning densitometer (Camag, Muttenz, Switzerland) equipped with a chart recorder; slit width, 1 mm; scanning speed, 20 mm/min; filter number, 826; λ = 510 nm; chart paper speed, 20 mm/min; range, 5 mV.

## 3. Results

### 3.1. Colour analysis–visible reflectance spectrometry

The reflectance intensity of reddish violet spots was measured using the Datacolor DC 3890 reflectance spectrometer. The on-plate remission spectrum was plotted for amino acid samples (Fig. 1). The wavelengths of the remission minimum (500 nm for glycine and 510 nm for alanine) were chosen for further determination. The calibration graphs of remission (%) against sample concentration, regression equations and correlation coefficients are given in Fig. 2.

The precision of quantitative analysis on zeolite layers was checked by applying on the same plate seven loadings of the mixed standard solutions (low concentration level, 30 μg/ml

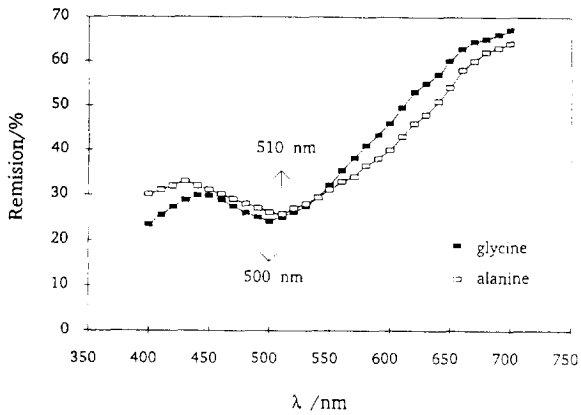


Fig. 1. Remission on-plate spectra of (□) alanine and (■) glycine obtained using the Datacolor DC 3890 visible reflectance spectrometer.

corresponding to 0.3 μg per spot; and high concentration level, 250 μg/ml corresponding to 2.5 μg per spot). Each spot was recorded five times and the relative standard deviation (R.S.D) was calculated. The results are summarized in Table 1. The error involved in the chromatographic and detection stages was estimated from the results of seven loadings (total R.S.D.). The instrumental R.S.D. corresponds to the error involved in the detection stage and it was obtained by multiple measurements of the same spot without resetting the screening position. This value represents the minimum error involved in the quantitative procedure and is attributable to the instrumentation system alone.

The detection limit (mass of substance that gives a response equal to twice the background) and the limit of quantification (mass of substance that allows the determination with 5% reliability) are given in Table 2. The results show that there is no significant difference between two amino acids and the values listed in table correspond for both alanine and glycine.

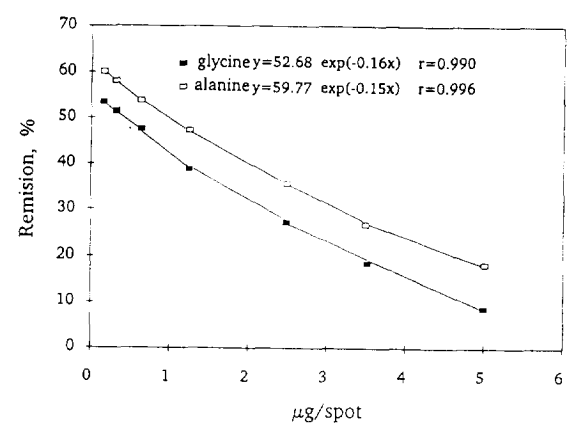


Fig. 2. Calibration graphs: colour analysis using the Datacolor DC 3890.

### 3.2. Image analysis

Calibration graphs of the spot area and spot intensity, determined with the Leco 2001 image analyser, as a function of sample concentration were plotted for each compound. Two sets of experiments were conducted. In one experiment samples were applied as a narrow band (length 15 mm) and in the other as a spot. The calibration graphs are given in Fig. 3A and B, respectively. Using an image analyser the chromatograms are imaged by a charged-coupled video camera and the information obtained is processed and documented in the form of a black-and-white picture. The spot area and spot intensity were determined using the "manual mode" software option for indication of spot edges. The measured value of the spot intensity is defined as the intensity of white colour in the spot within the range from 0 (100% black) to 255 (100% white).

### 3.3. Densitometry

For densitometric determination, calibration graphs of peak area against sample concentration were plotted (Fig. 4). A chromatogram of a mixed standard solution is given in Fig. 5. Each peak was recorded twice, and from the chromatographic profiles obtained areas under the chromatographic curves were determined using a planimeter as the mean value of three determinations. The R.S.D. of the planimetric determination of peak area was checked separately, and values of 0.5% for high concentration and 1.0% for low concentration were obtained on the basis

Table 1
Total and instrumental R.S.D. values

| Method of quantification | R.S.D. (%) | | | | |
|---|---|---|---|---|---|
| | Type | Alanine | | Glycine | |
| | | 2.5 μg per spot | 0.3 μg per spot | 2.5 μg per spot | 0.3 μg per spot |
| Color analysis: Datacolor | Total | 2.71 | 3.07 | 2.74 | 2.92 |
| 3890 | Instrumental | <0.1 | 0.2 | <0.1 | 0.2 |
| Densitometry: Camag Turner | Total | 3.53 | 12.08 | 3.52 | 12.05 |
| Fluorimeter 111 | Instrumental | 1.53 | 8.99 | 1.40 | 8.80 |
| Image analysis: | Spot area: | | | | |
| Leco 2001, sample applied | Total | 4.76 | BDL[a] | 4.67 | BDL |
| as band | Instrumental | 2.77 | BDL | 2.87 | BDL |
| | Spot intensity: | | | | |
| | Total | 3.03 | BDL | 3.37 | BDL |
| | Instrumental | 0.45 | BDL | 0.40 | BDL |
| Image analysis: | Spot area: | | | | |
| Leco 2001, sample applied | Total | 3.85 | 10.31 | 4.02 | 10.88 |
| as a spot | Instrumental | 2.00 | 7.40 | 2.10 | 7.00 |
| | Spot intensity: | | | | |
| | Total | 3.80 | 9.45 | 3.65 | 9.00 |
| | Instrumental | 0.90 | 5.90 | 1.00 | 5.35 |

[a] BDL = below detection limit.

of seven measurements. Errors associated with the various steps in the densitometry are given in Table 1. The measurement error (instrumental R.S.D. was determined by multiple scanning (5×) of a single lane without changing any experimental variables between scans.

## 4. Discussion

Modern TLC has introduced sophisticated mechanical scanning densitometers, electronic scanners and video systems in daily laboratory practice [2]. One of the prerequisites for their meaningful and successful application is meeting detailed set criteria in preliminary stages of quantitative (QTLC). These criteria include the use of commercial precoated plates with strictly controlled performance. The question is how to perform, if it is necessary, quantitative analysis on laboratory-prepared layers. These layers cannot compete with precoated plates in term of consistency and homogeneity. Consequently, the spots obtained on laboratory-prepared plates are

Table 2
Detection limit and limit of quantification for the methods used

| Method | Detection limit (μg per spot) | Limit of quantification (μg per spot) |
|---|---|---|
| Colour analysis | 0.08 | 0.16 |
| Image analysis: | | |
| Sample applied as band | 0.35 | 1.25 |
| Sample applied as spot | 0.20 | 1.00 |
| Slit-scanning densitometry | 0.20 | 0.50 |

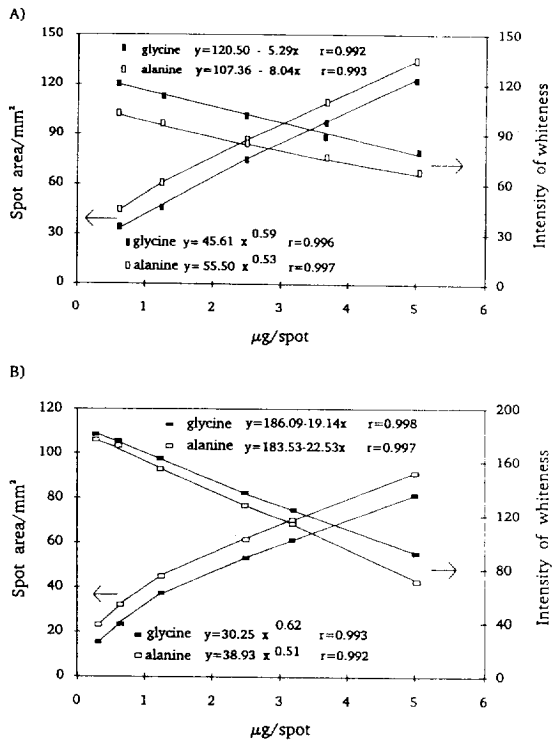


Fig. 3. Calibration graphs: image analysis using the Leco 2001 image analyser. (A) Sample applied as a 15-mm band; (B) sample applied as a spot.

more diffuse, making quantitative analysis more difficult even if an excellent separation is achieved.

The principal sources of error in QTLC are

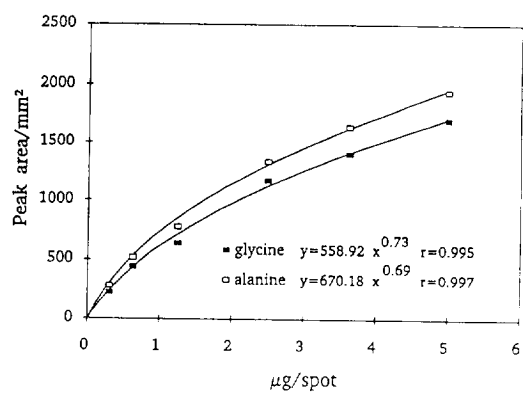


Fig. 4. Calibration graphs: densitometric scanning using Camag Turner Fluorimeter 111.

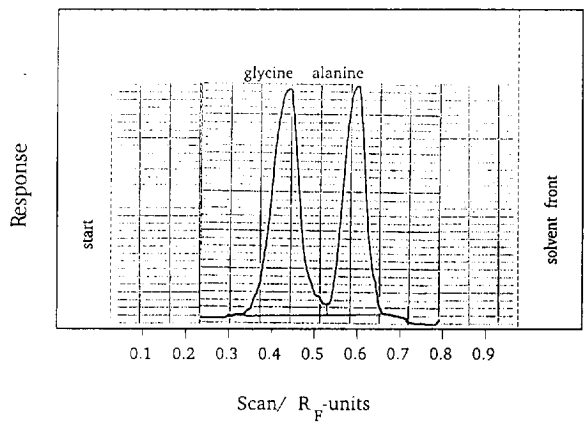


Fig. 5. Densitometric scan of a mixed standard solution Alanine and glycine concentrations, 0.25 µg/µl.

the reproducibility of sample application, reproducibility of the chromatographic conditions (heterogeneity of sorbent, deviations in layer thickness, non-linear solvent front) and reproducibility of the instrumental measurements.

The sample must be distributed homogeneously and the volume of sample applied to the layer must be accurately known. If possible, the sample application procedure should be automated using spray-on devices. However, many analytical laboratories, especially research laboratories dealing with small numbers of samples, use hand-held microsyringes. Although direct measurements of the precision of spotting procedure, relevant to this particular examination, were not conducted, some estimations on the basis of literature data can be made. The R.S.D. in spotting thin layers using a Hamilton microsyringe with a repeating dispenser, according to the literature [3,4], varies from 1.3 to 6.8% for delivery of 10 µl of ethanolic sample solution. This variation in the accuracy is due in part to the experience of the particular operator.

The difference between the total R.S.D. and instrumental R.S.D. corresponds to the error involved in the chromatographic stage. This error can be mainly attributed to the reproducibility of the spotting procedure, since the reproducibility of the chromatographic conditions was maintained by multiple determination

on a single plate. In all cases this value ranged between 2 and 3.3%.

A lower total R.S.D. was obtained by measuring reflectance with the Datacolor DC 3890: 2.7% for high concentration and 3% for low concentration with an instrumental R.S.D. of 0.1–0.2%. Transmission scanning (densitometry) and image analysis resulted in lower precision, especially at low concentration (total R.S.D. >10%), with a significant instrumental error.

The mechanical strength, durability and abrasion resistance of zeolite layers are comparable to those of conventional cellulose or silica gel layers. However, because of the considerably larger particle size of zeolite (<40 $\mu$m) and the high porosity of prepared layers, the spot edges were not sharp enough for automatic determination of spot size using the Leco 2001 image analyser. Working in the manual mode leads to a significant instrumental error and the results obtained are largely dependent on operator skill. An additional problem was associated with a reduced contrast between the spot and background due to the grey colour of the zeolite layer.

The main advantage of colour analysis using the Datacolor DC 3890 visible reflectance spectrometer is the possible analysis of irregular and diffuse spots. The parallel background colour analysis minimizes the problem of coloured and non-uniform layers. This method can be applied in laboratory practice for determination only in the visible range ($\lambda$ = 400–700 nm), but with low sensitivity at the edge of the wavelength range (light yellow spots). The advantage is fast data acquisition, simple instrument design and possible analysis of two-dimensional chromatograms which are difficult to scan using conventional slit-scanning densitometers.

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