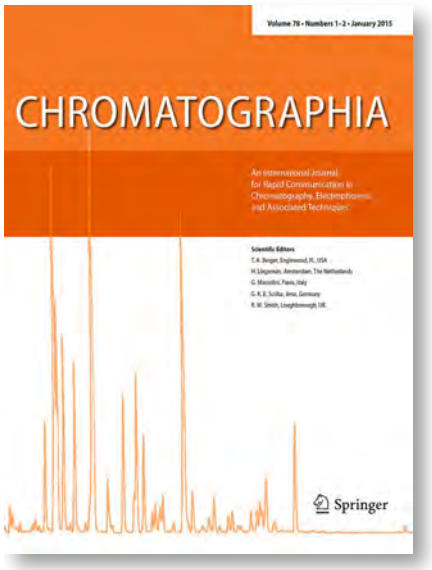

Chromatographia

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ORIGINAL

Studying Systematic Errors on Estimation Decision, Detection, and Quantification Limit on Micro-TLC

I Made Agus Gelgel Wirasuta · Ni Made Amelia Ratnata Dewi · Kadek Duwi Cahyadi · Luh Putu Mirah Kusuma Dewi · Ni Made Widi Astuti · I Nyoman Kadjeng Widjaja

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Abstract The decision limit ($CC\alpha$), capability of detection ($CC\beta$) and quantification limit (QL) are importance performance characteristics in method validation. The TLC-Scanner 3 from Camag provides the possibility to choose the slit dimension of light to determine the peak chromatogram of a substance. The influence of the slit dimension for determination of $CC\alpha$, $CC\beta$ and QL of paracetamol has been carried out. Paracetamol was spotted onto plates of AL-TLC Si G 60 F254 by linomat 4 in the range of 50–400 ng/spot and 10–400 ng/band, then on twin chambers eluted with TAEA (toluene:acetone-ethanol:conc.ammonia, 45 + 45 + 7 + 3 v/v) for 45 mm. Eluted spots were scanned in different slit dimensions at 248 nm. The $CC\alpha$, $CC\beta$ and QL of paracetamol were estimated through the linear regression (LRM) and signal-to-noise (S/N) methods. Slit lengths between 50 and 133 % of the band width of the spots, and with the noise factor of the slit under 2.6, produced good precision measurements of TLC-densitometry between plates, while slit lengths between 50 and 83 % of the band width of the spots introduced a higher sensitivity response of the detector. The estimated $CC\alpha$, $CC\beta$ and QL were determined by how the data were collected, the analytical optical setting, and the usage method for the estimation of both validation parameters.

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Keywords Thin layer chromatography · Decision limit · Capability of detection · Detection limit · Quantification limit, error

Introduction

The European Decision no. 657/2002/EC, concerning the performance of analytical methods and interpretation of results, recommends the calculation of two statistical limits, the decision limit ($CC\alpha$) and the capability of detection ($CC\beta$) [2, 3, 9]. The $CC\alpha$ is referred as the critical value and the limit for the non-existence of a certain component with a probability of first-order (α-error) to consider false-positive samples [3, 9]. The $CC\beta$ indicates the minimum content that can be proven with a high, given probability of second-order (β-error) to consider false-negative samples. The $CC\beta$ is also referred to as the detection limit (DL) [3]. The DL is known as the lowest analyte concentration that can be detected and identified with a given degree of certainty, and is also defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence [8]. These validation parameters are well known as not either robust or rugged parameters. They can be affected by minor changes in the analytical system.

TLC-densitometry is often used in screening tests for the testing of illicit drugs in seized materials and biological specimens. In screening tests, DL can be taken as the cut-off level [8]. The cut-off level is the minimum level at which the drug is consistently detected. Practically, estimations of DL and the quantitation limit (QL) based on LRM are often influenced by the range of concentration standard and the slit-dimension measurement. The implementation of a wider range of concentration standards often

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produces a non-linear regression of the calibration curve. Indrayanto et al. recommended using the *sdv* value for linearity as a criteria acceptance for the linearity of a calibration curve [7] and the minimum acceptance of *sdv* value should be not more than five [6]. Our laboratory's experience is that the value of the correlation coefficient (r) correlates with both DL and *QL*.

These validation parameters can be estimated by using the linear regression (LRM) and signal-to-noise (S/N) methods. The LRM is well described by Funk et al. [3]. The linear regression function, which is derived from the first-order calibration function, is:

$$y = a + bx, \tag{1}$$

where a is the intercept and b is the slope of the regression function. $CC\alpha$ and $CC\beta$ can be calculated as follows:

$$CC\alpha = \frac{Sy}{b}.t_{f,\alpha}\sqrt{\frac{1}{Na}+\frac{1}{Nc}+\frac{\bar{x}^2}{Qxx}} \tag{2}$$

[3] where Sy = residual standard deviations, Na = the original number of calibration data pairs, Nc = the number of calibration standards, Qxx = the sum of $(x_i - \bar{x})^2$, and $t_{f,\alpha}$ = the t value dependent upon both the acceptable error probability, α, and the number of degrees of freedom, f. For equal significance levels α and β, it follows that [3]:$t_\alpha = t_\beta = t$ and, therefore, $CC\beta = 2CC\alpha$

For estimation of DL and *QL* by using the S/N method, the peak-to-peak noise around the analyte is measured, and, subsequently, the concentration of the analyte that would yield a signal equal to a certain value of noise to signal is estimated. Mathematically, the analyte's signal at the DL, $(S_a)_{\mathrm{DL}}$, is:

$$(S_a)_{DL} = mN + 3sdN \quad [7] \tag{3}$$

$$CC\beta = DL\ [3], \text{therefore, } (S_a)_{CC\beta} = mN + 3sdN$$

$$CC\beta = CC\alpha + 1.64sdN\ [2], \text{ therefore, } (S_a)_{CC\alpha} = mN + 1.46sdN \tag{4}$$

$$(S_a)_{QL} = mN + 10sdN\ [7] \tag{5}$$

$$CC\alpha = \frac{(S_a)_{CC\alpha}}{S_s}C_s;\ CC\beta = \frac{(S_a)_{CC\beta}}{S_s}C_s \text{ and } QL = \frac{(S_a)_{QL}}{S_s}C_s \tag{6}$$

where mN is the mean of noise, sdN is the known standard deviation for noise, S_s is the signal of a certain concentration response, and C_s is the concentration of the measured response. The noise value may vary between the sample track and variation of the slit measurement.

The TLC Scanner 3-Camag provides many slit dimensions for quantitative analyses. The measuring slit is adjusted to give a light beam whose dimensions suit the size of the spot. Hahn-Deinstrop [4] has recommended that the prefered slit dimension for quantitative evaluation of an aliquot is 50–75 % of the spot band, which is taken from the central part of the band. The slit dimension determines the amount of the light beam, which will have an effect on the signal, noise, and AUC of the chromatogram peak [4].

In this study, we present the influence of slit dimension on the estimation of $CC\alpha$, $CC\beta$ and *QL*. The validation parameters were calculated using the LRM and S/N methods. The aim of this study was to compare the estimation methods for the limits of validation parameters based on the TLC-densitometry technique and to discover the optimum silt dimension for better qualitative and quantitative analysis.

Experimental

Chemicals and Materials

Chemicals (toluene, acetone, ethanol, conc. ammonia, methanol) were analytical grade from Merck, Darmstadt, Germany. AL-TLC silica 60G F_{254} (20 × 20 cm) were also from Merck. The plates in their original dimensions were cut into 10 × 5 cm pieces. Paracetamol was obtained from Pharmaceutical Industry (PT Samparindo, Semarang,Indonesia).

Sample Preparation and TLC-Densitometry

Before use, five plates were washed with methanol and dried in an oven at 120 °C for 30 min. The activated plates were equilibrated and stored in a desiccator.

The paracetamol was diluted in methanol and then applied to the TLC plates by means of a Linomat IV applicator (Camag, Muttenz, Switzerland) equipped with a 100-µL syringe. Nitrogen gas was used to disperse the solution into fine drops and to facilitate solvent evaporation. Samples were in the form of bands of 6 mm in length, for the first application $x = 10$ mm, $y = 10$ mm, the space between tracks was 10 mm. The amounts of paracetamol per spot were 50, 100, 200, and 400 ng/band for the first data series and 10, 20, 50, 100, 200, 400 ng/band for the second data series. The first data series were spotted on five different plates.

Every spotted sample was developed to a distance of 45 mm. Plates were on TAEA (toluene:acetone-ethanol:conc.ammonia, 45 + 45 + 7 + 3 v/v) at room temperature in glass twin-trough chambers (10 × 10 cm, with

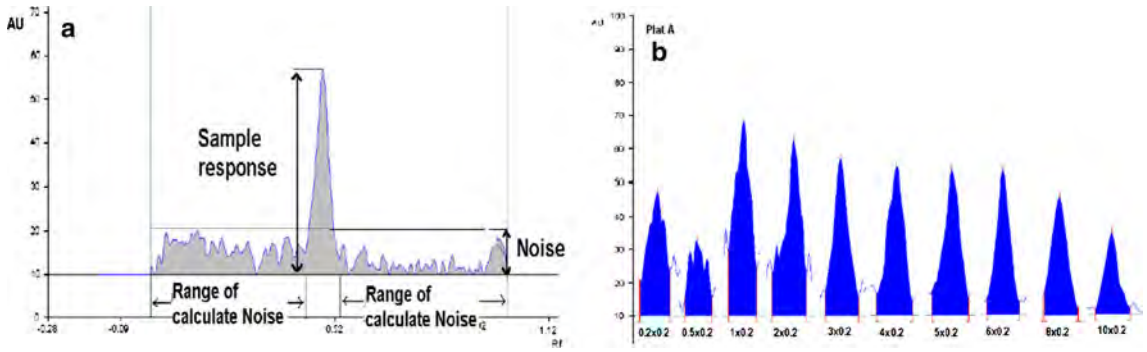

Fig. 1 Chromatogram peaks of 50 ng/spot paracetamol (**a**) and on different slit length dimensions (**b**)

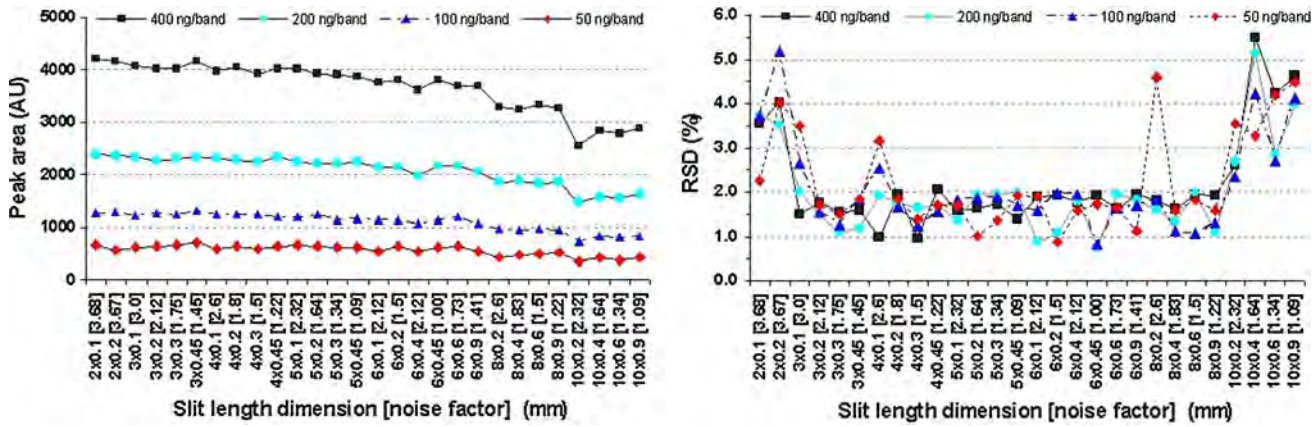

Fig. 2 The mean (*left*) and %RSD (*right*) of baseline-corrected area between plates of different slit dimension measurement

metal lids; Camag) previously saturated with mobile phase vapor for 30 min. After development, the plates were dried in an oven at 60 °C for 5 min.

Quantitative Evaluation of Chromatograms

Image capturing and data evaluation were performed with the TLC Scanner 3 operated with WinCATS–Planar Chromatography Manager v.1.4.2.8121 software (Camag). The chromatograms were captured under UV light ($\lambda = 248$ nm), with 28 different slit dimensions (see Fig. 2), scanning speed 20 mm s^{-1}, data resolution 100 µm/step. Parameters on integration of a peak for LRM were set on the filter factor: Savitsky-Golay 7, baseline correction: lowest slope, peak thresholds (minimum slope: 5, minimum height: 10 AU, minimum area: 50 AU, maximum height: 990 AU), integration limits of a standard peak was set between the start and end positions of the highest concentration peak; baseline correction was made. The S/N was obtained from all scanned tracks' concentration of the samples (Fig. 1a) by changing the parameters on integration of a peak. The peak thresholds were set at: minimum slope 1, minimum height 1 AU, minimum area 1 AU. The noise was collected from the spotted point to the response and from the response to the end of the elution point.

The following parameters were determined and calculated: baseline noise, evaluated from all empty line sample response, mean peak height of spot signal-to-noise ratio (shown in Fig. 1).

Results and Discussion

Peak Chromatogram on Difference Slit Dimension

The same amount of substance, which is scanned on different slit dimensions produced variation in chromatogram peaks (see Fig. 1b). The increasing of the slit length measurement tended to decrease the area of the standards (see Fig. 2). Great variation of the area for all concentrations between all plates was obtained on measurements under slit lengths of 2–10 mm. But under slits of 3.0 × 0.1 mm, 4.0 × 0.1 mm, and 8.0 × 0.2 mm, just concentrations of 100 and 50 ng/spot produced %RSD values of more than 2 (see Fig. 2). Based on %RSD values between plates, the quantitative measurement a spot should be done under slit lengths between 50 and 133 % of the band width of the spots and the noise factor of slit dimensions under 2.6.

Table 1 Linear regression data series (50–400 ng/band) of each plate for calibration plots, *CCα*, *CCβ* and *QL* estimation

	Range	Mean	%RSD
Plate A (*Na = 4; Nc = 1*)			
Slope	7.52–9.55	8.84	7.01
Intercept	166.48–326.28	245.23	20.11
Correlation coefficient (*r*)	0.9913–0.9984	0.9954	0.1921
Residual standard deviation (*sdv*) linear (%)	3.59–8.40	5.83	22.23
CCα 0.95 one side (ng/band)	24.65–58.69	41.51	21.83
CCβ 0.95 one side (ng/band)	49.30–117.39	83.02	21.83
QL (ng/band)	73.95–176.08	124.53	21.83
Plate B (*Na* = 4; *Nc* = 1)			
Slope	7.57–9.63	8.89	6.23
Intercept	150.43–369.77	256.10	21.23
Correlation coefficient (*r*)	0.9829–0.9980	0.9937	0.3750
Residual standard deviation (*sdv*) linear (%)	3.98–10.95	6.71	27.13
CCα 0.95 one side (ng/band)	28.15–82.46	48.01	28.68
CCβ 0.95 one side (ng/band)	56.30–164.93	96.01	28.68
QL (ng/band)	84.45–247.39	144.02	28.68
Plate C (*Na* = 4; *Nc* = 1)			
Slope	7.85–10.01	9.16	6.74
Intercept	147.90–317.41	229.05	21.09
Correlation coefficient (*r*)	0.9929–0.9988	0.9961	0.1684
Residual standard deviation (*sdv*) linear	3.14–8.34	5.64	26.01
CCα 0.95 one side (ng/band)	21.89–52.73	38.13	22.76
CCβ 0.95 one side (ng/band)	43.79–105.47	76.26	22.76
QL (ng/band)	65.68–158.20	114.38	22.76
Plate D (*Na* = 4; *Nc* = 1)			
Slope	7.74–9.84	9.13	6.61
Intercept	132.96–312.71	215.14	23.53
Correlation coefficient (*r*)	0.9918–0.9993	0.9969	0.1863
Residual standard deviation (*sdv*) linear	2.50–8.11	4.80	29.02
CCα 0.95 one side (ng/band)	16.73–56.87	33.48	29.96
CCβ 0.95 one side (ng/band)	33.45–113.74	66.96	29.96
QL (ng/band)	50.18–170.61	100.44	29.96
Plate E (*Na* = 4; *Nc* = 1)			
Slope	7.85–10.05	9.18	7.22
Intercept	150.49–300.36	216.44	18.10
Correlation coefficient (*r*)	0.9943–0.9989	0.9970	0.1458
Residual standard deviation (*sdv*) linear	2.95–6.81	4.76	24.15
CCα 0.95 one side (ng/band)	20.71–47.41	33.21	24.62
CCβ 0.95 one side (ng/band)	41.43–94.83	66.42	24.62
QL (ng/band)	62.14–142.24	99.63	24.62

DL and *QL* Estimation

LRM Method

The *CCα*, *CCβ* and *QL* were calculated based on calibration data pairs of each plate and all five plates, with the precision AUC between plates (%RSD) of not more than 2 %. Their linear regressions are listed in Tables 1, 2 and Fig. 3. The calculated *sdv* value based on data pairs of each plate ranged between 2.50 and 10.95 and the *r* value lay between 0.9829 and 0.9993. Camag described the *sdv* (residual standard deviation of the standard point) for expressing the fit of a calibration curve for TLC by using its CATS software. The lower the *sdv* value means, the

Table 2 Linear regression data series (50–400 ng/band) of five plates for calibration plots, *CCα, CCβ* and *QL* estimation

	Range	Mean	%RSD
All plates (A–E) ($Na = 4$; $Nc = 5$)			
Slope	7.80–9.82	9.04	6.65
Intercept	157.18–300.17	232.39	19.22
Correlation coefficient (r)	0.9924–0.9970	0.9952	0.14
$CC\alpha$ 0.95 one side (ng/band)	13.60–21.54	16.94	14.75
$CC\beta$ 0.95 one side (ng/band)	27.21–43.09	33.87	14.75
QL (ng/band)	40.81–64.63	50.81	14.75

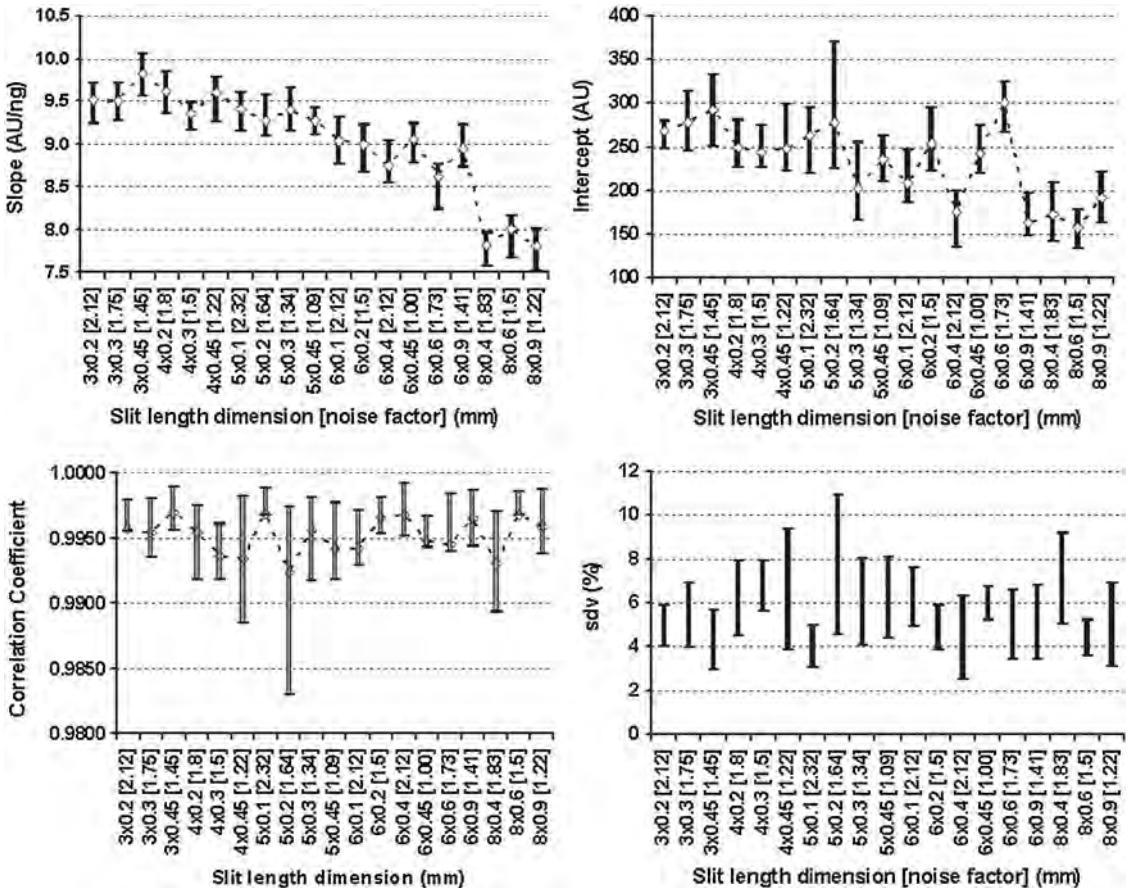

Fig. 3 The estimated parameters of calibration functions of data series of each plate (*filled whisker*) and all plates (*filled diamond*)

closer the points (measurement area) to the curve. The *sdv* is also referred to as residual standard deviations (*Sy*). The frequencies of *sdv* values vary between plate and slit dimension measurements, although all area of peaks presented good precision between plates.

Increasing the slit length measurement of a calibration series led to a decrease in the slope of matrix calibration (see Fig. 3). In the first-order calibration function, the slope is a measure of the sensitivity of the analytical procedure [3]. It means that an increase of the slit length measurement could decrease the analytical sensitivity. A measurement of the spot on a slit length between 50 and 83 % of the band width of the spot introduced higher sensitivity response of the detector.

Comparison of calculated calibration functions based on data series of each plate and all plates presented a relative closer function calibration between them (relative same slope and intercept) on the same slit length dimensions.

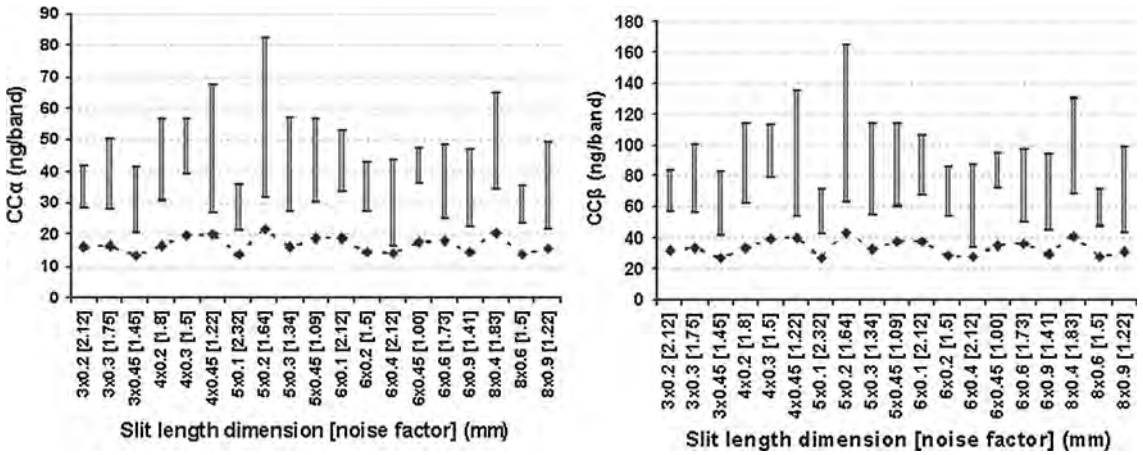

Fig. 4 Comparison of *CCα* and *CCβ* based on data series of each plate and all plates, *filled whisker* range of *CCα, CCβ* between each plate and *filled diamond CCα, CCβ* all plates

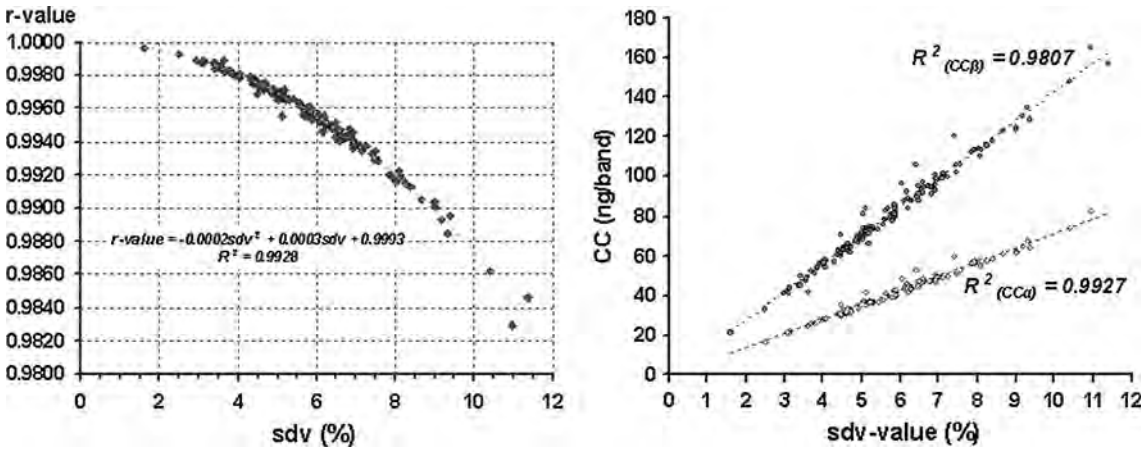

Fig. 5 Relationship between *sdv* value between *r* value (*left side*) and *CCα/CCβ* (*right side*)

Table 3 Linear regression data series (10–400 ng/band) for calibration plots, *CCα, CCβ* and *QL* estimation

Range of concentration (ng/band)	Calibration function	Correlation coefficient (r)	*sdv* (%)	*CCα* 0.95 one side (ng/band)	*CCβ* 0.95 one side (ng/band)	*QL* (ng/band)
10–200	$y = 18.04x - 24.39$	0.9998	1.28	3.84	7.68	13.60
10–400	$y = 16.42x + 74.38$	0.9973	7.05	21.37	42.73	87.29
50–400	$y = 16.11x + 164.76$	0.9966	5.83	36.29	72.58	108.87

The calculated slope, intercept, and *r* values of calibration functions based on the data series of all plates showed a mean value of those parameters, which were calculated based on data series of each plate (Fig. 3). But calculated *CCα*, *CCβ* and *QL* based on data series of all plates obtained lower values than those based on data series of each plate (Fig. 4). The lowering of these validation parameters was due to a decrease of the statistical *t* value, which was caused by a higher number of degrees of freedom (Nc).

The calculated *CCα*, *CCβ* and *QL* varied between slit dimension measurements and plates (Fig. 4; Tables 1, 2). The *sdv* values present a polynomial correlation to the *r* value and a linear correlation to both *CCα*, and *CCβ* (see Fig. 5). The closer points to the curve produced lower *CCα* and *CCβ*.

From the second data series (10–400 ng/band), the linear regression data, *CCα*, *CCβ* and *QL*, were calculated and are presented in Table 3. It is well known that the calibration range concentration influences the linearity of the

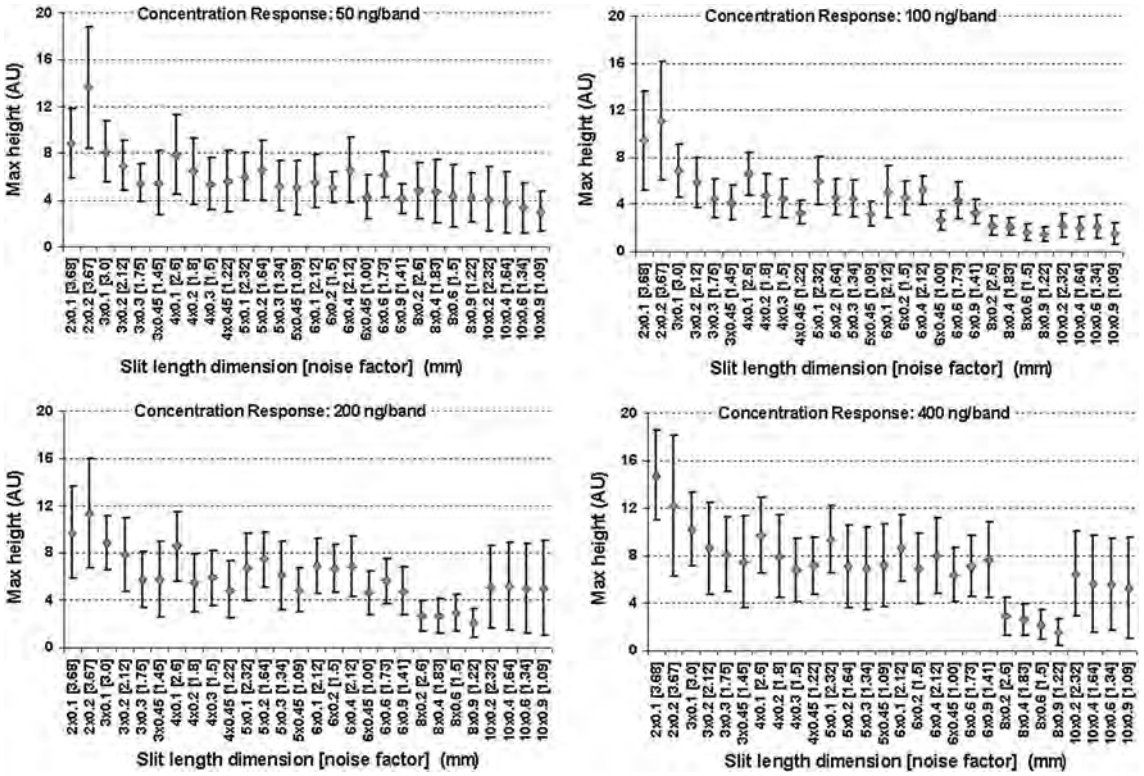

Fig. 6 The mean noise (mN ± sdN) of every concentration response on plate A

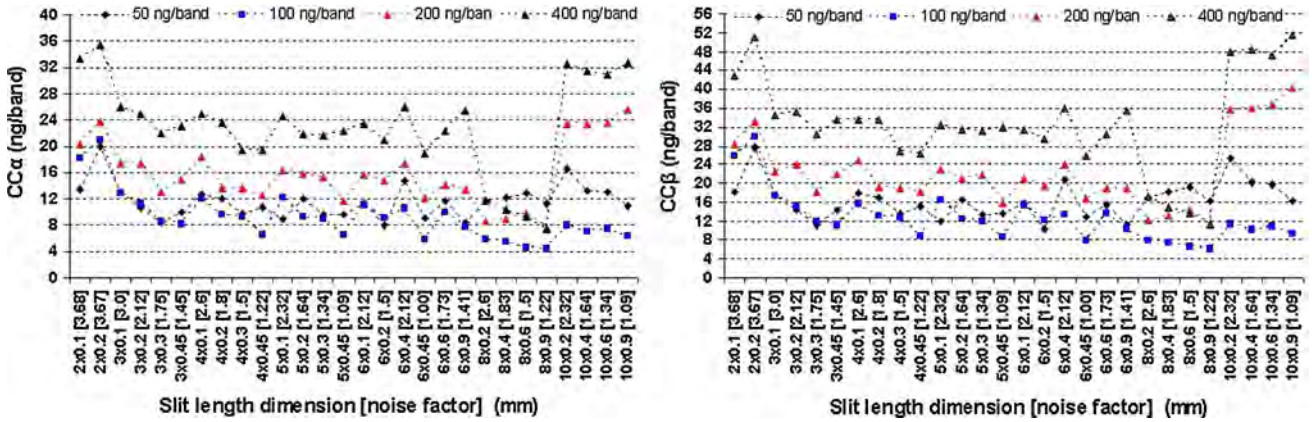

Fig. 7 The calculated *CCα* and *CCβ* based on the S/N method on different concentration responses under variable slit dimension measurements

regression calibration and also the *CCα*, *CCβ* and *QL*. The range concentration 10–200 ng/band showed the best linearity with the highest *r* and lowest *sdv* value. This introduced the lower *CCα*, *CCβ* and *QL*.

S/N Method

The noise value of every concentration response on plate A, which was measured under different slit lengths, are presented in Fig. 6. The signal and noise will decrease along the increasing area of the slit (see Figs. 1, 2, 6). These values were significantly different between slit measurements. Slit dimensions determine the amount of directed light to the plate and the covered surface area of the detected spot. The particle size variation of silica on the covered area influenced the direction of the reflected beam to the detector and the amount of detected light on the detector. This will deliver variation of noise between slit dimensions. The narrower slit introduced highly variable noise all over the measured track. This variation introduced

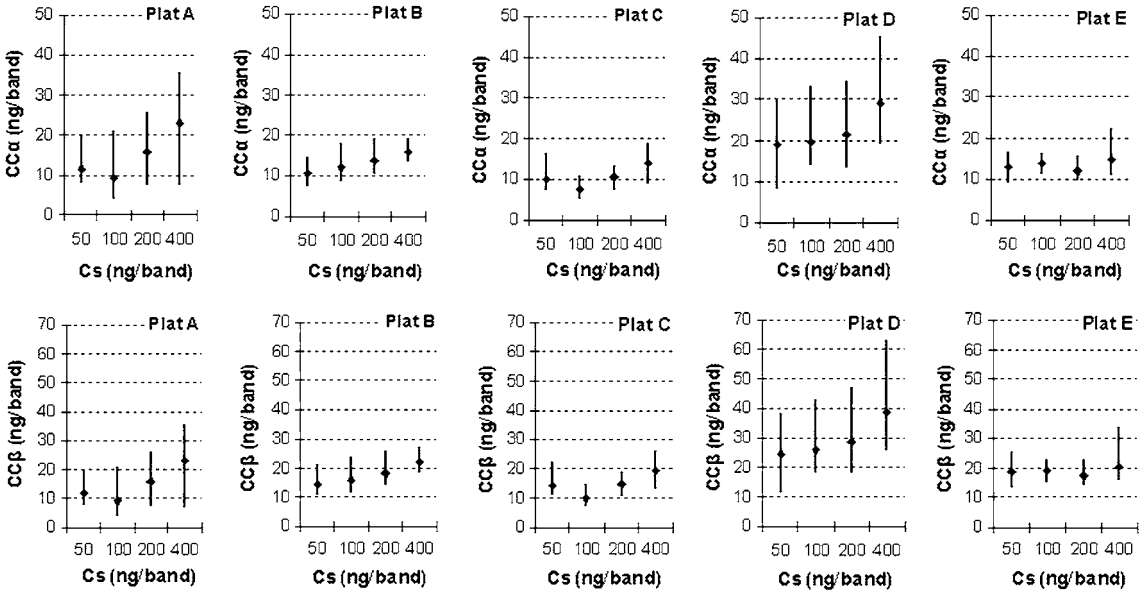

Fig. 8 The range and mean calculated $CC\alpha$ and $CC\beta$ based on the S/N method on different concentration responses between five plates

differences in $CC\alpha$ and $CC\beta$ values between slit measurements (Figs. 7, 8).

The range and mean of calculated $CC\alpha$ and $CC\beta$ based on the S/N method on different concentration response between five plates are shown in Fig. 8. Variations in the range and mean of these validation parameters could be caused by the different read-out of S/N values between slit measurements and plates of TLC. TLC is an open chromatogram system. The handling of the plates while being analyzed could introduce the variation in the detected noise.

$$\%\text{RSD} \approx \frac{50}{\text{S/N}} \tag{7}$$

Dolan [9] described the role of the S/N ratio in precision and accuracy, producing an equation to figure out the relationship between both validation parameters and the S/N ratio (Eq. 7) and used this equation to predict how small a peak can be and still generate usable data [1]. Rearranging Eq. (6), where $(S_a)_{CC\alpha} \approx N_{CC\alpha}$; $(S_a)_{CC\beta} \approx N_{CC\beta}$; $S_s \approx S$, we obtain:

$$CC\alpha = \frac{C_s}{S_s/(S_a)_{CC\alpha}} \quad ; \%\text{RSD} \approx \frac{C_s}{S_s/N_{CC\alpha}}$$

Based on this equation, the higher the S_s/N ratio, the better the precision and accuracy.

Conclusion

Slit lengths between 50 and 133 % of the band width of the spots, and with the noise factor of the slit under 2.6, produced good precision measurements of TLC-densitometry between plates, while slit lengths between 50 and 83 % of the band width of the spots introduced a higher sensitivity response of the detector. The estimated $CC\alpha$, $CC\beta$ and QL were determined by how the data were collected, the analytical optical setting, and the usage method for the estimation of both validation parameters.

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systematic error

by Gelgel Wirasuta

Chromatographia
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ORIGINAL

Studying Systematic Errors on Estimation Decision, Detection, and Quantification Limit on Micro-TLC

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Abstract The decision limit ($CC\alpha$), capability of detection ($CC\beta$) and quantification limit (QL) are importance performance characteristics in method validation. The TLC-Scanner 3 from Camag provides the possibility to choose the slit dimension of light to determine the peak chromatogram of a substance. The influence of the slit dimension for determination of $CC\alpha$, $CC\beta$ and QL of paracetamol has been carried out. Paracetamol was spotted onto plates of AL-TLC Si G 60 F254 by linomat 4 in the range of 50–400 ng/spot and 400 ng/band, then on twin chambers eluted with TAEA (toluene:acetone-ethanol:conc.ammonia, 45 + 45 + 7 + 3 v/v) for 45 mm. Eluted spots were scanned in different slit dimensions at 248 nm. The $CC\alpha$, $CC\beta$ and QL of paracetamol were estimated through the linear regression (LRM) and signal-to-noise (S/N) methods. Slit lengths between 50 and 133 % of the band width of the spots, and with the noise factor of the slit under 2.6, produced good precision measurements of TLC-densitometry between plates, while slit lengths between 50 and 83 % of the band width of the spots introduced a higher sensitivity response of the detector. The estimated $CC\alpha$, $CC\beta$ and QL were determined by how the data were collected, the analytical optical setting, and the usage method for the estimation of both validation parameters.

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Keywords Thin layer chromatography · Decision limit · Capability of detection · Detection limit · Quantification limit, error

Introduction

The European Decision no. 657/2002/EC, concerning the performance of analytical methods and interpretation of results, recommends the calculation of two statistical limits, the decision limit ($CC\alpha$) and the capability of detection ($CC\beta$) [2, 3, 9]. The $CC\alpha$ is referred as the critical value and the limit for the non-existence of a certain component with a probability of first-order (α-error) to consider false-positive samples [3, 9]. The $CC\beta$ indicates the minimum content that can be proven with a high, given probability of second-order (β-error) to consider false-negative samples. The $CC\beta$ is also referred as the detection limit (DL) [3]. The DL is known as the lowest analyte concentration that can be detected and identified with a given degree of certainty, and is also defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence [8]. These validation parameters are well known as not either robust or rugged parameters. They can be affected by minor changes in the analytical system.

TLC-densitometry is often used in screening tests for the testing of illicit drugs in seized materials and biological specimens. In screening tests, DL can be taken as the cut-off level [8]. The cut-off level is the minimum level at which the drug is consistently detected. Practically, estimations of DL and the quantitation limit (QL) based on LRM are often influenced by the range of concentration standard and the slit-dimension measurement. The implementation of a wider range of concentration standards often

produces a non-linear regression of the calibration curve. Indrayanto et al. recommended using the *sdv* value for linearity as a criteria acceptance for the linearity of a calibration curve [7] and the minimum acceptance of *sdv* value should be not more than five [6]. Our laboratory's experience is that the value of the correlation coefficient (r) correlates with both DL and *QL*.

These validation parameters can be estimated by using the linear regression (LRM) and signal-to-noise (S/N) methods. The LRM is well described by Funk et al. [3]. The linear regression function, which is derived from the first-order calibration function, is:

$$y = a + bx, \tag{1}$$

where a is the intercept and b is the slope of the regression function. $CC\alpha$ and $CC\beta$ can be calculated as follows:

$$CC\alpha = \frac{Sy}{b} \cdot t_{f,\alpha} \sqrt{\frac{1}{Na} + \frac{1}{Nc} + \frac{\bar{x}^2}{Qxx}} \tag{2}$$

[3] where Sy = residual standard deviations, Na = the original number of calibration data pairs, Nc = the number of calibration standards, Qxx = the sum of $(x_i - \bar{x})^2$, and $t_{f,\alpha}$ = the t value dependent upon both the acceptable error probability, α, and the number of degrees of freedom, f. For equal significance levels α and β, it follows that [3]: $t_\alpha = t_\beta = t$ and, therefore, $CC\beta = 2CC\alpha$

For estimation of DL and *QL* by using the S/N method, the peak-to-peak noise around the analyte is measured, and, subsequently, the concentration of the analyte that would yield a signal equal to a certain value of noise to signal is estimated. Mathematically, the analyte's signal at the DL, $(S_a)_{DL}$, is:

$$(S_a)_{DL} = mN + 3sdN \quad [7] \tag{3}$$

$CC\beta = DL$ [3], therefore, $(S_a)_{CC\beta} = mN + 3sdN$

$$CC\beta = CC\alpha + 1.64sdN \text{ [2], therefore, } (S_a)_{CC\alpha} = mN + 1.46sdN \tag{4}$$

$$(S_a)_{QL} = mN + 10sdN \text{ [7]} \tag{5}$$

$$CC\alpha = \frac{(S_a)_{CC\alpha}}{S_x} C_x;\ CC\beta = \frac{(S_a)_{CC\beta}}{S_x} C_x \text{ and } QL = \frac{(S_a)_{QL}}{S_x} C_x \tag{6}$$

where mN is the mean of noise, sdN is the known standard deviation for noise, S_x is the signal of a certain concentration response, and C_x is the concentration of the measured response. The noise value may vary between the sample track and variation of the slit measurement.

The TLC Scanner 3-Camag provides many slit dimensions for quantitative analyses. The measuring slit is adjusted to give a light beam whose dimensions suit the size of the spot. Hahn-Deinstrop [4] has recommended that the prefered slit dimension for quantitative evaluation of an aliquot is 50–75 % of the spot band, which is taken from the central part of the band. The slit dimension determines the amount of the light beam, which will have an effect on the signal, noise, and AUC of the chromatogram peak [4].

In this study, we present the influence of slit dimension on the estimation of $CC\alpha$, $CC\beta$ and *QL*. The validation parameters were calculated using the LRM and S/N methods. The aim of this study was to compare the estimation methods for the limits of validation parameters based on the TLC-densitometry technique and to discover the optimum silt dimension for better qualitative and quantitative analysis.

Experimental

Chemicals and Materials

Chemicals (toluene, acetone, ethanol, conc. ammonia, methanol) were analytical grade from Merck, Darmstadt, Germany. AL-TLC silica 60G F_{254} (20 × 20 cm) were also from Merck. The plates in their original dimensions were cut into 10 × 5 cm pieces. Paracetamol was obtained from Pharmaceutical Industry (PT Samparindo, Semarang,Indonesia).

Sample Preparation and TLC-Densitometry

Before use, five plates were washed with methanol and dried in an oven at 120 °C for 30 min. The activated plates were equilibrated and stored in a desiccator.

The paracetamol was diluted in methanol and then applied to the TLC plates by means of a Linomat IV applicator (Camag, Muttenz, Switzerland) equipped with a 100-µL syringe. Nitrogen gas was used to disperse the solute into fine drops and to facilitate solvent evaporation. Samples were in the form of bands of 6 mm in length, for the first application $x = 10$ mm, $y = 10$ mm, the space between tracks was 10 mm. The amounts of paracetamol per spot were 50, 100, 200, and 400 ng/band for the first data series and 10, 20, 50, 100, 200, 400 ng/band for the second data series. The first data series were spotted on five different plates.

Every spotted sample was developed to a distance of 45 mm. Plates were on TAEA (toluene:acetone-ethanol:concentrated ammonia, 45 + 45 + 7 + 3 v/v) at room temperature in glass twin-trough chambers (10 × 10 cm, with

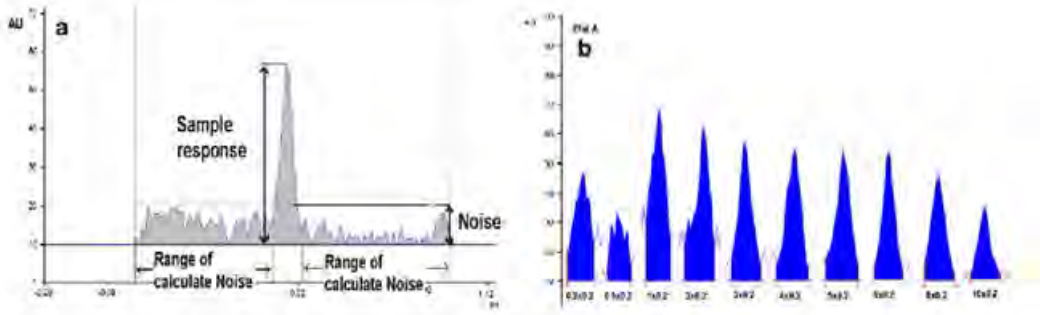

Fig. 1 Chromatogram peaks of 50 ng/spot paracetamol (**a**) and on different slit length dimensions (**b**)

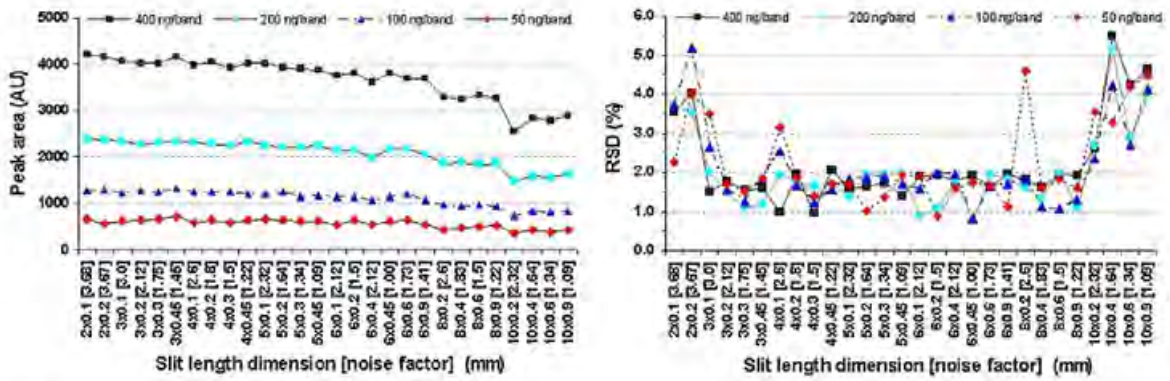

Fig. 2 The mean (*left*) and %RSD (*right*) of baseline-corrected area between plates of different slit dimension measurement

metal lids; Camag) previously saturated with mobile phase for 30 min. After development, the plates were dried in an oven at 60 °C for 5 min.

Quantitative Evaluation of Chromatograms

Image capturing and data evaluation were performed with the TLC Scanner 3 operated with WinCATS–Planar Chromatography Manager v.1.4.2.8121 software (Camag). The chromatograms were captured under UV light (λ = 8 nm), with 28 different slit dimensions (see Fig. 2), scanning speed 20 mm s^{-1}, data resolution 100 µm/step. Parameters on integration of a peak for LRM were set on the filter factor: Savitsky-Golay 7, baseline correction: lowest slope, peak thresholds (minimum slope: 5, minimum height: 10 AU, minimum area: 50 AU, maximum height: 990 AU), integration limits of a standard peak was set between the start and end positions of the highest concentration peak; baseline correction was made. The S/N was obtained from all scanned tracks' concentration of the samples (Fig. 1a) by changing the parameter integration of a peak. The peak thresholds were set at: minimum slope 1, minimum height 1 AU, minimum area 1 AU. The noise was collected from the spotted point to the response and from the response to the end of the elution point.

The following parameters were determined and calculated: baseline noise, evaluated from all empty line sample response, mean peak height of spot signal-to-noise ratio (shown in Fig. 1).

Results and Discussion

Peak Chromatogram on Difference Slit Dimension

The same amount of substance, which is scanned on different slit dimensions produced variation in chromatogram peaks (see Fig. 1b). The increasing of the slit length measurement tended to decrease the area of the standards (see Fig. 2). Great variation of the area for all concentrations between all plates was obtained on measurements under slit lengths of 2–10 mm. But under slits of 3.0 × 0.1 mm, 4.0 × 0.1 mm, and 8.0 × 0.2 mm, just concentrations of 100 and 50 ng/spot produced %RSD values of more than 2 (see Fig. 2). Based on %RSD values between plates, the quantitative measurement a spot should be done under slit lengths between 50 and 133 % of the band width of the spots and the noise factor of slit dimensions under 2.6.

Table 1 Linear regression data series (50–400 ng/band) of each plate for calibration plots, $CC\alpha$, $CC\beta$ and QL estimation

	Range	Mean	%RSD
Plate A ($Na = 4$; $Nc = 1$)			
Slope	7.52–9.55	8.84	7.01
Intercept	166.48–326.28	245.23	20.11
Correlation coefficient (r)	0.9913–0.9984	0.9954	0.1921
Residual standard deviation (sdv) linear (%)	3.59–8.40	5.83	22.23
$CC\alpha$ 0.95 one side (ng/band)	24.65–58.69	41.51	21.83
$CC\beta$ 0.95 one side (ng/band)	49.30–117.39	83.02	21.83
QL (ng/band)	73.95–176.08	124.53	21.83
Plate B ($Na = 4$; $Nc = 1$)			
Slope	7.57–9.63	8.89	6.23
Intercept	150.43–369.77	256.10	21.23
Correlation coefficient (r)	0.9829–0.9980	0.9937	0.3750
Residual standard deviation (sdv) linear (%)	3.98–10.95	6.71	27.13
$CC\alpha$ 0.95 one side (ng/band)	28.15–82.46	48.01	28.68
$CC\beta$ 0.95 one side (ng/band)	56.30–164.93	96.01	28.68
QL (ng/band)	84.45–247.39	144.02	28.68
Plate C ($Na = 4$; $Nc = 1$)			
Slope	7.85–10.01	9.16	6.74
Intercept	147.90–317.41	229.05	21.09
Correlation coefficient (r)	0.9929–0.9988	0.9961	0.1684
Residual standard deviation (sdv) linear	3.14–8.34	5.64	26.01
$CC\alpha$ 0.95 one side (ng/band)	21.89–52.73	38.13	22.76
$CC\beta$ 0.95 one side (ng/band)	43.79–105.47	76.26	22.76
QL (ng/band)	65.68–158.20	114.38	22.76
Plate D ($Na = 4$; $Nc = 1$)			
Slope	7.74–9.84	9.13	6.61
Intercept	132.96–312.71	215.14	23.53
Correlation coefficient (r)	0.9918–0.9993	0.9969	0.1863
Residual standard deviation (sdv) linear	2.50–8.11	4.80	29.02
$CC\alpha$ 0.95 one side (ng/band)	16.73–56.87	33.48	29.96
$CC\beta$ 0.95 one side (ng/band)	33.45–113.74	66.96	29.96
QL (ng/band)	50.18–170.61	100.44	29.96
Plate E ($Na = 4$; $Nc = 1$)			
Slope	7.85–10.05	9.18	7.22
Intercept	150.49–300.36	216.44	18.10
Correlation coefficient (r)	0.9943–0.9989	0.9970	0.1458
Residual standard deviation (sdv) linear	2.95–6.81	4.76	24.15
$CC\alpha$ 0.95 one side (ng/band)	20.71–47.41	33.21	24.62
$CC\beta$ 0.95 one side (ng/band)	41.43–94.83	66.42	24.62
QL (ng/band)	62.14–142.24	99.63	24.62

DL and QL Estimation

LRM Method

The $CC\alpha$, $CC\beta$ and QL were calculated based on calibration data pairs of each plate and all five plates, with the precision AUC between plates (%RSD) of not more than 2 %. Their linear regressions are listed in Tables 1, 2 and Fig. 3. The calculated sdv value based on data pairs of each plate ranged between 2.50 and 10.95 and the r value was between 0.9829 and 0.9993. Camag described the sdv (residual standard deviation of the standard point) for expressing the fit of a calibration curve for TLC by using its CATS software. The lower the sdv value means, the

Table 2 Linear regression data series (50–400 ng/band) of five plates for calibration plots, $CC\alpha$, $CC\beta$ and QL estimation

	Range	Mean	%RSD
All plates (A–E) ($Na = 4$; $Nc = 5$)			
Slope	7.80–9.82	9.04	6.65
Intercept	157.18–300.17	232.39	19.22
Correlation coefficient (r)	0.9924–0.9970	0.9952	0.14
$CC\alpha$ 0.95 one side (ng/band)	13.60–21.54	16.94	14.75
$CC\beta$ 0.95 one side (ng/band)	27.21–43.09	33.87	14.75
QL (ng/band)	40.81–64.63	50.81	14.75

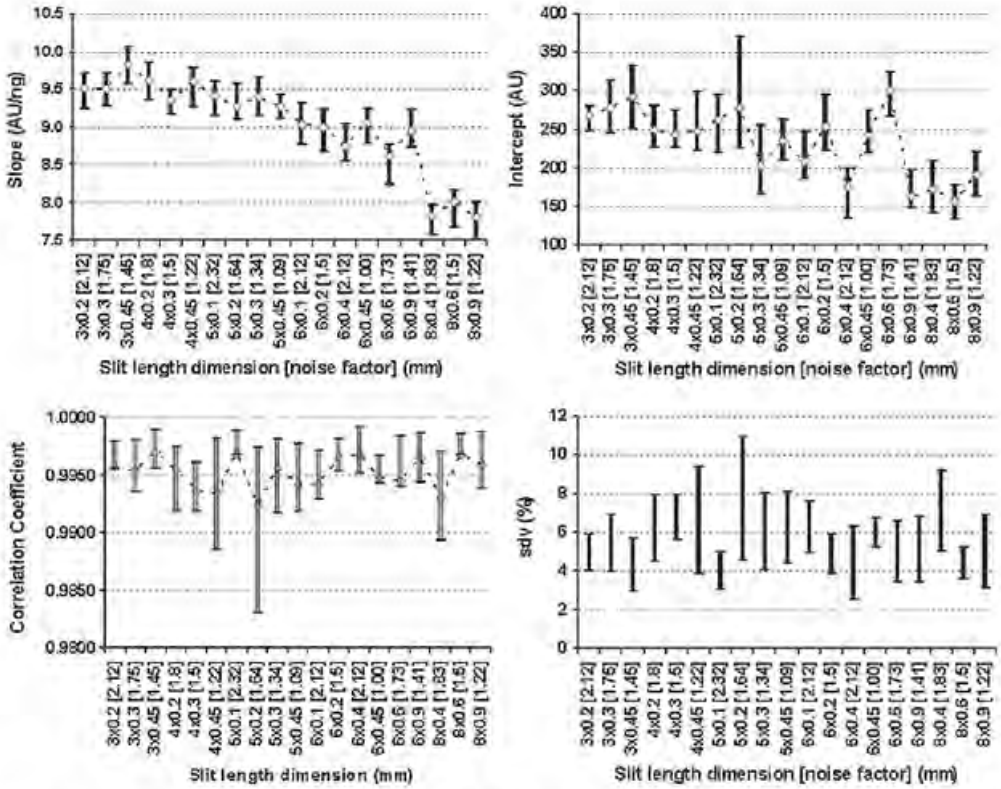

Fig. 3 The estimated parameters of calibration functions of data series of each plate (*filled whisker*) and all plates (*filled diamond*)

closer the points (measurement area) to the curve. The *sdv* is also referred to as residual standard deviations (Sy). The frequencies of *sdv* values vary between plate and slit dimension measurements, although all area of peaks presented good precision between plates.

Increasing the slit length measurement of a calibration series led to a decrease in the slope of matrix calibration (see Fig. 3). In the first-order calibration function, the slope measure of the sensitivity of the analytical procedure [3]. It means that an increase of the slit length measurement could decrease the analytical sensitivity. A measurement of the spot on a slit length between 50 and 83 % of the band width of the spot introduced higher sensitivity response of the detector.

Comparison of calculated calibration functions based on data series of each plate and all plates presented a relative closer function calibration between them (relative same slope and intercept) on the same slit length dimensions.

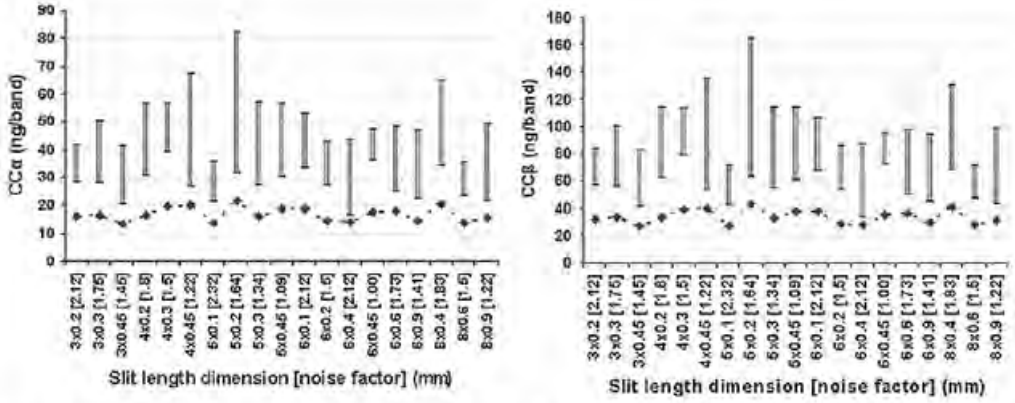

Fig. 4 Comparison of *CCα* and *CCβ* based on data series of each plate and all plates, *filled whisker* range of *CCα*, *CCβ* between each plate and *filled diamond CCα*, *CCβ* all plates

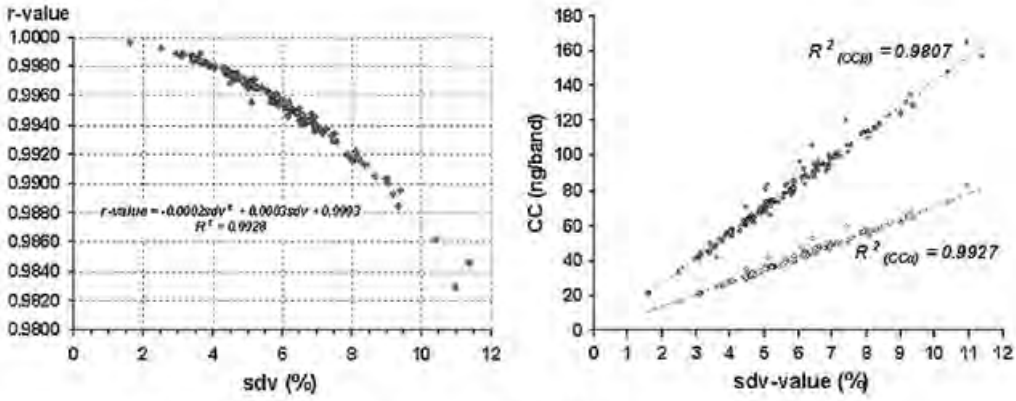

Fig. 5 Relationship between *sdv* value between *r* value (*left side*) and *CCα*/*CCβ* (*right side*)

Table 3 Linear regression data series (10–400 ng/band) for calibration plots, *CCα*, *CCβ* and *QL* estimation

Range of concentration (ng/band)	Calibration function	Correlation coefficient (r)	*sdv* (%)	*CCα* 0.95 one side (ng/band)	*CCβ* 0.95 one side (ng/band)	*QL* (ng/band)
10–200	$y = 18.04x - 24.39$	0.9998	1.28	3.84	7.68	13.60
10–400	$y = 16.42x + 74.38$	0.9973	7.05	21.37	42.73	87.29
50–400	$y = 16.11x + 164.76$	0.9966	5.83	36.29	72.58	108.87

The calculated slope, intercept, and *r* values of calibration functions based on the data series of all plates showed a mean value of those parameters, which were calculated based on data series of each plate (Fig. 3). But calculated *CCα*, *CCβ* and *QL* based on data series of all plates obtained lower values than those based on data series of each plate (Fig. 4). The lowering of these validation parameters was due to a decrease of the statistical *t* value, which was caused by a higher number of degrees of freedom (Nc).

The calculated *CCα*, *CCβ* and *QL* varied between slit dimension measurements and plates (Fig. 4; Tables 1, 2). The *sdv* values present a polynomial correlation to the *r* value and a linear correlation to both *CCα*, and *CCβ* (see Fig. 5). The closer points to the curve produced lower *CCα* and *CCβ*.

From the second data series (10–400 ng/band), the linear regression data, *CCα*, *CCβ* and *QL*, were calculated and are presented in Table 3. It is well known that the calibration range concentration influences the linearity of the

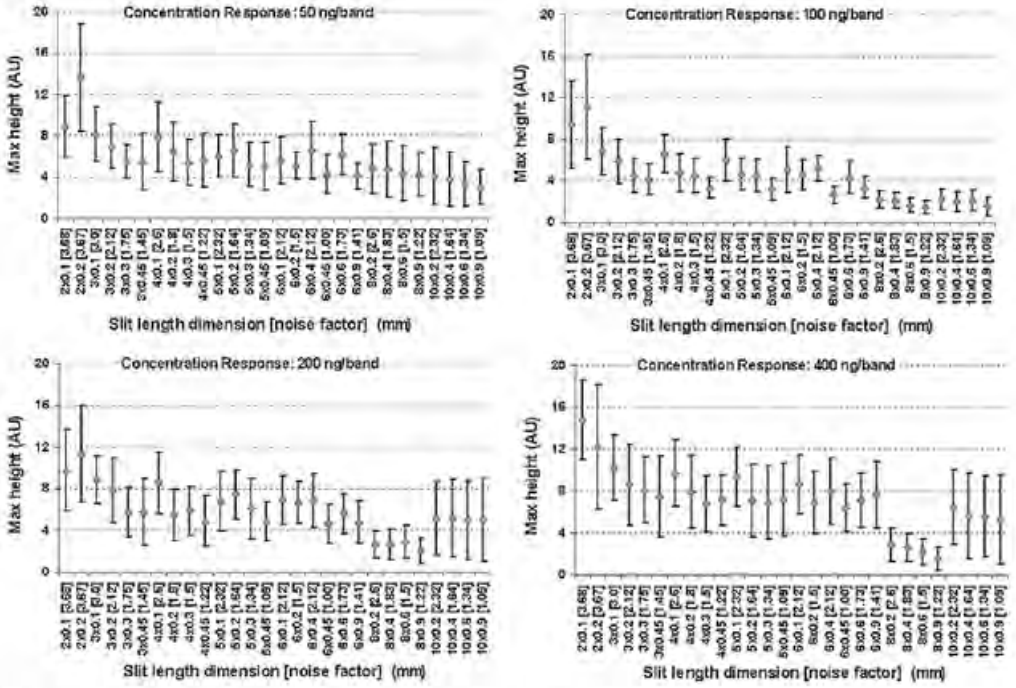

Fig. 6 The mean noise (mN ± sdN) of every concentration response on plate A

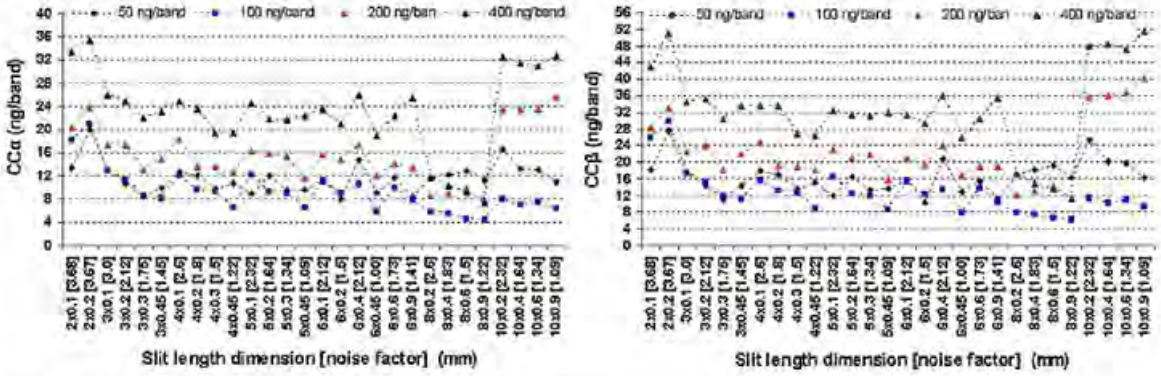

Fig. 7 The calculated *CCα* and *CCβ* based on the S/N method on different concentration responses under variable slit dimension measurements

regression calibration and also the *CCα*, *CCβ* and *QL*. The range concentration 10–200 ng/band showed the best linearity with the highest *r* and lowest *sdv* value. This introduced the lower *CCα*, *CCβ* and *QL*.

S/N Method

The noise value of every concentration response on plate A, which was measured under different slit lengths, are presented in Fig. 6. The signal and noise will decrease along the increasing area of the slit (see Figs. 1, 2, 6). These values were significantly different between slit measurements. Slit dimensions determine the amount of directed light to the plate and the covered surface area of the detected spot. The particle size variation of silica on the covered area influenced the direction of the reflected beam to the detector and the amount of detected light on the detector. This will deliver variation of noise between slit dimensions. The narrower slit introduced highly variable noise all over the measured track. This variation introduced

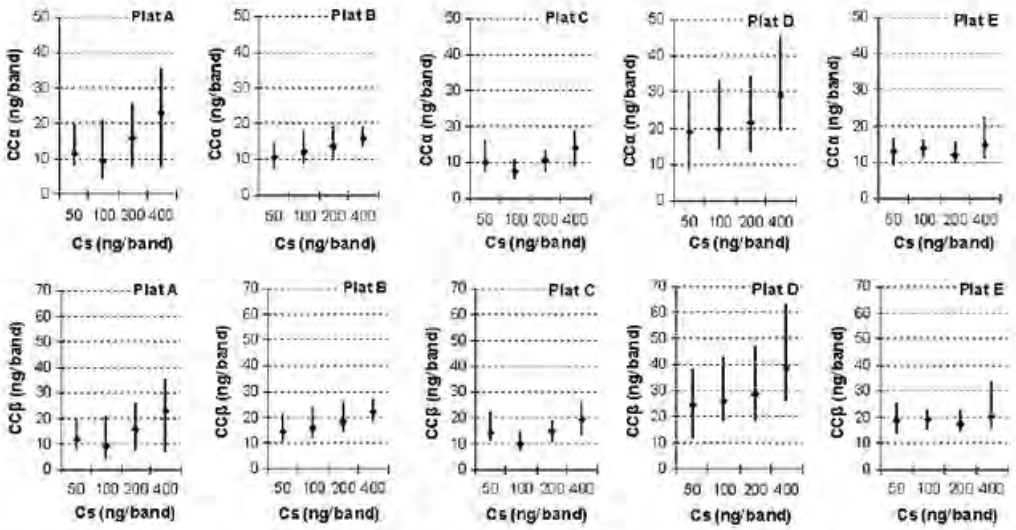

Fig. 8 The range and mean calculated $CC\alpha$ and $CC\beta$ based on the S/N method on different concentration responses between five plates

differences in $CC\alpha$ and $CC\beta$ values between slit measurements (Figs. 7, 8).

The range and mean of calculated $CC\alpha$ and $CC\beta$ based on the S/N method on different concentration response between five plates are shown in Fig. 8. Variations in the range and mean of these validation parameters could be caused by the different read-out of S/N values between slit measurements and plates of TLC. TLC is an open chromatogram system. The handling of the plates while being analyzed could introduce the variation in the detected noise.

$$\%\mathrm{RSD} \approx \frac{50}{\mathrm{S/N}} \tag{7}$$

Dolan [9] described the role of the S/N ratio in precision and accuracy, producing an equation to figure out the relationship between both validation parameters and the S/N ratio (Eq. 7) and used this equation to predict how small a peak can be and still generate usable data [1]. Rearranging Eq. (6), where $(S_a)_{CC\alpha} \approx N_{CC\alpha}$; $(S_a)_{CC\beta} \approx N_{CC\beta}$; $S_s \approx S$, we obtain:

$$CC\alpha = \frac{C_s}{S_s/(S_a)_{CC\alpha}} \quad ; \%\mathrm{RSD} \approx \frac{C_s}{S_s/N_{CC\alpha}}$$

Based on this equation, the higher the S_s/N ratio, the better the precision and accuracy.

Conclusion

Slit lengths between 50 and 133 % of the band width of the spots, and with the noise factor of the slit under 2.6, produced good precision measurements of TLC-densitometry between plates, while slit lengths between 50 and 83 % of the band width of the spots introduced a higher sensitivity response of the detector. The estimated $CC\alpha$, $CC\beta$ and QL were determined by how the data were collected, the analytical optical setting, and the usage method for the estimation of both validation parameters.

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