ENHANCED QUANTITATIVE EVALUATION OF THE HPTLC-BIOLUMINESCENCE DETECTION

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☐ *The HPTLC bioluminescence coupling is a method, which combines the separation ability of HPTLC with biodetection, applying the luminescent bacterium* Vibrio fischeri *that is also used in a cuvette assay for wastewater analysis according to a European norm method. As a result, a black and white image of the HPTLC plate is obtained, on which bioactive substances appear as dark spots on a brightly luminizing background.*

The evaluation of the image was usually done visually and, hence, qualitatively. Currently, available image evaluation programs showed to be unsuitable for quantitative evaluation because of insufficient background correction and/or tedious procedures. Furthermore, special corrections like a horizontal background correction and the recalculation of the sigmoid dose response relationship of the bacteria's reaction are needed. Available programs could not fulfill these requirements.

Therefore, a method was developed by using existing common or freeware programs with which the proper corrections could be accomplished. Steps were the selection of the regions of interest from the HPTLC image, the conversion of the image file into a text file, followed by the main calculation in the spreadsheet program Microsoft Excel. For calculation, adapted versions of the cuvette test calculations were used.

As Excel does not contain the necessary integration tools, two export methods were included. The first method allowed for a routine evaluation of chromatograms giving peak height and area as parameters. The second method enables an in-depth evaluation of chromatograms using an HPLC software leading to parameters like, e.g., signal-to-noise ratio, peak asymmetry, or peak width.

Results obtained with the method were convincing. With a macro bundle, the calculations were not very time consuming and could be applied for routine use.

Keywords chromatogram integration, HPTLC-bioluminescence coupling, quantitative image evaluation, *Vibrio fischeri*

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INTRODUCTION

HPTLC-Bioluminescence Coupling

The coupling of high performance thin-layer chromatography (HPTLC) with bioluminescence detection using the luminescent bacterium *Vibrio fischeri*[1] is derived from the waste water "cuvette test" according to a European norm method,[2] which is very well known in the field of water analysis.

As an effect directed method, the HPTLC-bioluminescence assay can reveal acute (cyto)toxic compounds and serve as a link between physical chemical analysis and toxicological relevance. The technique was developed and patented by Bayer AG.[3] *Vibrio fischeri* bacteria are inherently bioluminescent and emit blue–green light under suitable conditions. Because bioluminescence is directly linked to the bacteria's metabolism, a decrease in light intensity shows a disturbance of the metabolism.[4]

After application of the bacteria onto the HPTLC plate, bioactive substances appear as dark zones on a brightly glowing background. The method was already used for various applications, e.g., for the detection of melamine,[5] the biological activity of berberine containing drugs,[6] for the screening of waste water or the detection of unknown bioactive by-products,[7] screening for bioactive components of photodegraded UV filters in sunscreens,[8] and for screening for new bioactive components in marine sponges.[9]

As a result of the test, a black and white image of the HPTLC plate is taken for qualitative evaluation using a light sensitive CCD camera.

Quantitative evaluation can be done using an image evaluation software as mentioned in literature.[10] But unlike the cuvette test evaluation, image evaluation software does not consider that the bacteria do not show a linear dose response relationship. Furthermore, for HPTLC plates not perfectly having a homogeneous background, correction methods offered by the available software are not sufficient.

One approach adapted the cuvette test calculation method for the HPTLC-bioluminescence evaluation.[7] By this evaluation, interferences caused by β-fronting or inhomogeneous glowing on the HPTLC plate are compensated and the non-linear dose response relationship is taken into account. Track intensity is set in relation to the background on the left and on the right for each pixel row. The calculated value gives the inhibition in percent (I_{rel}). The ratio of inhibition to the remaining inhibition is then calculated resulting in the so called Γ-value. With this Γ-value, the effective concentration (EC) values can be calculated, where $\Gamma = 1$ and $\Gamma = 0.25$ represent EC_{50} and EC_{20}, respectively.

Problem and New Approach

For this effective background correction no software is available. Therefore, carrying out these calculations was a very complex and tedious task. The aim of this study was the implementation of a software tool that uses the calculations according to literature,[7] but does not require expensive, uncommon or too specialized software. It should also be as easy as possible to handle and offer efficient integration facilities.

The problem was solved using two freeware programs and Microsoft Excel for the calculations. Since Excel does not offer suitable integration tools, two export methods were created to integrate the curves in other, more dedicated programs. These were an image evaluation program for routine evaluation, giving the conventional parameters peak height and peak area, and an HPLC chromatography data system for the enhanced method evaluation, which permits the easy calculation of various parameters, among them signal-to-noise-ratio, peak width, and peak asymmetry.

To the best of our knowledge, this is the first time that an HPLC software was used for evaluating HPTLC plates.

EXPERIMENTAL

Evaluation Software

Operating system: Microsoft Windows XP Professional Version 2002, ServicePack 2.

- BioLuminizer 1.0 (CAMAG, Muttenz, Switzerland); CCD camera and software for taking photos of glowing HPTLC plates.
- Gimp 2.6.3; Image processing program; http://www.gimp.org, Freeware.
- ImageMagick 6.5.1-0 2009-03-30 Q16 OpenMP; program for creating, editing and composing bitmap images; http://www.imagemagick.org, Freeware.
- Microsoft Excel 2003; spreadsheet program with Microsoft Visual Basic 6.5, programming system.
- VideoScan (CAMAG); image evaluation software for general integration of HPTLC plates.
- ChromQuest 4.2.34 Version 3.1.6 (Thermo Scientific); data system for High-Performance Liquid Chromatography (HPLC).

Requirements

The method worked for RGB photos with a maximum amount of total 65.536 pixels and/or a width of at most 254 pixels after cutting (limits of Excel had to be taken into account).

For the application of substances it had to be considered that the width of the bands were wide enough to cover the homogeneous part of the spot with 7 pixels and the space between tracks had to be big enough that adjacent zones do not interfere.

General Calculations

1. A picture was taken from the HPTLC plate using the BioLuminizer (CAMAG) and saved as a PNG (*P*ortable *N*etwork *G*raphics) image file: foo.png
2. The photo was imported into the image editing application Gimp. A new "mask" layer (color: red, transparency: 45%) was created.

The previous steps 1 and 2 were implemented as Gimp macros.

3. From this mask, rectangular segments were cut out to be further processed. Segments for the track were taken from the middle part of each track. Segments for the background were taken from the space in between the tracks. The rectangle for each segment was layed out to cover the HPTLC run from the application area to the solvent front and was exactly 7 pixels wide.

The previous step 3 was carried out manually.

4. After selection, the file was saved in the native Gimp-file-format XCF to backup the layer and cutting information: foo-stripes.xcf
5. The transparency of the mask layer was increased to 100%, the image layer and the mask layer were merged and all red (masked) regions were cut away using the *Zealous Crop* command. The cut image was then saved as BMP-file: foo-stripes.bmp

The previous steps 4 and 5 were implemented as Gimp macros.

6. The image BMP file was converted to a text ASCII file using ImageMagick. For this, the Windows program *Command Prompt cmd.exe* was started and the directory chosen in which the files above were stored. The conversion was done using the command: convert foo-stripes.bmp foo-stripes.txt
7. Then, the file foo-stripes.txt was imported to Microsoft Excel in such a way that all words and all numbers were separated to different cells using commas and colons as separators. The result is a spreadsheet of 7 columns and several ten thousand rows. The first row represents the header of the ASCII file: The word *#ImageMagick* is followed by the width and height of the picture in pixels. Except for the first, each row represents one pixel. In the pixel rows, the first value represents the x-coordinate, the second one the y-coordinate, the third, forth and fifth are color values using the RGB system. All further values give additional information on the color which could be ignored.

8. With the information of the width and height of the picture in row one, the values of one channel were resorted in another sheet in such a way that it represented an array sorted like the original picture, now using numerical values (e.g. black $= 0$) instead of grayscale pixels.

9. Then, the average value of every seven cells in one row was calculated and written in an additional sheet. Column 1 was then the horizontal average of the left background of track 1, column 2 was the horizontal average of track 1, column 3 was the horizontal average of the background between tracks 1 and 2, column 4 was the horizontal average of track 2, and so forth.

10. In a next sheet, the first part of the calculation was done using Equation (1). Here, the average of the background on the left and on the right was calculated. This average background of each track was set at 100% and the track was calculated according to Equation (2). The resulting value was the inhibition in percent for this row of pixels.

$$BG = \frac{Background_{left} + Background_{right}}{2} \qquad (1)$$

$$I_{rel} \ in \ [\%] = \left(1 - \frac{Track}{BG}\right) \times 100 \qquad (2)$$

11. Last, in another sheet, the second calculation was made according to Equation (3) which gave the Γ-value.

$$\Gamma = \frac{I_{rel}}{100 - I_{rel}} \qquad (3)$$

The previous steps 6 to 11 were implemented as VisualBasic macros for Excel.

Export for Integration

I. Export as Bitmap for Evaluation with VideoScan

I-1. The absolute minimum Γ-value was added to all values.

I-2. The new values were converted into numbers from 0 to 255, whereas the former minimum value was set to zero. 255 equated to a (theoretical) Γ-value of 9 plus the absolute minimum Γ-value (Offset calculation).

I-3. In a next sheet, every value was written seven times side by side.

I-4. Additionally, in order to better discern between tracks and to define the offset with the absolute miniumum and maximum, a total of 5 rows of black and white were added alternately.

I-5. Then, a header was added which defined a PGM (<u>P</u>ixel <u>G</u>ray <u>M</u>ap) image: The first entry was "P2" followed by the number of converted values (=pixels) in x direction, then the y direction, and the last entry gave the color depth which was 255.

I-6. The sheet was saved as text-file: foo-stripes-BG-Gamma.txt

I-7. Next, ImageMagick was used for the conversion into an bitmap: convert foo.txt -type Truecolor -negate foo.bmp

The previous steps I-1 to I-7 were implemented as VisualBasic macros for Excel.

I-8. The image foo.bmp was imported to VideoScan, while the background correction was turned off. Then it could be evaluated like a photo of an HPTLC plate. The evaluation with VideoScan gave the parameters height and area.

II. Export of Tracks for ChromQuest

ChromQuest is able to import ASCII files with a specific header. Therefore, each track was saved as ASCII text file.

The header was as follows:

Version:	3	Version number of ChromQuest
Maxchannels:	—	
Sample ID:	foo	Name of the sample (arbitrary)
Vial Number:	—	
Data File:	—	
Method:	—	
Volume:	—	
Pretreat Name:	—	
User Name:	—	
Acquisition Date and Time:	01.11.2011	Date (arbitrary)
Sampling Rate:	1	—
Total Data Points:	231 Pts.	Has to be adapted! Number of values for the x-axis in ChromQuest.
X Axis Title:	—	
Y Axis Title:	Pixel	Caption of the y-axis (arbitrary).
X Axis Multiplier:	1	
Y Axis Multiplier:	1	
3.18	—	The values start from here on.
2.25	—	
9.71	—	

II-1. The values had to be written in reversed order.

II-2. The sheet was saved for each track as text file: foo-stripes-BG_Gamma_Track_x.asc

The previous steps II-1 and II-2 were implemented as VisualBasic macros in Excel.

II-3. In ChromQuest a new sequence was created. Each file was imported as one HPLC run.

II-4. Then, each track was opened as a curve and saved via "Utilities" → "Save trace..." as .dat-file: foo-stripes-BG_Gamma_Track_x.dat

The previous steps II-3 to II-4 might be implemented as macros using the ChromQuest Tool kit.

II-5. After this conversion, the HPTLC tracks were evaluated exactly like an HPTLC run.

The macro bundle and a step-by-step documentation (currently in German) are available from the authors on request.

HPTLC

Plates were 20×10 cm HPTLC silica gel plates (Merck, Darmstadt, Germany). Plates were precleaned by development to the top with methanol and dried for 30 minutes on a TLC heater (CAMAG, Muttenz, Switzerland). Application was performed using Linomat 5 (CAMAG) with a $100 \mu L$ syringe, application rate $150 nL/s$, bandwidth 6 mm, distance from the bottom of the plate was 8 mm, distance from the sides of the bronopol plate 9 mm, Kathon CG plate 10 mm, distance between tracks was 11.6 mm (bronopol) and 14.6 mm (Kathon CG), respectively.

Plates were developed with the appropriate solvent and preconditioning with methanol using the AMD2 (CAMAG), migration distance 50 mm.

For detection, luminising *Vibrio fischeri* bacteria were cultivated using the bioluminex kit (ChromaDex, Santa Ana, CA, USA). Plates were dried for 30–45 min at 120°C on a TLC plate heater (CAMAG, Muttenz, Switzerland) and then immersed for 1 s in the bacteria solution using the DC-Tauch-Fix II (Baron Laborgeräte, Insel Reichenau, Germany) and a glass dip tank (Desaga, Wiesloch, Germany). Excess bacteria solution was removed by placing a filter paper onto the wet TLC plate and wiping gently over it with a wiper. Images were taken using the BioLuminizer 1.0 (CAMAG), exposure time 55 s.

A standard of $1.1 \mu g/\mu L$ bronopol (bromo-2-nitro-1,3-propanediol, Sigma-Aldrich) in methanol was applied. Application from left to right was 1.71, 2.28, 2.85, 3.42 μg bronopol. The plate was developed with methanol/CH_2Cl_2/n-hexane (10/60/35).

Standard was Kathon CG (a mixture of 0.38% methylisothiazolinone and 1.22% methylchloroisothiazolinone, Fluka) with a concentration of 2.4 ng/μL methylisothiazolinone and 7.2 ng/μL methylchloroisothiazolinone. Applied amount was 12 ng and 36 ng, respectively, and the solvent was 2-propanol/CH_2Cl_2/n-hexane (1/4/2).

RESULTS AND DISCUSSION

Method Description

The method described was put into practice by using only programs which already existed at our laboratory (Excel, ChromQuest, VideoScan) or were freeware (Gimp, ImageMagick).

Integration was performed using two established programs, i.e., VideoScan and ChromQuest.

VideoScan is an image evaluation program for (HP)TLC, to which the corrected values could be imported as "image". This evaluation was suitable for routine applications. ChromQuest is an HPLC software used for in-depth evaluations; tracks can be tested for peak height, peak area, peak width, peak asymmetry, or signal-to-noise ratio. These parameters are very helpful for judging the quality of a separation method, which, however, cannot be calculated by VideoScan.

The principle of the workflow is shown in Figure 1.
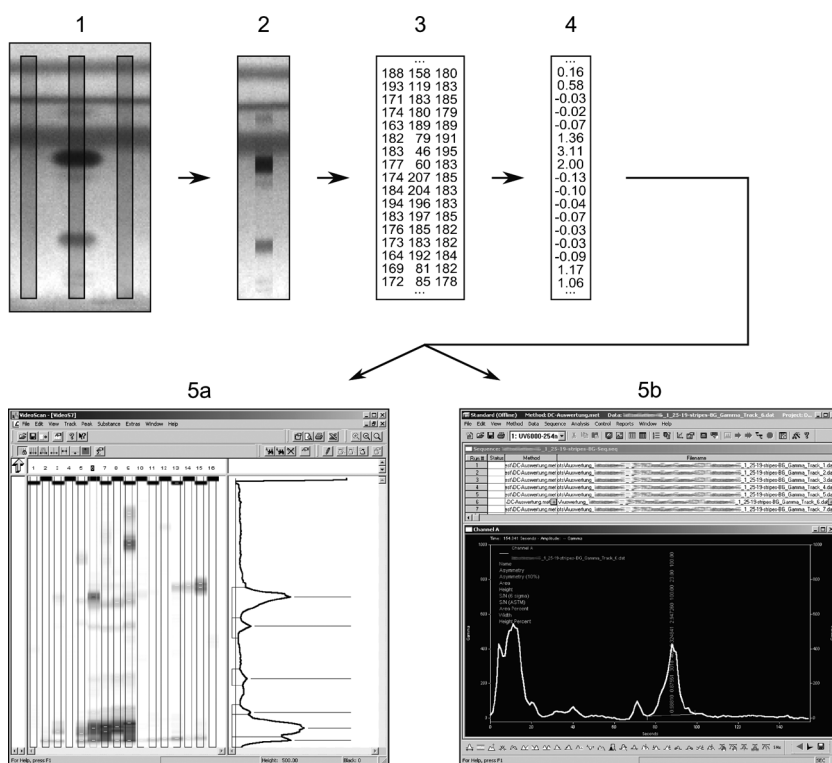
FIGURE 1 Principle of the workflow. 1. Track selection, 2. Cutting, 3. Conversion from image to numbers, 4. Calculation of the Γ-value, 5a. Export to VideoScan, 5b. Export to ChromQuest.

The new export procedures were of great advantage. Since Excel did not offer the necessary integration tools, evaluation could only be performed via setting maximum values as peak heights. Without any baseline correction, however, this was somewhat arbitrary, especially when the baseline was drifting or dipping below zero.

As an alternative for using VideoScan, other image evaluation software were tested. These were ImageJ (http://rsbweb.nih.gov/ij/) and the gel electrophoresis software AlphaEaseFC version 3.3.0 (Alpha Innotech). But the evaluation tools available in these programs were not considered to be enough user friendly. Also, a (background) correction via inhibition values, as originally intended, was not possible in these programs.

Practically all steps for which automation was possible were simplified by macros, so only track selection and the import into ChromQuest had to be done manually. With the macro bundle, the evaluation could be used routinely. A great advantage of the macro bundle apart from simplification was, that all intermediate steps and files were saved, rendering all steps traceable from a peak maximum in ChromQuest or VideoScan through the Excel sheets to the original image.

The additional manual steps which were necessary in ChromQuest could be simplified using the ChromQuest toolkit which can be purchased from the distributor.

Explanations on the Workflow

The width of 7 pixels for the tracks was chosen because it was a good compromise between getting a lot of information and still having a manageable amount of data. A reduction of data was necessary because Excel could only handle up to 65,536 rows and 256 columns. For example, a 20×10 cm HPTLC plate with 17 sample tracks and 18 background tracks and a height of 245 pixels (equal to 45 mm) needed 60.025 rows for each track due to the conversion of the image into a text file.

Since the images were black and white, RGB values for the Red, Green and Blue channel were the same. Only one channel was therefore used for the calculation of Γ-values.

After the calculation of Γ-values, only values of 9 or less could be converted to an image file. Higher values were not used because the Γ-values increased exponentially.

It was also not possible to keep the floating point of the Γ-values. They had to be rounded because image values have to be integer and positive.

Since no negative image values are possible, the minimum Γ-value was added to all Γ-values which set the minimum value to zero. All other values were calculated relatively.

Comparability

To check the comparability and correctness of the two methods, a test picture with clear zones of defined color and an even background was drawn in Gimp and then processed like a real photo including the calculation of Γ-values in Excel and exported to VideoScan and ChromQuest.

For each data pair Excel-VideoScan and Excel-ChromQuest a ratio f was calculated according to Equation (4a) (VideoScan) and Equation (4b) (ChromQuest). The factors and the test image itself are shown in Table 1.

$$f_{VideoScan} = \frac{\Gamma}{\frac{grayscale\ value}{6}} \cdot 100 \qquad (4a)$$

$$f_{ChromQuest} = \frac{\Gamma}{ChromQuest\ value} \cdot 100 \qquad (4b)$$

Inhibition values I_{rel} and Γ-values are linked by Equation (3) and were calculated in Excel. The question was, if the Γ-values and their ratios, respectively, remained the same after conversion for export into VideoScan and ChromQuest. This could be confirmed, especially for inhibition values higher than 10% the converted ratios for each data pair differed only slightly (Table 1).

The Correlation in Detail

For VideoScan, the ratio $f_{VideoScan}$ differed due to the conversion into 256 gray scales. In a sheet with inhibition values in steps of 0.5 from 2 to 90% (smaller values than 2% did not make sense since the calculation

TABLE 1 Test Picture for Compatibility Testing and its Maximum Values and the Differences due to Conversion. Maximum Heights were Obtained from the Excel Γ-value Spreadsheet by Looking for the Highest Values at the according Height. From VideoScan and ChromQuest, the Peak Heights were taken without any Correction. Track Width in VideoScan was 6 pixels

Peak No.	Excel (%)	Excel Γ	VideoScan	$f_{VideoScan}$	ChromQuest	$f_{ChromQuest}$
1	89.8	8.81	1500	881	3.52	1.00
2	80.4	4.10	696	410	3.53	1.00
3	70.6	2.40	408	240	3.53	1.00
4	60.8	1.55	264	155	3.52	1.00
5	51.0	1.04	174	104	3.59	1.00
6	41.2	0.70	120	70	3.50	1.00
7	31.4	0.46	78	46	3.52	0.99
8	21.6	0.28	48	28	3.44	0.98
9	11.8	0.13	24	13	3.33	1.03
10	9.0	0.10	18	10	3.30	0.99
11	2.0	0.02	6	2	2.00	1.00
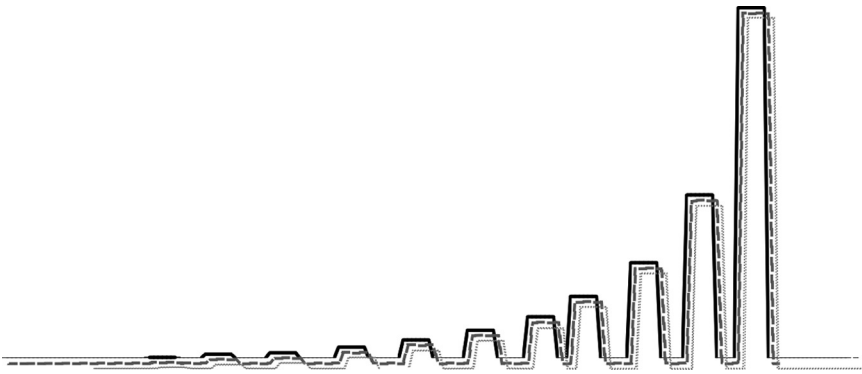
FIGURE 2 Overlay of the chromatograms obtained with Excel, VideoScan and ChromQuest. To overlay all curves, screenshots were taken, the curves scaled and the color was changed to patterns. Differences in line thickness are due to the processing steps, which were necessary for curve adjustment. Solid black curve: Excel, dashed curve: Chromquest, dotted curve: VideoScan.

was a division by zero), the average factor over all values was 3.54 with a standard deviation of 7.2% and a median of 3.53. These differences occurred mainly for Γ-values corresponding to less than 10% inhibition. According to literature,[2] only inhibition values between 10 and 90% were used for the cuvette test. Applying these limits here too, and calculating the factor only for values from 10% to 90%, the average was 3.53, but the
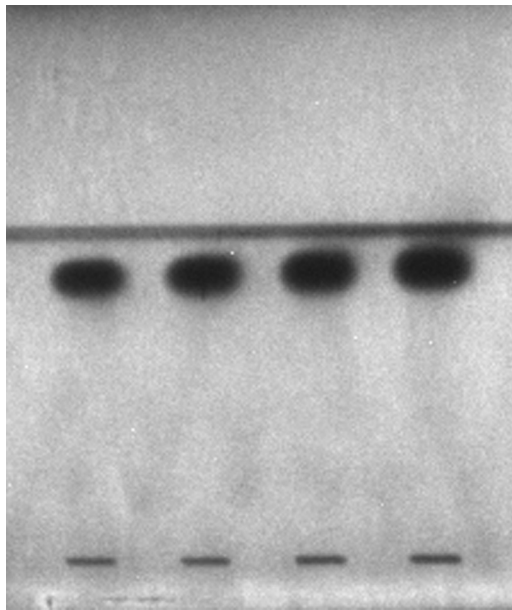
**FIGURE 3** HPTLC plate image of bronopol chromatography.

standard deviations shrunk to 2.68%. The median was then 3.53. It could be concluded that the differences due to conversion were negligible.

For ChromQuest, the deviations in factors were small. They occurred due to rounding which was only an issue for very small inhibition values. Using the sheet as described above for VideoScan, the average factor for all Γ-values from 2 to 90% was 1.00, the standard deviation 1.85%, and for values from 10 to 90% the average remained and the standard deviation was 0.7%. Therefore, the conversion did also not change the result in a relevant way.

By overlaying the chromatograms obtained with Excel, VideoScan, and ChromQuest, no differences could be observed (Figure 2).

Videoscan Correction vs. Method Described

An example for the effectiveness of the new method compared with the most suitable background correction available with the image evaluation program VideoScan is shown in the example of Figure 3. On this plate, bronopol
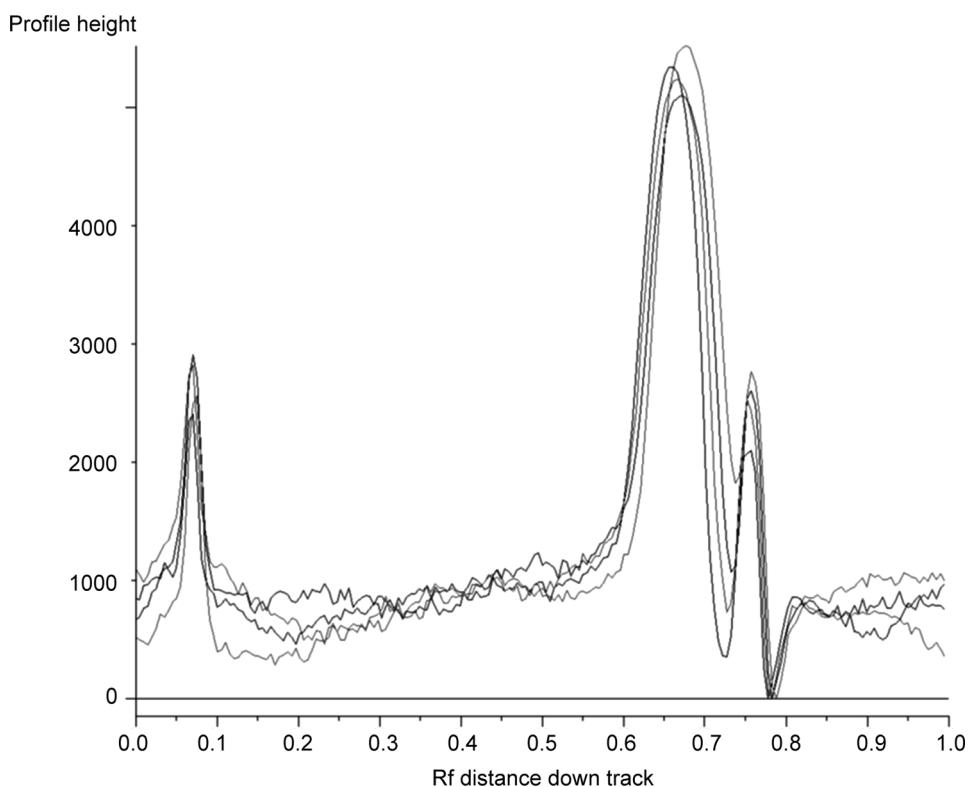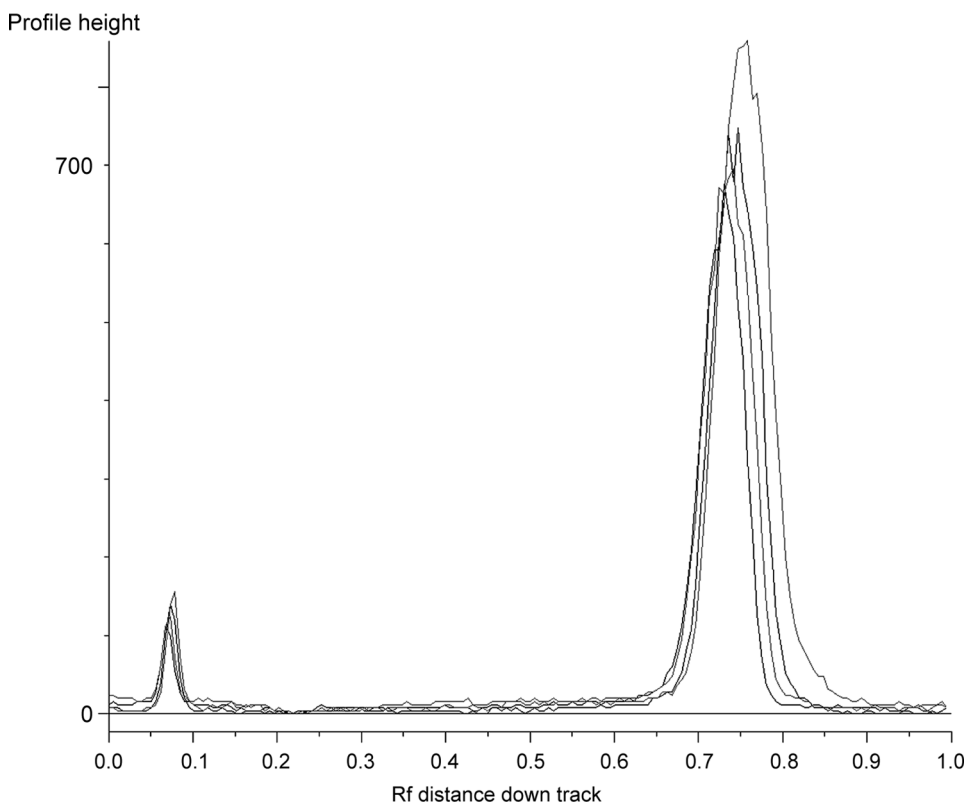

FIGURE 4 Overlay of track chromatograms of the bronopol run as shown in Figure 3. Evaluation was done using VideoScan with background correction. *Track Borders*. Track width was 38 pixels.

was applied in increasing concentrations. The substance migrates very close to the β-front of the solvent. It was expected, however, to give a viable calibration curve since the β-fronting was a horizontal interference over the whole HPTLC plate, which would be compensated for during calculation.

First, the original photo taken with the BioLuminizer was evaluated with VideoScan using the background correction *Track Borders*. Figure 4 shows an overlay of the chromatograms of the four tracks. The big bronopol peak could hardly be integrated because of positive and negative phantom peaks and other interferences which were not corrected. This made it very difficult to get a correct baseline.

After correcting the image using the method described in this paper, the curves were much smoother, the peaks more clearly defined, the run of the baseline was more easily visible; noise was reduced and the strong β-fronting was corrected in a way that it did not interfere with the peaks any more (Figure 5).


FIGURE 5 Overlay of track chromatograms of the bronopol run in Figure 3 after correction using the method described in this paper. Evaluated using VideoScan, background correction *None* and a track width of 4 pixels.

Figure 6 shows the peak heights and area and the calibration curve of the plate. After correction with VideoScan Track Borders, no calibration could be done with the curve drawn with the peak height because the values spread too much. Using area, the result was slightly better but not satisfying. However, calibration via peak height and area was very good using the described method.
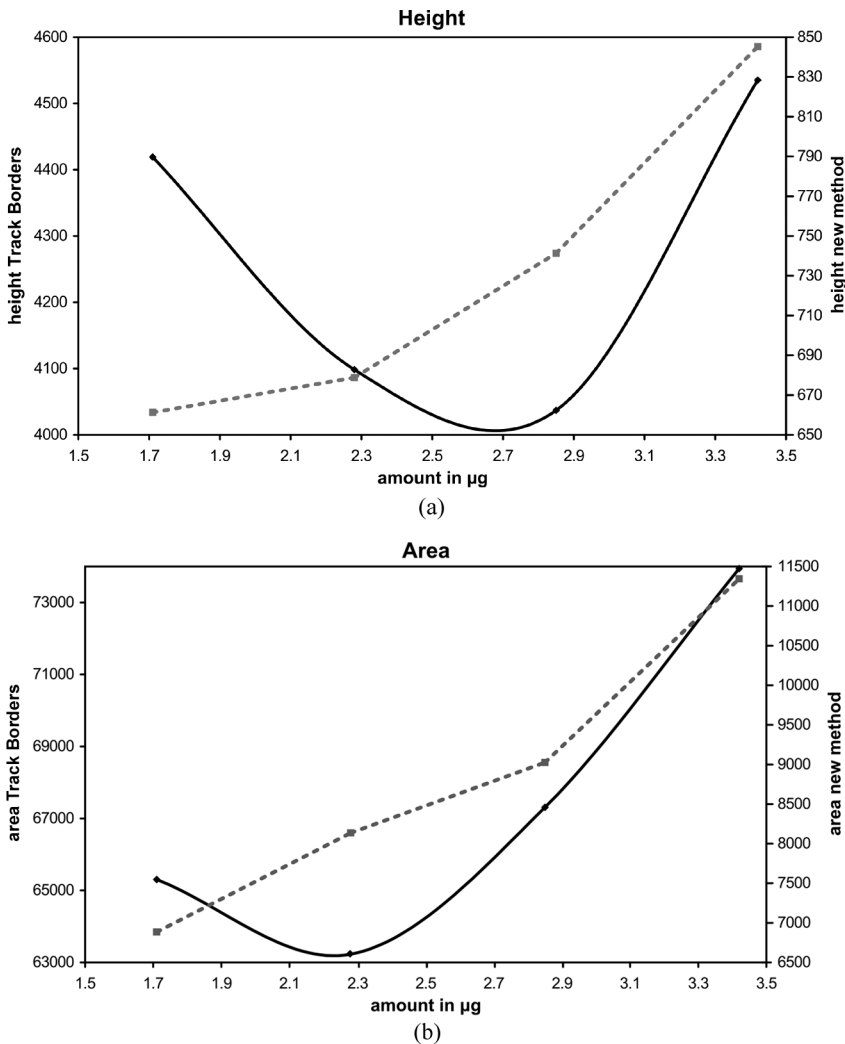
FIGURE 6 Comparison of the evaluation using the method described and the VideoScan correction Track Borders. In both cases, a manual baseline was set. The curves obtained with the new method could serve for calibration, the ones obtained with the Track Border correction were not satisfying. Top: Height values, bottom: area values. Solid black line: Track Borders correction, dotted gray line: new method.
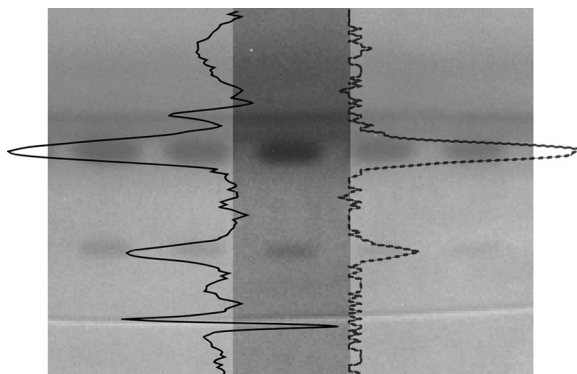
FIGURE 7 Curve obtained for the show track using VideoScan and the Track border correction (solid black line left) and the new method (dotted line right). For the new method the curve was obtained with VideoScan, correction None. Applied was a mixture of methylisothiazlinone (upper zone) and methylchloroisothiazolinone (lower zone).

Advantages Using ChromQuest

For method development it might be necessary to describe the separation in detail. For that purpose, the export to ChromQuest can be used. An example is given for the separation of methylisothiazolinone and methylchloroisothiazolinone (Figure 7). The HPTLC plate shows several horizontal interferences, which were perfectly compensated for. Peaks were only detected where a spot was actually visible.

The corresponding chromatographic parameters obtained from calculations by ChromQuest are presented in Table 2. The values height, area, and width depended on the amount of data points and could only be interpreted in comparison with other HPTLC tracks, whereas for the interpretation of asymmetry and signal-to-noise HPLC data from experience could

TABLE 2 A Selection of Parameters Evaluated using ChromQuest for the Track shown in Figure 7

	MI	MCI
amount in [μg]	1.2	3.6
Height	21	69
Area	124	570
S-N (6 sigma)	3.0	9.7
S-N (ASTM)	4.2	13.9
Asymmetry	1.08	0.98
Asymmetry (10%)	1.07	0.93
Width	13.0	19.0
Width at 5% height	12.4	15.2
Width at 10% height	11.8	13.3
Width at 50% height	5.4	8.0

be applied. Thus, an asymmetry of 1.08 for the small methylisothiazolinone peak and 0.98 for the methylchloroisothiazolinone peak were within a good range. This also applied to the signal-to-noise ratio, which was at least 3.0 for the methylisothiazolinone peak, thus the peak could clearly be identified.

CONCLUSIONS

An effective and reasonably fast quantitative evaluation makes HPTLC-bioluminescence coupling much more attractive for research and routine analysis. Now the method can be controlled and evaluated very effectively.

Furthermore, having the possibility to evaluate tracks with ChromQuest and thus having the same evaluation program as for HPLC increases the value of HPTLC in general and particularly its coupling with bioluminescence detection.

The method with the macro bundle is already in use at the laboratories of both authors.

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