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Rolling—A new application technique for luminescent bacteria on high-performance thin-layer chromatography plates

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ABSTRACT

High-performance thin-layer chromatography (HPTLC) coupled with bioluminescence detection using *Vibrio fischeri* bacteria can be used for screening for unknown substances. This is accomplished by dipping the HPTLC plate in an aqueous bacteria solution. Especially polar substances, however, can start to dissolve during this process, which leads to blurring and tailing of the zones on the plate. To overcome this disadvantage, we applied the bacteria solution by rolling. This method has been described for chemical derivatizations, but is very rarely used. The rolling device was made of commercially available household articles. Using octhilinone and methylparaben as test compounds, rolling was compared with dipping. Despite of performing the rolling process manually, the results were reproducible. Depending on the substance and its amount on the HPTLC plate, peaks were narrower, up to a factor of 4 higher and with a higher signal-to-noise ratio than after dipping.

1. Introduction

High-performance thin-layer chromatography (HPTLC) coupled with bioluminescence detection is a method for obtaining information on how a living organism, in this case *Vibrio fischeri* bacterium, responds to a substance. *V. fischeri* is a common marine bacterium, which luminizes under suitable conditions. It's respiratory chain is linked to the energy-consuming luminescence system. In case of an interference with the respiratory chain, e.g. because of exposure to toxins, the luminescence decreases [1]. Being robust and non-pathogenic, *V. fischeri* is a useful organism in an analytical laboratory, usually cultured in a saline liquid medium.

V. fischeri bacteria have been used for the waste water cuvette test according to DIN EN ISO 11348 [2], the result is a sum parameter of the cumulative effect of all substances in the sample. When screening for toxic compounds, this sum parameter can be misleading, because some substances inhibit the luminescence while others enhance it, which could result in a compensation of the effects. Major progress was achieved by coupling this test with a previous separation of compounds using HPTLC [3–6]. This not only allowed for a screening of individual compounds, but also provided the means to compare a biotest with conventional physicochemical detection. The method was used for various applications, e.g., for the detection of melamine [7], the biological activity of berberine containing drugs [8], the screening of waste water or the detection of unknown bioactive by-products [9], screening for bioactive components of photodegraded UV filters in sunscreens [10] and for screening for new bioactive components in marine sponges [5].

The workflow for HPTLC using bioluminescence detection consists of sample application and separation on the HPTLC plate, dipping of the dried plate in the bacteria solution and detection of the bioactive spots using a CCD camera. A black and white photo is obtained. Inhibiting substances appear as dark zones on a brightly luminizing background, enhancing substance zones luminize even brighter. The photo can be evaluated qualitatively (visually) or quantitatively [11,9].

Despite of a short dipping time, dark spots often show strong tailing and blurring when higher concentrations of compounds are applied (Fig. 1) [12,13].

Besides dipping, spraying with chemical derivatizing agents is commonly used to visualize zones of compounds [12,13]. This technique is not applicable for *V. fischeri*, because homogeneous application was not possible and the bacteria were harmed resulting in a poor luminescence [14]. A rather unknown method, the so-called overpressure derivatization is also described for chemical visualization, however, not for the biodetection of compounds [15–18].

To overcome the problems, we developed a superior, yet simple method of applying *V. fischeri* bacteria homogeneously on an HPTLC plate using a rolling device.

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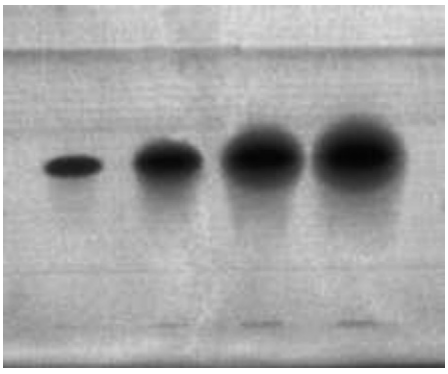

Fig. 1. HPTLC plate with increasing amounts of the preservative octhilinone after dipping. Amounts from left to right: 0.8 μg, 2.3 μg, 4.7 μg, and 7.8 μg.

2. Experimental

2.1. Rolling device and procedure

2.1.1. Materials

Wooden rolling pin, saran wrap (polyethylene), window cloth, half stocking (100% polyamide), cutting board (polyethylene), plastic tray, adhesive tape (all items commercially available in a supermarket); used HPTLC plate.

2.1.2. Implementation

The following numbers refer to Fig. 2, which shows a scheme of the rolling assembly and its components. The wooden rolling pin was coated with saran wrap to protect the wood from getting wet (1). Then, a piece of window cloth, size approximately 25 cm × 15 cm for 20 cm × 10 cm HPTLC plates, was applied as a pad onto the rolling pin (2) and held in place by encasing the complete roll into a stocking and fastening it with a knot.

A plastic cutting board was used as a support for the HPTLC plate (3). A stop bar was attached on the board using a strip of glass fixed with adhesive tape (4). For the rolling process, the HPTLC plate (5) was aligned with the stop bar layer side up with the application zone facing away from it. A glass plate roughened with carbide for better friction was used as a forerun. This was placed on the opposite side of the HPTLC plate (6). The roll was put on a tray where it was soaked with *V. fischeri* bacteria suspension. The tray served as containment and as a reservoir in which the roll could be gently rolled 3–4 times to achieve a homogeneous spreading of the bacteria suspension. The roll was then moved to the forerun in such a way, that it stood on the first few centimeters of the pad, the pad facing the HPTLC plate and stop bar. In one uniform movement applying adequate pressure (approx. 160 N) and adequate velocity (approx. 460 mm/s), the roll was completely rolled over the HPTLC plate until the end of the cutting board.

The HPTLC plate was taken from the cutting board and the back side cleaned with a tissue. The HPTLC plate was placed into the BioLuminizer, and the exposure time was started.

Between consecutive rolling, the cutting board and forerun were wiped dry and the roller wetted again with bacteria solution.

An assembled roller could be used for several HPTLC plates a day. If the time interval between two rolling procedures exceeded 30 min, the whole tray with the roll was stored in a refrigerator at about 8 ° C and taken out a couple of minutes before use. At the end of the day, everything was disassembled, thoroughly washed with tap water, rinsed with ethanol and left to dry at room temperature. For continued use, only the saran wrap was sometimes renewed and the pad exchanged once in a while due to its crumbling.

2.2. Process

2.2.1. Materials and equipment

20 cm × 10 cm HPTLC silica gel glass plates (Merck, Darmstadt, Germany); TLC heater III (CAMAG, Muttenz, Switzerland); Linomat 5 (CAMAG); automated multiple development device AMD2 (CAMAG); TLC Scanner 3 (CAMAG); SmartCut (CAMAG); immersion device (CAMAG); BioLuminizer 1.0 (CAMAG); LUMIStherm (Hach Lange, Rheineck, Switzerland); LUMIStox 300 (Hach Lange); filter paper 0.34 mm (Whatman, Bottmingen, Switzerland); ChromQuest HPLC software (Thermo Scientific); vortex (Bender & Hobein Genie); ultrasonic bath (Bandelin Sonorex); centrifuge (Heraeus Biofuge primo); flat bottom chromatography chamber; Derivapress (AR2i, Le Plessis Robinson, France).

2.2.2. Bacteria, chemical solution and sample

Bioluminex kit (ChromaDex, Santa Ana, CA, USA); octhilinone (2-octyl-4-isothiazoline-3-one, 99%, Fluka/Sigma–Aldrich); methylparaben (methyl 4-hydroxybenzoate, >98%, Fluka/Sigma–Aldrich); caffeine (Sigma–Aldrich, Buchs, Switzerland); methanol (Merck, Darmstadt, Germany); dichloromethane (Sigma–Aldrich); 2% aqueous sodium chloride solution; skin care cream (commercially available product) with methylparaben as the only declared preservative. Standard solutions were prepared by dissolving octhilinone (0.77 g/L), methylparaben (1.07 g/L), and caffeine (1.0 g/L)), respectively,

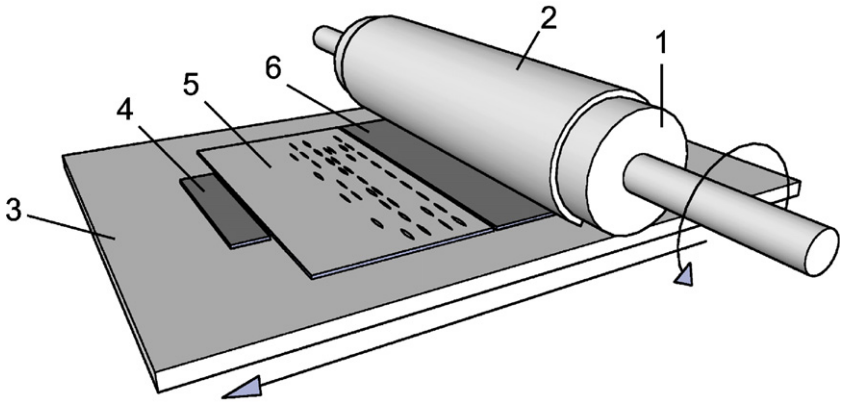

Fig. 2. Schematic of the rolling device: (1) saran wrapped roll, (2) pad, (3) cutting board, (4) stop bar, (5) HPTLC plate and (6) forerun.

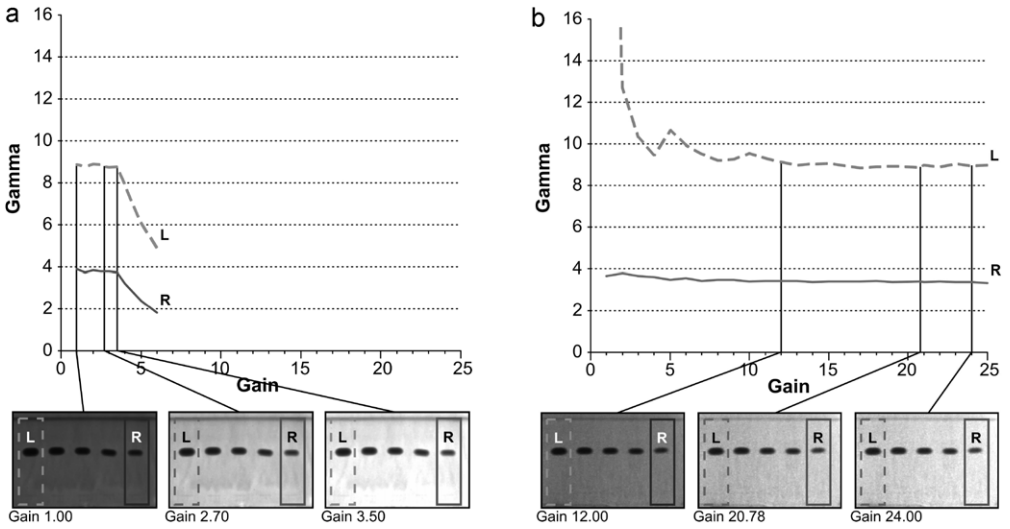

Fig. 3. Peak heights (gamma values) at different gain settings of (a) an originally bright (gain 2.7) and (b) originally dark (gain 20.78) HPTLC plate. Dashed line, L: left spot, highest amount; solid line, R: right spot, lowest amount (both without baseline correction). Amount from left to right: 3.0, 2.5, 2.0, 1.5 and 1.0 μg of octhilinone

in methanol. Skin care cream samples (0.5 g) were dissolved in 20 mL methanol using a Vortex and an ultrasonic bath. After centrifugation for 5 min at 4000 U/min, the supernatant was used for HPTLC analysis.

2.2.3. Chromatography for standard substances

HPTLC plates were precleaned in a chromatography chamber by development to the top with methanol and dried for 30 min at 120 ° C on a TLC heater. Application was performed using a Linomat 5 with a 100 mL syringe, application rate was 150 nL/s, bandwidth 6 mm, distance from the bottom of the plate was 8 mm, distance from the outer sides was 12 mm.

To achieve the same conditions for dipping and rolling, tracks were applied on a 20 cm × 10 cm plate, leaving an empty track in the middle where it could be cut in half after drying. Distance between tracks was between 12.1 mm and 14.1 mm, depending on the number of tracks.

Seven tracks per half were applied: six tracks being either octhilinone, methylparaben or the care cream solution, and one track being 2 μg of caffeine as a reference track.

For quantitative evaluation of high concentrations of octhilinone, only six tracks could be applied totally due to the large inhibiting zones.

Application was as follows:

- Octhilinone: 2 μg caffeine, 0.1 μg, 0.5 μg, 1.0 μg, 2.0 μg, 2.5 μg, 3.0 μg and – for evaluation of the high concentrations – 2 μg caffeine, 1.0 μg, 1.5 μg, 2.0 μg, 2.5 μg and 3.0 μg.
- Methylparaben: 2 μg caffeine, 0.1 μg, 0.5 μg, 0.7 μg, 1.0 μg, 1.5 μg and 2.0 μg.
- Skin care cream: 0.1 μg, 0.3 μg, 0.7 μg methylparaben standard solution as well as 3 sample tracks of 2 μL sample solution; track 1 (0.1 μg methylparaben) was overspotted with 2 μg caffeine.

Plates were developed using the AMD2. The mobile phase consisted of dichloromethane, preconditioning was performed with methanol, migration distance was 50 mm. Plates were dried for 60 min at 60 ° C on a TLC plate heater.

For skin care cream analysis, plates were scanned using the TLC Scanner at 260 nm, then cut in half using the SmartCut.

2.2.4. Biodetection and evaluation

Luminizing *V. fischeri* bacteria were cultivated from the bioluminex kit according to manual.

The luminescence of the bacteria was checked before use by adding 10 μL bacteria suspension to a cuvette containing 2 mL of a cooled 2 % sodium chloride solution (15°C, cooling block LUMIStherm). The relative luminescence capacity (Irel mode) of the suspension in the cuvette was measured immediately with a specific photometer (LUMIStox, measuring instrument for luminescent bacteria tests).

Dipping into the bacteria solution was performed with the immersion device at speed level 5 (4.5 cm/s), immersion time set at 0 s. Excess bacteria solution was removed by placing a filter paper onto the wet TLC plate and wiping gently over it with a wiper.

Rolling was performed according to Section 2.1.

Images were taken using the BioLuminizer 1.0, exposure time 55 s. The offset and gain corrections were set in the *automatic* mode. Gradient adjustment was black/white, Squeeze was set at zero.

The photos were evaluated quantitatively according to literature [11,9] with the ChromQuest HPLC software. Tracks, for which it was not possible to define a background without interference from adjacent zones were not used for evaluation. This was the case for the tracks 2.0 μg, 2.5 μg and 3.0 μg of the octhilinone scheme 1.

For calculation of the amount of methylparaben in the cream sample, three methylparaben standard tracks served for calibration on each plate. For each sample track, the measured value was entered into the calibration equation, and the result converted into percent methylparaben in the sample.

Table 1
Gain and offset values of all HPTLC plates.

Amount of tests	Offset		Gain	
	Dipping 30	Rolling 30	Dipping 30	Rolling 30
Minimum	1507	1510	2.47	2.70
Maximum	2157	1535	17.33	20.78
Median	1523	1519	9.33	7.24
Average	1549	1521	9.12	8.22
Standard deviation	116	7	3.83	3.83
Relative standard deviation	7.5 %	0.5 %	43.0 %	46.6 %

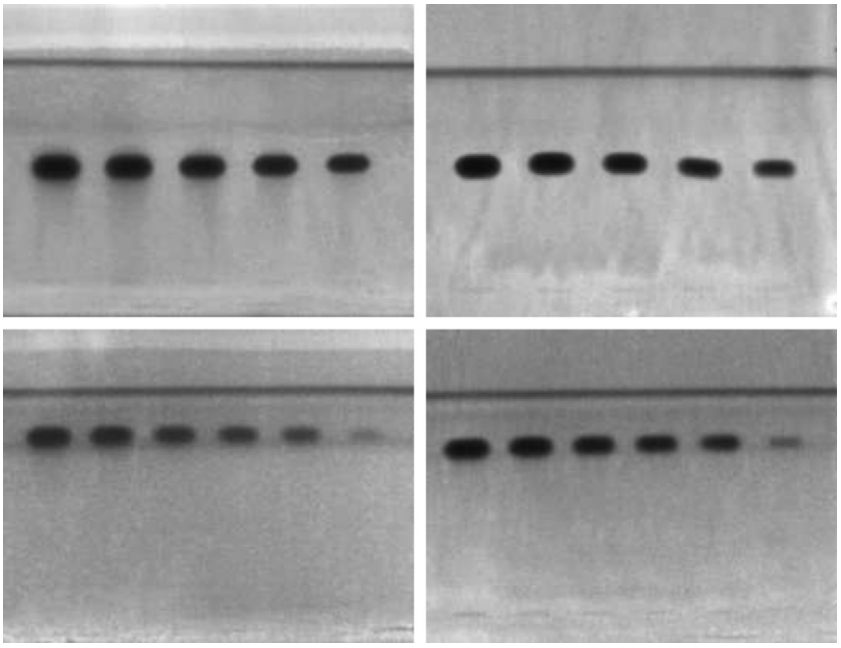

Fig. 4. Typical BioLuminizer images of octhilinone (top) and methylparaben (bottom) HPTLC plates after dipping (left) and rolling (right). Applied amount of octhilinone from left to right: 3.0, 2.5, 2.0, 1.5 and 1.0 μg; applied amount of methylparaben from left to right: 2.0, 1.5, 1.0, 0.7, 0.5 and 0.1 μg

2.2.5. System control and calibration

Before applying the bacteria onto the HPTLC plate, the relative luminescence capacity was measured. The bacteria could be used, if the Irel value was greater than 500. After taking the BioLuminizer image, the gain value indicated, if the bacteria luminized properly on the HPTLC plate. This was the case, when the image did not appear to be grainy or pixelized, and the gain value was about 5–20. Also, we routinely used 2 μg of caffeine as a reference track on all plates to check the detection quality and chromatography; the caffeine had to be visible as a gray zone.

3. Results and discussion

When analyzing polar substances, we encountered the problem that, despite of the shortest possible immersion time, some substances showed a strong tailing after dipping. Poor chromatography as a cause for peak tailing could be excluded, because dipping of an HPTLC plate without prior chromatography also showed tailing of the spot. The possibility, that tailing was an artifact of the wiping process was excluded by wiping in different directions.

In a first attempt, we tried to use the Derivapress, a device which is based on the stamping method [15–18]. But this method did not work for the purely aqueous bacteria suspension, as it did not wet the HPTLC plate homogeneously. However, with some effort it was possible to achieve at least a result showing that the observed tailing was definitely a problem of dipping.

3.1. Comparability of images

Originally, it was not intended to use the BioLuminizer for quantitative evaluations. Thus, it was necessary to ensure the comparability of the BioLuminizer photos. Our luminescence control ensured a minimal bacteria fitness, however, it was not possible to standardize the *V. fischeri* cultures. Therefore, the system varied and the HPTLC plates covered with the bacteria did not luminize equally.

The BioLuminizer software, however, converted the raw data from the camera not only into an image, but also normalized it in brightness and set the information *zero light* to black. This process uses two parameters: The gain value gave the spreading of the histogram and thus the level of amplification. The lower the value, the brighter the plate. The offset value shifted the whole color scale so that zones of no light detected corresponded to black in the image. It was mainly device and temperature dependent.

The offset values were practically the same for all plates both for dipping and rolling. Gain values of the evaluated plates varied more, but were within the same range (approximately 2–20) both for dipping and rolling (Table 1). The automatic gain value was sufficient, because (minor) deviations did not effect the gamma values, only unreasonably settings had an effect (photo completely over- or underexposed). Fig. 3 shows the gamma values of two peaks on a very bright and on a rather dark HPTLC plate in correlation with (manually changed) gain values.

For our work, we used evenly illuminated images. Usually, the gain value was between 5 and 12. Photos with excessive gain values tended to become grainy due to the high amplification of the luminescence. It could be assumed, that in this case something went wrong or the bacteria did not luminize properly. Such plates were not further evaluated.

For the comparison of the photos, it could be concluded, that the BioLuminizer in combination with the evaluation according to literature [11,9] could be used for quantitation. The obtained photos and the corresponding data were adequate for our purpose.

3.2. Handling in dipping and rolling

Normally, wiping is performed without a filter paper between the HPTLC plate and the wiper, but previous qualitative experiments in our laboratory showed, that the reproducibility was better when a filter paper was used. To make sure, that this had no effect on the result, two plates of octhilinone and methylparaben, respectively, were dipped and wiped with or without filter paper. The gamma values after quantitative evaluation for wiping without filter paper were within the distribution of values obtained by wiping with filter paper. No tendency to higher peaks could be observed. Thus we assume, that both ways a loss of substance occurred, either by the filter paper or by the wiper. Some wiping was necessary,

Table 2
Quantitative parameters of octhilinone HPTLC plates. Std. dev.: standard deviation; Rel. std. dev: relative standard deviation.

	Dipping				Rolling			
Amount per zone [μg]	Average	Std. dev.	Rel. std. dev. [%]	Number of plates	Average	Std. dev.	Rel. std. dev. [%]	Number of plates
Height								
0.5	163	22	13.2	9	138	31	22.5	9
1.0	281	52	18.4	18	358	58	16.2	18
1.5	384	29	7.6	9	542	37	6.7	9
2.0	445	17	3.9	9	668	45	6.7	18
2.5	467	52	11.1	9	769	49	6.4	18
3.0	511	68	13.3	9	859	63	7.3	18
Area								
0.5	1196	234	19.5	9	942	227	24.1	9
1.0	2524	531	21.0	18	2507	507	20.2	18
1.5	3832	505	13.2	9	4084	685	16.8	9
2.0	4664	530	11.4	9	5228	612	11.7	18
2.5	5543	638	11.5	8	6430	729	11.3	18
3.0	6565	771	11.7	8	7477	693	9.3	18
Width at 10% height								
0.5	0.20	0.01	7.1	9	0.19	0.02	8.6	9
1.0	0.24	0.02	7.8	18	0.20	0.02	10.2	18
1.5	0.28	0.03	9.9	9	0.20	0.02	9.1	9
2.0	0.30	0.03	8.6	9	0.21	0.01	6.8	18
2.5	0.33	0.03	9.6	9	0.23	0.02	6.6	18
3.0	0.35	0.04	12.3	9	0.23	0.01	6.3	18
Asymmetry (10%)								
0.5	1.01	0.14	14.1	9	0.95	0.07	7.4	9
1.0	0.92	0.06	6.9	18	0.94	0.04	4.5	18
1.5	0.91	0.08	8.4	9	0.95	0.12	12.1	9
2.0	0.91	0.05	5.8	9	0.95	0.05	5.3	18
2.5	0.90	0.06	6.5	9	0.96	0.05	5.0	18
3.0	0.95	0.08	8.7	9	0.95	0.09	9.3	18
S/N (ASTM)								
0.5	18.2	4.2	22.9	9	16.4	3.4	21.0	9
1.0	31.5	13.5	42.8	17	51.3	22.6	44.1	17
1.5	49.2	20.6	41.9	8	74.1	20.0	27.0	9
2.0	52.2	19.8	37.9	8	87.7	26.4	30.1	18
2.5	54.5	21.4	39.3	8	99.5	29.5	29.7	18
3.0	60.1	24.6	40.9	8	115.7	29.9	25.9	18
S/N (6 sigma)								
0.5	11.8	2.3	19.4	9	10.7	2.5	22.9	9
1.0	21.5	8.6	40.1	17	34.4	14.8	43.0	17
1.5	31.5	13.2	41.8	8	54.0	15.9	29.5	9
2.0	35.4	12.5	35.2	8	58.5	17.1	29.3	18
2.5	37.8	11.2	29.6	8	66.8	19.4	29.0	18
3.0	39.0	19.9	51.0	8	77.9	19.7	25.2	18

because without it the plate remained too wet and became unacceptably blurry.

For the dipping process, the highest speed was chosen to minimize the time of contact between plate and aqueous solution and, therefore, the risk of dissolving compounds from the plate.

To wet the rolling device properly for the rolling of 10 cm × 10 cm plates, approximately 80 mL of bacteria solution were needed. Once the device was wet, approximately 5 mL of bacteria solution were necessary to re-wet the pad for every additional plate. Excess solution from previous wetting or from the rolling process could be reused. For dipping, approximately 350 mL of *V. fischeri* solution were needed, which could be reused, too.

For all results, it has to be kept in mind, that rolling was always done manually, while dipping was performed using an automated device.

3.3. Standard substances

For the comprehensive evaluation of the rolling method and for comparison with dipping, the two preservatives octhilinone and methylparaben were applied in varying amounts.

The following quantitative parameters were evaluated using ChromQuest: Height, Area, Asymmetry (10 %), Width at 10 % height, S/N (ASTM) and S/N (6 sigma).

Statistical examination of the data series for each parameter showed, that values were approximately normally distributed (Lilliefors test [19], level of significance 99%). An *F*-test on the comparability of the variances at 5% displayed, that the variances of the data series were usually not comparable. Therefore, an adapted *t*-test for incomparable variances was performed (Welch test, [20], level of significance 95%).

Since the experiments to obtain the data for the standard substances were carried out on different days in different months and with different bacteria cultures, the calculated deviations not only considered repeatability but also reproducibility.

3.3.1. Octhilinone

The BioLuminizer images after dipping and rolling of the developed plates exhibited much sharper and clearer zones of octhilinone without any tailing, when the rolling device was used (Fig. 4).

The quantitative evaluation (Table 2) resulted in standard deviations of about 5–20% for most parameters and both methods.

Table 3
Quantitative parameters of methylparaben HPTLC plates. Std. dev.: standard deviation; Rel. std. dev: relative standard deviation.

	Dipping				Rolling			
Amount per zone [μg]	Average	Std. dev.	Rel. std. dev. [%]	Number of plates	Average	Std. dev.	Rel. std. dev. [%]	Number of plates
Height								
0.5	77	22	28.2	9	332	64	19.4	9
0.7	94	27	29.4	9	404	71	17.7	9
1.0	108	29	27.2	9	483	82	16.9	9
1.5	131	36	27.1	9	574	97	17.0	9
2.0	159	44	27.5	9	691	120	17.4	9
Area								
0.5	605	179	29.6	9	2424	486	20.0	9
0.7	788	224	28.4	9	3179	616	19.4	9
1.0	935	244	26.1	9	3959	678	17.1	9
1.5	1264	338	26.7	9	5212	934	17.9	9
2.0	1597	449	28.1	9	6408	1119	17.5	9
Width at 10% height								
0.5	0.24	0.02	9.8	9	0.20	0.01	4.3	9
0.7	0.25	0.05	20.0	9	0.21	0.01	4.6	9
1.0	0.24	0.02	8.2	9	0.22	0.01	4.5	9
1.5	0.27	0.02	7.3	9	0.24	0.01	3.7	9
2.0	0.27	0.01	4.5	9	0.24	0.01	3.6	9
Asymmetry (10%)								
0.5	1.01	0.14	13.6	9	0.93	0.08	8.2	9
0.7	0.93	0.11	11.3	9	0.92	0.09	10.2	9
1.0	0.88	0.10	11.4	9	0.91	0.07	7.2	9
1.5	0.89	0.10	11.7	9	0.84	0.07	8.7	9
2.0	0.86	0.08	9.0	9	0.87	0.06	7.0	9
S/N (ASTM)								
0.5	8.6	3.4	39.1	9	41.1	6.2	15.0	9
0.7	10.3	4.0	38.5	9	45.9	8.8	19.2	9
1.0	11.0	4.4	40.0	9	55.2	7.5	13.7	9
1.5	13.5	4.7	34.5	9	63.9	11.4	17.8	9
2.0	18.5	6.8	36.6	9	70.6	13.9	19.7	9
S/N (6 sigma)								
0.5	6.0	2.5	42.1	9	27.0	4.2	15.4	9
0.7	6.9	2.7	39.6	9	31.8	6.3	19.7	9
1.0	7.5	3.0	39.9	9	36.5	5.5	14.9	9
1.5	9.4	3.0	32.0	9	44.0	6.7	15.2	9
2.0	12.6	4.4	34.6	9	47.2	8.2	17.3	9

Table 4
Concentration of methylparaben in a care cream sample, determined by UV scan and *V. fischeri* assay using dipping and rolling of bacteria suspension.

		UV 260 nm				Dipping			
Plate	Track	Amount in %	Average in %	Std. dev.	Rel. std. dev. [%]	Amount in %	Average in %	Std. dev.	Rel. std. dev. [%]
A	1	0.32	0.32	0.003	1.1	0.31	0.33	0.065	19.4
	2	0.32				0.28			
	3	0.31				0.41			
B	1	0.31	0.29	0.021	7.1	0.24	0.27	0.030	11.0
	2	0.30				0.29			
	3	0.27				0.29			
C	1	0.26	0.24	0.029	12.3	0.30	0.25	0.052	20.5
	2	0.24				0.26			
	3	0.20				0.20			
		UV 260 nm				Rolling			
Plate	Track	Amount in %	Average in %	Std. dev.	Rel. std. dev. [%]	Amount in %	Average in %	Std. dev.	Rel. std. dev. [%]
A	1	0.33	0.30	0.038	12.7	0.32	0.31	0.013	4.3
	2	0.32				0.31			
	3	0.26				0.30			
B	1	0.32	0.32	0.004	1.3	0.31	0.32	0.019	5.9
	2	0.31				0.32			
	3	0.32				0.35			
C	1	0.29	0.29	0.002	0.6	0.30	0.30	0.002	0.8
	2	0.29				0.30			
	3	0.29				0.29			

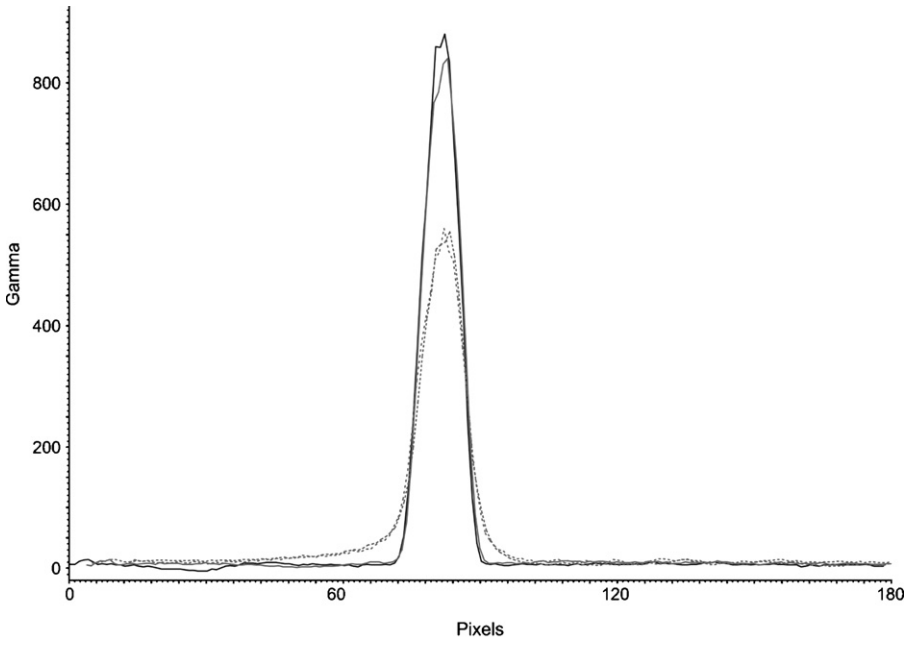

Fig. 5. Densitogram overlay of two tracks of dipping (dotted line) and two tracks of rolling (solid line); 3.0 μg of octhilinone

Only signal-to-noise ratios showed higher standard deviations, especially for dipping (dipping: approx. 40%, rolling: approx. 30%). Relative standard deviation values of this magnitude are acceptable for a biological system. Comparison of the evaluated parameters proved, that for high amounts of octhilinone (1.0 μg and higher), rolling was significantly better than dipping. The higher the applied amount of octhilinone, the stronger the advantage of rolling; peak heights increased up to a factor of 1.8. Ratios of peak width

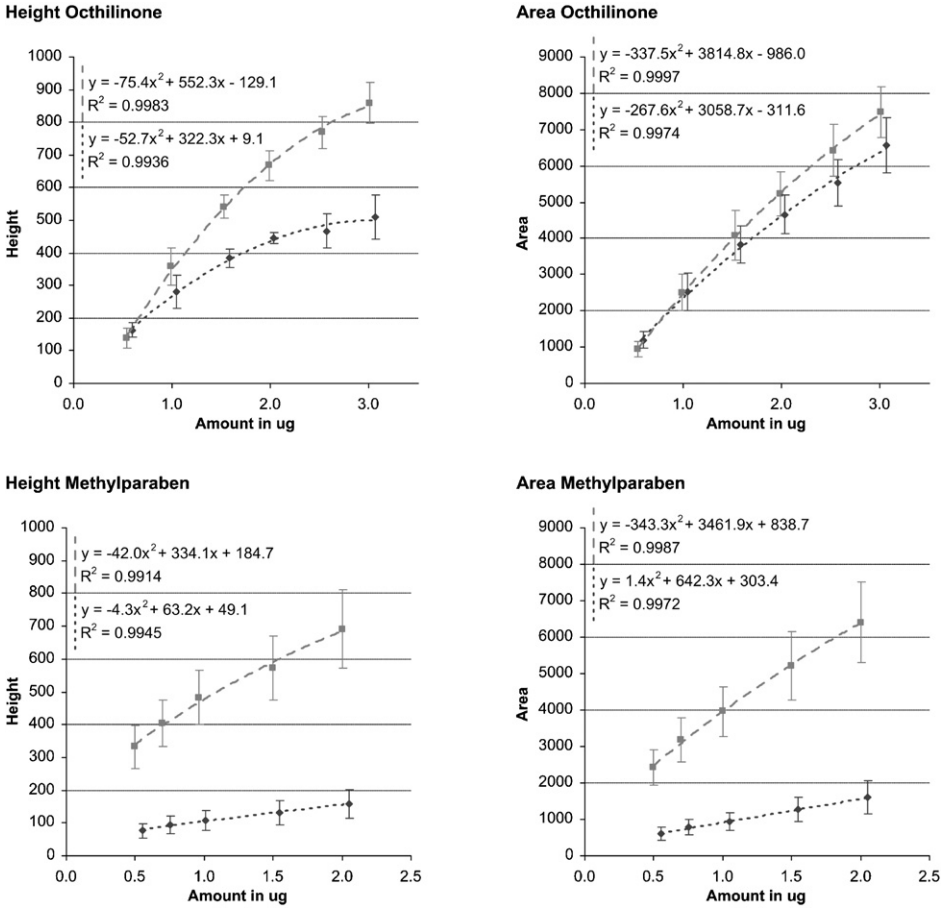

Fig. 6. Calibration curves of octhilinone (top) and methylparaben (bottom): peak height (left) and peak area (right) vs. amount per zone. Rolling: light grey points and dashed curves; dipping: dark grey points and dotted curves. Data series were very slightly shifted to make sure no points are hidden.

(dipping/rolling) were in favor of the rolling procedure: peak broadening was more pronounced with dipping, especially at higher concentrations, which is reflected in the blurring of the zones (Fig. 5). Signal-to-noise ratios were up to a factor of 2 higher after rolling than after dipping. Only minor differences could be observed for peak areas. Despite a visible tailing after dipping, this impression was not reflected by the asymmetry values.

Polynomial calibration using peak heights led to steeper curves for rolling, while the differences between the curves using peak area were only minor (Fig. 6). The steeper the curve, the better the sensitivity of the method. Coefficients of determination (R^2) of all curves were at least 0.99. The lowest applied amount of 0.1 μg was excluded from calibration, because peaks were too small for a reasonable integration.

We suppose, that octhilinone, being slightly soluble in water (0.48 g/L at 25 ° C [21]), starts to dissolve partially from the plate when dipped in the aqueous bacteria solution. This not only results optically in spot bleeding or blurring, but also leads to lower values, especially for peak heights. The phenomenon is more pronounced the higher the amount of compound applied. It can be explained with the lesser binding of octhilinone on the silica layer with increasing amounts. Peak area integration also covers the blurred parts of the zones, which was not the case for the peak height or signal-to-noise ratio, hence the peak area was closer to the values obtained with rolling.

3.3.2. Methylparaben

As found for octhilinone, the BioLuminizer images of the methylparaben plates presented quite blurry zones after dipping, whereas rolling resulted in much darker and clearer zones (Fig. 4).

The visual impression was confirmed by the results of the quantitative examination (Table 3). Due to very low signal intensities, the lowest applied amount of 0.1 μg methylparaben could also not be used for evaluation. All peak heights, peak areas and signal-to-noise ratios were significantly higher with rolling than with dipping (approximately a factor of 4). With dipping, the peaks were also usually slightly wider (factor 1.1–1.2 for parameter width at 10%), while there was no difference in the peak asymmetry for both methods. Standard deviations were usually higher with dipping than with rolling. With dipping, they were about 10–30%, depending strongly on the amount of methylparaben and the specific parameter, while for rolling, standard deviations were between 5 and 20% and did not depend very much on the amount per zone.

In contrast to octhilinone, the calibration diagrams of methylparaben are quite similar for peak height and peak area, respectively, while the curve for rolling was about a factor of 4 higher and also steeper than the curve for dipping. This effect can be explained with the water solubility of methylparaben (2.5 g/L at 25 °C [21]), being more readily soluble than octhilinone. Hence, independent from the amount applied, a certain fraction of methylparaben always dissolved in the bacteria suspension in the case of dipping. Subsequent dilution of methylparaben in the dip tank was probably very high, because an effect on the *V. fischeri* bacteria was not observed.

3.4. Cosmetic sample

To test the method with a real sample, a skin care cream was chosen, only containing methylparaben as a preservative. Our attempt was to determine the amount of methylparaben using HPTLC-Bioluminescence both with dipping and rolling and to compare these data with the results obtained with conventional HPTLC using a UV scan.

In general, the methylparaben concentrations obtained with dipping differed more from the corresponding UV value than those obtained with rolling (Table 4), regardless of the evaluation method (peak height or peak area). Furthermore, chromatograms obtained with rolling were more suitable for evaluation, because with higher peaks it was easier to establish a baseline, resulting in a higher reproducibility.

4. Conclusions and outlook

With the present study we introduced rolling as a very simple and yet superior alternative for the application of *V. fischeri* bacteria onto HPTLC plates. Although, up to now, rolling was only performed manually, results were much better concerning repeatability and sensitivity than those obtained with the immersion device for dipping. In cooperation with the University of Applied Sciences of Northwestern Switzerland, we are currently realizing a mechanical device for the rolling process. The aim is to achieve more precise adjustments concerning pressure and speed, and hence to increase reproducibility.

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