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ORIGINAL RESEARCH PAPER

Simple performance of the planar SOS-Umu-C–FLD genotoxicity bioassay shown for perfume and packaging material analysis

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Abstract

Planar bioassays are powerful, sustainable tools for nontargeted detection of hazardous compounds in complex samples. They provide more information on a sample than corresponding in vitro assays and are more sustainable in terms of plastic material and solvent consumption. However, instrument investment costs for high-performance thin-layer chromatography are high. Hence, the question arose of whether the sophisticated sensitive planar genotoxicity bioassay could be performed manually without instrumentation under simple conditions. Cheaper plate types were studied as well as manual application of the samples, cell suspension, and substrate solution. As a result, genotoxic compound zones were detected as rose-colored or orange fluorescent resorufin end-product formed upon contact of the genotoxins in tested perfume and packaging materials with a genetically modified *Salmonella* Typhimurium strain. The simple performance was found to be possible for low sample application volumes. Knowledge on neutral pH value and thickness of the adsorbent layer were further key aspects. Manual spraying was found to be superior to manual immersion if excess liquid was avoided. For high sample volumes and a higher level of standardization, the open-source 2LabsToGo system was proposed as excellent option for low investment costs. Its very low instrumental footprint and the straightforward prioritization strategy help analytical chemistry to balance between technology and nature/ecology to reduce the instrumental footprint and planetary overshoot.

Keywords Planar chromatography · High-performance thin-layer chromatography · HPTLC hyphenation · Planar biological assay · Simplicity · Sustainability · Dematerialization

1 Introduction

Among the many advantages of high-performance thin-layer chromatography (HPTLC) [1], one great benefit is the coupling to planar bioassays and thus the effect-directed detection of the separated compounds on the same adsorbent surface [2, 3]. The resulting effect-directed profiles generated for many samples in parallel allow prioritization on important active substances among the hundreds or even thousands of compounds present in a complex sample based on a specific effect. The most prominent compounds can straightforwardly be characterized by reagent sequences on the same bioautogram [4, 5] and further by direct elution to high-resolution mass spectrometry [6]. Although this is often overlooked in the field of liquid chromatographic hyphenations [7, 8], HPTLC provides comparatively much more information about the sample from a single analysis, reported as up to 12D hyphenation [9–11], and is more sustainable in terms of plastic material and solvent consumption [12] than effect-directed detection via cuvette or microtiter plate assays associated with column-based separation methods. It is also comparatively much faster and less laborious, given the fact that multiple information is obtained on the same plate, *i.e.* from screening and effect-directed detection of many samples in parallel to tentative assignment of molecular formulae of important active compounds. Since

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the effect detection and analysis are performed in a miniaturized way on the same surface and the straightforward prioritization on important active compounds requires less data evaluation and data storage [9, 13], hyphenated HPTLC contributes substantially to rethinking separation science and analytical methods to be more environmentally friendly and sustainable [14, 15].

An obstacle to the dissemination of this powerful hyphenated HPTLC technique, if not available in the laboratory, is the high investment cost. Although it depends on the level of automation and standardization chosen, the establishment of planar chromatography and biology laboratories, for example, can account for €160,000 in investment costs. In contrast, the miniaturized do-it-yourself open-source 2LabsToGo system [16] with investment costs of only €1717 [17] could be an excellent option to start instantly with this powerful technique. Given the fact that the current in vitro genotoxicity bioassay analysis of one sample costs more than €8000 in Germany, the investment costs of the 2LabsToGo system are amortized with one analysis. Nevertheless, the question that arises is whether sophisticated planar bioassays could also be performed manually under simple conditions without the least instrumentation to nontarget-detect hazardous compounds present in complex samples. As example of a worst-case scenario, a highly demanding and sensitive planar genotoxicity bioassay was selected, as well as one perfume and three packaging materials as very complex samples for analysis. Various plate types and more simple manual performance options for application, separation, and bioassay detection were studied. This would be helpful information when HPTLC equipment is not available, such as for biologists or toxicologists who wish to begin planar bioassays.

2 Experimental

2.1 Chemicals and materials

Bidistilled water was prepared with a Destamat Bi18E (Heraeus, Hanau, Germany). *Salmonella enterica* subspecies *enterica* Typhimurium bacteria strain TA1535 cryostock containing the plasmid pSK1002 (PTM *Salmonella* Typhimurium TA1535/pSK1002; DSM no. 9274) was purchased from DSMZ German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Sodium dihydrogenphosphate monohydrate (> 98%), magnesium sulfate heptahydrate (99.5%), D-glucose, lysogeny broth (Lennox) powder including 5 g/L sodium chloride, and resorufin-β-D-galactopyranoside, all from Sigma Aldrich, and HPTLC plates, 20 cm × 10 cm, as well as TLC aluminum foils, 20 cm × 20 cm, silica gel 60 with and without F_{254} were delivered by Merck, Darmstadt, Germany. Cyclohexane, ethanol, and methanol, all Chromasolv, were bought from Fisher Scientific, Seelze, Germany. Potassium dihydrogen phosphate (≥ 99%), potassium chloride (≥ 99.5%), sodium hydroxide (> 99%), and ampicillin sodium salt (> 99%) were purchased from Carl Roth, Karlsruhe, Germany. The 4-nitroquinoline 1-oxide (≥ 98%) was delivered by TCI, Eschborn, Germany. One perfume sample (ID 1) was purchased from a local discounter, and three packaging products (ID 2: packaging material, ID 3: recycled packaging material, and ID 4: raw material for packaging) were obtained for research purposes. Mineral oil saturated/aromatic hydrocarbons (ID 5, 1 mL/ampule MOSH/MOAH mixture consisting of *n*-undecane, *n*-tridecane, bicyclohexyl, 5-α-cholestane, 1-methylnaphthalene, 2-methylnaphthalene, *n*-pentylbenzene, perylene, and 1,3,5-tri-*tert*-butylbenzene; 150–600 µg/mL each in toluene) were supplied by Restek, Bad Homburg, Germany.

2.2 Sample solutions, enzyme substrate solution, and positive control solution

The perfume was used directly, without the least sample preparation. Two packaging products and one raw material for packaging sample (10 g each cut to 1–2 cm^2 pieces) were extracted with 200 mL ethanol for 6 h and concentrated to 5 mL (TurboVap 500, Zymark, Hopkiton, MA, USA) to obtain 2 g/mL sample solutions. From the resorufin-β-D-galactopyranoside substrate stock solution (20 mg/mL in dimethyl sulfoxide), 12.5 µL was dissolved in 2.5 mL phosphate buffer, prepared from disodium phosphate (4.3 g), potassium dihydrogen phosphate (4.1 g), potassium chloride (0.37 g), and magnesium sulfate heptahydrate (0.12 g) in 100 mL bidistilled water, adjusted to pH 7 with solid sodium hydroxide. For use as positive control standard for the bioassay, 4-nitroquinoline 1-oxide was dissolved in methanol (10 ng/µL).

2.3 Preparation of *Salmonella* cell suspension

As culture medium, lysogeny broth dissolved in bidistilled water (2 g/100 mL) was autoclaved at 120 °C for 20 min. Then, 1 mL each of aqueous glucose (100 mg/mL) and aqueous ampicillin (10 mg/mL) solution was added via a sterilizing 0.2 µm polytetrafluoroethylene syringe filter (VWR, Darmstadt, Germany). For a 16-h overnight culture, 25 µL *Salmonella* Typhimurium TA1535/pSK1002 cryostock (cell pellet of 10 mL 16-h *Salmonella* culture, in 10 mL fresh culture medium containing 10% glycerol, and frozen as 0.5 mL cryostock portions) was suspended in 30 mL culture medium in a 100-mL culture flask, and cultivated at 37 °C and 75 rpm in a mini-incubator (Cultura M, Carl Roth) positioned on a Miniature Shaker KM CO2-FL (Edmund Bühler, Bodelshausen, Germany).

To adjust an optical density at 600 nm (OD_{600} [18]) of 0.2, the 16 h overnight culture was 1:10 diluted in fresh culture medium.

2.4 Simple performance of the planar SOS-Umu-C genotoxicity bioassay

If necessary, TLC/HPTLC layers were cut to 5 cm × 10 cm and prewashed with methanol and dried for 10 min in an oven at 110 °C. Sample solutions were manually applied in an interrupted dosing mode using a 1-µL or 2-µL capillary, as spots with a track distance of 6 or 9 mm, distance from the lower edge 8 mm and left edge 10 mm. The plate was developed with 3 mL cyclohexane–ethanol 17:3 or toluene–ethyl acetate 2:3, all *V/V*, up to 70 mm, taking about 25 min (10 cm × 10 cm twin-trough chamber or similar glass vessel). During experiments, the relative humidity of the surrounding air was 57 ± 3%. After plate drying for 4 min (hairdryer), chromatograms were detected at 254 nm, 366 nm, and white light illumination (reflectance mode).

If necessary, the pH of the plate was controlled to be neutral (pH 7.0 ± 0.4) before bioassay application using a WTW SenTix Sur pH surface measurement electrode (Xylem Analytics, Weilheim, Germany). As positive control for the genotoxicity bioassay, 4-nitroquinoline-1-oxide solution (10 ng/µL) was applied as 0.2-µL and 1-µL spot or band on the upper plate part above the solvent front. The plate was manually immersed into 40 mL *Salmonella* Typhimurium suspension of OD_{660} 0.2 filled in a small glass dipping chamber (biostep, Burkhardtsdorf, Germany or similar small glass vessel), or it was manually sprayed with about 0.8 mL (2.5 mL for four plate pieces of 5 cm × 10 cm; 200 mL high-density polyethylene bottle with mist spray pump 24 mm neck; IndiaMART InterMESH, Uttar Pradesh, India or Glass Laboratory Sprayer, Macherey–Nagel, Düren, Germany) until visual plate wetness using a simple manual pump sprayer filled with sufficient *Salmonella* Typhimurium suspension. The wet plate was placed horizontally in a humid box with moistened filter paper lining (KIS 26.5 cm × 16 cm × 10 cm, ABM, Wolframs-Eschenbach, Germany), and the closed box was positioned in an incubator at 37 °C (Memmert, Schwabach, Germany). After incubation at 37 °C for 3 h and plate drying for 5 min, the plate was manually immersed in 40 mL or sprayed with about 0.8 mL (until visual plate wetness) resorufin-β-D-galactopyranoside substrate solution and incubated at 37 °C for 45 min, followed by plate drying and detection under white light illumination (reflectance mode) and FLD 366 nm, optionally also at FLD 254 nm (simple self-made 3D-printed box with LEDs, unpublished device, with image taken by a smartphone camera, or TLC Visualizer, CAMAG, Muttenz).

2.5 Comparison with performance using HPTLC instrumentation

Instrumentation from CAMAG, controlled by visionCATS software version 3.2 SP2, consisted of Automatic TLC Sampler 4, twin-trough chamber 10 cm × 10 cm, Derivatizer, TLC Visualizer 2, and TLC Plate Heater III. Sample solutions were applied analogously to manual application, but using the 100-µL syringe, 6-mm bands, track distance of 9 mm, and "fill only programmed volume" as setting, since only 1 mL of each sample solution was available. Cell suspension as well as substrate solution were piezoelectrically sprayed (2.5 mL, Derivatizer, level 4, red nozzle for cell suspension, yellow nozzle for substrate solution).

3 Results and discussion

3.1 Options for simple performance of the planar SOS-Umu-C genotoxicity bioassay

The SOS-Umu-C genotoxicity bioassay performed on the HPTLC surface is a good example of a sophisticated planar bioassay. The focus for simplification was laid on the use of cheaper TLC aluminum foils (compared to HPTLC glass plates) to reduce consumption material costs and on the manual application of the samples as well as bioassay cell suspension and substrate solution to save equipment costs of about €45,000 (Automatic TLC Sampler 4 and Derivatizer). Other steps of HPTLC were found not to be critical when performed simply, such as performing the development in a glass vessel instead of a fully automated development chamber. The absorbance/fluorescent measurement using a TLC scanner was not necessarily relevant, since chromatograms and bioautograms can be evaluated by videodensitometry [19, 20].

Perfume (ID 1 [21]), packaging material (ID 2), another one from recycled packaging material (ID 3), raw material for packaging (ID 4), and a MOSH/MOAH mixture (ID 5) were selected as complex samples to be analyzed in non-targeted fashion with regard to any genotoxins present. The perfume and MOSH/MOAH mixture were used directly, whereas the packaging materials were extracted with ethanol for any genotoxic migration products. After their application, the plate was developed with cyclohexane–ethanol 17:3 [21], or for a higher elution power, with toluene–ethyl acetate 2:3 in a small twin-trough chamber up to 70 mm. Chromatograms were detected at 254 nm, 366 nm, and white light illumination and then subjected to the genotoxicity bioassay. The positive control 4-nitroquinoline-1-oxide was applied on each upper plate part to prove proper bioassay performance.

For comparison and as a reference, the planar SOS-Umu-C genotoxicity bioassay was first performed with state-of-the-art instrumentation on HPTLC silica gel 60 plates (Fig. 1a). Since the end-product of the genotoxicity bioassay was also detected at FLD 254 nm, plates without fluorescence indicator F_{254} were used. Nevertheless, the separation was performed once on HPTLC plates silica gel 60 F_{254} to document any UV-absorbing compound zones present in the samples. After the separation of perfume and packaging material and application of the planar SOS-Umu-C genotoxicity bioassay, genotoxic substances appeared as rose resorufin zones on a bright background at white light illumination, or as orange fluorescent resorufin zones on a dark, slightly orange fluorescent background at FLD 366 nm, optionally at FLD 254 nm. The resorufin was released from the resorufin-β-D-galactopyranoside substrate via β-galactosidase produced by the genetically modified *Salmonella* Typhimurium bacteria in the presence of DNA-damaging compounds, such as genotoxins.

3.2 Manual application of sample solutions and separation on TLC aluminum foils

The simple performance of manual application using a capillary was found to be possible for low volumes of sample application volumes after some training in the best practice

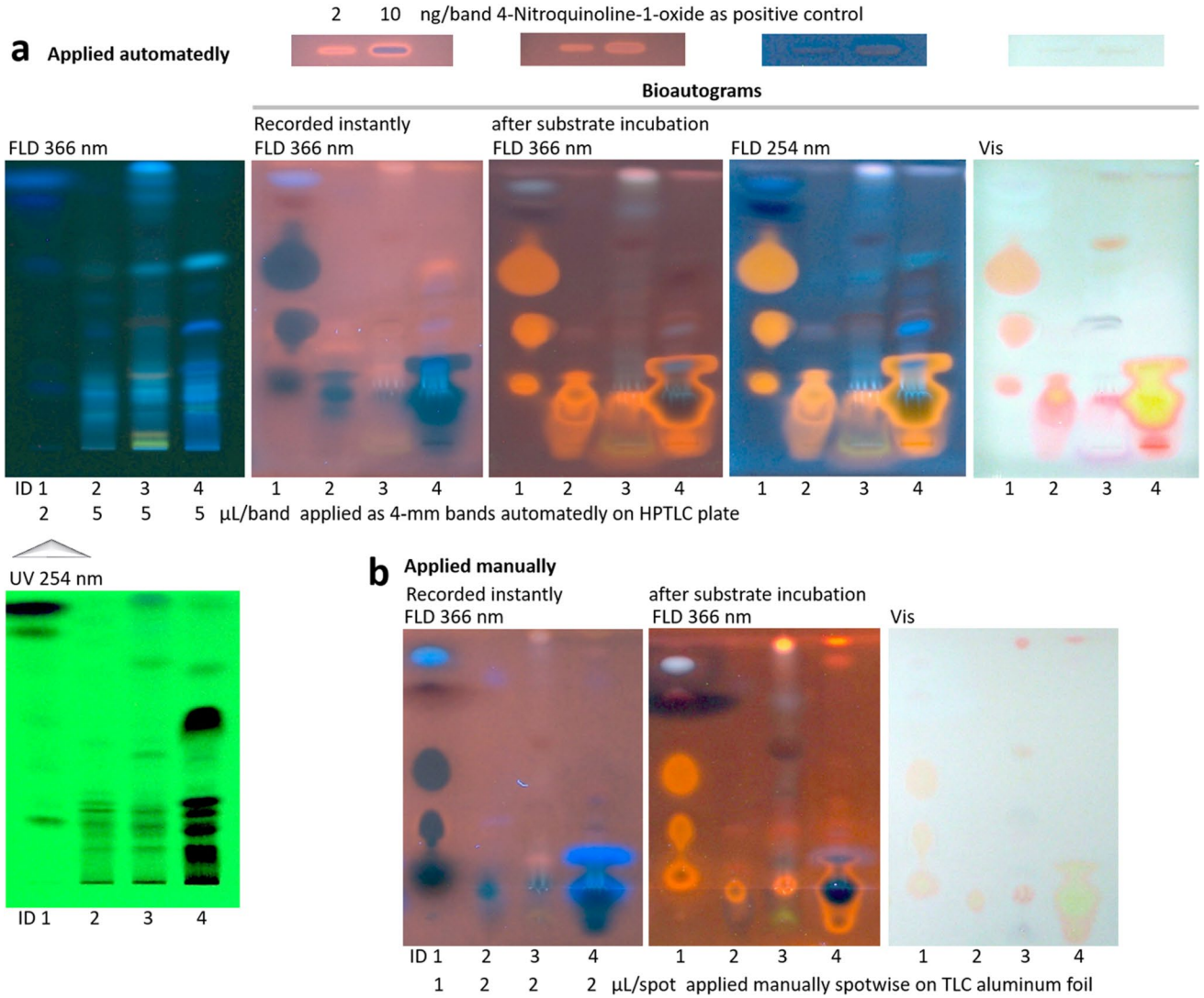

Fig. 1 Instrumental performance of the planar genotoxicity bioassay on HPTLC plate silica gel 60 with F_{254} using the resorufin-β-D-galactopyranoside substrate (**a**) *versus* manual, spotwise sample application on TLC aluminum foil silica gel 60 (**b**)**:** HPTLC/TLC chromatograms at UV 254 and FLD 366 nm developed with cyclohexane–ethanol 17:3, and after the bioassay performance, HPTLC/TLC bioautograms at FLD 366 nm (for comparison, recorded instantly after substrate application, and as usual, after 45 min incubation), FLD 254 nm, and white light illumination (Vis) of perfume (ID 1), packaging material (ID 2), recycled packaging material (ID 3), and raw material for packaging (ID 4)

of the manual performance. It was crucial that sample solutions were manually applied via interrupted dosing of each sample to obtain sharp start zones (Fig. 1b). Therefore, in an iterative mode, an aliquot of the sample liquid was flowing upon contact onto the adsorbent, then the capillary was lifted to allow drying of the application solvent, and the capillary was placed on the same position for dosing of the next aliquot.

The comparatively cheaper TLC aluminum foil silica gel 60 was selected. The control of the plate pH showed a neutral pH of 6.6 ± 0.2, which is a precondition for successful cell metabolism. After application and separation of perfume and packaging products, the applied planar SOS-Umu-C genotoxicity bioassay showed genotoxic substance zones comparable to the previous bioassay performed with state-of-the-art instrumentation on HPTLC plates silica gel 60 (Fig. 1a *versus* b). The successful manual application of the sample solutions on the comparatively cheaper TLC aluminum foil silica gel 60 was used for the following experiments.

3.3 Manual spraying *versus* manual immersion

The comparison of the manual spraying *versus* manual immersion of the cell suspension and substrate solution of the planar genotoxicity bioassay was investigated next. In addition to the previous samples, a MOSH/MOAH mixture (ID 5) was applied, since it was hypothesized that the very apolar genotoxic substance response in the solvent front of the samples from recycled packaging material (ID 3) and raw material for packaging (ID 4) could be caused by MOSH/MOAH contaminants. The chromatogram with the separated samples was manually sprayed with the *Salmonella* Typhimurium suspension until visual plate wetness (Fig. 2a). Two to three pump sprays were positioned outside the plate until the spray was stable, with which was then sprayed on the plate. The plate (5 cm × 10 cm) was too small to perform the meandering pattern, recommended for spray application. Typically, 2.5 mL was used for spraying four pieces of 5 cm × 10 cm plates, but the volume depended on the plate thickness and may need adjustment. Alternatively,

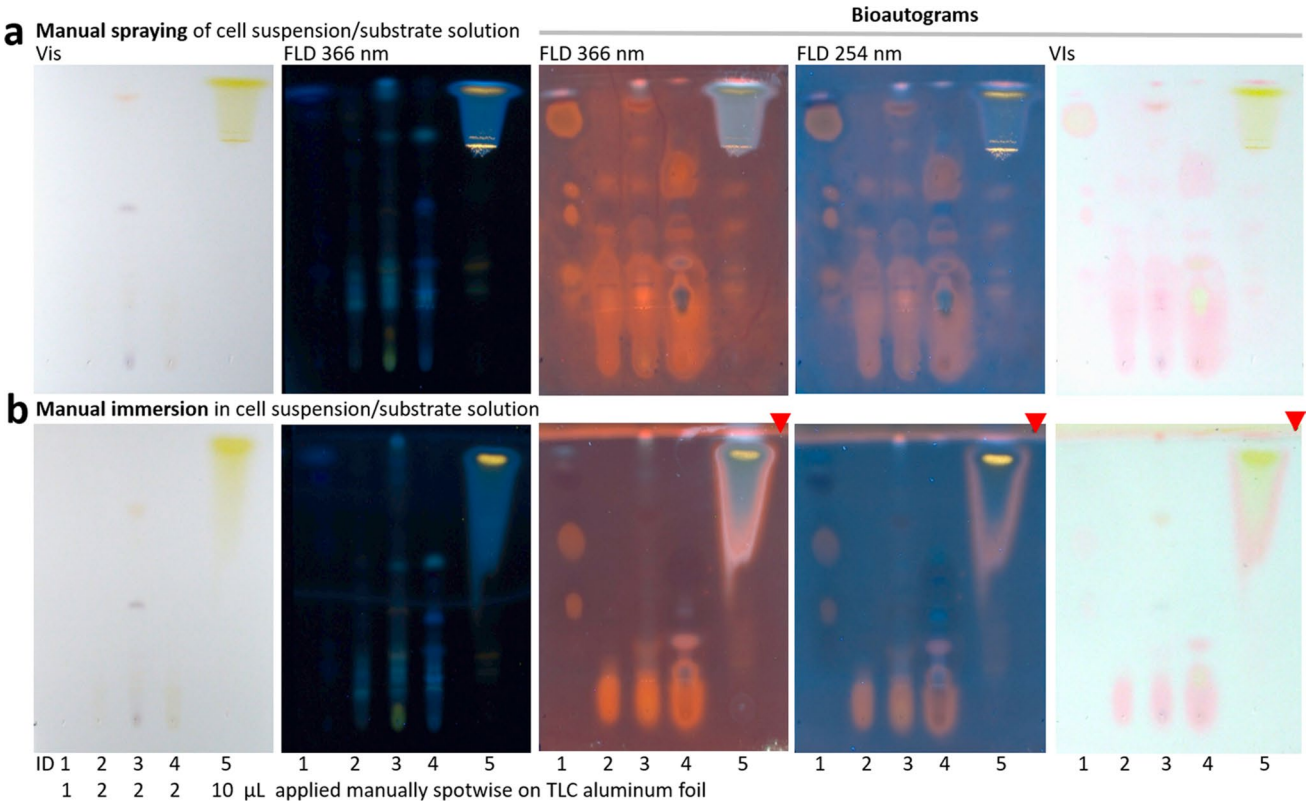

Fig. 2 Comparison of manual spraying **(a)** *versus* manual immersion (**b**) of the *Salmonella* Typhimurium cell suspension and resorufin-β-d-galactopyranoside substrate solution of the planar genotoxicity bioassay: TLC chromatograms at white light illumination (Vis) and FLD 366 nm, and after the bioassay performance, TLC bioautograms at FLD 366 nm, FLD 254, and white light illumination (Vis) of perfume (ID 1), packaging material (ID 2), recycled packaging material (ID 3), raw material for packaging (ID 4), and MOSH/MOAH mixture (ID 5), manually applied spotwise on TLC aluminum foil silica gel 60 developed with cyclohexane–ethanol 17:3 (**a** showed a slightly stronger elution than **b** owing to comparatively less drying of the start zones); solvent front with genotoxic signals (marked red) caused by immersion

the cell suspension was manually immersed into the *Salmonella* Typhimurium suspension (Fig. 2b). The wet plate was incubated at 37 °C for 3 h. Then, it was analogously treated with the resorufin-β-D-galactopyranoside substrate solution and incubated at 37 °C for 45 min. Both bioautograms showed genotoxic responses, whereby the manual spraying led to more blurred zones (Fig. 2a), most likely due to too much liquid on the surface, which means that less volume (2.2 mL instead of 2.5 mL) of the cell suspension and substrate solution should be used, since the volume needs to fit the layer thickness. Information on the layer thickness can be obtained in the product specification. Another difference was the striking genotoxic response in the solvent front only obtained by immersion (Fig. 2b, marked red). Obviously, part of the zones were eluted to the front during dipping. After the cell application, a lot of buffer salts are on the plate, which ease substance elution into the upwards-migrating substrate solution upon plate immersion. The MOSH/MOAH contaminants in the samples of recycled packaging material (ID 3) and raw material for packaging (ID 4) were confirmed by the co-applied MOSH/MOAH mixture (ID 5, 10 µL) in both bioautograms (Fig. 2), whereby the latter volume could be reduced to, *e.g.*, 3 µL. In addition, further even more intense genotoxic compound zones were detected in the samples.

3.4 Adjustment of application volume and mobile phase

Since the samples showed very intense genotoxic responses, only one-third of each sample volume was manually applied spotwise on the TLC aluminum foil silica gel 60. By development with toluene–ethyl acetate 2:3, the mobile phase that was comparatively higher in elution power, most genotoxic substance zones that previously remained at the start zones were now eluted, some even close to the solvent front. The elution power was too strong for the perfume sample, whereas the packaging material samples were spread along the migration distance, albeit with slight tailing, which was expected when using a neutral mobile phase for the lignin- and polyphenol-rich fibers. The manual immersion into the cell suspension and substrate solution was used to confirm the previous hypothesis. Again, the solvent front showed genotoxic signals obtained caused by the immersion technique (Fig. 3, marked red). Based on this outcome, manual spraying, with reduced excess liquid level, seemed superior to manual immersion.

The simple performance of the TLC–SOS-UmuC assay was successful, and the final workflow steps are summarized as an illustrative scheme in Fig. 4. Moreover, the miniaturized and portable open-source 2LabsToGo system [17, 22] is an excellent, sustainable instrument option with low material resource footprint and low investment costs, for both the chemistry and biology laboratory.

4 Conclusions

Performance of the HPTLC–SOS-UmuC assay was feasible without expensive HPTLC instrumentation. The contamination of recycled packaging material and raw material for packaging with MOSH/MOAH is very likely, as the MOSH/MOAH positive control samples showed the same retention time, apart from further, even more intense genotoxic signals detected in all samples. Crucial factors were the manual sample application of small volumes (< 2 µL)

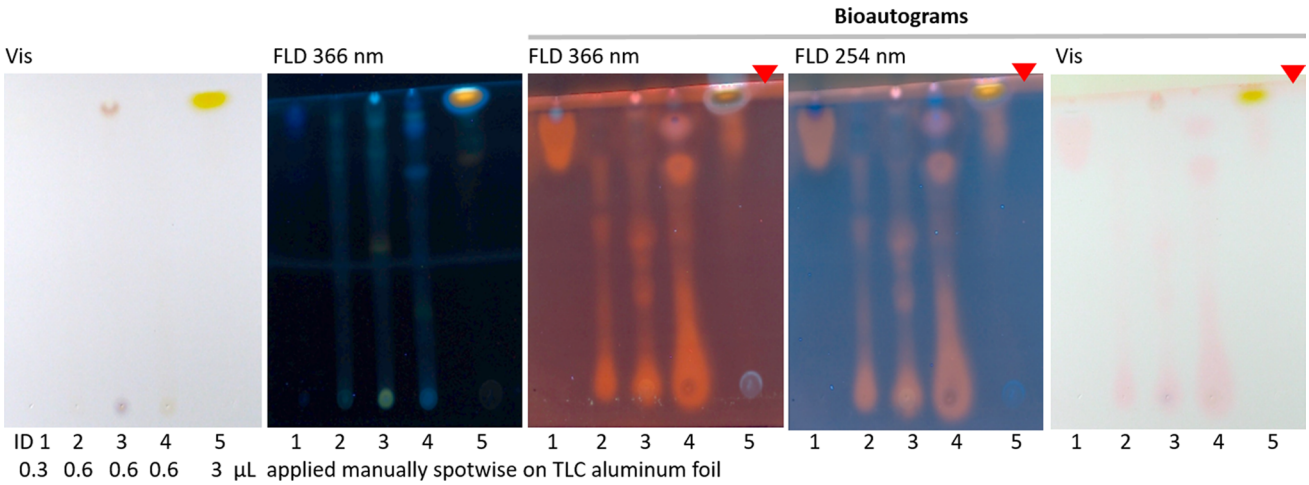

Fig. 3 Modifications to Fig. 2b: only one-third of the sample volume was manually applied spotwise on the TLC aluminum foil silica gel 60, developed with a mobile phase of higher elution power (toluene–ethyl acetate 2:3), and manually immersed into the cell suspension and substrate solution; solvent front with genotoxic signals (marked red) caused by the immersion technique

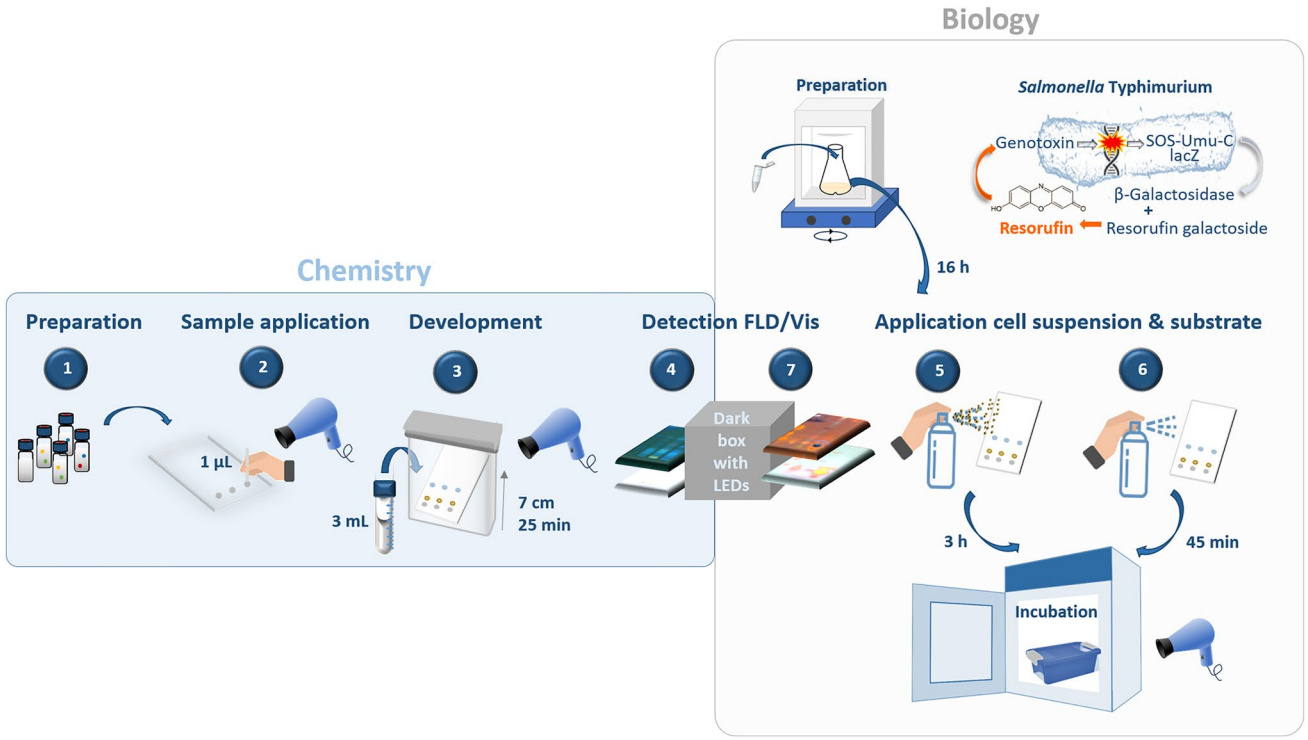

Fig. 4 Scheme of the final workflow of the simple TLC–SOS-UmuC bioassay performed in the chemistry and biology laboratory

in an interrupted dosing mode to obtain sharp start zones, the neutral pH value of the aluminum foil layer to ensure proper cell metabolism, and information on the layer thickness to adjust the volume of cell suspension and substrate solution and thus avoid excess liquid. Manual spraying as well as manual immersion of the plate resulted in positive genotoxic compound signals, although manual spraying was found to be superior. When high sample volumes have to be applied and high standardization is needed, the open-source 2LabsToGo system is an excellent sustainable instrumental option with low investment costs. Its very low instrumental footprint and the straightforward prioritization strategy help analytical chemistry to balance between technology and nature/ecology to reduce planetary overshoot.

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Declarations

Conflict of interest The author GM is member of the Editorial Board of the journal. Therefore, the submission was handled by a different member of the editorial board, and she did not take part in the review process in any capacity.

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