Research Article

Detection of Low Levels of Genotoxic Compounds in Food Contact Materials Using an Alternative HPTLC-SOS-Umu-C Assay

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Abstract

Food contact materials (FCMs) are perceived as major sources of chemical food contamination, bringing significant safety uncertainties into the food chain. Consequently, there has been an increasing demand to improve hazard and risk assessment of FCMs. High-performance thin-layer chromatography (HPTLC) coupled to a genotoxicity bioassay has been promoted as an alternative approach to assess food packaging migrates. To investigate the value of such a testing approach, a sensitive planar SOS-Umu-C assay has been developed using the *Salmonella* strain. The new conditions established based on HPTLC were verified by comparison with microtiter plate assays, the Ames and *Salmonella*-SOS-Umu-C assays. The lowest effective concentration of the genotoxin 4-nitroquinoline-1-oxide (0.53 nM; 20 pg/band) in the SOS-Umu-C assay was 176 times lower than in the microtiter plate counterpart. This was achieved by the developed chromatographic setup, including a fluorogenic instead of chromogenic substrate. As proof-of-principle, FCM extracts and migrates from differently coated tin cans were analyzed. The performance data highlighted reliable dose-response curves, good mean reproducibility, no quenching or other matrix effects, no solvent exposure limitations, and no need for a solid phase extraction or concentration step due to high sensitivity in the picomolar range. Although further performance developments of the assay are still needed, the developed planar assay was successfully proven to work quantitatively in the food packaging field.

1 Introduction

According to authorities such as the European Food Safety Agency (EFSA Scientific Committee, 2011), information on genotoxicity is a key component in risk assessment of chemicals, including those used in food and feed, consumer products, human and veterinary medicines, and other industrial and agricultural applications. It is considered that for some genotoxic chemicals (those acting through a direct DNA-reacting mechanism), no threshold may exist, and therefore no safe level can be established. Such chemicals are either not allowed for use or managed by the ALARA (as low as reasonably achievable) principle. Consequently, identifying genotoxic chemicals is critical.

Multi-component mixtures such as food contact materials (FCMs) are of special interest as they may release chemicals into foods, resulting in measurable human exposures. Some of these migrates are the primary chemicals the materials are made of. They are identified, characterized and can be efficiently managed. But side reaction products and impurities also may migrate into foods (Grob et al., 2006; Koster et al., 2015; Schilter et al., 2019). These substances can be numerous, with most of them being chemically unidentified and toxicologically uncharacterized. To address their health significance is very difficult since standard risk assessment methods are often not readily applicable. This causes substantial uncertainty with regard to safety in the food chain, triggering public, political and industrial concerns (Koster et al., 2015; Bopp et al., 2019; Schilter et al., 2019; European Parliament, Committee on the Environment, Public Health and Food Safety, 2016).

Packaging safety testing starts with migration studies in appropriate food simulants. The resulting migrates are multi-component mixtures of substances, many of them being chemically

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unknown (Koster et al., 2015; Bopp et al., 2019; Schilter et al., 2019). In principle, the best way to assess the safety of migrates would be to chemically identify and toxicologically test all constituents. However, because of the number of chemicals and relatively low concentrations involved, this is, in general, not feasible. The key challenge of packaging safety assessment is, therefore, the identification of the most relevant migrating substances to focus investigations (Koster et al., 2015; Bopp et al., 2019). In this frame, identifying mutagens appears of key importance, and genotoxicity bioassays are considered to play a major role for that purpose (Schilter et al., 2019).

The combination of analytical chemistry using universal detectors together with the concept of the threshold of toxicological concern (TTC) has been recently promoted as the most promising approach to prioritize unknown packaging migrants (Koster et al., 2011, 2014, 2015; Schilter et al., 2019). In practice, chemicals migrating at levels resulting in an exposure lower than the Cramer class III-TTC would be considered of low priority (Schilter et al., 2019). Since the Cramer Class III-TTC is only applicable to chemicals that do not possess any alerts for DNA-reactive mutagenicity, the major challenge of this approach is to provide enough evidence of the absence of such chemicals in the migrate under investigation (Schilter et al., 2019). Since direct DNA-reactive compounds must be excluded, the Ames assay has been proposed as the test of choice (Schilter et al., 2019). However, important limitations have been foreseen with the use of the standard Ames test. The main one is the limit of biological detection (LOBD) achieved (Rainer et al., 2018; Schilter et al., 2019). Previous reviews reported that only a small proportion of known mutagens would be expected to be detectable at a level compatible with safety (Rainer et al., 2018; Schilter et al., 2019). In addition, screening of FCMs using the liquid Ames MPF format showed that the matrix can significantly interfere with the test and prevent the proper detection of genotoxic compounds (Rainer et al., 2019).

In this context, methods coupling a separation step through high-performance thin-layer chromatography (HPTLC) with a bioassay may offer a ground-breaking possibility to solve these limitations. A newly developed bioassay workflow on reversed phases (RP) led to sharply bounded, separated bands of active chemicals (Klingelhöfer and Morlock, 2014) and improved the LOBD and resolution between bands, as well as reducing the matrix effect. Additionally, the possibility to elute/transfer the band from the HPTLC plate to high-resolution mass spectrometry (Jamshidi-Aidji and Morlock, 2018) or nuclear magnetic resonance spectroscopy (Yüce et al., 2019) should facilitate the identification of the substances responsible for the activity. The feasibility of performing the Ames assay on the TLC chromatogram was proposed (Bjorseth et al., 1982) but not pursued because of the intrinsic characteristics of the test.

Previous studies reported the use of the Umu-C assay (Baumann et al., 2003). The Umu-C assay is an indicator of genotoxicity shown to provide high concordance with the Ames test for detecting genotoxins including mutagens (Reifferscheid and Heil, 1996; Oda, 2016). It also has been used to address genotoxicity of complex environmental mixtures (Hamer et al., 2000). This assay is based on the induction of an SOS-DNA repair mechanism under genotoxic stress (Oda, 2016). It provides the advantages of requiring one bacterial strain and using a chromogenic detection system well suited to bioautography. The selected reporter assay is based on the *Salmonella typhimurium* TA1535[pSK1002] strain according to the ISO guidelines (ISO, 2000; Shakibai et al., 2019). The SOS-Umu response gene fused with the lacZ gene enables *Salmonella* to produce β-galactosidase that can convert *ortho*-nitrophenyl-β-galactoside (ONPG) into *ortho*-nitrophenol. So far, the feasibility of this approach has been evaluated only in a limited number of studies but with some encouraging results (Egetenmeyer and Weiss, 2017; Baumann et al., 2003; Stütz et al., 2019). Among these, responses were also observed as overlay assay, i.e., on a gauze pressed on the adsorbent (not the *in situ* adsorbent) (Egetenmeyer and Weiss, 2017). Recently, the assessment of model genotoxins and environmental samples in a test system constituted of HPTLC coupled to *Escherichia coli* strains carrying the SOS response gene fused with the *Photorhabdus luminescens* luxABCDE gene (Shakibai et al., 2019) further supported the promise of this approach to detect very low levels of genotoxins. Up to now, very little is available on the relevance of such an approach to serve FCM safety assessment.

In the present work, a newly developed RP-HPTLC-UV/Vis/FLD-*Salmonella*-SOS-Umu-C assay was tested for its application in FCM safety assessment. It was investigated whether a fluorogenic instead of chromogenic signal could improve the LOBD. The genotoxin 4-nitroquinoline-1-oxide (4-NQO), which can induce a response in the Umu-C in the absence of metabolic activation, was selected as reference. The assay performance was evaluated with respect to dose-response curves, working range, reproducibility, matrix effects, as well as LOBD and limit of biological quantification (LOBQ). Results obtained using the novel planar genotoxicity assay were verified by comparison to two liquid microtiter plate assays, the Ames MPF assay, and the *Salmonella*-SOS-Umu-C assay.

2 Materials and methods

Chemicals and materials

Six tin cans with six chemically different R&D coatings were provided by Ceritec SRL, Italy – Metlac Group in collaboration with Nestlé Research, Switzerland.

Materials used for the RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay (purity is specified, if available): *Salmonella typhimurium* bacteria strain TA1535, modified to contain the plasmid pSK1002 (PTM™ *Salmonella typhimurium* TA1535/pSK1002, cryostock) was obtained from Trinova Biochem, Giessen, Germany. Trans-1,2-cyclohexane-diaminetetraacetic acid monohydrate (CDTA, 98%) was bought from abcr, Karlsruhe, Germany. Ethyl acetate, ethanol and methanol (all HPLC quality) as well as dipotassium hydrogen phosphate (K_2HPO_4, > 98%), disodium hydrogen phosphate (Na_2HPO_4, > 98%), potassium dihydrogen phosphate (KH_2PO_4, > 98%), 4-methylumbelliferyl β-D-galactopyranoside (MUG), *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100, > 99%), glycerol (86%), potassium chloride (KCl, 98.5%), 2-amino-2-(hydroxymethyl)propan-1,3-diol

(TRIS, ≥ 99.9%) and sodium hydroxide (> 99%) were purchased from Carl Roth, Karlsruhe, Germany. Phenformin hydrochloride (98%), aflatoxin B1 (AFB1, > 98%), ampicillin sodium salt, D-(+)-glucose (99.5%), lysogeny broth (LB, Lennox) powder (including 5 g/L sodium chloride), D-mannitol (98%), *N*-nitroso-*N*-ethylurea (ENU, > 99%), dithiothreitol (DTT, for molecular biology) were purchased from Sigma-Aldrich, Steinheim, Germany. 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) and resorufin-β-D-galactopyranoside (RG) were purchased from trc, Toronto, Canada. Alosetron (98%) was purchased from APExBio, Houston, TX, USA. 4-Nitroquinolin 1-oxide (4-NQO, 98.0%) and hexachloroethane (HCE, 98%) were purchased from TCI, Eschborn, Germany. Citric acid monohydrate (p.a.), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$, 99.5%) and HPTLC plates RP-18 W (20 cm × 10 cm) were delivered by Merck, Darmstadt, Germany. For the plate batch used (No. HX86964996), a plate pre-treatment was required to harden the binder for the long-lasting aqueous bioassay procedure. A set of plates was heated at 120°C in the oven for 1 h, prewashed by development up to 9 cm first with methanol then with ethyl acetate (4 min drying in a cold stream of air after each prewashing step) and stored protected. Bidistilled water was prepared by a Destamat Bi 18E (Heraeus, Hanau, Germany). Lysis buffer concentrate was prepared from CDTA (0.73 g/L), TRIS (3 g/L), Triton X-100 (10 mL/L), DTT (0.31 g/L) and glycerol (100 mL/L) in bidistilled water. Phosphate buffer was prepared from KH_2PO_4 (40.8 g/L), Na_2HPO_4 (42.6 g/L), $MgSO_4 \cdot 7\ H_2O$ (1.2 g/L) and KCl (3.7 g/L), adjusted to pH 7 with solid sodium hydroxide. Alkaline citric acid phosphate buffer was prepared from citric acid monohydrate 6 g/L and Na_2HPO_4 10 g/L, adjusted to pH 12 with solid sodium hydroxide. The polypropylene box (26.5 cm × 16 cm × 10 cm) was from KIS, ABM, Wolframs-Eschenbach, Germany.

The SOS-Umu-C microtiter plate assay was provided by Xenometrix, Allschwil, Switzerland, including 4-NQO, ampicillin, B-buffer, stop reagent, *ortho*-nitrophenyl-β-D-galactoside (ONPG), 2-mercaptoethanol and *Salmonella typhimurium* strain TA1535[pSK1002].

The Ames MPF assay strains TA98[pKM101, hisD3052] and TA100[pKM101, hisG46], including the reagents and other components, were delivered by Xenometrix.

Standard solutions for HPTLC

As stock solutions, 4-NQO (10 mg/mL) in DMSO-methanol 1:1 and 1:100 diluted to 100 µg/ml were prepared. Thereof, standard solutions of 1, 10, 100, 1000 and 10000 ng/mL were prepared for spiking and assay development. Further stock solutions (1 mg/mL each) were prepared in methanol for AFB1 and ENU, in ethanol for PhIP, HCE, phenformin and D-mannitol, and in DMSO for alosetron. These stock solutions were diluted to 100 µg/mL, except AFB1, which was diluted to 10 µg/mL. All solutions were stored at -20°C.

Development of the planar SOS-Umu-C assay

On each pre-treated HPTLC RP-18 W plate, three 4-NQO track patterns (7 mm bands) were applied ranged from 4 to 1000 pg/band (Automatic TLC Sampler ATS4 with FreeMode option of winCATS software V.1.4.7, CAMAG, Muttenz, Switzerland). *Salmonella typhimurium* cells (20 µL cryostock) were prepared in 35 mL LB (20 g/L plus 1 g/L D-(+)-glucose and 106 mg/L ampicillin sodium) and incubated overnight for 16 h. As contamination control, the medium was incubated without cells in parallel, and only media showing no turbidity were used for cultivation. The 16-h overnight-cultured *Salmonella* cells were centrifuged (3000 x g, 10 min) and re-suspended in fresh culture medium to obtain six different OD_{660} between 0.2 and 0.7. An HPTLC plate with the applied 4-NQO track patterns was immersed in each suspension of a defined OD_{660} (immersion speed 3.5 cm/s, for 5 s using the Immersion Device III, CAMAG). Each plate was placed horizontally in a humid polypropylene box (pre-moistened with filter papers wetted with 35 mL water at room temperature for 30 min) and incubated at 37°C. Incubation times between 1-6 h were studied in 1-h steps for all ODs. Plate drying, MUG substrate application, incubation and evaluation was performed as follows in the new protocol.

Extraction of food cans and migration simulation

One of the six differently coated tin cans (ID 64) was used for the migration study performed as described (Veyrand et al., 2017) according to the European standards for fatty food (European Parliament, Committee on the Environment, Public Health and Food Safety, 2016). Briefly, the can was filled with 300 mL simulant (ethanol 95%), tightly closed with a 50 µm-thick aluminum foil (Korff, Oberbipp, Switzerland) and placed in an incubator at 60°C for 10 days. Then, the resulting migrates were applied directly onto the HPTLC plates.

For the extraction study, the other five cans were extracted with 300 mL *n*-hexane – acetone 1:1 (V/V) at 25°C for 16 h, tightly closed as mentioned. Procedural blanks were conducted analogously in glass bottles closed with a ground glass stopper.

HPTLC-UV/Vis/FLD SOS-Umu-C assay protocol

If not stated otherwise, the sample solutions (200 µL or 300 µL, taking 3 or 5 min) were applied as 7 mm x 10- or 20-mm area on pre-treated HPTLC RP-18 W plates using the ATS 4. The applied areas were focused by a two-fold front-elution with ethyl acetate up to the upper area edge (18 or 28 mm), followed by drying for 1 min (in a cold air stream using a hair dryer). The development was performed with toluene – ethyl acetate 8:5 (*V/V*) in the Twin-Trough Chamber (CAMAG) up to a developing distance of 70 mm, followed by drying for 5 min. The relative humidity of the ambient air was 30-50% during development. After neutralization with alkaline buffer (pH 12) and drying for 4 min (Klingelhöfer and Morlock, 2014), the chromatogram was immersed (immersion speed 3.5 cm/s, for 3 s) in the *Salmonella* suspension (OD_{660} of 0.2). The plate was horizontally placed in the humid polypropylene box and incubated at 37°C for 3 h. After plate drying for 4 min in a cold air stream, it was immersed (as before) either in the MUG-containing substrate buffer (10 mL lysis buffer concentrate, 30 mL alkaline buffer and 1 mL MUG solution (16 mg/mL in DMSO)) (Klingelhöfer and Morlock, 2014) or RG-containing substrate buffer (10 mL lysis buffer

concentrate, 30 mL phosphate buffer and 200 µL RG solution (20 mg/mL in DMSO)) (Schick and Schwack, 2017). After another 1-h incubation at 37°C and drying for 4 min, the bioautogram was documented at FLD 366 nm for 500 ms (DigiStore 2 Documentation System, CAMAG). The MU-fluorescence was measured at 366/ > 400 nm and the resorufin-fluorescence at 550/ > 580 nm (both mercury lamp, TLC Scanner 3, CAMAG). Data evaluation via peak area was performed using the winCATS software. HPTLC is an open system, and aerosol-forming operations must be performed in a fume hood in a room that must also provide a safe environment for handling *Salmonella* cells.

Performance study of the new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay

If not stated otherwise, samples were applied in triplicates per plate.

Evaluation of performance parameters:

i Dose response: The food migrate ID 64 and all extracts were spiked with 4-NQO at 100 to 1500 pg/200 µL applied as area.

ii Matrix effect: The food migrate ID 64 and extract ID 65 were spiked with 4-NQO at 300 and 600 pg/200 µL applied as area. In addition, the food simulant ID 64 was applied in the same way, but in a 5-mm overlapped mode with 4-NQO at 100 pg/area. Methanol solutions were subjected to the same procedure as blank. Methanol spiked with 4-NQO (100 pg/area) served as positive control.

iii LOBD/LOBQ: Application of 200 µL of the migration sample ID 64 was performed after spiking with 4-NQO to obtain 20 to 40 pg/200 µL applied as area (5 different amounts), whereas 300 µL of extracts ID 36-39 and 65 were applied after spiking with 4-NQO (20 to 100 pg/300 µL applied as area). Finally, 500 µL of extract ID 35 was applied after spiking with 4-NQO to obtain 20 to 100 pg/500 µL applied as area (5 different amounts). The visual LOBD (bioautogram) and the densitometric LOBD were evaluated for consistency. The LOBD and the LOBQ were calculated according to the ICH guidelines for validation of analytical procedures (European Medicines Agency, 1995):

$$LOBD = 3.3 * \frac{residual\ standard\ deviation\ of\ response}{slope\ of\ calibration\ curve}$$

$$LOBQ = 10 * \frac{residual\ standard\ deviation\ of\ response}{slope\ of\ calibration\ curve}$$

iv Upper working range limit: Application of 200 µL of the migration sample ID 64 was performed after spiking with 4-NQO to obtain a range from 1000 to 3000 pg/200 µL applied as area.

v Method precision: All six can matrices were spiked with 4-NQO at 300, 600 and 900 pg/200 µL and were applied five-fold at a 200-µL volume.

vi Assay specificity: Eight chemical solutions were selected and applied as track pattern in three different amounts, i.e., 0.3, 0.6 and 0.9 ng/band 4-NQO (1 ng/µL), 5, 10 and 15 ng/band AFB1, and 100, 300 and 500 ng/band ENU, PhIP, HCE, alosetron, D-mannitol and phenformin.

vii Lowest effective concentration (LEC): Application of 200 µL of methanol solution after spiking with 4-NQO to obtain a range from 0.5 to 200 pg/200 µL applied as area (12 different amounts).

Umu-C assay protocol

To perform the Umu-C assay in 96-well format, first LB medium (10 mL) was inoculated with 100 µL *Salmonella typhimurium* TA1535/pSK1002 in a 50-mL cell reactor tube (Greiner Bio-One CellStar, VWR, Dietikon, Switzerland), followed by 10 h incubation at 37°C and 250 rpm agitation using a shaker platform with speed control (Thermo Scientific, digital CO_2 resistant microplate shaker, Switzerland) installed with a timer control device (ThebenHTS, theben-timer 26, Germany). Before use, the OD600 of the culture was measured (JENWAY 6300 Spectrophotometer, Camlab, Cambridge, UK) until the bacterial density reached an OD600 between 2.0 and 3.0. The assay was performed according to Xenometrix UmuC Easy CS Instructions for Use with minor modifications. Briefly, the 10-h culture was diluted 1:7.5 with medium and incubated at 37°C and 150 rpm for 2 h using a shaker platform with speed control (Thermo Scientific, digital CO_2 resistant microplate shaker, Switzerland). This bacterial culture of 70-80% of the initial OD600 was used for the assay performed in a 96-well microtiter plate (Thermo Fisher Scientific, Roskilde, Denmark). Bacteria culture (50 µL) was added to each well and mixed with samples and controls at the corresponding concentrations. Plates were incubated at 37°C and 150 rpm for 2 h. After incubation, 30 µL product of each well were added to a new microtiter plate well and mixed with 270 µL fresh LB medium. The OD600 was measured using the microtiter plate reader (POLARstar OPTIMA, BMG LabTech, Germany), followed by incubation as before. Assessment of Umu-C induction was performed by adding 30 µL of each well to a new microtiter plate well. The B-buffer/ONPG mixture was prepared with B-buffer (30 mL), ONPG (2 mL) and 2-mercaptoethanol (82 µL). Plates were incubated at 37°C and 150 rpm for 30 min. Stop reagent (120 µL) was added and mixed. The OD420 was measured using a plate reader to evaluate the rate of β-galactosidase. The positive control of the test was 4-NQO at 0.5 µg/mL (without metabolic activation, Xeno No. 1801-1902, Xenometrics). Biological triplicates were performed testing blank, negative and positive controls in each microtiter plate. Data were analyzed using the average of the triplicates, considering dose-response effect and quality criteria achievement. The relative units (RU) were obtained at OD600 for the growth factor (G), OD600 and OD420 for β-galactosidase induction (UT), and, finally, OD420 for the induction ratio (IR). The quality criteria to classify a sample as genotoxic with respect to blank and negative controls are a $G \geq 0.5$ and an $IR \geq 1.5$.[1]

[1] Moltox. UMU Water – Waste water and concentrated and solid sample test kit instruction manual, 31-400, Version 07.02.2019. https://bit.ly/38mxUZc (accessed 23.01.2020)

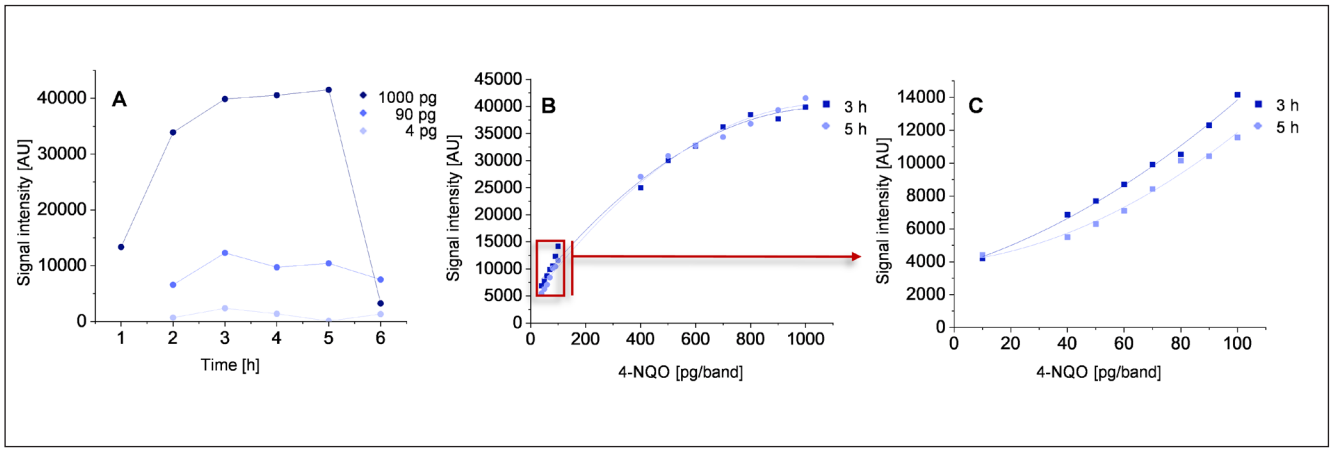

Fig. 1: Optimization of the planar SOS-Umu-C assay with regard to incubation time
Plots of the (A) highest achievable MU-signal intensities of 4-NQO against incubation times (1-6 h) and, in more detail, (B) MU-signal intensities against the 4-NQO amount ranged 4 to 1000 pg/band for a 3-h and 5-h incubation (C) with zoom on the lowest amount region of 4 to 100 pg/band (further data in Fig. S2[2]).

Liquid Ames MPF protocol

The liquid Ames MPF method was performed as recommended by Xenometrix (Flückiger-Isler and Kamber, 2012). Briefly, overnight grown *Salmonella* bacteria strains TA-98 for frameshift mutations and TA-100 for point mutations were exposed to 4-NQO (Xeno No. 1801-1902, Xenometrics) at increasing concentrations. Bacteria grown overnight were exposed using 24-well plates over 90 min at 37°C in medium containing histidine to allow two cell divisions. After exposure, bacteria were diluted into a pH indicator (bromocresol purple) medium lacking histidine using 384-well plates. A 48-h incubation at 37°C followed. The bromocresol purple from the indicator medium turned yellow as the pH dropped (pK_1 of 5.2) by catabolic activity of revertant cells, which grew in the absence of histidine. The number of wells containing revertant colonies was counted and compared to the vehicle control (DMSO). Biological triplicates were performed as described. Data were analyzed using the proprietary Xenometrix Calculation Sheet Version 3.23u 4/2017. Briefly, the mean number of positive (yellow) wells out of 48 wells per replicate and dose was compared with the number of spontaneous revertants obtained in the negative control samples. The fold increase (FI) above the baseline (mean of negative controls, $n = 3$, plus 1 standard deviation) was determined for each dose of test chemical (Flückiger-Isler and Kamber, 2012). Quality controls were applied for assay validity considering concentrations with FI ≥ 2.0 as genotoxic concentrations.

For comparison, the mean LEC of the new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay was calculated analogously to the microtiter plate assays described. The analysis was repeated on two different plates ($n = 2$, biological replicates). Microtiter plate and HPTLC data graphs were produced using GraphPad Prism 8.2.0 (GraphPad Software LLC, San Diego, CA, USA).

3 Results

3.1 Development of the RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay

Currently, the ONPG is used as a chromogenic substrate for the β-galactosidase in the *Salmonella*-Umu-C assay (ISO, 2000). Instead of generating an absorbance signal, different substrates producing a fluorescence signal were explored for the new bioassay. MUG was selected as a fluorogenic substrate. MUG is converted to the blue fluorescent 4-methylumbelliferone (MU) and already was proven to be superior over ONPG in our latest yeast-based bioassays (Klingelhöfer and Morlock, 2015, 2020).

Water-wettable (W), reversed phase (RP)-HPTLC plates RP-18 W were used. They showed almost no band diffusion, even after several hours of aqueous incubation (Klingelhöfer and Morlock, 2014).

As proof-of-concept, 4-NQO was selected as reference, as this compound is genotoxic in the absence of metabolic activation. Different amounts of 4-NQO were applied from 4 to 1000 pg/band to find the optimal incubation time and cell density for the planar assay. The densitometric results obtained for all different OD_{660} between 0.2 and 0.7 and incubation times between 1 and 6 h showed 4-NQO signals as low as 4 pg/band at OD_{660} 0.2. The track pattern response for OD_{660} 0.2 with a 3-h incubation period was the best condition to detect lowest 4-NQO amounts down to the 100 pg/band (Fig. 1). For 1-h incubation, the 4-NQO signal was only detectable down to the 400 pg/band (Fig. S1, S2[2]). At the highest incubation time (6 h), the background noise increased, affecting the sensitivity.

The resulting steps and selected parameters, as previously reported (Wöhrmann, 2019), of the newly developed RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay procedure are summarized in a

[2] doi:10.14573/altex.2006201s

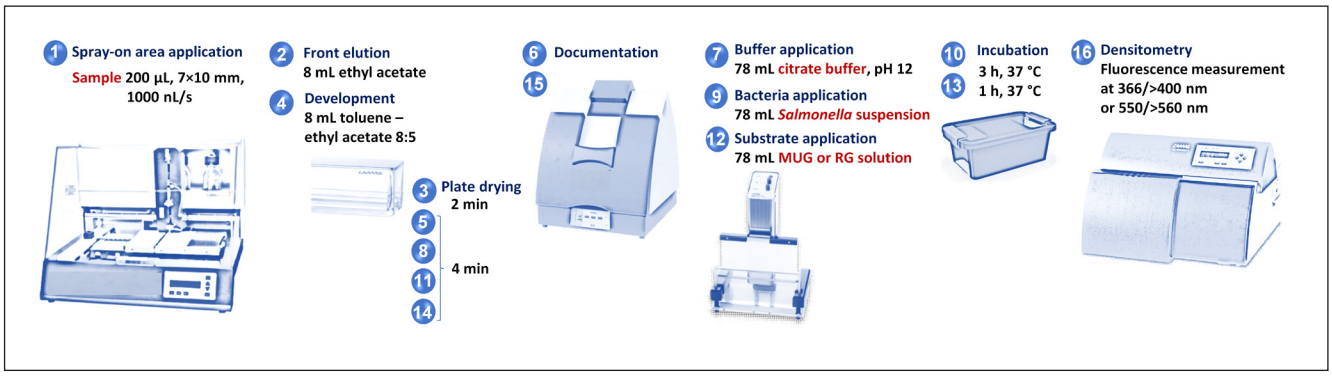

Fig. 2: Scheme of the newly developed RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay

The order of the individual steps is traceable by the increasing numbers, whereby some steps are performed repeatedly. The procedure on one RP-HPTLC plate took 5-6 h, was sensitive in detection and matrix-robust due to separation.

scheme (Fig. 2). As proof of the applicability of the new planar assay on RP plates with complex mixtures, samples of R&D tin can coatings were tested.

3.2 Performance of the new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay

Six tin cans (Fig. S3[2]) with six chemically different coatings were used for the migration and extraction study. The respective migrate or extract amounts in the low mg range were calculated as proof (Tab. S1[2]). The resulting migrate ID 64 sample and five extract samples of different tin can coatings IDs 35, 36, 37, 39 and 65 were investigated for potential genotoxic effects using the newly developed RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay. No genotoxic compounds or interferences were observed in any of the six different coating matrices (Fig. 3A) down to the given LOBD.

Since no direct spike of the tin cans was possible due to a technical limitation (volume needed to spike), the migration and the extract samples were divided into several portions and spiked with 4-NQO at different concentrations. Dose-response curves of 4-NQO between 100 and 1500 pg were investigated. One (for each of the five extract samples) and five (for the migrate sample) biological replicates were performed (Fig. 3). For the five different tin can coating extract IDs 35, 36, 37, 39, 65 (Fig. 3B-F), coefficients of correlation over five different concentrations were obtained between 0.987 and 0.997 with a mean precision of 7% (*%RSD* ranged between 5-9%, n = 3 technical replicates/plate, Tab. S2[2]). For the migrate ID 64 (Fig. 3G), the mean correlation coefficient over the five different concentrations was 0.992 with a mean precision of 8% (*%RSD*, n = 5 plates or days, n = 3 technical replicates/plate, Tab. S3[2]).

For LOBD and LOBQ, 200 µL food simulant migrate ID 64 spiked with 4-NQO was applied to obtain 20-40 pg/area. The LOBD was determined to be 13 pg/band. If referred to the applied volume (200 µL migrate), this LOBD is equal to 67 ng/L or 0.35 nM. The respective LOBQ of the migrate sample was 40 pg/band (202 ng/L or 1.06 nM). An additional dose-response curve for the migrate ID 64 spiked around the LOBD/LOBQ from 0.5 to 200 pg 4-NQO optically confirmed the low LOBD/LOBQ obtained (Fig. 4).

To evaluate the LOBD of the FCM samples, the maximal possible sample volume to be applied was tested. The LOBD/LOBQ determination for the five different tin can coating extracts was performed at application volumes ranging between 300-500 µL, each spiked between 20-100 pg/band 4-NQO, except extract ID 35, which was spiked between 30-100 pg/band 4-NQO. The LOBDs of 4-NQO in the five differently coated tin cans were determined to be between 32 and 71 ng/L (0.17-0.37 nM), depending on the maximal possible sample volume. The respective LOBQs of the five extract samples ranged between 98 and 215 ng/L (0.51-1.13 nM). In summary, the mean LOBD over six extract/migrate plates was 17 pg/band (13-21 pg/band, 32-71 ng/L, 0.17-0.37 nM) with a relative standard deviation of 16% (*%RSD*, n = 6 plates, each plate with n = 3 technical replicates, Tab. S4[2]).

Compared to the reported LOBD for 4-NQO (200 pg/spot after development) of the *Escherichia coli*-based bioluminescence assay applied for waste water (Shakibai et al., 2019), the newly developed *Salmonella*-based assay (mean LOBD of 17 pg/band for 4-NQO) is 12 times more sensitive. The detectable concentration of 4-NQO of the current assay is up to 1250 times more sensitive than the assay reported by Shakibai et al. (2019) (application of 5 µL of a 40 µg/L solution versus 200 µL of a 32 ng/L solution to obtain the LOBD). The lower working range started with the determined LOBQ. The mean LOBQ was 50 pg/band (40-65 ng/band, 98-215 ng/L, 0.51-1.13 nM) with a relative standard deviation of 16% (*%RSD*, n = 6 plates, each plate with n = 3 technical replicates, Tab. S4[2]).

The upper working range was studied by application of 200 µL migrate sample spiked with 1500 to 3000 pg 4-NQO. It was confirmed to be 1500 pg/band and thus 7.5 µg/L. Above this concentration, the saturation of the detector dominated over the increase in the biological signal response (Fig. S4[2]).

A potential matrix effect on the Umu-C response was investigated for the spiked migrate and the five extracts. Each of the six RP-HPTLC-SOS-Umu-C bioautograms of the different matrices showed a homogenous background and a high specificity for 4-NQO (Fig. 3). There was no influence of the six different

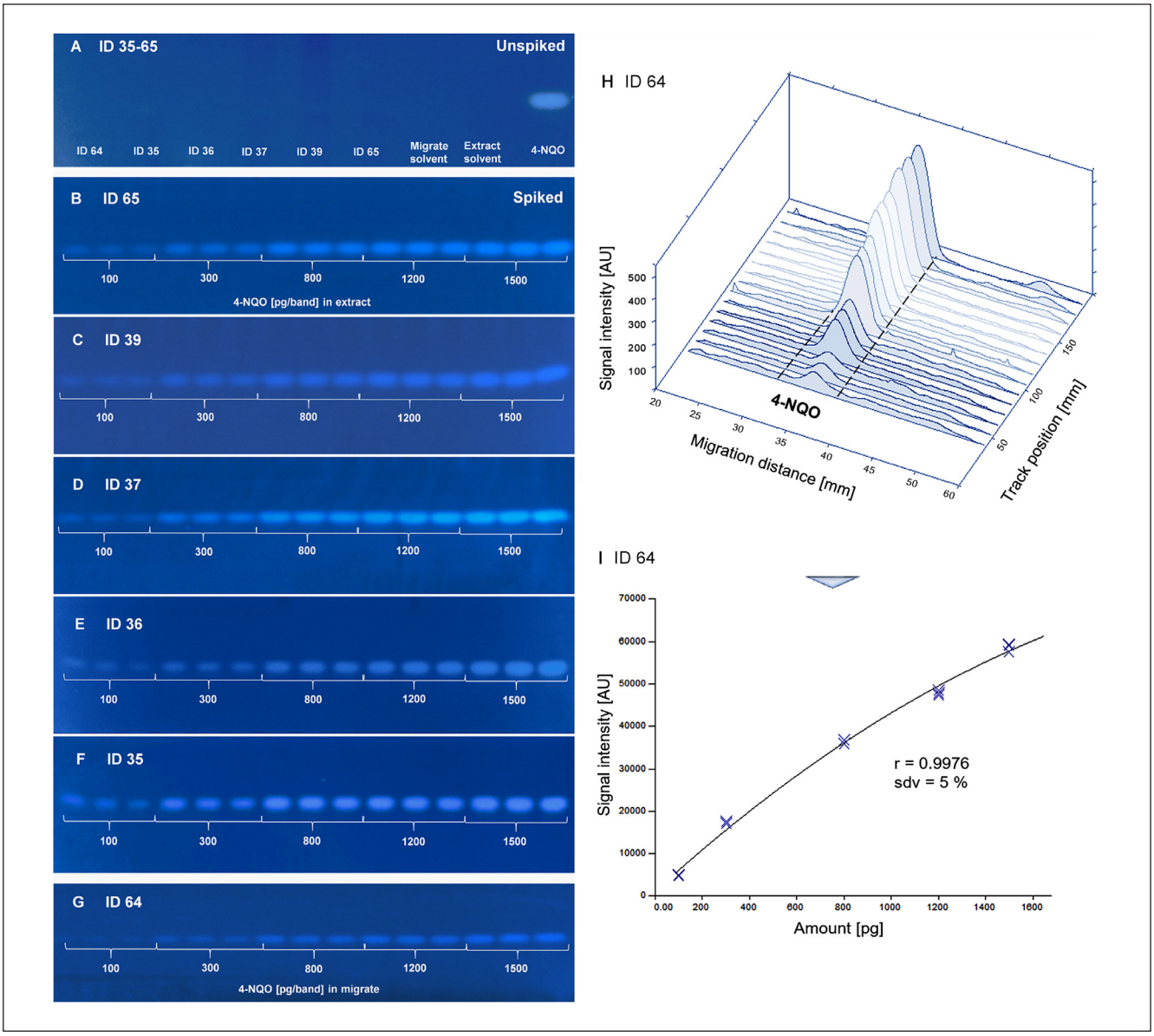

Fig. 3: Dose-response curve study

RP-HPTLC-SOS-Umu-C bioautograms at FLD 366 nm (A) of the food simulant migrate ID 64 and extracts of the five different tin can coatings ID 35, 36, 37, 39 and 65 showing no genotoxic compounds and (B-G) of the respective samples spiked with different amounts of 4-NQO (100-1500 pg/area. For the migrate, a representative example was depicted out of $n = 5$ plates or days). (H) Densitograms at FLD 366/ > 400 nm of food simulant migrate ID 64 and (I) its calibration curve via the biological response (dose-response curve).

tin can coating matrices observed on the 4-NQO hR_F value of 48 (Fig. S5[2]). Furthermore, migration sample ID 64 and can extract ID 65 were spiked with 300 pg and 600 pg of 4-NQO, analyzed and compared to their matrix-free replicates side by side on the same HPTLC plate. Neither the signal intensity nor the signal shape was influenced by the two different kinds of chemical matrices in the RP-HPTLC-SOS-Umu-C bioautogram (Fig. 5).

The mean precision (n = 5 technical replicates) of 4-NQO at three different concentrations (300, 600 and 900 pg/area) in the food simulant migrate ID 64 was 5% (*%RSD* ranged 2-5%), whereas it was 7% (*%RSD* ranged 5-8%) in the extracts. All achieved precisions were reliable for the given low picomolar range.

As proof of the specificity of the RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay, 8 chemicals exhibiting different mechanisms of genotoxicity were tested. The chemicals were chosen according to the Kirkland list (Kirkland et al., 2016) from 4 categories: (1) direct DNA-damage (AFB1, ENU and PhIP), expected pos-

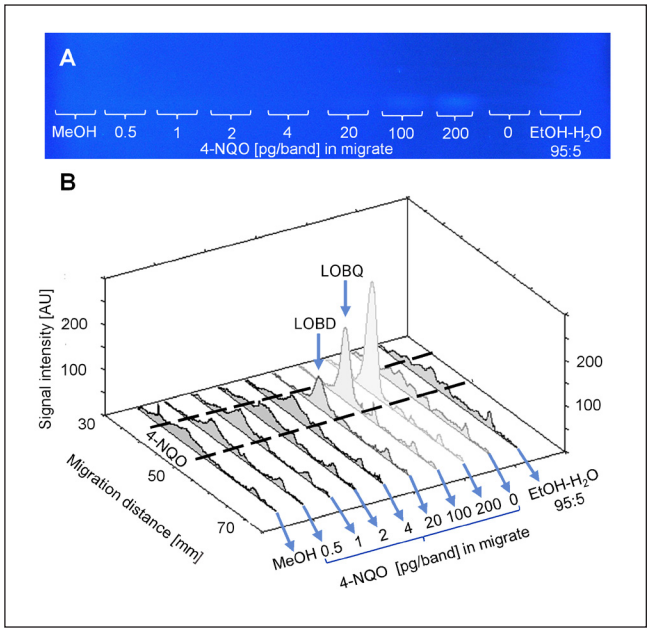

Fig. 4: LOBD/LOBQ study
(A) RP-HPTLC-SOS-Umu-C bioautogram at FLD 366 nm (enhanced image) of the food simulant migrate ID 64 (200 µL/area each) spiked with different amounts of 4-NQO (0-200 pg/area) and (B) corresponding densitogram at FLD 366/>400 nm, both confirming the LOBD and LOBQ at 20 and 100 pg/band, respectively; as control, no response for the migrate ID 64, migration simulant (ethanol – water, 95:5) and spike solution solvent (methanol).

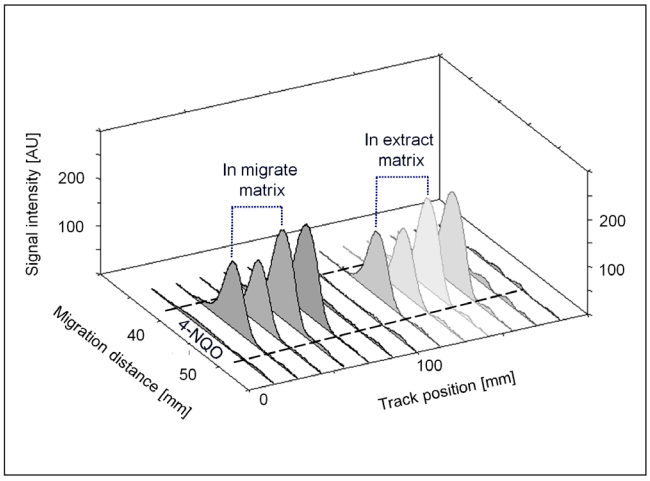

Fig. 5: Investigation of the matrix effect
Densitograms zoomed to the 4-NQO region of interest of the migration sample ID 64 and extract ID 65, showing 4-NQO at 300 and 600 pg/band, each side by side with and without matrix.

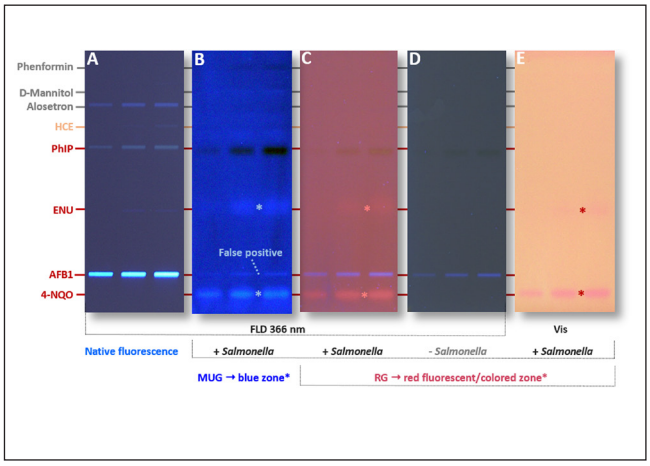

Fig. 6: Study on exchange of the enzyme substrate
Natively blue fluorescent compounds (false positive) afforded the change of the enzyme substrate (MUG substituted by RG) to improve the selectivity of the bioassay: (A) FLD image at 366 nm of 8 chemicals exhibiting different mechanisms of genotoxicity (applied threefold), (B/C) after the planar SOS-Umu-C assay with MUG/RG as substrate, (D) same procedure as C, but without *Salmonella* cells (negative control) and (E) Vis image of plate C (white light illumination); positive responses of 4-NQO and ENU are marked.

itive in *in vitro* testing, (2) equivocal genotoxicants (HCE), (3) non-genotoxicants (alosetron and D-mannitol), expected negative using *in vitro* assays and (4) unknown mechanism (phenformin, no *in vivo* data available). As expected for the chemical structure of AFB1, ENU, PhIP and alosetron, those molecules exhibited a blue native fluorescence at FLD 366 nm that was not distinguishable from the blue MU-fluorescence produced by the *Salmonella* (Fig. S6[2]). In order to avoid any interference by the florescence of an analyte (Fig. 6A,B), the fluorogenic substrate RG, which is excitable at a wavelength different to that of MUG/MU or the analytes, was selected to substitute the substrate MUG. The RG substrate is metabolized by β-galactosidase to red fluorescent resorufin, which can be measured at 550/ > 580 nm (Schick and Schwack, 2017). In comparison to the reported tungsten-halogen lamp, the use of the mercury lamp generated a signal increase of ca. 10%. This showed that the substrate that finally generates the response for the detection should be carefully selected to avoid method artefacts. The latter are easily discovered by multi-imaging of the plate.

The results of the specificity study proved the validity of the assay (without metabolic activation). The positive response represented by the red fluorescence/color of resorufin was selectively detected for 4-NQO and ENU (Fig. 6C,E, marked*). As a negative control, the whole protocol was performed without *Salmonella* cells, which proved the signal specificity, i.e., the signal was only generated in the presence of the bacteria (Fig. 6D). The reference controls AFB1 and PhIP were negative, as the test was performed in the absence of metabolic activation (only positive thereafter). Phenformin (no biodata available) as well as alosetron and D-mannitol (both negative controls) were negative. These results obtained for the 8 chemicals were in full agreement with their responses in the microtiter plate assays. This proved the reliability and good specificity of the assay.

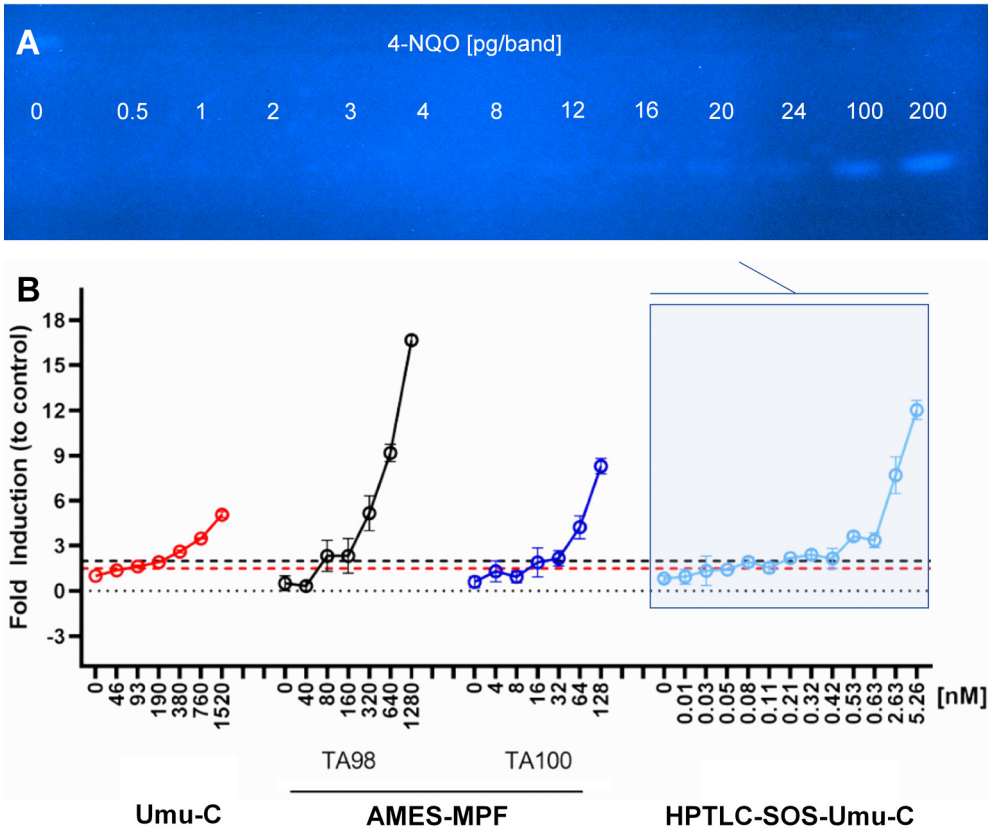

Fig. 7: Comparative LEC study using *in vitro* assays versus the new planar assay
(A) RP-HPTLC-SOS-Umu-C bioautogram at FLD 366 nm zoomed to the 4-NQO region of interest and for LEC determination, (B) comparison of the dose-response effects (nM) of 4-NQO via the SOS-Umu-C microtiter plate assay (threshold at FI 1.5; red dashed line), Ames MPF assay for TA98 and TA100 strains (FI 2.0; black dashed line) and new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay.

3.3 Comparison to Ames MPF assay and Umu-C microtiter plate assay

Two different microtiter plate assays were performed to compare and verify the data of the developed assay. In order to compare the molar concentration of 4-NQO obtained by the liquid assays with that of the HPTLC assay, the same nM unit was used for the HPTLC assay (conversion of pg/band to nM, Eq. S1[2]). Dose-response curves of 4-NQO were studied and plotted side by side (Fig. 7). In the absence of metabolic activation, 4-NQO exhibited a genotoxic effect (Flückiger-Isler and Kamber, 2012) in all three assays, which confirmed their performance. The LEC obtained by the Umu-C assay was determined to be 93 nM, and the dose range tested was 0-1520 nM. For the Ames MPF assay, dose-response curves of 4-NQO were performed for the strains TA98 and TA100. The LEC was determined to be 80 nM for TA98 and 32 nM for TA100, and the dose range tested was 0-1280 nM for TA98 and 0-128 nM for TA100.

The obtained LEC for the RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay was 0.53 nM, whereby the threshold FI was estimated to be 1.5 since the new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay uses the same *Salmonella* cells as the microtiter plate SOS-Umu-C assay. The dose range tested was 0-5.3 nM. Therefore, in comparison, the LEC obtained by the RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay was at least 176 times more sensitive than its counterpart, the SOS-Umu-C microtiter plate assay, as well as 151 and 60 times more sensitive than the Ames MPF assay (TA98 and TA100 strains, respectively).

Analogously, when the dose-response curve of 4-NQO was performed in matrix (200 µL food migrate ID 64, Fig. S7[2]), no matrix effect was observed. Even though neither the migration sample (ID64) nor the extractions were tested with the microplate Umu-C test in this study, potential artefacts caused by sample concentration and solvent exposure limitations (e.g., by DMSO) of the microtiter plate methods were not given for the developed RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay, as the migrates and extracts were applied directly. In contrast to the microtiter plate counterparts, the new assay points to single genotoxins in complex mixtures by the preceding planar separation. Thus, well-known quenching effects or other matrix effects can be avoided.

4 Discussion

There is an urgent need to improve methods to assess the safety of complex mixtures including FCM migrates and, in particular, to address the presence of unknown substances possibly migrating into food. This requires the availability of highly robust mutagenicity testing procedures allowing the detection of very low levels of DNA-damaging substances. This has been considered

mandatory to support the use of the TTC concept to prioritize chemically unknown migrating substances (Schilter et al., 2019). For this purpose, the Ames test has been identified as the most suitable assay because of its capability of detecting mutagens at relatively low concentrations; however, from a safety perspective, the achieved LOBDs are still unsatisfactory (Rainer et al., 2018; Schilter et al., 2019). In addition, no information can be obtained on the fractions/compounds inducing the biological effects. These are key factors to consider when assessing the safety of FCMs given the current uncertainties.

The performance of the developed RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay was convincingly good. Dose-response curves showed a reliable correlation and good reproducibility as tested with 4-NQO, even though the curve was not sigmoidal due to the limited range tested. Since HPTLC separates substances from their matrix, quenching effects or other matrix effects, potential artefacts during sample concentration, and any solvent exposure limitations were not observed. The determined LOBD/LOBQ and LEC were in the picomolar range. The mean relative standard variation of the LOBD over six plates was 16%. If analysis on several plates or days is compared, fluctuations are higher due to the RP plate quality and differences in the cell cul ture performance. The relative standard variation of the reproducibility of the LEC over two plates was 17%. The developed RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay was compared to two microtiter plate counterparts and provided a much lower LEC of 4-NQO. It was 60 and 176 times more sensitive compared to the Ames MPF assay and SOS-Umu-C microtiter plate assay, respectively, and 8 times more sensitive compared to Rainer et al. (2018, 2019). This makes it highly promising to detect the presence of low genotoxin concentrations in FCM migrates (Fig. S8[2]), FMC extracts and other multi-component mixtures. This may clearly provide an edge for the genotoxicity assessment of chemical mixtures.

The Umu-C assay is a genotoxicity test that responds to an array of genotoxicity mechanisms and not exclusively to mutagenicity. This is considered an important drawback in the context of the application of the Cramer class III TTC to prioritize the identification of unknown chemicals in complex mixtures. Indeed, only mutagens, defined as chemicals producing genotoxicity through a direct DNA reactivity mechanism, are excluded (Schilter et al., 2019). Consequently, the application of a test that is not selective to mutagenicity will likely overestimate alerts because of chemicals acting by other mechanisms. This means that an absence of genotoxicity would allow to apply the TTC Cramer class III. However, in case of a positive result, identification of the responsible chemical(s) by testing in an Ames test would be necessary to decide on the mechanism of genotoxicity involved and on applicability of the TTC Cramer class III.

The RP-HPTLC-coupled to Umu-C genotoxicity assay may become a breakthrough solution, not only in the packaging material field but also in genotoxicity testing of complex mixtures in general (e.g., environmental samples, cosmetics, commodities, foods, botanicals and medical devices). Our data represent a substantial contribution to the chemical food safety area. The detection conditions established feasibility to evaluate FCM to detect potential genotoxic compounds and to identify the bioactive molecule. The implementation of metabolic activation, also via rat-liver suspension cells produced without harming animals, and further genotoxic standards of differing potency along with further performance data in other matrices are still required.

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Conflict of interest

There is no conflict of interest.

Author contributions

DM performed experiments for RP-HPTLC assay development, data evaluation and wrote the manuscript draft. MMK supervised microtiter plate data analysis and provided substantial contributions to project and manuscript review. ED and PS/CC performed and evaluated microtiter plate assays (Umu-C and Ames MPF, respectively). BS contributed to the manuscript and the project rationale. GEM supervised the HPTLC assay development, data evaluation, provided resources, and substantially revised the manuscript.

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