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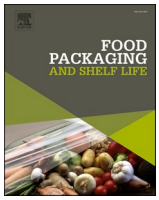

Towards detecting genotoxic chemicals in food packaging at thresholds of toxicological concern using bioassays with high-performance thin-layer chromatography

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ABSTRACT

High-performance thin-layer chromatography (HPTLC)-bioassays are promising new methods for detecting bioactive chemicals in food packaging. Here, we test whether direct-acting genotoxic chemicals are detectable in food contact materials (FCM) using HPTLC-bioassays. First, an interactive worksheet lays out steps to calculate needed detection limits in (bio)analytical methods from regulatory limits, including thresholds of toxicological concern (TTC). Second, we show that the sensitivity of a HPTLC-genotoxicity assay to low doses of chemicals, including food contact chemicals, is greater than a standardized microtiter plate version and in vitro assays already reported. Third, using HPTLC, we detected genotoxicity in extracts of FCM, and not in simulated migrates of FCM. Applying the worksheet to calculate needed detection limits in FCM migrates, we observed that seven of ten genotoxic chemicals would be detectable with HPTLC if present at the regulatory 10 ppb limit and two of ten at TTC for adults. With development, HPTLC-bioassays might become the best option for supporting safety assessment of genotoxicants in food packaging.

1. Introduction

While protecting food from various sources of contamination and extending shelf life, food packaging may itself contribute chemical contaminants to the packaged contents. Chemicals transferred to food from packaging can include oligomers of the base packaging structure and additives for adjusting material properties. In addition to these intentionally added substances, non-intentionally added substances (NIAS) are also present when formed during manufacturing or storage of food packaging, or from impurities in ingredients (Pack et al., 2021; Rusko et al., 2020). The resulting complex mixture present in food packaging may include toxic substances, potentially posing risks to a consumer. The regulatory requirement in the EU for any substance migrating from food contact materials (FCM) into food is set out in art. 3 of Regulation (EC) No 1935/2004, and states that no substance may migrate in amounts that could endanger human health (European Commission, 2004). Therefore, to protect human health, chemicals in, and migrating from, food packaging need to be characterized.

Abbreviations: CAS, Chemical Abstract Services; CF, concentration factor; DMSO, dimethyl sulfoxide; EC, European Commission; ECHA, European Chemicals Agency; ED_{10}, dose resulting in 10% of maximum effect; EU, European Union; FAU, formazine attenuation units; FCM, food contact material; HPTLC, high-performance thin-layer chromatography; IR, induction ratio; ISO, International Organization for Standardization; LEL, lowest effective level; MDL, method detection limit; MTBE, methyl *tert*-butyl ether; MUG, 4-methylumbelliferyl-β-D-galactoside; NIAS, non-intentionally added substance; NTP, U.S. EPA National Toxicology Program; ONPG, 2-nitrophenyl-β-D-galacto-pyranoside; Rf, retention factor; TGA, medium of tryptone, glucose, and ampicillin; TTC, threshold of toxicological concern.

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Chemicals without specific regulation, including NIAS, should be evaluated "in accordance with internationally recognised scientific principles on risk assessment" (European Commission, 2011). In the easiest case, chemicals with existing toxicological data can be assessed directly, but many chemicals may not yet have any toxicological data available. Those chemicals for which no toxicological data exist may be evaluated with a threshold of toxicological concern (TTC) approach (More et al., 2019; European Food Safety Authority EFSA; World Health Organization WHO, 2016). The TTC concept classifies concern for a chemical according to structural similarities to toxicologically characterized chemicals. The most stringent structure group in the TTC approach is, "potential DNA-reactive mutagens and/or carcinogens" which was updated from the former, "genotoxic substances" (Boobis et al., 2017; More et al., 2019).

However, the TTC approach was designed for chemicals of known structure (Kroes et al., 2004), so cannot be directly applied to unknown chemicals, of which many hundreds may migrate into food (Rusko et al., 2020). As one solution, bioassays have been recommended for inclusion in safety assessment of food packaging (Groh & Muncke, 2017; Koster et al., 2014). Bioassays detect substances based on their toxicological properties, rather than specific structure. For example, chemicals are commonly evaluated for mutagenicity with multiple strains of the Salmonella mutagenicity assay (Ames test) (Maron & Ames, 1983). Although considered the most suitable bioassay for testing food packaging migrates, the Ames test has also been described as still insufficiently sensitive to determine the safety of food packaging (Rainer et al., 2018; Schilter et al., 2019). Therefore, alternative tests for detecting low amounts of genotoxicants are desired.

In contrast with the Ames test, the umuC SOS response assay detects multiple genotoxic modes of action with one bacterial strain. Specifically, a reporter gene coupled to the SOS DNA repair pathway is induced in response to DNA damage that results in accumulation of single strand DNA (Janion, 2008). Importantly for the TTC requirement for "DNA reactive mutagens," the umuC assay has been shown to have good correspondence with the Ames test, in that one umuC strain (*S. typhimurium* TA1535 psk1002) agreed with the combined conclusions from five Ames test strains for 90% of 260 compounds with unequivocal results (Reifferscheid & Heil, 1996). However, chemicals that are DNA reactive through non-mutagenic modes of action may also be detected, making the umuC unspecific to the TTC category of DNA-reactive mutagens (More et al., 2019). Like the Ames test, the umuC does not cover all important genotoxicity endpoints, like some chromosomal damage that can be addressed with the micronucleus assay (Kirkland, Reeve, Gatehouse, & Vanparys, 2011).

The Ames and umuC are commonly performed in microtiter plates. However, the umuC can also be performed on high-performance thin-layer chromatography (HPTLC) plates. Bioassays on HPTLC plates take place directly on the silica layer of HPTLC plates after chromatographic separation of a chemical mixture (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020; Shakibai et al., 2019), thereby allowing detection of multiple biologically active compounds per sample. Chromatography can also reduce matrix interference by separating compounds of interest from cytotoxic or other confounding chemicals (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020). Some of the first TLC-genotoxicity assays were performed with Ames test strains, but with poor resolution of bioactive zones (Björseth et al., 1982). More recent HPTLC-bioassays have employed microorganism-based reporter gene assays, including the umuC assay (Egetenmeyer & Weiss, 2017; Riegraf et al., 2019). HPTLC-bioassays are promising for use with the TTC concept because they so far seem to detect lower doses of chemicals than their microtiter plate counterparts (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020; Meyer et al., 2020; Shakibai et al., 2019). However, despite general guidance (Koster et al., 2015), and limited examples in the literature (Debon et al., 2022; Rainer et al., 2018; Schilter et al., 2019), clear procedures have been lacking to determine how low the detection limits of bioassays need to be to detect genotoxicants coming from food packaging at TTC levels. Only a few studies have employed the new HPTLC-umuC (Debon et al., 2022; Egetenmeyer & Weiss 2017; Meyer et al., 2020), so more detailed evaluation of this promising bioassay is needed.

Towards the goal of being able to detect genotoxicants in food packaging at TTC levels, we first present an interactive calculation worksheet for determining needed detection limits in a bioassay. Second, we compare an umuC genotoxicity assay on normal phase HPTLC plates directly to a standardized umuC in 96-well plates using known genotoxicants and food contact chemicals. We compare the bioassay results to TTC levels and to a broader spectrum of published in vitro genotoxicity data. Third and finally, we apply the HPTLC-umuC to characterizing genotoxic hazards in a real example of FCMs. This work expands the data available for HPTLC-bioassays for direct-acting genotoxicants, and is the most extensive and clearest comparison to date of the new HPTLC-umuC bioassays in the context of food packaging.

2. Methods

2.1. Materials

Bacteria for umuC, *Salmonella typhimurium* TA1535 psk1002, were obtained from German Collection for Microorganisms and Cell cultures (Braunschweig, Germany).

Tryptone/glucose/ampicillin (TGA) medium was prepared according to ISO 13829:2000 (International Organization for Standardization ISO, 2000) with tryptone (peptone from casein, Chemical Abstracts Service Registration Number, CAS, 91079–40–2), D-(+)-glucose (CAS 50–99–7), and ampicillin (CAS 69–52–3) purchased from Sigma. The following materials were also purchased from Sigma: Sodium dodecyl sulfate (CAS 151–21–3), 2-mercaptoethanol (CAS 60–24–2), sodium carbonate (CAS 497–19–8), 2-nitrophenyl-β-D-galacto-pyranoside (ONPG, CAS 369–07–3), 4-methylumbelliferyl-β-D-galactoside (MUG, CAS 6160–78–7), dimethyl sulfoxide (DMSO, CAS 67–68–5), 4-nitroquinoline-1-oxide (4-NQO, CAS 56–57–5), 2-nitrofluorene (2-NF, CAS 607–57–8), methylnitronitrosoguanidine (MNNG, CAS 70–25–7), mitomycin C (MMC, CAS 50–07–7), etoposide (ETOP, CAS 33419–42–0), nalidixic acid (NA, CAS 389–08–2), triglycidyl isocyanurate (TIC, CAS 2451–62–9), 4-nitro-1,2-phenylenediamine (NOPD, CAS 99–56–9), nitrofurantoin (NF, CAS 67–20–9), 5-chloro-2-methyl-3(2 H)-isothiazolone (CMIT, CAS 26172–55–4), tris (2-ethylhexyl)phosphate (TEP, CAS 78–42–2), and C.I. Disperse Orange 25 (Orange 25, CAS 31482–56–1). Acetone (CAS 67–64–1), n-hexane (CAS 110–54–3), and normal phase HPTLC plates (20 x 10 cm Si gel 60 on glass backing, without fluorescence indicator) were purchased from Merck. Methanol (CAS 67–56–1) and ethyl acetate (CAS 141–78–6), were purchased from Fischer Scientific. All solvents were HPLC grade unless otherwise specified. Water (18 MΩ) was prepared with an ultrapurification system (Millipore). TENAX TA 60–80 (Sigma 11982), hexanes (technical mixture from VWR 24574.367, distilled in-house), methyl *tert*-butyl ether (MTBE, technical from Brenntag Schweizerhall AG 81208–156, distilled in-house), and methanol (Baker) were used in paperboard extraction and migration.

Standard chemicals were prepared by weighing stock powder or liquid into dilution solvent, methanol, ethanol, or acetone and stored in amber glass vials at 4 °C. These stock solutions were used as a consistent source for HPTLC and microtiter assays. Working dilutions (ten-fold steps for range finding or two-fold for refined tests) for HPTLC application were made with acetone or methanol in amber chromatography vials. For the microtiter umuC, standards were exchanged to 3% DMSO by evaporating a dilution of chemical in an amber glass vial under a gentle stream of nitrogen and external heating at 40 °C. DMSO was added as a keeper and the evaporated sample was brought to 3% DMSO by adding 18 MΩ water. If a precipitate was observed in 3% DMSO at the highest concentration (e.g. Orange 25, see Section 3.3), then the standard was diluted up to ten-fold in acetone or methanol before

exchanging the stock to 3% DMSO. The test substance in 3% DMSO was added to microtiter plates at the highest nominal test concentration and diluted with 3% DMSO to the other microtiter wells in ten-fold steps for range finding or two-fold for refined tests. Blank acetone and methanol served as process controls.

2.2. ISO standard umuC

Genotoxicity of standard chemicals and samples was evaluated with the umuC-SOS response test based on ISO standard 13829:2000 (International Organization for Standardization ISO, 2000). In summary, 200 μL aliquots of *S. typhimurium* TA1535 psk1002 that were grown overnight in TGA medium and adjusted to 500 formazine attenuation units (FAU) were stored in ampules at −80 °C in 20% glycerol. One ampule was thawed and added to 20 mL TGA media in a 250 mL Erlenmeyer flask the evening before a test and incubated at 37 °C, 220 rpm (Heidolph incubator 1000). After 10 ± 2 h, a morning culture was prepared with a 1:10 dilution of the overnight culture into TGA preheated to 37 °C. Samples and standards prepared in 3% DMSO were added to a microtiter plate (Plate A). Ten or two-fold dilutions were performed in microtiter plates with 3% DMSO to a volume of 180 μL. Then, 20 μL 10x TGA media was added to every well, followed by 70 μL bacteria morning culture adjusted to 350 FAU. Negative controls consisted of at least three wells each of vehicle controls (3% DMSO with bacteria), and absorbance blanks (3% DMSO with 70 μL TGA media instead of bacteria). Positive controls of 13.5 ng 4-NQO per well were included on each plate. Plate A was incubated for 2 h at 37 °C, 220 rpm (Heidolph incubator 1000). The contents of wells in Plate A were mixed with a multichannel pipettor, and 30 μL were transferred to Plate B, prepared with 270 μL warm TGA. Plate B was incubated for 2 h at 37 °C, 220 rpm (Heidolph incubator 1000). The contents of wells in Plate B were mixed with a multichannel pipettor and the absorbance at 600 nm (A_{600}) was measured (BioTek Synergy 2 microplate reader). The wells were mixed again and 30 μL were transferred to Plate C, which was already prepared with 120 μL B-buffer in each well. Then, 30 μL of ONPG solution (4.5 mg/mL in phosphate buffer) was added to each well. Plate C was incubated for 0.5 h at 28 °C (Heidolph). After this incubation, 120 μL of sodium carbonate solution (105.99 g/L) was added to each well. Absorbance at 420 nm (A_{420}) was measured in Plate C (BioTek Synergy 2 microplate reader). Growth and induction ratio (IR) were calculated according to equations 1 and 2.

$$Growth(G) = \frac{A_{600,treatment} - A_{600,blank}}{A_{600,vehicle} - A_{600,blank}} \quad (1)$$

$$Inductionratio(IR) = \frac{1}{G} \times \frac{A_{420,treatment} - A_{420,blank}}{A_{420,vehicle} - A_{420,blank}} \quad (2)$$

2.3. HPTLC plate handling and separation

HPTLC plates were washed by developing with methanol to about 90 mm in a twin trough chamber (CAMAG, Muttenz, Switzerland), incubated at 110 °C in a drying oven (WTC Binder, Tuttlingen, Germany) for 0.5 h, and stored in aluminum foil at ambient conditions (22 °C, 20–50% humidity). Standard chemicals and samples were applied to HPTLC plates in 6 mm bands with an Automated TLC Sampler 4 (CAMAG) at 10 mm from the plate bottom, at least 20 mm from the sides, and at least 10 mm between the centers of applied bands. Chromatographic development was performed with an Automated Multiple Development 2 (CAMAG). Atmospheric conditioning solution was 10 mL 25% NH_3 in 200 mL 18 MΩ water, with nitrogen gas supply. Chemical screening occurred with settings intended to chromatograph chemicals to a high retention factor but below the solvent front, namely, 3 × 100% methanol to 20 mm, 1 × 20:80 methanol:acetone to 60 mm, and 1 × 85:15 acetone:n-hexane to 80 mm. FCM samples were screened with 2 × 100% methanol to 20 mm, then 1:1 acetone:n-hexane to 80 mm. The chromatography was then refined to achieve better separation of genotoxic chemicals in FCM to 100% methanol to 20 mm, ethyl acetate to 35 mm, 2:1 ethyl acetate:n-hexane to 55 mm, and 1:1 ethyl acetate:n-hexane to 80 mm. Images of HPTLC plates were collected after every step with a Visualizer 2 (CAMAG) with white light and 366 nm illumination. Additional HPTLC device settings can be found in the supplementary information Tables S1 and S2.

2.4. HPTLC-umuC

Bacteria culturing prior to the bioassay was performed according to ISO 13829:2000 for umuC in microtiter plates (International Organization for Standardization ISO, 2000). The protocol diverged to replenish the TGA media before the HPTLC-bioassay, thereby ensuring the bacteria have enough nutrients during the assay. Specifically, an aliquot of the bacteria morning culture was centrifuged, the supernatant removed, and the bacteria pellet resuspended in fresh TGA media at a density of 380 ± 20 FAU. A Derivatizer (CAMAG) fitted for 20 × 10 cm HPTLC plates, with red nozzle, at spraying level 6, was used to spray 3 mL bacteria to a prepared HPTLC plate. After a settling period of the bacteria spray, the HPTLC plate was placed in a plastic box pre-heated to 37 °C, then placed on a middle shelf in an incubator at 37 °C for 2 h. Relative humidity inside the incubator was brought to approximately 65% with a tray of deionized water at the bottom. A box with an HPTLC-plate also contained two paper towels soaked with 50 mL deionized water to create greater than 90% relative humidity inside the plastic boxes. High relative humidity is required to prevent the plate from drying out, which can interfere with the bioassay response. After incubation, the HPTLC plate was removed and dried for approximately 5 min with a hair dryer (Satrap Classic 1250) at the lowest setting, with the dryer secured 40 cm above the plate in a ring stand. Images were collected with a Visualizer 2 as described above. The Derivatizer was used with the blue nozzle at spraying level 6 to spray 2 mL of MUG (0.5 mg/mL in B-buffer) onto the HPTLC plate, which was then placed back in the box at 37 °C and greater than 90% relative humidity for 0.5 h. The HPTLC plate was removed and dried with a hair dryer as before. To ensure an optimal signal from the product of MUG cleavage (4-methylumbelliferone) at 366 nm illumination (Müller et al., 2004; Zhi et al., 2013), the pH was increased by exposing the HPTLC plate to NH_3 vapor in a twin trough chamber. The plate sat for 10 min in a dry side of the chamber with the silica layer facing the opposing trough containing 20 mL deionized water and 3 mL 25% NH_3. Images were collected after every step with the Visualizer 2 as described above. The HPTLC plate tracks were scanned for fluorescence with the Scanner 3 (CAMAG) at 366 nm excitation and 400 nm filter. Solvents carrying standards (acetone, ethanol, or methanol) were applied as a negative control to every plate. Two levels (0.31 and 2.5 ng/band) of 4-NQO served as positive controls with each HPTLC-umuC plate.

2.5. Chemical screening

We selected twelve model chemicals for evaluation of the HPTLC-umuC (Table 1). Eight chemicals were chosen based on recommendations for testing genotoxicants (Kirkland et al., 2016), and for which data were present in other studies on genotoxicity assays (Meyer et al., 2020; Rainer et al., 2019; Rainer et al., 2018; Shakibai et al., 2019). The eight chemicals are 4-NQO, NF, 2-NF, NA, 4-NOPD, ETOP, MMC, MNNG. However, none of these eight chemicals is associated with food packaging, according to the Food Contact Chemical database (Groh, Geueke, & Muncke, 2020) and surveyed literature (Bengtstrom et al., 2016; Mertens et al., 2016; Mertens et al., 2017; Rusko et al., 2020; Van Bossuyt et al., 2017; Van Bossuyt et al., 2016, 2019). Four additional chemicals were included for their association with food packaging (Groh, Geueke, & Muncke, 2020): CMIT, TIC, Orange 25, and TEP. CMIT, TIC, and Orange are expected to be genotoxic based on experimental data in Ames assays (European Chemicals Agency, 2015;

Table 1
Chemicals tested in umuC assays.

CAS	Name	Abbreviation	Associated with food packaging[a]	Evidence for in vitro genotoxicity[b]	Active without (-) or with (+) m.a., or both (+-)[c]
607–57–8	2-nitrofluorene	2-NF	No	umuC, Ames	-
70–25–7	methylnitronitrosoguanidine	MNNG	No	umuC, Ames	-
50–07–7	mitomycin C	MMC	No	umuC, Ames	-
56–57–5	4-nitroquinoline-1-oxide	4-NQO	No	umuC, Ames	-
33419–42–0	etoposide	ETOP	No	Ames	-
389–08–2	nalidixic acid	NA	No	umuC, Ames	-
2451–62–9	triglycidyl isocyanurate	TIC	Yes	Ames	+ -
99–56–9	4-nitro-1,2-phenylenediamine	NOPD	No	umuC, Ames	+ -
31482–56–1	C.I. Disperse Orange 25	Orange 25	Yes	Ames	+ -
67–20–9	nitrofurantoin	NF	No	umuC, Ames	+ -
26172–55–4	5-chloro-2-methyl-3(2 H)-isothiazolone	CMIT	Yes	Ames	+ -
78–42–2	tris(2-ethylhexyl) phosphate	TEP	Yes	Not active	NA

Groh, Geueke, & Muncke, 2020 as described in Section 2.5
b Active in at least the umuC or one strain of the Ames test, or both (European Chemicals Agency, 2015; European Chemicals Agency (ECHA), 2022b; Kirkland et al., 2016; Rainer et al., 2021; Reifferscheid et al., 1996)
c m.a.: Metabolic activation with rat liver S9

European Chemicals Agency (ECHA), 2022a, b). The final chemical, TEP, is one of a set of chemicals recommended by Kirkland et al. (2016) as a non-genotoxic chemical to test that a true negative does not give a positive response (Kirkland et al., 2016). Further details are described in the supplementary information.

2.6. FCM preparation and testing

Samples of fresh fiber, printed, and recycled paperboard that never had been in contact with food were provided by a manufacturer (n = 1 for each sample type). To extract chemicals with a broad range of polarity from paperboard, samples were cut into pieces of 1–2 cm^2, and placed in with 45 mL methanol:hexane:MTBE 20:20:5 with all sides contacting solvent for 18 h at room temperature. The extracts were concentrated to 10 mL with Rotavapor at 55 °C and 500 mbar after filtration through a folded paper filter. The final concentration of paperboard in extract was 0.4 g/mL. The extracts were tested directly in HPTLC-umuC by applying 20 µL directly to HPTLC plates, and in the microtiter-umuC after exchanging to 3% DMSO as described for individual test chemicals. Furthermore, extracts were tested with and without overspiked 4-NQO at 1.5 ng/band (HPTLC-umuC) or 9 ng/well (microtiter umuC). The extractions were repeated one time to confirm results in the HPTLC-umuC.

The paperboard samples were also evaluated for migration to dry goods (European Commission, 2011). They were cut to reveal the inner (food contact) surface and 0.79 dm^2 was put in contact with 3.2 g TENAX®-TA (poly(2,6-diphenyl-*p*-phenylene oxide), BuChem BA, Apeldoorn, The Netherlands) in a migration cell for 10 days at 40 °C. The TENAX was extracted with 1 × 35 mL MTBE followed by 2 × 30 mL MTBE with shaking for approximately 1 min at room temperature. For each migrate, the TENAX extracts were filtered with folded filter paper, combined and concentrated at 45 °C at 600 mbar to a final migrate equaling 0.4 g paperboard/mL and 0.79 dm^2/mL. Blank solvents treated as samples (including filtering through filter paper), served as negative process controls for extraction and migration. Paperboard migrates were tested directly in HPTLC-umuC via application of 20 µL to HPTLC plates.

2.7. Data analysis and quality control

Criteria for evaluation of microtiter-umuC followed ISO 13829:2000 (International Organization for Standardization ISO, 2000). Growth had to be greater than 50%. Bioassay blanks and process blanks had to have IR less than 1.5. Samples inducing an IR greater than 1.5 were considered genotoxic in the microtiter assay. Criteria for a genotoxic response in HPTLC-umuC were: fluorescent bands had to have peak height three times greater than the adjacent noise in the same HPTLC lane and fluorescence over 10% of the maximum response of the positive control (2.5 ng 4-NQO) per plate. Some standard chemicals produced multiple fluorescent bands that, for e.g. NOPD, might be due to isomers in the product. In these cases, the most intense signal was used.

Data analysis and visualization was performed in R (ver. 4.1.0) using package drc (Ritz et al., 2015) (ver 3.0–1) for dose-effect modeling and ggplot2 (ver. 3.3.5) for visualization. Microtiter-umuC IRs of each chemical were modeled as a function of the nominal dose using four parameter logistic regression, with "bottom" set to one, equal to the negative control. Following Escher et al. (2014), only data up to IR of five were used in modeling. Maximum IR values are likely impossible to determine but IR at low doses still follows a log-logistic regression model (Escher et al., 2014). So, while we cannot interpret the "top" value of the dose-effect relationship, we interpolated the dose inducing an IR of 1.5 ($ED_{IR1.5}$, units: ng (per well)) with 95% confidence intervals. The HPTLC-umuC was also evaluated with a four parameter logistic regression using fluorescence peak height as response variable as a function of the nominal dose. The "bottom" value was set to zero, equal to the negative control. The dose interpolated at 10% effect (ED_{10}, units: ng (per band)), and its 95% confidence interval were calculated.

3. Results and discussion

3.1. Calculation of needed detection limits

To determine what detection limits are needed to detect chemicals at a regulatory or toxicological threshold, there are several assumptions about exposure and laboratory conditions that can be applied. Standard assumptions are suggested in food packaging regulations and case studies are present in primary literature, but the resulting detection limits are likely to be different for every scenario. We compiled assumptions and common laboratory conditions and lay out the many options in a customizable calculation worksheet (Supplementary information). As an example scenario, Fig. 1 demonstrates in six steps the calculation from TTC and regulatory limits to needed HPTLC-bioassay detection limits.

1. Choose a limit: The TTC for genotoxicants is 0.15 µg/person/day (Kroes et al., 2004). The TTC was derived assuming a 60 kg adult as consumer, which equals 0.0025 µg/kg body weight/day (More et al., 2019). A low assumption of infant body weight is 5 kg, which would equal a limit of 0.0125 µg/infant/day. A method detection limit (MDL) of 10 µg/kg food as stipulated by Regulation (EU) 10/2011 on plastic FCMs (European Commission, 2011) would enter the

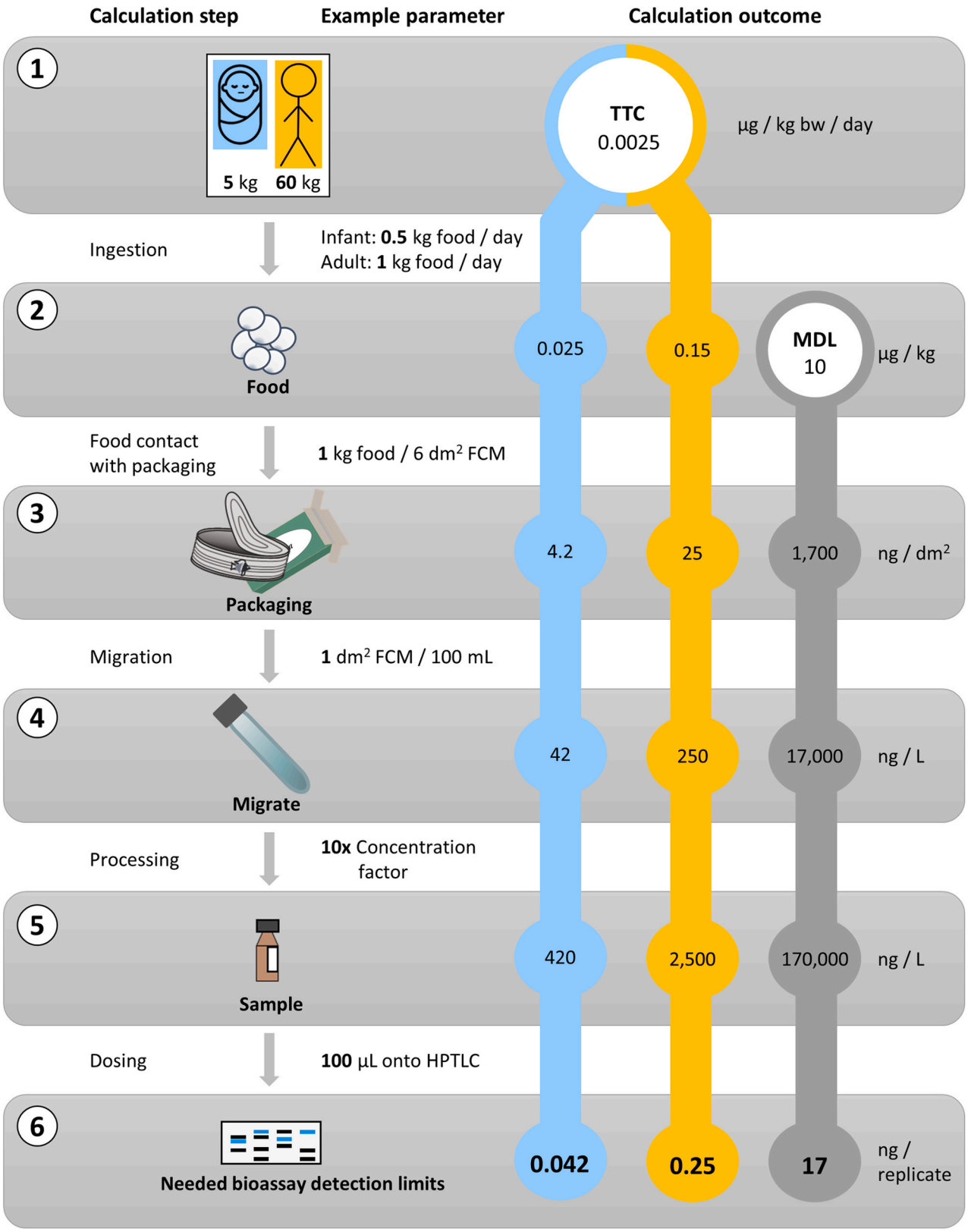

Fig. 1. Calculation of needed bioassay detection limits from threshold of toxicological concern (TTC) for DNA reactive mutagens. Example parameters are provided for each step, which are described in detail in Section 3.1 of the main text. The blue column tracks from TTC to needed detection limits for infants. Orange tracks from TTC to needed detection limits for adults. The MDL (dark grey column) is the needed detection limit set by European Commission regulation 10/2011 for substances without specific migration limits that migrate through a functional barrier and are not mutagenic, carcinogenic, or toxic to reproduction. The MDL has been employed by others in the context of genotoxicant migration into food as a pragmatic, although imperfect option (see Section 3.1 and Schilter et al., 2019). The calculations and additional parameter options are provided in a supplemental worksheet.

calculations after step 2, at which point the TTC has been converted to μg/kg. Although used by some as a pragmatic alternative to stringent TTC (Rainer et al., 2018; Schilter et al., 2019), the MDL is not based in analytical or toxicological data, and is therefore by regulation not appropriate for carcinogenic, mutagenic, or reproductive toxic chemicals (European Commission, 2011).

2. Estimate consumption: Assuming an intake of 1 kg packaged food/day for adults (European Commission, 2011), and 0.5 kg food/day for infants (U.S. Environmental Protection Agency (U.S. Environmental Protection Agency EPA, 2011), which we do in this example, the limits of a genotoxic substance in food would be 0.15 and 0.025 μg/kg, respectively. These intake assumptions are sometimes considered unrealistic and lower amounts of packaged food are used, for example 0.15 kg/day (Biedermann-Brem et al., 2016), or other case-specific scenarios (Brüschweiler, 2014; Koster et al., 2011).
3. Estimate food contact with packaging: A standard assumption for food contacting plastic packaging is 1 kg food/6 dm^2 packaging, provided the material volume is between 500 mL and 10 L (European Commission, 2011). This scenario yields 25 and 4.2 ng/dm^2 for adults and infants respectively. At low and high volumes, the actual surface area should be estimated.
4. Input laboratory migration conditions: Laboratory tests of chemical migration from food packaging use food simulants to mimic food properties but exclude potential matrix effects of food. Laboratory conditions may be controlled, or may be known, more precisely than actual food contact. Along with temperature adjustments to increase migration, surface area to volume could be adjusted. 100 mL simulant/dm^2 packaging is suggested as standard volume to surface area ratio for migrations of kitchenware (Simoneau, 2009) and is similar to a scenario described for plastic films (Koster et al., 2011).
5. Apply concentration factor of samples: This is the concentration factor as a result of laboratory handling such as rotary evaporation or solid phase extraction. The concentration factor may range from no concentration (CF = 1), in cases with very little sample volume to spare, up to CF= 1000 (Rainer et al., 2018). Increasing the concentration factor is an effective step to improve method detection limits, but may also increase concentrations of potentially interfering matrix components. A ten-fold concentration factor is a realistic compromise between sample availability, clean-up, and improved sensitivity, and yields a target sample concentration of 2500 and 420 ng/L, for adults and infants, respectively.
6. Apply dose in assay: The concentrated sample is applied in a bioassay. For HPTLC, two to hundreds of microliters could be sprayed in a band onto an HPTLC plate (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020; Meyer et al., 2020). Assuming 100% recovery throughout the laboratory procedures, an example of 100 μL results in needed HPTLC-bioassay detection limits of 0.042, 0.25, and 17 ng/band (or ng/replicate). In microtiter plate assays, the dose/replicate would be the mass of chemical in each well.

This exercise demonstrates examples of target detection limits in bioassays on a mass basis. However, chemicals can have very large differences in potencies for biological effects (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020; Shakibai et al., 2019). Differences in concentration factor have the greatest ability to achieve orders of magnitude differences in needed detection limits, assuming enough sample volume is available, recovery of the unknown chemicals is high, and potentially interfering chemicals are not also concentrated. We encourage the reader to access the calculation worksheet, explore the effect of various parameters, and to input values relevant to their own work.

3.2. Establishment of umuC assay

We established an HPTLC-umuC assay based on our experiences with a yeast estrogen screen (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020) and early trials in the literature (Egetenmeyer & Weiss 2017). Recently, other genotoxicity studies have been performed on HPTLC plates using umuC strain on reverse phase HPTLC plates (Meyer et al., 2020) and custom luminescent *Escherischia coli* on normal phase HPTLC plates (Shakibai et al., 2019). We encountered critical issues including ensuring humidity during the bioassay incubation to prevent the plates from drying out on the edges. After starting with a colorimetric indicator X-Gal (CAS: 7240–90–6) (Egetenmeyer & Weiss 2017), we settled on MUG as a main indicator to detect β-galactosidase production because it produces a sharp signal and follows other bioassays on HPTLC (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020; Buchinger et al., 2013; Meyer et al., 2020). We use resorufin-β-galactosidase as an alternative indicator because it can help confirm bioactivity in the case sample components are natively fluorescent at the same wavelength as MUG (Schick & Schwack, 2017). The final protocol is the one described in methods Section 2.4.

4-NQO was included as positive control at 0.31 and 2.5 ng with every plate. The HPTLC-umuC was consistent over a timeframe of about one year and on at least 24 different days: fluorescent heights of 0.31 and 2.5 ng 4-NQO were within typical ranges the historical mean for all of the results presented here (Fig. S1). Additionally, we observed no influence of chromatography on response: the dose-effect relationships of 4-NQO with and without chromatography overlapped along the entire distribution (Fig. S1).

Concerning the microtiter-umuC, it was at times difficult to reach the ISO stipulated positive control response of IR = 2 for 4-NQO at 13.5 ng/well. By controlling the bacteria culture quality with defined conditions for the frozen bacteria stocks, we improved the assay consistency, typically reaching approximately IR = 1.9, and proceeded with the assay format comparisons.

3.3. HPTLC is more sensitive than microtiter, and facilitates attaining regulatory thresholds

Ten of twelve chemicals were active in the HPTLC and the microtiter versions of the umuC assay (Fig. 2). The order of decreasing potency (median ED_{10}, 95% prediction interval) in HPTLC-umuC was 4-NQO (0.098 ng, 0.072 – 0.132) > NF (0.18 ng, 0.14 – 0.24) > MMC (0.48 ng, 0.11 – 2.0) > CMIT (0.51 ng, 0.42 – 0.63) > MNNG (1.2 ng, 0.35 – 4.0) > NOPD (3.3 ng, 2.0 – 5.3) > NA (7.0 ng, 4.9 – 9.9) > TIC

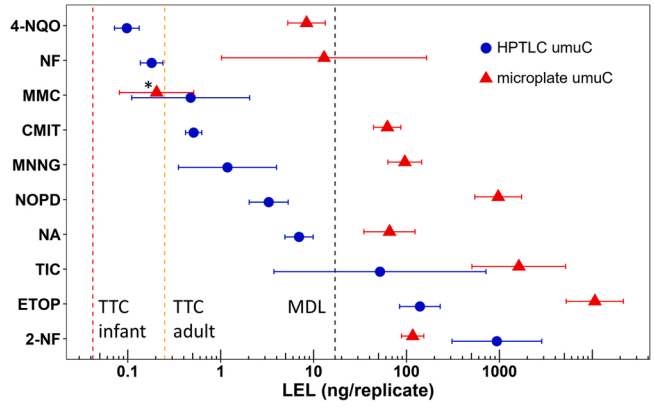

Fig. 2. Lowest effective level (LEL) of chemicals tested in umuC on HPTLC (blue) and microtiter (red) plates as effective dose at 10% effect (ED_{10}) and effective dose at induction ratio 1.5 ($ED_{IR1.5}$), respectively. Data points for each chemical are jittered to avoid overlap. Dashed lines mark needed detection limits according to regulatory requirements, as calculated in Fig. 1. Asterisk (*) indicates that $ED_{IR1.5}$ for MMC in microtiter extrapolated down from lowest dose of 0.54 ng. Red dashed line (TTC infant) represents 0.042 ng, from the TTC for infants; gold dashed line (TTC adult) is 0.25 ng, from the TTC for adults; black dashed line (MDL) equals 17 ng, from the method detection limit in EC 10/2011.(European Commission, 2011).

(99 ng, 5.9 – 1700) > ETOP (140 ng, 84 – 230) > 2-NF (940 ng, 310–2900). The order was different for microtiter-umuC (median $ED_{IR1.5}$, 95% prediction interval): MMC (0.20 ng, 0.081 – 0.51) > 4-NQO (8.4 ng, 5.3 – 13) > NF (13 ng, 1.0 – 160) > CMIT (62 ng, 44 – 87) > NA (66 ng, 35 – 120) > MNNG (96 ng, 63 – 150) > 2-NF (120 ng, 88 – 150) > 4-NOPD (970 ng, 540 – 1700) > TIC (1600 ng, 510 – 5200) > ETOP (11,000 ng, 5200 – 22,000). The individual dose-effect relationships are presented in Figs. S2 and S3. Two chemicals were inactive: Orange 25 and TEP.

A common positive control for mutagenicity and genotoxicity assays, 4-NQO, was the most potent chemical test with an ED_{10} in HPTLC-umuC of 0.098 ng. Another common positive control (2-NF) was one of the least potent chemicals. The fact that 2-NF is used at about ten-fold higher concentration than 4-NQO in Ames assays, e.g. Rainer et al. (2019), corroborates our observation that the $ED_{IR1.5}$ for 2-NF (120 ng) was also about ten-fold greater than 4-NQO (8.4 ng) in the microtiter umuC. However, the potencies of 2-NF and 4-NQO in the HPTLC-umuC were different by almost four orders of magnitude (940 and 0.098 ng, respectively).

Two of the four tested food contact chemicals were active in both HPTLC and microtiter umuC assays. CMIT was one of the most potent chemicals with an ED_{10} in the HPTLC-umuC of 0.51 ng. CMIT is reported to be mutagenic in Ames assays, however not considered an in vivo genotoxicant nor carcinogen (European Chemicals Agency, 2015). TIC, active in the HPTLC-umuC at 99 ng, is likewise considered by ECHA to be mutagenic, but not carcinogenic (European Chemicals Agency (ECHA), 2022a). Two food contact chemicals were not active in the umuC: TEP and Orange 25. TEP is expected to be non-genotoxic in vitro and one of multiple chemicals recommended for evaluating genotoxicity assays for true negatives (Kirkland et al., 2016), therefore providing accurate results in umuC assays. Orange 25 is expected to be active in the Ames test with and without metabolic activation based on experimental data submitted to ECHA (European Chemicals Agency (ECHA), 2022b). Testing of Orange 25 was limited by precipitation to about 100 μg/mL (approximately 20 μg/well) in the microtiter umuC assay. This is already below amounts of Orange 25 that were reported active for most Ames strains (European Chemicals Agency (ECHA), 2022b), but above any other chemical's LEL in an umuC assay (Fig. 2). Orange 25 was limited to about 10 μg in the HPTLC-umuC by an inhibition of fluorescence (dark zone) that may suggest cytotoxicity or another interference (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020).

In eight of ten cases, the HPTLC assay detected lower amounts of chemical than the microtiter format. For two exceptions, MMC and 2-NF, in which the microtiter format was as or more sensitive than HPTLC. We have previously observed for estrogens in a yeast-based HPTLC bioassay, that HPTLC was more sensitive than a microtiter assay, but that trend broke down for chemicals of the lowest potency (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020). We suspected that the trend for estrogens is linked to water solubility, which might also help explain why 2-NF is an exception for genotoxicants. However, we do not have an explanation for why MMC is not more potent in HPTLC than microtiter assay. Because genotoxicants are active in the umuC with multiple modes of action and have broad physicochemical properties (compared to estrogens), the trends we observe will remain merely suggestive until a much larger set of chemicals can be characterized.

The needed detection limits calculated in Section 3.1 are shown in Fig. 2 as dashed lines. Under the assumptions in this example, the HPTLC-umuC would be able to detect seven of ten chemicals at the MDL, two at the adult TTC, and none at the infant TTC. In contrast, the microtiter-umuC could at best detect three of ten chemicals at the MDL, and one at the adult TTC. More conservative assumptions may lead to even lower needed detection limits. For example, a 1:1 relationship between the packaging contact of actual food and the representative simulant (i.e. 1 kg food/dm^2 = 1 L migrate/dm^2) (Rainer et al., 2018) reduces the needed detection limits calculated from TTC for adults from 0.25 to 0.15 ng. At this reduction by a factor of 1.6, only one chemical would be detected by the HPTLC-umuC. Alternatively, the laboratory concentration factor could be as high as 1000 (Rainer et al., 2018; Schilter et al., 2019), leading to less stringent detection limits, i.e. 25 ng. At 25 ng, seven chemicals would be detectable by the HPTLC-umuC.

3.4. HPTLC more sensitive than other formats

HPTLC seems to be consistently more sensitive than other bioassay formats. Previous studies that directly compared HPTLC-bioassays to microtiter plate formats have mostly shown better sensitivity with HPTLC (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020; Meyer et al., 2020; Shakibai et al., 2019). In order to broaden that comparison, we searched for additional reports of lowest effective levels for other in vitro genotoxicity tests. Our results are shown in Fig. 3 for 4-NQO only because the greatest number of results were available for 4-NQO. At least 13 publications reported a detection limit for 4-NQO in an in vitro bioassay. For comparison, our focus was on bacteria based bioassays (De Flora et al., 1984; Egetenmeyer & Weiss 2017; Legault et al., 1994; Meyer et al., 2020; Nakamura et al., 1987; Rainer et al., 2021; Shakibai et al., 2019; Spiliotopoulos & Koelbert, 2020; Zwarg, Morales, Maselli, Brack and Umbuzeiro, 2018), but included three publications with human cell lines (Fig. S4) (Di Paolo et al., 2018; Hastwell et al., 2006; Jagger et al., 2009). Bioassay limits reported in each publication were converted from concentration (e.g. μg/mL) to dose (ng/replicate), provided total assay volumes were also reported (Table S4). The median LEL for HPTLC assays (0.098 ng) was about two orders of magnitude lower than the LELs for other assay formats such as microtiter plates (8 ng), or other (22 ng), which included test tubes and Petri dishes (Fig. 3). Results from literature for the other nine genotoxic chemicals are presented in Table S4 and Fig. S5. The trend that HPTLC is more sensitive than other formats is further suggested by these comparisons. Exceptions to this trend are MMC and 2-NF, which are discussed in Section 3.3 and for ETOP. However, the number of compared observations is very low for most chemicals, with only two to three data points per chemical. This literature search beyond HPTLC-genotoxicity assays was not comprehensive. It was further limited because many genotoxicity studies report only presence/absence of genotoxicity, rather than a limit of detection or LEL, as has been observed previously (Schilter et al., 2019).

HPTLC has low limits of detection compared to other bioassay formats. This may be because the final volume in which cells and sample interact is much lower. Although we do not have an accurate measure of

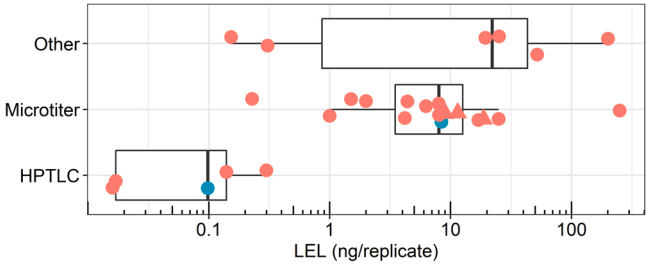

Fig. 3. Lowest effect levels (LEL) of 4-NQO in genotoxicity bioassays. Data were collected from literature (De Flora et al., 1984; Debon et al., 2022; Di Paolo et al., 2018; Egetenmeyer & Weiss 2017; Hastwell et al., 2006; Jagger et al., 2009; Legault et al., 1994; Nakamura et al., 1987; Rainer et al., 2021; Shakibai et al., 2019; Spiliotopoulos et al., 2020; Zwarg et al., 2018) (red) and compared to the present study (blue). Data points from bacterial tests are indicated with circles, human cell lines are indicated with triangles. Bioassays of category "Other" included assays on agar or in cuvettes. Box plots display the median (center line), 25 and 75th percentiles (hinges), and either 1.5 times the interquartile range or largest/smallest value within 1.5 x IQR (whiskers). The source and details of each data point are given in Fig. S4.

the volume of media in which a substance is dissolved on HPTLC plates, a glimpse at assays compiled for comparing 4-NQO in Fig. 3 suggest that sensitivity (ng/replicate) and total volume are correlated (Spearman's Rho = 0.67, supplementary information Fig. S6). HPTLC may essentially be miniaturizing the assay by bringing test organisms and chemicals together in a smaller volume. In other words, testing a higher, but undefined, concentration.

Although HPTLC-umuC seems to be sensitive to several genotoxicants, there may be chemical blind-spots specific to the HPTLC format such as volatile compounds or those that react with silica, as both aspects would reduce the availability of chemicals to bacteria cells. Further work should be done to characterize these and other possible limitations. Currently, an area in development is a combination of HPTLC-bioassays with metabolic activation (Debon et al., 2022; Egetenmeyer & Weiss 2017; Morlock et al., 2021). Strategies to incorporate detection of pro-genotoxicants should be further developed, whether directly with the bioassay on HPTLC plates, or as a preincubation step (Shao et al., 2020).

3.5. Genotoxicity detected in FCM

We detected multiple unknown direct-acting genotoxic chemicals in extracts of paperboard with HPTLC-umuC (Fig. 4 and supplementary information Fig. S7). In one example, four bands were detected in extracts of printed paperboard (Fig. 4). Microtiter-umuC results confirmed our observations of genotoxicity in paperboard samples (supplementary information Fig. S8). In a test of whether the extract matrices could suppress a known genotoxicant, no samples prohibited detection of spiked 4-NQO, compared to blank spiked with 4-NQO (Figs. S7 and S8). In migrates of paperboard samples, no genotoxicity was observed. Overall, our results demonstrate that unknown direct-acting genotoxicants can be detected in paperboard after extraction with organic solvents.

HPTLC acts like a sample clean-up step in which potentially interfering substances can be separated from chemicals of interest. Previously, we observed interferences caused by concentration dependent reduction in cell growth in a microtiter plate version of a yeast estrogen screen, whereas estrogenicity was detected when the bioassay was performed after chromatography on HPTLC plates (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020). In the current study in microtiter plates, we also observed a decrease in cell growth for the printed and recycled paperboard samples, although the reduction did not reach the ISO criterion of 50% to invalidate genotoxicity measurement. However, genotoxicity of native and 4-NQO-spiked samples was well detectable in each sample extract (supplementary information Figs. S7 and S8), so no interferences were apparent. Dark zones in an HPTLC-bioassay, sometimes centered in a fluorescent ring, indicates potential interference of the specific genotoxic response, such as through cytotoxicity (Bergmann, Simon, Schifferli, Schonborn, & Vermeirssen, 2020; Buchinger et al., 2013). Consistent with cytotoxicity, we observed that the largest dark zones in HPTLC-umuC and the greatest reduction in cell growth in microtiter-umuC occurred in the same samples: printed and recycled paperboard. Recent work has presented methods to determine cytotoxicity on HPTLC-plates with an indicator of cell viability (Riegraf et al., 2022). HPTLC did reveal that the samples contained multiple genotoxic chemicals, whereas a microtiter plate based bioassay provided a concentration-effect relationship for the whole mixture.

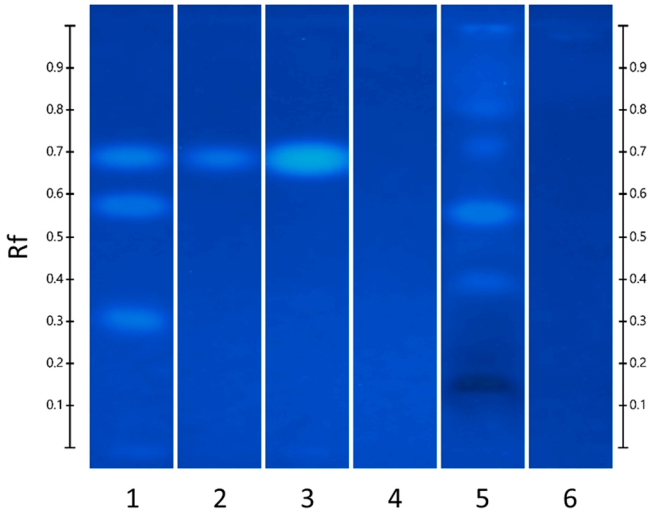

Fig. 4. HPTLC-umuC of quality control samples and FCM. Track (1) mixture of genotoxicants with visible bands of nitrofurantoin (Rf = 0.3), CMIT (Rf = 0.58), and 4-NQO (Rf = 0.7); (2) 4-NQO at 0.31 ng; (3) 4-NQO at 2.5 ng; (4) methanol; (5) printed paperboard extract; (6) printed paperboard migrate. Chromatography performed with methanol to 20 mm, ethyl acetate to 35 mm, 2:1 ethyl acetate:n-hexane to 55 mm, and 1:1 ethyl acetate:n-hexane to 80 mm. Fluorescence image taken with 366 nm illumination. Rf: retention factor.

We calculated the needed detection limits for these real FCM migrates using the calculation worksheet described in Section 3.1 and provided in the supplementary information. While the example in Fig. 1 shows generic and practical values, each migration scenario might be different. In an adaptation to Fig. 1, Fig. S9 shows the calculations for this real migration scenario. The migration conditions for printed paper board in this study (0.79 dm^2 migrated to TENAX beads, which were then extracted with organic solvents and concentrated to 1 mL) can be summarized as 0.79 dm^2/mL sample with a concentration factor of 1. Controlling genotoxicity at the MDL of 10 ppb (µg/kg food) (European Commission, 2011; Schilter et al., 2019), would therefore require a detection limit of 26 ng. At this level, seven out of ten genotoxic chemicals would be detectable in HPTLC-umuC. At the TTC for adults, 0.15 µg/person/day, the needed detection limit would be 0.4 ng, and two of ten genotoxicants would be detectable. We did not detect genotoxicity, nor cytotoxicity, in the migrates (e.g. Fig. 4). Therefore, we at least know that the genotoxicants detected in paperboard extracts leach at lower amounts under these migration conditions which are closer to real world use scenarios than exhaustive extraction.

3.6. Outlook and limitations

This study expands the available data on sensitivity of HPTLC-bioassays to low levels of genotoxic chemicals. Currently, the number of total chemicals tested, particularly those associated with food packaging is still small. The low number of chemicals is partly because the scope was technically limited to direct-acting genotoxicants. Many food contact chemicals are known to only be genotoxic after enzymatic oxidation (Rainer et al., 2018), so incorporating a metabolic activation step is critical for testing genotoxicity. Recent progress integrating metabolic activation in HPTLC-bioassays has been reported (Debon et al., 2022; Morlock et al., 2021) and further development and evaluation of these methods are needed. With or without metabolic activation, the umuC assay does not test directly for mutations. It instead captures a broader spectrum of genotoxicity but does not cover all chemicals active in the micronucleus assay. Therefore, the HPTLC-umuC can be used as a sensitive screening method to complement a recommended pairing of Ames and micronucleus (Debon et al., 2022; Kirkland, Reeve, Gatehouse, & Vanparys, 2011; Meyer et al., 2020).

We put our results and the results of others in context with the TTC for genotoxicity and a target MDL in Regulation 10/2011 (European Commission, 2011). Although HPTLC-bioassays detect lower amounts of genotoxicants than several other formats, chemicals of low potency will still go undetected, even if further developments to the assay could improve the sensitivity by additional orders of magnitude. Therefore, we recommend that different metrics than mass-based TTC be evaluated for assessing safety of food packaging. For example, bioassays could be

explored as a stand-alone measure, possibly following the effect-based threshold approach that has been developed for surface waters (Escher & Neale, 2021).

4. Conclusion

We established an HPTLC-umuC bioassay for direct-acting genotoxicants to add to the emerging field of genotoxicity detection on HPTLC plates. To compare to needed detection limits, we created and present a calculation worksheet illustrating the steps from TTC to bioassay detection. We determined that HPTLC-genotoxicity assays are sensitive to low levels of genotoxicants, both compared to other bioassay formats such as in microtiter plates, and to needed detection limits based on thresholds like the TTC. However, due to large differences in potency, the needed detection limits will remain difficult to achieve for many chemicals. These methods are clearly relevant because we show that direct-acting genotoxicants can be detected in extracts of paperboard with HPTLC-umuC. Although we did not detect genotoxicity in migrates of paperboard samples, by using a simple calculation worksheet we could define, and therefore better communicate, how many chemicals would have been detected at TTC levels. With further development and evaluation, especially in regards to incorporating metabolic activation, HPTLC-genotoxicity assays are promising for detecting unknown genotoxic chemicals that might migrate from food packaging.

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CRediT authorship contribution statement

Alan Bergmann: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Funding acquisition. **Milena Breitenbach:** Formal analysis, Investigation. **Celine Munoz:** Investigation. **Eszter Simon:** Conceptualization, Methodology, Supervision. **Maurus Biedermann:** Conceptualization, Methodology, Supervision. **Gregor McCombie:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Andreas Schönborn:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Etienne Vermeirssen:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of interest statement

A. Schönborn is co-founder of a university spin-off company offering services related to this research. A. Bergmann, E. Simon, M. Breitenbach, C. Muñoz, M. Biedermann, G. McCombie, and E. Vermeirssen declare that they have no conflict of interest.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2023.101052.

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