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Section **Fields (of activity)**

Validation and implementation of thin-layer chromatography based methods for identifying genotoxicants in broad assessment of food contact materials and drinking water

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Key words
food packaging, drinking water, high-performance thin layer chromatography (HPTLC), genotoxicity, threshold of toxicological concern (TTC), effect-directed analysis (EDA)

Aim of the study
We aimed to advance the development of an HPTLC-based genotoxicity (umuC) assay for routine application controlling, and chemical identification of toxic substances in, food packaging and drinking water. We aimed to improve the reliability of the HPTLC-umuC and characterize its sensitivity in context with regulatory thresholds. Throughout this project, we aimed to engage stakeholders with interest in applying HPTLC-bioassay methods, and with their help, evaluate case studies relevant to regulation and industry.

Material and methods
Bioassay development
Our methods built on the work of ToxSISTEM 1, in which we initially established the HPTLC-umuC. This work conducted bioassays on silica HPTLC plates after chromatographic separation of samples. Genotoxicity was assessed with *S. typhimurium* psk1002 after spraying onto the sample-containing HPTLC plates. The procedure was performed with a pipeline of HPTLC devices (CAMAG) for applying samples, performing chromatography, applying bacteria, and documenting results. We optimized several parameters of the bioassay to improve the intra- and interplate variability. We assessed dose response series of genotoxicants commonly used to validate genotoxicity tests, and some potential migrants from food packaging. We compared the sensitivity of the HPTLC-umuC to the standardized version in microtiter plates (ISO 13829_2000). We compared the assay sensitivities to thresholds of toxicological concern (TTC) by calculating needed bioassay detection limits from realistic exposure scenarios and laboratory conditions.

Stakeholder involvement
Project partners provided samples relevant to their industries. Zürich Cantonal Laboratory, St. Gallen Cantonal Laboratory, and Swiss Quality Testing Services provided samples of food packaging. Industrielle Werke Basel and Wasserversorgung Zürich provided samples related to drinking water. Samples were analyzed for genotoxicity with HPTLC-umuC.
We searched literature to create a custom list of chemicals relevant for validation of genotoxicity tests and likely genotoxic migrants from food packaging. Around this time, our project partner, the Food Packaging Forum, published the Food Contact Chemical Database (FCCdb), which we cross-referenced with our list.

Chemical identification

Non-target chemical analysis was performed at Eawag. Bioactive samples were fractionated with chromatography on HPTLC plates and zones of interest were eluted into sample vials for toxicity confirmation and chemical analysis. Chemical analysis with high-resolution mass spectrometry consisted of reversed phase high performance liquid chromatography coupled to a QExactive Plus Orbitrap mass spectrometer. Following preliminary experiments with model compounds, we focused on positive mode ionization at a resolution of 140,000. We tested the effectiveness of HPTLC fractionation in a mock-EDA using paperboard samples spiked with known genotoxicants. We investigated the strongest unknown genotoxic signals detected in food packaging samples. We explored advanced HPTLC techniques such as two-dimensional chromatography for better isolating responsible toxicants. The custom list of chemicals that we collected in a literature search served as a reference of suspected genotoxic migrants. Non-target workflows were implemented with Compound Discoverer (Thermo Scientific) to prioritize detected chemicals in extracted bands. Prioritized chemicals were obtained when possible and confirmed or deconfirmed as genotoxicants in HPTLC-umuC.

Results and significance

General outcomes

Funding from BLV allowed the equipment and methods for HPTLC bioassays to be established at the Ecotox Centre. It created and strengthened connections with cantonal authorities and industry. The methods will remain in use at the Ecotox Centre and contact with project partners will be maintained. We expect demand for HPTLC bioassays to grow as they continue to be improved, more widely used, and impetus from regulators increases. The Ecotox Centre will continue to be able to transfer methodological know-how to interested stakeholders in Switzerland and expand its use beyond the area of food packaging. A summary of results is provided here and “Umsetzungsziele” are further addressed in an attachment.

Bioassay development

HPTLC-umuC is now well developed and characterized. At the start, we obtained inconsistent results. By optimizing the conditions of the bioassay (e.g., spraying conditions of bacteria and incubation humidity) it is now better repeatable. HPTLC-umuC is sensitive when compared directly to a microtiter plate version, when it is compared to other formats of bioassays, and, conditionally, when compared to regulatory thresholds. We created an Excel worksheet to investigate the effect of food contact assumptions and laboratory conditions on needed bioassay detection limits in the framework of TTC. Sensitive detection of genotoxicants with HPTLC-umuC is the subject of the attached draft manuscript.

Stakeholder involvement

We collected samples from five stakeholder institutions to build experience with various materials and possible results. We received over 90 samples and substances of interest. They were tested for genotoxicity and archived. Information about sample types and bioassay results could help in future testing of food contact materials and drinking water by highlighting priority substances for identification. The database is stored at the Ecotox Centre but should not yet be distributed because of potentially confidential manufacturer/sample information. Our list focused on chemicals reported in extracts or migrates of packaging and chemicals expected to be genotoxic. We maintained additional collaboration with Planar-4, which is developing HPTLC bioassays for commercial use. We exchanged technical information and provided training to a representative of Planar-4.

Chemical identification

Trial experiments with spiked paperboard samples (mock-EDA) demonstrated that HPTLC fractionation and chemical analysis could successfully retain the responsible toxicants, leading to prioritization in chemical analysis from among a highly reduced suite of chemicals (10’s of chemicals compared to 1000’s in the unfractionated sample). The strongest and most consistent signals of genotoxicity that we observed were in samples from printed paperboard. We pursued these samples as a case study for toxicant identification. Through HPTLC fractionation, we reduced the number of possibly responsible chemicals by 98%. We employed our custom suspect list for genotoxic paperboard migrants to rule out many known chemicals. The strongest genotoxic signal could be attributed to 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT). We confirmed that CMIT was responsible by analyzing a purchased standard with chemical analysis and for genotoxicity in HPTLC-umuC. Unknown genotoxic chemicals remain in samples we investigated. Although the number of chemicals was reduced to 10’s of possible compounds, none matched the chemicals in our suspect list. Therefore, we eliminated from contention many of the more likely genotoxicants in paperboard. We investigated the unknown toxicants

further by performing a second dimension of chromatography. The number of possible chemicals was additionally reduced, but the responsible chemicals remain unidentified. Our obstacles included challenges with elucidating and confirming the structure of prioritized chemical features. This is not exclusive of HPTLC or food packaging. The work we have done to improve our theoretical success rate is application of relevant suspect lists and effective fractionation with HPTLC. We anticipate a publication concerning chemical identification in 2022.

Overall, our efforts have improved the ability to (1) detect genotoxicants in complex matrices at levels relevant to human health and (2) identify chemicals responsible for genotoxicity in food packaging and drinking water.

Publications, posters and presentations

Full list in attached Umsetzungsziele document

Publications

Alan J. Bergmann, Eszter Simon, Milena Breitenbach, Gregor McCombie, Celine Muñoz, Maurus Biedermann, Andreas Schönborn, Etienne Vermeirssen. HPTLC genotoxicity assay for detecting hazards in food packaging at thresholds of toxicological concern. To submit early 2022. Possibly: Food Add. and Contam. A.*

Anticipated: Effect-directed analysis of genotoxicants in paperboard packaging with HPTLC-umuC bioassay and high-resolution mass spectrometry. 2022.

Presentations

Alan J. Bergmann, Teresa Mairinger, Daniel Olbrich, Eszter Simon, Juliane Hollender, Andreas Schönborn, Etienne Vermeirssen. June 2020. Sensitive detection of toxic chemicals in food packaging. Webinar hosted by **Food Packaging Forum**. Zürich, Switzerland.

Alan J. Bergmann, Kasia Aturi, Daniel Olbrich, Eszter Simon, Juliane Hollender, Andreas Schönborn, Etienne Vermeirssen. February 2021. Sensitive detection of toxic chemicals in food packaging. **Knowledge transfer webinar hosted by Swiss Federal Food Safety and Veterinary Office**. Bern, Switzerland.

Alan J. Bergmann, Beat J. Brüschweiler, Eszter Simon, Gregor McCombie, Celine Muñoz, Maurus Biedermann, Juliane Hollender, Andreas Schönborn, Etienne Vermeirssen. HPTLC-bioassays for the detection and identification of toxic NIAS in food packaging. Abstract for oral presentation submitted to ILSI food packaging meeting. Postponed to May 2022.*

Posters

Alan Bergmann, Rebekka Merki, Vera Baumgartner, Heidi Moor, Etienne Vermeirssen, Thomas Gude. Challenging chemicals in genotoxicity assays: GLYMO as an example. Abstract for poster presentation submitted to ILSI food packaging meeting. Postponed to May 2022.*

Students advised

Moritz Walter. ZHAW Wädenswil, Switzerland. Bachelor's Semester project, October 2019 – January 2020.
Milena Breitenbach. Hochschule Fresenius Idstein, Germany. Bachelor's Thesis, Feb - August 2020.

** document is attached to report*

***Project** 4.20.a*

***Project duration** February 2020 – January 2022*