Institute of Nutritional Science
Chair of Food Science
Justus Liebig University Giessen

Development of Methods for Effect-Directed Detection and Quantification of Genotoxins and Endocrine Disrupting Chemicals in Complex Matrices

Cumulative dissertation

for the degree of

Doctor rerum naturalium (Dr. rer. nat.)

Submitted to the

Faculty of Agricultural Sciences, Nutritional Sciences, and Environmental Management

Submitted by

Daniel Meyer

from Bad Oeynhausen, Germany

Ludwigshafen, February 2023

With permission of the Faculty of Agricultural Sciences,
Nutritional Sciences, and Environmental Management,
Justus Liebig University Giessen

Examination committee:

1st Reviewer:	Prof. Dr. Gertrud Morlock
2nd Reviewer:	
3rd Examiner:	Prof. em. Dr. Wolfgang Schwack
3rd Examiner:	Prof. Dr. Sylvia Schnell

Day of the disputation:

Declaration

I declare: this dissertation submitted is a work of my own, written without any illegitimate help by any third party and only with the materials indicated in the dissertation. All passages in the text that have been taken verbatim or in spirit from published work of others, as well as all information relating to verbal communications, are identified as such. At any time during the investigations carried out by me and described in the dissertation, I followed the principles of good scientific practice as laid down in the “Justus Liebig University Giessen Statute for Ensuring Good Scientific Practice”.

Ludwigshafen,_____,_____

Table of contents

Scientific contributions

From each paper the author contributions sections were added.

Peer reviewed original research papers

I. Daniel Meyer, Maricel Marin-Kuan, Emma Debon, Patrick Serrant, Claudine Cottet-Fontannaz, Benoît Schilter and Gertrud E. Morlock, Detection of Low Levels of Genotoxic Compounds in Food Contact Materials Using an Alternative HPTLC-SOS-Umu-C Assay, ALTEX 38 (2021), 387–397. https://doi.org/10.14573/altex.2006201.

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.

II. Emma Debon, Paul Rogeboz, Hélia Latado, Gertrud E. Morlock, Daniel Meyer, Claudine Cottet-Fontannaz, Gabriele Scholz, Benoît Schilter and Maricel Marin-Kuan, Incorporation of Metabolic Activation in the HPTLC-SOS-Umu-C Bioassay to Detect Low Levels of Genotoxic Chemicals in Food Contact Materials, Toxics 10 (2022), 501. https://doi.org/10.3390/toxics10090501.

Author contributions: Conceptualization, G.E.M., B.S. and M.M.-K.; Data curation, P.R. and M.M.-K.; Formal analysis, E.D., P.R., H.L., C.C.-F. and M.M.-K.; Investigation, G.E.M., B.S. and M.M.-K.; Methodology, E.D., P.R., H.L., G.E.M., D.M., C.C.-F. and M.M.-K.; Project administration, M.M.-K.; Resources, G.S. and B.S.; Supervision, M.M.-K.; Validation, M.M.-K.; Visualization, H.L. and M.M.-K.; Writing — original draft, E.D., P.R., H.L., D.M. and M.M.-K.; Writing — review & editing, G.E.M., G.S. and B.S. All authors have read and agreed to the published version of the manuscript.

III. Daniel Meyer, Maricel Marin-Kuan, Elisa Mayrhofer, Christian Kirchnawy, Emma Debon, Helia Latado, Amaury Patin, Benoît Schilter, Gertrud Morlock, Effect-detection by planar SOS-Umu-C genotoxicity bioassay and chemical identification of genotoxins in packaging migrates, proven by microtiter plate assays SOS-Umu-C and Ames-MPF, Food Control 147 (2023), 109546. https://doi.org/10.1016/j.foodcont.2022.109546.

Author contributions: Conceptualization: DM, GEM, MMK; Methodology HPTLC: DM, ED, GEM; *in vitro* assays: EM, HL; Investigation: DM; Supervision: GEM, MMK, Funding acquisition: GEM, Project administration: GEM, Writing – original draft: DMWriting – review & editing: GEM, MMK, ED, HL, EM, CK, BS, AP

IV. Gertrud E. Morlock, Daniel Meyer, Designed genotoxicity profiling detects genotoxic compounds in staple food such as healthy oils, Food Control 408 (2023), 135253. https://doi.org/10.1016/j.foodchem.2022.135253.

Author contributions: Gertrud E. Morlock: Conceptualization, Methodology, Formal analysis, Supervision, Resources, Funding, Writing - original draft, Writing - review & editing. Daniel Meyer: Methodology, Investigation, Formal analysis, Writing - original draft.

V. Daniel Meyer, Maricel Marin-Kuan, Hélia Latado, Benoît Schilter and Gertrud E. Morlock, Planar 6-fold multiplex bioassay to differentiate endocrine agonist, antagonist, false positive antagonist, cytotoxin, anti-cytotoxin and false positive anti-cytotoxin, in submission

Author contributions: Daniel Meyer: Methodology, Investigation, Data Analysis, Writing – Original Draft. Maricel Marin-Kuan: Writing – Review and Editing. Hélia Latado: Investigation (ChromaJet and partially Derivatizer experiments in 3.2). Benoît Schilter: Writing – Review and Editing. Gertrud E. Morlock: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – Original Draft, Writing – Review and Editing.

Peer reviewed original research paper not in the scope of this thesis

VI. Daniel Meyer, Gertrud Morlock, HPTLC Analysis of Industrial Bamboo Tableware for Genotoxins, Melamine and Formaldehyde, in print.

Author contributions: Daniel Meyer: Methodology, Investigations, Data Analysis, Writing – Original Draft. Gertrud E. Morlock: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – Review and Editing

1. Introduction

Food for humans and feed for animals are potential gateways for harmful chemicals into humans and animals, which is why risk assessment is a key instrument for the safety of food, feed, other consumer products, medicine for humans/animals and in other industrial/agricultural applications [1]. For this reason, the European Parliament, the public and the industry have stressed the need for better methods [2–5]. Although information on intentionally added substances is available, there is still a possibility that unintentionally added substances (NIAS) are present as contaminants in end-use products. These substances can be process contaminants, products of unknown chemical reactions and can also migrate from packaging materials into food. It is very difficult to address their health significance. [2,4,6]

1.1. Genotoxin testing, genotoxicity and mutagenicity

The European Food Safety Agency stated that information on genotoxicity is a key component in risk assessment of chemicals. It should be considered that there might be no threshold for some genotoxic chemicals, so no safe level can be established. Such chemicals are either not allowed for use or are not managed by the as low as reasonably achievable principle. It is therefore important to detect those genotoxins. A variety of methods and technologies to detect genotoxins or mutagens exists. [1]

Testing chemicals directly on humans is not an option. Therefore, chemicals are tested on animals. In order to minimize animal suffering, the so so-called 3R rules have been introduced, which either refine the conditions for animals in animal testing, reduce the number of animal tests required or even lead to animal testing being replaced and thus avoided [7]. The Organization for Economic Co-operation and Development (OECD) published several guidelines for several assays that address genotoxicity and

mutagenicity. The Ames assay is used as a bacterial reversed mutation test and can detect mutations in the deoxyribonucleic acid (DNA) and serves as a replacement for animal experiments [8]. Another test is the hypoxanthine phosphorybosyl transferase test, which also refers to mutations, is based on mammalian cells and can also replace animal tests [9]. The micronucleus test based on mammalian cells is available for *in vivo* and for *in vitro* testing, the latter replacing animals and can detect structural and numerical chromosomal aberrations [10]. Testing for chromosomal aberrations is also possible with the chromosomal aberration test that follows OECD guideline 473. The tests uses mammalian cells and is used as an replacement for *in vivo* testing [11]. It is also possible to use a gene mutation test on unscheduled DNA synthesis *in vitro*. The test utilizes mammalian cells and detects gene damaging, DNA repair and replaces the same test used as *in vivo* test if performed according to OECD guideline 482 [12].

1.2. SOS-Umu-C assay in microtiter plate format

The SOS-Umu-C assay can detect genotoxins. It is based on the fusion of the umuC operon of *Escherichia coli* and the lacZ-gene. The umuC operon is induced by DNA damage caused by genotoxins. The lacZ gene is then induced as it is linked to the operon and leads to the production of a β-galactosidase, which can then convert a precursor of a chromophore i.e., *ortho*-nitrophenyl-β-galactoside (ONPG) into ortho-nitrophenol which is the active chromophore. The chromophore can then be detected. [13]

The assay procedure is standardized. Briefly, the *Salmonella* cells are exposed to different concentrations of the chemical to test, whereby the procedure also contains a positive control, a negative control and a blank. After an initial incubation the bacteria cells are transferred to fresh growth media and also diluted with this step. After a second incubation and if the umuC operon has been induced, the following generation

of the galactosidase is used to convert ONPG to the detectable chromophore which is then detected after cell lysis. Additionally, the optical density (OD) is measured at 660 nm wavelength before and after the growth phase and also the OD at 420 nm after the ONPG incubation to calculate the induction ratio of the genotoxin response and to calculate the growth factor as a quality control in order to detect cytotoxicity. The detection of cytotoxicity falsifies a negative test result. [14]

The assay provides high concordance with the Ames test [15,16], has the additional advantages of using only a single bacterial strain, using a chromogenic detection system for data collection, and the bacterial strain used (TA1535[pSK1002]) corresponds to ISO guidelines [14,17]. Although genotoxicity is not directly linked to cancer development in human cells, genotoxic effects of chemicals on the genome of bacteria have been correlated to mutagenicity and the generation of tumors in humans [18,19]. The bioassay has been well evaluated testing a variety of genotoxins over the past decades and is also known for usage in analysis of complex environmental samples *i.e.* for waste water analysis [13,14,20–22]. Other examples of the use of this bioassay are evaluations of photo-genotoxicity [23], aryl sulfonamide derivatives of (aryloxy)alkylamines [24], paper mill effluents [25], source water [26], fluoroquinolone antibiotics [27,28], drugs, disinfectants and other chemicals in hospital wastewaters [29], cyclophosphamide and degradation products [30] what shows the acceptance of the bioassay.

1.3. Endocrine assays based on yeast

In vitro assays for testing samples for endocrine disruptors are well known. The yeast androgen screen (YAS) and the yeast estrogen screen (YES) test for androgenic and estrogenic chemicals, respectively. In case of the androgen detecting bioassay, recombinant *Saccharomyces cerevisiae* cells are used that contain the DNA of a

human androgen receptor, which is then produced by the yeast cell accordingly and can be activated by androgens. The *lacZ* reporter gene is also part of the modification and responds to the activation of the receptor, resulting in the formation of β-galactosidase. This enzyme can then convert *e.g.* 4-methylumbelliferyl-β-D-galactopyranoside into the active chromophore methylembellyferone [31] or fluorescein-di-β-D-galactopyranoside (FDG) [32] into the active chromophore fluorescein, which can then be measured. The estrogen detecting bioassay works analogously, but with an estrogen receptor. [33–37]

1.4. Thin-layer and high-performance thin-layer chromatography

Thin-layer chromatography (TLC) is a technology to separate mixed substances utilizing a solid flat structure *e.g.*, a glass or an aluminum plate, coated with a stationary phase that can consist out of silica gel, silica gel that is modified, aluminum oxide, cellulose, modified cellulose, *etc.* [38]. Mobile phases in TLC are not subject to the limitations of a detection system, which can be disturbed by solvent absorption, for example, if solvents absorb at the same wavelength as the analyte. Solvents can be removed by evaporation prior to detection to circumvent these limitations. The detection of analytes can be done by imaging via visible light, UV light of different wavelengths, fluorescence detection and also by luminescence detection [39]. Quantification can be done by video-densitometrical evaluation of the images [40,41], by the preparation of densitograms via scanning [42,43] or coupling to Direct Analysis in Real Time mass spectrometry [44] or mass spectrometry (MS) in general [45]. By coupling to high-resolution mass spectrometry (HRMS) [46,47] and to nuclear magnetic resonance spectroscopy [46,48,49] structure elucidation is possible. Better results in planar chromatography are obtained by using small particle sizes of the stationary phase (5-7 µm), thin layers and a narrow size distribution of the particles

what also shortens the time consumption for development [50]. Since the performance of those plates is better than that of TLC plates, the term high-performance thin-layer chromatography (HPTLC) was introduced. Furthermore, application devices, devices for development of plates, for derivatization of plate contents and for plate analyzation were developed [51]. It was shown that up to 46 samples can be applied to one plate which was then developed and analyzed. Since all samples went through the procedure at once, the calculated time needed for each sample was respectively low, what also applied to the costs of the analysis including materials and solvents [52]. The matrix robustness of HPTLC plates is high and it was shown that up to 1 mL of liquid can be applied per sample onto a plate in an area shape [53]. This also reduces the efforts needed for sample preparation [39]. Because the matrix remains on the plate during the analysis of the sample as well as the analytes of the sample it is not excluded from the investigations that follow. Those investigations can be chemical derivatizations or effect directed analysis (EDA). EDAs can contain investigations of biochemistry in term of enzymatic induced reactions and inhibition of enzymes or bioautographically tests investigating biologically active compounds [39].

1.5. Planar endocrine and genotoxicity assays

HPTLC was used in detection of endocrine active substances [54]. The planar yeast estrogen screen (pYES) based on a wettable revered phase HPTLC plate and the same yeast cells as used in the YES assay was shown to detect estrogen-acting substances as sharp bands on the HPTLC plate what allowed for non-targeted analysis of compounds in complex mixtures [55]. Coupling the HPTLC to mass spectrometry allowed then substance identification [43,56]. Shortly after, the YAS assay was introduced to the planar format resulting in the pYAS bioassay creating the possibility of effect directed detection of androgens on the plate [57]. By applying the respective

agonist over the sample tracks in the form of strips, any anti-estrogens or anti-androgens present became detectable, as these reduced the signal intensity of the agonists, which was detectable [58]. The planar yeast-based antagonist androgen screen (pYAAS) and the antagonist estrogen screen (pYAES) lagged the possibility to differentiate between true antagonist detection (fluorescence reduction by antagonist) and cytotoxicity (fluorescence reduction because of damaged yeast cells). This possibility was added by the use of resazurin, which is converted by the yeast cells into resorufin, which is a different color, the detection of which is thus a detection of living cells and its absence presence of cytotoxicity [59,60]. The simultaneous detection of agonists, antagonists and false positive antagonists was achieved with the planar yeast antagonist-verified estrogen screen (pYAVES) [60] and the planar antagonist-verified androgen screen (pYAVAS) [61] by spraying a strip consisting of the fluorescent end product of the substrate used next to the agonist strip already applied. If the fluorescence was reduced by a putative antagonist on this strip, the fluorescence reduction was also due to quenching on the agonist strip and not due to antagonism [60,61]. Planar endocrine bioassays were successfully applied to a broad variety of samples *e.g.* thermal paper [58], surface and waste water [62–66], seed oils [67], cannabis [68], plant extracts [69], spices and herbs [70] and also to packaging material chemicals [71] showing their applicability.

Also, in the field of genotoxin detection, bioassays were tested on the HPTLC plate with promising results. Those bioassays were constructed as overlay assay (on a medicinal gauze, not direct on the HPTLC plate) [72], or utilized bioluminescence to detect genotoxins [17].

1.6. Packaging Materials and their hazards

Food packaging material or food contact material (FCM) can be made of many different materials. For example, these can be plastic (polymers), cellulose, peptides [73], metal, ceramic and others [74]. Among polymers, for example, polystyrene, polypropylene, polyvinyl chloride, polyethylene terephthalate and polyethylene are used as direct packaging materials [75]. It has become popular to use cellulose-based packaging material from bacterial production. In addition, there is often the possibility that the materials obtained from it are biodegradable. This also contributes to the fact that, in contrast to persistent materials, less waste is produced [75]. As a metal, aluminium is a material that is used directly as laminar packaging material in form of a foil or is coated with a polymer. There is also the possibility of using aluminum as a composite material together with other materials, especially when it comes to useability under high temperatures, while the packaging properties must be maintained [76]. Because the excellent properties of metal-based packaging materials have been and are being heavily advertised, metal is also being incorporated into packaging material plants in small-part format to eliminate weak points with pinpoint accuracy [77]. Seals made of metal are an example here [77]. At this small scale, but also in packaging where aluminum is used on a large scale, there are concerns about the migration of harmful substances such as bisphenol A, lead, cadmium, mercury, aluminum, iron, and nickel into the food [77]. Different foods require different packaging and because of the large number of possibilities, there are many different types of packaging, which often have different chemical modifications in order to adapt to the requirements [74]. Additives also contribute to the desired properties of the packaging materials. For materials from the polymer spectrum alone, many antioxidants, stabilizers, lubricants, antistatic chemicals and anti-blocking substances have been developed [78]. Also, combinations of these materials are well known and used. For example, many metal-

based packages are provided with coatings to adapt their properties to the respective food that can be acidic, which is why the metal-based can is in need of protection against the acid. The food is then also protected from the loosening of the metal, provided that the coating has no damage [79].

Some of these chemicals have also endocrine disruptive properties, which was proven by bioassays that detected endocrine agonistic [80,81] or antagonistic [82] activity of those chemicals in FCMs. Proof that FCMs can lead to human exposure to those chemicals exists [83]. The chemicals were also identified in food [84]. Genotoxic substances can migrate from packaging materials into food as well what poses an additional threat to humans and animals [1–6].

1.7. Registration, Evaluation, Authorization and Restriction of Chemicals

The Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) is a measure in the form of a regulation, provided by the European Union (EU), to protect human health and the environment from risks posed by chemicals. At the same time, the regulation is characterized by the fact that it is intended to increase the competitiveness of the EU chemical industry. It also proposes alternative methods for assessing the hazards of chemicals in order to reduce the number of animal tests. The regulation applies to all chemical substances and therefore also to chemicals of daily life and use. For this reason, it also affects most of the companies located in the EU. The regulation, which came into force on June 1, 2007, obliges companies to identify all risks associated with the substances they produce and to make these risks controllable. The companies must show how the respective substance can be used safely and, in addition, the users must be provided with sufficient risk management or information about the creation of such a management. [85]

Because authorities are authorized to restrict the use of a substance in case of unmanageable risks, the regulation leads in the long run to the replacement of the substance by a less problematic one or by several less problematic substances [85].

REACH regulates the recording and evaluation of properties of chemicals and their hazards. For this purpose, companies, which must also cooperate if they wish to register the same substance, must submit the data to the European Chemicals Agency, which then checks the data with regard to the requirements. The final evaluation of substances is then carried out by Scientific Committees of the European Chemicals Agency with the question of whether the risks of the substances can be controlled or not. [85]

The before mentioned control mechanisms have direct effects on companies involved in chemical production. It must comply with the requirements imposed by REACH whether the chemicals are supplied in-house or to others, which also applies to exports. In the other direction, the import of chemicals into the EU, the regulations of REACH also apply. Here, too, chemicals, but also furniture, clothing and plastic goods are regulated. This applies to imports of individual components and mixtures of substances. Even downstream users, even if they do not know in detail which chemicals they use, must meet the requirements of REACH. [85]

There is a great deal of effort for the industry. As a starting point in order to comply with the registration obligations for chemicals, so-called standard data requirements must be satisfied. In addition to the determination of physicochemical properties, chemicals must be examined for their toxicological properties. [86] Special attention is paid to potential mutagenic properties of the chemicals. If the chemicals in question have to withstand testing according to the requirements of REACH [87], an *in vitro* micronucleus test is required. This must be performed according to OECD Test Guideline 487 [10] and must contain two positive control substances, one to check the

assay for its ability to detect clastogenicity and one to check the assay for its ability to detect aneugenicity [88]. In general, no matter which *in vitro* test shows a positive result, an *in vivo* test must follow, with a few exceptions, which must be justified in detail [88]. When it comes to genotoxicity, REACH can ask for genotoxicity testing if sufficient *in vivo* data is not available. *In vivo* testing is required, if *in vitro* testing revealed a genotoxic activity of the tested compound. This indicates that *in vitro* testing should be performed before *in vivo* testing is chosen as an option to perform as less animal related experiments as possible. [89]

The REACH regulation addresses endocrine disruptors as well [89]. In addition, there is extensive guidance [90] on how to identify endocrine disruptors in the context of the placing of pesticides on the market [91] and the use of biocidal products [92], which highlights the importance and need for detection methods for genotoxins and also for endocrine disruptors.

1.8. Edible fats and oils

By the definition of the Bundesministerium für Ernährung und Landwirtschaft edible fats and oils are derived from the seeds, germs or fruits of plants, from fatty tissue and bones of slaughtered warm-blooded animals, intended for human consumption, or from fish or parts of fish. Edible fats and oils consist almost exclusively of the triglycerides of fatty acids and are practically anhydrous. They may contain small amounts of other substances from the starting material such as phospholipids, waxes, unsaponifiable components mono- and diacylglycerides and free fatty acids. [93]

Fatts are esters of glycerol with of a big variety of fatty acids. Especially the fatty acids oleic acid, linoleic acid and linolenic acid are known and promoted to the general public as healthy. How an oil is to be named is strictly regulated and depends on its origin (*e.g.* plant, region) and also the content of each oil is important for labeling. [93]

Since years effects of olive oils affecting the wing tissue of *drosophila* flies are known, leading to deformations during the genesis of the wings [94]. The so-called wing spot test is used for the detection of genotoxicity and thus it is reasonable to assume, that genotoxicity is a valid cause of those deformations [94]. It was already known that epoxides are a source of genotoxicity [95]. Those epoxides can be created if double bounds between two Carbon atoms are oxidized by oxygen. Because some fatty acids (*e.g.* oleic,- linoleic and linolenic acid) contain such double bounds they might be a possible source of epoxides in oils [96] which did then explain the outcome of the wing test.

Literature about the monitoring of genotoxic activity of heathy oils is not available simply because healthy oils are not monitored regarding their genotoxic behavior especially not when it comes to the epoxide content and its potentially dangerous effects. Technologies detecting the presence of epoxides in unsaturated carbon chains are known and also able to quantify the epoxide content of the samples. One utilized hydrogen bromide or comparable chemicals. This is problematical, because the identities of all epoxides present in the sample must be known before the evaluation begins. This is because the procedure works with reference materials that need to be chosen according to the epoxides present in the sample. Since, natural oils vary constantly in their composition of fatty acids content and perusing such an analytical approach is hard to do. Especially unknown food samples or oils generated out of different oils would mean a great effort. [97,98]

Another assay detected epoxides using carbamates within a high-performance liquid chromatography approach, which came with the disadvantage of being susceptible to metal ions present in the test system, which can again happen if food samples are investigated [99]. It was also possible to utilize nuclear magnetic resonance spectroscopy, what came with the downside, that in case the genotoxin can be very

potent but very low in concentration and therefore the technology may not detect the substance, especially when the genotoxic substance is contained within a complex oil sample matrix containing thousands of other substances [100–102].

The mentioned assays target only one substance class. But already at this point the efforts that have to be taken in order to detect this single substance class are quite big what is also true for further quantification steps. Since there are countless other substance classes potentially present in the healthy oils that can act genotoxic, the efforts that would have to be taken to detect every single genotoxin are too big to take. Since the direct approach is not an option to investigate all possible genotoxic compounds in oil samples, another approach should be adopted. If target-based analyses only work if the targets are known, but substances that are not expected are also to be included in the analyses, then a target-independent search is useful and can be carried out, for example, with the effect-directed analysis. This is also useful if it is possible to perform the analysis in the trace range at the same time. Genotoxin assays such as the Umu-C bioassay would be a conceivable approach here, also in combination with prior fractionation of the oil components. However, such a procedure can be very time-consuming because, although a genotoxic signal is detected in one of the fractions obtained after the first separation step, a mass spectrometric analysis that may then be carried out reveals that the substance is still a mixture. A new separation of the fractions under consideration would have to be carried out, whereby this separation step would possibly first have to be optimized in order to achieve sufficient separation. This would then be followed by the bioassay again. Even now, it would still not be certain that the genotoxin is present as a pure substance, so that the processes would have to be repeated again until the genotoxin was obtained as the sole substance and could be used for structural elucidation. [103,104]

2. Scope

Genotoxins in general are a threat to human and animal health. For substances intentionally added to packaging materials, care can be taken to ensure that no genotoxins are at risk of coming into contact with food. However, because substances can be added accidentally at different times during the production of packaging materials or, and because these substances can be genotoxic and can be unknown, there is a need for an analytical method that can detect unknown chemicals with genotoxic properties without having to search specifically for substance classes. The same applies to substances that are formed during the storage of materials due to external influences such as temperature, solar radiation or others. Especially when using novel packaging materials, it cannot be assumed that all physicochemical interactions between packaging and food are known. The problematic substances can migrate into food due to longer storage times and thus reach humans and animals. Existing EDA methods showed problems when it comes to the detection of trace amounts of genotoxins and had also to cope with solvent or other effects raising the detection limits what should also be solved for the prevention of false negative results. The main scope of this work is to develop a HPTLC bioassay method based on the SOS-Umu-C microtiter bioassay that is capable of detecting unknown and known genotoxins while not being susceptible to false-negative results due to solvent or other effects. Also, the new bioassay should reach very low limits of biological detection (LOBDs). This will be accomplished by transferring the microtiter plate bioassay to an HPTLC plate. By using HPTLC, the genotoxins should be separated from the matrix they would normally be in if a microtiter plate bioassay was used. Whatever is in the matrix that normally would interfere with the assay's response to a genotoxin, is then physically separated and cannot interfere anymore. To achieve this, it should first be

determined how best to transfer the *Salmonella* belonging to the bioassay to an HPTLC plate. Performance parameters such as LOBD, precision and others should be determined to evaluate functionality, with the bioassay on the plate already confronted with packaging material migrate, and any problems highlighted and resolved if possible. Also, the possibility of integrating liver metabolism and the associated risk of activation of otherwise harmless substances to potent genotoxins on the HPTLC plate should be investigated and demonstrated on several chemicals that are in need of metabolic activation to be genotoxic.

The developed bioassay is to be applied to different packaging material migrates to demonstrate its capabilities. Also demonstrated is how a coupling of HPTLC to mass spectrometry can be used to identify a discovered genotoxin. In addition, the HPTLC bioassay is compared with the original SOS-Umu-C bioassay and the Ames-MPF bioassay by examining the same migrates using these two bioassays, as well as the genotoxin determined by HPTLC-MS coupling.

Because healthy oils contain unsaturated fatty acid esters and unsaturated fatty acids are known to be a source of genotoxic epoxides, the newly developed assay is applied to healthy oils to check for their genotoxic potential and how it is generated by different storage conditions.

Packaging materials can potentially contain endocrine disruptive chemicals, which is why the pYAAS/pYAES endocrine bioassays should be used to investigate packaging materials. Furthermore, the pYAAS bioassay cannot simultaneously detect cytotoxic compounds and therefore not discriminate between anti-androgen and cytotoxic signals, which is why a bioassay that solves this problem is developed within this work and applied to food packing material migrate.

3. Progress achieved

3.1. Development of the HPTLC-SOS-Umu-C bioassay (Publication I)

There is a great need in public, in politics and in the industry for new methods for the detection of genotoxins in FCMs [2–4] since those genotoxins can migrate into food [2,4,6]. The SOS-Umu-C microtiter plate bioassay [16] is often used in genotoxin testing and also for complex environmental samples [105]. Although, there were initial attempts to transfer the bioassay to an HPTLC plate, these were not followed up [106]. In this work, the assay was transferred to the HPTLC plate for genotoxin testing and applied to FCMs to avoids matrix influence and provide a low limit of biological detection in FCMs. The first step was to optimize the conditions for the assay to take place on an HPTLC plate. Furthermore, the fluorescent chromophore methyl-umbelliferone was introduced into the assay format, which increased detection sensitivity over the common *o*-nitrophenol. The newly developed assay was verified by comparison of the new assay with the microtiter plate version of the assay and with the Ames assay, by utilizing the model genotoxin 4-nitroquinoline 1-oxide (4-NQO). The lowest effective concentration of the used genotoxin was 0.53 nM (20 pg/band) which was 176 times lower than in the microtiter plate version and between 60 and 151 times more sensitive than the Ames assay, depending on the strain. As a proof of principle, 4-NQO-spiked FCM extracts and migrates prepared from differently coated tin cans were analyzed. Dose-response-curves of 4-NQO between 100 and 1500 pg/band were prepared. For each of five extract samples, one curve was prepared. The correlation coefficients over five different amounts were obtained between R = 0.987 and 0.997. The mean precision was 7% (*%RSD* ranged between 5–9%, *n* = technical replicates/plate). For one migrate sample the determined curve had a correlation coefficient of R = 0.992. This experiment was repeated five times (*n* = 5 biological

replicates, 5 plates on five days; *n* = 3 technical replicates/plate). The mean precision was 8%. The LOBD of the migrate was found to be 13 pg/band. Referred to the applied volume (200 µL) this was equal to 67 ng/L (0.35 nM). The LOBDs of the five extracts were between 32 and 71 ng/L (0.17–0.37 nM). In summary, the LOBD for 4-NQO over six extract/migrate samples was 17 pg/band (13–21 pg/band, 32–71 ng/L, 0.17–0,37 nm). The relative standard deviation was 16 % (*%RSD*, *n* = 6 plates, *n* = 3 technical replicates). In terms of LOBD the newly developed assay is 12 times more sensitive and in terms of the lowest effective concentration 1250 times more sensitive than what can be found in literature [17]. The mean precision of the bioassay (tested via the migrate) was 5% (*%RSD*, *n* = 5 technical replicates, ranged 5–8%), whereas it was 7% (*%RSD*, ranged 5–8%) for the extracts. In addition, the newly developed bioassay was shown to completely avoid the influence of any matrix as well as the influence of solvents, which proved to be an advantage compared to the microtiter bioassays.

To proof the specify of the RP-HPTLC-UV/Vis/FLD-SOS-Umu-C bioassay, 8 chemicals exhibiting different genotoxic mechanisms according to the Kirkland list of genotoxicity [107] were tested. All chemicals behaved as expected, thus proving the bioassays specificity (without metabolic activation). Additionally, resorufin β-D-galactopyranoside was successfully tested as an alternative substrate in case one of the analytes shows fluorescence in the fluorescence region of 4-methylumbelliferone.

Based on the mentioned performance data it can be concluded that in this work it was achieved that in the future genotoxins in packaging materials can be detected in very small amounts and without the influence of interfering influences, which would represent an improvement of the evaluation possibilities of genotoxin exposure.

3.2. Introduction of on-surface metabolic activation to detect genotoxins in FCMs (Publication II)

Metabolic activation is an important part of genotoxin bioassays. Parent (not genotoxic) substances can be converted into genotoxins by liver metabolism, so not utilizing metabolic activation can result in false negative results. To include metabolic activation, S9 liver enzymes and associated cofactors were added to the *Salmonella* solution. This also completed the bioassay described in Publication I into a full-fledged alternative with the advantages outlined below. In addition, the cell suspensions used were applied to the HPTLC plate either by needle application or piezoelectric spraying, what also reduced the volume of needed cell suspension. The cell suspension was optimized with respect to its added amount of S9 mix, cofactors and buffer saline. In order to check the metabolic activation, Aflatoxin B1 (AFB1) was selected as test substance, since it shows no activity without metabolic activation. When comparing the total concentration of 1%, 5%, and 10% of the added S9 mixture to the assay suspension, 10% resulted in the strongest response of the bioassay to AFB1, so this concentration was chosen as the best. To provide a basis for evaluation of the successful integration of metabolic activation, various genotoxins acting through different genotoxic mechanisms were tested with optimized metabolic activation. 4-NQO as alkylant, AFB1 as adduct former requiring metabolic activation, mitomycin C (MMC) as inducer of DNA-DNA crosslinks and oxidative stress, and as alkylation reagent, methyl methanesulfonate (MMS) as potent clastogen, *N*-ethyl-nitrourea (ENU) as a potent gene mutation inducer, 7,12-dimethylbenzathracenes (DMBA), benzo(a)pyrene (B(a)P), 2-nitrofluorene (2-NF) and aminoanthracene (2-AA) as different DNA adduct formers all requiring metabolic activation. As negative controls, D-mannitol and melamine were chosen to support the display of the specificity of the assay.

Dose-response-curves were determined for all substances (D-mannitol and melamine showed no response as expected) and the correlations coefficients were all R > 0.99 except the ones for 2-AA and MMS, which were R > 0.96. Three biological replicates were made (n = 3). In the presence of metabolic activation, the *%RSD* of AFB1 was 12%, and of DMBA, ENU, 2-NF, 2-AA, and B(a)P, up to 20%. The *%RSD* was 23% for 4-NQO, and due to diffusion, 38% for MMS. When metabolic activation was not used, the *%RSD* was 24%, 20%, and 18% for 4-NQO, DMBA, and ENU, respectively, and approximately 30% for the other compounds. The ICH guidelines recommend a *%RSD* ≤ 20%. The results suggest that the overall assay performance suits the recommendations, except when it comes to MMS and MMC. This is probably due to diffusion of the chemicals on the plate and not due to the bioassay performance itself. Also, the LOBDs were determined with respect to the concentration the standards were applied from and ranged between 0.025 and 150 ng/L. Compared to the values obtained for an Ames assay (ranged between 35 and 2000000 ng/L) that was performed in parallel using the same substances, the HPTLC bioassay was between 40 to 18750 times more sensitive. Compared to literature values, the assay was 1.2 to 260 times more sensitive.

Through the described introduction of metabolic activation to the planar bioassay format, it was achieved in this work that the HPTLC-UV/Vis/FLD-(S9-) SOS-Umu-C-Vis/FLD bioassay has the potential to perform comprehensive analyses on packaging materials, as it has the functional scope of the ordinary SOS-Umu-C microtiter plate bioassay, but without being subject to negative effects from matrices and solvents.

3.3. Analysis of FCM migrates and identification of a genotoxin (Publication III)

The previous two publications showed the performance of the newly developed HPTLC−UV/Vis/FLD−(S9−) SOS-Umu-C−Vis/FLD bioassay on standard substances. In this work, the assay was applied to complex FCM migrates of paper, coating and plastic. Also, piezoelectrical spraying was compared with syringe-dosage application of *salmonella* cell suspension and substrate. The bioassay was applied with and without metabolic activation. The migrates were investigated on the normal and on the reversed phase plate. Up to 14 bioactive bands were found representing 14 different genotoxins. Metabolic activation showed that some chemicals present in the migrates are disactivated partially in terms of genotoxicity. Also, black bands were recorded, probably remainings of the sample matrices, that were potentially cytotoxic. A fact supporting this hypothesis was that the original SOS-Umu-C microtiterplate assay, which was also used to test the same samples, showed a negative result for the same sample which was probably a false negative result due to cytotoxicity. The band contents were evaluated via heated electrospray or atmospheric pressure chemical ionization (HRMS). In the paper migrate, linolenic acid epoxides were found. The genotoxicity of the linolenic acid epoxides was confirmed via chromatography of linolenic acid that was exposed to air. Additionally, the results were confirmed by an investigation of the samples and the linolenic acid via Ames-MPF^{TM}. This bioassay confirmed the findings. These results illustrated that the newly developed HPTLC−UV/Vis/FLD−(S9−) SOS-Umu-C−Vis/FLD bioassay is capable of preventing false negative results by separating the analytes from their matrix.

3.4. Analysis of edible plant oils and fats for genotoxins (Publication IV)

Lipids are susceptible to oxidation if they are exposed to air [96]. Epoxides can be formed by this oxidation and are a substance class that is known to be genotoxic [95].

Publication III showed that the HPTLC−UV/Vis/FLD−(S9−) SOS-Umu-C−Vis/FLD bioassay can detect linolenic acid epoxides. This makes the bioassay an ideal tool to screen lipids that are the main components in oils for the genotoxic potential of epoxides that may be present in the oils. 33 healthy oils were investigated for their genotoxic potential. Up to eight different genotoxic compounds were found. Linolenic acid oxides were found to be one cause of genotoxicity in all samples by HRMS. Genotoxic degradation products were found when fresh oils were compared with oils stored open and sealed for one month.

In addition, the metabolic activation procedure presented in Publication II was applied to oils and was found to deactivate most, but not all, genotoxins by metabolism.

One conclusion from this is that without detoxification via the liver, *e.g.,* with topical application of products containing oil, there is an increased genotoxic potential.

Food, feed, dietary supplements, and cosmetics as sources of genotoxicity can now be identified on the same surface. Simply by combining separation, effect detection and optional simulated metabolism on the same surface. The costs per plate were only 18 € without and 23 € with metabolic activation. For 17 samples/plate that made only 2.5 € (1.1 € and 1.4 € respectively) per sample. The low price made the developed method a valuable tool for genotoxin testing in products containing high oil or fat amounts, also for routine measurements.

In this work, it was achieved that edible oils and fats can be analyzed for genotoxins in a simple, time and cost saving manner.

3.5. Development of a 6-fold multiplex bioassay (Publication V)

The human endocrine system can be influenced by endocrine active substances. Prominent substance groups can interact with the human steroid receptor system and act like 17β-estradiol or testosterone or suppress the activity of these hormones [108–

110]. These interactions can even lead to the development of cancer in the reproductive organs of rats and in the female breast [111–113]. Methods to detect estrogens, anti-estrogens, androgens, anti-androgens, as well as methods that detect false-positive responses and also a method that can detect cytotoxicity were developed. Hence, a 6-fold multiplex bioassay was developed to differentiate cytotoxin, anti-cytotoxin and false positive anti-cytotoxin, endocrine agonist, antagonist, and false positive antagonist to detect endocrine disruptors in complex matrices such as FCMs. First, the robustness of half maximum inhibitory concentration IC_{50}, the limit of biological detection and dose-response curve of bisphenol A (BPA) was successfully demonstrated, using the pYAAS (2-fold) bioassay in two different laboratories utilizing two different application devices for substrate application. The test range was 10–490 ng/band and the bioassay was performed for six biological replicates (n = 6). The mean IC_{50} was 155 ng/band (n = 6, 103–267 ng/band, robustness *%RSD* of 38%). In literature the comparable IC_{50} value of 139 ng/spot was reported by an anti-androgenic assay (not a multiplex assay) [59]. This showed that the 2-fold bioassay of this study (agonist and antagonist) provided comparable values, whereas a 1-fold anti-androgenic bioassay provided only antagonistic information. The mean LOBD of BPA using the pYAAS assay was 21 ng/band (n = 6, 5–33 ng/band, robustness *%RSD* of 50%). Literature data about the LOBD of BPA determined via an anti-androgen bioassay were not available.

Then, the bioassay and the pYAES bioassay were applied to six differently coated tin cans. 19 potentially endocrine disrupting compounds with agonistic and antagonistic activity were found. With the addition of S9 enzymes and corresponding coenzymes, it was demonstrated that metabolism reduced the biological activity of all metabolic disruptors found in the migrates. By coupling of the HPTLC plate to HRMS molecule formulas were assigned to the compounds. For one biologically active band, several

molecular ions were found whose mass difference (neutral loss))corresponded to the molecular formula of butylparaben, which would explain the biological activity [114].

The antagonistic effects were observed mainly in the pYAAS assay, which is why a focus was placed here on the method development of a differentiation of true antagonistic effects and cytotoxic effects. In addition, this novel bioassay has been designed to provide a total of up to six different detections. An agonist strip applied along the sample tracks allowed the detection of antagonists, which could be detected together with the agonists, corresponding to the regular pYAAS bioassay (2-fold). The addition of resazurin to the yeast cell suspension allowed the detection of cytotoxic bands (3-fold). Also applied along the sample tracks were a cytotoxin stripe detecting anti-cytotoxins (4-fold) and two stripes containing the end-products of the respective enzyme-substrate reaction, which allowed for two false-positive detections (6-fold). The performance of the 6-fold multiplex bioassay was investigated by using it to determine dose-response curves of menadione (cytotoxin), flutamide (anti-androgen, antagonist), dihydrotestosterone (androgen, agonist), and quercetin (anti-cytotoxin). The respective values for menadione, flutamide and dihydrotestosterone were R^2 0.8431, 0.9197 and 0.9721. Considering the given assay complexity, the day-to-day variance of the yeast cell performance and the use of Degalan for fixation, these values are acceptable. Quercetin clearly showed anti-cytotoxic reactions, but the chemical was to polar to migrate in the polar phase and was too close to the start zone, which is why the dose-response curve was not determinable. The two false-positive detection stripes verified the responses of all tested chemicals.

The newly developed 6-fold bioassay was then applied to a migrate which was already investigated using the pYAAS bioassay. The results of the pYAAS bioassay (anti-androgenic activity) were confirmed, and furthermore, no cytotoxic reactions were

observed for the amounts of migrate tested, proving that the migrate indeed contains anti-androgenic substances.

In this work it was archived, that all important data about a sample (dose dependence of 6 different applied volumes) can be collected with only one plate. The procedure took 6 h for 1 analysis per plate (only 1 h labor transfer time between steps and short manual interventions). The costs were about 24 Euro per plate and sample. Without this new 6-fold assay, the effort for the same amount of information would be incomparably greater (bioassay-guided fractionation, analytical separation, 6 different *in vitro* assays, re-analysis for HRMS recording). This would need much more material and time. Additionally, complicated comparative evaluations are spared using the new bioassay, because it provides all the needed information on one compact plate.

4. References

[1] EFSA Scientific Committee, Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment, *EFSA J* 9 (2011) 2379. https://doi.org/10.2903/j.efsa.2011.2379.

[2] Koster S, Bani-Estivals M-H, Bonuomo M, Bradley E, Chagnon MC, Garcia ML, Godts F, Gude T, Helling R, Paseiro-Losada P, Pieper G, Rennen M, Simat T, Spack L, Guidance on best practices on the risk assessment of non intentionally added substances (NIAS) in food contact materials and articles, *ILSI Europe Report Series* (2015) 1–70.

[3] S.K. Bopp, A. Kienzler, A.-N. Richarz, S.C. van der Linden, A. Paini, N. Parissis, A.P. Worth, Regulatory assessment and risk management of chemical mixtures: challenges and ways forward, *Crit. Rev. Toxicol*. 49 (2019) 174–189. https://doi.org/10.1080/10408444.2019.1579169.

[4] B. Schilter, K. Burnett, C. Eskes, L. Geurts, M. Jacquet, C. Kirchnawy, P. Oldring, G. Pieper, E. Pinter, M. Tacker, H. Traussnig, P. van Herwijnen, A. Boobis, Value and limitation of in vitro bioassays to support the application of the threshold of toxicological concern to prioritise unidentified chemicals in food contact materials, Food Addit. Contam. 36 (2019) 1903–1936. https://doi.org/10.1080/19440049.2019.1664772.

[5] European Parliament, Committee on the Environment, Public Health and Food Safety (2016). Report on the implementation of the Food Contact Materials Regulation ((EC) No 1935/2004) (2015/2259(INI)). A8-0237/2016, 1-16. https://www.europarl.europa.eu/doceo/document/A-8-2016-0237_EN.pdf

[6] K. Grob, M. Biedermann, E. Scherbaum, M. Roth, K. Rieger, Food contamination with organic materials in perspective: packaging materials as the largest and least

controlled source? A view focusing on the European situation, *Crit. Rev. Food Sci. Nutr.* 46 (2006) 529–535. https://doi.org/10.1080/10408390500295490.

[7] The European Paarliament and the Council of the European Union, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, *OJEU* (2010). https://doi:10.3000/17252555.L_2010.276.eng

[8] Organisation for Economic Co-operation and Development, Test No. 471: Bacterial Reverse Mutation Test, OECD, 2020.

[9] Organisation for Economic Co-operation and Development, Test No. 476: In vitro Mammalian Cell Gene Mutation Test, OECD Publishing, 1997.

[10] Organisation for Economic Co-operation and Development, Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Publishing, Paris, 2016.

[11] Organisation for Economic Co-operation and Development, Test No. 473: In vitro Mammalian Chromosome Aberration Test, OECD Publishing, 1997.

[12] Organisation for Economic Co-operation and Development, Test No. 482: Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells in vitro, OECD Publishing, Paris, 1986.

[13] Y. Oda, S. Nakamura, I. Oki, T. Kato, H. Shinagawa, Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens, *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 147 (1985) 219–229. https://doi.org/10.1016/0165-1161(85)90062-7.

[14] International Standard Organization, ISO 13829:2000(en) Water quality — Determination of the genotoxicity of water and waste water using the umu-test. https://www.iso.org/obp/ui/#iso:std:iso:13829:ed-1:v1:en:sec:A (accessed 26 November 2021).

[15] G.D.A. Umbuzeiro, M. Heringa, E. Zeiger, In Vitro Genotoxicity Testing: Significance and Use in Environmental Monitoring, in: G. Reifferscheid, S. Buchinger (Eds.), in vitro Environmental Toxicology - Concepts, Application and Assessment, Springer International Publishing, Cham, 2017, pp 59–80.

[16] Y. Oda, Development and progress for three decades in umu test systems, *Genes. Environ.* 38 (2016) 24. https://doi.org/10.1186/s41021-016-0054-8.

[17] D. Shakibai, C. Riegraf, L. Moscovici, G. Reifferscheid, S. Buchinger, S. Belkin, Coupling High-Performance Thin-Layer Chromatography with Bacterial Genotoxicity Bioreporters, *Environ. Sci. Technol.* 53 (2019) 6410–6419. https://doi.org/10.1021/acs.est.9b00921.

[18] G.R. Mohn, Bacterial systems for carcinogenicity testing, *Mutat. Res. Rev. Genet. Toxicol*. 87 (1981) 191–210. https://doi.org/10.1016/0165-1110(81)90032-4.

[19] I. Purchase, An appraisal of predictive tests for carcinogenicity, *Mutat. Res. Rev. Genet. Toxicol*. 99 (1982) 53–71. https://doi.org/10.1016/0165-1110(82)90031-8.

[20] K. Yasunaga, A. Kiyonari, T. Oikawa, N. Abe, K. Yoshikawa, Evaluation of the Salmonella umu test with 83 NTP chemicals, *Environ. Mol. Mutagen*. 44 (2004) 329–345. https://doi.org/10.1002/em.20053.

[21] G. Reifferscheid, J. Heil, Y. Oda, R.K. Zahn, A microplate version of the SOS/umu-test for rapid detection of genotoxins and genotoxic potentials of environmental samples, *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 253 (1991) 215–222. https://doi.org/10.1016/0165-1161(91)90134-T.

[22] G. Reifferscheid, J. Heil, Validation of the SOS/umu test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data, *Mutat. Res. Genet. Toxicol.* 369 (1996) 129–145. https://doi.org/10.1016/S0165-1218(96)90021-X.

[23] T. Takamura-Enya, R. Ishii, Y. Oda, Evaluation of photo-genotoxicity using the umu test in strains with a high sensitivity to oxidative DNA damage, Mutagenesis 26 (2011) 499–505. https://doi.org/10.1093/mutage/ger008.

[24] B. Powroźnik, K. Słoczyńska, V. Canale, K. Grychowska, P. Zajdel, E. Pękala, Preliminary mutagenicity and genotoxicity evaluation of selected arylsulfonamide derivatives of (aryloxy)alkylamines with potential psychotropic properties, *J. Appl. Genet.* 57 (2016) 263–270. https://doi.org/10.1007/s13353-015-0322-5.

[25] D. Balabanič, M. Filipič, A. Krivograd Klemenčič, B. Žegura, Genotoxic activity of endocrine disrupting compounds commonly present in paper mill effluents, *Sci. Total. Environ.* 794 (2021) 148489. https://doi.org/10.1016/j.scitotenv.2021.148489.

[26] Y. Ye, J. Weiwei, L. Na, M. Mei, W. Donghong, W. Zijian, R. Kaifeng, Assessing of genotoxicity of 16 centralized source-waters in China by means of the SOS/umu assay and the micronucleus test: initial identification of the potential genotoxicants by use of a GC/MS method and the QSAR Toolbox 3.0, *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 763 (2014) 36–43. https://doi.org/10.1016/j.mrgentox.2013.11.003.

[27] A. Hartmann, A.C. Alder, T. Koller, R.M. Widmer, Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater, *Environ. Toxicol. Chem.* 17 (1998) 377–382. https://doi.org/10.1002/etc.5620170305.

[28] A. Hartmann, E.M. Golet, S. Gartiser, A.C. Alder, T. Koller, R.M. Widmer, Primary DNA damage but not mutagenicity correlates with ciprofloxacin concentrations in German hospital wastewaters, *Arch. Environ. Contam. Toxicol.* 36 (1999) 115–119. https://doi.org/10.1007/s002449900449.

[29] F. Giuliani, T. Koller, F.E. Würgler, R.M. Widmer, Detection of genotoxic activity in native hospital waste water by the umuC test, *Mutat. Res. Genet. Toxicol*. 368 (1996) 49–57. https://doi.org/10.1016/S0165-1218(96)90039-7.

[30] T. Steger-Hartmann, K. Kümmerer, A. Hartmann, Biological degradation of cyclophosphamide and its occurrence in sewage water, *Ecotoxicol. Environ. Saf.* 36 (1997) 174–179. https://doi.org/10.1006/eesa.1996.1506.

[31] M.B. Mueller, C. Dausend, C. Weins, F.H. Frimmel, A New Bioautographic Screening Method for the Detection of Estrogenic Compounds, *Chromatographia* 60 (2004). https://doi.org/10.1365/S10337-004-0315-8.

[32] C.N. Berger, S.S. Tan, K.S. Sturm, Simultaneous detection of beta-galactosidase activity and surface antigen expression in viable haematopoietic cells, *Cytometry* 17 (1994) 216–223. https://doi.org/10.1002/cyto.990170305.

[33] D.P. McDonnell, Z. Nawaz, C. Densmore, N.L. Weigel, T.A. Pham, J.H. Clark, B.W. O'Malley, High level expression of biologically active estrogen receptor in Saccharomyces cerevisiae, *J. Steroid Biochem* 39 (1991) 291–297. https://doi.org/10.1016/0960-0760(91)90038-7.

[34] D.P. McDonnell, Z. Nawaz, B.W. O'Malley, In situ distinction between steroid receptor binding and transactivation at a target gene, *Mol. Cell. Biol.* 11 (1991) 4350–4355. https://doi.org/10.1128/mcb.11.9.4350-4355.1991.

[35] W. Brack, S. Ait-Aissa, R.M. Burgess, W. Busch, N. Creusot, C. Di Paolo, B.I. Escher, L. Mark Hewitt, K. Hilscherova, J. Hollender, H. Hollert, W. Jonker, J. Kool, M. Lamoree, M. Muschket, S. Neumann, P. Rostkowski, C. Ruttkies, J. Schollee, E.L. Schymanski, T. Schulze, T.-B. Seiler, A.J. Tindall, G. de Aragão Umbuzeiro, B. Vrana, M. Krauss, Effect-directed analysis supporting monitoring of aquatic environments--An in-depth overview, *Sci. Total Environ.* 544 (2016) 1073–1118. https://doi.org/10.1016/j.scitotenv.2015.11.102.

[36] F.D.L. Leusch, P.A. Neale, A. Hebert, M. Scheurer, M.C.M. Schriks, Analysis of the sensitivity of in vitro bioassays for androgenic, progestagenic, glucocorticoid, thyroid and estrogenic activity: Suitability for drinking and environmental waters, *Environ. Int.* 99 (2017) 120–130. https://doi.org/10.1016/j.envint.2016.12.014.

[37] A.M. Soto, M.V. Maffini, C.M. Schaeberle, C. Sonnenschein, Strengths and weaknesses of in vitro assays for estrogenic and androgenic activity, Best Pract. *Res. Clin. Endocrinol. Metab.* 20 (2006) 15–33. https://doi.org/10.1016/j.beem.2005.09.001.

[38] A. Mohammad, A. Moheman (Eds.), TLC/HPTLC in biomedical applications, in: M. Srivastava (Ed.), High-Performance Thin-Layer Chromatography (HPTLC), Springer, Berlin Heidelberg, 2011, pp. 151–178.

[39] G. Morlock, W. Schwack, Hyphenations in planar chromatography, *J. Chromatogr. A* 1217 (2010) 6600–6609. https://doi.org/10.1016/j.chroma.2010.04.058.

[40] D. Fichou, G.E. Morlock, quanTLC, an online open-source solution for videodensitometric quantification, *J. Chromatogr. A* 1560 (2018) 78–81. https://doi.org/10.1016/j.chroma.2018.05.027.

[41] D. Fichou, P. Ristivojević, G.E. Morlock, Proof-of-Principle of rTLC, an Open-Source Software Developed for Image Evaluation and Multivariate Analysis of Planar Chromatograms, *Anal. Chem.* 88 (2016) 12494–12501. https://doi.org/10.1021/acs.analchem.6b04017.

[42] T.T. Häbe, G.E. Morlock, Challenges in quantitative high-performance thin-layer chromatography — Part 1: Influence of densitometric settings on the result, *J. Planar. Chromatogr. - Mod. TLC* 28 (2015) 426–435. https://doi.org/10.1556/1006.2015.28.6.2.

[43] I. Klingelhöfer, G.E. Morlock, Challenges in quantitative high-performance thin-layer chromatography — Part 2: Influence of the application mode on the result, *J.*

Planar. Chromatogr. - Mod. TLC 30 (2017) 411–417. https://doi.org/10.1556/1006.2017.30.5.11.

[44] T.T. Häbe, G.E. Morlock, Quantitative surface scanning by Direct Analysis in Real Time mass spectrometry, *Rapid Commun. Mass Spectrom.* 29 (2015) 474–484. https://doi.org/10.1002/rcm.7127.

[45] G.E. Morlock, N. Brett, Correct assignment of lipophilic dye mixtures? A case study for high-performance thin-layer chromatography-mass spectrometry and performance data for the TLC-MS Interface, *J. Chromatogr. A* 1390 (2015) 103–111. https://doi.org/10.1016/j.chroma.2015.02.011.

[46] E. Azadniya, G.E. Morlock, Bioprofiling of Salvia miltiorrhiza via planar chromatography linked to (bio)assays, high resolution mass spectrometry and nuclear magnetic resonance spectroscopy, *J. Chromatogr. A* 1533 (2018) 180–192. https://doi.org/10.1016/j.chroma.2017.12.014.

[47] I. Yüce, G.E. Morlock, All on one high-performance thin-layer chromatography plate: solvent-free nanomole-scaled on-surface synthesis, workup and online high-resolution mass spectrometry for elucidation of two new degradation products in an ifosfamide formulation, *J. Chromatogr. A* 1572 (2018) 145–151. https://doi.org/10.1016/j.chroma.2018.08.047.

[48] E. Azadniya, L. Goldoni, T. Bandiera, G.E. Morlock, Same analytical method for both (bio)assay and zone isolation to identify/quantify bioactive compounds by quantitative nuclear magnetic resonance spectroscopy, *J. Chromatogr. A* 1628 (2020) 461434. https://doi.org/10.1016/j.chroma.2020.461434.

[49] I. Yüce, M. Mayr, G.E. Morlock, Quantitative inkjet application on self-printed, binder-free HPTLC layers for submicromole-scaled analytical 1H NMR spectroscopy, *Anal. Chim. Acta* 1087 (2019) 131–139. https://doi.org/10.1016/j.aca.2019.08.013.

[50] P.S. Variyar, Patel, Mrunali R., Batel, Bharat G. (Ed.), Fundamentals and theory of HPTLC-based separation, in: M. Srivastava (Ed.), High-Performance Thin-Layer Chromatography (HPTLC), Springer, Berlin Heidelberg, 2011, pp. 27–40.

[51] D. Charegaonkar (Ed.), High-performance thin-layer chromatography: excellent automation, in: M. Srivastava (Ed.), High-Performance Thin-Layer Chromatography (HPTLC), Springer, Berlin Heidelberg, 2011, pp. 55–68.

[52] W. Schwack, G. Morlock, Planar chromatography — Back to the future?, *LC GT Europe* 21 (2008) 366–371.

[53] G.E. Morlock, S. Prabha, Analysis and stability of sucralose in a milk-based confection by a simple planar chromatographic method, J. Agric. Food Chem. 55 (2007) 7217–7223. https://doi.org/10.1021/jf071719u.

[54] G.E. Morlock, High-performance thin-layer chromatography combined with effect-directed assays and high-resolution mass spectrometry as an emerging hyphenated technology: A tutorial review, *Anal. Chim. Acta* 1180 (2021) 338644. https://doi.org/10.1016/j.aca.2021.338644.

[55] I. Klingelhöfer, G.E. Morlock, Sharp-bounded zones link to the effect in planar chromatography-bioassay-mass spectrometry, *J. Chromatogr. A* 1360 (2014) 288–295. https://doi.org/10.1016/j.chroma.2014.07.083.

[56] S.-S. Teh, G. Morlock, Analysis of Bioactive Components of Oilseed Cakes by High-Performance Thin-Layer Chromatography-(Bio)assay Combined with Mass Spectrometry, *Chromatography* 2 (2015) 125–140. https://doi.org/10.3390/chromatography2010125.

[57] C. Riegraf, G. Reifferscheid, S. Belkin, L. Moscovici, D. Shakibai, H. Hollert, S. Buchinger, Combination of yeast-based in vitro screens with high-performance thin-layer chromatography as a novel tool for the detection of hormonal and dioxin-

like compounds, *Anal. Chim. Acta* 1081 (2019) 218–230. https://doi.org/10.1016/j.aca.2019.07.018.

[58] I. Klingelhöfer, N. Hockamp, G.E. Morlock, Non-targeted detection and differentiation of agonists versus antagonists, directly in bioprofiles of everyday products, *Anal. Chim. Acta* 1125 (2020) 288–298. https://doi.org/10.1016/j.aca.2020.05.057.

[59] C. Riegraf, A.M. Bell, M. Ohlig, G. Reifferscheid, S. Buchinger, Planar chromatography-bioassays for the parallel and sensitive detection of androgenicity, anti-androgenicity and cytotoxicity, *J. Chromatogr. A* 1684 (2022) 463582. https://doi.org/10.1016/j.chroma.2022.463582.

[60] A. Ronzheimer, T. Schreiner, G.E. Morlock, Multiplex planar bioassay detecting estrogens, antiestrogens, false-positives and synergists as sharp zones on normal phase, *Phytomedicine* 103 (2022) 154230. https://doi.org/10.1016/j.phymed.2022.154230.

[61] T. Schreiner, A. Ronzheimer, M. Friz, G.E. Morlock, Multiplex planar bioassay with reduced diffusion on normal phase, identifying androgens, verified antiandrogens and synergists in botanicals via 12D hyphenation, *Food Chem.* 395 (2022) 133610. https://doi.org/10.1016/j.foodchem.2022.133610.

[62] A. Schoenborn, P. Schmid, S. Bräm, G. Reifferscheid, M. Ohlig, S. Buchinger, Unprecedented sensitivity of the planar yeast estrogen screen by using a spray-on technology, *J. Chromatogr. A* 1530 (2017) 185–191. https://doi.org/10.1016/j.chroma.2017.11.009.

[63] I. Klingelhöfer, G.E. Morlock, Bioprofiling of Surface/Wastewater and Bioquantitation of Discovered Endocrine-Active Compounds by Streamlined Direct Bioautography, *Anal. Chem.* 87 (2015) 11098–11104. https://doi.org/10.1021/acs.analchem.5b03233.

[64] D. Schick, W. Schwack, Planar yeast estrogen screen with resorufin-β-d-galactopyranoside as substrate, *J. Chromatogr. A* 1497 (2017) 155–163. https://doi.org/10.1016/j.chroma.2017.03.047.

[65] F. Brion, V. de Gussem, S. Buchinger, H. Hollert, M. Carere, J.-M. Porcher, B. Piccini, C. Féray, V. Dulio, S. Könemann, E. Simon, I. Werner, R. Kase, S. Aït-Aïssa, Monitoring estrogenic activities of waste and surface waters using a novel in vivo zebrafish embryonic (EASZY) assay: Comparison with in vitro cell-based assays and determination of effect-based trigger values, *Environ. Int.* 130 (2019) 104896. https://doi.org/10.1016/j.envint.2019.06.006.

[66] D. Schick, C. Oellig, Screening for estrogen active nonylphenols in surface waters by planar solid phase extraction-planar yeast estrogen screen, *Anal. Bioanal. Chem.* 411 (2019) 6767–6775. https://doi.org/10.1007/s00216-019-02053-0.

[67] S.-S. Teh, G.E. Morlock, Effect-directed analysis of cold-pressed hemp, flax and canola seed oils by planar chromatography linked with (bio)assays and mass spectrometry, *Food Chem*. 187 (2015) 460–468. https://doi.org/10.1016/j.foodchem.2015.04.043.

[68] G. Corni, V. Brighenti, F. Pellati, G.E. Morlock, Effect-directed analysis of bioactive compounds in Cannabis sativa L. by high-performance thin-layer chromatography, *J. Chromatogr. A* 1629 (2020) 461511. https://doi.org/10.1016/j.chroma.2020.461511.

[69] S. Hage, G.E. Morlock, Bioprofiling of Salicaceae bud extracts through high-performance thin-layer chromatography hyphenated to biochemical, microbiological and chemical detections, *J. Chromatogr. A* 1490 (2017) 201–211. https://doi.org/10.1016/j.chroma.2017.02.019.

[70] T. Schreiner, D. Sauter, M. Friz, J. Heil, G.E. Morlock, Is Our Natural Food Our Homeostasis? Array of a Thousand Effect-Directed Profiles of 68 Herbs and

Spices, *Front. Pharmacol.* 12 (2021) 755941. https://doi.org/10.3389/fphar.2021.755941.

[71] A.J. Bergmann, E. Simon, A. Schifferli, A. Schönborn, E.L.M. Vermeirssen, Estrogenic activity of food contact materials-evaluation of 20 chemicals using a yeast estrogen screen on HPTLC or 96-well plates, *Anal. Bioanal. Chem.* 412 (2020) 4527–4536. https://doi.org/10.1007/s00216-020-02701-w.

[72] N. Egetenmeyer, S.C. Weiss, Investigations for the detection of genotoxic substances on TLC plates, *J. Liq. Chromatogr. Relat. Technol.* 40 (2017) 69–74. https://doi.org/10.1080/10826076.2017.1284674.

[73] B. Cuq, N. Gontard, S. Guilbert, Proteins as Agricultural Polymers for Packaging Production, *Cereal Chem.* 75 (1998) 1–9. https://doi.org/10.1094/CCHEM.1998.75.1.1.

[74] L. Piergiovanni, S. Limbo (Eds.), Introduction to Food Packaging Materials.

[75] P. Cazón, M. Vázquez, Bacterial cellulose as a biodegradable food packaging material: A review, *Food Hydrocolloids* 113 (2021) 106530. https://doi.org/10.1016/j.foodhyd.2020.106530.

[76] M. Lamberti, F. Escher, Aluminium Foil as a Food Packaging Material in Comparison with Other Materials, *Food Rev. Int.* 23 (2007) 407–433. https://doi.org/10.1080/87559120701593830.

[77] G.K. Deshwal, N.R. Panjagari, Review on metal packaging: materials, forms, food applications, safety and recyclability, *J. Food Sci. Technol.* 57 (2020) 2377–2392. https://doi.org/10.1007/s13197-019-04172-z.

[78] O.W. Lau, S.K. Wong, Contamination in food from packaging material, *J. Chromatogr. A* 882 (2000) 255–270. https://doi.org/10.1016/s0021-9673(00)00356-3.

[79] G.K. Deshwal, N.R. Panjagari, Review on metal packaging: materials, forms, food applications, safety and recyclability, *J. Food Sci. Technol.* 57 (2020) 2377–2392. https://doi.org/10.1007/s13197-019-04172-z.

[80] B. Pinto, D. Reali, Screening of estrogen-like activity of mineral water stored in PET bottles, *Int. J. Hyg. Environ. Health* 212 (2009) 228–232. https://doi.org/10.1016/j.ijheh.2008.06.004.

[81] M. Wagner, J. Oehlmann, Endocrine disruptors in bottled mineral water: total estrogenic burden and migration from plastic bottles, *Environ. Sci. Pollut. Res. Int.* 16 (2009) 278–286. https://doi.org/10.1007/s11356-009-0107-7.

[82] M. Wagner, M.P. Schlüsener, T.A. Ternes, J. Oehlmann, Identification of putative steroid receptor antagonists in bottled water: combining bioassays and high-resolution mass spectrometry, *PLoS One* 8 (2013) e72472. https://doi.org/10.1371/journal.pone.0072472.

[83] J. Muncke, Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source?, *Sci. Total Environ.* 407 (2009) 4549–4559. https://doi.org/10.1016/j.scitotenv.2009.05.006.

[84] T.P. McNeal, J.E. Biles, T.H. Begley, J.C. Craun, M.L. Hopper, C.A. Sack (EDs), Determination of Suspected Endocrine Disruptors in Foods and Food Packaging in: L.H. Keith, T.L. Jones-Lepp, L.L. Needham (Eds), Analysis of Environmental Endocrine Disruptors, (1999), pp. 33–52. https://doi.org/10.1021/bk-2000-0747.ch004.

[85] European Chemical Agency, REACH verstehen, (2022). https://echa.europa.eu/de/regulations/reach/understanding-reach (accessed 29 December 2022).

[86] European Chemical Agency, Standarddatenanforderungen, 2022. https://echa.europa.eu/de/standard-information-requirements-recommendations (accessed 29 December 2022).

[87] REACH Online, ANNEX VII: Standard information requirements for substances manufactured or imported in quantities of one tinne or more. https://reachonline.eu/reach/en/annex-vii-8.html (accessed 29 December 2022).

[88] REACH Online, ANNEX VIII: Standard information requirements for substances manufactured or imported in quantities of 10 tommes or more, (2022). https://reachonline.eu/reach/en/annex-viii.html (accessed 29 December 2022).

[89] European Parliament and the Council of the European Union, Regulation (EC) No 1907/2006 - Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), (2006). https://osha.europa.eu/de/legislation/directives/regulation-ec-no-1907-2006-of-the-european-parliament-and-of-the-council (accessed 15 January 2023).

[90] N. Andersson, M. Arena, D. Auteri, S. Barmaz, E. Grignard, A. Kienzler, P. Lepper, A.M. Lostia, S. Munn, J.M. Parra Morte, F. Pellizzato, J. Tarazona, A. Terron, S. van der Linden, Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009, *EFSA J.* 16 (2018) e05311. https://doi.org/10.2903/j.efsa.2018.5311.

[91] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC, (2009), https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32009R1107 (accessed 29 December 2022).

[92] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal

products, (2012). https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32012R0528 (accessed 25.01.2023)

[93] Bundesministerium für Ernährung und Landwirtschaft, Leitsätze für Speisefette und Speiseöle, (2020). https://www.bmel.de/SharedDocs/Downloads/DE/_Ernaehrung/Lebensmittel-Kennzeichnung/LeitsaetzeSpeisefette.pdf (accessed 15 January 2023).

[94] R. Marcos, E.R. Carmona, The wing-spot and the comet tests as useful assays detecting genotoxicity in Drosophila, *Methods Mol. Biol.* 1044 (2013) 417–427. https://doi.org/10.1007/978-1-62703-529-3_23.

[95] A. Harder, B.I. Escher, P. Landini, N.B. Tobler, R.P. Schwarzenbach, Evaluation of bioanalytical assays for toxicity assessment and mode of toxic action classification of reactive chemicals, *Environ. Sci. Technol.* 37 (2003) 4962–4970. https://doi.org/10.1021/es034197h.

[96] A. Sevanian, J.F. Mead, R.A. Stein, Epoxides as products of lipid autoxidation in rat lungs, Lipids 14 (1979) 634–643. https://doi.org/10.1007/BF02533449.

[97] L.G. Hammock, B.D. Hammock, J.E. Casida, Detection and analysis of epoxides with 4-(p-Nitrobenzyl)-pyridine, *Bull. Environ. Contam. Toxicol.* 12 (1974) 759–764. https://doi.org/10.1007/BF01685927.

[98] S.C. Agarwal, B.L. van Duuren, T.J. Kneip, Detection of epoxides with 4-(p-nitrobenzyl) pyridine, *Bull. Environ. Contam. Toxicol.* 23 (1979) 825–829. https://doi.org/10.1007/BF01770049.

[99] C.L. Dupard-Julien, B. Kandlakunta, R.M. Uppu, Determination of epoxides by high-performance liquid chromatography following derivatization with N,N-diethyldithiocarbamate, *Anal. Bioanal. Chem.* 387 (2007) 1027–1032. https://doi.org/10.1007/s00216-006-1003-3.

[100] A.W. Claxson, G.E. Hawkes, D.P. Richardson, D.P. Naughton, R.M. Haywood, C.L. Chander, M. Atherton, E.J. Lynch, M.C. Grootveld, Generation of lipid peroxidation products in culinary oils and fats during episodes of thermal stressing: a high field 1H NMR study, *FEBS Letters* 355 (1994) 81–90. https://doi.org/10.1016/0014-5793(94)01147-8.

[101] H.A.J. Aerts, P.A. Jacobs, Epoxide yield determination of oils and fatty acid methyl esters using 1 H NMR, *J. Amer. Oil. Chem. Soc.* 81 (2004) 841–846. https://doi.org/10.1007/s11746-004-0989-1.

[102] C.H. Liao, Evaluation of Assays for Epoxides in Oxidized Lipids, New Brunswick, NJ, USA, (2013).

[103] D.W. Phillipson, K.E. Milgram, A.I. Yanovsky, L.S. Rusnak, D.A. Haggerty, W.P. Farrell, M.J. Greig, X. Xiong, M.L. Proefke, High-throughput bioassay-guided fractionation: a technique for rapidly assigning observed activity to individual components of combinatorial libraries, screened in HTS bioassays, *J. Comb. Chem.* 4 (2002) 591–599. https://doi.org/10.1021/cc020042e.

[104] N. Malviya, S. Malviya, Bioassay guided fractionation-an emerging technique influence the isolation, identification and characterization of lead phytomolecules, *IJHP* 2 (2017) 5. https://doi.org/10.28933/ijhp-2017-07-0901.

[105] B. Hamer, N. Bihari, G. Reifferscheid, R.K. Zahn, W. Müller, R. Batel, Evaluation of the SOS/umu-test post-treatment assay for the detection of genotoxic activities of pure compounds and complex environmental mixtures, *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 466 (2000) 161–171. https://doi.org/10.1016/S1383-5718(00)00016-4.

[106] U. Baumann, C. Brunner, E. Pletscher, N. Tobler, Biologische Detektionsverfahren in der Dünnschichtchromatographie, *Umweltwiss. Schadst. Forsch.* 15 (2003) 163–167. https://doi.org/10.1065/uwsf2001.12.080.

[107] D. Kirkland, P. Kasper, H.-J. Martus, L. Müller, J. van Benthem, F. Madia, R. Corvi, Updated recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests, *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 795 (2016) 7–30. https://doi.org/10.1016/j.mrgentox.2015.10.006.

[108] C. Sonnenschein, A.M. Soto, An updated review of environmental estrogen and androgen mimics and antagonists, *J. Steroid Biochem. Mol. Biol.* 65 (1998) 143–150. https://doi.org/10.1016/S0960-0760(98)00027-2.

[109] J. Mertl, C. Kirchnawy, V. Osorio, A. Grininger, A. Richter, J. Bergmair, M. Pyerin, M. Washüttl, M. Tacker, Characterization of estrogen and androgen activity of food contact materials by different in vitro bioassays (YES, YAS, ERα and AR CALUX) and chromatographic analysis (GC-MS, HPLC-MS), *PLoS One* 9 (2014) e100952. https://doi.org/10.1371/journal.pone.0100952.

[110] T. Colborn, F.S. vom Saal, A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, *Environ. Health Perspect.* 101 (1993) 378–384. https://doi.org/10.1289/ehp.93101378.

[111] I. Ceccarelli, D. Della Seta, P. Fiorenzani, F. Farabollini, A.M. Aloisi, Estrogenic chemicals at puberty change ERalpha in the hypothalamus of male and female rats, *Neurotoxicol. Teratol.* 29 (2007) 108–115. https://doi.org/10.1016/j.ntt.2006.10.011.

[112] S.C. Sikka, R. Wang, Endocrine disruptors and estrogenic effects on male reproductive axis, *Asian J. Androl.* 10 (2008) 134–145. https://doi.org/10.1111/j.1745-7262.2008.00370.x.

[113] J. Gray, N. Evans, B. Taylor, J. Rizzo, M. Walker, State of the Evidence: The Connection Between Breast Cancer and the Environment, *Int. J. Occup. Environ. Health* 15 (2009) 43–78. https://doi.org/10.1179/oeh.2009.15.1.43.

[114] J. Boberg, M. Axelstad, T. Svingen, K. Mandrup, S. Christiansen, A.M. Vinggaard, U. Hass, Multiple Endocrine Disrupting Effects in Rats Perinatally Exposed to Butylparaben, *Toxicol. Sci.* 152 (2016) 244–256. https://doi.org/10.1093/toxsci/kfw079.

5. Publication I

Research Article

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

Daniel Meyer[1], Maricel Marin-Kuan[2], Emma Debon[2], Patrick Serrant[2], Claudine Cottet-Fontannaz[2], Benoît Schilter[2] and Gertrud E. Morlock[1]

[1]Chair of Food Science, Institute of Nutritional Science, and TransMIT Center of Effect-Directed Analysis, Justus Liebig University Giessen, Giessen, Germany; [2]Chemical Food Safety Group, Société des Produits Nestlé SA – Nestlé Research, Vers-chez-les-Blanc, Switzerland

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

Received June 20, 2020; Accepted December 11, 2020;
Epub December 18, 2020; © The Authors, 2021.

ALTEX 38(3), 387-397. doi:10.14573/altex.2006201

Correspondence: Gertrud Morlock, PhD
Chair of Food Science, Institute of Nutritional Science
Justus Liebig University Giessen
Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
(gertrud.morlock@uni-giessen.de)

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)

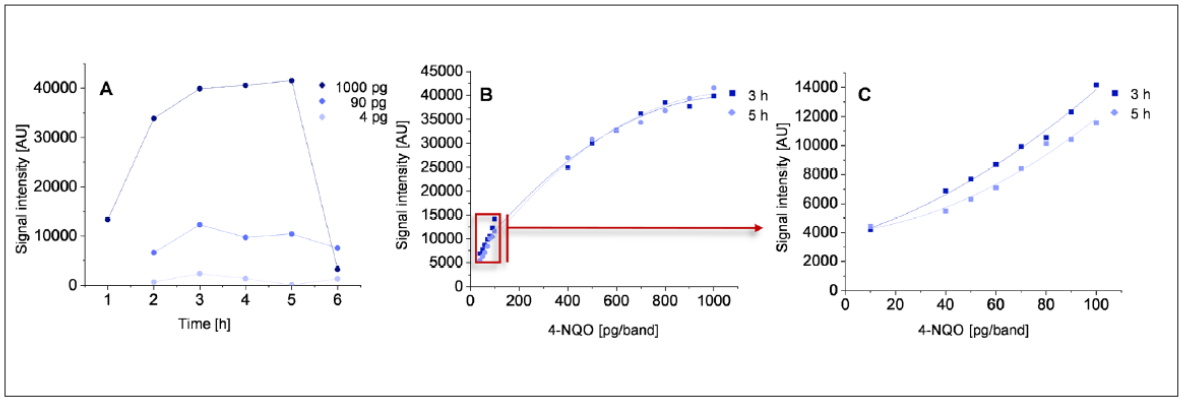


Fig. 1: Optimization of the planar SOS-Umu-C assay with regard to incubation time
Plots of the (A) highest achievable MU-signal intensities of 4-NQO against incubation times (1-6 h) and, in more detail, (B) MU-signal intensities against the 4-NQO amount ranged 4 to 1000 pg/band for a 3-h and 5-h incubation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

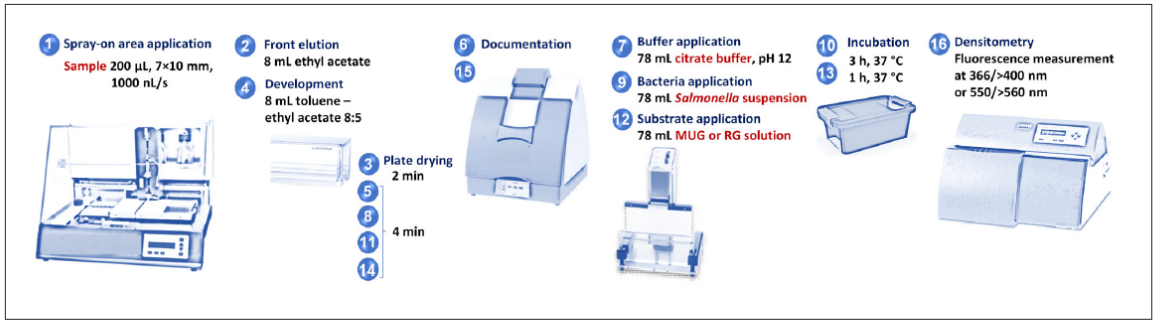


Fig. 2: Scheme of the newly developed RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay
The order of the individual steps is traceable by the increasing numbers, whereby some steps are performed repeatedly. The procedure on one RP-HPTLC plate took 5-6 h, was sensitive in detection and matrix-robust due to separation.

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

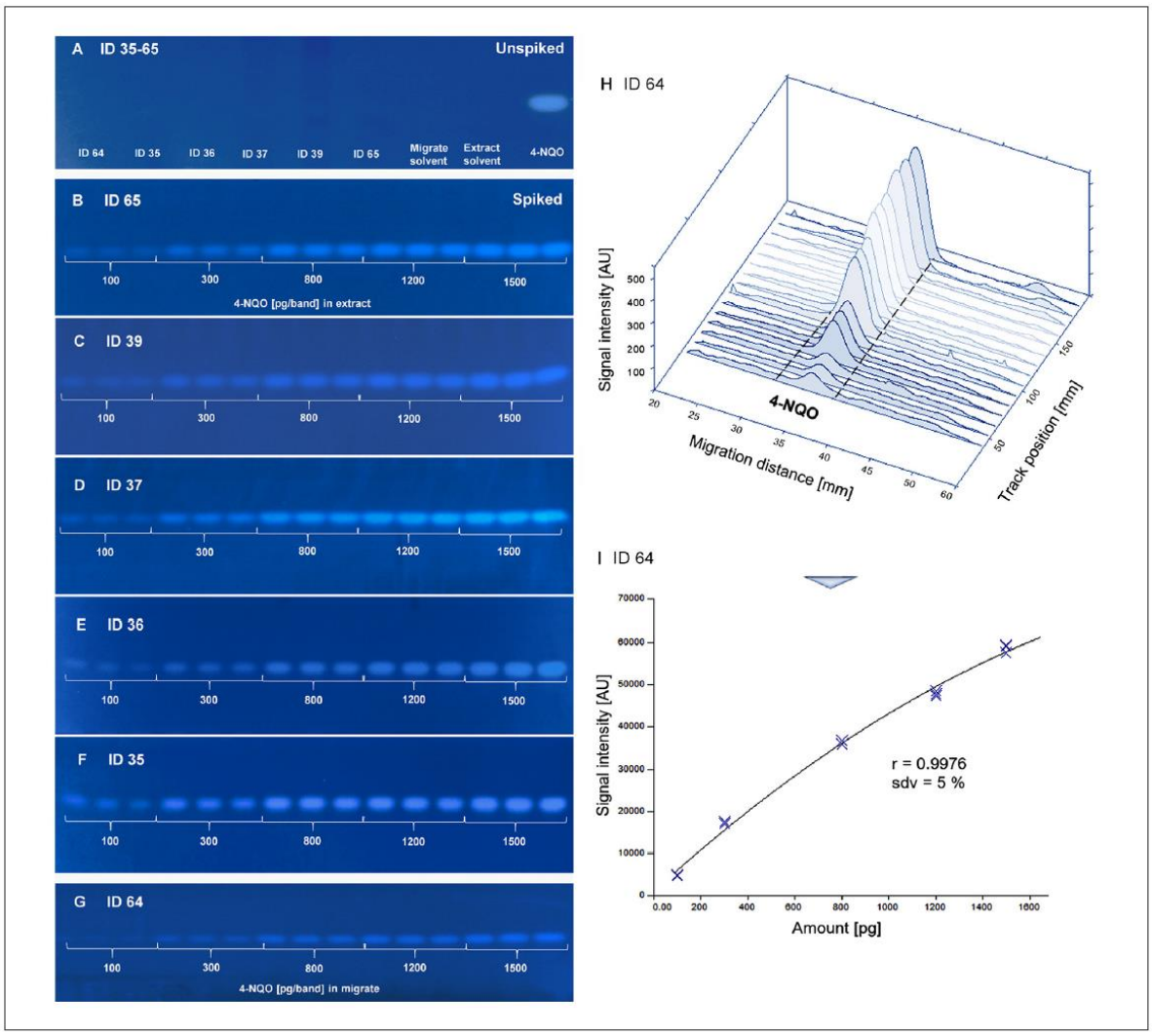


Fig. 3: Dose-response curve study

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-

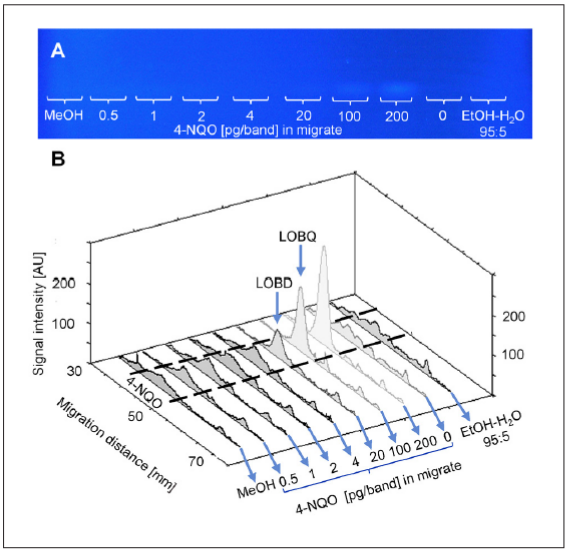


Fig. 4: LOBD/LOBQ study
(A) RP-HPTLC-SOS-Umu-C bioautogram at FLD 366 nm (enhanced image) of the food simulant migrate ID 64 (200 µL/area each) spiked with different amounts of 4-NQO (0-200 pg/area) and (B) corresponding densitogram at FLD 366/>400 nm, both confirming the LOBD and LOBQ at 20 and 100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

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).

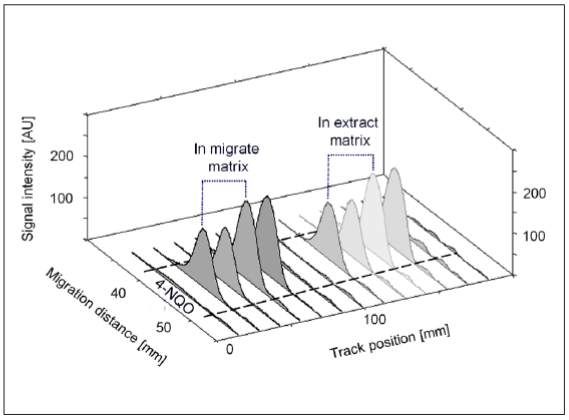


Fig. 5: Investigation of the matrix effect
Densitograms zoomed to the 4-NQO region of interest of the migration sample ID 64 and extract ID 65, showing 4-NQO at 300 and 600 pg/band, each side by side with and without matrix.

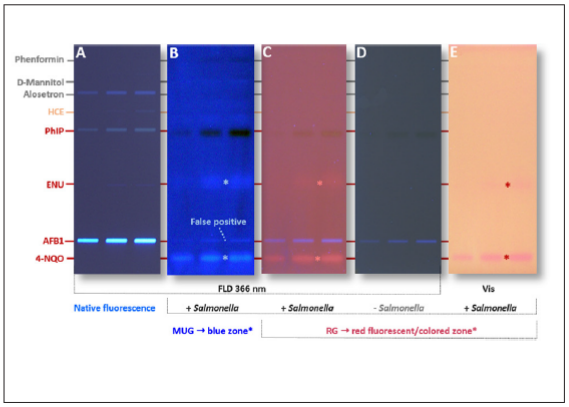


Fig. 6: Study on exchange of the enzyme substrate
Natively blue fluorescent compounds (false positive) afforded the change of the enzyme substrate (MUG substituted by RG) to improve the selectivity of the bioassay: (A) FLD image at 366 nm of 8 chemicals exhibiting different mechanisms of genotoxicity (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.

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.

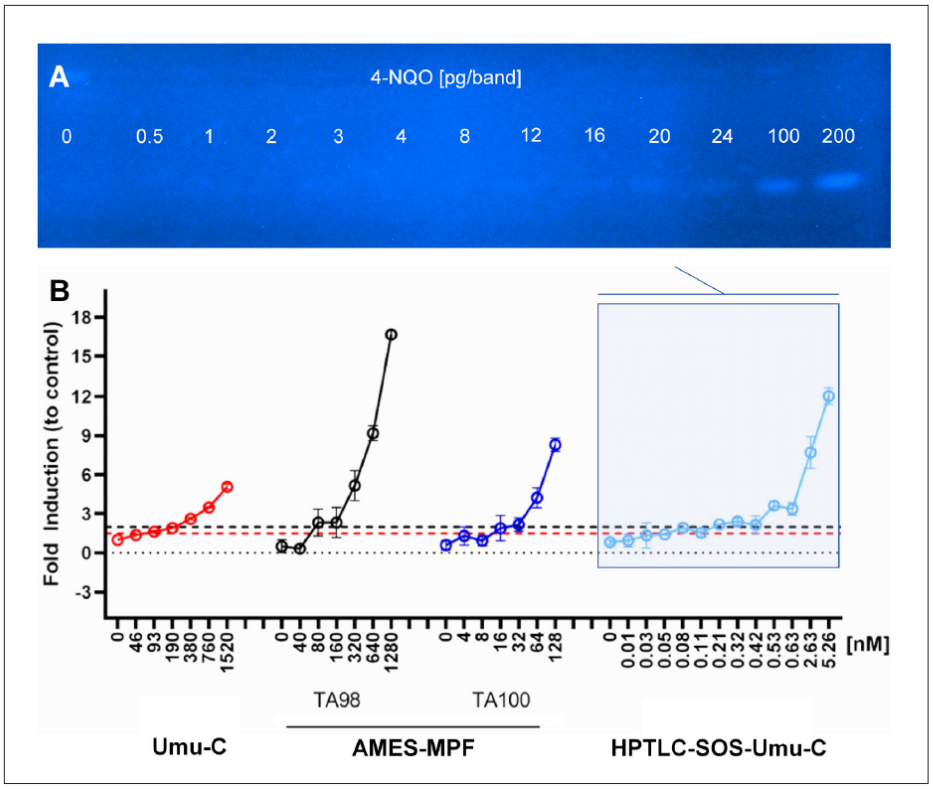


Fig. 7: Comparative LEC study using *in vitro* assays versus the new planar assay
(A) RP-HPTLC-SOS-Umu-C bioautogram at FLD 366 nm zoomed to the 4-NQO region of interest and for LEC determination, (B) comparison of the dose-response effects (nM) of 4-NQO via the SOS-Umu-C microtiter plate assay (threshold 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.

References

Baumann, U., Brunner, C., Pletscher, E. et al. (2003). Biologische Detektionsverfahren in der Dünnschichtchromatographie (Biological detection procedures using thin-layer chromatography). *UWSF – Z Umweltchem Ökotox 15*, 163-167. doi:10.1065/uwsf2001.12.080

Bjorseth, A., Eidsa, G., Gether, J. et al. (1982). Detection of mutagens in complex samples by the Salmonella assay applied directly on thin-layer chromatography plates. *Science 215*, 87-89. doi:10.1126/science.7031897

Bopp, S. K., Kienzler, A., Richarz, A.-N. et al. (2019). Regulatory assessment and risk management of chemical mixtures: Challenges and ways forward. *Crit Rev Toxicol 49*, 174-189. doi:10.1080/10408444.2019.1579169

EFSA Scientific Committee (2011). Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA J 9*, 2379. doi:10.2903/j.efsa.2011.2379

Egetenmeyer, N. and Weiss, S. C. (2017). Investigations for the detection of genotoxic substances on TLC plates. *J Liq Chromatogr Related Technol 40*, 69-74. doi:10.1080/10826076.2017.1284674

European Medicines Agency (1995). ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology. *CPMP/ICH/381/95*, 1-15. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf (accessed 06.11.2020)

European Parliament, Committee on the Environment, Public Health and Food Safety (2016). Report on the implementation of the Food Contact Materials Regulation ((EC) No 1935/2004) (2015/2259(INI)). A8-0237/2016, 1-16. https://www.europarl.europa.eu/doceo/document/A-8-2016-0237_EN.pdf

Flückiger-Isler, S. and Kamber, M. (2012). Direct comparison of the Ames microplate format (MPF) test in liquid medium with the standard Ames pre-incubation assay on agar plates by use of equivocal to weakly positive test compounds. *Mutat Res Fundam Mol Mech Mutagen 747*, 36-45. doi:10.1016/j.mrgentox.2012.03.014

Grob, K., Biedermann, M., Scherbaum, E. et al. (2006). Food contamination with organic materials in perspective: Packaging materials as the largest and least controlled source? A view focusing on the European situation. *Crit Rev Food Sci Nutr 46*, 529-535. doi:10.1080/10408390500295490

Hamer, B., Bihari, N., Reifferscheid, G. et al. (2000). Evaluation of the SOS/umu-test post-treatment assay for the detection of genotoxic activities of pure compounds and complex environmental mixtures. *Mutat Res Genet Toxicol Environ Mutagen 466*, 161-171. doi:10.1016/S1383-5718(00)00016-4

ISO – International Organization for Standardization (2000). Determination of the genotoxicity of water and waste water using the umu-test. https://www.iso.org/obp/ui/#iso:std:iso:13829:ed-1:v1:en:sec:A

Jamshidi-Aidji, M. and Morlock, G. E. (2018). Fast equivalency estimation of unknown enzyme inhibitors in situ the effect-directed fingerprint, shown for bacillus lipopeptide extracts. *Anal Chem 90*, 14260-14268. doi:10.1021/acs.analchem.8b03407

Kirkland, D., Kasper, P., Martus, H.-J. et al. (2016). Updated recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests. *Mutat Res Genet Toxicol Environ Mutagen 795*, 7-30. doi:10.1016/j.mrgentox.2015.10.006

Klingelhöfer, I. and Morlock, G. E. (2014). Sharp-bounded zones link to the effect in planar chromatography-bioassay-mass spectrometry. *J Chromatogr A 1360*, 288-295. doi:10.1016/j.chroma.2014.07.083

Klingelhöfer, I. and Morlock, G. E. (2015). Bioprofiling of surface/wastewater and bioquantitation of discovered endocrine-active compounds by streamlined direct bioautography. *Anal Chem 87*, 11098-11104.

Klingelhöfer, I., Hockamp, N. and Morlock, G. E. (2020). Non-targeted detection and differentiation of agonists versus antagonists, directly in bioprofiles of everyday products. *Anal Chim Acta 125*, 288-298. doi:10.1016/j.aca.2020.05.057

Koster, S., Boobis, A. R., Cubberley, R. et al. (2011). Application of the TTC concept to unknown substances found in analysis of foods. *Food Chem Toxicol 49*, 1643-1660. doi:10.1016/j.fct.2011.03.049

Koster, S., Rennen, M., Leeman, W. et al. (2014). A novel safety assessment strategy for non-intentionally added substances (NIAS) in carton food contact materials. *Food Addit Contam 31*, 422-443. doi:10.1080/19440049.2013.866718

Koster, S. Bani-Estivals, M., Bonuomo, M. et al. (2015). Guidance on best practices on the risk assessment of non-intentionally added substances (NIAS) in food contact materials and articles. *ILSI Europe Report Series*, 1-70.

Oda, Y. (2016). Development and progress for three decades in umu test systems. *Genes Environ 38*, 24. doi:10.1186/s41021-016-0054-8

Rainer, B., Pinter, E., Czerny, T. et al. (2018). Suitability of the Ames test to characterise genotoxicity of food contact material migrates. *Food Addit Contam Part A – Chem 35*, 2230-2243. doi:10.1080/19440049.2018.1519259

Rainer, B., Mayrhofer, E., Redl, M. et al. (2019). Mutagenicity assessment of food contact material migrates with the Ames MPF assay. *Food Addit Contam Part A – Chem 36*, 1419-1432. doi:10.1080/19440049.2019.1634841

Reifferscheid, G. and Heil, J. (1996). Validation of the SOS/umu test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. *Mutat Res Genet Toxicol 369*, 129-145. doi:10.1016/S0165-1218(96)90021-X

Schick, D. and Schwack, W. (2017). Planar yeast estrogen screen with resorufin-β-d-galactopyranoside as substrate. *J Chromatogr A 1497*, 155-163. doi:10.1016/j.chroma.2017.03.047

Schilter, B., Burnett, K., Eskes, C. et al. (2019). Value and limitation of in vitro bioassays to support the application of the threshold of toxicological concern to prioritise unidentified chemicals in food contact materials. *Food Addit Contam 36*, 1903-1936. doi:10.1080/19440049.2019.1664772

Shakibai, D., Riegraf, C., Moscovici, L. et al. (2019). Coupling high-performance thin-layer chromatography with bacterial genotoxicity bioreporters. *Environ Sci Technol 53*, 6410-6419. doi:10.1021/acs.est.9b00921

Stütz, L., Leitner, P., Schulz, W. et al. (2019). Identification of genotoxic transformation products by effect-directed analysis with high-performance thin-layer chromatography and non-target screening. *J Planar Chromatogr 32*, 173-182. doi:10.1556/1006.2019.32.3.1

Veyrand, J., Marin-Kuan, M. and Bezencon, C. et al. (2017). Integrating bioassays and analytical chemistry as an improved approach to support safety assessment of food contact materials. *Food Addit Contam 34*, 1807-1816. doi:10.1080/19440049.2017.1358466

Wöhrmann, U. (2019). *Entwicklung einer HPTLC-basierten Nachweismethode gentoxischer Substanzen durch Salmonella typhimurium*. Master thesis, Justus Liebig University Giessen.

Yüce, I., Mayr, M. and Morlock, G. E. (2019). Quantitative ink-jet application on self-printed, binder-free HPTLC layers for submicromole-scaled analytical 1H NMR spectroscopy. *Anal Chim Acta 1087*, 131-139. doi:10.1016/j.aca.2019.08.013

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.

Acknowledgements

Thank is owed to students Ulf Wöhrmann and Jasmin Bahmer for support during assay development at JLU. Instrumentation was partially funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – INST 162/471-1 FUGG; INST 162/536-1 FUGG. The JLU research project RDLS017728 (Application of HPTLC-coupled to bioassays system to address safety of complex food-related mixtures) was funded by Nestlé Research, Switzerland. The authors thank Alessandro Pistone, Ceritec SRL, Italy – Metlac Group for providing the R&D can coating material for the study.

Meyer et al.:

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

Supplementary Data

Tab. S1: Data of extracted/migrated amount
Data on extraction (IDs 35-37, 39 and 65) and migration (ID 64) samples; tin cans were weighed before and after extraction/migration

Tin can sample	Mass of extracted or migrated material (mg)	Amount (mg/dm^2)
ID 35	7.80	3.73
ID 36	12.16	5.82
ID 37	34.90	16.70
ID 39	34.90	16.70
ID 65	17.80	8.52
ID 64	8.00	3.83

Tab. S2: Data of the dose-response study (extract samples)
Data for the dose-response curves obtained by the new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay for the five different *n*-hexane – acetone extracts

Tin can sample	Correlation coefficient	Relative standard deviation [%]
ID 35	0.987	9
ID 36	0.995	6
ID 37	0.997	5
ID 39	0.988	8
ID 65	0.993	7
Mean	**0.992**	**7**

doi:10.14573/altex.2006201s

Tab. S3: Data of the dose-response study
Data for the repeated dose-response curves (n = 5) obtained by the new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay for the 95%-ethanol migration sample ID 64

Plates for ID 64	Correlation coefficient	Relative standard deviation [%]
1	0.990	8
2	0.998	5
3	0.995	7
4	0.989	11
5	0.990	9
Mean	**0.992**	**8**

Tab. S4: Data of LOBD/LOBQ study
Data on determination of the LOBD and LOBQ of 4-NQO in six spiked tin can coatings via the new RP-HPTLC-UV/Vis/FLD-SOS-Umu-C assay

Tin can ID	LOBD [pg/band]	LOBD [ng/L]	LOBD [nmol/L]	Mean LOBD [pg/band] Precision [*%RSD*]
64	13	67	0.35	**17** **16%**
35	16	32	0.17	
36	18	59	0.31	
37	21	71	0.37	
39	16	54	0.28	
65	15	50	0.26	
Tin can ID	**LOBQ [pg/band]**	**LOBQ [ng/L]**	**LOBQ [nmol/L]**	**Mean LOBD [pg/band] Precision [*%RSD*]**
64	40	202	1.06	**50** **16%**
35	49	98	0.51	
36	53	177	0.93	
37	65	215	1.13	
39	49	163	0.86	
65	45	150	0.79	

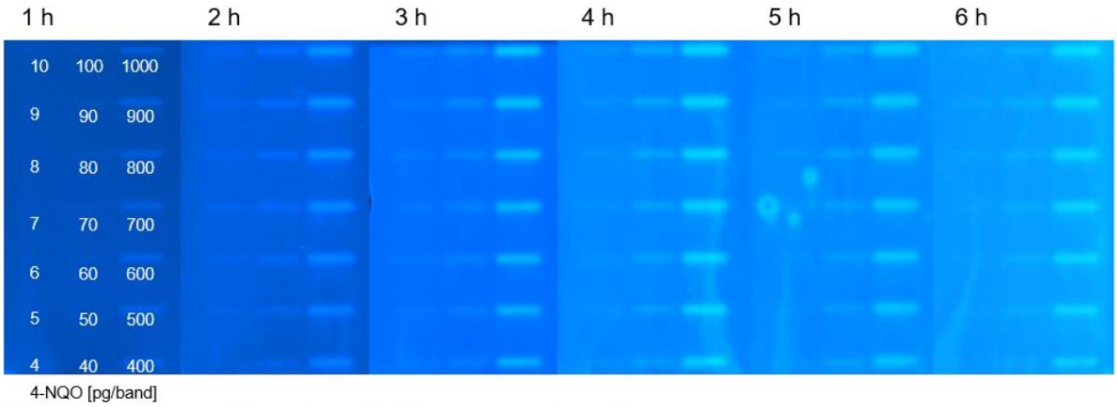


Fig. S1: Influence of incubation times of 1-6 h on response (images)
Planar SOS-Umu-C assay image at FLD 366 nm for different *Salmonella* incubation times of 1 h to 6 h on the adsorbent, all at OD_{660} of 0.2.

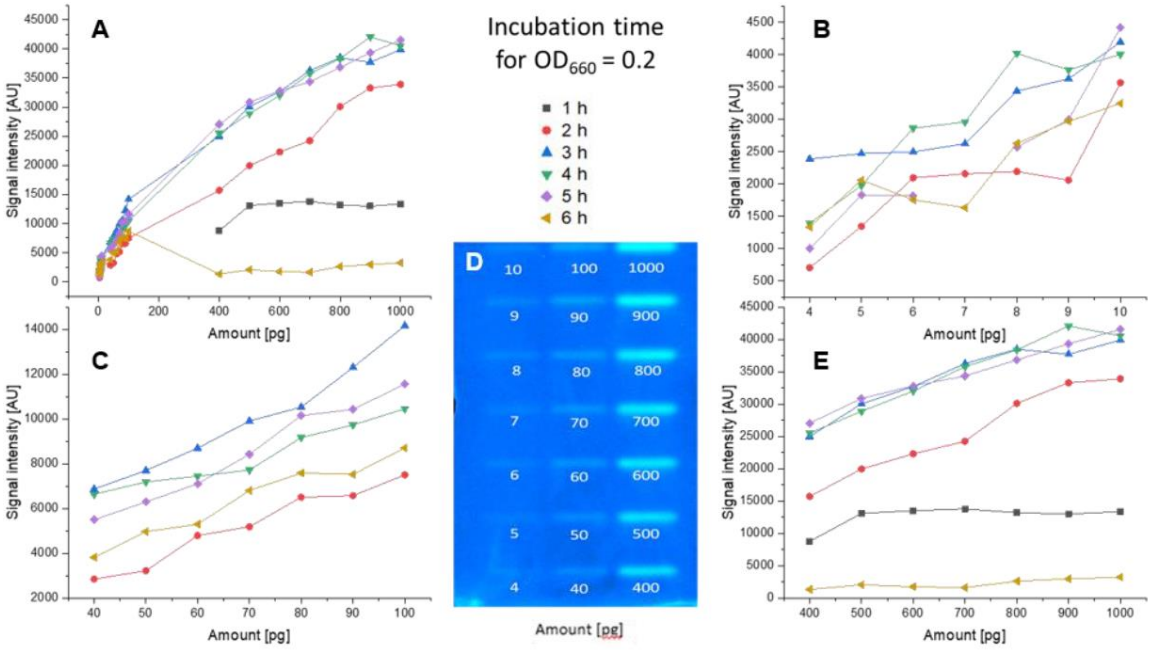


Fig. S2: Influence of incubation times on response for different amount ranges
Plots of signal intensities in densitograms at FLD 366/>400 nm of the planar SOS-Umu-C assay (image at FLD 366 nm after a 3-h incubation, D) against the applied 4-NQO amount for incubation times of 1-6 h (different colors): (A) 4-1000 pg/band region; zoom to the (B) 4-10 pg/band, (C) 40-100 pg/band and (E) upper 400-1000 pg/band region.

Fig. S3: Investigated tin cans with different coatings
FCM model studied consisting of five different tin can coatings (ID 35-37, 39 and 65), kindly provided for research purposes by the packaging supplier to Nestlé Research, Switzerland.

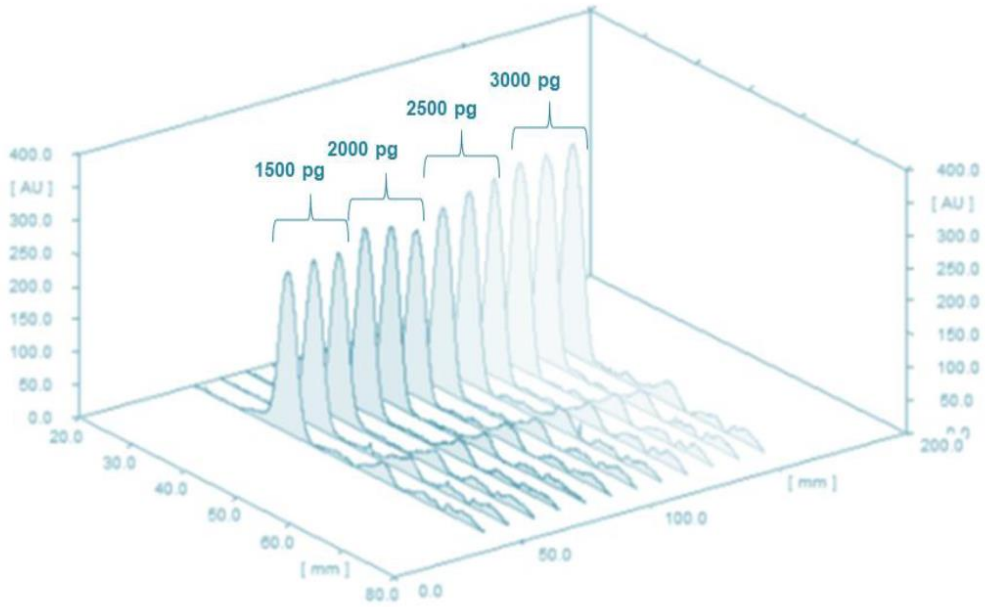


Fig. S4: Determination of the upper working range
3D densitogram (366/>400 nm) of the RP-HPTLC-SOS-Umu-C bioautogram for determination of the upper working range showing no substantial increase in signal intensity (peak area) above 1500 pg/band.

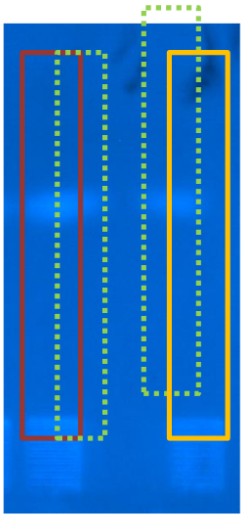


Fig. S5: Proof of absence of any matrix influence on separation
RP-HPTLC-SOS-Umu-C bioautogram at FLD 366 nm of a spiked (200 µL/area each, red box) *versus* original migrate sample ID 64 (orange box), partially oversprayed with 100 pg/area 4-NQO (green dotted box) showing no impact of the can matrix on the hR_F of 4-NQO at 48.

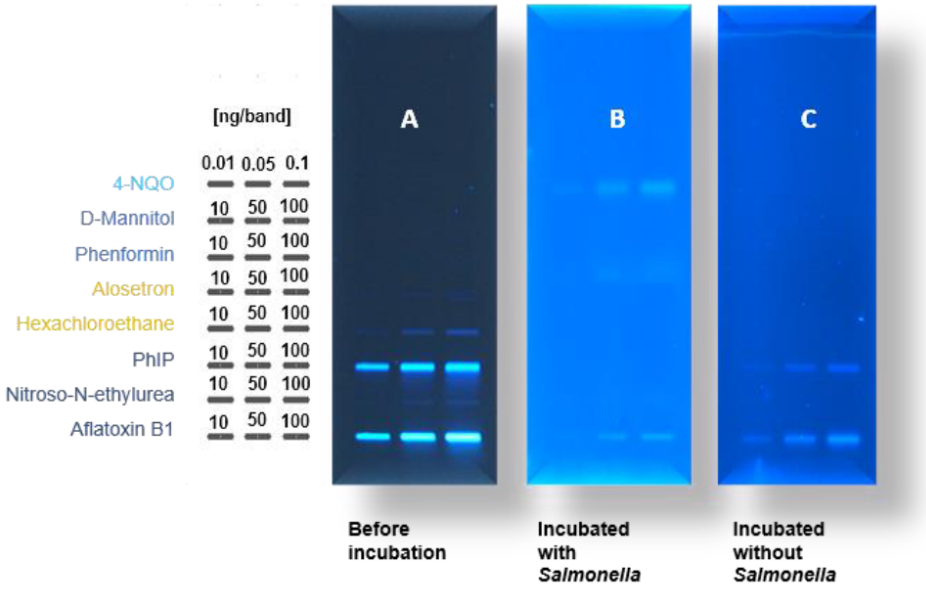


Fig. S6: Investigation of false positive responses
Images at FLD 366 nm showing fluorescent bands of the different substances, each applied on the HPTLC plate RP-18 W in three different amounts: (A) native fluorescence, (B) after the planar SOS-Umu-C assay and (C) same procedure as B but without *Salmonella* clearly identifies aflatoxin B1 as a false positive caused by its native fluorescence.

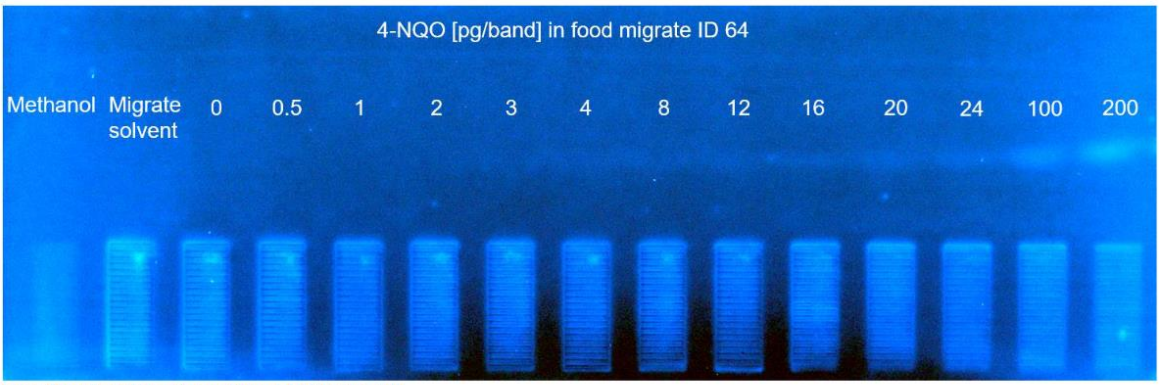


Fig. S7: Confirmation of the LEC in matrix
RP-HPTLC-SOS-Umu-C bioautogram at FLD 366 nm in matrix, showing food migrate ID 64 (200 µL each) spiked with 0 to 200 pg 4-NQO and applied as 7 mm x 20-mm area on the pretreated HPTLC RP-18 W plate (comparatively more cells settled down in the rills of the start area caused during spray-on application); it confirmed the LEC of 4-NQO in matrix (3 pg/band, 0.08 nM; experiment was performed twice).

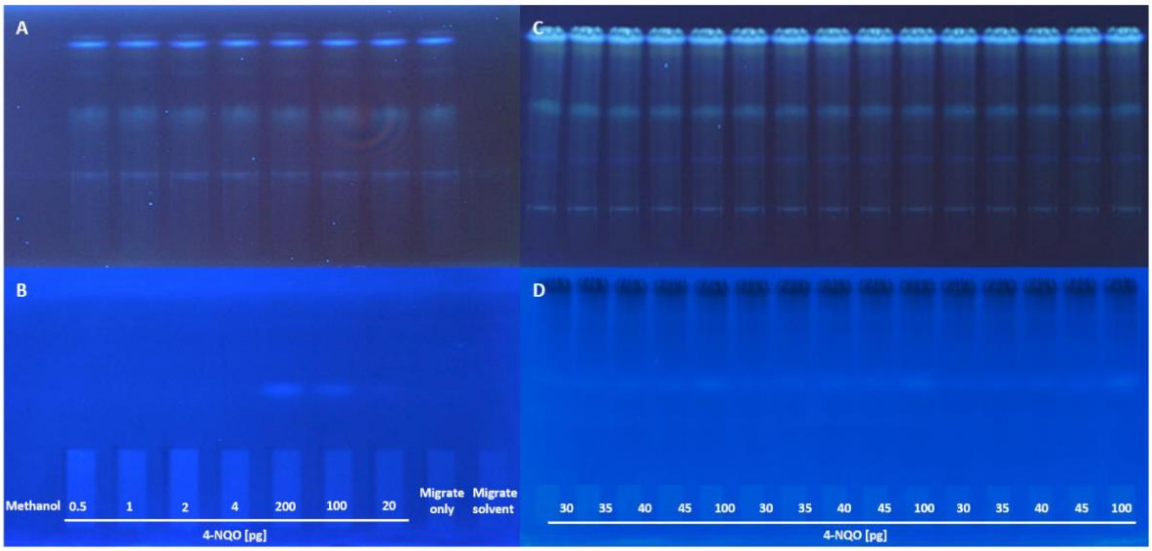


Fig. S8: Comparison of high and low level of migrated/extracted compounds
RP-HPTLC-chromatograms (A,C) and RP-HPTLC-SOS-Umu-C bioautograms (B,D) at FLD 366 nm, showing food migrate ID 64 (A,B; 200 µL each area) spiked with 0 to 200 pg 4-NQO and applied as 7 mm x 20 mm area (A,B), and food extract ID 39 (C,D; 500 µL each area) spiked with 30 to 100 pg 4-NQO and applied as 7 mm x 10 mm area on the pretreated HPTLC RP-18 W plate, together with respective negative controls (A,B). The migrate ID 64 is an example for the lowest level of migrated compounds (8 mg/can) and the extract ID 39 for higher levels of extracted compounds (35 mg/can, Tab. S1).

Eq. S1: Conversion of the units for the LEC determination.
Conversion of the amount on the HPTLC plate (pg/band) into nM, as used for the LEC determination experiment: 4-NQO was solved in methanol or migration sample. Twelve concentrations were prepared ranging from 0.5 to 200 pg/200 µL, whereby 200 µL of methanol or migration sample were applied, respectively. The following formula was used to calculate the nM concentration for each amount:

$$nM = \frac{m(4-NQO[pg])}{M(4-NQO[pMol])*200\,\mu L} * 5 * 1000 * \frac{1}{1000} = \frac{nMol}{L} \qquad \text{(Eq.S-1)}$$

with $\frac{m(4-NQO[pg])}{M(4-NQO[pMol]*200\,\mu L}$ = mass of 4-NQO in pg solved in 200 µL divided by the mol mass of 4-NQO in pMol, a factor of 5 taking into account the conversion from 200 µL to 1 mL, a factor of 1000 for the conversion of 1 mL to 1 L and a factor of 1/1000 to convert pmol into nmol.

6. Publication II

toxics

MDPI

Article

Incorporation of Metabolic Activation in the HPTLC-SOS-Umu-C Bioassay to Detect Low Levels of Genotoxic Chemicals in Food Contact Materials

Emma Debon [1], Paul Rogeboz [1], Hélia Latado [1], Gertrud E. Morlock [2], Daniel Meyer [2], Claudine Cottet-Fontannaz [1], Gabriele Scholz [1], Benoît Schilter [1] and Maricel Marin-Kuan [1,*]

[1] Food Safety Research Department, Société des Produits Nestlé SA—Nestlé Research, Vers-chez-les-Blanc, 1000 Lausanne, Switzerland
[2] Institute of Nutritional Science, Chair of Food Science, and TransMIT Center of Effect-Directed Analysis, Justus Liebig University Giessen, 35392 Giessen, Germany
* Correspondence: maricel.marin-kuan@rdls.nestle.com

Abstract: The safety evaluation of food contact materials requires excluding mutagenicity and genotoxicity in migrates. Testing the migrates using in vitro bioassays has been proposed to address this challenge. To be fit for that purpose, bioassays must be capable of detecting very low, safety relevant concentrations of DNA-damaging substances. There is currently no bioassay compatible with such qualifications. High-performance thin-layer chromatography (HPTLC), coupled with the planar SOS Umu-C (p-Umu-C) bioassay, was suggested as a promising rapid test (~6 h) to detect the presence of low levels of mutagens/genotoxins in complex mixtures. The current study aimed at incorporating metabolic activation in this assay and testing it with a set of standard mutagens (4-nitroquinoline-*N*-oxide, aflatoxin B1, mitomycin C, benzo(a)pyrene, *N*-ethyl nitrourea, 2-nitrofluorene, 7,12-dimethylbenzanthracene, 2-aminoanthracene and methyl methanesulfonate). An effective bioactivation protocol was developed. All tested mutagens could be detected at low concentrations (0.016 to 230 ng/band, according to substances). The calculated limits of biological detection were found to be up to 1400-fold lower than those obtained with the Ames assay. These limits are lower than the values calculated to ensure a negligeable carcinogenic risk of 10^{-5}. They are all compatible with the threshold of toxicological concern for chemicals with alerts for mutagenicity (150 ng/person). They cannot be achieved by any other currently available test procedures. The p-Umu-C bioassay may become instrumental in the genotoxicity testing of complex mixtures such as food packaging, foods, and environmental samples.

Keywords: hazard identification; in vitro genotoxicity; planar chromatography coupled with bioassay; food contact material; limit of biological detection; metabolic activation

Citation: Debon, E.; Rogeboz, P.; Latado, H.; Morlock, G.E.; Meyer, D.; Cottet-Fontannaz, C.; Scholz, G.; Schilter, B.; Marin-Kuan, M. Incorporation of Metabolic Activation in the HPTLC- SOS-Umu-C Bioassay to Detect Low Levels of Genotoxic Chemicals in Food Contact Materials. *Toxics* **2022**, *10*, 501. https://doi.org/10.3390/toxics10090501

Academic Editor: Panagiotis Georgiadis

Received: 28 July 2022
Accepted: 24 August 2022
Published: 27 August 2022

1. Introduction

The safety of complex chemical mixtures containing a substantial proportion of unidentified components is difficult to assess [1]. This is illustrated by food contact materials (FCMs) containing multiple chemicals that can potentially migrate into food and result in consumer exposure. Some of these substances are intentionally added for technological purposes. They are chemically and toxicologically well characterized. Others can be side products from manufacturing. They are referred as to non-intentionally added substances (NIAS). Among these, many may be unknown. Because not all NIAS require a thorough safety assessment, the key question is how to identify the most relevant substances on which toxicological investigations should be focused. In this context, detecting the presence of mutagenic/genotoxic/chemicals would be invaluable [2,3]. Several tests using either bacterial or mammalian cell models have been used to assess the mutagenicity/genotoxicity of FCM migrates, but in general, without establishing the suitability of the

test systems used to detect sufficiently low, safety-relevant, levels of mutagens. According to previous evaluations of the suitability of mammalian cell-based reporter gene assays targeting DNA-damage endpoints, these methods lack the analytical sensitivity to fully cover genotoxic effects [4]. The bacterial reverse mutation assay, known as the Ames assay test, using *Salmonella typhimurium* or *Escherichia coli* strains as indicator organisms for direct mutagenic effects, has been considered the best option to assess FCM, even though clear limitations have been highlighted, and it cannot be used as standalone method for evaluating the genotoxic potential of FCM migrates [3–7]. Indeed, the assumed high potency of genotoxic/mutagenic substances requires the use of high-performance tests to detect DNA-damaging substances at very low levels. This is not achievable by any of the currently available bioassays, including the Ames test [3–7]. In addition, the nature of the current assay formats does not allow for the elucidation of the chemical(s) responsible for the mutagenic/genotoxic activity of a mixture, consequently complicating risk assessment. Therefore, alternative methods are needed.

High-performance thin-layer chromatography (HPTLC) methods coupled with bioassays constitutes a very promising approach to address the limits of biological detection (LOBD). In addition, these methods provide a new, improved avenue to identify active chemicals [8,9]. HPTLC has been reported to provide an excellent separation, qualitative, and quantitative analysis of a wide range of compounds [10]. Important features of HPTLC are the application of large sample volumes and almost no solvent limitation due to evaporation before the development step and bioassay application. The chemical profiling of mixtures and the identification of bioactive bands directly from the bioautogram, with good detectability and reliability, was recently reported as eight-dimensional hyphenation [11]. The bacterial-based SOS-Umu-C bioassay was first applied on a TLC plate via a gauze-gel layer [12] and later, by spraying on the HPTLC plate [13]. In particular, the latest HPTLC coupled to SOS Umu-C (p-Umu-C) bioassay [8] has been shown as a promising solution to detect genotoxicity at low levels, e.g., the lowest effective concentration of 4-nitroquinoline-1-oxide (4-NQO) at the 20 pg/band, in complex samples, such as FCM migrates. Such a sensitive planar assay offers the potential for many further successful applications (e.g., cosmetics, new drugs, environmental and foods samples) [14]. As previously shown for environmental samples, the SOS-Umu-C test exhibits a strong concordance with the Ames test for the detection of genotoxic compounds [15,16], including mutagens.

However, the developed planar genotoxicity bioassays still suffer from important limitations. Foremost, they lack the integration of metabolic activation. Indeed, to fully assess the DNA-damage potential requires the evaluation of both parent compounds, as well as potential reactive metabolites. This addresses the fact that compounds may not be genotoxic as such and therefore, require biotransformation into reactive metabolites, for example, through cytochrome P450-mediated reactions. In in vitro test systems, metabolic activation is provided by the addition of the liver S9-fraction from rats treated with P450-inducers. Although the use of the S9-fraction has been shown to be applicable to planar assays [11,17,18], it has not been reported yet for HPTLC coupled with genotoxicity bioassays. Moreover, previously, the p-Umu-C has only been applied to a very limited number of chemicals [8]. The predictive capacity of any improved or new genotoxicity assay should be established with characterized reference substances covering different chemical classes and compared with standard tests addressing the same endpoints. Such data have not yet been reported for the p-Umu-C assay.

The main objective of this work was to incorporate the metabolic activation condition in the p-Umu-C bioassay. In addition, to address the predictive capacity of the new assay, 11 reference compounds [19] representing a broad spectrum of genotoxic mechanisms of action were tested in the presence and absence of metabolic S9-activation. To verify the potential of the p-Umu-C bioassay to detect low levels of genotoxic/mutagenic chemicals, the limits of biological detection (LOBDs) for each compound are reported and compared with those obtained in the Ames MPF test and the microtiter plate SOS Umu-C assay.

Additionally, the calculated LOBDs are interpreted from the perspective of the qualifications required to document negligible carcinogenic risks.

2. Materials and Methods

2.1. Chemicals and Materials

The bacteria strain *Salmonella typhimurium* TA1535, modified to contain the plasmid pSK1002 (PTM™ *Salmonella typhimurium* TA1535/pSK1002, cryostock), ampicillin, rat liver S9 fraction (phenobarbital/β-naphthoflavone), nicotinamide adenine dinucleotide phosphate (NADP), D-glucose-6-phosphate (G-6-P), and buffer salts solution (phosphate buffer, $MgCl_2$, KCl) were obtained from Xenometrix, Allschwil, Switzerland. Ethyl acetate, methanol, and toluene (all solvents of HPLC quality), lysogeny broth (LB), D-(+)-glucose, sodium hydrogen phosphate (Na_2HPO_4, ≥99.0%), sodium hydroxide, potassium dihydrogen phosphate (KH_2PO_4, ≥99.0%), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$, ≥98%), potassium chloride (KCl, ≥99.0%), 4-nitroquinoline-*N*-oxide (4-NQO, CAS N° 56-57-5), aflatoxin B1 (AFB1, CAS N° 1162-65-8), mitomycin C (MMC, CAS N° 50-07-7), benzo(a)pyrene (B(a)P, CAS N° 50-32-8), *N*-ethyl nitrourea (ENU, CAS N° 759-73-9), 2-nitrofluorene (2-NF, CAS N° 607-57-8), 7,12-dimethylbenzanthracene (DMBA, CAS N° 57-97-6), 2-aminoanthracene (2-AA, CAS N° 613-13-8), methyl methanesulfonate (MMS, CAS N° 66-27-3), melamine (CAS N° 108-78-1), D-mannitol (CAS N° 69-65-8), dimethyl sulfoxide (DMSO, CAS N° 67-68-5), and fluorescein-di-β-D-galactopyranoside (FDG, CAS N° 17817-20-8) (all Sigma-Aldrich), as well as HPTLC silica gel 60 plates were purchased from Merck, Darmstadt, Germany. The plates were prewashed by elution with methanol (twin trough chamber 20 cm × 10 cm, CAMAG, Muttenz, Switzerland) up to the plate top, heated at 110 °C for 15 min (Plate Heater III, CAMAG, Muttenz, Switzerland), and stored protected from light until use.

The Umu-C assay, including 4-NQO, 2-AA, B-buffer, stop reagent, *ortho*-nitrophenyl-β-D-galactoside (ONPG, CAS N° 369-07-3), and *Salmonella typhimurium* TA1535[psK1002], and the AMES MPF assay, including the strains TA98[pKM101, hisD3052], and TA100[pkM101, hisG46], as well as the reagents and other components, were provided by Xenometrix. The BacTiter-Glo™ Microbial Cell Viability Assay (#G8230) was obtained from Promega, Dübendorf, Switzerland.

2.2. Standard Solutions and Buffers

Each stock solution was prepared at 100 mM in DMSO. Standard solutions were obtained by dilution with methanol: AFB1 at 10 and 100 pg/µL; B(a)P and MMC at 1 and 10 ng/µL; 4-NQO at 1, 10, and 100 pg/µL; ENU at 1, 10, and 100 ng/µL; MMS, D-mannitol, and melamine at 100 ng/µL; 2-NF at 0.1, 1, and 10 ng/µL; 2-AA at 10 ng/µL; and DMBA at 10 and 100 ng/µL. Phosphate buffer was prepared in purified water (H_2O) using 40.8 g/L KH_2PO_4, 42.6 g/L Na_2HPO_4, 1.2 g/L $MgSO_4 \cdot 7\ H_2O$, and 3.7 g/L KCl, adjusted to pH 7 with solid sodium hydroxide.

2.3. Salmonella Typhimurium Culture

To perform the p-Umu-C assay and the liquid SOS-Umu-C assays, first, an overnight (ON) culture of the *Salmonella typhimurium* TA1535[psK1002] strain is started using 10 mL of LB medium (20 g/L LB, 1 g/L D-glucose and 50 mg/L ampicillin) inoculated with 100 µL of *Salmonella typhimurium* TA1535. The incubation is performed in a 50 mL Greiner Bio-One CellStar cell reactor tube (VWR International, Dietikon, Switzerland) for 10 h at 37 °C and 250 rpm in a shaker (Thermo Scientific digital CO_2 resistant microplate shaker, Reinach, Switzerland). The ON incubation time of 10 h is ensured with the installation of an LED with a timer control device (ThebenHTS, theben-timer 26, Effretikon, Switzerland) in the incubator. The next day, an aliquot of the ON culture is recovered to measure the optical density (OD_{600}) of the culture. The OD_{600} should be between 2.0 and 3.0 (JENWAY 6300 Spectrophotometer, Staffordshire, UK).

2.4. Chromatography

Standard solutions were applied as bands onto prewashed HPTLC silica gel 60 plates with the following settings: band length—8 mm, dosage speed—80 nL/s, application volume—between 1–8 µL, syringe installed—10 µL. The development was performed with a mixture of toluene—ethyl acetate, 2:3 (v/v) up to 80 mm after pre-conditioning with toluene, with a pump power of 40% for 150 s, followed by drying for 5 min. For the MMC, the mobile phase was toluene—ethyl acetate—methanol 2:1:1 ($v/v/v$), up to 80 mm, without preconditioning. The humidity was controlled at 0% during both elutions using a molecular sieve. The application and elution were performed using HPTLC PRO (CAMAG). The bioautograms were documented (TLC Visualizer 2, CAMAG, Muttenz, Switzerland) at fluorescence light detection (FLD) 254 nm and 366 nm, along with white light illumination.

2.5. Planar Umu-C Bioassay

The ON culture described above was subdivided by 1:7.5 dilution with LB medium and incubated at 37 °C and 150 rpm for 2 h. This bacterial culture of 70–80% of the initial OD_{600} was centrifuged (3000× g, 10 min) and re-suspended in LB medium to obtain a *Salmonella* suspension with an OD_{600} of 0.2. The bacteria suspension was sprayed onto the plate using the Chromajet DS20 (Biostep, Burkhardtsdorf, Germany) as follows: reagent quantity—4.01 mL, spray cycles–3, width—200 mm, length—100 mm. The plate was incubated in a dark plastic box with nearly 100% relative humidity for 3 h at 37 °C and dried for 4 min in a stream of cold air. For detection of the β-galactosidase activity, a phosphate buffer (10 mL) containing FDG (100 µL, 5 mg/mL in DMSO/H_2O 1:1, v/v) was sprayed, as before. After 15 min incubation in a dark plastic box with 100% relative humidity at 37 °C, the fluorescence of the fluorescein was measured at 485/>500 nm (tungsten lamp, TLC Scanner 3, CAMAG, Muttenz, Switzerland)). The bioautograms were documented at a fluorescence light detection (FLD) of 254 nm and 366 nm, along with white light illumination.

2.6. Metabolic S9-Activation

For the test condition in presence of metabolic activation, a mixture of bacteria suspension and S9-mix (0.5 mL, containing, according to the manufacturer, 1.916 mL buffer salts, 0.084 mL G-6-P, and 0.332 mL NADP, *i.e.*, 18% S9) was sprayed, as previously described.

For the S9 optimization, different amounts of AFB1 from 10 to 800 pg/band, a methanol solvent control, and 4-NQO (500 pg/band) as a positive control were applied on three different plates. Therefore, three S9-mixtures containing *Salmonella* suspensions (OD = 0.2) were prepared by adding 0.1, 0.5, and 1 mL S9 (1%, 5% and 10% S9 respectively), to bacteria suspension to obtain 10 mL of the mixture, following the protocol as described.

2.7. SOS Umu-C Assay Protocol

The ON culture was prepared as described. The assay was performed according to the ISO guideline [20], as stated by Xenometrix, with minor modifications. Briefly, the bacterial culture of 70–80% of the initial OD_{600} was used for the assay. For each well of the 96-well plate (Thermo Fisher Scientific, Roskilde, Denmark), test substances, positive (4-NQO at 0.5 µg/mL in the absence, and 2-AA at 2 µg/mL in the presence, of metabolic activation) and negative controls (only bacteria suspension) were tested in biological triplicates. Bacteria culture was added to each well and mixed with the samples and controls. For the metabolic activation, a mixture of 30% S9-mix in bacteria culture was added to each well instead. The two plates were incubated at 37 °C and 150 rpm for 2 h. Then, bacteria were diluted 10 times in a new plate with fresh media. The absorbance at 600 nm was measured. The plates were incubated for another 2 h under the same conditions. Bacteria were again diluted ten times in a new plate, mixed with a mixture of B-Buffer/ONPG, and incubated for 30 min at 37 °C and 150 rpm. After adding a stop

reagent into each well, the absorbance at 420 nm was measured to evaluate the conversion rate of β-galactosidase.

2.8. Liquid Ames MPF Protocol

The liquid Ames MPF method was applied, as recommended by the supplier [21]. Briefly, overnight grown *Salmonella* bacteria strains TA-98 for frameshift mutations and TA-100 for point mutations were exposed, in medium containing histidine in 24-well plates, to compounds at increasing concentrations in the presence or absence of metabolic activation at 37 °C for 90 min. Then, bacteria were diluted into a pH indicator (bromocresol purple) medium lacking histidine using 384-well plates and incubated at 37 °C for 48 h. The bromocresol purple turned yellow as the pH dropped due to the catabolic activity of revertant cells, which grew in the absence of histidine. The number of wells containing revertant colonies were counted and compared to the solvent control (DMSO). The cytotoxicity of the compounds tested in the AMES MPF test was estimated with the BacTiter-Glo™ Microbial Cell Viability assay by adding a single reagent directly to the medium containing exposed bacteria, measuring the luminescence. The luminescent signal is proportional to the amount of the ATP present, which is directly proportional to the number of viable cells in the culture.

2.9. Data and Statistical Analysis

The HPTLC biodensitograms were evaluated considering the peak height (visionCATS 3.0 software, CAMAG, Muttenz, Switzerland). Data were analyzed using the average of triplicates performed for each pure compound, considering the dose–response effect. Twenty peaks in the solvent control track were used as the mean blank. The results were expressed as induction ratio (*IR*) determined by the average of the normalized peaks. The *IR* was calculated according to Equation (1), where H_n is the peak height for the three compound replicates, and AVGb + Sd is the average peak height plus standard deviation of 3 blanks at a similar hR_F to the analyte.

$$IR = \left(\left(\frac{\text{H1}}{\text{AVGb} + \text{Sd}}\right) + \left(\frac{\text{H2}}{\text{AVGb} + \text{Sd}}\right) + \left(\frac{\text{H3}}{\text{AVGb} + \text{Sd}}\right)\right)/3 \quad (1)$$

The data obtained were evaluated according to the guidelines of the International Conference on Harmonization [22]. The experiments were conducted in biological triplicates for each compound, in the presence and absence of metabolic activation. The repeatability was expressed as the relative standard deviation of peak height (%RSD) calculated using the ratio between the standard deviation and the average of the triplicates. The linearity was fitted with the first five doses of the dose–response curve, confirming the coefficient of determination for acceptable linear relationship ($r^2 \geq 0.99$).

For the liquid Umu-C assay, biological triplicates were performed. The relative units (RU) for each replicate were obtained at OD_{600} for the growth factor (*G*), and OD_{420} for the IR. Data were analyzed using the average of the triplicates performed for each compound and the corresponding standard deviation. The quality criteria to classify a sample as genotoxic with respect to the blank and negative controls is a $G \geq 0.5$ and $IR \geq 1.5$, as recommended by the supplier.

For the liquid Ames MPF assay, biological triplicates were performed. Data were analyzed using the proprietary Xenometrix Calculation Sheet Version 3.23u 4/2017. Briefly, the mean number of positive wells (yellow) out of 48 wells per replicate and dose was compared with the number of spontaneous revertant wells 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 a test chemical. Quality controls were applied for assay validity, considering concentrations with FI $\geq$ 2.0 (for TA100) and FI $\geq$ 3.0 (for TA98) as mutagenic concentrations.

The limit of detection (LOD) and the limit of quantification (LOQ) of selected compounds were calculated to determine the lowest activity that can be detected with acceptable

precision (LOD) and that can be quantitated with a degree of certainty (LOQ), according to the International Conference on Harmonization (ICH) [22] guidelines using the three- and ten-fold standard deviation of blank peak heights divided by the slope of the regression line. The lowest effective concentration (LEC) is the lowest concentration with an IR above the 2-fold threshold, according to supplier's recommendation. To ensure comparability with the LECs of the Umu-C microtiter and AMES MPF bioassays at $IR \geq 1.5$ and 2.0, respectively, the LEC of the HPTLC-SOS-Umu-C bioassay was set as the lowest concentration with an IR above 2.0. Data graphs were produced using GraphPad Prism 9.0.0 (GraphPad Software LLC, San Diego, CA, USA).

The limit of biological detection (LOBD) was applied, as previously reported [3,6]. This concept proposes the consideration of not only the above analytical detection capability, but also the contribution of the conditions applied for sample preparation and bioassay exposure, including the migration protocol, migrate concentration, and final exposure to the bioassays. This approach considers the concentration and dilution factors during sample preparation and the corresponding factor applied to the LEC. In the case of the Ames test, this corresponds to a concentration factor of 40× for 1 L of migrate concentrated 1000×, followed by a 25× dilution during cell treatment. To allow for direct comparisons, a maximum 1000× migrate concentration factor (1 L migrate concentrated 1000× to 1 mL directly loaded on the HPTLC plate), along with the measured LECs, was considered for HPTLC-SOS-Umu-C.

The target limit of biological detection (tLOBD) is defined as the LOBD required to comply with a predefined level of health risk (carcinogenicity). The tLOBD was estimated, considering the total daily dose calculated to produce an excess carcinogenic risk of 1 in 100,000 applied to 1 kg of packaged food for a 60 kg adult [3].

2.10. Selection of Reference Compounds

The developed method was applied to 11 pure compounds to demonstrate the repeatability and the validity of the protocol. To avoid false positive results, the selection of the test substances was based on the recommendations regarding chemicals that would be appropriate for evaluating the sensitivity and specificity of the genotoxicity test [19]. Based on those recommendations, for the selection of compounds for the HPTLC coupled with the Umu-C assay, the following criteria were applied: applied DNA-damage with diverse mechanisms of actions, including direct DNA-binding, indirect DNA-damage, and negative controls (Table 1).

Table 1. Selected reference compounds with different mechanisms of action.

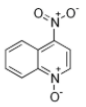
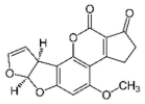
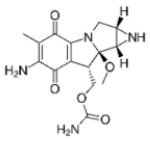
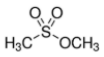
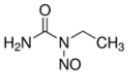
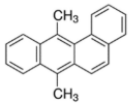
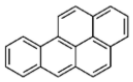
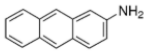
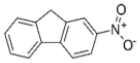
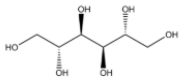
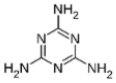
Compounds	CAS No.	Chemical Structure	Comments	Classification
4-Nitroquinoline-*N*-oxide (4-NQO)	56-57-5		Alkylating agent, forms DNA adducts	In-vivo genotoxins which should be detected with positive response in in-vitro genotoxicity tests
Aflatoxin B1 (AFB1)	1162-65-8		Requires metabolic activation; forms various adducts	
Mitomycin C (MMC)	50-07-7		Inducing DNA–DNA crosslinks and oxidative damage; alkylating activity	

Table 1. *Cont.*

Compounds	CAS No.	Chemical Structure	Comments	Classification
Methyl methanesulfonate (MMS)	66-27-3		Strong clastogen	
N-ethyl-nitrourea (ENU)	759-73-9		Strong gene mutation	
7,12-Dimethylbenzanthracene (DMBA)	57-97-6		Requires metabolic activation. Forms bulky adducts	
Benzo(a)pyrene (B(a)P)	50-32-8		Requires metabolic activation; forms bulky adducts	
2-Aminoanthracene (2-AA)	613-13-8		Requires metabolic activation; forms bulky adducts [23]	
2-Nitrofluorene (2-NF)	607-57-8		Forms DNA adducts [24]	
D-mannitol (D-man)	69-65-8		*n/a*	Non-DNA reactive chemicals that should give negative results in in-vitro genotoxicity tests
Melamine	108-78-1		*n/a*	

3. Results

3.1. Incorporation of a Metabolic Activation Step

The incorporation of a metabolic activation step in the latest HPTLC-genotoxicity bioassay [8] was undertaken by spraying a mixture of bacteria and rat liver S9-fraction, containing P450 enzymes and required cofactors, directly onto the chromatogram. The ratio between the S9 and the bacteria was investigated to obtain optimal genotoxic signals. Biological triplicates of S9-*Salmonella* preparations, with fixed amounts of bacteria but increasing levels of S9-mix, corresponding to final concentrations of 1%, 5%, and 10%, were evaluated on plates loaded with AFB1 (requiring S9-activation) and 4-NQO (inactivated by S9). With AFB1, no response was visible on the bioautograms and densitograms of the plates sprayed with 1% S9 mixture (Figure 1A). However, clear dose-dependent signals were observed at higher S9 concentrations, with the best response obtained at 10%. With the standard control 4-NQO, a signal was found on the plate sprayed with 1% S9-mix, but not at higher S9-concentrations, indicating a metabolic inactivation at the dose tested (Figure 1B,C).

To confirm these results and to assess the repeatability of the test, dose-response curves in the absence (−S9) and presence (+S9, 10%) of metabolic activation were generated in biological triplicates. As expected for the AFB1 (10−800 pg/band; Figure 2), a dose-dependent genotoxic response was only observed in the presence of metabolic activation. A genotoxic signal for 4-NQO (1–64 pg/band without S9, 10–800 pg/band; Figure 3) was detected in both the absence and presence of the S9-mix. However, for this chemical, the dose–response curves obtained in the presence of metabolic activation were clearly shifted towards the right, confirming the S9-dependent inactivation. With both substances, the results were highly reproducible. The LECs in the presence of metabolic activation for AFB1 and

4-NQO were determined at 25 (0.08 pmol) and 250 pg/band (1.3 pmol), respectively. The LEC of 4-NQO in the absence of metabolic activation was determined at 16 pg/band (0.08 pmol).

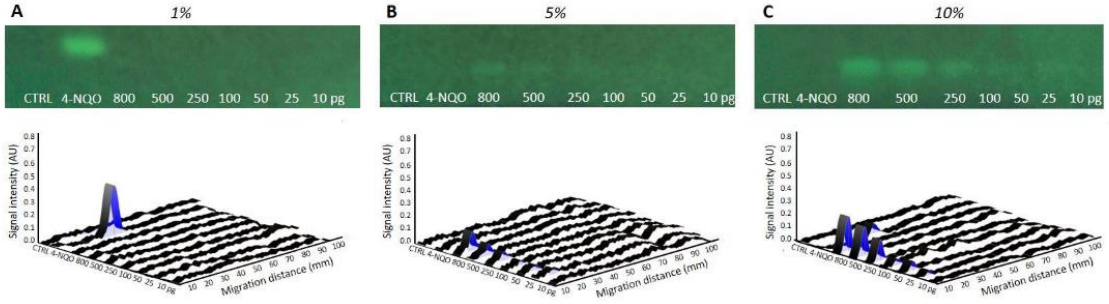


Figure 1. Bioautogram at FLD 254 nm showing the region of interest for optimization of the metabolic activation varying percentage (%) of mixtures of the S9 fraction, with co-factors, at (**A**) 1%, (**B**) 5%, and (**C**) 10%. Biodensitograms at 485/>500 nm of AFB1 (10−800 pg/band), along with the positive control 4-NQO (500 pg/band) and methanol solvent control (CTRL, 10 µL/band).

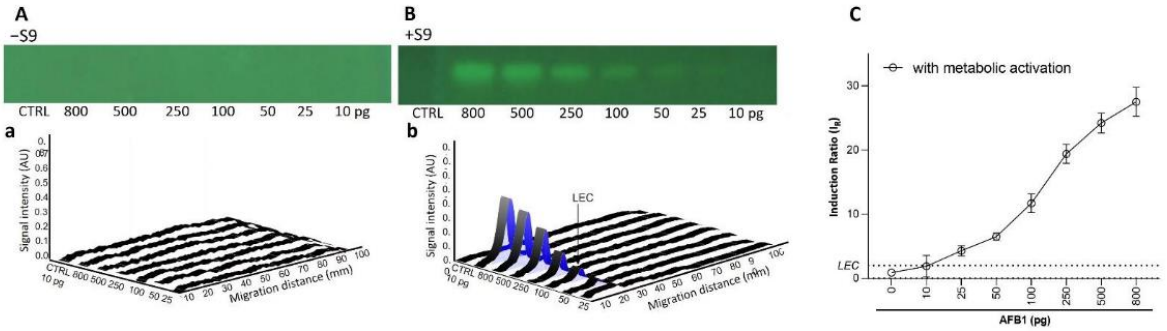


Figure 2. Dose–response effect for AFB1. Bioautograms at FLD 254 nm (region of interest shown) in absence (−S9) (**A**) or presence (+S9) (**B**) of metabolic activation, with respective biodensitograms at 485/>500 nm (a and b). Dose–response curves with LEC (black dotted line at IR 2.0) (**C**).

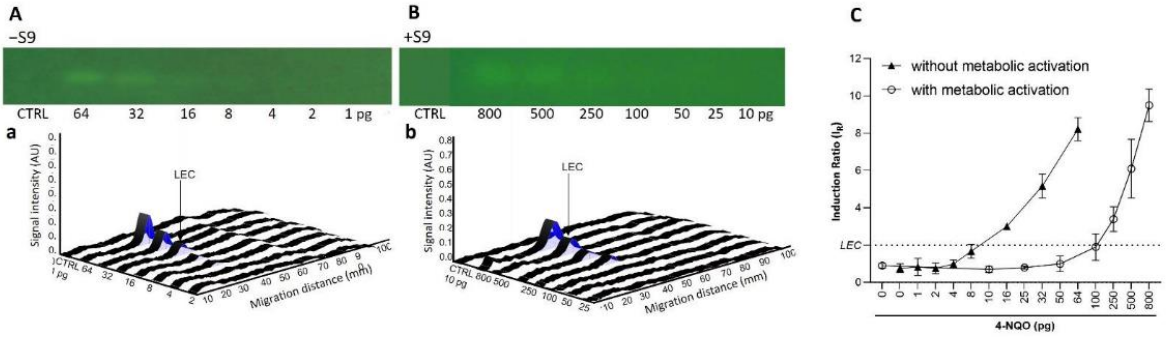


Figure 3. Dose–response effect for 4-NQO. Bioautograms at FLD 254 nm (region of interest shown) in absence (−S9) (**A**) or presence (+S9) (**B**) of metabolic activation, with respective biodensitograms at 485/>500 nm (a and b). Dose–response curves with LEC (black dotted line at IR 2.0) (**C**).

D-mannitol and melamine (100−800 ng/band) were selected as negative controls to demonstrate the specificity of the test. As expected, no genotoxic response was ob-

served in the corresponding bioautograms and densitograms for both substances (Figure 4). The expected responses were observed with AFB1 and 4-NQO, at 500 and 800 pg/band respectively, confirming the validity of the test.

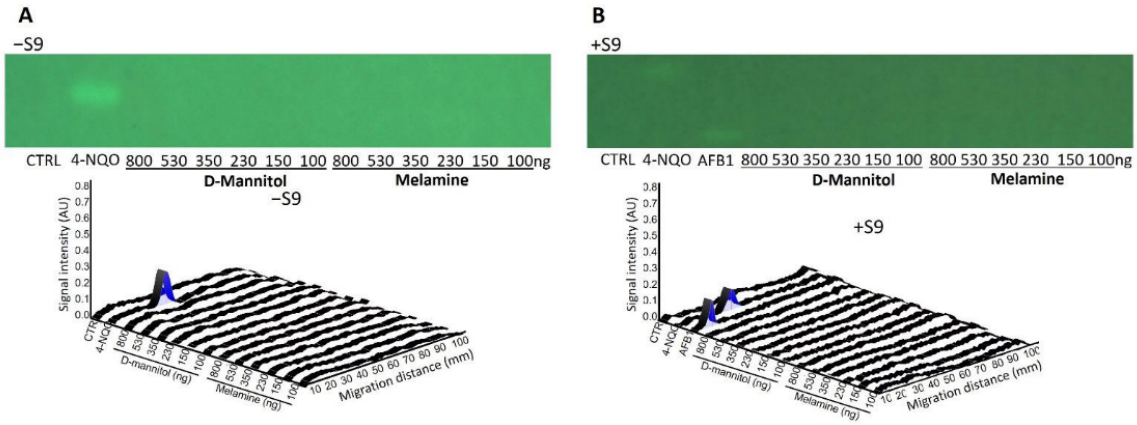


Figure 4. Bioautograms at FLD 254 nm (region of interest shown) and biodensitograms at 485/>500 nm showing the negative controls D-mannitol, melamine (100−800 ng/band each), and with the positive control 4-NQO (500 pg/band) (**A**) in absence (−S9) and (**B**) in presence (+S9) of metabolic activation.

3.2. Performance of the HPTLC-SOS-Umu-C Assay

Seven additional compounds (Table 1) were selected to further demonstrate the predictive value of the full p-Umu-C test protocol, in the presence and absence of the S9-mix. The selection was based on reported ECVAM recommendations for validation of new or modified genotoxicity tests [19], including chemicals causing DNA damage through diverse mechanisms of actions. The dose–response effects were studied in the presence and absence of metabolic activation and in triplicates (Figure 5A; one representative bioautogram replicate is shown). The applied amount was adjusted according to responses obtained in preliminary experiments (1−64 ng/band for MMC, ENU, and B(a)P; 25–800 ng/band for DMBA; 15–75 ng/band for 2-AA; 1–64 and 0.25–16 ng/band for 2-NF). The obtained fluorescence signals were normalized to the solvent control background signal to calculate the induction ratio and LEC (Figure 5B). A clear dose–response effect was observed for six test compounds in the presence and absence of metabolic activation. The genotoxic response of 2-AA was weak; however, an inhibitory dark signal was noticed on the bioautogram, possibly reflecting bacterial toxicity. B(a)P and DMBA both showed positive signals in the absence and presence of metabolic activation.

Variable band diffusion was observed, possibly complicating the data analysis. This is illustrated by the data obtained with MMS in both the presence and absence of metabolic activation (Figure 6). Despite the impaired band visualization on the bioautograms at 254 nm, the dose–response curves were still usable in the respective densitograms of the fluorescence measurement at 485/>500 nm. Measuring the peak area instead of the height did not provide any improvement, significantly increased variability.

The data obtained were evaluated according to the guidelines of the International Conference on Harmonization [22]. The experiments were conducted in biological triplicates for each compound, in the presence and absence of metabolic activation. The data were used to establish LODs, LOQs, and method repeatability (Table 2). The repeatability was expressed as the relative standard deviation of peak height (*%RSD*) calculated using the ratio between the standard deviation and the average of the triplicates. The linearity was fitted with the first five doses of the dose–response curve, confirming the acceptable linear relationship ($r^2 \geq 0.99$), with two exceptions ($r^2 \geq 0.96$ for 2-AA and MMS) (data not shown). In the presence of metabolic activation, the *%RSD* of AFB1 was 12%, and of

DMBA, ENU, 2-NF, 2-AA, and B(a)P, up to 20%. The *%RSD* was 23% for 4-NQO, and due to diffusion, 38% for MMS. In the absence of metabolic activation (AFB1 excluded), the *%RSD* was 24%, 20%, and 18% for 4-NQO, DMBA, and ENU, respectively, and approximately 30% for the other compounds. The acceptance criteria established by the ICH guidelines is $\%RSD \leq 20\%$, suggesting an overall acceptable performance of the HPTLC SOS Umu-C assay. However, some exceptions were observed for MMC and MMS, which are more related to the analytes' features and not necessarily to the performance of the assay.

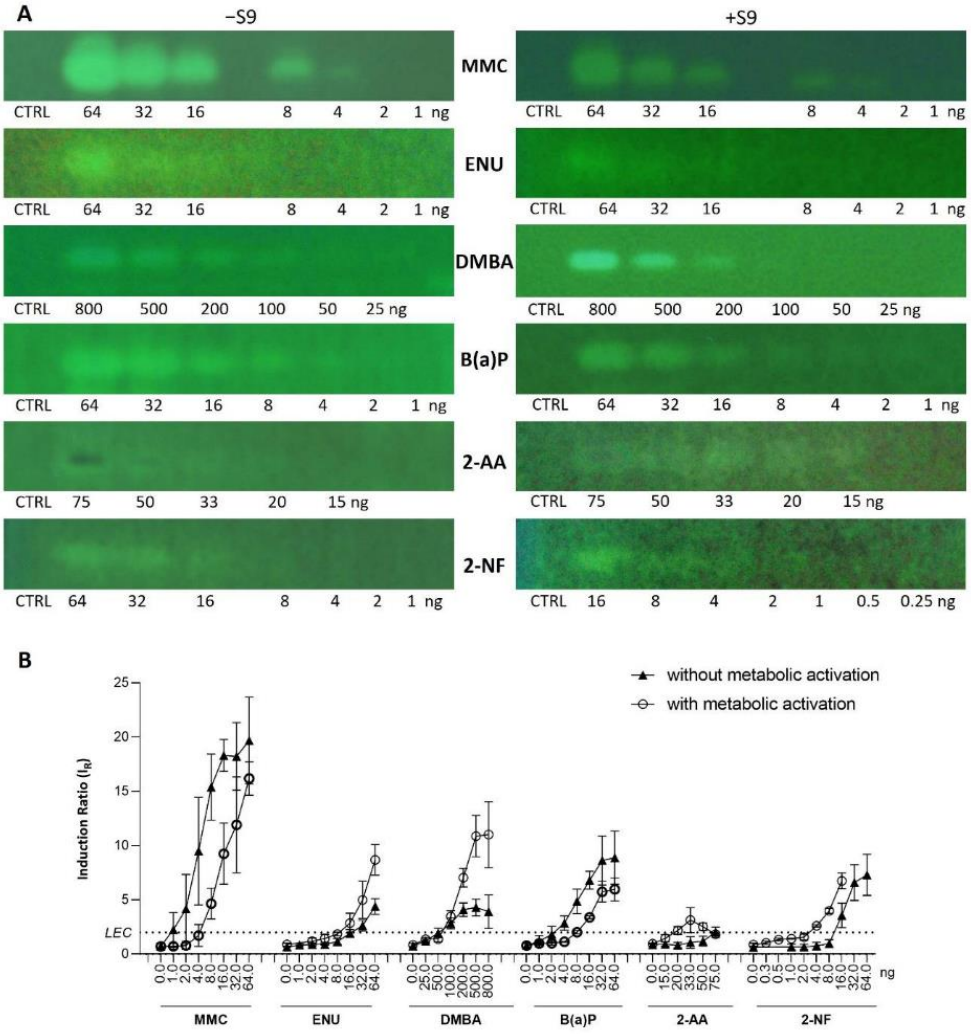


Figure 5. Dose–response study extended to 6 compounds (Table 1) in absence (−S9) and presence (+S9) of metabolic activation, along with methanol solvent control (CTRL). (**A**) Bioautograms at FLD 254 nm (region of interest shown) and (**B**) dose–response curves with LECs (black dotted line at IR 2.0).

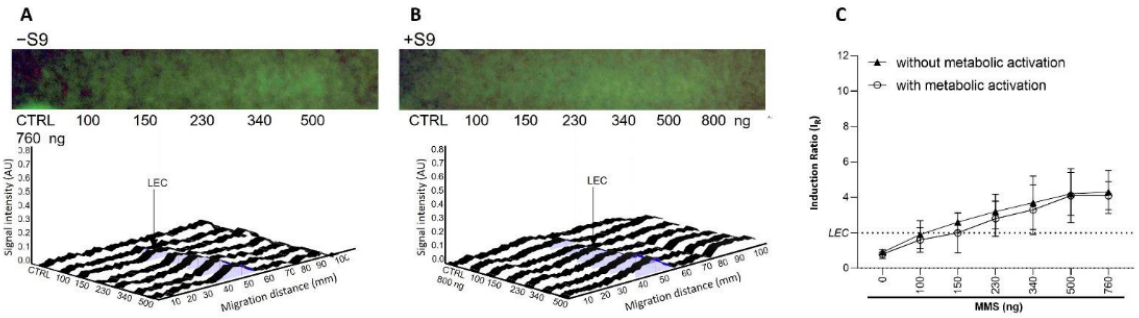


Figure 6. Dose–response study for MMS (100–760 ng/band) (**A**) in absence (−S9) and (**B**) in presence (+S9) of metabolic activation. Bioautograms at FLD 254 nm (region of interest shown), biodensitograms at 485/>500 nm and (**C**) dose–responses curves with LEC (black dotted line at IR 2.0).

Table 2. Performance data for 9 reference compounds tested in the HPTLC-SOS-Umu-C assay in the presence and absence of metabolic activation.

Substances	Without Metabolic Activation						With Metabolic Activation					
ng/band	LEC	LOD	LOQ	Linearity Dose Range	r^2	% RSD	LEC	LOD	LOQ	Linearity Dose Range	r^2	% RSD
4-NQO	0.016	0.006	0.021	0.001–0.016	0.997	17	0.25	0.056	0.186	0.01–0.25	0.996	23
AFB1	–	–	–	*n/a*	*n/a*	–	0.025	0.006	0.022	0.01–0.25	0.994	17
MMC	1.0	0.40	1.32	1–16	0.991	33	4	1.19	3.95	1–16	0.997	35
MMS	150	119.74	399.14	100–500	0.966	29	230	116.49	388.30	100–500	0.986	33
ENU	16	8.70	28.99	1–16	0.992	18	16	6.97	23.24	1–16	0.992	19
DMBA	50	37.48	124.94	25–500	0.992	20	100	33.35	111.15	25–500	0.995	16
B(a)P	4	1.42	4.72	1–16	0.993	28	8	3.37	11.24	1–16	0.993	19
2-AA	75	37.23	124.10	15–75	0.961	32	20	14.73	49.12	15–75	0.986	17
2-NF	16	5.66	18.87	1–16	0.995	32	4	2.14	7.13	0.25–4	0.994	9

(−): negative in absence of metabolic activation; LEC: lowest effective concentration; LOD: limit of detection; LOQ: limit of quantification; (% *RSD*): relative standard deviation; r^2: coefficient of determination.

3.3. Performance Comparison with Standard Multi-Well Liquid Format Assays

LECs for 4-NQO and AFB1 obtained in the AMES MPF and Umu-C microtiter plate assays (expressed in ng/well) were compared to those generated using the HPTLC−S9-Umu-C bioassay (Table 3). Data show consistently lower LECs for the HPTLC method, suggesting a high potential for this new methodology to significantly reduce the limit of biological detection of genotoxic substances in complex mixtures, such as packaging migrates.

Table 3. Comparison of the LECs of the p-Umu-C, SOS Umu-C and AMES-MPF (TA98 and TA100) assays. For liquid test formats, data are expressed in ng/well. For p-UmuC, data are expressed in ng/band.

Condition	LEC (ng) without Metabolic Activation				LEC (ng) with Metabolic Activation			
Assays	HPTLC Umu-C	Liquid Umu-C	AMES-MPF		HPTLC Umu-C	Liquid Umu-C	AMES-MPF	
Strains			TA98	TA100			TA98	TA100
4-NQO	0.016	25	15.5	6.25	0.25	50	395 *	79 *
AFB1	–	–	–	–	0.025	47.8	0.06	0.24

(−): negative in absence of metabolic activation; * data kindly provided by Xenometrix.

To estimate the potential improvement in detecting low levels of genotoxic substances in FCM migrates requires considering not only the intrinsic analytical capability of the test (reflected in the LECs), but also the sample preparation protocol, including migrate

concentration and dilution prior to bioassay exposure. This allows for the calculation of the limits of biological detection (cLOBDs) in migrate samples [3,7]. To do this, the approach previously developed to assess the standard Ames test was used [3,7]. Data on the chemicals for which all necessary information was available are provided in Table 4. Ratios between calculated cLOBDs for Ames ($cLOBDs_{Ames}$) and HPTLC ($cLOBDs_{HPTLC}$) assays highlight that $cLOBDs_{HPTLC}$ were, in general, orders of magnitude lower than those anticipated according to the use of the standard Ames test. The $cLOBDs_{HPTLC}$ were also compared with target LOBDs (tLOBDs) established to limit an excess carcinogenic risk of 1 in 10^5 [3]. Ratios between tLOBDs and $cLOBDs_{HPTLC}$ range from 1.2 (MMC) to 260 (MMS), indicating that in general, the $cLOBDs_{HPTLC}$ are equal to or lower than what would be required from a safety perspective. Interestingly, all chemicals were detected at a concentration corresponding to the threshold of toxicological concern for substances presenting structural alerts for genotoxicity (150 ng/person, converted in 150 ng per band [3]).

Table 4. Comparison of the calculated LOBDs for theHPTLC−S9-Umu-C versus the standard Ames bioassays for selected genotoxic substances.

Genotoxic Substances	$cLOBD_{HPTLC}$ (ng/L)	* $cLOBD_{Ames}$ (ng/L)	$cLOBD_{Ames}/cLOBD_{HPTLC}$	* tLOBD (ng/L)	$tLOBD/cLOBD_{HPTLC}$
AFB1	0.025	35	1400	3.9	156
MMC	1	40	40	1.2	1.2
MMS	150	175	1.167	39,000	260
ENU	16	300	18.750	1000	63
DMBA	50	200,000	4000	100	2
B(a)P	20	5000	250	1100	55
2-NF	20	5000	250	340	17

* From Schilter et al. [3]; cLOBD: (calculated LOBD); tLOBD: (targeted LOBD).

4. Discussion

In vitro bioassays aimed at determining genotoxic/mutagenic potential are standard tests required for chemical risk assessment [25]. They have been designed and successfully applied for evaluating pure substances. However, their suitability for characterizing complex chemical mixtures containing unidentified components is being challenged [3–7]. Indeed, an adequate test should possess the capability to detect genotoxicants/mutagens at levels low enough to be compatible with negligible carcinogenic risk [3]. Currently, there is no test available exhibiting such a high level of performance. This lack was recently highlighted in the field of packaging safety [3,5]. Although the Ames assay is recommended as the best possible choice to test packaging migrates, it is also recognized that it suffers from significant weaknesses with respect to limits of biological detection [3]. Research efforts are therefore necessary to address this important limitation. In this context, the development of alternative methodologies using HPTLC coupled with genotoxicity assays, such as the SOS-Umu-C assay, has been encouraging [7], providing potentially improved detection limits compared to standard methods. However, until now, the reported protocols have been applied mainly to 4-NQO in the absence of metabolic activation. Thus, there is a need to confirm the promises of the p-Umu-C test through the detection of more chemicals acting through different mechanisms of action and to incorporate metabolic activation.

The current study expands the number of chemicals tested in the p-Umu-C test. The data provides new evidence confirming the capability of this test to detect low levels of DNA-damaging substances. The main key contribution of the study is the development and implementation of metabolic activation into the previously published protocol [7]. AFB1 was used as a prototypical reference compound requiring bioactivation. Standard post-mitochondrial S9-fraction was employed as a source of xenobiotic metabolizing enzymes. The generated data demonstrated the efficacy of the developed S9-mediated bioactivation protocol, with reduced assay time as compared to other classical methods (~6 h/without considering the ON culture). Altogether, these data provide assurance that pro-mutagens requiring bioactivation to express their genotoxic potential can be detected with the p-Umu-

C assay. With some other substances, such as 4-NQO and MMC, a shift in the genotoxic activity dose–responses to the right was observed, suggesting a possible S9-mediated inactivation. The establishment and incorporation of an S9-protocol in the p-Umu-C approach may be considered as a breakthrough step for the acceptability of this method as a credible genotoxicity test.

Among the reference genotoxic chemicals used, 4 were polycyclic aromatic hydrocarbons (PAHs, *i.e.*, 2-NF, 2-AA, DMBA, and B(a)P). In agreement with published data, 2-NF induced genotoxic activity in both the presence and absence of S9 [24]. However, the 3 others, which are documented to require bioactivation to exert their genotoxic potential, were also active in the absence of S9 treatment. The role of the metabolic activation of PAHs has been extensively studied. For example, it is well known that in biological systems, B(a)P must be converted to oxygenated metabolites to exert its mutagenic activity. Cytochrome P-450 oxidase-mediated activation is involved in these reactions, forming the DNA-reactive metabolites, *e.g.*, the 7,8-diol-9,10-epoxide [26]. However, evidence is available that the non-enzymatic transformation of PAHs such as B(a)P may also occur, for example, through photo-activation by irradiation with UV light [27,28]. In this type of reaction, genotoxic derivatives are formed, such as quinones, which can either directly bind to DNA, or induce damages through the production of H_2O_2 by redox cycling [29–31]. The physicochemical conditions present in the separation step of the p-Umu-C assay or the recording of UV images after the separation may be favorable to the non-enzymatic oxidation of PAHs and therefore, constitute a plausible explanation for the induction of genotoxic activity observed with PAHs in the absence of S9. This deserves further attention and investigations.

The main promise of HPTLC coupled with genotoxicity assays is its high potential for application to complex mixtures containing active substances at very low levels. Indeed, because of their possible high potency, low levels of genotoxic chemicals may still be of significant safety relevance and require identification. Currently, no satisfactory test is available for this purpose [3–7]. The HPTLC-bioassay approach was chosen to specifically address this issue. LECs for AFB1 and 4-NQO obtained in the p-Umu-C assay were found to be much lower than those observed in liquid formats of the SOS Umu-C and Ames tests, suggesting a real potential for this approach to achieve the detection of levels of genotoxic chemicals that are compatible with safety. This was confirmed with the theoretical LOBDs in migrates calculated for 7 of the tested chemicals. These were orders of magnitude lower than those established for the standard Ames test. Importantly, the calculated LOBDs obtained with the p-Umu-C assay were in line with levels associated with negligible carcinogenic risk, as calculated based either on TD50s, or on the threshold of toxicological concern (TTC) for substances bearing alerts regarding genotoxicity [3]. Although the application of the method to case studies involving packaging matrices is still required, the data generated up to this point indicate that the p-Umu-C bioassay may contribute toward filling the actual gaps and satisfying the other limitations of microplate-based in vitro testing for decision-making and prioritization purposes.

It must be acknowledged that the p-Umu-C test cannot readily replace the Ames test, since it does not specifically detect mutagens. This can be seen as a limitation regarding packaging safety assessment [3]. However, the active bands on HPTLC-plates can be recovered and then either identified chemically by high resolution mass spectrometry or/and directly tested in a suitable Ames assay format. This should provide highly relevant information regarding mutagenic potentials of the chemicals present in the band.

In conclusion, the p-Umu-C incorporated with the S9 metabolic activation condition has the potential to become the most suitable approach to identify genotoxic/mutagenic substances in complex mixtures, such as packaging migrates, or food and environmental samples. It appears to be the tool of choice to support the application of the TTC Cramer class III threshold to prioritize unidentified substances in migrates, as recently proposed.

Author Contributions: Conceptualization, G.E.M., B.S. and M.M.-K.; Data curation, P.R. and M.M.-K.; Formal analysis, E.D., P.R., H.L., C.C.-F. and M.M.-K.; Investigation, G.E.M., B.S. and M.M.-K.; Methodology, E.D., P.R., H.L., G.E.M., D.M., C.C.-F. and M.M.-K.; Project administration, M.M.-K.; Resources,

G.S. and B.S.; Supervision, M.M.-K.; Validation, M.M.-K.; Visualization, H.L. and M.M.-K.; Writing — original draft, E.D., P.R., H.L., D.M. and M.M.-K.; Writing — review & editing, G.E.M., G.S. and B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank imitrios Spiliotopoulos for his support with the Umu-C test and providing the AMES MPF data.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Agency:, E.F.S. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA J.* **2011**, *9*, 2379.
2. Koster, S.; Bani-Estivals, M.-H.; Bonuomo, M. Guidance on best practices on the risk assessment of on-intentionally added substances (NIAS) in food contact materials and articles. *ILSI Eur. Rep. Ser.* **2015**, *2015*, 1–70.
3. Schilter, B.; Burnett, K.; Eskes, C.; Geurts, L.; Jacquet, M.; Kirchnawy, C.; Oldring, P.; Pieper, G.; Pinter, E.; Tacker, M.; et al. Value and limitation of in vitro bioassays to support the application of the threshold of toxicological concern to prioritise unidentified chemicals in food contact materials. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **2019**, *36*, 1903–1936.
4. Pinter, E.; Rainer, B.; Czerny, T.; Riegel, E.; Schilter, B.; Marin-Kuan, M.; Tacker, M. Evaluation of the Suitability of Mammalian In Vitro Assays to Assess the Genotoxic Potential of Food Contact Materials. *Foods* **2020**, *9*, 237.
5. Rainer, B.; Mayrhofer, E.; Redl, M.; Dolak, I.; Mislivececk, D.; Czerny, T.; Kirchnawy, C.; Marin-Kuan, M.; Schilter, B.; Tacker, M. Mutagenicity assessment of food contact material migrates with the Ames MPF assay. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **2019**, *36*, 1419–1432.
6. Rainer, B.; Pinter, E.; Czerny, T.; Riegel, E.; Kirchnawy, C.; Marin-Kuan, M.; Schilter, B.; Tacker, M. Suitability of the Ames test to characterise genotoxicity of food contact material migrates. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **2018**, *35*, 2230–2243.
7. Rainer, B.; Pinter, E.; Prielinger, L.; Coppola, C.; Marin-Kuan, M.; Schilter, B.; Apprich, S.; Tacker, M. Direct Comparison of the Lowest Effect Concentrations of Mutagenic Reference Substances in Two Ames Test Formats. *Toxics* **2021**, *9*, 152.
8. Meyer, D.; Marin-Kuan, M.; Debon, E.; Serrant, P.; Cottet-Fontannaz, C.; Schilter, B.; Morlock, G.E. Detection of low levels of genotoxic compounds in food contact materials using an alternative HPTLC-SOS-Umu-C assay. *ALTEX* **2021**, *38*, 387–397.
9. Morlock, G.E. High-performance thin-layer chromatography combined with effect-directed assays and high-resolution mass spectrometry as an emerging hyphenated technology: A tutorial review. *Anal. Chim. Acta* **2021**, *1180*, 338644.
10. Sherma, J. Modern thin-layer chromatography. *J. AOAC Int.* **2008**, *91*, 1142–1144.
11. Schreiner, T.; Morlock, G.E. Non-target bioanalytical eight-dimensional hyphenation including bioassay, heart-cut trapping, online desalting, orthogonal separations and mass spectrometry. *J. Chromatogr. A* **2021**, *1647*, 462154. [PubMed]
12. Baumann, U.; Brunner, C.; Pletscher, E.; Tobler, N. Biologische Detektionsverfahren in der Dünnschichtchromatographie. *Umweltwiss. Und Schadst. Forsch.* **2003**, *15*, 163–167.
13. Shakibai, D.; Riegraf, C.; Moscovici, L.; Reifferscheid, G.; Buchinger, S.; Belkin, S. Coupling High-Performance Thin-Layer Chromatography with Bacterial Genotoxicity Bioreporters. *Environ. Sci. Technol.* **2019**, *53*, 6410–6419.
14. Oda, Y. Development and progress for three decades in umu test systems. *Genes Environ.* **2016**, *38*, 24.
15. Reifferscheid, G.; Heil, J. Validation of the SOS/umu test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. *Mutat. Res. Genet. Toxicol.* **1996**, *369*, 129–145.
16. Yasunaga, K.; Kiyonari, A.; Oikawa, T.; Abe, N.; Yoshikawa, K. Evaluation of the Salmonella umu test with 83 NTP chemicals. *Environ. Mol. Mutagen.* **2004**, *44*, 329–345. [CrossRef] [PubMed]
17. Azadniya, E.; Mollergues, J.; Stroheker, T.; Billerbeck, K.; Morlock, G.E. New incorporation of the S9 metabolizing system into methods for detecting acetylcholinesterase inhibition. *Anal. Chim. Acta* **2020**, *1129*, 76–84.
18. Morlock, E.G.; Drotleff, L.; Brinkmann, S. Miniaturized all-in-one nanoGIT(+active) system for on-surface metabolization, separation and effect imaging. *Anal. Chim. Acta* **2021**, *1154*, 338307.
19. Kirkland, D.; Kasper, P.; Martus, H.-J.; Müller, L.; van Benthem, J.; Madia, F.; Corvi, R. Updated recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **2016**, *795*, 7–30.
20. *ISO 13829:2000*; Water Quality—Determination of the Genotoxicity of Water and Waste Water Using the Umu-Test. ISO: Geneva, Switzerland, 2000; pp. 3–5.
21. Fluckiger-Isler, S.; Kamber, M. Direct comparison of the Ames microplate format (MPF) test in liquid medium with the standard Ames pre-incubation assay on agar plates by use of equivocal to weakly positive test compounds. *Mutat. Res.* **2012**, *747*, 36–45. [PubMed]

22. ICH Harmonised Triopartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1). Available online: https://database.ich.org/sites/default/files/Q2%28R1%29Guideline.pdf (accessed on 23 August 2022).
23. Veres, Z.; Török, G.; Vereczkey, L.; Jemnitz, K. The spectrum of enzymes involved in activation of 2-aminoanthracene varies with the metabolic system applied. *Mutat. Res.* **2005**, *586*, 18–27.
24. Beije, B.; Moller, L. 2-Nitrofluorene and related compounds: Prevalence and biological effects. *Mutat. Res.* **1988**, *196*, 177–209.
25. European Food Safety Authority (EFSA). Genotoxicity assessment of chemical mixtures. *EFSA J.* **2019**, *17*, e05519.
26. Shi, Q.; Godschalk, R.W.L.; van Schooten, F.J. Inflammation and the chemical carcinogen benzo[a]pyrene: Partners in crime. *Mutat. Res. Rev. Mutat. Res.* **2017**, *774*, 12–24.
27. Platt, K.L.; Aderhold, S.; Kulpe, K.; Fickler, M. Unexpected DNA damage caused by polycyclic aromatic hydrocarbons under standard laboratory conditions. *Mutat. Res.* **2008**, *650*, 96–103.
28. Zhao, X.; Hu, X.; Hwang, H.M. Effects of riboflavin on the phototransformation of benzo[a]pyrene. *Chemosphere* **2006**, *63*, 1116–1123.
29. Gower, J.D.; Wills, E.D. The generation of oxidation products of benzo[a]pyrene by lipid peroxidation: A study using gamma-irradiation. *Carcinogenesis* **1984**, *5*, 1183–1189. [CrossRef]
30. Gower, J.D.; Wills, E.D. The oxidation of benzo[a]pyrene mediated by lipid peroxidation in irradiated synthetic diets. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **1986**, *49*, 471–484.
31. Lee-Ruff, E.; Kazarians-Moghaddam, H.; Katz, M. Controlled oxidations of benzo[a]pyrene. *Can. J. Chem.* **1986**, *64*, 1247–1253.

7. Publication III

Food Control 147 (2023) 109546

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Effect-detection by planar SOS-Umu-C genotoxicity bioassay and chemical identification of genotoxins in packaging migrates, proven by microtiter plate assays SOS-Umu-C and Ames-MPF

Daniel Meyer [a], Maricel Marin-Kuan [b], Elisa Mayrhofer [c], Christian Kirchnawy [c], Emma Debon [b], Helia Latado [b], Amaury Patin [b], Benoît Schilter [b], Gertrud Morlock [a,*]

[a] Institute of Nutritional Science, Chair of Food Science, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392, Giessen, Germany
[b] Chemical Food Safety Group, Société des Produits Nestlé SA - Nestlé Research, Vers-chez-les-Blanc, Switzerland
[c] Department for Microbiology and Cell Culture, Austrian Research Institute for Chemistry and Technology, Franz-Grill Straße 5, 1030, Vienna, Austria

ARTICLE INFO

Keywords:
Food contact material
Paper
Plastic
Coating
SOS-Umu-C
Ames-MPF™

ABSTRACT

Recently shown to meet regulatory requirements for safety assessment, high-performance thin-layer chromatography hyphenated with the SOS-Umu-C bioassay and S9 metabolization was applied to various food contact material prototypes, such as paper, plastic and coating samples. Studying reversed *versus* normal phase separation and piezoelectrical spraying *versus* syringe dosage of the bioassay, 14 genotoxic bands were detected. Three epoxides of linolenic acid were identified as genotoxic candidates in the paper migrate by heated electrospray and atmospheric pressure chemical ionization high-resolution mass spectrometry. These were confirmed by co-chromatography with linolenic acid epoxides. Comparatively poorer detection limits of *in vitro* microtiter plate bioassays and opposing effects/signal responses in the sample explained false-negative responses for paper migrate. However, the *in vitro* SOS-Umu-C microtiter plate bioassay confirmed the genotoxicity of a simulated linolenic acid epoxidation, and after sample concentration, the paper migrate mutagenicity was confirmed by the Ames-MPF™ bioassay.

1. Introduction

Food contact materials (FCMs) can release chemicals into food. Safety assessment of FCMs is challenging because hundreds of compounds can be present intentionally or non-intentionally. The chemicals may originate from the raw materials and the manufacturing process through to final packaging including non-intentionally added substances (NIAS) which are not always characterized or identified. These NIAS may be derived from unknown chemical side reactions and impurities. To address their health significance is challenging and standard risk assessment methods are often not readily applicable for migrates, which are multi-component mixtures of chemicals of unknown identity. The resulting uncertainty about safety is a concern for policymakers, industry and consumers. Those chemicals for which direct identification is not feasible are numerous and present at low levels. The importance of those chemicals is underlined by the fact, that they are potentially introduced into the materials during a lot of stages in the production process of FCMs. Hence, packaging safety assessment is best practiced by the exclusion of genotoxic compounds (Koster et al., 2015; Schilter et al., 2019). In absence of toxicological information, it is requested to exclude genotoxicity potential via thresholds for safety evaluation. The Threshold of Toxicological Concern (TTC) concept (Bopp et al., 2019) can then be applied for prioritization of unknown packaging migrants.

However, current analytical methods are not sufficient to reliably exclude that genotoxic substances are potentially present in FCMs. Even if *in vitro* microtiter plate bioassays were included in the safety assessment of FCMs, genotoxic effects of migrating substances have not easily been detectable in the past. The limits of detection of available *in vitro* bioassays are not adequate and matrices or opposing effects/signal responses may also interfere *in vitro*. The evaluation solely as a sum value hampered the respective risk assessment. Hence, a pressing challenge for the exclusion of a genotoxic potential is, on the one hand, the detection limit of the standard mutagenicity testing (AMES test) and, on the other hand, the assignment of compounds responsible for the effect. Several chemicals in the migrates identified by GC/MS that were already known to be mutagenic should have been mutagenic, but were not active in the

* Corresponding author.
E-mail address: Gertrud.Morlock@uni-giessen.de (G. Morlock).

https://doi.org/10.1016/j.foodcont.2022.109546
Received 17 July 2022; Received in revised form 30 October 2022; Accepted 29 November 2022
Available online 30 November 2022
0956-7135/

Ames bioassay (Monarca et al., 1994). Food migrate simulants that were spiked with mutagenic chemicals showed partially a matrix influence in the Ames bioassay resulting in biased data (Rainer et al., 2019). It is not known whether this was due to problems with the detection limit or other limitations, but it underscores the need to investigate more appropiate methods.

To counteract these obstacles, high-performance thin-layer chromatography (HPTLC) was explored in combination with a genotoxic or mutagenic bioassay. Early attempts using the Ames bioassay on a thin-layer plate were not pursued further due to cumbersome implementation (Bjorseth et al., 1982). A recently developed sensitive HPTLC−Vis/FLD−SOS-Umu-C bioassay was more applicable. This method was considered groundbreaking because it allowed discrimination of genotoxic compounds in multicomponent mixtures separated on a planar adsorbent and lower detection limits than those previously reported in *in vitro* bioassays (Meyer et al., 2020). The metabolic activation via the S9 mixture simulating the liver system was also implemented in this sensitive genotoxicity bioassay (Debon et al., 2022). Recently, the piezoelectric spraying was demonstrated as a new technique for the application of bacterial strains on the adsorbent (Azadniya & Morlock, 2019; Klingelhöfer et al., 2020; Morlock & Heil, 2020; Shakibai et al., 2019), but up to now this had not been tested for *Salmonella*.

In this study the objective was to investigate the genotoxic hazard of paper, coating and plastic migrates. The piezoelectric spraying of the *Salmonella typhimurium* bacteria strain TA1535 suspension onto the reversed phase (RP) and normal phase (NP) separation with and without metabolic activation via the S9 mixture was investigated. The potential of identifying the detected genotoxic substances via high-resolution mass spectrometry (HRMS) was studied. Therefore, both RP- and NP-HPTLC−(S9)–SOS-Umu-C−Vis/FLD bioassays (Meyer et al., 2020; Debon et al., 2022) were applied to the analysis of one migrate each from paper, coating and plastic granulate prototypes. As the genotoxicity bioassay has been shown to be 162 times more sensitive than state-of-the-art *in vitro* assays (Meyer et al., 2020), genotoxic responses were detected at substantially lower genotoxin concentrations present. However, a low concentration of a substance made identification extremely difficult, even with powerful HRMS systems (Jamshidi-Aidji & Morlock, 2016). Therefore, coupling of the RP/NP-HPTLC−(S9)–SOS-Umu-C−Vis/FLD bioassay to HRMS using atmospheric pressure chemical ionization (APCI) or heated electrospray ionization (HESI) as well as two separation selectivities (NP/RP, which can be helpful in signal detection) were explored for the feasibility to identify genotoxins detected at the trace level.

2. Materials and methods

2.1. Chemicals and materials

Salmonella typhimurium bacteria strain TA1535, modified to contain the plasmid pSK1002 (PTM™ *Salmonella typhimurium* TA1535/pSK1002, cryostock) was obtained from Trinova Biochem, Giessen, Germany (for HPTLC-S9-SOS-Umu-C and microtiter plate SOS-Umu-C). The S9 fraction from phenobarbital/ß-naphthoflavone-induced lyophilized rat liver (proteins at 25–35 mg/mL) and *Salmonella typhimurium* bacteria strains TA98 and TA100 (for AMES MPF™) were acquired from Xenometrix, Allschwil, Switzerland. Ethyl acetate, ethanol, and methanol (all gradient grade) 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), potassium chloride (KCl, 98.5%) and sodium hydroxide (>99%) were purchased from Carl Roth, Karlsruhe, Germany. Aflatoxin B1 (AFB1, >98%), ampicillin sodium salt, D-(+)-glucose (99.5%), lysogeny broth (Lennox) powder (including 5 g/L sodium chloride), benzo[a]pyrene (B[a]P, ≥96%), 7,12-dimethyltetrapene (DT, ≥95%), mitomycin C (MC, BioReagent) and hexamethyl cyclotrisiloxane (D3) were purchased from Sigma-Aldrich, Steinheim, Germany. Linolenic acid, linoleic acid and oleic acid (all 99%) were supplied by Acros Organics, Fair Lawn, NJ, USA. Resorufin-β-D-galactopyranoside (RG) was from Toronto Research Chemicals, Toronto, ON, Canada. 4-Nitroquinoline 1-oxide (4-NQO, ≥98%) was purchased from TCI, Eschborn, Germany. Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$, 99.5%), ethanol (LiChrosolv), HPTLC plates silica gel 60 (normal phase, NP) and RP-18 W (water-wettable reversed phase), both 20 cm × 10 cm, were delivered by Merck, Darmstadt, Germany. Bidistilled water was prepared by a Destamat Bi 18 E (Heraeus, Hanau, Germany).

The SOS-Umu-C microtiter plate bioassay was provided by Xenometrix, including 4-nitroquinoline 1-oxide (4-NQO) and 2-aminoanthracene (2-AA), ampicillin, B-buffer, stop reagent, *o*-nitrophenyl-β-D-galactoside (ONPG), 2-mercaptoethanol. For the Ames MPF™ bioassay, 4-NQO (≥98%), 2-nitrofluorene (2-NF; 98%), 2-AA (≥96%), ampicillin sodium salt, magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$, 99–102%), D-glucose-6-phosphate sodium salt (G6P), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), citric acid monohydrate (≥99%), L-histidine (≥99%) and bromocresol purple sodium salt (90%) were purchased from Sigma-Aldrich, Steinheim, Germany. Dimethyl sulfoxide (DMSO, ≥99%), KCl, $MgSO_4{\bullet}7H_2O$, K_2HPO_4 and sodium ammonium hydrogen phosphate tetrahydrate ($NaNH_4HPO_4{\bullet}4H_2O$) were provided by Merck. Nicotinamide adenine dinucleotide phosphate sodium salt (NADP) was purchased from AppliChem, Darmstadt, Germany. Nutrient broth no. 2 powder was obtained from Oxoid, Basingstoke, UK. D-Glucose was provided by VWR Chemicals BDH, Leuven, Belgium. D-Biotin (≥99%) was delivered by VWR Life Science, Solon, OH, USA. Ultra-pure water was obtained from a MilliQ water preparation system from Merck. Various FCM research prototypes were tested, such as paper, plastic granulate and coating samples.

2.2. Salmonella typhimurium culture as well as substrate and standard solutions

Salmonella culture medium was prepared from lysogeny broth (20 g/L) in bidistilled water and autoclaved. To this solution an aqueous solution (3 mL) of D-glucose (1 g/3 mL) and ampicillin-Na salt (106 mg/3 mL) was added via a sterilizing syringe filter. A *Salmonella* suspension (100 µL *Salmonella* TA1535/pSK1002 cryoctock suspended in 30 mL culture medium, 37 °C, 16 h, 75 rpm) was centrifuged (3000 × *g*, 10 min) and resuspended in culture medium to obtain an optical density OD_{660} of 0.2. For metabolic activation (Debon et al., 2022), the S9 enzyme suspension (500 µL) and solution of cofactors, *i.e.* NADP (166 µL), G6P (42 µL) and buffer salt solution (953 µL), were added to the *Salmonella* suspension (3334 µL) prepared in the same way and adjusted to the same OD. The substrate solution was prepared by solving RG (20 mg/mL in DMSO; 15 µL in 3 mL) or FDG (5 mg/mL in DMSO; 25 µL in 2.5 mL) in phosphate buffer. 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. The two positive controls 4-NQO (500 pg/µL) and AFB1 (100 pg/µL) as well as the reference linolenic acid (5 µg/µL) were prepared in methanol.

2.3. Migration simulation

Migration of selected FCM was performed based on the regulation EU 10/2011 (The European Commission, 2011). One plastic granulate, one paper and one coating sample were each completely immersed in simulant (ethanol 95%, aqueous) and stored at 60 °C in a glass bottle closed tightly with polytetrafluoroethylene-coated screw caps for 10 days. A mass to volume ratio of 10 g/100 mL was used for the plastic granulate, and a surface to volume ratio of 2 dm^2/100 mL for the paper and coating. As migration simulant blank, 95%-ethanol (aqueous) was prepared analogously. Either 30, 50 or 300 mL of these migrates were subjected to evaporation. For the 30- or 50-mL migrates, forced nitrogen

flow (room temperature) was used, followed by dissolving each residue in 1 mL migration simulant (95%-ethanol), whereas a pressure gradient (>70 mbar at 60 °C until < 5 mL ethanol was left to add DMSO as keeper for evaporation > 50 mbar) was applied for the 300 mL migrate.

2.4. Remarks on planar bioassay workflow

Plates were dried (cold air stream, hair dryer) after application for 1 min and between other steps for 4–5 min, if not stated otherwise. For the HPTLC RP-18 W plate batch no. HX86964996, a pretreatment was required to enforce the binder hardening for the long aqueous bioassay procedure. A set of plates was heated at 120 °C on a plate heater for 1 h and prewashed by development up to 9 cm first with methanol, then with ethyl acetate and dried. HPTLC plates silica gel 60 were prewashed by development up to 9 cm with methanol and dried. HPTLC instrumentation (Automated TLC Sampler 4 for application, Derivatizer for piezoelectric spraying via yellow nozzle and level 3, if not stated otherwise, Twin Trough Chamber for development, TLC Visualizer for documentation and TLC Scanner 3 for densitometry; HPTLC-PRO system for application and development in 2.5, all operated by visionCATS software version 3.0) was from CAMAG, Muttenz, Switzerland. Bioassay syringe dosage was performed using the ChromaJet, Biostep, Burkhardtsdorf, Germany. Incubation of the plate was performed at 37 °C in a humid polypropylene box (26.5 cm × 16 cm × 10 cm, KIS, ABM, Wolframs-Eschenbach, Germany). Experiments were repeated at least 3 times to confirm reproducibility.

2.5. Genotoxicity screening performed via Derivatizer versus ChromaJet

The 50 × migrate concentrates (2, 4 and 8 μL/band each) and the two positive controls 4-NQO (500 pg/band) and AFB1 (500 pg/band for Derivatizer; 80 ng/band for ChromaJet) were applied on the HPTLC plates silica gel 60 and dried. The adsorbent was activated over a molecular sieve for 10 min to contain maximal 5% relative humidity. After exposure to a toluene-enriched gas phase for 2.5 min, the plate was developed with toluene – ethyl acetate 2:3, *V/V*, up to 80 mm, followed by drying. The *Salmonella typhimurium* bacteria strain TA1535/pSK1002 suspension was applied on the plate using the Derivatizer (2.8 mL) or ChromaJet (4.0 mL in 3 cycles), incubated for 3 h and dried. The FDG substrate solution was applied using the Derivatizer (2.5 mL) or ChromaJet (4.0 mL in 3 cycles), incubated for 15 min and dried. Images were documented at FLD 254 nm. Separation was also performed on HPTLC plates silica gel 60 RP-18 W or with further mobile phases adjusted for recording of mass spectra, as described in 2.7. The RG substrate solution was used analogously but incubated for 1 h.

2.6. Artefact exclusion by 2D development

For proof of artefact generation, linolenic acid (5 μg/band) was applied as 2-mm band on the HPTLC plate silica gel 60, dried and 2D developed using a 2-step development with first toluene – ethyl acetate 3:2, *V/V*, up to 50 mm, and then *n*-hexane – toluene 4:1, *V/V*, up to 70 mm. After plate drying and a waiting time of 15 min, the plate was turned by 90° and the same 2-step development was repeated. The dried 2D chromatogram was subjected to the SOS-Umu-C bioassay using the Derivatizer, as described in 2.5. For confirmation, the whole experiment was repeated twice.

2.7. Identification of potential genotoxic substances in FCM migrates

For flow-injection analysis (FIA)–HESI-HRMS, each of the 50 × migrate concentrates was applied six times (8 μL each) on the HPTLC plates silica gel 60, developed with first toluene – ethyl acetate 3:2, *V/V*, up to 50 mm, then *n*-hexane – toluene 4:1, *V/V*, up to 70 mm and dried after each development. One plate piece containing each FCM type was cut and subjected to the NP-HPTLC–SOS-Umu-C bioassay as described (Debon et al., 2022). On the other plate piece, the zones of interest were marked on the residual 5 migrates on the chromatogram at FLD 366 nm by comparison with the bioautogram at FLD 254 nm (Fig. S2). Five bands at the same position were eluted (Plate Express, Advion, Ithaca, NY, USA) each with ethyl acetate (300 μL) into the same vial. After evaporation of the solvent (1.5 mL), the residue was taken up in methanol (200 μL), whereof 10 μL were injected for FIA–HESI-HRMS. The instrument parameters were + 3.5 kV and − 3.2 kV, 270 °C capillary and 200 °C probe heater temperature, resolution 280000, *m/z* 100–1500, and automatic maximum injection time 10/200 ms for positive/negative ionization (QExactive Plus mass spectrometer, Thermo Fisher Scientific, Dreieich, Germany).

For RP-HPTLC–APCI-HRMS, the 30 × migrate concentrates (100 μL/area each) as well as the positive controls 4-NQO (1 ng/area) and AFB1 (100 pg/area) were applied on the RP-18 W plate as area of 7 mm × 10 mm. The areas were focused by front elution up to 21 mm with methanol – ethyl acetate 1:1, *V/V*. A 2-step development was performed, *i. e.* first with *n*-hexane – ethyl acetate 4:5, *V/V*, up to 50 mm, and then *n*-hexane – ethyl acetate 8:1, *V/V*, up to 70 mm, whereby the plate was dried after each development. Bands of interest were eluted each for 1 min with methanol containing 4 mM ammonium formate and 0.1% formic acid at a flow rate of 0.2 mL/min directly into the high-resolution mass spectrometer and recorded using the same parameters as above. As control of the proper positioning of the elution head on the zones, the stamped plate was subjected to the RP-HPTLC–SOS-Umu-C bioassay as described (Meyer et al., 2020). Another plate was prepared analogously (migrates applied twice as two sample sets), but developed with *n*-hexane – ethyl acetate 4:0.1, *V/V*. After plate cut, one sample set was used for the bioassay and the other for APCI-HRMS recording (same parameters, but ± 5.0 kV, 250 °C capillary temperature, 350 °C probe heater temperature, automated maximum injection time 10/100 ms for positive/-negative ionization).

2.8. Confirmation via co-chromatography of standards and HRMS spectra

For NP-HPTLC–HESI-HRMS, 50 × concentrates of the paper migrate (8 μL/band) and migration simulant blank (5 μL/band) as well as linolenic acid, linoleic acid, and oleic acid (5 μg/band each) were applied together with the positive control 4-NQO (500 ng/band), all applied twice as two sample sets. A 2-step development was performed, *i. e.* first with toluene – ethyl acetate 3:2, *V/V*, up to 50 mm and then *n*-hexane – toluene 4:1, *V/V*, up to 70 mm. After plate cut, one sample set was used for the bioassay and the other for HESI-HRMS recording (except methanol as eluent).

2.9. Confirmation via Ames-MPF™ bioassay

The Ames MPF tests were performed according to a procedure recommended by Xenometrix (Xenometrix, 2018), originally based on the OECD guideline 471 (Organisation for Economic Co-operation and Development, 2020) but specifically adapted in this case for migration of food contact materials (Rainer et al., 2019). The number of strains was reduced to two (TA98/TA100) since it is known that these two strains already detect 93% of the genotoxins (Williams et al., 2019). In short, bacterial cell cultures of *Salmonella typhimurium* (TA98/TA100) were grown overnight in growth medium (25 g/L Oxoid nutrient broth No. 2 powder in ultra-pure water) supplemented with 50 μg/mL ampicillin at a cell density of about $1.5–2{\cdot}10^9$ CFU/mL. An aliquot (10 μL) of each 300 × concentrate of paper, coating and plastic migrate or DMSO solutions of pure substances (linolenic acid at a top concentration of 50 mg/mL and D3 at 0.7125 mg/mL) were mixed with 240 μL of a 5 *V*% (TA100) or 10 *V*% (TA98) suspension of overnight culture in exposure medium in 24-well plates. Each sample was analyzed in triplicates following the supplier protocol with adaptations described and mixed with reversion indicator medium followed by a final incubation in

384-well plates for 48–72 h. Exposure medium (ISO 11350) was prepared from $MgSO_4 \bullet 7H_2O$ (0.2 g/L), citric acid monohydrate (2 g/L), K_2HPO_4 (10 g/L), $NaNH_4HPO_4 \bullet 4H_2O$ (3.5 g/L), D-glucose (4 g/L), D-biotin (0.73 mg/L) and L-histidine (1 mg/L) in ultra-pure water (pH 7.0 ± 0.2). Reversion indicator medium (ISO 11350) was prepared from $MgSO_4 \bullet 7H_2O$ (0.2 g/L), citric acid monohydrate (2.2 g/L), K_2HPO_4 (10.8 g/L), $NaNH_4HPO_4 \bullet 4H_2O$ (3.8 g/L), D-glucose (4.3 g/L) bromocresol purple sodium salt (0.03 g/L) and D-biotin (2.6 mg/L) in ultra-pure water. After incubation, revertant wells were scored. Each sample was tested in the presence and absence of an external metabolic activation system (S9 fraction from phenobarbital/β-naphthoflavone-induced rat liver) to account for metabolic processes. A 30 *V*% S9 mixture was prepared in an S9-co-factor providing aqueous solution with NaH_2PO_4 (102 mM), KCl (33 mM), $MgCl_2$ (8 mM), NADP (4 mM) and G6P (5 mM). This mixture was added to the bacterial cells suspended in exposure medium at a concentration of 15 *V*%, resulting in a final S9 concentration of 4.5 *V*%. Inhibiting effects were assessed by spiking the exposure mixtures with the Ames positive controls 4-NQO (TA100 −S9), 2-NF (TA98 −S9) and 2-AA (TA100 +S9 and TA98 +S9) at a final concentration of 0.1 μg/mL (TA100 −S9), 2 μg/mL (TA98 −S9), 1.25 μg/mL (TA100 +S9) and 0.5 μg/mL (TA98 +S9), respectively.

2.10. Confirmation via microtiter plate SOS-Umu-C bioassay

SOS-Umu-C assay (ISO 13829) was performed with minor modifications as described (Meyer et al., 2020) using *Salmonella typhimurium* TA1535/pSK1002 cells. Briefly, a bacterial cell culture with 70–80% of the initial OD_{600} (overnight culture with 10 mL of growth medium and 100 μL of bacteria, 10 h incubation, 250 rpm) was used. For each 96-well plate, positive control substances were added to confirm the validity of the test, *i.e.* 4-NQO at 12.5 μg/mL (without metabolic activation) and 2-AA at 2 μg/mL at 100 μg/mL (with metabolic activation). Bacterial cell culture was added to each well and mixed with samples and controls. For metabolic activation, a mixture of 30 *V*% S9-mix was added to each well. Plates were incubated for 2 h at 37 °C shaking at 150 rpm. A dilution of the plates was performed followed by an incubation for 2 h. The assessment of the Umu-C induction was performed by adding a buffer containing ONPG followed by a 30-min incubation and the addition of stop reagent.

2.11. Data and statistical analysis

Concerning the Ames-MPF™ bioassay, a sample was considered Ames positive if the arithmetic mean of revertant wells (mean value from triplicate) showed at least a 2-fold increase compared to baseline value, which was calculated as the arithmetic mean of the solvent control (triplicate) plus one standard deviation. A sample was considered inhibiting, if the spiked sample showed less than 60% of revertant recovery compared to a spiked solvent control (mean value from triplicate).

Concerning the microtiter plate SOS-Umu-C bioassay, the OD_{420} was measured to evaluate the rate of β-galactosidase formation. Relative units were obtained at OD_{600} for the growth factor (G) and OD_{420} for the induction ratio (IR). The quality criteria to classify a sample (triplicate) as genotoxic with respect to blank (triplicate) and negative controls (triplicate) is a $G \geq 0.5$ and $IR \geq 1.5$. The data was analyzed using the average of the triplicates and the corresponding standard deviation.

All calculations including data plots were done using Microsoft Excel 2019 (Microsoft, Redmond, WA, USA) and OriginPro2018 (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1. Genotoxicity screening performed via Derivatizer versus ChromaJet

The validity of the planar bioassay was proven and confirmed via solvent controls (to detect process contamination) and the SOS-Umu-C positive controls (pc) 4-NQO and AFB1, used in absence and presence of metabolic activation, respectively. In the migrates of the plastic granulate, paper and coating prototypes, altogether 14 genotoxic bands were detected by NP-HPTLC−UV/Vis/FLD−SOS-Umu-C−Vis/FLD without any metabolic activation (Fig. 1A). The present response of 4-NQO (**pc**) and absent responses in the solvent and migration simulant blanks (indicating a contamination-free workflow) confirmed the quality performance of the bioassay. For the first time, the bacterial cell suspension with and without metabolic activation as well as the substrate solution were successfully applied to the RP and NP separations via piezoelectric spraying (Derivatizer). The resulting bioautograms were compared with those obtained by syringe dosage (ChromaJet).

The bioautograms obtained without metabolic activation via piezoelectric spraying (Fig. 1A) showed five genotoxic bands for the coating migrate (**1**–**5**), another five genotoxic bands for the paper migrate (**6**–**10**), and four genotoxic bands for the plastic granulate migrate (**11**–**14**). Thereof, the eleven most intense genotoxic bands (**1**–**5, 6**, **8, 9, 11**–**13**) showed a decrease in the response for lower levels applied (dose-response criterion for genotoxicity), whereas the very weak genotoxic bands **7**, **10** and **14** did not show a clear dose-response relationship, probably due to the very low amounts present in the samples. For the coating, paper and plastic migrates exposed to metabolic activation, only band **5** of the coating migrate, band **6** of the paper migrate and bands **11** and **12** of the plastic migrate showed responses satisfying the dose-response criterion.

In contrast, fewer bands were observed in the bioautogram without metabolic activation using the syringe dosage (Fig. 1C). For the coating migrate only band **5**, for the paper migrate only band **6,** and for the plastic migrate only bands **11** and **12** were obtained, all fulfilling the dose-response criterion. Only band **14** did not meet the dose-response criterion. If metabolic activation was used (Fig. 1D), the same bands were active, and band **14** now also showed a dose-response effect. However, this was in contradiction to the corresponding piezoelectric spraying (Fig. 1B), which instead showed a darkened band. This instrumental difference needs to be clarified by studying further samples.

All four bioautograms (Fig. 1) had in common that black bands occurred at the application zone of the paper migrate, possibly as a result of a cytotoxic effect. Both bioassay application techniques had in common that a decrease in the genotoxic responses was observed under metabolic activation condition, suggesting a detoxification reaction. Small differences in the effect response may occur more readily when the genotoxins are present at very low levels. Although instrumental differences were minor, a comparatively more sensitive genotoxic response was evident in the bioautogram obtained without metabolic activation via piezoelectric spraying (Fig. 1A *versus* C), whereas syringe dosage led to better responses in the bioautogram with metabolic activation (Fig. 1D *versus* B). An additional advantage of the planar assay approach over *in vitro* assays is that the matrix is separated from the analytes prior to the assay performance, but not excluded from the assay evaluation. In this way, possible interfering effects of the matrix on the assay are prevented, understood and the matrix is simultaneously examined for its genotoxic potential.

3.2. Identification of unknown genotoxins in the migrates

HPTLC was combined with HRMS in two different modes to identify low-level genotoxic substances detected in the paper, plastic and coating migrates. Either respective genotoxic zones of several samples on the NP plate were pooled (selectively eluted with ethyl acetate into the same vial and re-dissolved in methanol) for analysis by FIA-HRMS or one zone (applied at higher amount) was directly eluted from the RP plate to APCI/HESI-HRMS. Orthogonal selectivity (NP/RP) for compound separation can change the impact of coeluting compounds which can be useful for mass selective detection. The sample pooling procedure

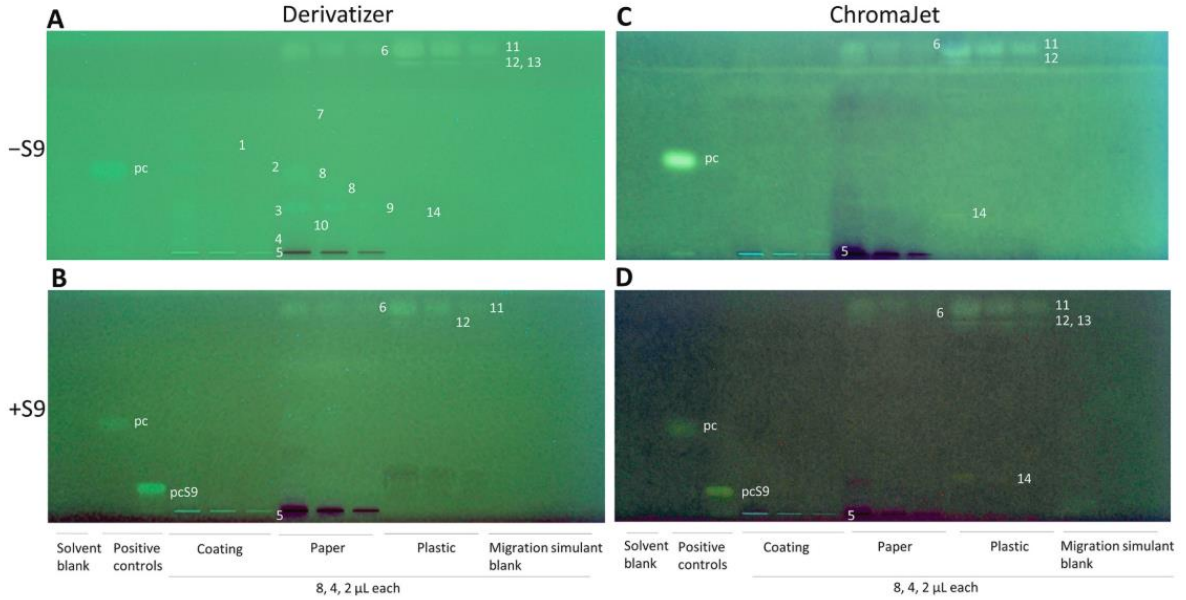


Fig. 1. Genotoxic screening of FCM migrates: Bioautograms at FLD 254 nm of the 50 × migrate concentrates of the coating, paper, plastic sample and migration simulant blank, the two positive controls 4-NQO (**pc**) and AFB1 (**pcS9**), each 500 pg/band, as well as their solvent control (8 µL/band) on HPTLC plate silica gel 60, developed with toluene – ethyl acetate 2:3, *V/V*, up to 80 mm and subjected to the SOS-Umu-C bioassay using the piezoelectric spraying via the Derivatizer without (**A**) and with metabolic activation (**B**) as well as the syringe dosage via the ChromaJet without (**C**) and with metabolic activation (**D; pcS9** only 80 ng/band). Note that the hR_F value of the genotoxic band **8** depends on the amount applied.

(elution and collection of several identical zones into the same vial) is a general advantage of the planar technique, since several sample separations can be done simultaneously. In contrast, this would take for HPLC approaches several runs and the respective summed time.

For NP-FIA–HESI-HRMS, the mobile phase of the genotoxic screening was adjusted to sharpen genotoxic bands (Fig. 2A and S1). As the detected genotoxins were present at very low amounts or traces in the samples, the following enrichment of the traces was helpful to improve signal intensity. From five applied 50 × migrates (8 µL each), 5 bands at the same position were eluted with 300 µL ethyl acetate each and pooled in the same vial (Fig. S2). Ethyl acetate was used to discriminate polar interfering compounds by their solubility, and thus, reduce polar background signals coming from the plate or matrix. The ethyl acetate was evaporated, which was three times faster than the typically used methanol. As preferred solvent for electrospray ionization, the residue was taken up in methanol, whereof an aliquot was injected for FIA–HESI-HRMS (Table S3). In band **9** of the paper migrate, linolenic acid and its epoxides were preliminary assigned to the detected mass signals (Fig. 2A). Additional molecular ions (Table S1) were assigned to genotoxic bands but were not further investigated by coelution of standards because the performance of the assay coupled to HRMS has already been demonstrated by the investigation of band **9** and will be further substantiated in the next chapter. For band **6** of the paper migrate, diisodecyl phthalate or dicecyl phthalate were presumably assigned for the found molecular ion at *m/z* 447.34670 (Δppm 1.754), for band **9** nordehydroabietane at *m/z 257.22630* (Δppm −2.550), (methylethyl)phenantrene at *m/z* 255.21070 (Δppm −2.413) and abietic acid at *m/z* 303.23750 (Δppm −0.352), all known in literature to be present in paper (Ozaki et al., 2006; Rosenmai et al., 2017; Wilkins & Panadam, 1987; Yunker et al., 2011). For bands **11** and **12** of the plastic migrate possible breakdown products of the antioxidant Irganox 1076 or 1010 at *m/z* 293.17612 (Δppm 0.746), which are known not-intentionally added substances (Dorival-Garcia et al., 2020; Jenke et al., 2005), and at *m/z* 473.28207 (Δppm 1.000) a possible fragment of the antioxidant Irgafos (Gong et al., 2021; Hermabessiere et al., 2020) as well as two oxidized derivatives (Table S1) were found. For band **13** of the plastic migrate, UV-light absorbing stabilizers are likely to be present. Triazines or benzotriazoles and oxidation products were found at *m/z* 440.16052 (Δppm 0.085), *m/z* 448.15015 (Δppm −1.585), *m/z* 412.12915 (Δppm −0.079), *m/z* 446.13611 (Δppm 0.788), *m/z* 462.13101 (Δppm −0.990), *m/z* 474.16732 (Δppm 0.572) and *m/z* 490.16232 (Δppm 0.707) (Keck et al., 1998; Cormack et al., 2019; Olson et al., 2015).

For RP-HPTLC–APCI/HESI-HRMS, mass spectra were recorded from one zone only. Therefore, the genotoxic compound amount per band had to be increased, and 100 µL of the respective 30 × migrate concentrate, or alternatively, 60 µL of the 50 × migrate concentrate, were applied (Table S3). The RP-HPTLC–S9–SOS-Umu-C bioautogram showed altogether three clear genotoxic bands for the paper and plastic migrates (Fig. S4), whereas the coating migrate did not show any genotoxic response for the tested amounts. Less genotoxic bioactive zones were detected on the RP bioautogram, which were not as sensitively detected as on the NP bioautogram. Methanol containing 4 mM ammonium formate and 0.1% formic acid was used as eluent for good ionization. RP-HPTLC–HESI/APCI-HRMS spectra were recorded from the genotoxic band **15** in the paper migrate near the solvent front (Fig. S4), which indicated the presence of hexamethylcyclotrisiloxane (also named D3) at *m/z* 237.06198 (Δppm −7.623) as well as polydimethylsiloxane clusters. Since polydimethylsiloxane polymers are regulated food additives, no further investigations were performed for these substances (Europäische Datenbank für Lebensmittelzusatzstoffe, 2019). Hexamethylcyclotrisiloxane had already been reported to be DNA-damaging by the comet bioassay (Farasani & Darbre, 2017). However, although genotoxicity and mutagenicity of the respective standard compound were investigated, it could not be verified via the 96-well plate SOS-Umu-C bioassay and Ames MPF™ bioassay (data not shown). This was explained by the limited solubility of hexamethylcyclotrisiloxane (only 0.7125 mg/mL soluble in DMSO). However, reference amounts up

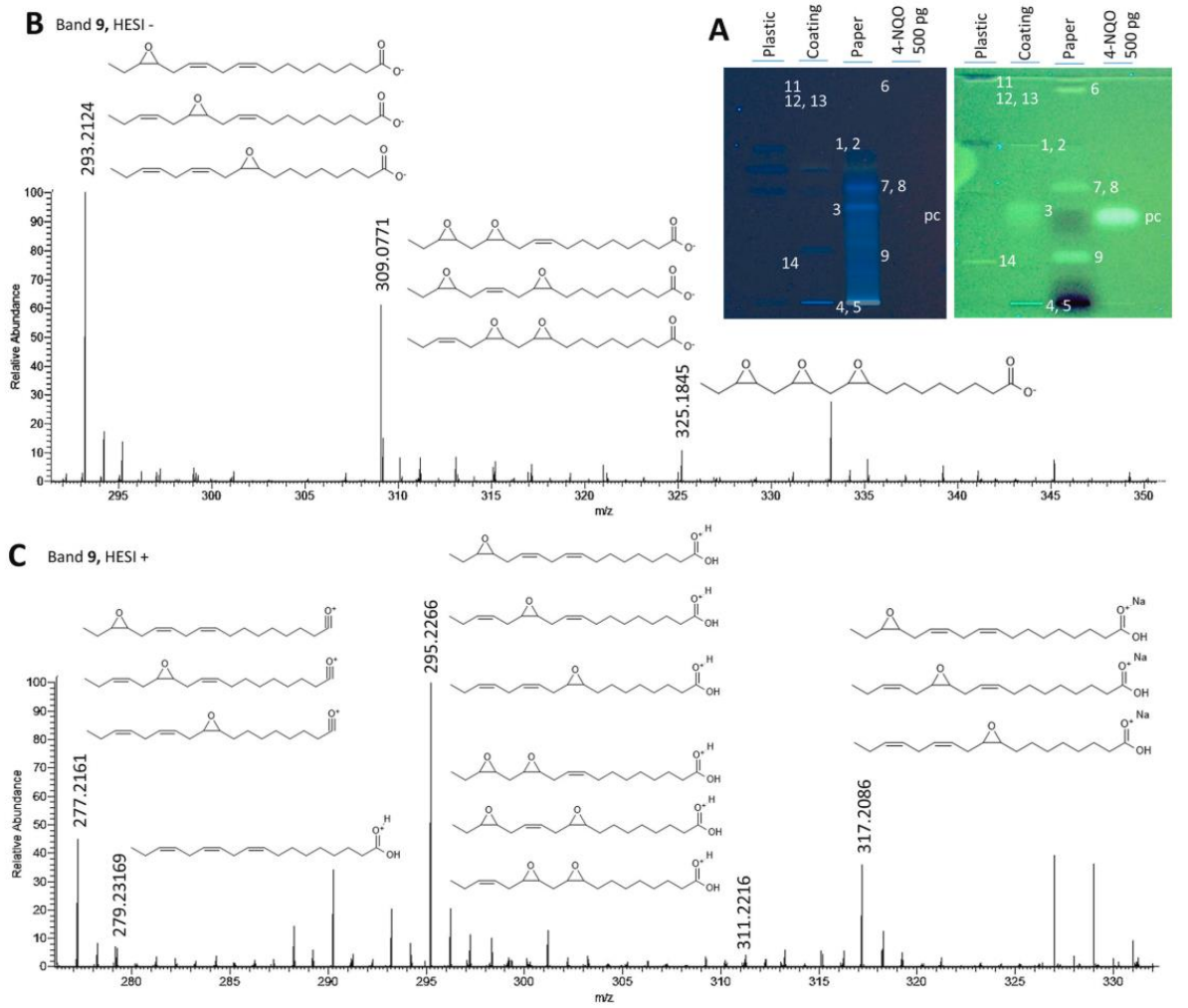


Fig. 2. Identification of the genotoxic band 9: Chromatogram at 366 nm and bioautogram at FLD 254 nm of the 50 × migrate concentrates of the paper sample and migration simulant blank (8 μL/band each) applied on HPTLC plate silica gel 60, developed twice with, *i. e.* first *n*-hexane – ethyl acetate 3:2, *V/V*, up to 50 mm and then *n*-hexane – toluene 4:1, *V/V*, up to 70 mm, and subjected to the SOS-Umu-C bioassay (**A**); HPTLC–HESI-HRMS spectra of band **9** in the negative (**B**) and positive (**C**) ionization modes.

to 5.0 μg/band tested via the RP-HPTLC–S9–SOS-Umu-C bioassay showed no response (Fig. S5). As a proof of principle, the two positive controls were recorded by HPTLC-HESI-HRMS. At a substance amount of about 1 ng/band, the 4-NQO was difficult to detect (Fig. S4). This showed that even if a compound has a strong genotoxic signal, it is not necessarily detectable in HRMS at such low amounts. On the other hand, this challenge in the identification of low-level genotoxins underlines the very good biological detectability. In addition, selectivity, solubility and ionization settings can influence the signals and those observations deserve further attention.

Comparing both coupling approaches made for recording of mass spectra, the solution transferred via a vial from the NP-HPTLC plate to FIA–HESI-HRMS was about 4.5-fold less concentrated (Table S3), but poor in polar compounds due to the more selective elution with ethyl acetate. In contrast, the elution from the RP plate directly to HESI-/APCI-HRMS via acidified methanol was more polar. Hence, differences in compound solubility within a zone during elution to HRMS (selective compound solubility) can be exploited advantageously for assignment of mass signals. As a conclusion for trace analysis, it is recommended to elute the sample not only with methanol (preferred for ionization), but also to pool several sample zones while using a less polar elution solvent. Still, the latter needs to be capable for elution of the substances in the zone, which after evaporation and redissolution in methanol, are transferred to HRMS. Another option is the recently developed 8D hyphenation (Schreiner & Morlock, 2021) or 12 D hyphenation (Schreiner et al., 2022) of the bioautogram with an integrated desalting step and liquid chromatography before recording of mass spectra. Note that the recording of mass spectra from all zones of interest on the HPTLC plate can be performed fully automated as sequence, as recently shown in an open-source modified interface (Mehl et al., 2021).

3.3. *Confirmation of the genotoxic bioactive band 9 as linolenic acid epoxides*

The genotoxic band **9** was preliminary assigned to be linolenic acid epoxides. For proof of a potential epoxide formation on the plate and

thus artefact generation by the open chromatographic system itself, the reference linolenic acid was analyzed by a 2D development. The same mobile phase system was used for both dimensions, and the 2D chromatogram was subjected to the SOS-Umu-C bioassay. Genotoxic responses were only observed on the diagonal line, which proved the absence of any artefact generation (Fig. 4). In contrast, genotoxic responses located outside the diagonal line would have indicated artefacts generated by the chromatographic system.

Together with the paper migrate the reference compounds linolenic, linoleic and oleic acids were co-developed and analyzed via the HPTLC–SOS-Umu-C assay to further proof that band **9** is caused by linolenic acid. In the bioautogram (Fig. 3), linolenic acid showed four genotoxic responses (**8**–**10**, **15**) and linoleic acid one genotoxic response (**16**), whereas oleic acid showed none. In addition, the genotoxic signals were observed at the same hR_F value and the HPTLC–HESI-HRMS signals of the genotoxic band **9** in the paper migrate matched to those of linolenic acid and their epoxides (Figs. 2 and 3, S3, Table S2). This clearly confirmed the preliminary assignment.

It can be assumed that linolenic acid epoxides are present in the paper because paper contains plant material. In plants, the formation of fatty acid epoxides via peroxygenase for defense mechanisms is well described (Blee & Schuber, 1990). Especially for oat, a peroxygenase pathway was found producing linolenic acid epoxide (Meesapyodsuk & Qiu, 2011). Additionally, powder coatings, containing epoxidized linolenic acid esters in form of seed oil epoxides, had been proposed to be used as crosslinker in coatings (Overeem et al. 1999). These may also have technical applications as paper coatings. Also, soybean-oil-based epoxy acrylate emulsion stabilized by silanized nanocrystalline cellulose was recently demonstrated to be a sustainable paper coating (Tian et al., 2022). This could also explain the D3 and silicium containing polymer found via reversed phase separation.

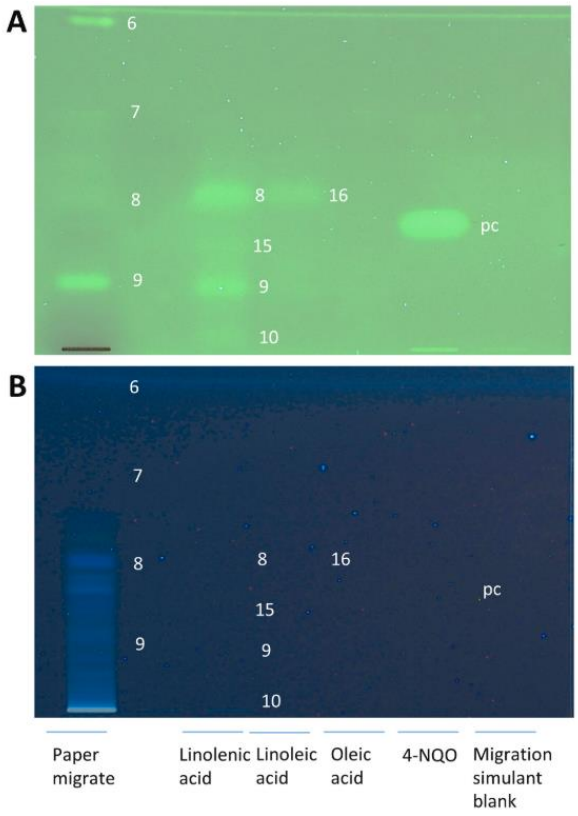


Fig. 3. Study of genotoxicity of unsaturated fatty acid epoxides and paper migrate: Bioautogram at FLD 254 nm (**A**) of the 50 × migrate concentrates of the paper sample and migration simulant blank (8 μL/band each) as well as the references linolenic acid, linoleic acid, and oleic acid (5 μg/band each), and the positive control 4-NQO (**pc**, 500 pg/band), applied on HPTLC plate silica gel 60 developed twice with, *i. e.* first toluene – ethyl acetate 3:2, *V/V*, up to 50 mm, and then, *n*-hexane – toluene 4:1, *V/V*, up to 70 mm, and subjected to the SOS-Umu-C bioassay, and corresponding chromatogram at FLD 366 nm (**B**).

3.4. Migrates and linolenic acid studied via Ames-MPF™ microtiter plate bioassay

The 300 × concentrates of the plastic, paper and coating migrates, which were 6- or 10-fold more concentrated compared to the planar bioassay procedure, were analyzed with the Ames MPF™ bioassay with and without metabolic activation to follow up the results from the planar bioassay. Both, the plastic and coating migrate scored negative in an initial screening (Table S4). A weak positive result was recovered for the undiluted paper migrate when using the strain/S9 combination TA100 –S9 (Fig. 5G), however, no dose response relationship was obtained. Other strains did not show a response (Fig. 5 E/F/H). As oxidation products of linolenic acid were identified as a potential source of the genotoxic signal, a concentrated DMSO solution of this pure substance was subjected to the Ames MPF™ testing. To trigger oxidation, the pure substance was exposed to air in the dark overnight for about 20 h before dissolving in DMSO at 50 mg/mL. This was done without stirring and heating to prevent the formation of hydroperoxides, which were otherwise observed with linolenic acid when heated and stirred (Yamaguchi & Yamashita, 1979). The Ames MPF™ bioassay now revealed weak positive effects in several strain S9 combinations (TA98 –S9, TA98 +S9; TA100 –S9, Fig. 5), overall supporting the hypothesis that epoxy groups could trigger the obtained responses.

For all assay experiments performed, the pronounced signals of the positive controls proved the successful performance of the bioassay. Both positive controls used, *i.e.* the 4-NQO without metabolic activation and the 2-AA with metabolic activation, were able to induce at least 12-fold the induction limit.

3.5. Migrates and linolenic acid studied via SOS-Umu-C microtiter plate bioassay

The SOS-Umu-C microtiter plate bioassay with and without metabolic activation did not show any genotoxic response for 300 × concentrates of the paper, coating and plastic migrates (Fig. 5K), however, the positive controls showed positive responses, which proved the proper functioning of the assay (Fig. S6). Linolenic acid did not show any genotoxic activity whether metabolic activation was used or not (Fig. 5I). However, after exposure of linolenic acid to air for about 20 h, the therefore partially oxidized linolenic acid derivatives exhibited a weak genotoxic activity without any metabolic activation (Fig. 5J). These results are a further proof of the epoxide formation found out via NP-HPTLC–UV/Vis/FLD–SOS-Umu-C–Vis/FLD (Fig. 1A), which is more sensitive in the detection compared to state-of-the-art *in vitro* bioassay counterparts. Considering the probably cytotoxic effects (Fig. 1, dark band **5**) of some constituents present in the paper migrate, two opposing effects/signal responses are differentiated on the planar surface and evident due to the planar separation. This may explain the false-negative responses for the paper matrix in the sum value assays. This highlights the important advantage of the NP-HPTLC–UV/Vis/FLD–SOS-Umu-C–Vis/FLD method, which is the separation of complex samples and thus differentiation of opposing effects/signal responses.

For all assay experiments performed, both positive controls, *i.e.* the 4-NQO without metabolic activation and the 2-AA with metabolic activation, were able to induce the transcription of the SOS Umu-C gene (5.9- and 2.0-fold induction, respectively) confirming the quality criteria to accept the assay.

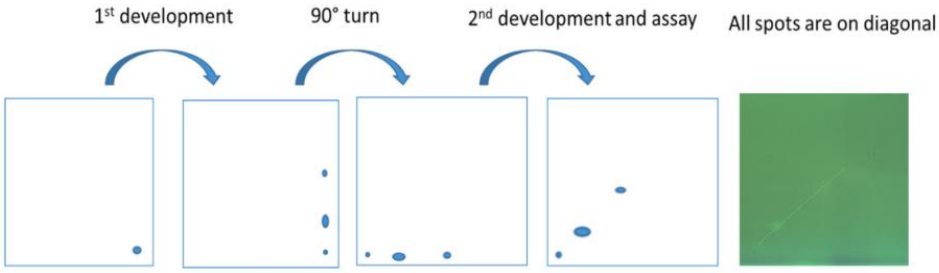


Fig. 4. 2D separation of linolenic acid to exclude artefacts: 2D separation scheme and resulting bioautogram at FLD 254 nm of linolenic acid (5 μg/band) on HPTLC plate silica gel 60 after a 2-step development with first toluene – ethyl acetate 3:2, *V/V*, up to 50 mm, and then, *n*-hexane – toluene 4:1, *V/V*, up to 70 mm. After waiting for 15 min, the plate was turned by 90°, developed again via the 2-steps and subjected to the SOS-Umu-C bioassay. Genotoxic responses (bright green fluorescent zones) are only observed on the diagonal line, whereas any artefact generated by the chromatographic system (potential epoxide formation on the plate) would have been positioned outside the diagonal line.

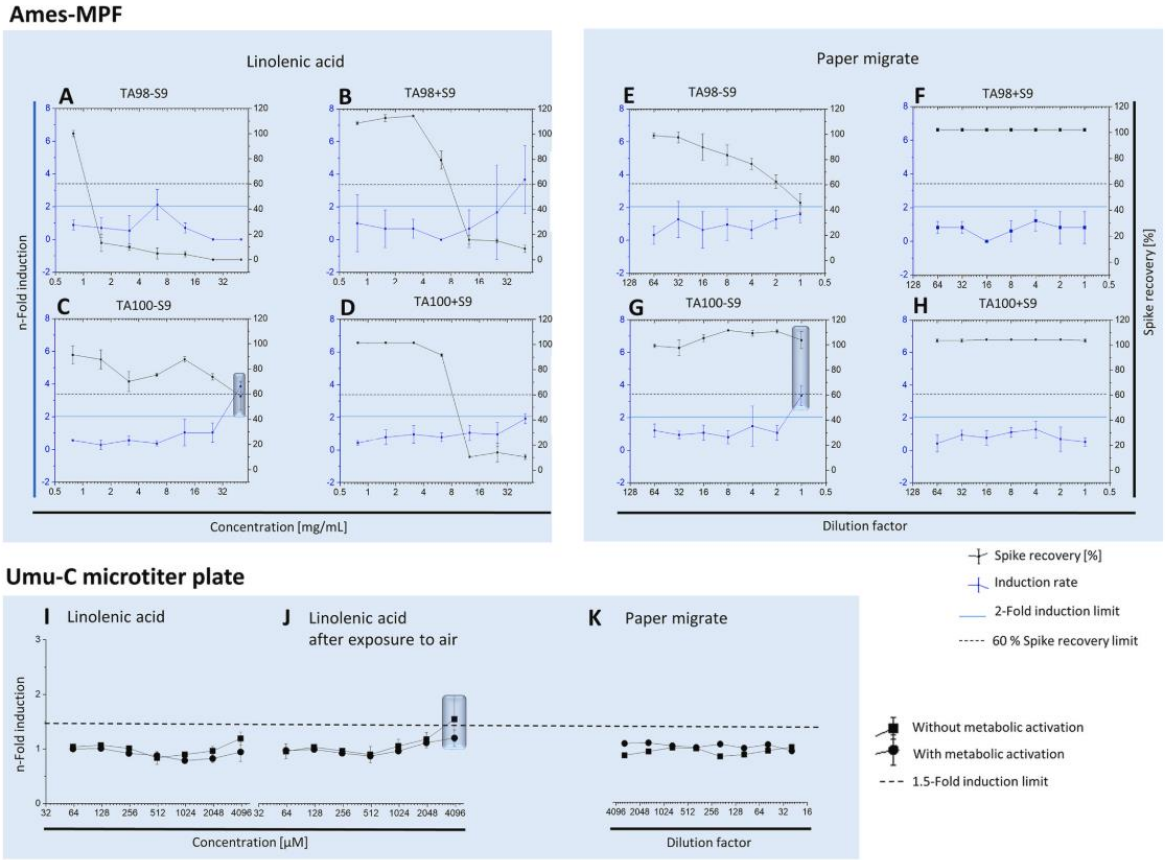


Fig. 5. Confirmation of the identified genotoxin via two *in vitro* assays: Results of the *in vitro* Ames-MPF™ and SOS-Umu-C microtiter plate assays for linolenic acid and paper migrate. The n-fold induction of the respective mechanism is referred either to the concentration of the reference compound linolenic acid or dilution factor of the paper migrate.

4. Conclusions

The newly developed RP/NP-HPTLC–(S9)–SOS-Umu-C bioassay with piezoelectrical spraying of the *Salmonella typhimurium* TA1535/pSK1002 suspension was demonstrated to have solved the challenges regarding detection limit, matrix effects (occurring in *in vitro* microtiter plate bioassay counterparts) and chemical identification of hazards of concern to fully assess the safety of new packaging. The advantage of directly eluting zones for HRMS was shown in the rapid preliminary identification of linolenic acid epoxides, as the genotoxic compounds present in the paper migrate. By the analysis of the linolenic acid reference compound that was exposed to air to generate the epoxides,

this preliminary assignment was proven by NP-HPTLC−SOS-Umu-C bioassay and confirmed via the SOS-Umu-C microtiter plate bioassay. Additionally, the Ames-MPF™ bioassay revealed the mutagenicity of the linolenic acid after exposure to air and of the paper migrate, however, being not so sensitive in the detection, higher concentration (300 × concentrates) were required. Although several low-level genotoxic bands could not be assigned to any compounds by direct HPTLC-HESI/APCI-HRMS, the developed selective elution, substance enrichment via zone pooling and redissolvation for flow injection was shown as a new successful workflow to improve HRMS detectability for trace-level genotoxins. Although a higher sample amount is needed on the RP plate, the use of both phases (NP/RP) is beneficial due to the orthogonal separation. Additional ionization techniques should be used in the future to enlarge the scope of ionizable and thus identifiable molecules.

Human and animal rights and informed consent

The article does not contain any studies with animals or human performed by any of the authors.

CRediT authorship contribution statement

Daniel Meyer: Methodology, Investigation HPTLC, Writing – original draft. **Maricel Marin-Kuan:** Conceptualization, Supervision, Writing – review & editing. **Elisa Mayrhofer:** Investigation Ames MPF assay, Writing – review & editing. **Christian Kirchnawy:** Writing – review & editing. **Emma Debon:** Investigation HPTLC confirmation, Writing – review & editing. **Helia Latado:** Investigation in vitro SOS-Umu-C assay, Writing – review & editing. **Amaury Patin:** Writing – review & editing. **Benoît Schilter:** Writing – review & editing. **Gertrud Morlock:** Conceptualization, Methodology, Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Instrumentation was partially funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - INST 162/471-1 FUGG; INST 162/536-1 FUGG. The JLU research project RDLS017728 (Application of HPTLC-coupled to bioassays system to address safety of complex food-related mixtures) was funded by Nestlé Research, Switzerland.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109546.

References

Azadniya, E., & Morlock, G. E. (2019). Automated piezoelectric spraying of biological and enzymatic assays for effect-directed analysis of planar chromatograms. *Journal of Chromatography A, 1602*, 458–466. https://doi:10.1016/j.chroma.2019.05.043.

Bjorseth, A., Eidsa, G., Gether, J., Landmark, L., & Moller, M. (1982). Detection of mutagens in complex samples by the Salmonella assay applied directly on thin-layer chromatography plates. *Science, 215*(4525), 87–89. https://doi:10.1126/science.7031897.

Blee, E., & Schuber, E. (1990). Stereochemistry of the epoxidation of fatty acids catalyzed by soybean peroxygenase. *Biochemical and Biophysical Research Communications, 173*(3), 1354–1360. https://doi:10.1016/S0006-291X(05)80937-4.

Bopp, S. K., Kienzler, A., Richarz, A.-N., van der Linden, S. C., Paini, A., Parissis, N., & Worth, A. P. (2019). Regulatory assessment and risk management of chemical mixtures: Challenges and ways forward. *Critical Reviews in Toxicology, 49*(2), 174–189. https://doi:10.1080/10408444.2019.1579169.

Cormack, P. A. G., Erdemli, O. C., & Sankey, S. W. (2019). Polymerizable UV absorbers for the UV stabilization of polyesters. I. Design, synthesis and polymerization of a library of UV absorbing monomers. *ARKIVOC (Gainesville, FL, United States) NO VOL. NO., 6*, 148–173. https://doi:10.24820/ark.5550190.p011.575.

Debon, E., Rogeboz, P., Latado, H., Morlock, G. E., Meyer, D., Cottet Fontannaz, C., Scholz, G., Schilter, B., & Marin-Kuan, M. (2022). Incorporation of metabolic activation in the HPTLC-SOS-Umu-C bioassay to detect low amounts of genotoxic chemicals. *Toxics, 10*, 501 . https://doi.org/10.3390/toxics10090501.

Dorival-Garcia, N., Galbiati, F., Kruell, R., Kovasy, R., Dunne, S. O., D'Silva, K., & Bones, J. (2020). Identification of additives in polymers from single-use bioprocessing bags by accelerated solvent extraction and ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry. *Talanta, 219*, Article 121198. https://doi:10.1016/j.talanta.2020.121198.

Europäische Datenbank für Lebensmittelzusatzstoffe. (2019). *E 900 Dimethyl polysiloxane*. https://webgate.ec.europa.eu/foods_system/main/index.cfm?event=substance.view&identifier=292&sector=FAD&auth=SANCAS. (Accessed 4 April 2022) Accessed.

Farasani, A., & Darbre, P. D. (2017). Exposure to cyclic volatile methylsiloxanes (cVMS) causes anchorage-independent growth and reduction of BRCA1 in non-transformed human breast epithelial cells. *Journal of Applied Toxicology, 37*(4), 454–461. https://doi:10.1002/jat.3378.

Gong, X., Zhang, W., Zhang, S., Wang, Y., Zhang, X., Lu, Y., Sun, H., & Wang, L. (2021). Organophosphite antioxidants in mulch films are important sources of organophosphate pollutants in farmlands. *Environmental Science & Technology, 55*(11), 7398–7406. https://doi:10.1021/acs.est.0c08741.

Hermabessiere, L., Receveur, J., Himber, C., Mazurais, D., Huvet, A., Lagarde, F., Lambert, C., Paul-Pont, I., Dehaut, A., Jezequel, R., Soudant, P., & Duflos, G. (2020). An Irgafos 168 story: When the ubiquity of an additive prevents studying its leaching from plastics. *Science of the Total Environment, 749*, Article 141651. https://doi:10.1016/j.scitotenv.2020.141651.

International Organization for Standardization ISO 13829. (2000). *2000-03, Water quality - determination of the genotoxicity of water and waste water using the umu-test.* Berlin, Germany: Beuth Verlag.

International Organization for Standardization ISO 11350. (2012). *2012-05, Water quality - determination of the genotoxicity of water and waste water - Salmonella/microsome fluctuation test (Ames fluctuation test)*. Berlin, Germany: Beuth Verlag.

Jamshidi-Aidji, M., & Morlock, G. E. (2016). From bioprofiling and characterization to bioquantification of natural antibiotics by direct bioautography linked to high-resolution mass spectrometry: Exemplarily shown for *Salvia miltiorrhiza* root. *Analytical Chemistry, 88*(22), 10979–10986. https://doi:10.1021/acs.analchem.6b02648.

Jenke, D. R., Jene, J. M., Poss, M., Story, J., Tsilipetros, T., Odufu, A., & Terbush, W. (2005). Accumulation of extractables in buffer solutions from a polyolefin plastic container. *International Journal of Pharmaceutics, 297*(1–2), 120–133. https://doi:10.1016/j.ijpharm.2005.03.010.

Keck, J., Roessler, M., Schroeder, C., Stueber, G. J., Waiblinger, F., Stein, M., LeGourrierec, D., Kramer, H. E. A., Hoier, H., Henkel, S., Fischer, P., Port, H., Hirsch, T., Rytz, G., & Hayoz, P. (1998). Ultraviolet absorbers of the 2-(2-Hydroxyaryl)-1,3,5-triazine class and their methoxy derivatives: Fluorescence spectroscopy and X-ray structure analysis. *Journal of Physical Chemistry B, 102*(36), 6975–6985. https://doi:10.1021/jp9818380.

Klingelhöfer, I., Hockamp, N., & Morlock, G. E. (2020). Non-targeted detection and differentiation of agonists versus antagonists, directly in bioprofiles of everyday products. *Analytica Chimica Acta, 1125*, 288–298. https://doi:10.1016/j.aca.2020.05.057.

Koster, S., Bani-Estivals, M.-H., Bonuomo, M., Bradley, E., Chagnon, M. C., Garcia, M. L., Godts, F., Gude, T., Helling, R., Paseiro-Losada, P., Pieper, G., Rennen, M., Simat, T., & Spack, L. (2015). Guidance on best practices on the risk assessment of non intentionally added substances (NIAS) in food contact materials and articles. *ILSI Europe Report Series*, 1–70.

Meesapyodsuk, D., & Qiu, X. (2011). A peroxygenase pathway involved in the biosynthesis of epoxy fatty acids in oat. *Plant Physiology, 157*(1), 454–463. https://doi:10.1104/pp.111.178822.

Mehl, A., Schwack, W., & Morlock, G. E. (2021). On-surface autosampling for liquid chromatography−mass spectrometry. *Journal of Chromatography A, 1651*, Article 462334. https://doi:10.1016/j.chroma.2021.462334.

Meyer, D., Marin-Kuan, M., Debon, E., Serrant, P., Cottet-Fontannaz, C., Schilter, B., & Morlock, G. E. (2020). Detection of low levels of genotoxic compounds in food contact materials using an alternative HPTLC-SOS-Umu-C assay. *Alternatives to Animal Experimentation, 38*(3), 387–397. https://doi:10.14573/altex.2006201.

Monarca, S., Fusco, R. de, Biscardi, D., Feo, V. de, Pasquini, R., Fatigoni, C., Moretti, M., & Zanardini, A. (1994). Studies of migration of potentially genotoxic compounds into water stored in PET bottles. *Food and Chemical Toxicology, 32*(9), 783–788. https://doi:10.1016/0278-6915(94)90153-8.

Morlock, G. E., & Heil, J. (2020). HI-HPTLC-UV/Vis/FLD-HESI-HRMS and bioprofiling of steviol glycosides, steviol, and isosteviol in *Stevia* leaves and foods. *Analytical and Bioanalytical Chemistry, 412*(24), 6431–6448. https://doi:10.1007/s00216-020-02618-4.

Olson, D. B., Savu, P. M., & North, D. (2015). *Copolymers including a triazine group and compositions including them*, Article US20170198119A1.

Organization for Economic Co-operation and Development. (2020). *Test No. 471: Bacterial reverse mutation test: Oecd*. https://doi.org/10.1787/20745788

Overeem, A., Buisman, G. J. H., Derksen, J. T. P., Cuperus, F. P., Molhoek, L., Grisnich, W., & Goemans, C. (1999). Seed oils rich in linolenic acid as renewable feedstock for environment-friendly crosslinkers in powder coatings. *Industrial Crops and Products, 10*(3), 157–165. https://doi:10.1016/S0926-6690(99)00018-7.

Ozaki, A., Ooshima, T., & Mori, Y. (2006). Migration of dehydroabietic and abietic acids from paper and paperboard food packaging into foodsimulating solvents and Tenax TA. *Food Additives & Contaminants, 23*(3), 854–860. https://doi:10.1080/02652030600743813.

Rainer, B., Mayrhofer, E., Redl, M., Dolak, I., Mislivececk, D., Czerny, T., Kirchnawy, C., Marin-Kuan, M., Schilter, B., & Tacker, M. (2019). Mutagenicity assessment of food contact material migrates with the Ames MPF assay. *Food Additives & Contaminants: Part A, 36*(9), 1419–1432. https://doi:10.1080/19440049.2019.1634841.

Rosenmai, A. K., Bengtström, L., Taxvig, C., Trier, X., Petersen, J. H., Svingen, T., Binderup, M., van Vugt-Lussenburg, B. M. A., Dybdahl, M., Kit, G., & Vinggaard, A. M. (2017). An effect-directed strategy for characterizing emerging chemicals in food contact materials made from paper and board. *Food and Chemical Toxicology, 106*, 250–259. https://doi:10.1016/j.fct.2017.05.061.

Schilter, B., Burnett, K., Eskes, C., Geurts, L., Jacquet, M., Kirchnawy, C., Oldring, P., Pieper, G., Pinter, E., Tacker, M., Traussnig, H., van Herwijnen, P., & Boobis, A. (2019). Value and limitation of in vitro bioassays to support the application of the threshold of toxicological concern to prioritise unidentified chemicals in food contact materials. *Food Additives & Contaminants: Part A, 36*(12), 1–34. https://doi:10.1080/19440049.2019.1664772.

Schreiner, T., & Morlock, G. E. (2021). Non-target bioanalytical eight-dimensional hyphenation including bioassay, heart-cut trapping, online desalting, orthogonal separations and mass spectrometry. *Journal of Chromatography, A, 1647*, Article 462154. https://doi:10.1016/j.chroma.2021.462154.

Schreiner, T., Ronzheimer, A., Friz, M., & Morlock, G. E. (2022). Multiplex planar bioassay with reduced diffusion on normal phase, identifying androgens, verified antiandrogens and synergists in botanicals via 12D hyphenation. *Food Chemistry, 395*, 1336110. https://doi.org/10.1016/j.foodchem.2022.133610.

Shakibai, D., Riegraf, C., Moscovici, L., Reifferscheid, G., Buchinger, S., & Belkin, S. (2019). Coupling high-performance thin-layer chromatography with bacterial genotoxicity bioreporters. *Environmental Science & Technology, 53*(11), 6410–6419. https://doi:10.1021/acs.est.9b00921.

The European Commission. (2011). *Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food*. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020R1245&from=EN. (Accessed 4 April 2022) Accessed.

Tian, X., Wu, M., Wang, Z., Zhang, J., & Lu, P. (2022). A high-stable soybean-oil-based epoxy acrylate emulsion stabilized by silanized nanocrystalline cellulose as a sustainable paper coating for enhanced water vapor barrier. *Journal of Colloid and Interface Science, 610*, 1043–1056. https://doi:10.1016/j.jcis.2021.11.149.

Wilkins, A. L., & Panadam, S. (1987). Extractable organic substances from the discharges of a New Zealand pulp and paper mill. *Appita, 40*(3), 208–212.

Williams, R. V., DeMarini, D. M., Stankowski, L. F., Jr., Escobar, P. A., Zeiger, E., Howe, J., Elespuru, R., & Cross, K. P. (2019). Are all bacterial strains required by OECD mutagenicity test guideline TG471 needed? *Mutation Research, Genetic Toxicology and Environmental Mutagenesis, 848*, Article 503081. https://doi.org/10.1016/j.mrgentox.2019.503081

Xenometrix. (2018). *Ames MPF™ 98/100 microplate format mutagenicity assay*. Switzerland: Allschwil. https://www.xenometrix.ch/shop/mediafiles/Xeno%20Dateien/Short%20Protocol/Ames/Ames%20MPF%2098-100%20Short%20Protocol_2.0.pdf. (Accessed 4 April 2022) Accessed.

Yamaguchi, T., & Yamashita, Y. (1979). Mutagenic activity of autoxidized linolenic and linoleic acid. *Agricultural and Biological Chemistry, 43*(10), 2225–2226. https://doi:10.1271/bbb1961.43.2225.

Yunker, M. B., Lachmuth, C. L., Cretney, W. J., Fowler, B. R., Dangerfield, N., White, L., & Ross, P. S. (2011). Biota - sediment partitioning of aluminum smelter related PAHs and pulp mill related diterpenes by intertidal clams at Kitimat, British Columbia. *Marine Environmental Research, 72*(3), 105–126. https://doi:10.1016/j.marenvres.2011.06.004.

Supporting information

Effect-detection by planar SOS-Umu-C genotoxicity bioassay and chemical identification of genotoxins in packaging migrates, proven by microtiter plate assays SOS-Umu-C and Ames-MPF

Daniel Meyer[a], Maricel Marin-Kuan[b], Elisa Mayrhofer[c], Christian Kirchnawy[c] Emma Debon[b], Helia Latado[b], Amaury Patin[b], Benoît Schilter[b], Gertrud Morlock[a,*]

[a]Institute of Nutritional Science, Chair of Food Science, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

[b]Chemical Food Safety Group, Société des Produits Nestlé SA - Nestlé Research, Vers-chez-les-Blanc, Switzerland

[c]Department for Microbiology and Cell Culture, Austrian Research Institute for Chemistry and Technology, Franz-Grill Straße 5, 1030 Vienna, Austria

*Corresponding author: Tel. +49-641-99-39141; fax: +49-641-99-39149.

E-mail address: Gertrud.Morlock@uni-giessen.de

Table of Contents

plate together with the positive control 4-NQO (500 ng/band). The plate was developed twice, *i. e*. first with *n*-hexane − ethyl acetate 4:5 up to 50 mm and then with *n*-hexane − ethyl acetate 8:1 up to 70 mm. One plate piece was subjected to the NP-HPTLC−SOS-Umu-C bioassay to obtain the bioautogram at FLD 254 nm (**A**), and from the other plate piece (chromatogram at FLD 366 nm), the respective bands **1**–**5** were eluted with methanol (**B**) and transferred to NP-HPTLC−HESI-HRMS.

Page S-11 **Fig. S4** HPTLC-APCI/HESI-HRMS spectrum of band 15 and positive controls: RP-HPTLC−S9-SOS-Umu-C bioautogram at FLD 254 nm of the plastic, coating and paper migrates (30x concentrate, 100 µL), two positive controls 4-NQO (0.7 ng/band), AFB1 (140 ng/band) and migration simulant blank as well as HPTLC-APCI(+)-HRMS spectra of the paper migrate band **15** near the solvent front and its zoom on *m/z* 220-223 showing the signal of protonated hexamethyl-cyclotrisiloxane as well as dimethylpolysiloxane clusters. In comparison, the obtained HPTLC-HESI(+)-HRMS spectra of 4-NQO and AFB1. No ions were detected from the genotoxic zones of the plastic and coating migrates.

Page S-12 **Fig. S5** Testing of the assumed candidate hexamethylcyclotrosiloxane: RP-HPTLC-SOS-Umu-C bioautograms at FLD 254 nm of paper migrate (30× concentrate, 100 µL) showing a positive response for the silicon compound band even without metabolic activation (**A**), whereas the assumed candidate hexamethylcyclotri-siloxane did not show any genotoxic response (**B**).

Page S-13 **Fig. S6** Microtiter plate SOS-Umu-C bioassay results for the 300× concentrates of the plastic and coating migrates with and without metabolic activation.

Table S1 Molecular ions found during the NP–FIA–HESI-HRMS experiments for the paper and plastic migrates (for zone ID in Fig. S2), to which substance have been assigned by literature search or which remain unknown.

Zone ID	$[M]^+$	Corresponding molecular formular	Δ *m/z* [ppm]	$[M]^-$	Corresponding molecular formular	Δ *m/z* [ppm]
Paper 1	447.34670	C28H47O4	-1.754	397.22684 to 1465.96899	unknown polymer	-
	684.20287 to 1426.38875	silicon polymers				
Paper 2	257.22630	C19H29	-2.55	151.04007	C8H7O3	0.017
	255.21070	C19H27	-2.413	165.05571	C9H9O3	-0.045
	303.23750	C20H31O2	-0.352	167.03497	C8H7O4	-0.163
				179.03503	C9H7O4	0.268
				181.05072	C9H9O4	0.486
Plastic 3				293.17612	C17H25O4	2.865
				473.28281	C28H42O4P	1.564
				489.27789	C28H42O5P	1.839
				505.27310	C28H42O6P	2.735
Plastic 4	440.16052	C26H22O4N3	0.085	446.13611	C24H20O6N3	0.788
	1102.60718	C69H84O11N	2.488	462.13101	C24H20O7N3	-0.990
	448.15015	C24H22O6N3	-1.585	322.07227	C18H12O5N	0.541
	412.12915	C24H18O4N3	-0.079	294.07736	C17H12O4N	0.608
				278.08231	C17H12O3N	0.156
				474.16733	C26H24O6N3	0.572
				490.16232	C26H24O7N3	0.707

Table S2 Mass signals detected via HPTLC–HESI-HRMS in the positive ionization mode for linolenic, linoleic, and different epoxides (for zone ID in Fig. S3)

ID	$[M+Na]^+$	Corresponding fatty acid and error	Δ *m/z* [ppm]	Intensity
1	301.21378	Linolenic acid	-0.071	8.51E+07
	317.20868	Epoxide of linolenic acid	-0.113	2.12E+07
	333.20355	Diepoxide of linolenic acid	-0.242	9.65E+06
	349.19855	Triepoxide of linolenic acid	-0.014	8.53E+06
2	301.21368	not found	-	-
	317.20859	Epoxide of linolenic acid	-0.397	2.87E+06
	333.20349	Diepoxide of linolenic acid	-0.422	3.24E+06
	349.19855	Triepoxide of linolenic acid	0.186	2.12E+06
3	301.21356	Linolenic acid	-0.801	9.04E+04
	317.20880	Epoxide of linolenic acid	0.265	9.79E+04
	333.20352	Diepoxide of linolenic acid	-0.332	7.33E+06
	349.19849	Triepoxide of linolenic acid	-0.158	1.49E+06
4	301.21390	Linolenic acid	0.327	1.17E+06
	317.20874	Epoxide of linolenic acid	0.076	1.88E+06
	333.20370	Diepoxide of linolenic acid	0.208	4.09E+06
	349.19858	Triepoxide of linolenic acid	0.100	4.59E+07
5	307.26077	Linoleic acid	0.060	1.26E+05
	321.23987	Epoxide of linoleic acid	-0.455	2.22E+05
	335.21915	Diepoxide of linoleic acid	-0.300	1.14E+06

Table S3 Estimation of the migrate concentration transferred for recording of HPTLC-HRMS spectra

NP-HPTLC–FIA–HESI-HRMS
Subsequently: bioassay as proof for proper zone elution
Five eluted 8-µL bands of the 50× concentrate in 200 µL methanol, whereof 10 µL were injected within 10 s at a 200-µL/min flow rate: 5 x 8 x 50 / 200 = 10 → 10× concentrate/µL injected over 10 s at a flow rate of 200 µL/min

RP-HPTLC–HESI-HRMS
In parallel with bioassay for match of zone positioning
One eluted 100-µL band of the 30x concentrate eluted in 67 µL (volume calculated: sample ions visible for 20 s during ms-experiment at a flow rate of 200 µL/min = 67 µL) at a 200-µL/min flow rate directly by TLC-MS interface into the HESI-HRMS: 1 x 100 x 30 / 67 = 45 → 45x concentrate/µL injected over 20 s at flow rate of 200 µL/min

Table S4 Ames MPF^{TM} results for the 300× concentrates of the coating and plastic migrates

		n-fold increase		Spike recovery [%]	
		Mean	Std. dev.	Mean	Std. dev.
		Coating			
TA98	-S9	1.3	1.1	98.6	1.2
	+S9	0.5	0.5	104.3	0.0
TA100	-S9	1.7	0.6	101.4	3.3
	+S9	1.4	0.9	100.0	0.0
		Plastic			
TA98	-S9	0.3	0.0	89.7	5.6
	+S9	0.7	0.6	100.3	0.0
TA100	-S9	0.7	0.1	99.3	1.2
	+S9	0.7	0.1	119.8	2.9

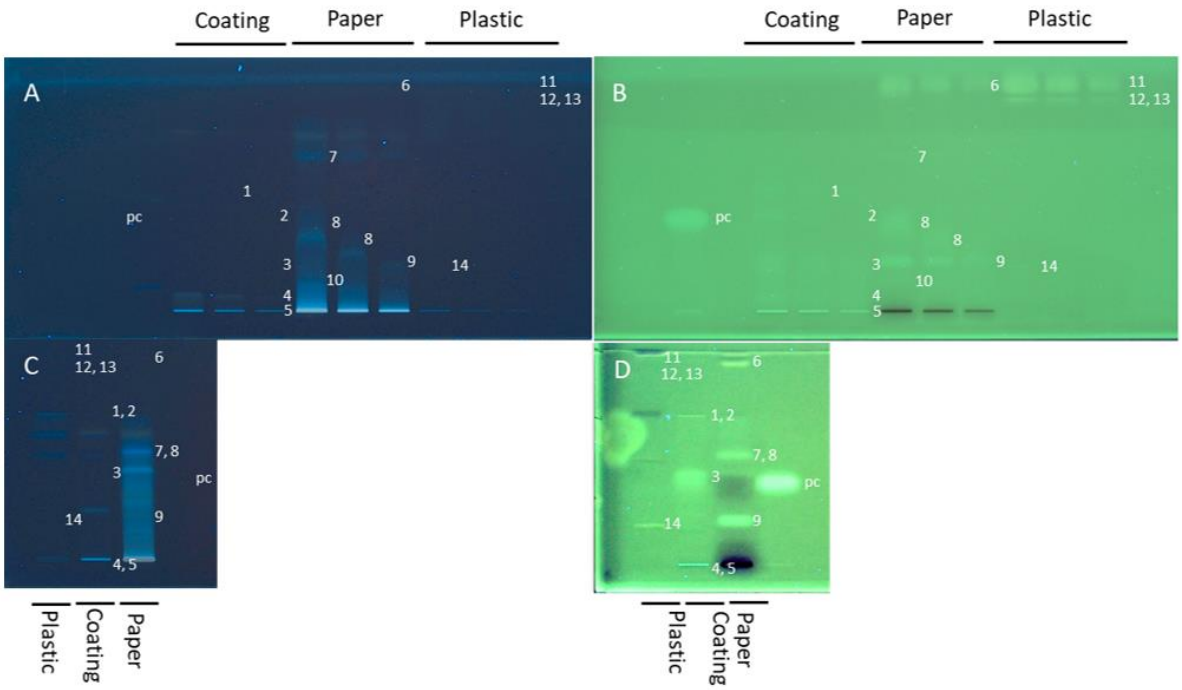


Fig. S1 Comparison of two different mobile phases: toluene − ethyl acetate 2:3, *V/V*, up to 80 mm (**A**/**B**) and first toluene − ethyl acetate 3:2, *V/V*, up to 50 mm, and then *n*-hexane − toluene 4:1, *V/V*, up to 70 mm (**C**/**D**), chromatograms at FLD 366 (**A**/**C**) and corresponding bioautograms at FLD 254 (**B**/**D**) of the 50× migrate concentrates (8, 4 and 2 µL/band) using the experimental conditions as for Fig. 1 (**A**/**B**) *versus* Fig. 2A (**C**/**D**; 8 µL/band).

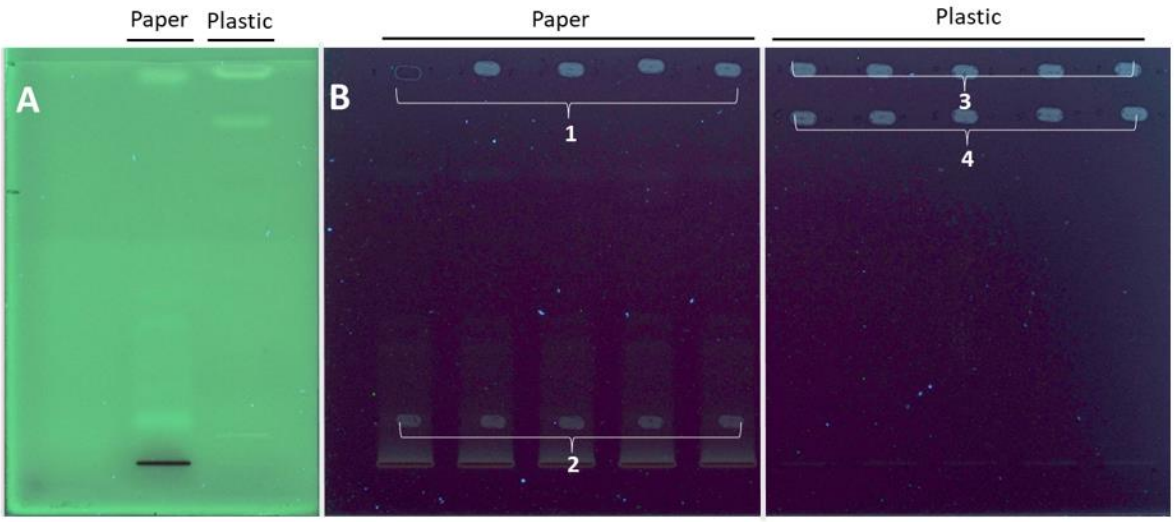


Fig. S2 Identification of unknown genotoxins in migrates via FIA-HESI-HRMS: The 50× migrate concentrates of the paper and plastic samples (8 µL/band each) were 6-fold applied on the NP plate, developed twice (first with toluene – ethyl acetate 3:2 up to 50 mm and then *n*-hexane – ethyl acetate 4:1 up to 70 mm), and cut into two pieces. One was subjected to the bioassay to obtain the bioautogram at FLD 254 nm (**A**), and from the other chromatogram at FLD 366 nm (**B**), the respective five bands at the same position were eluted each with 300 µL ethyl acetate and collected/pooled in the same vial. This was performed for the marked zones **1**–**4**. Each solvent (1.5 mL) was evaporated, and each residue was taken up in 200 µL methanol. These four solutions were used for FIA-HESI-HRMS.

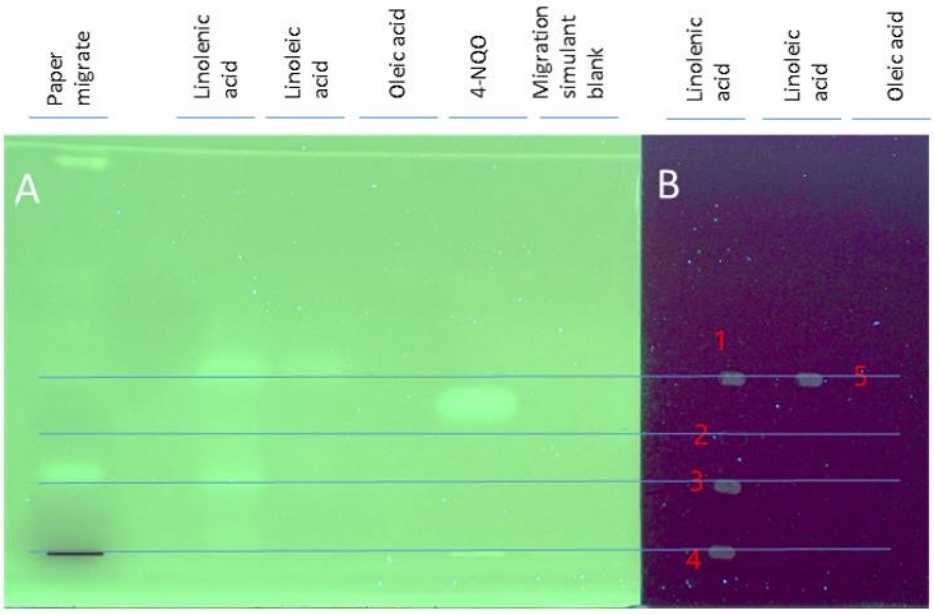


Fig. S3 Study of genotoxicity of unsaturated fatty acid epoxides and paper migrate via NP-HPTLC-HESI-HRMS and NP-SOS-Umu-C bioassay: 50× migrate concentrates of the paper sample and migration simulant blank (8 µL/band each) as well as linolenic acid, linoleic acid, and oleic acid (5 µg/band each) were applied on the NP-HPTLC plate together with the positive control 4-NQO (500 ng/band). The plate was developed twice, *i. e.* first with *n*-hexane – ethyl acetate 4:5 up to 50 mm and then with *n*-hexane – ethyl acetate 8:1 up to 70 mm. One plate piece was subjected to the NP-HPTLC–SOS-Umu-C bioassay to obtain the bioautogram at FLD 254 nm (**A**), and from the other plate piece (chromatogram at FLD 366 nm), the respective bands **1**–**5** were eluted with methanol (**B**) and transferred to NP-HPTLC–HESI-HRMS.

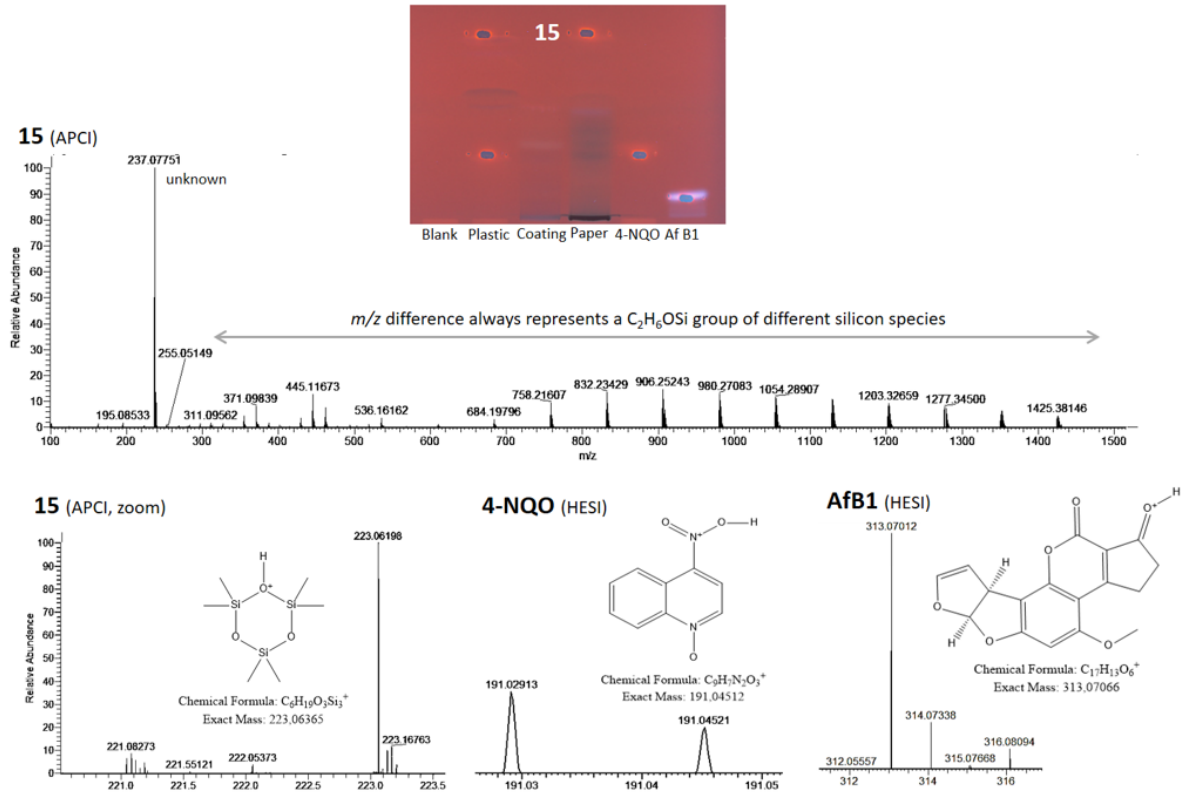


Fig. S4 HPTLC-APCI/HESI-HRMS spectrum of band 15 and positive controls: RP-HPTLC–S9-SOS-Umu-C bioautogram at FLD 254 nm of the plastic, coating and paper migrates (30x concentrate, 100 µL), two positive controls 4-NQO (0.7 ng/band), AFB1 (140 ng/band) and migration simulant blank as well as HPTLC-APCI(+)-HRMS spectra of the paper migrate band **15** near the solvent front and its zoom on *m/z* 220-223 showing the signal of protonated hexamethylcyclotrisiloxane as well as dimethylpolysiloxane clusters. In comparison, the obtained HPTLC-HESI(+)-HRMS spectra of 4-NQO and AFB1. No ions were detected from the genotoxic zones of the plastic and coating migrates.

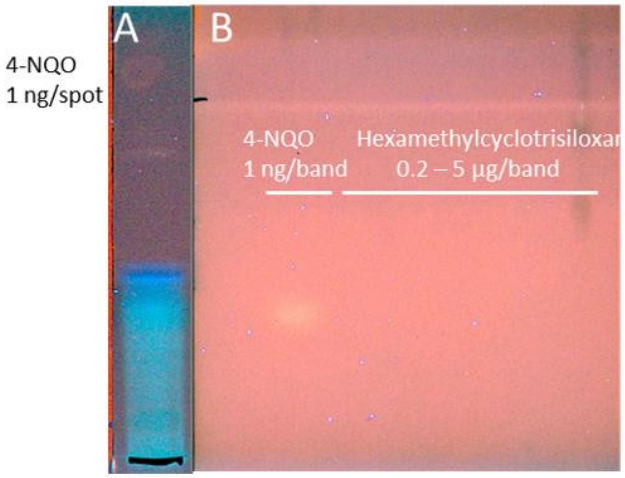


Fig. S5 Testing of the assumed candidate hexamethylcyclotrisiloxane: RP-HPTLC-SOS-Umu-C bioautograms at FLD 254 nm of paper migrate (30× concentrate, 100 µL) showing a positive response for the silicon compound band even without metabolic activation (**A**), whereas the assumed candidate hexamethylcyclotrisiloxane did not show any genotoxic response (**B**).

8. Publication IV

Food Chemistry 408 (2023) 135253

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Designed genotoxicity profiling detects genotoxic compounds in staple food such as healthy oils

Gertrud E. Morlock [*,1], Daniel Meyer

Institute of Nutritional Science, Chair of Food Science, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

ARTICLE INFO

Keywords:
Lipid
Oil
Unsaturated fatty acid
Planar SOS-Umu-C assay
S9 liver system detoxification
High-performance thin-layer chromatography

ABSTRACT

Current techniques used in food analysis overlook genotoxic compounds. This urgently calls for a paradigm shift in analytics towards non-target planar genotoxicity profiling that can detect genotoxins. Up to eight different genotoxins (*i.e.*, genotoxic compound zones) have been detected in 33 oils used for healthy diets. A comparison of fresh oils with oils stored open and closed for one month identified genotoxic degradation products. Characterization of genotoxic zones via high-resolution mass spectrometry revealed oxidized linolenic acid as a source of genotoxicity in all samples. Detoxification via on-surface S9 liver metabolization was investigated, which showed a reduction in most, but not all, genotoxins. Food, feed, dietary supplements, and cosmetics as sources of genotoxicity can now be identified by combining separation, effect detection and optionally simulated metabolization on the same surface. The application of the planar genotoxicity profiling will improve the understanding on food and its impact as well as risk assessment and derived recommendations.

1. Introduction

Little is known about non-monitored genotoxic compounds in the food we consume daily. This is because our foods and feed are complex mixtures consisting of thousands of different compounds, of which most minor compounds are unknown (Morlock, 2021). Lipids (fats and oils) are an important component of staple foods and animal feeds. However in particular, the healthy unsaturated fatty acids are known to be susceptible to oxidation, resulting in the formation of epoxides (Sevanian et al., 1979), a substance class known to be genotoxic for a long time (Harder et al., 2003). Assays to detect and quantify epoxides are based on the reaction with hydrogen bromide or other chemicals (Hammock et al., 1974; Agarwal et al., 1979). Knowledge of the identity of each epoxide present is important since the reference standards must be chosen according to the analyte chain length, but this can be problematic with food samples of unknown composition. Another approach uses a carbamate assay coupled to high-performance liquid chromatography, which can lead to problems with metal ions in the system (Dupard-Julien et al., 2007). Nuclear magnetic resonance spectroscopy is another tool (Claxson et al., 1994; Aerts & Jacobs, 2004; Liao, 2013) but is very limited, especially when applied to highly potent genotoxins present at very low amounts in a complex sample. All that effort is necessary only to detect epoxides and does not even cover other substance classes that might also represent genotoxins.

Therefore, instead of target analysis, non-target trace analysis methods are required. The bioassay-guided fractionation is a tedious procedure involving several consecutive fractionation and bioassay testing steps, and therefore prone to genotoxin loss and also not suitable for routine food control (Phillipson et al., 2002; Malviya & Malviya, 2017). Genotoxic effects are also measured by commonly used *in vitro* microtiter plate assays. However, they are most likely not sensitive enough to detect genotoxin traces, as shown recently (Meyer et al., 2021, 2023). Also, they may easily provide false results for complex food samples due to interferences caused by the food matrix, since *in vitro* microtiter plate assays cannot discriminate between opposing signals (Meyer et al., 2021, 2023; Ronzheimer, Schreiner, & Morlock, 2022; Schreiner, Ronzheimer, Friz, & Morlock, 2022). Effect signals can be canceled out by the opposing signal responses caused by anti-effects, cytotoxicity, and physicochemical signal reduction. Therefore, the scientist may be misled in the interpretation of test results obtained with commonly used *in vitro* assays as only a sum value is obtained for a complex sample without the least differentiation of effects. Also a series of confirmative assays (excluding anti-effects, cytotoxicity, and physicochemical signal reduction) and their offsetting against each other may

* Corresponding author.
E-mail address: Gertrud.Morlock@uni-giessen.de (G.E. Morlock).
[1] Dedicated to the late Prof. Dr. Helmut Jork and to Prof. Dr. Heinz Engelhardt, both Saarland University, Germany.

https://doi.org/10.1016/j.foodchem.2022.135253
Received 28 September 2022; Received in revised form 7 December 2022; Accepted 17 December 2022
Available online 20 December 2022

not function properly, also due to the variances from assay to assay which nullifies smaller differences indicating a genotoxic response.

This explains that our food is not controlled regarding genotoxic compounds in general but is only controlled for selected known target compounds. All genotoxic degradation products and metabolites are not necessarily known, either. Potential contaminations along the global food chain are another challenge to cope with. For example, vegetable oils are key ingredients of foods, feeds, and cosmetics, important for consistency, nutritional value, added value, mouthfeel, taste, flavor, *etc*. Their quality and safety primarily depend not only on the sort and origin of the oil but also on the applied processing. Even though numerous contaminants from a large number of chemical classes (oxidized lipids, waxes, alkanes, mineral oil saturated/aromatic hydrocarbons, polyaromatic hydrocarbons, pesticides, pyrollizidine alkaloids, dialkyl ketones, glycidyl esters, monochloropropane diol esters, cyanogenic glycosides, residual solvents, *etc*.) are known, most others are still unknown since influences are not under control due to the global production chain.

We hypothesize that our daily food may also contain genotoxins. We also pose the hypothesis that genotoxic compounds are overlooked in current analytical techniques, both because target analysis is very limited and because non-target assays are not sensitive enough and effect signals can be cancelled by opposing signal responses. Therefore, we also hypothesize that a sensitive planar genotoxicity bioassay combining separation with effect detection on the same surface allow for effect differentiation and may be able to clarify the presence of genotoxins in food. To prove our hypotheses, 33 oils recommended for a healthy diet were sampled from surrounding households. These were also studied under different household storage conditions. Multi-imaging high-performance thin-layer chromatography combined with a genetically modified *Salmonella typhimurium* strain equipped with the SOS-Umu-C repair mechanism (HPTLC–UV/vis/FLD–SOS-Umu-C genotoxicity bioassay) (Meyer et al., 2021) was studied for separation of genotoxins from the interfering oil matrix and sensitive detection in the oils. The planar genotoxicity bioassay (applied on the adsorbent surface) was recently shown to reliably detect epoxidized fatty acids directly in complex mixtures (Meyer et al., 2021, 2023). Detoxification of detected genotoxic compounds was also studied via the simulated S9 liver system metabolization on the same surface (Debon et al., 2022). This would indicate whether a healthy liver can detoxify the genotoxins taken up.

2. Methods

2.1. Chemicals and materials

Purity is stated if known. *Salmonella typhimurium* bacteria strain TA1535 modified to contain the plasmid pSK1002 (PTM *Salmonella typhimurium* TA1535/pSK1002, cryostock) was obtained from Trinova Biochem, Giessen, Germany. The S9 fraction from phenobarbital/β-naphthoflavone-induced lyophilized rat liver strain, glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP), and buffer salt solution were purchased from Xenometrix, Allschwil, Switzerland. 4-Nitroquinoline 1-oxide (≥98 %) was purchased from TCI, Eschborn, Germany. Aflatoxin B1 (>98 %), ampicillin sodium salt, D-(+)-glucose (99.5 %), lysogeny broth (Lennox) powder (including 5 g/L sodium chloride), diethyl ether (Chromasolv) and phosphomolybdic acid were purchased from Sigma-Aldrich, Steinheim, Germany. Ethanol (LiChrosolv), magnesium sulfate heptahydrate (99.5 %) and HPTLC plates silica gel 60, 20 cm × 10 cm, were delivered by Merck, Darmstadt, Germany. Bidistilled water was prepared with a Destamat Bi 18E (Heraeus, Hanau, Germany). *n*-Hexane, chloroform, ethyl acetate, isopropanol, and methanol (all Chromasolv, Honeywell Riedel-de-Haën) were bought from Fisher Scientific, Seelze, Germany. Toluene (Rotisolv), dimethyl sulfoxide (≥99.0 %), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), disodium phosphate (≥99.0 %), potassium dihydrogen phosphate (≥99 %), potassium chloride (≥99.5 %), and sodium hydroxide (>99 %) were purchased from Carl Roth, Karlsruhe, Germany. Linolenic acid (99 %) was purchased from Acros Organics, Fair Lawn, NJ, USA. Rhodamin 6B was purchased from Alfa Aesar, Ward Hill, MA, USA. Fluorescein di(β-D-galactopyranoside) (FDG) was bought from Santa Cruz Biotechnology, Dallas, TX, USA. The 33 oils were obtained from regular households in Giessen, Germany (Table 1).

2.2. Cell suspension and enzyme substrate solution

As *Salmonella typhimurium* culture medium, lysogeny broth was dissolved in bidistilled water (20 g/L) and autoclaved (20 min, 121 °C). An aqueous solution (3 mL) containing glucose (1 g/3 mL) and ampicillin (106 mg/3 mL) was added via a sterilizing syringe filter (0.2 μm polytetrafluoroethylene filter, VWR, Darmstadt, Germany). An overnight culture of *Salmonella* (100 μL *Salmonella* TA1535/pSK1002 cryostock suspended in 30 mL culture medium, 37 °C, 16 h, 75 rpm, orbital shaker, Edmund Bühler, Hechingen, Germany) was centrifuged (3,000 × *g*, 10 min, Labofuge 400, Heraeus, Hanau, Germany) and re-suspended in fresh culture medium to obtain an optical density OD_{660} of 0.2. In the case of metabolization, S9 liver enzyme mixture (500 μL) as well as the solutions of NADP (166 μL), glucose-6-phosphate (42 μL), and buffer salts (953 μL) were added to the *Salmonella* suspension (3334 μL) of the same OD (Debon et al., 2022). The enzyme substrate solution was prepared by solving 25 μL pre-dissolved FDG (5 mg/mL in dimethyl sulfoxide) in 2.5 mL phosphate buffer, prepared from potassium dihydrogen phosphate (40.8 g/L), disodium phosphate (42.6 g/L), magnesium sulfate heptahydrate (1.2 g/L), and potassium chloride (3.7 g/L), adjusted to pH 7 with solid sodium hydroxide.

2.3. Chromatographic method

HPTLC plates were prewashed by developing up to 9 cm with methanol (simultaneously performed for 7 plates in the Simultan Separation Chamber, Biostep, Burkhardtsdorf, Germany), dried and stored protected. Diluted oil samples (1:10 in isopropanol, 0.1 mL/mL) were applied (1 μL/band, Automated TLC Sampler 4, CAMAG, Muttenz). All plates were developed (Twin Trough Chamber or Automated Developing Chamber 2, CAMAG) first with chloroform–ethanol (5:1, *V/V*) up to 20 mm, then with *n*-hexane–diethyl ether (2:1, *V/V*) up to 40 mm, and finally with *n*-hexane–toluene (1:2, *V/V*) up to 60 mm. The plates were dried after each development (6 min, cold stream of air, hairdryer) and finally documented at white light illumination, FLD 254 nm, and FLD 366 nm (Visualizer 2, CAMAG).

2.4. Planar genotoxicity bioassay

The positive control 4-nitroquinoline 1-oxide (500 pg/μL in methanol), or aflatoxin B1 (100 pg/μL in methanol) in case of S9 metabolization, was applied (1 μL/band each) on the plate top. The *Salmonella* suspension with or without the S9 mixture was piezoelectrically sprayed onto the HPTLC plate (yellow nozzle, level 6, Derivatizer, CAMAG). After incubation at 37 °C for 3 h (humid box with moistened filter paper lining, KIS 26.5 cm × 16 cm × 10 cm, ABM, Wolframs-Eschenbach, Germany), the plate was dried (4 min, cold air stream) and FDG was piezoelectrically sprayed on it (yellow nozzle, level 4, Derivatizer, CAMAG). After incubation (37 °C, humid box, 15 min), the plate was dried (4 min, cold air stream), documented at FLD 254 nm (Visualizer 2, CAMAG), and densitometrically evaluated (485/>500 nm, TLC Scanner 4, CAMAG).

2.5. Planar cytotoxicity bioassay

MTT solution (0.2 % in phosphate buffer) was sprayed (800 μL, blue nozzle, level 4, Derivatizer, CAMAG) onto the still wet plate after the incubation with the *Salmonella* culture. The plate was then incubated (37 °C, humid box, 18 h), followed by drying in a stream of cold air and

Table 1
The 33 oil samples (31 plant oils, and for comparison, one clarified butter and one fish oil food supplement) on the German market that have been tested for genotoxicity.

ID	Oil sort	Brand name and manufacturer
>2 a old oils		
O1	Olive oil	Olivenöl, Epikouros, Kalamata, Greece
T	Thistel oil	Diestelöl Gut & Günstig, EDEKA Zentrale, Hamburg, Germany
S1	Sunflower seed oil	Sonenblumenöl Gut & Günstig, EDEKA Zentrale, Hamburg, Germany
Soj	Soybean oil	Sojala soybean oil, Vandemoortele, Ghent, Belgium
W1	Walnut oil	Wallnussöl geröstet, Rapunzel Naturkost, Legau, Germany
L1	Linseed oil	Leinöl, Erfurter Ölmühle Werner Fischer, Erfurt, Germany
R1	Rapeseed oil	Rapskernöl Gut & Günstig, EDEKA Zentrale, Hamburg, Germany
Ses	Sesame oil	Sesamöl kaltgepresst, Bio-Zentrale Naturprodukte, Wittibreut, Germany
C	Coconut oil	Kokosöl Edeka Bio, EDEKA Zentrale, Hamburg, Germany
B	Clarified butter	Butterschmalz Butaris, Dairy Fine Food, Uelzen, Germany
<1 a old oils		
L2	Linseed oil	Leinöl, Schneekoppe, Buchholz in der Nordheide, Germany
O2	Olive oil	Olivenöl nativ extra, Naturata, Marbach, Germany
R2	Rapeseed oil	Rapsöl Rapso, VOG, Linz, Austria
O3	Olive oil	Olio extra vergine di oliva, Luglio, Molfetta, Italy
P2	Peanut oil	Erdnussöl Bamboo Garden, Theodor Kattus, Dissen, Germany
L3	Linseed oil	Leinöl, Schneekoppe, Buchholz in der Nordheide, Germany
R3	Rapeseed oil	Rapsöl nativ kaltepresst Bellasan, Aldi Süd, Mühlheim an der Ruhr, Germany
O4	Olive oil	LaMusta Not specified
Pl1	Plant oil	Becht's Spezial "S" reines Pflanzenöl, Peter Kölln, Elmshorn, Germany
R4	Rapeseed oil	Pflanzenöl aus Raps Gut & Günstig, EDEKA Zentrale, Hamburg, Germany
O5	Olive oil	Natives Olivenöl extra Ja!, REWE, Köln, Germany
R5	Rapeseed oil	Natives Rapsöl kaltgepresst Ja!, REWE, Köln, Germany
O6	Olive oil	Natives Olivenöl extra Edeka Bio, EDEKA Zentrale, Hamburg, Germany
R6	Rapeseed oil	Reines Rapsöl Thomy, Neuss, Germany
S2	Sunflower seed oil	Reines Sonnenblumenöl, Thomy, Neuss, Germany
O7	Olive oil	Natives Olivenöl extra, REWE, Köln, Germany
L4	Linseed oil	Leinöl nativ, Rapunzel Naturkost, Legau, Germany
Pl2	Plant oil	Omega-3 Pflanzenöl classic Becel, Upfield Deutschland, Hamburg, Germany
O8	Olive oil	Primadonna natives Olivenöl Lidl, Neckarsulm, Germany
Sup	Fish oil food supplement	Vital Pur Plus+, OMEGA 3–rTG 80, capsules Eva Szalontai, Homburg, Germany
H	Hemp seed oil	Hanföl, Bio-Zentrale Naturprodukte, Wittibreut, Germany
W2	Walnut oil	Walnussöl kaltgepresst, Bio-Zentrale Naturprodukte, Wittibreut, Germany
O9	Olive oil	Natives Olivenöl extra, Bio-Zentrale Naturprodukte, Wittibreut, Germany

documented at white light illumination (TLC Visualizer 2, CAMAG).

2.6. Reagent sequence

Confirmative detection of aliphates was performed via a reagent sequence on the same plate. The Rhodamine 6B reagent (2.5 mL, 100 mg/L ethanol) was first sprayed onto the plate (blue nozzle, level 3, Derivatizer, CAMAG), dried, and documented at FLD 366 nm. Via the preferred physisorption of Rhodamine 6B reagent to aliphates, these were highlighted as yellow fluorescent zones. Then, the phosphomolybdic acid reagent was sprayed onto the same plate (2.5 mL, 100 mg/mL bidistilled water, blue nozzle, level 3, Derivatizer, CAMAG), heated at 120 °C for 10 min, and documented at white light illumination (TLC Visualizer 2, CAMAG). The phosphomolybdic acid reagent detected the apolar compounds as dark zones against a redish plate background. All HPTLC devices were operated with visionCATS software version 3.0 (CAMAG).

2.7. High-resolution mass spectrometry

Linseed oil was applied sixfold onto the plate and separated. One plate part was cut containing one track of linseed oil and the positive control (applied on the plate top) and subjected to the planar SOS-Umu-C bioassay. At the position of the genotoxic band, the equivalent bands (same hR_F value) on the five tracks on the other plate part were eluted via ethyl acetate (flow rate 200 µL/min, open source modified autoTLC-MS interface, Mehl et al. 2021) and pooled into the same sampler vial. Ethyl acetate was selected for its good solubility for lipids, still acceptable elution power of the lipids from the adsorbent, and good volatility. After evaporation of the ethyl acetate, the residue was dissolved in methanol (100 µL), selected for a good electrospray ionization, and analyzed via flow injection analysis (FIA; methanol, flow rate 300 µL/s) using a heated electrospray ionization high-resolution mass spectrometer (HESI-HRMS; QExactive Plus, Thermo Fisher Scientific, Dreieich, Germany) with the following settings: + 3.5 kV and −3.5 kV spray voltage, 270 °C capillary and 200 °C probe heater temperature, resolution 280,000, m/z 100–1,500, and automatic maximum injection time 10/200 ms for positive/negative ionization.

3. Results and discussion

3.1. Study design

Different plant oils were collected from surrounding households to represent the status of oil use in households (Table 1). There were no choice rules for the oils regarding their origin, manufacturer, country, *etc*. The oils were divided into two categories regarding the storage period, *i.e.* stored for less than one year and for more than two years. It did not make sense to split the less-than-one-year category because it was difficult for the consumers to remember the date of first opening. For the oils stored in the dark for more than two years, the beginning of storage in the respective household was similar. A clarified butter sample (as a frequently used product) and a fish oil food supplement sample (as a popular dietary trend product) were added for comparison. A 7D hyphenation for the non-target screening of oils for genotoxins was developed which combined sample separation with genotoxicity detection on the same surface (Fig. 1).

The 33 samples (Table 1) were only diluted and no further sample preparation was performed. Thus, the sample was as original as possible and no sample part was lost. Only 0.1 millionth of a gram (0.1 μg) of each household oil was applied to the HPTLC plate and separated in a three-step development. The first step was for the separation of polar, the second one for middle polar, and the last one for apolar components. Afterwards, the planar genotoxicity bioassay was performed on the same surface, and as a result, genotoxic compounds were evident in the bioautogram at FLD 254 nm as green fluorescent zones (fluorescein as the end product of the galactosidase–substrate reaction) against a slightly green fluorescent plate background. During method development, selected oil samples were repeatedly analyzed, which confirmed the bioassay results multiple times (multifold technical/biological replicates). For the screening, each of the 33 samples was analyzed once (one technical/biological replicate), and the bioassays were all performed with the same cell culture to allow a direct comparison of the signal intensities. Thus, the differences in the genotoxic responses are actually related to the genotoxin amount or potency and not due to a different cell culture performance. Note that the validation of the planar genotoxicity bioassay was recently reported for food contact materials (Debon et al., 2022; Meyer et al., 2021, 2023).

3.2. Screening and evaluation of oils for presence of genotoxins

Our first hypothesis that our daily food may contain genotoxins, unfortunately, was proven to be correct. In the genotoxicity bioautogram (Fig. 2), several green fluorescent genotoxin bands were detected in the samples. When oils were stored longer than 2 years, the number and the signal intensity of the mid-polar genotoxin bands **1**–**5** were higher compared to oils stored for less than one year (Fig. 2). Around the most intense dark triacylglycerol zone, a genotoxic response surrounding it (**6**) was observed, which was more or less pronounced but present in all oil samples. The essential need for a separation of the oils before bioassay application and detection was evident by the dark band detected in each oil sample, showing responses in opposite to the green fluorescence of the genotoxins. Genotoxins can easily be overlooked via such a strong and dominant fluorescence signal reduction using *in vitro* microtiter plate or cuvette assays, since the genotoxic response may easily be extinguished by the strong opposing dark response in the obtained sum value. Such a problem is also expected for other enzyme substrate combinations. Hence, sum values obtained for complex samples may mislead the interpretation of genotoxicity and respective decisions and thus risk assessment. It was previously shown that linolenic and linoleic acid epoxides formed via air contact have genotoxic properties (Meyer et al., 2023), which is why it is plausible to assume that the genotoxic response is the result of epoxide formation of linolenic acid and its esters such as acylglycerols. In the apolar range, up to two further genotoxin bands (**7** and **8**) were detected. Mainly plant-based oils were

Fig. 1. Scheme of the developed 7D hyphenation for genotoxicity screening: HPTLC method combined with the planar genotoxicity bioassay with and without S9 liver system metabolization and genotoxic zone characterization, *i.e.* HPTLC–UV/Vis/ FLD−(S9)-SOS-Umu-C–HRMS/MS.

screened and for comparison one clarified butter sample (Fig. 2, B) and one fish oil food supplement sample (Sup). The fish oil food supplement sample showed a green fluorescent halo shine around the intense dark triacylglycerol zone, and also around the diacylglycerol zone below. This indicated that the genotoxicity was most likely caused by the oxidized unsaturated fatty acids. The clarified butter sample and the fish oil supplement sample showed also a green fluorescent halo shine around the intense dark triacylglycerol zone.

Our second hypothesis that opposing signal responses can lead to wrong sum values and mislead in the interpretation of results using *in vitro* microtiter plate assays was also proven to be correct, unfortunately. With no effect differentiation and only a sum value, this explains why we have not seen staple food containing genotoxins before. Using *in vitro*

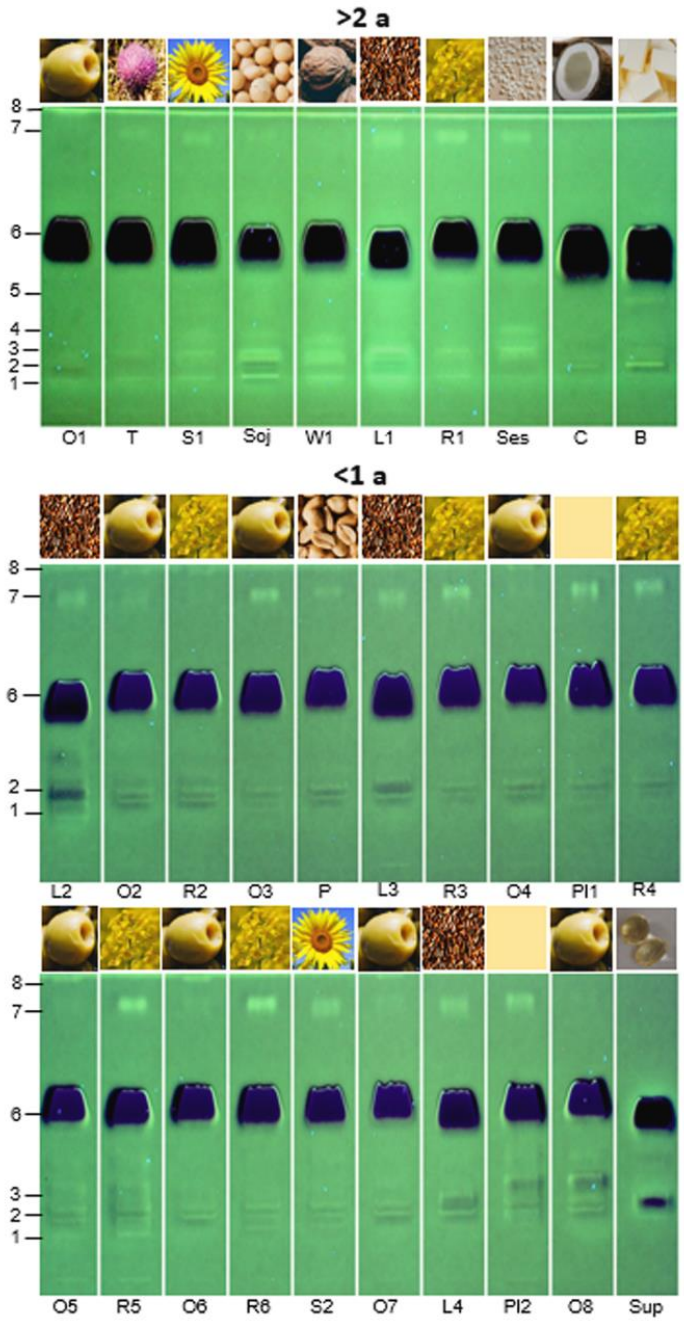


Fig. 2. Screening of oils for genotoxins detected as green fluorescent zones: HPTLC–SOS-Umu-C–FLD bioautograms at 254 nm of different oil sorts (0.1 μg each) taken from households stored in bottles in the dark for more than two years (**>2 a**) and for less than one year (**<1 a**). Olive oils (O1–8), thistle oil (T), soybean oil (Soj), walnut oil (W1), linseed oils (L1–4), rapeseed oils (R1–6), sesame oil (Ses), coconut oil (C), clarified butter (B), peanut oil (P), plant oils (Pl1–2), and a fish oil food supplement (Sup). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

assays, the solubility of all oil constituents in the very polar bioassay medium (rich in buffer salts) might also be difficult. This and whatever further reasons (such as the huge time/cost effort of bioassay-guided fractionation) have led to the situation that our food is not controlled as it should be. In just 0.1 millionths of a gram of each household oil, several genotoxic compounds were observed. The average consumption of oils and fats was about 25 kg per capita in Germany in 2020 (Koptyug, 2021) which calculates into an average consumption of 69 g oil/fat per capita per day. This means a 690 million higher genotoxic response is taken up daily than the response observed in the bioautogram (Fig. 2). One can imagine that our findings do matter.

The situation may be similar for many other countries and samples. Considering the current analytical situation, the worldwide misalignment becomes obvious. On the one hand, we search strongly regulated for the least genotoxic migrant or contaminant in packaging material (Meyer et al., 2021). On the other hand, staple food that we eat every

day showed orders of magnitude higher genotoxic potential. The question arises to why this discrepancy exists and why food is not regularly investigated regarding genotoxic compounds. It is explained by the fact that we do not have fast enough and powerful enough screening tools for routine analysis of complex mixtures and that the huge potential of planar bioassays is ignored or overlooked.

3.3. Study of oil degradation

The influence of time on the genotoxicity of some oils has been previously reported. Olive oil is especially known to become genotoxic over time as detected within a *Drosophila* wing spot test (Anter et al., 2010). This increases the need for an analytical tool enabling the industry to identify critical steps of processing, and this calls for more stable product formulations and for a better practice regarding the storage of oils, also in one's own houshold. To test the suitability of the HPTLC–(S9)-SOS-Umu-C genotoxicity bioassay for the analysis of oil degradation, olive, walnut, and hemp oils were selected as three popular oils used in healthy diets. These were exposed to different storage conditions commonly found in households and restaurants. Samples of the three oils were stored at −20 °C as reference (fresh), at room temperature inside their original glass bottles in the dark (closed) and in a glass container that allowed air exchange in the dark (open). The oxidation triggered by light (photooxidation) is expected to produce even more degradation products than observed here. After one month, all nine samples were analyzed via the planar SOS-Umu-C genotoxicity bioassay. A period of one month was chosen since a genotoxic increase was expected after this time. Longer exposure times under the given conditions are conceivable, and an even more dramatic effect on the oils is expected for longer storage times. The storage study showed that olive oil was most prone to oxidation over time among the studied olive, walnut, and hemp oils (Fig. 3a). In comparison to the two olive oil samples stored closed, the genotoxin band **1** increased in its genotoxic response when stored open under air exchange as practiced in many restaurants. This outcome was additionally illustrated by comparing the densitograms of the closed and open stored oils (Fig. 3b). This underscores the critical effect of air on the oil, despite well-known antioxidative minor compounds present. The genotoxic response of band **1** in the other two oils remained constant. However, an additional dark band (*) representing the diacylglycerols appeared on the track of the hemp oil sample that was exposed to air. This additional degradation via hydrolysis showed that hemp oil is particularly susceptible to the influence of the humidity of the surrounding air.

3.4. Study of metabolic detoxification

One important question arose: Why we are still alive? A potential detoxification of the detected genotoxic compounds (presupposed a healthy liver) was assumed and clarified via the simulated metabolization via the S9 liver system on the same adsorbent surface. Therefore, the S9 liver system was added to the prepared cell culture suspension and the following oil analysis was according to the same workflow. The genotoxin band **1** was found in all oils; however, it was not detectable anymore after metabolic conversion (Fig. 3a *vs* 3d). In the case of epoxides, this is expected because epoxides are metabolized into alcohols (Greene et al., 2000). Therefore, when the liver has a healthy constitution and it comes to resorption, the oil genotoxins can be detoxified. However, the hemp oil additionally exhibited a second genotoxin response (**7**) that partially remained genotoxic after S9 metabolization (Fig. 3d). Hence, as evident from the screening, storage and metabolization experiments, the genotoxic potential of oils used for a healthy diet cannot be ignored anymore and has to be considered and discussed in future risk assessment.

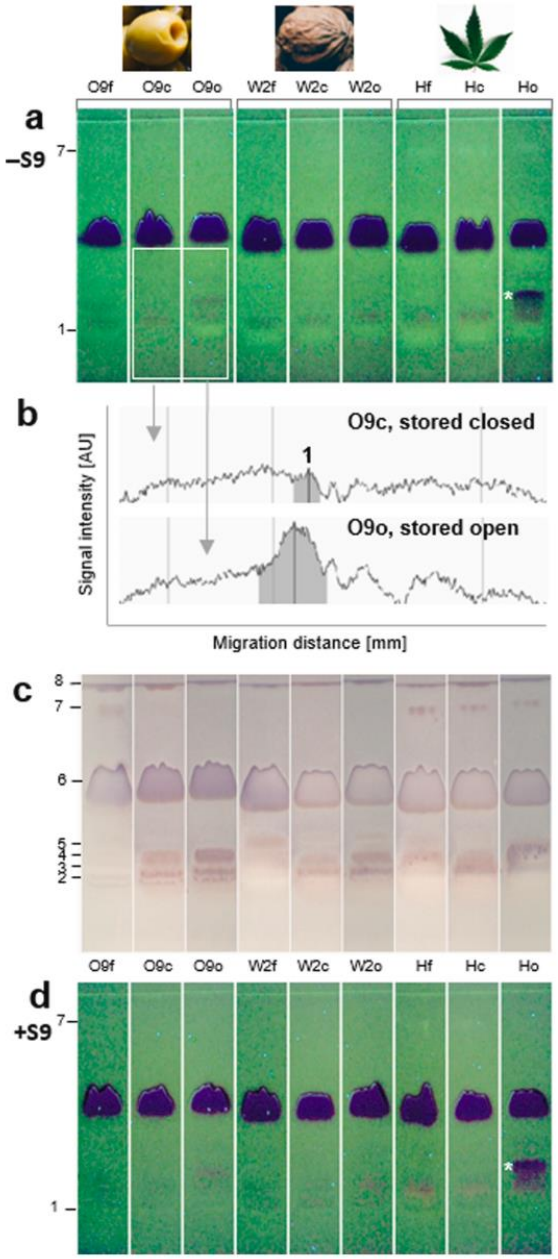


Fig. 3. Comparison of three selected oils stored differently for the presence of genotoxins and their metabolization: HPTLC–SOS-Umu-C–FLD bioautograms at 254 nm (**a**) of olive oil (O9), walnut oil (W2), and hemp oil (H), showing degradation (*) of fresh oils (f) *versus* stored closed (c) and open (o) for one month; densitograms at 485/>500 nm of two track sections (**b**); exclusion of cytotoxicity: bioautogram at white light illumination of the same oils showing cell metabolism as purple color (**c**); S9 liver metabolization (**d**, +S9) of the same oils, showing a clear reduction in the genotoxic response. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Exclusion of cytotoxicity

Dark bands (especially zone **6**) were detected in the bioautograms of all oils, indicating a possible cytotoxicity which has to be excluded. Since cytotoxicity can counteract the genotoxic signal detection, MTT was used as the enzyme substrate for detecting a proper functioning of cell metabolism, resulting in purple dye formation. Any cytotoxic compounds are evident as colorless zones against a purple plate background. The MTT cytotoxicity bioautogram (Fig. 3c) showed more or less purple bands on the positions that appeared dark in the previous genotoxicity bioautograms, thereby identifying the purple bands as non-cytotoxic (*e.g.*, samples O9c and O9o). The other zones being brighter toward the center indicated cytotoxicity, which nullifies the detectability of a genotoxic effect and requires further clarification. The walnut and hemp oil samples showed purple edges of the zones (indicating vivid cells), which underscores our previous genotoxicity findings at the edges.

3.6. Confirmative detection of aliphatic molecules

The degradation study revealed some changes in the composition of olive and hemp oils. To further characterize the changes in oil composition, a detection sequence on the same plate was utilized. The first applied Rhodamine 6B reagent highlighted aliphatic substances (Fig. 4a). The bands **1**–**6** that were previously reported as genotoxic were also identified as aliphatic compounds. However, the most apolar compound bands **7** and **8** were hardly detectable, indicating structurally cyclic moieties. On the same plate, the derivatization with the phosphomolybdic acid reagent, indicating also lipophilic molecules with double bonds, confirmed the results (Fig. 4b). Thus, zones **7** and **8** are assumed to contain cyclic and double bond-containing molecules. The change in the composition of the oils under different storage conditions was also evident as the intensity of band **5** increased for the sample stored open under air exchange (Fig. 4, marked *).

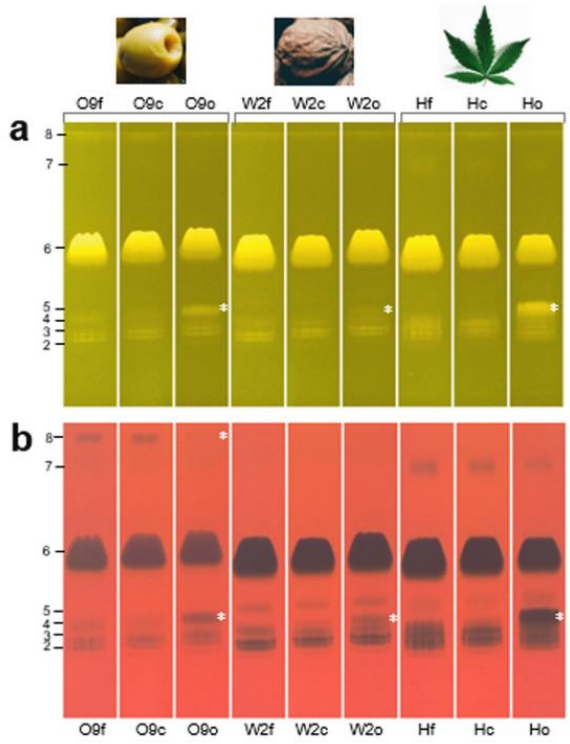


Fig. 4. HPTLC chromatograms for physicochemical comparison of the three oils stored differently: aliphatic components of the same oils as in Fig. 3 detected at FLD 366 nm via physisorption of rhodamine 6B (**a**) and subsequently (reagent sequence on the same plate) at white light illumination after derivatization with the phosphomolybdic acid reagent (**b**), clearly confirming the degradation (*marked) of the oils stored over one month when compared to the fresh ones.

If the different detectabilities and polarities (hR_F value) of the substances are taken into account, bands **7** and **8** could result from the presence of alkylfuranes and mineral oil aromatic hydrocarbons since representatives are already known to be genotoxic and present in oils (Arcella et al., 2019). In the middle polar range, hydroperoxides or epoxides that have been reported as degradation products of fats (Esterbauer, 1993) are conceivable. This would explain the genotoxic responses **1**–**5** discussed. To further assign bands to substances other methods should be applied such as nuclear magnetic resonance spectroscopy or high-resolution mass spectrometry. Only the latter technique was found suited to detect highly potent genotoxin traces in a complex sample.

3.7. Characterization of the detected genotoxic compounds

Linseed oil L1 was selected for mass spectrometric evaluation because it showed the strongest reactions of all oils. Normally, the genotoxic compound zone is directly eluted from the bioautogram for high-resolution tandem mass spectrometry (HRMS/MS) recording (Schreiner et al., 2021, 2022; Ronzheimer et al., 2022). However, for very low amounts of highly potent genotoxins present in the zone, the transferred amount is too low. Even the powerful HRMS recording does not provide clear signals, since the planar genotoxicity bioassay is very sensitive in the detection. It was proven to detect 176 times better than the state-of-the-art microtiter plate counterpart (Meyer et al., 2021). Hence, picogram amounts per genotoxic compound band can be expected, and several zones were pooled for HRMS analysis. Briefly, the linseed oil L1 was applied sixfold and separated. One edge track was cut from the plate and subjected to the genotoxicity bioassay. On other plate part, the five bands at the same hR_F value as the genotoxic band **2** were online eluted and pooled into the same sampler vial and then analyzed via FIA-HESI-HRMS. The base peak signal at *m/z* 353.2661 ($C_{19}H_{38}O_4Na$, Δ *ppm* −0.257) was assigned to glyceryl palmitate (Fig. 5a). This seems reasonable since monoacylglycerols are a common minor degradation compound in oils.

The genotoxic response was explained by several oxidized hydrocarbon molecules found in the positive ionization mode and represented by signals at *m/z* 185.1149 ($C_8H_{18}O_3Na$, Δ *ppm* 0.510), *m/z* 345.2036 ($C_{19}H_{30}O_4Na$, Δ *ppm* −0.147) and *m/z* 381.2975 ($C_{21}H_{42}O_4Na$, Δ *ppm* −0.212), whereby for all these mass signals a mass signal lighter by one oxygen atom was also found in each case (not shown because of too low signal intensity). This supports the assumption that epoxides were present in the oil sample. Epoxides are known to be genotoxic, which was recently confirmed for fatty acid epoxides on the HPTLC plate (Meyer et al., 2023). Considering the stronger polarity of the oxidized fatty acids and monoacylglycerols compared to the main triaclyglycerols, their position on a normal phase plate was expected at a low hR_F value in the bioautogram. Moreover, though at a much lower signal response, five different oxidized linolenic acid species were found in the genotoxic compound band **2** of the linseed oil sample. All evident as respective sodium adduct signals, the diepoxide and/or hydroperoxide of linolenic acid (Fig. 5b) were found at *m/z* 333.2036 (Δ *ppm* −0.062), the triepoxide and/or a molecule containing a hydroperoxide and an epoxide group (Fig. 5c) at *m/z* 349.1985 (Δ *ppm* 0.014), a molecule containing either two epoxide groups and one hydroperoxide groups or two hydroperoxide groups (Fig. 5d) at *m/z* 365.1936 (Δ *ppm* 0.284), the linolenic acid (Fig. 5e) at *m/z* 301.2135 (Δ *ppm* − 0.901), and its epoxide (Fig. 5f) at *m/z* 317.2087 (Δ *ppm* 0.076).

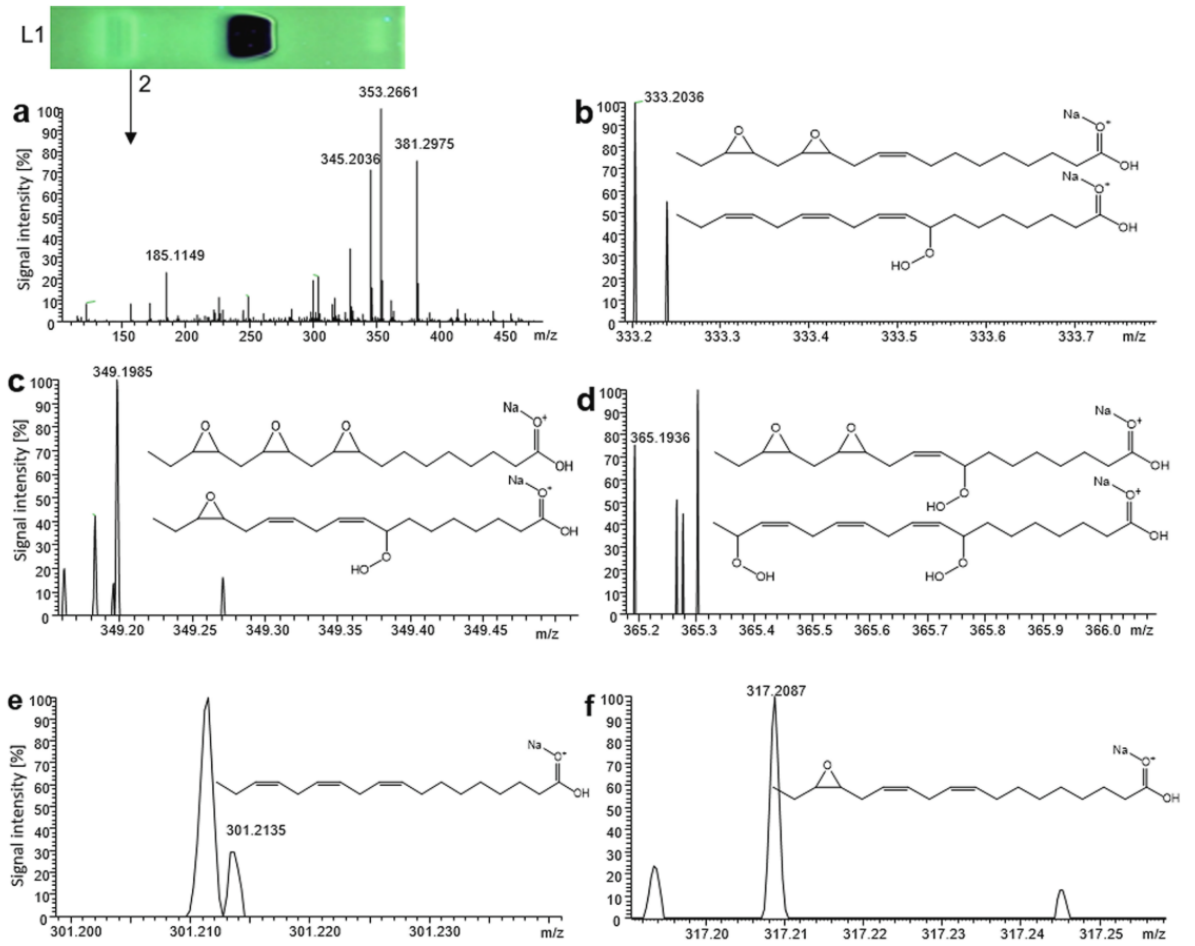


Fig. 5. Characterization of detected genotoxic zones: HPTLC-HRMS spectrum of the genotoxic zone **2** in linseed oil L1 (**a**), showing the glyceryl palmitate mass signal at *m/z* 353.2661 as base peak; however, the genotoxic response is explained by additionally detected oxidized molecule ions at *m/z* 185.1149, *m/z* 345.2036, and *m/z* 381.2975, as well as oxidized linolenic acid mass signals present though at a low signal intensity (**b**–**f**).

The same procedure as for band **2** was applied for band **7**. Apparently, the obtained HRMS signals revealed that epoxides or hydroperoxides were present within this band, which would explain the genotoxic response. However, structures could not clearly be assigned to the obtained mass signals. In the negative ionization mode, the detected molecular ions were at *m/z* 337.1660 ($C_{18}H_{25}O_6$, *Δ ppm* 0.974), 325.1656 ($C_{17}H_{25}O_6$, *Δ ppm* −0.221), 321.171 ($C_{18}H_{25}O_5$, *Δ ppm* 0.663), 309.1709 ($C_{17}H_{25}O_5$, *Δ ppm* 0.591), 293.1761 ($C_{17}H_{25}O_4$, *Δ ppm* 0.878), 291.1604 ($C_{17}H_{23}O_4$, *Δ ppm* 0.850), 277.1810 ($C_{17}H_{25}O_3$, *Δ ppm* 0.404), 275.1655 ($C_{17}H_{23}O_3$, *Δ ppm* 0.844), and 396.2182 ($C_{24}H_{30}O_4N$, *Δ ppm* 0.349). In the positive ionization mode, the ions were found at *m/z* 314.2685 ($C_{18}H_{36}O_3N$, *Δ ppm* −1.529), 298.2737 ($C_{18}H_{36}O_2N$, *Δ ppm* −1.26), 282.2788 ($C_{18}H_{36}ON$, *Δ ppm* −1.28), 439.3722 ($C_{29}H_{49}O_2$, *Δ ppm* −1.135), 413.3772 ($C_{29}H_{49}O$, *Δ ppm* −2.688) and 397.3824 ($C_{29}H_{49}$, *Δ ppm* −1.153). Underscored by this HRMS data, our third hypothesis that planar bioassays allows for effect differentiation and may be able to clarify the presence of genotoxins in food was true.

3.8. Suitability of the planar bioassay methodology for routine use

Analysis is the basis of all knowledge; however, the proper analytical tool has to be used. Otherwise progress and understanding are poor. This strongly calls for an paradigm shift in analytics towards non-target planar bioassay screening that can cope with sample complexity, save costs, be rapid and be used as sustainable screening in routine food control. Planar separations tolerate heavy matrix loading, so sample preparation is minimal, which is also an extremely important precondition with regard to sample integrity for non-target analysis. This also saves time and costs, as sample preparation is often the most tedious step. Here, <1 mL solvent per sample was needed for the sample dilution. No other sample preparation step was needed. The HPTLC analysis is step-automated, which means that each step is fully automated, except for the plate transfer to the next device. This implies, in contrast to TLC, that only few operator minutes are required for plate transfer and manual interventions in routine, such as press next-step-button in software. Step-automation offers the chance of intermediate control (plate check) and is thus robust and time-saving in routine due to early detection of instrument errors/defects/failures. It also provides good fallback and compensation options, since the entire instrument chain is not affected and a complete system stop is not forced.

The time required for the step-automated HPTLC–UV/vis/FLD–(S9)–SOS-Umu-C genotoxicity analysis is about 17 min per sample, whereof the share of manual operations is about 10 % (1.7 min per sample). Calculated for one plate (for analysis of 17 samples in parallel),

it took 4.9 h. As mentioned, the workflow with or without S9 metabolization differs only in that the S9 mixture is added to the cell suspension, which takes only 1–2 min extra per plate. Briefly, application took 20 min, development 40 min, cell suspension preparation 15 min, cell application and incubation 190 min, plate drying 4 min, substrate application and incubation 20 min, plate drying 4 min and documentation 2 min, summing up to 295 min, whereof 30 min were manual operations per plate, mostly for the bioassay part. Regarding the development time (40 min), this oil application is considered as a worst case scenario. In normal scenarios, HPTLC development is trimmed to 20 min. Therefore, the given calculation is very conservative and leaves room for higher sample throughput (or for mobile phase optimization). Due to the step-based instrumentation, the next plate (plate 2) can already be started/handled when the application of plate 1 is finished. Thus, the double sample throughput (34 samples) or the two plates with and without S9 metabolization are performed in almost the same time. For a staggered (30-min time-shifted) handling about 6 plates containing ca. 100 samples can be performed/screened per day.

The analysis costs without and with S9 metabolization are below €1.1 and €1.4 per sample, respectively. Calculated for one plate (for analysis of 17 samples in parallel), it was €18 and €23, respectively. Briefly, the plate cost (€8) is always the most expensive part. This oil application is considered as a worst case scenario regarding the solvent costs, having used three developments plus one plate prewashing (€3 for 28 mL solvents). In normal scenarios, HPTLC is very sustainable using 7 mL solvent for one plate development. With regard to the costs for the biological detection (bioassay), the materials consumed for the cell culture preparation are low (€1), however, the costs for the substrate (€6) and S9 mixture (€5) are the most expensive items. The use of another cheaper substrate or cheaper S9 mixture would reduce the aforementioned costs substantially. For example, the 4-methylumbelliferyl-galactopyranoside substrate (Meyer et al., 2021) costs only €1 instead of the expensive FDG substrate used here, which costs €6 – 12 per plate depending on the supplier. Therefore, the given calculation is very conservative and leaves room for cost reduction.

The planar screening strategy is ready to be applied to other complex samples. For example, the workflow (with adjusted mobile phase system depending on the sample) can be used to analyze fish oil food supplements rich in unsaturated fatty acids, parfumes often used in direct contact with the skin, essential oil products known to contain also genotoxins and so on. However, most surprise can be obtained by analyzing products that are considered safe. The planar workflow can also be used for mechanistic studies, *i.e.* under which conditions the genotoxins are preferentially formed, or under which processing parameters or formulation conditions the products are more stable with regard to the formation of genotoxins during the product shelf life.

4. Conclusions

We are inevitably exposed to food, but our food is not controlled as it should be. The power of the planar screening strategy was highlighted, and we postulate an analytical paradigm shift and propose non-target planar genotoxicity screening, which can be used in routine analysis to detect genotoxins in foods, feeds, dietary supplements and cosmetics. Clearly, a bioautogram has much more significance than a number (sum value). Oxidized molecules, among them also epoxidized unsaturated fatty acids, were shown to be potentially genotoxic in commonly used healthy oils. The number and/or amount of the genotoxic compounds in those oils rise with ongoing storage while being exposed to air. Hence, it might be better to eat the linseeds (freshest oil by the mouth/teeth press) instead of the oil. When the liver has a healthy constitution and it comes to resorption, the oil genotoxins can be detoxified in the liver, which is good news. However, the question arose as to what happens on the way through the gastrointestinal tract to metabolization in the liver. For topical applications of oils rich in unsaturated fatty acids, the genotoxic compounds also come into direct contact with the skin surface, which is highly exposed to oxygen and light. Therefore, further studies need to be conducted to find out good processing practices, safe product formulations, and proper storage conditions to ensure the stability of the oil-containing foods, feeds, dietary supplements, and cosmetics over the recommended product shelf life. In analogous non-target screenings, further compounds with neurotoxic, cytotoxic, antimicrobial, and endocrine effects can be analyzed in complex samples. Powerful multiplex planar assays have recently been decribed and are ready to be transferred and applied to complex samples to learn more about their secrets.

CRediT authorship contribution statement

Gertrud E. Morlock: Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Formal analysis, Writing – original draft, Supervision, Resources. **Daniel Meyer:** Methodology, Investigation, Formal analysis, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Thanks is owed to Merck, Darmstadt, for supplying plates. Instrumentation was partially funded by the Deutsche Forschungsgemeinschaft (INST 162/536-1 FUGG and INST 162/471-1 FUGG).

References

Aerts, H. A. J., & Jacobs, P. A. (2004). Epoxide yield determination of oils and fatty acid methyl esters using 1 H NMR. *Journal of the American Oil Chemists' Society, 81*, 841–846. https://doi.org/10.1007/s11746-004-0989-1

Agarwal, S. C., van Duuren, B. L., & Kneip, T. J. (1979). Detection of epoxides with 4-(*p*-nitrobenzyl) pyridine. *Bulletin of Environmental Contamination and Toxicology, 23*, 825–829. https://doi.org/10.1007/BF01770049

Anter, J., Campos-Sánchez, J., Hamss, R. E., Rojas-Molina, M., Muñoz-Serrano, A., Analla, M., & Alonso-Moraga, A. (2010). Modulation of genotoxicity by extra-virgin olive oil and some of its distinctive components assessed by use of the Drosophila wing-spot test. *Mutation Research, 703*, 137–142. https://doi.org/10.1016/j.mrgentox.2010.08.012

Arcella, D., Baert, K., & Binaglia, M. (2019). Rapid risk assessment on the possible risk for public health due to the contamination of infant formula and follow-on formula by mineral oil aromatic hydrocarbons (MOAH). *EFSA, 16*. https://doi.org/10.2903/sp.efsa.2019.EN-1741

Claxson, A. W., Hawkes, G. E., Richardson, D. P., Naughton, D. P., Haywood, R. M., Chander, C. L., … Grootveld, M. C. (1994). Generation of lipid peroxidation products in culinary oils and fats during episodes of thermal stressing: A high field 1 H NMR study. *FEBS letters, 355*, 81–90. https://doi.org/10.1016/0014-5793(94)01147-8

Debon, E., Rogeboz, P., Latado, H., Morlock, G. E., Meyer, D., Cottet-Fontannaz, C., … Marin-Kuan, M. (2022). Incorporation of metabolic activation in the HPTLC-SOS-Umu-C bioassay to detect low levels of genotoxic chemicals in food contact materials. *Toxics, 10*, 501. https://doi.org/10.3390/toxics10090501

Dupard-Julien, C. L., Kandlakunta, B., & Uppu, R. M. (2007). Determination of epoxides by high-performance liquid chromatography following derivatization with *N,N*-diethyldithiocarbamate. *Analytical and Bioanalytical Chemistry, 387*, 1027–1032. https://doi.org/10.1007/s00216-006-1003-3

Esterbauer, H. (1993). Cytotoxicity and genotoxicity of lipid-oxidation products. *The American Journal of Clinical Nutrition, 5*, 769–786. https://doi.org/10.1093/ajcn/57.5.779S

Greene, J. F., Newman, J. W., Williamson, K. C., & Hammock, B. D. (2000). Toxicity of epoxy fatty acids and related compounds to cells expressing human soluble epoxide hydrolase. *Chemical Research in Toxicology, 13*, 217–226. https://doi.org/10.1021/tx990162c

Hammock, L. G., Hammock, B. D., & Casida, J. E. (1974). Detection and analysis of epoxides with 4-(p-Nitrobenzyl)-pyridine. *Bulletin of Environmental Contamination and Toxicology, 12*, 759–764. https://doi.org/10.1007/BF01685927

Harder, A., Escher, B. I., Landini, P., Tobler, N. B., & Schwarzenbach, R. P. (2003). Evaluation of bioanalytical assays for toxicity assessment and mode of toxic action classification of reactive chemicals. *Environmental Science & Technology, 37*, 4962–4970. https://doi.org/10.1021/es034197h

Koptyug, E. (2021). Per capita consumption of oils and fats in Germany 1970-2020: Statista. Retrieved from: www.statista.com/statistics/514686/per-capita-consumption-of-oils-and-fats-germany. Accessed August 13, 2022.

Liao, C.H. (2013). Evaluation of Assays for Epoxides in Oxidized Lipids. Rutgers University Community Repository, New Brunswick, NJ, USA: The State University of New Jersey.

Malviya, N., & Malviya, S. (2017). Bioassay guided fractionation - an emerging technique influence the isolation, identification and characterization of lead phytomolecules. *IJHP*, *2*, 5. 10.28933/ijhp-2017-07-0901.

Mehl, A., Schwack, W., Morlock, G. E. (2021). On-surface autosampling for liquid chromatography−mass spectrometry, *J. Chromatogr. A*, *1651*, 462334. 10.1016/j.chroma.2021.462334.

Meyer, D., Debon, E., Cottet-Fontannaz, C., Marin-Kuan, M., Morlock, G. E., Mayerhofer, E., … Schilter, B. (2023). Effect-detection by planar SOS-Umu-C genotoxicity bioassay and chemical identification of genotoxins in packaging migrates, proven by microtiter plate assays SOS-Umu-C and Ames-MPF. *Food Control*. https://doi.org/10.1016/j.foodcont.2022.109546

Meyer, D., Marin-Kuan, M., Debon, E., Serrant, P., Cottet-Fontannaz, C., Schilter, B., & Morlock, G. E. (2021). Detection of low levels of genotoxic compounds in food contact materials using an alternative HPTLC-SOS-Umu-C assay. *ALTEX, 38*, 387–397. https://doi.org/10.14573/altex.2006201

Morlock, G. E. (2021). High-performance thin-layer chromatography combined with effect-directed assays and high-resolution mass spectrometry as an emerging hyphenated technology: A tutorial review. *Analytica Chimica Acta, 1180*, Article 338644. https://doi.org/10.1016/j.aca.2021.338644

Phillipson, D. W., Milgram, K. E., Yanovsky, A. I., Rusnak, L. S., Haggerty, D. A., Farrell, W. P., … Proefke, M. L. (2002). High-throughput bioassay-guided fractionation: A technique for rapidly assigning observed activity to individual components of combinatorial libraries, screened in HTS bioassays. *Journal of Combinatorial Chemistry, 4*, 591–599. https://doi.org/10.1021/cc020042e

Ronzheimer, A., Schreiner, T., & Morlock, G. E. (2022). Multiplex planar bioassay detecting estrogens, antiestrogens, false-positives and synergists as sharp zones on normal phase. *Phytomedicine, 103*, Article 154230. https://doi.org/10.1016/j.phymed.2022.154230

Schreiner, T., Ronzheimer, A., Friz, M., & Morlock, G. E. (2022). Multiplex planar bioassay with reduced diffusion on normal phase, identifying androgens, verified antiandrogens and synergists in botanicals via 12D hyphenation. *Food Chemistry, 395*, Article 133610. https://doi.org/10.1016/j.foodchem.2022.133610

Schreiner, T., Sauter, D., Friz, M., Heil, J., & Morlock, G. E. (2021). Is Our Natural Food Our Homeostasis? Array of a Thousand Effect-Directed Profiles of 68 Herbs and Spices. *Frontiers in Pharmacology, 12*, Article 755941. https://doi.org/10.3389/fphar.2021.755941

Sevanian, A., Mead, J. F., & Stein, R. A. (1979). Epoxides as products of lipid autoxidation in rat lungs. *Lipids, 14*, 634–643. https://doi.org/10.1007/BF02533449/

9. Publication V

1

2

3 **Planar 6-fold multiplex bioassay to differentiate endocrine agonist,**

4 **antagonist, false positive antagonist, cytotoxin, anti-cytotoxin, and**

5 **false positive anti-cytotoxin**

6

7 Daniel Meyer[a,#], Maricel Marin-Kuan[b], Hélia Latado[b], Benoît Schilter[b] and

8 Gertrud E. Morlock[a,#,*]

9

10

11 [a]Institute of Nutritional Science, Chair of Food Science, Justus Liebig University

12 Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

13 [b]Chemical Food Safety Group, Société des Produits Nestlé SA - Nestlé Research,

14 Vers-chez-les-Blanc, Switzerland

15

16 [#]Authors contributed equally.

17

18 *Corresponding author. Tel.: +49 641 9939141, fax: +49 641 9939149, E-mail

19 address: Gertrud.Morlock@uni-giessen.de (G.E. Morlock)

Abstract

The 2-fold multiplex endocrine planar bioassay method was applied to and validated for food contact materials. The robustness of the half-maximal inhibitory concentration IC_{50}, the limit of biological detection, and the dose-response curve of bisphenol A were successfully proven. Applied to six differently coated tin cans, 19 potentially endocrine-disrupting compounds with agonistic and antagonistic activity were found and molecule formulas were tentatively assigned via coupling to high-resolution mass spectrometry. However, cytotoxicity had to be excluded for the detected antagonists. Hence, an advanced concept of a planar multiplex bioassay for differentiation of up to 6 responses in one analysis was developed and applied to migrates from food packaging materials. In contrast to *in vitro* bioassays providing for complex samples with opposing signal responses most likely falsified sum values, the planar multiplex bioassay allows differentiation of up to 6 responses and assignment of responsible substances in complex samples, bypassing matrix effects by the integrated planar separation. Cytotoxin bands were detected by adding the substrate resazurin to the yeast cell culture, and on the same plate, endocrine-active compound bands were detected with the respective second substrate applied thereafter. The anti- and false-positive responses were distinguished via signal reduction on 4 stripes applied along each separated sample track before the bioassay application. The agonist stripe detects antagonists, the cytotoxin stripe anti-cytotoxins, and the two end-product stripes of the respective enzyme–substrate reaction each detect false positives. Determined dose-response curves of agonists and antagonists proved the performance of the 6-fold multiplex planar bioassay.

Keywords High-performance thin-layer chromatography; Agonist; Antagonist; Hormone; Endocrine Disruptor; Effect-directed analysis; Cancer; Bisphenol A

1. Introduction

The human endocrine system can be affected by endocrine active substances. Prominent substance groups can interact with the human steroid receptor system and act like 17β-estradiol or testosterone, or prohibit the activity of those hormones. [1–3] Evidence of consequential damage was recorded for male rats [4], and cancer developments in male reproduction organs [5] and female breasts [6]. Food contact materials are known as a source of endocrine active substances for humans [7]. The migration of endocrine active substances into food was analyzed via the *in vitro* yeast estrogen [8,9] and antagonist screens [10]. For identification of the antagonistic compounds, the strongest high-resolution mass spectrometry (HRMS) signals were assumed to represent the endocrine active compounds, which might not be true. Further, no distinction was made between signal reduction by receptor antagonism and cytotoxicity [9,11]. This is likely to result in a mismatch between HRMS and bioassay results and false-positive antagonists assigned [2]. A detected signal reduction requires clarification about the mechanistic source and verification of whether it is a true antagonist (biological signal reduction by an endocrine effect), a false positive (physico-chemical signal reduction), or a cytotoxic effect (biological signal reduction by reduced cell viability). To understand the effect mechanisms of a complex sample, bioassay-guided fractionation, analytical separation, fraction collection, compound enrichment, re-solubilization, the performance of several bioassays, and combinatory

67 evaluation of all effects is required, surely a long procedure not suited for routine food
68 control and food safety analysis.

69 Effect-directed analysis using a high-performance thin-layer chromatography (HPTLC)
70 separation combined with a planar bioassay seems comparatively simple. On the
71 same adsorbent surface, it allows for sample enrichment during the application,
72 separation of bioactive components from interfering matrix in complex mixtures, non-
73 target biological effect detection, effect differentiation, straightforward compound
74 characterization, and tentative structural assignment [12]. The online elution of
75 interesting active compound zones directly from the bioautogram into an HRMS
76 system streamlines the molecule identification process [13–16]. This hyphenated
77 strategy, especially due to separation and effect differentiation resulting in meaningful
78 bioprofiles, allows us to draw correct conclusions about endocrine responses in
79 complex mixtures in contrast to *in vitro* assays, in which merely sum value numbers
80 are determined.

81 Planar cytotoxicity bioassays were recently demonstrated using resazurin [17,18] or a
82 tetrazolium [19] substrate for the detection of cytotoxins. Planar endocrine bioassays
83 have been used among others for the analysis of cosmetics and thermal paper [16],
84 waste and surface water [20–24], seed oils [25], cannabis [26], plant extracts [27],
85 herbs and spices [28], and packaging material [29]. The planar yeast estrogen screen
86 (pYES) [13,21,22] and planar yeast androgen screen (pYAS) [17] bioassays were
87 developed further to planar multiplex bioassays. The planar yeast antagonist estrogen
88 screen (pYAES) [16,30] and antagonist androgen screen (pYAAS) [16,30] bioassays
89 detect agonists and antagonists, whereas the planar yeast antagonist-verified estrogen
90 screen (pYAVES) [18] and antagonist-verified androgen screen (pYAVAS) [30]
91 bioassays detect agonists, antagonists and false-positive antagonists in one analysis.

However, it would be helpful for verification of endocrine effects and progress in understanding to obtain additional information on the cytotoxicity of the same complex sample. Hence, it was hypothesized that an all-in-one workflow is possible due to the flexibility of the open planar format. In one analysis, all important endocrine and cytotoxicity responses (*i.e.,* agonistic, antagonistic, false-positive antagonistic, cytotoxic, anti-cytotoxic, and false-positive cytotoxic responses) should be distinguishable. This work is the first to find out the potential of such an all-in-one concept and to study a comprehensive multiplex bioassay for the differentiation of up to six important responses. As samples in the field of food contact materials, differently coated tin cans were exemplarily studied for potential endocrine active and/or cytotoxic migrants coming from these packaging materials.

2. Material and methods

2.1. Materials and chemicals

If available, purities are stated. Dimethyl sulfoxide (≥ 99.8%), disodium phosphate (≥ 99.0%), solid sodium hydroxide (> 99%), potassium dihydrogen phosphate (≥ 99%), and potassium chloride (≥ 99.5%) were bought from Carl Roth (Karlsruhe, Germany). HPTLC plates silica gel 60, citric acid monohydrate (≥ 99.5%), and sodium chloride (≥ 99.5%) were bought from Merck (Darmstadt, Germany). Methanol (100%) was bought from VWR (Darmstadt, Germany). D-Glucose (≥ 99.5%), ethanol (≥ 99.8%), copper (II) sulfate (≥ 98%), yeast nitrogen base without amino acids, resazurin disodium salt (resazurin), menadione (K3, analytical standard), flutamide, testosterone (≥ 99.0%), fluorescein disodium salt (fluorescein), Tween 20 and magnesium sulfate heptahydrate (> 99%) was bought from Fluka Sigma Aldrich (Darmstadt, Germany).

116 Toluene (≥ 99.8%) was bought from Fisher Scientific (Schwerte, Germany). Ethyl
117 acetate (≥ 99.8%, Chemsolute) was bought from Th. Geyer (Renningen, Germany).
118 17β-Estradiol (≥ 98.5%) was bought from Dr. Ehrenstorfer (Augsburg, Germany).
119 *Saccharomyces cerevisiae* BJ3505 (YES) were obtained from D. P. McDonnel *et al.*
120 [31,32]. XenoScreen YAS (*Saccharomyces cerevisiae* BJ1991) was bought from
121 Xenometrix (Allschwil, Switzerland). Amino acids for the respective solution were
122 described elsewhere [31,32]. Fluorescein di-(β-D-galactopyranoside, FDG) was
123 bought from Santa Cruz Biotechnology (Dallas, TX, USA). Dihydrotestosterone (DHT,
124 > 99.0%) and resorufin (> 99.5%) were purchased from TCI (Eschborn, Germany).
125 Bisphenol A (BPA, > 97%) was bought from Alfa Aesar (Karlsruhe, Germany).
126 Quercetin hydrate (quercetin, 95%) was bought from Acros Organics (Geel, Belgium).
127 Degalan™ P 28 N was donated by Röhm (Darmstadt, Germany). Six differently coated
128 R&D tin cans from Ceritec SRL, Italy (Metlac Group) were obtained in collaboration
129 with Nestlé Research, Vers-chez-les-Blanc, Switzerland. Bidistilled water was
130 produced using the Destamat Bi 18E (Heraeus, Hanau, Germany).

131 2.2. Yeast cell suspensions

132 Adenine (200 mg), L-arginine (200 mg), L-aspartic acid (1000 mg), L-glutamic acid
133 (1000 mg), L-histidine (200 mg), L-isoleucine (300 mg), L-leucine (1000 mg), L-lysine
134 (300 mg), L-methionine (200 mg), L-phenylalanine (500 mg), L-serine (4000 mg), L-
135 threonine (2000 mg), L-tyrosine (300 mg), and L-valine (1500 mg) were dissolved in 1
136 L bidistilled water and afterward sterile filtrated into a sterile bottle stored at 4 °C until
137 use (amino acid solution). As culture medium, D-glucose (10 g for YES and 5 g for
138 YAS) and yeast nitrogen base without amino acids (6.8 g) were dissolved in bidistilled
139 water (100 mL), which was then sterile filtered into bidistilled water (800 mL) and the
140 amino acid solution (100 mL) was added (two filtration steps required, otherwise

viscosity too high). Cells (1 mL cryostock) were suspended in the culture medium (29 mL for YES and 39 mL for YAS) and cultivated (30 °C, 16 h, 100 rpm, rotatory horizontal shaker SM-30, Edmund Bühler, Bodelshausen, Germany). Depending on the cell density determined using a Thoma chamber (VWR, Darmstadt, Germany), a volume of cell suspension was withdrawn, centrifuged (2500 × *g*, 5 min), and resuspended in respective fresh medium (supplemented with 100 µL of an aqueous 200 µM copper(II) sulfate solution per 30 mL medium) to yield a cell density of 8.0 x 10^7 cells/mL.

2.3. Substrate solutions

For the endocrine response detection, FDG (5 mg) was dissolved in dimethyl sulfoxide (1 mL) and an aliquot (25 µL) was dissolved in 2.5 mL phosphate buffer (40.8 g potassium dihydrogen phosphate, 42.6 g disodium phosphate, 1.2 g magnesium sulfate, and 3.7 g potassium chloride dissolved in 1 L bidistilled water and adjusted to pH 7 with solid sodium hydroxide). For cytotoxicity detection, 2 mg resazurin were dissolved in 4 mL cell suspension.

2.4. Standard solutions and fixation solutions

Ethanol stock solutions of DHT, quercetin, flutamide, K3, testosterone, fluorescein, and resorufin were prepared (1 mg/mL) and diluted with ethanol to 100 pg/µL DHT, 100 ng/µL flutamide, 10 ng/µL and 100 ng/µL K3, 1.5 ng/µL testosterone, 50 ng/µL fluorescein and 50 ng/µL resorufin. For substance fixation, Degalan™ P28 N was dissolved in *n*-hexane to obtain a 0.25% solution. Tween 20 was dissolved in a 9:1 mixture of ethanol and 10 mM aqueous sodium chloride solution to obtain a 0.05% solution.

165 2.5. Migrate preparation

166 The migrates were prepared by filling the cans with food simulant solvent (300 mL,
167 ethanol 95%), closed with a 50 µm-thick aluminum foil (Korff, Oberbipp, Switzerland),
168 and placed iSSSn an incubator at 60 °C for 10 days. The 30-fold migrate concentrates
169 were prepared by evaporating the solvent from a 30-mL aliquot (nitrogen atmosphere)
170 and resolving the residues of each migrate in 1 mL food simulant solvent (ethanol
171 95%).

172 2.6. HPTLC–UV/Vis/FLD–multiplex bioassay method

173 The HPTLC plate silica gel 60 was prewashed by front elution using methanol (up to
174 90 mm, Twin Trough Chamber). HPTLC instrumentation was from CAMAG, Muttenz,
175 Switzerland, if not stated otherwise. The migrate concentrates (3–35 µL/band) and a
176 positive control mixture (generated via overspraying of 1–10 µL/band depending on the
177 compound) were applied (Automatic TLC Sampler 4) as 8-mm bands (for 2-fold
178 multiplex bioassay with 1 stripe) or 28-mm bands (for 6-fold multiplex bioassay with 4
179 stripes). The plate was developed with toluene – ethyl acetate (6:1, *V/V*) up to 70 mm
180 (Twin Trough Chamber). After drying in a cold stream of air (8 min), the chromatogram
181 was documented at white light illumination, FLD 254 nm, and FLD 366 nm (TLC
182 Visualizer 2).

183 Depending on the multiplicity of the bioassay, testosterone (4 µL), estradiol (5.7 µL),
184 K3 (3.2 µL), fluorescein (2 µL), and resorufin (2 µL, better 4 µL) solutions were applied
185 as vertical stripes (0.1 mm × 80 mm, width × height) on each separated track
186 (FreeMode option of winCATS software). The plate was horizontally immersed into a
187 Degalan solution [18,30], let dry at room temperature for 8 min, and Tween 20 solution

was sprayed onto the plate (2.5 mL, blue nozzle, level 4, Derivatizer). After drying in a cold stream of air (4 min), the chromatogram was optionally documented again.

Either (I) cell suspension (8.0 x 10^7 cells/mL), or for metabolization, (II) cell suspension containing the S9 system (prepared by adding 500 µL S9 liver enzyme suspension mixture, 162 µL nicotinamide adenine dinucleotide phosphate solution, 42 µL D-glucopyranose 6-phosphate solution and 953 µL buffer salt solution to 3334 µL yeast bioassay suspension [33]), or for cytotoxicity detection, (III) cell suspension containing the resazurin (2 mg per 4 mL cell suspension) was sprayed onto the chromatogram (2.8 mL, red nozzle, level 6, Derivatizer), incubated in a humid box at 30 °C for 4 h (YAS) or 3 h (YES) and plate drying in a cold stream of air for 4 min followed. In the case of cytotoxicity evaluation, the bioautogram was documented at white light illumination, and for later comparison with the second substrate, also at FLD 254 nm. The FDG substrate solution was either piezoelectrically sprayed onto the plate (2.5 mL, yellow nozzle, level 3, Derivatizer) or syringe-dosed (4.0 mL, spray cycles 3, width 200 mm, length 100 mm, Chromajet DS20, Biostep, Burkhardsdorf, Germany). The plate was then incubated in a humid box at 37 °C for 15 min, dried in a cold stream of air for 4 min, and documented at FLD 254 nm (for comparison, also at FLD 366 nm and white light illumination, TLC Visualizer 2).

2.7 Statistical evaluation

The measurement signals were evaluated via peak area using videodensitometry (VideoScan software, green channel, absorption mode). Dose-response curves were determined via Excel software (Microsoft, Redmond, WA, USA), and thereof, half maximal inhibitory concentration IC_{50} values of BPA were calculated according to AAT Bioquest, IC50 Calculator [34] and its limit of biological detection (LOBD) according to

212 ICH guideline (3.3 × residual standard deviation of the calibration curve to slope of the
213 calibration curve) [35].

214 2.8 Zone characterization by HRMS

215 For characterization, interesting endocrine-active compound zones were eluted from
216 the HPTLC chromatogram and online transferred to heated electrospray ionization
217 high-resolution mass spectrometry (HPTLC–HESI-HRMS). Briefly, the respective
218 migrate was applied to a normal phase plate two times (10 µL/band) and the plate was
219 developed afterward using toluene – ethyl acetate (6:1, *V/V*) up to 70 mm (Twin Trough
220 Chamber). The plate was cut into two pieces. One piece was subjected to the pYAAS
221 bioassay. On the other plate piece, each zone of interest (corresponding to each active
222 zone on the bioassay plate) was eluted (200 µL/s, methanol) into the HESI-HRMS
223 system (QExactive Plus mass spectrometer, Thermo Fisher Scientific, Dreieich,
224 Germany) via the PlateExpress interface (Advion, Ithaca, NY, USA). In case of signal
225 intensity had to be increased, the sample was applied six times at the same quantity
226 as before, developed as before, and cut into two pieces (containing one sample and
227 five samples). As a control, the plate piece containing one sample was subjected to
228 the pYAES bioassay. From the other plate piece containing five samples, the zones of
229 interest were then eluted (200 µL/min, 30 s/band, ethyl acetate used for a good zone
230 solubility and selectivity) and pooled into the same vial. The ethyl acetate solvent was
231 evaporated in a nitrogen stream (ambient temperature) and the residue afterward
232 dissolved in methanol (100 µL) needed for a better ionization. Afterward, the sample
233 (20 µL) was injected via flow-injection analysis (FIA, 200 µL/min) into the HESI-HRMS
234 system (HPTLC–FIA–HESI-HRMS). The instrument parameters were + 3.5 kV and −
235 3.2 kV, 270 °C capillary and 200 °C probe heater temperature, resolution 280 000, m/z

100−1500, and automatic maximum injection time 10/200 ms for positive/negative ionization.

3. Results and discussion

3.1 Outline of the study

The existing HPTLC−pYAAS/pYAES bioassay−FLD method [18,30] was applied to food contact materials and validated according to [36]. Due to the identical procedure except for the receptor in the yeast cell, an analogous result of the method performance is expected for both bioassays. Since the determination of the signal reduction by antagonists (reverse signal, observed via fluorescence reduction on the testosterone agonist stripe) was more challenging than the determination of a direct agonist signal, the HPTLC–pYAAS bioassay–FLD method was selected. It was exemplarily studied for the analysis of BPA, known as an anti-androgenic food contaminant from packaging materials. Instead of the reversed phase [16], the pYAAS bioassay was investigated on the normal phase plate, and the results of the method performance obtained by two different laboratories were compared.

Next, the validated method was exemplarily applied to six selected R&D tin cans with different coatings. Respective migrates were produced using 95% ethanol as a food simulant. The food packaging migrate samples (30-mL aliquots) were 30-fold concentrated by solvent evaporation, followed by dissolving the residues in 1 mL food simulant solvent. Planar multiplex bioassays bypassing matrix effects by the integrated planar separation were selected since these are superior to *in vitro* assays when it comes to separation from the matrix, opposing response differentiation, and

clarification of the specific effects of individual endocrine active substances in a complex sample. Zones of interest were further characterized by HRMS.

However, an antagonistic effect can falsely be assigned, if cytotoxicity is the underlying effect (both reduce the measurement signal). Hence, in the case of detected antagonists in a sample, cytotoxicity has to be proven and excluded, too. The viability of the yeast cells on the HPTLC plate was studied using resazurin as substrate, resulting in an HPTLC−cytotoxicity bioassay. Finally, the cytotoxicity testing was integrated into the existing HPTLC−pYAAS/pYAES bioassay−FLD workflow. The resulting planar 6-fold multiplex bioassay was studied using respective positive controls and was able to differentiate up to 6 responses required to comprehensively evaluate complex samples, exemplarily shown for migrate 39.

3.2. Interlaboratory validation of the HPTLC−pYAAS bioassay−FLD method

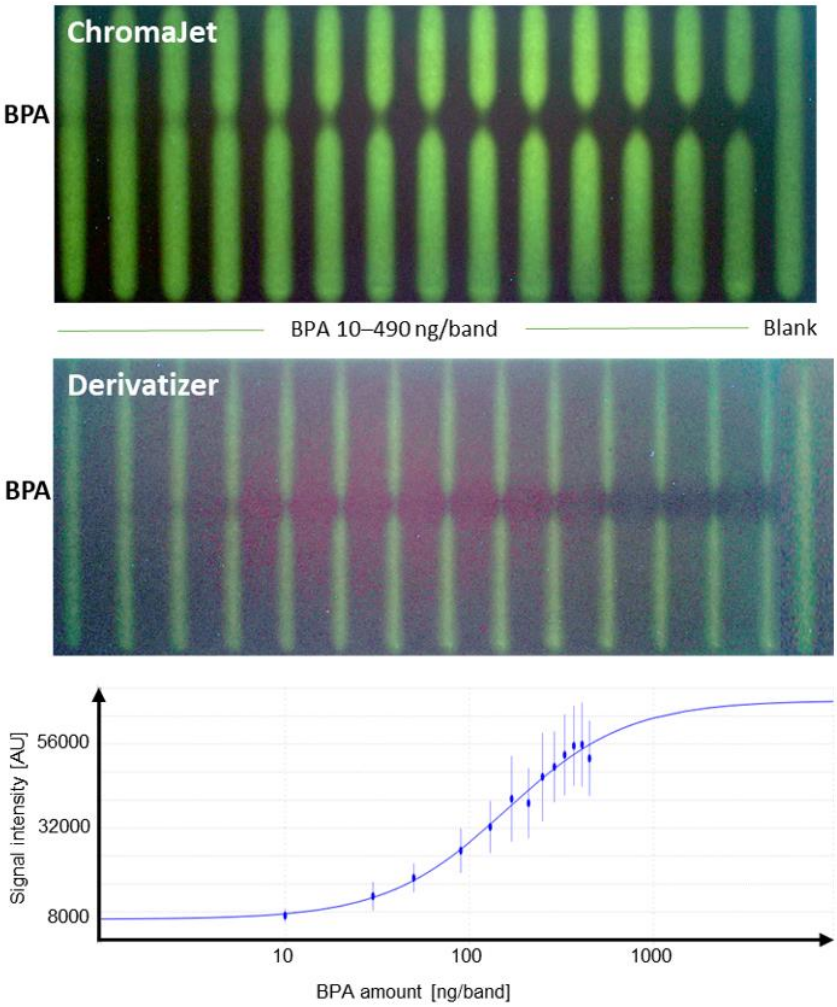
The method validation was performed in two different laboratories to determine the robustness of the dose-response curve, IC_{50} and LOBD. Any difference in the dose-response curves applied in the range of 10–490 ng/band or 10–270 ng/band (Figures 1 and S1) was studied for six replicates using each time freshly prepared cell cultures, six different days, two different operators, two different laboratories and instrumentation, and a variation in the device technology (four times the Derivatizer and two times the ChromaJet for piezoelectrically sprayed and syringe-dosed substrate application, respectively). So, the precision values of the method robustness considered a different cell culture performance, operator, instrumentation, and time.

Calculating the interlaboratory robustness over 6 experiments, the mean IC_{50} value of BPA was 155 ng/band (n = 6, 103–267 ng/band, *%RSD* 38%) for the 2-fold multiplex HPTLC−pYAAS bioassay−FLD method. A similar IC_{50} value (139 ng/spot) was

283 reported by an anti-androgenic bioassay (no multiplex bioassay), in which testosterone
284 was applied on the whole plate format for detection of the antagonistic response [17].
285 These comparable results showed that the time-saving multiplex bioassay, which
286 provides the double (agonist and antagonist) information at the same time, leads to a
287 BPA result comparable to a single anti-androgenic bioassay. The mean LOBD of BPA
288 was determined to be 21 ng/band (*n* = 6, 5–33 ng/band, *%RSD* 50%). Literature data
289 (LOBD of BPA with anti-androgen bioassay) have not been available for comparison.

290 Broken down to the used instruments, the experiments performed via the Derivatizer
291 showed a mean LOBD of 21 ng/band (*n* = 4, 5–33 ng/band, *%RSD* 57%), and the
292 respective experiments done via the ChromaJet provided a mean LOBD of 20 ng/band
293 (*n* = 2, 12 and 27 ng/band, *%RSD* 54%). The mean IC_{50} via the Derivatizer was 164
294 ng/band (*n* = 4, 103–267 ng/band, *%RSD* 44%) and via the ChromaJet 136 ng/band
295 (*n* = 2, 117 and 155 ng/band, %*RSD* 20%). The relative standard deviation tended to
296 be smaller for the ChromaJet. This was explained by the fact that the ChromaJet was
297 used in only one lab, whereas the Derivatizer was used in both labs. So, the impact
298 from different cell performances was bigger for the values generated with the
299 Derivatizer.

300 All in all, the BPA results obtained in two different laboratories were highly comparable,
301 which highlights the good performance of the HPTLC−pYAAS bioassay−FLD method
302 under the different conditions regarding cell culture preparation and biological growth,
303 operator influence, day variance, laboratory, and instrument influence.



304

305 **Figure 1** Validation of the HPTLC−pYAAS bioassay−FLD method: Two representative
306 example bioautograms at FLD 254 nm with different amounts of bisphenol A (BPA,
307 ranging 10–490 ng/band and 10–270 ng/band) and a solvent blank separated on
308 HPTLC plate silica gel 60 with toluene – ethyl acetate 6:1; a testosterone agonist stripe
309 (4 µL) was applied for antagonist detection via the bioassay, using either the
310 Derivatizer or ChromaJet device for substrate application, as well as corresponding
311 mean dose-response curve.

312

313 3.3 Application of the HPTLC−pYAAS/pYAES bioassay−FLD method

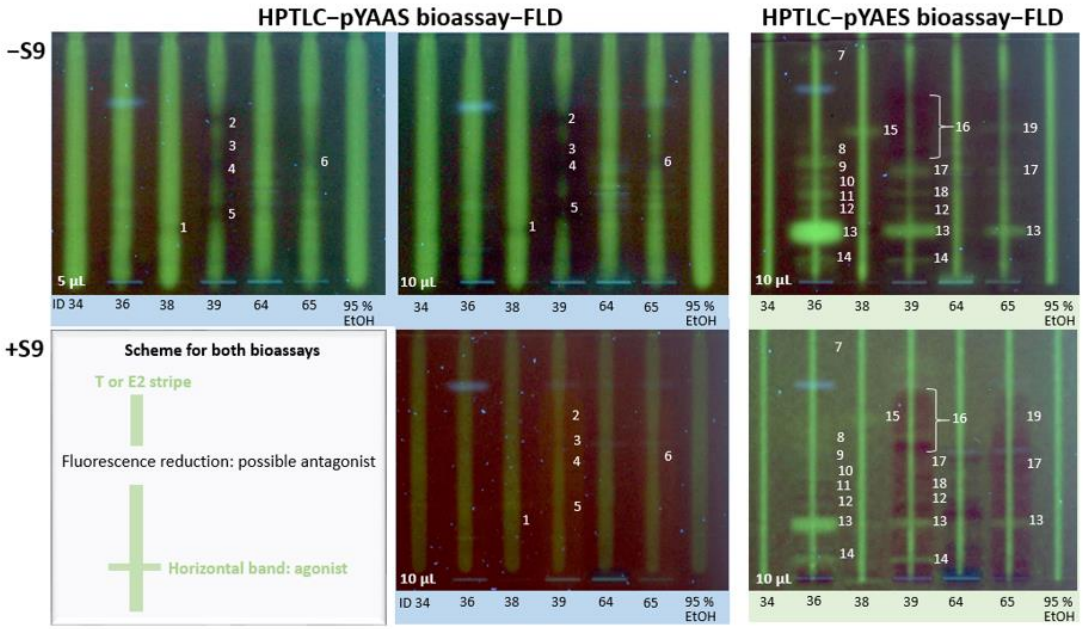
314 The validated method was next applied to food contact materials. After application of
315 the 6 different migrate concentrates (5 or 10 µL/band) on the HPTLC plate, their
316 separation, respective agonist stripe application, and bioassay detection, 19 potentially
317 endocrine disrupting compounds with agonistic and antagonistic activity were found
318 (Figure 2, -S9). The FDG substrate was used for both bioassay methods, which
319 generated a green fluorescence when it was cleaved by glucosidase, released upon
320 receptor binding of an agonist. Thus, a green fluorescent band was detected when an
321 androgenic or estrogenic substance (agonist) was present. This substrate was
322 advantageous because the sample did not originally show green fluorescent bands
323 that could interfere with the interpretation of the results. Antagonists were observed via
324 the fluorescence reduction on the agonist stripe (testosterone or estradiol stripe),
325 applied along each separated sample track before the bioassay application. When an
326 antagonist was present, the green fluorescent stripe was partially interrupted because
327 the effect of the corresponding agonist (producing the biologically induced
328 fluorescence signal) was reduced.

329 The pYAAS bioautogram revealed several antagonists but no agonists (Figure 2, -S9).
330 In migrate concentrate 38, one antagonist (**1**) was found. Migrate concentrate 39
331 contained four further antagonists (**2**–**5**). The applied 5-µL volume resulted in better
332 resolution of the bands since bands **3** and **4** were not differentiable anymore for the
333 applied 10-µL volume. This highlighted that less sample volume should also be used
334 to check if more than one band is present in a broad band. Migrate concentrate 65
335 contained one antagonist (**6**). The simulated metabolic conversion via the S9 liver
336 system (Figure 2, +S9) reduced the antagonistic activity for all bands. Also evident was
337 the overall fluorescence reduction on the testosterone stripe through the

338 metabolization of the testosterone by the S9 enzymes, resulting in less testosterone
339 being available for receptor activation.

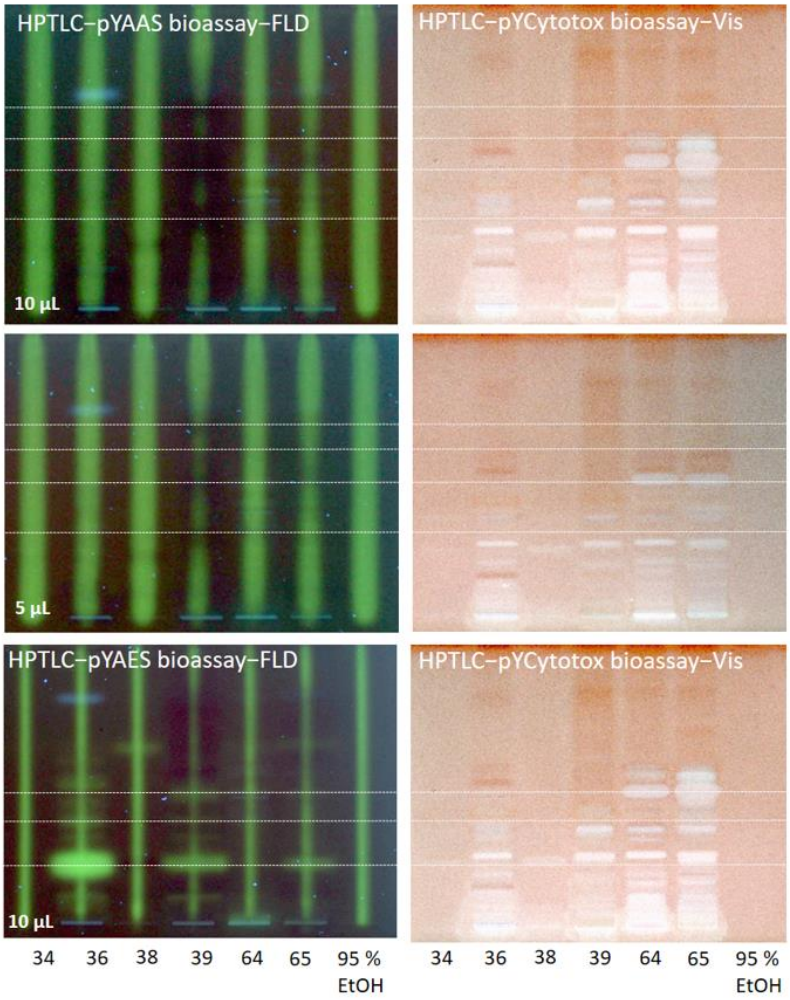
340 The pYAES bioautogram (Figure 2) showed eight agonist bands (**7**–**14**) for migrate
341 concentrate 36, one (**15**) for migrate concentrate 38, a range indicating several
342 antagonists (**16**) for migrate concentrate 39 as well as two further agonistic bands (**17**
343 and **18**), and again the three agonistic bands **12**–**14** that were already present in
344 migrate concentrate 36. Migrate concentrate 65 showed one unique agonist band (**19**)
345 and two agonist bands **13** and **17**, previously detected in migrate concentrates 36 or
346 39. When the simulated metabolic conversion via the S9 liver system was studied and
347 applied, the intensities of all fluorescent bands were reduced again. The previously
348 weaker agonist signals were not detected anymore for the same given sample amount.
349 For both bioassays, the comparison showed that the S9 metabolization converted
350 endocrine disruptors into substances with less activity or reduced their amount and
351 impact on the respective human receptor.

352 For all endocrine active compounds detected in the 6 migrate concentrates, cytotoxicity
353 had to be excluded, and thus, was proven using the respective cell suspension
354 containing the resazurin (planar yeast cytotoxicity bioassay, pYCytotox bioassay). For
355 the same sample amount applied, a cytotoxicity of the endocrine active compounds
356 was not observed (Figure 3, dotted line), which confirmed their endocrine mechanism.



357

358 **Figure 2** Application of the HPTLC–pYAAS/pYAES bioassay–FLD methods to food
359 contact materials: Bioautograms at FLD 254 nm of six different migrate concentrates
360 (IDs 34–65; 5 and 10 µL/band) and a food simulant blank (separated as in Figure 1) as
361 well as applied testosterone (T, 4 µL) or estradiol (E2, 5.7 µL) agonist stripes to detect
362 respective antagonists, shown for the bioassay applied with (+S9) and without (–S9)
363 metabolization.



364

365 **Figure 3** Exclusion of cytotoxicity via the HPTLC–pYCytotox bioassay–Vis method:
366 Bioautograms at white light illumination of the six different migrate concentrates (IDs
367 34–65; 5 and 10 µL/band), showing no cytotoxicity (dotted line) of the endocrine active
368 zones as in Figure 2.

369

370 3.4. Assignment of molecular formulas to endocrine active migrants

371 The coupling to HRMS allowed the determination of molecular formulas, exemplarily
372 performed for the most endocrine active bands in the most active migrate concentrates.

373 The pYAAS bioassay showed four strong anti-androgenic compounds (**2**–**5**) in migrate
374 concentrate 39, and the pYAES bioassay one strong estrogenic substance (**13**) in
375 migrate concentrates 36, 39, and 65. These endocrine active migrants were
376 investigated using migrate concentrates 36 and 39, applied 4 times, followed by plate
377 development and plate cut to obtain 4 replicates, each containing the two migrate
378 concentrates. Since the bands of interest were not detectable at UV/Vis/FLD, one plate
379 piece was subjected to the pYAAS bioassay and another to the pYAES bioassay to
380 confirm the endocrine responses and obtain reference positions for zone marking on
381 the third and fourth plate pieces, followed by zone elution to HRMS. However, the
382 signals obtained were too low in intensity. Since the bioassay is very sensitive in the
383 detection, low picogram amounts can be expected to be present in the zone, too low
384 for successful HRMS recording.

385 Thus, to obtain a better signal intensity, migrate concentrate 36 was applied five times
386 and the respective agonist band **13** was five times eluted with ethyl acetate (for zone
387 solubility and selectivity reasons) and pooled into the same vial. After solvent
388 evaporation, the residue was re-dissolved in methanol (for good ionization) and flow-
389 injected into the HRMS system. Thanks to the selective elution and enrichment, the
390 active zones were further characterized and assigned to molecular formulas (Table 1).
391 Several mass signals with a mass difference of 162 Da representing a neutral loss of
392 $C_{11}H_{14}O$ were found for band **2**. A possible explanation of this repetitive neutral loss
393 was the presence of a polymer coating of the can or an oligomer side product that
394 migrated into the food simulant solvent exposed to 60 °C for 10 days. Considering that
395 the molecular formula of this fragment is close to that of butylparaben ($C_{11}H_{14}O_3$), a
396 butylparaben-like substructure could cause the observed anti-androgenic activity. For

397 bands **3**–**5** and **13,** substance structures could not be proposed to the molecular
398 formulas found (Table 1).

399 **Table 1** Mass signals detected and assigned molecular formulas

Band	Signal	*m/z*	Molecular formula	Error ± [ppm]
2	$[M+H]^+$	337.2161	$C_{23}H_{19}O_2$	0.14
	$[M+Na]^+$	289.1773	$C_{16}H_{26}O_3Na$	0.05
	$[M+Na]^+$	435.2873	$C_{27}H_{40}O_3Na$	0.59
	$[M+Na]^+$	451.2820	$C_{27}H_{40}O_4Na$	0.02
	$[M+Na]^+$	613.3866	$C_{38}H_{54}O_5Na$	0.42
	$[M+Na]^+$	775.4912	$C_{49}H_{68}O_6Na$	0.54
	$[M-H]^-$	193.0871	$C_{11}H_{13}O_3$	0.89
	$[M-H]^-$	265.1810	$C_{16}H_{25}O_3$	0.42
	$[M-H]^-$	427.2857	$C_{27}H_{39}O_4$	0.70
	$[M-H]^-$	589.3907	$C_{38}H_{53}O_5$	1.43
	$[M-H]^-$	751.4952	$C_{49}H_{67}O_6$	1.88
3	$[M+Na]^+$	395.2193	$C_{23}H_{32}O_4Na$	1.34
	$[M-H]^-$	355.1918	$C_{22}H_{27}O_4$	0.98
	$[M-H]^-$	501.3016	$C_{33}H_{41}O_4$	1.21
4	$[M+Na]^+$	349.1774	$C_{21}H_{26}O_3Na$	0.05
	$[M+Na]^+$	365.1722	$C_{21}H_{26}O_4Na$	0.49
	$[M+Na]^+$	463.1362	$C_{24}H_{24}O_8Na$	0.37
	$[M-H]^-$	341.1760	$C_{21}H_{25}O_4$	0.37
	$[M-H]^-$	683.3594	$C_{24}H_{51}O_8$	1.17
5	$[M+Na]^+$	443.1677	$C_{22}H_{28}O_8Na$	1.12
	$[M-H]^-$	403.1553	$C_{25}H_{25}O_5$	1.94
13	$[M+H]^+$	282.2790	$C_{18}H_{36}ON$	0.32
	$[M+H]^+$	298.2740	$C_{18}H_{36}O_2N$	0.12
	$[M+Na]^+$	304.2609	$C_{18}H_{35}ONNa$	0.41
	$[M+H]^+$	314.2685	$C_{18}H_{36}O_3N$	1.43
	$[M+H]^+$	330.2641	$C_{18}H_{36}O_4N$	0.92
	$[M-H]^-$	293.1762	$C_{17}H_{25}O_4$	1.19
	$[M-H]^-$	309.1711	$C_{17}H_{25}O_5$	2.75

400

401 3.5. Advanced concept of a multiplex bioassay differentiating up to 6 responses

402 The anti-androgenic reaction is not a reaction in the sense of receptor activation, but a
403 blocking of receptor activation. Since also cytotoxicity leads to a reduction in the
404 response, a clear differentiation of anti-androgenicity from cytotoxicity is required. In
405 order to comprehensively evaluate complex mixtures, a 6-response differentiation was
406 aimed. To upgrade the existing 2-/3-fold multiplex bioassays to a 6-fold multiplex
407 bioassay, the use of a respective cell viability indicating substrate and four/two further
408 stripes were required to differentiate also cytotoxicity responses. As a substrate,
409 resazurin was selected as a metabolic indicator to detect cell metabolism. The
410 resazurin substrate was simply added to the yeast cell culture. After its application on
411 the plate and incubation, the resulting bioautogram was recorded under white light
412 illumination to detect cytotoxin bands and also documented at FLD 254 nm for
413 comparison with the subsequent substrate. On the same plate as a substrate
414 sequence, the second FDG substrate was applied, and after a short incubation,
415 endocrine agonist bands were detected at FLD 254 nm.

416 For differentiation of anti-effects, altogether 4 stripes (androgen/agonist testosterone,
417 cytotoxin K3, both end products fluorescein and resorufin) had to be applied along
418 each separated sample track before the bioassay application. Or, in addition to the
419 stripes used in the existing pYAVAS [30] and pYAVES [18] bioassays, the cytotoxin
420 K3 and the end product of the enzyme–substrate reaction resorufin were applied to
421 differentiate anti-cytotoxins (biological signal reduction) from false positives (physico-
422 chemical signal reduction). Since 4 stripes were required on each separated sample
423 track and all zones/stripes had to remain sharp during the long bioassay incubation for
424 good evaluation, substance diffusion was reduced by fixation with a Degalan solution.
425 This made the layer apolar and the plate was no longer homogeneously water-

426 wettable. Therefore, a Tween solution was subsequently sprayed onto the plate, which
427 acted as a surfactant to improve the penetration of the following salt-rich aqueous
428 bioassay into the layer. Note that the use of lower amounts of Degalan (0.1% instead
429 of 0.25% Degalan solution), allowed a better wettability, but again led to higher
430 diffusion (Figure S2).

431 The main procedural steps of the 6-fold multiplex bioassay are illustrated (Figure 4). In
432 a laboratory routine with many different multiplex bioassays performed in various
433 projects, sound terminology is the precondition for clear communication. Since Y
434 stands for the yeast strain, A for the anti-effect detection, and V for the false-positive
435 detection in the already existing terms, the resulting 6-fold multiplex bioassay workflow
436 was named HPTLC−pYAVCytotox−pYAVAS/pYAVES bioassay−Vis/FLD.

437

438 **Figure 4** Developed 6-fold multiplex planar bioassay: Procedure steps of the
439 HPTLC−pYAVCytotox−pYAVAS/pYAVES bioassay−Vis/FLD method, taking 6 h per
440 plate, whereof labor time takes 1 h.

441

442 3.6. Proof of the 6-fold multiplex bioassay using positive control standards

443 Again, an analogous performance is expected for both HPTLC−pYAVCytotox−
444 pYAVAS/pYAVES bioassay−Vis/FLD methods, since both pYAAS and pYAES

445 bioassay workflows are identical except for the receptor in the yeast cell. The
446 HPTLC−pYAVCytotox−pYAVAS bioassay−Vis/FLD method was selected due to the
447 high relevance for endocrine-disrupting anti-androgens and reproduced on three
448 different days (Figure S3). Positive control standard solutions (K3, quercetin, DHT, and
449 flutamide) commonly used in *in vitro* bioassays [2, 37−40] were applied in increasing
450 amounts by spraying each solution on the same application zone to generate an
451 oversprayed mixture. The overspray application was more flexible with regard to an
452 adjustment of the individual amounts. After their separation, application of the 4 stripes
453 (Figure 5A/B), substance fixation, application of the cell suspension with the added
454 substrate resazurin, incubation at 30 °C for 4 h, and detection at white light illumination,
455 the cytotoxic, anti-cytotoxic, and false-positive anti-cytotoxic responses were observed
456 in the resulting bioautogram (Figure 5C). After application of the second substrate
457 FDG, incubation at 37 °C for 15 min and detection at FLD 254 nm, the androgenic,
458 anti-androgenic, and false-positive anti-androgenic responses were observed in the
459 resulting bioautogram (Figure 5F). The dose-response curves of K3, flutamide, and
460 DHT (Figure 5G), which were generated from the data obtained on three different days
461 and cell cultures (n = 3), showed determination coefficients R^2 of 0.8431, 0.9197, and
462 0.9721, respectively. This was worse than usually expected but explained by the given
463 bioassay complexity and day-to-day variances of the yeast cell performance and
464 substance fixation via the Degalan.

465

466 3.6.1 Cytotoxic response evaluation

467 The K3 was selected as a representative cytotoxin since it was proven as a suited
468 positive control in *in vitro* cytotoxicity assays [37]. When the substrate resazurin is not

469 metabolized due to cytotoxicity, depending on the surrounding pH value, orange (< pH
470 6.5) or purple (> pH 6.5) cytotoxic bands can be expected on a reddish (purple)
471 resorufin plate background. The K3 was evident as an increasing orange cytotoxin
472 band (unreacted resazurin) surrounded by a very strong colorless (white) halo on a
473 purple background (Figure 5C). The orange K3 bands were even stronger detectable
474 after the second substrate application (Figure 5E). The selected K3 amounts led to an
475 overloaded response, however, were needed to detect at white light illumination the
476 cytotoxicity-indicating orange band inside the colorless halo. The amount-dependent
477 white halo was explained by the further reduction of the generated red resorufin to the
478 colorless dihydroresorufin. This is plausible because the cytotoxic potential of K3
479 arises from oxidative stress [37] against which cells can defend themselves by
480 chemical reduction. The reduction equivalents necessarily formed by this could also
481 react with resorufin, which would explain the production of dihydroresorufin. Due to this
482 plausible explanation, the amount of K3 can be reduced substantially in future
483 applications.

484 Anti-cytotoxic substances were detected via the applied cytotoxic K3 stripe, which
485 again showed a strong white halo around the weak sharp orange line. Any anti-
486 cytotoxic substance present would interrupt the cytotoxic K3 stripe and could thus be
487 detected. Although many different anti-mechanisms exist, quercetin was exemplarily
488 selected as a positive control standard since it was described as anti-cytotoxic [38]. At
489 low quercetin amounts, the quercetin band was clearly interrupted by the colorless
490 cytotoxic K3 stripe (Figure 5C), however, for increasing amounts, the quercetin
491 increasingly mitigated the oxidative cell stress (no colorless white interruption of the
492 quercetin band anymore). Thus, the effect-dose dependency of the anti-toxic effect of
493 quercetin was given. The dose-response curve was not determined since quercetin

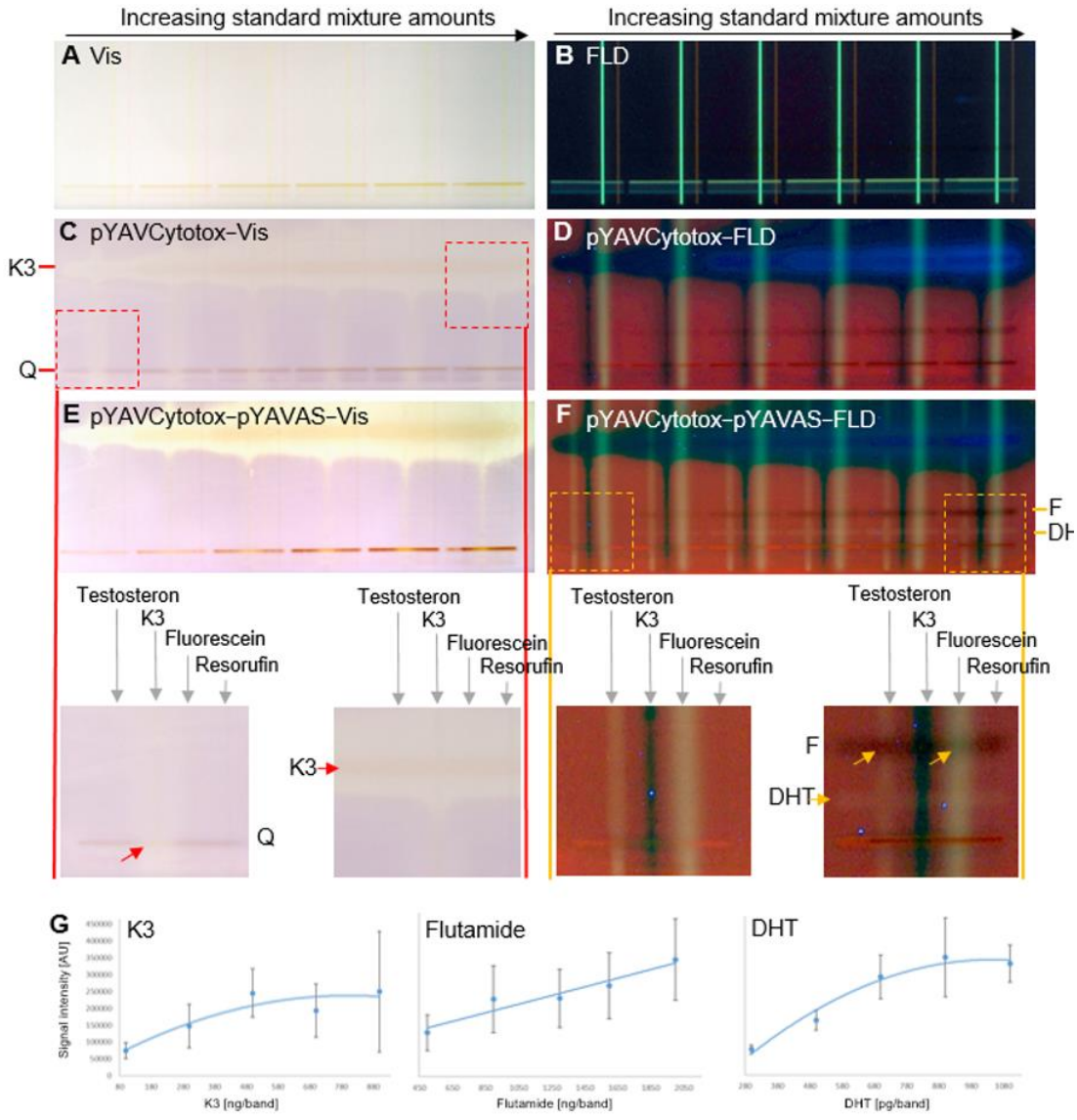
was too polar to migrate in the mobile phase system for the food packaging material and thus was too close to the start zone. It could be substituted by a more apolar anti-cytotoxin in the future. As proof for false positives, the resorufin end-product stripe of the respective enzyme–substrate reaction was not influenced by the quercetin which verified the anti-toxic effect (Figure 5C). Note that the resorufin end-product stripe was hardly visible and could be applied at the double amount (4 µL instead of 2 µL stripe) in the future.

3.6.2 Endocrine response evaluation

After the application of the second FDG substrate solution and incubation, the resulting bioautogram at FLD 254 nm was evaluated for the three respective endocrine responses (Figure 5F). The androgen detection was studied and proven using the well-known androgen DHT, already used as a positive control in the androgen screening of packaging migrates [2]. DHT was observed as an ochre fluorescent band. The normally expected green FDG fluorescence changed to ochre, which hue shift was caused by the resorufin substrate (respective end-product of the previous enzyme reaction with the resazurin substrate) already present on the layer.

The same hue change to ochre was observed for the testosterone agonist stripe, which was required for the detection of anti-androgens. Flutamide was used as a positive control for an anti-androgen, as proven in *in vitro* assays [39,40]. The anti-androgenic effect of flutamide was shown by its fluorescence reduction on the testosterone stripe (Figure 5F). Any false-positive anti-androgenic response (physico-chemical fluorescence reduction) that could falsely be interpreted as cytotoxic or anti-androgenic was clarified via the vertically applied respective resorufin and fluorescein end-product stripes. A fluorescence reduction of these stripes was not observed for flutamide in the

518 bioautogram (Figure 5F). This was expected for flutamide which was not described as
519 acting false-positive, however, at higher amounts, flutamide altered the fluorescein
520 response.



521

522 **Figure 5** Proof-of-principle of the HPTLC–pYAVCytotox–pYAVAS bioassay–Vis/FLD
523 method: Chromatograms/bioautograms at white light illumination (Vis; **A**/**C**/**E**) and at
524 254 nm (FLD; **B**/**D**/**F**) of the positive control standards cytotoxin K3 (30–900 ng/band,
525 0.3–9 µL, 100 ng/µL), anti-cytotoxic acting quercetin (700–1700 ng/band, 0.7–1.7 µL),

anti-androgen flutamide (100–2000 ng/band, 0.1–2.0 µL), and androgen DHT (100–1100 pg/band, 1.0–11 µL) applied (oversprayed on the same start zone) and separated as in Figure 1; four stripes (testosterone 4 µL, K3 3.2 µL, fluorescein 2 µL and resorufin 2 µL) applied on each separated track, followed by the bioassay performance; respective dose-response curves determined via videodensitometry.

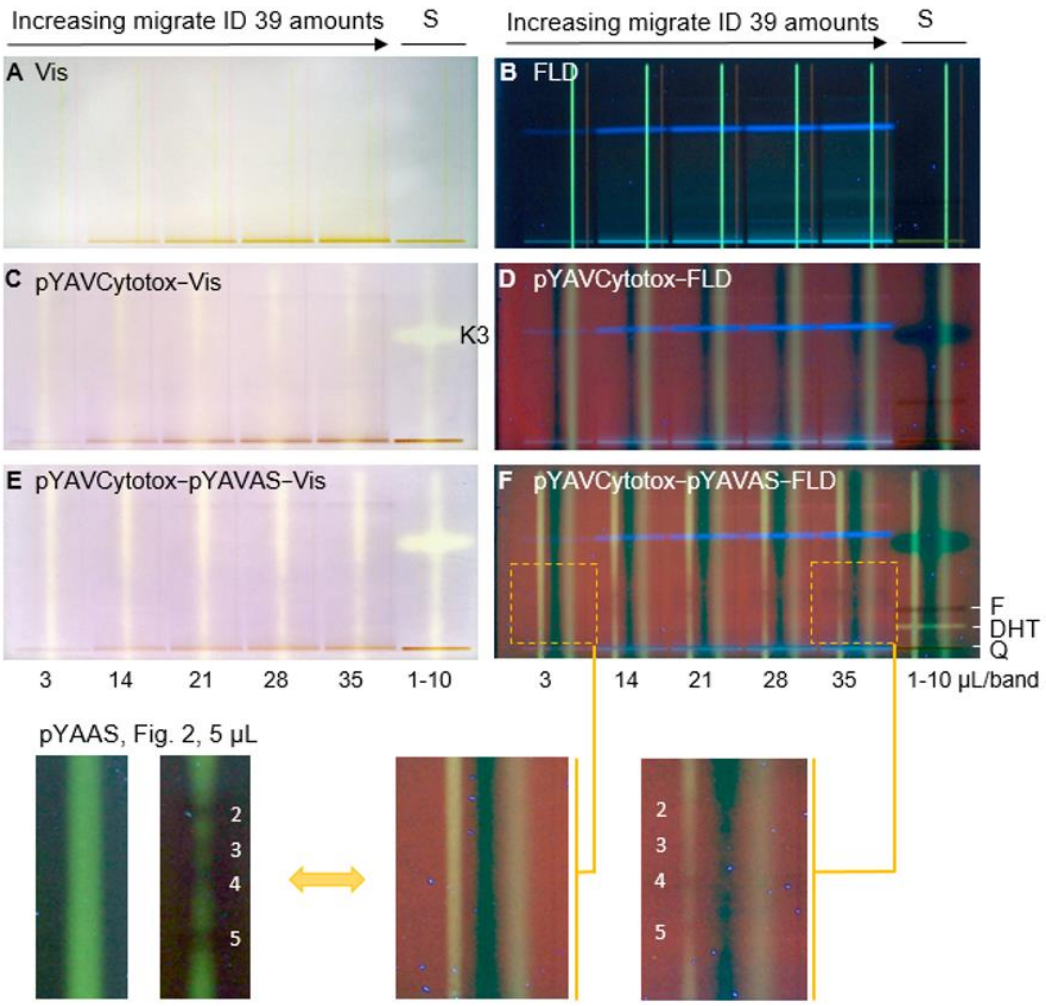
3.7. Application of the 6-fold multiplex bioassay to migrate 39 and cost/time calculation

The HPTLC–pYAVCytotox–pYAVAS bioassay–Vis/FLD method was exemplarily applied to the analysis of migrate concentrate 39, as it showed the most prominent response in the HPTLC–pYAAS bioautogram, *i.e.* the four anti-androgenic bands **2**–**5** (Figure 2). Five different amounts of the migrate concentrate 39 were applied on the plate as well as the positive control mixture (DHT, flutamide, quercetin, and K3). Since K3 resulted in the formation of the colorless (white) dihydroresorufin indicating cytotoxicity as discussed, the applied K3 amount was reduced (and could be even further reduced in the future). After the 6-fold multiplex bioassay, the positive controls indicated that the bioassay worked as expected. For the four anti-androgenic bands **2**–**5**, dose-response-dependent effects were observed (Figure 6). No cytotoxic band was found for the studied migrate, which means that the anti-androgenic bands **2**–**5** in the migrate concentrate 39 were not cytotoxic in the tested dose-response range and were therefore true anti-androgens.

By this, all important information about a complex sample and its dose-response dependency (5 different amounts) was obtained from one plate and thus from one analysis. On the same surface, the planar 6-fold multiplex bioassay method brings together all information in form of an image worth a thousand words. The procedure

550 took about 6 h per plate (Table S1), whereof only 1 h was labor work, *i.e.* transfer time
551 between the steps and short manual operations. Four plates can be handled in parallel
552 per 8-h day. The costs were about € 24 per plate, mainly due to the cost of the plate
553 (€ 8) and the expensive substrates (€ 12), which, however, can be substituted by low-
554 cost ones. In contrast, imagine how much effort is required, how much material is
555 consumed, how much time is needed, and how complicated the combinatory
556 evaluation of the obtained sum values is for bioassay-guided fractionation, analytical
557 separation, 6 different *in vitro* assays at 5 studied sample amounts, and re-analysis for
558 HRMS recording.

559



560

561 **Figure 6** Application of the HPTLC–pYAVCytotox–pYAVAS bioassay–Vis/FLD
562 method: Chromatograms/bioautograms at white light illumination (Vis; A/C/E) and at
563 254 nm (FLD; B/D/F) of the migrate concentrate ID 39 at increasing amounts (3–35
564 µL/band) and the positive control mixture (S; quercetin 1 µg/band, 1 µL; DHT 1
565 ng/band, 10 µL; flutamide 1 µg/band, 10 µL and K3 20 ng/band, 2 µL, 10 ng/µL),
566 analyzed as in Figure 5.

567

568

4. Conclusions

The HPTLC−UV/Vis/FLD−(S9)−pYAAS/pYAES bioassays with and without metabolization showed an acceptable performance, which makes them a promising tool for the control of food and food contact materials for endocrine-disrupting compounds. The newly developed concept of a 6-fold multiplex bioassay, *i.e.* the HPTLC−pYAVCytotox−pYAVAS/pYAVES bioassay−Vis/FLD method, demonstrated in one analysis all the important endocrine and cytotoxicity responses, *i.e.* endocrine agonist, antagonist, false positive antagonist, cytotoxin, anti-cytotoxin, and false positive anti-cytotoxin responses. This allowed the fast and cost-saving differentiation of individual substance effect mechanisms in a complex sample. The new 6-fold multiplex bioassay concept can be applied adjusted in many variations with regard to the selected microorganisms, substrates, stripes, and positive controls to suit other purposes.

CrediT authorship Contribution Statement

Daniel Meyer: Methodology, Investigation, Data Analysis, Writing – Original Draft. **Maricel Marin-Kuan**: Writing – Review and Editing. **Hélia Latado**: Investigation (ChromaJet and partially Derivatizer experiments in 3.2). **Benoît Schilter**: Writing – Review and Editing. **Gertrud E. Morlock**: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – Original Draft, Writing – Review and Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Thank is owed to Röhm, Darmstadt, Germany, for providing Degalan™ P 28 N, respectively. Instrumentation was partially funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - INST 162/471-1 FUGG; INST 162/536-1 FUGG.

598 **References**

599 [1] C. Sonnenschein, A.M. Soto, An updated review of environmental estrogen and
600 androgen mimics and antagonists, The Journal of Steroid Biochemistry and Molecular
601 Biology 65 (1998) 143–150. https://doi.org/10.1016/S0960-0760(98)00027-2.
602 [2] J. Mertl, C. Kirchnawy, V. Osorio, A. Grininger, A. Richter, J. Bergmair, M. Pyerin, M.
603 Washüttl, M. Tacker, Characterization of estrogen and androgen activity of food contact
604 materials by different in vitro bioassays (YES, YAS, ERα and AR CALUX) and
605 chromatographic analysis (GC-MS, HPLC-MS), PLoS One 9 (2014) e100952.
606 https://doi.org/10.1371/journal.pone.0100952.
607 [3] T. Colborn, F.S. vom Saal, A.M. Soto, Developmental effects of endocrine-disrupting
608 chemicals in wildlife and humans, Environ. Health Perspect. 101 (1993) 378–384.
609 https://doi.org/10.1289/ehp.93101378.
610 [4] I. Ceccarelli, D. Della Seta, P. Fiorenzani, F. Farabollini, A.M. Aloisi, Estrogenic
611 chemicals at puberty change ERalpha in the hypothalamus of male and female rats,
612 Neurotoxicol. Teratol. 29 (2007) 108–115. https://doi.org/10.1016/j.ntt.2006.10.011.
613 [5] S.C. Sikka, R. Wang, Endocrine disruptors and estrogenic effects on male reproductive
614 axis, Asian J. Androl. 10 (2008) 134–145. https://doi.org/10.1111/j.1745-
615 7262.2008.00370.x.
616 [6] J. Gray, N. Evans, B. Taylor, J. Rizzo, M. Walker, State of the Evidence: The
617 Connection Between Breast Cancer and the Environment, International Journal of
618 Occupational and Environmental Health 15 (2009) 43–78.
619 https://doi.org/10.1179/oeh.2009.15.1.43.
620 [7] J. Muncke, Endocrine disrupting chemicals and other substances of concern in food
621 contact materials: an updated review of exposure, effect and risk assessment, The
622 Journal of Steroid Biochemistry and Molecular Biology 127 (2011) 118–127.
623 https://doi.org/10.1016/j.jsbmb.2010.10.004.

624 [8] B. Pinto, D. Reali, Screening of estrogen-like activity of mineral water stored in PET
625 bottles, Int. J. Hyg. Environ. Health 212 (2009) 228–232.
626 https://doi.org/10.1016/j.ijheh.2008.06.004.
627 [9] M. Wagner, J. Oehlmann, Endocrine disruptors in bottled mineral water: total estrogenic
628 burden and migration from plastic bottles, Environ. Sci. Pollut. Res. Int. 16 (2009) 278–
629 286. https://doi.org/10.1007/s11356-009-0107-7.
630 [10] M. Wagner, M.P. Schlüsener, T.A. Ternes, J. Oehlmann, Identification of putative steroid
631 receptor antagonists in bottled water: combining bioassays and high-resolution mass
632 spectrometry, PLoS One 8 (2013) e72472.
633 https://doi.org/10.1371/journal.pone.0072472.
634 [11] L. Zimmermann, Z. Bartosova, K. Braun, J. Oehlmann, C. Völker, M. Wagner, Plastic
635 Products Leach Chemicals That Induce In Vitro Toxicity under Realistic Use Conditions,
636 Environ. Sci. Technol. (2021). https://doi.org/10.1021/acs.est.1c01103.
637 [12] G.E. Morlock, High-performance thin-layer chromatography combined with effect-
638 directed assays and high-resolution mass spectrometry as an emerging hyphenated
639 technology: A tutorial review, Anal. Chim. Acta (2021) 338644.
640 https://doi.org/10.1016/j.aca.2021.338644.
641 [13] I. Klingelhöfer, G.E. Morlock, Sharp-bounded zones link to the effect in planar
642 chromatography-bioassay-mass spectrometry, J. Chromatogr. A 1360 (2014) 288–295.
643 https://doi.org/10.1016/j.chroma.2014.07.083.
644 [14] G.E. Morlock, I. Klingelhöfer, Liquid chromatography-bioassay-mass spectrometry for
645 profiling of physiologically active food, Anal. Chem. 86 (2014) 8289–8295.
646 https://doi.org/10.1021/ac501723j.
647 [15] S.-S. Teh, G. Morlock, Analysis of Bioactive Components of Oilseed Cakes by High-
648 Performance Thin-Layer Chromatography-(Bio)assay Combined with Mass
649 Spectrometry, Chromatography 2 (2015) 125–140.
650 https://doi.org/10.3390/chromatography2010125.

651 [16] I. Klingelhöfer, N. Hockamp, G.E. Morlock, Non-targeted detection and differentiation of
652 agonists versus antagonists, directly in bioprofiles of everyday products, Anal. Chim.
653 Acta 1125 (2020) 288–298. https://doi.org/10.1016/j.aca.2020.05.057.
654 [17] C. Riegraf, A.M. Bell, M. Ohlig, G. Reifferscheid, S. Buchinger, Planar chromatography-
655 bioassays for the parallel and sensitive detection of androgenicity, anti-androgenicity
656 and cytotoxicity, Journal of Chromatography A 1684 (2022) 463582.
657 https://doi.org/10.1016/j.chroma.2022.463582.
658 [18] A. Ronzheimer, T. Schreiner, G.E. Morlock, Multiplex planar bioassay detecting
659 estrogens, antiestrogens, false-positives and synergists as sharp zones on normal
660 phase, Phytomedicine 103 (2022) 154230.
661 https://doi.org/10.1016/j.phymed.2022.154230.
662 [19] D. Meyer, G.E. Morlock, Designed genotoxicity profiling detects genotoxic compounds in
663 healthy staple food, Food Chem. (2022).
664 [20] A. Schoenborn, P. Schmid, S. Bräm, G. Reifferscheid, M. Ohlig, S. Buchinger,
665 Unprecedented sensitivity of the planar yeast estrogen screen by using a spray-on
666 technology, J. Chromatogr. A 1530 (2017) 185–191.
667 https://doi.org/10.1016/j.chroma.2017.11.009.
668 [21] I. Klingelhöfer, G.E. Morlock, Bioprofiling of Surface/Wastewater and Bioquantitation of
669 Discovered Endocrine-Active Compounds by Streamlined Direct Bioautography, Anal.
670 Chem. 87 (2015) 11098–11104. https://doi.org/10.1021/acs.analchem.5b03233.
671 [22] D. Schick, W. Schwack, Planar yeast estrogen screen with resorufin-β-d-
672 galactopyranoside as substrate, J. Chromatogr. A 1497 (2017) 155–163.
673 https://doi.org/10.1016/j.chroma.2017.03.047.
674 [23] F. Brion, V. de Gussem, S. Buchinger, H. Hollert, M. Carere, J.-M. Porcher, B. Piccini,
675 C. Féray, V. Dulio, S. Könemann, E. Simon, I. Werner, R. Kase, S. Aït-Aïssa, Monitoring
676 estrogenic activities of waste and surface waters using a novel in vivo zebrafish
677 embryonic (EASZY) assay: Comparison with in vitro cell-based assays and

678 determination of effect-based trigger values, Environ. Int. 130 (2019) 104896.
679 https://doi.org/10.1016/j.envint.2019.06.006.
680 [24] D. Schick, C. Oellig, Bestimmung estrogen aktiver Nonylphenole in
681 Oberflächengewässern mittels pSPE-pYES, Lebensmittelchemie 73 (2019) S106-S106.
682 https://doi.org/10.1002/lemi.201951106.
683 [25] S.-S. Teh, G.E. Morlock, Effect-directed analysis of cold-pressed hemp, flax and canola
684 seed oils by planar chromatography linked with (bio)assays and mass spectrometry,
685 Food Chem. 187 (2015) 460–468. https://doi.org/10.1016/j.foodchem.2015.04.043.
686 [26] G. Corni, V. Brighenti, F. Pellati, G.E. Morlock, Effect-directed analysis of bioactive
687 compounds in Cannabis sativa L. by high-performance thin-layer chromatography, J.
688 Chromatogr. A 1629 (2020) 461511. https://doi.org/10.1016/j.chroma.2020.461511.
689 [27] S. Hage, G.E. Morlock, Bioprofiling of Salicaceae bud extracts through high-
690 performance thin-layer chromatography hyphenated to biochemical, microbiological and
691 chemical detections, J. Chromatogr. A 1490 (2017) 201–211.
692 https://doi.org/10.1016/j.chroma.2017.02.019.
693 [28] T. Schreiner, D. Sauter, M. Friz, J. Heil, G.E. Morlock, Is Our Natural Food Our
694 Homeostasis? Array of a Thousand Effect-Directed Profiles of 68 Herbs and Spices,
695 Front. Pharmacol. 12 (2021) 755941. https://doi.org/10.3389/fphar.2021.755941.
696 [29] A.J. Bergmann, E. Simon, A. Schifferli, A. Schönborn, E.L.M. Vermeirssen, Estrogenic
697 activity of food contact materials-evaluation of 20 chemicals using a yeast estrogen
698 screen on HPTLC or 96-well plates, Anal. Bioanal. Chem. 412 (2020) 4527–4536.
699 https://doi.org/10.1007/s00216-020-02701-w.
700 [30] T. Schreiner, A. Ronzheimer, M. Friz, G.E. Morlock, Multiplex planar bioassay with
701 reduced diffusion on normal phase, identifying androgens, verified antiandrogens and
702 synergists in botanicals via 12D hyphenation, Food Chem. 395 (2022) 133610.
703 https://doi.org/10.1016/j.foodchem.2022.133610.
704 [31] D.P. McDonnell, Z. Nawaz, C. Densmore, N.L. Weigel, T.A. Pham, J.H. Clark, B.W.
705 O'Malley, High level expression of biologically active estrogen receptor in

706 Saccharomyces cerevisiae, The Journal of Steroid Biochemistry and Molecular Biology
707 39 (1991) 291–297. https://doi.org/10.1016/0960-0760(91)90038-7.
708 [32] D.P. McDonnell, Z. Nawaz, B.W. O'Malley, In situ distinction between steroid receptor
709 binding and transactivation at a target gene, Mol. Cell. Biol. 11 (1991) 4350–4355.
710 https://doi.org/10.1128/MCB.11.9.4350.
711 [33] E. Debon, P. Rogeboz, H. Latado, G.E. Morlock, D. Meyer, C. Cottet-Fontannaz, G.
712 Scholz, B. Schilter, M. Marin-Kuan, Incorporation of Metabolic Activation in the HPTLC-
713 SOS-Umu-C Bioassay to Detect Low Levels of Genotoxic Chemicals in Food Contact
714 Materials, Toxics 10 (2022). https://doi.org/10.3390/toxics10090501.
715 [34] AAT Bioquest, IC50 Calculator, 2022. https://www.aatbio.com/tools/ic50-calculator.
716 [35] European Medicines Agency, ICH Topic Q 2 (R1) Validation of Analytical Procedures:
717 Text and Methodology, 1995. https://www.ema.europa.eu/en/documents/scientific-
718 guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf
719 (accessed 13 January 2023).
720 [36] J. Veyrand, M. Marin-Kuan, C. Bezencon, N. Frank, V. Guérin, S. Koster, H. Latado, J.
721 Mollergues, A. Patin, D. Piguet, P. Serrant, J. Varela, B. Schilter, Integrating bioassays
722 and analytical chemistry as an improved approach to support safety assessment of food
723 contact materials, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.
724 34 (2017) 1807–1816. https://doi.org/10.1080/19440049.2017.1358466.
725 [37] H. Thor, M.T. Smith, P. Hartzell, G. Bellomo, S.A. Jewell, S. Orrenius, The metabolism
726 of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the
727 implications of oxidative stress in intact cells, Journal of Biological Chemistry 257 (1982)
728 12419–12425. https://doi.org/10.1016/S0021-9258(18)33730-X.
729 [38] D. Xu, M.-J. Hu, Y.-Q. Wang, Y.-L. Cui, Antioxidant Activities of Quercetin and Its
730 Complexes for Medicinal Application, Molecules 24 (2019).
731 https://doi.org/10.3390/molecules24061123.
732 [39] E.M. Hill, K.L. Evans, J. Horwood, P. Rostkowski, F.O. Oladapo, R. Gibson, J.A. Shears,
733 C.R. Tyler, Profiles and some initial identifications of (anti)androgenic compounds in fish

734 exposed to wastewater treatment works effluents, Environ. Sci. Technol. 44 (2010)
735 1137–1143. https://doi.org/10.1021/es901837n.
736 [40] R. Urbatzka, A. van Cauwenberge, S. Maggioni, L. Viganò, A. Mandich, E. Benfenati, I.
737 Lutz, W. Kloas, Androgenic and antiandrogenic activities in water and sediment samples
738 from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses,
739 Chemosphere 67 (2007) 1080–1087.
740 https://doi.org/10.1016/j.chemosphere.2006.11.041.
741

Planar 6-fold multiplex bioassay to differentiate endocrine agonist, antagonist, false positive antagonist, cytotoxin, anti-cytotoxin, and false positive anti-cytotoxin

Daniel Meyer[a,#], Maricel Marin-Kuan[b], Hélia Latado[b], Benoît Schilter[b] and Gertrud E. Morlock[a,#,*]

[a]Institute of Nutritional Science, Chair of Food Science, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

[b]Chemical Food Safety Group, Société des Produits Nestlé SA - Nestlé Research, Vers-chez-les-Blanc, Switzerland

[#]Authors contributed equally.

*Corresponding author. Tel.: +49 641 9939141, fax: +49 641 9939149, E-mail address: Gertrud.Morlock@uni-giessen.de (G.E. Morlock)

Table S1 Calculation of costs and time for the 6-fold multiplex bioassay analysis

Materials	**Costs [€]**	**Step**	**Time [min]**
HPTLC plate	8	Sample application	25
Solvents	3	Development and substance fixation	30
Cell suspension	1	Preparation cell suspension	20
Substrate solutions	12	Cell application/incubation	250
		Plate drying	4
Total costs per plate	**24**	Substrate application/incubation	25
		Plate drying	4
		Documentation	2
		Total time for 1 plate	**6 h**

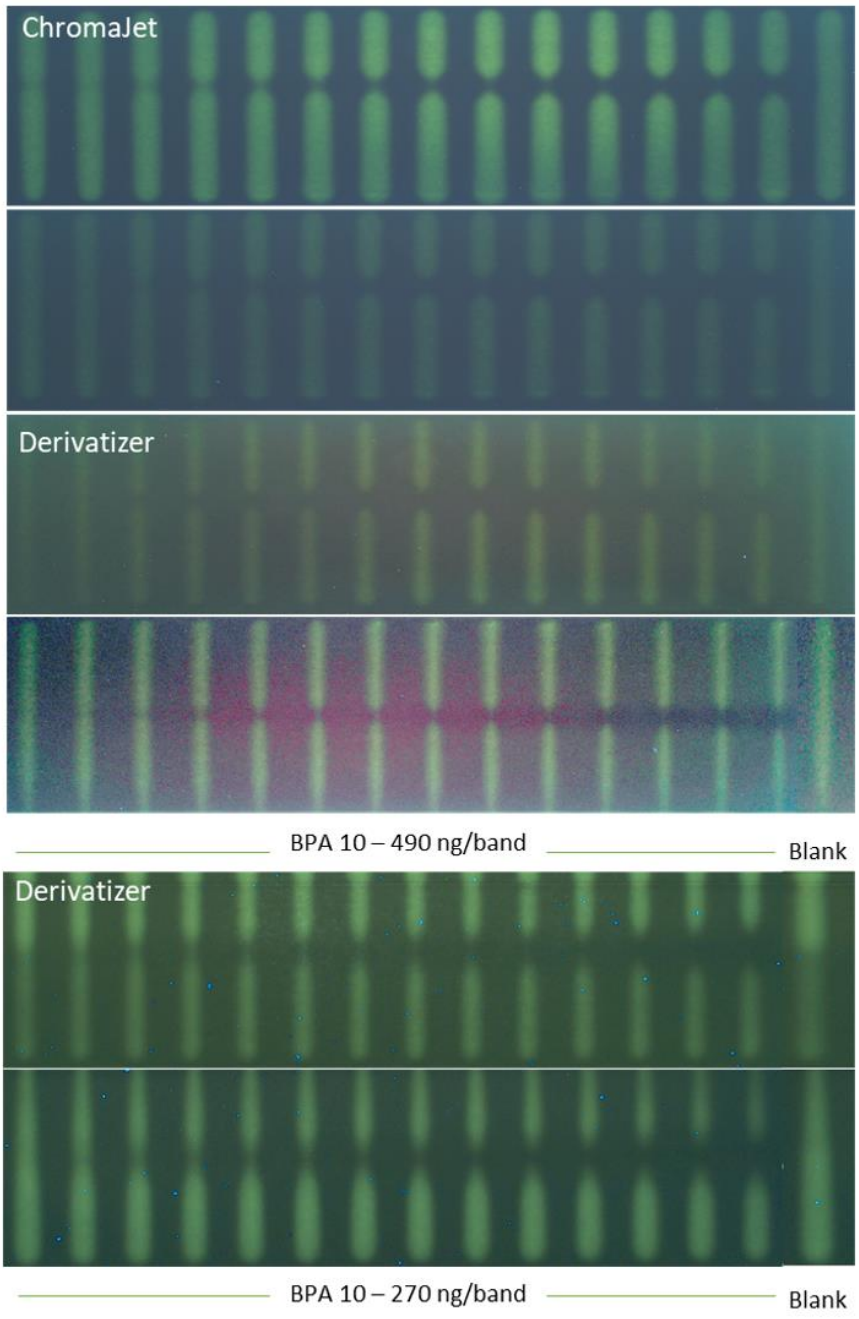


Figure S1 Validation of the HPTLC−pYAAS bioassay−FLD method: Six bioautograms at FLD 254 nm with different amounts of bisphenol A (BPA, 10–490 ng/band and 10–270) and a solvent blank separated on HPTLC plate silica gel 60 with toluene – ethyl acetate 6:1; a testosterone stripe (4 µL) was applied for antagonist detection via the bioassay using either the Derivatizer or ChromaJet device for substrate application as well as corresponding overall dose-response curve.

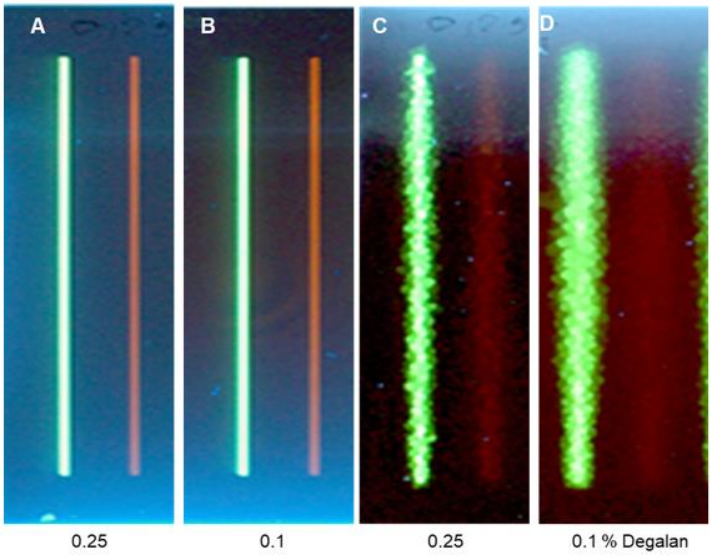


Figure S2 Influence of Degalan on the diffusion: Example image at FLD 254 nm of fluorescein and resorufin, each applied as 400 ng per stripe of 80 mm on the HPTLC plate silica gel 60. One stripe pair each was horizontally immersed into (**A**) 0.25% and (**B**) 0.1% Degalan solution (in *n*-hexane). After plate drying (solvent evaporation for 5 min), the YAS medium was sprayed onto the plates (red nozzle, level 6, Derivatizer) and the plates were incubated at 37 °C for 4 h in a humid box. After drying in a cold stream of air for 4 min, less diffusion was observed for both stripes when immersed into the (**C**) 0.25% than (**D**) 0.1% Degalan solution.

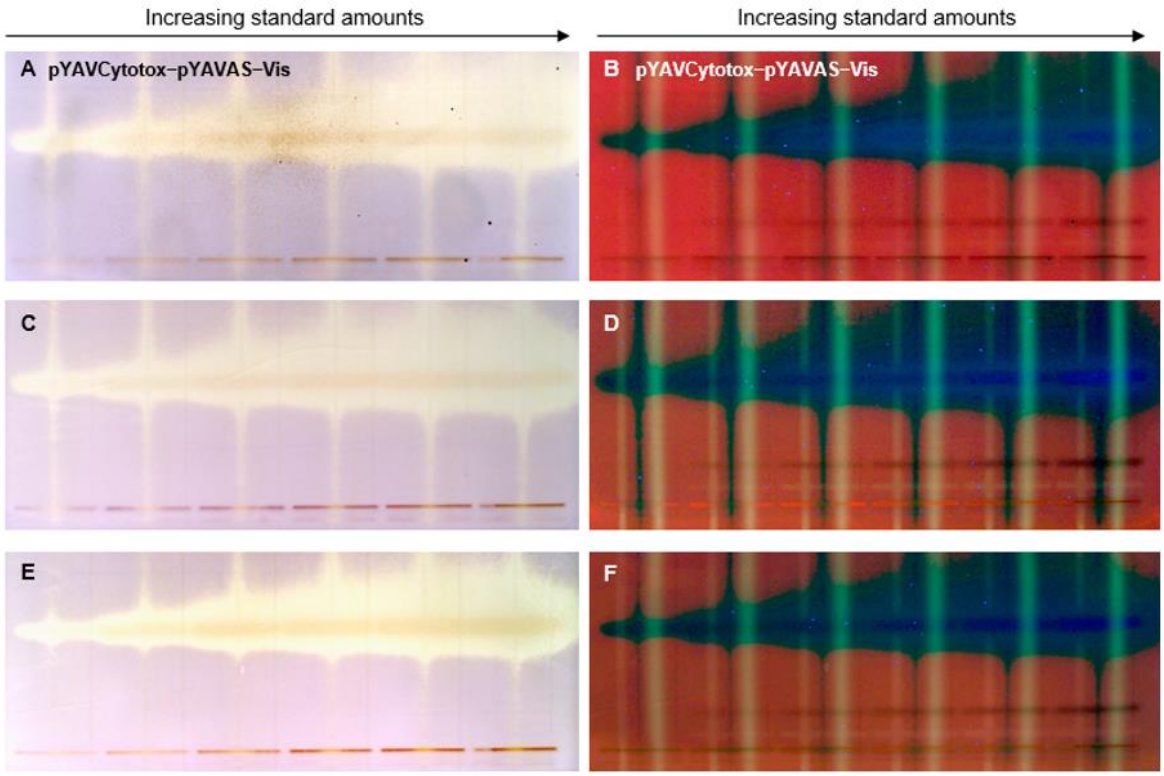


Figure S3 Bioautograms at white light illumination (Vis; **A**/**C**/**E**) and at 254 nm (FLD; **B**/**D**/**F**) of the positive control standards cytotoxin K3 (30–900 ng/band, 0.3–9 µL, 100 ng/µL), anti-cytotoxic acting quercetin (700–1700 ng/band, 0.7–1.7 µL), anti-androgen flutamide (100–2000 ng/band, 0.1–2.0 µL), and androgen DHT (100–1100 pg/band, 1.0–11 µL) applied (oversprayed on the same start zone) and separated as in Figure 1; four stripes (testosterone 4 µL, K3 3.2 µL, fluorescein 2 µL and resorufin 2 µL) applied on each separated track, followed by the bioassay performance.

Summary

Consumers, policy makers and industry need more powerful technologies for the detection of genotoxins and endocrine disruptors in complex matrices. Existing methods do not allow the analysis of unknown sample compositions or are based on effect-directed analysis with microorganisms, but this can give false negative results due to cytotoxicity or physico-chemical quenching effects and is not sensitive enough. For the first time, an HPTLC–(S9)-SOS-Umu-C bioassay was developed that also takes into account the metabolic activation of non-genotoxic substances. It was shown that the new bioassay has never reached detection limits. For packaging material migrates, HPTLC was shown to separate genotoxins from the cytotoxic matrix, making them detectable, which was not possible with the SOS-Umu-C microplate bioassay. Genotoxic linolenic acid epoxides were identified by coupling with HRMS. Analysis of several healthy vegetable oils for which the same fatty acid was shown to be a source of genotoxicity, among other genotoxins, showed that air exclusion can reduce genotoxin formation. The pYAES/pYAAS bioassays were used to detect endocrine disruptors in packaging material migrates. For detection enhancement, a 6-fold multiplex assay for simultaneous detection of agonists, antagonists, false positive antagonists, cytotoxins, anti-cytotoxins, and false positive anti-cytotoxins was developed and verified. A packaging agent migrate could be screened for endocrine disruptors and their disruptive properties could be verified in the same bioassay. The bioassays could also be used for risk assessment and authorization procedures under REACH and then improve product safety, as well as save time and costs.

Zusammenfassung

Verbraucher, politische Entscheidungsträger und die Industrie brauchen leistungsstärkere Technologien zum Nachweis von Gentoxinen und endokrinen Disruptoren in komplexen Matrices. Bestehende Methoden erlauben nicht die Analyse unbekannter Probenzusammensetzungen oder basieren auf der wirkungsorientierten Analyse mit Mikroorganismen, die jedoch aufgrund von Zytotoxizität oder physikalisch-chemischer Quencheffekte falsch negative Ergebnisse liefern kann und nicht empfindlich genug ist. Zum ersten Mal wurde ein HPTLC─(S9)-SOS-Umu-C-Bioassay entwickelt, der auch die metabolische Aktivierung von nicht gentoxischen Substanzen berücksichtigt. Gezeigt wurde, dass der neue Bioassay nie erreichte Detektionslimits hat. Für Verpackungsmittelmigrate wurde gezeigt, dass die HPTLC Genotoxine von der cytotoxischen Matrix trennt und somit nachweißbar macht, was mit dem SOS-Umu-C-Mikrotiterplatten-Bioassay nicht gelang. Durch Kopplung mit HRMS wurden genotoxische Linolensäureepoxide identifiziert. Die Analyse verschiedener gesunder Pflanzenöle, für die dieselbe Fettsäure neben anderen Genotoxinen als Quelle von Genotoxizität nachgewiesen wurde, zeigte, dass Luftausschluss die Bildung von Genotoxinen verringern kann. Mit den pYAES/pYAAS Bioassays wurden endokrine Disruptoren in Verpackungsmittelmigraten nachgewiesen. Zur Detektionsverbesserung wurde ein 6-facher Multiplexassay zum gleichzeitigen Nachweis von Agonisten, Antagonisten, falsch positiven Antagonisten, Zytotoxinen, Antizytotoxinen und falsch positiven Antizytotoxinen entwickelt und verifiziert. Ein Verpackungsmittelmigrat konnte auf endokrine Disruptoren untersucht und deren disruptive Eigenschaften im selben Bioassay verifiziert werden. Die Bioassays könnten auch für Risikobewertung und Zulassungsverfahren unter REACH verwendet werden und dann Produktsicherheiten verbessern, was auch Zeit und Kosten sparen würde.

Danksagung

Zuerst möchte ich mich bei Prof. Dr. Gertrud Morlock bedanken, die mir ermöglicht hat im Bereich der wirkungsbezogenen Analytik zu arbeiten. Danke auch für das zukunftsorientierte Promotionsthema und für all die Hilfestellung in den letzten Jahren und für die Begutachtung dieser Arbeit.

Für die Übernahme des Zweitgutachtens danke ich dem oder der zum Zeitpunkt des Drucks dieser Arbeit noch unbekannten Zweitprüfer oder Zweitprüferin, sowie Prof. em Dr. Wolfgang Schwack und Prof. Dr. Sylvia Schnell für die Drittprüfung.

Auch dem Team des Kooperationspartners Nestlé aus der Schweiz möchte ich danken und hier besonders Dr. Maricel Marin-Kuan für die vielen konstruktiven Gespräche. Dem Team von OFI aus Österreich danke ich für die Anfertigung der Assays und der Proben.

Bei den Professurangehörigen der Professur für Lebensmittelwissenschaften aus Gießen möchte ich mich für viele Jahre der Zusammenarbeit und der Arbeitsteilung bei Routineaufgaben in den Laboren bedanken. Danke auch an Simone Cutts für die Bearbeitung aller Formalien und auch an Julia Heil für die Hilfe bei den Bioassays.
Ulf Wöhrmann, René Vogt, Katharina Weber, Christopher Look, Jasmin Bahmer, Julia Küllmer, Marilena Semmler, Sophie Arnold und Sophia Hörnlein möchte ich für ihre Unterstützung im Labor während ihrer Bachelor- und Masterarbeiten danken.

Natürlich möchte ich mich auch bei meiner Familie bedanken, die mich in den letzten Jahren und auch von Zwischenstation zu Zwischenstation bis hierher unterstützt hat.