TECHNISCHE UNIVERSITÄT WIEN
Vienna | Austria

Dissertation

Determination of Genotoxic Effects of Food Packaging Materials with *in vitro* Bioassays

carried out for the purpose of obtaining the degree of Doctor *rerum naturalium* (Dr. rer. nat.), submitted at TU Wien, Institute of Chemical, Environmental and Bioscience Engineering, by Bernhard Rainer, MSc

Bernhard Rainer, MSc

Mat.Nr.: 11713964

under the supervision of

Univ. Doz. Mag. Dr. Manfred Tacker

Institute of Chemical, Environmental and Bioscience Engineering

Vienna, January 2022

This work was supported by FFG, the Austrian Research Promotion Agency within the framework of the project MIGRATOX and PolyCycle.

I confirm, that going to press of this thesis needs the confirmation of the examination committee.

Affidavit

I declare in lieu of oath, that I wrote this thesis and performed the associated research myself, using only literature cited in this volume. If text passages from sources are used literally, they are marked as such.

I confirm that this work is original and has not been submitted elsewhere for any examination, nor is it currently under consideration for a thesis elsewhere

City and Date *Signature*

Acknowledgements

First of all, I would like to thank my PhD supervisor Manfred Tacker for guiding me through the process of writing this thesis. Especially in the early stages of my doctoral studies, I have greatly appreciated your constant motivation and suggestions.

Next, I must mention my colleagues at FH Campus Wien who have supported me the most during the last five years. Dear Elisabeth, Verena, Lukas and Silvia, without your help and support this thesis would not have been possible. Also a big thank you to the rest of the team, it has always been a pleasure to be part of the Packaging and Resource Management Department.

Not to forget all the students who had to do their bachelor and master theses under my supervision and helped me with the experimental work in the lab. Dear Miriam, Irene, Martina, Tobias, Isabella 1&2, Xaver, Chiara, Markus, Eva and all those I forgot to mention, if we ever meet again in real life, the first drink is always on me!

In addition, the OFI team, including Christian and Elisa, helped me a lot and always gave valuable suggestions and support to advance our joint research projects.

Last but not least, I would like to thank my family, my parents, but also my grandparents who are no longer around, and my girlfriend Birgit.

Honorable mentions go to the Victoria and Simon, as it is their fault that I started this work in the first place.

Table of Contents

Abstract

The safety assessment of food packaging materials presents an ongoing challenge for the packaging and food industry. In particular, non-intentional added substances are a major issue, because their chemical and toxicological properties are unknown and they might be of concern for the health of consumers. Substances with genotoxic properties are the most problematic, since they can cause adverse health effects in extremely low concentration, when chronically ingested. The current state of the art for the safety assessment of packaging materials is to conduct migration experiments, followed up by analytical chemistry. These techniques are however not suitable to detect trace amounts of mutagenic substances, so there is an urgent research need to find a new and more sensitive approach.

For this purpose, the present doctoral thesis focuses on the application of *in vitro* bioassays for the safety assessment of food contact material migrate samples, with a particular focus on the bacterial reverse mutation assay, or Ames test. The thesis consists of three peer-reviewed papers that tackle several important questions, on how this type of assay should be applied. In paper number one it is concluded, based on a literature review, that the Ames test can be a highly valuable tool for food contact material safety assessment, even though the limits of detection are not low enough to fully cover current regulatory requirements. In paper number two, a combination of a standardized sample preparation approach with a specifically optimized Ames MPF protocol is applied for several real-world packaging samples and the results are presented and discussed. The third paper tackles the question what type of Ames test format should be applied for the safety assessment of samples that contain very low concentrations of genotoxic substances.

Overall, it is concluded that the application of the Ames test, in particular the Ames MPF protocol, offers a major improvement over the current state of the art. Even though chemical analysis based on chromatography and mass spectrometry can hardly detect any genotoxic contaminations, the application of in vitro assays offers the possibility to consistently detect the effects of low concentrations of genotoxins in food contact material migrates. However, to fully comply with current regulatory requirements, based on the threshold of toxicological concern, further research has to be conducted to allow for even lower limits of detection.

Kurzfassung

Die Sicherheitsbewertung von Lebensmittelverpackungsmaterialien ist eine Herausforderung für die Verpackungs- und Lebensmittelindustrie. Insbesondere die nicht absichtlich zugefügten Stoffe stellen ein Problem dar, weil ihre chemischen und toxikologischen Eigenschaften unbekannt sind und sie für die Gesundheit der Verbraucher bedenklich sein könnten. Genotoxische Substanzen sind am problematischsten, da sie bei chronischer Aufnahme bereits in extrem niedrigen Konzentrationen gesundheitsschädliche Auswirkungen haben können. Der derzeitige Stand der Technik für die Sicherheitsbewertung von Verpackungsmaterialien sind Migrationsstudien in Kombination mit chemischer Analytik. Diese Techniken sind jedoch nicht geeignet, um Spuren von mutagenen Stoffen nachzuweisen, so dass ein dringender Forschungsbedarf besteht, einen neuen und empfindlicheren Ansatz zu entwickeln.

Zu diesem Zweck befasst sich die vorliegende Doktorarbeit mit der Anwendung von *in vitro* Bioassays für die Sicherheitsbewertung von Migratproben aus Materialien, die mit Lebensmitteln in Berührung kommen. Ein besonderer Schwerpunkt liegt dabei auf dem bakteriellen Rückmutationsassay oder Ames-Test. Die Dissertation besteht aus drei, in wissenschaftlichen Journalen publizierten, Arbeiten, die sich mit mehreren wichtigen Fragen zur Anwendung dieser Art von Test befassen. Im ersten Beitrag wird auf der Grundlage einer Literaturrecherche der Schluss gezogen, dass der Ames-Test ein äußerst wertvolles Instrument für die Sicherheitsbewertung von Materialien, die mit Lebensmitteln in Berührung kommen, sein kann, auch wenn die Nachweisgrenzen nicht niedrig genug sind, um die derzeitigen gesetzlichen Anforderungen vollständig zu erfüllen. Im zweiten Beitrag wird eine Kombination aus einem standardisierten Probenvorbereitungsansatz und einem speziell optimierten Ames MPF Protokoll mit mehreren Verpackungsproben angewandt und die Ergebnisse werden vorgestellt und diskutiert. Der dritte Beitrag befasst sich mit der Frage, welche Art von Ames Testformat für die Sicherheitsbewertung von Proben angewendet werden sollte, die sehr geringe Konzentrationen genotoxischer Substanzen enthalten. Zusammenfassend wird festgestellt, dass die Anwendung des Ames-Tests, insbesondere des Ames MPF Protokolls, eine wesentliche Verbesserung gegenüber dem derzeitigen Stand der Technik darstellt. Auch wenn chemische Analysen auf der Grundlage von Chromatographie und Massenspektrometrie kaum genotoxische Verunreinigungen nachweisen können, bietet die Anwendung von *in vitro* Tests die Möglichkeit, die Auswirkungen niedriger Konzentrationen von Genotoxinen in Lebensmittelkontaktmaterialien durchgängig nachzuweisen. Um jedoch die aktuellen gesetzlichen Anforderungen, die auf dem sog. Threshold of Toxicological Concern-Konzept beruhen, vollständig zu erfüllen, müssen weitere Forschungsarbeiten durchgeführt werden, um noch niedrigere Nachweisgrenzen zu ermöglichen.

Published Articles

This cumulative doctoral thesis consists of the following articles, published in peer-reviewed journals:

Paper number one:

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 migrate. Food Addit. Contam. Part A. 2018, 1–14.

I have provided the following contributions to this paper:

- Conceptualization
- Investigation
- Writing – original draft
- Visualization

Paper number two:

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 2019, 36, 1419–1432.

I have provided the following contributions to this paper:

- Conceptualization
- Experimental work (Ames test, chemical analysis was performed by OFI) and Investigation
- Writing – original draft
- Visualization

Paper number three

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.

I have provided the following contributions to this paper:

- Conceptualization and Methodology
- Investigation and Experimental work
- Writing – original draft
- Visualization

List of Figures

List of Tables

List of Abbreviations

BaP	benzo[a]pyren
2,4-DAT	2,4-diaminotoluene
DMSO	dimethyl sulfoxide
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
ENU	N-ethyl-N-nitrosourea
FCM	food contact material
IAS	intentionally added substances
ILSI	International Life Science Institute
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IQ	2-Amino-3-methyl-3H-imidazo[4,5-F]quinoline
LEC	lowest effect concentration
LOD	limit of detection
NIAS	non-intentionally added substances
TTC	threshold of toxicological concern
OECD	organisation for Economic Co-operation and Development
OFI	Österreichisches Forschungsinstitut für Chemie und Technik
WHO	world health organisation

1. Introduction

1.1. Safety of Food Contact Materials

The safety of food contact materials (FCMs) is a global issue for packaging- and food manufacturers. Over recent decades, concerns have been raised, whether chemicals that threaten the health of consumers, could leech from packaging and contaminate the contained foodstuff – a process that is referred to as migration. Previous publications, which found the migration of hormone active substances started a big debate among the public, the industry and regulatory authorities on whether our food is safe from contamination with toxic chemicals [1,2]. However, since packaging is essential for our supply chains and to protect food from outside contaminations, as well as to reduce potential food loss/waste, methods must be developed to allow for a comprehensive safety testing of packaging materials. For hormone active substances, there are already sufficient methods available [3,4], on the other hand the critical endpoint of genotoxicity/mutagenicity was shown to be of even higher concern [5–7].

1.1.1. Current Regulatory Requirements

The current EU regulation No. 1935/2004 states under article three that, under no normal or anticipated conditions the FCM may release substances into the packaged good that could threaten the human health or change the food in a way that is unacceptable for the consumer, which includes organoleptic properties [8]. While this regulation does not provide any specific details on how to assess the safety of FCM, or defines what could be accepted, as reasonably safe, more specific regulations exist both on European and on national levels. Currently, the most important piece of legislation on more specific aspects it the Commission Regulation EU 10/2011 on plastic materials and articles intended to come into contact with food [9]. It provides a general guideline on how to approach FCM safety testing and defines specific technical requirements. These include conditions for compliance testing in the form of migration with food simulants, limits for overall migration, as well as migration limits for individual substances. While this regulation was designed specifically with plastic products in mind, it also serves as a basis for safety testing of other types of packaging materials, such as coatings or paperboard, since they are not specifically covered by other EU regulations.
It is of high importance that the EU commission states that substances that fall under the categories mutagenic, carcinogenic or toxic for reproduction must not be present in FCM. A

limit of detection of 0.01 mg/kg should be achieved by analytical methods that are applied for compliance tests. As will be outlined in the following chapters, there are still major technical challenges that must be overcome, to allow FCM producers to fully comply with these specific requirements.

1.1.2. Non-Intentionally Added Substances

One of issue is the presence of non-intentionally added substances (NIAS), which are the sum of all constituents of a FCM that are present in the final product, but not accounted for by the producers. NIAS can be formed during the production process as side/degradation products, or can be contaminations that occur during the storage or shelf life of the product [10]. In addition, not all manufacturers state all details about the recipe of their products, so some of the information on the chemical composition of a product can be lost along the supply chain. The most frequent methods that are applied for chemical analysis of FCM migrate samples are GC or LC-MS based, however their use is limited since these measurements frequently result in a so-called “forest of peaks”, many of which cannot be clearly identified or related to a specific structural group of chemicals [11–13].

Yet, to fully comply with current regulations a complete identification and characterisation of all substances would be necessary, a process which is not feasible due to time and resource constraints. Even if all substances could be fully identified, their safety must be assessed on an individual basis by comparing them to toxicological data, which is frequently unavailable. *In vivo* testing with feeding studies could be an alternative, but both ethical and practical concerns, such as producing enough migrate for conducting such studies, makes them an undesirable solution [7]. A tiered approach that helps with addressing the issue of unknown toxicants, such as NIAS, will be outlined in the next chapter.

1.1.3. Threshold of Toxicological Concern and Genotoxicity

The concept of the threshold of toxicological concern (TTC) is a toxicological tool for the risk assessment of substances, for which no toxicological information is available [14,15]. Based on statistical evaluation of thousands of chemicals, thresholds were derived, below of which the risk for adverse health effects is acceptably low. If no information at all is available for a substance, a worst-case assumption has to be taken: the unknown substance is DNA-reactive and could be carcinogenic. For this critical substance group the TTC is very low, but if the daily exposure is less than 0.0025 µg/kg bodyweight, than even the risk of DNA-reactive carcinogens can be regarded as acceptable according to EFSA and WHO [14].

Currently, for the risk assessment of unidentified substances migrating from food contact materials, this highly conservative threshold should be applied, because it cannot be completely ruled out that a substance could be a DNA-reactive carcinogen. However, a recent report by EFSA and WHO confirms [14], that a 600-times higher toxicological threshold (the so-called Cramer Class III) could be applied, if the mixture contains no direct DNA-reactive carcinogens. Other substances, even genotoxic chemicals that are not directly DNA-reactive, are considered an acceptable risk up to to 90 µg/person/day (derived from 0.15 µg/person/day for a person with 60 kg b.w.). For most of the other exclusion classes, like heavy metals, dioxins or polyhalogenated biphenyls, there are reliable and sensitive chemical analysis methods established [11]. Other exclusion groups such as steroids can be considered as a negligible risk due to very low likelihood of occurrence in food contact materials. The following chapter will present a brief overview of *in vitro* bioassays and their potential to cover the risk of genotoxic substances.

1.2. In Vitro Bioassays

1.2.1. In vitro Bioassays for Genotoxicity

A potential solution for the problems of conventional analytical methods, is the application of *in vitro* bioassays as complementary methods. This type of assays applies living cells, that interact with target substances and yield a measureable signal, which allows for an effect-based detection of adverse outcomes. Their use is often times an important part of international guidelines for safety testing of chemicals, food additives or pharmaceutical products [16–19]. Various mechanisms of action cause genetic damage. This includes direct interaction on a DNA-level (e.g. point mutations), chromosomal damage (e.g. clastogenicity) or indirect effects, such as damage to the process of DNA synthesis [20]. For this reason, a wide range of different types of in vitro assays has been developed over the last decades to cover all relevant mechanisms. These includes a large variety of cell culture-based assays, such as the micronucleus assay [21], chromosomal aberration assays [17] or comet assay [22]. In vitro reporter gene assays, such as the p53-Calux or the Bluescreen assay are a more novel development, but also cover a wide range of endpoints [23,24]. In addition, a range of bacteria-based genotoxicity assays are available, such as the UMUc test [25], the Rec-assay [26] or, most importantly, the Ames test [27–29]. In general, these types of *in vitro* bioassay have the advantage that they allow for an effect-based detection, without the need to know the exact chemical properties or structures of the test item. This makes them well-suited for the

assessment of unknown complex mixtures [30], or for the exclusion of critical substance groups according to the TTC concept [7,10,14]. Nevertheless, these assays come with certain disadvantages: a.) No in vitro assay can cover all potential mechanisms of action, which can cause genetic damage. b.) Performing a large test battery is expensive and time consuming. c.) While being applicable for the assessment of complex mixtures, they were mostly developed for testing individual chemicals in the scope of regulatory studies according to industry guidelines.

The most commonly used test that is included in all international guidelines for genotoxicity studies, is the before-mentioned Ames test [18]. While its use is most frequently recommended in conjunction with at least one additional mammalian cell culture-based assay, it was found that for the specific application of packaging safety testing, it could be applied on its own [7]. In addition, it is stated in the ICH M7 guideline (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use), that for the detection of genotoxic impurities in low concentration, the Ames test can be applied as a standalone method [16]. Paper number one, which was submitted as part of the present doctoral thesis, "Suitability of the Ames test to characterise genotoxicity of food contact material migrates", [31] discusses this issue in more detail.

1.2.2. Sample preparation

The successful application of in vitro bioassays requires proper sample preparation methods. This process is particularly challenging for genotoxicity studies, since the target substances consist of a wide range of chemicals with various physiochemical properties [7,32].

The first step for the preparation of packaging migrate samples is the migration or extraction process. In the EU guideline 10/2011, applicable testing conditions are outlined. This includes appropriate temperature, contact time and solvent that should be applied. This is followed by a concentration step, to allow for an easier detection of genotoxic substances, that generally occur in very low concentrations [7,32]. This step is of high importance, since the LECs (lowest effect concentrations) of *in vitro* bioassays are a limiting factor. Afterwards, a solvent exchange is necessary, to transfer the target substances into a bio-compatible vehicle, such as DMSO (dimethyl sulfoxide). During all of these steps, it is important to keep potential substances losses to a minimum.

This topic is addressed in paper number two: "Mutagenicity assessment of food contact material migrates with the Ames MPF assay", where a suitable approach is introduced and validated with chemical analytical methods [32].

1.2.3. Limits of Detections of *in vitro* Bioassays

As already outlined in a previous chapter about the TTC concept, the LODs (limits of detection) that should be achieved with methods that are used for genotoxicity tests of packaging materials are very low (0.15 µg/L [15]). This a major challenge, not only for analytical chemistry, but also for *in vitro* bioassays. Most commonly, these assays are applied for regulatory studies of pure substances, such as industrial chemical or pharmaceuticals, where the LOD is not a real concern, since the substance is available in relatively large quantities [22]. This however changes completely, when low levels of genotoxic impurities must be detected in complex mixtures.

When discussing the capability of an *in vitro* test to detect a substance in low concentrations, most commonly the so-called LEC is applied. This term refers to the lowest concentration of a substance that can be picked up by the assay, when compared to an appropriate negative control sample. The concentration can then be roughly translated into a corresponding limit of bio detection (LOBD) [7,31]. However to figure out the overall performance of the method, two more factors have to be included: 1.) Living cells cannot be applied incubated with pure samples material, since this type of exposure would be lethal for them. Instead, a dilution in nutrient media is necessary, which indirectly increases the LOD. 2.) As already mentioned in the previous chapter, a concentration step should be included. This step is critical to reduce the overall LOD of the *in vitro* test method.

Another major factor, that was rarely discussed in previous publications [5,6], is the fact that packaging material migrates can contain chemicals that are toxic for bacteria or mammalian cells. This leads to false negative results, since a dead organism does not yield any response in an *in vitro* test. To address this challenge, methods had to be developed to detect and quantify toxic matrix effects and to test whether packaging material migrates are toxic or inhibiting for bacterial/cellular growth.

The issues that are outlined in this chapter are addressed in all three publications that are part of this thesis, since they are the core challenge that have to be overcome to establish *in vitro* bioassays for FCM safety assessment.

1.2.4. Project MIGRATOX and PolyCycle

The issues that are outlined in the introduction are within the scope of two FFG-funded research projects (Austrian Research Promotion Agency). The project funding has directly contributed to the creation of the present thesis.

The first project **FFG Migratox** (grant number: 866854), addresses the challenge of testing FCM for genotoxic NIAS with *in vitro* bioassays. It took place from 06/2018 until (expected) 06/2023 and was submitted in the framework of the 7th "Coin Aufbau" grant with an overall project volume of approximately 1.6 mio. €. It is carried out with research partners at OFI Vienna (Österreichisches Forschungsinstitut für Chemie und Technik), as well as a large consortium of industry partners, from the food and packaging sector. The research that was performed for this thesis was an integral part for establishing the relevant methods over the first four project years. At the point of writing this thesis, the project is currently in its fourth year and the current focus is on proper validation of the *in vitro* methods with ring trials, as well as on large-scale sample screenings.

The second project **FFG/CORNET PolyCycle** (grant number: 874379), focuses on the development of methods for the safety assessment of recycled plastics from post-consumer waste. It took place from 01/2020 until (expected) 06/2022 and was submitted in the framework of the CORNET cooperation projects with an overall volume of approximately 0.5 mio. € for the Austrian research partners. It is carried out with research partners from the IVV Fraunhofer in Freising and OFI Vienna, as well as a large consortium of industry partners, mostly from the recycling sector. The research that was performed for this thesis laid the groundwork for the application of the Ames test for the safety assessment of recycled plastics.

2. Aims and Structure

The overall goal of the present thesis is to develop a safety assessment method for packaging migrate samples that is based on the Ames test. For this purpose, three articles were published in peer-reviewed journals:

Paper number one: As an initial step, a **literature review** conducted, to gain a better understanding of the potential of the Ames test for FCM safety testing applications. For this paper multiple critical points of the test system such as available formats, limits of detection and previous research that was conducted with the assay on packaging migrates, were addressed. A summary of the most important findings is presented in chapter 4.1 and the full text is available in chapter 7.

Paper number two: The next steps, was to **develop a methods** than can be applied for real world samples and that yields reliable and reproducible results. It was not only necessary to develop and optimise the test procedure to detect toxic matrix effects, but also to improve and validate the sample preparation methods. A small subset of genotoxic standard substances was tested to get a better understanding of the detection limits that can be obtained with the Ames MPF assays. Finally, the second paper presents the test results of several real world packaging samples, including some positive results for genotoxicity. The paper is briefly summarized in chapter 4.2 and the full text can be found in chapter 7.

Paper number three: The last part of this thesis was to test whether the Ames MPF assay is as suitable for the application in the field of packaging safety assessment, as the standard agar-based Ames test. To address this issue, a **comparison study** was conducted, that compared the LECs of both assays for a set of standard test substances. The paper is summarized in chapter 4.3 and the full text is displayed in chapter 8.

This leads to the following **main research questions** for this thesis:

1.) Is the Ames test in its current form applicable for the safety testing of FCM for genotoxicity?

2.) Can the Ames test detect genotoxicity in real world packaging samples?

3.) What type of Ames test format should be selected for FCM sample screenings?

3. Methods

3.1. Test principle of the Ames test

The following chapter will describe the principle of the Ames test system, as well as the most important aspects that have to be taken into account for FCM migrate samples. Exact details, such as volumes and concentrations that were applied for the individual experiments, are listed in the publications and will not be outlined in this chapter.

3.1.1. Test principle

The Ames test allows for the detection of substances that interact directly with the DNA and cause changes to its structure. This is critical, because permanent alterations of the genetic material, which are called mutations, can cause issues with fertility or even cancer in humans. Bruces Ames and colleagues developed the basic test procedure in the early 1970s. Over the decades, large corporations and international institutions acknowledged the value of the test system. At present, the test is applied on a global scale for regulatory studies, because it is capable of predicting rodent carcinogenicity and is comparatively cheap and fast [33].
For the Ames test, bacteria that are unable to synthesize and essential amino acid, such as histidine, are applied. This is caused by a mutation that leads to auxotrophy, making them unable to grown in media that do not contain the essential nutrient. However, when a mutation occurs at the site of the pre-existing DNA-damage, the function of the gene can be restored. This allows the organism to regain the ability of synthesizing the essential and grow on deficient media, which leads to the formation of visible colonies [22].
Multiple *Salmonella* strains were developed, which carry mutations in genes in the histidine operon. Each of the strains is responsive to mutagenic substances that act with a different mechanism. In addition, these bacteria were altered to make them more sensitive to DNA-reactive substances [29]. These alterations include repair deficiencies, via deletion of the uvrB repair system, which prevents an error-free nucleotide repair. Additionally the rfa mutation leads to a higher permeability of the cell membrane for larger molecules. Finally, plasmids such as pKM101 can be introduced, that increases the chance of errors in the DNA repair process, by coding for the umuD polymerases, that is normally absent in *Salmonella* [33].

3.1.2. Agar-based Ames Test

The earliest version of the Ames test followed the so-called plate incorporation protocol. For this approach, the sample and the tester strains are mix with the agar and plated directly without an initial exposure phase [27]. Over the years, a more sensitive test protocol was developed, the so-called preincubation test. It is considered an improvement over the standard plate incorporation protocol, in particular with regards to the detection of positive substances in presence of an external metabolization system (see chapter 3.1.6). The agar-based preincubation protocol is still the most commonly applied variant for regulatory studies and is well known and accepted all around the world [22].

The test procedure for the pre-incubation test is the following [22]:

- An overnight culture with the individual tester strains (e.g. *Salmonella* TA98) is prepared.
- Histidine-deficient base agar plates are prepared by pouring the nutrient media in standard 90 mm petri dishes.
- A semi-solid top agar is prepared, which contains small quantities of histidine, to allow for a few cell divisions of the auxotrophic bacteria.
- The samples (either pure test substances or complex mixtures) are prepared and diluted in DMSO, or any other biocompatible solvent.
- The overnight culture is mixed with phosphate buffer and the sample. All strains are tested individually and the S9 mix is added as needed.
- Bacteria are exposed to the test item in an orbital shaker at 37 °C for 20 to 90 minutes, depending on the specific protocol.
- A small quantity of the semi-solid top agar is added to the preincubation mix, briefly homogenized and immediately plated on bottom-agar plates.
- The solidified petri dishes are incubated at 37 °C for 48 to 72 hours.
- The plates are scored by manually counting the revertant colonies and comparing them to negative control plates.

For a regulatory Ames study, at least five tester strains (see 3.1.5) and the addition of an S9 protocol are required. All plates must be tested in (at least) triplicates and both a positive and a negative control must be included for each run. Any test item concentration that causes a significant increase in the revertant count, is determined to be mutagenic for bacteria. To proof that a substance is either negative or positive, a dilution series is prepared and a dose-dependent response should be observed. However, toxic effects, or precipitation of the test

item, can cause a non-linear response. The bacteria that grow in the background cause the agar to become cloudy, which is also known as the background lawn, an effect which can be used to detect toxic effects of the sample on the bacteria. If the hazy background is either fully or partly clear, then a toxic effect has occurred that could reduce the response of the bacteria to mutagenic effects.

3.1.3. Limitations of the Standard Test Protocol

While the agar-based Ames protocol is well established and yields reliable and reproducible results in a strict, quality controlled laboratory environment, it also comes with certain drawbacks, which are relevant specifically for FCM safety assessment.

- For one test sample at least 18 petri dishes are needed per. When factoring in at least two test strains, the presence/absence of S9, spike experiments to measure toxicity (see 3.1.8), as well as at least one repetition for each run, this results in a minimum of 288 petri dishes for one sample, not including any controls. This makes the test unsuitable for a high throughput sample screenings.
- The standard Ames test requires about 650 µL of sample material for one dilution series. When factoring in all the above-mentioned factors, this results in at least 10.4 mL of sample material, not including any dead volume or human error. The sample preparation process includes a concentration step by at least a factor of 300. This results in at least 3120 mL of migrate that must be prepared for each sample. Since the sample preparation process is time-consuming and expensive, this is neither practical, nor feasible.

The points that are mentioned above made it necessary to find a test system that lowers both the sample material requirements and allows for a faster screening.

3.1.4. Ames MPF Assay

The variant of the Ames test, which was applied for this thesis, is the so-called Ames MPF assay. It is currently sold by the company Xenometrix from Allschwil, Switzerland. The basic principle of the Ames test, that was outlined in chapter 3.1.1, still applies to this variation of the test system. The major change, when compared to the standard method, based on the OECD guideline, is that instead of agar-based nutrient media, liquid media are applied. Instead of plating petri dishes and scoring revertant colonies, which is both time and material consuming, 384-well plates are used and scored based on a colour shift. This effect occurs, because the indicator medium contains bromocresol purple, a substance that shifts from

purple to yellow when the pH drops due acidification caused by bacterial growth. Therefore, a yellow well represents a revertant colony (see Figure 1).

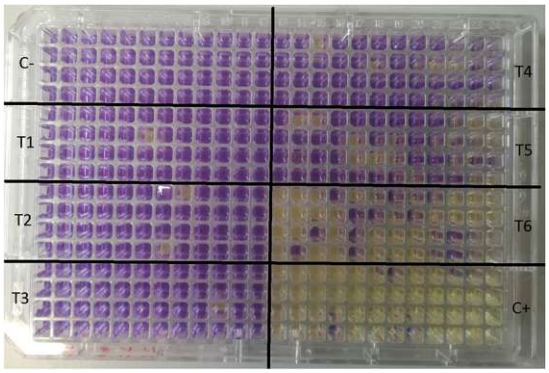


Figure 1: A 384-well test plate that is applied for the Ames MPF assay. The higher the ratio of yellow to purple wells in a zone on the plate, the higher the mutagenic activity.

While the test is very similar to the standard, agar-based protocol, it has several advantages, which makes its application for FCM safety assessment preferable from a practical standpoint.

1.) It requires five times less sample material than the standard Ames test, which leads to significantly reduced costs in the sample preparation process.
2.) The throughput of the liquid format, when automatic multichannel pipettes are used, is significantly higher than for the agar-based protocol. A single operator can process up to three times more samples in the same time.
3.) The scoring of the plates is much faster and they require less space in the incubator.
4.) As will be outlined in paper number three, the test has slightly better LECs than the standard agar-based test system.

Previous publications found that the test is has a concordance of about 90% with the standard Ames protocol [34,35]. While the Ames MPF assay has several advantages, there are also a few limitations that must be highlighted:

1.) At present, the Agar-based preincubation protocol is the international standard and alternative Ames formats might not be accepted for regulatory studies.
2.) The so-called dynamic range of the Assay is lower. Instead of scoring a petri dish, which can have >500 revertant colonies, the scoring of the Ames MPF assay is limited to 48 wells. This means that small changes in the revertant count, in particular in the negative control, can affect the result of the assay.
3.) Tester strains that have a higher background mutation rate are problematic in the Ames MPF assay. If the background mutation rate increases too much, the test can quickly become invalid. This makes pre-screenings of the bacterial cultures for background reversion rates necessary.

3.1.5. Strain Selection

As already mentioned earlier, for a full Ames study, multiple tester strains are required [18,21,33]. Over the last decades many such strains were developed, but the following are applied most frequently for genotoxicity studies: *Salmonella* TA98, TA100, TA1535, TA1537, TA102, TA97 and E. coli WP2 [33,36]. According to the current OECD guideline 471, a full Ames study needs to include at least five tester strains: 1.) TA 1535 2.) TA1537 or TA97 or TA97a 3.) TA98 4.) TA100 5.) *E.coli* WP2 or TA102. The choice of strains is based on their reversion sites in their DNA. While certain strains, such as TA98 detect frameshift mutagens, other strains, such as TA100 or TA1537 are sensitive for point mutations. E. coli WP2 and TA102 are different in a way, that allow for the detection of mutation in AT-rich DNA-regions (adenine & thymine), while most other strains have their primary reversion site in a GC-rich site (guanine & cytosine). This variance allows a set of five test strains, to detect a wide range of substances with multiple modes of action.
However, it is common praxis for research studies that are not submitted to regulatory authorities, to only use two out of five strains, namely TA98 and TA100. This is mostly because the material and time requirements increase significantly with more bacterial strains and most Ames-positive substances can be covered with only these two strains. While the application of too few tester strains is a common reason for the rejection of Ames studies, the review by Richard V. Williams offers a deeper insight into the matter of appropriate strain selection [36]. They evaluated a large database of over 10000 substances and concluded, that only TA98 and TA100 cover about 93% of all substances that can be picked up with a full regulatory strain battery according to the OECD TG471. The resulting loss of sensitivity for about 7% of Ames-positive substances seems to be acceptable, given that the sample throughput can be increased by a factor of 2.5-fold, when using only two, instead of five strains.

3.1.6. Metabolic activation

In contrast to mammals, bacteria do not have a xenobiotic metabolism that converts DNA-reactive substances to their active form. These mutagens are also referred to as “indirect” and cannot be detected without the addition of an external metabolic activation system [22]. Bruce Ames and his team developed a protocol in the early 1970s for which they used induced rat liver S9 in combination with co-factors, to simulate a xenobiotic metabolism. The term S9 refers to a fraction of the liver homogenate after a centrifugation at 9000 g. To apply this

external metabolism, the S9-mix is plated together with the sample material and bacteria. A similar method is still applied today [27]. Subsequently, for the detection of short lived metabolites, a pre-incubation version of the plate incorporation Ames test was developed [37]. All Ames studies for regulatory submissions must include test runs in both the presence and the absence of an S9-mix [16,18,22]. A wide range of highly mutagenic substances, such as aflatoxin B1 or benz[a]pyrene yield negative results, if tested without the addition of an S9-mix. For the metabolic activity of the enzymes in the liver homogenate a co-factor mix must be added. It consists of glucose-6-phosphate, potassium- and magnesium chloride and NADP [22].

The most frequent source of S9 is produced from male rats that is pre-treated with either Aroclor 1254, or phenobarbital/β-naphthoflavone. The pre-treatment allows for an increase of the xenobiotic enzymes, before the rats are sacrificed and their liver extracted [22]. In rare cases, S9 can be derived from other mammals, such as hamsters, mice or even humans. However, the production of Aroclor 1254 is banned in most countries, because it is a highly toxic polychlorinated biphenyl, which leads to a global supply shortage and makes a suitable replacement product necessary. This issue is addressed in paper number three.

3.1.7. Scoring and Control Samples

All tester strains that are used for the Ames test each have a chance to mutate spontaneously and form revertant colonies. Each strain has a characteristic range (e.g. 0-5 spontaneous revertants per 48 wells in the Ames MPF assay for TA98 -S9) for these events to occur. Therefore it is critical to include at least one negative control sample, which consists of the solvent for the test samples (e.g. DMSO), in each test run. In addition, a positive control sample with a strain-specific standard mutagen must be included as well. Most laboratories that perform the Ames test on a regular basis, have a historical background database, where the revertant counts for positive and negative controls are routinely documented. Experiments that fall outside of this range are usually considered to be invalid and must therefore be repeated.

There are multiple approaches to evaluate whether a sample causes a mutagenic response in the literature, which includes complex statistical tests [18,38]. However, a simple and practical approach, that was chosen for all of the studies that were conducted for this thesis is the following [39]: 1.) The negative control results for each test run are scored and the mean/standard deviation are calculated. 2.) Based on the result a so-called positive threshold is calculated by multiplying the sum of the mean revertant count plus one standard deviation

by a factor of two. 3.) For each test sample the mean revertant count is calculated and if exceeds the positive threshold, the sample is considered positive. In some cases, in particular when the negative control yields a very low positive threshold, false-positive results can occur more frequently. Theoretically, for TA98 without the addition of S9, this threshold can be below one in the Ames MPF assay, which makes it necessary to artificially increase it to at least two, to avoid this effect.

When a test sample gives a weak positive response, barely exceeding the positive threshold, that cannot be reproduced in a follow-up run, the sample is considered to be equivocal (neither a clear positive, nor a clear negative results). Additional tests must be conducted and three negative results are considered to cancel out one weak positive. Only if the sample yields both weak positive and negative results again, it is considered to be either equivocal, or a weak positive. The choice ultimately depends on the expert opinion and the strength of the positive effects, as well as the strains and S9 conditions are taken into account for the final interpretation [38].

3.1.8. Spike Protocol and Cytotoxicity

When testing FCM-migrate samples, toxic or growth-inhibiting effects can cause major interferences with the Ames test results. For the standard Ames protocol, toxicity of the test items is assessed by looking at the formation of the so-called background lawn. This hazy layers of micro colonies is formed during the incubation step [22]. An absence of these micro colonies indicates cytotoxic effects on the bacteria, which can cause false negative results, due to the inability of the cells to divide and for mutations to manifest. However, under real world conditions with packaging migrate samples this approach was found to be insufficient due to two factors:

1.) The scoring of cytotoxic effects by checking the growth of the background layer requires a lot of experience and a highly trained operator and is susceptible to human error.
2.) The strength of the toxic effect is hard to quantify and even harder to proof/document, due to the poor visibility of the effect on photos.

For this purpose a so called “spike protocol” was developed for the Ames MPF Assay to allow for easier quantification and documentation of the effect strength. This allows for the definition of a threshold that has to be met, in terms of revertants, for the test run to be accepted under quality-controlled conditions. A brief overview of the method is presented below:

1.) During the preparation of the exposure plates for the Ames MPF protocol, all plates are duplicated for each tester strain and S9 condition.
2.) The exposure medium of the second plate is spiked with the respective positive control substance for the specific strain (e.g. 2 µg/mL of 2-nitrofluoerene for TA98)
3.) The plates are exposed and incubated in parallel with the non-spiked plates. All other conditions should be identical to the first plate (same operator, test sample and bacterial pre-culture as well as addition of S9).
4.) After the incubation, the revertant wells are counted and scored as usual. Due to the addition of the positive control spike to the second plate, all/most wells should score positive if there is no cytotoxic effect.
5.) The revertant count of the samples on the spiked plate is compared to with the positive control on the non-spiked plate. If the spike results in a revertant count that is lower than 60% of the positive control plate, the sample is considered cyotoxic/inhibiting.
6.) If no valid result (above the 60% threshold) is detected, the experiment must be repeated. Further dilutions must be included in subsequent tests.

For FCM migrate samples, this approach was shown to be highly valuable, because inhibiting/cytotoxic effects occur relatively frequently. The same approach could theoretically be applied to the standard agar-based Ames test, but the time and material requirements are a limiting factor. The spike control method is presented and discussed in paper number two.

3.1.9. Limitations of the Ames test

While the Ames test has many advantages for FCM migrate safety assessment and is a well-accepted assay by regulatory authorities, it also has some inherit limitations. The most important issue is that bacteria, unlike humans, do not have chromosomes. This means that bacteria-based genotoxicity assays cannot detect DNA damage that is happening purely on a chromosomal level [21]. This makes it impossible to detect genetic effects that, for instance, damage the spindle apparatus during the cell division. While the overall ability of the Ames test to predict rodent carcinogenicity is high, the standard tests for pure substances, such as pharmaceuticals or pesticides, usually include at least one mammalian cell-based assay. Based on a paper by David Kirkland an colleagues [21], a combination with an *in vitro* micronucleus assay is sufficient to detect most carcinogenic chemicals.

This suggests that for food packaging safety assessment, the addition of a mammalian cell based assay must also be considered. While this approach was followed during the initial phase of the Migratox project (publications by Pinter and colleagues: [40,41]), it was found

that the LECs of mammalian cell-based *in vitro* tests are significantly worse than for the Ames test.

4. Summary of the Published Articles

4.1. Suitability of the Ames Test to Characterise Genotoxicity of Food Contact Material Migrates

4.1.1. Background and Aim

In the initial stages of the Migratox project, collection of data on the state of the art of current *in vitro* bioassay methods was necessary. For this purpose a literature review was conducted and subsequently published [31]. The review focuses on the theoretical applicability of the Ames test for the safety assessment of FCM. In addition, it reviewed all Ames-based studies that dealt with food packaging, or related materials. Different variants of the Ames test are also presented and a theoretical target LEC based on the EU10/2011 guideline was calculated. Finally, data from FCM-related genotoxic substances, as well as standard test substances was gathered and compared to the threshold that was calculated.

In parallel to this study, a second literature review was performed on the applicability of mammalian assays [40]. However, the second review was set as a lower priority, since it quickly became apparent that mammalian cell-based *in vitro* bioassays had a lower performance in terms of overall LODs.

4.1.2. Results and Discussion

Frist a literature survey was conducted, that targeted different variants of the Ames test, which could be applied for FCM safety assessment (Table 1). Amongst them, it was found that the Ames MPF had the most advantages, when compared to the classic agar-based Ames protocol.

The concept of LOD does not apply to *in vitro* bioassay methods directly, since the term stems from analytical chemistry. Instead, the LEC, the lowest substance concentration that yields a measureable effect when compared to a control group, is used. However, for complying with the current guidelines EU10/2011 and the TTC, it is important to calculate the overall detection limit of the whole test procedure [9,14]. Not only the LECs of test substances are important, but also factors that are applied during the sample preparation process, such as a hypothetical concentration/dilution factor. Based on the target concentration of 0.01 mg kg^{-1}, a 1000-fold concentration factor, a 4% solvent tolerance of the Ames test, as well as a (overly optimistic) 100% recovery-rate, the target LEC was calculated

to be at 0.4 mg L^{-1}. This means, that every genotoxic substance that can be detected below this threshold with the Ames test, could be picked up during a migration test. It has to be mentioned that the relevant overall target LOD that was addressed in this publication, is 10 ppb or 0,01 mg kg^{-1}. This is based on technical limitations that are outlined in the EU10/2011 guideline [9] and has no toxicological relevance. The second issue is that both the 1000-fold concentration, as well as the 100% recovery rate are unrealistic. This resulted in a target LEC that was too optimistic. In retrospective, this is one of the biggest shortcomings of this publication, which is further discussed in publication two and three [32,42].

Table 1: Overview of variations of the Ames test, based on Escobar et al. 2013 [31,43]

Name	Incubation	Incubation vessel	Readout	Special feature	Source
Plate incorporation assay	Solid	Petri dishes	Growth of revertant cultures	-	[44]
Pre-incubation Assay	Solid	Petri dishes	Growth of revertant cultures	-	[44]
Miniscreen	Solid	6 well plates	Growth of revertant cultures	-	[45]
Micro Ames	Solid	24/96 well plates	Growth of revertant cultures	-	[33]
Mini Ames	Solid	Petri dishes	Growth of revertant cultures	Mixed strains for higher throughput	[43]
Fluctuation test	Liquid	Test tubes	Colourimetric readout	-	[46]
Ames II	Liquid	384 well plates	Colourimetric readout	Mixed strains for higher throughput	[47]
Ames MPF	Liquid	384 well plates	Colourimetric readout	-	[34]
5FU assay	Liquid	384 well plates	Colourimetric readout	Forwarded mutation for 5 FU resistance	[48]
Bioluminescent Ames	Solid	24 well plates	Bioluminescence of revertant cultures	-	[49]

Subsequently, LEC data was gathered on positive standard substances from literature sources. For this purpose, the so-called ECVAM-list (EU Reference Laboratory for alternatives to animal testing) was chosen and the selected substances [50] were compared with peer-reviewed Ames studies. Out of the 16 chemicals, eight have an LEC of lower than the calculated threshold 0.4 mg L^{-1} and could therefore be detected during FCM migration testing (Table 2). Additional LEC data was compiled on substances that were detected in FCM related sources from paper, coating or polymers, such as PET (see Table 3 in the full text [31]). In general, these real-world contaminants have higher LECs in the Ames test, than the standard substances from the ECVAM list and the target threshold can be achieved for four out of 40 substances.

Table 2: LEC values of Ames standard substances that are part of the ECVCAM-List. All values were converted into concentrations in mg L^{-1} for comparison with the 0.4 mg L^{-1} threshold. Source: [31]

Substance	CAS Nr.	LEC [µg plate^{-1}]	LEC [mg L^{-1}]	Strain[1]	S9[2]	Source
2-Acetylaminofluorene	53-96-3	0.27	0.1	TA98	+	[51]
Aflatoxin B1	1162-65-8	0.0038	0.0014	TA98, TA100	+	[51]
Benzo-α-pyrene	50-32-8	0.57	0.21	TA100	+	[51]
p-Chloroaniline	106-47-8	333	123	TA98	+	[52]
Cisplatin	15663-27-1	1.0	0.37	TA98, TA100	-	[53](
Cyclophosphamide	6055-19-2	2	0.74	TA1535	+	[54]
2,4-Diaminotoluene	95-80-7	20	7.4	TA1538	+	[55]
7,12 Dimethylbenzanthracene	57-97-6	25	9.26	TA100	+	[56]
Dimethyl nitrosamine	62-75-9	20	7.4	YG7108	+	[57]
ENU (N-Ethyl nitrosourea)	759-73-9	33	12.2	TA98, TA100	-	[53]
Etoposide	33419-42-0	500	185	TA98, TA100	±	[58]
IQ (2-Amino-3-methyl-3H-imidazo[4,5-f]quinoline)	76180-96-6	0.005	0.0019	TA98	+	[57]
Methyl Methanosulphonate	66-27-3	19	7.04	WP2	-	[51]
Mitomycin C	50-07-7	-[3]	0.0015	WP2	-	[59]
4-Nitroquinoline-N-Oxide	56-57-5	0.01	0.004	TA100	-	[60]
PhIP.HCl (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine)	105650-23-5	0.01	0.004	TA98	+	[61]

Finally, the literature sources were reviewed for studies that applied the Ames test for migration testing of food contact materials (see table 4 in paper number one [31]). Only very few publications (13) were found and they were hard to compare. A very wide array of methods have been applied in the past, which includes multiple variants of the Ames test. Additionally, the sample preparation methods varied widely. Due to incomplete method descriptions, the overall concentration factors that were achieved, could not be calculated. Additionally, the issue of detection limits (LOD or LEC) was barely ever discussed. Most papers did not find any positive results, but also did not discuss potential shortcomings of their approach, that could cause false negatives.

The most important conclusion of the literature review is that the Ames test is a highly promising method, when it comes to the assessment of FCM for genotoxicity. It has many advantages, when compared to other *in vitro* bioassays, such as low LECs and higher tolerance to toxic effects. However, there are also several limitations that have to be addressed: 1.) The LODs that can be achieved with Ames-based methods are mostly too high to cover the 0.4 mg L^{-1} threshold. 2.) There is little knowledge about potential toxic matrix effects of FCM migrate samples. 3.) Sample preparation methods need to be optimised and validated to achieve reproducible results. Nevertheless, the approach to use the Ames test in combination with data from analytical chemistry and product manufacturers would be a significant improvement over the current state of the art.

4.2. Mutagenicity Assessment of Food Contact Material Migrates with the Ames MPF Assay

4.2.1. Background and Aim

In paper number one, it was established that the Ames test can be a highly valuable tool for FCM safety testing, when applied in conjunction with proper sample preparation methods. However, almost all previous publications that applied the Ames test with FCM migrate samples did not address several important points. These include, but are not limited to: 1.) There are theoretical limits of detection in context with target thresholds that have to be met, to fulfil regulatory requirements. 2.) Proper sample preparation methods are needed, that are optimised and validated for FCM migration tests. 3). The issue of toxic matrix effects, that could mask genotoxicity, has to be addressed.

For this purpose, paper number two presents a method, that can be applied for FCM safety assessment with the Ames test. Since preliminary tests that were conducted, found that the Ames MPF format had advantages over the OECD method, it was selected as a test of choice for the publication. The main goal of the paper, was to prove that the test procedure is applicable for real world samples and capable to detect both positive and negative results reliably. In addition, the recovery rates of the sample preparation method were tested with HPLC-MS and GC-MS methods (high performance liquid chromatography and gas chromatography coupled with mass spectrometry). To achieve a better understanding of the LECs that are achievable with the Ames MPF assay, a small subset of mutagenic standard substances was tested and the results were compared with literature sources from paper number one.

4.2.2. Results and Discussion

The test items that were used for this study were received from industry partners from the MIGRATOX project. All samples were virgin materials and had not been in contact with food prior to testing. Overall, 28 packaging samples from three categories (plastics, composite materials and coatings), were tested. Two of the samples, specifically two paper/plastic compound materials were also migrated “inside out”, which is important for the discussion of the final results.

The first part of the methods section covers the sample preparation process. Migration experiments were conducted according to the EU 10/2011 guideline with slight adaptations.

Whenever possible, the packaging was migrated “as intended”, by filling the container with the food simulant. Otherwise, closed bottles with PTFE screwcaps, or special migration chambers were used. The final surface to volume ratio was one dm^2 per 100 mL of food simulant, which consisted of 95% ethanol. After the migration, the samples underwent a concentration step via rotary evaporation and a solvent exchange to DMSO (final concentration factor: ~300). Substance recoveries of the method were determined with a set of standard test substances and it was found that for most substance classes (non/intermediate volatile) a 60-80% recovery was achieved. Only highly volatile substances are lost (0% recovery).

The Ames MPF assay was selected as the Ames format for this study, since it has multiple advantages over the classic Ames test. In particular, the reduced sample volume requirements (by at least a factor of five) are highly important, since the sample preparation process is both expensive and time consuming. Two *Salmonella* strains were applied (TA98 and TA100) in presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9). Since some samples caused cytotoxicity, which could mask genotoxic effects, all tests were duplicated. One test was conducted with pure samples and the second test was conducted in presence of a positive control spike. A reduction in revertant count of the positive control spike (<60% revertant recovery) was counted as an inhibitory effect, which required higher sample dilution factors. In addition, a set of standard mutagenic substances were tested to find out how the data that is generated with the Ames MPF Assay compares with literature sources (see Table 3). While the LECs of the Ames MPF assay seem to be very close to the literature data, most substances cannot be picked up the 0.15 ppb threshold that is required, according to the TTC-concept.

Table 3: Limits of detection of ten mutagenic standard substances in the Ames MPF assay. The LECs were converted to an LOD in the migrate based on Rainer et al. 2018 [31].

Substance	CAS	Strain	S9	Tested LEC Bioassay [µg/mL]	Literature LEC Bioassay [µg/mL]	Volatility (log H [e])	Estimated LOD Migrate [µg/mL]
2-acetylaminofluorene	53-96-3	TA98	+	0.03	0.10	non-volatile (-4.71 [c])	0.003
Benz[a]anthracene	56-55-3	TA100	+	1.56	1.56	intermediate (0.09 [a])	0.18
Benzo[a]pyrene	50-32-8	TA98	+	0.20	0.21	intermediate (-1.33 [b])	0.02
7, 12-dimethylbenzo[a]anthracene	57-97-6	TA100	+	6.25	9.26	intermediate (-0.42 [d])	0.73
2, 4-diaminotoluene	95-80-7	TA98	+	25.00	7.40	non-volatile (-4.12 [c])	2.79
N-ethylnitrosurea	759-73-9	TA100	-	12.50	12.20	non-volatile (-4.88 [c])	1.40
Methyl methanesulfonate	66-27-3	TA100	-	10.00	7.04	intermediate (-0.39 [c])	1.17
4-nitroquinoline 1-oxide	56-57-5	TA100	-	0.003	0.004	non-volatile (-8.56 [c])	0.0003
Phenyl glycidyl ether	122-60-1	TA100	-	3.10	3.70	intermediate (-1.08 [d])	0.36
Tris-(2, 3-epoxypropyl) isocyanurate	2451-62-9	TA98	+	25.00	3.70	non-volatile (-15.02 [c])	2.79

From the 30 items that were tested, two show clear signs of inhibiting bacterial growth (one coating and one paper sample). However, the inhibitory effects were no longer present in higher dilutions (see Figure 2). Three out of 30 samples showed positive results for genotoxicity (Figure 3). Two out of the three positive, the compound material samples, were migrated "inside out". While this type of migration is not realistic, the packaging samples still do not comply with current guidelines, since in the EU regulation 10/2011, it is specifically stated that mutagenic substances may not be present in FCM, even when behind a barrier layer.

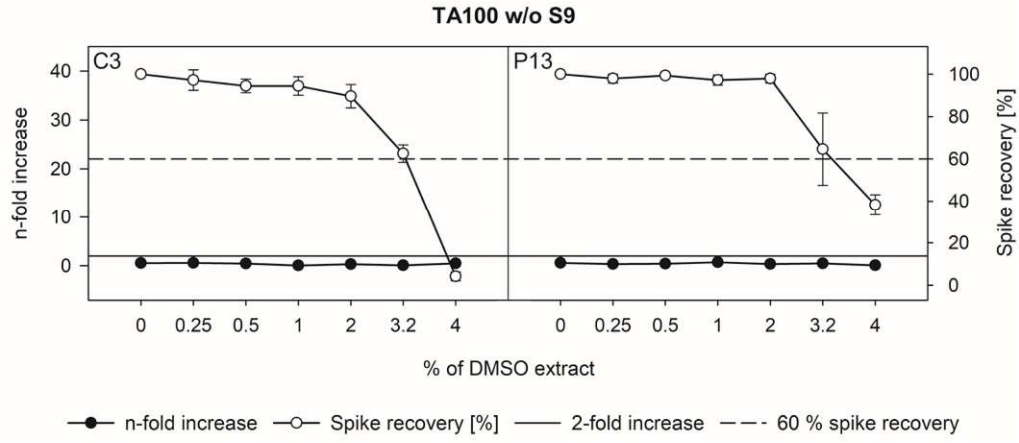


Figure 2: Inhibitory effects of two packaging samples in strain TA100 without metabolic activation. Sample C3: metal coating, sample P13: paperboard

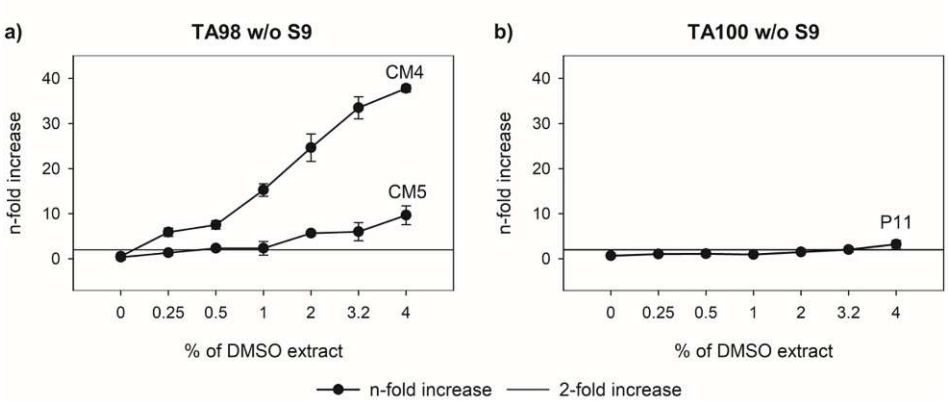


Figure 3: Dose-response curves of positive packaging migrate samples tested in the Ames MPF assay with TA98 and TA100. Sample CM 4&5 are compound materials, which were migrated "inside out" and sample P11 is a plastic material.

Overall, the most important points that were highlighted by the publication are the following:

1.) Even though the LODs/LECs of the Ames MPF assay are insufficient to comply with the TTC threshold of 0.15 ppb, the method is still able to detect positive results in real-world FCM migrate samples.
2.) The achievable concentration factor of 1000, which was assumed in paper number one, was overly optimistic. A factor of ~300 could be achieved reliably.
3.) Sample concentration with rotary evaporation of 95% ethanol lead to satisfactory recovery rates of >60% and only highly volatile substances cannot be recovered.
4.) Further research must be conducted to improve the limits of detection of the Ames MPF assay.

4.3. Direct Comparison of the Lowest Effect Concentrations of Mutagenic Reference Substances in Two Ames Test Formats

4.3.1. Background and Aim

Paper number one and two established that the Ames test, while being a highly promising approach, had insufficient LECs/LODs to cover the required safety thresholds that are set by the TTC concept [15]. Multiple attempts were made to improve the LODs for genotoxic substances in the Ames MPF assay, but they remained unsuccessful (data not published). These attempts included multiple variations in the standard test procedure, such as bacteria, solvent and histidine concentration, as well as variations in incubation/exposure time and temperature. While some changes yielded good results for individual substances and strain combination, the same changes caused massive reductions in LEC/LOD for different combinations. Initially, it was planned to publish a paper that describes an improved Ames MPF method, this effort was however discontinued, due to limited success.

In parallel, multiple formats of the Ames test were assessed for their performance. This included the standard petri-dish based Ames test, according to OECD [18], as well as multiple miniaturized variants, such as the Ames MPF assay [34]. This lead to the overall conclusion, that the Ames MPF assay has significant advantages over the agar-based Ames variants, while still applying the tester strains that are required in the OECD guideline [18]. However, there was no publication that showed that the Ames MPF assay performed equally well, when testing for low levels of genotoxic contaminations. The importance of comparing multiple variants of the Ames test and identifying the best option was also highlighted in an ILSI publication (International Life Science Institute, [7]). For this purpose, an in-depths comparison study of the Ames MPF assay and the standard agar-based Ames test, according to OECD was conducted. The main aim of the paper was to find out which assay has better performance in terms of LEC/LOD. In addition, two different sources of S9 (Aroclor 1254- and phenobarbital/ß-naphtoflavone-induced) where compared, to see whether the S9 activation affects the results.

4.3.2. Methods

A set of 21 standard test substances was selected from the ECVAM-list [50] as well as paper number one [31]. This included melamine, which was used as a negative control substance. All of these test items were assessed with two strains, TA98 and TA100, in the Ames MPF test and the standard agar-based Ames test. A major focus was to control as many parameters as possible, such as bacteria count in the pre culture and potential pipetting errors during the dilution series. For this purpose, two operators conducted the tests at the same time (workflow see Figure 4). The scoring was also done in parallel and a positive threshold of a two-fold increase over the negative control (including one standard deviation) was applied. For a subset of eleven test substances, which required metabolic activation in the form of rat liver S9, a second test series was conducted. The performance of Arcolor 1254-induced rat liver S9 was compared to the performance of phenobarbital/ß-naphtoflavone-induced S9, to find out if the type of S9 has an effect on the LECs. Due to the lower S9 volumes that are required, the second comparison was only run in the Ames MPF assay.

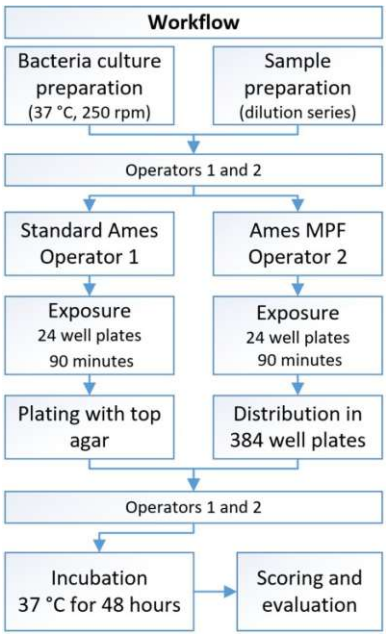


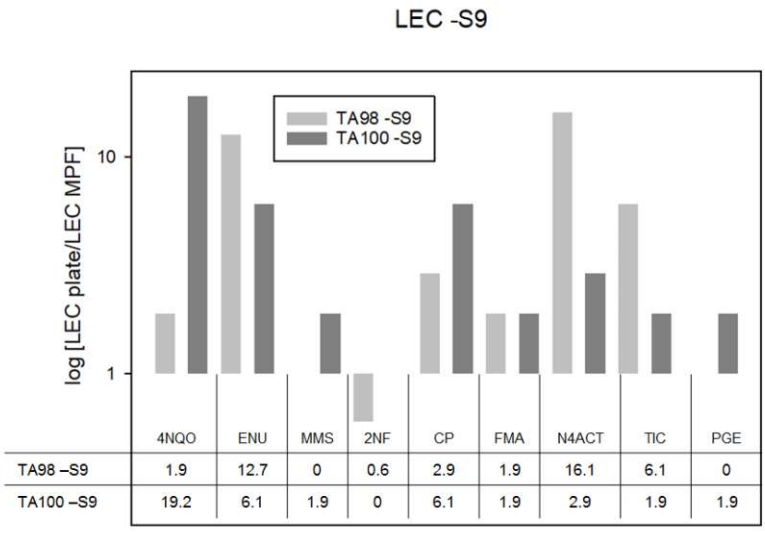
Figure 4: Work flow of the Ames comparison study. Two operators conducted the experiments in parallel.

All data was compiled and the differences in LEC, of the assay/S9-formats, were tested for statistical significance with a paired sample t-test. Since the data did not follow a normal distribution, all LEC values were changed to their decadic logarithm.

4.3.3. Results and Discussion

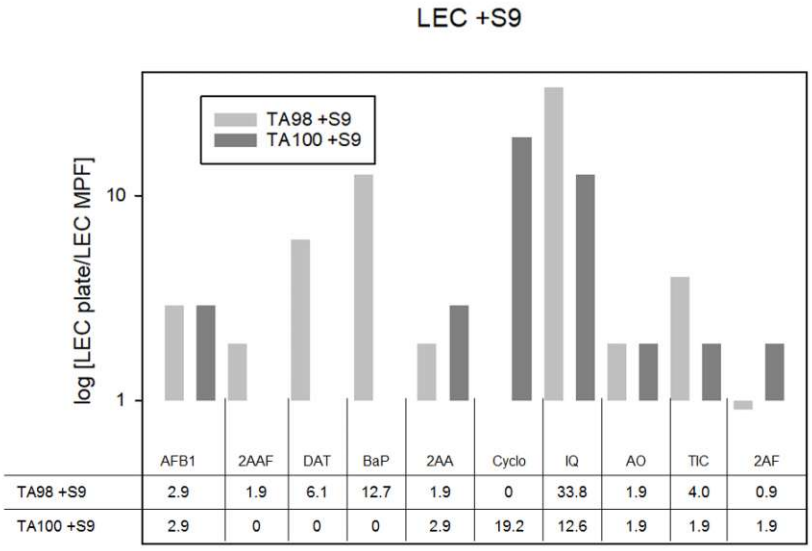
Of the 21 test substances, 19 yielded concordant results in the Ames MPF assay, as well as the agar-based Ames test. A test chemical that gave discordant results was sodium azide, which scored negative in the Ames MPF assay, but positive in the agar-based Ames test. For benzo[a]anthrazene, it was the other way around and it tested only positive in the Ames MPF assay. In both cases, toxic effects on the bacteria, can most likely explain the discordant results. Some test items, namely 2-nitrofluorene, 2-acetylaminofluorene and benzo[a]pyrene gave discordant results in only one of the two test strains. Overall, this concordance of ~90% agrees with findings that were published in previous studies [34,35].

When it comes to the comparison of the LECs, the Ames MPF assay generally, yielded better results (see Figure 5 and Figure 6). When dividing the LECs of the agar-based Ames test and the Ames MPF assay, it was found that for most substances, the MPF assay has lower LECs by two- to 33.8-fold. Only one substances, namely 2-nitrofluoerene, performed better in the agar-based Ames test. The paired sample t-test showed that the MPF assay has significantly better overall LECs with $p < 0.001$, when comparing the concentrations that were applied during the exposure step. When comparing the overall LODs, which had to figure in the sample concentration that yields a positive result, this gap slightly closes, since the sample dilution factors that are applied for the assay differ slightly (25-fold for the MPF assay and 13-fold for the agar-based Ames test). Yet, the statistical test still shows that the MPF assay has lower overall LODs with $p < 0.001$.



	4NQO	ENU	MMS	2NF	CP	FMA	N4ACT	TIC	PGE
TA98 –S9	1.9	12.7	0	0.6	2.9	1.9	16.1	6.1	0
TA100 –S9	19.2	6.1	1.9	0	6.1	1.9	2.9	1.9	1.9

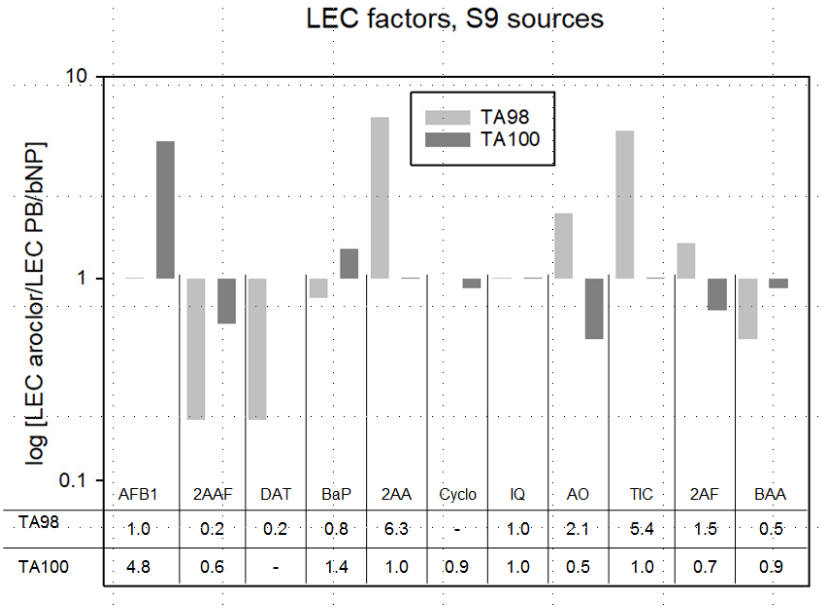
Figure 5: Differences of the LECs in the Ames MPF assay and the standard agar-based Ames test on a substance by substance basis The results are shown in logarithmic scale and were calculated by dividing the LEC of the agar-based test, by the LEC of the MPF variant. All tests were run without the addition of rat liver S9.



	AFB1	2AAF	DAT	BaP	2AA	Cyclo	IQ	AO	TIC	2AF
TA98 +S9	2.9	1.9	6.1	12.7	1.9	0	33.8	1.9	4.0	0.9
TA100 +S9	2.9	0	0	0	2.9	19.2	12.6	1.9	1.9	1.9

Figure 6: Differences of the LECs in the Ames MPF assay and the standard agar-based Ames test on a substance by substance basis. The results are shown in logarithmic scale and were calculated by dividing the LEC of the agar-based test, by the LEC of the MPF variant. All tests were run with the addition of rat liver S9.

When comparing the two S9-sources (see Figure 7), it was found that they do not have a significant impact on the LECs. While there might be differences for individual substances, the paried t-test showed there is no statistical significant difference ($p = 0.65$). These results are relevant, since Aroclor 1254-induced rat liver S9 was the most frequently used product for *in vitro* studies over the last decades and is no longer produced.



	AFB1	2AAF	DAT	BaP	2AA	Cyclo	IQ	AO	TIC	2AF	BAA
TA98	1.0	0.2	0.2	0.8	6.3	-	1.0	2.1	5.4	1.5	0.5
TA100	4.8	0.6	-	1.4	1.0	0.9	1.0	0.5	1.0	0.7	0.9

Figure 7: Differences of the LECs that were obtained with Arocor 1254-induced S9 vs. phenobarbital/ß-naphtoflavone-induced S9. The results are shown in logarithmic scale and were calculated by dividing the LECs. A higher bar means a better performance of the phenobarbital/ß-naphtoflavone induced S9 source.

Overall, it was concluded in this study, that the Ames MPF assay performs better in terms of achievable LECs, which directly translate into lower LODs for a bioassay-based safety

assessment approach of complex mixtures. While the performance of both assays is relatively similar in terms of concordance, 17 out of 21 substances can be picked up in lower concentrations in the MPF format. However, when comparing the LECs that were measured in this study and comparing them to the target LODs, that were established in paper number one, it becomes obvious that even the Ames MPF assay cannot consistently reach the 0.15 ppb threshold that is required according to the TTC concept [14,15].

When Taking practical considerations into concern, the MPF assay has multiple advantages over the standard format. In particular, the amounts of sample and reagents that are required to perform the assay are significantly lower (~5-fold). In addition, the throughput that a single operator can handle is about threefold higher.

The most important shortcoming of the Ames MPF assay is that the so-called "dynamic range" of the assay is limiting in specific scenarios. Since the scoring takes place in a 384-well plate, instead of a petri-dish, the maximum amounts of revertants is limited. In theory, a single well in a 384-well plate could contain multiple revertant colonies, which would be visible as individual spots on a petri-dish, but can no longer be differentiated by the colorimetric readout. This means that when the background mutation rate rises, the Ames MPF assay becomes less sensitive towards relative revertant increases. This effect hardly ever occurs for the agar-based Ames test. This makes pre-screening of the pre-cultures , as well as the selection of test strains with low base revertant counts, crucial to achieve optimal results.

The most important conclusions of the paper are the following:

1. The Ames MPF assay has lower LECs than the standard agar-based Ames variant.
2. The selection of the assay format should take regulatory and technical requirements (such as availability of sample material) into account.
3. The application of Aroclor 1254-induced S9 and phenobarbital/ß-naphtoflavone-induced S9 results in similar LECs.
4. The critical 0.15 ppb threshold cannot be reached consistently, in terms of overall LOD. More research is required to find improvements for, or viable alternatives to the Ames test in the future.

5. Discussion and Outlook

5.1. Improvements Over the State of the Art

As already mentioned, the detection of genotoxic NIAS in FCM, as well as recycled plastics, is a major ongoing challenge. Even though, it was found that the Ames test does not provide sufficient detection limits, in terms of LECs and the resulting LODs, its application is a major improvement over the current state of the art. Over the last decades, safety testing of packaging materials was mostly based on chemical analytical methods [11–13], which detected a wide range of unknown substances that could neither be identified, nor fully assessed for their toxicological properties. Even if modern *in silico* approaches can give important insights into the potential toxicological properties of unknown substances [62], they can only be applied if the structure is at least partly known. This means, that if the regulatory requirements were enforced more strictly this would mean that most packaging material would no longer qualify as safe for the consumer and should be taken off the market. Even if the likelihood of each individual NIAS to be mutagenic is relatively low, in case of unknown contaminants a worst-case scenario must be assumed. This is obviously neither feasible from a practical standpoint, nor would it lead to improved food safety, because of the highly important barrier properties and the protection of food items from both chemical and biological contaminations.

The application of the Ames test, in conjunction with a suitable sample preparation approach, was shown to be the most efficient way to detect low concentrations of genotoxic/mutagenic substances. In the scope of the Migratox project, a wide range of in vitro bioassays were assessed for their ability to detect low levels of genotoxic substances and it was continuously found that the performance of the Ames tests (in particular the MPF protocol) hast major advantages over other currently available test system [31,40–42]. This is mostly due to the ability of the Ames test to respond to relatively low concentrations of individual mutagens. In addition, bacterial test organisms tolerate toxic effects much better and therefore higher sample concentrations are applicable. The Ames test is also recommended by ILSI to be applied for FCM safety assessment and is considered an important step in an approach that is based on multiple pillars of evidence. It must however be mentioned that the Ames test should never be applied on its own, but instead should be combined with chemical analytical data on the migrate samples, as well as data on the material properties and composition that must be passed along the supply chain.

The outcome of the first research question, that came up during the initial stages of the thesis: "Is the Ames test in its current form applicable for the safety testing of FCM for genotoxicity?" is therefore answered with a clear yes. Even if slight adaptations, such as a switch to the Ames MPF protocol, are necessary and the inclusion of a spike protocol is highly recommended (see chapter 3.1.8), it was repeatedly shown [7,31,32] that the assay is a valuable addition to any test battery. It provides additional toxicological information about the FCM migrate samples, that could not be obtained with the current state of the art. The necessary switch to the Ames MPF protocol also answers the third research question, which assay format is best suited for the study of FCM (see chapter 5.3 for a more detailed discussion).

The second research questions on whether the Ames test is capable to detect genotoxicity in real world packaging samples, was mostly addressed in paper number two, as well as in the scope of the PolyCycle project (results will be published in 2022). Even though the detection limits of the Ames test are not yet sufficient to comply with regulatory/TTC standards, several samples scored positive [32]. Therefore, the second research question can also be answered with an unambiguous yes. More important aspects that came up during the thesis, such as how the LECs of the Ames test could be improved and how the research in the field of packaging safety assessment with *in vitro* bioassays will evolve in the future, will be discussed in the remaining chapter.

5.2. Establishing a Standardized Test Protocol

An important point that was found during the initial literature review was that no standardized approach on how to conduct genotoxicity studies with *in vitro* bioassay for FCM existed. Instead a wide range of methods, with very little consideration to standardization and detection limits was applied [5,6,31]. The research partners at OFI, with their focus on proper sample preparation methods, tackled this important aspect and came up with a relatively straightforward protocol that allows for an efficient and reliable way to conduct migration experiments for subsequent *in vitro* studies [32]. In short, the protocol is based on the EU10/2011 guideline and the migration experiments are followed up by a concentration step via rotary evaporation and a solvent exchange to DMSO. While this approach already yields mostly satisfactory results, further improvements must be made, in particular when it comes to the recovery of volatile substances.

In addition to the sample preparation, a standardized approach for the Ames test protocol must be presented. While the methodical approach is presented in chapter 3. Methods, a brief

summary of the protocol, as well as the most important criteria for the respective decisions is presented below:

1.) Strain selection: For the selection of the tester strains, a combination of at least TA98 and TA100 is suggested. While this does not fully cover the standard five strain test battery, it was shown that only two strains can cover ~93% of all Ames positive substances [36].
2.) Metabolic activation: The addition of rat liver S9, or an alternative metabolic activation system of equal performance, must be included in all FCM safety assessment studies.
3.) Ames format selection: The Ames MPF format provides several advantages over the standard agar-based approach. Until an assay format with better performance is identified, the Ames MPF assay is recommended for FCM safety assessment.
4.) Detection of toxic effects: False negative results, due to sample toxicity, can be an major issue for *in vitro* bioassays. For this purpose, it is recommended to apply a spike control protocol, as is outlined in paper number two, to quantify these effects.
5.) Positive results: A sample must be considered genotoxic when two independent test runs give positive results. If results are equivocal, additional tests must be run until a decision can be made, based on expert opinion.

In the author's opinion, the list above presents the minimum requirements that should be taken into consideration for all future studies that apply the Ames test for material safety assessment. While it would be possible to apply a full-scale regulatory standard Ames test, that also includes spike controls with five tester strains, the cost and time requirements, as well as the shear amount of migrate sample that has to be prepared is cost prohibitive for screening large volumes of packaging samples.

5.3. Acceptance of Novel Ames Formats

Since the development of the Ames test in the 1970s, it has been established as an industry standard for toxicological studies of chemicals and pharmaceutical products (xx). The standard agar-based Ames test is the format of choice and it is directly or indirectly mentioned in the respective guidelines [16,18]. While it works very well and yields reliable results with a high prediction rate for rodent carcinogenicity [21], there was little push for development of new and improved assays formats. Even though, most alternative Ames formats are still not yet accepted for regulatory studies, there have were many developments over the last decades. One of them, the assay of choice for the present thesis, is the Ames MPF assay.

Even though regulatory authorities are relatively inflexible when it comes to changes in accepted test formats, from the perspective of the author, the MPF assay offers considerable advantages over the standard test protocol. Not only does it work with by exact same molecular mechanisms as the standard test, it is also faster, easier and cheaper to handle.
As already mentioned, one major limitation of the Ames test for NIAS safety assessment is the LODs/LECs of the assay. In paper number three, a wide range of standard Ames positive test was tested under multiple conditions, to see how the Ames MPF assay and the standard agar-based Ames test compare in terms of detections limits. It was found that the MPF protocol consistently detects most substances in lower quantities than the standard Ames test. This difference was highly statistically significant, in terms of overall LECs, for a set of 21 test substances [42]. While this gap narrowed, when the concentration in the sample is considered, instead of the concentration during the exposure step, the difference remains significant. Possible explanations for this effect could be ad/absorption of genotoxic substances by the agar matrix, or an uneven distribution of the test item in the petri dishes [34,35]. Therefore the author concludes, that the Ames MPF assay should be selected as an assay of choice, until a better alternative format can be found. This, again, answers research questions number three – Which assay format should be selected for the safety assessment of FCM.

5.4. Improvement of the Limits of Detection

One of the major weak points of the Ames test is, that all studies that were conducted concluded the the LECs and derived LODs of the method are insufficient to cover the requirements that are set by the TTC concept: a detection limit of 0.15 µg/kg for genotoxic substances. Even if you assume a best case scenario for the sample preparation, a 300fold concentration, with a 100% recovery rate, this threshold only increases to 45 µg/kg. For migrate samples this can be converted into a required detection limit of 0.045 µg/mL. When comparing this extremely low threshold to the detection limits that were found in paper number one, two and three, it can be seen that only very few substances could be picked up. This includes IQ (2-Amino-3-methyl-3H-imidazo[4,5-F]quinoline) and Aflatoxin B1, although aflatoxins fall into its own category and must undergo specific assessment, when compared with other substances with genotoxic activity [15]. Even though the TTC concept presents a highly conservative approach, that is based on the assumption that genotoxic substances have a strictly linear dose-response relationship down to the ng/kg range, it is unlikely that the regulatory/toxicological experts will change their requirements in the near

future. Therefore, it is important to develop methods that allow for a more sensitive detection of genotoxins. For the Migratox project, multiple attempts have been made to come up with relatively simple changes to the test protocol (such as changes in bacteria concentration or incubation timings) to make the Ames test more sensitive for lower concentrations of genotoxic substances. Unfortunately, these attempts were unsuccessful, because any change that lowered the LEC for one test chemical, also lead to a higher LEC for other test substances. There could be however, still be opportunities to improve the detection limits by a significant factor by one, or all of the following approaches:

1.) A development of a novel Ames test format that either changes the tester strains on a molecular level, or makes significant changes to the assay protocol is the first possibility. Currently there are developments in both directions. However, they cannot be discussed in this thesis, because they fall under non-disclosure agreements with research partners.
2.) Another option is the coupling of a separation step for the test substances on via chromatographic methods, with a follow-up bioassay. This approach was already highlighted in several research papers [63–66] and shows some promise. However, these studies did not yet proof that they can reliably detect low concentration of a wide range of test items, since they mostly apply highly potent compounds, such as aflatoxin B1 or 4-nitroquinoline-n-oxide.
3.) Any improvement that could be achieved for the sample preparation methods directly translates into improved overall detection limits for the subsequent Ames test. Even if the concentration via rotary evaporation is limited, due to precipitation and loss of volatiles, other approaches, such as an optimised solid-phase extraction method, could still yield significant improvements.

5.5. Outlook

There are still lots of research opportunities in the field of packaging material safety assessment. The following chapter will provide a brief outline on the specific issues that will be addressed by follow-up publications in the near future. Since the Migratox project is still ongoing until 07/2023, multiple publications are planned for the remainder of the project:

1.) The first and most important part is that the standardized testing approach, that was outlined in chapter 5.2 still needs to be fully validated. While in-house studies at FH Campus Wien and OFI (data not published) already yielded satisfactory results, a major multicentre study, which also includes several partners from the food and

packaging industry, is planned for the near future. For this purpose, standardized test materials will be produced by OFI and analysed by at least four European partner institutes.

2.) So far, mostly conventional packaging materials, such as paperboard and bulk polymers (PE, PP, PET), as well as metal coatings were assessed for their genotoxic potential. The next step will be to apply the Ames test for the safety assessment of bio-based materials, such as PLA or starch-based coatings.

3.) A method that allows for the fractionation of the packaging migrate samples and subsequent analysis of the individual factions, could help to gain important insights into the chemical properties of genotoxic NIAS. For this purpose, a study is in preparation that deals with the separation of packaging migrate samples with thin layer chromatography and their subsequent analysis in the Ames test.

Another research field that was found to be highly promising is the safety assessment of recycled plastic polymers, in particular polyolefins (PE and PP). There is a lot of pressure on the plastics industry by the EU to enforce higher recycling quotas. However, safety concerns still are a major hurdle that must be overcome. For this purpose, further studies will be conducted on recycled plastics to gain an insight on whether state of the art recycling methods can produce materials that are suitable for food contact applications.

Both the PolyCycle and the Migratox projects focus on food contact materials. Further research opportunities are in the field of pharmaceutical packaging materials, as well as materials that come into contact with drugs during the production process. While the pharmaceutical industry has already built up a lot of expertise in the field of safety assessment, the addition of the Ames test could play an important role to test for NIAS.

Lastly, only very little research was conducted in the field of cosmetics packaging safety assessment. Here recycled materials are already being used, even though it remains unclear whether they are safe for the consumer. Both pharmaceutical products and cosmetics are consumed/applied in significantly lower quantities than foods or drinks. This results in favourable exposure scenarios, which could make the application of the Ames test even more promising, even if a strict TTC-based approach is applied.

Overall, the research needs for the application of *in vitro* bioassay for material safety assessment are still extensive and will keep both researchers and industrial partners busy in the coming decade.

6. References

1. Muncke, J. Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source? Science of The Total Environment 2009, 407, 4549–4559, doi:10.1016/j.scitotenv.2009.05.006.

2. Muncke, J. Endocrine disrupting chemicals and other substances of concern in food contact materials: An updated review of exposure, effect and risk assessment. The Journal of Steroid Biochemistry and Molecular Biology 2011, 127, 118–127, doi:10.1016/j.jsbmb.2010.10.004.

3. Kirchnawy, C.; Mertl, J.; Osorio, V.; Hausensteiner, H.; Washüttl, M.; Bergmair, J.; Pyerin, M.; Tacker, M. Detection and Identification of Oestrogen-Active Substances in Plastic Food Packaging Migrates. Packag. Technol. Sci. 2014, 27, 467–478, doi:10.1002/pts.2047.

4. Mertl, J.; Kirchnawy, C.; Osorio, V.; Grininger, A.; Richter, A.; Bergmair, J.; Pyerin, M.; Washüttl, M.; Tacker, M. Characterization of Estrogen and Androgen Activity of Food Contact Materials by Different In Vitro Bioassays (YES, YAS, ERa and AR CALUX) and Chromatographic Analysis (GC-MS, HPLC-MS). PLoS One 2014, 9, doi:10.1371/journal.pone.0100952.t001.

5. Souton, E.; Severin, I.; Le Hegarat, L.; Hogeveen, K.; Aljawish, A.; Fessard, V.; Marie-Christine, C. Genotoxic effects of food contact recycled paperboard extracts on two human hepatic cell lines. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 2017, 1–12, doi:10.1080/19440049.2017.1397774.

6. Groh, K.J.; Muncke, J. In Vitro Toxicity Testing of Food Contact Materials: State-of-the-Art and Future Challenges. Comprehensive Reviews in Food Science and Food Safety 2017, 293, 463, doi:10.1111/1541-4337.12280.

7. 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, doi:10.1080/19440049.2019.1664772.

8. Verordnung über Materialien und Gegenstände, die dazu bestimmt sind mit Lebensmitteln in Berührung zu kommen und zur Aufhebung der Richtlinien 80/590/EWG und 89/109/EWG: EU 1935/2004, 2004.

9. 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. EU 10/2011 (Jan. 14, 2011).

10. Koster, S.; Bani-Estivals MH; Bonuomo M; Bradley E; Chagnon MC; Garcia ML; Godts F; Gude T; Helling R; Paseiro-Losada P; et al. 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.

11. Koster, S.; Rennen, M.; Leeman, W.; Houben, G.; Muilwijk, B.; van Acker, F.; Krul, L. A novel safety assessment strategy for non-intentionally added substances (NIAS) in carton food contact materials. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 2014, 31, 422–443, doi:10.1080/19440049.2013.866718.

12. Rennen, M.A.J.; Koster, S.; Krul, C.A.M.; Houben, G.F. Application of the threshold of toxicological concern (TTC) concept to the safety assessment of chemically complex food matrices. Food Chem. Toxicol. 2011, 49, 933–940, doi:10.1016/j.fct.2010.12.017.

13. Veyrand, J.; Marin-Kuan, M.; Bezencon, C.; Frank, N.; Guérin, V.; Koster, S.; Latado, H.; Mollergues, J.; Patin, A.; Piguet, D.; et al. Integrating bioassays and analytical chemistry as an improved approach to support safety assessment of food contact materials. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 2017, 34, 1807–1816, doi:10.1080/19440049.2017.1358466.

14. EFSA; WHO. Review of the Threshold of Toxicological Concern (TTC) approach and development of new TTC decision tree. EFS3 2016, 13, 893, doi:10.2903/sp.efsa.2016.EN-1006.

15. Kroes, R.; Renwick, A.G.; Cheeseman, M.; Kleiner, J.; Mangelsdorf, I.; Piersma, A.; Schilter, B.; Schlatter, J.; van Schothorst, F.; Vos, J.G.; et al. Structure-based thresholds of toxicological concern (TTC): Guidance for application to substances present at low levels in the diet. Food and Chemical Toxicology 2004, 42, 65–83, doi:10.1016/j.fct.2003.08.006.

16. ICH Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential potential carcinogenic risk: ICH M7, 2014.

17. OECD guideline for the testing of chemicals: In vitro mammalian chromosomal abberation test: OECD 473, 2016.

18. OECD Guideline for Testing of Chemicals, Bacterial Reverse Mutation Test; OECD 471; OECD: Paris, France, 2020.

19. EFSA Scientific Committee. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFS2 2011, 9, doi:10.2903/j.efsa.2011.2379.

20. Parry, J.M.; Parry, E.M. Genetic Toxicology; Springer New York: New York, NY, 2012, ISBN 978-1-61779-420-9.

21. Kirkland, D.; Reeve, L.; Gatehouse, D.; Vanparys, P. A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. Mutat. Res. 2011, 721, 27–73, doi:10.1016/j.mrgentox.2010.12.015.

22. Genetic Toxicology Testing: A Laboratory Manual; Proudlock, R., Ed., 2016.

23. van der Linden, Sander C; von Bergh, Anne R M; van Vught-Lussenburg, Barbara M A; Jonker, L.R.A.; Teunis, M.; Krul, C.A.M.; van der Burg, B. Development of a panel of high-throughput reporter-gene assays to detect genotoxicity and oxidative stress. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 2014, 760, 23–32, doi:10.1016/j.mrgentox.2013.09.009.

24. Andrew Knight, Chris Hughes and Matthew Tate. Gentronix Limited, Manchester, UK. BlueScreen HC - A Luminescence Based, High-Throughput, In Vitro Genotoxicity Assay.

25. 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. Mutation Research/Genetic Toxicology 1996, 369, 129–145, doi:10.1016/S0165-1218(96)90021-X.

26. Takigami, H.; Matsui, S.; Matsuda, T.; Shimizu, Y. The Bacillus subtilis rec-assay: a powerful tool for the detection of genotoxic substances in the water environment. Prospect for assessing potential impact of pollutants from stabilized wastes. Waste Management 2002, 22, 209–213, doi:10.1016/s0956-053x(01)00071-x.

27. Ames, B.N.; Durston, W.; Yamasaki, E.; Lee, F.D. Carcinogens are Mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Nat. Acad. Sci. USA 1973, 2281–2285, doi:10.1073/pnas.70.8.2281.

28. Ames, B.N.; Lee D., F.; Durston E., W. An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens. Proc. Nat. Acad. Sci. 1973, 782–786.

29. Mortelmans, K.; Zeiger, E. The Ames Salmonella/microsome mutagenicity assay. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 2000, 455, 29–60, doi:10.1016/S0027-5107(00)00064-6.

30. Agurell, E.; Stensman, C. Salmonella mutagenicity of three complex mixtures assayed with the microsuspension technique. A WHO/IPCS/CSCM study. Mutation Research/Reviews in Genetic Toxicology 1992, 276, 87–91, doi:10.1016/0165-1110(92)90057-G.

31. 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 migrate. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 2018, 1–14, doi:10.1080/19440049.2018.1519259.

32. 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, doi:10.1080/19440049.2019.1634841.

33. Proudlock, R.; Evans, K. The micro-Ames test: A direct comparison of the performance and sensitivities of the standard and 24-well plate versions of the bacterial mutation test. Environ. Mol. Mutagen. 2016, 57, 687–705, doi:10.1002/em.22065.

34. Flückiger-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, doi:10.1016/j.mrgentox.2012.03.014.

35. Spiliotopoulos, D.; Koelbert, C. Assessment of the miniaturized liquid Ames microplate format (MPF™) for a selection of the test items from the recommended list of genotoxic and non-genotoxic chemicals. Mutat. Res. 2020, 856-857, 503218, doi:10.1016/j.mrgentox.2020.503218.

36. Williams, R.V.; DeMarini, D.M.; Stankowski, L.F.; Escobar, P.A.; Zeiger, E.; Howe, J.; Elespuru, R.; Cross, K.P. Are all bacterial strains required by OECD mutagenicity test guideline TG471 needed? Mutat. Res. 2019, 848, 503081, doi:10.1016/j.mrgentox.2019.503081.

37. Helmut Bartsch, Christian Malaveille and Ruggero Montesano. In Vitro Metabolism and Microsome-mediated Mutagenicity of Dialkylnitrosamines in Rat, Hamster, and Mouse Tissues. Cancer Research 1975.

38. Levy, D.D.; Zeiger, E.; Escobar, P.A.; Hakura, A.; van der Leede, B.-J.M.; Kato, M.; Moore, M.M.; Sugiyama, K.-I. Recommended criteria for the evaluation of bacterial mutagenicity data (Ames test). Mutat. Res. Genet. Toxicol. Environ. Mutagen. 2019, 848, 403074, doi:10.1016/j.mrgentox.2019.07.004.

39. Xenometrix. Ames MPF™ 98/100 Microplate Format Mutagenicity Assay, Instructions for use 2015.

40. 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, doi:10.3390/foods9020237.

41. Pinter, E.; Friedl, C.; Irnesberger, A.; Czerny, T.; Piwonka, T.; Peñarroya, A.; Tacker, M.; Riegel, E. HepGentox: a novel promising HepG2 reportergene-assay for the detection of genotoxic substances in complex mixtures. PeerJ 2021, 9, e11883, doi:10.7717/peerj.11883.

42. 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, doi:10.3390/toxics9070152.

43. Escobar, P.A.; Kemper, R.A.; Tarca, J.; Nicolette, J.; Kenyon, M.; Glowienke, S.; Sawant, S.G.; Christensen, J.; Johnson, T.E.; McKnight, C.; et al. Bacterial mutagenicity screening in the pharmaceutical industry. Mutat. Res. 2013, 752, 99–118, doi:10.1016/j.mrrev.2012.12.002.

44. Maron, D.M.; Ames, B.N. Revised methods for the Salmonella mutagenicity test. Mutat. Res. 1983, 173–215, doi:10.1016/0165-1161(83)90010-9.

45. Brooks, T.M.; Priston, R.A.; Wright, A.S.; Watson, W.P. Evaluation of modified bacterial mutagenicity assays for the genotoxicity testing of mineral oils. Mutagenesis 1995, 409–415, doi:10.1093/mutage/10.5.409.

46. Bridges, B.A. The fluctuation test. Arch. Toxicol. 1980, 46, 41–44, doi:10.1007/BF00361244.

47. Gee, P.; Sommers, C.H.; Melick, A.S.; Gidrol, X.M.; Todd, M.D.; Burris, R.B.; Nelson, M.E.; Klemm, R.C.; Zeiger, E. Comparison of responses of base-specific Salmonella tester strains with the traditional strains for identifying mutagens: the results of a validation study. Mutat. Res. 1998, 115–130, doi:10.1016/S1383-5718(97)00172-1.

48. Miller, J.E.; Vlasakova, K.; Glaab, W.E.; Skopek, T.R. A low volume, high-throughput forward mutation assay in Salmonella typhimurium based on fluorouracil resistance. Mutat. Res. 2005, 578, 210–224, doi:10.1016/j.mrfmmm.2005.05.023.

49. Aubrecht, J.; Osowski, J.J.; Persaud, P.; Cheung, J.R.; Ackerman, J.; Lopes, S.H.; Ku, W.W. Bioluminescent Salmonella reverse mutation assay: a screen for detecting mutagenicity with high throughput attributes. Mutagenesis 2007, 22, 335–342, doi:10.1093/mutage/gem022.

50. 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, doi:10.1016/j.mrgentox.2015.10.006.

51. Kenyon, M.O.; Cheung, J.R.; Dobo, K.L.; Ku, W.W. An evaluation of the sensitivity of the Ames assay to discern low-level mutagenic impurities. Regul. Toxicol. Pharmacol. 2007, 48, 75–86, doi:10.1016/j.yrtph.2007.01.006.

52. Zeiger, E. Mutagenicity of 42 chemicals in Salmonella. Environ. Mol. Mutagen. 1990, 16, doi:10.1002/em.2850160504.

53. Zeiger, E.; Anderson, B.; Haworth, S.; Lawlor, T.; Mortelmans, K. Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. Environ. Mol. Mutagen. 1992, 19, doi:10.1002/em.2850190603.

54. Eliopoulos, P.; Mourelatos, D.; Dozi-Vassiliades, J. Comparative study on Salmonella mutagenicity and on cytogenetic and antineoplastic effects induced by cyclophosphamide and 3-aminobenzamide in cells of three transplantable tumours in vivo. Mutation Research/Genetic Toxicology 1995, 342, 141–146, doi:10.1016/0165-1218(95)90023-3.

55. Ames, B.N.; Kammen, H.O.; Yamasaki, E. Hair Dyes Are Mutagenic: Identification of a Variety of Mutagenic Ingredients: (hair dyes as possible human carcinogens and mutagens/2,4-diaminoanisole/2,4-diaminotoluene). Proc. Nat. Acad. Sci. USA 1975, Vol. 72, 2423–2427, doi:10.1073/pnas.72.6.2423.

56. Kaden, D.A.; Hites, R.A.; Hilly, W.G. Mutagenicity of soot and associated polycyclic aromatic hydrocarbons to salmonella typhimurium. Cancer Research 1979, 4152–4159.

57. Hakura, A.; Suzuki, S.; Sawada, S.; Sugihara, T.; Hori, Y.; Uchida, K.; Kerns, W.D.; Sagami, F.; Motooka, S.; Satoh, T. Use of human liver S9 in the Ames test: Assay of three procarcinogens using human S9 derived from multiple donors. Regulatory Toxicology and Pharmacology 2003, 37, 20–27, doi:10.1016/S0273-2300(02)00024-7.

58. Nakanomyo, H.; Hirokam, M.; Shiraya, M. Mutagenicity Tests of Etoposide and Teniposide. The Journal of Toxicological Science 1986, 11, 301–310.

59. Green, M.H.L.; Murial, W.J.; Bridges, B.A. Use of a simplified fluctuation test to detect low levels of mutagens. Mutat. Res. 1976, 33–42, doi:10.1016/0165-1161(76)90077-7.

60. Blahová, M.; Lahitová, N.; Sokolík, J. Mutagenicity of 4-nitroquinoline N-oxide after its complexation with copper(II) 2-chlorophenoxyacetate: Brief Report. Polia Microbiology 1997, 42, 401–402, doi:10.1007/BF02816957.

61. Smith, C.; Payne, V.; Doolittle, D.J.; Debnath, A.K.; Lawlor, T.; Hansch, C. Mutagenic activity of a series of synthetic and naturally occurring heterocyclic amines in Salmonella. Mutation Research/Genetic Toxicology 1992, 279, 61–73, doi:10.1016/0165-1218(92)90267-4.

62. Wichard, J.D. In silico prediction of genotoxicity. Food Chem. Toxicol. 2017, 106, 595–599, doi:10.1016/j.fct.2016.12.013.

63. 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 2020, doi:10.14573/altex.2006201.

64. Buchinger, S.; Spira, D.; Bröder, K.; Schlüsener, M.; Ternes, T.; Reifferscheid, G. Direct coupling of thin-layer chromatography with a bioassay for the detection of estrogenic compounds: applications for effect-directed analysis. Anal. Chem. 2013, 85, 7248–7256, doi:10.1021/ac4010925.

65. 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, doi:10.1021/acs.est.9b00921.

66. A Bjorseth, G Eidsa, J Gether, L Landmark, M Moller. Detection of mutagens in complex samples by the Salmonella assay applied directly on thin-layer chromatography plates.

9. Full Text: Paper III

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.

toxics

MDPI

Article

Direct Comparison of the Lowest Effect Concentrations of Mutagenic Reference Substances in Two Ames Test Formats

Bernhard Rainer [1,*], Elisabeth Pinter [1], Lukas Prielinger [1], Chiara Coppola [1], Maricel Marin-Kuan [2], Benoit Schilter [2], Silvia Apprich [1] and Manfred Tacker [1]

[1] Department of Applied Life Sciences, University of Applied Sciences, FH Campus Wien, 1030 Vienna, Austria; elisabeth.pinter@fh-campuswien.ac.at (E.P.); lukas.prielinger@fh-campuswien.ac.at (L.P.); Chiara.Coppola@fh-campuswien.ac.at (C.C.); silvia.apprich@fh-campuswien.ac.at (S.A.); manfred.tacker@fh-campuswien.ac.at (M.T.)

[2] Nestle Research-Chemical Food Safety, 1000 Lausanne, Switzerland; maricel.marin-kuan@rdls.nestle.com (M.M.-K.); benoit.schilter@rdls.nestle.com (B.S.)

* Correspondence: bernhard.rainer@fh-campuswien.ac.at

Abstract: The Ames assay is the standard assay for identifying DNA-reactive genotoxic substances. Multiple formats are available and the correct choice of an assay protocol is essential for achieving optimal performance, including fit for purpose detection limits and required screening capacity. In the present study, a comparison of those parameters between two commonly used formats, the standard pre-incubation Ames test and the liquid-based Ames MPF™, was performed. For that purpose, twenty-one substances with various modes of action were chosen and tested for their lowest effect concentrations (LEC) with both tests. In addition, two sources of rat liver homogenate S9 fraction, Aroclor 1254-induced and phenobarbital/β-naphthoflavone induced, were compared in the Ames MPF™. Overall, the standard pre-incubation Ames and the Ames MPF™ assay showed high concordance (>90%) for mutagenic vs. non-mutagenic compound classification. The LEC values of the Ames MPF™ format were lower for 17 of the 21 of the selected test substances. The S9 source had no impact on the test results. This leads to the conclusion that the liquid-based Ames MPF™ assay format provides screening advantages when low concentrations are relevant, such as in the testing of complex mixtures.

Keywords: complex mixtures; mutagenicity; genotoxicity; Ames assay; food contact materials; bacterial reverse mutation; lowest effective concentration (LEC); S9 comparison

Citation: Rainer, B.; Pinter, E.; Prielinger, L.; Coppola, C.; Marin-Kuan, M.; Schilter, B.; Apprich, S.; Tacker, M. Direct Comparison of Lowest Effect Concentrations of Mutagenic Reference Substances in Two Ames Test Formats. *Toxics* **2021**, *9*, 152. https://doi.org/10.3390/toxics9070152

Academic Editor: Isabelle Severin

Received: 21 May 2021
Accepted: 25 June 2021
Published: 29 June 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

In multiple fields dealing with chemical safety, the Ames test plays an important role for the detection of DNA-reactive genotoxic substances (mutagens) and is recommended to be included as part of a battery of genetic toxicology tests by EFSA [1]. The fields of application also include environmental toxicology, where soil, air or water sample testing is concerned [2–5]. In addition, the detection of mutagenic impurities in pharmaceutical drugs, as outlined in the ICH M7 guideline [6,7], or in the frame of the development of novel medical products are major topics [8], requires the use of the Ames test. Further applications include food safety assessment [9], safety evaluation of packaging materials [10,11], testing of medical plant extracts [12] or testing materials of importance for the chemical industry such as mineral oils [13]. Overall, those areas raise a common issue, which is the need to assess the mutagenicity of low-level contaminants potentially present in complex mixtures.

The lowest effect concentration (LEC) achieved in the assay, reflecting the limit of detection of mutagens, is the key attribute of the test to address this challenge. Indeed, it has to be low enough to meet regulatory/safety requirements and this in the presence of

complex sample matrices, which may interfere with the test results. In this context, the LEC refers to the lowest measured concentration of a mutagenic substance that causes a measurable effect on the test bacteria strains. Together with a (hypothetical) concentration factor that can be achieved during sample preparation, LEC values can be converted into limits of biological detection (LOBD) of the test procedure, which refers to the lowest concentration of a substance that can be detected in a sample [10].

1.1. Relevance for Packaging Safety Assessment

Previous investigations [14–16] focused on the applicability of in vitro genotoxicity assays for packaging migrate safety assessment. Packaging migrates are typical complex mixtures which could contain low levels of genotoxic chemicals. It was found that the Ames test is currently the most appropriate in vitro bioassay to address the challenges of direct DNA-reactive substances potentially migrating from food contact materials (FCM) into product simulants. Its use has been recommended by an expert group of the International Life Science Institute [10] as part of a comprehensive safety assessment strategy. Compared to other in vitro tests based on mammalian cells, the Ames test exhibits several advantages, such as lower LEC values [14,16] for most substances, possibly resulting from tolerance to higher solvent concentrations [17,18].

1.2. Ames Test Protocols and S9 Selection

Different Ames tests formats are available with potential impact on LECs/LOBDs for mutagenic substances [10]. However, the question regarding the most suitable Ames test protocol for detecting very low concentrations of direct DNA-reactive genotoxic contaminants is still open. The initial version of the assay, based on agar media and Petri dishes, is still widely considered the standard format, as it is recommended for regulatory testing and is part of the OECD guideline No 471 [19]. Over the years, many miniaturized formats have emerged [20–25]. Most of them still use agar-based media and rely on counting revertant colonies, while new approaches based on respiratory activity measurement [26] are currently being developed. In this context, the Ames MPF™ assay, a liquid incubation format followed by a colorimetric readout, has been promoted as an alternative. This type of liquid incubation assay has been widely applied for testing pharmaceutical substances [7] and herbal formulations [27]. Moreover, recent results showing the feasibility of screening small volumes of FCM migrate samples [15], prompted a detailed look at this version of the assay.

Therefore, the present study compares the LEC values of two Ames formats, namely the pre-incubation standard Petri dish agar Ames test and the Ames MPF™ test. The question of concordance in terms of sensitivity/specificity of these formats was previously addressed [24,28]. However, the performance of these test protocols in terms of achievable LECs/LOBDs has never been directly compared. The LEC refers to the lowest measured concentration of the testing substance able to induce the growth of revertant colonies at equal or higher levels to the threshold established for each bacterial strain according the spontaneous revertant colonies of the solvent control. For this purpose, both test protocols were performed in parallel with 21 chemicals, and the results were compared.

Other than the assay protocol itself, another factor that could theoretically affect the LECs is the metabolic activation system. The production of the most commonly used S9 from Aroclor 1254-induced rat liver homogenate is being phased out, since the production of polychlorinated biphenyls was banned in the late 1970s [29] and stocks are now running out. An efficient and comparable alternative is essential to provide reliable test results in the long term. There are several replacement products on the market, however phenobarbital/β-naphthoflavone (PB/βNF)-induced S9 in particular stands out as a potential promising candidate. To determine the impact of changing the metabolic activation system on the bacterial response and therefore on the LEC values, several Ames-positive test substances were tested with different sources of S9 fractions.

2. Materials and Methods

2.1. Test Substances, Chemicals and Reagents

Twenty substances classified as mutagenic were analysed for the comparison of the LEC values of the standard pre-incubation Petri dish agar-based Ames and the Ames MPF™. They were mainly selected from the EU Reference Laboratory for Alternatives to Animal Testing (ECVAM) list of recommended chemicals [30]. Another substance, namely melamine, was included as an Ames negative substance and is a known non-genotoxic carcinogen classified as ECVAM category III. Additional substances, which are not part of the ECVAM list, were included to cover other properties, such as higher volatility (formaldehyde) or the interference of coloured substances with the colour shift of the MPF medium (acridine orange). Standard positive control substances (e.g., 2-aminoanthracene or 2-nitrofluorene) were included to allow for an easy comparison of the results with the data of other laboratories, due to general availability. Lastly, two packaging-related substances (phenylglycidyl ether and triglycidyl isocyanurate) as well as a weak positive substance, with a tendency to cause cytotoxic effects and precipitation in higher concentrations during dose-finding experiments (benzo[a]anthracene), were included. All test substances were dissolved and diluted in DMSO. Information about the supplier and purity of the substance is listed in Table 1. Reagents for the Ames MPF™ Assay, namely the exposure and indicator media, were supplied by Xenometrix (Allschwil, Switzerland). For the standard pre-incubation Petri dish agar-based Ames, the protocol by Proudlock [31] was followed and all chemicals were obtained from Carl Roth (Karlsruhe, Germany), except for nutrient broth No 2, which was purchased from Thermo Fisher (Waltham, MA, USA).

Table 1. List of test substances used for the direct LEC comparison as well as the S9 comparison.

Chemical	Abbreviation	CAS No.	Purity [%]	Supplier	Selection Criteria/Mode of Action
2,4-Diaminotoluene	DAT	95-80-7	99.5	SCB [1]	Aromatic amine, requires metabolic activation [29]
2-Acetylaminofluorene	2AAF	53-96-3	≥98	Sigma Aldrich	Hydroxylated by CYP1A2and then acetylated. Forms C^8 adduct on guanine [29]
2-Amino-3-methylimidazol[4,5-f]quinoline	IQ	76180-96-6	98	SCB [1]	Heterocyclic amine with potent genotoxicity, requires metabolic activation [29]
4-Nitroquinoline 1-oxide	4NQO	56-57-5	≥98	Sigma Aldrich	Alkylating agent, forms DNA adducts [29]
Aflatoxin B1	AfB1	1162-65-8	≥98	Fermentek	Activated by CYP3A4. Forms various adducts [29]
Benzo[a]pyrene	BaP	50-32-8	≥96	Sigma Aldrich	Requires metabolic activation (CYP 1A1, 1B1, epoxide hydrolase), forms bulky adduct [29]
Cisplatin	CP	15663-27-1	n.s.	Sigma Aldrich	Cross-linking agent [29]
Cyclophosphamide monohydrate	Cyclo	6055-19-2	≥97	SCB [1]	Requires metabolic activation (CYP2B6) [29]
Melamine	Mel	108-78-1	99	Sigma Aldrich	Ames negative, causes bladder and ureteral carcinomas [29]
Methyl methanesulfonate	MMS	66-27-3	99	Sigma Aldrich	Strong clastogen (N^7 alkylation) [29]
N-ethyl-*N*-nitrosourea	NEU	759-73-9	56	SCB [1]	Strong gene mutagen (O^6 alkylation) [29]
2-Aminoanthracene	2AA	613-13-8	96	Carl Roth	Positive control, activated mainly by CYP1A2, DNA binding [32]
2-Aminofluorene	2AF	153-78-6	98	Sigma Aldrich	Positive control, formation of C^8-AF adducts [33]
2-Nitrofluorene	2NF	607-57-8	>99	TCI [2]	Positive control, adduct formation [34]
N4-Aminocytidine	N4ACT	57294-74-3	≥95	SCB [1]	Positive control, DNA incorporation, AT to GC transition [35]
Sodium azide	SA	26628-22-8	≥99.5	Sigma Aldrich	Positive control, A.T to G.C base pair transition and transversion [36]

Formaldehyde	FM	50-00-0	37	SCB [1]	Volatile, N-hydroxymethyl mono-adducts on guanine, adenine and cytosine, N-methylene crosslinks [37]
Acridine Orange	AO	494-38-2	n.s.	SCB [1]	Strong coloring agent, DNA intercalation [38]
Benzo[a]anthracene	BAA	56-55-3	99	Sigma Aldrich	S9 Weak positive, adduct formation, oxidative DNA damage [39]
Phenylglycidyl ether	PGE	204-557-2	99	Sigma Aldrich	Packaging related [14]
Triglycidyl isocyanurate	TIC	2451-62-9	≥98	SCB[1]	Packaging related [14]

[1] SCB: Santa Cruz Biotechnology, [2] TCI: Tokyo Chemical Industry, n.s.: not specified by the supplier.

2.2. Test Strains and Pre-Culture

Two strains were used for the present study: *Salmonella typhimurium* TA98 and TA100, which were supplied by Xenometrix AG. They were grown in an environmental shaker at 250 rpm in Nutrient broth No.2 (Thermo Fisher) with 50 μg/mL ampicillin, until they reached an OD_{600} of 2–2.5 measured with a UV/VIS spectrometer Lambda 265 (Perkin Elmer, Waltham, MA, USA). For the strain TA100, the overnight cultures were pre-screened to test whether the spontaneous background reversion rate was in an acceptable range, according to the Ames MPF™ protocol.

2.3. Metabolic Activation

For the comparison test runs, the induced rat liver post-mitochondrial supernatant S9 fractions from phenobarbital/β-naphthoflavone (PB/βNF) and 1254 aroclor (both purchased from Xenometrix) were used. The co-factors were prepared according to Proudlock, 2016 [31]: 5 mM glucose-6-phosphate, 4 mM NADP, 8 mM $MgCl_2$, 33 mM KCl in 100 mM sodium phosphate buffer at a pH of ~7.4.

2.4. Test Conditions for the Direct Comparison

The following testing workflow (outlined in Figure 1) was chosen to minimise sources of external variation and to ensure a direct comparison is possible: All dilutions were performed in half-logarithmic steps (factor 3.16) and eight concentrations were applied in both assays. The results were scored and documented at the same time point (after 42–54 h). For an assay to be considered valid, the spontaneous revertant background and the positive control response had to be within the confirmed reported range.

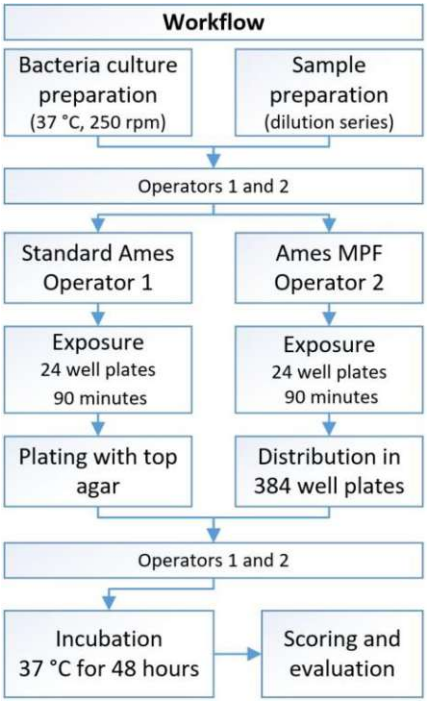


Figure 1. Visualization of the workflow for the comparison of the different Ames protocols.

2.5. Ames MPF™ Test Protocol

The Ames MPF™ test protocol was performed, according to the method's supplier protocol (Xenometrix), with minor adaptations. As solvent control, DMSO was applied. As a positive control, for TA98 without S9 50 µg/mL 2-nitrofluorene (2NF), for TA100 without S9 2.5 µg/mL 4-nitroquinoline-1-oxide (4NQO) and for TA98 and TA100 with S9 50 µg/mL 2-aminoanthracene (2AA) was applied. Exposures were performed in triplicates in 24-well plates and 10 µL of the test substance or the controls were used per well. The pre-culture was mixed with exposure medium (10% bacteria *v*/*v* for TA98 and 5% *v*/*v* for TA100) and then 240 µL of this mix was added to each well. After 90 min of incubation at 37 °C at 250 rpm in an orbital shaker, 2.6 mL of indicator medium (Xenometrix) were added. The content of the 24-well plates was distributed into three 384-well plates. For the metabolic activation, a 15% S9 mix was prepared and kept on ice, until use and consisted of either PB/β-NF, or Aroclor 1254-induced rat liver S9 and the co-factor mix (see metabolic activation section). The 15% S9 and the co-factor mix were added as required, resulting in a final concentration of 2.25% S9 during the exposure.

2.6. Agar-Based Ames Test Protocol

The pre-incubation Petri dish agar-based Ames test protocol was conducted according to the methods described by [31], with minor adaptations. The bacteria were grown as described above and the exposure was done in 24-well plates, in triplicates, containing 100 µL of pre-culture, 500 µL of phosphate buffer (0.2 M, pH 7.4) and 50 µL of the test substance dissolved in DMSO. For the negative control, pure DMSO was applied. As a positive control, for TA98 without S9 50 µg/mL 2NF, for TA100 without S9 2.5 µg/mL 4NQO and for TA98/100 with S9 25 µg/mL 2AA were applied. After 90 min of exposure the mixture was pipetted into 2 mL molten top agar (5 µM histidine and biotin), which was melted and kept at 48 °C in a water bath. The agar was then poured onto Petri dishes containing histidine free minimal glucose agar (MGA; 0.4% glucose). For the metabolic activation, a 1% S9 mix was prepared and kept on ice, until use. It consisted of PB/β-NF-induced rat liver S9 and the co-factor mix (see chapter metabolic activation). The 1% S9 and the co-factor mix were applied instead of the phosphate buffer as required, resulting in a final concentration of 0.77% S9 during the exposure.

2.7. Scoring Criteria and Interpretation

The following scoring criteria were applied for both assays: The mean of the solvent control plus one standard deviation was multiplied by a factor of two. This established 2×-factor was set as a positive threshold and test concentrations, for which the mean of revertant/positive wells surpassed this threshold, were considered positive. Toxicity was routinely assessed by checking the background lawn as well as any colour change or bubble formation for the Ames MPF™ protocol.

2.7.1. Statistical Analysis

To test whether the mean LECs of the assay formats, or the mean LECs that were obtained with two different S9 sources, are significantly different from each other a statistical analysis was conducted. For this purpose a paired sample *t*-test was performed. In order to achieve normal distribution, the LEC values were transformed to their decadic logarithm. Substances for which the assays yielded non-concordant results were excluded.

3. Results

3.1. Concordance of the Assay Results

The concordance of the positive/negative results was ~90% (19/21 test chemicals). However, two test items yielded discordant results: sodium azide (SA) tested negative in the Ames MPF™, but positive in the standard pre-incubation Petri dish agar-based Ames test (top sample concentration: 25,000 µg/mL, toxicity was observed at higher doses). Incubation with benzo[a]anthrazene (BAA) did not produce a positive test result in the standard pre-incubation Petri dish agar-based Ames test, but tested positive in the Ames MPF™ (top sample concentration: up to 5000 µg/mL, precipitation was observed after adding buffer at the highest dose). The following substances yielded discordant results in only one strain: 2-nitrofluorene (2NF) tested negative in the Ames MPF™ with TA100 – S9, 2-acetylaminofluorene (2AAF) tested negative in the standard pre-incubation Petri dish agar-based Ames test in TA100 + S9 and benzo[a]pyrene (BaP) tested negative in TA100 + S9 in the standard pre-incubation Petri dish agar-based Ames test.

3.2. Direct LEC Comparisons

Overall, the 20 standard substances, as well as melamine as negative control, were tested and their LEC values determined in the *Salmonella* strains TA98 and TA100 in both the Ames MPF™ assay and the standard pre-incubation Petri-dish agar-based formats. The mean results of two test runs are listed in Table 2. A more detailed table, which includes the top concentrations for each test run, is provided in the annex (Table A1).

Table 2. Comparison of the Ames MPF™ protocol with the standard pre-incubation Petri-dish agar-based Ames test. Each substance was tested twice with the same dilution series and pre-culture. The concentration in µg/mL refers to the substance concentration during the exposure step. Each substance was tested in eight concentrations in half logarithmic dilution steps. (**a**) test results without metabolic activation, (**b**) results including metabolic activation with PB/ßNP induced S9.

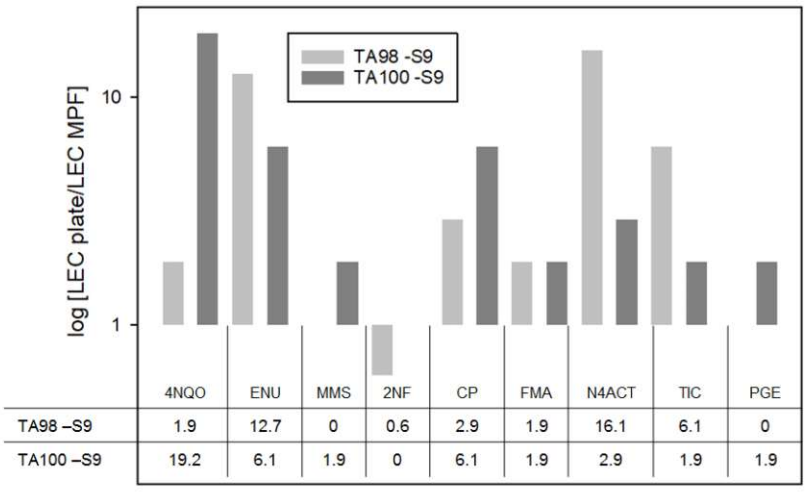
(a)					
Substance	**CAS**	**Strain TA98 – S9 [µg/mL]**		**Strain TA100 – S9 [µg/mL]**	
		Plate	**MPF**	**Plate**	**MPF**
4NQO	56-57-5	0.08	0.04	0.08	0.004
ENU	759-73-9	320	25	154	25
MMS	66-27-3	–	–	77	40
2NF	607-57-8	0.38	1	12	–
CP	15663-27-1	12	4	8	1
FMA	50-00-0	8	4	12	6
SA	26628-22-8	–	–	1.2	–

N4ACT	57294-74-3	67	4	0.012	0.0042
Mel	108-78-1	–	–	–	–
TIC	2451-62-9	127	21	192	100
PGE	204-557-2	–	–	12	6

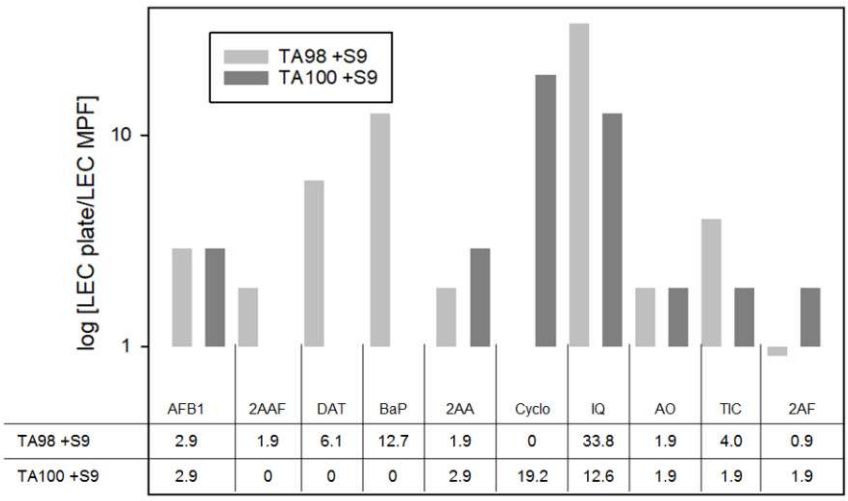
(**b**)

Substance	CAS	Strain TA98 + S9 [µg/mL]		Strain TA100 + S9 [µg/mL]	
		Plate	MPF	Plate	MPF
AFB1	1162-65-8	0.0025	0.0008	0.0077	0.0026
2AAF	53-96-3	0.38	0.2	–	2
DAT	95-80-7	160	26	–	–
BaP	50-32-8	3	0.2	–	0.64
2AA	613-13-8	0.02	0.01	0.2	0.1
Cyclo	6055-19-2	–	–	689	36
IQ	76180-96-6	0.001	0.00002	0.08	0.006
AO	494-38-2	0.19	0.1	1.92	1
Mel	108-78-1	–	–	–	–
TIC	2451-62-9	127	31.6	192	100
2AF	153-78-6	0.038	0	0.8	0.4
BAA	56-55-3	–	35	–	2

For 81% of the substances (17 out of 21), the arithmetic mean of two independent test runs of the Ames MPF™ yielded lower LEC values, in terms of µg/mL concentration during the incubation, than the standard pre-incubation Petri-dish agar plate Ames test. The mean LEC values for nine out of 11 substances were at least five times lower. Relative differences, for substances which led to positive results in both assay formats, are displayed in Figure 2. Examples of dose response curves, which result in major differences, are shown in Figure 3. The results of both individual test runs can be found in the annex (see annex, Table A1). A statistical analysis of the LEC values for the two assays was conducted including all test runs with concordant results (paired sample *t*-test) and resulted in a highly significant difference ($p < 0.001$). Due to the importance of the LOBD, the overall LECs were also compared in terms of sample concentration (LEC × 25 for the Ames MPF assay and LEC × 13 for the plate Agar assay, see Table A2) and likewise showed highly significant differences ($p < 0.001$). This indicates, that the LEC values obtained with the MPF assay were significantly lower compared to those of the standard pre-incubation petri dish assay and would translate in lower LOBDs.



(**a**)



	AFB1	2AAF	DAT	BaP	2AA	Cyclo	IQ	AO	TIC	2AF
TA98 +S9	2.9	1.9	6.1	12.7	1.9	0	33.8	1.9	4.0	0.9
TA100 +S9	2.9	0	0	0	2.9	19.2	12.6	1.9	1.9	1.9

(**b**)

Figure 2. Relative differences in LECs between the Ames MPF and the standard pre-incubation Petri-dish agar-based Ames test are shown in logarithmic scale. The factor was calculated by dividing the mean LEC of the agar-based Ames test by the mean LEC of the Ames MPF. Therefore, a higher factor means a better performance of the Ames MPF assay. (**a**) Test results without metabolic activation, (**b**) results including metabolic activation with PB/ßNP induced S9.

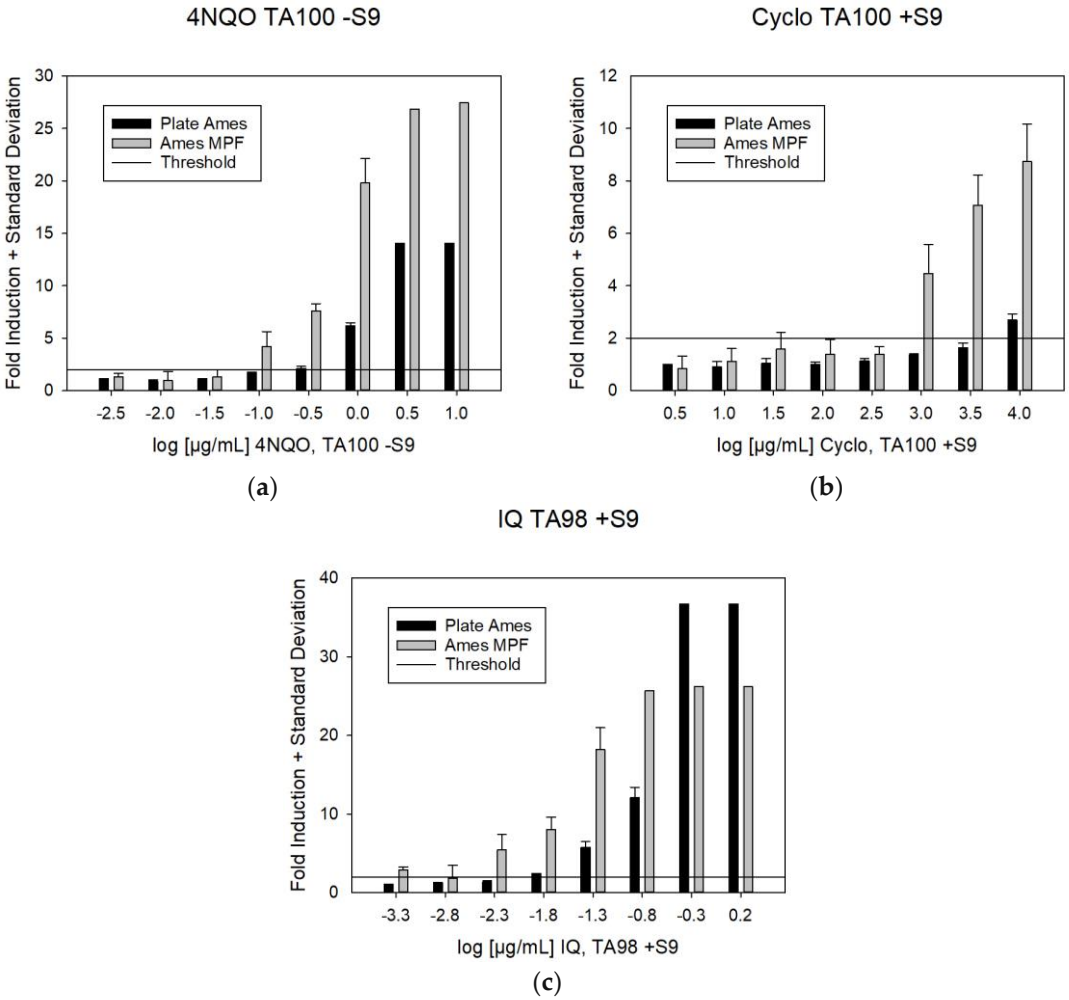
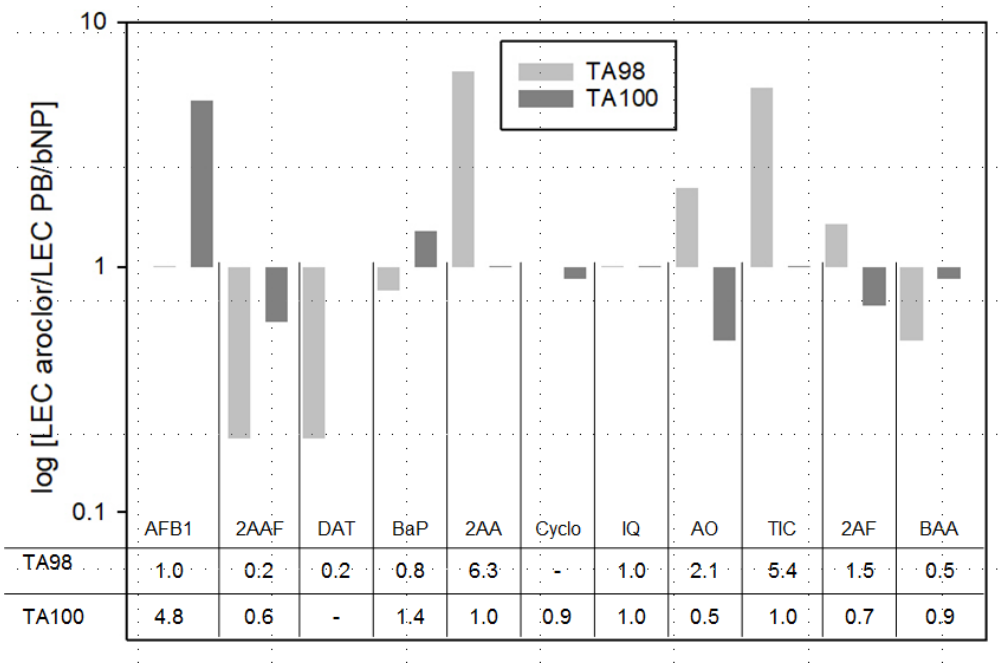


Figure 3. Fold induction of the dose-response testing illustrating differences of dose-responses across test protocols. The bar charts show example results for the dose response curves, obtained with the Ames MPF™ and the standard pre-incubation Petri-dish agar-based Ames assay. The line across the chart indicates the positive threshold, which refers to a

two-fold induction over the mean negative control results, including one standard deviation. (**a**) 4NQO tested with TA100, without metabolic activation, (**b**) Cyclo tested with TA100 with metabolic activation and (**c**) IQ tested in TA98 with metabolic activation.

3.3. S9-Source Comparison

Eleven substances requiring metabolic activation were tested in the Ames MPF™ assay with both Aroclor 1254 and PB/β-NF-induced rat liver S9 fractions. The relative differences in the obtained LECs are shown in Figure 4. All substances tested positive with both Aroclor 1254 and PB/β-NF-induced S9. As expected, slightly different LEC values were obtained for individual substances in the two groups, however statistical analysis revealed no significant overall difference ($p = 0.65$). More detailed information on the individual test runs and exact LECs are shown in the Appendix A (Table A3).



	AFB1	2AAF	DAT	BaP	2AA	Cyclo	IQ	AO	TIC	2AF	BAA
TA98	1.0	0.2	0.2	0.8	6.3	-	1.0	2.1	5.4	1.5	0.5
TA100	4.8	0.6	-	1.4	1.0	0.9	1.0	0.5	1.0	0.7	0.9

Figure 4. Relative differences in LECs for eleven substances tested with either Aroclor 1254 or PB/β-NP-induced S9, in the Ames MPF™ assay, are shown in logarithmic scale. The factor was calculated by dividing the mean LEC of the Aroclor 1254-induced S9 by the mean LEC of the PB/β-NP-induced S9.

4. Discussion

4.1. Assay Concordance

While previous publications addressed the issue of assay concordance with a wider range of test substances, the findings in our study, an overall concordance of 90%, align well with previous results [24,28]. Specifically, only 2/21 substances yielded discordant results, namely sodium azide (SA) and benzo[a]anthracene (BAA). The discordance of the results for SA, might be due to a higher bioavailability in the liquid media. The substance is not only mutagenic in bacteria, but also commonly used as an antimicrobial agent, which works by binding to heme-iron (e.g., cytochrome oxidase). However, it is recommended by the OECD 471 guideline as a positive control substance for the strain TA100 [40]. BAA on the other hand showed a tendency during initial experiments, to cause only very weak positive results and increased toxicity, while precipitating in the highest concentrations. More narrow dilution steps, as well as an increase in S9-concentration might have allowed for the detection of this substance in the standard pre-incubation Petri-dish agar-based Ames assay.

4.2. LEC According to the Test Protocol

When comparing the LEC values for 20 mutagenic substances, it appears that most of them are detected at a lower concentration with the Ames MPF™ protocol. The mean LECs range from 0.6-fold (2NF) to 33.8-fold (IQ) lower in the Ames MPF™ protocol as compared to values obtained with the standard pre-incubation Petri-dish agar-based Ames protocol. For 81% of the substances (17 out of 21), the Ames MPF™ protocol yielded lower LEC values and for 43% (9 out of 21) of the substances, the difference was at least 5-fold. Overall, these differences are statistically significant with a p-value < 0.001. A possible explanations for the lower LECs of the Ames MPF™ protocol could be (according to a publication by Xenometrix, [28]) either potential adsorption effects of the agar therefore reducing bacteria exposure in the standard version and/or an uneven distribution of the test chemicals during the incubation.

There is a significant difference in S9 mix concentration for the Ames MPF™ (2.25%) and the standard pre-incubation Petri-dish agar-based Ames exposure (0.77%). However, in preliminary experiments (data not shown) it was found that lower S9 concentrations yielded slightly better LECs for the agar-based version. Further, studies by Belser et al. [41] and Zeiger et al. [42] showed that less S9 led to an improved detection of BaP and 2AA at lower concentrations. However, they concluded that some substances present at higher concentrations were not as easily detected with less S9. In the study, the aim was to detect at low concentrations to obtain the lowest LECs, therefore it was concluded that the application of less S9 is more suitable.

4.3. S9 Fraction Comparison

The results of the S9 comparison (see Figure 4) indicate that both Aroclor 1254-induced, and PB/β-NF-induced rat liver S9 worked equally well. An older study that compared these S9-types, as well as the respective Cyp enzyme activities, came to a similar conclusion [43]. Overall, the differences that can be seen in the substance per substance response in Figure 4 can most likely be explained by varying Cyp activities. While the mean LEC values detected varied from 0.2-fold (2AF) up to 6.3-fold (2AA), the overall differences in the results were inconsistent and not statistically significant in term of overall LEC values (p = 0.65). It has to be mentioned, that for the purpose of this study only two batches of S9 were compared and both were subjected to quality control by the supplier. Previous studies have found that significant variations between different types of S9 products are possible [44,45]. However, the present dataset indicates that Aroclor 1254-induced S9, which will not be available anymore in the near future, can likely be adequately replaced by PB/β-NF induced S9, without any anticipated negative impact on the LEC values. In the long term an animal-free S9 source may become preferable [46]. While initial results looks highly promising [44], more data needs to be provided, before such material can be considered a valid alternative.

4.4. Implication for the LOBD

As already mentioned, the LOBD for the Ames test, refers to the substance concentration that can be detected in the sample. When comparing the sample concentrations instead of the concentration during the exposure step (in the incubation medium), the differences decrease, but the Ames MPF™ still yields significantly lower results (see Table A3). This type of comparison is however only relevant, when the sample quantities applied during the exposure (4%-Ames MPF and ~8%-standard pre-incubation Petri-dish agar-based Ames test) remain constant. The applicable sample concentration can vary widely, depending on the solvents and can reach up to 70% or more for protocols for water testing, such as in the Ames Aqua [47].

4.5. Practical Considerations

When it comes to the practical applicability, the Ames MPF™ protocol offers major advantages compared to the standard pre-incubation Petri dish agar-based Ames test: (i) The amount of sample material required is considerably lower (10 µL per data point for the Ames MPF™ protocol vs. at least 50 µL per data point for the standard pre-incubation Petri dish agar-based Ames test protocol). (ii) Lower amounts of S9 and other consumables are required. (iii) The handling time is much shorter and a single operator can handle at least twice as many samples in the same time.

For the detection of toxic effects of test substances or sample materials, the standard approach is to assess the growth of the bacterial background lawn [31]. While this is not possible for the Ames MPF™ version, colour changes and bubble formation can be an indicator for toxicity [28]. When sample toxicity is a concern, which is the case when testing FCM migrate samples, it has been suggested to use a spiking approach with a well characterized mutagen, a procedure easily applicable to the Ames MPF™ protocol [10,15]. It has to be acknowledged that the procedure can be applied in the standard pre-incubation Petri-dish agar-based Ames test as well, but would lead to an increased requirement in sample volume and material which often is not feasible with complex mixtures and especially for packaging migrants. The spiking approach could also detect other sources of inhibition than cytotoxicity in complex mixtures, since inhibitory effects that are based on other effects (e.g., adsorption on matrix particles) could also be detected.

A disadvantage for the Ames MPF™ assay is the limited number of wells scored, precisely 48 per data point. This results in a non-linear response when the revertant count increases, since multiple events (mutations) can occur in a single well. Therefore, a slight increase in background reversion rate, mostly with TA100, can have an impact on the assay performance, which also negatively affects the LEC and the LOBD. This makes pre-screening of the bacteria pre-cultures for low spontaneous reversion rates a useful tool, in particular when reproducible and low LEC values are of importance.

4.6. Relevance for FCM Safety Assessment

According to the threshold of toxicological concern concept (TTC) [48], the suggested acceptable limit of direct DNA reactive mutagenic substances is 0.15 µg/L in the migrate sample. This limit is very conservative and poses a challenge, for both chemical analytical methods and bio-detection approaches [10]. When comparing this to the LEC results, as shown in Table 2, it can be seen that only the two most potent substances, namely IQ and AFB1, could be picked up at such low levels. Specifically, these are highly potent mutagens, which cannot be expected to occur in complex mixtures, such as packaging samples under realistic conditions. However, in a previous publication it was demonstrated that the Ames MPF™ assay is capable to detect mutagenic activity under realistic conditions in FCM migrate samples [15]. Alternative approaches have been proposed combining chemical and bioassays solutions as high performance thin-layer chromatography (HPTLC). Most probably a breakthrough improvement of LOBDs would require a very different test system design such as the coupling of bioassay with high performance thin-layer chromatography [49–51]. Finally, when considering the fact that the Ames MPF™ has not only practical advantages, but also provides significantly lower LEC values and LOBDs, its use might be preferred over the standard pre-incubation Petri-dish agar-based Ames test. However, the Ames MPF™ is still not the ideal solution, since further improvements must be made to allow for a more consistent and sensitive detection of low levels of mutagenic contamination, in order to fulfil regulatory requirements.

5. Conclusions

- According to the conditions and data analysis applied, the LEC values of the Ames MPF™ assay are significantly lower when compared to the LEC values obtained with

the standard pre-incubation Petri-dish agar-based Ames protocol. This is expected to result in lower LOBDs for mutagens in complex mixtures.

- In addition to LEC values, the choice of assay protocol should be based on regulatory requirements as well as technical considerations such as availability of sample material and consumables required.
- The use of either Aroclor 1254-induced S9 or PB/β-NF-induced S9 has no major impact on LEC values.
- The assay protocols show a concordance of over 90% for the set of test chemicals that were chosen for this study.
- Safety assessment of packaging migrate material: Neither protocol can consistently detect DNA reactive substances at a concentration range of 0.15 µg/kg, a limit which is derived from the TTC concept for substances with alert for mutagenicity. More research is needed to achieve such low a level of detection.

Based on the present comparison study, it can be concluded that the Ames MPF™ assay is a suitable approach for screening samples for low concentrations of genotoxic substances. This is of importance, when assessing complex mixtures, such as packaging samples, for low-level contaminations.

Author Contributions: Conceptualization, B.R. and E.P.; Investigation, B.R., E.P., L.P. and C.C.; Supervision, S.A. and M.T.; Writing–original draft, B.R.; Writing–review & editing, E.P., M.M.-K., B.S., S.A. and M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research is funded by the FFG—The Austrian Research Promotion Agency, as part of the project MIGRATOX (project number: 866854).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Acknowledgments: We would like to thank Thomas Czerny and Elisabeth Riegel for their highly valuable inputs for the data analysis and data interpretation. We also thank Mary Wallis for proof reading of the draft.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Table A1. Comparison of individual results of two independent test runs in the Ames MPF™ protocol with the standard agar-based Ames test. The concentration in µg/mL represents the concentration during the exposure step. The top dose is presented as µg/mL in the sample. Each substance was tested in eight concentrations in half logarithmic dilution steps.

Strain TA98-Tests Run without Metabolic Activation (−S9)								
Substance	CAS	Run 1 [µg/mL]		Top Dose [µg/mL]	Run 2 [µg/mL]		Top Dose [µg/mL]	Factor
		Plate	MPF		Plate	MPF		
4NQO	56-57-5	0.08	0.04	10	0.08	0.04	10	1.9
ENU	759-73-9	487	25	20,000	154	25	20,000	12.7
MMS	66-27-3	–	–	10,000	–	–	10,000	–
2NF	607-57-8	0.4	0.6	5000	0.4	0.6	500	0.6
CP	15663-27-1	12	2	5000	12	6	500	2.9
FMA	50-00-0	12	6	5000	4	2	5000	1.9
SA	26628-22-8	–	–	5000	–	–	25,000	–
N4ACT	57294-74-3	12	6	5000	122	2	5000	16.1
Mel	108-78-1	–	–	25,000	–	–	25,000	–
TIC	2451-62-9	192	10	25,000	61	32	7906	6.1

PGE	204-557-2	–	–	5000	–	–	5000	–
Strain TA100-Tests Run without Metabolic Activation (–S9)								
Substance	**CAS**	**Run 1 [µg/mL]**		**Top Dose [µg/mL]**	**Run 2 [µg/mL]**		**Top Dose [µg/mL]**	**Factor**
		Plate	**MPF**		**Plate**	**MPF**		
4NQO	56-57-5	0.08	0.004	10	0.077	0.004	10	19.2
ENU	759-73-9	154	25	20,000	154	25	20,000	6.1
MMS	66-27-3	77	40	10,000	77	40	10,000	1.9
2NF	607-57-8	12	–	5000	12	–	500	–
CP	15663-27-1	3.8	0.6	5000	12	2	500	6.1
FMA	50-00-0	12	6	5000	12	6	5000	1.9
SA	26628-22-8	0.38	–	5000	1.9	–	25,000	–
N4ACT	57294-74-3	0.012	0.006	5	0.012	0.002	5	2.9
Mel	108-78-1	–	–	25,000	–	–	25,000	–
TIC	2451-62-9	192	100	25,000	192	100	25,000	1.9
PGE	204-557-2	12	6	5000	12	6	5000	1.9
Strain TA98-Tests Run with Metabolic Activation (+S9)								
Substance	**CAS**	**Run 1 [µg/mL]**		**Top Dose [µg/mL]**	**Run 2 [µg/mL]**		**Top Dose [µg/mL]**	**Factor**
		Plate	**MPF**		**Plate**	**MPF**		
AFB1	1162-65-8	0.0025	0.0004	10	0.0025	0.0013	1	2.9
2AAF	53-96-3	0.38	0.2	500	0.38	0.20	500	1.9
DAT	95-80-7	77	40	10,000	243	13	10,000	6.1
BaP	50-32-8	1.2	0.2	5000	3.8	0.2	500	12.7
2AA	613-13-8	0.024	0.013	10	0.024	0.013	10	1.9
Cyclo	6055-19-2	–	–	10000	–	–	10,000	–
IQ	76180-96-6	0.0012	0.000019	15	0.00012	0.000019	0.15	33.8
AO	494-38-2	0.19	0.10	2500	0.19	0.10	250	1.9
Mel	108-78-1	–	–	25,000	–	–	7906	–
TIC	2451-62-9	61	32	25,000	192	32	7906	4.0
2AF	153-78-6	0.038	0.020	50	0.038	0.063	50	0.9
BAA	56-55-3	–	6	5000	–	63	5000	–
Strain TA100-Tests Run with Metabolic Activation (+S9)								
Substance	**CAS**	**Run 1 [µg/mL]**		**Top Dose [µg/mL]**	**Run 2 [µg/mL]**		**Top Dose [µg/mL]**	**Factor**
		Plate	**MPF**		**Plate**	**MPF**		
AFB1	1162-65-8	0.008	0.004	1	0.0077	0.0013	1	2.9
2AAF	53-96-3	–	2	5000	–	2	5000	–
DAT	95-80-7	–	–	10,000	–	–	10,000	
BaP	50-32-8	–	0.64	5000	–	0.64	500	–
2AA	613-13-8	0.24	0.13	10	0.24	0.04	10	2.9
Cyclo	6055-19-2	769	40	10,000	608	32	25,000	19.2
IQ	76180-96-6	0.036	0.006	15	0.115	0.006	15	12.6
AO	494-38-2	1.9	1.0	250	1.9	1.0	250	1.9
Mel	108-78-1	–	–	25,000	–	–	7906	–
TIC	2451-62-9	192	100	25,000	192	100	25,000	1.9
2AF	153-78-6	0.385	0.632	50	1.2	0.2	50	1.9
BAA	56-55-3	–	2	5000	–	2	5000	–

Table A2. Comparison of individual results of two independent test runs in the Ames MPF™ protocol with the standard agar-based Ames test. The concentration in µg/mL represents the substance in the sample. Each substance was tested in eight concentrations in half logarithmic dilution steps.

Strain TA98-Tests Run without Metabolic Activation (–S9)								
Substance	**CAS**	**Run 1 [µg/mL]**		**Top Dose**	**Run 2 [µg/mL]**		**Top Dose**	**Factor**
		Plate	**MPF**	**[µg/mL]**	**Plate**	**MPF**	**[µg/mL]**	
4NQO	56-57-5	1	1	10	1	1	10	1.0
ENU	759-73-9	6325	632.5	20,000	2000	632.5	20,000	6.6
MMS	66-27-3	–	–	10,000	–	–	10,000	–
2NF	607-57-8	5	15.8	5000	5	15.8	500	0.3
CP	15663-27-1	158.1	50	5000	158.1	158.1	500	1.5
FMA	50-00-0	158.1	158.1	5000	50	50	5000	1.0
SA	26628-22-8	–	–	5000	–	–	25,000	–
N4ACT	57294-74-3	158.1	158.1	5000	1581.1	50	5000	8.4
Mel	108-78-1	–	–	25,000	–	–	25,000	–
TIC	2451-62-9	2500	250	25,000	791	791	7906	3.2
PGE	204-557-2	–	–	5000	–	–	5000	–
Strain TA100-Tests Run without Metabolic Activation (–S9)								
Substance	**CAS**	**Run 1 [µg/mL]**		**Top Dose**	**Run 2 [µg/mL]**		**Top Dose**	**Factor**
		Plate	**MPF**	**[µg/mL]**	**Plate**	**MPF**	**[µg/mL]**	
4NQO	56-57-5	1	0.1	10	1	0.1	10	10.0
ENU	759-73-9	2000	632.5	20,000	2000	632.5	20,000	3.2
MMS	66-27-3	1000	1000	10,000	1000	1000	10,000	1.0
2NF	607-57-8	158.1	–	5000	158.1	–	500	–
CP	15663-27-1	50	15.8	5000	158.1	50	500	3.2
FMA	50-00-0	158.1	158.1	5000	158.1	158.1	5000	1.0
SA	26628-22-8	5	–	5000	25	–	25,000	–
N4ACT	57294-74-3	0.158	0.158	5	0.158	0.05	5	1.5
Mel	108-78-1	–	–	25,000	–	–	25,000	–
TIC	2451-62-9	2500	2500	25,000	2500	2500	25,000	1.0
PGE	204-557-2	158.1	158.1	5000	158.1	158.1	5000	1.0
Strain TA98-Tests Run with Metabolic Activation (+S9)								
Substance	**CAS**	**Run 1 [µg/mL]**		**Top Dose**	**Run 2 [µg/mL]**		**Top Dose**	**Factor**
		Plate	**MPF**	**[µg/mL]**	**Plate**	**MPF**	**[µg/mL]**	
AFB1	1162-65-8	0.032	0.01	10	0.032	0.032	1	1.5
2AAF	53-96-3	5	5	500	5	5	500	1.0
DAT	95-80-7	1000	1000	10,000	3162	316	10,000	3.2
BaP	50-32-8	15.8	5	5000	50	5	500	6.6
2AA	613-13-8	0.316	0.316	10	0.316	0.316	10	1.0
Cyclo	6055-19-2	–	–	10,000	–	–	10,000	–
IQ	76180-96-6	0.015	0.00047	15	0.0015	0.00047	0.15	17.6
AO	494-38-2	2.5	2.5	2500	2.5	2.5	250	1.0
Mel	108-78-1	–	–	25,000	–	–	7906	–
TIC	2451-62-9	791	791	25,000	2500	791	7906	2.1
2AF	153-78-6	0.5	0.5	50	0.5	1.581	50	0.5
BAA	56-55-3	–	158.1	5000	–	1581.1	5000	–
Strain TA100-Tests Run with Metabolic Activation (+S9)								
Substance	**CAS**	**Run 1 [µg/mL]**		**Top Dose**	**Run 2 [µg/mL]**		**Top Dose**	**Factor**
		Plate	**MPF**	**[µg/mL]**	**Plate**	**MPF**	**[µg/mL]**	
AFB1	1162-65-8	0.1	0.1	1	0.1	0.032	1	1.5
2AAF	53-96-3	–	50	5000	–	50	5000	–
DAT	95-80-7	–	–	10,000	–	–	10,000	–
BaP	50-32-8	–	16	5000	–	16	500	–
2AA	613-13-8	3.162	3.162	10	3.162	1	10	1.5
Cyclo	6055-19-2	10,000	1000	10,000	7906	791	25,000	10.0
IQ	76180-96-6	0.47	0.15	15	1.5	0.15	15	6.6

AO	494-38-2	25	25	250	25	25	250	1.0
Mel	108-78-1	–	–	25,000	–	–	7906	–
TIC	2451-62-9	2500	2500	25,000	2500	2500	25,000	1.0
2AF	153-78-6	5	15.811	50	15.811	5	50	1.0
BAA	56-55-3	–	50	5000	–	50	5000	–

Table A3. Individual results of two independent test runs in the Ames MPF assay with PB/ßNP-induced S9 vs. Aroclor 1254-induced S9. LECs are presented in µg/mL in the exposure medium. The factor is calculated by dividing the mean LEC of the results obtained with Aroclor 1254 by the results obtained with PB/β-NP-induced S9.

TA98						
Substance	**CAS**	**Arclor 1254 LEC [µg/mL]**		**PB/ßNF LEC [µg/mL]**		**Factor**
		Run 1	**Run 2**	**Run 1**	**Run 2**	
AFB1	1162-65-8	0.0013	0.0004	0.0004	0.0013	1.0
2AAF	53-96-3	0.06	0.03	0.20	0.20	0.2
DAT	95-80-7	8	25	126	13	0.2
BaP	50-32-8	0.20	0.12	0.20	0.20	0.8
2AA	613-13-8	0.12	0.04	0.013	0.013	6.3
IQ	76180-96-6	0.000019	0.000019	0.000019	0.000019	1.0
AO	494-38-2	0.1	0.3	0.1	0.1	2.1
TIC	2451-62-9	316	25	32	32	5.4
2AF	153-78-6	0.06	0.06	0.02	0.06	1.5
BAA	56-55-3	20	13	6	63	0.5
TA 100						
Substance	**CAS**	**Arclor 1254 LEC [µg/mL]**		**PB/ßNF LEC [µg/mL]**		**Factor**
		Run 1	**Run 2**	**Run 1**	**Run 2**	
AFB1	1162-65-8	0.013	0.013	0.004	0.001	4.8
2AAF	53-96-3	0.25	2.00	2	2	0.6
BaP	50-32-8	1.0	0.2	0.6	0.6	0.9
2AA	613-13-8	0.13	0.31	0.13	0.04	2.6
Cyclo	6055-19-2	32	100	40	32	1.8
IQ	76180-96-6	0.006	0.006	0.006	0.006	1.0
AO	494-38-2	0.32	0.32	1	1	0.3
TIC	2451-62-9	100	100	100	100	1.0
2AF	153-78-6	0.2	0.6	0.6	0.2	1.0
BAA	56-55-3	1.6	1.6	2	2	0.8

References

1. EFSA. Recent developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials. *EFSA J.* **2016**, *14*, 4357, doi:10.2903/j.efsa.2016.4357.
2. Claxton, L.D.; Umbuzeiro, G.d.A.; DeMarini, D.M. The Salmonella mutagenicity assay: The stethoscope of genetic toxicology for the 21st century. *Environ. Health Perspect.* **2010**, *118*, 1515–1522, doi:10.1289/ehp.1002336.
3. Umbuzeiro, G.d.A.; Rech, C.M.; Correia, S.; Bergamasco, A.M.; Cardenette, G.H.L.; Flückiger-Isler, S.; Kamber, M. Comparison of the Salmonella/microsome microsuspension assay with the new microplate fluctuation protocol for testing the mutagenicity of environmental samples. *Environ. Mol. Mutagen.* **2010**, *51*, 31–38, doi:10.1002/em.20504.
4. Verschaeve, L. Genotoxicity studies in groundwater, surface waters, and contaminated soil. *Sci. World J.* **2002**, *2*, 1247–1253, doi:10.1100/tsw.2002.338.
5. Reifferscheid, G.; Maes, H.M.; Allner, B.; Badurova, J.; Belkin, S.; Bluhm, K.; Brauer, F.; Bressling, J.; Domeneghetti, S.; Elad, T.; et al. International round-robin study on the Ames fluctuation test. *Environ. Mol. Mutagen.* **2012**, *53*, 185–197, doi:10.1002/em.21677.
6. ICH. *ICH Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Potential Carcinogenic Risk*: *ICH M7*; ICH: Geneva, Switzerland, 2014.
7. Escobar, P.A.; Kemper, R.A.; Tarca, J.; Nicolette, J.; Kenyon, M.; Glowienke, S.; Sawant, S.G.; Christensen, J.; Johnson, T.E.; McKnight, C.; et al. Bacterial mutagenicity screening in the pharmaceutical industry. *Mutat. Res.* **2013**, *752*, 99–118, doi:10.1016/j.mrrev.2012.12.002.
8. International Organization for Standardization. *ISO10993-3. Biological Evaluation of Medical Devices—Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity*; International Organization for Standardization: Geneva, Switzerland, 2003.

9. EFSA Scientific Committee. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA J.* **2011**, *9*, 2379, doi:10.2903/j.efsa.2011.2379.
10. 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, doi:10.1080/19440049.2019.1664772.
11. Bolognesi, C.; Castoldi, A.F.; Crebelli, R.; Barthélémy, E.; Maurici, D.; Wölfle, D.; Volk, K.; Castle, L. Genotoxicity testing approaches for the safety assessment of substances used in food contact materials prior to their authorization in the European Union. *Environ. Mol. Mutagen.* **2017**, *58*, 361–374, doi:10.1002/em.22094.
12. Sponchiado, G.; Adam, M.L.; Silva, C.D.; Soley, B.S.; de Mello-Sampayo, C.; Cabrini, D.A.; Correr, C.J.; Otuki, M.F. Quantitative genotoxicity assays for analysis of medicinal plants: A systematic review. *J. Ethnopharmacol.* **2016**, *178*, 289–296, doi:10.1016/j.jep.2015.10.026.
13. Mackerer, C.R.; Griffis, L.C.; Grabowski, J.S.; Reitman, F.A. Petroleum mineral oil refining and evaluation of cancer hazard. *Appl. Occup. Environ. Hyg.* **2003**, *18*, 890–901, doi:10.1080/10473220390237467.
14. 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 migrate. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **2018**, 1–14, doi:10.1080/19440049.2018.1519259.
15. 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, doi:10.1080/19440049.2019.1634841.
16. 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, doi:10.3390/foods9020237.
17. Timm, M.; Saaby, L.; Moesby, L.; Hansen, E.W. Considerations regarding use of solvents in in vitro cell based assays. *Cytotechnology* **2013**, *65*, 887–894, doi:10.1007/s10616-012-9530-6.
18. Hakura, A.; Mochida, H.; Yamatsu, K. Dimethyl sulfoxide (DMSO) is mutagenic for bacterial mutagenicity tester strains. *Mutat. Res. Lett.* **1993**, *303*, 127–133, doi:10.1016/0165-7992(93)90025-Q.
19. OECD. *OECD Guideline for Testing of Chemicals, Bacterial Reverse Mutation Test*; OECD 471; OECD: Paris, France, 2020.
20. Diehl, M.S.; Willaby, S.L.; Snyder, R.D. Comparison of the results of a modified miniscreen and the standard bacterial reverse mutation assays. *Environ. Mol. Mutagen.* **2000**, *36*, 72–77, doi:10.1002/1098-2280(2000)36:1<72:AID-EM10>3.0.CO;2-Y.
21. Flückiger-Isler, S.; Baumeister, M.; Braun, K.; Gervais, V.; Hasler-Nguyen, N.; Reimann, R.; van Gompel, J.; Wunderlich, H.-G.; Engelhardt, G. Assessment of the performance of the Ames II assay: A collaborative study with 19 coded compounds. *Mutat. Res.* **2004**, *558*, 181–197, doi:10.1016/j.mrgentox.2003.12.001.
22. Miller, J.E.; Vlasakova, K.; Glaab, W.E.; Skopek, T.R. A low volume, high-throughput forward mutation assay in Salmonella typhimurium based on fluorouracil resistance. *Mutat. Res.* **2005**, *578*, 210–224, doi:10.1016/j.mrfmmm.2005.05.023.
23. Aubrecht, J.; Osowski, J.J.; Persaud, P.; Cheung, J.R.; Ackerman, J.; Lopes, S.H.; Ku, W.W. Bioluminescent Salmonella reverse mutation assay: A screen for detecting mutagenicity with high throughput attributes. *Mutagenesis* **2007**, *22*, 335–342, doi:10.1093/mutage/gem022.
24. Flückiger-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, doi:10.1016/j.mrgentox.2012.03.014.
25. Proudlock, R.; Evans, K. The micro-Ames test: A direct comparison of the performance and sensitivities of the standard and 24-well plate versions of the bacterial mutation test. *Environ. Mol. Mutagen.* **2016**, *57*, 687–705, doi:10.1002/em.22065.
26. Kauffmann, K.; Werner, F.; Deitert, A.; Finklenburg, J.; Brendt, J.; Schiwy, A.; Hollert, H.; Büchs, J. Optimization of the Ames RAMOS test allows for a reproducible high-throughput mutagenicity test. *Sci. Total Environ.* **2020**, *717*, 137168, doi:10.1016/j.scitotenv.2020.137168.
27. Chandrasekaran, C.V.; Sundarajan, K.; David, K.; Agarwal, A. In vitro efficacy and safety of poly-herbal formulations. *Toxicol. In Vitro* **2010**, *24*, 885–897, doi:10.1016/j.tiv.2009.11.021.
28. Spiliotopoulos, D.; Koelbert, C. Assessment of the miniaturized liquid Ames microplate format (MPF™) for a selection of the test items from the recommended list of genotoxic and non-genotoxic chemicals. *Mutat. Res.* **2020**, *856–857*, 503218, doi:10.1016/j.mrgentox.2020.503218.
29. National Oceanic and Atmospheric Administration. What Are PCBs? Available online: https://oceanservice.noaa.gov/facts/pcbs.html (accessed on 22 April 2021).
30. 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, doi:10.1016/j.mrgentox.2015.10.006.
31. Proudlock, R. (Ed.) *Genetic Toxicology Testing*: *A Laboratory Manual*; Elsevier: London, UK, 2016.
32. Veres, Z.; Török, G.; Tóth, E.; 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. doi:10.1016/j.mrgentox.2005.05.009.

33. Feng, Y.; Jiang, W.; Hein, D.W. 2-Aminofluorene-DNA Adduct Levels in Tumor-Target and Nontargetorgans of Rapid and Slow Acetylator Syrian Hamsters Congenic at the NAT2 Locus. *Toxicol. Appl. Pharmacol.* **1996**, *141*, 248–255. doi:10.1016/S0041-008X(96)80030-5.
34. Beije, B.; Möller, L. 2-nitrofluorene and related compounds: Prevalence and biological effects. *Mutat. Res./Rev. Genet. Toxicol.* **1988**, *196*, 177–209, doi:10.1016/0165-1110(88)90019-x.
35. Negishi, K.; Takahashi, M.; Yamashita, Y.; Nishizawa, M.; Hayatsu, H. Mutagenesis by N4-aminocytidine: Induction of AT to GC transition and its molecular mechanism. *Biochemistry* **1985**, *24*, 7273–7278, doi:10.1021/bi00346a038.
36. Khan, S.; Al-Quirainy, F.; Anwar, F. Sodium azide: A chemical mutagen for enhancement of agronomic traits of crop plants. *Environ. We Int. J. Sci. Technol.* **2009**, *2009*, 1–21.
37. Kawanishi, M.; Matsuda, T.; Yagi, T. Genotoxicity of formaldehyde: Molecular basis of DNA damage and mutation. *Front. Environ. Sci.* **2014**, *2*, 173, doi:10.3389/fenvs.2014.00036.
38. Armstrong, R.W.; Kurucsev, T.; Strauss, U.P. The interaction between acridine dyes and deoxyribonucleic acid. *J. Am. Chem. Soc.* **1970**, *92*, 3174–3181, doi:10.1021/ja00713a041.
39. Seike, K.; Murata, M.; Oikawa, S.; Hiraku, Y.; Hirakawa, K.; Kawanishi, S. Oxidative DNA damage induced by benzaanthracene metabolites via redox cycles of quinone and unique non-quinone. *Chem. Res. Toxicol.* **2003**, *16*, 1470–1476, doi:10.1021/tx034103h.
40. Winter, C.; Kerros, M.-E.; Weinbauer, M.G. Effects of sodium azide on the abundance of prokaryotes and viruses in marine samples. *PLoS ONE* **2012**, *7*, e37597, doi:10.1371/journal.pone.0037597.
41. Belser, W.L.; Shaffer, S.D.; Bliss, R.D.; Hynds, P.M.; Yamamoto, L.; Pitts, J.N.; Winer, J.A. A standardized procedure for quantification of the ames salmonella/mammalian-microsome mutagenicity test. *Environ. Mutagen.* **1981**, *3*, 123–139, doi:10.1002/em.2860030204.
42. Zeiger, E.; Chhabra, R.S.; Margolin, B.H. Effects of the hepatic S9 fraction from aroclor-1254-treated rats on the mutagenicity of benzo[a]pyrene and 2-aminoanthracene in the salmonella/microsome assay. *Mutat. Res.* **1979**, *64*, 379–389, doi:10.1016/0165-1161(79)90108-0.
43. Callander, R.D.; Mackay, J.M.; Clay, P.; Elcombe, C.R.; Elliott, B.M. Evaluation of phenobarbital/beta-naphthoflavone as an alternative S9-induction regime to Aroclor 1254 in the rat for use in in vitro genotoxicity assays. *Mutagenesis* **1995**, *10*, 517–522, doi:10.1093/mutage/10.6.517.
44. Brendt, J.; Crawford, S.E.; Velki, M.; Xiao, H.; Thalmann, B.; Hollert, H.; Schiwy, A. Is a liver comparable to a liver? A comparison of different rat-derived S9-fractions with a biotechnological animal-free alternative in the Ames fluctuation assay. *Sci. Total Environ.* **2021**, *759*, 143522, doi:10.1016/j.scitotenv.2020.143522.
45. Hakura, A.; Suzuki, S.; Sawada, S.; Sugihara, T.; Hori, Y.; Uchida, K.; Kerns, W.D.; Sagami, F.; Motooka, S.; Satoh, T. Use of human liver S9 in the Ames test: Assay of three procarcinogens using human S9 derived from multiple donors. *Regul. Toxicol. Pharmacol.* **2003**, *37*, 20–27, doi:10.1016/S0273-2300(02)00024-7.
46. Shao, Y.; Schiwy, A.; Glauch, L.; Henneberger, L.; König, M.; Mühlenbrink, M.; Xiao, H.; Thalmann, B.; Schlichting, R.; Hollert, H.; et al. Optimization of a pre-metabolization procedure using rat liver S9 and cell-extracted S9 in the Ames fluctuation test. *Sci. Total Environ.* **2020**, *749*, 141468, doi:10.1016/j.scitotenv.2020.141468.
47. ISO. *ISO 11350:2012. Water Quality—Determination of the Genotoxicity of Water and Waste Water—Salmonella/Microsome Fluctuation Test (Ames Fluctuation Test)*; ISO: Geneva, Switzerland, 2012.
48. Kroes, R.; Renwick, A.G.; Cheeseman, M.; Kleiner, J.; Mangelsdorf, I.; Piersma, A.; Schilter, B.; Schlatter, J.; van Schothorst, F.; Vos, J.G.; et al. Structure-based thresholds of toxicological concern (TTC): Guidance for application to substances present at low levels in the diet. *Food Chem. Toxicol.* **2004**, *42*, 65–83, doi:10.1016/j.fct.2003.08.006.
49. 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 Altern. Anim. Exp.* **2020**, doi:10.14573/altex.2006201.
50. 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, doi:10.1021/acs.est.9b00921.
51. Stütz, L.; Leitner, P.; Schulz, W.; Winzenbacher, R. Identification of genotoxic transformation products by effect-directed analysis with high-performance thin-layer chromatography and non-target screening. *J. Planar Chromatogr. Modern TLC* **2019**, *32*, 173–182, doi:10.1556/1006.2019.32.3.1.