Sense Ames – A Miniaturized Ames Test with Superior Detection Limits

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Abstract. The Ames test is a widely used *in vitro* bioassay for the assessment of direct DNA-reactive effects. However, the limits of biological detection (LOBD) for many genotoxic substances are not sufficient to reach the threshold of toxicological concern (TTC) limit, which is 0.15 µg per person/day for food contact materials (FCMs). The Ames test has been improved over the years in terms of lower sample volumes, higher throughput and achieving lower LOBD-values for many genotoxins. The liquid media-based Ames MPFTM is already an improvement over the agar-based plate Ames test. The Ames MPFTM is a fluctuation assay performed in liquid media. Instead of counting colonies as indication for mutagenicity, DNA reactivity is assessed visually through a colour change in the indicator medium. However, despite the improved sensitivity the Ames MPFTM offers, the desired TTC threshold has still not been reached for many genotoxins (Rainer et al., 2021). An in-house developed liquid media-based Ames test, called Sense Ames, was modified to detect genotoxins at even lower LOBD and with lower sample volumes. In this study, ten recycled plastic extracts were tested with a *Salmonella* Typhimurium strain TA98 with and without metabolic activation (+/–S9) and the performance of two Ames test formats (Ames MPFTM vs. Sense Ames) was compared by their lowest effect concentration (LEC) values. In six out of ten samples, the Sense Ames showed lower LEC values than the Ames MPFTM. Therefore, the Sense Ames is a promising bioassay for reaching lower LOBD values compared to other existing Ames test formats and an important step towards achieving the desired TTC threshold for genotoxins.

Keywords: Sense Ames, Ames MPFTM, miniaturized Ames test, *in vitro* bioassays, genotoxins, toxicology, TTC concept, safety assessment, plastics, recycling, extracts

Introduction

In order to use recycled plastics in food contact materials (FCMs), they must comply with EU regulations 1935/2004 (EU, 2004), 10/2011 (EU, 2011) and 2022/1616 (EU, 2022). FCMs are materials or articles which are intended to come into contact with food, typically used as packaging materials such as plastics. To assess the potential mutagenic activity of non-intentionally added substances (NIAS) in recycled plastics, *in vitro* bioassays such as the Ames test play a crucial role in complementing chemical analysis (EFSA, 2019; Schilter et al., 2019). The Plate Ames test according to OECD 471 guideline (OECD, 2020) has been modified and improved over the years, for example though the development of the Ames MPFTM from Xenometrix AG (Xenometrix, 2018). These advantages include reduced sample volume, lower LEC values, and higher throughput (Rainer et al., 2021). The LEC (lowest effective concentration) value is defined as the lowest concentration at which a measurable positive result is detectable in the bioassay. The LOBD (limit of biological detection) value describes the lowest concentration of an analyte that can be reliably detected in a bioassay-based method, meaning it can be distinguished from a blank (Schilter et al., 2019). Another in-house developed Ames test, called Sense Ames, has been optimized for the analysis of pure mutagenic reference substances and recycled plastic extracts. The Sense Ames has been established as a novel miniaturized version of the Ames test, which is optimized for the analysis of pure substances and migrates. Additionally, it offers improved detection limits, operates with lower sample volumes and fully automated analysis when compared with both the agar-based plate Ames test and the Ames MPFTM (Xenometrix, 2018).

The aim of this study is to compare the performance of the Sense Ames with the Ames MPFTM by testing ten recycled plastic extracts and comparing their LEC values.

Materials and Methods

Ten recycled plastic extracts were tested including samples which were not compliant with EU regulation 2022/1616 (EU, 2022). For the sample preparation, migration simulations were carried out based on EU regulation 10/2011 (EU, 2011) and Rainer et al. (2019). The samples were then tested with the Ames MPFTM and Sense Ames. The Ames MPFTM was performed according to the Ames MPFTM protocol from Xenometrix (Xenometrix, 2018). A sample volume of 10 µl was used, limited by the tolerance of DMSO. As the Ames test makes use of *Salmonella* bacteria, it is necessary to avoid any

conditions or chemicals impairing bacterial viability. DMSO is the solvent used to prepare dilutions with, but at higher concentrations, DMSO is toxic for bacteria, affecting the overall performance of the assay (Rainer et al., 2018). For the Sense Ames, an optimized protocol, which is only available to the authors, was used. The sample volume used was 2 µl. For the experiments, the bacterial strain *Salmonella Typhimurium* TA98 with and without metabolic activation (+/–S9) was used, as most of the recycled plastic samples only showed positive results with the strain TA98 with metabolic activation (+S9) (Mayrhofer et al., 2023). All samples were tested with both Ames variants and samples which exceeded the positive threshold were classified as mutagenic. The presented dilutions are expressed as percentages (%) as it makes comparison with related literature on mutagenicity assessment easier. It also improves the clarity of the results produced, as it gives an immediate overview of the concentrations at which mutagenic activity was observed.

Results

In Table 1, an overview of the results with Ames MPFTM and Sense Ames with bacterial strain TA98 with and without metabolic activation (+/–S9) can be seen, these results are also depicted in Figure 1. Two samples (RP5 and RP8) showed negative results in both test conditions with the Ames MPFTM and Sense Ames. Using the bacteria strain TA98 without metabolic activation (–S9), all ten samples tested negative with the Ames MPFTM. Using the Sense Ames, the samples RP4 (Figure 1e) and RP6 showed a weak positive result without metabolic activation (–S9). Samples RP1 (Figure 1a) and RP3 only tested positive with the Sense Ames with TA98 with metabolic activation (+S9). Six samples showed positive results in both Ames test variations. Four samples showed positive results in both Ames test formats, but the Sense Ames reached lower LEC values. As an example, sample RP10 was positive until 25 % dilution in the Ames MPFTM and positive until 6.25 % in the Sense Ames (Figure 1d).

Table 1: Overview of the Ames MPFTM and Sense Ames results of ten recycled plastic extracts. Samples were tested with bacterial strain TA98 without and with metabolic activation (+/– S9). The column "Result" shows the measured effect for mutagenicity. A "+" indicates a positive result for mutagenicity and a "-" indicates a negative result. The column "LEC" shows the lowest dilution of the extract with a positive result. Author's own representation based on experimental study generated in this study.

Sample	TA98 –S9				TA98 +S9			
	Ames MPF		Sense Ames		Ames MPF		Sense Ames	
	Result	LEC	Result	LEC	Result	LEC	Result	LEC
RP1	–	–	–	–	–	–	+	50 %
RP2	–	–	–	–	+	100 %	+	50 %
RP3	–	–	–	–	–	–	+	100 %

Sample	TA98 –S9				TA98 +S9			
	Ames MPF		Sense Ames		Ames MPF		Sense Ames	
	Result	LEC	Result	LEC	Result	LEC	Result	LEC
RP4	–	–	+	100%	+	0.39%	+	0.39%
RP5	–	–	–	–	–	–	–	–
RP6	–	–	+	50%	+	6.25%	+	12.5%
RP7	–	–	–	–	+	25%	+	12.5%
RP8	–	–	–	–	–	–	–	–
RP9	–	–	–	–	+	6.25%	+	3.13%
RP10	–	–	–	–	+	25%	+	6.25%

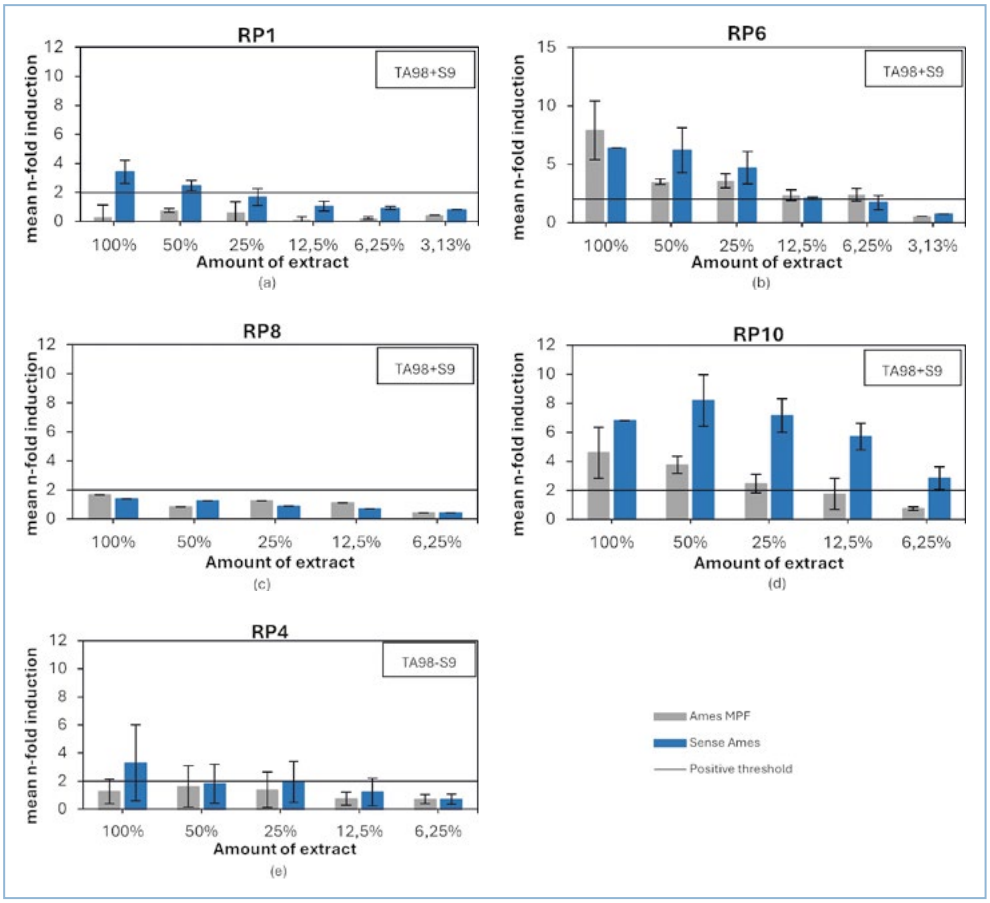

Figure 1: Results of five recycled plastic extracts tested with the Ames MPFTM and Sense Ames analysed with TA98 +S9 and TA98 -S9. The samples RP1 (a), RP6 (b), RP8 (c), RP10 (d) and RP4 (e) are shown. The x-axis provides information about the dilution (from 100% meaning undiluted sample, until 3,13% dilution with DMSO) while the y-axis gives information about the mean n-fold induction (representing a mutagenic event). The horizontal line indicates a positive threshold level, above which a result is deemed mutagenic.

Discussion

The Sense Ames is a highly promising bioassay for the screening of direct DNA-reactive effects in recycled plastic extracts. The results have shown that the Sense Ames generally achieves lower LOBD values than the Ames MPFTM. The Sense Ames also provides several other advantages, including smaller sample volume (2 µl instead of 10 µl) and less manual work. All samples that tested positive for mutagenicity in the Ames MPF™ were confirmed, and two samples that tested negative in the Ames MPFTM were positive in the Sense Ames. Therefore, the Sense Ames facilitates improvements in reducing the number of false negatives, potentially advancing the safety assessment of recycled plastics. The Sense Ames was also able to detect more positive samples without metabolic activation (–S9), meaning that the risk of inhibition causing false negative results is lower than with the Ames MPFTM. The Sense Ames is an effect-based bioassay for hazard identification that ideally can be used in combination with chemical analysis for the identification of unknown mutagenic substances for example in post-consumer recycled (PCR) plastic materials (Koster et al., 2015; Mayrhofer et al., 2023; Prielinger et al., 2024).

References

EFSA (2019). Guidance on the use of the threshold of toxicological concern approach in food safety assessment. *EFSA Journal*, *17*(6), e05708. https://doi.org/10.2903/j.efsa.2019.5708

Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food Text with EEA relevance, L 12/1, Document 32011R0010 (2011). https://eur-lex.europa.eu/eli/reg/2011/10/oj/eng (retrieval date 01/12/2025)

Commission Regulation (EU) 2022/1616 on recycled plastic materials and articles intended to come into contact with foods, and repealing Regulation (EC) No 282/2008 (2022). https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32022R1616 (retrieval date 01/12/2025)

Koster, S., Bani-Estivals, M. H., Bonuomo, M., Bradley, E., Chagnon, M. C., García, M. L., Godts, F., Gude, T., Helling, R., Paseiro-Losada, P., Pieper, G., Rennen, M., Simat, T., & Spack, L. (2015). *Guidance on best practices on the risk assessment of non-intentionally added substances (NIAS) in food contact materials and articles.* ILSI Europe, D/2015/10.996/39. https://ilsi.org/europe/wp-content/uploads/sites/3/2016/04/2015-NIAS_version-January-2016.pdf (retrieval date 01/12/2025)

Mayrhofer, E., Prielinger, L., Sharp, V., Rainer, B., Kirchnawy, C., Rung, C., Gruner, A., Juric, M., & Springer, A. (2023). Safety assessment of recycled plastics from post-consumer waste with a combination of a miniaturized Ames test and chromatographic analysis. *Recycling*, *8*(6), 87.

OECD (2020). *Test no. 471: Bacterial reverse mutation test. OECD guidelines for the testing of chemicals, section 4.* Paris: OECD Publishing. https://doi.org/10.1787/9789264071247-en

Prielinger, L., Bandyopadhyay, S., Ortner, E., Novak, M., Radusin, T., Annfinsen, S., Sharmin, N., Rainer, B., & Pettersen, M. K. (2024). Investigation of the effectiveness of barrier layers to inhibit mutagenic effects of recycled LDPE films, using a miniaturized Ames test and GC-MS analysis. *Recycling*, *9*(4), 57.

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

Rainer, B., Pinter, E., Czerny, T., Riegel, E., Kirchnawy, C., Marin-Kuan, M., Schilter, B., & Tacker, M. (2018). Suitability of the Ames test to characterise genotoxicity of food contact material migrates. *Food Additives & Contaminants: Part A*, *35*(11), 2230–2243. https://doi.org/10.1080/19440049.2018.1519259

Rainer, B., Pinter, E., Prielinger, L., Coppola, C., Marin-Kuan, M., Schilter, B., Apprich, S., & Tacker, M. (2021). Direct comparison of the lowest effect concentrations of mutagenic reference substances in two Ames test formats. *Toxics*, *9*(7), 152. https://doi.org/10.3390/toxics9070152

Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing directives 80/590/EEC and 89/109/EEC (2004). https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32004R1935&from=de (retrieval date 01/12/2025)

Schilter, B., Burnett, K., Eskes, C., Geurts, L., Jacquet, M., Kirchnawy, C., Oldring, P., Pieper, G., Pinter, E., Tacker, M., Traussnig, H., van Herwijnen, P., & Boobis, A. (2019). Value and limitation of in vitro bioassays to support the application of the threshold of toxicological concern to prioritise unidentified chemicals in food contact materials. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, *36*(12), 1903–1936. https://doi.org/10.1080/19440049.2019.1664772

Xenometrix (2018). *Ames MPF™ 98/100 microplate format mutagenicity assay.* Short protocol, Version 2.0. https://www.xenometrix.ch/shop/mediafiles/Xeno%20Dateien/Short%20Protocol/Ames/Ames%20MPF%2098-100%20Short%20Protocol_2.0.pdf (retrieval date June 12, 2025).