How low can you go?

An analysis of lowest effective dose in the Ames test.

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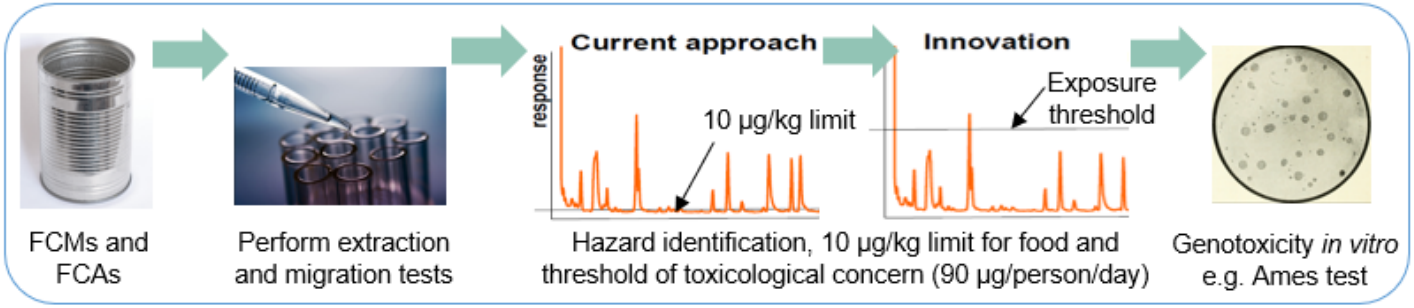
The Ames Test and the Food Contact Industry

The food industry requires risk assessments to be performed on all substances migrating from Food Contact Articles (FCAs) and Food Contact Materials (FCMs). This includes non-intentionally added substances (NIAS).



Performing in vitro testing may prove difficult due to:

- Insufficient sensitivity of the test methods at low doses.
- The small amount of migrate produced.

The Ames test is a sensitive assay and is one of the most common tests used for assessing the mutagenicity of impurities.
Is the conventional Ames test sufficiently sensitive to detect toxicity at very low doses?

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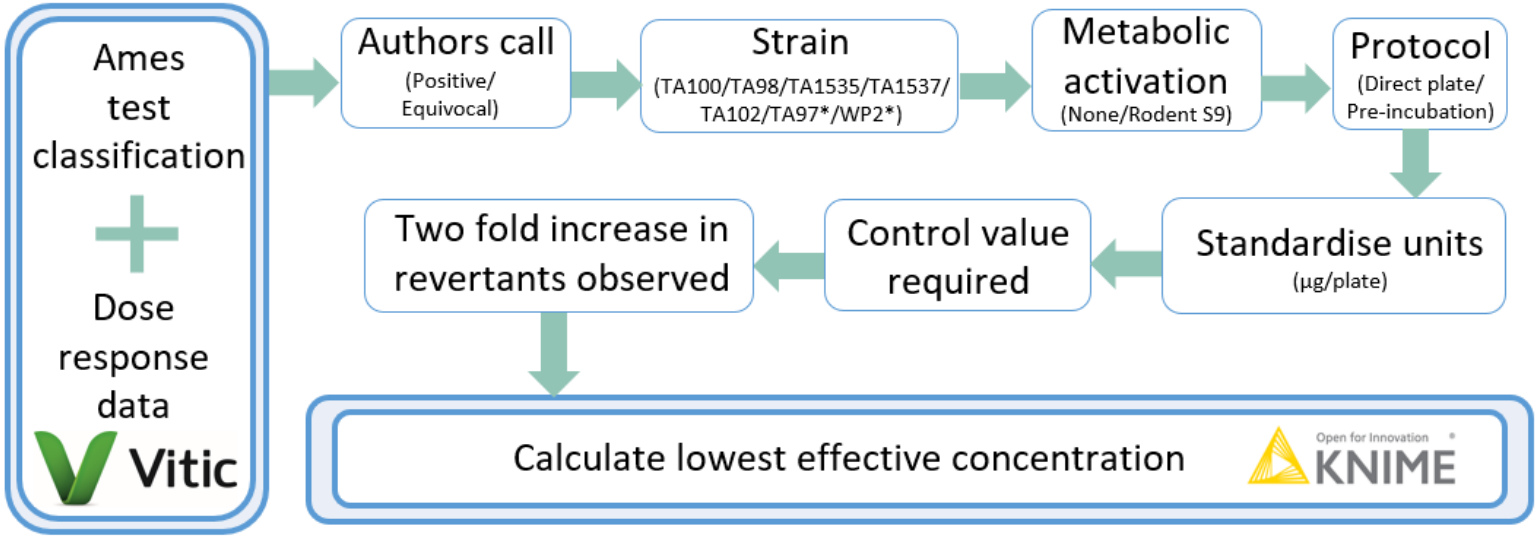
Method

To investigate this question, publicly available Ames test and dose response data for more than 4,300 substances was collated from the Vitic® database.



Using the Knime Analytics Platform®, data analytics was applied to determine the lowest effective dose (LED) at which a mutagenic response could be detected.

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Results

The data analytics produced a dataset of 1,222 mutagenic substance with dose-response data in TG471 compliant strains.

- The lowest effective doses (LEDs) ranged from 0.001-20,000 µg/plate.

- Mean, minimum and maximum LEDs for each substance were derived and the primary focus was on the minimum lowest effective dose.

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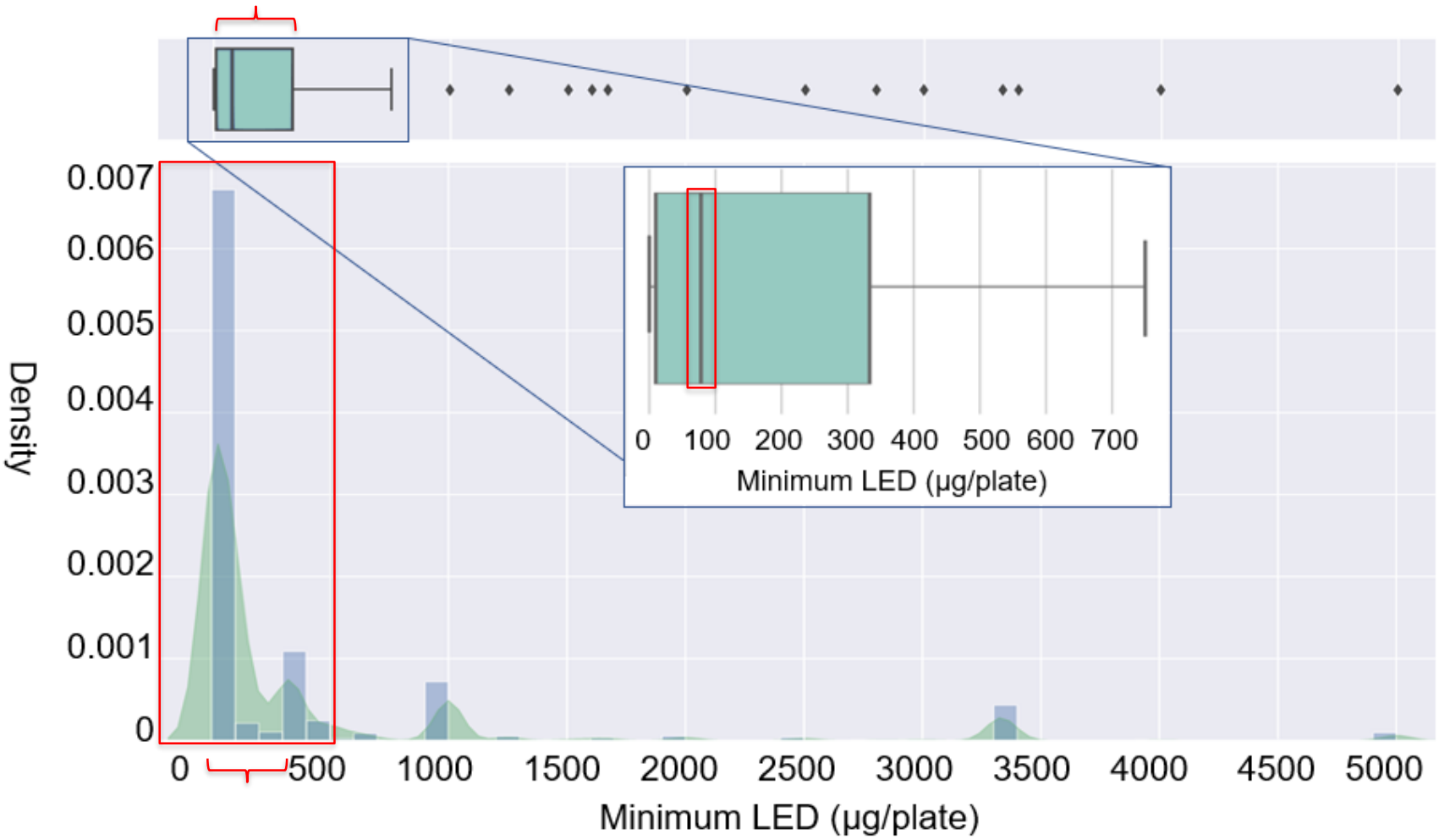
Minimum lowest effective dose



The Ames test can detect mutagens at very low doses.

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Potent substances

The 15 most potent substances as judged by their minimum lowest effective dose. *The "Yes" indicates a lower dose was not tested in this dataset.*

Substance	Minimum LED (µg/plate)	Highest fold increase in revertants	Lowest tested dose (µg/plate)	
2,4,7-Trinitrofluorenone	0.001	14.0	0.001	Yes
Furylfuramide	0.001	3.46	0.001	Yes
3-Chloro-4-(dichloromethyl)-5-hydroxy-2-furanone	0.0025	2.1	0.00125	
(-)-Aflatoxin B1	0.003	2.5	0.001	
2,5-Dinitrofluorene	0.003	2.36	0.003	Yes
2-(Formylamino)-4-(5-nitro-2-furyl)thiazole	0.003	2.49	0.001	
2,7-Dinitro-9-fluorenone	0.01	6.5	0.01	Yes
Altertoxin III	0.018	2.32	0.018	Yes
3-Chloro-4-(chloromethyl)-5-hydroxy-2(5H)-furanone	0.02	2.0	0.01	
Acrolein	0.03	2.13	0.01	
Ethidium bromide	0.03	2.45	0.03	Yes
Sodium azide	0.03	4.76	0.03	Yes
2-Aminoanthracene	0.03	2.0	0.01	
Nitrofurantoin	0.03	2.24	0.01	
3-Methyl-3H-imidazo[4,5-f]quinolin-2-amine	0.03	5.0	0.03	Yes

Further investigation could result in even lower lowest effective doses being derived.

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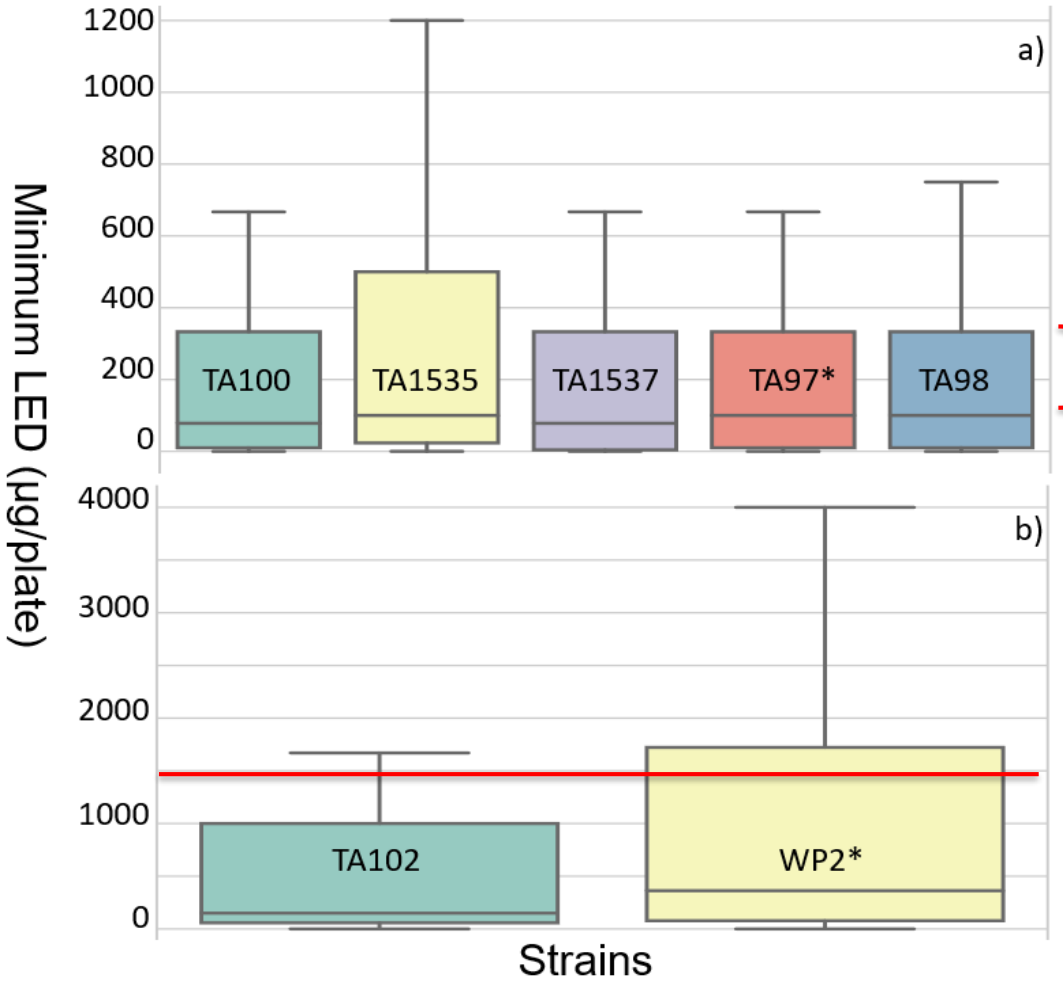
Strain analysis



Average strain minimum lowest effective dose median and upper quartile.

Average strain minimum lowest effective dose upper quartile.

Most strains had a very similar sensitivity and could be used interchangeably.

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Conclusion - How low can you go?

- For non-intentionally added substances (NIAS) a self-regulated threshold of 10 µg/kg (10ppb) in food has been set by industry and, if exceeded, further risk assessment needs to be performed.

Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2018 Nov;35(11):2230-2243. doi: 10.1080/19440049.2018.1519259. Epub 2018 Sep 26.

Suitability of the Ames test to characterise genotoxicity of food contact material migrates.

Rainer B[1], Pinter E[1], Czerny T[1], Riegel E[1], Kirchnawy C[2], Marin-Kuan M[3], Schilter B[3], Tacker M[1].

⊕ Author information

- A study on 40 genotoxic FCM migrants, highlighted that only 10% could be detected by the Ames test.

- Using the same methodology, our larger dataset resulted in 11.3% of substances theoretically detected.

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Conclusion - How low can you go?

- Is the conventional Ames test sufficiently sensitive to detect toxicity at very low doses?
  - *The Ames test can detect potent mutagens.*

- Is this sufficient for the safety studies of food contact materials
  - *The food contact material industry could incorporate the Ames test and Ames test data to detect and/or confirm potential mutagenicity safety hazards.*

- Work is ongoing to cross-reference our data with results from other assays.

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Acknowledgements

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How low can you go?
An analysis of lowest effective dose in the Ames test.

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Introduction

The food industry requires risk assessments to be performed on all substances migrating from Food Contact Articles (FCAs) and Food Contact Materials (FCMs). This includes non-intentionally added substances (NIAS), which often form a significant part of the overall migrate [1]. Genotoxicity risk assessments strongly rely on the availability of information but the unknown properties of NIAS present a significant challenge.
Performing *in vitro* assay testing might prove difficult, partly due to questions regarding which test methods provide sufficient sensitivity and if the small amount of migrate produced is insufficient for toxicological testing. The results of *in vitro* tests could potentially be used to assess FCM migrant's potential to cause toxicological hazard [2], see figure 1.

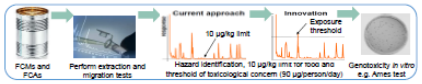

Figure 1: Workflow showing FCMs and FCAs, extraction of migrate, hazard identification and incorporation of genotoxicity assays as part of the risk assessment.

The Ames test is a sensitive assay for mutagen detection and is one of the most common tests used for assessing the mutagenicity of impurities [3]. Thus the question is whether the conventional Ames test is sufficiently sensitive to detect toxicity at very low doses?

Method

To investigate this question, publicly available Ames test and dose response data for more than 4,300 substances was collated from the Vitic ® database [4]. Using the Knime Analytics Platform® [5], data analytics was applied to determine the lowest effective dose (LED) at which a mutagenic response could be detected. Figure 2 shows a workflow of how the dataset was selected and filtered based on the experimental design being consistent with the OECD 471 guideline [6]. Following an initial review, it was decided that only data with an author's call of positive and/or equivocal would be included in the dataset. The calculated LED was defined as the dose that produced at least a 2-fold increase in revertant count compared to control.

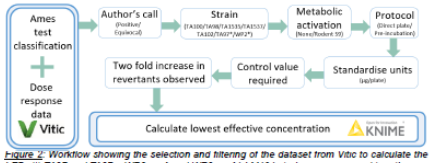

Figure 2: Workflow showing the selection and filtering of the dataset from Vitic to calculate the LED. (*) TA97 and TA97a, WP2 urvA and WP2 urvA/pkM101 strains were grouped together.

Results

Following the analysis, the calculated LEDs ranged from approximately 0.001-20,000 µg/plate. Substances with LEDs higher than 5,000 µg/plate were not included in the dataset since this is the upper dose requirement in the OECD 471 guideline, resulting in a dataset of 1,222 mutagenic substances. Summary statistical parameters such as mean, minimum and maximum were derived from the LED for each substance and the primary focus was on the minimum LED.

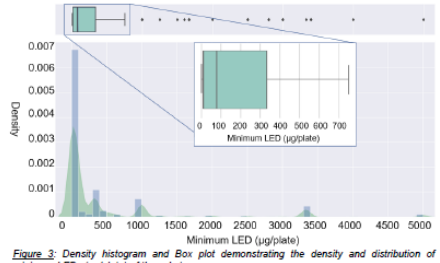

Figure 3: Density histogram and Box plot demonstrating the density and distribution of minimum LEDs (µg/plate) of the substances.

The density histogram in figure 3 shows the density of minimum LEDs of substances in the dataset. 83.7% of results are clustered at concentrations between 0.001-500 µg/plate. A Box plot, also shown in figure 3, presents a summary of the distribution of the minimum LEDs. The median minimum LED was 78.1 µg/plate and the interquartile range was 10-333 µg/plate.

Substance	Minimum LED (µg/plate)	Highest fold increase in revertants	Lowest tested dose (µg/plate)	
2,4,7-Trinitrofluorenone	0.001	14.0	0.001	Yes
Furylfuramide	0.001	3.46	0.001	Yes
3-Chloro-4-(dichloromethyl)-5-hydroxy-2-furanone	0.0025	2.1	0.00125	
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Nitrofurantoin	0.03	2.24	0.01	
3-Methyl-3H-Imidazo[4,5-f]quinolin-2-amine	0.03	5.0	0.03	Yes

Table 1: Substance, minimum LEDs (µg/plate), highest fold increase in revertants and the lowest tested dose (µg/plate) for most potent 15 results. The "Yes" indicates a lower dose was not tested in this dataset.

Results (continued)

Table 1 shows the 15 most potent substances as judged by their minimum LED. A proportion of these substances had the minimum LED being the lowest dose tested i.e. it is possible that a lower dose may also produce a positive result, therefore a lower LED could be produced. However, in most cases the fold increase in revertant count for the substances was close to 2-fold, suggesting that lower does would produce insignificant responses.
The dataset was also analysed per strain, to establish if any strains were more sensitive than the others. Figure 4a shows that the strains detecting mutations at "GC" base pairs have a similar sensitivity (minimum LED median ~100 µg/plate, upper quartiles 333 µg/plate). Figure 4b shows the strains detecting mutations at "AT" base pairs have a larger distribution of minimum LEDs (upper quartiles ~1,460 µg/plate) and therefore were less sensitive than GC" base pairs. The WP2* strains were less sensitive than the TA102 strain (medium minimum LED ~362.5µg/plate compared to ~150µg/plate).

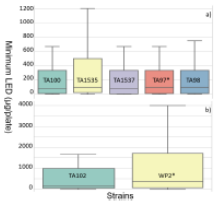

Figure 4: Box plot without outliers showing the distribution of minimum LEDs (µg/plate) by strain. (*) TA97 and TA97a, WP2 urvA and WP2 urvA/pkM101 strains were grouped together.

Conclusion

The Ames test can detect mutagens at very low doses. A minimum LED as low as 1 ng/plate was observed and the majority of substances had minimum LEDs between 10-333 µg/plate. Most strains had a very similar sensitivity and could be used interchangeably. It was interesting that in this dataset several substances' LEDs were also the lowest tested dose and further investigation could result in even lower LEDs being produced. For NIAS a self-regulated threshold of 10 µg/kg (10ppb) in food has been set by industry [7] and, if exceeded, further risk assessment needs to be performed.
A recent study on a small number of genotoxic FCM migrants, highlights that only 10% could be detected by Ames test [8] and using the same methodology with our larger dataset resulted in 11.3% of substances theoretically detected.
Thus, the Ames test can detect potent mutagens and work is ongoing to cross-reference this data with results from other assays. The FCM industry could incorporate the Ames test and Ames test data to detect and/or confirm potential mutagenicity safety hazards.

References

[1] Groh KJ. 2014. Work plans to get out of the deadlock for the safety assurance of migration from food contact materials? A proposal. Food Control 46:312–318. [2] Groh KJ. 2017. In Vitro Toxicity Testing of Food Contact Materials: State-of-the-Art and Future Challenges. Comprehensive Reviews in Food Science and Food Safety 16:1123–1150. [3] Kenyon MO. 2007 An evaluation of the sensitivity of the Ames assay to discern low-level mutagenic impurities. Regulatory Toxicology and Pharmacology 48:75-86. [4] Vitic (2018.1.0), Lhasa Limited https://www.lhasalimited.org/products/vitic.htm [5] KNIME.com AG (version 3.4.2). Available at: https://www.knime.org/ [6] https://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en [7] Geueke B. 2018 Dossier - Non-intentionally added substances (NIAS). Food Packaging Forum. DOI: 10.5281/zenodo.1285331. [8] Rainer B. 2018. Suitability of the Ames test to characterise genotoxicity of food contact material migrates. Food Additives & Contaminants: Part A 35:2230-2243.

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