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Mutagenicity Screening of Selected Water-Based Dispersions and Materials Utilized in Cardboard and Wood Coatings Using the Standard and 384-Well Ames Tests

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Abstract

Novel biobased materials and processing techniques are actively developed for sustainable coatings. This study investigated the potential mutagenicity of novel materials and derived dispersions used for biobased paper and wood coatings using a pilot 384-well test. The standard Ames test was performed for selected materials to compare and validate the results with the non-standard 384-well test. *Salmonella* Typhimurium strains (TA100 and TA98) were used for testing. Experimental dispersions were prepared using suberin and betulin extracted from outer birch bark. The test set of seven samples (n = 7) included commercial reference samples and additives. Both test methods were suitable for these samples but also highlighted method-specific differences and challenges. For suberin-derived materials and betulin at 0.5–1% concentration, neither of the tests indicated mutagenicity. In the case of some industrial samples, the 384-well test and the standard Ames test gave clearly contradictory results. These can be explained by the test limitations, such as the sample color or compositional instability of dispersions. To summarize, this study indicated the need to test the novel coating materials with multiple concentrations, and several bacterial strains carrying different types of genetic mutations, as well as to use complementary genotoxicity tests for a more accurate toxicity profile.

Keywords: 384-well test; standard Ames test; biobased materials; suberin-derived dispersions; betulin; mutagenicity

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1. Introduction

Today, coating manufacturers have a need to find new and sustainable solutions to replace fossil-based raw materials and further the progress of sustainable development. One solution to this is to develop biobased coatings, which also offer numerous environmental benefits [1]. In many cases, the aim is to replace traditional solvent-based solutions with water-borne or -dispersed materials, which may not cause health hazards or harm to the environment or to humans [2]. However, biobased materials and coatings have drawbacks, such as challenges related to compromised properties, price, availability of raw materials,

and the safety of coatings and coating materials [1,3]. Furthermore, it is well known that if mixed biomasses or recycled materials are used to process biobased components for products, they may contain harmful residues due to poor quality or contamination of input feedstock material. Particularly challenging are applications where biobased coatings are used in food packaging applications, which are very strictly regulated to ensure consumer safety. Water-based dispersion processing is challenging, and different kind of additives are needed such as polyvinyl alcohol (PVA), surfactants, stabilizers, preservatives, and biocides. These potentially harmful additives or their derivatives may accumulate in the feedstock, for example, if a certain fraction of a recycled material is included in the raw materials [3–5].

Various plant-based materials, such as birch betulin and suberin, can be used in biobased coatings [5,6]. Although the origin of natural biobased materials is nature, and solutions and products made from them are advertised as better alternatives to fossil-based non-biodegradable solutions, we cannot rule out the safety concerns of biobased materials. For example, Catalán and Norppa [7] pointed out that there are conflicting studies on the safety of biobased nanomaterials, and Oliveira et al. [8] also suggest that there are conflicting results in the literature regarding mutagenicity of betulin. The literature also indicates that adverse response to nanoparticles is dose-dependent and can vary between different materials [6]. Based on the literature, harmful compounds have also been found in plant-based and biobased and/or biodegradable materials [9,10]. Therefore, their safety aspects for humans and the environment must be evaluated comprehensively by utilizing proper, preferably well-established, standard procedures. The safety assessment must also consider the manufacturing of biobased coatings, as production parameters (different techniques, solvents used, etc.) also affect the safety of the final product and can change the original input compositions, even for the worse [9,11–14]. It should be noted that sensitivity to different chemical substances varies depending on their exposure routes and chemical mode of action [7,15]. Thus, as biobased materials become more common, the need for their testing also increases, and therefore it is necessary to screen the toxicity of new coating solutions already in the research and product development phase. Then potential problems can be identified and resolved early, and international guidelines can be followed accordingly [16].

The Ames test is a bacterial reverse mutation test designed to identify chemicals or compounds that cause one or more point mutations. The Ames test is a well-established standard test method, and it is still the primary and most important prescreening method used when developing industrial chemicals, food additives, drugs or other chemicals for regulatory approval, although the test has gradually evolved from the original test method. The test uses *Salmonella* and *E. coli* strains that have been mutated to lack the ability to synthesize an essential amino acid for their growth. The principle of the test is that the test detects the existence of mutations that restore the mutations in the test strain and thus restore the bacteria's ability to synthesize the essential amino acid [17–19]. When bacteria are exposed to a test compound in an environment where only a little essential amino acid is available, and if the exposure leads to a specific mutation in the DNA of the bacterial cell, then the ability of the bacteria to synthesize this essential amino acid is restored. This allows the mutated bacteria to grow, which can be observed, for example, as colony growth or color change. In the case of such an observation, the test substance is considered to give a positive result, i.e., to be mutagenic [17,19].

The standard Ames test is performed using Petri dishes, in which all the necessary test chemicals and the sample are placed at the same time. In this standard method, the result numbers are based on counting the number of growing colonies [17]. However, the standard Ames test is very laborious to perform and requires a lot of sample material,

which is why smaller-sized versions have been developed alongside the standard method, especially for industrial needs. They are used as prescreening methods to predict the results of the standard Ames test [19,20].

One of the smaller-scale tests is a 384-well test which is performed entirely in liquid medium in a 384-well plate. This test allows testing with very small amounts of the test substance. Mutagenicity indication also differs from the standard Ames test, as this test is based on a color change from purple to yellow. The test medium contains bromocresol purple, in which a color change occurs due to a decrease in pH caused by the metabolic processes of the replicating bacteria during the test. The advantages of this method compared to the standard Ames test are good sensitivity and suitability for small sample quantities. In addition, the test reduces the amount of work and time, the number of reagents used, and the amount of waste. In addition, the reading of the test results is easier and faster, decreasing risk for interpretation errors. This test is also considered to have good compatibility with the standard Ames test [19,21–23].

As in the case of the standard Ames test, these smaller-scale versions have limitations and weaknesses. In general, the Ames test is not suitable for chemicals that are known to kill bacteria (e.g., certain antibiotics) [17]. In addition to this, the small-scale tests cannot necessarily utilize all the bacterial strains that are used in the standard Ames test. This is due to the fact that the miniaturization can make the spontaneous reverse mutation measurement difficult. However, this can be overcome by using an alternative strain with a higher spontaneous reversion rate or using a larger number of replicate wells [20,21]. The principal limitation of the 384-well test format is that it becomes technically challenging when dealing with low-solubility compounds. It is only suitable for water-soluble or water-dispersed materials, although small amounts of additional components such as ethanol, DMSO (dimethyl sulfoxide), or surfactant may be used to improve solubility or dispersion stability [20,24]. Furthermore, color change from purple to yellow may be difficult to observe for colorful test materials. There is also less information available on the small-scale tests than on the standard Ames test in the literature, although these are mentioned in the OECD guideline [20,22].

The aim of this study was to investigate whether selected novel biobased materials and dispersions used for biobased paper coatings show signs of mutagenicity in the 384-well test. The particle size distributions of dispersions studied here are in the range 1–100 μm, an average particle size of about 10–20 μm, and the dispersions have been utilized in laboratory and pilot-scale coating trials [25]. They have been shown to provide required uniformity, surface roughness and other characteristics for protective layers in packaging applications. In addition, the standard Ames test was carried out for selected materials to compare the results with the 384-well test. The testing was performed for different kind of material solutions which have potential for industrial paper coating applications, such as suberin-derived dispersions, betulins, spruce sugar and novel acrylate polymer coating materials. These are real coating dispersions and relevant materials from an application point of view, i.e., these materials are not finely dispersed nanomaterials but micron-scale dispersions. The aim was to reveal the potential feasibility of the 384-well test for preliminary screening of water-based dispersions in the development phase and its possible limitations compared to the standard Ames test.

The novelty of this article is that the mutagenicity of novel biobased barrier dispersion coating materials is assessed using two different Ames tests, which provide up-to-date information on the safety of coating materials under development. In addition, the comparison of the methods provides some new understanding of the readability and applicability of the methods. The 384-well test is developed for rapid screening before the final approval testing of the product using several standard tests offered by service providers like

Eurofins Ltd. The test enables more efficient and higher-throughput analytics to support industrial research and development processes. Therefore, both standard tests and novel developments in the research phase are needed.

2. Materials and Methods

2.1. Samples and Initial Preparations

The samples consisted of suberin-derived dispersions, betulins, and industrial biobased coating materials; details are presented in Table 1. For preparation of the suberin-derived dispersions, the preparation conditions and procedures were identical to Hu et al. [25]. The industrial materials are extracted using industrial biorefining methods from specific biomasses. In the suberin-derived dispersion (Sub-ST), Span 80 (Sorbitan monooleate) and Tween 80 (Polysorbate 80) (Merck KGaA, Darmstadt, Germany) were used as non-ionic surfactants for dispersing suberin into an aqueous dispersion. The surfactants were mixed in a 0.65:0.35 ratio. This mixture was added to water at a 2% addition level relative to dry suberin. The pH was adjusted to 12–13. Suberin was gradually added to this surfactant solution and mixed thoroughly using a Silverson L5M-A mixer (Silverson, East Longmeadow, MA, USA). After addition of all the suberin, mixing continued for 30 min with an ultramix head, followed by another 30 min using a fine emulsifier screen. The rotation speed was increased stepwise from 3000 to 5000. This was carried out to prevent spillage during the start of the mixing process. The final content of solids was 22%, and the pH was adjusted to 7. Another suberin-derived dispersion (Sub-CNF) was prepared in the same way as Sub-ST, but dispersion used amphiphilic cellulose nanofibrils (CNF) as a surfactant. The addition level of the CNF was 0.1% with respect to dry suberin. The final content of solids was 15% for Sub-CNF. More detailed information on the preparation of suberin-derived dispersion is available in Hu et al. [25], which includes suberin characterization. The composition of suberin in the current study can be found in Appendix A.1. The samples of this study are presented in Table 1.

Table 1. The samples and their descriptions used for both Ames tests (the standard Ames test and the 384-well test).

Sample	Description
Suberin-ST (Sub-ST)	Suberin-derived dispersion stabilized by the mix of Span 80 and Tween 80 surfactant; more detailed information in Hu et al. [25].
Suberin-CNF (Sub-CNF)	Suberin-derived dispersion stabilized by amphiphilic CNF; more detailed information in Hu et al. [25].
Coating-1 (Co-1)	Industrial water-based coating material (acrylate polymer).
Coating-2 (Co-2)	Industrial water-based coating material (acrylate polymer).
SpruceSugar™ (Spru)	Industrial hemicellulose spruce extract for barrier coating.
Betulin (Bet-1)	Laboratory betulin extract from Natural Resources Institute Finland (Luke).
Betuinno.™ Betulin (Bet-2)	Industrial betulin sample from Innomost Ltd.

The samples were prepared for the tests as follows. The betulin samples were provided by the Natural Resources Institute Finland (Luke) and an industrial partner (Innomost Oy, Kokkola, Finland) as powder. Then, betulin solutions 5% (w/v) were prepared from the powders obtained using 1.5 g betulin powder, 0.6 mL (2%) Tween 80 (Sigma-Aldrich, Darmstadt, Germany), 1.5 mL (5%) DMSO (Fisher Scientific, Loughborough, UK), and 26.4 mL ultrapure water. More detailed composition of betulin powders can be found in Appendix A.2. Similar additives are used in other micro-organism tests such as bacterial and cell tests, and in general they do not interfere with testing at these concentrations [24]. The Spru-sample was prepared from industrially available powder. A 20% solution was

prepared by adding 7.0 g SpruceSugar™ powder (Boreal Bioproducts, Turku, Finland) and 28.0 mL ultrapure water. Suberin dispersions were obtained with 15 and 22% dry solid weight content in solutions and tested concentrations refer to final percentage in the incubation media. Co-1 and Co-2 samples were tested as they were obtained from industry, by diluting the original 100% solution to concentrations 1, 10 and 20% in the incubation media. All samples were prepared just before the start of the test. In particular, the homogeneity of the suberin samples was ensured by vortexing and visual inspection. Each sample was subjected to two different mutagenicity tests (the standard Ames test and the 384-well test) with two different *Salmonella* Typhimurium strains (TA98 and TA100). Both tests are described in more detail below, and the concentrations of the samples tested in both tests are shown in Table 2. For the 384-well test, concentrations of 10% and 20% were prepared using industrial Co-1 and Co-2 samples, to reveal the option for applying this test type to colorful samples at higher concentrations.

Table 2. Samples tested in both tests and their final concentrations in the incubation media.

Sample	Tested Concentrations of Samples	
	The Standard Ames Test	The 384-Well Test
Sub-ST	0.5%	0.5%
Sub-CNF	0.5%	0.5%
Co-1	1.0%	1.0%, 10.0%, 20.0%
Co-2	1.0%	1.0%, 10.0%, 20.0%
Spru	0.5%, 1.0%	0.5%, 1.0%
Bet-1	1.0%	1.0%
Bet-2	1.0%	1.0%

2.2. Storage of Bacterial Strains

The bacterial strains used in both tests were *Salmonella* Typhimurium TA98 and TA100. Bacterial strains stored in a deep freezer (−80 °C) were streaked onto tryptic soy agar (TSA) plates (VWR International, Leuven, Belgium) and incubated at +37 °C for 24 h. The TSA-plates were stored at +4 °C and renewed weekly.

2.3. Preparation of Bacterial Suspensions

From the TSA plates, one colony of each used strain was transferred into individual test tubes containing 10 mL of Nutrient broth (VWR International, Leuven, Belgium). The tubes were then placed on a shaker incubator overnight (+37 °C, 150 rpm, 16 h). Following incubation, 100 µL of each bacterial suspension was transferred to test tubes containing 25 mL Nutrient broth and 63 µL ampicillin (MP Biomedicals, Illkirch, France) solution (10 mg/mL). The test tubes were then placed on a shaker incubator overnight (+37 °C, 150 rpm, 16 h).

2.4. The Standard Ames Test

The test was performed according to the OECD guideline for the testing of chemicals, test number 471 with slight modifications [17]. The test was performed with and without metabolic activation. Briefly, each of the samples tested, along with 100 µL bacterial suspension, were added to triplicate test tubes containing 2 mL top agar. To another set of triplicates of test tubes, 500 µL of S9 mix, and cofactors, was added for testing in the presence of metabolic activation. Negative and positive controls were also included in the test. Negative controls were prepared as described above but did not include test samples. Since some of the samples tested contained a solvent, negative controls both with and without the solvent were prepared. Positive controls contained strain-specific mutagens listed below. In some instances, more than one positive control was used. Tubes were

mixed by vortexing and poured over minimum glucose agar plates. Plates were tilted to allow the agar to spread evenly, allowed to dry and incubated at +37 °C for 48 h, after which the number of revertant colonies on the plates were counted.

The strain-specific mutagens (Merck KGaA, Darmstadt, Germany) used as positive controls in the standard Ames test were:

- TA100 with metabolic activation: 4-nitroquinoline-1-oxide 10 µg/plate;
- TA100 without metabolic activation: 4-nitroquinoline-1-oxide 1 µg/plate;
- TA98 with metabolic activation: 2-aminoanthracene 20 µg/plate;
- TA98 without metabolic activation: 2-nitrofluorene 50 µg/plate or 2-aminoanthracene 20 µg/plate.

2.5. The 384-Well Test

The test was performed according to ISO Standard 11350:2012, both with and without metabolic activation [26]. Briefly, 600 µL of five times (5×) concentrated medium, sample, and 10 µL bacterial suspension were mixed in wells of a 24-well plate. For testing with metabolic activation, 300 2NFL S9 mix was also added. Sterile water was added to bring the total volume of each well to 3 mL. Negative control wells were prepared as test wells but did not contain a test sample. As with the standard Ames test assay, separate negative controls were prepared for the samples containing water and samples also containing solvents. Positive controls containing strain-specific mutagens (listed below) were also prepared. A sterility control well, containing only medium and sterile water, as well as a S9 sterility control, containing medium, water and S9 mix, were also prepared.

The 24-well plates were pre-incubated at +37 °C for 2 h. After the incubation, the contents of each well on the 24-well plate were pipetted onto a 384-well plate, 50 µL per well, so that the contents of one well on the 24-well plate was distributed into 48 wells on the 384-well plate. The 384-well plate was covered with plastic film to prevent evaporation and incubated at +37 °C for 5 days. After the incubation period, the number of wells with color change from purple to yellow, indicating growth of bacteria, was counted.

The strain-specific mutagens used as positive controls in the 384-well test were:

- TA100 with metabolic activation: 4-nitroquinoline-1-oxide 1.5 µg/well;
- TA100 without metabolic activation: 4-nitroquinoline-1-oxide 0.15 µg/well;
- TA98 with metabolic activation: 2-aminoanthracene 3 µg/well;
- TA98 without metabolic activation: 2-nitrofluorene 7.5 µg/well or 2-aminoanthracene 3 µg/well.

2.6. Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics version 29.0.2.0 (IBM Corp., Armonk, NY, USA). Due to the small sample size, group comparisons were conducted using the Kruskal–Wallis test. A two-tailed p value of <0.05 was considered statistically significant.

3. Results

This study tested the mutagenicity of different dispersion samples (Table 1) using two different Ames tests (the standard Ames test and the 384-well test) and two different *Salmonella* Typhimurium strains (TA100 and TA98) with and without metabolic activation. Samples were also tested at different concentrations. In addition, this study compared the tests to each other with identical samples.

It was possible to prepare properly stable samples with one phase only at concentrations of 0.5% or 1% for all the samples. Both tests were performed in triplicate, from which either the colony counts or the number of wells that had changed color were tab-

ulated, and mean, standard deviation, and fold response (i.e., the number of revertant colonies or wells divided by that of the negative control) were calculated. The results are also partially presented in Figures 1–4. For the standard Ames test, the mean and standard error of mean values for the fold response are shown in Figures 1–4. In the case of 384-well test data, higher-order Taylor series expansion as described in Holmes et al. [27] was used to estimate the error of the mean fold response. The numerical results for the standard Ames test are presented in Appendices B.1 and B.2, and for the 384-well test in Appendices C.1 and C.2. The relevant data related to the negative and positive control samples are in Appendices D.1–D.4, E.1 and E.2.

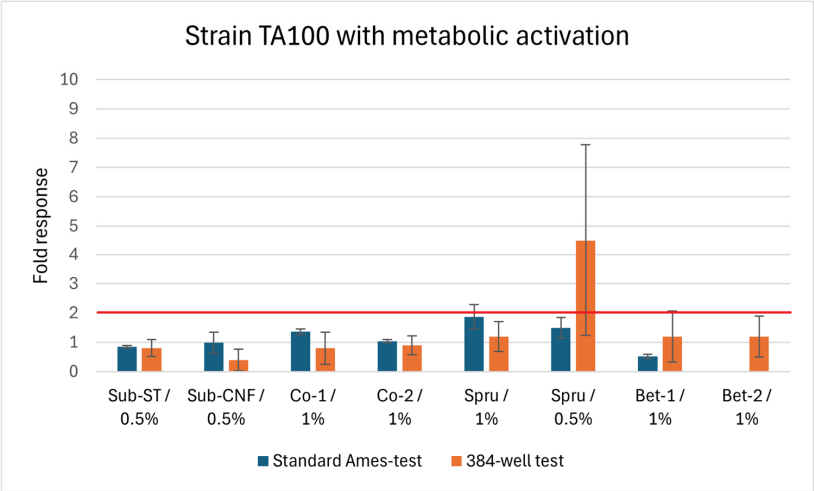

Figure 1. Results of the standard Ames test and the 384-well test for strain TA100 with metabolic activation. Results are reported as averages of replicates of fold response with standard error. Sample Bet-2 has not undergone the standard Ames test. The red line represents the value of two times the negative control.

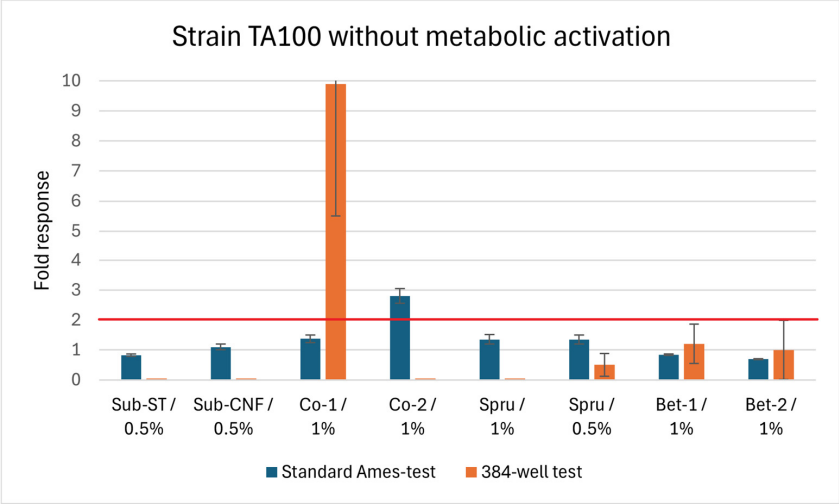

Figure 2. Results of the standard Ames test and the 384-well test for strain TA100 without metabolic activation. Results are reported as averages of replicates of fold response with standard error. The red line represents the value of two times the negative control.

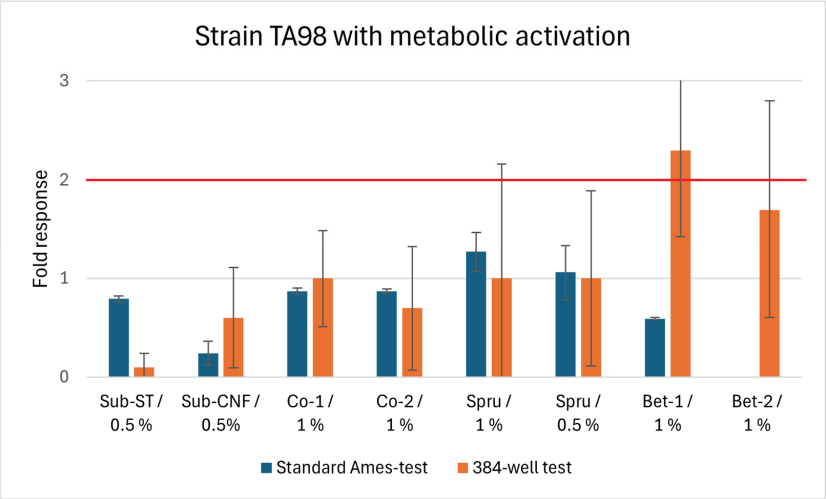

Figure 3. Results of the standard Ames test and the 384-well test for strain TA98 with metabolic activation. Results are reported as averages of replicates of fold response with standard error. Sample Bet-2 has not undergone a standard Ames test. The red line represents the value of two times the negative control.

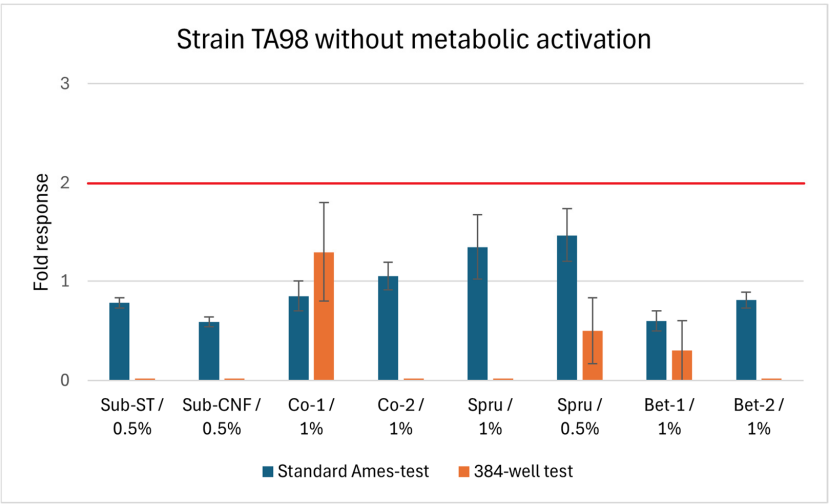

Figure 4. Results of the standard Ames test and the 384-well test for strain TA98 without metabolic activation. Results are reported as averages of replicates of fold response with standard error. The red line represents the value of two times the negative control.

Figures 1–4 do not include the results of both Co-samples for concentrations 10% and 20% with the 384-well test, because all these results (both strains with and without metabolic activation) are nulls, except for the result of sample Co-1 at the concentration of 10% with strain TA98 with metabolic activation, for which the fold response was 0.66. In addition, one of two betulin samples (Bet-2) could not be tested with metabolic activation for the TA98 and TA100 strains in the standard Ames test, because the white milky color of the sample made it impossible to reliably calculate the number of colonies on the plate. Colony counting was still possible in tests without metabolic activation, as the sample volume added to the test tubes is smaller than in tests with metabolic activation. For the other betulin sample (Bet-1) colonies were countable for some, but not all, of the plates.

In the standard Ames test, only one of the samples, Co-2, showed a slightly elevated mutagenicity compared to baseline with strain TA100 without metabolic activation (Figure 2). None of the other samples showed any sign of mutagenicity in the standard Ames test. In the 384-well test, the Spru sample also shows an elevated mutagenicity

compared to the twofold rule at a concentration of 0.5% with strain TA100 with metabolic activation (Figure 1). However, evidently this is not statistically significant. The Bet-1 sample showed a value just on the twofold rule line with strain TA98 with metabolic activation (Figure 3). Neither of the suberin samples or the Bet-2 sample showed any indication of mutagenicity in either of the tests.

When comparing the Ames tests applying the twofold rule, some differences in the results were noticed. The Co-1 sample shows mutagenicity at a concentration of 1% in the 384-well test with strain TA100 without metabolic activation, but not in the standard Ames test (Figure 2). This mutagenicity result with the 384-well test is statistically significant. In turn, the Co-2 sample shows mutagenicity at a concentration of 1% in the standard Ames test with strain TA100 without metabolic activation, but not in the 384-well test (Figure 2). The Spru sample shows mutagenicity at a concentration of 0.5% in the 384-well test with strain TA100 with metabolic activation, but not in the standard Ames test (Figure 1). The results also show that for all the samples tested at a concentration of 0.5% and 1%, there was no statistically significant difference in the fold response of either of the Ames tests vs. concentration (Appendices B.1, B.2, C.1 and C.2).

4. Discussion

The results of this study, based on two different types of Ames test, suggest that the possibility of mutagenicity of some of the dispersion samples cannot be ruled out. Therefore, it would be of value to test a wider range of concentrations, but the challenge of color and water solubility needs to be solved first with some of the dispersions, or alternatively, another type of test should be used. Moreover, utilizing a wider set of test strains would aid in confirming the findings, even though TA100 and TA98 are widely used in extrapolation of sample mutagenicity. The tested concentrations were chosen to correspond to the ones that may occur in industrial use or are slightly higher. Two different Ames tests were used in this study, namely, the traditional standard Ames test and the 384-well test, which were also compared with each other. Both test methods proved to be applicable to these samples but also highlighted method-specific differences, limitations, and challenges. It should be noted that this study focused on testing the relevant materials suitable for paper coatings and not using high-purity or nanosize biobased materials, which are not used for these kinds of coatings in industry. It was also generally observed that different Ames tests give slightly different results, which is affected by the composition of the samples and their preparation. Despite these differences, importantly both novel suberin and betulin dispersions did not indicate mutagenicity in either the standard or the 384-well test at 1% concentration level, contrasting with some findings for nanosized suberins and betulins in the literature [7].

The standard Ames test is a conventional method that has long been the standard method for assessing the mutagenicity of substances [18]. However, its use is limited, especially in situations where the test substance is in short supply, or rapid screening is required. The standard Ames test is relatively laborious, time-consuming, and requires a larger number of test substances [19,20]. For this reason, the 384-well test has been developed alongside the standard Ames test, due to its better sensitivity and cost-effectiveness. This test is particularly suitable for early screening, as it allows the analysis of a larger number of samples with smaller volumes. Thus, the higher throughput of non-OECD 471 TG assays, such as the 384-well assay, can be used for prescreening purposes [21]. Furthermore, a modified version of the 384-well test may provide an alternative in EU standard tests or improvements in the test guidelines.

However, the 384-well test has its limitations, the most significant of which is related to the physicochemical properties of samples such as water solubility of the substances

typically used for packaging barrier coatings or colored samples that may affect the interpretation of results. For example, the Co- and Bet-samples were thick and whitish mixtures. In addition, the Bet-, Spru-, and Sub-samples had a tendency to form precipitates, which hindered their uniform dissolution and testing at higher concentration. The higher (10% and 20%) concentrations of Co-1 and Co-2 samples required mixing within the well on the 24-well plates in the standard Ames test in order to dissolve the sample evenly. The functionality of the 384-well test is particularly limited to at least partly water-soluble materials, as the test is conducted in a water-based liquid environment in wells. Both betulin and suberin are natural high-molecular-weight polymers that are virtually insoluble in water and thus require the addition of proper solvents such as DMSO, or surfactants to improve suitability for Ames testing. This was carried out, for example, with Bet-samples by adding 2% Tween 80 and 5% DMSO to the samples, causing significant improvement in dissolution. The homogeneity of the samples was ensured by vortexing and visual inspection. Potentially, suberin and betulin polymers may even form large precipitates or flakes and physically envelop bacterial cells and cause their death, which would result in a false negative. Therefore, after the Ames test, the viability of the bacteria was confirmed by plating the test strains along with the samples, and the results showed that the bacteria were alive and no evident growth inhibition was observed. Furthermore, the possible effects of the solvents were tested separately. However, in the future, more effort should be devoted to sample dissolving, because even after this, the samples tended to form a small precipitate at the bottom of the well.

There are also differences in sample preparation and different formulations, which can ultimately significantly affect the test results, as observed between the Bet-1 and Bet-2 samples. The Bet-2 sample is purer in terms of betulin than the Bet-1 sample (Appendix A.2). However, the Ames results indicate only minor differences, which are not statistically significant. In higher concentrations, colony counts could not be obtained for the Co-samples due to the samples' white color, which is why the 10% and 20% concentrations were tested in the 384-well method but not in the standard Ames test. For the Bet-2 sample, colonies could not reliably be counted on the plates in the standard Ames test with metabolic activation due to the samples' white color obscuring colonies. However, if we look at the trends of the standard Ames test, it seems that metabolic activation does not play a major role. However, based on this study, it was found that the whiter the color of the sample, the more difficult it is to interpret the results. Therefore, it is possible that metabolic activation had an effect, and due to the color we were unable to detect this effect in Bet-2 samples. For the Bet-1 sample, which had a more yellowish, beige color, colony counting was possible but challenging, since the sample was a slightly different color than the Bet-2 sample. This would suggest that the whiter the sample, the more difficult it is to test with the standard Ames test. In addition, the Sub- and Spru-samples were light brown or beige in color, which made it difficult to observe color changes in the 384-well test at higher concentrations.

Several trends and differences were observed between the tests, related to both the Ames test method and the bacterial strains used (TA100 and TA98). In general, in the 384-well test, metabolic activation was required for almost all samples to generate the same response in both strains. However, exceptions were the Spru-sample at a lower concentration (TA98), Bet-1 (TA100/+S9) and Co-1 (TA100/−S9 and TA98/−S9), where the response deviated from the general trend. This might be related to the details of the composition of these samples. In the standard Ames test, the effect of metabolic activation was less pronounced in both strains. For some samples, only weak changes in responses were observed in the TA100 strain, and no clear mutagenicity occurred. A greater decrease in the response with metabolic activation was observed only in the Co-2 sample. In turn, in

the TA98 strain, the responses even slightly decreased in several samples with activation. Differences were observed between the results of the 384-well test and the standard Ames test, which were due to both the test method and the bacterial strain: in the 384-well test, most samples required metabolic activation to generate a response, while in the standard Ames test, the effect of activation was less significant, and only in individual samples did the responses clearly deviate from this general trend.

Both *Salmonella* Typhimurium TA100 and TA98 strains carry a pKM101 plasmid, which enhances error-prone repair of DNA damage, making these strains more sensitive to mutagenic agents. This is why these strains were selected for this study, as they are a good pair for screening mutagenicity, especially since they contain different types of mutations: TA100 a base pair substitution and TA98 a frameshift mutation. In this study, more samples showed signs of mutagenicity with strain TA100 than with strain TA98, which indicates they induce base pair substitutions. The Co-1 and Spru (0.5%) samples showed possible mutagenicity in the 384-well test with the TA100 strain, but the same samples did not show mutagenicity in the standard Ames test. It has also been reported in the literature that when comparing different Ames tests with each other, the tests give opposite positive responses to the same samples in different Ames tests [23,28]. This indicates that the evaluation of the results must consider the physicochemical properties of the samples, the test method, the use of metabolic activation, and the test strain.

Furthermore, it was found that for both Co-samples, no results were obtained at higher concentrations (10% and 20%) due to inhibited bacterial growth, which may indicate a bacteriostatic or bacteriocidic effect of the samples, especially since both samples showed some indications of mutagenicity (Figure 2) at the lower concentration of 1% and bacteria remained viable. Therefore, in general, the use of any biocides in the samples must also be taken into account in Ames testing, as has also been observed in the literature [29]. The inhibition of bacterial growth limited the results obtained at the highest concentrations and suggests that in the future it would be important to widen the range of the test concentrations and include a wider range of strains and different test methods.

The results were evaluated using the so-called twofold rule, in which a sample is interpreted as potentially mutagenic if the number of revertant colonies or wells is at least twice that of the negative control. This criterion is based on the assumption that the number of background mutations remains constant, and a significant increase indicates a mutagenic effect. In some cases, a broader threefold rule response limit is also used, especially with even more sensitive strains. According to international recommendations, an increase in response alone is not sufficient to demonstrate mutagenicity, but a broader overall assessment, including dose response, is needed. Testing with up to five different bacterial strains is also recommended [30–32]. Based on this study alone, it is therefore not possible to definitively conclude whether the samples are mutagenic or not. For that, further testing, including a wider range of dilutions, is required. The interpretation of the Ames test can vary, and there is no unambiguous, generally accepted assessment method despite all the testing and development efforts of numerous institutions around the world [30].

For suberin-derived microparticle dispersions, no previous published data were found in the literature regarding the Ames test. Our study may therefore represent the first published results on the potential mutagenicity of suberin-derived microparticle dispersions. The results suggest that the tested concentrations of suberin-derived material had no mutagenic effect on either *Salmonella* Typhimurium test strains in this test, regardless of having metabolic activation or not. Despite the microdispersed nature of the samples (10–100 μm), the viability of the test strains was confirmed by plating the test suspension and achieving adequate bacterial growth. For nanocellulose, the literature found that nanocellulose, especially nanofibril, is not mutagenic at the concentrations used in the Ames test. However,

the literature shows that genotoxicity has been observed in other in vitro tests in certain cell lines and with certain material properties. The literature also indicates that attention must be paid to nanomaterials (i.e., shape, size, surface chemical modifications, interaction, and route of exposure), as the safety of these materials depends on several factors and these can have an impact on the harmfulness of the sample, and the Ames test may not detect these effects. This highlights the importance of test design and exposure conditions, and thus the overall risk assessment of a sample requires the combination of multiple tests, typically with several bacterial and cell lines selected specifically for each case [33,34].

There are few studies in the literature on the mutagenicity of betulin, and even these contain some results that are contradictory [8,35]. Therefore, our study provides important new information on the safety profile of the substance, as betulin is of interest in biorefining, although the molecule has its own challenges in isolation techniques, processing and especially in the Ames testing as a water-insoluble sample. According to the literature, betulin has not been shown to be mutagenic in the Ames test with TA98 or TA100 strains, regardless of the use of S9 activation [35,36]. This is consistent with the findings of this study, although the Bet-1 sample did reach a value barely exceeding that of the baseline in the 384-well test using strain TA98 with metabolic activation. This may be due to other components in the Bet-1 samples as shown in Appendix A.2. It is known that the other components in a solution can modulate the response [23]. To determine whether this result is truly an indication of mutagenicity, a wider range of betulin concentrations of the sample would need to be tested.

The toxicity of betulinic acid derivatives may increase with increasing water solubility [36]. The dose window is also important, as at certain concentrations betulin can be harmful to animal fibroblast cells [37]. It is therefore important to note that a single Ames result does not exclude the possibility of betulin genotoxicity in eukaryotic systems or in vivo. Thus, building a broader safety profile would therefore require combining several different test methods. This also applies to betulin and the other samples in our study, as all samples are more or less mixtures.

Biobased phenols from birch can be separated and purified with great precision as suberin and betulin [38]. They may provide antibacterial improvements. The preliminary aim of this study included testing purified biobased phenolic mixtures, but already at a concentration of 1%, high toxicity to *Salmonella* strains was observed, as also discussed in the literature [39]. Therefore, they were omitted in this study. This observation supports the idea that the bioactivity of the samples can lead to toxicity without actual mutagenicity. The composition of the samples and the isolation methods are therefore critical factors for the reliability of the tests. In addition, the color of phenolic compounds poses a challenge, especially in the interpretation of the 384-well tests.

The physicochemical properties and preparation methods of the samples affected the success and results of both Ames tests, influencing the ability to draw a conclusive statement of the possible mutagenicity of the samples. Improving water solubility, especially for betulin, is a key development target. However, the OECD test guidelines allow testing of poorly soluble substances at concentrations that cause precipitation, as long as it does not interfere with the interpretation of the results. Thus, small precipitations do not invalidate the results, but the test guideline also requires confirmation of cytotoxicity in this case [17,40].

The mutagenicity screening and safety investigation of biobased coating materials investigated in this study are in line with a key perspective highlighted in a recent review of sustainable chemistry [14]. The greenness of various chemicals is not based solely on their origin, renewability, or improved manufacturing process, but on a comprehensive assessment including various safety aspects. Future studies should also test the cytotoxicity

properties of the samples, using several test concentrations with five different bacterial strains, and use complementary genotoxicity tests to obtain a more accurate toxicity profile. Further studies are needed for betulin and suberin to comprehensively assess their safety. Although the size distribution of suberin samples has been determined in Hu et al. [25], further research is needed to determine the particle size distribution for other samples and surface chemistry for all samples, as these properties also have an impact on the safety assessments of the materials. In addition, nanoparticle dispersions of these materials should be studied and their particle interaction with bacteria such as *Salmonella*, including membrane damage or stress to cells. In general, nanoparticles have high active surface area and therefore they may cause significant effects on bacteria even at very low mass concentrations compared to microparticle dispersions studied in this article [41].

5. Conclusions

This study compared the suitability of the standard Ames test and the 384-well test for the evaluation of mutagenicity of dispersion materials from the bio and forest industry. The main aim of this study was to ensure mutagenicity screening and safety verification of biobased dispersion coating materials align with the core viewpoint emphasized in recent reviews. Both test methods were suitable for the evaluation of the substances, even when the physicochemical properties of the samples, especially solubility, color, and precipitation, had a notable influence on the tests, resulting in method-specific differences, limitations, and challenges. The results showed that some of the samples could potentially be mutagenic, but confirming these findings requires further studies such as studies of cytotoxicity. Importantly, high antibacterial activity prevents the assessment of mutagenicity at high concentrations. The improvement of water solubility and more extensive follow-up tests of bacterial growth details are necessary to verify the results. Based on this study, a definite conclusion on the safety of the samples studied cannot be made, as further focused studies of these dispersions and comprehensive characterization of particles and particle size profiles as well as certainty about the cytotoxicity of the samples are needed to assess the safety of the dispersions and their components.

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Abbreviations

The following abbreviations are used in this manuscript:

Bet-1	Betulin
Bet-2	Betuinno.^{TM} Betulin
CNF	Cellulose nanofibrils
Co-1	Coating-1
Co-2	Coating-2
DMSO	Dimethyl sulfoxide
PVA	Polyvinyl alcohol
Span 80	Sorbitan monooleate
Spru	SpruceSugar^{TM}
Sub-CNF	Suberin-derived dispersion with cellulose nanofibrils
Sub-ST	Suberin-derived dispersion
TA100	*Salmonella* Typhimurium strain
TA98	*Salmonella* Typhimurium strain
TSA	Tryptic soy agar
Tween 80	Polysorbate 80
+S9	With metabolic activation
−S9	Without metabolic activation

Appendix A

Appendix A.1

Table A1. Compositions of suberin samples.

Monomer Class	Suberin (mg/g)
Mid-chain functionalized acids	295.00
ω-Hydroxy acids	153
α, ω-Dicarboxylic acids	68.61
Triterpenoids (Extractives)	143.04
Fatty acids	12.76
Phenolics	3.96
Alcohols	1.92
Others/Unidentified	40.63

Appendix A.2

Table A2. Compositions of betulin samples.

Component	Bet-1 (Luke)		Bet-2 (Innomost)	
	Average (mg/g)	Standard Deviation (mg/g)	Average (mg/g)	Standard Deviation (mg/g)
Lupa-2,20(29)-dien-28-ol	4.36	0.88	-	-
Unidentified triterpenyl alcohol	10.99	5.12	-	-
Lupenone	1.56	0.93	-	-
Sitosterol	2.62	0.96	-	-
β-amyrin	1.08	0.01	-	-
Butyrospermol	2.02	0.20	-	-
Lupeol	43.24	0.35	-	-
Erythrodiol (olean-12-ene-3,28-diol)	25.32	1.58	1.24	0.15
Betulone	1.81	0.21	-	-
Betulinol	621.00	14.76	837.03	9.37
Betulinic acid	25.03	2.90	-	-
Monogynol A (lupane-3,20-diol)	40.76	1.49	-	-
Oleanolic acid, acetate	1.87	1.27	-	-
Lupene-3,20-diol	35.76	5.83	3.79	0.24

Appendix B

Appendix B.1

Table A3. Test results of standard Ames test with strain TA100.

Sample Concentration	Metabolic Activation	Number of Revertants			Average	Standard Deviation	Fold Response
Sub-ST 0.5%	−S9	55	62	52	56.33	5.13	0.77
		64	59	66	63.00	3.61	0.90
		55	55	49	53.00	3.46	0.82
Sub-ST 0.5%	+S9	33	35	39	35.66	3.06	0.77
		35	46	45	42.00	6.08	0.86
		44	41	59	48.00	9.64	0.91
Sub-CNF 0.5%	−S9	73	85	77	78.33	6.11	1.02
		68	68	72	69.33	2.31	1.30
		63	70	80	71.00	8.54	0.97
Sub-CNF 0.5%	+S9	43	57	44	48.00	7.81	1.07
		42	42	48	44.00	3.46	1.57
		17	18	26	20.33	4.93	0.33
Co-1 1%	−S9	87	99	95	93.66	6.11	1.28
		101	116	127	114.66	13.05	1.63
		103	77	53	77.66	25.00	1.19
Co-1 1%	+S9	78	59	70	69.00	9.54	1.48
		52	69	53	58.00	9.54	1.19
		78	57	84	73.00	14.18	1.39
Co-2 1%	−S9	138	279	300	239.00	88.10	3.27
		284	130	100	171.33	98.72	2.43
		115	112	300	175.66	107.69	2.70
Co-2 1%	+S9	39	41	51	43.66	6.43	0.94
		56	41	70	55.66	14.50	1.14
		45	65	52	54.00	10.15	1.03
Spru 0.5%	−S9	89	94	100	94.33	5.51	1.23
		83	63	114	86.66	25.69	1.63
		88	79	70	79.00	9.00	1.15
Spru 1%	−S9	101	89	119	103.00	15.10	1.34
		87	77	94	86.00	8.54	1.62
		71	84	65	73.33	9.71	1.07
Spru 0.5%	+S9	38	42	50	43.33	6.11	0.96
		65	71	45	60.33	13.61	2.15
		87	79	85	83.66	4.16	1.36

Table A3. *Cont.*

Sample Concentration	Metabolic Activation	Number of Revertants			Average	Standard Deviation	Fold Response
Spru 1%	+S9	73	66	74	71.00	4.36	1.57
		74	93	58	75.00	17.52	2.67
		80	74	98	84.00	12.49	1.36
Bet-1 1%	−S9	54	64	54	57.33	5.77	0.90
		59	50	48	52.33	5.86	0.81
		59	46	66	57.00	10.15	0.81
Bet-1 1%	+S9	26	26	-	26.00	0.00	0.63
		18	-	-	18.00	-	0.41
		21	-	-	21.00	-	0.51
Bet-2 1%	−S9	51	44	41	45.33	5.13	0.71
		48	40	49	45.66	4.93	0.70
		45	42	50	45.66	4.04	0.65
Bet-2 1%	+S9	-	-	-	-	-	-
		-	-	-	-	-	-
		-	-	-	-	-	-

Appendix B.2

Table A4. Test results of standard Ames test with strain TA98.

Sample Concentration	Metabolic Activation	Number of Revertants			Average	Standard Deviation	Fold Response
Sub-ST 0.5%	−S9	14	20	11	15.00	4.58	0.84
		14	19	14	15.66	2.89	0.69
		13	14	13	13.33	0.58	0.81
Sub-ST 0.5%	+S9	10	7	18	11.66	5.69	0.85
		14	-	8	11.00	4.24	0.75
		14	10	9	11.00	2.65	0.76
Sub-CNF 0.5%	−S9	3	4	9	5.33	3.21	0.69
		6	8	9	7.66	1.53	0.57
		5	6	5	5.33	0.58	0.51
Sub-CNF 0.5%	+S9	1	2	0	1.00	1.00	0.12
		1	2	0	1.00	1.00	0.10
		3	1	4	2.66	1.53	0.47
Co-1 1%	−S9	13	15	21	16.33	4.16	0.92
		12	8	18	12.66	5.03	0.56
		21	12	20	17.66	4.93	1.08
Co-1 1%	+S9	7	14	16	12.33	4.73	0.90
		9	11	16	12.00	3.60	0.82
		11	12	16	13.00	2.65	0.91

Table A4. *Cont.*

Sample Concentration	Metabolic Activation	Number of Revertants			Average	Standard Deviation	Fold Response
Co-2 1%	−S9	18	24	27	23.00	4.58	1.30
		25	12	19	18.66	6.51	0.83
		21	12	17	16.66	4.51	1.02
Co-2 1%	+S9	15	10	9	11.33	3.21	0.82
		17	9	14	13.33	4.04	0.91
		15	10	13	12.66	2.52	0.89
Spru 0.5%	−S9	19	14	13	15.33	3.21	1.99
		13	12	20	15.00	4.35	1.12
		13	15	12	13.33	1.52	1.28
Spru 1%	−S9	12	19	15	15.33	3.51	2.00
		11	20	14	15.00	4.58	1.12
		10	8	11	9.66	1.53	0.94
Spru 0.5%	+S9	6	7	10	7.66	2.08	0.96
		4	9	5	6.00	2.64	0.64
		5	12	10	9.00	3.60	1.58
Spru 1%	+S9	9	4	10	7.66	3.21	0.96
		13	8	13	11.33	2.88	1.21
		9	11	8	9.33	1.52	1.64
Bet-1 1%	−S9	7	6	10	7.66	2.08	0.39
		9	13	10	10.66	2.08	0.69
		7	9	12	9.33	2.51	0.69
Bet-1 1%	+S9	8	5	6	6.33	1.52	0.59
		15	4	6	8.33	5.85	0.56
		10	2	5	5.66	4.04	0.61
Bet-2 1%	−S9	12	19	12	14.33	4.04	0.72
		19	12	14	15.00	3.60	0.97
		8	12	9	9.66	2.08	0.72
Bet-2 1%	+S9	8	4	5	5.66	2.08	0.53
		7	4	7	6.00	1.73	0.40
		5	5	8	6.00	1.73	0.64

Appendix C

Appendix C.1

Table A5. Test results of 384-well test with strain TA100.

Sample Concentration	Metabolic Activation	Number of Positive Wells			Average	Standard Deviation	Fold Response
Sub-ST 0.5%	−S9	0	0	0	0.00	0.00	0.00
Sub-ST 0.5%	+S9	2	3	4	3.00	1.00	0.75
Sub-CNF 0.5%	−S9	0	1	0	0.33	0.58	0.00
Sub-CNF 0.5%	+S9	4	0	1	1.66	2.08	0.42
Co-1 20%	−S9	0	0	0	0.00	0.00	0.00
Co-1 10%	−S9	0	0	0	0.00	0.00	0.00
Co-1 1%	−S9	24	30	15	23.00	7.55	9.86
Co-1 20%	+S9	0	0	0	0.00	0.00	0.00
Co-1 10%	+S9	0	0	0	0.00	0.00	0.00
Co-1 1%	+S9	4	3	3	3.33	0.58	0.83
Co-2 20%	−S9	0	0	0	0.00	0.00	0.00
Co-2 10%	−S9	0	0	0	0.00	0.00	0.00
Co-2 1%	−S9	0	0	0	0.00	0.00	0.00
Co-2 20%	+S9	0	0	0	0.00	0.00	0.00
Co-2 10%	+S9	0	0	0	0.00	0.00	0.00
Co-2 1%	+S9	4	4	3	3.66	0.58	0.92
Spru 0.5%	−S9	3	8	4	5.00	2.65	0.50
Spru 1%	−S9	0	0	0	0.00	0.00	0.00
Spru 0.5%	+S9	14	11	29	18.00	9.64	4.50
Spru 1%	+S9	5	6	10	7.00	2.64	1.24
Bet-1 1%	−S9	3	2	3	2.66	0.57	1.15
Bet-1 1%	+S9	10	3	0	4.33	5.13	1.17
Bet-2 1%	−S9	1	6	0	2.33	3.21	0.98
Bet-2 1%	+S9	0	5	8	4.33	4.04	1.17

Appendix C.2

Table A6. Test results of 384-well test with strain TA98.

Sample Concentration	Metabolic Activation	Number of Positive Wells			Average	Standard Deviation	Fold Response
Sub-ST 0.5%	−S9	0	0	0	0.00	0.00	0.00
Sub-ST 0.5%	+S9	1	0	0	0.33	0.57	0.13
Sub-CNF 0.5%	−S9	0	0	0	0.00	0.00	0.00
Sub-CNF 0.5%	+S9	0	2	1	1.00	1.00	0.60
Co-1 20%	−S9	0	0	0	0.00	0.00	0.00
Co-1 10%	−S9	0	0	0	0.00	0.00	0.00
Co-1 1%	−S9	2	1	2	1.66	0.57	1.27
Co-1 20%	+S9	0	0	0	0.00	0.00	0.00
Co-1 10%	+S9	3	0	3	2.00	1.73	0.66
Co-1 1%	+S9	3	3	1	2.33	1.15	0.99
Co-2 20%	−S9	0	0	0	0.00	0.00	0.00
Co-2 10%	−S9	0	0	0	0.00	0.00	0.00
Co-2 1%	−S9	0	0	0	0.00	0.00	0.00
Co-2 20%	+S9	0	0	0	0.00	0.00	0.00
Co-2 10%	+S9	0	0	0	0.00	0.00	0.00
Co-2 1%	+S9	4	1	0	1.66	2.08	0.72
Spru 0.5%	−S9	0	1	1	0.66	0.57	0.52
Spru 1%	−S9	0	0	0	0.00	0.00	0.00
Spru 0.5%	+S9	1	3	3	2.33	1.15	0.99
Spru 1%	+S9	3	0	0	1.00	1.73	1.00
Bet-1 1%	−S9	1	0	0	0.33	0.57	0.30
Bet-1 1%	+S9	2	3	4	3.00	1.00	2.25
Bet-2 1%	−S9	0	0	0	0.00	0.00	0.00
Bet-2 1%	+S9	0	4	3	2.33	2.08	1.72

Appendix D

Appendix D.1

Table A7. Negative controls of standard Ames test with strain TA100.

Control	Metabolic Activation	Related to the Sample	Number of Revertants			Average	Standard Deviation
H_2O	−S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%)	67	72	80	73.0	6.6
			68	78	65	70.3	6.7
			68	65	62	65.0	3.0
		Sub-CNF (0.5%), Spru (0.5%, 1.0%)	63	88	78	76.3	12.6
			51	59	49	53.0	5.3
		Sub-CNF (0.5%)	76	71	71	72.7	2.9
		Spru (0.5%, 1.0%)	62	77	66	68.3	7.8
Tween-DMSO	−S9	Bet-1 (1.0%), Bet-2 (1.0%)	84	62	45	63.7	19.6
			65	67	65	65.7	1.2
			69	69	73	70.3	2.3
H_2O	+S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%)	58	40	41	46.3	10.1
			48	45	53	48.7	4.0
			48	59	50	52.3	5.9
		Sub-CNF (0.5%), Spru (0.5%, 1.0%)	58	41	36	45.0	11.5
			34	26	24	28.0	5.3
			51	69	64	61.3	9.3
Tween-DMSO	+S9	Bet-1 (1.0%), Bet-2 (1.0%)	33	49	41	41.0	8.0
			39	37	57	44.3	11.0
			31	51	42	41.3	10.0

Appendix D.2

Table A8. Negative controls of standard Ames test with strain TA98.

Control	Metabolic Activation	Related to the Sample	Number of Revertants			Average	Standard Deviation
H_2O	−S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%)	21	11	21	17.7	5.8
			16	26	26	22.7	5.8
			15	13	21	16.3	4.2
		Sub-CNF (0.5%), Spru (0.5%, 1.0%)	8	10	5	7.7	2.5
			9	14	17	13.3	4.0
			10	10	11	10.3	0.6
Tween-DMSO	−S9	Bet-1 (1.0%), Bet-2 (1.0%)	21	23	15	19.7	4.2
			24	10	12	15.3	7.6
			16	12	12	13.3	2.3
H_2O	+S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%)	15	11	15	13.7	2.3
			17	16	11	14.7	3.2
			13	13	17	14.3	2.3
		Sub-CNF (0.5%), Spru (0.5%, 1.0%)	8	7	9	8.0	1.0
			8	12	8	9.3	2.3
			7	7	3	5.7	2.3
Tween-DMSO	+S9	Bet-1 (1.0%), Bet-2 (1.0%)	13	17	2	10.7	7.8
			11	17	16	14.7	3.2
			12	8	8	9.3	2.3

Appendix D.3

Table A9. Negative controls of 384-well test with strain TA100.

Control	Metabolic Activation	Related to the Sample	Number of Positive Wells			Average	Standard Deviation
H_2O	−S9	Sub-ST (0.5%)	1	1	3	1.7	1.2
		Sub-CNF (0.5%)	6	8	4	6.0	2.0
		Spru (0.5%)	10	2	18	10.0	8.0
		Spru (1.0%)	2	2	5	3.0	1.7
		Co-1 (1.0%), Co-2 (1.0%)	1	2	4	2.3	1.5
Tween-DMSO	−S9	Bet-1 (1.0%), Bet-2 (1.0%)	21	23	15	19.7	4.2
H_2O	+S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%)	5	5	2	4.0	1.7
		Sub-CNF (0.5%)	6	4	2	4.0	2.0
		Spru (0.5%)	1	7	4	4.0	3.0
		Spru (1.0%)	6	8	3	5.7	2.5
Tween-DMSO	+S9	Bet-1 (1.0%), Bet-2 (1.0%)	5	3	3	3.7	1.2

Appendix D.4

Table A10. Negative controls of 384-well test with strain TA98.

Control	Metabolic Activation	Related to the Sample	Number of Positive Wells			Average	Standard Deviation
H_2O	−S9	Sub-ST (0.5%)	1	0	0	0.3	0.6
		Sub-CNF (0.5%), Spru (0.5%)	0	0	2	0.7	1.2
		Spru (1.0%)	1	1	1	1.0	0.0
		Co-1 (1.0%), Co-2 (1.0%)	1	2	0	1.0	1.0
Tween-DMSO	−S9	Bet-1 (1.0%), Bet-2 (1.0%)	0	0	0	0.0	0.0
H_2O	+S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%)	3	3	1	2.3	1.2
		Sub-CNF (0.5%)	3	0	0	1.0	1.7
		Spru (0.5%)	5	1	1	2.3	2.3
		Spru (1.0%)	0	1	1	0.7	0.6
Tween-DMSO	+S9	Bet-1 (1.0%), Bet-2 (1.0%)	1	1	2	1.3	0.6

Appendix E

Appendix E.1

Table A11. Positive controls of standard Ames test.

Control	Strain	Metabolic Activation	Related to the Sample	Number of Revertants *		
4NQO 10 μg/plate	TA100	−S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%), Bet-1 (1.0%), Bet-2 (1.0%)	TNC	TNC	TNC
4NQO 1 μg/plate	TA100	−S9	Sub-CNF (0.5%), Spru (0.5%, 1.0%)	TNC	TNC	TNC
4NQO 10 μg/plate	TA100	+S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%), Bet-1 (1.0%), Sub-CNF (0.5%), Spru (0.5%, 1.0%)	TNC	TNC	TNC
2NF 50 μg/plate	TA98	−S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%), Bet-1 (1.0%), Bet-2 (1.0%)	TNC	TNC	TNC
2AA 20 μg/plate	TA98	−S9	Sub-CNF (0.5%), Spru (0.5%, 1.0%)	61	205	58
2AA 20 μg/plate	TA98	+S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%), Bet-1 (1.0%)	43	43	43
			Sub-CNF (0.5%), Spru (0.5%, 1.0%)	130	152	TNC

* too numerous to count.

Appendix E.2

Table A12. Positive controls of 384-well test.

Control	Strain	Metabolic Activation	Related to the Sample	Number of Positive Wells *		
4NQO 0.15 μg/well	TA100	−S9	Sub-ST (0.5%), Sub-CNF (0.5%), Co-1 (1.0%), Co-2 (1.0%), Spru (0.5%, 1.0%), Bet-1 (1.0%), Bet-2 (1.0%)	48	48	48
4NQO 1.5 μg/well	TA100	+S9	Sub-ST (0.5%), Sub-CNF (0.5%), Co-1 (1.0%), Co-2 (1.0%), Spru (0.5%, 1.0%), Bet-1 (1.0%), Bet-2 (1.0%)	48	48	48
2NF 7.5 μg/well	TA98	−S9	Co-1 (1.0%), Co-2 (1.0%)	30	20	30
			Sub-ST (0.5%), Bet-1 (1.0%), Bet-2 (1.0%)	31	31	31

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Table A12. *Cont.*

Control	Strain	Metabolic Activation	Related to the Sample	Number of Positive Wells *		
2NF 7.5 µg/well	TA98	−S9	Spru (1.0%)	41	44	38
2AA 3 µg/well	TA98	−S9	Sub-CNF (0.5%), Spru (0.5%)	2	16	13
2AA 3 µg/well	TA98	+S9	Sub-ST (0.5%), Co-1 (1.0%), Co-2 (1.0%), Bet-1 (1.0%), Bet-2 (1.0%)	48	48	48
2AA 3 µg/well	TA98	+S9	Sub-CNF (0.5%)	48	43	41
			Spru (0.5%)	48	48	48
			Spru (1.0%)	46	48	48

* too numerous to count.

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