Assessment of genotoxic potential of fragrance materials in the Chicken Egg Assays

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# Abstract

The genotoxic and clastogenic/aneugeneic potentials of four α, ß-unsaturated aldehydes, 2-phenyl-2-butenal, nona-2-trans-6-cis-dienal, 2-methyl-2-pentenal and p-methoxy cinnamaldehyde, which are used as fragrance materials, were assessed in avian fetal livers using the Chicken Egg Genotoxicity Assay (CEGA) and the Hen’s egg micronucleus (HET-MN) assay, respectively. Selection of materials was based on their chemical structures and the results of their assessment in the regulatory *in vitro* and/or *in vivo* genotoxicity test battery. Three tested materials, 2-phenyl-2-butenal, nona-2-trans-6-cis-dienal and 2-methyl-2-pentenal, were negative in both, CEGA and HET-MN assays. These findings were congruent with the results of regulatory *in vivo* genotoxicity assays. In contrast, p-methoxy cinnamaldehyde, which was also negative in the *in vivo* genotoxicity assays, produced evidence of DNA damage, including DNA strand breaks and DNA adducts in CEGA, however, no increase in the micronucleus formation in blood was reported in the HET-MN study. Pretreatment with a glutathione precursor, N-acetyl cysteine, negated positive outcomes produced by p-methoxy cinnamaldehyde in CEGA, indicating that difference in response observed in the egg and rodent models can be attributed to rapid glutathione depletion. Additionally, the dosing protocols for both HET-MN and CEGA assays are different, which can also be an important contributing factor. Overall, our findings support the conclusion that CEGA and/or HET-MN can be considered as a potential alternative to animal testing as follow-up strategies for assessment of genotoxic potential of fragrance materials with evidence of genotoxicity *in vitro*.

Key words:

# INTRODUCTION

The Scientific Committee on Consumer Safety (SCCS) 7th amendment to the EU Cosmetics Directive (EC 2003) prohibits marketing of cosmetic ingredients which have undergone animal safety testing after March 2013. Accordingly, cosmetic and fragrance materials which produce positive results in the standard *in vitro* genotoxicity test battery are prevented from further development. Nevertheless, traditional *in vitro* mutagenicity and genotoxicity assays often produce false positive results, in part due to a lack of sufficient phase II metabolic detoxification (Kirkland et al., 2007). Thus, according to Ates et al (2014), non-confirmed or “misleading” positive results occurred in up to 93% of *in vitro* genotoxicity assays with cosmetic ingredients. Hence, there is a need for a reliable non-animal alternative assay to be used as a follow-up testing strategy for compounds that produces positive results in the in vitro genotoxicity assays.

The Chicken and related Turkey Egg Genotoxicity Assays (CEGA and TEGA, respectively) (Williams et al., 2014; Iatropoulos et al., 2017; Kobets et al., 2018b; Kobets et al., 2016; 2018a), were developed as metabolically competent (Kobets et al., 2018b; Perrone et al., 2004) non-animal screening assays for genotoxicity testing to potentially replace short-term in vivo studies required for human safety assessment. CEGA evaluates two different endpoints indicative of DNA damage produced by either direct or indirect mechanisms including nuclear DNA adducts by the means of the nucleotide 32P-postlabeling (NPL) assay (Phillips and Arlt, 2014; Randerath et al., 1981; Reddy and Randerath, 1986) and DNA strand breaks using the alkaline single cell gel electrophoresis (comet) assay (Brendler-Schwaab et al., 2005; OECD, 2016; Tice et al., 2000). Both techniques are widely used for the evaluation of chemical-induced DNA damage in animal models as well (Himmelstein et al., 2009). Thus, elucidation of the mechanism of action of chemicals is possible.

CEGA uses fertilized, specific pathogen free eggs from the white leghorn chicken of undetermined sex. The average incubation period until hatching for chicken egg is 21 days. Termination of the embryos in CEGA is conducted on day 11, well before hatching, and is considered not to cause discomfort to the organism, as the nervous system of the embryos is not completely formed (Hughes 1953). Thus, in compliance with Animals (Scientific Procedures) Act 1986, the assay is not considered to be an animal model.

Another in ovo model, Hen’s egg test for micronucleus induction (HET-MN) is utilized to assess the clastogenic properties of chemicals. The assay was first described in 1997 by Wolf et. al., and has been subsequently optimized by different working groups (Kerstein et al., 2019). In comparison with other alternative micronucleus assays, the physiological conditions of the incubated fertilized chicken egg may be more similar to the conditions present in animals than in any in vitro system. The hematopoietic system and the metabolic competency of the incubated chicken egg resemble much more the situation of mammals in vivo. The HET-MN combines the use of the commonly accepted genetic endpoint “formation of micronuclei” with the well characterized and complex model of the incubated chicken egg, which enables metabolic activation, elimination, and excretion of xenobiotics – including those that are mutagens or pro-mutagens. The incidence of micronuclei in interphase cells provides an indirect but easy and rapid measure of chromosomal damage. Micronuclei arise from acentric chromosomal fragments or whole chromosomes induced by clastogens or agents affecting the spindle apparatus. Avian erythrocytes are nucleated and oval which is different from mammalian erythrocytes. Additionally, avian erythrocytes survive 35 days in circulation as compared to 120 days for mammals (el-Mekawi et al.,1993).

In the fertilized chicken egg, blood cells are formed as early as about 1 day after the start of incubation. Just a couple of hours later the differentiation of blood vessels starts in the yolk sac. Formation of the chorioallantoic membrane (CAM), occurs on day 5 of incubation. CAM is highly vascularized and contains the main volume of extraembryonic peripheral blood of the incubated hen’s egg. Different from adult animals, there are two different cell lines in the peripheral blood of the chick embryo: primitive erythrocytes (E I) and definite erythrocytes (E II). The latter appear in diverse maturation stages as early, middle and late polychromatic erythrocytes, and as normochromatic erythrocytes

In order to evaluate the potential of both CEGA COMET/NPL and HET-MN in detecting genotoxic events set of four fragrance materials with α, β-unsaturated aldehyde structures, 2-phenyl-2-butenal, nona-2-trans-6-cis-dienal, 2-methyl-2-pentenal and p-methoxy cinnamaldehyde, were evaluated in these models. Alpha, beta-unsaturated carbonyl compounds are a class of organic molecules that contain a carbonyl group (C=O) attached to a carbon-carbon double bond (C=C) at the alpha and beta positions. Some examples of these compounds include acrolein, crotonaldehyde, and acrylamide. These compounds are known to be genotoxic and mutagenic, meaning they can cause DNA damage and mutations (Li et al., 2021). The molecular mechanisms of DNA damage initiated by alpha, beta-unsaturated carbonyl compounds involve several processes such as formation of DNA adduct, deamination of adenine, formation of DNA cross-links, induction of oxidative stress, interference in DNA self-repair processes, etc. (Eder et al., 1990). However, sometimes this class of materials tends to produce biologically non-relevant misleading positive outcomes in the in vitro genotoxicity testing battery. The main purpose of the current study was to verify whether CEGA and HEN-MT are reasonable follow up assays to the in vitro genotoxicity assays. Furthermore, analysis of DNA adduct formation can add additional weight of evidence in supporting a genotoxicity safety assessment for safety assessment of fragrance materials.

# MATERIALS AND METHODS

# Selection of test materials

The four α, β-unsaturated aldehydes selected for testing previously produced positive outcome in at least one of the traditional in vitro Ames and/or in vivo micronucleus assays and had been tested in the in vivo comet and/or micronucleus assay as a follow-up to the in vitro assays.

2-Phenyl-2-butenal (CAS: 4411-89-6; > 97% pure) was supplied by Symrise (Holzminden, Germany). Nona-2 trans- 6-cis-dienal (CAS: 557-48-2; > 98% pure) was provided from Bedoukian Research Inc. (Danbury, CT, USA). 2-Methyl-2-pentenal (CAS: 623-36-9) was delivered from International Flavors & Fragrances (New York City, NY, USA). p-Methoxy cinnamaldehyde (CAS: 1963-36-6; > 96% pure) was contributed by Hasegawa Co., LTD. (Tokyo, Japan). Chemical structures and tested doses for the chemicals selected for testing are provided in **Table 1**.

# Control test materials

Solutol HS15 (Kolliphor HS15) (CAS: 70142-34-6), obtained from Sigma-Aldrich (St Louis, MO, USA) prepared as a 20 % aqueous solution (20% HS15) was used as the vehicle in CEGA. Isopropylmyristate (IPM), (CAS: 110-27-0, batch: STBF9929V), obtained from Sigma Aldrich (St Louis, MO, USA) was used as a vehicle control in HET-MN studies.

Quinoline (CAS: 91-22-5; 99% pure), purchased from Acros Organics (Bridgewater, NJ, USA), was used as a positive control in CEGA at the total dose of 5 mg/egg. Cyclophosphamide (CPA, CAS Number: 6055-19-2, batch: MKBX1822V); obtained from Sigma Aldrich (St Louis, MO, USA) at a dose of 0.191 μmol/65 g egg (0.05 mg CPA/egg), dissolved in 300 μL sterile water (local water tap) and was used as a positive control in the HET-MN study.

# CEGA

EXPERIMENTAL DESIGN

Fertilized white leghorn chicken (*Gallus gallus*) eggs were sourced from Charles River Laboratories (North Franklin, CT, USA). The eggs were numbered, weighed, and randomly divided into control and dosed groups (minimum 10 eggs per group). Incubation began on Day 0 in Styrofoam incubators, maintaining conditions at 37 ± 0.5 °C and 60 ± 5% humidity. Viability assessments were performed on Day 8 through transillumination, and non-developing eggs were discarded. Test compounds were administered in 0.15 ml/egg volumes via three daily injections into the air sac on incubation days 9 through 11. For a study assessing effects of glutathione depletion, pre-treatment with N-acetyl cysteine (1mg/egg) was conducted 1-hour prior to p-methoxy cinnamaldehyde dosing on day 8 before the first dosing. Three hours after the last injection on incubation day 11, eggs were terminated, and livers were collected for analysis in either NPL or comet assay. Viability, body and liver weights were recorded. Only groups with viability above 50% (at least half of the fetuses in the group were viable upon termination) were selected for the analyses. In each assay 3 liver samples per group were analyzed, owing to the limitation in the number of samples which can be analyzed simultaneously.

NPL  
In the Nucleotide 32P-postlabeling (NPL) assay, only groups receiving the highest tested doses that did not reduce viability by more than 50% were analyzed, following the approach recommended by Phillips and colleagues (Phillips et al., 2000). DNA isolation from liver samples utilized QIAGEN G100 columns, and the isolated DNA's purity was assessed. A 10 μg portion of purified DNA was used for NPL assessment following the method of Randerath et al. (1981). The assay's high sensitivity allowed the detection of a single adduct in 109 normal nucleotides. Enzymatic digestion and nuclease P1 enrichment were employed before labeling the adducts with [γ-32P]-adenosine-5'-triphosphate (32P-ATP). Two-directional thin-layer chromatography (TLC) was used for adduct resolution, and results were analyzed using a Molecular Dynamics Storm system. Positive outcomes were determined by visual comparison with controls, and quantitation was achieved using the Relative Abundance Level (RAL) formula. A positive result in the NPL assay strongly suggests direct DNA damage, providing robust evidence for genotoxic potential in CEGA.

COMET

Comet analysis (Tice et al., 2000) was conducted according to OECD guidelines (OECD, 2016) following previously described protocol (Williams et al., 2014). Specifically, 200 mg of each liver sample (3 samples per group) were minced and suspended in ice-cold Hank’s balanced salt solution (HBSS) containing 20 mM EDTA and 10% dimethylsulfoxide (DMSO). Cell suspensions (105 x 5 cells in 10 µl) underwent comet analysis following OECD guidelines. Aliquots were embedded in 1.0% low melting point agarose on slides, lysed, and electrophoresed at 20 V. DNA migration was visualized using ethidium bromide and analyzed with Comet Score software. Results, presented as mean + SD, underwent statistical analysis with one-way ANOVA and Tukey's method for multiple comparisons, and linear regression analysis was used to determine dose-related trends. Positive results were based on predefined criteria, including a statistically significant increase in DNA-in-tail, dose-related DNA damage, and values outside the historical control range. The evaluation criteria for determining positive comet results is described in detail in Kobets et al. (2018a) Compounds with positive comet outcomes were considered potentially genotoxic with either direct or indirect DNA reactivity.

# HET-MN assay

The assay protocol, based on Reisinger et al. (2018), utilized fertilized eggs from VALO Biomedia GmbH, Germany, implementing a thermal selection process followed by pre-warming and incubation. Eggs were turned automatically until day eight, and those meeting viability criteria were selected for further testing. A solubility experiment was conducted using different vehicles, and the maximum concentration for the main experiment was determined based on pre-screen toxicity data. Primitive erythrocytes were not considered relevant target cells, and a mutagenic treatment after day 7 was expected to have no impact on them. A dingle dose treatment was applied on day 8, with termination on day 11. Viability calculations excluded doses with <40% viability, and blood samples were collected for analysis. Blood smears were stained, and at least 1000 erythrocytes were analyzed per egg for micronuclei occurrence. Cytotoxic effects were assessed through the ratio of polychromatic to normochromatic erythrocytes. To evaluate the results, criteria described in Reisinger et al., were followed. Statistical analyses was conducted which included linear regression and Mann-Whitney tests, to assess micronucleus frequency and its statistical significance. Positive outcomes required a dose-related increase in micronuclei frequency above historical controls, while negative results indicated no significant or dose-related increase. The study design aimed to determine the potential genotoxic potential of the test material.

# RESULTS

2-Phenyl-2-butenal

### Effect on Viability

Viability in the groups dosed with 2-phenyl-2-butenal were >50%. Groups that received a total dose of 1, 2, 2.5, 3 and 5 mg/egg of the compound had viability of at least 83%, thus, these groups were used for the comet and NPL assays. Groups that received a dosage of and above 7.5 mg/egg had survival rate of less than 8%, and thus, were not utilized for the analyses. The viability percentage in the groups that received either vehicle or positive control, was 100%.

### DNA adducts

The results of the NPL test are demonstrated in Figure 1. No background adducts were detected in the vehicle control group (**Fig. 1 (A)**). No adducts were observed in the group that received 5 mg/egg of 2-phenyl-2-butenal (the highest tested dose that did not reduce viability below 50%) (**Fig. 1 (A)**). In contrast, 5 mg/egg of the positive control, quinolone produced approximately 6.07 DNA adducts in 108 of normal nucleotides (**Fig. 1 (A)**).

### DNA strand breaks

The results of the comet test are represented in **Figure 1 (B)**. In the comet assay, 2-phenyl-2-butenal was tested at the total doses of 1, 2, 2.5, 3 and 5 mg/egg, which did not significantly affect viability levels. No statistically significant difference was observed in the percentage of DNA in tail between control and dosed groups with the exception of 2 mg/egg, which is indicative of absence of DNA strand breaks. The group that received 2 mg/egg showed slight statistically significant increase in the percent of DNA in tail observed, however the higher doses did not show any dose dependent increases and there were no dose-dependent increases observe, which suggest this increase may not be biologically relevant. In contrast, the positive control, quinolone, produced statistically significant 2.6-fold increase in DNA strand breaks in the chicken fetal livers (**Figure 1 (B)**).

### HET-MN

The results of the micronucleus test are represented in **Figure 1 (C)**. In the micronucleus test, 2-phenyl-2-butenal was tested at the total doses of 0.5, 1, 2, and 2.5 mg/egg. Since there was significant toxicity at 2.5 mg/egg resulting in 33 and 27% viability, respectively, in two experiments it was not included in MN analysis. Doses of 0.5,1 and 2 were analyzed for MN, which did not significantly affect viability levels. No statistically significant difference was observed in the percentage micronuclei between control and dosed groups, which is indicative of absence of clastogenic potential. In contrast, the positive control, cyclophosphamide, produced a statistically significant increase in MN frequency (**Figure 1 (C**).

# Nona-2 trans- 6-cis-dienal

### Effect on Viability

Viability in the groups dosed with nona-2 trans-6-cis-dienal was >50%. Groups that received a total dose of 0.05, 0.1, 0.25 and 0.5 mg/egg had viability of at least 89%. Due to the limitation in the number of samples which can be analyzed simultaneously, only groups dosed with the total dose of 0.25 and 0.5 mg/egg were analyzed in the assay. Groups that received a total dose of 0.75 and 0.9 mg/egg had low survival rate, 25 and 33%, respectively. Thus, these groups were not utilized for the analyses. The viability percentage in the groups that received either vehicle or positive control, was 100%.

### DNA adducts

The results of the NPL test are summarized are demonstrated in Figure 2. No background adducts were detected in the vehicle control group (**Fig. 2 (A)**). No adducts were observed in the group that received 0.5 mg/egg of nona-2 trans-6-cis-dienal (the highest tested dose that did not reduce viability below 50%). In contrast, 5 mg/egg of the positive control, quinolone produced approximately 7.3 DNA adducts in 108 of normal nucleotides (**Fig. 2 (A)**).

### DNA strand breaks

The results of the comet test are summarized in **Figure 2 (B)**. In the comet assay, nona-2 trans-6-cis-dienal was tested at the total doses of 0.25 and 0.5 mg/egg, which did not significantly affect viability levels. No statistically significant difference was observed for the percentage of DNA in tail between control and dosed groups, which is indicative of absence of DNA strand breaks. In contrast, the positive control, quinolone, produced statistically significant 2.9-fold increase in DNA strand breaks in the chicken fetal livers (**Figure 2 (B)**).

### HET-MN

The results of the micronucleus assay are represented in **Figure 2 (C)**. In the micronucleus assay, nona-2 trans-6-cis-dienal was tested at the total doses of 0.05, 0.1, 0.25, and 0.5 mg/egg. Since there was toxicity at 0.5 mg/egg resulting in 40 and 60% viability, respectively, in two experiments it was included in MN analysis considering it was in the acceptable toxicity range. Doses of 0.1,0.25 and 0.5 were analyzed for MN, which did not significantly affect viability levels. No statistically significant difference was observed in the percentage micronuclei between control and dosed groups, which is indicative of absence of clastogenic potential. In contrast, the positive control, cyclophosphamide, produced statistically significant increase in MN frequency (**Figure 2 (C)**).

# 2-Methyl-2-pentenal

### Effect on Viability

Viability in the groups dosed with 2-methyl-2-pentenal was >50%. Groups that received a total dose of 5, 6, 7, and 8 mg/egg had viability of at least 90%. Due to the limitation in the number of samples which can be analyzed simultaneously, only groups dosed with the total dose of 7 and 8 mg/egg of 2-methyl-2-pentenal were analyzed in the assay. Group that received a total dose of 10 mg/egg had 45% viability rate and was still analyzed in the comet assay. Groups that were dosed with 15 and 20 mg/egg had very low survival rate, 18 and 0%, respectively. Thus, these groups were not utilized for the analyses. The viability percentage in the groups that received either vehicle or positive control, was 100%.

### DNA adducts

The results of the NPL test are depicted in Figure 3. No background adducts were detected in the vehicle control group (**Fig. 3 (A)**). No adducts were observed in the group that received 8 mg/egg of 2-methyl-2-pentenal (the highest tested dose that did not reduce viability below 50%) (**Fig. 3 (A)**). In contrast, 5 mg/egg of the positive control, quinolone produced approximately 7.33 DNA adducts in 108 of normal nucleotides (**Fig. 3 (A)**).

### DNA strand breaks

The results of the comet test are summarized in **Figure 3 (B)**. In the comet assay, 2-methyl-2-pentenal was tested at the total doses of 7 and 8 mg/egg, which did not significantly affect viability levels, as well as at 10 mg/egg, which decreased survival rate just below 50%. No statistically significant difference was observed for the percentage of DNA in tail between control and dosed groups, even at 10 mg/egg, which is indicative of absence of DNA strand breaks. In contrast, the positive control, quinolone, produced a statistically significant 2.9-fold increase in DNA strand breaks in the chicken fetal livers (**Figure 3 (B)**).

### HET-MN

The results of the micronucleus assay are represented in **Figure 3 (C)**. In the micronucleus test, 2-methyl-2-pentenal was tested at the total doses of 1, 3, 5, and 7 mg/egg. Since there was significant toxicity at 7 mg/egg resulting in 20 and 13% viability, respectively, in two experiments it was not included in MN analysis. Doses of 1,3 and 5 were analyzed for MN, which did not significantly affect viability levels. No statistically significant difference was observed in the percentage micronuclei between control and dosed groups, which is indicative of absence of clastogenic potential. In contrast, the positive control, cyclophosphamide, produced statistically significant increase in MN frequency (**Figure 3 (C)**).

# p-Methoxy cinnamaldehyde

### Effect on Viability

Viability in the groups dosed with p-methoxy cinnamaldehyde was >50%. Groups that received a total dose of 1.5, 2 and 2.5 mg/egg had viability of at least 83%. Thus, all groups were analyzed in the assay. The viability percentage in the groups that received either vehicle or positive control, was 88%.

### DNA adducts

The results of the NPL test are depicted in Figure 4. No background adducts were detected in the vehicle control group (**Fig. 4 (A)**). The NPL assay detected and average of 17 DNA adducts in 108 of normal nucleotides produced by 2.5 mg/egg of p-methoxy cinnamaldehyde (the highest tested dose that did not reduce viability below 50%) (**Fig.4 (A)**). The positive control, quinolone, at the total dose of 5 mg/egg, also produced DNA adducts, approximately 6.54 in 108 of normal nucleotides (**Fig.4 (A)**).

### DNA strand breaks

The results of the comet test are summarized in **Figure 4 (B)**. In the comet test, p-methoxy cinnamaldehyde was tested at the total doses of 1.5, 2 and 2.5 mg/egg, which did not significantly affect viability levels. Comet assay results confirmed findings in NPL assay, in all dosed groups, p-methoxy cinnamaldehyde produced dose-related significant 3- to 3.8-fold increase in the percentage of DNA in tail compared to control group. Positive control, quinolone, also produced statistically significant 3.3-fold increase in DNA strand breaks in the chicken fetal livers (**Figure4 (B)**).

### HET-MN

The results of the micronucleus (MN) assay are represented in **Figure 4 (C)**. In the micronucleus test, 2-methyl-2-pentenal was tested at the total doses of 0.75, 1, 1.25, and 1.5 mg/egg. Since there was no significant toxicity at 1.5 mg/egg, in two experiments it was included in MN analysis. Doses of 1,1.25 and 1.5 were analyzed for MN, which did not significantly affect viability levels. No statistically significant difference was observed in the percentage micronuclei between control and dosed groups, which is indicative of absence of clastogenic potential. In contrast, the positive control, cyclophosphamide, produced statistically significant increase in MN frequency (**Figure4 (C)**).

# p-Methoxy cinnamaldehyde follow-up Study with N-acetyl cysteine pretreatment

### Effect on Viability

Groups were dosed with p-methoxy cinnamaldehyde (2.5 mg/egg) and single injection pre-treatment of N-acetyl cysteine (1.25mg/egg) followed by p-methoxy cinnamaldehyde treatment (2.5 mg/egg). Groups that received a total dose of 2.5 mg/egg had viability of at least 100%. Thus, all groups were analyzed in the assay. The viability percentage in the groups that received either vehicle or treatment or positive control, was 100%.

### DNA strand breaks

The results of the comet test are summarized in **Figure 5**. In the comet test, p-methoxy cinnamaldehyde was tested at total doses of 2.5 mg/egg, which did not significantly affect viability levels. Comet assay results, in dosed group, p-methoxy cinnamaldehyde produced significant 3- fold increase in the percentage of DNA in tail compared to control group. To validate if this was a true DNA damage event due to chemical treatment or depletion of glutathione at higher doses of treated chemical, we repeated the study by pre-treatment with N-acetyl cysteine. However, when single injection of N-acetyl cysteine followed by p-methoxy cinnamaldehyde injections at total doses of 2.5 mg/egg had no significant increases in percentage DNA in tail compared to control group. Positive control, quinolone, also produced statistically significant 4-fold increase in DNA strand breaks in the chicken fetal livers (**Figure 5**).

DNA adduct formation and HET-MN studies were not conducted using N-acetyl cysteine pre-treatment protocol.

# CONCLUSIONS AND DISCUSSION

Three out of four tested materials, 2-phenyl-2-butenal, nona-2 trans-6-cis-dienal, 2-methyl-2-pentenal produced negative results in both, the NPL and comet assays, indicating lack of DNA damaging potential. These findings correlate with the lack of genotoxicity for these chemicals in the comet and micronucleus assays in vivo (**Table 2**). *In vitro*, nona-2 trans-6-cis-dienal and 2-methyl-2-pentenal produced positive results, which were not confirmed in vivo.

One chemical, p-methoxy cinnamaldehyde, produced DNA adducts and strand breaks in CEGA, indicating DNA damaging potential. These results are different from the outcomes of the in vivo comet and micronucleus assays, which were negative for p-methoxy cinnamaldehyde (**Table 2**). The findings in CEGA might indicate the ability of chicken fetal liver to bioactivate the compound. These findings indicate the importance of more detailed investigation of the metabolic activities of chicken fetus and comparison of the metabolic enzyme activities between chicken, rodent and human hepatocytes. Additionally, as per prediction model (OASIS TIMES v2.28.1.6) the primary detoxification pathway for p-Methoxycinnamaldehyde is considered to be via glutathione conjugation (**Figure 5**) hence the variability in the results could possibly be due to glutathione depletion in chicken egg assay. Additional follow-up studies were conducted to verify this by pretreatment of the eggs with N-acetyl cysteine (Kumar et al., 2021) to determine whether the response was affected. The results for eggs pretreated with N-acetyl cysteine were negative for statistically significant increases in % DNA tail intensity in the COMET assay (**Figure 6**), this further supports the conclusion that the initial positive response in the CEGA-COMET was due to glutathione depletion.

No DNA damage was detected in control groups that received vehicles. The absence of background adducts in the vehicle control group is consistent with the previous findings in the egg models (Kobets et al., 2016; 2018a; Williams et al., 2014). This may reflect the age of the organism or absence of environmental exposures, e.g. diet. The positive control, quinolone, consistently yielded positive results in both NPL and comet assay, confirming reliability of the assays. In the NPL assay chromatographically similar spots were obtained in all liver samples tested with quinoline. Average levels of DNA adduct, and DNA strand breaks were also similar between the positive control groups from different experiments.

Overall, outcomes in CEGA with one exception correlated better with the in vivo data than with in vitro genotoxicity findings. Even one which was found positive in the CEGA COMET/NPL study, was concluded to be negative when eggs were pretreated with N-acetyl cysteine. Hence in order to verify if the response is biologically relevant or not one can potentially use in silico tools and repeat the study with appropriate cofactors based on the outcome from *in silico* prediction models to verify the response.

For HET-MN all four fragrance materials of alpha, beta unsaturated class of aldehydes were negative which had 100% concordance with the in vivo study results. The HET-MN data was produced with a slightly different study design than CEGA-COMET/NPL study. The dosing regimen for CEGA-COMET/NPL is 3 doses starting from day 9-11, the doses were distributed in three different injections of 50µl/egg (total volume of 150 µl) on three days to make total concentration/egg shown in the results. However, the dosing for HET-MN was a single dose of 50µl/egg on day-8 with 72-hour incubation period and MN analysis. This also helps explain the difference of doses tested in the two models, the doses tested in the HET-MN study were slightly lower than the ones tested in the CEGA-COMET/NPL studies. The DRF studies conducted in the HET-MN studies had one concentrated dose of final concentration in the test model, but in CEGA studies this dose was divided into three different dosing intervals which can help test doses at higher concentrations. Additionally, for p-Methoxycinnamaldehyde the glutathione depletion issue as discussed before may be more prominent in CEGA-COMET with different dosing for 3 days and evaluation of tissue 3-hour post final injection, when compared to a single injection then 72-hour before sample collection in HET-MN studies. The longer post treatment time before sample collection in HET-MN studies may lead to sufficient glutathione generation to detoxify alpha-c-oxidation product formed by p-methoxycinnamldehyde.

Even with different dosing and incubation periods post dosing the results were quite like the in vivo studies and more biologically relevant compared to the outcomes observed in the current regulatory approved in vitro test battery. Hence, Chicken egg COMET/NPL assay could potentially be considered as an animal alternative and more biologically relevant follow-up assay to address misleading in vitro Ames studies.

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**Figures and Legends**

|  |  |  |
| --- | --- | --- |
| A close-up of a black and white photo of a pipe  Description automatically generated | A blurry black and white photo of a hammer  Description automatically generated | A black arrow in the air  Description automatically generated  6.07±1.99\*108 nucleotides |
| Vehicle, 20% HS 15 | 2-Phenyl-2-butenal,5mg/egg | Quinoline, 5 mg/egg |

(A)

 

(B) (C)

|  |
| --- |
| **Figure 1.** (A) Representative nucleotide ³²P-postlabeling (NPL) chromatograms from the chicken fetal liver samples after exposure to the vehicle, 2-phenyl-2-butenal, or positive control. Analysis of DNA adducts from the livers (3 samples (eggs) per group) was obtained using NP1 enrichment. Adducts were resolved in the second and third directions of chromatography and are indicated by arrows. Dose represents cumulative dose, administered in 3 daily injections. (B) DNA-in-tail percentage in the livers of chicken fetuses exposed to the vehicle, 2-phenyl-2-butenal, or positive control. (C) Micronucleus percentage in the blood of chicken fetuses exposed to the vehicle, 2-phenyl-2-butenal, or positive control. |

|  |  |  |
| --- | --- | --- |
| A blurry image of a person's hand  Description automatically generated | A blurry black and white photo of a person's head  Description automatically generated | A black arrow pointing towards a black circle  Description automatically generated  7.33±0.55\*108 nucleotides |
| Vehicle, 20% HS 15 | Nona-2 trans-6-cis-dienal, 0.5 mg/egg | Quinoline, 5 mg/egg |

(A)

 

(B) (C)

**Figure 2.** (A) Representative nucleotide ³²P-postlabeling (NPL) chromatograms from the chicken fetal liver samples after exposure to the vehicle, nona-2 trans-6-cis-dienal, or positive control. Analysis of DNA adducts from the livers (3 samples (eggs) per group) was obtained using NP1 enrichment. Adducts were resolved in the second and third directions of chromatography and are indicated by arrows. Dose represents cumulative dose, administered in 3 daily injections. (B) DNA-in-tail percentage in the livers of chicken fetuses exposed to the vehicle, nona-2 trans-6-cis-dienal, or positive control. (C) Micronucleus percentage in the blood of chicken fetuses exposed to the vehicle, nona-2 trans-6-cis-dienal, or positive control.

|  |  |  |
| --- | --- | --- |
| A blurry black and white photo of a tall pole  Description automatically generated | A blurry black and white photo of a person's head  Description automatically generated | A black arrow pointing to the sky  Description automatically generated with medium confidence  7.33±0.55\*108 nucleotides |
| Vehicle, 20% HS 15 | 2-Methyl-2-pentenal, 8 mg/egg | Quinoline, 5 mg/egg |

(A)

 

(B) (C)

**Figure 3.** (A) Representative nucleotide ³²P-postlabeling (NPL) chromatograms from the chicken fetal liver samples after exposure to the vehicle, 2-methyl-2-pentenal, or positive control. Analysis of DNA adducts from the livers (3 samples (eggs) per group) was obtained using NP1 enrichment. Adducts were resolved in the second and third directions of chromatography and are indicated by arrows. Dose represents cumulative dose, administered in 3 daily injections. (B) DNA-in-tail percentage in the livers of chicken fetuses exposed to the vehicle, 2-methyl-2-pentenal, or positive control. (C) Micronucleus percentage in the blood of chicken fetuses exposed to the vehicle, 2-methyl-2-pentenal, or positive control.

|  |  |  |
| --- | --- | --- |
| A blurry black and white photo of a light  Description automatically generated | A blurry image of a black arrow  Description automatically generated  17.04±4.39\*108 nucleotides | A black arrow in the air  Description automatically generated  6.54±0.38\*108 nucleotides |
| Vehicle, 20% HS 15 | p-Methoxy cinnamaldehyde, 2.5 mg/egg | Quinoline, 5 mg/egg |

(A)

 

(B) (C)

**Figure 4.** (A) Representative nucleotide ³²P-postlabeling (NPL) chromatograms from the chicken fetal liver samples after exposure to the vehicle, p-methoxy cinnamaldehyde, or positive control. Analysis of DNA adducts from the livers (3 samples (eggs) per group) was obtained using NP1 enrichment. Adducts were resolved in the second and third directions of chromatography and are indicated by arrows. Dose represents cumulative dose, administered in 3 daily injections. (B) DNA-in-tail percentage in the livers of chicken fetuses exposed to the vehicle, p-methoxy cinnamaldehyde, or positive control. (C) Micronucleus percentage in the blood of chicken fetuses exposed to the vehicle, p-methoxy cinnamaldehyde, or positive control.



**Figure 5:** DNA-in-tail percentage in the livers of chicken fetuses exposed to the vehicle, p-methoxy cinnamaldehyde, N-acetyl cysteine with p-methoxy cinnamaldehyde or positive control.

A black and white drawing of a molecule

Description automatically generated

p-Methoxycinnamaldehyde

A structure of a chemical formula

Description automatically generatedA structure of a chemical formula

Description automatically generated A molecule structure with letters and numbers

Description automatically generated

Alpha-C-oxidation product

(Glutathione Conjugation) (Mercapturic acid formation)

A structure of a chemical formula

Description automatically generated

(Glutathione Conjugation)

**Figure 6:** Modified Predicted metabolism pathway of p-Methoxycinnamaldehyde using OASIS TIMES (v2.28.1.6)

**Tables**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Table 1.** Characterization of chemicals tested in CEGA and HET-MN | | | |  |
| **Chemical Name** | **Chemical Structure** | **LD50 (rat oral), mg/kg bw a** | **Doses tested in CEGA, mg/egg b** | **Doses tested in HET\_MN, mg/eggc** |
| Vehicle | | | |  |
| Solutol HS15d | A chevron of a chemical formula  Description automatically generated with medium confidence |  | 20% aqueous solution |  |
| Isopropyl myristate (IPM) e | A black line on a white background  Description automatically generated |  |  |  |
| Test Chemicals | | | |  |
| 2-Phenyl-2-butenal | A structure of a chemical compound  Description automatically generated | N/A | 1  2  2.5  3  5 | 0.5  1  2 |
| Nona-2 trans- 6-cis-dienal | A diagram of a chemical formula  Description automatically generated | 5,000 | 0.25  0.5 | 0.1  0.25  0.5 |
| 2-Methyl-2-pentenal | A structure of a chemical formula  Description automatically generated | 4,290 | 7  8  10 | 1  3  5 |
| p-Methoxy cinnamaldehyde | A black and white drawing of a molecule  Description automatically generated | N/A | 1.5  2.0  2.5 | 1.0  1.25  1.5 |
| Positive Control | | | |  |
| Quinoline d | A black and blue chemical structure  Description automatically generated | 331 | 5 |  |
| Trans-2-Hexenal d | A black line drawing of a graph  Description automatically generated | 780 | 0.25  0.5  1 |  |
| Cyclophosphamide e | A structure of a molecule  Description automatically generated |  |  | 0.05 |

a, values obtained from ChemIDplus Advanced database (<https://chem.nlm.nih.gov/chemidplus/>)

b, cumulative dose administered in CEGA in 3 consecutive daily injections in 0.15 ml

c, Single dose administered in HET-MN assay on Day 8

d, control for GEGA

e, control for HET-MN

CEGA, Chicken Egg Genotoxicity Assay, HET-MN, Hen Egg micronucleus induction test, N/A, not available

The results of testing for all four compounds are summarized in Table **2** below**.**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Chemical Name | CAS # | CEGA | | HET-MN | In vitro*a* | | In vivo b | |
| NPL | Comet |  | Ames | In vitro MNT | Comet | MNT |
| 2-Phenyl-2-butenal | 4411-89-6 | - | - | - | **+** |  | **-** | **-** |
| Nona-2 trans- 6-cis-dienal | 557-48-2 |  | - | - |  | **+** | **-** | **-** |
| - |
|  |
| 2-Methyl-2-pentenal | 623-36-9 | - | - | - | **-** | **+** | **-** | **-** |
| p-Methoxy cinnamaldehyde | 1963-36-6 | + | **+** | - | **+** | **+** | **-** | **-** |

a values obtained from RIFM for in vitro studies

b, values obtained from RIFM for in vivo studies

MNT, micronucleus assay

+, positive; -, negative

**Table 2. Summary of the results of α, ß-unsaturated aldehyde e class of fragrance materials in CEGA, HET-MN and comparison to available in vitro and in vivo genotoxicity data.**