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Assessment and characterization of DNA adducts produced by alkenylbenzenes in fetal turkey and chicken livers

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ABSTRACT

Formation of DNA adducts by five alkenylbenzenes, safrole, methyl eugenol, eugenol, and asarone with either α- or β-conformation, was analyzed in fetal avian livers in two *in ovo* models. DNA reactivity of the carcinogens safrole and methyl eugenol was previously demonstrated in the turkey egg model, whereas non-genotoxic eugenol was negative. In the current study, alkenylbenzenes were also tested in the chicken egg model. Injections with alkenylbenzenes were administered to fertilized turkey or chicken eggs for three consecutive days. Three hours after the last injection, liver samples were evaluated for DNA adduct formation using the ^{32}P-nucleotide postlabeling assay. DNA samples from turkey livers were also analyzed for adducts using mass spectrometry. In both species, genotoxic alkenylbenzenes safrole, methyl eugenol, α- and β-asarone produced DNA adducts, the presence and nature of which, with exception of safrole, were confirmed by mass spectrometry, validating the sensitivity of the ^{32}P-postlabeling assay. Overall, the results of testing were congruent between fetal turkey and chicken livers, confirming that these organisms can be used interchangeably. Moreover, data obtained in both models is comparable to genotoxicity findings in other species, supporting the usefulness of avian models for the assessment of genotoxicity as a potential alternative to animal models.

1. Introduction

Phytochemicals from the class of alkenylbenzenes are widely utilized by manufacturers and consumers for their fragrance, flavor and pharmacological properties. Some of the members of this class, e.g., safrole and methyl eugenol, however, have carcinogenic activity in rodents (IARC, 1976; NTP, 2000). The mechanism of liver tumor induction by alkenylbenzenes is associated with their genotoxicity. Specifically, DNA-reactivity stems from formation of a carbenium ion as a result of side-chain hydroxylation catalyzed by cytochromes P450 enzymes (CYPs) and biotransformation to 1′-sulfooxy metabolites in the presence of sulfotransferase (Miller et al., 1979; Miller and Miller, 1983; Rietjens et al., 2005; Smith et al., 2002). DNA binding has been detected in rodents after exposure to safrole, methyl eugenol and estragole (Phillips et al., 1984; Randerath et al., 1984). Findings by several investigators (Howes et al., 1990; Miller et al., 1979, 1983; Rietjens et al., 2014; Smith et al., 2002; Tsai et al., 1994), including a study in fetal turkey liver (Kobets et al., 2016), indicate that the genotoxic activity of alkenylbenzenes is dependent on several structural features. The presence of a double bond at the terminal position in the alkenyl side chain and/or the absence of a free phenolic hydroxyl group (Fig. 1) is a common feature of alkenylbenzenes that exhibit genotoxic properties. In contrast, non-genotoxic alkenylbenzenes, such as eugenol, possess structural features more favorable to detoxication (Fig. 1) (Kobets et al., 2016). The genotoxicity of eugenol was only recorded at high cytotoxic doses, indicating the saturation of the detoxication pathways (EFSA, 2009). Genotoxicity of safrole and methyl eugenol was previously reported in an *in ovo* genotoxicity model, the Turkey Egg Genotoxicity Assay (TEGA) while eugenol was negative in the model (Kobets et al., 2016, 2018a).

Trans- and *cis*-isomers of the alkenylbenzene 1-propenyl-2,4,5-trimethoxybenzene, α- and β-asarone, naturally occur in plants, including *Acorus calamus and Asarum gramineus* (Berg et al., 2016). The widespread use of these materials as flavoring ingredients or constituents of some medicinal products raises safety concern, since both asarone isomers produced cancer in the liver and duodenum of rodents (SCF, 2002; JECFA, 1981; Wiseman et al., 1987). Findings in various *in vitro* studies support a genotoxic and mutagenic mode of action of asarone isomers (Chellian et al., 2017; Göggelmann and Schimmer, 1983; Hasheminejad and Caldwell, 1994; Haupenthal et al., 2017; Kim et al.,

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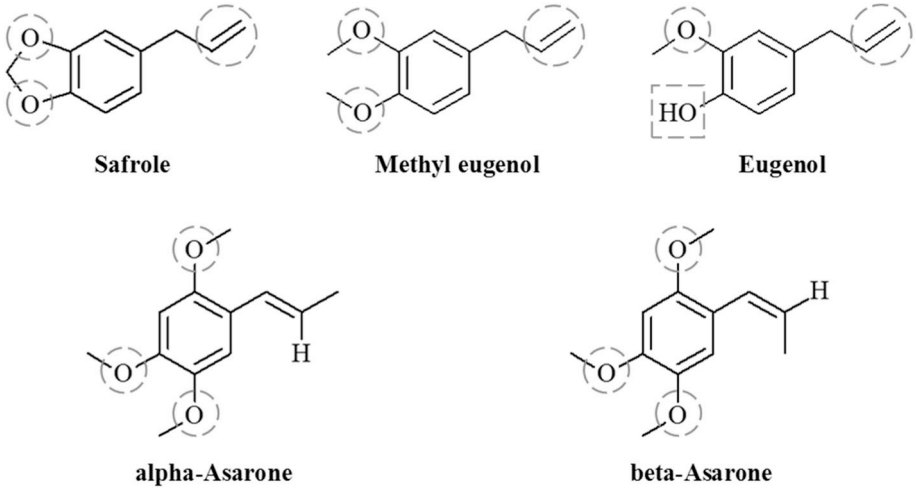

Fig. 1. Chemical structures of tested alkenylbenzenes. Grey circle indicates structural feature favorable for bioactivation. Grey square indicates structural feature favorable for detoxication.

1999). Moreover, *in vivo,* α- and β-asarone form highly reactive and mutagenic side-chain epoxides after bioactivation (Berg et al., 2016; Cartus et al., 2015; Cartus and Schrenk, 2016). The CYP inhibitor, cimetidine, decreased genotoxicity of asarones (Hasheminejad and Caldwell, 1994), indicating the importance of CYPs in bioactivation of asarones and consequently their carcinogenicity, similar to other carcinogenic alkenylbenzenes. Moreover, both isomers of asarone have cytotoxic potential, and in many studies α-asarone was found to be more cytotoxic compared to the β-isomer (Haupenthal et al., 2017; Unger and Melzig, 2012).

The current study was designed to evaluate the DNA reactivity potentials of the aforementioned alkenylbenzenes (Fig. 1) in fetal turkey and chicken livers in order to compare the responses of the two *in ovo* models under slightly different experimental protocols (Fig. 2) and to examine the utility of developing avian livers from two different species in genotoxicity testing. Avian egg models (Kobets et al., 2016, 2018a, 2018b; Williams et al., 2014) were developed as alternative to animal testing tools for the assessment of various hazardous effects of environmental chemicals to potentially replace short-term animal studies required for human safety assessment. The egg models utilize metabolically competent (Hamilton et al., 1983; Kobets et al., 2018b; Perrone et al., 2004; Wolf and Luepke, 1997) livers of avian fetuses, which are terminated prior to hatching (Fig. 2) and as such are not yet considered to be animals.

Among the endpoints assessed in the *in ovo* assays is the ability of a test substance to form DNA adducts. Formation of DNA adducts is a

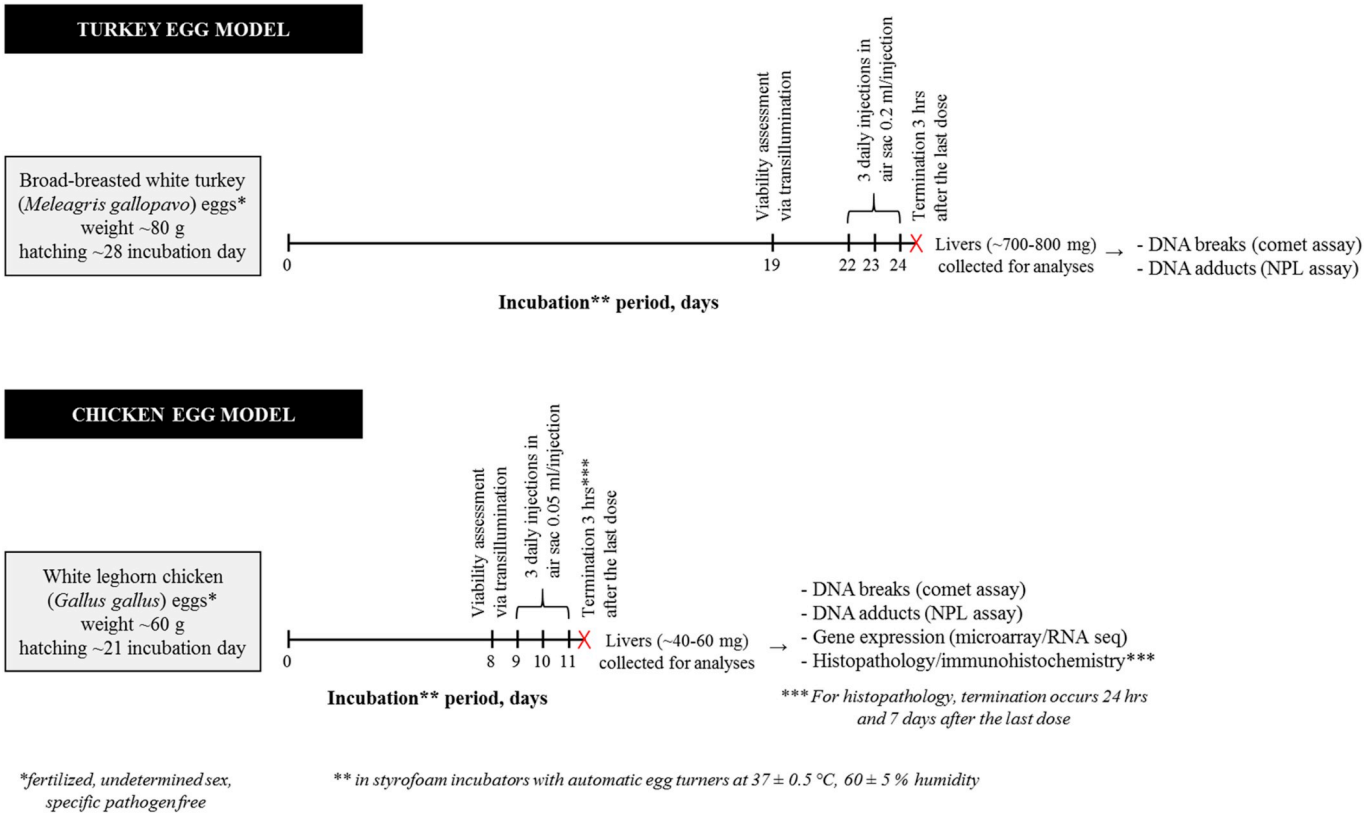

Fig. 2. Experimental design of *in ovo* models.

Table 1
Dose levels of alkenylbenzenes tested in the fetal turkey and chicken livers.

Compound	Dose, mg/egg[a]	Average weight of the egg, g[b]	Dose, mg/kg[c]	Molecular weight, g/mol	Dose, μmol[d]
Turkey					
Safrole	1	73.7	14	164.2	83.7
	2	69.6	29		177.1
Methyl eugenol	2	81.3	25	178.2	138
	4	79.3	50		283.1
Eugenol	1	77	13	164.2	79.1
	2.5	78.7	32		193.6
α-Asarone	5	86.3	58	208.3	278.3
	10	83.2	120		577.4
β-Asarone	2.5	72.1	35	208.3	166.6
	5	72.6	70		335.5
Chicken					
Safrole	6	58.5	103	162.2	632.4
Methyl eugenol	2	51.3	39	178.2	218.7
Eugenol	3	51.2	59	164.2	356.8
α-Asarone	8	59.2	135	208.3	648.4
β-Asarone	0.5	59.8	8	208.3	40.2

[a] , administered in 3 daily injections.
[b] , average weight in each group upon termination.
[c] , based on the average weight of the eggs in each group on termination day.
[d] , based on the mg/kg dose.

crucial step in the carcinogenicity of DNA-reactive chemicals, and as such, it can be used as a sensitive biomarker, not only of exposure to the chemical, but also of preneoplastic effect (Williams et al., 2013; Kobets et al., 2019). The ^{32}P-postlabeling assay (Randerath et al., 1981; Phillips and Arlt, 2014) is an appropriate and widely used method for the detection of DNA adducts. In order to validate the sensitivity of the postlabeling assay, in the current study DNA samples from turkey livers were also analyzed for adducts using ultra high performance liquid chromatography electrospray ionization tandem mass spectrometry (UHPLC-ESI$^+$-MS/MS).

2. Material and methods

2.1. Experimental design

2.1.1. Chemicals

Chemicals selected for testing as well as tested doses are listed in Table 1. The chemical structures of the selected materials are shown in Fig. 1. Safrole (CAS: 94-59-7, 97%) and eugenol (CAS: 97-53-0, 99%) were obtained from Sigma-Aldrich (St Louis, MO, USA). Methyl eugenol (CAS: 93-15-2, 98% pure as reported by the supplier) was purchased from Vigon International (East Stroudsburg, PA, USA). Alpha-asarone (α-asarone) (CAS: 2883-98-9, 98%) was purchased from AlfaAesar by Thermo Fisher Scientific (Karlsruhe, Germany). Beta-asarone (β-asarone) (CAS: 5273-86-9, 98.8%) was isolated by column chromatography (EtOAc/hexane 1:4 on silica gel 60) from *Acorus Calamus* oil at the University of Kaiserslautern (Kaiserslautern, Germany). 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 a vehicle, based on the polarity of the tested chemicals. Properties of the tested chemicals, including solubility and toxicity data, were obtained from Material Safety Data Sheets (MSDS) and online database ChemID plus (https://chem.nlm.nih.gov/chemidplus/). All solutions were freshly prepared not more than 3 days prior to dosing. Solutions were stored away from direct light to avoid photo-degradation.

2.1.2. Egg handling and dosing protocols

The protocols (Fig. 2) are described in detail in Williams et al. (2014) and Kobets et al. (2016). Fertilized broad-breasted white turkey (*Meleagris gallopavo*) eggs of undetermined sex were purchased from Aviagen Turkeys, Inc. (Lewisburg, WV, USA). Specific pathogen free (SPF), fertile white leghorn chicken (*Gallus gallus*) eggs of undetermined sex were obtained from Charles River Laboratories (North Franklin, CT, USA). Eggs were incubated in 2GIF Styrofoam incubators with automatic egg turners (Murray McMurray Hatchery Inc, Webster City, IA, USA) at 37 ± 0.5 °C and 60% ± 5% humidity. Viability was assessed 1–3 days prior to the commencement of dosing by transillumination, eggs that did not develop were eliminated. Control and dosed (n > 10 eggs per group) eggs were separated to avoid any possible airborne cross contamination. The repeated dose regimen consisted of 3 daily injections with vehicle or test substances administered into the air sac in total volume of 0.6 ml/egg (0.2 ml/egg/day) (turkey) or 0.15 ml/egg (50 μl/egg/day) (chicken) on incubation days 22 through 24 (turkey) or 9 to 11 (chicken). For each chemical the cumulative dose given in a course of 3 days was expressed as mg/egg total (Table 1). Dose ranges for testing were selected based on the data from pilot studies. In case of low solubility of a compound, a serial dilution was prepared to detect the solubility limit, which determined the highest dose selected for testing. Eggs were terminated 3 h after the last injection. Eggs were weighted (Fig. 3A), egg shells were opened, fetuses were removed and decapitated. Fetal livers were harvested and frozen at −80 °C for a subsequent DNA adduct analysis. Fetal body and liver weights (Fig. 3B and C) as well as viability were recorded. Groups chosen for analysis had viability level higher than 50% (at least half of the fetuses in the group are viable upon termination) in order to avoid false positive results due to cytotoxicity. In each assay 3 liver samples per group were analyzed, owing to the limitation in the number of samples which can be analyzed simultaneously. For each procedure, appropriate New York Medical College-Chemical Safety Laboratory Standard Operating Procedures were followed.

2.2. DNA adducts analyses

2.2.1. ^{32}P-nucleotide postlabeling (NPL) assay

The NPL assay (Phillips and Arlt, 2014; Randerath et al., 1981) was used as previously described in detail (Williams et al., 2014). DNA extraction from three (3) frozen liver samples per group was conducted using genomic QIAGEN G100 columns (Valencia, CA, USA) following the manufacturer's protocol. Only samples that received the highest tested doses were analyzed, as they are most likely to reveal the presence of adducts, which, according to Phillips and colleagues, is adequate for identifying a positive response (Phillips et al., 2000). Cary 1E UV/visible spectrophotometer (Varian Optical Spectroscopy Instruments, Mulgrave, Victoria, Australia) was used to measure the purity of extracted DNA. 10 μg of DNA per sample with adequate purity (260/280 and 260/230 nm absorbance ratios equal or greater than 1.8 and 2.0, respectively), was analyzed.

NPL analysis was conducted with nuclease P_1 (NP_1) digestion (Reddy and Randerath, 1986). In case when adducts were not detected, hydrophilic-lipophilic balance (HLB) column enrichment (Gupta, 1985) was used, as previously described in detail (Jeffrey et al., 2002; Williams et al., 2014). Labeled modified bases were then resolved using two- or three-directional thin-layer chromatography (TLC) systems. The radioactivity on the TLC plates was detected using a Molecular Dynamics Storm 860 system with exposure times of 2 h to overnight (GE Health Care Life Sciences, Edison, NJ, USA).

Interpretation of the results was based on visual comparison of the chromatograms of samples from dosed groups with corresponding vehicle control group, which were run simultaneously. A positive result was recorded if the chromatogram from exposed sample displayed any extra spot(s) compared to the negative control group. A negative result was recorded if there were no other spot(s) than the naturally occurring ones in the controls. If the test substance showed a positive response, quantitation was achieved by integrating the individual spots, using the Molecular Dynamics Imager software. DNA adducts in 10^8 of normal

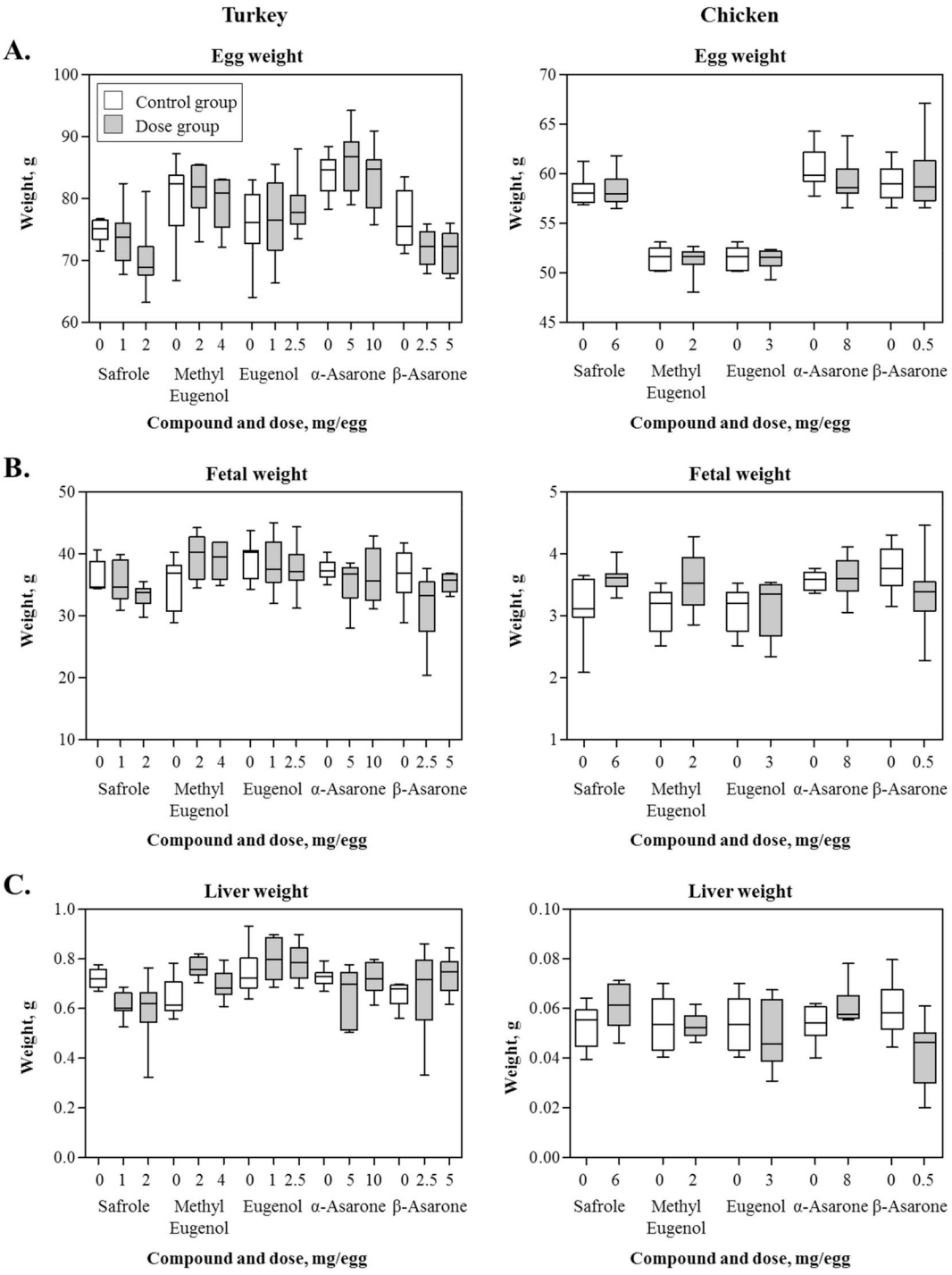

Fig. 3. Box-plot representation of turkey and chicken egg (A), fetal (B) and liver (C) weights upon termination. Grey boxes represent groups dosed with alkenylbenzenes, white boxes represent corresponding control groups. Data is presented as mean weight in g, whiskers correspond to minimum and maximum values observed.

nucleotides were ascertained (threshold for NPL assay in the laboratory is 10^9), and a mean value per group was calculated. Values obtained are considered comparative rather than absolute since many assumptions are made in the calculations and no internal standard was included.

A positive outcome in the NPL provides very strong evidence for direct DNA damage since, owing to the multiple enzyme steps involved in the digestion of the purified DNA and kinase reaction of the resulting deoxyribonucleoside 3′-phosphates, few false positives occur and then only under very extreme conditions. Thus, a compound positive in NPL assay is considered positive in the *in ovo* assay. However, some DNA adducts can poorly be phosphorylated or are difficult to separate from the normal nucleotides present.

2.2.2. Ultra high performance liquid chromatography (UHPLC)-electrospray ionization (ESI+)-tandem mass spectrometry (MS/MS) method

Previously described methodology (Stegmüller et al., 2018) was used for characterization and quantification of DNA adducts using an Agilent 1290 infinity UHPLC system (Agilent, Waldkirch, Germany) equipped with an UHPLC column (U-VDSpher PUR C18-E 1.8 µm;

50 × 4.6 mm; VDS Optilab, Berlin, Germany) coupled to a Sciex QTrap 5500 MS (Sciex, Darmstadt, Germany). For the quantification of the dG amount of DNA samples, an Agilent 1100 series system equipped with an RP18 column (LiChrospher®, 5 µm, 4 × 125 mm, Merck, Darmstadt, Germany) coupled to a triple quadrupole mass spectrometer (API, 2000; PE SCIEX, USA) was used. Due to the larger organ size, only turkey livers were analyzed, as the chicken livers samples did not yield sufficient amount of DNA for analyses. All dose groups (3 samples (eggs) per group) were analyzed in the assay.

Standard chemicals and solvents were purchased in biochemical grade, analytical grade or the grade appropriate for the intended use from commercial suppliers. Safrole-derived DNA adducts were synthesized via reaction of 1′-acetoxysafrole with either dG, dA, or $^{15}N_5$-dG and subsequent purification via preparative HPLC according to the methods described by Herrmann et al. (2012) for methyl eugenol DNA adducts. Unlabeled and $^{15}N_5$-isotope labeled 2′-deoxynucleoside internal standards of the DNA adducts of methyl eugenol (according to Herrmann et al., 2012) as well as α-asarone and β-asarone (Stegmüller et al., 2018) were synthesized at University of Kaiserslautern by the methods given in the references. Methanol (LC-MS gradient grade) was obtained from Promochem (Wesel, Germany). Deionized and afterwards double distilled water (ddH_2O) was used for biochemical and chromatographic purposes. RNAse was purchased from Carl Roth (Karlsruhe, Germany), proteinase K and alkaline phosphatase (calf intestine) were obtained from Sigma-Aldrich (Taufenkirchen, Germany), and phosphodiesterase II and micrococcal nuclease were purchased from Worthington (Lakewood, NJ, USA). Glass vials and inserts were obtained from Wicom (Heppenheim, Germany), and all consumables from Greiner Bio-One (Frickenhausen, Germany).

DNA isolation, digestion into nucleosides and sample preparation was conducted using the fetal turkey livers of the same group which were frozen using liquid nitrogen, pooled and mechanically homogenized. DNA isolation was conducted using a phenol-chloroform extraction modified to Stegmüller et al. (2018) and Herrmann et al. (2014) to enhance DNA yield and purity from turkey fetal liver tissue. Briefly, 250 µL TAE buffer (40 mM TRIS, 0.5 mM Na_2EDTA, 20 mM acetic acid) was added to each 50 mg liver sample and sonified for 3 s on ice. After addition of 25 µL SDS solution (10%), 13 µL triton-X-100-solution (10%), 5 µL proteinase K (10 mg/mL), and 2.5 µL RNase (10 mg/mL) the samples were incubated for 15–18 h at 55 °C for further lysis. 340 µL of extraction solution 1 (phenol:chloroform:isoamyl alcohol, 25:24:1) was added and each samples was mixed and centrifuged (14000 rpm, 10 min, 4 °C). The aqueous phase was separated and incubated again with 1.9 µL RNase (10 mg/mL) for 20 min at room temperature. 300 µL of extraction solution 2 (chloroform:isoamyl alcohol, 24:1) was added for a second extraction. Each sample was again mixed and centrifuged, and the aqueous phase was separated. 800 µL ethanol (−20 °C) was added to precipitate DNA. After centrifugation, the remaining DNA pellet was dissolved in 170 µL ddH_2O. DNA was precipitated again by addition of 17 µL sodium acetate solution (3 M) and 170 µL isopropanol (−20 °C). After centrifugation the DNA pellet was washed with ethanol (70% in ddH_2O, −20 °C), dried at room temperature and dissolved in 50 µL ddH2O. Purity and concentration of the DNA solution was assessed spectrophotometrically using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, NC, USA). The digestion of DNA into nucleosides was performed according to the method of Schumacher et al. (2013) using 50 µg DNA of each sample, spiked with 30 fmol of each $^{15}N_5$-labeled DNA adduct and 600 pmol $^{15}N_5$-dG, and finally taken up in a final volume of 30 µL methanol (methanol:ddH_2O, 75:25). Isotope labeled standards were added in a defined concentration and DNA was enzymatically digested into nucleosides.

DNA adduct detection and quantification was performed using UHPLC-ESI$^+$-MS/MS methods. An overview of the structures of the investigated DNA adducts is given in Fig. 4.

The compound specific parameters for the MS instrumentation of the safrole DNA adducts are given in Table 2. The methyl eugenol adducts N^6-MIE-dA and N^2-MIE-dG were analyzed with the same UHPLC-MS/MS system and chromatographic settings as the asarone adducts, but with compound specific parameters given in Table 2 modified from Cartus et al. (2012). The α- and β-asarone adducts N^6-1′–OH–2H-A-dA and N^2-1′–OH–2H-A-dG were analyzed according to the method previously described (Stegmüller et al., 2018). Possible eugenol-derived DNA adducts were investigated using both, a neutral loss scan with an m/z range of 400–450 Da (with a loss of 116 Da, i.e. the loss of deoxyribose) and a Q1 scan for m/z ratios which putative eugenol-derived DNA adducts might have (430.160 Da, 416.440 Da, 448.180 Da, 434.200 Da). To determine adducts per 10^8 nucleosides, the efficiency of the DNA digestion was examined. The dG concentration of each sample was measured according to Stegmüller et al. (2018) and used to calculate the number of DNA adducts related to 10^8 nucleosides.

3. Results

Summary of the results of testing is provided in Table 3. Average viability in the control groups was 84% for turkeys and 100% for chickens. In all dosed groups analyzed, the viability of fetuses was at least 67% (Table 3). Results of testing of safrole, methyl eugenol and eugenol in turkey model are described in Kobets et al. (2016, 2018a). Briefly, safrole at the total dose of 2 mg/egg and methyl eugenol at 4 mg/egg produced DNA adducts, while eugenol up to 2.5 mg/egg was negative in the assay.

The total doses of the alkenylbenzenes were converted to mg/kg, based on the average egg weight at termination, in order to compare the administered dosages (Table 1). However, these calculations do not account for any changes over the 3-days injection period or the distribution of the compound to the fetus from the air sac by circulation. Methyl eugenol and α-asarone were administered at similar dose levels to the turkey and chicken eggs. Due to lower toxicity of safrole and eugenol to chicken fetuses, higher dosages were achieved for these compounds in the chicken egg. In contrast, β-asarone produced higher toxicity in chicken egg, thus, doses tested in chicken eggs were lower compared to those in turkey eggs.

3.1. Quantification of DNA adducts

3.1.1. NPL assay results

The NPL assay did not detect background adducts in any of the vehicle control groups (Fig. 5, Table 3). Similar to previous findings in the turkey livers, safrole and methyl eugenol produced DNA adducts in the chicken livers detected by the NPL assay (Fig. 5B). Specifically, safrole at a cumulative dose of 6 mg/egg produced approximately 1.16 adducts per 10^8 of normal nucleotides, while 2 mg/egg of methyl eugenol produced about 314 adducts per 10^8 of normal nucleotides (Table 3). In contrast, no DNA adducts were detected in the groups that received a total dose of 3 mg of eugenol per egg (Fig. 5B).

DNA adducts were also observed in turkey and chicken livers after dosing with either α- or β-asarone (Fig. 5). In the turkey livers, the highest tested dose of α-asarone of 10 mg/egg produced about 1.6 adducts per 10^8 of normal nucleotides, while in the chicken livers 8 mg/egg produced about 1.3 adducts per 10^8 of normal nucleotides. β-Asarone in the turkey livers at 5 mg/egg produced approximately 4 adducts per 10^8 of normal nucleotides, and in chicken livers at 0.5 mg/egg this compound produced 0.2 adducts per 10^8 of normal nucleotides (Table 3). As evident from chromatograms (Fig. 5), safrole, methyl eugenol and asarones produced 2 spots, indicative of two different types of DNA adducts. Additionally, the chromatographic patterns of adducts formed by α- and β-asarone were similar between turkey and chicken livers (Fig. 5).

The total level of adducts at the highest tested dose was normalized to the molecular weight of the compounds (Fig. 6, Table 3) in order to compare the DNA-binding potency of tested alkenylbenzenes. These

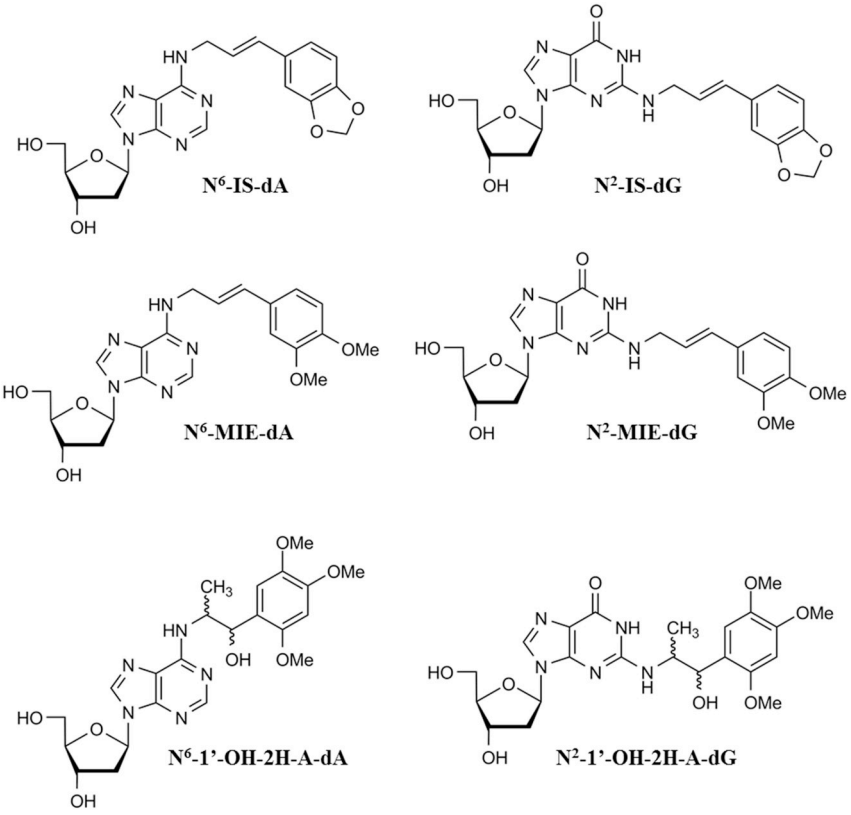

Fig. 4. Chemical structures of the investigated DNA adducts of safrole, methyl eugenol and asarone isomers.

Table 2
Compound-specific mass spectrometric parameters[a] for the methyl eugenol- and safrole-derived DNA adducts.

	Q1	Q3	DP	EP	CE	CXP
N^2-IS-dG	428.100	312.1	41	8.5	19	26
	428.100	164.1	41	8.5	33	4
[$^{15}N_5$]N^2-IS-dG	433.030	317.1	41	8.0	19	26
	433.030	169.1	41	8.0	35	4
N^6– IS -dA	412.250	296.2	56	10.5	21	24
	412.250	148.3	56	10.5	33	4
N^6-MIE-dA	428.170	177.0[b]	71	10	35	14
	428.170	312.1	71	10	21	22
N^2-MIE-dG	444.166	328.1[b]	46	10	17	34
	444.166	177.1	46	10	33	12
[$^{15}N_5$]N^6-MIE-dA	433.094	177.1[b]	56	10	33	12
	433.094	317.1	56	10	21	18
[$^{15}N_5$]N^2-MIE-dG	499.078	333.1[b]	61	10	15	24
	449.078	177.1	61	10	41	12

[a] , Q1, quadrupole 1 (*m/z*); Q3, quadrupole 3 (*m/z*); DP, declustering potential [V]; EP, entrance potential [V]; CE, collision cell energy [eV]; CXP, cell exit potential [V].

[b] , mass transitions used as quantifier.

calculations were done with the understanding that adduct formation might not be linear and that the recoveries (enrichment) could be different.

In fetal turkey livers, methyl eugenol exhibited the highest DNA binding potential, followed by safrole (0.65 and 0.02 adducts/μmol, respectively), β-asarone was more potent than α-asarone (0.01 and 0.003 adducts/μmol, respectively) (Fig. 6). In chicken livers, methyl eugenol was also the most potent (1.4 adducts/μmol), followed by β-asarone (0.005 adducts/μmol), the potency of α-asarone and safrole was similar (~0.002 adducts/μmol).

3.1.2. UHPLC-ESI + -MS/MS results

The formation of DNA adducts in the fetal turkey liver after administration of methyl eugenol, α- and β-asarone was confirmed using the aforementioned UHPLC-ESI$^+$-MS/MS methods. The limit of detection (LOD) and the limit of quantification (LOQ) for the putative major DNA adduct of safrole, N^2-IS-dG and its [$^{15}N_5$]-isotope labeled standard were 3 and 22 adducts per 10^8 nucleosides. The LOD for N^2-MIE-dG and N^6-MIE-dA were 1 and 16 adducts per 10^8 nucleosides with LOQ of 4 and 78 adducts per 10^8 nucleosides. The LOD for N^2-1′–OH–2H-A-dG and N^6-1′–OH–2H-A-dA was 1 and 2 adducts per 10^8 nucleosides and LOQ was of 2 and 11 adducts per 10^8 nucleosides, respectively. In any of the vehicle control groups the adduct levels were below the LOD for all investigated DNA adducts.

No quantifiable DNA adducts were detected with UHPLC-ESI$^+$-MS/MS in the livers of turkey fetuses dosed with either 1 or 2 mg/egg of safrole despite an attempt to re-extract the DNA hydrosylates using butanol to concentrate them or the use of $^{15}N_5$-labeled standards for quantification.

DNA adduct levels determined by the isotope dilution UHPLC-MS/MS method were higher compared to the total adduct amounts determined by NPL assay. For methyleugenol (4 mg/egg) values were approximately 8 times higher and in case of α-asarone (10 mg/egg) and β-asarone (5 mg/egg) the DNA adduct levels determined by mass spectrometry were approximately 500 times and 250 times higher, respectively. The amount of deoxyguanosine (dG) adducts exceeded the amount of deoxyadenosine (dA) adducts in all samples (Fig. 7, Table 3). At the tested doses, the highest amount of epoxide-derived DNA adducts was detected in groups that received 2.5 mg/egg of β-asarone, over 2000 N^2-1′–OH–2H-A-dG and 1460 N^6-1′–OH–2H-A-dA adducts per 10^8 nucleosides, while the DNA adduct levels detected in the higher dose group (5 mg/egg) were considerably lower, with approximately 600 N^2-1′–OH–2H-A-dG and 400 N^6-1′–OH–2H-A-dA adducts per 10^8 nucleosides (Fig. 7C, Table 3). For methyl eugenol and α-asarone the formation of DNA adducts was dose-dependent (Fig. 7A and B).

Table 3
Levels of DNA adducts in the fetal turkey and chicken livers.

Compound	Dose, mg/egg[a]	Viability, %	NPL results		UHPLC-ESI$^+$-MS/MS results	
			DNA adducts X 10^8 nts[e]	Adducts/μmol[f]	N^2-dG X 10^8 nts[e]	N^6-dG X 10^8 nts[e]
Turkey						
Vehicle, 20% HS15	0	84[c]	ND	ND	ND	
Safrole	1	67[d]	N/T	0.017 ± 0.003[d]	ND	ND
	2	92[d]	3.07 ± 0.47[d]		ND	ND
Methyl eugenol	2	67[d]	N/T	0.65 ± 0.124[d]	241 ± 2.46	213 ± 4.83
	4	88[d]	185 ± 34.9[d]		946 ± 55.0	491 ± 24.4
Eugenol	1	78[d]	N/T	ND[d]	ND	ND
	2.5	90[d]	ND[d]		ND	ND
α-Asarone	5	78	N/T	0.003 ± 0.001	201 ± 18.7	158 ± 34.0
	10[b]	78	1.61 ± 0.51		429 ± 59.3	420 ± 28.01
β-Asarone	2.5	83	N/T	0.012 ± 0.005	2006 ± 1701	1461 ± 149.1
	5[b]	67	4.05 ± 1.68		599 ± 30.4	400 ± 34.12
Chicken						
Vehicle, 20% HS15	0	100[c]	ND	ND	N/T	
Safrole	6	100	1.16 ± 0.10	0.002 ± 0.0002	N/T	
Methyl eugenol	2	100	314 ± 29.7	1.44 ± 0.14	N/T	
Eugenol	3	70	ND	ND	N/T	
α-Asarone	8	100	1.33 ± 0.6	0.002 ± 0.001	N/T	
β-Asarone	0.5[b]	92	0.22 ± 0.16	0.005 ± 0.004	N/T	

N/T, not tested; N/D, not detected; nts, nucleotides.
[a] , administered in 3 daily injections.
[b] , the next tested dosage caused ≥50% decrease in viability.
[c] , value presented as average across all control groups (viability in each control group from studies in turkeys at least 60%, in chickens was 100%).
[d] , results published in Kobets et al. (2016).
[e] , mean ± standard deviation.
[f] , based on dose in μmol provided in Table 1.

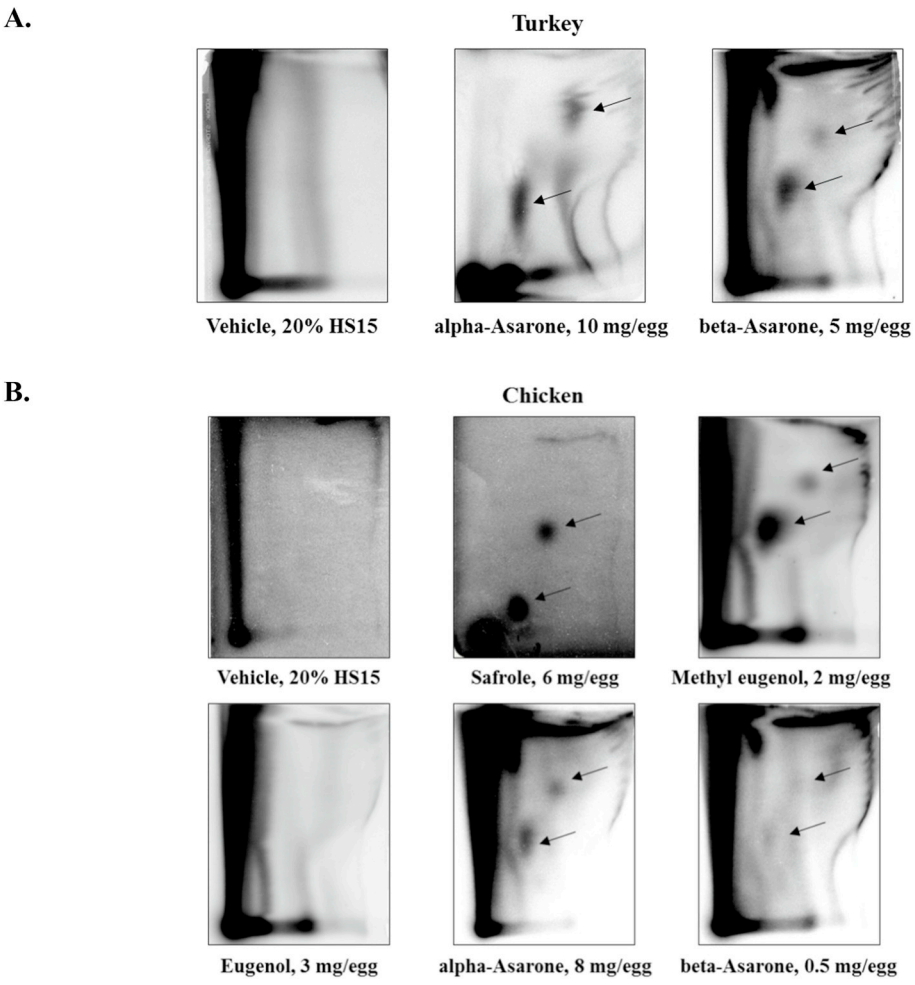

Fig. 5. Nucleotide ^{32}P-postlabeling (NPL) chromatograms. Analysis of DNA adducts in the fetal turkey (A) or chicken (B) livers 3 h after the last injection with tested alkenylbenzenes obtained with NP_1 enrichment. Adducts were resolved in the second and third directions of chromatography and are indicated by arrows. Corresponding spots are not visible for the livers in the control group. NPL chromatograms obtained from the analysis of DNA adducts in fetal turkey livers after dosing with safrole, methyl eugenol and eugenol are published in Kobets et al. (2016).

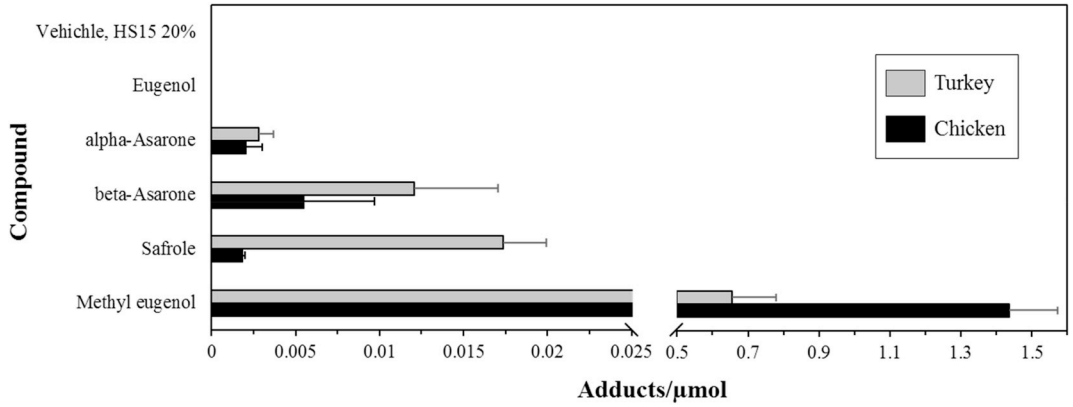

Fig. 6. DNA binding potential of tested alkenylbenzenes in fetal turkey (grey bars) and chicken (black bars) livers. Total levels of DNA adducts detected using NPL assay were normalized to the molecular weight of the compound and the tested dose. Values are provided in Table 3.

Specifically, methyl eugenol at a total dose of 2 mg/egg produced approximately 241 N^2-MIE-dG and 213 N^6-MIE-dA adducts per 10^8 nucleosides, while at 4 mg/egg 946 N^2-MIE-dG and 491 N^6-MIE-dA adducts per 10^8 nucleosides were detected. α-Asarone at 5 mg/egg produced 200 N^2-1′–OH–2H-A-dG and 158 N^6-1′–OH–2H-A-dA adducts per 10^8 nucleosides, and at 10 mg/egg 430 N^2-1′–OH–2H-A-dG and 420 N^6-1′–OH–2H-A-dA adducts per 10^8 nucleosides (Table 3).

Eugenol-derived DNA adducts, e.g. formed from 1′-hydroxylation and subsequent *O*-sulfonation (i.e. methyl eugenol-analogue adducts with dG and dA), as well as adducts derived from side-chain epoxidation, were not detected in any turkey liver dosed with eugenol (data not shown).

4. Discussion

Overall, formation of DNA adducts in fetal avian livers from two different species was confirmed for the established DNA-reactive alkenylbenzenes, safrole, methyl eugenol, α- and β-asarone, while non-genotoxic eugenol did not produce DNA adducts (Table 3). The absence of background adducts in the control groups is consistent with the previous studies in the in ovo models (Kobets et al., 2016, 2018a; Williams et al., 2014) and can be possibly attributed to the lack of environmental exposures other than tested materials, rigorous conditions of incubation and the young age of the avian organisms. For all tested compounds, with the exception of safrole, formation of two types of DNA adducts detected by NPL assay was confirmed with the UHPLC-ESI$^+$-MS/MS method, validating the sensitivity of the NPL assay for chemical-induced genotoxicity. The failure of the mass spectrometric method to detect safrole-derived DNA adducts could be on the one hand due to the comparatively lower sensitivity of the method, and, on the other hand, due to an enormous ion suppression observed for the investigated safrole adducts when analyzed in matrix (i.e. DNA hydrosylate solution) compared to the synthesized standards. The LOD and LOQ for the putative major DNA adduct of safrole in the used UHPLC-ESI$^+$-MS/MS method were considerably lower compared to that of NPL assay (LOD of 1 per 10^9 nucleotides). In NPL, 3.07 ± 0.47 safrole DNA adducts per 10^8 nucleosides were detected in turkey livers, which is equal to the LOD of the MS method. Taken into account that the NPL likely detects virtually all DNA adducts at once, whereas the MS method aims to quantify two specifically safrole DNA adducts, it is not surprising that the detection was not feasible by the method used. In a recent *in vitro* study with HepG2 cells, several DNA adducts were detected after exposure to safrole with LC-ESI-MS/MS methodology using a non-targeted DNA adductomic approach (Takeshita et al., 2019). In this study, putative N^2-IS-dG was detected in traces, besides other till then unknown safrole-derived DNA adducts, which were not analyzed in the present method and may indicate again the low sensitivity compared to the NPL assay. For example, in a study by Gupta et al. (1993), NPL assay was successfully used to determine dose- and time-related formation of safrole-DNA adducts in mice. The authors described two major 5′-monophospate adducts, N^2-IS-dG and N^2-(safrol-1′-yl)-dG, which were also detected in the livers of patients with hepatocellular carcinoma (Chung et al., 2008).

Nevertheless, most DNA adduct level determined by the isotope dilution UHPLC-MS/MS method in the samples that received methyl eugenol, α- and β-asarone, were higher compared to the NPL assay. Similar findings were observed in previous investigations comparing results from the NPL assay with mass spectrometric methods, e.g. by analyzing DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) (Goodenough et al., 2007), benzo[a]pyrene (Singh et al., 2006) or 1-methylpyrene (Monien et al., 2008). However, variations between the two methods in these studies were lower with a range of 3–25 (MS/NPL ratio) and can be attributed to the varying efficiencies of sample preparation in both methods, e.g. during DNA digestion or nucleotide labeling.

In the turkey liver, a total dose of 4 mg/egg methyleugenol produced 946 ± 55 N^2-MIE-dG adducts per 10^8 nucleosides. Assuming an average weight of the turkey egg is 80 g, this dose is equivalent to 50 mg/kg (Table 1). In mice the same dose administered orally resulted in the formation of 735 ± 342 N^2-MIE-dG adducts after 6 h (Herrmann et al., 2014). Applied to cell culture medium, a concentration of 44.5 mg/L (250 µM) methyleugenol resulted in the formation of 193 ± 14 N^2-MIE-dG adducts after 12 h in primary rat hepatocytes (Cartus et al., 2012). Although these comparisons are very limited, e.g. due to different enzyme activities and amounts, administration routes and treatment times, etc., they all are within an order of magnitude with respect to the formed DNA adduct level. The methyl eugenol derived N^6-MIE-dA and N^2-MIE-dG adducts were also detected using isotope-dilution UPLC-MS/MS in the human liver and lung samples, with dG adduct levels beeing higher compared to the levels of dA adduct (Herrmann et al., 2013; Monien et al., 2015). The nature and the levels of adducts confirms that avian fetal liver possesses the necessary enzymes for the bioactivation of methyl eugenol and that the bioactivation is similar to that in other species.

For β-asarone, higher DNA adduct levels were detected compared to α-asarone in both species using the NPL assay and in turkey livers using UHPLC-ESI$^+$-MS/MS methods. The same was true in incubations of primary rat hepatocytes with both compounds (Stegmüller et al., 2018). Asarone-isomers administered in cell culture medium at a concentration of 41.6 mg/L (200 µM) resulted in the formation of approximately 600 and 1700 of the corresponding dG adducts after 48 h in primary rat hepatocytes for α-asarone or β-asarone, respectively (Stegmüller et al., 2018). These DNA adduct level are of the same order of magnitude as those determined in the turkey livers, once again confirming the

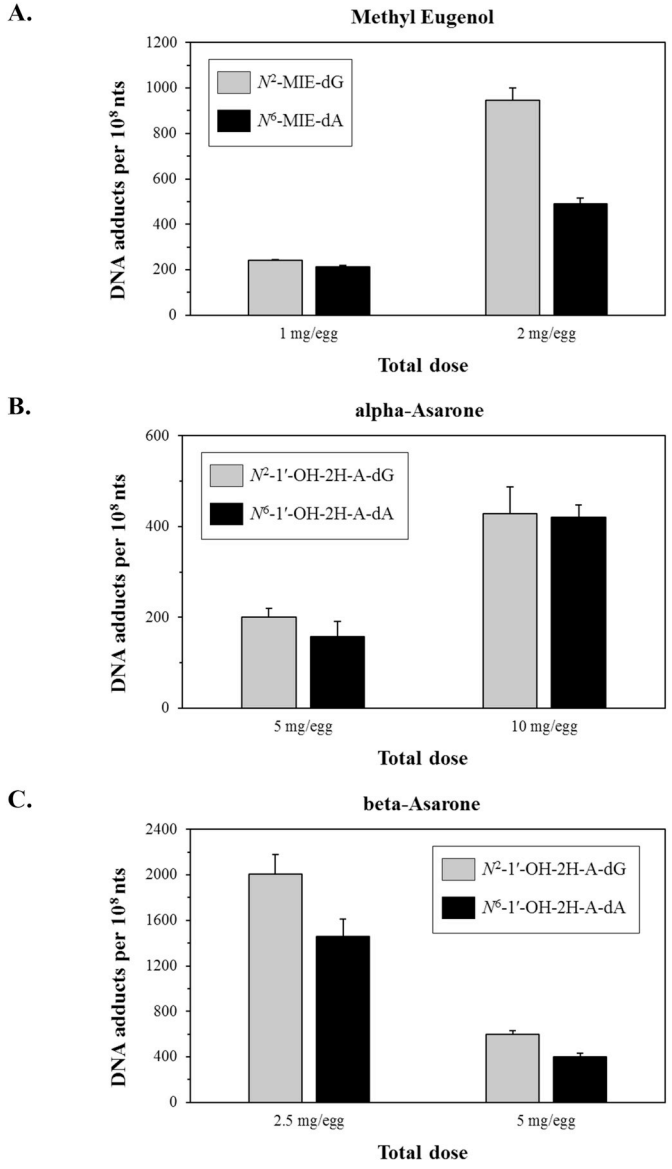

Fig. 7. DNA adduct levels in fetal turkey livers after dosing with methyl eugenol (A), alpha-asarone (B) or beta-asarone (C) obtained using UHPLC-ESI$^+$-MS/MS method. Data represent means + range of two independent measurements of three pooled liver samples from two independent sample preparations. DNA adduct levels of the corresponding control groups were below the LOD (data not shown). nts, nucleotides.

biotransformation capacities of the fetal avian livers.

Although DNA adduct formation was dose-dependent for methyleugenol and α-asarone, the adduct level in the β-asarone treatments were about 3.5 times higher at the lower dose (2.5 mg/egg, viability 83%) as compared to the higher dose (5 mg/egg, viability 67%). This may be due to increased cytotoxicity reflected in the lower viability in the β-asarone high dose group.

The formation of any putative eugenol-derived DNA adducts were not detected using UHPLC-ESI$^+$-MS/MS methods in livers of turkeys that received eugenol, which is in accordance with the absence of DNA adducts as determined by the NPL assay, and the overall lack of genotoxicity of eugenol in other models. However, it should be noted that in contrast to the determination of asarone and methyleugenol DNA adducts, the measurement of eugenol-derived DNA adducts followed an untargeted approach, which likely is much less sensitive than a targeted approach using isotope-labeled DNA adduct standards.

5. Conclusions

Overall, the findings for tested alkenylbenzenes in fetal turkey and chicken livers provide evidence of DNA reactivity of safrole, methyl eugenol, α- and β-asarone, congruently with genotoxicity findings in other species (Berg et al., 2016; Hasheminejad and Caldwell, 1994; Phillips et al., 1984; Randerath et al., 1984). In both species, formation of DNA adducts measured by NPL was confirmed by the UHPLC-ESI$^+$-MS/MS, with exception of safrole, for which adducts were found only by NPL. These results confirm that the investigated turkey and chicken *in ovo* models can be used interchangeably and are valuable alternatives to animal models as a tool for genotoxicity assessment, e.g. for regulatory purposes.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Berg, K., Bischoff, R., Stegmüller, S., Cartus, A., Schrenk, D., 2016. Comparative investigation of the mutagenicity of propenylic and allylic asarone isomers in the Ames fluctuation assay. Mutagenesis 31 (4), 443–451.

Cartus, A.T., Herrmann, K., Weishaupt, L.W., Merz, K.H., Engst, W., Glatt, H., Schrenk, D., 2012. Metabolism of methyleugenol in liver microsomes and primary hepatocytes: pattern of metabolites, cytotoxicity, and DNA-adduct formation. Toxicol. Sci. 129 (1), 21–34.

Cartus, A.T., Schrenk, D., 2016. Metabolism of the carcinogen alpha-asarone in liver microsomes. Food Chem. Toxicol. 87, 103–112.

Cartus, A.T., Stegmüller, S., Simson, N., Wahl, A., Neef, S., Kelm, H., Schrenk, D., 2015. Hepatic metabolism of carcinogenic β-asarone. Chem. Res. Toxicol. 28 (9), 1760–1773.

Chellian, R., Pandy, V., Mohamed, Z., 2017. Pharmacology and toxicology of α- and β-Asarone: a review of preclinical evidence. Phytomedicine 32, 41–58.

Chung, Y.T., Chen, C.L., Wu, C.C., Chan, S.A., Chi, C.W., Liu, T.Y., 2008. Safrole-DNA adduct in hepatocellular carcinoma associated with betel quid chewing. Toxicol. Lett. 183 (1–3), 21–27.

European Food Safety Authority (EFSA), 2009. Scientific opinion of the panel on food additives, flavourings, processing aids and materials in contact with food on a request from commission on consideration of eugenol and related hydroxyallylbenzene derivatives evaluated by JECFA (65th meeting) structurally related to ring-substituted phenolic substances evaluated by EFSA in flavouring group evaluation (FGE).22. EFSA J. 965, 1–53.

Goodenough, A.K., Schut, H.A., Turesky, R.J., 2007. Novel LC-ESI/MS/MSn method for the characterization and quantification of 2'-deoxyguanosine adducts of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by 2-D linear quadrupole ion trap mass spectrometry. Chem. Res. Toxicol. 20 (2), 263–276.

Göggelmann, W., Schimmer, O., 1983. Mutagenicity testing of beta-asarone and commercial calamus drugs with Salmonella typhimurium. Mutat. Res. 121 (3–4), 191–194.

Gupta, K.P., van Golen, K.L., Putman, K.L., Randerath, K., 1993. Formation and persistence of safrole-DNA adducts over a 10,000-fold dose range in mouse liver. Carcinogenesis 14 (8), 1517–1521.

Gupta, R.C., 1985. Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen: DNA adducts. Cancer Res. 45, 5656–5662.

Hamilton, J.W., Denison, M.S., Bloom, S.E., 1983. Development of basal and induced aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in the chicken embryo *in ovo*. Proc. Natl. Acad. Sci. U.S.A. 80, 3372–3376.

Hasheminejad, G., Caldwell, J., 1994. Genotoxicity of the alkenylbenzenes alpha- and beta-asarone, myristicin and elimicin as determined by the UDS assay in cultured rat hepatocytes. Food Chem. Toxicol. 32 (3), 223–231.

Haupenthal, S., Berg, K., Gründken, M., Vallicotti, S., Hemgesberg, M., Sak, K., Schrenk, D., Esselen, M., 2017. In vitro genotoxicity of carcinogenic asarone isomers. Food Funct. 8 (3), 1227–1234.
Herrmann, K., Engst, W., Appel, K.E., Monien, B.H., Glatt, H., 2012. Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in Salmonella typhimurium and detection of associated DNA adducts using UPLC-MS/MS methods. Mutagenesis 27 (4), 453–462.
Herrmann, K., Schumacher, F., Engst, W., Appel, K.E., Klein, K., Zanger, U.M., Glatt, H., 2013. Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human liver samples. Carcinogenesis 34, 1025–1030.
Herrmann, K., Engst, W., Meinl, W., Florian, S., Cartus, A.T., Schrenk, D., Appel, K.E., Nolden, T., Himmelbauer, H., Glatt, H., 2014. Formation of hepatic DNA adducts by methyleugenol in mouse models: drastic decrease by Sult1a1 knockout and strong increase by transgenic human SULT1A1/2. Carcinogenesis 35 (4), 935–941.
Howes, A.J., Chan, V.S., Caldwell, J., 1990. Structure-specificity of the genotoxicity of some naturally occurring alkenylbenzenes determined by the unscheduled DNA synthesis assay in rat hepatocytes. Food Chem. Toxicol. 28, 537–542.
International Agency for Research on Cancer (IARC), 1976. Some naturally occurring substances safrole, isosafrole and dihydrosafrole. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 10. IARC, Lyon, France, pp. 231–244.
Joint FAO/WHO Expert Committee on Food Additives (JECFA), 1981. Monograph on β-asarone. WHO Food Additive Series, vol. 16.
Jeffrey, A.M., Luo, F.Q., Amin, S., Krzeminski, J., Zech, K., Williams, G.M., 2002. Lack of DNA binding in the rat nasal mucosa and other tissues of the nasal toxicants roflumilast, a phosphodiesterase 4 inhibitor, and a metabolite, 4-amino-3,5-dichloropyridine, in contrast to the nasal carcinogen 2,6-dimethylaniline. Drug Chem. Toxicol. 25 (1), 93–107.
Kim, S.G., Liem, A., Stewart, B.C., Miller, J.A., 1999. New studies on trans-anethole oxide and trans-asarone oxide. Carcinogenesis 20 (7), 1303–1307.
Kobets, T., Duan, J.D., Brunnemann, K.D., Etter, S., Smith, B., Williams, G.M., 2016. Structure-activity relationships for DNA damage by alkenylbenzenes in Turkey egg fetal liver. Toxicol. Sci. 150 (2), 301–311.
Kobets, T., Duan, J.D., Brunnemann, K.D., Iatropoulos, M.J., Etter, S., Hickey, C., Smith, B., Williams, G.M., 2018a. *In ovo* testing of flavor and fragrance materials in Turkey Egg Genotoxicity Assay (TEGA), comparison of results to in vitro and in vivo data. Food Chem. Toxicol. 115, 228–243.
Kobets, T., Iatropoulos, M.J., Duan, J.D., Brunnemann, K.D., Iacobas, D.A., Iacobas, S., Vock, E., Deschl, U., Williams, G.M., 2018b. Expression of genes encoding for xenobiotic metabolism after exposure to dialkylnitrosamines in the chicken egg genotoxicity alternative model. Toxicol. Sci. 166 (1), 82–96.
Kobets, T., Iatropoulos, Williams, G.M., 2019. Mechanisms of DNA-reactive and epigenetic chemical carcinogens: applications to carcinogenicity testing and risk assessment. Toxicol Res. 8, 123–145.
Miller, J.A., Miller, E.C., 1983. The metabolic activation and nucleic acid adducts of naturally-occurring carcinogens: recent results with ethyl carbamate and the spice flavors safrole and estragole. Br. J. Canc. 48, 1–15.
Miller, E.C., Swanson, A.B., Phillips, D.H., Fletcher, T.L., Liem, A., Miller, J.A., 1983. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res. 43, 1124–1134.
Miller, J.A., Swanson, A.B., Miller, E.C., 1979. The metabolic activation of safrole and related naturally occurring alkenylbenzenes in relation to their carcinogenesis by these agents. In: Miller, E.C. (Ed.), Naturally Occurring Carcinogens- Mutagens and Modulators of Carcinogenesis. Japanese Scientific Societies Press, Tokyo and University Park Press, Baltimore, pp. 111–125.
Monien, B.H., Müller, C., Engst, W., Frank, H., Seidel, A., Glatt, H., 2008. Time course of hepatic 1-methylpyrene DNA adducts in rats determined by isotope dilution LC-MS/MS and ^{32}P-postlabeling. Chem. Res. Toxicol. 21 (10), 2017–2025.
Monien, B.H., Schumacher, F., Herrmann, K., Glatt, H., Turesky, R.J., Chesné, C., 2015. Simultaneous detection of multiple DNA adducts in human lung samples by isotope-dilution UPLC-MS/MS. Anal. Chem. 87 (1), 641–648.
National Toxicology Program (NTP), 2000. NTP toxicology and carcinogenesis studies of methyleugenol (CAS NO. 93-15-2) in F344/N rats and B6C3F1 mice (gavage studies). National Toxicology Program Toxicity Report Series Number 491 NIH publication, pp. 1–412 No. 00-3950.
Perrone, C.E., Ahr, H.J., Duan, J.D., Jeffrey, A.M., Schmidt, U., Williams, G.M., Enzmann, H.G., 2004. Embryonic Turkey liver: activities of biotransformation enzymes and activation of DNA-reactive carcinogens. Arch. Toxicol. 78 (10), 589–598.
Phillips, D.H., Reddy, M.V., Randerath, K., 1984. ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5 (12), 1623–1628.
Phillips, D.H., Farmer, P.B., Beland, F.A., Nath, R.G., Poirier, M.C., Reddy, M.V., Turteltaub, K.W., 2000. Methods of DNA adduct determination and their application to testing compounds for genotoxicity. Environ. Mol. Mutagen. 35, 222–233.
Phillips, D.H., Arlt, V.M., 2014. ^{32}P-postlabeling analysis of DNA adducts. Methods Mol. Biol. 1105, 127–138.
Randerath, K., Reddy, M.V., Gupta, R.C., 1981. ^{32}P-labeling test for DNA damage. Proc. Natl. Acad. Sci. Unit. States Am. 78 (10), 6126–6129.
Randerath, K., Haglund, R.E., Phillips, D.H., Reddy, M.V., 1984. ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5 (12), 1613–1622.
Reddy, M.V., Randerath, K., 1986. Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis 7, 1543–1551.
Rietjens, I.M., Boersma, M.G., van der Woude, H., Jeurissen, S.M., Schutte, M.E., Alink, G.M., 2005. Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk. Mutat. Res. 574 (1–2), 124–138.
Rietjens, I.M., Cohen, S.M., Fukushima, S., Gooderham, N.J., Hecht, S., Marnett, L.J., Smith, R.L., Adams, T.B., Bastaki, M., Harman, C.G., Taylor, S.V., 2014. Impact of structural and metabolic variations on the toxicity and carcinogenicity of hydroxy- and alkoxy-substituted allyl- and propenylbenzenes. Chem. Res. Toxicol. 27, 1092–1103.
Schumacher, F., Herrmann, K., Florian, S., Engst, W., Glatt, H., 2013. Optimized enzymatic hydrolysis of DNA for LC-MS/MS analyses of adducts of 1-methoxy-3-indolylmethyl glucosinolate and methyleugenol. Anal. Biochem. 434 (1), 4–11.
Scientific Committee on Food (SCF), 2002. Opinion of the Scientific Committee on Food on the Presence of β-asarone in Flavourings and Other Food Ingredients with Flavouring Properties. SCF/CS/FLAV/FLAVOUR/9 ADD1 Final.
Singh, R., Gaskell, M., Le Pla, R.C., Kaur, B., Azim-Araghi, A., Roach, J., Koukouves, G., Souliotis, V.L., Kyrtopoulos, S.A., Farmer, P.B., 2006. Detection and quantitation of benzo[a]pyrene-derived DNA adducts in mouse liver by liquid chromatography-tandem mass spectrometry: comparison with ^{32}P-postlabeling. Chem. Res. Toxicol. 19 (6), 868–878.
Smith, R.L., Adams, T.B., Doull, J., Feron, V.J., Goodman, J.I., Marnett, L.J., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Rogers, A.E., Caldwell, J., Sipes, I.G., 2002. Safety assessment of allylalkoxybenzene derivatives used as flavouring substances – methyl eugenol and estragole. Food Chem. Toxicol. 40, 851–870.
Stegmüller, S., Schrenk, D., Cartus, A.T., 2018. Formation and fate of DNA adducts of alpha- and beta-asarone in rat hepatocytes. Food Chem. Toxicol. 116, 138–146.
Takeshita, T., Tao, F., Kojima, N., Kanaly, R.A., 2019. Triple quadrupole mass spectrometry comparative DNA adductomics of Hep G2 cells following exposure to safrole. Toxicol. Lett. 300, 92–104.
Tsai, R.S., Carrupt, P.A., Testa, B., Caldwell, J., 1994. Structure-genotoxicity relationships of allylbenzenes and propenylbenzenes: a quantum chemical study. Chem. Res. Toxicol. 7 (1), 73–76.
Unger, P., Melzig, M.F., 2012. Comparative study of the cytotoxicity and genotoxicity of alpha- and beta-asarone. Sci. Pharm. 80 (3), 663–668.
Williams, G.M., Iatropoulos, M.J., Jeffrey, A.M., Duan, J.D., 2013. Methyleugenol hepatocellular cancer initiating effects in rat liver. Food Chem. Toxicol. 53, 187–196.
Williams, G.M., Duan, J.D., Brunnemann, K.D., Iatropoulos, M.J., Vock, E., Deschl, U., 2014. Chicken fetal liver DNA damage and adduct formation by activation-dependent DNA-reactive carcinogens and related compounds. Toxicol. Sci. 141 (1), 18–28.
Wiseman, R.W., Miller, E.C., Miller, J.A., Liem, A., 1987. Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F1 mice. Cancer Res. 47 (9), 2275–2283.
Wolf, T., Luepke, N.P., 1997. Formation of micronuclei in incubated hen's eggs as a measure of genotoxicity. Mutat. Res. 394, 163–175.