# General outline

# Introduction

## General problem setting

* EC
* Growing body of evidence concerning adverse health effects
* Toxicity testing on animals costly and toxicity data not available given it is a GRAS (not necessary to test)
* Mechanistic understanding of the toxicological risk through new approach methodologies (NAMS)
* In vitro toxicity testing to elucidate mechanism of aldehydes
* PBK modelling for QIVIVE as well as to assess kinetics and toxicity of data poor chemicals

An important public health act that shapes how such additives are used currently was the Food Additives Amendments to the Federal Food, Drug, and Cosmetic Act. This United States (US) act meant that each substance intentionally added to food in the US had to be pre cleared by the Federal Food and drug Authority (FDA). The GRAS program is run by The Flavor and Extract Manufactures Association of the United States (FEMA).

A decline in cigarette use has been noted by the WHO in almost all regions across the planet(WHO., 2021). This can be ascribed to a number of factors such as increased public awareness of the risk of health effects, due to the introduction of nicotine patches and by the rise of Electronic cigarettes (EC)(Hartmann-Boyce et al., 2021). With EC a consumer is no longer exposed to the burning of tabaco. EC work by the vaporization and inhalation of a blend of nicotine, propylene glycol and/or glycerol. Furthermore, EC mixtures are often enhanced further with the addition of flavoring agents(Omaiye et al., 2019; Page & Goniewicz, 2021). Examples of such flavoring agents are Vanillin (Vanila), Benzaldehyde (Almond) and Cinnamaldehyde (Cinnamon). CNMA is a Generally Regarded as Safe (GRAS) food additive. A GRAS food ingredient is having shown adequately through scientific procedures to be safe under the conditions of intended use. CNMA is an examples are of a subclass of reactive chemicals known as aldehydes. Aldehydes are compounds which possess a carbonyl group with a substituent Hydrogen atom (Lopachin & Gavin, 2014). These carbonyl groups are known for the electrophilic characteristics and associated reactivity. An assortment of aldehydes are associated with adverse health outcomes. These include the known cancer causing agents: Formaldehyde, Acrolein and Crotonaldehyde IARC Working Group on the Evaluation of Carcinogenic Risks to Humans., 2021). Cinnamaldehyde (CNMA) is present in multiple vape products at concentrations up to 343mg/ml with possible high retention after exposure. (Khachatoorian et al., 2022; Omaiye et al., 2019). CNMA is noted to induce toxicity in in vitro systems including oxidative stress, inflammatory response and reductions in cell viability(Bhattacharya et al., 2021; Clapp et al., 2019; Gerloff et al., 2017; Muthumalage et al., 2018)

A considerable part of the appeal of EC is removal of a host of toxic and carcinogenic compounds found in cigarette smoke. Yet, a mounting body of evidence suggest adverse health effects can be seen after use of EC (Chatham-Stephens et al., 2014; Hua & Talbot, 2016). The exact cause of these adverse health effects is as of yet unknown. The reactive aldehyde CNMA is an interesting possible candidate that might contribute to adverse health effects. Even though it is GRAS ingredient the main exposure pathway considered by FEMA evaluations is oral exposure. Inhalation exposure is not an exposure pathway that has to be considered even for volatile chemicals (Hallagan & Hall, 2009). In order to investigate the risk of adverse health effects occurring due to exposure to CNMA in EC liquids a Physiological based kinetics model will be used. The model in question is an oral exposure model for CNMA that was used to estimate the DNA adduct formation in the liver (Kiwamoto et al., 2016). The aim of this paper is the add inhalation exposure to this model and evaluate if inhalation exposure to CNMA can lead to CNMA concentration in the body that are expected to lead to adverse health effects.

# Methods

## Section 1: model structure

As the basis for an inhalation model of CNMA, a previously developed oral exposure model by Kiwamoto *et al* (Kiwamoto et al., 2016) was used. The base code used in this model was used together with the metabolic parameters. Physiological parameters, chemical parameters and the following compartments where changed. DNA adduct formation and CNMA-GSH metabolism in the small intestine where removed as they were shown by (Kiwamoto et al., 2016) to be negligible. Added to the model was an inhalation compartment. This compartment was coded as described in (Jongeneelen & Berge, 2011a). This involves inhalation into the lungs into an alveolar air compartment from which CNMA is absorbed into the blood and enters circulation. CNMA can also enter the alveolar air and be exhaled. The model was coded in R (version 4.1.1) and R Studio (version 1.4.1717) using the Rxode, ggplot2 and PKNCA, packages(Fidler et al., 2022).

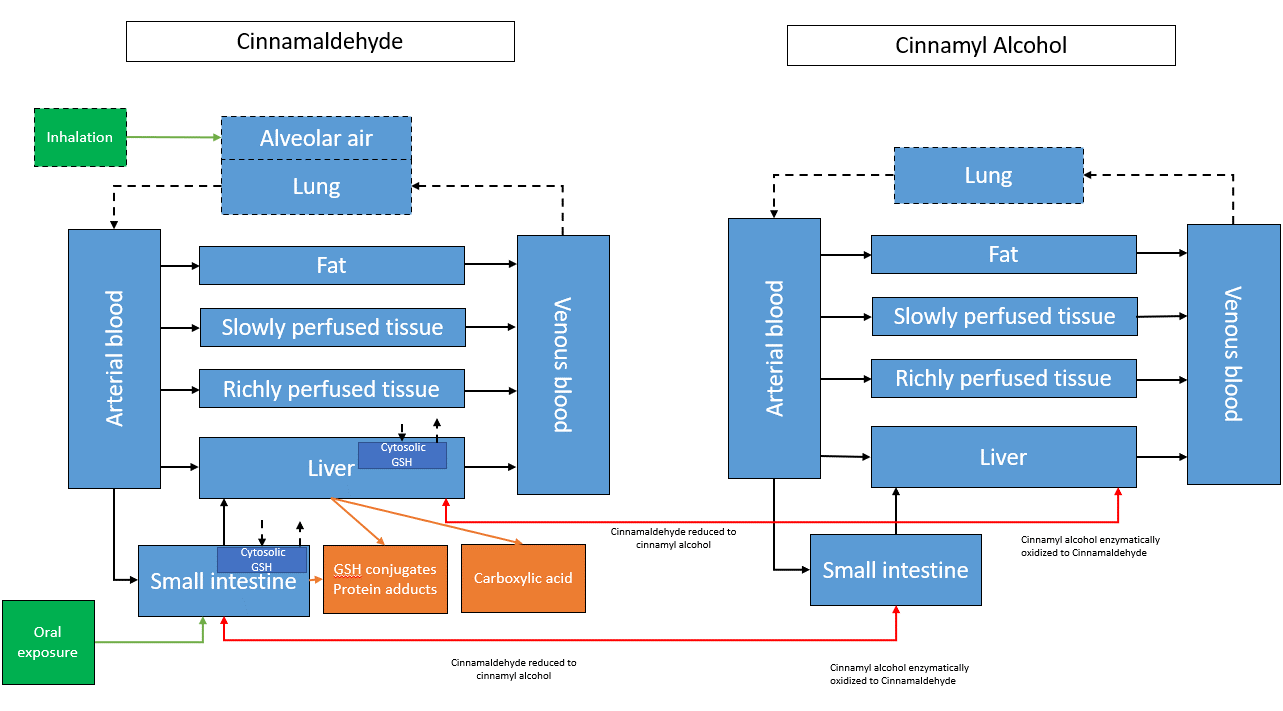


Figure : Structure of the inhalation CNMA model in Humans. Dotted lines represent additions to the model compared to Kiwamoto et al. Exposure routes are coloured green. Elimination routes are present in both the liver and small intestine and are coloured orange. In both the small intestine and the liver cytosolic Glutathione concentrations are modelled. Transformations of CNMA to Cinnamyl alcohol and back are represented by red lines.

## Section 2: Parameters

### Rat

The physiological parameters used for the rat model were adapted from Kiwamoto *et al*. Some parameters had minor changes. Details can be found in the supplementary data. Pulmonary parameters were based (Brown et al., 1997). The logKow was calculated using EPIsuite (Version 4.5 SP1).The partition coefficients where calculated based on this LogKow using both (Dejongh et al., 1997; Jongeneelen & Berge, 2011b). Kiwamoto *et al* also used Episuite and Dejongh *et al* to calculate the partition coefficients yet the values differ significantly. Sadly, Kiwomato *et al* provides no specifics as to how they were derived and thusly the reason for the difference is unexplained. The uptake constant Ka was calculated based on a method shown byYu et al,1999 (Yu & Amidon, 1999). The complete list of sources/calculations can be found in the supplementary data.

### Single Human model

The same approach was used with the the single human model as with the rat model. This means adopting the parameters as used by Kiwamoto *et al* and supplementing these parameters with additional parameters as needed. Pulmonary ventilation was derived from IRCP values (Alexaklrin Obninsk et al., 2003). Pulmonary blood flow as set as equal to cardiac output. The following parameters where calculated. Partition coefficients and the uptake rate constant. The LogKow values where calculated using EPIsuite (Version 4.5 SP1). Based on the LogKow the partition coefficients where calculated using both (Dejongh et al., 1997; Jongeneelen & Berge, 2011b). Similar unexplained differences in partition coefficients could be found as with rats. The uptake rate was calculated in the same way as in rat. The complete list of sources/calculations can be found in the supplementary data.

### Population based model

To more accurately model possible variation between individuals it was decided to create a population based model. As a basis for this model the following service was used. Popgen is a web based application which generates a population of individuals with necessary parameters for pbk modeling based on several initial parameters(Willmann et al., 2007). Using Popgen twee data sets consisting of 1000 individuals were generated one female data set and one male data set. To this data set the following parameters where added. Pulmonary ventilation, and the volume of arterial and venous blood. Pulmonary ventilation was based on (Alexaklrin Obninsk et al., 2003) the male value for the data set is based on a normal distribution and 3 standard deviations (99.7%) of variance based on the male mean of 540 L/h. the female value for the data set is based on a normal distribution and 3 standard deviations (99.7%) of variance based on the female mean of 390 L/h. The blood compartment volumes were based on calculations shown in (Price et al., 2003). A complete overview of initial parameters and calculations can be found in the supplementary data.

## Model validation

As the PBK model used as the basis for the inhalation model is an oral absorption model validation of the inhalation component should be considered. Validation based on in vivo exposure data is the preferred method to do this. Unfortunately exceedingly little inhalation exposure data that is coupled with PBK relevant parameters is available (plasma concentration, tissue concentrations etc). Oral sensitization is an end point considered for human exposures. In order to bridge this gap a read across approach using the similar chemical Benzaldehyde was considered. Unfortunately, this chemical is similarly data poor. General performance of the model structure will be assessed based on the RAT models performance for oral exposure and the human metabolic data.

## Exposure modeling

Limited date is available on possible human exposure to CNMA from E-Cig liquids. (Khachatoorian et al., 2022)

Exposure was modeled as being a 3 minute ‘smoke’ break using a E-Cig every 30 minutes for 6 hours. A total of 12 exposures. As Khachatorian *et al* (2022) has shown that the a E-Cig user consumes on average 567mg of E-cig liquid per session. If we assume a worst case scenario with a CNMA concentration of 343mg/ml (Omaiye et al., 2019) this would yield an exposure of 197.6 mg of CNMA per session. For an average person weighing this would yield approximately 2.8mg/kg-BW.

## Section 3: Global sensitivity analysis

To evaluate the sensitive parameters of the models global sensitivity analyses where performed. This was done using a method demonstrated by Kasteel et al 2021(Kasteel et al., 2021). This involves the `soboljansen` function from the Package `sensitivity´(Bertrand Iooss et al., 2022). This method requires the following input. Two data sets from which parameter values can be sampled for use in the analysis. These two data sets where generated based on the single human parameter set. As an upper bound the parameter values +1% and as a lower bound the parameter values -1% where used. Between these bounds the parameter values where normally distributed. The output of the analysis is a ranking of influential parameter using two indexes. The total effect and main effect. The total effects has cumulative value of 1 representing the contribution of a parameter to the overall change in output of interest. The main effect similarly has a cumulative value of 1 representing the contribution of this parameter independent of its effects on other parameters. The total and main effects for alle parameters where investigated at multiple time points for both oral and inhalation exposures. The corresponding code can be found in the supplementary data section.

# Results

## Sensitivity analysis

Multiple global sensitivity analysis where performed for both the human and rat models. In rats the sensitive parameters determining the CNMA concentration in blood with oral and inhalation exposure were investigated. The results are presented in Figure 2 and Figure 3. During oral exposure the two most important parameters excluding T= 30 min where the uptake rate constant (Ka) and cardiac output (QC). At T=30 min the initial concentration of Gluthation in the liver (init\_GSH\_L) is the second most important parameters followed by the first order rate constant for the conjugation GSH with CNMA(k\_L\_GST). It can be noted that after 90 min the partition coefficient between air and blood for CNMA (PB) and pulmonary ventilation (PV) steadily increase in relevance. Body weight(BW) has a moderate impact at all time points. The sensitive parameters for inhalation exposure are noted to be considerably different. The two most important parameters are PB followed by BW. Both blood flow to Richly perfused tissue (Q\_RP) and volume of slowly perfused tissue (V\_SP) are moderately important at all time points. The sensitive parameters in the Human model where investigated using two different exposures . Firstly, a high dose of 250mg/kg-BW to compare to the Rat model and secondly a 2.8mg/kg-BW dose comparable to a high E-Cig exposure dose. Secondly, we considered both the concentration of CNMA in blood and the concentration in lung If we consider the two different exposures. It can be noted that in both generally the same set of parameters is important. From these PB and PV are the most important parameters. Following in slightly different order are the following parameters; V\_SP, Q\_SP, Q\_RP and V\_Pu. Absent are parameters responsible for metabolic processes. This suggest that CNMA concentrations are dictated by perfusion dynamics.The sensitive parameters for oral exposure in Humans can be found in the supplementary data.

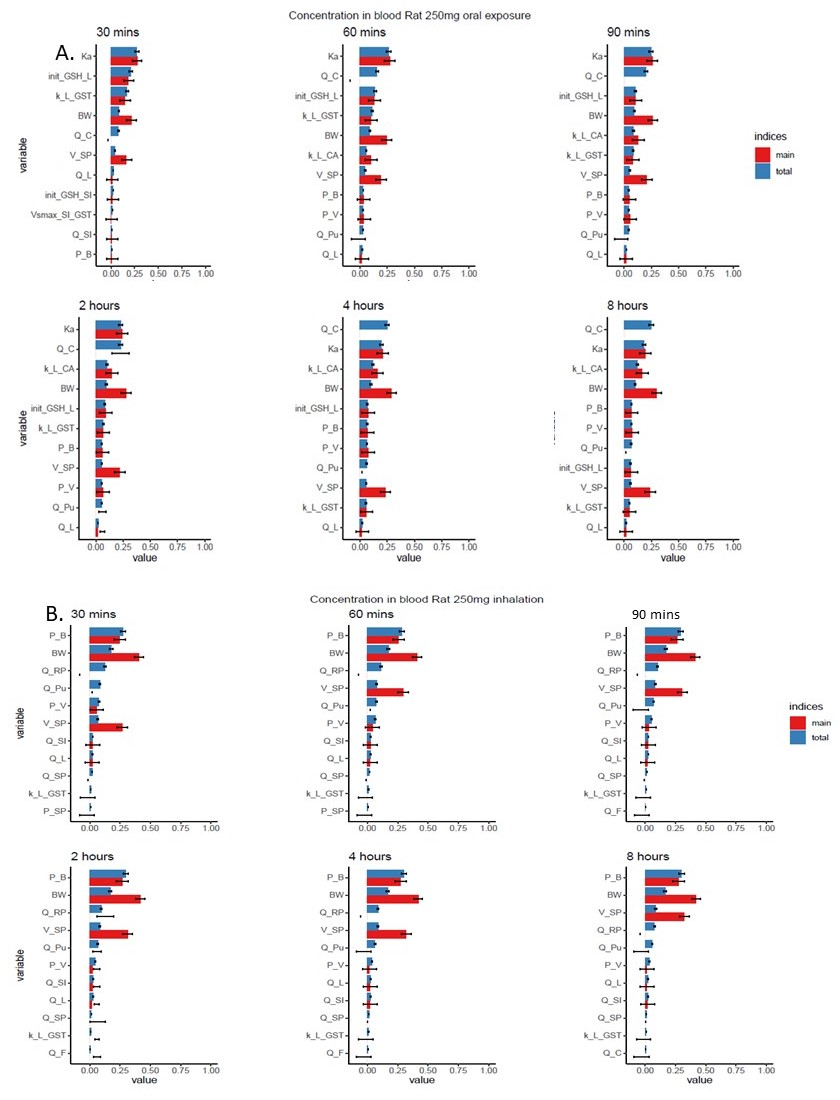


Figure : Global sensitivity analyses results for CINMA exposure both inhalation and oral in Rat 250mg -kg/BW. (A) top ten sensitive parameters influencing the concentration of CNMA in the liver.

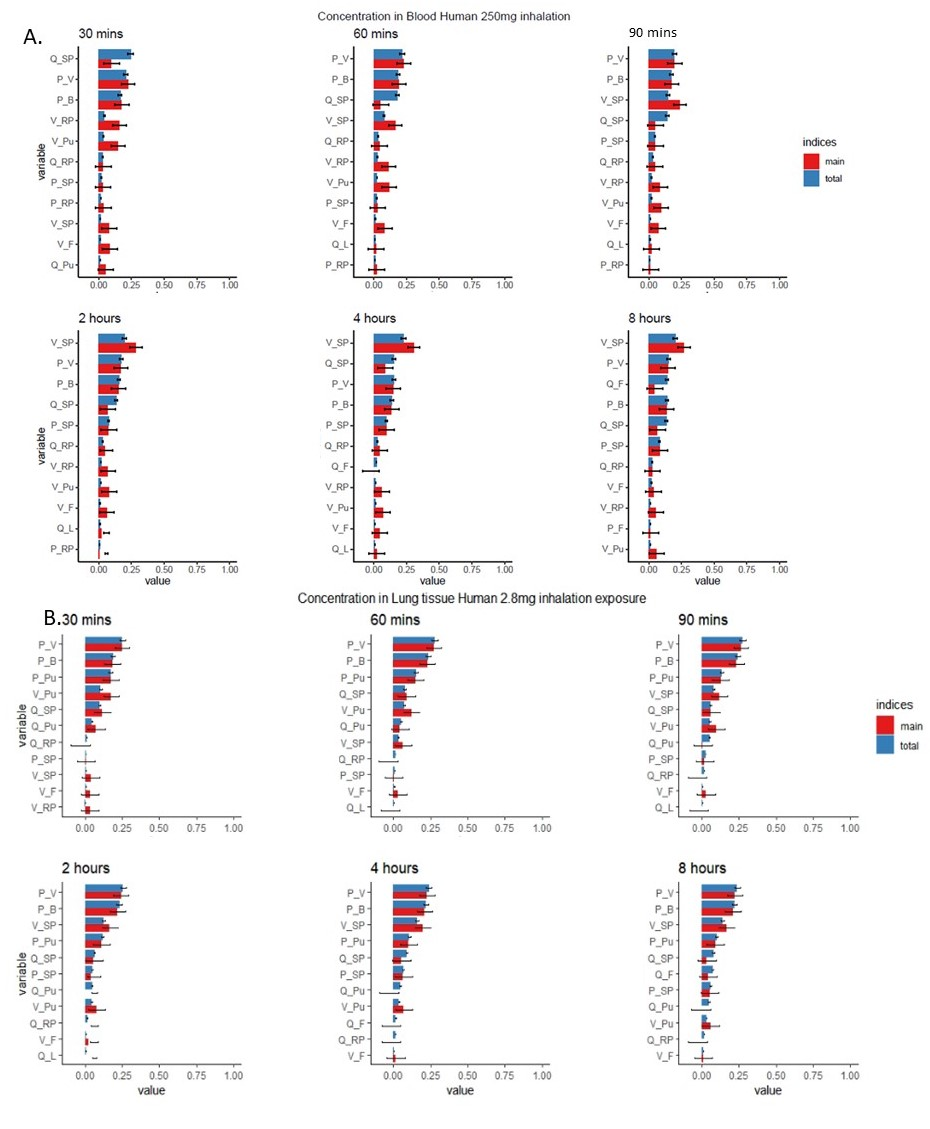


Figure : Global sensitivity analyses results for inhalation exposure with the human model. A; top ten sensitive parameters for inhalation exposure to a 250 mg/kg-BW dose. B; top ten sensitive parameters for inhalation exposure to a 2.8 mg/kg-BW dose.

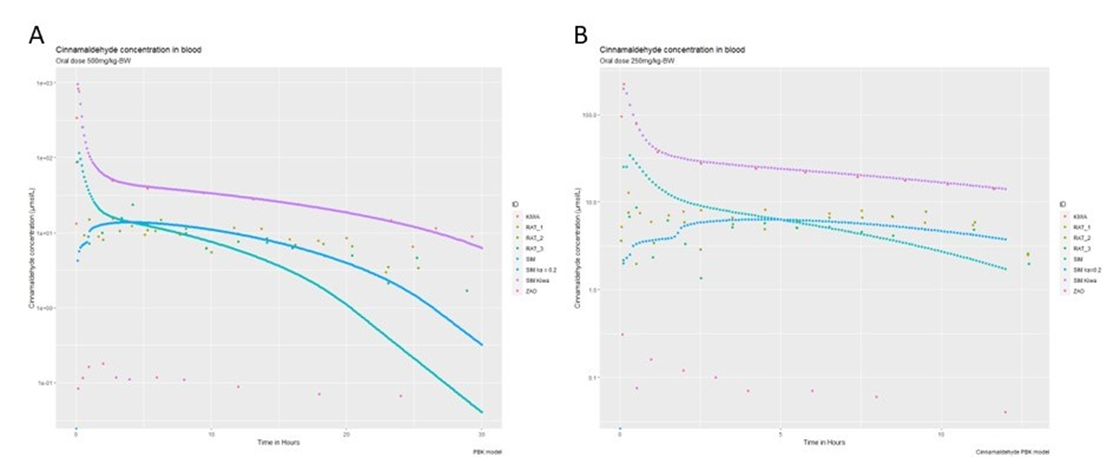


Figure : CNMA blood concentration comparison between simulated and Kiwamoto.

## Evaluation Rat model

As the inhalation model is based on an existing model the first objective was seeing if it was possible to recreate the results of this model using the translated model in R code. In order to do this oral and IV exposure data was used from the literature. Different exposure doses are available from literature these include 500mg/kg-BW, 250mg/kg-BW oral exposures (Yuan et al., 1992; Zhao et al., 2014) already considered in the Kiwamoto report and more recent pharmacokinetic study’s concerning 375 mg/kg-BW, 15mg/kg-BW and 50mg/kg-BW exposures(Dong et al., 2022; Ji et al., 2015; Yong et al., 2020). Lastly two Iv exposures will be considered 10 and 2050mg/kg-BW. The plasma concentration of CNMA as measured in (Zhao et al., 2014) (Zao) and (Yuan et al., 1992)(RAT\_1,2,3) are presented in Figure 4. Also presented in Figure 4 are the simulation results as presented in Kiwamoto *et al* (Kiwa) and the simulation results found when running the inhalation model (SIM). As expected the results of the Kiwamoto model and the inhalation model can be seen to differ substantially with a Cmax value of 829 umol/L and 116 umol/L respectively. A 7.15 fold higher Cmax value with the Kiwamoto model. To evaluate whether this difference was due to errors in the R code a new simulation was run this time using all of the parameters as specified in Kiwamoto *et al* (SIM kiwa). This resulted in a Cmax value of 769 umol/L or a 0.93 fold difference compared to Kiwamoto. The inhalation model performance compared to the experimental data found in Yao *et al*. A 6.56 fold difference can be found between the inhalation model 116 umol/L)and the Yao data 17.69 umol/L. This represents a improvement compared to model performance found in Kiwamoto et al from 56 fold compared to 6.56 fold. As the model still overpredicts in the time points shortly after exposure. Another model run was done now with a lower absorption rate constant to simulate a slower uptake rate in the small intestine. This resulted in a inhalation model Cmax 13.81 umol/L that was 0.78 fold that of the Yao data 17.60 umol/L. The remaining data is presented in Figure 5, in this figure predicted vs outcome plots are presented for the different exposures. If relevant the results of the inhalation model with adapted Ka are also shown. To compare the results between the inhalation model and adapted model the Root mean square deviations are shown. This is a measure of the size of the deviation from the observed values.

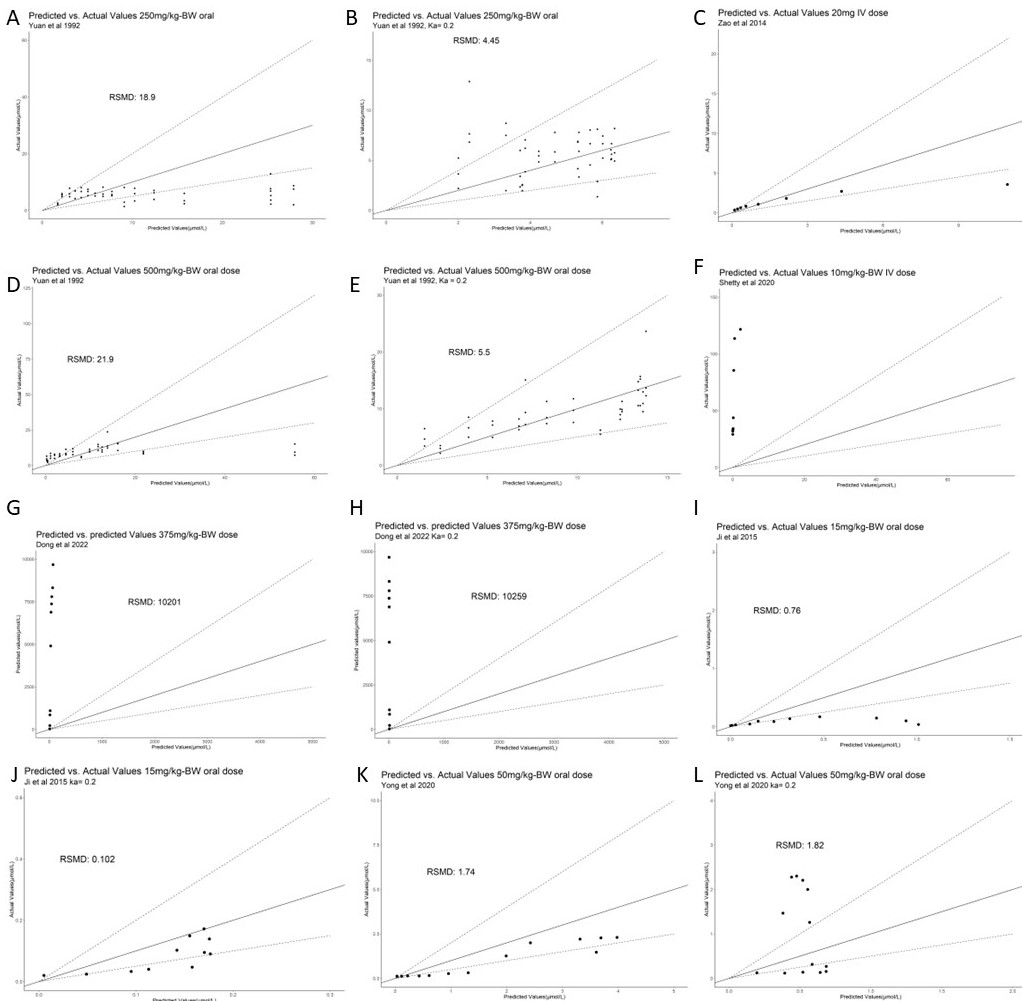
 IE the higher the value the worse a model corresponds to the observed data. Adapting the model with a reduced Ka value improved model performance in 3 of the 5 cases with oral absorption and had a negligible or slight negative impact on the remaining two cases. With 500, 250, 20 and 15 mg/kg-BW doses most data points where within a 2 fold difference of the observed values. 50mg/kg-BW values remained within a 5 fold difference and 15 mg/kg-BW doses within a 10 fold difference. 375 and 10mg/kg-BW doses differed greatly from predicted values.

Figure : Predicted vs outcome plots of experimental data compared to the inhalation model in R. Plots (A,B,D,E,G,H,I, J, K, L) show oral data. Plots (C and F) show IV data. The diagonal solid lines in the graph represents the line at which predicted vs actual values are one 1. The dotted lines represent a 2 fold difference. To compare similar exposure the Root square mean deviation (RSMD) is added.

## Human model

In order evaluate the performance of the single and population based inhalation model only very limited metabolic data is available. In the study two male participants were given 0.7mg/kg-BW CNMA orally. This resulted in 96.2% and 96.5% of the administered dose being excreted in the urine as hippuric acid, benzoyl glucronide, HPPA or benzoyl acid al of which are downstream products of the Cinnamic acid metabolite(Maria & Peters, 1993) . In Figure 6 the results of 0.7mg/kg-BW oral exposure simulation using the human population model can be seen. The median percentage metabolized for both females and males 97.6% after 24h. with 99.72 percent of results laying between 98.42% and 96.3%.

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Figure : percentage of 0.7mg/kg-BW oral dose metabolized to cinnamic acid metabolites using the Human population based model. Individual male and female results are represented as dots.

As no read across data of in vivo data was available no further validation steps were performed. In order to evaluate whether inhalation exposure leads to differences in sensitive organs two simulation where performed. In Figure 7 the results of these simulation can be seen. If we considered oral exposure we can see that apart from the small intestine no organs statistically differ from each other. Small intestine is the organ with the highest CNMA concentration. If compared to inhalation exposure it can be noted that the organs differ between each other and that the fat is now the organ with the highest CNMA concentration.

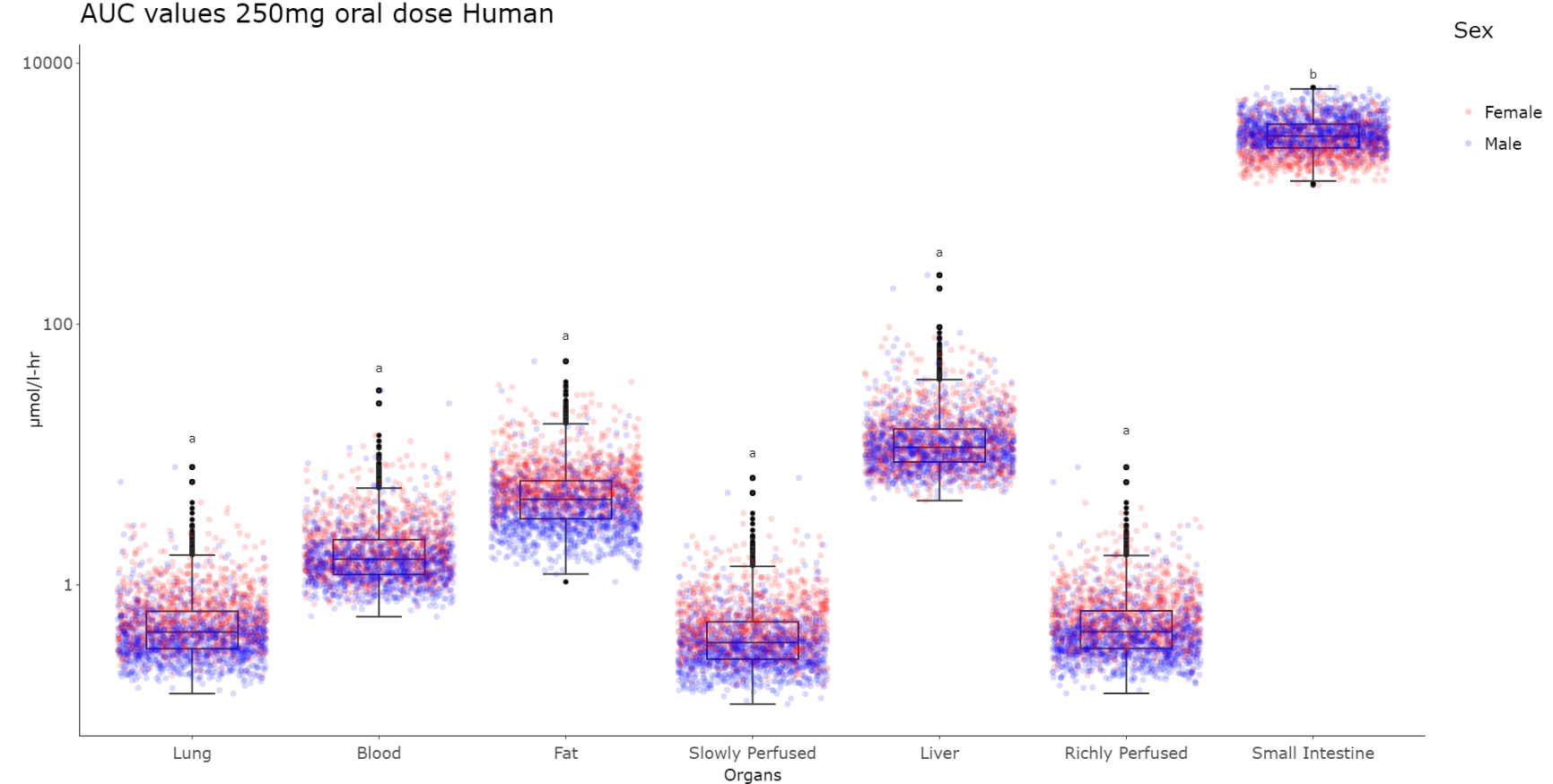
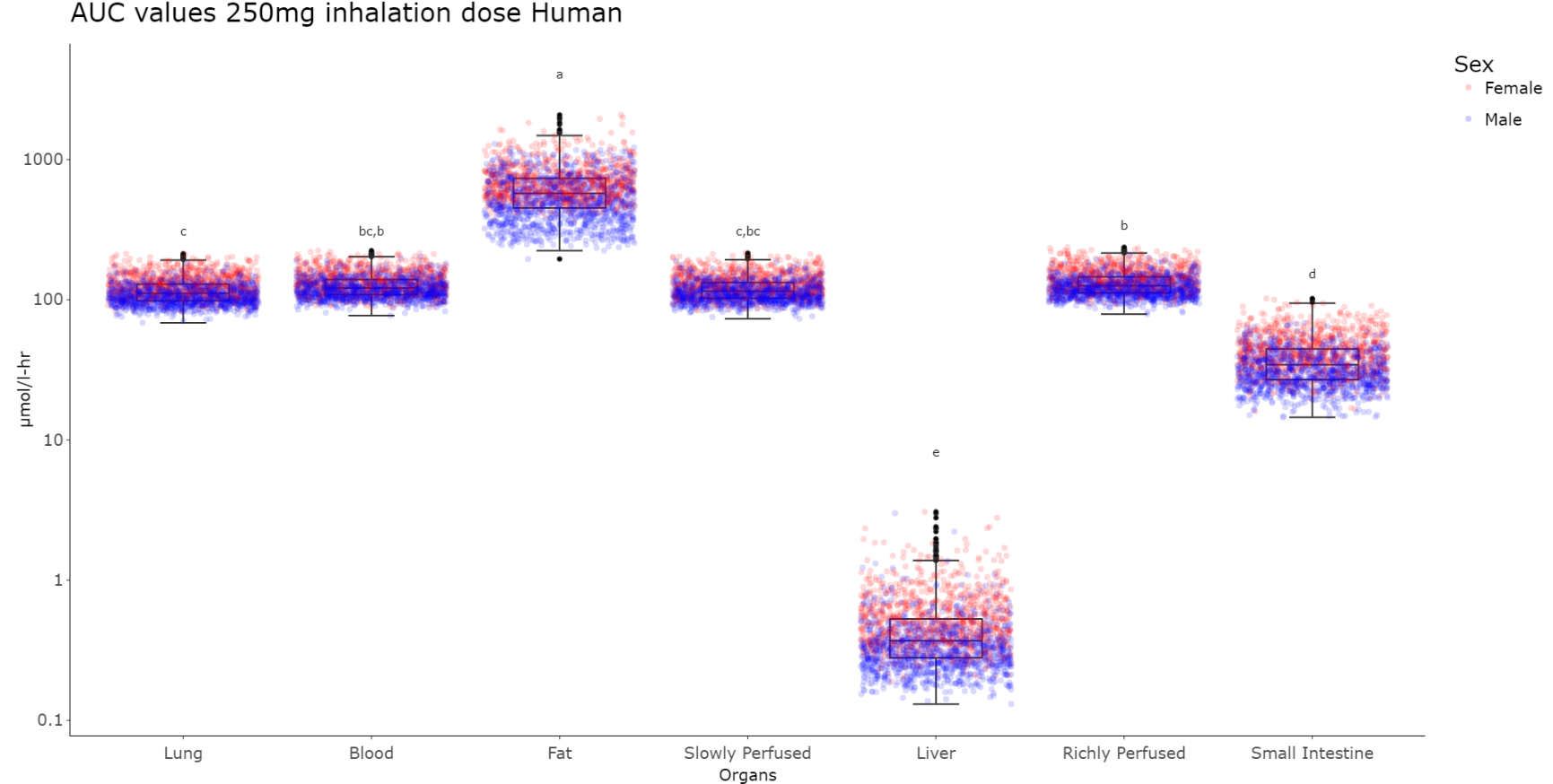
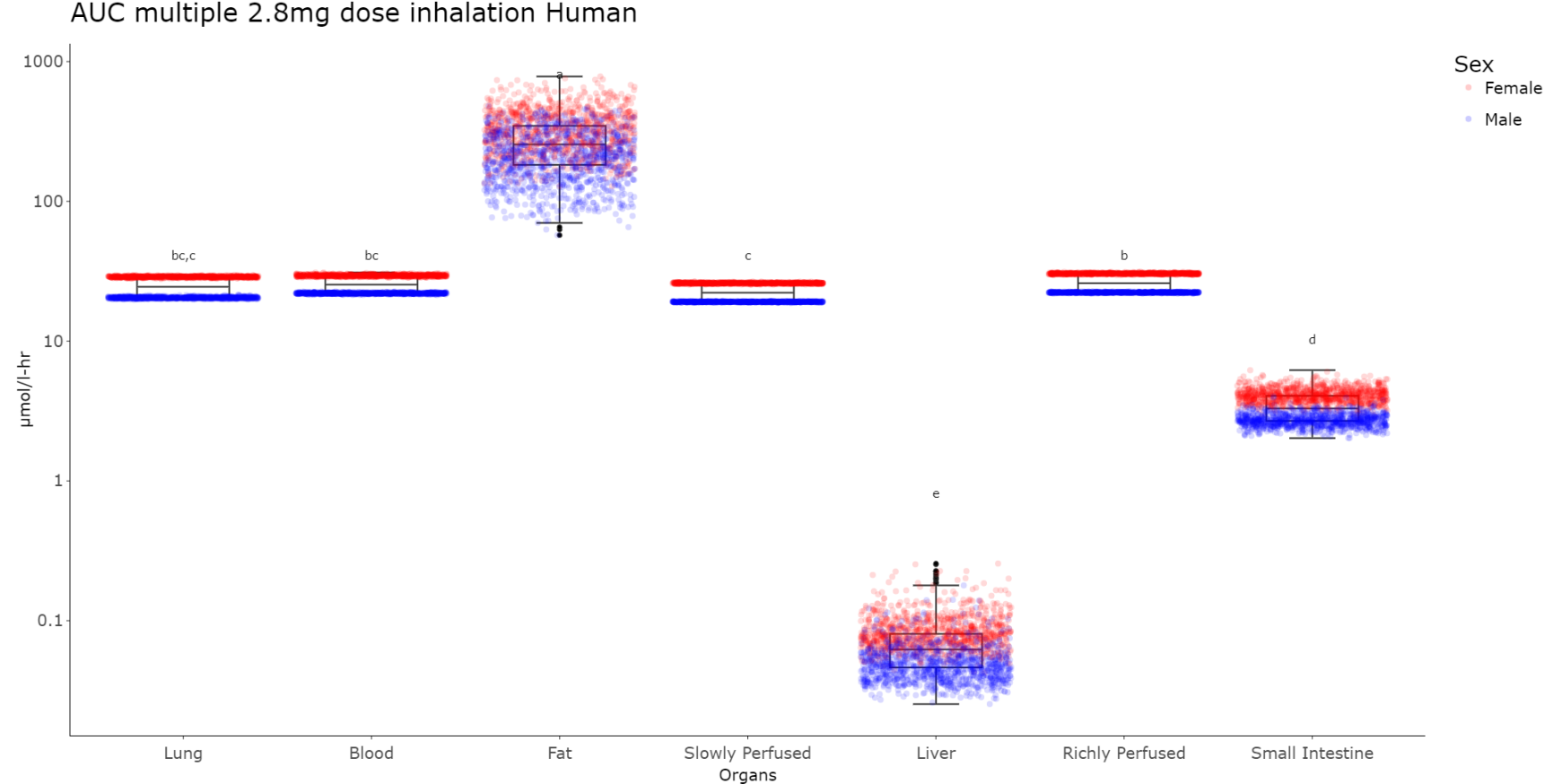


Figure : Area under the curve value results for a 250mg/kg-BW and inhalation dose using the human inhalation population model. Significance differences where calculated between the different organs. Organs that do not differ significantly share the same significance letter. If two significancy letters are present the first one represents female results and the second male results.



## Outcome

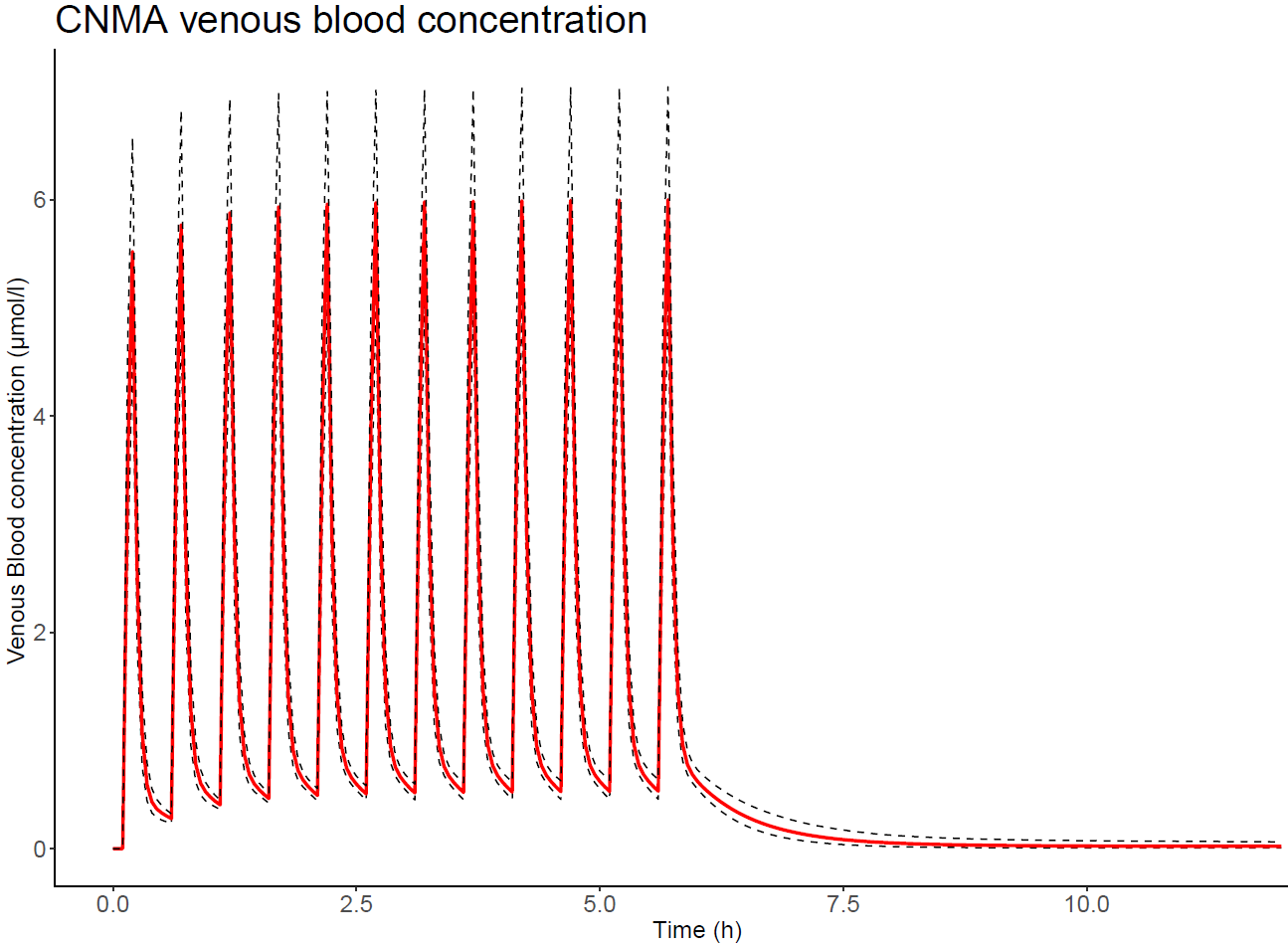
* Sensitive organs
  + Comparison between the three variants of the model
* General range of exposure as currently known

## Validating model

# Discussion

## Issues to be solved

This means that the differences between the Kiwamoto model and the R model are likely due to differences in parameters definition and not due to errors in the R script.

* GHS metabolism in the Lung?
* Iterate that no validation data is available to assess cinnamaldehyde predictions in tissue, explain how you still trust the simulations (based on chemical, biological applicability domains)
* Explain how oral and inhalation routes lead to differences in tissue levels of cinnamaldehyde and consequences for toxicity
* Explain how species differ in the kinetics of cinnamaldehyde exposure through inhalation and consequences for toxicity
* Comparison of model to rat data
* Lack of Human Data

## Next steps

Measure metabolic profile in rat and humans of benzaldehyde in vitro, being extra careful given the volatility of the chemical

Expand with other aldehydes to make it a generic model for aldehydes

Assess mixture effects for aldehydes

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