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PII: S0378-4274(16)30023-6
DOI: http://dx.doi.org/doi:10.1016/j.toxlet.2016.02.001
Reference: TOXLET 9315

To appear in: *Toxicology Letters*

Received date: 27-10-2015
Revised date: 29-1-2016
Accepted date: 2-2-2016

Please cite this article as: Filser, Johannes Georg, Gelbke, Heinz-Peter, An evaluation of concentrations of styrene-7,8-oxide in rats and humans resulting from exposure to styrene or styrene-7,8-oxide and potential genotoxicity.Toxicology Letters http://dx.doi.org/10.1016/j.toxlet.2016.02.001

An evaluation of concentrations of styrene-7,8-oxide in rats and humans resulting from exposure to styrene or styrene-7,8-oxide and potential genotoxicity

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Highlights

- Genotoxic risk of styrene-7,8-oxide (SO) after oral styrene (ST) intake was evaluated.
- Study was based on published results on inhalation genotoxicity of ST or SO in rats.
- A physiological toxicokinetic model predicted blood burdens of SO in rats and humans.
- Genotoxic risk of SO was linked to the SO blood burden.
- A genotoxic risk of SO for humans resulting from ST from food containers is excluded.

Abstract

There is potential for oral exposure of humans to styrene (ST) such as from migration of residual levels in polystyrene food containers. After absorption, ST is metabolised to styrene-7,8-oxide (SO), an alkylating epoxide. Hence, a comparison of blood burdens of SO resulting from oral exposures to ST was made with SO burdens possibly warranting genotoxic concern. A validated physiological toxicokinetic model was used for the assessment. Model calculations predicted for exposures to ST that maximum concentrations of SO in venous blood of rats and humans should not exceed 0.33 μg/ml and 0.036 μg/ml, respectively, because of saturation of the SO formation from ST. The daily area under the concentration-time curve of SO in venous blood (AUC_{SO}) was directly proportional to the dose of ST (mg/kg body weight; BW), independent of the exposure route (inhalation or oral exposure). In resting humans, the daily AUC_{SO} was about half that in rats at the same amount of ST/kg BW (calculated up to 100 mg ST/kg BW in humans). Taking into account the results of cytogenetic studies in ST-exposed rats, it was deduced that no genotoxic effects of SO are to be expected in ST-exposed humans, at least up to a daily amount of 100 mg ST/kg BW, which is equivalent to 100 times the amount originating from the Overall Migration Limit for ST migrating from food contact plastics in the EU. Therefore, no potential genotoxic concern is predicted for ST uptake from food packaging, based on the reported combined measured and modelled data.

Abbreviations

ACB_{SO}, lifetime average concentration of SO in venous blood; AUC, area under a concentration-time curve; AUC_{ST}, area under the concentration-time curve of ST in venous blood; AUC_{SO}, area under the concentration-time curve of SO in venous blood; BW, body weight; C_{maxSO}, maximum (peak) concentration of SO in venous blood; FPG, formamidopyrimidine DNA glycosylase; LEC, lowest effective SO concentration resulting in a genotoxic/mutagenic effect; OML, Overall Migration Limit in the EU, maximum permitted amount of a substance released from a material or article into food; SD, standard deviation; ST, styrene; SO, styrene-7,8-oxide; V_{maxmo}, maximum rate of metabolic elimination of ST catalysed by cytochrome P450-dependent monooxygenase.

Keywords: Styrene; Styrene-7,8-oxide; Physiological toxicokinetic model; Rat; Human; Genotoxic risk.

1. Introduction

1.1 Background

Under the European Regulation 793/93 for risk assessment of existing chemicals the UK submitted a comprehensive evaluation of the whole toxicological database for styrene (ST). It was concluded, "there are no concerns for … mutagenicity or carcinogenicity" (UK RAR, 2008). This was the basis for the non-classification of ST for both of these endpoints within the EU (EU, 2008). On the other hand, IARC (2002) concluded that ST is "possibly carcinogenic to humans" (Group 2B). The Scientific Committee on Health and Environmental Risks (SCHER, 2008) evaluated the UK RAR (2008). Although SCHER agreed to the major parts of the UK RAR regarding genotoxicity and lung carcinogenicity, they questioned whether the lung is the only potential target for carcinogenicity.

The total database for ST shows that most of the studies for carcinogenicity, mutagenicity and the mode of action refer to the inhalation route of exposure. This represents the highest potential exposure to humans, namely for workers exposed in glass-fibre reinforced industries. On the other hand the vast majority of the population may be orally exposed to ST, albeit to a much lesser extent. By this route ST will be nearly completely metabolised to styrene-7,8-oxide (SO) by first-pass liver metabolism and will enter blood circulation. Therefore, regarding other potential target organs for carcinogenicity SCHER (2008) agreed "with the conclusion of IARC (2002), that, based on the observations in human workers regarding blood styrene 7,8-oxide, DNA adducts and chromosomal damage, it cannot be excluded that this and other mechanisms are important for other organs". Such considerations may have led to the request of the Working Group on Genotoxicity of EFSA at its 3rd and 4th meetings (March 22-23 and 24-25, 2012; EFSA, 2012a) "that additional in vivo investigations by oral route should be performed in order to clarify styrene genotoxicity by oral route". The basis for this request was explained in a note of the EFSA working group specifying that although a number of in vitro and in vivo genotoxicity studies exist, the database was not considered to be "adequate to clear genotoxicity of styrene in vivo after oral exposure since most of the in vivo genotoxicity studies were conducted by different route of administration" (EFSA, 2012b). In support of its request the group refers to an in vivo comet assay in the mouse by the intraperitoneal route with positive findings in several organs like bone marrow, blood, liver and kidney (Vaghef and Hellman, 1998). The EFSA working group noted the significant differences in metabolism of ST between species and tissues and concluded, based on the toxicokinetic data described in the UK RAR

(2008), that rats represent a better model for humans than mice. It was therefore proposed to carry out the in vivo comet assay in rats after oral administration of ST. This note of EFSA (2012b) was the basis for a request of the European Commission (EC, 2012) to conduct such a study.

Although IARC (2002) and SCHER (2008) addressed the possibility of a carcinogenic response in other organs than the lung of mice, it should be taken into consideration that only the lung of mice has been identified as the target organ for carcinogenicity or precursor effects by inhalation of ST as well as by oral exposure to ST. Thus, similar to lung tumour induction in mice by inhalation (Cruzan et al., 2001), former non-guideline studies showed some indications for lung tumours also by the oral route (Ponomarkov and Tomatis, 1978; NCI, 1979). In addition, short-term exposure resulted in increased cell replication in the terminal bronchioles of mice by the oral (Green, 1999a) and the inhalation route (Green, 1999b). Cruzan et al. (2009) presented a mode of action explaining why ST leads to lung tumours in mice but not in rats similar to some other chemicals with a comparable carcinogenic profile. Lung tumour formation was specifically related to metabolism of ST by the cytochrome P450-dependent monooxygenase CYP2F2, present in the lung of mice but not of rats or humans.

To address the request of EFSA (2012b) and the conclusion of SCHER (2008) the present assessment investigates

- whether and to what extent the blood burden of SO after oral ST exposure may be increased compared to inhalation exposure to ST;
- whether and under what exposure conditions ST may lead to the formation of SO in concentrations sufficiently high to give reasons for concerns for systemic genotoxic effects and whether such blood burdens of SO may be attained after oral exposure to ST.

To this aim, genotoxicity in blood cells was selected as a sensitive surrogate for systemic genotoxicity.

1.2 Selection of the database

The EFSA working group, when requiring an oral in vivo comet assay in rats, noted that "the rat would likely represent a more realistic scenario in terms of metabolic body burden than the mouse" with respect to the human ST exposure situation. Therefore the UK RAR (2008) and the IUCLID (last update Sept. 2014) were searched for mutagenicity/genotoxicity data in rats. The literature compiled in the IUCLID was based on the UK RAR (2008) completed by an overlapping literature search from 1998 up to September 2014. The following databases were searched for toxicology information: CHEMLIST, REGISTRY, EMBASE and TOXCENTER. The search profile included: acute, subacute, subchronic, chronic toxicity, irritation to skin and eyes, sensitization, carcinogenicity, teratogenicity, reproductive toxicity, neurotoxicity immunotoxicity, endocrine effects, genotoxicity, mutagenicity, metabolism, alternative methods. One of the authors (HPG) was responsible for identifying the studies relevant for the IUCLID (2014) and for the present paper.

For assessment of SO blood burden and its potential genotoxicity after oral exposure of rats, two rat studies with repeated inhalation exposures to ST, which measured several different genotoxicity endpoints, were selected (Sinha et aI., 1983 and Kligerman et aI., 1993), based on the UK RAR (2008). In addition, a more recent investigation reported by Gaté et al. (2012), with inhalation exposure to ST and SO and measured blood levels of ST and SO, was included in this analysis. The database is briefly described below:

Sinha et al. (1983)

Seven to eight-week old male and female Sprague-Dawley rats were repeatedly exposed by inhalation to ST at concentrations of 600, and 1000 ppm (6 h/d, 5 d/w, 12 months). Thereafter, the mitotic index as well as the frequency of metaphase aberrations (chromatid and chromosome type) was determined in femoral bone marrow cells. At both ST concentrations, there was no statistically significant increased incidence of chromosomal abnormalities compared to the non-exposed controls. Higher frequencies of chromatid gaps were not dose-related. This finding was considered not to be of toxicological significance in the UK RAR (2008) concluding that the result was overall negative. Thus, ST was not clastogenic in the rat up to the exposure concentration of 1000 ppm.

Kligerman et al. (1993)

Female Fischer 344 (F344) rats were exposed by inhalation to ST at concentrations of 125, 250, and 500 ppm for 6 h/d for 14 consecutive days. Thereafter, peripheral blood lymphocytes were cultured for analyses of sister chromatid exchange, chromosomal aberrations, and the micronucleus frequencies in cytochalasin B-induced binucleated cells. The frequencies of micronuclei were also determined in femoral bone marrow normochromatic erythrocytes. DNA single-strand breaks were determined in peripheral blood lymphocytes by means of the alkaline comet assay. Compared to non-exposed controls, there was neither an increase in chromosomal aberrations nor one in micronucleus induction frequencies. Also, DNA strand breakage did not increase. However, ST was a weak inducer of sister chromatid exchange.

Gaté et al., 2012

Male F344 rats were exposed over 6 h to atmospheric concentrations of SO (25, 50, or 75 ppm) or of ST (75, 300, or 1000 ppm). The SO concentrations in blood resulting from 1000 ppm ST were in between those determined after SO exposures to 25 and 50 ppm. In order to examine genotoxic effects of ST or SO, to the exposure schedule for ST and SO was 6 h/d over 3d or 6 h/d, 5 d/w over 4 weeks. At the end of the exposures, the induction of micronuclei in circulating reticulocytes was studied by means of flow cytometry and DNA strand breaks in leukocytes caused by DNA adducts by using the "standard" alkaline comet assay as an indicator test. In addition, the formamidopyrimidine DNA glycosylase (FPG) modification was applied that is specifically sensitive for identifying DNA modifications induced by oxidative damage or alkylating agents (e.g. Smith et al., 2006). The micronucleus test and the "standard" comet assay were clearly negative for both test substances at both time points. On the other hand, by the FPG modification of the comet assay an increased DNA migration was observed after 3 days of exposure to all three ST concentrations. Over the whole exposure range of 75-1000 ppm ST, the effect was quantitatively very similar with no dose-response relationship. No genotoxic effects were identified in blood from rats exposed to ST or SO for 28 days.

1.3 Experimental procedure

An extensively validated physiological toxicokinetic (PT) model for ST and SO in rodents and humans (Csanády et al., 1994) was used in order to gain quantitative information

about the blood burden of the genotoxic ST-metabolite SO resulting from the inhalation studies in rats and from oral as well as inhalation intake of ST in rats and humans. The following exposure scenarios were modelled: Inhalation exposures of rats to ST at concentrations of 500 ppm (6 h/d, over 14 consecutive days) according to Kligerman et al., 1993) and of 75 as well as 1000 ppm (6 h/d, 5 d/w, up to 4w; according to Gaté et al., 2012), and to SO at a concentration of 75 ppm (6 h/d, 5 d/w, up to 4 w; according to Gaté et al., 2012). An extension of the modelled exposure duration of up to one year according to the study of Sinha et al. (1983) was not done considering it not to be required. However, the SO burden in venous blood of a male Sprague-Dawley rat (BW 612 g), exposed for four weeks (6 h/d, 5 d/w) to 1000 ppm of ST, was simulated by the PT model. The value of the BW was the estimated average over the 12-months exposure period in the study of Sinha et al. (1983). It was calculated by using the information on body weight gain of male Sprague-Dawley rats fed NIH-31 diet ad libitum (Lewis et al., 2003). Additionally, inhalation exposures of rats and humans to ST at concentrations of 9 ppm (rats) and 7 ppm (humans) were modelled. The exposure conditions (6 h in rats and 8 h in humans) result in the intake of the same daily amounts of ST as the lowest modelled amounts of ST taken in orally (see below). This modelling procedure enables comparison of the SO burden resulting from two routes of exposure to low amounts of ST. Oral dosing of ST was modelled as daily intake of a bolus dose and as daily intake of the same dose divided into three equal parts administered in time intervals of 3 h to approximate the schedule of food intake of humans. The cornerstone for modelling oral intake of ST in humans was the Overall Migration Limit in the EU for substances that can migrate from food packaging into the foodstuff (OML) of 60 mg/kg food according to EU (2011) since a Specific Migration Limit (SML) has not been established. Considering ST-induced ototoxicity, reproductive toxicity, and effects on colour discrimination, it has previously been demonstrated that the OML would be sufficiently protective for human health (Gelbke et al., 2014). Assuming for an adult a body weight (BW) of 70 kg and a daily food consumption of 1 kg (EC SCF, 2001), the OML corresponds to a daily oral exposure of approximately 1 mg ST/kg BW. This was taken as the lowest modelling dose for humans and 100 mg ST/kg BW/d as the highest modelling dose. The doses for oral exposure of rats (BW, 0.25 kg) were obtained from those of humans (BW, 70 kg) by allometric scaling based on $BW^{3/4}$. This procedure led to 4 mg ST/kg BW/d and 400 mg ST/kg BW/d. As an intermediate dose, 40 mg ST/kg BW/d was chosen for rats. As intermediate doses for humans, those of the rat (4 and 40 mg ST/kg BW/d) were

directly transferred. Based on the data obtained, the question was addressed whether the systemic SO burdens resulting from oral exposures to ST may become sufficiently high to give rise to concern of genotoxic effects.

2 Material and methods

2.1 PT model

Two PT models developed by J. G. Filser's research group describe the fate of ST and SO in rodents and humans (Csanády et al., 1994; 2003). Both deal with the blood burdens of ST and SO, the newer one additionally with the metabolism and the burdens of both substances in the airways. Because the goal of the present work was to predict blood burdens of SO solely, the earlier less complex PT model was chosen for the present work. Here, the body is subdivided into 4 compartments consisting of the very richly perfused tissues and organs, the moderately perfused muscle group, the slowly perfused adipose tissue, and the liver. Organ- and tissue-compartments of the PT model are interconnected by the arterial and venous blood flow. The liver compartment contains a sub-compartment for SO, the endoplasmic reticulum. ST or SO are taken in by inhalation or by the oral route.

Metabolism is assigned exclusively to the liver. ST is metabolically converted to SO by cytochrome P450-dependent monooxygenase. SO is hydrolysed to its glycol by endoplasmic epoxide hydrolase. It is also conjugated with glutathione by cytosolic glutathione S-transferase according to an ordered sequential ping-pong mechanism. Turnover of cytosolic glutathione is described by zero order production and first-order elimination. The sub-compartment endoplasmic reticulum was required in order to reflect the interaction between cytochrome P450-dependent monooxygenase and microsomal epoxide hydrolase. As a result of the functional cooperation of both enzymes in the endoplasmic reticulum, hydrolysis of SO is more effective with SO formed from ST than with SO coming from outside across the liver compartment. The non-hydrolysed part of SO in the endoplasmic reticulum is modelled to be at steady state with the SO concentration in the liver compartment where SO can be metabolically eliminated via conjugation with glutathione. Non-metabolized SO enters the venous blood. The omission of the lung as metabolizing organ is justified because ST-metabolism in this organ does not contribute significantly to the systemic SO burden, as was shown for rats and mice by Hofmann et al. (2006). In human lungs, metabolic ST oxidation is very small if there is any (Nakajima et al., 1994; Carlson et al., 2000).

The values of the physiological and biochemical parameters used in the model were identical to those given in Tables 1 and 2 of Csanády et al. (1994), with the following exceptions: Three apparent Michaelis constants were changed for rats: the new values of K_{mmo}, K_{mih}, and K_{mapp} (for abbreviations, see **Table 1**) were taken from Csanády et al. (2003) who had revised the original K_m-values of Csanády et al. (1994). The new parameter values, which were between 77% and 200% of the older ones, enabled a better agreement of model predictions with measured data. **Table 1** summarizes the used biochemical parameters and their values.

In the 2-week study of Kligerman et al. (1993), female rats were exposed to ST. The animals were 8 weeks old at the start of the study. In 8–9 week old female rats, the cytochrome-P450 content per g liver was about 75% of the value in equally old male rats (read from Figure 2 in Chengelis et al., 1988a). A linear extrapolation of the NADPH-dependent hepatic microsomal activity towards ST in male and female rats measured at weeks 3 and 18 of life (Table 1 in Kishi et al., 2005) to week 8.5 gave for females 61% of the value for males. The activities of hepatic microsomal epoxide hydrolase towards SO and of hepatic cytosolic glutathione S-transferase towards p-nitrobenzyl chloride were per g liver of 8–9 week old female rats about 76% and about 80%, respectively, of the values in equally old male rats (read from Figures 2 and 5 in Chengelis et al., 1988b). Consequently, the values of V_{maxmo}, V_{maxeh}, and V_{maxGST}, all given in Table 1, were multiplied with 0.70, 0.76, and 0.80, respectively, in order to model the study of Kligerman et al. (1993).

The PT model does not contain any change in the bioavailability of ST as a result of repeated exposures. This is in agreement with findings of Filser et al. (1993) that repeated exposures of rats to 150 or 500 ppm of ST (6 h/d, 5 d) did not influence the kinetics of ST as compared to that in naïve rats. Also Mendrala et al. (1993), when comparing concentration-time courses of ST and SO in venous blood of orally ST-dosed (500 mg/kg BW) rats, did not find statistically significant ($P \leq 0.05$) differences in the areas under the concentration-time curves (AUC) for ST (AUC_{ST}) or SO (AUC_{SO}) in venous blood between animals pre-treated with ST (1000 ppm; 6h/d, 4 d) or naïve ones.

The PT model was described by a series of mass balance differential equations (see Csanády et al., 1994). In the cited publication, there was a typographical error in equation 5. The correction is shown in equation 13 in Csanády et al. (2003). The differential equations were solved numerically on a Mac computer using the program Berkeley Madonna X (version 8.3.22).

BWs of F344 rats in dependence of age (up to 13 weeks) were obtained from the Gene Editing Rat Resource Center (2013). For rat ages that were between the reported ones, BWs were linearly interpolated. If the BW of a rat differed from the reference value of 0.25 kg, its influence on physiological parameters and maximum rates of ST and SO metabolism was taken into account by allometrically scaling cardiac output, alveolar ventilation, and the maximum rates V_{maxmo}, V_{maxeh}, and V_{maxGST} (see **Table 1**) using a body surface scaling factor of $BW^{2/3}$ (e.g., Filser, 1992). The influence of physical activity on alveolar ventilation, cardiac output, and blood flow through the liver was taken into account by revising the values of these parameters according to Csanády and Filser (2001).

AUCs of ST or SO in blood from t = zero to infinity were calculated up to the last data point of the concentration-time curve using GraphPad Prism, version 6 for Macintosh (GraphPad Software, La Jolla California USA) and adding the ratio of the last data point to the rate constant of the final elimination phase. The same program was used to calculate linear regression curves.

2.1.1 Validation of the PT model

The quality of the model was demonstrated for humans by comparing predicted concentrations of ST and SO in venous blood of ST-exposed workers against values measured by Korn et al. (1994); see **Figure 1**. For rats, model-predicted concentrations of SO in venous blood were plotted against corresponding experimental data obtained following inhalation of ST (Kessler et al., 1992; Filser et al., 1992; Cruzan et al., 1998; Gaté et al., 2012); see **Figure 2**. Also, model-predicted concentration-time curves of ST and SO in venous blood resulting from oral administration of ST were compared with values measured by Mendrala et al. (1993); see **Figure 3**. An additional comparison is given in **Table 2** that shows measured and PT model-predicted concentrations of SO in venous blood of rats that were single-exposed (6 h) by inhalation to various concentrations of SO or ST and SO in venous blood of rats that were exposed (6 h) to 1000 ppm of ST (Gaté et al., 2012). All of the predicted blood concentrations in **Table 2** were within the standard deviations (SD) of the measured data with the exception of the lowest predicted SO concentration that was little above the reported SD range.

Figures 1 and **2** and **Table 2** demonstrate that the model-predicted concentrations of ST or SO in venous blood of humans and rats, which result from inhalation exposures to ST (humans) and to both compounds (rats), were in agreement with the measured data. Cruzan et al. (1998), when determining SO in the blood of 95-week-old ST-exposed rats, found somewhat lower SO concentrations than the other authors (**Figure 2**). The difference might result from the higher activity of hepatic epoxide hydrolase towards SO in senescent as compared to young male rats (Birnbaum et al., 1979; Chengelis 1988b). A publication most relevant to the present investigation was that of Mendrala et al. (1993; see **Figure 3**) because it enabled a direct comparison of measured and model-predicted concentration-time courses and of AUCs of both ST and SO in venous blood of rats resulting from oral administration of ST. The AUC_{ST} of the predicted concentration-time curve of ST (723 µg·h/ml) was about twice as large as that reported on the basis of measured data (367 ± 81 µg·h/ml), the predicted concentration-time curve of the metabolite SO had an AUC_{SO} (4.39 µg·h/ml) that was within the SD range of the reported one (3.32 ± 1.20 µg·h/ml).

Given the agreement between measured and predicted data in rats and humans, the PT model was applied for the following modelling exercise.

3 Results and Discussion

3.1 Inhalation studies of Kligerman et al. (1993) and Gaté et al. (2012)

3.1.1 Kligerman et al. (1993)

The authors exposed female F344 rats by inhalation to atmospheric concentrations of ST of up to 500 ppm for 6 h/d for 14 consecutive days. In order to acquire information on the SO blood burden resulting form the exposure to 500 ppm ST, the exposure scenario and the resulting concentration-time curve of SO in venous blood was calculated by the PT model (**Figure 4**). At each exposure day, the concentration of SO in venous blood increases continuously during the 6-h exposure to ST until a sharp peak of 0.252 µg SO/ml blood is reached. Immediately thereafter, it declines rapidly until reaching the baseline 18 h later. Similarly, the concentration of the metabolic precursor ST in the venous blood rises during the daily 6-h exposure to a short peak of 41 µg/ml blood (first week of exposure). Then it drops rapidly to baseline at the end of each 24-h period. During the second week, ST-peaks are a little less high (calculated concentration-time curve of ST not shown). Under such exposure conditions, SO does not accumulate in the rat, thus the daily SO peaks are level.

3.1.2 Gaté et al. (2012)

Gaté et al. (2012) exposed rats to vaporous ST (75, 300, or 1000 ppm; 6 h/d, 3d or 6 h/d, 5 d/w, 4 w) or to SO (25, 50, or 75 ppm; 6 h/d, 3d or 6 h/d, 5 d/w, 4 w). Of these scenarios, the 4-week exposures to the lowest and the highest concentrations of ST and to the highest concentration of SO were simulated by means of the PT model. Model-calculated concentration-time curves of ST and SO in venous blood of rats exposed to 75 ppm and 1000 ppm of ST are shown in **Figures 5 and 6**. **Figure 7** depicts the model-calculated concentration-time curve of SO in the venous blood of a rat that is exposed over the same time period to 75 ppm of SO. ST concentrations in venous blood of the animals exposed to 75 ppm of ST rise to peak concentrations of 1.57 µg/ml during the first week of exposure (**Figure 5**). The maximum peaks of ST reached during the first week of exposure to 1000 ppm of ST are 94.6 µg/ml (**Figure 6**). The disproportionate increase in the blood concentrations of ST from 75 to 1000 ppm results from the saturation kinetics of the ST metabolism. At the high exposure concentration, the metabolic elimination of ST is relatively slower and takes longer than at the low exposure concentration. As a consequence, ST enriches disproportionately in the body, predominantly in the fatty tissue, an effect demonstrated by Filser et al. (1993). The less than proportional increase in the maximum SO concentration in venous blood (C_{maxSO}) from exposures to 75 and 1000 ppm ST (**Figure 5 and 6; Table 3**) results also from the saturation kinetics of ST: The SO formation is relatively slower at the high ST-concentration than it is at the low one. Therefore, the curve in **Figure 2** showing the SO concentration in rat blood in dependence of the ST exposure concentration reaches a plateau at high exposure concentrations when the maximum rate of the metabolism of ST to SO is reached.

Considering that the calculated C_{maxSO} value of the SO exposed animals (**Figure 7**) was 24- and almost 4-fold higher than the C_{maxSO} values in the rats that were exposed to 75 and 1000 ppm of ST (**Figures 5**, **6,** and **Table 3**), it becomes evident that the DNA damage observed in the presence of FPG in circulating leukocytes of the 3-day ST-exposed but not of the SO exposed rats (Gaté et al., 2012) could not result from metabolically produced SO. The maximum concentrations of ST in venous blood of the animals exposed to 75 ppm of ST were calculated by the PT model to be 60 times lower than of those exposed to 1000 ppm of ST (compare **Figures 5A** and **6A**). However, the rather small DNA damage (in average 1.9 or 6.9 times higher than the damages found in

two independent FPG-containing controls) was not dose-dependent, although ST exposure concentrations ranged from 75 up to 1000 ppm. It is hard to explain how the DNA damage could result from ST. In order to interpret their findings of increased DNA strand breaks after 3 days of exposure to ST but neither after 20 days of exposure to ST nor after 3 or 20 days of exposure to SO, Gaté et al. (2012) speculated "a possible cellular adaptation to genotoxic insults". The authors suggested also oxidative stress induced by ST or by an ST-metabolite other than SO as the source of the DNA damage found after 3 days in the presence of FPG. However, the fact that the extent of this damage was not dependent on the ST concentration does not support these hypotheses. One possible explanation is that the statistically significant difference from the controls is actually a random result. (A more detailed assessment of the comet assay performed by Gaté et al. (2012) in circulating white blood cells in the presence of FPG is given in the **Appendix**). Whatever might be the biological significance of the DNA damage described for ST after 3 days of exposure in the FPG modification, this study clearly shows that SO does not lead to systemic genotoxicity in peripheral blood.

During the exposure to 75 ppm of ST, the rate of ST oxidation is by far below its maximum value (V_{maxmo}); Concentrations of ST and its metabolite SO in venous blood decrease rapidly after the end of each exposure (**Figure 5**). At exposures to 1000 ppm of ST (**Figure 6**), the metabolic elimination of ST is almost saturated. As a consequence of the limited metabolic ST elimination, ST enriches to relatively higher concentrations in the organism and the enrichment phase is longer as compared to lower exposure concentrations at which metabolism is not saturated. Additionally, the allometrically scaled alveolar ventilation (a measure of the gas exchange rate between the exposed organism and the surrounding air) is relatively smaller in heavier compared with lighter animals. Hence, during the 6-h exposures to 1000 ppm of ST, lower maximum concentrations of ST are reached in the venous blood of the older (heavier) than in that of the younger (lighter) rats (**Figure 6**). At low exposure concentrations of ST, this effect is less evident (**Figure 5**). The SO peak curves at exposures to 1000 ppm of ST (**Figure 6**) due not drop to the baseline at the end of each exposure day in contrast to those at exposures to 500 or 75 ppm of ST (**Figures 4** and **5**). However, even at 1000 ppm of ST, there is almost no SO accumulation of the daily exposures. Also, after exposure to 75 ppm of SO the epoxide does not accumulate in spite of the much higher SO concentrations reached at the end of each exposure period (**Figure 7**).

A comparison of the model-calculated declines of SO after the end of the daily exposures in **Figures 4** to **6** with those in **Figure 7** demonstrates much slower decline rates following exposure to ST than after exposure to SO. Obviously, the elimination rate of ST limits the elimination rate of SO when it is formed metabolically. **Figure 7** shows about 4 times higher SO maxima from exposure to only 75 ppm of SO than those that are reached during exposure to 1000 ppm of ST (see also **Table 3**).

3.2 SO in blood after oral intake of ST

Rat: **Figures 8** and **9** show PT-model-calculated concentration-time curves of SO in venous blood of a rat with a BW of 250 g. Several conditions of ST intake were modelled: either daily (5 d) single oral bolus doses of 400, 40, or 4 mg of ST/kg BW (**Figure 8**) or daily (5 d) three times in time intervals of 3 h repeated orally administered doses of each 400/3, 40/3 or 4/3 mg of ST/kg BW (**Figure 9**). Daily bolus doses of ST result in higher values of C_{maxSO} than divided doses. The effect is less evident when comparing the largest single dose with the largest divided one because metabolism of ST to SO is almost saturated after the second administration of the divided dose. The SO peaks resulting from the largest dose of ST (administered as a bolus or as a divided dose) are broader than those from the smaller doses. This is a consequence of the saturation of the ST metabolism: it takes a while until the concentration of ST in the liver is low enough so that its elimination and, as a consequence, that of SO follows first-order kinetics. There is no accumulation of SO after multiple oral dosing, even after the largest bolus dose of 400 mg ST/kg BW at which the highest SO concentration is found.

Human: Concentration-time curves of SO in blood of a resting adult human (70 kg) who receives single or divided oral doses of ST of 100, 40, 4, or 1 mg/kg BW for 2 weeks were calculated by the PT model and are shown in **Figures 10** and **11**. The time period of 14 days of repeated exposures was chosen because there is some SO accumulation in humans in contrast to rats. It requires about 12 days until steady state is reached. This is because the final, first-order elimination phase of ST, representing the elimination from its storage, the adipose tissue, is by far longer in humans (elimination half-life of 55 h as calculated by the PT model for a 70-kg human) than in rats (elimination half-life of 1.8 h, calculated by the PT model for a 250-g rat). These data are in agreement with published final elimination half-lives of the lipophilic ST of 2–4 days in the adipose tissue of humans (Engström et al., 1978) and of 3.6±2.76 h in rats (Ramsey and Young, 1978). A

very large difference in the species-specific half-lives of the last elimination phase can also be expected from allometrical scaling by taking into account the species-specific volumes of the adipose tissue in both species and the available (human or rat) elimination rate constant which is linked to the corresponding final elimination half-life of ST (see equation given in Table 1 on page 7 of Filser, 1992).

Generally, the findings in humans resemble those in rats. Bolus doses result in higher values of C_{maxSO} than divided ones except the largest daily doses (100 mg/kg BW). The SO peaks are wider after the largest dose of ST (bolus or divided) as compared to the smaller doses.

3.3 C_{maxSO} and AUC_{SO} resulting from inhalation or oral exposures to ST

Table 3 shows daily doses of ST taken in by rats or humans exposed to ST by inhalation or the oral route. Also given are PT-model calculated C_{maxSO} values and daily AUC_{SO} values resulting from exposures to ST or SO.

3.3.1 C_{maxSO}

3.3.1.1 Oral intake of ST

Rat: Model-calculated values of C_{maxSO} are proportional to the oral dose of ST for the two lowest doses of 4 and 40 mg/kg BW/d, administered once or divided. The C_{maxSO} at the highest dose of 400 mg ST/kg BW/d (bolus or divided dose; about 0.3 µg/ml), however, is only 3 times higher than that from the bolus dose of 40 mg ST/kg BW/d or 6 times higher than that resulting from the divided doses of 3·40/3 mg ST/kg BW/d (see also **Figures 8 and 9**) although the doses differ by a factor of 10. Obviously, saturation kinetics of the SO formation becomes relevant. The model-predicted C_{maxSO} that cannot be surpassed is in the rat 0.33 µg/ml (see **Figure 2**). It is almost reached at 400 mg ST/kg BW, the highest modelled oral dose.

Human: After both the bolus dose and the three times divided dose of 100 mg ST/kg BW/d, the PT model predicts the same C_{maxSO} of about 0.031 µg/ml in venous blood. Based on calculations by means of the PT model, it is predicted that the concentration of metabolically produced SO in venous blood cannot surpass 0.036 µg/ml. Of this concentration, 95% are reached in a resting human with a BW of 70 kg at a daily oral dose of 200 mg/kg BW. The predicted maximum possible SO concentration in ST-exposed humans is by a factor of 9 lower than the corresponding one in rats. In humans, saturation kinetics of ST becomes apparent even at the second highest bolus dose of 40 mg/kg BW/d because the C_{maxSO} of 0.0248 µg/ml is only about half the concentration that is obtained by a linear extrapolation of the values of C_{maxSO} that were calculated by the PT model for the two lower bolus doses of 4 and 1 mg ST/kg BW/d. At these two doses, single or divided dosing results in an about 2-fold difference in the values of C_{maxSO}. These values are proportional to the dose of ST following daily single or divided intake. When 40 mg ST/kg BW/d is taken in, the difference in the C_{maxSO} values resulting from single and divided ST intake shrinks to a factor of about 1.2. This is because saturation kinetics of the metabolism of ST to SO becomes evident following intake of the bolus dose of ST. However, after intake of 40 mg ST/kg BW/d in 3 divided doses of 13.33 mg ST/kg BW/d each, the proportionality

between C_{maxSO} and dose seen at the two lowest divided doses still exists. When 100 mg ST/kg BW/d are taken in, dividing the dose has almost no effect on C_{maxSO}. Here, the ST concentration in the liver remains high enough for several hours for the SO formation rate to become rather near to its maximum value and without major changes as a result from the saturation of its metabolic formation from ST, independently of whether the dose of ST is taken in undivided or divided (see also **Figures 10** and **11**).

3.3.1.2 Inhalation of ST or SO versus oral intake of ST

Rat: In the venous blood of rats that are exposed to ST (6 h/d) by inhalation, predicted values of C_{maxSO} increase roughly linearly up to a ST concentration of 350 ppm. At higher concentrations of ST in air, values of C_{maxSO} approach a plateau because of the saturation of the ST metabolism (**Figure 2**). After administering daily an oral bolus of 4 mg ST/kg BW to rats, the resulting C_{maxSO} is almost twice as high as that resulting from a 6-h intake of the same ST dose by inhalation (exposure to 9 ppm). This is also the case when comparing C_{maxSO} resulting from daily oral intake of an ST bolus of 40 mg/kg BW with that from daily 6-h exposures to 75 ppm of ST (daily SO dose of 36.6 mg/kg BW). Dividing the orally administered amounts in three equal parts results in almost the same values of C_{maxSO} per mg of ST per kg BW as obtained after ST inhalation of similar amounts thus showing the resemblance between divided oral administrations and continuous inhalation intake of ST. The highest oral dose of 400 mg ST/kg BW differs by about 20% from the doses inhaled during 6-h exposures to 1000 ppm of ST (**Table 3**). Because of saturation of the ST metabolism, both routes of exposure lead to almost the same C_{maxSO} of about 0.3 µg/ml. Saturation of the metabolism of ST is one cause for the fact that C_{maxSO} values reached during exposure to 1000 ppm of ST are nearly 4-fold lower than the C_{maxSO} values resulting from inhalation exposure to 75 ppm of SO (see also **Figures 6** and **7**).

Human and comparison with rat: In humans, the picture is similar to that in rats (**Table 3**): A daily dose of ST of 1 mg/kg BW leads to higher C_{maxSO} values when administered orally (as bolus or as a divided one) than when inhaled by a resting human during an 8-h period (exposure to 7 ppm of ST).

A comparison of the C_{maxSO} values between humans and rats resulting from exposures to equal ST doses/kg BW shows lower values in humans. Following inhalation exposures to low concentrations of ST (rat, 6 h/d; human, 8 h/d) model-calculated C_{maxSO} values are 4 times lower in the venous blood of humans than in that of rats which inhale the same

amount of ST per kg BW (e.g., 0.0053/3.87/(0.000354/1.02) ≈ 4; see **Table 3**). The same difference results from a daily single bolus of 40 mg ST/kg BW. It diminishes to a factor of about two following the daily oral intake of divided ST doses of 40 mg/kg BW or of single and divided ST doses of 4 mg/kg BW. At oral ST doses higher than 40 mg/kg BW, the difference increases again because ST metabolism in humans is saturated at a lower dose of ST/kg BW than in rats.

In summary, the values of C_{maxSO} cannot surpass an upper limit because of the saturation kinetics of ST. C_{maxSO} values are higher following oral bolus doses of ST than after inhalation of the same amounts of ST. Divided doses of ST result in lower C_{maxSO} values than single bolus doses except at high doses of ST at which its metabolism is saturated. At the same doses of ST per kg BW, C_{maxSO} values are higher in rats than in humans.

3.3.2 Daily AUC_{SO} values

The area under a concentration-time curve of a directly DNA-alkylating substance like SO (e.g., González-Pérez et al., 2014) in the target tissue is generally considered as a more appropriate dose metric than a peak concentration (e.g., Ehrenberg et al., 1974; EPA, 2006). Daily AUC_{SO} values in venous blood of rats and humans, calculated for various scenarios of exposure to ST (and for one to SO; rats only), are summarized in **Table 3** together with daily doses of ST taken in per kg BW. By using this data set, two linear regression curves through the origin were constructed (**Figure 12**). In rats, the daily AUC_{SO} [µg SO·h/ml blood] = 0.01057 times the daily dose of ST [mg/kg BW] with a standard error of the slope of ± 0.000520 (µg SO·h/ml)/(mg ST/kg BW). The linear regression curve that is based on 11 calculated AUC_{SO}-values for rats is valid for up to a dose of ST of at least 488 mg/kg BW. In humans, the corresponding slope with its standard error is 0.00457 ± 0.0000728 (µg SO·h/ml)/(mg ST/kg BW). The linear regression curve that is based on 9 calculated AUC_{SO}-values for humans is valid for up to a dose of ST of at least 100 mg/kg BW. The relative standard error of the slope is larger in rats (± 4.92%) than in humans (± 1.59%). The difference stems largely from the fact that the modelled BWs of the animals were variable (between 0.136 and 0.612 kg) whereas a constant BW of 70 kg was used for humans (see **Table 3**).

The daily AUC_{SO} in a rat at rest is 2.3-fold that in a resting human if related to the same dose of ST/kg BW. It is independent of the exposure regimen (inhalation, undivided or divided oral dosing). Saturation kinetics of ST doesn't show an influence on the daily AUC_{SO} in the investigated dose ranges of up to 488 (rat) or 100 (human) mg ST/kg

BW/d. This observation results from the following: At high atmospheric ST concentrations at which ST metabolism is saturated, ST enriches to relatively higher levels in the organism than at lower concentrations. Consequently, SO formation lasts over longer time periods at high formation rates than at low ST concentrations at which the SO formation rate is limited by the actual ST concentration (compare **Figures 5** and **6**). As a result, the AUC_{SO} (not the concentration of SO) may further increase linearly with the dose of ST in spite of the saturation kinetics of ST.

In rats or humans when receiving a defined dose of ST either by inhalation at rest or by oral exposure, the species-specific daily AUC_{SO} values are the same for both exposure scenarios (**Table 3**).

3.4 Is a genotoxic risk by SO to be expected from oral intake of ST?

SO was mutagenic in most bacterial assays without an exogenous metabolic system, in Schizosaccharomyces, Allium cepa, and Drosophila melanogaster (summarized in IARC (1994). The lowest effective SO concentration (LEC) was generally rather high (≥ 24 µg/ml). Only in four out of 39 clearly positive mutagenicity studies with SO, lower LECs were reported. In Drosophila Melanogaster, sex-linked recessive lethal mutations were found at an atmospheric LEC of 1 µg/ml. Because SO enriches drastically in exposed organisms (mean value of the mammalian blood:air-partition coefficient: 2370 at 37°C; Csanády et al., 1994), the LEC within the exposed flies was surely much higher than the SO concentration in the atmosphere. IARC (2002) reports a study with Drosophila melanogaster, in which the LEC leading to somatic mutation was 600 µg SO/ml in feed. IARC (1994) stated that Vainio et al. (1976) should have detected LECs of 0.6 µg/ml in the Salmonella typhimurium strains TA 100 and TA 1535 and of 6.0 µg/ml in the strain TA 1538. However, the SO concentrations are given in the original publication in mol/plate but not per ml (Vainio et al., 1976). Also, Vainio et al. (1976) reported that SO "was not mutagenic to …TA 1538". Obviously, the information given in IARC (1994) is incorrect.

According to the literature summarized in IARC (1994, 2002), the LEC in non-human mammalian cells in vitro was 90 µg/ml for both chromosomal aberration and micronucleus formation, 3.6 µg/ml for DNA strand breaks, and 13.8 µg/ml for gene mutation. The LECs leading in human cells in vitro to micronucleus formation, chromosomal aberrations, and to DNA strand breaks were 12 µg/ml, 3 µg/ml (interpreted by IARC (1994) from a figure in the publication of Pohlová et al., 1985), and 1.2, respectively. The LEC for gene mutation in human cells in vitro was 24 µg/ml (IARC, 2002). Laffon et al. (2001; 2002) investigated the genotoxicity of SO in a concentration range of between 1.2 and 24 µg SO/ml (10 and 200 µmol/L) using leukocytes of four healthy non-smoking donors (2 females, 2 males). The authors evaluated genotoxicity by means of the alkaline comet assay, micronucleus formation and sister-chromatid exchanges. LECs for significant increases in the frequencies of DNA damage (comet assay), micronuclei, and sister-chromatid exchanges were 2.4 µg/ml (20 µmol/L), 12 µg/ml (100 µmol/L) , and 2.4 µg/ml (20 µmol/L), respectively.

The C_{maxSO} that can be reached for a short period of time during daily 6-h exposures of rats to 75 ppm SO (according to Gaté et al., 2012) is 1.16 µg/ml (**Figure 7**; **Table 3**). The model-calculated C_{maxSO} values in rat and human that can be reached during exposures

to ST cannot surpass 0.33 µg/ml and 0.036 µg/ml, respectively (see above). These maximum possible SO concentrations are 3.6-fold and 33-fold smaller than the lowest LEC value of 1.2 µg/ml. Therefore, it is not surprising that the in-vivo inhalation studies of Sinha et al. (1983), Kligerman et al. (1993) and Gaté et al. (2012) with ST concentrations of up to 1000 ppm were negative with respect to the induction of micronuclei, chromosomal aberrations, and DNA single-strand breaks. A small increase occurred in the frequency of sister chromatid exchange (Kligerman et al., 1993). However, the mechanism of the formation and the biological significance of small increases in sister chromatid exchanges is uncertain (Henderson and Speit, 2005). The small, not dose-dependent increase in DNA damage observed in the comet assay in the presence of FPG after three days of exposure to ST but not to SO (Gaté et al., 2012) was likely a random result (see *3.1.2* and **Appendix**).

Internal dose metrics that can be useful for estimating the SO-related genotoxic risk in rats and humans are C_{maxSO}, AUC_{SO}, and ACB_{SO}, the lifetime average concentration of SO in venous blood (in analogy to EPA, 2006).

C_{maxSO}: Daily oral administrations of a single dose of ST of 400 mg/kg BW to rats result in a calculated C_{maxSO} of 0.308 µg SO/ml (**Table 3**). A value of C_{maxSO} of 1.16 µg SO/ml (**Table 3**) was obtained by the PT model for the SO exposure-study of Gaté et al. (2012) that was negative for micronucleus formation and negative in the comet assay with or without FPG. Oral exposure of humans to 100 mg ST/kg BW/d (factor of 100 above the exposure at the OML) divided into 3 equal daily doses would lead to a C_{maxSO} of 0.0315 µg/ml (**Table 3**). This concentration is 36.8 times lower than the C_{maxSO} associated with daily inhalation exposure (6 h) of rats to 75 ppm of SO. In addition, it should be taken into consideration that the C_{maxSO} values after a daily oral intake of 100 mg ST/kg BW by a human are already very close to the PT model-calculated C_{maxSO} value that cannot be exceeded in humans by any ST exposure level. Daily oral ST intake of 1 mg/kg BW in 3 divided doses of 0.33 mg/kg BW each was calculated to lead in humans to a value of C_{maxSO} of 0.000494 mg/ml (**Table 3**) which is 2350-fold smaller than that resulting from exposure of rats to 75 ppm SO for 6 h.

AUC_{SO}: When rats or humans at rest receive ST orally instead of inhaling it, the daily species-specific AUC_{SO} is the same for both exposure scenarios based on the amount of ST absorbed (see *3.3.2*). When using the AUC_{SO} as dose metric, it follows that any genotoxic risk of SO in the target tissue blood should be the same, independently

whether the exposure to ST is by inhalation at rest or by the oral route, provided that the same amount of ST is taken in. The highest daily AUC_{SO} of 6.98 µg·h/ml (**Table 3**) was calculated for the repeated exposures of rats to 75 ppm of SO (Gaté et al., 2012). The daily AUC_{SO} calculated for the one-year exposures to 1000 ppm of ST (Sinha et al., 1983) was 65% of this value. In the four-week exposures to 1000 ppm of ST (Gaté et al., 2012) and the two-week exposures to 500 ppm of ST (Kligerman et al., 1993), the daily AUC_{SO} values were 63% and 39% of that obtained for the exposures to 75 ppm of SO. The AUC_{SO} values of the four studies (**Table 3**) support each other with respect to the negative genotoxicity of SO. Assuming that the same daily AUC_{SO} is linked to the same genotoxic risk of SO in rats and in humans, no genotoxic effects are to be expected in humans up to a daily AUC_{SO} of 6.98 µg·h/ml. This value is 15.3 and 1530 times larger than the daily AUC_{SO} values of 0.457 and 0.00457 µg·h/ml calculated for a human by means of the linear regression (**Figure 12**) for a daily oral ST intake of 100 and 1 mg/kg BW, respectively.

ACB_{SO}: The study of Sinha et al. (1983) with ST concentrations of 600 and 1000 ppm had the longest exposure period (6 h/d, 5 d/w, 12 m) of the three negative genotoxicity studies with ST (Sinha et al., 1983; Kligerman et al., 1993; Gaté et al., 2012). Taking into account a rat life expectancy of 2 years, the ACB_{SO} for the one-year exposure of rats to 1000 ppm of ST (Sinha et al., 1983) is calculated according to Reitz et al. (1988) from the daily AUC_{SO} (= 4.52 µg SO·h/ml; **Table 3**) to $ACB_{SO} = 4.52/24 \cdot 5/7 \cdot 52/104 \approx 0.067$ µg SO/ml. The ACB_{SO} values in a human (70 kg BW) who is orally exposed for the whole lifetime to daily amounts of ST of 100 or 1 mg ST/kg BW are 0.019 and 0.00019 µg SO/ml, respectively (ACB_{SO} = daily $AUC_{SO}/24$, with daily AUC_{SO} values of 0.457 µg SO·h/ml (daily 100 mg ST/kg BW) and 0.00457 µg·h/ml (daily 1 mg ST/kg BW) calculated by means of the linear function given in **Figure 12B**). These ACB_{SO} values are 3.53- and 353-fold smaller, respectively, than that resulting from an ST exposure concentration of 1000 ppm in the one-year rat study of Sinha et al. (1983).

The safety factors of the three dose surrogates for genotoxic effects of SO for human exposures to ST relative to those in rat studies are summarized in Table 4.

In conclusion, independently of the dose metric for SO chosen (C_{maxSO}, AUC_{SO}, or ACB_{SO}), the dose level that can be reached after oral intake of daily amounts of ST of 100 mg/kg

BW or less is below that reached in negative genotoxicity studies with ST or SO. Considering that daily amounts of ST taken in according to the OML are 100 fold lower, a genotoxic risk of SO to humans from oral exposure to ST can be excluded.

4 Appendix

Comments to the results of the comet assay in the presence of FPG (Gaté et al., 2012)

The study of Gaté et al. (2012) clearly shows that high systemic concentrations of ST and SO do not induce clastogenic effects or DNA damage in the "standard" comet assay. On the other hand, the positive results obtained with ST in the FPG modification of the comet assay might indicate potential oxidative damage after a short-term exposure to ST. Therefore, in addition to the above formulated criticism on this particular study (see 3.1.2), a more detailed interpretation of the findings obtained by the FPG modification is necessary taking into account methodological aspects of this relatively new method as well as details of the study results.

Although the FPG modification is now becoming more popular, the FPG modification is not mentioned in the validated OECD guideline for the alkaline comet assay (OECD, 2014). Thus, the "standard" comet assay may be considered as a well-established routine method but not its FPG modification. The FPG modification of the comet assay specifically enables detection of oxidative DNA damage (Collins et al., 1996; Speit et al., 2004), by incubation of cellular DNA with the FPG enzyme. But apart from these oxidation products also DNA modifications, like alkylations, showing up in the "standard" comet assay are detected as well (e.g. Speit et al., 2004; Smith et al., 2006).

The European Comet Assay Validation Group (ECVAG) undertook a major effort to standardize the FPG modification of the comet assay. These investigations showed that a substantial number of participating laboratories, although rating themselves as having high or medium experience with the comet assay, had problems in performing this assay (Forchhammer et al., 2012; Ersson et al., 2013; Godschalk et al. 2014). This corresponded to results of an earlier inter-laboratory trial of the European Standards Committee on Oxidative DNA Damage (ESCODD, 2003). In a later trial a better performance was reported for most of the participating laboratories (Johansson et al., 2010) but this could be explained by differences in cells provided as reference materials.

A comparison of two different ESCODD studies (ESCODD, 2003; 2005) showed large (10 times) differences in the levels of FPG sensitive sites between the laboratories (Møller, 2006). It should be taken into consideration that none of these inter-laboratory comparisons used typical chemical mutagens apart from RO19-8022, a substance generating specific oxidative DNA damage after light-exposure. To our knowledge, other chemicals suspected as mutagens have not yet been included in such trials.

As the FPG modification is still under validation and an OECD guideline does not exist, in contrast to the "standard" comet assay, data by the FPG assay should be analysed with caution. It can be concluded that even laboratories with good experience with the "standard" assay may obtain unsatisfactory results by the FPG modification.

Taking into account general methodological problems associated with the FPG assay and the incompatibilities of the results obtained by Gaté et al. (2012) when using FPG with the blood burdens of ST and SO (see 3.1.2), this study must be interpreted with caution and not too much weight can be given to the positive findings in the FPG assay with ST.

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Figure Captions

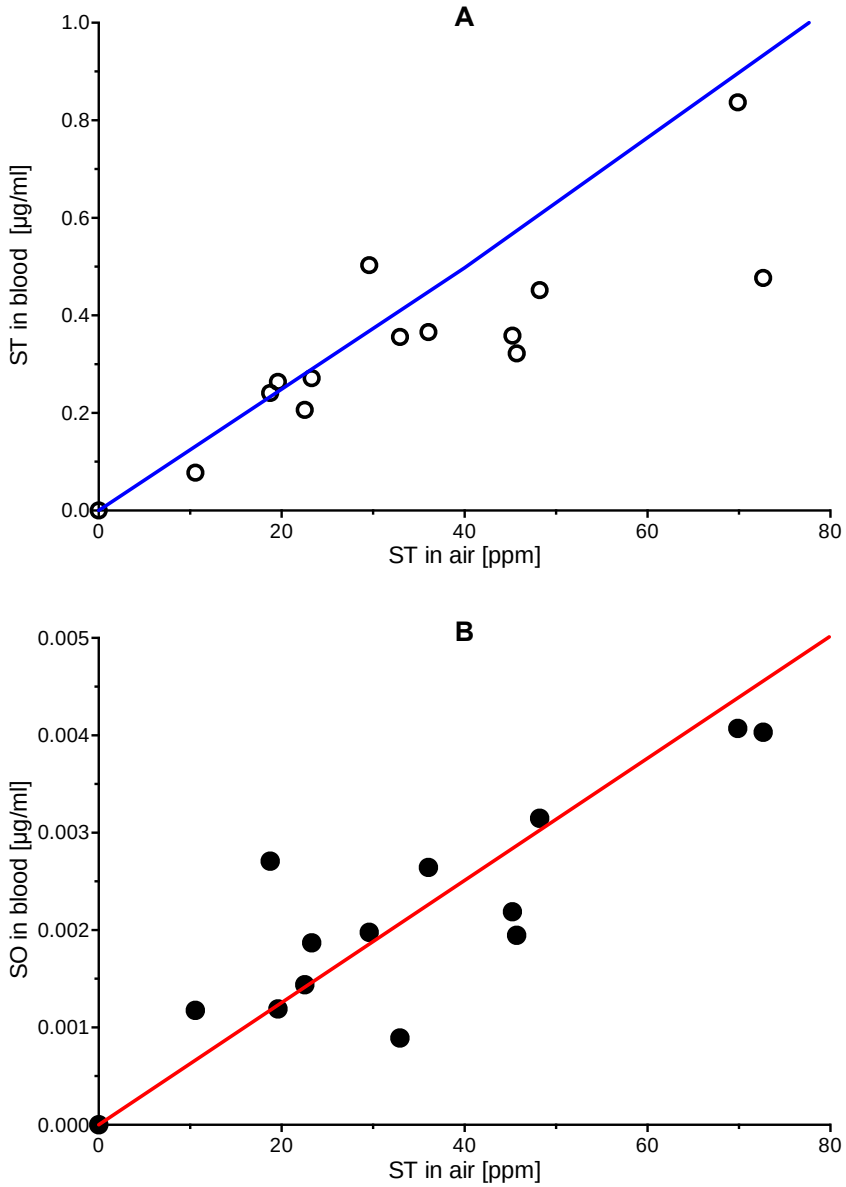

Figure 1. Styrene (ST, **A**) and styrene-7,8-oxide (SO, **B**) in venous blood of 13 male workers exposed to vaporous ST (10.6–72.6 ppm) for time frames of between 248 and 325 min. Meas0ured individual data points (taken from Korn et al., 1994) are symbolised by ❍ for ST and by ● for SO. The curves were predicted by the PT model for a human of 70 kg BW with an alveolar ventilation of 410 L/h. Exposure to constant concentrations of ST was modelled to last 287 min. The data points at the origin were from unexposed persons.

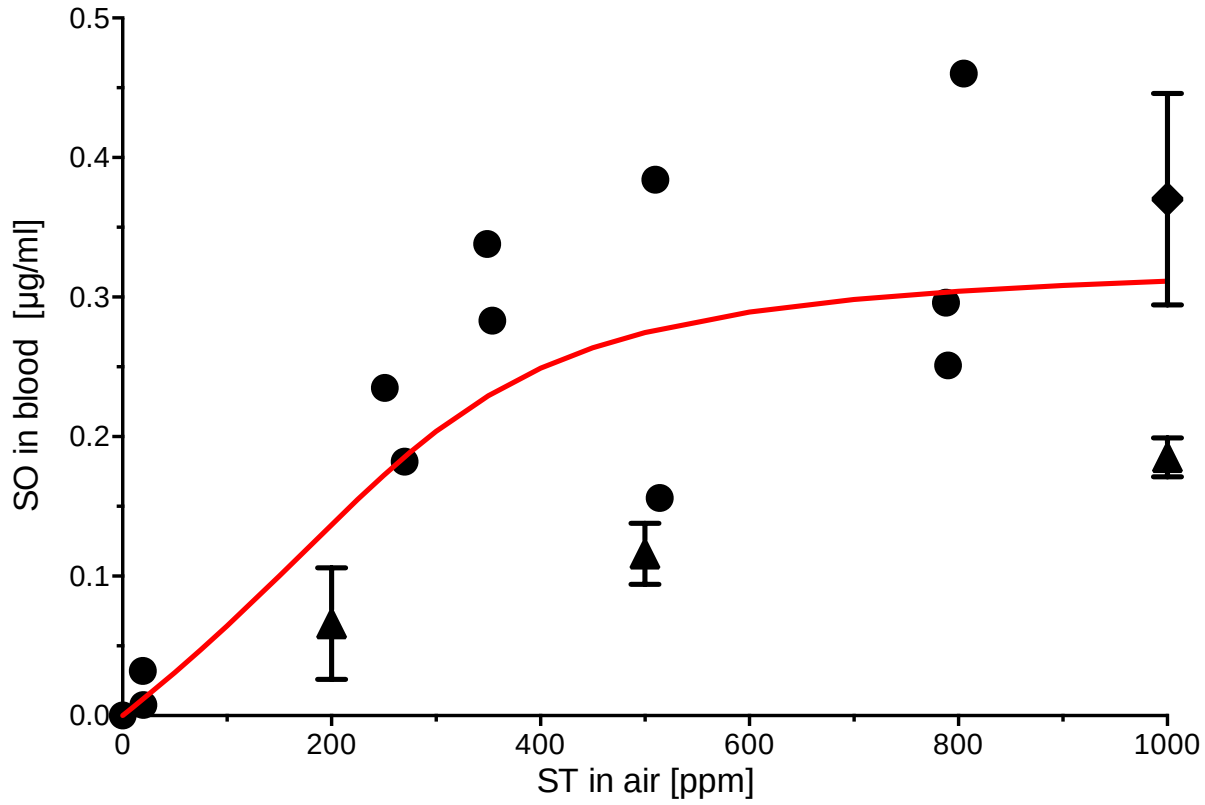

Figure 2. Concentrations of styrene-7,8-oxide (SO) in venous blood of male rats exposed for 6 h to atmospheric concentrations of styrene (ST) ranging from 0 to 1000 ppm. Measured data had been published (Kessler et al., 1992 and Filser et al., 1992, Sprague-Dawley rats ●; Cruzan et al., 1998, Sprague-Dawley rats ▲; Gaté et al., 2012, F344 rats ◆). Bars represent reported SDs. The curve was predicted by the PT model for a rat with a BW of 225 g, the average of the BWs of the rats used by Kessler et al. (1992) and Filser et al. (1992).

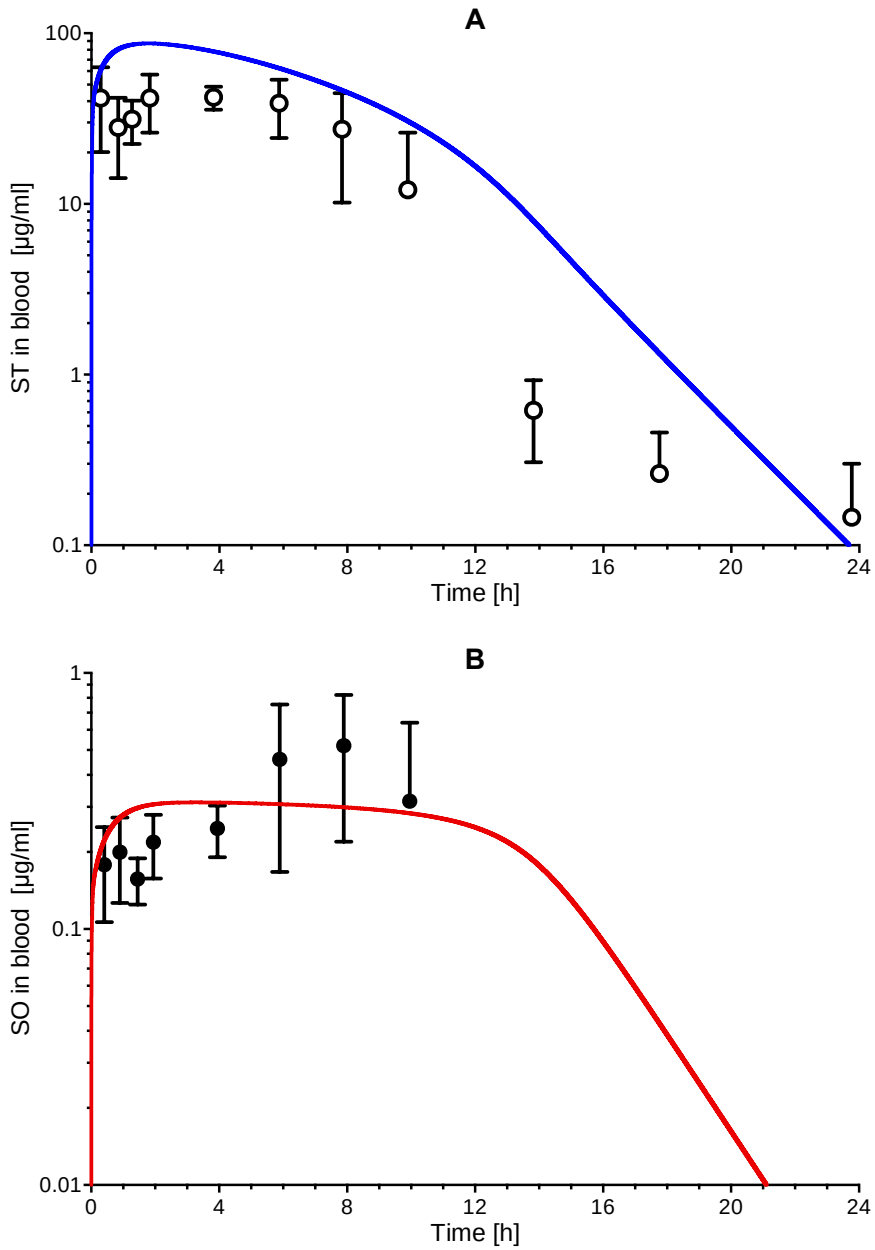

Figure 3. Concentration-time courses of styrene (ST, **A**) and styrene-7,8-oxide (SO, **B**) in blood of male F344 rats after oral administration of 500 mg ST/kg BW. Measured data (ST: ❍; SO: ●) ± SDs from Mendrala et al. (1993). Minus SDs that were smaller than the smallest value on the y-axis are not shown. The curves were predicted by the PT model for a rat with a BW of 174 g, the average of the BWs of the rats used by Mendrala et al. (1993).

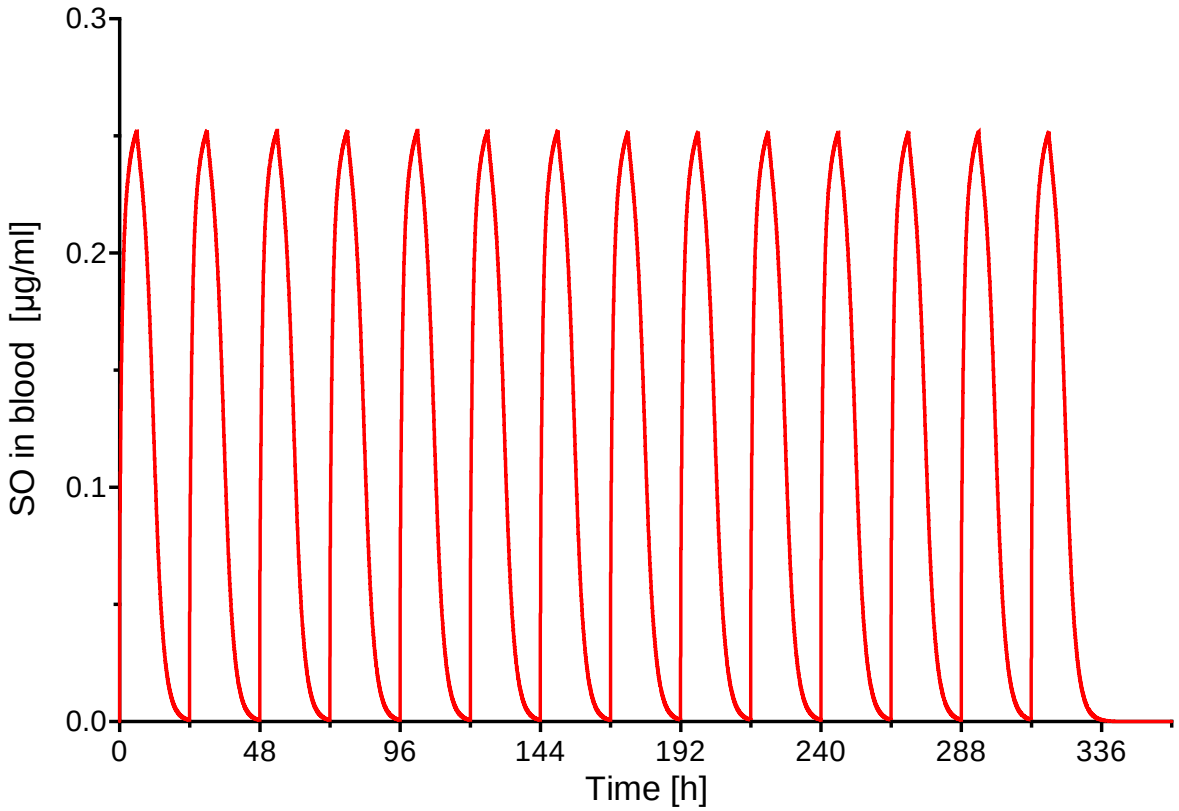

Figure 4. PT-model-calculated concentration-time curve of styrene-7,8-oxide (SO) in venous blood of a female F344 rat exposed by inhalation to vaporous styrene (ST) at a concentration of 500 ppm for 6 h/d for 14 consecutive days (according to the experimental design of Kligerman et al., 1993). At the start of the study, the rat was 8 weeks old. BW was set to 130 g and 141 g for the first and the second week of exposure, respectively.

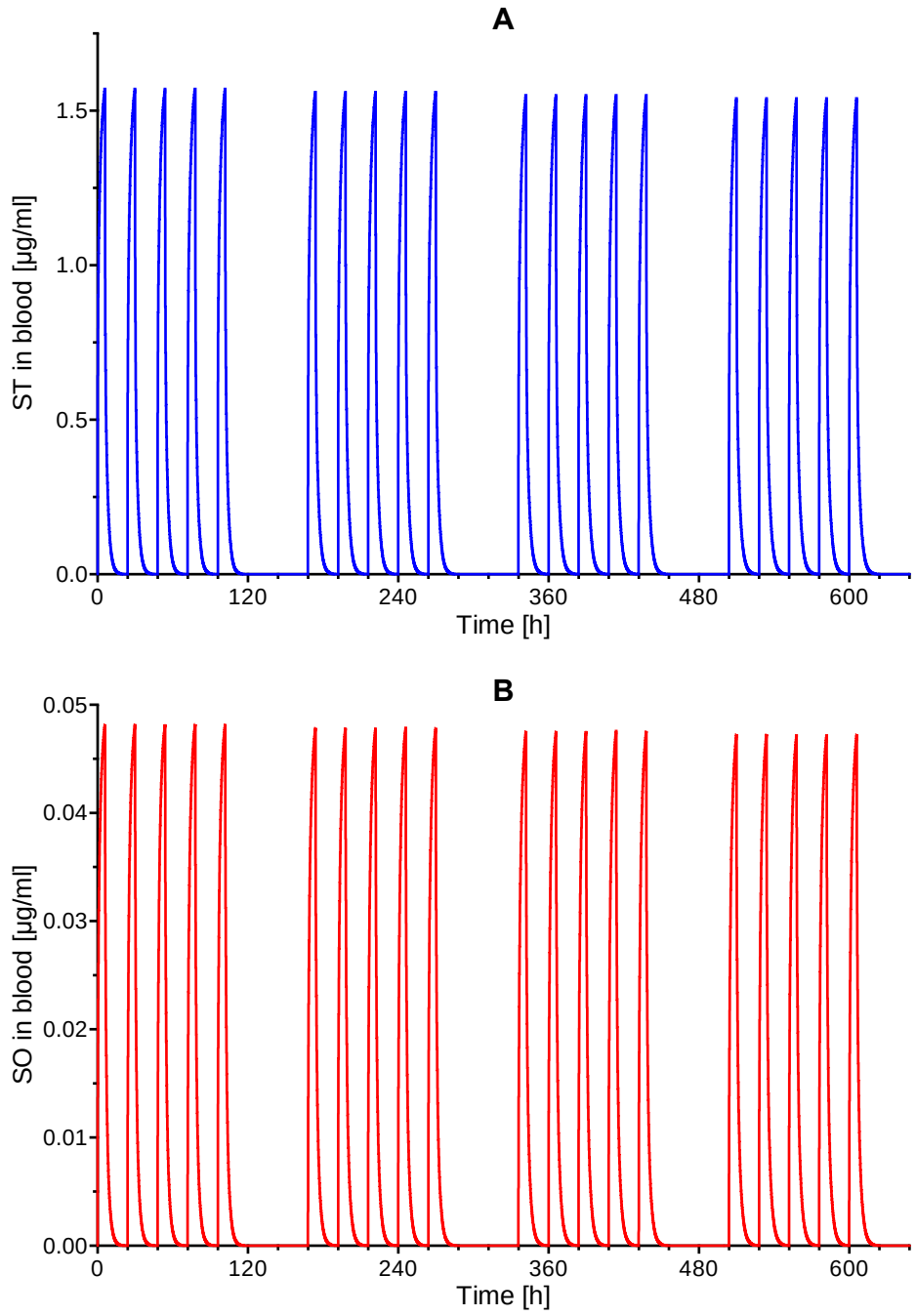

Figure 5. PT-model-calculated concentration-time curves of styrene (ST, **A**) and styrene-7,8-oxide (SO, **B**) in venous blood of a male F344 rat exposed repeatedly (6 h/d, 5 d/w, 4 w) by inhalation to vaporous ST at a concentration of 75 ppm (according to the experimental design of Gaté et al., 2012). At the start of the study, the rat was 6 weeks old. BW was set to 130 g, 157 g, 184 g, and 211 g for the first, second, third, and fourth week of exposure, respectively.

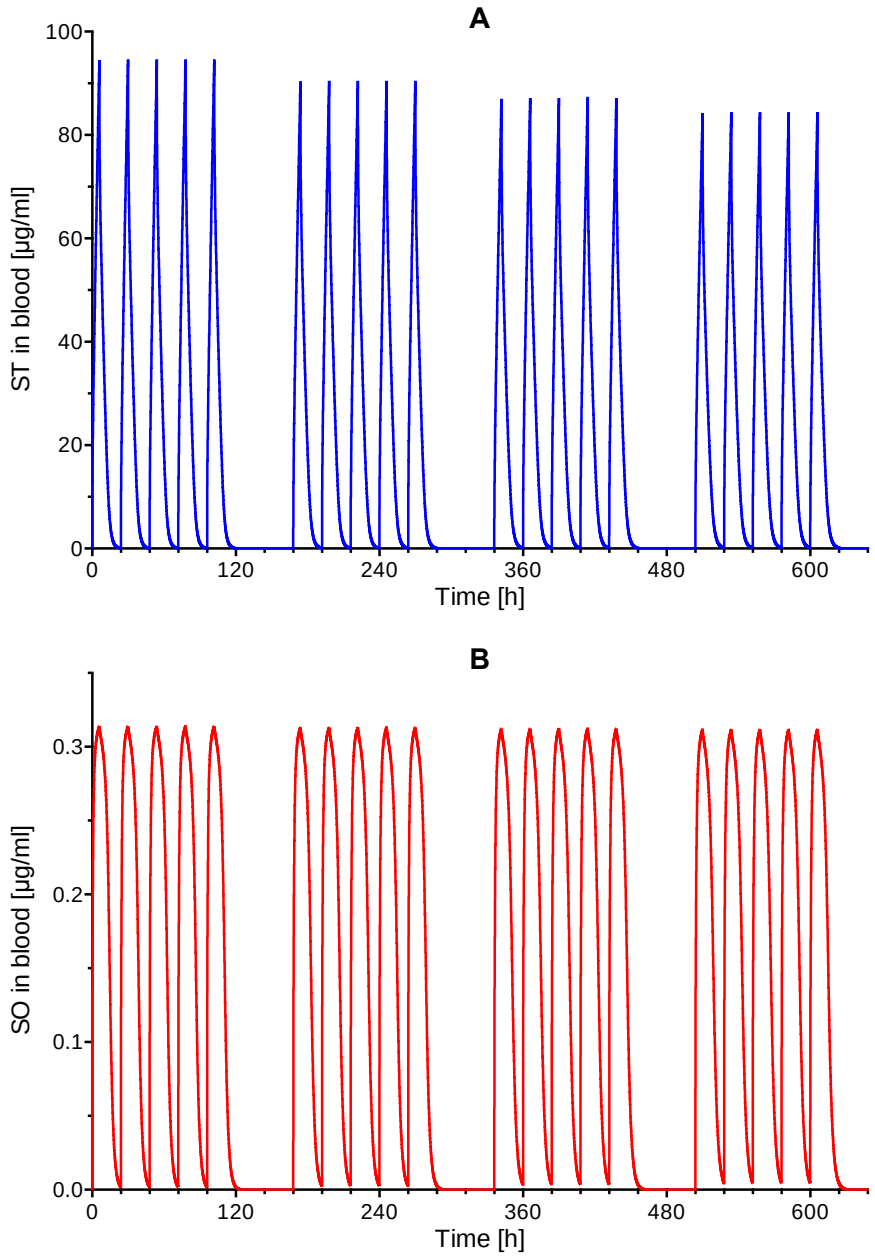

Figure 6. PT-model-calculated concentration-time curves of styrene (ST, **A**) and styrene-7,8-oxide (SO, **B**) in venous blood of a male F344 rat exposed repeatedly (6 h/d, 5 d/w, 4 w) by inhalation to vaporous ST at a concentration of 1000 ppm (according to the experimental design of Gaté et al., 2012). At the start of the study, the rat was 6 weeks old. BW was set to 130 g, 157 g, 184 g, and 211 g for the first, second, third, and fourth week of exposure, respectively.

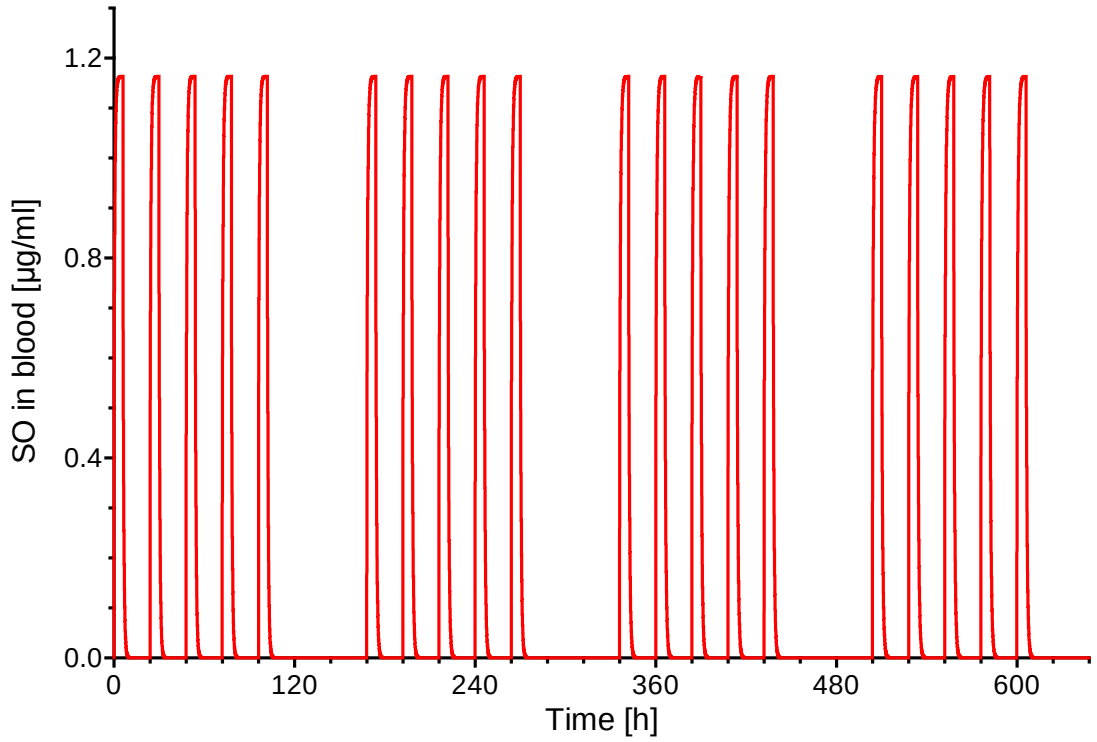

Figure 7. PT-model-calculated concentration-time curve of styrene-7,8-oxide (SO) in venous blood of a male F344 rat exposed repeatedly (6 h/d, 5 d/w, 4 w) by inhalation to vaporous SO at a concentration of 75 ppm (according to the experimental design of Gaté et al., 2012). The rat was 6 weeks old at the start of the study. BW was set to 130 g, 157 g, 184 g, and 211 g for the first, second, third, and fourth week of exposure, respectively.

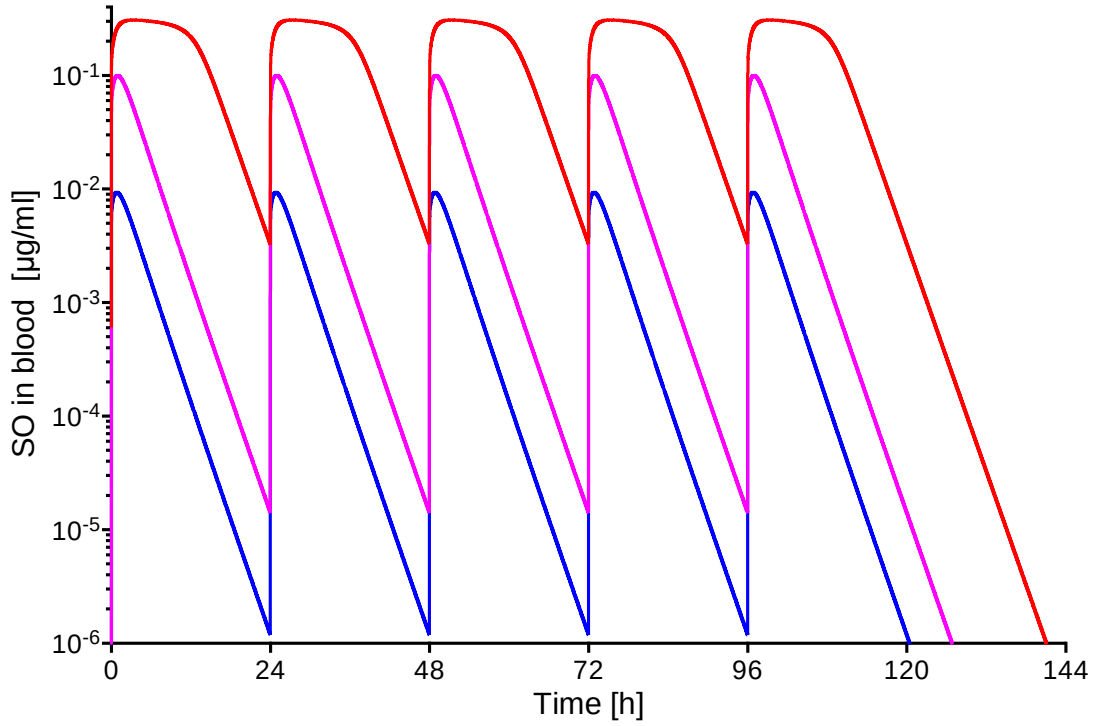

Figure 8. PT-model-calculated concentration-time curves of styrene-7,8-oxide (SO) in venous blood of a rat (BW: 250 g) resulting from daily (5 d) repeated oral administrations of doses of styrene (ST) of 400 (red), 40 (purple), or 4 (blue) mg/kg BW.

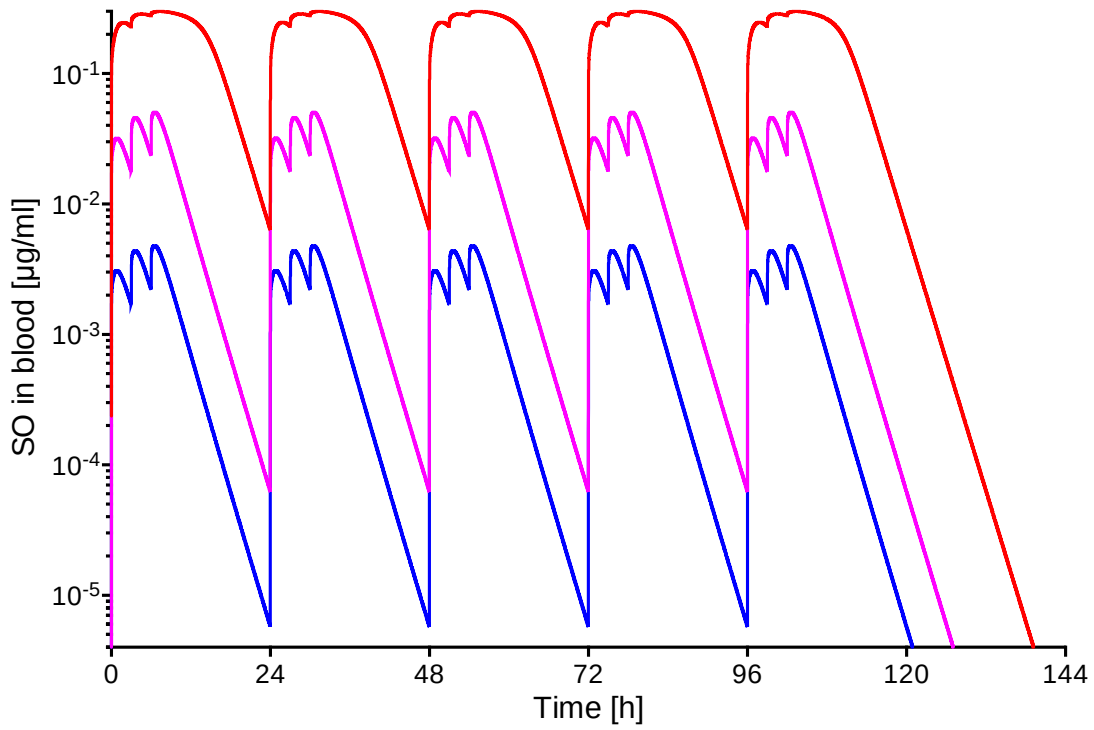

Figure 9. PT-model-calculated concentration-time curves of styrene-7,8-oxide (SO) in venous blood of a rat (BW: 250 g) resulting from three times (time intervals of 3 h) daily (5 d) repeated oral administrations of doses of styrene (ST) of 400/3 (red), 40/3 (purple), or 4/3 (blue) mg/kg BW.

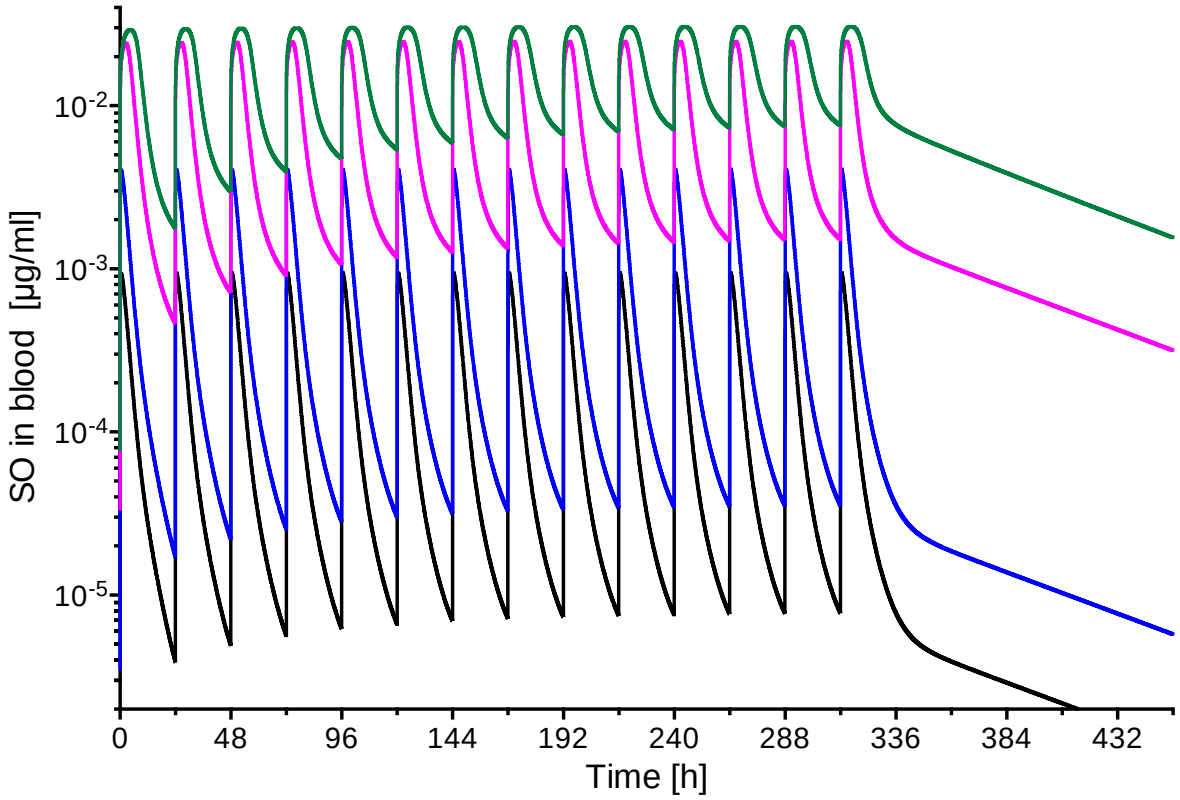

Figure 10. PT-model-calculated concentration-time curves of styrene-7,8-oxide (SO) in venous blood of a human at rest (BW: 70 kg) resulting from daily (14 d) oral intake of doses of styrene (ST) of 100 (green), 40 (purple), 4 (blue), or 1 (black) mg/kg BW.

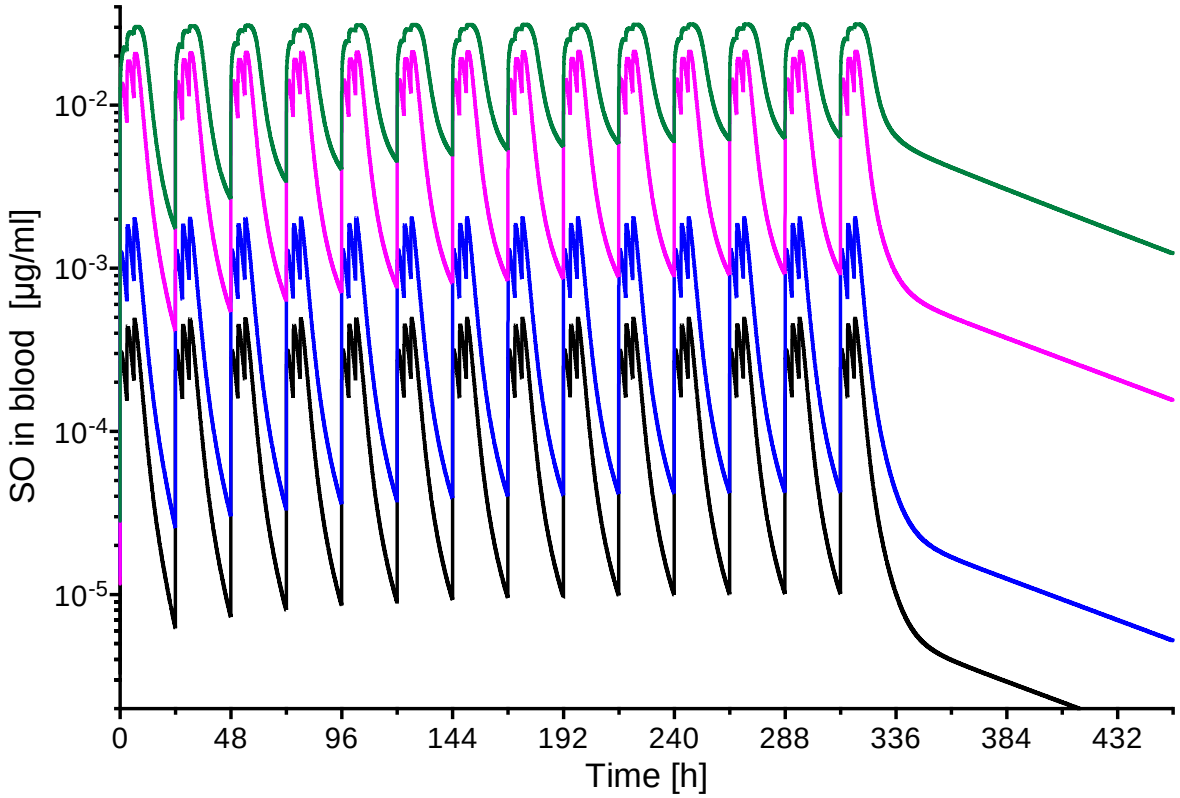

Figure 11. PT-model-calculated concentration-time curves of styrene-7,8-oxide (SO) in venous blood of a human at rest (BW: 70 kg) resulting from three times (time intervals of 3 h) daily (14 d) repeated oral intake of doses of styrene (ST) of 100/3 (green), 40/3 (purple), 4/3 (blue), or 1/3 (black) mg/kg BW.

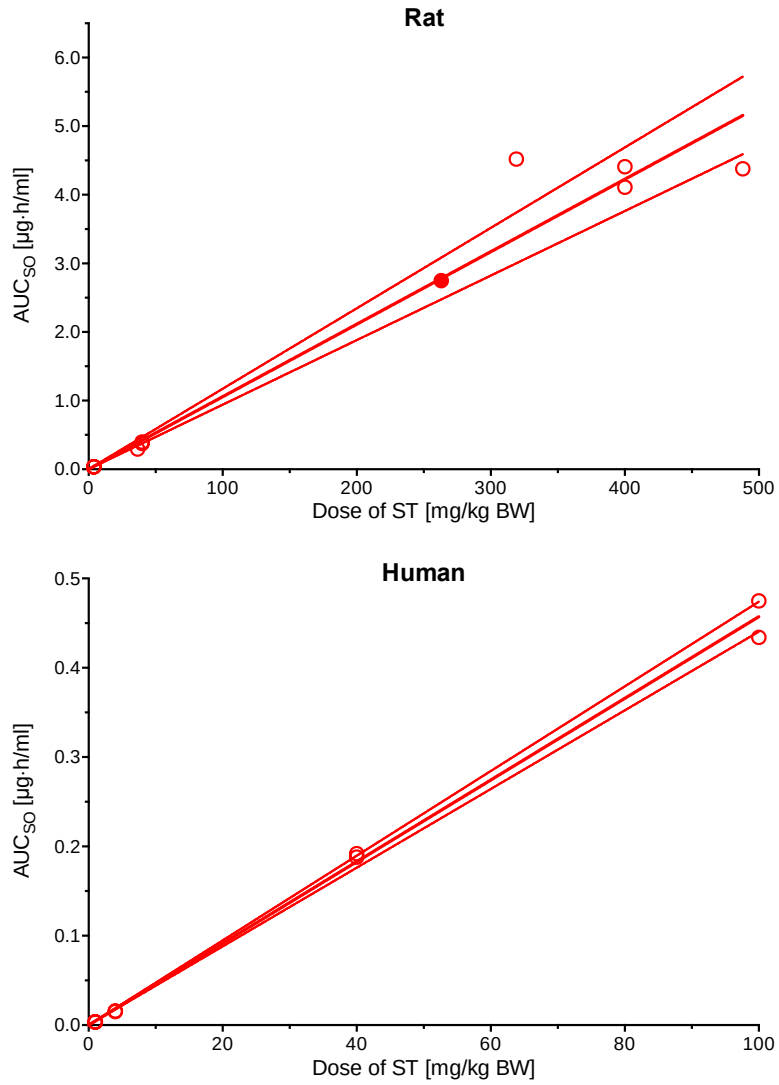

Figure 12. Daily areas under the PT model-calculated concentration-time curves of styrene-7,8-oxide (SO) in venous blood (AUC_{SO}) of male and female rats and of humans versus the doses of styrene (ST) taken in. Data points (10 for male rats, 1 (full symbol) for female rats, and 9 for humans; some not visible because of overlapping) were taken from **Table 3**. Curves are linear regression lines through the origin (solid) ± 95% confidence bands of the fitted lines (dashed). The slopes m of the linear functions (AUC_{SO} = m · dose of ST) and their standard errors are m = 0.01057 ± 0.000520 [(µg SO·h/ml)/(mg ST/kg BW)] in rats and m = 0.00457 ± 0.0000728 [(µg SO·h/ml)/(mg ST/kg BW)] in humans. The coefficients of determination (r^2) are 0.9533 (rats) and 0.9966 (humans).

Tables

Table 1. Biochemical parameters used in the PT model for a male rat and a male human with reference body weights of 0.25 kg and 70 kg, respectively

Hepatic enzyme	Parameter	Rat	Human	Unit
Cytochrome P450 monooxygenase	V_{maxmo}	0.0056	0.002	mmol/h per g liver
	K_{mmo}	0.03*	0.01	mmol/L
Epoxide hydrolase	V_{maxeh}	0.011	0.0045	mmol/h per g liver
	K_{mih}	0.021*	0.001	mmol/L
	K_{mapp}	0.1*	0.01	mmol/L
Glutathione-S-transferase	V_{maxGST}	0.37	0.028	mmol/h per g liver
	K_{mGSH}	0.1	0.1	mmol/L
	K_{mSO}	2.5	2.5	mmol/L
	C_{GSH0}	5.5	5.9	mmol/L
	k_{dGSH}	0.2	0.2	h^{-1}
Oral intake	k_{po}^{ST}	0.50	0.65*	h^{-1}

All values are from Csanády et al. (1994) except those marked by * which are from Csanády et al. (2003). Abbreviations: ST, styrene; SO, styrene-7,8-oxide; GSH, glutathione; V_{maxmo}, maximum rate of metabolic elimination of ST catalysed by cytochrome P450-dependent monooxygenase; V_{maxeh}, maximum rate of SO hydroxylation catalysed by microsomal epoxide hydrolase; V_{maxGST}, maximum rate of SO conjugation with GSH catalysed by cytosolic GSH S-transferase (GST); K_{mmo}, apparent Michaelis constant of ST oxidation related to the ST concentration in venous blood leaving the liver; K_{mih}, intrinsic Michaelis constant of SO hydroxylation related to the SO concentration in the liver compartment; K_{mapp}, apparent Michaelis constant of SO hydroxylation related to the SO concentration in the liver compartment; K_{mGSH}, apparent Michaelis constant of the reaction of GSH with GST, related to the GSH concentration in the liver compartment; K_{mSO}, apparent Michaelis constant of the reaction of SO with GST, related to the SO concentration in the liver compartment; C_{GSH0}, initial concentration of GSH in the liver compartment; k_{dGSH}, first order elimination rate constant of GSH turnover; k_{po}^{ST}, first order absorption rate constant of ST intake from the gastrointestinal tract.

Table 2. Measured (Gaté et al., 2012) and PT model-predicted concentrations of styrene (ST) or styrene-7,8-oxide (SO) in venous blood of 6 weeks old male F344 rats (estimated average BW in the seventh week: 130 g), 6 h after starting a single inhalation exposure to a constant concentration of ST or SO

Exposure	Compound determined	Concentration (μg/g blood)	
		Measured ± SD (n=6)	Predicted
SO (25 ppm)	SO	0.239 ± 0.098	0.388
SO (50 ppm)	SO	0.601 ± 0.193	0.776
SO (75 ppm)	SO	0.859 ± 0.430	1.16
ST (1000 ppm)	ST	85.5 ± 11.3	94.5
ST (1000 ppm)	SO	0.370 ± 0.076	0.313

Table 3. Daily doses of styrene (ST), PT-model-calculated maximum concentrations of styrene-7,8-oxide (SO) in venous blood (C_{maxSO}), and areas under the model-calculated concentration-time curves of SO in venous blood from t = zero to infinity (AUC_{SO}) of resting rats or resting humans resulting from various exposures to ST or SO

Reference (Figure)	Species (kg BW)	Exposure	Daily dose of ST† [mg/kg BW]	C_{maxSO} [µg/ml]	AUC_{SO} per day of exposure [µg · h/ml]
Sinha et al., 1983 (no Figure)	Rat (0.612)[a]	ST, inhalation, 6 h/d, 5 d/w, 12 m; 1000 ppm	319*	0.306	4.52
Kligerman et al., 1993 (Figure 4)	Rat (0.136)[b]	ST, inhalation, 6 h/d, 14 d; 500 ppm	263*	0.252	2.75
Gaté et al., 2012 (Figure 5)	Rat (0.171)[b]	ST, inhalation, 6 h/d, 5 d/w, 4 w; 75 ppm	36.6*	0.0482	0.295
Gaté et al., 2012 (Figure 6)	Rat (0.171)[b]	ST, inhalation, 6 h/d, 5 d/w, 4 w; 1000 ppm	488*	0.313	4.38
Gaté et al., 2012 (Figure 7)	Rat (0.171)[b]	SO, inhalation, 6 h/d, 5 d/w, 4 w; 75 ppm	—	1.16	6.98
Present work (no Figure)	Rat (0.250)	ST, inhalation, 6 h; 9.0 ppm	3.87*	0.00530	0.0334
Present work (Figure 8)	Rat (0.250)	ST, orally, once daily, 5 d	400 40 4	0.308 0.100 0.00935	4.11 0.398 0.0358
Present work (Figure 9)	Rat (0.250)	ST, orally, three times daily, 5 d	3 · 400/3 3 · 40/3 3 · 4/3	0.300 0.0507 0.00480	4.41 0.376 0.0356

Present work (no Figure)	Human (70.0)	ST, inhalation, 8 h/d, 5 d; 7.0 ppm	1.02*	0.000354	0.00333
Present work (Figure 10)	Human (70.0)	ST, orally, once daily, 14 d	100 40 4 1	0.0307 0.0248 0.00408 0.000946	0.434 0.188 0.0159 0.00372
Present work (Figure 11)	Human (70.0)	ST, orally, three times daily, 14 d	3 · 100/3 3 · 40/3 3 · 4/3 3 · 1/3	0.0315 0.0214 0.00204 0.000494	0.475 0.192 0.0151 0.00367

[a]: Estimated average body weight (BW) over the exposure period, calculated for a male Sprague-Dawley rat fed NIH-31 diet ad libitum by using the body weight gain given in Lewis et al. (2003); [b]: BW averaged over the exposure period; †: days free of exposure not considered; *: calculated as the product of the atmospheric concentration of ST with the alveolar ventilation and the time span of exposure, then related to kg BW (example for a 6-h exposure of a 250-g rat to 1000 ppm of ST: 1000 (ppm in air) / 24450 (ml; molar volume of an ideal gas at 25°C at 101.3 kPa) · 104 (µg; micro-molecular weight of ST) · 4210 (ml/h; alveolar ventilation of a 250-g rat at rest; Csanády et al., 1994) · 6 (h; exposure period) · 1000 / 250 (1000/BW of the exposed rat->µg per kg BW) / 1000 (µg->mg) = 429.8 mg/kg BW; the alveolar ventilation for a BW differing from 250 g was scaled allometrically by $BW^{2/3}$, for instance the alveolar ventilation of a 130-g rat is $4210 \cdot (130/250)^{2/3}$ = 2722 ml/h).

Table 4. Safety factors of the dose surrogates for genotoxic effects of styrene-7,8-oxide (SO) in rats and humans: maximum concentration of SO in venous blood (C_{maxSO}), area under the concentration-time curve of SO in venous blood (AUC_{SO}), and lifetime average concentration of SO in venous blood (ACB_{SO})

	Rat		Human	
Exposure	SO inhalation (75 ppm, 6 h/d, 5 d/w, 4 w)*		Oral ST intake (100 mg/kg BW/d)	Oral ST intake (1 mg/kg BW/d)†
Safety factor related to C_{maxSO}	1		36.8	2350
Safety factor related to AUC_{SO} per exposure day	1		15.3	1530
Exposure	ST inhalation (1000 ppm, 6 h/d, 5 d/w, 12 m)**		Oral ST intake (100 mg/kg BW/d)	Oral ST intake (1 mg/kg BW/d)†
Safety factor related to ACB_{SO}	1		3.53	353

*: Study of Gaté et al. (2012), no genotoxic effect, calculated C_{maxSO} = 1.16 µg/ml, calculated AUC_{SO} per exposure day = 6.98 µg · h/ml, study chosen because of the highest values of C_{maxSO} and AUC_{SO} of all of the studies dealt with (see **Table 3**); **: Study of Sinha et al. (1983), no genotoxic effect, calculated ACB_{SO} = 0.067 µg/ml, study chosen because of the highest value of ACB_{SO} of all of the studies dealt with; †: Estimated maximum daily ST intake according to the Overall Migration Limit in the EU, the maximum permitted amount of a substance released from a material or article into food