



June 10, 2014

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Dear Mr. Carpenter:

I am writing on behalf of the Hydrocarbon Solvents Panel of the American Chemistry Council (ACC). The Panel represents the major US producers of hydrocarbon solvents, including trimethylbenzenes¹. The Panel is hereby providing comments in response to the Federal Register notice announcing meetings of the Chemical Assessment Advisory Committee Augmented for the Review of the Draft Trimethylbenzenes Assessment (CAAC-TMB Panel) (79 Fed. Reg. 16324, March 25, 2014). The EPA's draft Integrated Risk Information System (IRIS) Toxicological Review of Trimethylbenzenes dated August 2013 (Draft Assessment) is to be assessed at the CAAC-TMB Panel peer review meeting to be held June 17-19, 2014.

The Panel appreciates the opportunity to comment on EPA's Draft Assessment of Trimethylbenzenes (TMBs). The Panel strives to ensure appropriate product stewardship; and, as part of its mission, the Panel addresses important science, regulatory and public policy issues related to the hydrocarbon solvents industry, such as EPA's Draft IRIS Assessment.

I. Introduction

As stated in the Draft Assessment, trimethylbenzenes are aromatic hydrocarbons with the chemical formula C_9H_{12} , of which three separate isomers exist. Apart from the structural similarity, trimethylbenzene isomers are similar in terms of physical/chemical, toxicity and

¹ Members of the ACC Hydrocarbon Solvents Panel are Chevron Phillips, CITGO, ExxonMobil Chemical Company, and Sasol North America.



metabolic profiles. This similarity has been utilized by the EPA in considering data from one or more isomers as representative of other trimethylbenzene isomers and in read-across justification for applying reference values calculated from a study of one isomer to others. As EPA indicates, trimethylbenzenes are primarily produced as a complex C₉ aromatic fraction (containing other structurally similar aromatic hydrocarbons with nine carbons such as ethyltoluenes and propylbenzenes) by catalytic reforming and are used either as blending agents in gasoline or as solvents. Vehicle emissions are the major anthropogenic source of trimethylbenzene exposure.

These comments cover three major topics, each addressed in an attachment:

- Attachment I: Justification for considering the “pain sensitivity” neurotoxicity endpoint as evidence of acute CNS effects with no persistent effects with continuous exposure
- Attachment II: Justification for the inclusion of complex C₉ aromatic fraction in the draft IRIS assessment for trimethylbenzenes
- Attachment III: Justification for employing the Adenuga et al. (2014) study as the basis for the derivation of a reference dose (RfD)

Within each attachment, the Panel addresses specific EPA charge questions.

II. The Draft IRIS Assessment Contains Major Deficiencies

The EPA Draft Assessment of TMBs contains key deficiencies that range from a lack of a scientifically sound rationale for inclusion/exclusion of studies to the failure to utilize the best available science, as well as a failure to use a ‘weight-of-evidence’ approach that considers all relevant information and its quality in the Draft Assessment. Briefly, the major deficiencies highlighted in our comments include:

- Flawed assessment of “pain sensitivity” as the critical endpoint for derivation of the reference concentration (RfC),
- Exclusion of the TSCA Section 4(a) guideline studies where complex C₉ aromatic fractions were tested, and
- Unwarranted use of additional uncertainty factors.



A. *Flawed assessment of “pain sensitivity” as the critical endpoint for derivation of the reference concentration (RfC)*

The Panel agrees with the EPA that both acute and long-term effects of trimethylbenzenes are important and that any derived reference value should be protective of both effects. The selection of the “pain sensitivity” endpoint in the Korsak and Rydzynski (1996) study is an appropriate selection for the derivation of an RfC, as it is both amenable to benchmark dose (BMD) analysis and also covers the transient and systemic effects of trimethylbenzene exposure. However, the Panel disagrees with the EPA’s assertion that the “pain sensitivity” endpoint is indicative of a persistent effect following subchronic exposure to trimethylbenzenes. As detailed in Attachment I, the EPA wrongly conflates two completely different models of evaluating neurotoxicity (“pain sensitivity” and “conditioned analgesia”) to give the impression of persistency. As is shown in the Korsak and Rydzynski (1996) study, effects on pain sensitivity were only significant with exposure when the animals were tested using the hot plate method immediately after the last exposure. When the animals were tested 2-weeks after exposure, no exposure-related effects were noted with 123- or 124-trimethylbenzene. This is consistent with all other studies on individual trimethylbenzene isomers or complex C9 aromatic fraction. On the other hand, the studies where footshock is applied before or during exposure to the hot plate stimuli should be considered models of “conditioned analgesia” with no relevance to pain sensitivity measured without the application of footshock. Overall, weight-of-evidence considerations of the “pain sensitivity” and other neurotoxicity and neuropathology endpoints support the fact that trimethylbenzenes cause only an acute central nervous system (CNS) response with no evidence for persistence with prolonged exposure.

B. *Exclusion of the TSCA Section 4(a) guideline studies where complex C9 aromatic fractions were tested*

In setting out criteria for the selection and/or exclusion of studies for the toxicological review of trimethylbenzenes, the EPA indicated that studies where complex C9 aromatic fractions were tested and/or were not in the English language would not be included in the review. However, a rationale for this decision was not included in the main document and application of these criteria was inconsistent. For example, Battig et al. (1956), cited as evidence for neurotoxic effects of trimethylbenzene exposure in humans, was not written in the English language. In addition, Hissink et al. (2007), on which the PBPK model employed in the Draft Assessment was based, was originally developed for a complex substance (white spirits).

As detailed in Attachment II, the complex C9 aromatic substances should be integrated into the main review document as they represent a critical set of data that can be used for weight-of-evidence evaluation of critical endpoints of concern while also addressing the EPA’s database insufficiency concerns. Contrary to the EPA’s comments, the composition of these complex C9



aromatic fractions are well characterized and consist of C9 aromatic constituents that are structurally, toxicologically and metabolically similar to trimethylbenzenes. While we agree with the EPA that exposure to individual trimethylbenzenes do occur, the manufacture and use conditions (as spelled out by the EPA) clearly indicate that exposures to trimethylbenzenes primarily occur in the context of a combined exposure to the complex C9 aromatic fraction rather than to individual isomers in isolation.

C. Unwarranted use of additional uncertainty factors

As described in Attachment I (response to Charge Question 4), the overwhelming evidence on the “pain sensitivity” endpoint clearly indicate that this is a transient acute response which does not become progressively more severe with prolonged exposure. Hence, the use of an additional subchronic to chronic uncertainty factor (U_F) of 3 is not justified and should be removed.

In addition, the EPA has included an U_F of 3 to account for database insufficiency (i.e. lack of standard reproductive/developmental toxicity studies). However, this “insufficiency” only occurs because the EPA has chosen to ignore studies where the complex C9 aromatic fractions have been tested. For example, and as explicitly detailed in Attachment II, a well-conducted 3-generation reproductive toxicity assay in mice, two developmental toxicity (mice and rats) assays and one developmental neurotoxicity (rats) assay are available for which the test substance was a complex C9 aromatic substance consisting predominantly of isomeric mixtures of trimethylbenzene and ethyltoluene. These should be incorporated into the main text of the assessment and an U_F for database insufficiency should be excluded.

III. The 90-Day Oral Toxicity Study of 135-Trimethylbenzene is the Most Appropriate Study for the Derivation of a Reference Dose (RfD)

In the Draft Assessment, the EPA considered a valid 90-day oral toxicity study of 135-trimethylbenzene, conducted according to EPA guideline 798.2650, as unsuitable for use in the development of the reference dose². EPA’s rationale for this decision was that the study did not identify any adverse effects because it evaluated “insensitive endpoints” and did not evaluate neurobehavioral and respiratory endpoints. As detailed in Attachment III, these reasons are not justified.

- First, the study was mandated by the EPA to be used in the development of Health Advisories (HAs) for drinking water contamination under the Safe Drinking Water Act (SDWA), and is therefore the most appropriate for the development of an oral RfD.

² The 90-day oral toxicity study was cited by EPA in the draft assessment as Koch Industries 1995b, but is now published as Adenuga et al. 2014 (see Attachment III).



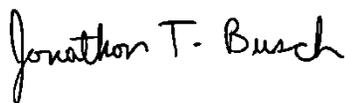
- Second, the argument that an endpoint is only “sensitive” when an adverse response is observed is flawed, as the goal of a toxicity test is to identify a threshold for a safe response.
- Third, the overall weight-of-evidence does not support the validity of the neurological and respiratory endpoints in this oral study. For example, the respiratory effects observed in the inhalation studies are “portal of entry” effects (irritation of the respiratory tract) that would be unreproducible in an oral study. Furthermore, acute neurological effects with oral exposure to trimethylbenzenes and other structurally similar aromatic hydrocarbons occur at exposure doses several fold higher than the highest dose in this study. In other words, the NOAEL in the oral toxicity study of 135-trimethylbenzene is conservative and protective of any potential neurological effects that may be of concern.

IV. Conclusion

EPA should substantially revise the Draft IRIS Assessment of TMBs to accurately incorporate the best available science. As set forth in these comments, the Draft Assessment does not accurately represent the health effects associated with exposure to TMB. The Draft Assessment should utilize a consistent and transparent data evaluation procedure for evaluating and weighing the full body of evidence in compliance with the Information Quality (IQ) Guidelines. The comments presented herein offer specific improvements that should be made to the Draft Assessment.

If you have any questions relating to these comments, please contact me.

Sincerely,



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Comments of the ACC Hydrocarbon Solvents Panel
Attachment I

Justification for considering the “pain sensitivity” neurotoxicity endpoint as evidence of acute CNS effects with no persistent effects with continuous exposure

The comments of the ACC Hydrocarbon Solvents Panel are organized according to EPA’s Charge Questions to the Science Advisory Board (SAB) for the draft IRIS Toxicological Review of Trimethylbenzenes (Draft Assessment). These comments focus on accuracy, objectivity and transparency of EPA’s analyses of primarily the neurotoxicity endpoints because they are considered to be the critical effect for risk assessment.

GENERAL CHARGE QUESTIONS

1. NRC (2011) state that “all critical studies need to be thoroughly evaluated with standardized approaches that are clearly formulated” and that “strengthened, more integrative, and more transparent discussions of weight of evidence are needed.” NRC also indicated that the changes suggested would involve a multiyear process. Please comment on EPA’s success thus far in implementing these recommendations.

ACC Comments:

EPA’s summary tables in the text and in Appendix B are significant improvements over the manner in which data was tabulated and presented 5 to 10 years ago. The presentation of information makes it easier to review EPA’s assessment of the studies. As will be discussed in greater detail in response to Charge Question C1, there were inaccuracies and serious omissions of key results in the summary tables in Appendix B, which translated into incorrect evaluation of the weight of evidence. In addition, the summaries do not capture important methodological limitations that affect study quality and interpretation of consistency of results. From this perspective, the guidance for evaluating the weight of evidence in the preamble has not been fully utilized. This discussion focuses on the section on neurotoxicity because the point of departure is based on pain sensitivity.

4. EPA solicited public comments on the draft IRIS assessment of trimethylbenzenes and has revised the assessment to respond to the scientific issues raised in the comments. A summary of the public comments and EPA’s responses are provided in Appendix F of the Supplemental Information to the Toxicological Review of Trimethylbenzenes. Has EPA adequately addressed the scientific issues?

ACC Comments:

EPA has not adequately addressed earlier comments from ACC that the C9 toxicity studies should be included in the trimethylbenzene (TMB) review. Although EPA argues they have a risk management need to regulate TMBs independent of other aromatic hydrocarbons (F-2), it does not follow that all mixed C9 isomer (i.e., approximately 55% TMB, 28% ethyl toluene isomers) toxicity studies should be dismissed as irrelevant to the scientific weight of evidence for TMB. In fact, EPA included discussion of findings from animal and human neurotoxicity studies on other substances, including toluene, xylene and white spirit, which are less closely related to the TMB isomers than the commercial mixed C9 isomeric substances (pp 1-1, 1-2, 1-23; 2-7). Compared to white spirit (which contains 15-25% aromatic

constituents), the C9 aromatic naphtha used in the neurotoxicity study is more relevant to TMB risk assessment because it contains ~ 87% aromatic hydrocarbons with nine carbon atoms, primarily ethyl toluene (ET; 28%) and TMB (53%) (Douglas et al. 1993). The reason this is an important point is that the C9 aromatic naphtha neurotoxicity study, in particular, contributes data on latency to paw lick in the hot plate test, which is directly relevant to evaluating the reversibility of the critical endpoint that was selected as the basis for the point of departure.

EPA's rationale for excluding the mixed C9 isomer studies stems from the EPA's observation that "multiple peer-reviewed studies have been published that demonstrate that individual TMB isomers do elicit clearly adverse toxicological effects", whereas the C9 fraction studies generally "failed to observe clear measures of toxicity in the systems investigated" (F-3). In other words, EPA made an *a priori* assumption that the findings from primarily one laboratory are incontrovertible, and excludes the mixed C9 isomers studies because of largely negative results (F-3) in the neurotoxicity studies evaluating the potential for persistent effects. This is not an objective approach to evaluating the weight of evidence; nor is this correct. For the neurotoxicity studies, it is more likely that the differences in findings are related to different behavioral tests, duration of exposures, time of test relative to last exposure, whether time of testing was balanced across dose groups, and the extent to which experimental bias and environmental factors affecting behavioral testing were controlled. Additional rationale supporting the relevance of mixed C9 isomer studies to the TMB risk assessment will be discussed below in comments to Charge Question B1 below.

Chemical-Specific Charge Questions

B. Literature Search Strategy/Study Selection

1. Please comment on the whether the literature search approach, screening, evaluation, and selection of studies for inclusion in the assessment are clearly described and supported. Please identify any additional peer-reviewed studies from the primary literature that should be considered in the assessment of noncancer and cancer health effects of 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB.

ACC Comments:

A detailed justification for the inclusion of the studies on complex C9 aromatic substances is provided in Attachment II.

The EPA clearly describes the literature search approach, screening, and selection of studies for inclusion in the assessment, **but does not provide any rationale for excluding studies on complex C9 solvent mixtures, such as C9 aromatic fraction containing TMBs in the main body of the report**. A rationale can be found buried in Appendix F "Resolution of Public comments" of the Supplemental Material, but this is not transparent unless included in the Executive Summary and the Literature Search Strategy Section. EPA's decision to exclude C9 studies lacks consistency and objectivity because EPA includes papers that do not meet EPA's criteria for relevant test chemicals, such as Hissink et al. (2007); Battig et al. (1956, 1958); Chen et al. (1999); Lammers et al. (2007) (rats and humans exposed to white spirit); Lee et al. (2005); Norseth et al. (1991); and Sulkowski et al. (2002).

As discussed in our response to General Charge Question 4, a risk management need to regulate TMB alone does not imply that relevant scientific literature on similar group of chemicals or solvent mixtures should be ignored, particularly when these groups of chemicals are present together in the environment. The scientific basis for EPA's decisions will be strengthened if the mixed C9 isomer studies currently

summarized in Appendix E of the supplementary material are evaluated similarly to those included in Appendix B and integrated into the appropriate sections of the main report. Just as EPA indicates that the rats in the Lammers et al. (2007) study were exposed to 1,2,4-TMB as a constituent of white spirit (Table B-34; p. B-123), EPA can indicate that the rats in the Douglas et al. (1993); McKee et al. 1990 and Schreiner et al. (1989) studies were exposed to 1,2,4-TMB as a constituent of C9 Aromatic Naphtha. This will improve the objectivity and transparency of EPA's selection of studies for inclusion in the assessment and include all the relevant scientific evidence.

EPA suggests that the negative results of the C9 mixture toxicity studies could be due to interactive effects between the constituents of the C9 mixture and that biological systems could alter the ADME of TMB (F-13 line 4). This hypothesis has not been tested directly, but acute toxicity and PK data suggest that this is not likely to be the case. In a 3-day acute exposure study by McKee et al. (2010), a complex C9 aromatic solvent produced similar, but perhaps more profound effects than did 1,2,4-TMB in tests of operant visual discrimination, functional observation battery and motor activity. This study clearly documented the timing of behavioral observations (functional observational battery and motor activity) relative to end of exposure and counterbalanced the dose groups across time of testing and testing device for the operant 2-choice visual discrimination performance test. Based on a pilot PK study in humans, Järnberg et al. (1998) concluded that exposure to 1,2,4-TMB in white spirit appear to increase the concentration of 1,2,4-TMB in blood and its metabolites in urine compared to exposure to 1,2,4-TMB alone. Since it is known that aromatic constituents induce their own metabolism, this apparent difference was likely due to increased metabolism of 1,2,4-TMB with co-exposure to other C9 aromatics in white spirits, including other TMB isomers. Apart from this, the disposition of 1,2,4-TMB did not differ whether exposure was to 1,2,4-TMB alone or in white spirit (Tables 2 and 3 in Järnberg et al.,1998). Thus, from an acute toxicity and pharmacokinetic perspective, these data support EPA's earlier assumption that "assessing the toxicity of the C9 mixture as a complete entity should provide a reasonable upper bound" for the toxicity of TMB [isomers] in the C9 mixture (EPA, 1985).

In addition, EPA's PBPK modeling validation and optimization suggest that there may be only modest impacts of other constituents of white spirit, a hydrocarbon solvent (approximately 80% aliphatic and 20% aromatic) containing more constituents other than TMB compared to complex C9 aromatic solvents. The PBPK model EPA uses for the Draft Assessment is based on a model developed by Hissink et al. (2007) following single day of exposure of rats and humans to white spirit containing only 7.8% TMB (after spiking) and having an overall aromatic content of 25.6%. Relatively modest changes in $V_{max}C$ and K_m values were needed to optimize fit of this PBPK model to produce acceptable simulation of venous blood 1,2,4-TMB for repeated exposures to 1,2,4 TMB by inhalation. In fact, EPA reported that a $V_{max}C$ value of 3.39, which is 3% different from Hissink's original value, improved the model fit for humans exposed to TMB only, and was not significantly different from EPA's selected value of 4.17.

Table 1. Chemical specific parameters used for each step to optimize the white spirit PBPK model for exposures to 1,2,4-TMB only.

	Original Hissink et al. 2007 parameters (8 hr exposure to white spirit)	Step 1. Optimization to Hissink et al. 2007 (8 hr exposure to white spirit)	Step 2. Optimization to Swiercz et al. 2003 (6 hr/day, 5 days/4 weeks to TMB only)	Step 3. Optimization to human data exposed to TMB only
EPA reference	Table B-5, B-6; pp. B-21-26	p. B-34	Table B-7; p B-36	p. B-43

V _{max} C (% difference from 3.5) mg/hr/kg ^{0.70}	3.5	3.08 (-12%)	4.17 (20%)	4.17 (20%) 3.39 (-3%) (improved model fit, but not significantly from 4.17)
Km (% difference from 0.25) mg/L	0.25	0.050 (80%)	0.322 (20%)	0.322 (20%)

Note: % difference is in comparison with original Hissink et al. 2007 PBPK model parameters based on TMB in white spirit; final EPA model parameters are in bold.

In summary, EPA's decision to exclude mixed C9 isomer studies lacks accuracy, objectivity and transparency when considering that EPA appropriately included animal and human studies for solvent mixtures and other solvents in the weight of evidence *and also as the fundamental basis for PBPK modeling for TMB alone*. In addition, direct comparison of the acute central nervous system (CNS) effects of exposure to individual C9 isomers with that from exposure to a complex C9 substance show no substantial differences. Thus, EPA's rationale for excluding the C9 mixture studies is not supported by the available data. **As stated earlier, a detailed justification for the inclusion of the TSCA Section 4(a) studies conducted on complex C9 aromatic substances is provided in Attachment II.**

C. Hazard Identification

Synthesis of Evidence

1. A synthesis of the evidence for trimethylbenzene toxicity is provided in Chapter 1, Hazard Identification. Please comment on whether the available data have been clearly and appropriately synthesized for each toxicological effect. Please comment on whether the weight of evidence for hazard identification has been clearly described and scientifically supported.

ACC Comments:

EPA's summary tables in the text and in Appendix B are clearly presented. However, the summaries do not capture important methodological limitations that affect study quality and interpretation of results. Table 2 of these comments highlights experimental design issues and summarizes available historical control data. In addition, EPA's reporting of statistical significance is inaccurate in several instances. Table 3 summarizes the results of statistical analysis including important results of the main ANOVA analyses that EPA did not include in any of the Supplementary or Main report summary tables. Table 3 also reports the post-hoc comparisons between treatment level and control because in some cases EPA mistakenly reported within group statistical significance as between group statistical significance. Due to these issues, the weight of evidence for the neurotoxicity section has not been accurately described, and the guidance for evaluating the weight of evidence in the preamble has not been followed.

Of special concern is that all of the studies by Korsak and colleagues did not indicate if time of testing was balanced across exposure level and devices or if the subjective measures were conducted by the same or different observers without knowledge of exposure level. In addition, the original studies did not clearly report all the statistical analyses conducted for each of the variables. In some cases statistical

comparisons were made across trials within a treatment group (i.e. L3 vs. L1) and inferences were made regarding significance across a treatment group (treated L3 vs control L3). This is confusing and may have contributed to errors in EPA's summary table.

Regarding the Korsak and Rydinski (1996) study, ACC appreciates EPA's need to select a sensitive endpoint that is amenable to BMD analysis and agrees that both acute and long-term effects of repeated exposures to TMB are important. However, ACC does not agree with EPA's conclusions regarding consistency of the acute pain sensitivity finding with effects reported 50-51 days after repeated exposure for 4 weeks. This is important because it impacts the selection of uncertainty factors. (See in particular response "2" below in which we show that latency to paw lick in the hot plate tests, a measure of pain sensitivity, is different from the results of the hot plate-foot shock tests, which are a measure of active avoidance.)

1. **The EPA's tables do not accurately report the statistical results or the total number of comparisons.** Comments below are focused on EPA's tables for studies conducted by Korsak, Gralewicz, Wiaderna and colleagues on pain sensitivity and conditioned analgesia. Based on the types of errors EPA made for these endpoints, EPA should check summaries of statistical results for other endpoints involving repeated trials. The statistical analyses conducted by Korsak and colleagues included 2-way ANOVA (4 dose groups and 3 trials as factors; reported as repeated measures in some but not all papers); 1-way ANOVAs for each trial across dose groups (to investigate significant interactions) and ratios of trials; and post-hoc comparisons of the direct measures (L1, L2, L3) and ratios (L2/L1, L3/L1). The post-hoc comparisons were conducted between (e.g. TMB vs. control) and within (e.g. L3 vs L2 for each TMB level) treatment groups. At a minimum, the Supplementary Appendix B tables should be revised to include significant and non-significant results of the 2-way and 1-way ANOVA analyses (considered to be the main analyses by the authors) and post-hoc comparisons, and include explanation of whether significant findings are comparisons between or within groups and if they are for direct measures or ratios. For this purpose, we summarize the results of ANOVA analyses focused on comparisons between control and treated groups (Table 3).
 - 1.1. **Table 1-1 for 1,2,4-TMB Gralewicz et al. (1997) (p. 1-10) incorrectly reports that statistical significance was achieved for latency after trial 3 (L3).** Only the ratio of L3/L1 was statistically significant when comparing high (250 ppm) and mid (100 ppm) doses with controls. The direct measure L3 was not reported by Gralewicz et al. (1997) to be statistically significant in any for the exposed groups compared to controls.
 - 1.2. **Table 1-1 for 1,2,4-TMB Gralewicz and Wiaderna (2001) (p. 1-10) is accurate, but more discussion is needed in the report text.** EPA's tabulation of data as percent of concurrent control can be misleading to the casual reader if the data are not discussed rigorously in the report. For example, the post-shock trial 3 (L3) is reported as a 191% difference for Gralewicz and Wiaderna (2001) but the pre-shock value (L1) was also 206% of control. This indicates a lack of an effect of the shock to produce an analgesic effect but an overall increase in latency across all trials. However, these results conflict with those from Gralewicz et al. (1997). This lack of consistency is discussed and illustrated in greater detail below (Figure 1).
 - 1.3. **Table 1-1 for 1,2,3-TMB Wiaderna et al. (1998) (p. 1-12) incorrectly reports statistical significance at the mid-dose compared to controls for response at 51 days post-exposure (24 hr. after foot shock).** The report is inaccurate because there was no statistically significant difference between the mid-dose and control group in the direct measurement L3. The significance was in reference to L1 (i.e. L3 vs. L1) within each treatment group and not in comparison to the concurrent control L3. This error is discussed in greater detail below.

- 1.4. **EPA's table B-42 Figure 4 from Wiaderna et al. (1998) (1,2,3-TMB: hot plate immediately before and after foot shock) is not transparent because EPA does not report important negative results and does not provide sufficient explanation of statistically significant results.** EPA did not include the footnote for the upper panel that “*p<0.05 compared with L1 in the same group” (note that the original authors correctly indicated p<0.5). EPA also did not include the results that were reported in this study as not statistically significant, namely: “Post hoc comparisons revealed no differences between groups within successive trials”. This led to the error in EPA's summary Table 1-1 (p. 1-12) of the main report, incorrectly indicating significance at the mid dose compared to control.

Table 2. Description of and Control Data for Pain Sensitivity and Conditioned Analgesia Studies

		Age or weight of animal at start of study	Duration of exposure	Test time interval since last exposure	Shock from test prior to hot plate test	Hot Plate Shock immediately after Trial 1	Balance time of testing across dose level	Latency trial 1 control mean/error	Latency trial 2 Control mean/error	Latency trial 3 Control mean/error
Pain (Thermal) Sensitivity										
1,2,4-TMB	Korsak, 1996	250-300g	3 mo	Immediate	Rotarod 2mA	No shock	No	15.4 / (s.d.)=5.8 EPA assumed s.d.	n.a.	n.a.
1,2,3-TMB	Korsak, 1996	250-300g	3 mo	Immediate	Rotarod 2mA	No shock	No	9.7 / (s.d.)=2.1 EPA assumed s.d.	n.a.	n.a.
Solvents	Korsak, 1994	330 g	3 mo	Immediate	Rotarod 2mA	No shock	No	12.2/ s.d.=3.1	n.a.	n.a.
C-9 aromatic naphtha mixture (55% TMB)	Douglas, 1993	300 g	3 mo	2 days	None	No shock	Yes, random	Wk 0, 5, 9, 13, respectively: 8 / s.d.=2.7 12.2 /s.d.=4.8 10.2/ s.d.=3.8 10.9/s.d. = 4.2	n.a.	n.a.
								Wk 0 lab historical control 9.5-12.2		
THERMAL SENSITIVITY (Trial 1) and CONDITIONED ANALGESIA WITH SHOCK (Trial 2, 3)										
1,2,4-TMB	Gralewicz 1997	5 mo	4 wk	50-51d	Passive Avoidance 100 ms 4 mA: 1 Hz 10s	100 ms 2 mA: 0.5 Hz for 2 min	No	23 / (sem)=3	44 / (sem)=4	25 / (sem)=4
1,2,4-TMB	Gralewicz 2001	5 mo	4 wk	50-51 d	Passive Avoidance 100 ms 2 mA: 1 Hz for 10s	100 ms, 2 mA: 0.5 Hz for 2 min	No	9 / sem=1	41 / sem=6	11 / sem=2
1,2,3-TMB	Wiaderna 1998	5 mo	4 wk	50-51 d	Passive Avoidance 100 ms 2 mA: 1 Hz for 10s	100 ms, 2 mA: 0.5 Hz for 2 min	No	16 / (sem)=4	34 (sem)=4	18 (sem)=5
1,3,5-TMB	Wiaderna 2002	5 mo	4 wk	50-51 d	Passive Avoidance 100 ms 4 mA: 1 Hz 10s	100 ms, 4 mA: 0.5 Hz for 2 min	No	22 / (sem)=2	43 (sem)=3	22 (sem)=3

Table 3. Statistical results of Pain sensitivity (Hot Plate) and Conditioned Analgesia Tests (Hot plate paired with shock)

		2-way ANOVA (4-dose;3trial; sometimes reported as repeated measures ANOVA)			1-way ANOVA (4 dose)			Group comparisons with control			Group comparisons with control	
TMB isomer	First author, year	Dose	Dose x trial	trial	L1	L2	L3	L1	L2	L3	L2/L1	L3/L1
1,2,4 acute effect	Korsak 1996	n.a.	n.a.	n.a.	-	n.a.	n.a.	*100↑ *250↑	n.a.	n.a.	n.a.	n.a.
1,2,4	Gralewicz 1997	Θ ^a [L1&L2]	Θ ^a [L1&L2]	* ^a [L1&L2]	(Θ)	(Θ) [ANOVA L2/L1: Θ]	(Θ) [ANOVA L3/L1: *]	(Θ)	(Θ)	(Θ)	(Θ)	*100↑ *250↑
1,2,4 (100 ppm ^b)	Gralewicz 2001	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Θ	Θ	*100↑	-	-(Θ)
1,2,3 acute effect	Korsak 1996	n.a.	n.a.	n.a.	-	n.a.	n.a.	*25↑ *250↑	n.a.	n.a.	n.a.	n.a.
1,2,3	Wiaderna 1998	Θ	(*) ^c	*	Θ	Θ	Θ	Θ	Θ	Θ	(Θ)	(Θ)
1,2,3 (100 ppm ^b)	Gralewicz 2001	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Θ	Θ	*100↑	-	-(Θ)
1,3,5 (100 ppm ^b)	Gralewicz 2001	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Θ	Θ	Θ	-	-
1,3,5	Wiaderna 2002	Θ	*	*	Θ	Θ	*	Θ	Θ	*100↑	Θ	Θ
C9 (55% TMB)	Douglas 1993	n.a.	n.a.	n.a.	-	n.a.	n.a.	Θ	n.a.	n.a.	n.a.	n.a.

Notes: Korsak and colleagues reported numerous comparisons within a group (i.e. Trial 1 vs Trial 3 within each exposure level) and these are not reported in this table. EPA's tables sometimes mistakenly reported within group comparisons as between group comparisons (control vs. treated). For example, Wiaderna et al. 1998 did not report any group comparisons with control to be significant, analysis was always between trials within a dose group. This table only reports post-hoc comparisons conducted between treatment group and controls. This table also reports results of ANOVA that were reported to be conducted in at least one of the publications.

^a 2-way ANOVA reported for trials 1 and 2. It is likely that analyses for all 3 trials were also conducted but not reported.

^b Only 100 ppm level tested for each test chemical. This study tested multiple chemicals, but only one exposure level.

^c For 1,2,3-TMB, Wiaderna et al. 1998 reported that the dose x trial interaction was not statistically significant, but based on *p* value it is likely to be significant

L1, L2, L3 = latency for trials 1, 2, 3, respectively, for the conditioned analgesia test with shock immediately after trial 1;

– “authors did not report results of an analysis, and it is not clear if this analysis was conducted

“**n.a.**” statistical analysis is not applicable to the experimental design because there was only one trial

Θ not statistically significant

(Θ) It is likely analysis was conducted based on methods section and/or post-hoc tests reported, but statistically insignificant results were not reported

* indicates the ANOVA was significant for the specified factor or interaction

The columns for post-hoc comparisons of treated with control lists the dose levels that were statistically significant and indicates the direction of change from control

- 1.5 EPA's Table B-43 Figure 2 for Wiaderna et al. (2002) (1,3,5-TMB : hot plate immediately before and after foot shock) did not report the results of the 2-way ANOVA and large number of mostly non-statistically significant comparisons.** Of the 27 comparisons (9 between group for each trial + 12 within group comparisons of trials + 6 between group for proportions L2/L1 and L3/L1), the only significant result was an increase in L3 when comparing the mid-dose with control and low-dose. This should not be considered an effect on pain sensitivity because there were no significant increases in L1 at any dose level compared to controls. In the results section the authors state, "in none of the groups did the reaction latencies in trial 3 differ significantly from those determined in trial 1" and "no significant differences were detected between the groups in the values of the proportions L2/L1 and L3/L1". This indicates that there were no effects on the conditioned analgesia test, and the biological significance of the increase in L3 at the mid-dose level is uncertain.
- 2. EPA's assessment of pain sensitivity conflates two different behaviors involving hot plate into a single "pain sensitivity phenotype". This incorrectly gives the impression of persistency and consistency of finding.**

The test of "Pain Sensitivity" (pp. 1-2 to 1-4) should be divided into two sections – one on "pain sensitivity" that discusses the hot plate test without shock, and the other on "conditioned analgesia" for the hot plate combined with shock paradigm.

The pain sensitivity test evaluates the response to noxious stimuli (hot temperature) as an unconditioned stimulus. In the hot plate shock paradigm, only the first of three trials is identical to the pain sensitivity test because the animals have not been shocked. Trials 2 and 3 are models of "conditioned analgesia" in which an unconditioned (shock) and/or possibly conditioned (hot plate environment) aversive stimuli are used to induce analgesia before or during exposure to a noxious stimulus (hot temperature) (Butler and Finn, 2009; Miguez et al. 2014). There are different hypotheses regarding whether an increase in latency during Trial 2 or 3 is due to fear, stress, or improved memory (for trial 3). Although these hypotheses are speculative, none of these behaviors would be considered relevant to the pain sensitivity test that is measured without shock. These behaviors should be discussed separately in different sections entitled "pain sensitivity" and "conditioned analgesia".

Indeed, one of the EPA's external peer-reviewers for the 1995 Koch Industries study report on the 90-day oral toxicity study of 1,3,5-TMB¹ highlighted this exact same inconsistency in the final report submitted to the EPA. In discussing his findings regarding the Korsak "pain sensitivity" studies, the peer reviewer suggested that the Korsak studies on "pain sensitivity" with the addition of footshock "*may not be a valid or equivalent paradigm usage for classical conditioning*". It is not clear why this reviewer's comments were ignored by the EPA.

There were a large number of statistical comparisons conducted by Korsak, Gralewicz, Wiaderna and colleagues. Based on a review of all of these papers, the standard approach by this laboratory is to conduct 2-way ANOVAs, large number (up to 27) different post-hoc comparisons within and between groups and additional 1-way ANOVAs. The authors focused on reporting the significant findings. Table 3 selects only the group comparisons between treatment and control groups because this is the standard of comparison for risk assessment purposes.

¹ Peer Review Report – External Peer Review of the 1995 Koch Industries Study Report. 90-Day Oral Gavage Toxicity Study of 1,3,5-Trimethylbenzene in Rats with a Recovery Group. Page 19.

3. **Gralewicz and colleagues did not replicate their own results for the effects of 100 ppm 1,2,4-TMB on pain sensitivity.**

Figure 1 below illustrates the lack of concordance in the results from two different experiments on the effect of 100 ppm 1,2,4-TMB on pain sensitivity and conditioned analgesia. The results of the statistical analyses are reported in Table 3. The Gralewicz and Wiaderna (2001) study (left panel of Figure 1) shows that 1,2,4-TMB increases pain sensitivity (L1; not statistically significant from control) but has no effect on conditioned analgesia because the ratio of L3 to L1 are relatively the same for control and chemical. The Gralewicz (1997) study (right panel) shows a different result. There is no effect of 1,2,4-TMB on pain sensitivity, but there is an effect of the shock (i.e. effect on conditioned analgesia) based on differences between L3 and L1. The Gralewicz (2001) study measured the effect of one single concentration (50 ppm) of xylene, 1,2,4-TMB, 1,2,3-TMB and 1,3,5-TMB. In this study, the results for 1,2,4-TMB indicated there were increases in L1 that altered the “baseline” behavior just prior to the shock. This change in pre-shock “baseline” (L1) was not observed in the 3 other studies evaluating the effects of multiple doses on these same TMB isomers. There were no significant effects of 1,2,4-TMB on the ratio L3/L1, which therefore indicates there is no effect of TMB on conditioned analgesia. Thus, there is conflicting evidence of the effect on conditioned analgesia at 100 ppm. This, together with limitations in study design (discussed below) reduces the utility of this endpoint for risk assessment.

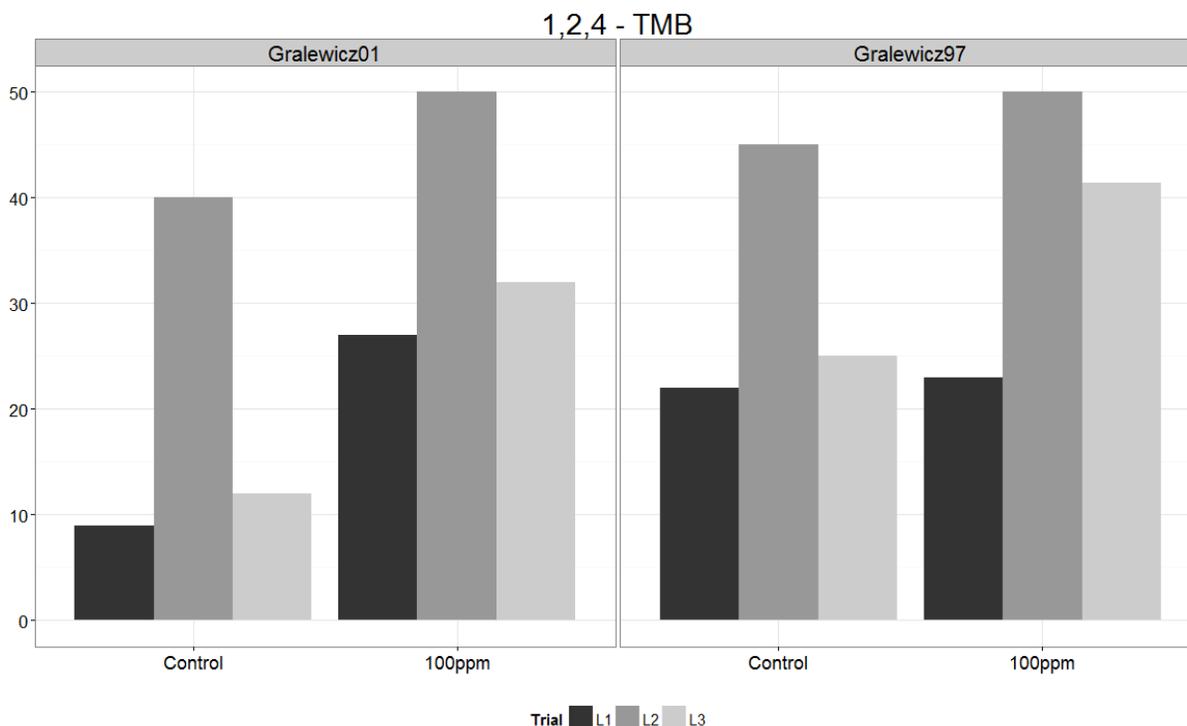


Figure 1: Results from two studies that show inconsistent results for the effect of 100 ppm 1,2,4-TMB. Data were estimated from the graphs of the original papers. Statistical results are summarized in Table 3 of these comments. Although there is an apparent increase in L3 in both studies, the pattern of effects are inconsistent with each other. The left panel shows no effect on conditioned analgesia, the right panel shows an effect on conditioned analgesia. The left panel shows a numerical increase in pain sensitivity (L1), the right panel does not.

4. Interpretation of the biological significance of effects on L3 or L3/L1 is made more difficult by (a) the confounding effects of the passive avoidance test (animals associate leaving a platform with shock) that was completed just 2 days prior to the hot plate test and (b) the absence of a control “sham” group for the shock .

The objectivity of this discussion can be improved by including discussion of limitations in interpreting changes in L3 or L3/L1 and overall lack of replication of findings (Table 3 of our comments). For example, an increase in L3 could reflect improved memory of the conditioned (hot plate environment) aversive stimuli that was associated with receiving a shock for two minutes 24 hours earlier. It could also be due, in part, to the fact that the rats were just tested two days earlier on a passive avoidance paradigm in which the rats were required to suppress their normal tendency to step down off of a platform. Following trial 3 of the passive avoidance test, the animals receive shocks for 10s. This prior testing with passive avoidance confounds the interpretation of the hot plate-shock test that uses the same aversive stimuli for 2 minutes (see Table 2). In addition, Wiaderna et al. (1998) observed that in the 1,2,4-TMB study “licking the hind-paw, was usually preceded by attempts to get out of the plastic enclosure. The more persistent were the attempts, the longer was the paw-lick latency.” This would suggest that the increased latency may not necessarily be an adverse effect. This reduces the level of concern for changes in L3 or L3/L1 parameters 50 days after four weeks of exposure to TMB, and increases confidence that the BMDL for the reversible Korsak et al. paw-lick finding will be protective of equivocal effects.

5. EPA does not follow the guidance in the preamble to evaluate the quality of experimental studies including control of other variables that could influence the occurrence of effect, assessment of study quality characteristics and examination of historical control data from the same laboratory. In general, the studies by Korsak and colleagues had several weaknesses in study design which were not described or considered in the weight of evidence:

5.1. Subjective measures were not conducted blind to treatment level

5.2. The time of testing was not randomized (or balanced) across dose group (Table 2)

Counterbalancing all the relevant factors within and across test sessions is a basic requirement of EPA’s neurotoxicity test guideline and expectation for quality behavioral studies. If the different dose groups were tested in sequential order (i.e. all control animals, then all low dose, etc.), statistically significant differences between treated and control group could be due to factors other than exposure level. The interpretation is confounded, and no statistical method could disentangle group and time effects (Maurissen, 2010).

5.3. Historical control data from the laboratory indicates wide range of “normal” latency on hind limb paw lick (Table 2). EPA should discuss the wide range of “normal” latency and discuss observations by Wiaderna et al. (1998) that animals that react to pain stimuli by trying to escape have longer latencies.

5.3.1.L1 values for 4-week exposure studies conducted in 5-month old animals and tested 50 days later range from **9 – 22** (Table 2 of our comments).

5.3.2.L1 values for 3-month studies conducted in rats weighing 250-300 g range from **9.7 -15.4**. Hence the experimental value of 11.8 ± 3.8 for 25 ppm 1,2,3-TMB, although statistically different from the concurrent control value of 9.7 ± 2.1 (Korsak and Ryzdzyński, 1996) falls within the range of control values and should not be considered as a treatment-related effect. These data support a NOAEL of 100 ppm for 1,2,3-TMB and 1,2,4-TMB because the L1 values were within this range of control values (Korsak and Ryzdzyński, 1996).

5.3.3.One of the reasons why latency to hindlimb paw lick (trial 1) can be variable is that the rats may have different strategies for escaping the aversive heat stimuli which is not captured by measuring hindlimb paw lick.

6. EPA's discussion of behavior treats conjecture as scientific evidence for incorrect conclusions regarding behavioral measures. The most significant examples are in the Hazard Assessment sections on "Motor Function and/or anxiety" and "Pain Sensitivity". This severely weakens the scientific credibility of the TMB review.

- 6.1. **The motor activity tests should not be interpreted as effects on anxiety.** The tests for motor function were not designed or validated to measure "anxiety". Thus changes in motor behavior cannot be interpreted as an effect on "anxiety". All references to "anxiety" should be removed from this section, including the title of this section.

The Douglas et al. (1993) neurotoxicity study of C9 aromatic naphtha has relevant data for TMB risk assessment, including hot plate latency, automated startle response, hind foot splay, grip strength and an especially strong evaluation of relevant tissues for neuropathology. The neuropathology included evaluation of perfusion fixed peripheral and central nerve tissue with H&E stains and luxol fast blue stain for myelin degeneration, cross and longitudinal sectioning and nerve teasing. The automated 30-minute motor activity test measured a large number of variables, but the data are too variable.

- 6.2. Our comments to General Charge Question 4 discuss in detail why EPA's decision to exclude this study from the TMB assessment is not scientifically sound or consistent with EPA's inclusion of studies involving white spirit.

- 6.3. **The Douglas et al. neurotoxicity study provides additional evidence that the increased latency to hind paw lick (without shock) is reversed within two days of approximately 4, 8 and 13 weeks of inhalation exposures to C9 aromatic naphtha mixture containing 55% TMB.**

Summary and Evaluation

- 1. Does EPA's hazard assessment of noncancer human health effects of trimethylbenzenes clearly integrate the available scientific evidence (i.e., human, experimental animal, and mechanistic evidence) to support the conclusions that trimethylbenzenes pose potential hazards to the nervous system, respiratory system, the developing fetus, and the circulatory system (i.e., blood)?**

ACC Comments:

The EPA's summary and evaluation is clearly written. There were no studies designed to measure effects of TMB isomers on anxiety, and reference to this should be omitted from line 9 of page 1-52. By excluding the C9 aromatic naphtha neurotoxicity study, EPA fails to integrate all the available relevant scientific evidence (see ACC comments to Charge Question B.1 for detailed discussion). This is important because the C9 aromatic naphtha neurotoxicity study indicates that there are no effects on hot plate hind limb paw lick when measured two days following 4, 8 or 12 weeks of exposure, which is consistent with the results of Korsak and Ryzdzyński (1996). In addition, there are no effects on a very thorough neuropathology evaluation following 3 months of exposure. **This data should be added to the weight of evidence because it reduces concern that the sensitive critical effect will increase in severity with increased duration of exposure.**

EPA's characterization of the TMB effect on the hot plate foot shock results give the impression that these results are the same type of effect as the hot plate results without the foot shock. EPA describes the hot plate-foot shock results as a measure of pain sensitivity following environmental challenge. This

would be akin to characterizing active avoidance as primarily a measure of activity following an environmental challenge. In addition, the potential confounding effect of the passive avoidance test just prior to the hot plate-foot shock test decreases the utility of the hot plate-footshock test for risk assessment.

2. Does EPA's hazard assessment of the carcinogenicity of trimethylbenzenes clearly integrate the available scientific evidence to support the conclusions that under EPA's *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), there is "inadequate information to assess the carcinogenic potential" of trimethylbenzenes?

ACC Comments:

The charge question may be misleading. The EPA's *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005) require that all relevant information be reviewed. Although there are no cancer studies of individual TMB isomers, there are repeated dose (90 days, 12 months) of C9 aromatic naphtha in which there is no evidence of pre-neoplastic lesions (Clark et al., 1989). Additionally, the C9 aromatic naphtha was evaluated in a battery of *in vitro* and *in vivo* tests and found to be non-genotoxic (Schreiner et al., 1990). The EPA reviewed this information and concluded that no additional carcinogenesis testing was necessary (Meranda, 1988). Thus the statement that there is "inadequate information to assess the carcinogenic potential" of trimethylbenzenes does not convey the information that the relevant data were reviewed by the EPA and determined to be sufficient for hazard characterization purposes.

D. Toxicokinetics and Pharmacokinetic Modeling

1. Please comment on whether the selected PBPK model (Hissink et al., 2007) with EPA's modifications adequately describe the toxicokinetics of 1,2,4-TMB (Appendix B). Was the PBPK modeling appropriately utilized and clearly described? Are the model assumptions and parameters scientifically supported and clearly described? Are the uncertainties in the model structure adequately characterized and discussed?

2. The internal dose metric selected for use in the derivation of the RfC and RfD for 1,2,4-TMB was the steady-state weekly average venous blood concentration (mg/L) of 1,2,4-TMB for rats exposed for 6 h/day, 5 days/week. Please comment on whether the selection of this dose metric is scientifically supported and clearly described. If a different dose metric is recommended for deriving the RfC, please identify this metric and provide scientific support for this choice. Are the uncertainties in the selected dose metric adequately characterized and discussed?

ACC Comments:

The EPA's description of model assumptions and parameters are clearly described. We note that *when EPA optimized the PBPK model based on white spirit to fit data from TMB exposures only very modest changes in chemical-specific parameters were required (see detailed response to Charge Question B4). From a pharmacokinetic perspective, this would suggest the other constituents of C9 (other than TMB) do not have a major influence on the pharmacokinetics of TMB based on this indirect evidence.*

E. Inhalation Reference Concentration (RfC) for 1,2,4-TMB

1. A 90-day inhalation toxicity study of 1,2,4-TMB in male rats (Korsak and Rydzyński, 1996) was selected as the basis for the derivation of the RfC. Please comment on whether the selection of this study is scientifically supported and clearly described. If a different study is recommended as the basis for the RfC, please identify this study and provide scientific support for this choice.

2. Decreased pain sensitivity (measured as an increased latency to paw lick response after a hotplate test) in male Wistar rats was concluded by EPA to be an adverse effect on the nervous system and was selected as the critical effect for the derivation of the RfC. Please comment on whether the selection and characterization of this critical effect is scientifically supported and clearly described. If a different endpoint(s) is recommended as the critical effect(s) for deriving the RfC, please identify this effect and provide scientific support for this choice.

3. In order to characterize the observed dose-response relationship comprehensively, benchmark dose (BMD) modeling was used in conjunction with dosimetric adjustments for calculating the human equivalent concentration (HEC) from a rat and human PBPK model (Hissink et al., 2007) to identify the point of departure (POD) for derivation of the RfC. Please comment on whether this approach is scientifically supported for the available data, and clearly described. a. Has the modeling been appropriately conducted and clearly described, based on EPA's *Benchmark Dose Technical Guidance* (U.S. EPA, 2012)? b. Has the choice of the benchmark response (BMR) for use in deriving the POD (i.e., a BMR equal to 1 standard deviation change in the control mean for the latency to paw lick response) been supported and clearly described?

ACC Comments:

The use of the Korsak and Rydzyński (1996) study is a sensitive endpoint that is amenable to BMD analysis that will adequately be protective of the other behavioral effects that have been reported for TMBs by primarily one laboratory. The BMR equal to 1 standard deviation change in the control mean is appropriate given the wide variability in control values across studies.

However, results from a subchronic toxicity study do not necessarily indicate that the observed effects are persistent or increase in severity with exposure. For example, the hot plate latency to paw lick responses in the Korsak and Rydzyński (1996) studies are essentially the same whether the rats were exposed once for 4 hours or continuously for 3 months. In addition, the response observed after the 3 month exposure period is seen only when the rats are tested immediately after the last exposure. When tested 2 weeks after the last exposure, no statistically significant effects relative to control are reported by the authors. This response clearly indicates an acute reversible CNS effect and should not be confused with persistent effects based on difference in duration of exposure alone.

4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfC for 1,2,4-TMB. Are the UFs appropriate based on the recommendations described in Section 4.4.5 of *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002), and clearly described? If changes to the selected UFs are proposed, please identify and provide scientific support for the proposed changes.

ACC Comments:

The additional subchronic to chronic UF of 3x is unnecessary because there is strong weight of evidence that there are no effects of repeated exposure on pain sensitivity as measured by latency to paw lick on a hot plate. Using EPA's guidance on weight of evidence, there are five dose response studies supporting the lack of a long lasting effect on paw-lick latency two weeks after a 4-week exposure (Korsak and Rydzynski, 1996; Gralewicz et al. 1997; Gralewicz and Wiaderna 2001; Wiaderna et al. 1998, 2002). In addition, a neurotoxicity study on C9 aromatic naphtha (55% TMB) reported no effect on pain sensitivity to hot plate following 4, 8 and 12 weeks of exposure (animals were tested after a two-day holiday to C9). This C9 mixture neurotoxicity study also did not find any treatment related histopathology findings based on a thorough evaluation that exceeded EPA test requirements by including longitudinal of peripheral nerves and spinal cord and teased nerve fibers. In the 1,2,4-TMB dose response study, which should carry greater weight than the studies with only one dose level of 1,2,4-TMB isomer, there were no long lasting effects on conditioned analgesia (or stress-induced analgesia) as reflected by lack of consistent statistically significant effects on the proportion L3/L1. Taken together, these data support a reduction of the subchronic to chronic UF from 3 to 1.

In addition, the EPA included a UF of 3x to account for database insufficiency. In support of this, the EPA cites the lack of a multigenerational reproductive/developmental toxicity study as a weakness in the database. However, this weakness only exists because the EPA has chosen to ignore the existing data on the complex C9 aromatic substance. As summarized in Appendix E of the supplement to the EPA draft assessment, a 3-generation reproductive toxicity study in mice, 2 developmental toxicity studies in mice and rats and one developmental neurotoxicity study in rats, in which the complex C9 aromatic substance was tested, is available. On this basis, the inclusion of the UF for database insufficiency is not justified.

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Comments of the ACC Hydrocarbon Solvents Panel
Attachment II

**Justification for the inclusion of complex C9 aromatic fraction in the draft IRIS assessment
for trimethylbenzene**

Chemical-Specific Charge Questions

B. Literature Search Strategy/Study Selection

1. Please comment on the whether the literature search approach, screening, evaluation, and selection of studies for inclusion in the assessment are clearly described and supported. Please identify any additional peer-reviewed studies from the primary literature that should be considered in the assessment of noncancer and cancer health effects of 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB.

ACC Comments:

In setting out the literature search strategy and criteria for the selection and/or exclusion of studies for the toxicological review of trimethylbenzene, the EPA indicated that certain references were excluded (via manual review) because they either involved the use of complex solvent mixtures or were not available in English. **This is inconsistent, as the Battig et al. (1956) papers, cited as evidence for neurotoxic effects in humans, involved exposure to a mixed solvent (80% mixed trimethylbenzene isomers) and were not published in English.** In essence, employing these criteria eliminated a critical set of data on mixed C9 aromatic fractions, primarily consisting of trimethylbenzene and ethyltoluene isomers (Table 1), that would have enriched the existing database for trimethylbenzene, provided information addressing database insufficiency issues raised by the EPA¹ and also providing for a more robust weight of evidence in the consideration of critical endpoints. **For example, the EPA cites the “lack of a multi-generation reproductive/developmental toxicity study” as a weakness of the database. However, as shown in Table 1, a well-conducted 3-generation reproductive toxicity assay in mice, two developmental toxicity assays (mice and rats), and one developmental neurotoxicity assay (rats) are available for which the test substance was a complex C9 aromatic substance consisting predominantly of isomeric mixtures of trimethylbenzene and ethyltoluenes.** Most importantly, the results from the reproductive/developmental toxicity studies of the complex C9 aromatic substances are virtually identical to those that are available for constituents tested individually (1,3,5-trimethylbenzene, 1,2,4-trimethylbenzene, ethyltoluene isomers, propylbenzene isomers, butylbenzene, o-, m- and p-xylene) and are discussed in subsequent sections.

¹ Section 2.1.3, Page 2-13, lines 4 – 5 and 10-11 of the Draft Assessment

Table 1: Available database of studies utilizing complex C9 aromatic substances

Test	Assay or Doses	Results	Reference
Genetic Toxicity	Ames <i>Salmonella</i> assay CHO HGPRT forward mutation CHO chromosome aberration CHO -SCE Rat chromosome aberration	All studies negative results for gene mutation (<i>Salmonella</i> , CHO/HGPRT mutation) or cytogenetic effects. C9 aromatics unlikely to be genotoxic carcinogen	Schreiner et al., 1989‡
Subchronic Neurotoxicity - Rats	100, 500, 1500ppm (500, 2500, 7500mg/m ³) 6hr/day, 5 days/wk for 90 days	No adverse effects for motor activity, functional observation battery or neuropathology	Douglas et al., 1993‡
3-generation reproductive/Developmental toxicity – Mice Female	100, 500, 1500ppm (500, 2500, 7500mg/m ³) 6hr/day from gestational days 6-15	<ul style="list-style-type: none"> • 1500ppm – 50% mortality • 500ppm - maternal and fetal body weights reduced • 100ppm – no effects 	McKee et al., 1990‡
Developmental toxicity – CFY Rats	600, 1000, 2000 mg/m ³ 24 hrs/day from gestational days 7-15	<ul style="list-style-type: none"> • 2000 mg/m³ – enlarged liver weight in dams. Increased incidence of internal organ retardations and skeletal retardations in foetus • 1000 mg/m³ - Increased incidence of internal organ retardations and skeletal retardations in foetus • 600 mg/m³ – no effects <p>Note – The internal organ and skeletal retardations did not appear to be functionally relevant as offspring from the 2000 mg/m³ dose groups showed no adverse effects when sacrificed at post natal day 90.</p>	Ungvary et al., 1983#
Developmental neurotoxicity – CFY Rats	600, 1000, 2000 mg/m ³ 24 hrs/day from gestational days 7-15	<p>Tested – body reflexes (time to correction of gait etc.) at day 21</p> <p>Open field spontaneous locomotive activity at day 23, 36 and 90</p> <p>Amphetamine sensitivity at day 37</p> <p>Association and learning ability assessments – at day 42</p> <p>Results – the authors found no evidence for developmental neurotoxicity with exposure to C9 aromatic fraction.</p>	Lehotzky et al., 1985#
Reproductive Toxicity Rats 30M,30F/group parental	100, 500, 1500ppm (500, 2500, 7500mg/m ³) 6hr/day, 7 days/wk 10 wks pre-mating, 2 wks	No adverse effects on reproductive parameters. Maternal and offspring	McKee et al., 1990‡

	mating (both sexes) females GD0 to GD20 Females not exposed to postnatal day 4 to weaning at LD21. Offspring began exposure after weaning.	body weight effects at 1500ppm	
Repeated dose toxicity Rats	1800, 3700 or 7400 mg/m ³ 5d/week for 13 weeks.	Primary effects were liver and kidney weight increases in female rats at mid and high doses with no adverse pathological correlates. Low grade anemia was observed in all exposed females.	Reported in Clark et al., 1989‡
Repeated dose toxicity Rats^a	450, 900, 1800 mg/m ³ 5d/week for 12 months	Primary effect liver weight increase with no adverse pathologic correlate at 1800 mg/m ³	Clark et al., 1989‡

a- EPA considered this study sufficient to fulfill the repeat dose requirement and did not require an additional repeated dose study in the C9 test rule program.

‡ - Studies were conducted under a 1985 EPA test rule program.

- Independently conducted studies (in Hungarian).

Although the criteria for study exclusion is stated in Figure LS-1², no clear rationale for excluding studies utilizing complex C9 mixtures and/or studies available in languages other than English was provided in the document. However the EPA attempted to address public comments to this regard in Appendices E and F³. We respond to the EPA justification with the following points:

- **The compositions of the complex C9 aromatic fractions are known and detailed gas chromatographic analysis of the constituents are available in the study reports. In addition, these constituents are all alkylbenzenes with structural similarity to trimethylbenzene.**
- **The toxicological profile (acute, subchronic and reproductive/developmental) of the complex C9 aromatic fractions is identical to those of the individual constituents.**
- **The manufacture, use and exposure to trimethylbenzene occurs primarily in the form of complex C9 aromatics (as acknowledged by the EPA) and hence the use of the data on complex C9 aromatic fractions is useful in the development of a hazard assessment for trimethylbenzenes.**

[1] **Composition and the problem of impurities**

One criticism of the studies involving complex C9 aromatics is that they are mixtures of multiple constituents, many of which are unknown. For example, the EPA, in its response to earlier public comments indicated that the complex fraction reported in Douglas et al. (1993), McKee et al.

² Literature Search Strategy/Study Selection and Evaluation; Page xlvi of the Draft Assessment

³ Appendix E and F – Summary of available C9 aromatic hydrocarbon fraction toxicity studies and resolution of public comments; Page E1 – F3 of the Supplement to the Draft Assessment

(1990) and Schreiner et al. (1989) contained up to 6% of unknown C10 constituents while that reported in Clark et al. (1989) was comprised of 9% unidentified impurities⁴. In fact, the EPA had indicated that although a comparison of sufficient toxicokinetic and toxicological similarity had been used to support the adoption of reference values for the individual isomers of trimethylbenzene, such a comparison could not be extended to the C9 aromatic fractions because some of the constituents (such as the C10 constituents) were not identified in the compositional analysis, in reference to the Douglas et al. (1983) study⁵. In addition, the EPA indicated that the Lehotzky et al. (1985) and Ungvary et al. (1983) studies were not included in the toxicological review because the compositional make-up of the test substance was not available.

However, although the detailed compositional analysis for the C9 aromatic fraction was not provided in the published studies, this data was available in the original study reports which were provided to the EPA. Table 2 provides an overview of the composition of three different complex C9 fractions that were used in the studies mentioned in Table 1. In the Douglas et al. (1983) study, the C10 constituents (comprising 8.3% of total mixture) were mainly comprised of isomers of dimethyl-ethylbenzene, isomers of methyl-propylbenzene, isomers of butylbenzene (including 0.82% n-butylbenzene), 1,2,4,5- and 1,2,3,5-tetramethylbenzene, 2% diethylbenzene isomers and 0.02% naphthalene. In other words, the C10 component of the C9 fraction in the Douglas et al. (1983) study was not different from that reported for the Clark et al. (1989) study as shown in Table E-2⁶.

From Table 2, the C11 constituent of the complex substance in the Clark et al. (1989) study are most likely < 1% similar to that obtained in the Douglas et al. (1993) study. The 8% unaccounted for in the Clark et al. (1989) study is likely to include isopropylbenzene and other C10 constituents similar to those reported in the Douglas et al. (1993) study but for which proportions were not readily available. Although C10 and C11 constituents were not reported in the Lehotzky and Ungvary studies, the proportions of these constituents could not be > 2% since at least 98% of constituents are accounted for.

The C9 fraction employed in Ungvary et al. (1983) and Lehotzky et al. (1985) did not contain a significant amount of constituents \geq C10 compared to those in the other studies based on differences in manufacturing processes. While the C9 fractions in the Clark et al. (1989) and Douglas et al. (1993) studies are derived primarily from catalytic reforming of petroleum feedstocks, the fraction used in the Ungvary et al. (1983) and Lehotzky et al. (1985) studies undergo additional refining steps in order to meet product specifications for use as solvents (Firth, 2008). **However, as will be shown in the subsequent section, the presence or absence of the \geq C10 fraction had no effect on toxicity endpoints where a direct comparison can be**

⁴ Appendix E, pages E-1 and E-2 of the Supplement to the Draft Assessment.

⁵ Table E-1, page E-1 of the Supplement to the Draft Assessment.

⁶ Table E-2, page E-2 of the Supplement to the Draft Assessment.

made between the C9 aromatic fractions and individual trimethylbenzene and ethyltoluene isomers.

Independent of source, the C9 fractions had a similar composition (> 76% trimethylbenzenes and ethyltoluenes with xylene and propylbenzene isomers being the other constituents present at more than trace levels). In addition, the constituents present in minute proportions appear to be well characterized and are also structurally similar to trimethylbenzenes (alkylated benzenes), such that a comparison of toxicokinetic and toxicological similarity can be made.

Table 2: Composition of complex C9 fractions used in various mixed C9 constituent studies

Constituents	Weight (%)		
	Clark et al., 1989	Douglas et al., 1993¶	Ungvary et al., 1983 and Lehotzky et al., 1985‡
Non-aromatics	0.46	< 0.10	0.04
o-xylene	2.27	3.17	2.69
m-xylene	NR	0.05	NR
p-xylene	NR	0.02	NR
Isopropylbenzene	NR	2.76	2.11
n-propylbenzene	4.05	3.95	6.99
4-ethyltoluene	16.60	6.13	31.25
3-ethyltoluene	7.14	15.85	
2-ethyltoluene	7.22	5.78	7.21
1,2,4-trimethylbenzene	32.70	39.18	33.7
1,2,3-trimethylbenzene	2.76	5.49	5.52
1,3,5-trimethylbenzene	9.35	8.09	8.80
C10	8.31*	8.32	NR
C11	NR	0.14	NR

‡ Substance reported as Aromatol (Complex C9 solvent mixture).

¶ Identical substance was used in the Schreiner et al (1989) and McKee et al (1990) studies.

NR – Proportion was not reported.

* Predominantly comprised of 1-methyl-3-n-propylbenzene, 1,2-diethylbenzene and 1-ethyl-3,5-dimethylbenzene

[2] Similarity of toxicity

Aside from the presence of unknown contaminants in the C9 aromatic fraction, the EPA had also indicated that a major reason for the exclusion of the studies on the C9 aromatic fraction was that they failed to observe clearly adverse effects (except for the reproductive/developmental toxicity study of McKee et al., 1990)⁷ in contrast to the studies on the individual isomers. The implication of this statement is that there is the possibility of interactive effects where certain mixed constituents may be masking the potentially adverse effects of the trimethylbenzene isomers. However, as will be shown in this section, the data on the complex C9 aromatic fractions are virtually identical to those of the individual isomers of trimethylbenzene and ethyltoluene. For the sake of brevity, data presented on the individual constituents are limited to those present in a significant proportion in the various complex C9 fractions. For example, taking the composition of C9 fraction from Douglas et al (1993), it is immediately apparent **that isomers of trimethylbenzene, ethyltoluene, propylbenzene and xylenes make up at least**

⁷ Appendix E, page E-8. Lines 27-28 of the Supplement to the Draft Assessment.

90% of the C9 fraction (Table 2). The similarity in the toxicological profile of each constituent and the complex C9 fraction would then suggest that:

- **The presence of the $\leq 10\%$ C10-C11 component of the C9 fraction does not mask the potential toxicity of the entire C9 fraction, does not potentiate the toxic effect, and does not introduce a unique toxic effect that is not seen in the key individual constituents.**

i. Neurotoxicity

Table 3 lists the results of neurotoxicity studies on individual constituents of a typical complex C9 aromatic fraction. The results are compared with neurotoxicity and developmental toxicity tests on a complex C9 aromatic fraction. Analyses of the results, both with individual constituents or in the complex C9 aromatic fractions, show a consistent pattern of acute central nervous system (CNS) depression immediately following exposure with complete recovery following cessation of exposure.

In the Korsak studies (heavily relied upon by the EPA in the Draft Assessment), a few statistically significant responses are observed. However, many of these responses are characterized by wide variations, lack of dose-response and the complete absence of temporal concordance. As an example, Gralewicz et al. (1997) reported statistically significant effects in passive avoidance tests following a 4-week exposure to 1,2,4-trimethylbenzene in the mid and high dose groups at 48 days post exposure, 7 days after footshock (0, 123, 492, 1230 mg/m³; 0, -20, -79*, -49%). No significant effects were observed in three other paradigms attempted (Table 1-1)⁸. However, a subsequent study (Gralewicz & Wiaderna, 2001) with 492 mg/m³ 1,2,4-trimethylbenzene reported no statistically significant effect in all four passive avoidance tests attempted. Further details regarding concerns with EPA's interpretation of the statistical significance of study results are provided in Attachment I.

In Appendix E of the Supplement to the Draft Assessment, the EPA concluded that the Douglas et al. (1993) study was not reliable because the lack of neurotoxic effects was not compatible with the neurotoxic effects of pre-mating exposures in McKee et al. (1990) even though similar exposure concentrations were employed. In McKee et al. (1990), pregnant and non-pregnant adult mice were reported to show signs of neurotoxicity, including abnormal gait, decreased motor activity and slight ataxia, which is consistent with acute CNS depression. However, this criticism misses two key points:

- a. The effects reported in McKee et al. (1990) were seen immediately after exposure. However, in the Douglas et al. (1993) study, rats were tested 48 hours **after** last-exposure to avoid confounding acute effects. An example of how time at which observations are made may

⁸ Table 1-1, page 1-11 of the Draft Assessment.

affect clinical observations is noted in Table 3. In the NTP oral subchronic study of m-xylene, mice administered 2000 mg/kg-day showed acute neurological effects, including abnormal gait, tremors and ataxia (NTP, 1986); similar to those observed in inhalation-exposed mice in McKee et al. (1990). What is most important however is that in the NTP study, the observed neurological effects were only seen within the first hour of m-xylene administration, followed by complete reversal of acute CNS effects.

- b. Although the highest exposure concentrations in the Douglas et al. (1993) and McKee et al. (1990) studies were identical (7500 mg/m³), the studies employed different animal species. In Douglas et al. (1993), rats were used (standard body weight – 350 g) while McKee et al. (1990) tested mice (standard body weight – 25 g). The 14-fold difference in body weight would most likely have led to a much larger exposure/unit mass in the mouse compared to the rat.

Overall, comparison of the neurotoxicity data for both individual constituents and complex C9 aromatic substance revealed no evidence for unique differences such as to preclude the use of data on the complex C9 aromatic substances from weight of evidence considerations.

Table 3: Neurotoxicity studies on complex C9 fractions and key individual constituents

Test	Substance tested	Assay/Doses	Results	Reference
Studies with the major individual constituents of complex C9 aromatic fractions				
Acute neurotoxicity test in rats (inhalation).	1,2,3-, 1,2,4- and 1,3,5-trimethylbenzene	250 – 2000 ppm (1230 – 9840 mg/m ³) once for 4 hours.	<ul style="list-style-type: none"> No deaths reported. Dose dependent increase in response to rotarod performance test and latency to paw lick (hot plate method) when tested immediately after exposure. 	(Korsak & Ryzdzynski, 1996)
Subchronic neurotoxicity test in rats (inhalation).	1,2,3- and 1,2,4-trimethylbenzene	25, 100 or 250 ppm (123, 500 or 1230 mg/m ³), 6h/day, 5 days/week for 3 months.	<ul style="list-style-type: none"> No deaths reported. No significant clinical observations made. Increased latency to paw lick was observed when tested immediately after last exposure. No effects on latency to paw lick when tested 2 weeks after last exposure. 	(Korsak & Ryzdzynski, 1996)
Subacute neurotoxicity test in rats (inhalation)	1,2,3-, 1,2,4- and 1,3,5-trimethylbenzene	123 – 1230 mg/m ³ 6h/day, 5 days/week for 4 weeks.	<ul style="list-style-type: none"> Very few statistically significant responses when tested ≥ 2 weeks after exposure. Wide and inconsistent variations were noted in statistically significant responses reported. These were not dose-responsive and temporal concordance could not be established for any of the responses. 	(Gralewicz & Wiaderna, 2001; Gralewicz et al., 1997; Lutz et al., 2010; Wiaderna et al., 1998; Wiaderna et al., 2002)
Subacute neurotoxicity test in rats (inhalation)	m-xylene	100 ppm, 6h/day, 5 days/week for 4 weeks.	<ul style="list-style-type: none"> No effect in radial maze tests 14-18 days post-exposure. No effect on open field activity 25 days post-exposure. 	(Gralewicz & Wiaderna, 2001)

			<ul style="list-style-type: none"> • No effect on active avoidance tests 54-60 days post-exposure. • Significant effects in 1 of 6 trials in a passive avoidance test 39-48 days post-exposure. • Significant effects in paw-lick latency only with footshock employed 50-51 days post-exposure. 	
Subchronic toxicity test in mice (oral)	Technical xylene‡	125, 250, 500, 1000 or 2000 mg/kg-day, 5 days/week for 13 weeks.	<ul style="list-style-type: none"> • Lethargy, unsteady gait, tremors and paresis at 2000 mg/kg-day within 5-10 minutes of dosing for up to 1 hour. 	(NTP, 1986)
Acute neurotoxicity test in rats (inhalation).	Isopropylbenzene	500 – 6000 mg/m ³ for 6 hours.	<ul style="list-style-type: none"> • Alterations in FOB with a NOAEC of 500 mg/m³. 	(Cushman et al., 1995)
Subchronic neurotoxicity test in rats (inhalation).	Isopropylbenzene	250, 500, 2500 or 6000 mg/m ³ , 6h/day, 5 days/week for 3 months followed by a 4-week recovery period.	<ul style="list-style-type: none"> • Unsteady gait in rats within 1 hour post-exposure. No effects reported after 6 hours post-exposure. • No exposure-related changes in FOB, auditory brain stem response, brain measurements or histopathology of nervous system tissues. 	(Cushman et al., 1995)
Studies with complex C9 aromatic fractions				
Subchronic neurotoxicity test in rats (inhalation).	C9 aromatic substance (See Table 2 for composition)	500, 2500 or 7500 mg/m ³ , 6h/day, 5 days/week for 3 months.	<ul style="list-style-type: none"> • Rats tested 24-48 hrs post-exposure to avoid confounding acute effects. • No exposure-related effects on motor activity and FOB tests. • No histopathological effect on nervous system tissue. 	(Douglas et al., 1993)
Acute neurotoxicity test in rats (inhalation).	Mixed isomer C9 aromatic solvent.	200, 1000 or 5000 mg/m ³ once for 8 hours each on 3 consecutive days.	<ul style="list-style-type: none"> • Effects on gait, hunched body positions, motor activity and slight ataxia reported within first hour after first exposure in the 5000 mg/m³ exposure group alone. • Some statistically significant effects in the visual discrimination test were observed in the 1000 and 5000 mg/m³ exposure groups after the first 8-hr exposure. These effects were reversed 24-hours post last exposure. 	(McKee et al., 2010)
Developmental neurotoxicity test in rats (inhalation)	C9 aromatic fraction (See Table 2 for compositional information)	600, 1000 or 2000 mg/m ³ , 24h/day from gestation days 7-15.	<ul style="list-style-type: none"> • No effects with open field spontaneous locomotive activity tests in 23, 36 and 90-day old pups. • No effects on amphetamine sensitivity tests in 37-day old pups. • No statistically significant effects in learning ability tests conducted on male pups from postnatal day 42. 	(Lehotzky et al., 1985)

‡ Contains 60% m-xylene, 13.6[^] p-xylene, 17% ethylbenzene and 9.1% o-xylene.

ii. **Subchronic/chronic toxicity**

As shown in Table 4, the systemic effects of prolonged exposures to either individual C9 alkylbenzenes or as complex C9 fractions are basically identical. Overall, the general effects include body weight decreases, mild increases in liver and kidney weights (although histopathological changes indicative of tissue injury were not observed), hematological changes (mostly decreases in RBCs with increased leukocyte counts) and upper respiratory tract irritation (associated with increased inflammatory cell counts in bronchoalveolar lavage fluid). In addition, the variations in the C8 and/or C10 alkyl benzene components in the complex fraction did not change the rodent systemic response.

Table 4: Subchronic toxicity studies on complex C9 fractions and key individual constituents

Test	Substance tested	Assay/Doses	Results	Reference
Studies with the major individual constituents of complex C9 aromatic fractions				
Subchronic toxicity in rats (inhalation)	1,2,4-trimethylbenzene	123, 492 and 1230 mg/m ³ . 6 h/day, 5 days/week for 3 months	<ul style="list-style-type: none"> • Low-grade anemia (decreased RBC and reticulocytes at 1230 mg/m³). • Increased SDH activity at all exposure levels. • Decreased clotting time at 492 and 1230 mg/m³ with no dose-response pattern. • Statistically significant increase in pulmonary lesions at 492 and 1230 mg/m³. No incidence data available. 	(Korsak et al., 2000a)
Subchronic toxicity in rats (inhalation)	1,2,4-trimethylbenzene	123, 492 and 1230 mg/m ³ . 6 h/day, 5 days/week for 3 months	Increase in total cell count and macrophage cell count in bronchoalveolar (BAL) fluid at all exposure levels.	(Korsak et al., 1997)
Subchronic toxicity in rats (inhalation)	1,2,3-trimethylbenzene	123, 492 and 1230 mg/m ³ . 6 h/day, 5 days/week for 3 months	Increased liver weight associated with slight increase in SDH activity in high exposure male rats. Increased number of goblet cells and interstitial lung parenchyma infiltration in high exposure males and females.	(Korsak et al., 2000b)
Subchronic toxicity in rats (oral)	1,3,5-trimethylbenzene	50, 200 and 600 mg/kg/day. 5 days/week for 90 days.	Increased liver weights.	(Adenuga et al., 2014)
Subchronic toxicity in rats (oral)	<i>p</i> -ethyltoluene	100, 300 and 900 mg/kg/day. Once daily for 94 days.	<ul style="list-style-type: none"> • Dose-related mortality and decreased body weights. • Increased liver weights associated with increases in ALP, albumin and ALT in 300 and 900 mg/kg dose groups. 	(USEPA, 2009)
Subchronic toxicity in	<i>p</i> -ethyltoluene	477 or 2337 mg/m ³ , 6 h/day, 5 days/week for	<ul style="list-style-type: none"> • Statistically significant increase in total cells, 	(Swiercz et al., 2000)

rats (inhalation)		4 weeks.	macrophages, neutrophils and lymphocytes in BAL fluid from high dose male rats. <ul style="list-style-type: none"> Increased number of rats with pulmonary lesions in high exposure group. 	
Subchronic toxicity in rats (inhalation)	Isopropylbenzene	492, 2438 or 5909 mg/m ³ for 6 h/day, 5 days/week for 13 weeks.	<ul style="list-style-type: none"> Statistically significant increase in kidney, liver and adrenal weights. Low grade anemia with concentration-dependent increase in leukocyte count. 	(Cushman et al., 1995) Virtually identical results are also reported in (Fabre et al., 1955; Jenkins et al., 1970)
Subchronic toxicity in rats (inhalation)	<i>m</i> -xylene	<i>Study 1</i> - 100 ppm, 6 h/day, 5 days/week for 6 months or 1000 ppm for 3 months. <i>Study 2</i> - 50 or 100 ppm, 6 h/day, 5 days/week for 3 months	<ul style="list-style-type: none"> Decreased lymphocyte differential counts and increased monocyte counts in study 1. Low-grade anemia with increased leukocyte counts in study 2 (exposure to 100 ppm). 	2 Korsak studies (Korsak et al., 1992; Korsak et al., 1994) were summarized in USEPA, 2003
Studies with complex C9 aromatic fractions				
Subchronic toxicity in rats (inhalation)	C9 aromatic substance (See Table 2 for composition)	1800, 3700 or 7400 mg/m ³ for 13 weeks.	<ul style="list-style-type: none"> Increased liver and kidney weights in high exposure females. Low-grade anemia in females at all exposure levels. 	Summarized in Clark et al., 1989
Subchronic toxicity in rats (inhalation)	C9 aromatic substance (See Table 2 for composition)	450, 900 or 1800 mg/m ³ , 6h/day, 5 days/week for 12 months.	<ul style="list-style-type: none"> Reduced body weight gain in high exposure rats. Increased liver and kidney weights in high exposure males. Various statistically significant hematological changes at 6 months but not at 12 months. 	(Clark et al., 1989)
Subchronic toxicity in rats (inhalation)	C9-C10 alkyl aromatic fraction‡.	200 ppm, 8h/day, 5 days/week for 90 exposures.	<ul style="list-style-type: none"> No persistent or significant peripheral blood changes, weight gains, bone marrow or eye lens changes were observed in the rats. 	(Nau et al., 1966)
Subchronic toxicity in rats (inhalation)	C9-C10 alkyl aromatic fraction‡.	460, 1100 or 2200 mg/m ³ ,	<ul style="list-style-type: none"> Reduced body weight in high exposure groups. Statistically significant reduction in BUN in high exposure rats. 	(Carpenter et al., 1975)

‡These fractions contained about 30-45% C9 alkylbenzenes.

iii. Developmental toxicity

As shown in table 5, there was virtually no difference in the developmental toxicity of individual constituents of C9 aromatic fractions and the complex substance. Overall, none of the constituents or complex substances caused malformations in the various species tested. Fetotoxicity appeared to be associated with maternal toxicity and as has been reported earlier, severity of maternal toxicity and fetotoxicity appeared to be influenced more by differences in study design (USEPA, 2003). For example, the highest severity of effects was seen in the studies where exposure occurred over a 24-hour time period in contrast to the more typical 6-hour exposures.

Table 5: Developmental toxicity studies on complex C9 fractions and key individual constituents

Test	Substance tested	Assay/Doses	Results	Reference
Studies with the major individual constituents of complex C9 aromatic fractions				
Developmental toxicity in rats (inhalation)	1,2,4-trimethylbenzene	100, 300, 600 or 900 ppm 6 h/day on gestational days 6-20.	<ul style="list-style-type: none"> • Statistically significant decrease in maternal body weight gain and food consumption from 600 ppm. • Significant reduction in fetal body weight from 600 ppm. • No evidence of teratogenic effects. 	(Saillenfait et al., 2005)
Developmental toxicity in rats (inhalation)	1,3,5-trimethylbenzene	100, 300, 600 or 1200 ppm 6 h/day on gestational days 6-20.	<ul style="list-style-type: none"> • Statistically significant decrease in maternal body weight gain and food consumption from 300 ppm. • Significant reduction in fetal body weight from 600 ppm. • No evidence of teratogenic effects. 	(Saillenfait et al., 2005)
Developmental toxicity in rats (oral)	<i>p</i> -ethyltoluene	25, 100 or 200 mg/kg/day from gestation days 6-19.	<ul style="list-style-type: none"> • No evidence of maternal and fetal effects. 	(USEPA, 2009)
Developmental toxicity in rabbits (oral)	<i>p</i> -ethyltoluene	25, 125, 200 or 250 mg/kg/day from gestation days 6-27.	<ul style="list-style-type: none"> • 12/16 dams died in highest dose group. • Increased incidence of fetuses with 13th full ribs in the 125 mg/kg dose group. • Increased incidence of fetuses with 13th rudimentary rib at 200 mg/kg dose group. • No other reproductive 	(USEPA, 2009)

			<p>and fetal effects were reported.</p> <ul style="list-style-type: none"> • Developmental parameters could not be evaluated meaningfully due to the high mortality in the 250 mg/kg dose group. 	
Developmental toxicity in rats (inhalation)	Isopropylbenzene	487, 2399 or 5935 mg/m ³ , 6h/day on gestation days 6-15.	<ul style="list-style-type: none"> • Statistically significant decrease in maternal body weight gain on gestation days 6-9. • No significant adverse effect on reproductive parameters and fetal development was reported. 	<p>(Darmer et al., 1997).</p> <p>A follow-up study in rabbits showed a non-significant increase in early resorptions and non-significant decrease in percent of live fetuses associated with a statistically significant decrease in body weight gain and increased relative liver weight in dams following exposure to 11,300 mg/m³ isopropylbenzene (Darmer et al, 1997).</p>
Developmental toxicity in rats (inhalation)	<i>o</i> -, <i>m</i> - and <i>p</i> -xylene	150, 1500 or 3000 mg/m ³ , 24h/day from gestation day 7-14.	<ul style="list-style-type: none"> • The authors reported a dose-dependent increase in the incidence of fetal retardation at concentrations that caused maternal effects. • The authors reported that none of the isomers were teratogenic. 	(Ungváry et al., 1980)
Developmental toxicity in rats (inhalation)	<i>o</i> -, <i>m</i> - and <i>p</i> -xylene	100, 500, 1000 or 2000 ppm (434, 2167, 4335 or 8670 mg/m ³), 6h/day for gestation days 6-20.	<ul style="list-style-type: none"> • No evidence of teratogenicity was found with exposure to any of the xylene isomers. • Significant decreases in fetal body weight were associated with significant decrease in maternal body weight gain and food consumption. 	(Saillenfait et al., 2003)
Studies with complex C9 aromatic fractions				
Developmental toxicity in rats (inhalation)	C9 aromatic fraction (See Table 2 for compositional information)	600, 1000 or 2000 mg/m ³ , 24h/day from gestation days 7-15.	<ul style="list-style-type: none"> • Liver weight enlargement in dams. Authors reported slight toxic effects in the dams. • Increased incidence of internal organ and skeletal retardations were reported in the 	(Ungváry et al., 1983)

			fetus from 1000 mg/m ³ . However, these changes had largely disappeared by post natal day 90 indicating a lack of toxicological relevance for the mild changes seen on gestation day 21.	
3-generation reproductive/Developmental toxicity in mice (inhalation)	C9 aromatic fraction (See Table 2 for compositional information)	(500, 2500, 7500mg/m ³ 6hr/day from gestational days 6-15	<ul style="list-style-type: none"> • 1500ppm – 50% maternal mortality • 500ppm - maternal and fetal body weights reduced • 100ppm – no effects 	(McKee et al., 1990)

iv. Reproductive toxicity

Unlike other endpoints, the database for reproductive toxicity of individual constituents was not as robust. A search of existing databases revealed a 2-generation reproductive toxicity study of n-butylbenzene (a C10 component of C9 aromatic fractions), a 1-generation reproductive toxicity study of mixed xylenes and a reproductive toxicity screening study of 1,4-diethylbenzene (C10 isomers present in C9 aromatic fractions at about 2%) (See Table 2). In all three cases, there were no treatment-related effects on reproductive and fertility indices. With the exception of maternal effects such as increased mortality and reductions in body weight gain, inhalation exposure to a complex C9 aromatic fraction (see Table 2 for compositional information) had no effect on reproductive and fertility indices in a 3-generation reproductive toxicity study in mice. Overall, the lack of effects in the complex C9 fraction was consistent with the lack of effects noted in the existing data on individual constituents.

Table 6: Reproductive toxicity studies on complex C9 fractions and key individual constituents

Test	Substance tested	Assay/Doses	Results	Reference
Studies with the major individual constituents of complex C9 aromatic fractions				
2-generation reproductive toxicity in rats (oral)	n-butylbenzene	30, 100 or 300 mg/kg/day over 2 generations.	• No effects on reproductive fertility in males or females.	(Izumi et al., 2005)
1-generation reproductive toxicity in rats (inhalation)	Mixed xylenes‡	60, 250 or 500 ppm, 6h/day for 131 days pre-mating, 20 day mating period, gestation and lactation.	• No effects on pregnancy and fertility indices in males and females.	Study report summarized in (OEHHA, 2012)
Reproductive toxicity screening test in rats (oral) Similar to OECD TG (422)	1,4-diethylbenzene	30, 150 or 750 mg/kg	• No treatment-related effects on reproductive and developmental toxicity.	Robust study summary provided in (OECD, 1994)
Studies with complex C9 aromatic fractions				
3-generation reproductive/Developmental	C9 aromatic fraction (See	(500, 2500, 7500mg/m ³	• No evidence of treatment-related	(McKee et al., 1990)

toxicity in mice (inhalation)	Table 2 for compositional information)	6hr/day from gestational days 6-15	effects on reproductive and fertility indices. Maternal effects such as increased mortality and reduced body weight gain were observed in the mid and high dose groups.	
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‡ Details on constituents were not provided but this was likely a mixture of xylene isomers and ethylbenzene based on compositional information on other technical xylenes.

[3] Manufacture, use and exposure considerations

As the EPA has stated, trimethylbenzene isomers are primarily “*produced during petroleum refining and production of aromatic hydrocarbons with nine carbons (i.e., C9 aromatic fraction)*” and that the “*vast majority of the C9 fraction is used as a component of gasoline*”⁹. In the presentation made by the EPA to the trimethylbenzene augmented Chemical Assessment Advisory Committee, the EPA identifies the primary use of trimethylbenzenes as part of the C9 fraction used as blending agents in gasoline formulations, as industrial solvents and as paint thinners¹⁰. With regard to exposure considerations, the EPA indicates that “*vehicle emissions are expected to be the major anthropogenic source of trimethylbenzenes*” and that exposures could also occur through occupational exposures in oil/gas extraction and printing industries (see footnotes).

Based on the manufacture use and exposure conditions described above, it is clear that trimethylbenzenes are primarily produced and utilized, not as individual isomers, but as part of a complex substance consisting of C9 alkylbenzenes that may also include smaller percentages of C8-C10 aromatic hydrocarbons (Firth, 2008). If this is true, then it stands to reason that the primary exposure to trimethylbenzenes would occur, not as individual isomers, but as part of a complex containing predominantly C9 isomers. This is in line with the EPA’s conclusion that general population exposures to trimethylbenzenes occur through emissions from refining activities (manufacture of aromatic C9 fraction), automobile combustion (aromatic C9 fraction blended into gasoline) and in printing ink industries (where aromatic C9 fractions are used as printing ink solvents) (Firth, 2008).

The EPA indicated that data on individual trimethylbenzene isomers were used exclusively because “*current information demonstrates that trimethylbenzene isomers are released to and persist in the environment and that human populations are exposed to trimethylbenzenes in occupational and residential settings*”¹¹. The EPA bolsters this argument by citing the data on the yearly emissions data on 1,2,4-trimethylbenzene. **We agree with the EPA that isomers of**

⁹ Executive Summary (Occurrence and Health Effects) – Lines 2-5, page xxxiv of the Draft Assessment.

¹⁰ Slide 4, EPA presentation on “*Overview of the Draft IRIS Assessment of Trimethylbenzenes*”. May 22nd, 2014.

¹¹ EPA response to public comments – Appendix F, line 4-7, page F-3 of Supplement to Draft Assessment.

trimethylbenzene are indeed released into the atmosphere where potential human exposure can occur. However, it should be noted that the Toxics Release Inventory (TRI) data that the EPA cites does not take into account the source of the 1,2,4-trimethylbenzene. Based on the manufacture, use and exposure considerations outlined here, we believe that the complex C9 aromatic fraction are the primary source of the 1,2,4-trimethylbenzene. As has been outlined in prior sections, the individual trimethylbenzene isomers are structurally similar to the alkylbenzenes present in the C9 aromatic fraction and are toxicologically identical. Hence, the decision to exclude the large amount of federally mandated data on the toxicity of the complex C9 aromatics is not justified in light of these and potential exposure conditions.

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Comments of the ACC Hydrocarbon Solvents Panel
Attachment III

Justification for employing the Adenuga et al (2014) study (cited as Koch Industries, 1995b) as the basis for the derivation of a reference dose (RfD)

Chemical-Specific Charge Questions

B. Literature Search Strategy/Study Selection

1. Please comment on the whether the literature search approach, screening, evaluation, and selection of studies for inclusion in the assessment are clearly described and supported. Please identify any additional peer-reviewed studies from the primary literature that should be considered in the assessment of noncancer and cancer health effects of 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB.

ACC Comments:

The 90-day oral subchronic toxicity study of 1,3,5-trimethylbenzene (cited as *Koch Industries, 1995b* in the Draft Assessment) (Adenuga et al., 2014) was conducted in response to a TSCA Section 4(a) test rule (58 Fed. Reg. 59667 (1993) in support of the “EPA’s efforts to develop Health Advisories (HAs) for unregulated drinking water contaminants that are monitored under section 1445 of the Safe Drinking Water Act (SDWA)”. As an oral study, it is directly relevant for to an RfD determination compared to the inhalation studies the EPA has used since it does not require route-to-route extrapolation. **The principal reason this study was rejected was that it did not identify any adverse neurological or respiratory effects. As will be explained in more detail below, the respiratory effects observed with trimethylbenzenes (either as individual isomers are as complex substances) are local “portal of entry” effects that would not be associated with exposure in drinking water and are assessed in inhalation studies that are directly relevant to the RfC. In addition, evaluation of oral exposures causing acute central nervous system (CNS) effects with 1,3,5-trimethylbenzene and other structurally similar alkylbenzenes (such as xylenes) show that neurological effects are not expected at the highest dose employed in the study.** In other words, the study provides a more conservative NOAEL estimate that is also protective of systemic effects more appropriate to oral exposures. Specifically, our comments on the validity of this study for the determination of an RfD are as follows:

[1] Lack of objectivity in EPA independent peer-review

As the study report was not published at the time the Draft Assessment was developed, the EPA sought external peer review to assess study reliability. The EPA indicated that the results of the external peer review led them to “conclude that this study was not suitable to serve as a principal study with which to derive human health reference doses”¹ and that it provided only “limited toxicological information”². Although this was the conclusion of two of three peer reviewers, this conclusion is **not** based on the quality of the study itself but in context of the neurotoxicity endpoints evaluated in the inhalation studies. In essence, the EPA concluded that the TSCA test rule-mandated study, conducted in accordance with

¹ Section 2.6.1, lines 6-19, page 2-48 in Draft Assessment

² EPA response to public comments. Appendix F, lines 3-13, page F-4 of the Supplement to the Draft Assessment.

existing EPA guidelines, was not “suitable” only because it did not evaluate the EPA’s pre-determined critical endpoints that are more appropriate for inhalation exposure. This bias is reflected in the misleading charge question presented to the peer reviewers for their review of the 1995 study report. Rather than request that the external peer reviewers independently assess the quality of the study, the EPA framed charge question 1b as follows:

In consideration of the toxicological properties of trimethylbenzenes reported in the provided contextual references (Wiaderna et al., 2002; Gralawicz and Wiaderna, 2001; Korsak et al., 200a, b; Wiaderna et al., 1998; Gralawicz et al., 1997a; Gralawicz et al., 1997b; Korsak et al., 1997; Korsak and Rydzynski, 1996; Korsak et al., 1995), please comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation³.

Considering that the existing EPA guideline at the time this study was conducted did not specifically call for neurotoxicity evaluation other than the standard clinical observations, this charge question could only have led to one conclusion. **As indicated by all three peer reviewers, the study quality was high and “all the elements required by the EPA 798.2650 guidelines were included”**. According to one of the peer reviewers (citing the current EPA OCSSP Harmonized Test Guideline 870.3100), such a study should include a functional observation battery (FOB), if the two-week repeat study had clinical signs of depression of the CNS. Indeed, a 2-week oral toxicity study of 1,3,5-trimethylbenzene was available (cited as *Koch Industries, 1995a* in the Draft Assessment). Clinical observations of treated rats in this study (administered 60, 150 or 600 mg/kg, 7 days/week for 2 weeks) revealed no signs of CNS depression, hence including a full functional observation battery in the 90-day subchronic toxicity study was not justified. The 90-day study has since undergone rigorous peer review and is now published as Adenuga et al., 2014.

[2] The NOAEL is a valid conservative estimate of safe exposure levels through the oral route

In Appendix F (response to comments)⁴, the EPA cited a concern raised by one of the external peer reviewers that the NOAEL identified in the study report was “*most likely an artifact of the study investigating insensitive endpoints (i.e., body weights, gross pathology)*”. We strongly disagree with this comment. Not only was the study conducted strictly according to existing EPA guidelines at the time, this statement implies that an endpoint is only “sensitive” when an adverse effect is observed. This statement also ignores that the goal of subchronic toxicity tests is not merely to identify adverse effects, but to determine levels at which exposure to a substance can be considered safe.

In the 1,3,5-trimethylbenzene oral toxicity study, several statistically significant effects were noted, particularly in the high dose group (600 mg/kg-day). These included clinical chemistry changes such as an increase in phosphorus levels, alkaline phosphatase, in high dose male rats and increased liver weights in males and females. In humans and rodents, sustained elevations of serum phosphorus are a sensitive

³ Peer Review Report – External Peer Review of the 1995 Koch Industries Study Report. 90-Day Oral Gavage Toxicity Study of 1,3,5-Trimethylbenzene in Rats with a Recovery Group. Page 2.

⁴ EPA response to public comments. Appendix F, lines 9-11, page F-14 of the Supplement to the Draft Assessment.

indicator of decreased renal elimination (such as would be expected in patients with renal insufficiency), increased phosphate load (such as could occur through hemolysis or muscle breakdown) and increased reabsorption, an indicator of hypoparathyroidism. In addition, other general clinical chemistry and gross pathological changes are highly sensitive indicators of adverse effects on tissues such as the liver or kidney while significant decrease in body weight is a sensitive indicator of adverse maternal systemic effects in developmental toxicity studies for example. The relevance of effects observed in rats in the oral 90-day study of 1,3,5-trimethylbenzene was rigorously adjudicated during the publication peer review process, especially as relates to the selection of an appropriate NOAEL. All three manuscript peer reviewers agreed that the effects (clinical chemistry and tissue weights) were accidental and not toxicologically relevant.

[3] Neurological and respiratory endpoints are not appropriate endpoints on which to judge the validity of the 90-day oral toxicity study of 1,3,5-trimethylbenzene

As stated in above, the EPA's major criticism of the reliability of the Adenuga et al (2014) study was that it did not include an evaluation of a neurotoxicity endpoint. The EPA, citing one of the external peer reviewers of the original Koch Industries study report, indicated that a lower NOAEL would have been identified had the study investigated endpoints "*more pertinent to human health*" (e.g., behavioral, respiratory or electrophysiological endpoints). This is conjecture and not consistent with the study design and the rationale for the study, which was to develop a reference value for drinking water contamination.

Firstly, it is hard to understand how evaluating a respiratory endpoint could have been considered "pertinent to human health" based on an oral study in this case. Inhalation toxicity studies of individual isomers of trimethylbenzene, ethyltoluene, isopropylbenzene etc. indicate that the respiratory effects seen are largely local portal of entry effects and hence would not be expected in an oral toxicity study. In the 3-month inhalation studies of 1,2,3- and 1,2,4-trimethylbenzene for example, the respiratory effects were limited to irritation of the respiratory tract, observed as increased inflammatory cells in bronchoalveolar (BAL) fluid and goblet cell hyperplasia (Korsak et al., 1997; Korsak et al., 2000). Certainly these effects would not be expected via oral exposure.

Secondly, the EPA cites two studies of acute oral exposure to 1,3,5-trimethylbenzene that evaluated both electrophysiological and locomotor activity in rats. In the first study, acute exposures to 250, 1000 or 4000 mg/kg 1,3,5-trimethylbenzene resulted in slight dose-dependent **increases** in animal locomotor activity (Tomas et al., 1999), certainly not evidence of CNS depression. In the second study, gavage administration of 250, 1000 or 4000 mg/kg 1,3,5-trimethylbenzene resulted in changes in electrocortical activity (Tomas et al., 2000). However, the changes were observed within 60 minutes of solvent administration which would be indicative of an acute CNS effect but not a persistent neurological effect. In fact, a similar acute CNS effect was noted in an oral 90-day subchronic toxicity study of m-xylene. In this study, oral administration of 2000 mg/kg-day consistently resulted in abnormal gait, tremors and ataxia in rats within 5 minutes of administration. These effects wore off within 1-hour of exposure and no long-term neurological effects were noted at study termination (NTP, 1986). The highest administered dose in the Adenuga et al (2014) study was 600 mg/kg-day. No clinical evidence of acute CNS depression was reported in this study and the 600 mg/kg-day dose level is several fold lower than doses where oral

administration of 1,3,5-trimethylbenzene (albeit in 2 acute studies) and xylene caused acute effects in rodents. In other words, the weight of the evidence does not support the potential for a neurological effect at the doses tested in the Adenuga et al. (2014) study and the use of this endpoint as a rationale for excluding this study in the development of an RfD for trimethylbenzenes is not justified.

In summary, the 90-day oral toxicity study of 1,3,5-trimethylbenzene was conducted to fulfill the goal of developing a reference value to regulate drinking water exposure to trimethylbenzenes. It was conducted according to EPA guidelines and identifies a point of departure (POD) for oral exposures in rats. This POD of departure takes into account all possible endpoints appropriate for an oral exposure and is thus appropriate for the derivation of an RfD. In addition, the use of this study eliminates the increased uncertainty that comes with extrapolating from an inhalation study as has been done in the Draft Assessment.

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