Acrolein/PC 000701
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EPA Reviewer: Jeremy Leonard, PhD **Signature:** Jeremy Leonard
Risk Assessment Brand IV, Health Effects Division (7509P) **Date:** 05/21/2021
EPA Secondary Reviewer: Jessica Kidwell, MS **Signature:** Jessica Kidwell
Risk Assessment Branch IV, Health Effects Division (7509P) **Date:** 05/21/2021

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DATA EVALUATION RECORD – Supplemental
See TXR # [insert number] for root DER

STUDY TYPE: Non-guideline Acute Inhalation Toxicity – Human

PC CODE: 000701 **DP BARCODE: D458866**

TEST MATERIAL (PURITY): Acrolein (≥99% a.i.)

SYNONYMS: 2-propenal

CITATION: Dwivedi, A *et al*. (2015) Acute effects of acrolein in human volunteers during controlled exposure. Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden. October 27, 2015. MRID 51570802. *Inhalation Toxicology* 27(14): 810-821. https://doi.org/10.3109/08958378.2015.1115567

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EXECUTIVE SUMMARY:

In an acute inhalation toxicity study (MRID 51570802) Acrolein (≥99% a.i., batch/lot # not provided) was administered to 9 women and 9 men for 2 hours via whole-chamber exposures at 0, 0.05, and 0.1 ppm, either alone or in combination with 15 ppm ethyl acetate (EA) to mask the potential influence of acrolein odor. Symptoms related to irritation and central nervous system effects were rated on 100-mm Visual Analog Scales. Additionally, measurements of pulmonary and nasal airway parameters and blink and breathing frequency were evaluated, and markers of inflammation were analyzed in blood and sputum samples.

There was no effect of acrolein on markers of inflammation or coagulation in the blood or on cell count, differential cell count, and interleukin levels in sputum samples, and there were no differences between sexes for any markers. There was also no effect of acrolein exposure on throat irritation, breathing frequency, or on the function of pulmonary and nasal airway parameters.

There was a significant dose-dependent increase in ratings of eye irritation from 0 mm, 1.5 mm, and 8 mm at 118 minutes of exposure to the control, 0.05 and 0.1 ppm acrolein treatments, respectively. The verbal equivalents were ratings of "not at all" for the control and lowest dose to "a little more than hardly at all" for the highest dose, and exposures to EA did not affect these ratings. Median ratings for nose irritation were highest for the 0.1 ppm acrolein + EA treatment, at 6 mm, but showed little change between treatments over time. Ratings for smell were

immediately increased upon entering the exposure chamber and were highest for EA treatments. Ratings of fatigue were increased at all timepoints, with no influence of acrolein or EA, while the ratings of other symptoms were not affected by acrolein, EA, or any combination of the two. In general, no sex-specific differences were seen between the ratings. The most sensitive subjects (within the 75th percentile) showed a significant association between ratings of eye irritation and the serum amyloid A ratio. This was the only significant association between any ratings and blood inflammatory markers, and further analyses showed that symptom ratings and exposure levels were not correlated. There were no differences between sexes for the symptom ratings and eye blink frequency. Sex-specific differences in airway measures included higher ratios of expiratory volume and vital capacity in the lungs of females and higher volumes and cross-sectional areas within the first few mm from the nostril opening in the nasal passageway of males, regardless of exposure condition.

This acute inhalation toxicity study in humans is **acceptable/non-guideline**.

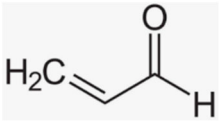
COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were not provided, as this is a literature study.

I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test material: Acrolein

Description: Not reported
Lot/batch #: Not reported; Sourced from FLUKA Analytical, Sigma Aldrich, Buchs, Switzerland
Purity: ≥99 % a.i.
Compound stability: Not reported
CAS # of TGAI: 107-02-8
Structure:



2. Vehicle and/or positive control: ≥99% ethyl acetate (EA); Merck, Darmstadt, Germany

3. Subjects:

Species: Human: 9 male and 9 female volunteers
Strain: N/A
Age/weight at study initiation: 21-38 years of age for men and 20-26 years of age for females; Body weights not reported
Source: Students recruited by advertisement at Karolinska Institutet
Chamber temperature: 23.9-24.1°C
Chamber relative humidity: 29-31.4%
Chamber air exchange rate: 18-20 times/hour

B. STUDY DESIGN:

1. In life dates: Not reported

2. Subject assignment and participant data collection: All subjects participated in the same dosing regimen (Table 1) that involved exposure of up to three subjects at the same time in a chamber for 2 hours on six different occasions. Each exposure session was separated by at least one exposure-free week. Subjects were healthy non-smokers, and females were verified as non-pregnant by a pregnancy test administered immediately before each exposure. Ethyl acetate was used to mask the potential influence of acrolein odor while seated in the exposure chamber.

TABLE 1: Study design and measured concentrations in exposure chamber

	Acrolein (ppm)		Ethyl Acetate (EA; ppm)	
Test group	Nominal Concentration (ppm)	Measured Concentration (ppm)	Nominal Concentration (ppm)	Measured Concentration (ppm)
Control (clean air)	0	0	0	0
Low acrolein	0.05	0.051 ± 0.003	0	0
High acrolein	0.1	0.11 ± 0.007	0	0
EA	0	0	15	15.0 ± 0.25
Low acrolein + EA	0.05	0.047 ± 0.002	15	14.6 ± 0.30
High acrolein + EA	0.1	0.098 ± 0.006	15	14.8 ± 0.64

Measured values are given as arithmetic means with standard deviations.
Data from Table 1 on page 812 of MRID 51570802

3. **Dose selection rationale:** A pilot study was conducted with 4 males and 4 females to determine limits of odor and irritation, with the goal to allow this data to set the high and low exposures for the main study. Volunteers were exposed to increasing concentrations of acrolein at 0.02, 0.04, 0.07, 0.1, 0.2, and 0.3 ppm in an exposure chamber for 10 minutes, where they rated symptoms on visual analog scales (as described in Methods section below). Ratings of smell increased immediately after entering the chamber, while further increases in concentration had no effect. Logistic quantile regression analyses suggested dose-related effects for throat irritation ($p<0.01$) for the 50th percentile and of eye irritation ($p=0.066$) for the 75th percentile. However, no clear effect thresholds could be established. The symptoms ratings for the pilot study are provided in the Appendix below. Based on results of the pilot study, and for ethical reasons, exposure levels were set at 0.1 and 0.05 ppm in the main study. These concentrations represented the Swedish 8-hour occupational exposure level for the high concentration and half that as the low concentration, respectively.

4. **Generation of the test atmosphere / chamber description:** Exposures were carried out in a 20 m^3 dynamic exposure chamber with controlled climate. Outlet airflow rate was set higher than inlet rate to avoid leakage of vapors into the surrounding laboratory. Acrolein and EA vapors were generated by injecting liquid acrolein (0.1%) and EA into inlet air via a high-pressure chromatography piston pump, and the inlet air was dispersed in the chamber ceiling. Gas chromatography coupled to a flame ionization detector was used to monitor acrolein and EA concentrations in the chamber after sucking air with an air pump from the upper (20 cm below ceiling) central part of the exposure chamber. The limit of detection was approximately 0.01 ppm for both acrolein and EA.

   Measured concentrations of acrolein in the chamber were similar to target concentrations (within 10%) and varied (coefficient of variation; CV) by 6-7% between exposure sessions. EA measured concentrations also were close to target concentrations with low variability (2-4% CV).

C. METHODS:

1. **Rating symptoms:** Volunteers rated symptoms on a 0-100 mm visual analog scale (VAS) that was graded from "not at all", "hardly at all", "rather", "quite", "very", and "almost unbearable) in a questionnaire containing ten questions. The questions involved: 1) burning, discomfort, or irritation of eyes; 2) burning, discomfort, or irritation of the nose; 3) discomfort in the throat or airway; 4) dyspnea (difficult or labored breathing); 5) smell; 6) headache; 7) fatigue; 8) nausea; 9) dizziness; and 10) feeling of intoxication. The volunteers rated symptoms immediately prior to exposure, during exposure (3, 60, and 118 minutes after exposure began), and after exposure ended at 20 minutes, 3 hours, and 22 hours.

2. **Measuring eye blink:** Two skin electrodes on the *orbicularis oculi* muscle and one reference electrode on the cheek bone were used to measure the blink movements of the left eye by electromyography (EMG). Blink frequency was continuously recorded from 2 minutes prior to exposure to the end of the exposure period. Blinks during exposure

were counted in 20-minute intervals via a dedicated software program developed in Borland C+++ using one filter for amplitude and one filter for latency, with blinding to exposure conditions. A complete blink was considered to be a sharp positive signal peak immediately followed by a negative peak with less amplitude approximately 50 milliseconds later.

3. **Measuring pulmonary airway parameters:** Pulmonary function parameters were measured prior to, immediately after, and 3.5 hours after exposure using a spirometer and a designated software program (Spirotrac 3, v 2.0, Buckinham, UK). The parameters evaluated included vital capacity (VC), forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), peak expiratory flow (PEF), and forced expiratory flow at 25%, 50%, and 75% of the FVC (FEF25, FEF50, FEF75). The highest values of the three slow and three forced exhalations were used. Breathing frequency was measured via respiratory inductive plethysmography, using a flexible belt around the volunteer's chest area. Breathing frequencies were counted by visual inspection of breathing curves on a computer using designated software (Variograph, v 4.70, Karlsruhe, Germany), with blinding to exposure conditions.

4. **Measuring nasal swelling and nasal airway resistance:** Nasal swelling was evaluated by acoustic rhinometry prior to, immediately after, and 3.5 hours after exposure using a SRE2000 rhinometer (Assens, Denmark) and corresponding software (Rhinosan, v 2.6, Assens, Denmark). Volumes of the nasal cavity were measured between 0 and 22 mm (VOL1) and between 23 and 54 mm (VOL2) from the opening of the nostril, along with the minimum nasal cross-sectional areas between 0 and 22 mm (MCA1) and between 23 and 54 mm (MCA2). The average of three measurements from each side of the nose, taken from a sitting position with the head placed in a frame, was used for subsequent analysis. The blocking index representing nasal airway resistance was calculated as the difference between the mouth and nasal PEF values, divided by the mouth PEF value, with nasal and mouth PEF rates measured using a PEF meter (Mini-Wright, Clement Clarke Internation, Ltd, London, UK). The flow meter was connected to a face mask during nasal exhalation, with the subject exhaling maximally into the meter with mouth closed, and measurements were taken prior to, immediately after, and 3.5 hours after exposure; the highest of the three measurements was recorded for each occasion.

5. **Measuring inflammatory markers:** Venous plasma or serum was collected from volunteers prior to, 3.5 hours after, and 22 hours after exposure for enzyme linked immunosorbent assay (ELISA) analysis of interleukin-6 (IL-6), C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, factor VIII, von Willebrand factor, and Clara cell protein (CC16). Additionally, IL-6 and interleukin-8 (IL-8) were analyzed in the supernatant of sputum collected at 6 hours from the start of exposure in the control and high acrolein exposure treatments and kept at -70°C until analysis, if the samples collected contained less than 30% squamous cells. To assess differential cell counts, sputum weight was determined and an equal volume of dithiothreitol 0.1% was added, the sample was rocked in a 37°C water bath for 15-20 minutes, the cell pellet was resuspended in 2 mL phosphate-buffered saline after centrifuging the sample 10 minutes at 280 g, and cytocentrifuge-prepared slides were stained with May-Grünwald Giemsa stain and 300 cells; total cell count and viability were also assessed using Türk and Trypan blue.

2. Statistics: A Friedman test was used to explore differences in symptom ratings between exposure levels for the non-normally distributed VAS rating data. A Mann-Whitney *U* test was used to examine gender differences in symptom ratings. Inflammatory markers in the sputum were evaluated using a non-parametric Wilcoxon matched pair test. Logistic quantile regression (25^{th}, 50^{th}, and 75^{th} percentiles) was also used for statistical analysis of irritation ratings and to analyze correlations between irritation ratings and inflammatory markers in the blood, with sampling error estimated via a cluster bootstrap resampling technique accounting for the repeated-measures study design. Blood inflammatory markers and blocking index were log transformed prior to analysis of variance (ANOVA), while all other analyses were conducted by repeated measure ANOVA, using a significance level of 0.05 for all tests. The effect of exposure on inflammatory markers was expressed as ratios by dividing the value at 3.5 hours after exposure by the pre-exposure value for each subject and exposure level. The ratios obtained for each acrolein treatment were then divided by the ratio for the control treatment to assess the change in inflammatory marker level under each exposure condition.

HED identified several deficiencies in the statistical analyses, and the investigators provided some raw data for further review (See Appendix).

II. RESULTS:

A. Symptoms ratings: Select symptoms ratings for volunteers are provided in Table 2, and their trends over time are presented in the Appendix (Figure A.1). There was a dose-dependent increase ($p<0.001$) in ratings of eye irritation from 0 mm, 1.5 mm, and 8 mm at 118 minutes of exposure to the control, 0.05 and 0.1 ppm acrolein treatments, respectively. The verbal equivalents were ratings of "not at all" for the control and low dose to "a little more than hardly at all" for the high dose, and exposures to EA did not affect these ratings. Median ratings for nose irritation were highest for the 0.1 ppm acrolein + EA treatment, at 6 mm, but showed little change between treatments over time. There was no effect of acrolein or EA on throat irritation, while ratings for smell were immediately increased upon entering the exposure chamber and were highest for EA treatments. Ratings of fatigue were increased at all timepoints, with no influence of acrolein or EA, while the ratings of other symptoms were not affected by acrolein, EA, or any combination of the two. No sex-specific differences were seen between the ratings, other than spurious findings of higher ratings by females to 0.05 ppm 60 minutes after exposure and to 0.1 ppm acrolein + EA 22 hours after exposure.

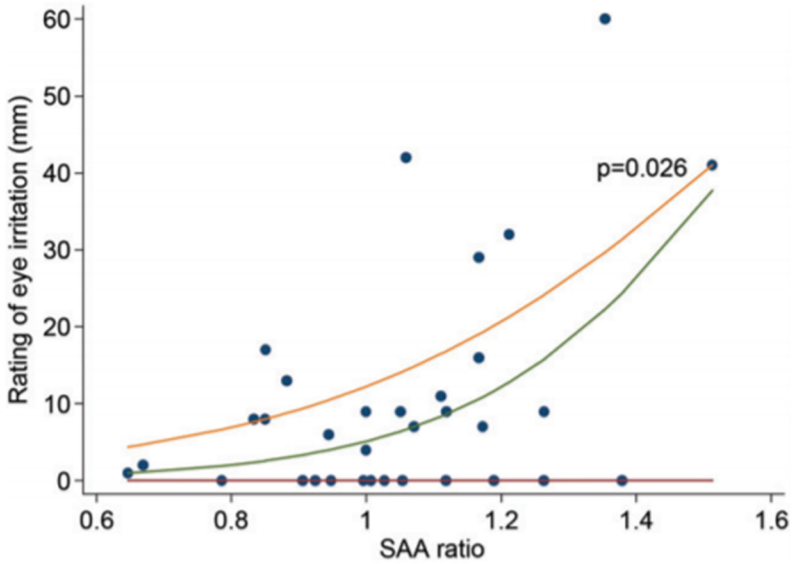
Table 2: Select symptom ratings of 18 volunteers (median and ranges) measured before and 3, 60, 118, 140, 330, and 1440 minutes after a 2-hour exposure to acrolein (ACR) and ethyl acetate (EA).

Rating parameter	Before (baseline)	3 min	60 min	118 min	140 min	330 min	1440 min
Discomfort in Eyes							
0 ppm ACR	0 (0-26)	1.5 (0-37)	0 (0-51)	0 (0-42)	0 (0-10)	0 (0-33)	0 (0-36)
0.05 ppm ACR	0 (0-8)	0 (0-34)	1 (0-49)	1.5 (0-71)	0 (0-26)	0 (0-15)	0 (0-26)
0.1 ppm ACR	0 (0-28)	0 (0-34)	6 (0-75)	8 (0-71)	0 (0-13)	0 (0-34)	0 (0-19)
EA	0.5 (0-6)	0 (0-26)	0.5 (0-38)	0.5 (0-31)	0.5 (0-8)	0 (0-26)	0 (0-26)
EA + 0.05 ppm ACR	0.5 (0-6)	0 (0-26)	0.5 (0-49)	3 (0-50)	0 (0-26)	0.5 (0-25)	0 (0-9)
EA + 0.1 ppm ACR	0.5 (0-16)	0.5 (0-25)	2 (0-70)	4.5 (0-78)	0 (0-16)	0 (0-32)	0 (0-47)
Discomfort in Nose							
0 ppm ACR	0 (0-20)	1 (0-26)	1 (0-35)	1.5 (0-49)	0 (0-26)	0 (0-26)	1.5 (0-26)
0.05 ppm ACR	0 (0-30)	1 (0-33)	2.5 (0-26)	1.5 (0-49)	1 (0-49)	0 (0-49)	0.5 (0-26)
0.1 ppm ACR	0 (0-28)	0.5 (0-24)	3.5 (0-26)	3 (0-49)	2.5 (0-49)	0 (0-26)	0 (0-32)
EA	0 (0-26)	3 (0-39)	1.5 (0-31)	0.5 (0-37)	0.5 (0-32)	0 (0-26)	0 (0-23)
EA + 0.05 ppm ACR	1.5 (0-26)	1 (0-26)	1 (0-49)	0.5 (0-49)	1.5 (0-48)	0.5 (0-38)	0 (0-26)
EA + 0.1 ppm ACR	2.5 (0-30)	4 (0-48)	3 (0-49)	4.5 (0-37)	3.5 (0-50)	0.5 (0-26)	0.5 (0-26)
Discomfort in Throat							
0 ppm ACR	1.5 (0-20)	0.5 (0-23)	0 (0-43)	3 (0-40)	1 (0-61)	0 (0-32)	0 (0-64)
0.05 ppm ACR	1.5 (0-23)	0 (0-24)	3.5 (0-28)	3.5 (0-22)	7 (0-50)	0 (0-25)	0 (0-25)
0.1 ppm ACR	2 (0-25)	0 (0-18)	5 (0-20)	1 (0-28)	2.5 (0-23)	0 (0-6)	0 (0-6)
EA	0 (0-16)	0 (0-15)	0 (0-49)	0 (0-34)	1 (0-35)	0 (0-15)	0 (0-14)
EA + 0.05 ppm ACR	3 (0-58)	2 (0-28)	1 (0-57)	0 (0-38)	3 (0-68)	0 (0-37)	1 (0-70)
EA + 0.1 ppm ACR	0 (0-26)	0 (0-17)	0 (0-16)	0 (0-31)	1.5 (0-26)	0 (0-11)	0 (0-35)
Solvent Smell							
0 ppm ACR	0 (0-6)	5 (0-50)	0.5 (0-26)	1.5 (0-37)	0 (0-29)	0 (0-10)	0 (0-6)
0.05 ppm ACR	0 (0-9)	6 (0-34)	5 (0-58)	2.5 (0-26)	0 (0-6)	0 (0-11)	0 (0-14)
0.1 ppm ACR	0 (0-6)	6 (0-49)	5.5 (0-48)	3 (0-26)	0 (0-6)	0 (0-11)	0 (0-14)
EA	0 (0-6)	26 (0-70)	15.5 (0-48)	23 (0-35)	0 (0-6)	0 (0-16)	0 (0-10)
EA + 0.05 ppm ACR	0 (0-6)	24.5 (0-63)	6 (0-60)	6 (0-53)	0 (0-26)	0 (0-6)	0 (0-3)
EA + 0.1 ppm ACR	0 (0-4)	7 (0-70)	6 (0-53)	5.5 (0-56)	0 (0-10)	0 (0-5)	0 (0-1)
Fatigue							
0 ppm ACR	5.5 (0-26)	2.5 (0-32)	3 (0-51)	11 (0-55)	5 (0-28)	4.5 (0-49)	4.5 (0-49)
0.05 ppm ACR	7 (0-49)	8 (0-49)	22.5 (0-60)	9.5 (0-49)	6 (0-30)	5 (0-42)	10.5 (0-36)
0.1 ppm ACR	6 (0-28)	4.5 (0-70)	6 (0-93)	6 (0-90)	4 (0-49)	6 (0-62)	2.5 (0-35)
EA	6 (0-71)	6 (0-72)	6 (0-100)	5.5 (0-100)	0.5 (0-90)	1.5 (0-73)	1 (0-26)
EA + 0.05 ppm ACR	6 (0-49)	5.5 (0-45)	13.5 (0-71)	16 (0-68)	5 (0-47)	4 (0-48)	3 (0-51)
EA + 0.1 ppm ACR	6 (0-49)	4.5 (0-54)	6 (0-50)	6 (0-34)	3.5 (0-26)	2.5 (0-55)	3.5 (0-29)

Data extracted from Appendix B on pages 820-821 of MRID 51570802

B. <u>Inflammatory markers:</u> There was no effect of acrolein on markers of inflammation or coagulation in the blood (Appendix Table A.2). Additionally, cell count and differential cell count, IL-6, and IL-8 in the sputum were unaffected by exposure to acrolein (Appendix Table A.3), and there were no differences between sexes for any markers. The most sensitive subjects showed a significant association (75th percentile, $p<0.05$) between ratings of eye irritation and the SAA ratio (Figure 1). This was the only significant association between any ratings and blood inflammatory markers, and further analyses showed that symptom ratings and exposure levels were not correlated.

Figure 1. Relation between average rating of eye irritation during exposure (3, 60, and 118 minutes) and serum amyloid A ratio[a].



[a] Each dot represents one subject and one exposure. Curves represent the 25th, 50th, and 75th percentiles.
Copied from Figure 3 on page 815 of MRID 51570802

C. <u>Effects on blink frequency, airway, and nose:</u> Blink frequency was slightly but significantly ($p<0.05$) increased at the highest acrolein concentration during the last 20 minutes of exposure compared to the first 20 minutes, which was not seen in the controls or low concentration (Table 3). In contrast, this same response was not seen with the acrolein and EA combined exposures, and there were no differences between sexes. Therefore, the toxicological significance of the change with the acrolein-only exposure is unclear. There was no effect of acrolein exposure on breathing frequency or in the pulmonary function or nasal airway parameters (Tables A.4 and A.5). Sex-specific differences included higher ratios of FEV1/VC and FEV1/FVC in the lungs of females and higher VOL1 and MCA1 in the nasal passageway of males, regardless of exposure condition.

Table 3: Blink frequency of 18 volunteers (mean ± SD) measured in the beginning and end of the 2-hour exposure to acrolein (ACR) and ethyl acetate (EA), as blinks per 20 minutes.

Exposure condition	0-20 minutes	100-120 minutes
Control (0 ppm)	11.7 ± 2.4	10.9 ± 2.5
0.05 ppm ACR	11.5 ± 1.5	10.9 ± 1.9
0.1 ppm ACR	11.7 ± 1.7	12.6 ± 2.7*
EA	11.9 ± 3.5	11.6 ± 1.9
EA + 0.05 ppm ACR	11.2 ± 1.6	12.5 ± 3.0
EA + 0.1 ppm ACR	11.7 ± 1.4	11.9 ± 2.4

* *p=0.049* according to ANOVA compared to the first 20 minutes of exposure
Data extracted from Table 2 on page 815 of MRID 51570802

III. DISCUSSION AND CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS (Pages 814-818 of MRID 51570802)**:** This study showed minor subjective eye irritation at exposure to acrolein at 0.1 ppm and no such effect at 0.05 ppm, with its purpose to increase the tools to study low level acrolein exposures. In the pilot study, volunteers experienced the smell to acrolein immediately at 0.02 ppm when entering the chamber, with a median rating of 13 mm, but rapidly adapted to the smell with ratings drops to about 5 mm despite increased exposure concentrations. In the main study the ratings of smell were higher with co-exposure to EA than with acrolein alone, and in general ratings of smell decrease while ratings for eye irritation increase over time, and the latter do not increase when EA is added. Altogether, this suggests that the increased ratings for eye irritation from acrolein are not explained by the smell of acrolein itself, nor EA. Eye irritation was the most prominent effect observed during the 2-hour exposures to acrolein and increased during the exposure in a dose-dependent manner. As these ratings were the only ones that were significantly increased, eye irritation should be considered as the critical effect. However, because the median rating reached only 8 mm at 0.1 ppm, which is only just slightly higher than a verbal assignment of "hardly at all" (6 mm), this effect must be considered as minor. While it is difficult to characterize sensory irritation over time due to inconsistent behaviors depending on the test compound, a marginal increase in ratings for eye irritation was seen after 3 minutes compared to pre-exposure ratings, with these ratings continuing to increase especially during the first hour of acrolein exposure. Blink frequency, another measure of eye irritation, was significantly higher during the last 20 minutes compared to the first 20 minute of exposure to 0.1 ppm acrolein alone, but was not affected by combined exposures with EA. No significant effect in ratings or on measurements indicating nasal irritation was found. Although no effect in ratings for throat irritation or on airway measurements was also shown in the main study, up to 0.1 ppm acrolein, quantile regression in the pilot study suggested a dose-effect relation in throat irritation using concentrations up to 0.3 ppm. Quantile regression analysis also revealed a positive association between eye irritation and the inflammatory marker SAA in the volunteers with the highest ratings of irritation, suggesting an up-regulation of the SAA response in these sensitive subjects.

B. REVIEWER COMMENTS: There was no effect of acrolein on markers of inflammation or coagulation in the blood or on cell count, differential cell count, and interleukin levels in sputum samples, and there were no differences between sexes for any markers. There was also no effect of acrolein exposure on throat irritation, breathing frequency, or on the function of pulmonary and nasal airway parameters.

There was a dose-dependent increase in ratings of eye irritation from 0 mm, 1.5 mm, and 8 mm at 118 minutes of exposure to the control, 0.05 and 0.1 ppm acrolein treatments, respectively. The verbal equivalents were ratings of "not at all" for the control and low dose to "a little more than hardly at all" for the high dose, and exposures to EA did not affect these ratings. Median ratings for nose irritation were highest for the 0.1 ppm acrolein + EA treatment, at 6 mm, but showed little change between treatments over time. Ratings for smell were immediately increased upon entering the exposure chamber and were highest for EA treatments. Ratings of fatigue were increased at all timepoints, with no influence of acrolein or EA, while the ratings of other symptoms were not affected by

acrolein, EA, or any combination of the two. In general, no sex-specific differences were seen between the ratings. The most sensitive subjects (within the 75th percentile) showed a significant association between ratings of eye irritation and the serum amyloid A ratio. This was the only significant association between any ratings and blood inflammatory markers, and further analyses showed that symptom ratings and exposure levels were not correlated. There were no differences between sexes for the symptom ratings and eye blink frequency. Sex-specific differences in airway measures included higher ratios of expiratory volume and vital capacity in the lungs of females and higher volumes and cross-sectional areas within the first few mm from the nostril opening in the nasal passageway of males, regardless of exposure condition.

C. STUDY DEFICIENCIES:

The following deficiencies were noted but do not alter the conclusions or interpretation of results for this study:

1) Only two exposure concentrations of acrolein were used in this study – minor for study purposes
2) The majority of volunteers were young (average of 24 years), which may have influenced response to the irritating effects of acrolein (i.e., lower) compared to older individuals which might have a less robust eye tear film – minor
3) Several deficiencies were identified in the statistical analyses used by the investigators. Further details are provided in the Appendix.

Appendix

Statistical Summary

The statistical analyses presented in the publication may not have in all cases been optimal or necessarily met some of the assumptions of the statistical tests used, and other alternative approaches may have been somewhat more appropriate. For example, this includes the investigators' use of repeated measures ANOVA, the testing for interactions, and the use of the non-parametric Friedman test. More specifically:

- Repeated Measures ANOVA: While use of repeated measures ANOVA is not uncommon in evaluating toxicology studies, certain statistical assumptions need to be met for such tests to be most valid[1]. Here, pulmonary function, nasal swelling, and blink frequency were analyzed by repeated measure ANOVA, but it is unclear to the extent that the repeated measures ANOVA requisite assumption of sphericity[2] for these data was met. The use of repeated measure ANOVA assumes that the correlation between measurements of the same subject between any two time points has the same correlation, whereas in reality correlation might be expected to be higher for more chronologically similar time points (e.g., two vs. five consecutive intervals of separation). Rather than repeated measures ANOVA, a mixed-effects model would have been more appropriate and tends to be more favored by statisticians as this approach allows for more options for variance-covariance matrices to account for the random effects (such as subject effect and day effect) and for various correlation structures (such as compound symmetry, unstructured, or first-order autoregressive analyses) between measurements of same subject within each day.

- Testing of Interactions: The investigators did not mention or report on the presence of an interaction between ethyl acetate (2 levels of yes or no) and acrolein (3 levels of 0, 0.05, and 0.1 ppm). If the interaction was not significant, then the comparisons of each acrolein treatment level against the control for many endpoints could have been conducted by combining the "with" and "without" ethyl acetate data, thus lowering the degrees of freedom and increasing the sample size and the associated statistical power of the tests. Increasing the statistical power would mean that it would have been less likely for the investigators to have "missed" a true association, if one indeed existed.

- Friedman Test: For the irritation rating data, the authors used two different approaches to analyze the data: non-parametric Friedman test and a quantile logistic regression model. It is unclear how the authors applied the Friedman test, which is typically used for one-way repeated measures data with complete block designs to analyze the rating data of this study design which had 3 factors (acrolein levels, ethyl acetate levels, and different timepoints). Also, the Friedman Test requires measurements on all levels in each block, and it is unclear from the publication how the authors addressed the missing data (two subjects did not have data of all exposure levels) when using the Friedman test.

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1 Alternatively, certain statistical adjustments (e.g, Greenhouse-Geiser or Huynh-Feldt) can be made

2 Repeated-measures ANOVA assumes sphericity which is the condition where that the variances of the differences between all possible pairs of within-subject conditions are equal. If sphericity is violated, then the F-ratio is inflated, which means that the Type I error rate is increased and the analyst is more likely to conclude that there is a difference among treatments when in reality there is not.

- Interpretation of figure: While not specifically related to the statistical analyses, it is noted that graphs presented in Figure 1A below, taken from the literature study, are misleading in that the distances between the times are quite different (e.g., the distance between 3 minutes and 60 minutes is much smaller than between 140 minutes and 330 minutes; however, they show up misleadingly as same distance in graph). The slopes of the lines connecting the times, thus, are not depicted accurately and it can be difficult to visually discern differences or trends. The connecting lines should not have been inserted, and the X-axis should be more clearly marked as being categorical. It is also unfortunate that there are not indications of uncertainty (like error bars) which makes visual interpretation difficult.

The investigators, upon request, kindly provided raw data for blink frequency, inflammatory markers in the blood, pulmonary airway measurements, nasal measures, and visual analog scale (VAS) ratings. The data for blink frequency and VAS rating for "discomfort in eyes"/eye irritation (but not any of the others) are currently undergoing reevaluation by HED. It is important to note that that although HED will be using these two data sets for different statistical methods than used by the study authors in their publication, this does not necessarily mean that the conclusions reached regarding the outcomes will differ.

Select Study Data

Table A.1. Symptom ratings (median and ranges) in the pilot study for eight volunteers measured before and every 10 minutes after stepwise increasing concentrations of acrolein.

Rating parameter	Before (baseline)	0.02 ppm	0.04 ppm	0.07 ppm	0.1 ppm	0.2 ppm	0.3 ppm
1. Discomfort in Eyes	0 0-13	1.5 0-10	0 0-33	0 0-36	0 0-26	0 0-13	0 0-61
2. Discomfort in Nose	3 0-31	2 0-28	2 0-38	2 0-49	0 0-45	0 0-48	0 0-51
3. Discomfort in Throat	0 0-27	1.5 0-47	3 0-60	3 0-48	3 0-38	5.5 0-29	6 0-59
4. Breathing Difficulty	0 0-5	0 0-13	0 0-7	1.5 0-16	1 0-38	0 0-26	0 0-21
5. Solvent Smell	0 0-6	13 0-51	6 0-29	6 0-16	4 0-38	6 0-39	4 0-48
6. Headache	0 0-24	0 0-24	0 0-16	0 0-19	0 0-25	0 0-15	0 0-16
7. Fatigue	2 0-30	0 0-12	3 0-17	1 0-27	8 0-26	7.5 0-32	11.5 0-39
8. Nausea	0 0-7	0 0-15	0 0-18	0 0-11	0 0-9	0 0-11	0 0-7
9. Dizziness	0 0-3	0 0-2	0 0-3	0 0-11	0 0-12	0 0-6	0 0-9
10. Feeling of Intoxication	0 0-3	0 0-6	0 0-3	0 0-9	0 0-12	0 0-8	0 0-9

Data extracted from Appendix A on page 820 in MRID 51570802

Table A.2. Inflammatory and coagulation markers (arithmetic means ± SD) in blood samples collected from 18 volunteers before, immediately after, and 3.5 hours after exposure to 0, 0.05 (low), and 0.1 (high) ppm acrolein.

	Exposure condition		
	0 ppm	Low ACR	High ACR
C-reactive protein, mg/l			
Before	0.7 (1.1)	0.8 (1.6)	1.3 (2.8)
After	0.6 (0.9)	0.8 (1.4)	1.2 (2.4)
3.5 h after	0.7 (1.0)	0.6 (1.0)	0.8 (1.4)
Serum amyloid A protein, mg/l			
Before	3.8 (5.3)	3.1 (3.8)	3.8 (3.6)
After	3.5 (4.7)	2.9 (3.2)	3.4 (2.7)
3.5 h after	3.2 (3.8)	3.0 (2.6)	2.5 (1.0)
Fibrinogen, g/l			
Before	2.6 (0.5)	2.5 (0.5)	2.5 (0.5)
After	2.6 (0.5)	2.6 (0.5)	2.6 (0.5)
3.5 h after	2.6 (0.5)	2.5 (0.4)	2.6 (0.4)
Factor VIII, kiE/l			
Before	1.2 (0.3)	1.2 (0.3)	1.3 (0.4)
After	1.1 (0.3)	1.2 (0.4)	1.2 (0.3)
3.5 h after	1.3 (0.4)	1.2 (0.4)	1.2 (0.3)
von Willebrand factor, kiE/l			
Before	0.9 (0.3)	0.9 (0.3)	1.0 (0.3)
After	0.9 (0.3)	0.9 (0.3)	0.9 (0.3)
3.5 h after	1.0 (0.3)	1.1 (0.7)	0.9 (0.3)
Clara cell protein CC16, ng/ml			
Before	19.3 (10.7)	21.4 (10.1)	19.6 (9.1)
After	19.2 (9.4)	20.0 (9.0)	19.6 (9.1)
3.5 h after	21.7 (10.8)	18.6 (9.0)	20.1 (12.4)

	0 ppm	High ACR
Interleukin-6, pg/ml		
Before	0.8 (0.4)	0.9 (0.4)
After	0.7 (0.3)	0.7 (0.3)
3.5 h after	0.9 (0.5)	0.7 (0.3)

Copied from Table 5 on page 817 of MRID 51570802

Table A.3. Inflammatory markers and cell counts (median and ranges) in sputum samples collected from 18 volunteers after 2 hours of exposure to 0 ppm and 0.1 ppm (high) acrolein.

	0 ppm	High ACR
Exposure condition		
Sputum cells, cells/mg	328 (62–1146)	280 (60–2585)
Interleukin-6, pg/ml	15.4 (1.0–46.1)	15.8 (2.5–133.4)
Interleukin-8, pg/ml	439 (107–2545)	426 (163–1573)
Differential cell count (%)		
Macrophages	56.0 (33.5–63.5)	55.3 (30.0–63.5)
Neutrophils	36.1 (28.5–51.0)	37.9 (20.0–63.0)
Eosinophils	1.8 (0–37.5)	2.0 (0–20.5)
Lymphocytes	5 (1.0–7.7)	4.3 (1.0–16.1)

Copied from Table 6 on page 818 of MRID 51570802

Table A.4. Pulmonary function and blocking index of 18 volunteers (arithmetic means ± SD) measured before, immediately after, and 3.5 hours after a 2-hour exposure to 0, 0.05 (low), and 0.1 (high) ppm acrolein (ACR) and ethyl acetate (EA).

	Exposure condition					
	0 ppm	Low ACR	High ACR	EA	Low ACR + EA	High ACR + EA
Vital capacity (VC), l						
Before	5.0 (1.7)	5.0 (1.6)	5.0 (1.7)	5.0 (1.7)	4.9 (1.6)	5.0 (1.6)
After	5.0 (1.7)	5.0 (1.7)	5.1 (1.8)	4.9 (1.9)	4.9 (1.7)	5.1 (1.7)
3.5 h after	5.0 (1.7)	5.1 (1.6)	5.2 (1.8)	5.1 (1.8)	5.0 (1.6)	5.1 (1.7)
Forced vital capacity (FVC), l						
Before	5.0 (1.7)	5.1 (1.6)	5.0 (1.7)	5.0 (1.7)	4.9 (1.6)	5.0 (1.7)
After	5.0 (1.7)	5.1 (1.6)	5.1 (1.7)	5.1 (1.7)	4.9 (1.6)	5.0 (1.7)
3.5 h after	5.0 (1.7)	5.1 (1.7)	5.1 (1.7)	5.2 (1.8)	4.9 (1.6)	5.1 (1.7)
Forced expiratory volume in 1 s (FEV1), l						
Before	4.1 (1.3)	4.1 (1.3)	4.1 (1.3)	4.2 (1.3)	4.0 (1.2)	4.1 (1.3)
After	4.1 (1.4)	4.2 (1.3)	4.2 (1.3)	4.2 (1.3)	4.1 (1.2)	4.1 (1.3)
3.5 h after	4.1 (1.3)	4.3 (1.3)	4.2 (1.3)	4.2 (1.3)	4.0 (1.2)	4.2 (1.3)
FEV1/VC, %						
Before	84 (6.5)	83 (7.4)	83 (6.5)	83 (6.8)	83 (7.0)	82 (6.1)
After	84 (6.1)	83 (7.5)	83 (7.0)	84 (7.9)	84 (6.3)	83 (6.5)
3.5 h after	83 (5.7)	83 (3.5)	83 (5.2)	84 (6.3)	82 (6.7)	83 (5.5)
FEV1/FVC, %						
Before	83 (6.1)	82 (6.7)	83 (7.0)	84 (6.6)	83 (6.8)	83 (6.5)
After	83 (6.1)	82 (6.5)	83 (6.3)	83 (6.9)	83 (6.4)	83 (7.2)
3.5 h after	83 (5.2)	83 (7.0)	83 (5.6)	84 (6.7)	84 (6.3)	84 (6.8)
Peak expiratory flow (PEF), l/min						
Before	500 (141)	511 (145)	499 (138)	465 (67)	502 (143)	503 (148)
After	500 (143)	504 (149)	509 (150)	510 (142)	497 (138)	502 (147)
3.5 h after	504 (138)	518 (145)	519 (137)	510 (142)	498 (136)	504 (135)
Forced expiratory flow at 25% of FVC (FEF25), l/s						
Before	7.1 (1.7)	7.3 (2.0)	7.3 (1.8)	7.3 (1.9)	7.1 (1.9)	7.1 (2.0)
After	7.2 (1.8)	7.3 (2.1)	7.3 (1.9)	7.3 (1.9)	7.2 (2.0)	7.3 (2.0)
3.5 h after	7.3 (1.8)	7.6 (2.1)	7.4 (1.8)	7.5 (1.9)	7.0 (1.9)	7.4 (1.9)
Forced expiratory flow at 50% of FVC (FEF50), l/s						
Before	4.7 (1.5)	4.6 (1.6)	4.7 (1.6)	4.8 (1.6)	4.5 (1.5)	4.7 (1.7)
After	4.6 (1.5)	4.7 (1.7)	4.8 (1.6)	4.8 (1.6)	4.8 (1.5)	4.8 (1.6)
3.5 h after	4.7 (1.5)	5.0 (1.6)	4.8 (1.6)	5.0 (1.8)	4.6 (1.5)	4.9 (1.7)
Forced expiratory flow at 75% of FVC (FEF75), l/s						
Before	2.1 (0.7)	2.1 (1.0)	2.0 (1.0)	2.2 (0.9)	2.0 (0.7)	2.1 (0.9)
After	2.1 (0.9)	2.1 (0.9)	2.1 (1.0)	2.2 (1.0)	2.1 (0.8)	2.3 (1.7)
3.5 h after	2.0 (0.8)	2.3 (0.9)	2.1 (0.9)	2.2 (1.0)	2.1 (0.8)	2.2 (0.9)
Blocking index (BI)						
Before	0.53 (0.13)	0.51 (0.17)	0.51 (0.17)	0.52 (0.09)	0.49 (0.14)	0.50 (0.15)
After	0.54 (0.12)	0.53 (0.15)	0.53 (0.15)	0.54 (0.09)	0.50 (0.13)	0.53 (0.15)
3.5 h after	0.53 (0.12)	0.53 (0.15)	0.53 (0.15)	0.54 (0.10)	0.50 (0.14)	0.53 (0.14)

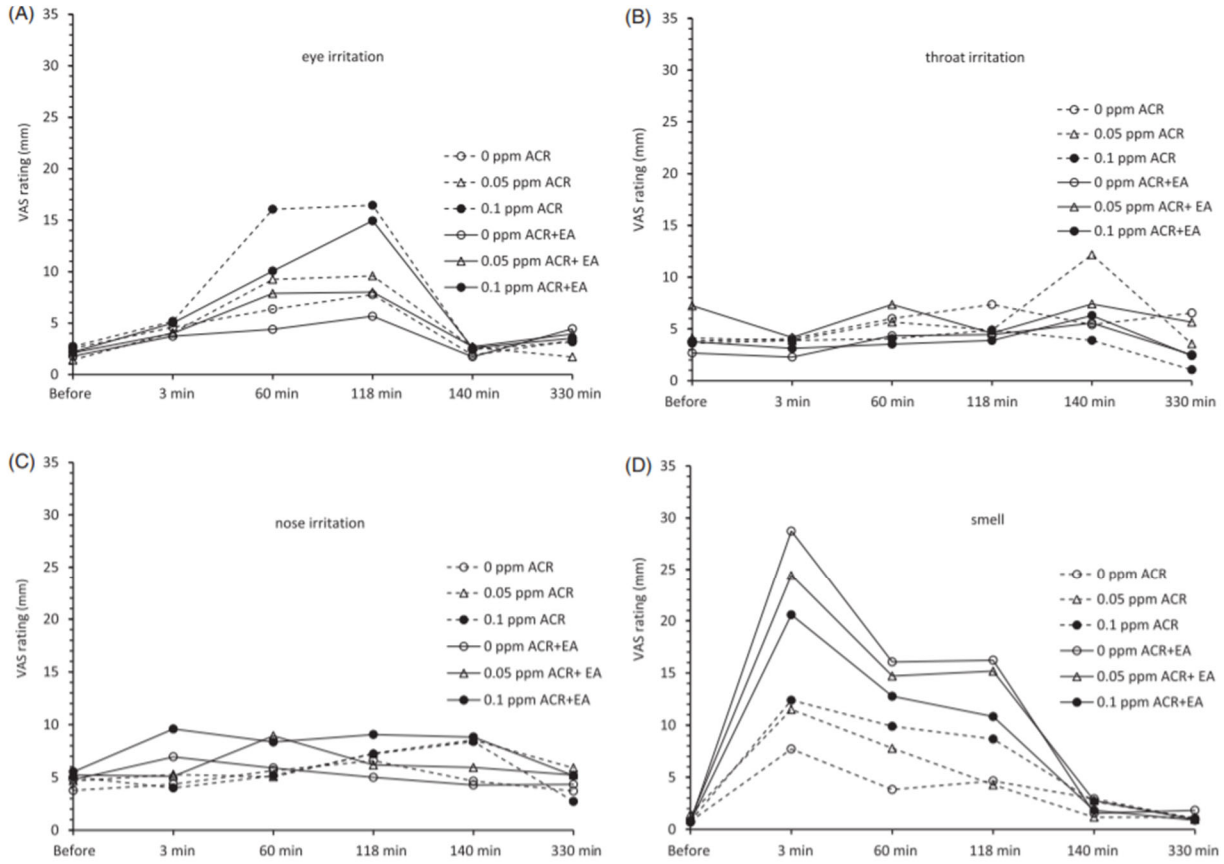
Copied from Table 3 on page 816 of MRID 51570802

Table A.5. Acoustic rhinometry measurements of 18 volunteers (arithmetic means ± SD) measured before, immediately after, and 3.5 hours after a 2-hour exposure to 0, 0.05 (low), and 0.1 (high) ppm acrolein (ACR) and ethyl acetate (EA).

	Exposure condition					
	0 ppm	Low ACR	High ACR	EA	Low ACR + EA	High ACR + EA
Minimum cross-sectional area 0–22 mm (MCA1), cm^2						
Before	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)	1.2 (0.3)	1.2 (0.2)
After	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)
3.5 h after	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)	1.1 (0.2)	1.2 (0.2)	1.2 (0.2)
Minimum cross-sectional area 23–54 mm (MCA2), cm^2						
Before	1.6 (0.6)	1.6 (0.6)	1.6 (0.3)	1.6 (0.5)	1.5 (0.4)	1.6 (0.4)
After	1.7 (0.5)	1.7 (0.6)	1.7 (0.5)	1.7 (0.5)	1.6 (0.4)	1.7 (0.6)
3.5 h after	1.6 (0.5)	1.5 (0.6)	1.6 (0.5)	1.7 (0.5)	1.5 (0.4)	1.6 (0.5)
Volume 0–22 mm (VOL1), ml						
Before	4.1 (0.7)	4.1 (0.7)	4.2 (0.6)	4.2 (0.7)	4.1 (0.7)	4.1 (0.6)
After	4.0 (1.1)	4.2 (0.6)	4.1 (0.6)	4.2 (0.7)	4.1 (0.6)	4.2 (0.7)
3.5 h after	3.9 (1.1)	4.1 (0.6)	4.1 (0.7)	3.9 (0.9)	4.0 (0.6)	4.1 (0.6)
Volume 23–54 mm (VOL2), ml						
Before	12.0 (3.5)	12.7 (6.1)	13.6 (2.8)	12.6 (3.3)	11.6 (2.9)	12.3 (2.8)
After	12.1 (3.4)	12.4 (5.6)	12.0 (3.5)	12.0 (3.5)	11.8 (3.4)	11.9 (4.4)
3.5 h after	16.6 (20.6)	10.7 (4.2)	11.5 (2.9)	11.6 (3.4)	11.1 (3.0)	11.3 (3.4)

Copied from Table 4 on page 817 of MRID 51570802

Figure A.1. Select symptom ratings of 18 volunteers measured before and 3, 60, 118, 140, 330, and 1440 minutes after a 2-hour exposure to acrolein (ACR) and ethyl acetate (EA).



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