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### **Hydroprocessed Esters and Fatty Acids (HEFA) Bio-Based Jet Fuels: Sensory Irritation Study and Human Health Hazard Assessment**

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## PREFACE

Funding for this project was provided through the Alternative Fuels Certification Office (AFLCMC/WNN). This research was conducted either under contract FA8650-10-2-6062 with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF) or under Navy work unit number H1104 under the management of Naval Medical Research Unit – Dayton (NAMRU-D). The program manager for the HJF contract was David R. Mattie, PhD (711 HPW/RHDJ), who was also the technical manager for this project. The technical manager for NAMRU-D was Karen Mummy, PhD.

The sensory irritation study protocol was designed to be in compliance with the ASTM, International (formerly known as the American Society for Testing and Materials, West Conshohocken PA) Method E 981-04: Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals (ASTM, 2004). This study was not performed in a Good Laboratory Practice (GLP) Standards certified laboratory, and therefore there is no certification of compliance with GLP regulations (40 CFR Part 792). However, this study was conducted with an effort to follow the intent and purpose of GLP requirements.

The sensory irritation study protocol was approved by the Wright-Patterson AFB Installation Animal Care and Use Committee (IACUC) as protocol number F-WA-2010-0125-A. The sensory irritation study and all studies reviewed in this report were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011).

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## 1.0 SUMMARY

The purpose of this technical report is two-fold. First, this technical report presents the results of a sensory irritation assay conducted in male Swiss-Webster mice exposed to different concentrations of hydroprocessed esters and fatty acids (HEFA) bio-based jet fuels, HEFA-Camelina (HEFA-C) and HEFA-Tallow (HEFA-T). The mice were exposed via a modified nose-only plethysmograph tube to each fuel, administered as an aerosol/vapor combination. This fuel type was previously termed as hydro renewable jet (HRJ) fuel. Results of the breathing rates were correlated with the exposure concentration to predict a concentration that produces a 50 percent reduction in respiratory rate.

The concentrations of the HEFA-C aerosol/vapor were 1371, 4603, 5670, 8878 and 8513 mg/m<sup>3</sup>, with the corresponding percent decrease in respiratory rate during the first 5 minutes of exposure at 31, 45, 33, 30 and 38 percent, respectively. The concentrations of the HEFA-T aerosol/vapor were 1794, 4795, 8094, and 13,306 mg/m<sup>3</sup>, with the corresponding percent decrease in respiratory rate during the first 5 minutes of exposure at 24, 26, 33 and 11 percent, respectively, for the four aerosol/vapor exposures; for the vapor only exposure, the concentration of HEFA-T was 4299 mg/m<sup>3</sup> and the corresponding decrease in respiratory rate was 19 percent. The exposure concentrations were well tolerated by the mice and did not produce a classic sensory irritation response.

For HEFA-C, the predicted concentration that produces a 50 percent decrease in respiratory rate (RD<sub>50</sub>) was greater than 11,755 mg/m<sup>3</sup>, the highest concentration for these exposures, but was not verified experimentally because the highest attainable concentration before saturation of the analytical method was 14,920 mg/m<sup>3</sup>. For HEFA-T, the predicted RD<sub>50</sub> was greater than 13,306 mg/m<sup>3</sup>, but again was not experimentally verified due to saturation of the analytical method.

This technical report also summarizes toxicity data for HEFA fuels, including HEFA-C, HEFA-T and a HEFA fuel with a feedstock of mixed animal fats and oils (HEFA-F). HEFA toxicity data are then compared with toxicity data from synthetic paraffinic kerosene (SPK) alternative jet fuel and petroleum derived JP-8. The toxicity data are used to develop a comparative occupational exposure limit (OEL) for HEFA fuels that concurs with the current JP-8 OEL (200 mg/m<sup>3</sup>).

## 2.0 INTRODUCTION

The Office of the Secretary of Defense Assured Fuels Initiative is pursuing domestically produced alternative fuels for military use to decrease dependence on foreign oil sources (Blackwell, 2007). Biobased jet fuels are being formulated to complement or replace petroleum-derived JP-8, the traditional military fuel. As the overall chemical composition is different from JP-8, the potential health effects must be studied during development of the alternative jet fuels. The toxicity of an alternative fuel called synthetic paraffinic kerosene (SPK) made from natural gas using the Fischer-Tropsch (F-T) process was recently evaluated by Hinz *et al.* (2012); the occupational exposure limit (OEL) for SPK fuel was proposed to be equivalent to the JP-8 OEL of 200 mg/m<sup>3</sup> (NRC, 2003).

A type of bio-based jet fuel is also being considered for military use; this fuel is called hydroprocessed esters and fatty acids (HEFA) jet fuel, formerly known as hydro renewable jet (HRJ) fuel. HEFA fuels are produced from renewable biological feedstocks using a process called hydro-treatment (water and high pressure). Feedstocks include rendered animal fat (tallow, HEFA-T), mixed fats and oils (HEFA-F) and plant oil, such as that from the camelina plant (*Camelina sativa*, HEFA-C).

Inhalation is one of the primary routes of exposure for fuels, so it is essential to evaluate the pulmonary effects of breathing concentrations of jet fuel. Jet fuel exposures may occur as vapor only or both vapor and aerosol (fuel droplets) in combination. Upper airway sensory irritation is the involuntary decrease in respiration when an animal is exposed to an irritant. It is expressed as the RD<sub>50</sub> or the concentration that produces a 50 percent decrease in respiratory rate (Bos *et al.*, 2002).

The purpose of this report is two-fold. First, two fuels from different feedstocks, HEFA-C and HEFA-T, are investigated for sensory irritation potential. Second, the toxicity of the HEFA fuels is reviewed and an OEL is proposed for use in handling HEFA fuels alone or in a blend with petroleum-derived JP-8.

## 3.0 METHODS: SENSORY IRRITATION ASSAY

The complete study protocol is found in Appendix A.

### 3.1 Study Design

Animals were exposed to five concentrations of HEFA-C or five concentrations of HEFA-T. The exposure concentrations consisted of a mixture of aerosol and vapor, except for a single exposure concentration of HEFA-T that was vapor only. An initial exposure level of 2000 mg/m<sup>3</sup> was selected based on literature results of previous inhalation studies with jet fuels of similar chemical composition. This concentration has been used in acute, five-day, ten-day and 90-day studies with HEFA fuels (Mattie *et al.*, 2011a; Wong *et al.*, 2013). This concentration has also been utilized in toxicity studies of other fuels, including acute, two-week, and 90-day

inhalation toxicity studies of F-T jet fuel (Mattie *et al.*, 2011a and 2011b) and in two 14-day JP-8 studies (Sweeney *et al.*, 2013). Results obtained from the initial exposure level of 2000 mg/m<sup>3</sup> were used to guide additional target exposure concentrations by determining the percent decrease in respiratory rate and by comparing the change in respiratory rate to the previous jet fuel studies. Subsequent concentrations were increased to establish a dose curve that identifies the level that produces 50 percent reduction in respiratory rate.

### **3.2 Animals and Animal Husbandry**

A total of 48 male Swiss-Webster (*Mus musculus*) mice [CrI:CFW(SW)] at approximately 4 weeks old and 16 to 21 g bodyweight were purchased from Charles River Laboratories (Wilmington MA). Eight mice were used for training and 20 were used for each HEFA jet fuel (5 concentrations, 4 mice per concentration). Food and water were made available *ad libitum* during periods of non-exposure. Mice were acclimated to the facility for seven days. Animals were delivered from the Vivarium under a tarp to the exposure laboratory each morning/afternoon prior to exposures and returned to the Vivarium for euthanasia after completion of exposure.

### **3.3 Test Substance**

The HEFA-C and HEFA-T jet fuels were obtained from the manufacturer (UOP LLC, a Honeywell Company, Des Plaines IL) by the Air Force Research Laboratory (AFRL) Fuels Branch at Wright Patterson Air Force Base (AFB) OH. An additive package consisting of chemicals normally added to JP-8 jet fuel was mixed with each HEFA fuel by the Fuels Branch. The combination of HEFA-C fuel with additives was designated as POSF log book number 6183 by the Fuels Branch. The combination of HEFA-T fuel with additives was designated as POSF log book number 6308.

### **3.4 Inhalation Exposure System**

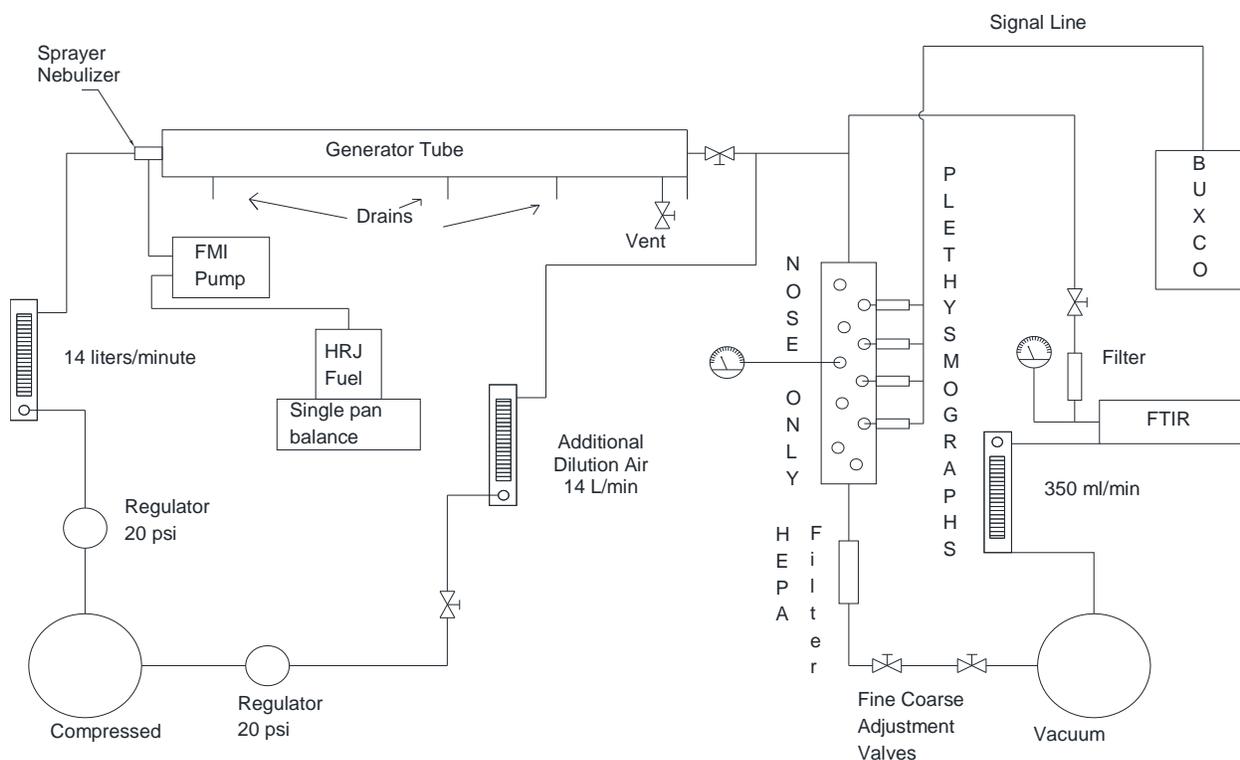
Mice were exposed using a 52-position Cannon nose-only exposure system (Lab Products, Maywood NJ). One nose-only exposure system (NOES) was used for all exposures. Mice were exposed in nose-only plethysmographs (Buxco Research Systems, Wilmington NC) with a neck dam sealing the head chamber from the body chamber. Neck dams were a latex material with either an 8 or 9 mm hole for the neck seal. The head chamber was attached to the NOES and the body chamber was attached to the head chamber. Respiratory rates were monitored with Buxco BioSystem FinePoint Software (Buxco Research Systems) using a differential pressure transducer connected to a pneumotachograph with a fine-screen pressure plate to detect pressure changes resulting from volume changes from the inspirations and expirations of the mouse.

The exposure atmosphere flowed from the generator at a total flow rate of approximately 14 L/minute through the central, inner plenum and out through the delivery nozzles into the breathing zone of each animal at approximately 0.27 L/minute per open port. The NOES was

fitted with connections for a differential pressure gauge to monitor static pressure at an open port. The outer plenum of the NOES carried the animal's exhaled breath and excess test atmosphere to an exhaust set at a flow rate of approximately 14 L/minute. The NOES operated as a push-pull system where the air supply was positive and the exhaust flow was negative. The exhaust was set at the target flow rate and the supply was adjusted to maintain a static pressure (Magnehelic® Gage, Dwyer Instrument Co., Michigan City IN) in the range of 0.00 to -0.10 inches of water.

Temperature and relative humidity were measured by a hygrometer (Model 445703, Extech Instruments, Inc., Nashua NH) located near the NOES. The target temperature range was 64 to 79 °F and the target relative humidity range was 30 to 70 percent.

A fluid metering pump (Fluid Metering Inc. (FMI), Model QG20, Syosset NY) was used to deliver one of the HEFA fuels from a small, closed, amber glass bottle to a nebulizer (Model SU1A-SS, Spray Systems, Milford OH) and was adjusted to deliver a flow corresponding to the desired concentration of HEFA fuel. The FMI pump flow rates were calibrated for a range of flows expected to create target concentrations for the HEFA exposures. FMI pump calibrations are shown in Appendix B. The generator tube diameter was stepped down in stages from four inches to one half inch at the outlet. Site gauges with drain valves allowed removal of condensed fuel, while the vent valve allowed the generator to be brought up to the target concentration prior to the start of the exposure. A shut off valve at the end of the generator allowed it to be isolated from the exposure system, while an alternate compressed air line provided clean air to the tower before and after the exposure. The fine and coarse adjustment valves maintain a slight sub-ambient pressure in the nose only tower. The Buxco system records real time data from the plethysmographs. Figure 1 shows a diagrammatic representation of the exposure system.

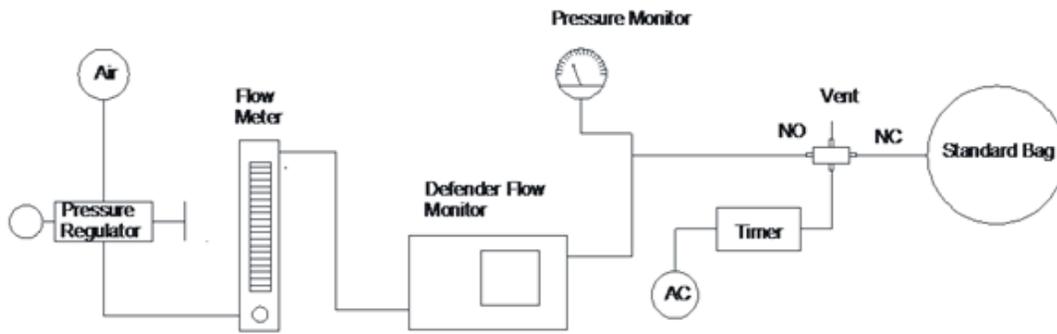


**Figure 1. Diagrammatic Representation of the Exposure System**

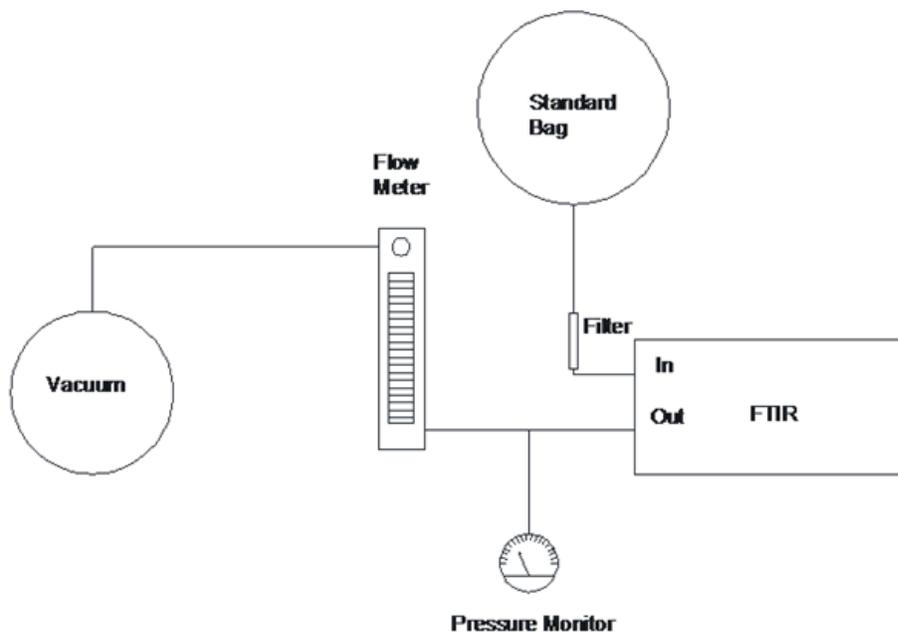
### 3.5 Test Atmosphere Monitoring

A Fourier Transform Infrared Spectrophotometer (FTIR, Model 380, Thermo Scientific, Waltham MA) was calibrated using standard bag methodology (Figure 2). Tedlar® (DuPont, Wilmington DE) bags containing 10 liters of air were prepared using a standard bag preparation station. A timer was used with a flow monitor to fill the standard bag with a known volume of air. The standard calibration bags were injected with sufficient HEFA fuel to yield the desired concentrations. After injection the bag was warmed with a hot air gun to assure that the fuel was totally volatilized. The bags were allowed to cool for approximately five minutes to ambient temperature, in order to establish equilibrium between the ambient aerosol and vapor components. The bags were agitated to assure thorough mixing.

A sample of the bag was drawn through a filter and into the FTIR at a flow rate of 350 mL per minute to obtain a steady absorbance value that was correlated with the standard calibration bag nominal concentration (Figure 3). This procedure was replicated three times for each concentration. The 47 mm glass fiber filter was used to remove aerosol particles prior to entering the FTIR analytical cell. The FTIR was equipped with a 10-cm glass gas analysis cell. FTIR calibration data are found in Appendix C.



**Figure 2. Standard Calibration Bag Preparation System.** AC indicates the power source (120 volts alternating current); NO indicates a normally open solenoid while NC is a normally closed solenoid.



**Figure 3. FTIR Calibration System**

The measured maximum absorption versus bag concentration ( $\text{mg}/\text{m}^3$ ) was recorded for the 5 concentrations (15 calibration points) and an average for each of the 3 values for each concentration was calculated. The data were plotted and a best-fit regression equation was determined. An estimated concentration was calculated from the nominal concentration applied to the data regression equation. The comparison of the nominal bag concentration to the estimated concentration, expressed as a ratio, is an indication of how well the data regression will predict real time samples.

For HEFA-C, the highest point of the calibration curve was 14,920 mg/m<sup>3</sup>. A higher concentration calibration bag standard was made at 18,650 mg/m<sup>3</sup>, but there was very little difference in the resulting FTIR absorbance values. It was assumed that the 10-cm FTIR cell had reached a level of saturation for HEFA-C.

For HEFA-T, the highest point of the calibration curve was 11,250 mg/m<sup>3</sup>. A higher concentration calibration bag standard was made at 15,000 mg/m<sup>3</sup>, but again there was very little difference in the resulting FTIR absorbance values and saturation was assumed for HEFA-T. Concentrations higher than 11,250 mg/m<sup>3</sup> were measured using the FTIR by extending the linear regression line past the highest calibration point.

Concentration estimations were made prior to the study start for each of the FMI pump calibration settings and trial runs were conducted. The FTIR was used to sample the nose only exposure system during each trial run and the actual concentration was determined based on the regression equation. The ratio of the expected concentration to actual concentration was calculated to show the efficiency of the HEFA exposure system. Trial run data are shown in Appendix D.

### **3.6 Exposure Data Collection**

All mouse respiratory data were collected and stored electronically by the Buxco BioSystem FinePoint Software (Buxco Research Systems, Wilmington NC).

Temperature, humidity and NOES static pressure were manually recorded in the study file at least 3 times during the 50-minute trial. At the end of each exposure, the average value, standard deviation, minimum value, maximum value, and total number of data values were calculated for each environmental parameter for exposure. Supply air flow and exhaust flow rate were recorded at least once and maintained at the same setting during the exposure. The FMI pump flow rate was recorded when changes occurred; otherwise the FMI pump was maintained at the recorded setting. To help reach the target concentration as quickly as possible during the 30-minute exposure, the FMI pump flow rate was initially set higher than the anticipated flow rate needed to achieve the target concentration and was reduced to the final flow rate.

FTIR data were collected approximately every 20 seconds during the 10 minute pre exposure period, the 30 minute exposure period and the 10 minute recovery period. Once the exposure ended, the data were transported into a Microsoft Excel file and the data were evaluated. An average concentration was determined for the time period of HEFA fuel concentration stability to represent the exposure concentration.

### **3.7 Respiratory Rate Measurement during Exposure**

Prior to exposure, animals were loaded into modified nose-only plethysmograph tubes and placed onto the NOES. Respiratory rates were monitored and recorded for a 10 minute acclimation period, during which animals were breathing high-efficiency particulate air (HEPA)-filtered house air. Following the acclimation period, the respiratory rates were monitored and recorded for a 30 minute exposure and a 10 minute post exposure recovery period.

More specifically, all respiratory rates were sampled at 1 second intervals. A data point was collected every 1 second for the entire time the mice were in the plethysmographs. At the end of the exposure, data were transferred into a spreadsheet where average respiratory rates for each mouse were calculated. During the 10-minute baseline period, 1 minute averages were calculated during the first 8.5 minutes and every 15 seconds for the final 1.5 minutes. During the 30-minute exposure period, 15 second averages were calculated during the first 5 minutes and every 3 minutes for the final 25 minutes. During the 10-minute recovery period, 1 minute averages were calculated for the entire 10 minute period. The mean of four individual averages was taken to determine the final respiratory rates for each concentration.

### **3.8 Exposure Day**

Up to two exposures were completed within a calendar day. For each exposure, the animals were delivered from the Vivarium in polycarbonate domiciliary cages on a cart under a tarp to the exposure laboratory. Mice were loaded into the modified nose-only plethysmograph tubes and onto the exposure tower within 30 minutes after arrival at the exposure laboratory. Immediately following the baseline, exposure and recovery periods, individual animals were unloaded from the nose only tubes and returned to its domiciliary cage. Animals were returned to the Vivarium and euthanized within 1 hour after the end of the exposure by placing them individually in an enclosed chamber for carbon dioxide inhalation as anesthesia, followed by cervical dislocation.

On the day of exposure, clinical observations were performed twice pre-exposure, during the exposure, and post-exposure. Clinical observations included examination of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia, as well as evaluations of respiration, circulatory effects, autonomic effects, central nervous system effects and reactivity to handling and sensory stimuli. Following euthanasia, a complete macroscopic gross necropsy was performed on all animals.

## 4.0 RESULTS: SENSORY IRRITATION

### 4.1 Animals

*4.1.1 HEFA-C.* The mice were weighed on the day after receipt, during group assignment, and on the day of exposure prior to transport. On the day after arrival, the mice weighed 19.56 g to 22.14 g (average of 20.86 g). During group assignment, the mice weighed 22.54 g to 28.26 (average of 25.52 g). The mice were divided into five groups of four mice per group. Due to the increased weight of some of the mice, the four heaviest mice were assigned to the first exposure group, the next four heaviest mice to the second, and so on. The four lightest mice were assigned to fifth group. On exposure days, the mice weighed 24.01 g to 29.83 g, with an average of 27.07 g. Individual body weights are tabulated in Appendix E.

No overt signs of toxicity were observed. The only non-normal observations noted were “Wet around mouth” and “Wet around mouth and head” during the exposure (8 out of 20 mice) and post-exposure (6 out of 20 mice). During the 1979 mg/m<sup>3</sup> exposure, one animal was noted as having a fogged-up tube 11 minutes following the start of the exposure period. One animal from the 5182 mg/m<sup>3</sup> exposure group was noted as being “Wet on head.” All other animals were reported as being “Normal (No abnormal observations).”

*4.1.2 HEFA-T.* The mice were weighed on the day after arrival, during group assignment and on the day of exposure prior to transport. On the day after arrival, the mice weighed 16.82 g to 19.44 g (average 17.78 g). During group assignment, the mice weighed 22.57 g to 27.59 (average 24.45 g). The mice were divided into five groups of four mice per group. The four heaviest mice were assigned to the first exposure group, the next four heaviest mice to the second, and so on, as above. The four lightest mice were assigned to the aerosol only exposure. On exposure days, the mice weighed 23.68 g to 27.50 g, with an average of 25.36 g.

No overt signs of toxicity were observed. The only non-normal observation noted was “Wet around mouth” during the exposure (2 out of 19 mice) and post-exposure (2 out of 19 mice). During the 5670 mg/m<sup>3</sup> group baseline exposure, one mouse was observed as “Wet around mouth.” Following the 1371 mg/m<sup>3</sup> exposure, one animal appeared “Normal” when removed from the tube, but was gasping and prostrate 2 minutes after removal from the nose-only tube, moribund 6 minutes following removal, and found dead 13 minutes after removal (animal died during transport to Vivarium for euthanasia). The Vivarium staff performed a necropsy on this mouse and no findings were noted. Prior to the baseline of the aerosol only exposure, one animal was found dead in the nose-only tube; therefore, averages were based on observations from only three mice.

A complete macroscopic gross necropsy was performed on all animals to include observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia. All animals were reported as being “Normal (No abnormal observations).” The animal that died in

the nose-only tube prior to the baseline of aerosol only exposure did not have a necropsy performed since this animal was found dead prior to the beginning of the exposure to HEFA-T.

## 4.2 Test Atmosphere Generation

The FMI pump settings versus final concentrations shown in Appendix D were used as a starting point for each experiment. In order to assure a more rapid rise of the initial concentration, the FMI pump was set to deliver a somewhat higher than desired final concentration for the first few minutes and then reset to the final setting for the anticipated target concentration.

## 4.3 Exposure Concentrations

**4.3.1 HEFA-C.** Thirty-minute average concentrations in the HEFA-C exposures were calculated to be 1979, 5182, 7048, 9698 and 11755 mg/m<sup>3</sup>. The average aerosol/vapor concentrations of HEFA-C for the first 5 minutes of exposure were 1371, 4603, 5670, 8878 or 8513 mg/m<sup>3</sup>, respectively. The highest attainable concentration before saturation was reached for HEFA-C was approximately 14,920 mg/m<sup>3</sup>. Oxygen levels were checked during the prestudy testing at the highest concentration (approximately 12000 mg/m<sup>3</sup>) and were 20.6 percent O<sub>2</sub> with room air levels measuring 20.9 percent O<sub>2</sub>. The mass median aerodynamic diameter determined by cascade impactor samples during each exposure ranged from 3.5 to 3.9 μm. The vapor only exposure was not completed because of the inconsistent respiratory decrease results obtained in the first four vapor/aerosol exposures; instead a fifth vapor/aerosol exposure was performed. FTIR graphs for the 30-minute exposures are shown in Appendix F.

**4.3.2 HEFA-T.** Thirty-minute average concentrations in the HEFA-T exposures were calculated to be 2107, 5983, 9690 and 15043 mg/m<sup>3</sup>, while the 30-minute vapor only concentration average was 5955 mg/m<sup>3</sup>. The highest attainable concentration before saturation was reached for HEFA-C was approximately 11,250 mg/m<sup>3</sup>. Oxygen levels checked during the prestudy testing at the highest concentration (about 10,000 mg/m<sup>3</sup>) remained the same as before (20.6 percent O<sub>2</sub> inside the chamber and 20.9 percent O<sub>2</sub> in the room). The mass median aerodynamic diameter determined by cascade impactor samples during each exposure ranged from 3.9 to 4.5 μm. FTIR graphs for the 30-minute exposures are shown in Appendix F.

## 4.4 Exposure Conditions

**4.4.1 HEFA-C.** The data for environmental parameters for each exposure are provided in Table 1. The particle size distribution of the aerosol portion was higher than expected with mass median aerodynamic diameters (MMAD) that ranged from 3.5 to 3.9 μm and geometric standard deviation (GSD) values that ranged from 2.4 to 3.5 μm. This could mean that a portion of the aerosol component of the jet fuel exposures was not in the respirable range for a mouse, which is

generally considered to be between 2 and 3  $\mu\text{m}$ . The aerosol portion of the jet fuel test atmosphere ranged from 14 to 22 percent of the vapor portion determined by comparing the gravimetric filter results to the measurements by the FTIR.

**4.4.2 HEFA-T.** The data for environmental parameters for each exposure are provided in Table 2. The particle size distribution of the aerosol portion again was higher than expected, with MMAD ranging from 3.9 to 4.5  $\mu\text{m}$  and GSD values ranging from 2.9 to 4.4. A portion of the aerosol component of the jet fuel exposures was likely not in the respirable range for a mouse, which is generally considered to be between 2 and 3  $\mu\text{m}$ . The aerosol portion of the jet fuel test atmosphere ranged from 15 to 22 percent of the vapor portion determined by comparing the gravimetric filter results from the cascade impactor to the measurements by the FTIR.

The higher than expected particle size range may be due to some evaporation of HEFA-T from the individual cascade impactor stages, thereby creating artificial masses. To check the cascade impactor results, samples were taken using an aerosol particle sizer spectrometer (Model APS3321, TSI Inc., Shoreview MN). The particle sizes ranged from 2.5 to 3.0  $\mu\text{m}$  and GSD values ranged from 1.5 to 1.7. These results may support the theory that the cascade impactor provided particle size results that were higher than the actual particle size of the HEFA-T aerosol. Additional work needs to be conducted to compare these different particle sizing methods.

**Table 1. Inhalation Exposure Summary Data for HEFA-C Assay: Exposure System Environmental Parameters**

			Group 1	Group 2	Group 3	Group 4	Group 5
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> ) [a]	Mean	1979	11755	9698	5182	7048
		St Dev	333	1748	1116	464	759
		Min	161	3348	1068	1604	2660
		Max	2164	12880	10771	5507	7875
		Count	89	88	89	88	89
Temp	(°F)	Mean	64.6	65.6	64.3	66.7	65.5
		St Dev	0.3	0.3	0.5	0.3	0.6
		Count	3	3	2	3	2
Humidity	(%)	Mean	32	28	28	26	29
		St Dev	1	1	0	0	1
		Count	3	3	2	3	2
Static Pressure	(inches H <sub>2</sub> O)	Mean	-0.10	-0.13	-0.10	-0.10	-0.05
		St Dev	0.00	0.06	0.00	0.00	0.00
		Count	3	3	2	3	2
Supply Air Flow Rate	(L/min)		14	14	14	14	14
Exhaust Flow Rate	(L/min)		16	16	16	16	16
Pump Flow Rate Initial	(mL/min)		0.031	0.187	0.140	0.065	0.099
Pump Flow Rate Final	(mL/min)		0.025	0.174	0.124	0.059	0.096
Gravimetric Filter	(mg/m <sup>3</sup> )		492	2387	1643	1128	1609
% Aerosol	(%)		14	19	17	21	22
Particle Size [b]	(µm)	MMAD	3.5	3.5	3.9	3.6	3.8
		GSD	2.4	3.0	3.5	2.9	2.9

[a]: Average for the 30-minute exposure; [b]: Determined by Cascade Impactor during each exposure; count = number of readings; GSD: geometric standard deviation; H<sub>2</sub>O: water; Max: maximum; min: minutes; Min: minimum; MMAD: mass median aerodynamic diameters; St Dev: standard deviation

**Table 2. Inhalation Exposure Summary Data for HEFA-T Assay: Exposure System Environmental Parameters**

			Group 1	Group 2	Group 3	Group 4	Group 5 (Vapor)
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> ) [a]	Mean	<b>2107</b>	<b>15043</b>	<b>9690</b>	<b>5983</b>	<b>5955</b>
		St Dev	210	1295	1458	669	965
		Min	713	5965	405	2740	666
		Max	2256	16439	10672	7171	6922
		Count	88	87	88	87	88
Temp	(°F)	Mean	<b>73.9</b>	<b>74.2</b>	<b>75.2</b>	<b>74.9</b>	<b>75.7</b>
		St Dev	0.1	0.5	0.2	0.3	0.2
		Count	3	2	3	3	3
Humidity	(%)	Mean	<b>19</b>	<b>19</b>	<b>22</b>	<b>36</b>	<b>37</b>
		St Dev	0	0	0	0	0
		Count	3	2	3	3	3
Static Pressure	(inches H <sub>2</sub> O)	Mean	<b>-0.10</b>				
		St Dev	0.00	[c]	[c]	[c]	[c]
		Count	3				
Supply Air Flow Rate	(L/min)		14	14	14	14	14
Exhaust Flow Rate	(L/min)		14	14.5	14.5	14.5	14.5
Pump Flow Rate Initial	(mg/min)		31	243	146	77	108
Pump Flow Rate Final	(mg/min)		27	231	146	85	108
Gravimetric Filter	(mg/m <sup>3</sup> )		305	2544	1787	1290	27
% Aerosol	(%)		15	17	18	22	0
Particle Size [b]	(µm)	MMAD	4.0	4.2	4.5	3.9	[d]
		GSD	4.4	3.2	3.9	3.0	

[a]: Average for the 30-minute exposure; [b]: Determined by Cascade Impactor during each exposure; [c]: No data collected; [d]: No aerosol collected on the cascade impactor stages, indicating no aerosol present; count = number of readings; GSD: geometric standard deviation; H<sub>2</sub>O: water; Max: maximum; min: minutes; Min: minimum; MMAD: mass median aerodynamic diameters; St Dev: standard deviation

## 4.5 Animal Response Data

ASTM International (2004) defines the calculations for the respiratory decrease as the baseline minus the exposure induced decrease divided by the baseline times 100. The baseline and exposure induced decrease were determined as follows: the baseline data were the average of 15 second averages for the last 90 seconds of the 10 minute baseline period for each mouse; the exposure induced decrease in respiratory rate was determined by selecting the lowest 15 second average during the first 5 minutes of exposure to the test chemical. The recovery data were the average of the ten 1-minute averages for the 10 minute recovery period.

### 4.5.1 HEFA-C.

Tables 3 and 4 show the summary data for decreases in respiratory rate following HEFA-C exposures. The respiratory data were reviewed and some anomalies were discovered in how the Buxco FinePoint software counted the individual breaths. Some of the higher respiratory rates (greater than 400 breaths per minute (BPM), as determined for a one second interval) appear to be caused by body movement or non-normal breathing functions such as sniffing. The data presented in Table 3 represent a data set with all breathing frequencies of 400 breaths or greater removed before data collation. The data presented in Table 4 represent a data set with all breathing frequencies. Figures 4 through 8 show the individual respiratory rates during the baseline, exposure and recovery periods for each exposure of 1979, 5182, 7048, 9698 or 11755 mg/m<sup>3</sup>, respectively.

To estimate the RD<sub>50</sub> for HEFA-C, respiratory rates of 400 breaths per minute and greater were removed from the data set prior to analysis. Table 3 (breaths of 400 breaths per minute excluded) and Figure 9 show that a respiratory decrease of 50 percent was determined to be greater than 11,755 mg/m<sup>3</sup>. This could not be determined directly because the highest attainable concentration before saturation of the analytical method was approximately 14,920 mg/m<sup>3</sup>. If the lowest respiratory decrease (breaths of 400 breaths per minute excluded) during the entire 30 minute exposure period is considered, the RD<sub>50</sub> would also be greater than 11,755 mg/m<sup>3</sup>. The extrapolated RD<sub>50</sub> values do not change even if all breaths (Table 4) were used in the dataset.

**Table 3. Summary Data for HEFA-C Assay: Summary of Mouse Breathing Rates (excluding breathing rates of 400 BPM or greater)**

			Group 1	Group 2	Group 3	Group 4	Group 5
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [a]	1,371	8,513	8,878	4,603	5,670
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [b]	1,979	11,755	9,698	5,182	7,048
Baseline	(BPM)	[c]	305	279	295	275	255
Exposure	(BPM)	[d]	215	173	207	148	171
Exposure	(BPM)	[e]	204	171	171	123	150
% Decrease	(%)	[d]	-31	-38	-30	-45	-33
% Decrease	(%)	[e]	-34	-39	-42	-56	-41
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[d]	>11,755				
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[e]	>11,755				
Recovery	(%)		83	79	71	71	66

[a]: Average aerosol/vapor concentration for first 5-minutes of exposure; [b]: Average aerosol/vapor concentration for 30-minutes of exposure; [c]: BPM = breaths per minute; [d]: Lowest average breathing rate for a 15 second period during the first 5 minutes of exposure was used for this calculation; [e]: Lowest average breathing rate for any period during the 30 minutes of exposure was used for this calculation

**Table 4. Summary Data for HEFA-C Assay: Summary of Mouse Breathing Rates (not excluding breathing rates of 400 BPM or greater)**

			Group 1	Group 2	Group 3	Group 4	Group 5
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [a]	1,371	8,513	8,878	4,603	5,670
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [b]	1,979	11,755	9,698	5,182	7,048
Baseline	(BPM)	[c]	310	301	300	277	257
Exposure	(BPM)	[d]	219	182	214	153	173
Exposure	(BPM)	[e]	208	179	173	128	150
% Decrease	(%)	[d]	-30	-39	-29	-44	-32
% Decrease	(%)	[e]	-34	-40	-43	-55	-42
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[d]	>11,755				
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[e]	>11,755				
Recovery	(%)		83	74	71	71	66

[a]: Average aerosol/vapor concentration for first 5-minutes of exposure; [b]: Average aerosol/vapor concentration for 30-minutes of exposure; [c]: BPM is breathes per minute; [d]: Lowest average breathing rate for a 15 second period during the first 5 minutes of exposure was used for this calculation; [e]: Lowest average breathing rate for any period during the 30 minutes of exposure was used for this calculation

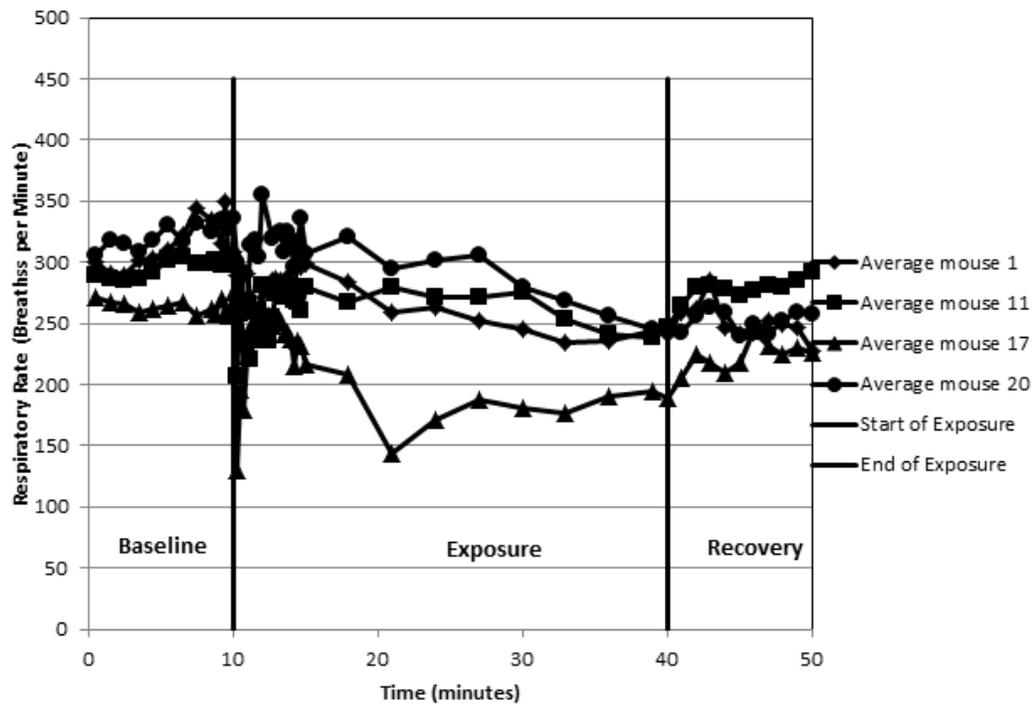


Figure 4. Animal Response to 1,979 mg/m<sup>3</sup> HEFA-C Exposure

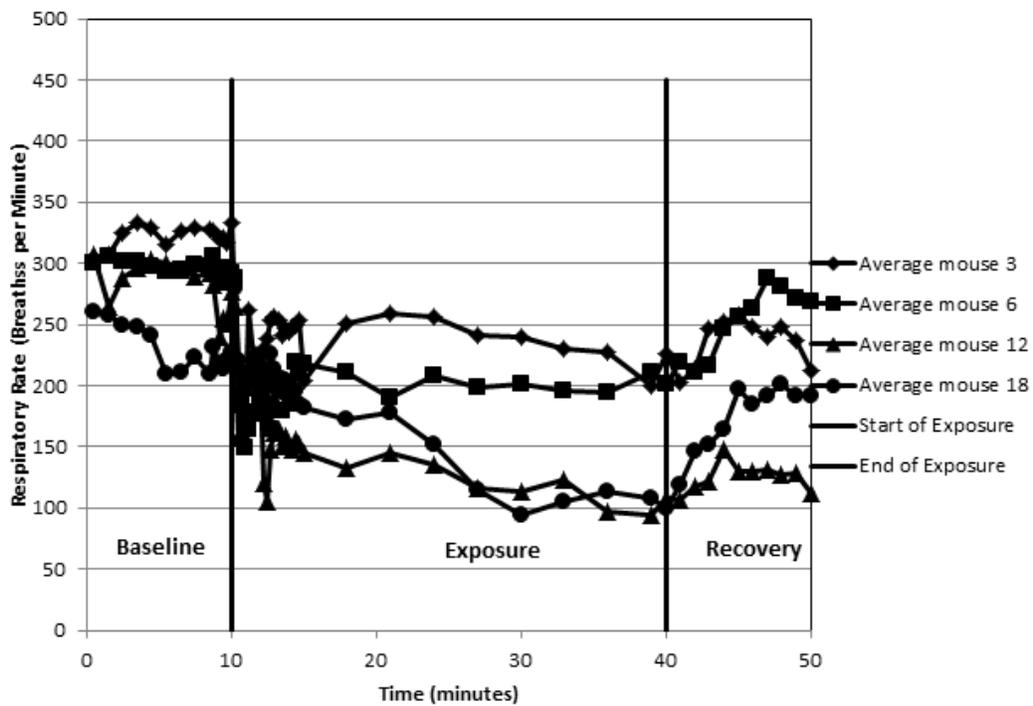
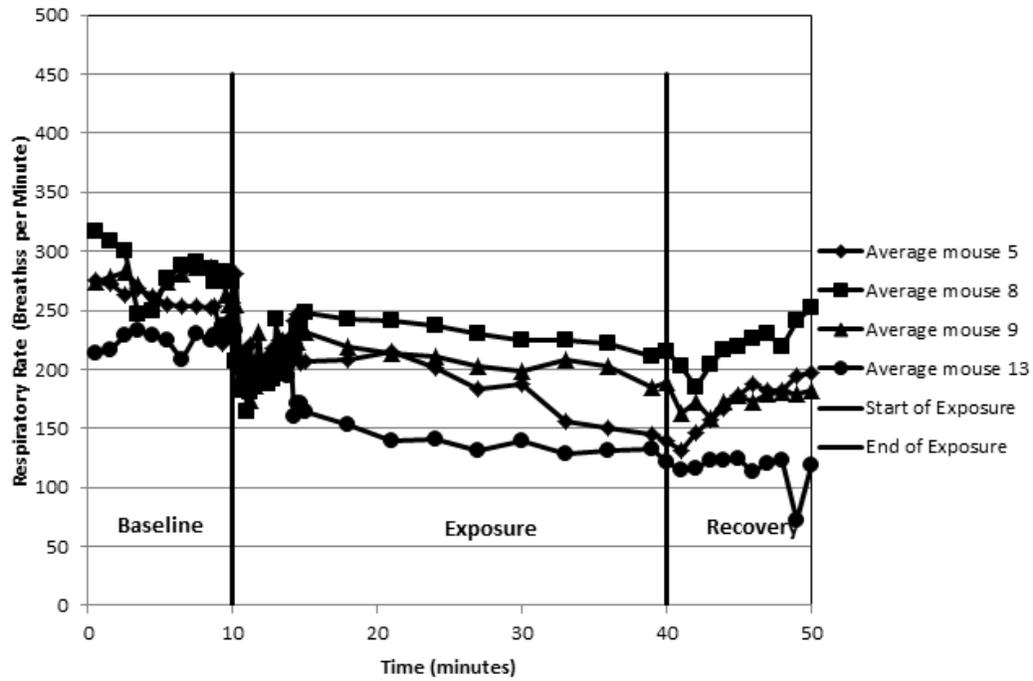
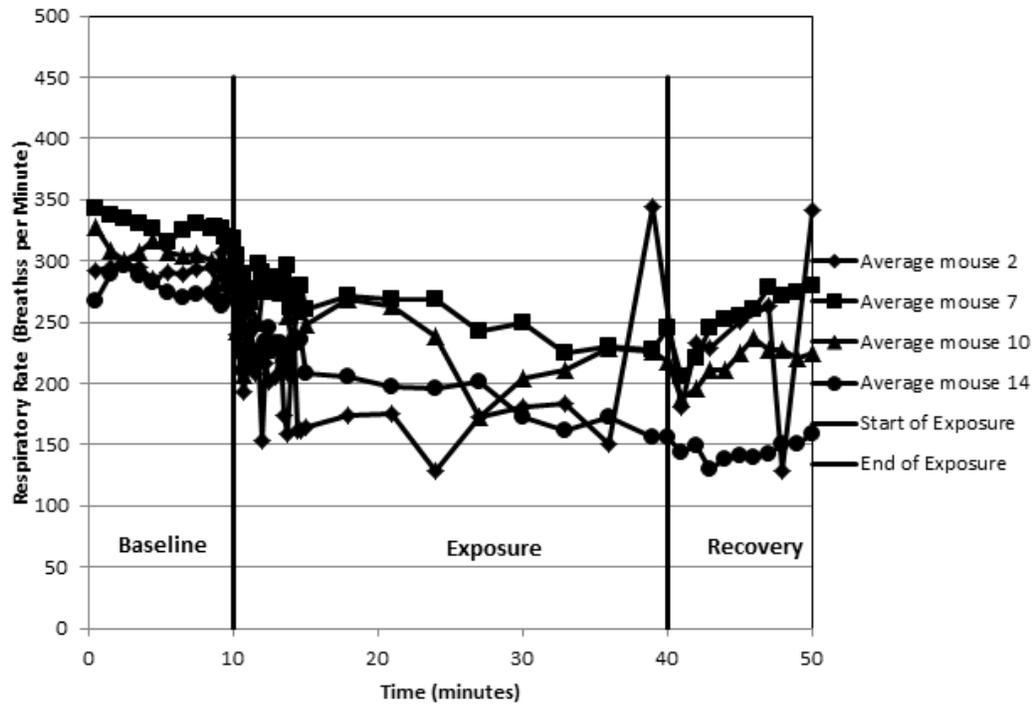


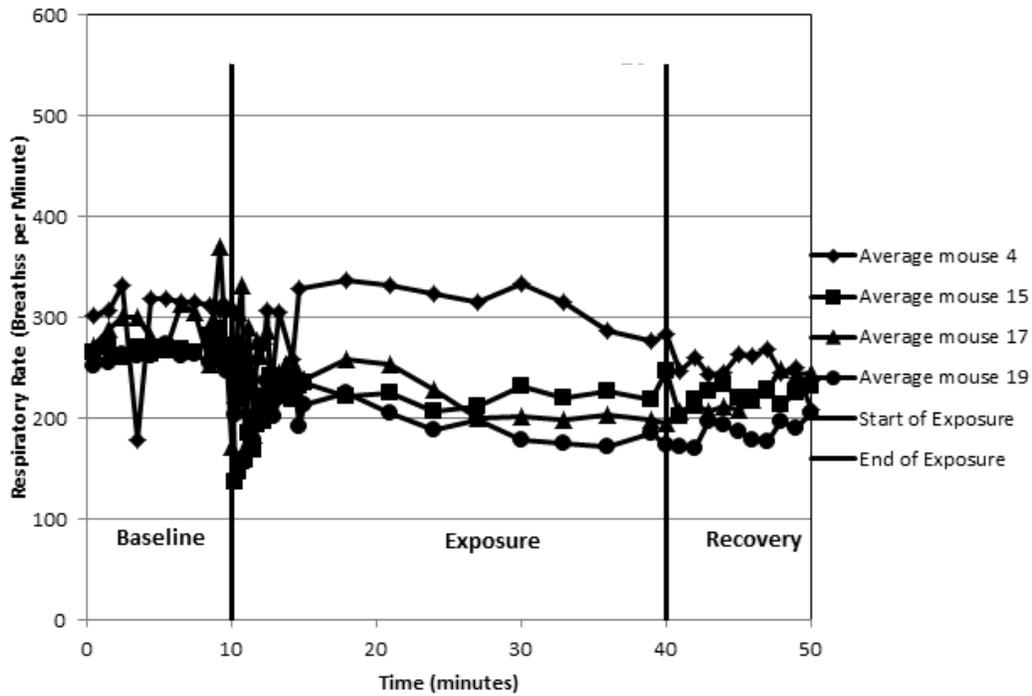
Figure 5. Animal Response to 5,182 mg/m<sup>3</sup> HEFA-C Exposure



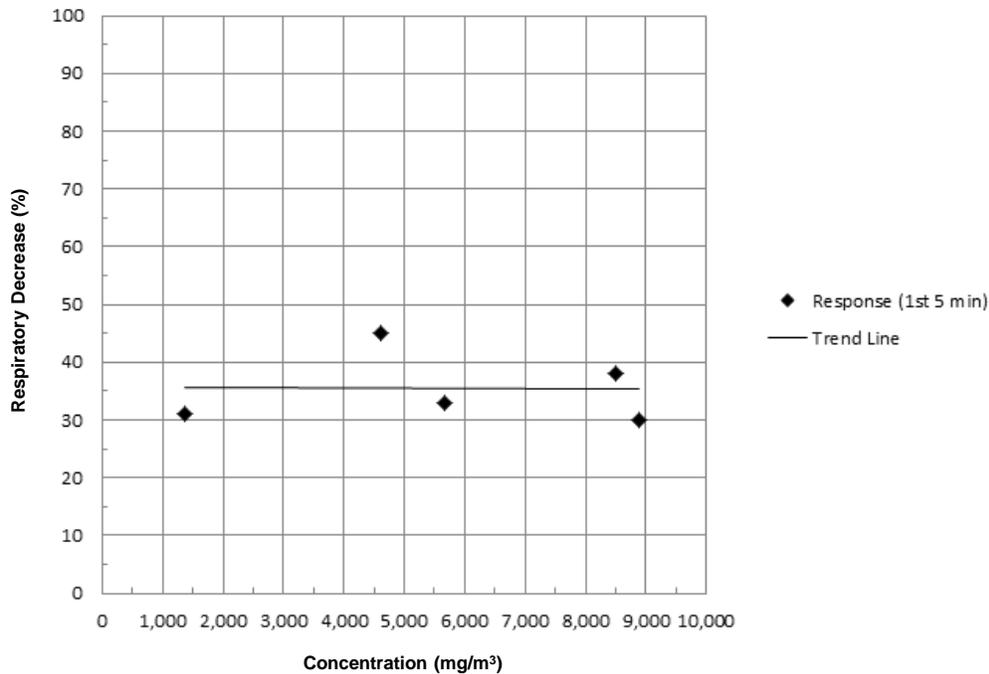
**Figure 6. Animal Response to 7,048 mg/m<sup>3</sup> HEFA-C Exposure**



**Figure 7. Animal Response to 9,698 mg/m<sup>3</sup> HEFA-C Exposure**



**Figure 8. Animal Response to 11,755 mg/m<sup>3</sup> HEFA-C Exposure**



**Figure 9. Correlation of HEFA-C Exposure Concentration to Mouse Breathing Rates.** Correlation excludes breathing rates of 400 BPM or greater. The estimated RD<sub>50</sub> is greater than or equal to 11,755 mg/m<sup>3</sup>.

**4.5.2 HEFA-T.** Tables 5 and 6 show the summary data for the decrease in respiratory rate for each exposure group mouse according to the HEFA-T exposure concentration. As with the HEFA-C data, some of the higher respiratory rates (> 400 breaths per minute, measured for a one second interval) appeared to be caused by body movement or non-normal breathing functions such as sniffing. The data presented in Table 5 represent a data set with all breathing frequencies of 400 breaths or greater removed before data collation. The data presented in Table 6 represent a data set with all breathing frequencies. Figures 10 through 14 show the individual respiratory rates during the baseline, exposure and recovery periods for 30-minute aerosol/vapor concentrations of 2107, 5983, 9690 or 15043 mg/m<sup>3</sup>, and the 30-minute vapor only concentration of 5955 mg/m<sup>3</sup>, respectively.

To determine the estimated RD<sub>50</sub> for HEFA-T, respiratory rates of 400 breaths per minute and greater were excluded from the data set prior to analysis. Table 5 and Figure 15 show that a respiratory decrease of 50 percent was determined to be greater than 13,306 mg/m<sup>3</sup>. This could not be determined directly because the highest attainable concentration before saturation of the analytical method was approximately 11,250 mg/m<sup>3</sup>. If the lowest respiratory decrease (breaths of 400 breaths per minute excluded) during the entire 30 minute exposure period is considered, the RD<sub>50</sub> would be greater than 15,043 mg/m<sup>3</sup>. If all of the breathing data are used (Table 6), the estimated HEFA-T RD<sub>50</sub> values do not change.

**Table 5. Summary Data for HEFA-T Assay: Summary of Mouse Breathing Rates (excluding breathing rates of 400 BPM or greater)**

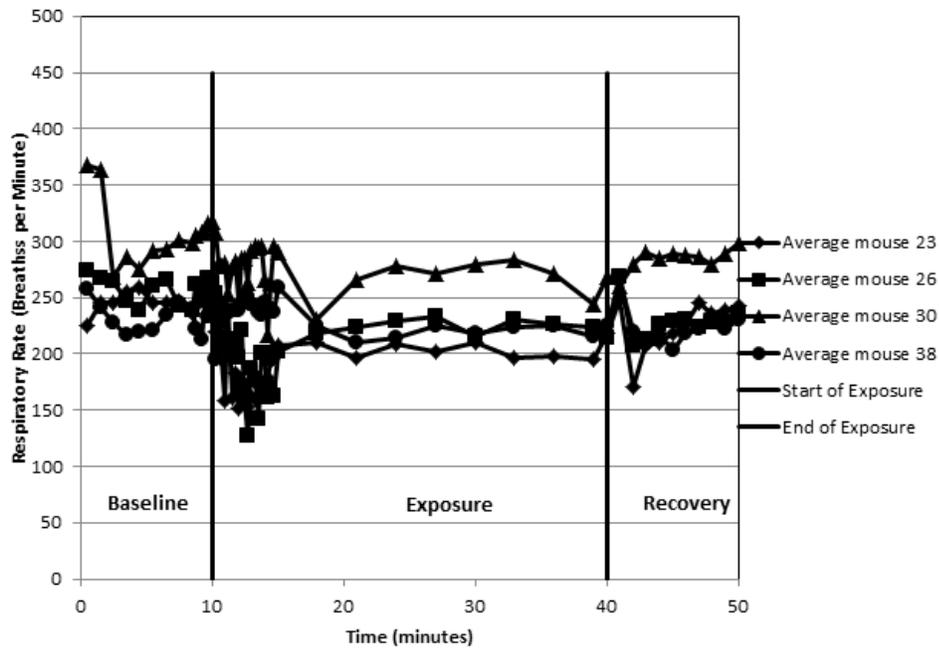
			Group 1	Group 2	Group 3	Group 4	Group 5 (Vapor)
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [a]	1,794	13,306	8,094	4,795	4,299
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [b]	2,107	15,043	9,690	5,983	5,955
Baseline	(BPM)	[c]	261	272	266	251	266
Exposure	(BPM)	[d]	199	241	177	186	216
Exposure	(BPM)	[e]	199	205	177	186	200
% Decrease	(%)	[d]	-24	-11	-33	-26	-19
% Decrease	(%)	[e]	-24	-25	-33	-26	-25
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[d]	>15,043				
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[e]	>15,043				
Recovery	(%)		92	68	77	90	83

[a] Average aerosol/vapor concentration for first 5-minutes of exposure; [b] Average aerosol/vapor concentration for 30-minutes of exposure; [c] BPM is breathes per minute; [d] Lowest average breathing rate for a 15 second period during the first 5 minutes of exposure was used for this calculation; [e] Lowest average breathing rate for any period during the 30 minutes of exposure was used for this calculation

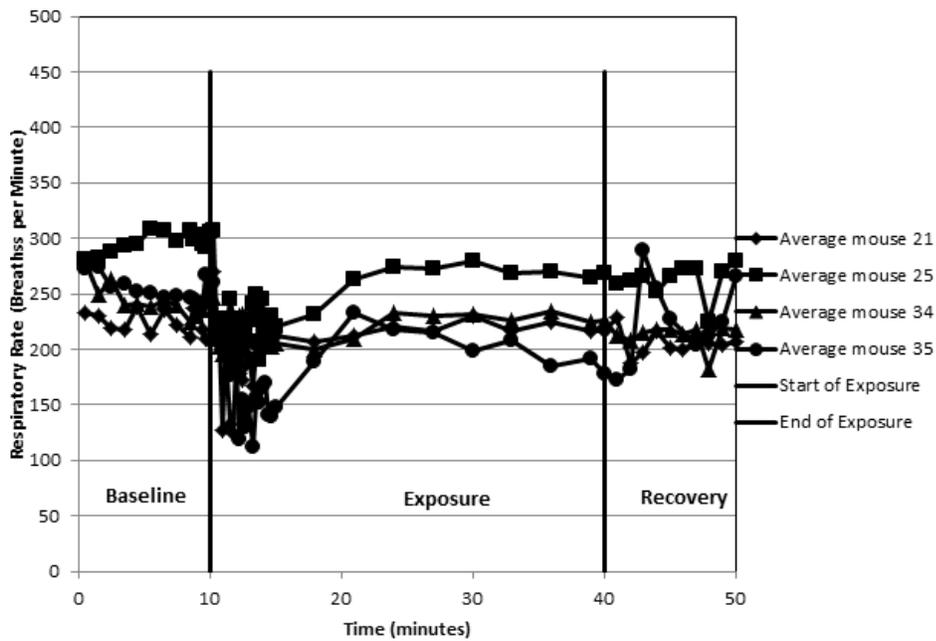
**Table 6. Summary Data for HEFA-T Assay: Summary of Mouse Breathing Rates (not excluding breathing rates of 400 BPM or greater)**

			Group 1	Group 2	Group 3	Group 4	Group 5 (Vapor)
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [a]	1,794	13,306	8,094	4,795	4,299
Aerosol/ Vapor Concentration	(mg/m <sup>3</sup> )	Mean [b]	2,107	15,043	9,690	5,983	5,955
Baseline	(BPM)	[c]	262	277	266	252	270
Exposure	(BPM)	[d]	199	241	178	188	216
Exposure	(BPM)	[e]	199	210	178	188	201
% Decrease	(%)	[d]	-24	-13	-33	-25	-20
% Decrease	(%)	[e]	-24	-24	-33	-25	-25
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[d]	>15,043				
RD <sub>50</sub>	(mg/m <sup>3</sup> )	[e]	>15,043				
Recovery	(%)		93	68	80	93	85

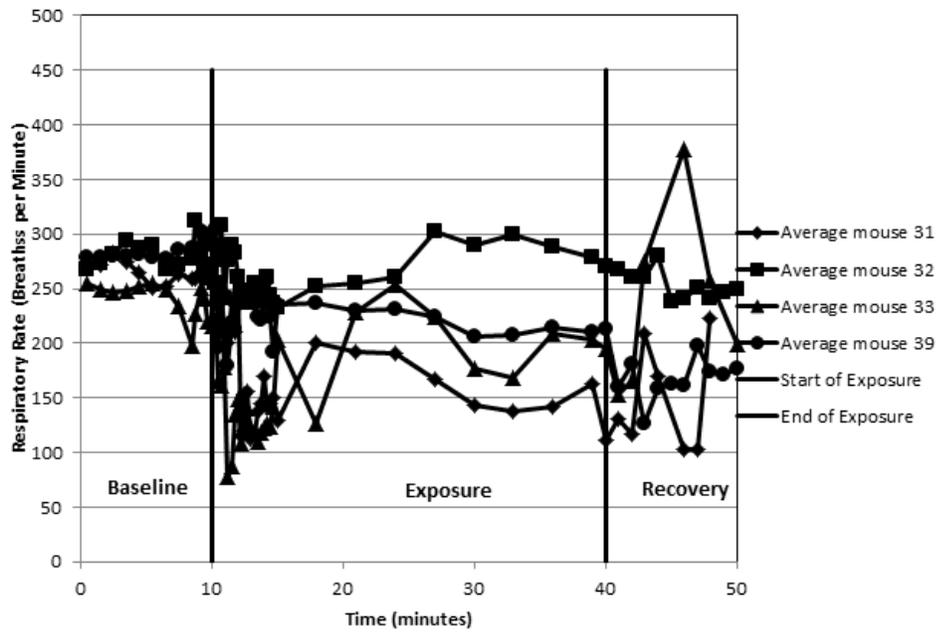
[a] Average aerosol/vapor concentration for first 5-minutes of exposure; [b] Average aerosol/vapor concentration for 30-minutes of exposure; [c] BPM is breathes per minute; [d] Lowest average breathing rate for a 15 second period during the first 5 minutes of exposure was used for this calculation; [e] Lowest average breathing rate for any period during the 30 minutes of exposure was used for this calculation



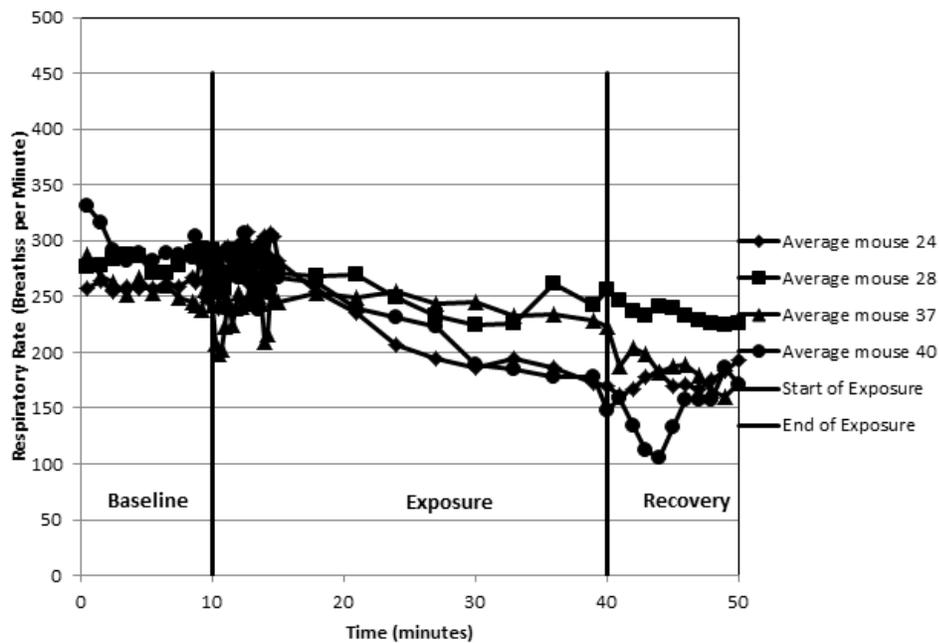
**Figure 10. Animal Response to 1,794 mg/m<sup>3</sup> HEFA-T Exposure**



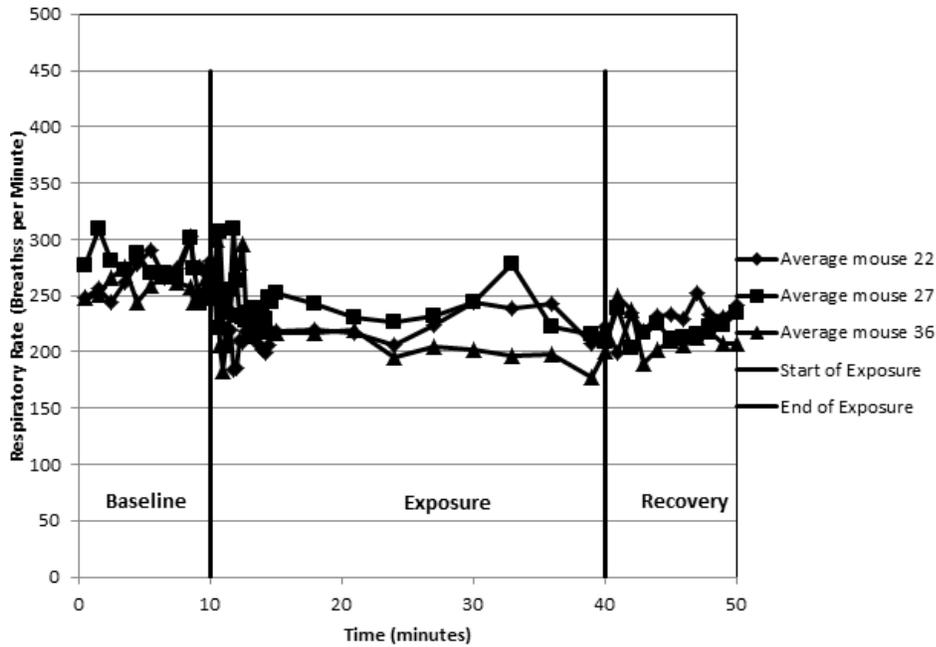
**Figure 11. Animal Response to 4,795 mg/m<sup>3</sup> HEFA-T Exposure**



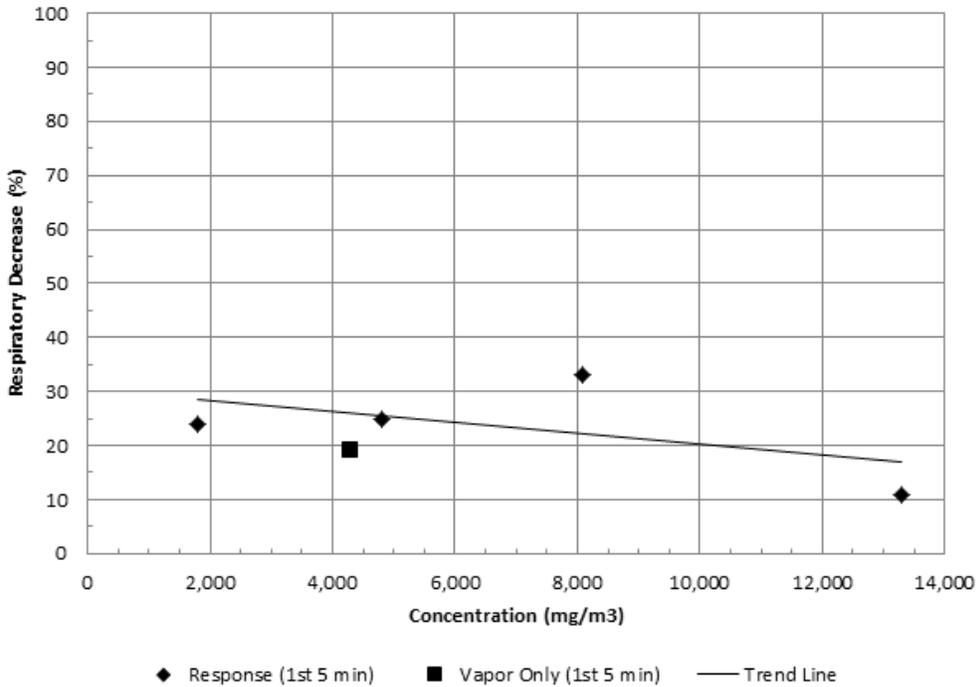
**Figure 12. Animal Response to 8,094 mg/m<sup>3</sup> HEFA-T Exposure**



**Figure 13. Animal Response to 13,306 mg/m<sup>3</sup> HEFA-T Exposure**



**Figure 14. Animal Response to 4,299 mg/m<sup>3</sup> HEFA-T Vapor Only Exposure**



**Figure 15. Correlation of HEFA-T Exposure Concentration to Mouse Breathing Rates.** Correlation excludes breathing rates of 400 BPM or greater. The estimated RD<sub>50</sub> is greater than or equal to 13,306 mg/m<sup>3</sup>.

## 5.0 DISCUSSION: SENSORY IRRITATION ASSAY

The purpose of the sensory irritation assay was to determine the concentrations of HEFA-C and HEFA-T that elicit a 50 percent decrease in the respiratory rate (RD<sub>50</sub>) of mice caused by sensory irritation of the test chemical. The RD<sub>50</sub> for HEFA-C was estimated to be greater than 11,755 mg/m<sup>3</sup>, but could not be determined exactly because the highest attainable concentration before saturation was approximately 14,920 mg/m<sup>3</sup>. The HEFA-T RD<sub>50</sub> was estimated to be greater than 13,306 mg/m<sup>3</sup>, but could not be determined precisely because the highest attainable concentration before saturation was approximately 11,250 mg/m<sup>3</sup>. Concentrations higher than 11,250 mg/m<sup>3</sup> were determined by extending the linear calibration regression line.

The estimate RD<sub>50</sub> values for HEFA-C and HEFA-T are higher than the RD<sub>50</sub> determined for SPK (Hinz *et al.*, 2012) and more than four times higher than that of JP-8 (Whitman and Hinz, 2001). These values are compared in Table 7. Sensory irritation by the HEFA fuels is not a likely outcome of an inhalation exposure level. The lack of irritancy may be the reason that there appears to be only minor differences in the respiratory decreases recorded for these mice over a wide range of concentrations, resulting in essentially flat slopes from the regression analyses (Figures 9 and 15). The mice detected the test atmosphere when it was presented and responded, but the response appeared to be due more to the detection of a strange odor, pungency or aerosol, than an involuntary response to an irritating chemical. Lower and upper 95 percent confidence limits were not calculated since an RD<sub>50</sub> values were not quantified. This wide range is reflective of the lack of response and resulted in the nearly flat slopes from the regression analysis.

Table 7 also compares the RD<sub>x</sub> values found for each fuel at/near 2000 mg/m<sup>3</sup>. This is the initial exposure level for sensory irritation evaluation of a jet fuel, per experience and the protocol (Appendix A). It is based on the highest exposure levels in several fuel studies for HEFA, SPK and JP-8 (Mattie *et al.*, 2011a, 2011b, 2012; Sweeney *et al.*, 2013; Wong *et al.*, 2013); and is the single test point for the sensory irritation limit test (Mattie *et al.*, 2012). Comparing the limit test values allows all three HEFA feedstock fuels to be addressed together.

**Table 7. Comparison of Sensory Irritation Levels between Alternative Fuels and JP-8**

	HEFA-F	HEFA-C	HEFA-T	SPK (F-T)	JP-8
<b>Full Sensory Irritation Assay</b>					
<b>RD<sub>50</sub> (mg/m<sup>3</sup>)</b>	NP	>11755	>15043	10939	2876
<b>Sensory Irritation Limit Test</b>					
<b>Maximum RD (%)</b>	23	30-31	24	20	46
<b>Concentration (mg/m<sup>3</sup>)</b>	1916	1371	1794	2225	1837
<b>Reference</b>					
	Mattie <i>et al.</i> (2012)	Tables 3 & 4	Tables 5 & 6	Hinz <i>et al.</i> (2012)	Whitman & Hinz (2001)

Note: NP = not performed

The limit test for HEFA-F (Mattie *et al.*, 2012) was performed to determine if a single sensory irritation run could be informative as to a fuel's sensory irritation potential. In comparing the RD<sub>x</sub> data for HEFA fuels, SPK and JP-8, it is clear that little information can be gained from this single run approach. All fuels in Table 7 are moderately irritating at limit test values; severity is categorized as slight (12 to 19 percent respiratory reduction), moderate (20 to 49 percent) and extreme (50 percent or more) (Whitman and Hinz, 2001). Although JP-8 has a higher response (RD<sub>46</sub>) at the limit concentration, one would expect that an RD<sub>50</sub> could be calculated for each of these fuels. Instead, the calculated RD<sub>50</sub> values for HEFA-C and HEFA-T exceed the highest atmosphere generable by the exposure system.

In conclusion, HEFA-C and HEFA-T did not evoke a typical sensory irritation response. Given the similarity of values at limit test concentrations, all three HEFA fuels are therefore not considered to be true sensory irritants.

## 6.0 COMPARATIVE HUMAN HEALTH HAZARD ASSESSMENT

### 6.1 Background

The U.S. Air Force (USAF) alone purchases upwards of 2.5 billion gallons of JP-8 yearly (Starosta, 2012). Since it is the Department of Defense's (DoD's) universal battlefield fuel, additional fuel is used annually in airplanes, tanks, stoves and heaters across the military branches (Edwards *et al.*, 2001). Alternative jet fuels such as the HEFA fuels and SPK are being certified to fly in Air Force planes in order to reduce dependence on foreign petroleum, to decrease the carbon footprint of using solely petroleum derived fuels, and to help steady the fluctuation of the USAF jet fuel budget, since the cost of the alternative fuels are not affected by petroleum price per gallon variability (Starosta, 2012).

Due to the immense volume of jet fuel used each year, the opportunity for occupational exposure to JP-8 and alternative fuels is high among airmen. Personnel are most frequently exposed by dermal and inhalation routes to fuel vapors and/or aerosols. Toxicological evaluation of all fuels is necessary to determine appropriate occupational exposure levels (OELs) to protect worker health.

SPK was the first alternative fuel to undergo a comparative health hazard assessment (HHA) with JP-8. A list of toxicity studies was compiled in order to assess the baseline risk of SPK and determine if further testing was necessary following completion of the list. The list included:

- Dermal irritation test (SPK vs. JP-8 vs. 50/50 blend)
- *In vitro* genotoxicity tests
- Acute inhalation study
- Two-week inhalation rangefinder study
- *In vivo* genotoxicity test in tandem with an *in vivo* study
- 90-day inhalation toxicity study
- Sensory Irritation Assay

The list was developed in conjunction with and with financial support from the Alternative Fuels Certification Office (AFLCMC/WNN) located at Wright-Patterson AFB OH. Each test was designed to follow a U.S. Environmental Protection Agency (EPA) Office of Prevention, Pesticides and Toxic Substances (OPPTS) health effects test guideline, a European Organisation for Economic Co-operation and Development (OECD) guideline for testing of chemicals or an appropriate ASTM International standard test method. All studies were conducted under Good Laboratory Practice (GLP) Standards (40 CFR Part 792) or followed the intent and purpose of GLP requirements (Hinz *et al.*, 2012).

Toxicity studies for a second alternative fuel (HEFA) were planned following the SPK HHA. The HEFA fuels study list was designed to align HEFA toxicity data with JP-8 and F-T, in order to compare potential human toxicity. The acute inhalation study and the two-week inhalation rangefinder were combined to include an acute recovery period as well as a five-day exposure, in order to take a closer look at the short-term effects of jet fuel inhalation (Mattie *et al.*, 2012).

## 6.2 JP-8 and SPK OELs

An occupational exposure limit for JP-8 was first recommended in 1996 by the National Research Council (NRC), Committee on Toxicology (COT), Subcommittee on Permissible Exposure Levels for Military Fuels. This subcommittee proposed an 8-hour time weighted average threshold limit value (TLV-TWA) of 350 mg/m<sup>3</sup> and a 15-min short-term exposure limit (STEL) of 1000 mg/m<sup>3</sup>. Their report identified major data gaps in JP-8 toxicological studies (NRC, 1996). At that time, the Air Force accepted these proposed values as interim OELs. Shortly thereafter, the Centers for Disease Control and Prevention, Agency for Toxic Substances and Disease Registry (CDC-ATSDR), prepared the Toxicology Profile for Jet Fuels (JP-5 and JP-8) (ATSDR, 1998). This document also identified toxicity data gaps and concluded that the underlying physiological mechanisms of JP-8 toxicity are not well defined or understood.

A second expert panel was convened by the NRC COT to review the occupational JP-8 exposure level of 350 mg/m<sup>3</sup>. The panel concluded that JP-8 was potentially toxic to the immune system, respiratory tract and nervous system at exposure concentrations near the OEL (NRC, 2003). In response, the American Conference of Governmental Industrial Hygienists (ACGIH) proposed a TLV of 200 mg/m<sup>3</sup> for kerosene and jet fuel vapors in 2003. A TLV of 5 mg/m<sup>3</sup> for kerosene and middle distillate fuel aerosols was concurrently recommended by the NRC COT, based on the value set by ExxonMobil Biosciences (NRC, 2003). These values were in turn adopted by the U.S. Air Force. The National Institutes for Occupational Safety and Health (NIOSH) advised a recommended exposure level (REL) TWA of 100 mg/m<sup>3</sup> for the less refined kerosene (NIOSH, 2011). The U.S. Air Force's OEL and the ACGIH TLV-TWA for JP-8 remains at 200 mg/m<sup>3</sup>.

In 2011, the U.S. EPA OPPTS, National Academies Council on Acute Exposure Guidelines Levels (NAC-AEGL) published guidelines for JP-5 and JP-8 acute inhalation exposure. The Acute Exposure Guideline 1 (AEGL-1) for non-disabling exposure was determined to be 290 mg/m<sup>3</sup> at all durations (10 or 30 minutes, 1, 4 or 8 hours) (NAC-AEGL, 2011). This

concentration was based on 10 percent of the sensory irritation RD<sub>50</sub> (0.1 x 2876 mg/m<sup>3</sup>; Whitman and Hinz, 2001).

Since SPK fuel was being certified for use in the Air Force as a 50/50 blend of FT jet fuel with JP-8, the recommended OEL for this alternative fuel was the same as JP-8 (200 mg/m<sup>3</sup> vapor, 5 mg/m<sup>3</sup> aerosol). This approach allows airmen to treat either neat fuel or the blend the same in terms of personal protective equipment use and exposure monitoring. Although SPK fuel appears to be less hazardous than JP-8 based on the toxicity data, a conservative approach such as this allows the more hazardous fuel to define how the fuels are handled. These guidelines are established and familiar to all personnel (Hinz *et al.*, 2012). It is the purpose of the remainder of this document to determine if a similar approach is appropriate for HEFA bio-based fuels.

### 6.3 HEFA Dermal Irritation/Toxicity

Multiple jet fuels have been tested for dermal irritation using rabbits as an animal model. In this assay, each animal serves as its own control as there are up to six sites per rabbit on which to apply individual fuels. The assay was run with two sets of rabbits, occluded and semi-occluded, as these conditions affect fuel irritation results (Hinz *et al.*, 2012). All three HEFA jet biofuels tested were, at most, slightly irritating (Table 8) based on the Primary Dermal Irritation Index (PDII) (Mattie *et al.*, 2013). HEFA fuels were less irritating than SPK, petroleum-derived JP-8 or the 50:50 blend of the two when previously tested (Hurley *et al.*, 2011; Hinz *et al.*, 2012). HEFA biofuels are not expected to result in additional dermal irritation when handled alone or used in a 50:50 mixture with petroleum-derived JP-8.

**Table 8. Dermal Irritation Scores for JP-8 and Three HEFA Biofuels Compared with Historical Data for JP-8 and SPK**

Test Substance* POSF#	Exposure	PDII	Descriptive Rating	Reference
<b>JP-8</b> 4658	Occluded	0.8	Slightly Irritating	Mattie <i>et al.</i> (2013)
	Semi-Occluded	0.8	Slightly Irritating	
<b>HEFA-C</b> 6152	Occluded	0.9	Slightly Irritating	
	Semi-Occluded	0.6	Slightly Irritating	
<b>HEFA-T</b> 6308	Occluded	0.6	Slightly Irritating	
	Semi-Occluded	0.2	Slightly Irritating	
<b>HEFA-F</b> 5469	Occluded	0.3	Slightly Irritating	
	Semi-Occluded	0	Nonirritating	
<b>SPK (S-8)</b> 5109	Occluded	2.3	Moderately Irritating	Hurley <i>et al.</i> (2011);
	Semi-Occluded	0.8	Slightly Irritating	
<b>JP-8</b> 4658	Occluded	2.1	Moderately Irritating	Hinz <i>et al.</i> (2012)
	Semi-Occluded	1.8	Slightly Irritating	
<b>50:50 JP-8:SPK Blend</b> 4658:5109	Occluded	1.9	Slightly Irritating	
	Semi-Occluded	1.5	Slightly Irritating	

Note: \*Alternative jet fuel names evolve over time. The current name may be listed with the name used in the publication in parentheses.

#### **6.4 HEFA Mutagenicity and Genotoxicity**

All HEFA biofuels originating from different feedstocks (i.e., HEFA-C, HEFA-F and HEFA-T) have been found negative for mutagenicity by means of the *Salmonella-Escherichia coli*/microsome plate incorporation assay (Ames test) (Ricchio *et al.*, 2010; Mattie *et al.*, 2013). The *in vivo* rat micronucleus assay was negative for HEFA-C. In this assay, male and female rats exposed to 200, 700 and 2000 mg/m<sup>3</sup> HEFA-C vapor and aerosol for two weeks showed no significant difference in the percentage of reticulocytes compared with air-exposed controls. Although there were slight differences in the percentage of micronucleated reticulocytes, the difference was not dose related. Therefore, HEFA-C was considered to be non-genotoxic by means of the micronucleus assay (Wong *et al.*, 2013). These negative results for both mutagenic and carcinogenic outcomes increases the evidence that exposure to the HEFA jet fuels will not result in carcinogenic outcomes.

Table 9 compares mutagenicity and carcinogenicity assay outcomes for all tested HEFA biofuels, as well as JP-8 jet fuel and SPK alternative fuel. Exposure to any of these fuels is not expected to result in increased cancer findings.

**Table 9. Mutagenicity and Genotoxicity Assay Outcomes for HEFA Biofuels Compared with JP-8 and SPK**

Fuel* POSF #	Exposure	Result	Reference
<b>Ames Salmonella-Escherichia coli/Microsome Plate Incorporation Assay</b>			
HEFA-C 6152	**	Non-mutagenic	Mattie <i>et al.</i> (2013)
HEFA-T 6308	**	Non-mutagenic	Mattie <i>et al.</i> (2013)
HEFA-F 5469	**	Non-mutagenic	Mattie <i>et al.</i> (2013)
HEFA-F (R-8) 5469	**	Non-mutagenic	Riccio <i>et al.</i> (2010)
JP-8 NR	**	Non-mutagenic	Brusick & Matheson (1978)
SPK (F-T) 5109	**	Non-mutagenic	Mattie <i>et al.</i> (2011c)
SPK (S-8) 4734	**	Non-mutagenic	Riccio <i>et al.</i> (2010)
<b>Human Lymphocyte Chromosomal Aberration Test</b>			
SPK (F-T) 5109	**	Non-clastogenic	Mattie <i>et al.</i> (2011c)
<b>In Vivo Rat Micronucleus Assay (2 Week Inhalation Exposure)</b>			
HEFA-C 6152	200, 700, 2000 mg/m <sup>3</sup>	Non-genotoxic	Wong <i>et al.</i> (2013)
SPK (F-T) 5109	500, 1000, 2000 mg/m <sup>3</sup>	Non-genotoxic	Mattie <i>et al.</i> (2011a)
<b>In Vivo Mouse Micronucleus Assay (Dermal Exposure)</b>			
JP-8 3509	50, 100, 300 µL, unoccluded, once or repeated 3 days	Non-genotoxic	Vijayalaxmi <i>et al.</i> (2006) Vijayalaxmi, 2011
Jet A 3404	50, 100, 300 µL, unoccluded, once or repeated 3 days	Non-genotoxic	Vijayalaxmi <i>et al.</i> (2006) Vijayalaxmi, 2011

Note: \*Alternative jet fuel names evolve over time. The current name may be listed with the name used in the publication in parentheses. \*\*Exposure concentrations listed for *in vivo* assays only.

## 6.5 HEFA Inhalation Toxicity

**6.5.1 Acute.** An acute inhalation exposure was conducted in male and female F-344 rats with HEFA-F biofuel. Exposures concentrations used were 0, 200, 700 or 2000 mg/m<sup>3</sup> mixed aerosol and vapor HEFA-F. Average aerosol concentrations were 14.3, 147.7 and 551.7 mg/m<sup>3</sup>, resulting in aerosol percentages of 7, 22 and 28, respectively, among the three fuel exposures. Two groups of rats were utilized; both were exposed over a single 6 hour period but one group was allowed an 11-day recovery period (Mattie *et al.*, 2012). Results are found in Table 10. In a similar study, male and female F-344 rats were exposed to a single high concentration (2000 mg/m<sup>3</sup>) of SPK jet fuel for a single 4-hour period (Mattie *et al.*, 2011a). Rats were euthanized

following a 14-day recovery period. In a JP-8 study, F-344 rats were exposed to a single high concentration of either JP-8 vapor (3430 mg/m<sup>3</sup>) or JP-8 vapor plus aerosol (4440 mg/m<sup>3</sup>); exposures were followed by a 14-day recovery period (Wolfe *et al.*, 1996).

**Table 10. Acute Inhalation Exposure Outcomes for HEFA Biofuels Compared with JP-8 and SPK**

Fuel* PSOF #	Exposure mg/m <sup>3</sup> (% aerosol)	Exposure Duration	NOAEC LOAEC mg/m <sup>3</sup>	Result	Reference
<b>Gross Observations</b>					
HEFA-F 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr	2000 no LOAEC	No biologically significant changes in gross observation, bodyweight or food consumption (M&F)	Mattie <i>et al.</i> (2012)
HEFA-F 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr, RECOVERY: 11 dy	2000 no LOAEC	No biologically significant changes in gross observation, bodyweight or food consumption (M&F)	Mattie <i>et al.</i> (2012)
SPK (F-T) 5109	2000 (29)	4 hr, RECOVERY: 14 dy	2000 no LOAEC	No lethality or adverse clinical signs	Mattie <i>et al.</i> (2011a)
JP-8 NR	3430 vapor only (0)	4 hr, RECOVERY: 14 dy	vapor: 3430 no LOAEC	No lethality; Signs of eye or upper respiratory tract irritation during exposure (M&F)	Wolfe <i>et al.</i> (1996)
JP-8 NR	4440 (41)	4 hr, RECOVERY: 14 dy	4440 no LOAEC	No lethality or adverse clinical signs (M&F)	Wolfe <i>et al.</i> (1996)
<b>Gross Pathology, Histopathology</b>					
HEFA-F 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr	no NOAEC 200	Minimal olfactory epithelial degeneration, not dose dependent (M&F)	Mattie <i>et al.</i> (2012)
HEFA-F 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr, RECOVERY: 11 dy	200 700	Minimal olfactory epithelial degeneration, not dose dependent (M&F)	Mattie <i>et al.</i> (2012)
HEFA-F 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr, RECOVERY: 11 dy	200 700	Alveolus inflammation (M&F)	Mattie <i>et al.</i> (2012)

Note: \*Alternative jet fuel names evolve over time; the current name may be listed with the name used in the publication in parentheses. dy = day. F = female. hr = hour. LOAEC = lowest observed adverse effect concentration. M = male. NA-Hmn = not applicable to human health risk. NOAEC = no observed adverse effect concentration. NR = not reported. wk = week.

**6.5.2 Short-Term.** In conjunction with the acute study (Section 6.3.1), additional male and female F-344 rats were exposed to 0, 200, 700 or 2000 mg/m<sup>3</sup> HEFA-F in two short-term exposures. One group was exposed 6 hours/day for 5 consecutive days. In the other exposure, rats were exposed for 6 hours/day for 5 consecutive days, given a 2-day break and exposed again on 5 consecutive days (Mattie *et al.*, 2012). Results are found in Table 11 and compared to short-term studies with SPK and JP-8. In the SPK exposure, male and female F-344 rats were exposed to 0, 500, 1000 or 2000 mg/m<sup>3</sup> SPK fuel for 6 hours per day, 5 days per week, over 2 weeks. Two Jet A studies were reported by Sweeney *et al.* (2013). Female rats of two strains

(F-344 and Sprague-Dawley (SD)) were exposed to 0, 500, 1000 or 2000 mg/m<sup>3</sup> Jet A for 4 hours per day over a 14 day period. A 14-day recovery period followed.

**Table 11. Short-Term Inhalation Exposure Outcomes for HEFA Biofuels Compared with JP-8 and SPK**

Fuel* POSF #	Exposure mg/m <sup>3</sup> (% aerosol)	Exposure Duration	NOAEC LOAEC mg/m <sup>3</sup>	Result	Reference
<b>Gross Observations</b>					
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>5 dy</b>	2000 no LOAEC	No lethality or adverse clinical signs	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>10 dy</b>	NA-Hmn	Increased right kidney weight consistent with α2-microglobulin accumulation (M)	Mattie <i>et al.</i> (2012)
<b>SPK</b> (F-T) 5109	0, 500, 1000, 2000 (0, 12, 14, 30)	6 hr/dy, <b>10 dy</b>	1000 2000	Nasal discharge observed; Decreased bodyweight (M=11%, F=5%)	Mattie <i>et al.</i> (2011a)
<b>Jet A</b> 4658	0, 500, 1000, 2000 (0, 4, 6, 15)	4 hr/dy, <b>14 dy</b>	1000 2000	Potential upper airway inflammation (elevated protein and lactate dehydrogenase in nasal lavage fluid) at 7 dy post-exposure (F SD)	Sweeney <i>et al.</i> (2013)
<b>Jet A</b> 4658	0, 500, 1000, 2000 (0, 6, 12, 19)	4 hr/dy, <b>14 dy</b>	1000 2000	Decreased bodyweight (F F344); Some lung lavage fluid markers increased at 1 dy post-exposure	Sweeney <i>et al.</i> (2013)

**Table 11 (continued).**

Fuel* POSF #	Exposure mg/m <sup>3</sup> (% aerosol)	Exposure Duration	NOAEC LOAEC mg/m <sup>3</sup>	Result	Reference
<b>Gross Pathology, Histopathology</b>					
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>5 dy</b>	no NOAEC 200	Minimal olfactory epithelial degeneration, not dose dependent (M&F)	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>10 dy</b>	no NOAEC 200	Minimal olfactory epithelial degeneration, not dose dependent (M&F)	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>5 dy</b>	700 2000	Minimal lung interstitium fibrosis (M&F)	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>10 dy</b>	700 2000	Minimal lung interstitium fibrosis (M&F)	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>5 dy</b>	200 700	Alveolus inflammation (M&F)	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>10 dy</b>	200 700	Alveolus inflammation (M&F)	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>5 dy</b>	NA-Hmn	Hyaline droplet observation (M)	Mattie <i>et al.</i> (2012)
<b>HEFA-F</b> 5469	0, 200, 700, 2000 (0, 7, 22, 28)	6 hr/dy, <b>10 dy</b>	NA-Hmn	Hyaline droplet observation (M)	Mattie <i>et al.</i> (2012)
<b>SPK</b> <b>(F-T)</b> 5109	0, 500, 1000, 2000 (0, 12, 14, 30)	6 hr/dy, <b>10 dy</b>	500 1000	Minimal to mild olfactory epithelial degeneration; Lung inflammatory cell infiltration (M&F); Hyaline droplet accumulation (M)	Mattie <i>et al.</i> (2011a)
<b>Jet A</b> 4658	0, 500, 1000, 2000 (0, 4, 6, 15)	4 hr/dy, <b>14 dy</b>	2000 no LOAEC	No evidence of upper airway inflammation; No effect on spleen immune cell population (F SD)	Sweeney <i>et al.</i> (2013)
<b>Jet A</b> 4658	0, 500, 1000, 2000 (0, 6, 12, 19)	4 hr/dy, <b>14 dy</b>	2000 no LOAEC	No evidence of upper airway inflammation; No histological changes in lungs, nasal cavities, other tissues (F SD & F344)	Sweeney <i>et al.</i> (2013)

Note: \*Alternative jet fuel names evolve over time; the current name may be listed with the name used in the publication in parentheses. dy = day. F = female. F344 = Fischer 344. hr = hour. LOAEC = lowest observed adverse effect concentration. M = male. NA = not applicable. NA-Hmn = not applicable to human health risk. NOAEC = no observed adverse effect concentration. NR = not reported. wk = week. SD = Sprague-Dawley.

**6.5.3 90-Day.** Male and female F-344 rats were exposed by inhalation to an aerosol and vapor combination of HEFA-C fuel. Animals were exposed to three target concentrations, 200, 700 and 2000 mg/m<sup>3</sup>, or a control exposure of clean air. Exposures were conducted for six hours/day, five days/week resulting in a total of 71 exposure days. The average analytical total

concentrations were  $0.9 \pm 2.4$ ,  $194.8 \pm 15.3$ ,  $702.5 \pm 29.8$  and  $1990.5 \pm 52.4$  mg/m<sup>3</sup> for the 0, 200, 700 and 2000 mg/m<sup>3</sup> exposure groups, respectively. The average aerosol concentrations were  $0.0 \pm 0.0$  (background),  $0.0 \pm 0.0$ ,  $4.6 \pm 3.0$  (0.7 percent) and  $242.7 \pm 34.6$  (12 percent) mg/m<sup>3</sup> for the 0, 200, 700 and 2000 mg/m<sup>3</sup> exposure groups, respectively (Wong *et al.*, 2013). A summary of the findings are found in Tables 12 through 14.

Table 12 compares general findings between the HEFA-C 90-day study and the SPK 90-day study. Like the HEFA-C study, male and female F-344 rats were exposed 6 hours/day, 5 days/week over 90 days to 0, 200, 700 or 2000 mg/m<sup>3</sup> SPK jet fuel. Actual exposure concentrations were found to be  $173.2 \pm 8.2$ ,  $598.9 \pm 44.6$  and  $2046.8 \pm 76.8$  mg/m<sup>3</sup>, while aerosol concentrations were measured at  $0.11 \pm 0.12$ ,  $1.28 \pm 0.54$ ,  $81.8 \pm 14.2$  and  $656.4 \pm 67.7$  mg/m<sup>3</sup> (Mattie *et al.*, 2011b). Further, a JP-8 continuous inhalation exposure study is also compared. Male and female F-344 rats were exposed to 0, 500 or 1000 mg/m<sup>3</sup> JP-8 vapor, continuously, for 90 consecutive days (Mattie *et al.*, 1991). An aerosol exposure appears to be required to produce changes in nasal cavity tissues and lung epithelium.

**Table 12. Gross Findings from 90-Day Inhalation Exposures to HEFA Biofuels Compared with JP-8 and SPK**

Fuel* POSF #	Exposure mg/m <sup>3</sup> (% aerosol)	Exposure Duration	NOAEC LOAEC mg/m <sup>3</sup>	Result	Reference
<b>Gross Observations</b>					
<b>HEFA-C</b> 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk	700 2000	Decreased body weight (M, 5%)	Wong <i>et al.</i> (2013)
<b>HEFA-C</b> 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk	700 2000	Decreased body weight (F, 3%)	Wong <i>et al.</i> (2013)
<b>SPK</b> <b>(F-T)</b> 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	700 2000	Decreased body weight (M, 12%)	Mattie <i>et al.</i> (2011b)
<b>SPK</b> <b>(F-T)</b> 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	700 2000	Decreased body weight (F, 5%)	Mattie <i>et al.</i> (2011b)
<b>SPK</b> <b>(F-T)</b> 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	700 2000	Decreased food consumption (M & F)	Mattie <i>et al.</i> (2011b)
<b>JP-8</b> NR	0, 500, 1000 vapor only (0)	23 hr/dy continuous	no NOAEC vapor: 500	Decreased body weight (M, 4.9%)	Mattie <i>et al.</i> (1991)

**Table 12 (continued).**

Fuel* POSF #	Exposure mg/m <sup>3</sup> (% aerosol)	Exposure Duration	NOAEC LOAEC mg/m <sup>3</sup>	Result	Reference
<b>Gross Pathology, Histopathology</b>					
HEFA-C 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk	700 2000	Olfactory epithelial degeneration & goblet hyperplasia in nasal airways (M&F)	Wong <i>et al.</i> (2013)
SPK (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	700 2000	Olfactory epithelial degeneration & respiratory epithelial hyperplasia in nasal airways; Lung inflammatory cell infiltration (M&F)	Mattie <i>et al.</i> (2011b)
JP-8 NR	0, 500, 1000 vapor only (0)	23 hr/dy continuous	NA-Hmn	Increased relative kidney weight & kidney lesions consistent with $\alpha_2$ - microglobulin nephropathy (M)	Mattie <i>et al.</i> (1991)
<b>Clinical Chemistry, Hematology</b>					
HEFA-C 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk	2000 no LOAEC	No dose-dependent changes in clinical chemistry or hematology	Wong <i>et al.</i> (2013)
SPK (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	2000 no LOAEC	No dose-dependent changes in clinical chemistry or hematology	Mattie <i>et al.</i> (2011b)
JP-8 NR	0, 500, 1000 vapor only (0)	23 hr/day continuous	vapor: 1000 no LOAEC	No dose-dependent changes in clinical chemistry or hematology	Mattie <i>et al.</i> (1991)
<b>Alpha 2-Microglobulin</b>					
HEFA-C 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk	NA-Hmn	Dose-dependent increase in kidney $\alpha_2$ - microglobulin concentration	Wong <i>et al.</i> (2013)
SPK (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	NA-Hmn	No significant $\alpha_2$ -microglobulin nephropathy	Mattie <i>et al.</i> (2011b)

Note: \*Alternative jet fuel names evolve over time; the current name may be listed with the name used in the publication in parentheses. dy = day. F = female. hr = hour. LOAEC = lowest observed adverse effect concentration. M = male. NA = not applicable. NA-Hmn = not applicable to human health risk. NOAEC = no observed adverse effect concentration. NR = not reported. wk = week.

**6.5.4 Reproductive Outcomes.** In conjunction with the 90-day inhalation studies, HEFA-C and SPK have been evaluated for specific reproductive endpoints. Table 13 compares these findings. JP-8 was evaluated more extensively as part of a 90-day oral study in F-344 rats; the 90-day study was first reported in Mattie *et al.* (1995) and reproductive results were reported later (Mattie *et al.*, 2000). In the first experiment, male rats were given 0, 750, 1500 or 3000 mg/kg neat JP-8 daily by gavage for 70 days prior to mating with naïve females to assess fertility and

sperm parameters. In the second reproductive experiment, general toxicity, fertility and reproductive endpoints were assessed in female rats dosed with neat JP-8 (0, 325, 750 or 1500 mg/kg) daily by gavage for a total of 21 weeks (90-days plus mating with naïve males, gestation and lactation). None of these jet fuels resulted in findings that would indicate adverse outcomes targeting the reproductive system.

**Table 13. Reproductive Outcomes from 90-Day Exposures to HEFA Biofuels Compared with JP-8 and SPK**

Fuel* POSF #	Exposure mg/m <sup>3</sup> **(% aerosol)	Exposure Duration	NOAEC LOAEC mg/m <sup>3</sup>	Result	Reference
HEFA-C 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk	2000 no LOAEC	No significant differences in male reproductive endpoints	Wong <i>et al.</i> (2013)
HEFA-C 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk	2000 no LOAEC	No alteration of estrus cycle (vaginal cytology endpoints)	Wong <i>et al.</i> (2013)
SPK (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	2000 no LOAEC	No significant differences in male reproductive endpoints	Mattie <i>et al.</i> (2011b)
SPK (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	2000 no LOAEC	No alteration of estrus cycle (vaginal cytology endpoints)	Mattie <i>et al.</i> (2011b)
JP-8 NR	ORAL (0, 750, 1500, 3000 mg/kg)	M ONLY 1 dose/dy, 90 dy	3000 mg/kg (NOAEL) no LOAEL	No significant changes for pregnancy rate, gestation length, sperm parameters	Mattie <i>et al.</i> (2000)
JP-8 NR	ORAL (0, 325, 750, 1500 mg/kg)	F ONLY 1 dose/dy, 146 dy	1500 mg/kg (NOAEL) no LOAEL	No significant changes for gestation length, pregnancy rate, number of pups/litter	Mattie <i>et al.</i> (2000)
JP-8 NR	ORAL (0, 325, 750, 1500 mg/kg)	F ONLY 1 dose/dy, 146 dy	750 mg/kg (NOAEL) 1500 mg/kg (LOAEL)	Decreased pup weight related to decreased maternal weight	Mattie <i>et al.</i> (2000)

Note: \*Alternative jet fuel names evolve over time; the current name may be listed with the name used in the publication in parentheses. \*\*Some exposures were oral; units are specified when different. dy = day. F = female. hr = hour. LOAEC = lowest observed adverse effect concentration. LOAEL = lowest observed adverse effect level. M = male. NA = not applicable. NOAEC = no observed adverse effect concentration. NOAEL = no observed adverse effect concentration. NR = not reported. wk = week.

**6.5.5 Neurobehavioral Function.** During the HEFA-C 90-day exposure, animals were assessed for neurobehavioral effects utilizing a functional observation battery (FOB) after the 13<sup>th</sup> week of exposure and motor activity assay after the 14<sup>th</sup> week (Wong *et al.*, 2013). Results are compared with outcomes from the SPK 90-day study and multiple JP-8 studies. Changes in behavioral response were observed in two studies where male SD rats were exposed to 0, 500 or 1000 mg/m<sup>3</sup> JP-8 vapor for 6 hours/day 5 days a week for 6 weeks (Ritchie *et al.*, 2001). In a second study using the same exposure methods, animals were tested in a large battery of neurobehavioral tasks (Rossi *et al.*, 2001).

**6.5.6 Conclusions from Inhalation Exposures.** Multiple inhalation studies with HEFA fuels indicate that their toxicity, in general, is similar to that of petroleum-derived JP-8. Comparisons of gross observations from acute (4 to 6 hours) studies (Table 10) indicate no difference between HEFA-F, SPK and JP-8 fuels. Histopathology is not normally performed in these acute assays, leaving no results with which to compare the HEFA-F findings. It is likely that the minimal olfactory and alveolar effects are inherent to aerosol inhalation.

Again, comparisons of short term study gross observations (Table 11) are very similar between fuels. Some differences between fuels are noted in the histopathology data. Minimal olfactory epithelial degeneration is seen in even the lowest HEFA-F exposure group; however, the effect was not dose dependent, again indicating an aerosol irritation effect that may not be specific to the actual compounds in the exposure. Alveolar inflammation occurred in both HEFA-F and SPK exposures, but was not found in the Jet A exposure. The occurrence of lung interstitium fibrosis is a potential concern; however, fibrosis was not found in the 90-day study (Table 12), indicating that this change may resolve over further exposure time.

Comparison of 90-day exposures shows even fewer differences between fuels (Table 12). Decreased bodyweight by a small percentage is a common occurrence; HEFA-C resulted in the lowest average bodyweight change, followed by JP-8 and then SPK. Minor olfactory and respiratory histopathological changes occurred with both alternative fuels but not with JP-8; JP-8 exposures were vapor only, however, giving further indication that these changes are related to aerosol exposures.

Further, the 90-day studies were accompanied by reproductive and neurobehavioral assays in order to better characterize the toxicity of each fuel without the cost of an additional exposure. No adverse reproductive endpoints were found with any of the fuels (Table 13). No neurobehavioral effects were found with HEFA-C exposure; sporadic changes in the open field test (rearing behavior) and motor activity assay were found among the highest exposure group of SPK exposed rats (Table 14). Neurobehavioral effects from JP-8 exposure likely derive from the aromatic content of JP-8, of which the alternative fuels have little or none.

Overall, transient histopathological changes were seen with the lowest exposure concentration of HEFA-F (200 mg/m<sup>3</sup>) fuel. These changes resolve over time and only the highest concentration (2000 mg/m<sup>3</sup>) continued to exhibit effects from HEFA-C exposure for 90 days.

**Table 14. Neurobehavioral Outcomes from 90-Day Exposures to HEFA Biofuels Compared with JP-8 and SPK**

Fuel* POSF #	Exposure mg/m <sup>3</sup> (% aerosol)	Exposure Duration	NOAEC LOAEC mg/m <sup>3</sup>	Result	Reference
<b>FOB</b>					
<b>HEFA-C</b> 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk, 90 dy	2000 no LOAEC	No significant changes (M&F)	Wong <i>et al.</i> (2013)
<b>SPK</b> (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk, 90 dy	700 2000	Reduced rearing behavior (F)	Mattie <i>et al.</i> (2011b)
<b>Motor Activity</b>					
<b>HEFA-C</b> 6152	0, 200, 700, 2000 (0, 0, 0.7, 12)	6 hr/dy, 5 dy/wk, 90 dy	2000 no LOAEC	No significant changes (M&F)	Wong <i>et al.</i> (2013)
<b>SPK</b> (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk	700 2000	Reduced total activity (M)	Mattie <i>et al.</i> (2011b)
<b>SPK</b> (F-T) 5109	0, 200, 700, 2000 (0, 0.6, 12, 33)	6 hr/dy, 5 dy/wk, 90 dy	700 2000	Reduced initial exploratory activity (F)	Mattie <i>et al.</i> (2011b)
<b>JP-8</b> 3509	70, 500, 1000 vapor only (0)	6 hr/dy, 5 dy/wk, 90 dy	vapor: 1000 no LOAEC	No significant changes	Rossi <i>et al.</i> (2001)
<b>Other Neurobehavioral Tests</b>					
<b>JP-8</b> 3509	70, 500, 1000 vapor only (0)	6 hr/dy, 5 dy/wk, 90 dy	vapor: 500 vapor: 1000	Deficits in acquisition, performance of moderate to difficult tasks (stimulus reversal, incremental repeated acquisition)	Ritchie <i>et al.</i> (2001)
<b>JP-8</b> 3509	70, 500, 1000 vapor only (0)	6 hr/dy, 5 dy/wk, 90 dy	vapor: 1000 no LOAEC	No difficulty with simple learning tasks (lever acquisition, fixed ration, lever spatial reversal)	Ritchie <i>et al.</i> (2001)
<b>JP-8</b> 3509	70, 500, 1000 vapor only (0)	6 hr/dy, 5 dy/wk, 90 dy	vapor: 1000 no LOAEC	No difficulty with acoustic startle, forelimb grip strength, nociception, social interaction, forced swim test, passive avoidance, Morris water maze	Rossi <i>et al.</i> (2001)
<b>JP-8</b> 3509	70, 500, 1000 vapor only (0)	6 hr/dy, 5 dy/wk, 90 dy	vapor: 500 vapor: 1000	Longer duration spent on appetitive stimulus approach sensitization assay	Ritchie <i>et al.</i> (2001)

Note: \*Alternative jet fuel names evolve over time; the current name may be listed with the name used in the publication in parentheses. dy = day. FOB = functional observation battery. hr = hour. LOAEC = lowest observed adverse effect concentration. NA = not applicable. NOAEC = no observed adverse effect concentration. NR = not reported. wk = week.

## 7.0 HEFA OCCUPATIONAL EXPOSURE LEVEL RECOMMENDATION

Similar to the certification described above for SPK (Section 6.0), HEFA alternative fuels will be certified for use in a 50/50 blend with petroleum-derived JP-8. Air Force personnel will potentially come into contact with the whole HEFA fuel prior to mixing or to the blend. The comparative HHA in Section 6 indicates that the HEFA fuels are not expected to be more hazardous than JP-8 to handle. Therefore, an OEL of 200 mg/m<sup>3</sup> for vapor and 5 mg/m<sup>3</sup> for aerosol is recommended at this time.

A single OEL is recommended for HEFA fuels of the feedstocks featured in Section 6.0 (Camelina, Mixed Fats and Oils, Tallow). This recommendation is justified due to the similarity of chemical composition, as presented in the analytical summary in Table 15. See Appendix G for a more detailed analysis. The results of toxicity tests in which all three feedstocks were tested (Sections 6.3 HEFA Dermal Irritation/Toxicity and 6.4 HEFA Mutagenicity and Genotoxicity) were also similar and provide additional confirmation.

**Table 15. HEFA Fuels Composition and Comparison to Jet A**

<b>FUEL</b>	<b>Jet A</b>	<b>HEFA-C</b>	<b>HEFA-T</b>	<b>HEFA-F</b>
POSF	POSF-4658	POSF-6152	POSF-6308	POSF-5469
<b>Aromatics</b>				
Total Alkylbenzenes	13.69	0.26	0.20	0.46
Total Alkylnaphthalenes	1.76	<0.01	0.02	<0.01
Total Cycloaromatics	5.79	0.06	0.09	0.10
<b>Total Aromatics</b>	<b>21.25</b>	<b>0.33</b>	<b>0.31</b>	<b>0.56</b>
<b>Aliphatics</b>				
Total iso-Paraffins	31.34	84.18	87.21	81.70
Total n-Paraffins	19.00	11.41	11.61	14.62
Total Cycloparaffins	28.42	4.09	0.86	3.12
<b>Total Aliphatics</b>	<b>78.75</b>	<b>99.67</b>	<b>99.69</b>	<b>99.44</b>

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## **APPENDIX A. RD<sub>50</sub> STUDY PROTOCOL**

**U.S. AIR FORCE SPONSORED ANIMAL RESEARCH PROPOSAL  
SIGNATURE COORDINATION SHEET**

**I. NAME OF FACILITY:**

Naval Medical Research Unit (NAMRU) – Dayton, Wright-Patterson AFB, OH

**II. PROTOCOL NUMBER: F-WA-2010-0125-A**

**III. PROTOCOL TITLE: Sensory Irritation Study of Two Hydro-Treated Renewable Jet (HRJ) Fuels in Mice (*Mus musculus*)**

**IV. PRINCIPAL INVESTIGATOR:**

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MICHAEL L. GARGAS (Printed Name)      Michael L. Gargas (Signature)      17 Feb 2011 (Date)

**V. SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.**

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Karen L. Mumy (Printed Name)      Karen Mumy (Signature)      15 Feb 2011 (Date)

**VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensured that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.**

**U.S. AIR FORCE SPONSORED ANIMAL RESEARCH PROPOSAL  
SIGNATURE COORDINATION SHEET**

**I. NAME OF FACILITY:**

Naval Medical Research Unit (NAMRU) – Dayton, Wright-Patterson AFB, OH

**II. PROTOCOL NUMBER: F-WA-2010-0125-A**

**III. PROTOCOL TITLE: Sensory Irritation Study of Two Hydro-Treated Renewable Jet (HRJ) Fuels in Mice (*Mus musculus*)**

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**V. SCIENTIFIC REVIEW: This animal use proposal received appropriate peer scientific review and is consistent with good scientific research practice.**

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**VI. STATISTICAL REVIEW: A person knowledgeable in biostatistics reviewed this proposal and ensured that the number of animals used is appropriate to obtain sufficient data and/or is not excessive, and the statistical design is appropriate for the intent of the study.**

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**VII. ATTENDING VETERINARIAN:** In accordance with Animal Welfare Regulations, the Attending Veterinarian was consulted in the planning of procedures and manipulations that may cause more than slight or momentary pain or distress, even if relieved by anesthetics or analgesics.

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**PROTOCOL TITLE**

Sensory Irritation Study of Two Hydro-Treated Renewable Jet (HRJ) Fuels in Mice (*Mus musculus*)

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17 Feb 2011  
(Date)

## **I. NON-TECHNICAL SYNOPSIS**

The Office of the Secretary of Defense Assured Fuels Initiative is pursuing domestically produced alternative fuels for military use to decrease dependence on foreign oil sources. There are now two biobased jet fuels called hydro-treated renewable jet (HRJ) produced from the biological sources (*renewable*) animal fat and plant oil using a process called hydrotreatment (water and high pressure; *hydro-treated*). Inhalation is one of the primary routes of exposure for fuels so it is very important to study all effects on the lungs as a result of breathing a chemical mixture such as jet fuel. This study will investigate the sensory irritation potential of two HRJ jet fuels, Camelina and Tallow. Sensory irritation is expressed as the RD<sub>50</sub> or the concentration that produces a 50% decrease in respiratory rate or how fast you are breathing (breaths per minute). To determine the RD<sub>50</sub>, each HRJ will be administered by inhalation exposure at five concentrations with 2000 mg/m<sup>3</sup> as the starting level. The other four concentrations (at least one higher and at least one lower) will be determined based on the results of each previous concentration. The last concentration will be vapor only while the other four will be a vapor/aerosol mix. The vapor only level will match one of the mixed concentrations to determine if there is a difference between a vapor/aerosol mix and vapor only. Male mice (four per concentration) will be exposed by nose only to 10 minutes of air to establish a control value then receive 30 minutes of HRJ exposure followed by 10 more minutes of air only to determine if the respiratory rate returns to control levels. The total number of mice will be forty-eight with eight for training and twenty for each HRJ jet fuel (five concentrations × four mice × two fuels). Mice will be euthanized after their exposure to jet fuel. Results will be used to develop the health hazard assessment for each HRJ jet fuel and to set safe exposure levels for short-term occupational exposures.

## **II. BACKGROUND**

### **II.1. Background**

The Office of the Secretary of Defense Assured Fuels Initiative is pursuing domestically produced alternative fuels for military use to decrease dependence on foreign oil sources. These fuels would potentially be used in military aircraft and ground vehicles, as well as ships. Fuels are among the most common sources of military occupational exposures. Dermal contact and inhalation are generally the primary routes of exposure. Preliminary analysis of the new fuels shows that many of the ingredients are the same as JP-8, the traditional military fuel, but the composition is still different in each fuel. Therefore, the health effects associated with exposure to each alternative fuel may also be significantly different than JP-8.

One of the alternate fuels currently produced in the United States is a Fischer-Tropsch (F-T) fuel made from natural gas (S-8). RHPB conducted a Toxicology Program for this Fischer-Tropsch (F-T) fuel that along with JP-8 will be the baseline for comparing all future biobased alternative fuels. There are already two biobased jet fuels that need to be examined as they undergo further development and certification. Until we learn more about the new alternative fuels, we need to examine each one for potential hazards to DoD personnel.

Respiratory tract sensory irritation was examined for JP-4, JP-8 and JP-8+100 in male Swiss-Webster mice. Sensory irritation is expressed as the RD<sub>50</sub> or the concentration that produces a 50% decrease in respiratory rate. Alarie (1973) demonstrated a dose response for breathing rate depression related to irritation of the respiratory system. Alarie (1981) then established a correlation of the RD<sub>50</sub> value with existing occupational exposure limits. In a previous study, groups of mice were exposed nose only to JP-4 (685, 956, 1888 or 11430 mg/m<sup>3</sup>), JP-8 (681, 708, 1090, 1837 or 3565 mg/m<sup>3</sup>) or JP-8+100 (777, 1519 or 2356 mg/m<sup>3</sup>) for 30 minutes. The calculated concentration at which the respiratory rate decreased 50% (RD<sub>50</sub>) was 4842, 2876 and 1629 mg/m<sup>3</sup> for each fuel, respectively (Whitman and Hinz, 2001). More recently the RD<sub>50</sub> for F-T jet fuel was determined to be 10,939 mg/m<sup>3</sup> meaning that F-T is much less irritating than JP-8 (Mattie *et al.*, 2010). The HRJ jet fuels are also expected to be less irritating than JP-8 but there is not enough data available to predict the actual values without conducting the animal study.

## **II.2. Literature Search for Duplication**

### **II.2.1. Databases Searched**

Biomedical Research Database (BRD), Research Portfolio Online Reporting Tool (RePORTER), Federal Research in Progress (FEDRIP), AltWeb, Cambridge Scientific Abstracts, PubMed, DTIC technical reports and research summaries, Engineering Village 2 (Compendex, NTIS and INSPEC), and Dialog databases: BIOSIS Previews, Agricola, EMBASE, Toxfile, Dissertation Abstracts Online, Inside Conferences, International Pharmaceutical Abstracts, IPA Toxicology, MEDLINE, BIOSIS Toxline.

### **II.2.2. Number, Date, and Resources of Search**

2011152, December 28, 2010, and Carol Reed, Reference Librarian, D'Azzo Research Library

### **II.2.3. Period of Search**

All years for each database

### **II.2.4. Key Words of Search**

Kerosene, Jet A, jet fuel, JP-8, HRJ, hydrorenewable jet, HRJ Camelina, HRJ plant oils, HRJ Tallow, HRJ Animal Fats and Oils, biofuel, biobased/bio-based, toxicity/toxicology, animal, rat, mouse/mice, micronucleus, inhalation, acute, two-week, 90-day, subchronic, 13-week, sensory irritation (in lungs so respiratory tract), RD<sub>50</sub>, Alarie

### **II.2.5. Results of Search**

The results of this literature search found no articles for the sensory irritation assay with HRJ jet fuels, although related articles were found for JP-8. Therefore, duplication of research efforts proposed for this project was not identified.

## **III. OBJECTIVE / HYPOTHESIS**

HRJ jet fuels have the potential for both vapor and aerosol (fuel droplets) inhalation exposure. This study is designed to assess the sensory irritation potential of HRJ jet

fuels when administered via inhalation exposure to mice once for 30 minutes duration; that is obtain an RD<sub>50</sub> value, a 50% decrease in respiratory rate. The effect of HRJ jet fuels on respiratory rates have not been measured in any previous inhalation study. Based on the RD<sub>50</sub> value for JP-8, JP-4 and F-T (S-8), our hypothesis is that the RD<sub>50</sub> for the two HRJs will be higher than JP-8 and lower than S-8.

#### **IV. MILITARY RELEVANCE**

The military is pursuing a number of alternate fuels aimed at increasing domestic fuel production. Two of the alternate fuels currently produced in the United States are the hydrorenewable jet or HRJ jet fuels. These fuels will be used in Air Force aircraft and ground vehicles, as well as Navy ships and Army tanks. Certification of the Air Force fleet to fly using HRJ fuel is already in progress. Fuels are among the most common sources of military occupational exposures. Inhalation is one of the primary routes of exposure, so it is very important to study effects on the lungs and body. Preliminary analysis of the new fuel suggests that the components are similar to JP-8, the traditional military fuel, but the overall composition is significantly different; therefore, the health effects associated with exposure to the alternative fuel may also be significantly different than JP-8. The Air Force Research Laboratory Applied Biotechnology Branch (711 HPW/RHPB) and the Naval Medical Research Unit-Dayton (NAMRU-D) have designed a toxicity testing program for these alternative fuels. The program represents toxicity testing required to develop a health hazard assessment for the fuel in order to develop occupational exposure limits. The Air Force Research Laboratory Applied Biotechnology Branch (AFRL/711 HPW/RHPB) has been asked by the Air Force Alternative Fuels Certification Office and the Air Force Research Laboratory Fuels Branch to determine the potential health effects of the alternative fuels under development and certification. The Sensory Irritation Study or RD<sub>50</sub> Test will be used to help develop the health hazard assessment and to establish acute exposure guidelines (AEGs) for DoD personnel.

##### **IV.1. Funding Agency**

U. S. Air Force, AFMC, Alternative Fuels Certification Office, ASC/WNN

#### **V. MATERIALS AND METHODS**

##### **V.1. Experimental Design and General Procedures**

###### Test Guideline

The RD<sub>50</sub> study design is based on the ASTM International (American Society for Testing and Materials) guideline, Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals, Designation: E 981-04 (ASTM, 2004). Mice each will be exposed to HRJ jet fuel in nose-only plethysmograph tubes for monitoring respiratory rate.

###### Number of Animals

The number of male mice (n=4) per concentration group has been shown to be adequate for calculating RD<sub>50</sub> values for a large number of chemical irritants (ASTM, 2004).

The number of mice is only four because the ASTM method requires the use of only one sex and strain of mice and a very narrow weight range all of which reduce variability and the need for larger numbers of animals. This method was used to determine sensory irritation for JP-4, JP-8 and JP-8+100 (Whitman and Hinz, 2001).

#### Test Articles

Test Article #1 Identification/Lot Number:  
HRJ Camelina/ (UOP) - POSF 6152

Test Article #2 Identification/Lot Number:  
HRJ Tallow/ (UOP) - POSF 6308

#### Route, Duration and Frequency of Administration

The test articles, HRJ jet fuels, will be administered as an aerosol/vapor combination and as vapor only via a modified nose-only plethysmograph tube. The inhalation route is one of the potential routes of human exposure to this test substance. The mice will be exposed for 10-min to air, followed by 30-min HRJ jet fuel, followed by 10-min air. Each mouse will serve as its own control and will only be exposed to a single HRJ for one 30 minute period. At the conclusion of the exposure, all mice will be euthanized within approximately one hour.

#### **V.1.1. Experiment 1 - Training**

Personnel are familiar with exposing rats in nose-only exposure systems. One person has conducted RD<sub>50</sub> studies at another laboratory. A new system has been purchased to conduct RD<sub>50</sub> studies in either building 824 or 837. In order to ensure everyone is familiar with the ASTM E 981-04 procedure and the new BUXCO respiratory measurement system, 8 mice are requested for training of personnel and to validate the system under normal, non-exposure conditions. The mice will always be treated the same as in this protocol which is per the ASTM guideline so the maximum time in the nose-only exposure system will be 50 minutes per day. These mice will be euthanized after training personnel and establishing baseline respiratory data unless they can be transferred to another protocol not involving RD<sub>50</sub> measurements.

#### **V.1.2. Experiment 2**

##### Exposure Levels

Animals will be exposed to five concentrations of each HRJ jet fuel; four concentrations will be mixed aerosol and vapor and one concentration will be repeated as vapor only. An initial exposure level of 2,000 mg/m<sup>3</sup> was selected based on literature results of previous inhalation studies with jet fuels of similar chemical composition. This concentration has been used at The Hamner Institute in acute, two-week, and thirteen-week inhalation toxicity studies of S-8 and by the NAMRU-Dayton in two two-week JP-8

studies. Results obtained from the initial exposure level of 2,000 mg/m<sup>3</sup> will be used to guide additional target exposure concentrations by determining the per cent decrease in respiratory rate and by comparing the change in respiratory rate to the previous jet fuel studies. Subsequent concentrations must then be higher or lower to establish a dose curve that identifies the level that produces 50% reduction in respiratory rate.

#### Respiratory Rate Measurement during Exposure

Prior to exposure animals will be loaded into modified nose-only plethysmograph tubes and placed onto the exposure tower. Respiratory rates will be monitored and recorded for a 10 minute acclimation period, during which animals will be breathing HEPA-filtered house air. Following the acclimation period the respiratory rates will be monitored and recorded for a 30 minute exposure and a 10 minute post exposure recovery period. All data will be collected and stored electronically by the Buxco plethysmography system using the Buxco BioSystem FinePoint Software (Buxco Research Systems, Wilmington NC). Methods will be documented in the technical report.

#### Observations

Animals will be observed before, during and after exposures for overt signs of toxicity.

#### Study Summary

<b>Group</b>	<b>Exposure Level</b>	<b>Number of Animals</b>	<b>Number of Animals</b>	<b>Number of Animals</b>	
	<b>mg/m<sup>3</sup></b>		<b>HRJ 1</b>	<b>HRJ 2</b>	
Training		8			
Intermediate 1	2000 mg/m <sup>3</sup>		4	4	
Intermediate 2	TBD		4	4	
Low	TBD		4	4	
High	TBD		4	4	
	TBD vapor only		4	4	
<b>Total</b>		<b>8</b>	<b>20</b>	<b>20</b>	<b>48</b>

#### Acclimation Period

Shortly after their arrival at the laboratory, the animals will be transported to a room selected for the study for acclimation. The animals will be removed from the shipping cartons and examined. All animals with evidence of disease or physical abnormalities will be euthanized. If an unusually large number of animals show evidence of disease or physical abnormalities, the entire shipment of animals will be rejected for use in the study. Animals will be acclimated to the facility for at least 7 days. Prior to assignment to study, all animals will be examined by an animal care staff member to ascertain

suitability for study. They will be weighed, randomized by mean body weights to the dose groups and ear tagged for identification. Individual weights of animals placed on test will range from 22-28 grams. This is a tight weight range for outbred mice. A weight outside of this range (but no less than 20 grams or greater than 30 grams) will be acceptable on occasion. To reduce stress during exposure, mice will be acclimated to modified nose-only plethysmograph tubes for 50 minutes on the day preceding exposure and at least two additional times prior to the day before acclimatization. All four mice in a concentration group will be acclimated at the same times.

#### Test Substance Administration

A nose-only exposure system (Lab Products, Seaford, DE) will be used. Mice will be restrained in nose-only tubes that attach to the exposure system. Chamber airflow will be maintained at a minimum of 1.5 times the mouse's estimated minute ventilation, or approximately 50 ml/min per mouse. The test substance will be administered as an aerosol and vapor combination for 4 concentrations. An additional vapor only concentration will match one of the combined exposure levels. The exposure atmosphere will be generated first in a stainless steel and glass inhalation chamber (H640 or H1000). The atmosphere of this chamber will be drawn into the nose-only chamber for delivery to mice. Procedures will be determined during pre-study trials. The trials will be performed to evaluate the optimal set of conditions and equipment to generate a stable atmosphere at the target exposure levels. Methods will be described in the raw data of the study and in a technical report.

#### Generation of Test Substance

The test atmosphere will be generated by a spray nozzle using procedures developed during pre-study trials and used for the previous jet fuel studies. Trials will be performed to evaluate the optimal set of conditions and equipment to generate a stable atmosphere at the target exposure levels. The method will be described in the raw data of the study and in the technical report.

#### Monitoring of Test Substance Concentration

A nominal exposure concentration will be calculated. The flow of air through the chamber will be monitored using appropriate, calibrated equipment. The test substance consumed during the exposure and the total volume of air passing through the chamber (volumetric flow rate times total exposure time) will be used to calculate the nominal concentration.

During the exposure, measurements of airborne concentrations will be performed at the inlet to the nose only exposure system and will be made at least once using an appropriate sampling procedure and analytical method. The analytical method will be the same as developed for previous jet fuel inhalation studies. The sampling methods and procedures developed in the pre-study trials will be documented in the technical report.

#### Particle Size distribution

Prior to each exposure, a particle size determination will be performed using an optical particle sizing instrument and/or cascade impactor.

#### Monitoring of Environmental Conditions

Chamber temperature, humidity, airflow rate and static pressure will be monitored continuously and recorded during the exposure. Chamber temperature and relative humidity will be maintained, to the maximum extent possible, between 20 to 24°C and 30 to 70%, respectively.

#### **In-Life Evaluation Observations**

##### Viability Checks

Animals will be observed for morbidity, mortality, general appearance and signs of severe toxic or pharmacological effects before, during and after the exposure. Animals will be observed at least once during the day prior to exposure. Animals in extremely poor health or in a possible moribund condition will be identified for further monitoring and possible euthanasia.

##### Clinical Observations

Each animal will be examined at least twice pre-exposure and once after the exposure. Examinations will include observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia as well as evaluations of respiration, circulatory effects, autonomic effects, central nervous system effects, and reactivity to handling or sensory stimuli.

##### Body Weight

Body weights will be recorded the day after arrival, at randomization and the day of exposure.

#### **Postmortem Observations**

##### Moribund and Humane Euthanasia

Animals showing signs of severe debility, particularly if death appears imminent, will be euthanatized to prevent unnecessary suffering.

##### Terminal Necropsy

Euthanasia and gross necropsy of all surviving animals will be performed on the day of the last exposure within approximately one hour of the end of the exposure.

##### Gross Necropsy

A complete macroscopic examination will be performed on all animals, including all scheduled and unscheduled deaths; all abnormal observations will be recorded. The necropsy will include observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia. A full necropsy will not be performed unless requested by the Principal Investigator or a Co-Investigator based on results of the physical examination.

## **V.2. Data Analysis**

### **V.2.1.**

#### **Respiratory Rates and Mean Body Weights**

For respiratory rate, individual animal responses will be evaluated either manually counting the respiratory rate or by using an automatic rate counter (minus body movement artifacts). Each group of four mice serves as its own control. In general, the control respiratory rate is the average of six 15-sec intervals immediately preceding the test agent exposure period. Respiratory rate for each 15-sec interval of the first five minutes of exposure and at 3-min intervals for the remainder of the exposure period will be calculated. Rates will be calculated at 1 min intervals for the post exposure period. Data sets will be used to prepare a concentration-response regression to calculate an RD<sub>50</sub> value (the concentration required to reduce the respiratory rate by 50%) and 95% confidence limits (Armitage, 1971; ASTM, 2004).

Mean body weight values will also be calculated and comparison between exposure day weight and the two pre exposure weights will be made by analysis of variance (Shirley, 1977; Williams, 1971 and 1972).

For all experiments, results will be considered significant when  $p < 0.05$ .

## **V.3. Laboratory Animals Required and Justification**

### **V.3.1. Non-animal Alternatives Considered**

There are still no adequate non-animal alternatives to *in vivo* inhalation studies and the sensory irritation assay. Toxicity assessments in cell lines to eliminate or reduce the use of animals exposed by inhalation have been conducted by various researchers. However, there is still little correlation between *in vitro* and *in vivo* studies of lung toxicity (Sayes, Reed and Warheit, 2007). Living animal models must still be used due to the complex nature of the lungs and intricate interaction between the central and peripheral nervous systems involving sensory irritation and the effect on respiratory rate.

### **V.3.2. Animal Model and Species Justification**

The purpose of this study is to provide sensory irritation data associated with acute inhalation exposure of the test substance. The mouse is used as a surrogate for humans in estimating sensory irritation potential and is a species in which known chemical irritants have been detected. The experimental design for this protocol uses the procedures and standards required by the ASTM International (American Society for Testing and Materials) guideline, Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals, Designation: E 981-04 (ASTM, 2004). This guideline specifically identifies the Swiss-Weber strain of male mice for use in the sensory irritation study. Historical control data are also available with the Swiss-Webster stock (male) for comparative evaluation.

### **V.3.3. Laboratory Animals**

#### **V.3.3.1. Genus / Species**

*Mus musculus*

#### **V.3.3.2. Strain / Stock**

Swiss-Webster Crl:CFW(SW)

#### **V.3.3.3. Source / Vendor**

Charles River Laboratories, Wilmington, MA, 01887-1000, USDA Number 14-B-013

#### **V.3.3.4. Age**

At receipt: 4 weeks

At start of exposures: 5-6 weeks

#### **V.3.3.5. Weight**

22-28g (5 weeks of age)

#### **V.3.3.6. Sex**

Male. ASTM guideline, E 981-04 requires only male mice as it has been established that sensory irritation is not sex specific (ASTM, 2004)

#### **V.3.3.7. Special Considerations**

None

### **V.3.4. Number of Animals Required (by Species)**

48 Mice

### **V.3.5. Refinement, Reduction, Replacement (3 R's):**

#### **V.3.5.1. Refinement**

This study has been refined and harmonized by the ASTM International (American Society for Testing and Materials) guideline, Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals, Designation: E 981-04 (ASTM, 2004).

Although mice will be acclimated to reduce stress during exposure for 50 minutes on the day preceding exposure to be consistent with past RD<sub>50</sub> studies conducted for Air Force jet fuels, a further refinement will be to acclimate at least two additional times. This will ensure the mice are not under stress from the exposure tube.

#### **V.3.5.2. Reduction**

The number of animals selected is the minimum required to satisfy the guideline. This study is designed with each animal serving as its own control which eliminates a control group and reduces the number of animals used. Data collected during the first ten minutes in the nose-only chamber determines the control level of respiratory rate that is compared to the respiratory rate during exposure after 30 minutes of jet fuel exposure.

### **V.3.5.3. Replacement**

Currently there is no *in vitro* system that can substitute for an animal inhalation model. No non-animal systems are capable of addressing the research questions being asked in this protocol. There is still little correlation between *in vitro* and *in vivo* studies of lung toxicity (Sayes, Reed and Warheit, 2007). Animal systems, specifically the mouse, are necessary for determining the sensory irritation effect of HRJ jet fuels on the respiratory system.

## **V.4. Technical Methods**

### **V.4.1. Pain / Distress Assessment**

#### **V.4.1.1. APHIS Form 7023 Information**

##### **V.4.1.1.1. Number of Animals**

**V.4.1.1.1.1. Column C:** ..... 8 (# of animals)

**V.4.1.1.1.2. Column D:** ..... \_\_\_\_\_ (# of animals)

**V.4.1.1.1.3. Column E:** ..... 40 (# of animals)

##### **V.4.1.2. Pain Relief / Prevention**

###### **V.4.1.2.1. Anesthesia / Analgesia / Tranquilization**

The use of anesthetics, analgesics and tranquilizers is not planned for this study as they are not part of the ASTM guideline and may interfere with the results of the study. Based on the results of previous RD<sub>50</sub> studies with jet fuels (Whitman and Hinz, 2001; Mattie *et al.*, 2010), the mice should not experience any pain and any distress should be minimal. However, since there is a minimal degree of stress associated with placing animals in nose-only chambers that cannot be alleviated by any anesthetic, analgesic or tranquilizer, the exposed mice were placed in Column E.

###### **V.4.1.2.2. Pre- and Post-procedural Provisions**

###### **Viability Checks**

Animals will be observed for morbidity, mortality, general appearance and signs of severe toxic or pharmacological effects before, during and after the exposure. Animals will be observed at least once during the day on non-exposure days prior to exposure. Animals in extremely poor health or in a moribund condition will be identified for further monitoring and possible euthanasia. The veterinary staff and animal caretakers will observe all animals daily.

###### **Clinical Observations**

Each animal will be examined at least twice pre-exposure and once on the day of each exposure. Examinations will include observations of general condition, skin and fur,

eyes, nose, oral cavity, abdomen and external genitalia as well as evaluations of respiration, circulatory effects, autonomic effects, central nervous system effects, and reactivity to handling or sensory stimuli.

Animals that experience severe or chronic pain or distress will be painlessly euthanized. The decision to terminate an animal before the end of the study will be made by the Attending Veterinarian and/or Principal Investigator. The Co-Investigators will be advised by the Principal Investigator of all circumstances which could lead to this action in as timely a manner as possible.

#### **V.4.1.2.3. Paralytics**

None

#### **V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures**

##### **V.4.1.3.1. Databases Searched**

AGRICOLA, PubMed, BIOSIS, Animal Welfare Information Center (AWIC) web site and Johns Hopkins Center for Alternatives to Animal Testing (Altweb/Center for Alternatives to Animal Testing), Bibliography on Alternatives to the Use of Live Vertebrates in Biomedical Research and Testing (Altbib), Non-animal Methods for Toxicity Testing (AltTox), Dialog, DTIC and IR & D

##### **V.4.1.3.2. Number, Date, and Resources of Search**

2011153, December 28, 2010, and Carol Reed, Reference Librarian, D'Azzo Research Library

##### **V.4.1.3.3. Period of Search**

All years for each database

##### **V.4.1.3.4. Key Words of Search**

pain, alternatives, distress, rat, mouse/mice, micronucleus, inhalation, acute, two-week, 90-day, subchronic, 13-week, sensory irritation (in lungs so respiratory tract), RD<sub>50</sub>, Alarie, *in vitro*, cell culture plus kerosene, Jet A, jet fuel, JP-8, HRJ, hydrorenewable jet, HRJ Camelina, HRJ plant oils, HRJ Tallow, HRJ Animal Fats and Oils, biofuel, biobased/bio-based

##### **V.4.1.3.5. Results of Search**

The literature search did not yield any alternatives to the RD<sub>50</sub> assay. One study was found that tried to correlate *in vivo* mouse inhalation exposures to JP-8 with *in vitro* rat lung slices. Similar changes were seen between the two studies but the method was never validated (Hays, Lantz, and Witten, 2003). We were aware of this work but there are serious questions about the actual exposure concentration used in the studies conducted in Witten's laboratory. In addition they didn't even repeat any of the *in vivo* studies (or show any *in vivo* photos) but compared the *in vitro* data to *in vivo* studies reported in 1998 and 2000. So there is still little correlation between *in vitro* and *in vivo* studies of lung effects and toxicity (Sayes, Reed and Warheit, 2007).

#### **V.4.1.4. Unalleviated Painful or Distressful Procedure Justification**

All procedures used in this study have been designed to avoid discomfort or distress to the animals. It is anticipated based on previous studies that there will be no pain caused by the administration of the jet fuel. The mice may experience **more than slight or momentary distress** during the exposure but should quickly return to a normal state. The use of anesthetics, analgesics and tranquilizers could interfere with the results of the study as they could potentially block the development of the respiratory rate changes the study is trying to quantify.

#### **V.4.2. Prolonged Restraint**

A nose-only inhalation exposure system will be used to expose the animals to the HRJ jet fuel. During exposures, the rats will be restrained in nose-only exposure tubes. The mice will only be exposed once, and the exposure will not exceed 50 minutes. During each transfer animals will be observed for any signs of significant stress prior to loading animals into nose-only exposure tubes and after exposure. Prior to the beginning of exposures, the animals will be acclimatized and trained to these tubes (See section V.1.2. Experiment 2, Acclimation Period on page 6).

The rats will only be exposed once, and the exposure will not exceed 4 hours.

#### **V.4.3. Surgery**

##### **V.4.3.1. Pre-surgical Provisions**

N/A

##### **V.4.3.2. Procedure(s)**

N/A

##### **V.4.3.3. Post-surgical Provisions**

N/A

##### **V.4.3.4. Location**

N/A

##### **V.4.3.5. Surgeon**

N/A

##### **V.4.3.6. Multiple Major Survival Operative Procedures**

N/A

###### **V.4.3.6.1. Procedures**

N/A

###### **V.4.3.6.2 Scientific Justification**

N/A

#### **V.4.4. Animal Manipulations**

N/A

##### **V.4.4.1. Injections**

N/A

##### **V.4.4.2. Biosamples**

N/A

##### **V.4.4.3. Adjuvants**

N/A

##### **V.4.4.4. Monoclonal Antibody (MAb) Production**

N/A

##### **V.4.4.5. Animal Identification**

Each animal will be assigned an identification number and cage location upon receipt. Each animal will further be identified with an ear tag. The identification number will comprise the unique animal number for each animal. Cage identification cards will indicate cage assignment by the animal identification number.

##### **V.4.4.6. Behavioral Studies**

N/A

##### **V.4.4.7. Other Procedures**

N/A

##### **V.4.4.8. Tissue Sharing**

Tissues not needed for data analysis will be made available to WPAFB researchers upon request.

#### **V.4.5. Study Endpoint**

The study will end after the inhalation exposure when all surviving animals will be euthanized within approximately an hour following exposure, as described in section

**V.4.6. Euthanasia** below.

#### **V.4.6. Euthanasia**

Moribund animals will be removed from study and humanely euthanized. All animals will be euthanized at the end of the study. Animals to be euthanized will be deeply anesthetized with sodium pentobarbital (intraperitoneal injection, 30 mg/kg, using either a 1 or 3 ml syringe with a 21 or 23 gauge needle) and exsanguinated by transection of the abdominal aorta.

#### **V.5. Veterinary Care**

### **V.5.1. Husbandry Considerations**

Upon arrival at Bldg 838, WPAFB Area B, animals will be housed, fed, and watered in accordance with the following RSC SOP: 603 (rats and mice). New animals will be segregated from the current population for a quarantine and acclimation period of 7 days. Animal rooms will be maintained at a temperature and relative humidity in accordance with the recommendations of the NRC's *Guide for the Care and Use of Laboratory Animals*, with approximately 15 complete air changes per hour, and a 12hr:12hr electronically controlled light:dark cycle. Animal caging will be cleaned in accordance with the above SOPs, and all animals will be observed twice daily by RSC personnel for any signs of pain, distress, or any other abnormalities.

#### **V.5.1.1. Study Room**

The inhalation exposure for less than one hour will be conducted in Room 264, Bldg 837 or in building 824, Rooms 111 or 114.

#### **V.5.1.2. Special Husbandry Provisions**

N/A

#### **V.5.1.3. Exceptions**

N/A

### **V.5.2. Veterinary Medical Care**

#### **V.5.2.1. Routine Veterinary Medical Care**

Animals will be observed twice daily for signs of distress and the observations will be recorded by RSC staff. The PI will be contacted if an animal is discovered in a moribund condition or appears to be in intense pain during duty hours (0730-1600, Monday-Friday). At that time, the PI will consult with the Attending Veterinarian as to appropriate actions to be taken for the well-being of the animal. If any unexpected animal deaths occur, the PI will immediately notify the Attending Veterinarian (or alternate) for consultation as to the cause of death, any immediate corrective actions to institute, and the need for a necropsy.

#### **V.5.2.2. Emergency Veterinary Medical Care**

During normal duty hours, animal health care emergencies should be reported to the RSC Facility Manager (937-255-7210) or Attending Veterinarian (937-255-8510). After normal duty hours, weekends, and holidays, animal health care emergencies should be reported as described in the memorandum document "Emergency Veterinary Medical Care" describing procedures for contacting emergency personnel. This document is posted on the bulletin board across from the Attending Veterinarian's office (Room 59). During off-duty hours, the PI will authorize the RSC staff, at the discretion of the Attending Veterinarian, to euthanize any animal that is found moribund, or appears to be in intense, unrelievable pain. The carcass of the animal will be placed in a plastic bag along with its cage card. The bag will then be placed in the walk-in refrigerator in Necropsy, room 67, Bldg 838. The RSC staff will then alert the PI by email as to the condition of the animal and the animal number on the cage card.

**V.5.3. Environmental Enrichment**

**V.5.3.1. Enrichment Strategy**

Mice will be group housed.

**V.5.3.2. Enrichment Restrictions**

None

**VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING**

<b>Activity</b>	<b>Name</b>	<b>Qualifications</b>	<b>Training</b>
Principal Investigator, animal handling, clinical evaluations, euthanasia	Michael Gargas, PhD	>25 years experience in animal handling and experimentation	WPAFB animal handlers training.
Co-Investigator, study design and protocol writing only	David R. Mattie, PhD	DABT Over 30 years of experience in toxicology research. He is currently a Senior Research Toxicologist.	Investigator course refresher training (20 Oct 05) GLP training (attended again in July 05 and Feb 09) RCRA training WPAFB animal handlers training
Co-Investigator, animal handling, euthanasia	Brian Wong, PhD	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course
Animal handling, clinical evaluations, euthanasia	Chet Gut, MS	>2 years live animal handling experience, including clinical observations, neurobehavioral testing, anesthesia, euthanasia, necropsy, and tissue harvest	WPAFB animal handlers training

<b>Activity</b>	<b>Name</b>	<b>Qualifications</b>	<b>Training</b>
Animal handling, clinical evaluations, euthanasia	Michelle Okolica, BS	> 13 years live animal handling of mammals, birds, and reptiles. Five years combined professional euthanasia necropsy, and tissue harvest experience with birds and mammals	WPAFB animal handlers training
Animal handling, clinical evaluations, euthanasia	Sue Prues, BS	Combined total of 23 years doing biomedical research many projects involve the handling and husbandry required for research animals	Purina Laboratory Animal Care Course Certification. WPAFB animal handlers training
Animal handling, clinical evaluations, euthanasia	Tracy Doyle-McInturf, BA	> 5 years animal handling experience	WPAFB animal handlers training
Animal handling, clinical evaluations, euthanasia	Shawn McInturf, MS	12 years practical animal handling experience. Trained in anesthesia (CO2), euthanasia (to include guillotine), pup handling/manipulation , and rodent necropsy. Expert in conducting animal neurobehavior testing (NHRC NTAB WPAFB protocol).	WPAFB animal handlers training. RCRA training (biohazards and chemical)
Animal Handling, clinical evaluations	Arden James, BA	29 years of experience in basic research and laboratory management of inhalation toxicology studies using rodents or non-human primates exposed to test chemicals by nose only, intra-tracheal or whole body inhalation procedures.	Various laboratory training courses including intra-laboratory Annual Animal Care and Handling and Animal Care and Use; also on-the-job training loading rodents in nose only exposure tubes or whole body wire mesh cages for inhalation exposures.

<b>Activity</b>	<b>Name</b>	<b>Qualifications</b>	<b>Training</b>
Animal handling, clinical evaluations	Jim Reboulet, MS	18 years experience in biomedical research using laboratory animals including mice, guinea pigs and rats	Purina Laboratory Animal Care Course – NMRI/TD – 1994. On the job training loading rats into nose only exposure tubes and whole body exposure cages and chambers – from Dr. E.C. Kimmel –1990 to present. Administration of CO2 for anesthesia and euthanasia -from Dr. Kimmel - 1993 to present. .
Animal handling, euthanasia	Pedro Ortiz, PhD	> 5 years animal handling experience including clinical observations, neurobehavioral testing, anesthesia, euthanasia, necropsy, and tissue harvest	WPAFB Investigator Training Course
Animal handling, euthanasia	Karen Mummy, PhD	> 5 years animal handling experience including clinical observations, neurobehavioral testing, anesthesia, euthanasia, necropsy, and tissue harvest	WPAFB Investigator Training Course
Animal handling, euthanasia	Richard Erickson, MS	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course
Animal handling, euthanasia	Dan Hardt, MS	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course

<b>Activity</b>	<b>Name</b>	<b>Qualifications</b>	<b>Training</b>
Animal handling, euthanasia	Lisa Sweeney, PhD	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course
Animal handling, euthanasia	Michael Grimm, BS	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course
Animal handling, euthanasia	Angie Hulgan	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course
Animal handling, euthanasia	Brian Sharits	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course
Animal handling, euthanasia	Jessica Sharits	limited animal handling experience and will only work under the supervision of the more experienced study personnel	WPAFB Investigator Training Course

<b>Activity</b>	<b>Name</b>	<b>Qualifications</b>	<b>Training</b>
husbandry, animal care, animal handling, clinical evaluations, viability, body weights, euthanasia	Dick Godfrey	Mr. Godfrey has 38 years of laboratory animal research experience and is certified as a Lab Animal Technologist through AALAS. Mr. Godfrey is highly experienced in Toxicology studies and is proficient in all forms of dosing, blood draws, injections and methods for anesthesia, euthanasia, and necropsies.	Certified AALAS Animal Technologist - 1980 Laboratory Animal Medicine and Science Series - 1980 Purina Animal Care WPAFB animal handlers training RCRA training (biohazards and chemical)
husbandry, animal care, animal handling, clinical evaluations, viability, body weights, euthanasia	Tim Bausman, BS	Mr. Bausman has a BS in Education and 32 years of laboratory animal research experience in Reproductive Toxicology studies and is proficient in all forms of dosing, blood draws, injections and methods for anesthesia, euthanasia, and necropsies.	Certified AALAS Lab Animal Technologist Certified X-Ray Technologist WPAFB animal handlers training Purina Laboratory Animal Care Course Certification RCRA training (biohazards and chemical)

All personnel involved in the protocol have attended the WPAFB Investigator Training Course, or are scheduled to take the next available one offered.

#### **VII. BIOHAZARDS/SAFETY:**

At minimum, appropriate gloves, eye protection and long sleeves (lab coat) will be worn during dose administration. Working with jet fuel is not expected to pose any undue hazards to personnel used to working with very toxic chemicals.

#### **VIII. ENCLOSURES**

1. Literature searches: Available upon request
2. References: Attached
3. Hazardous Agent Summary Form for HRJ Camelina
4. Hazardous Agent Summary Form for HRJ Tallow

5. Material Safety Data Sheet for Bio-oil Derived SPK (Bio-oil Derived SPK is the general class name for HRJ jet fuels so it includes both Camelina and Tallow.)

## **IX. ASSURANCES**

**PROTOCOL TITLE:** Sensory Irritation Study of two HRJ Jet Fuels in Mice, (*Mus musculus*)

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

**A. Animal Use:** The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

**B. Duplication of Effort:** I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

**C. Statistical Assurance:** I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

**D. Biohazard / Safety:** I have taken into consideration and made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

**E. Training:** I verify that the personnel performing the animal procedures / manipulations / observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures / manipulations.

**F. Responsibility:** I acknowledge the inherent moral, ethical, and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely, "Responsibility," which the DoD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

**G. Scientific Review:** This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

**H. Painful Procedure(s):** (A signature for this assurance is required by the Principal Investigator if the research being conducted has the potential to cause more

than momentary or slight pain or distress even if an anesthetic or analgesic is used to relieve the pain and/or distress.)

I am conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress **WILL NOT** be relieved with the use of anesthetics, analgesics, and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

MICHAEL L. GARGAS (Principal Investigator Printed Name)      Michael L. Gargas (Principal Investigator Signature)      17 FEB 2011 (Date)

**I. OCCUPATIONAL HEALTH PROGRAM:** I acknowledge the inherent risks associated with animal contact, such as allergies and zoonoses. I have made a reasonable, good faith effort to ensure all persons with animal contact, working on this protocol are enrolled in an Occupational Health Program.

Name	Enrollment Date	Provider
Michael Gargas	02/2010	Base Occ Health
David Mattie	07/1978	Base Occ Health
Chet Gut	06/2010	Kettering Worker's Care
Michelle Okolica	10/2009	WorkCare/Concentra
Sue Prues	09/2009	WorkCare/Concentra
Tracey Doyle-McInturf	10/2009	WorkCare/Concentra
Shawn McInturf	10/2009	WorkCare/Concentra
Arden James	01/2010	WorkCare/Concentra
Jim Reboulet	10/2009	WorkCare/Concentra
Pedro Ortiz	06/2009	Base Occ Health
Karen Mummy	05/2009	WorkCare/Concentra
Richard Erickson	09/2009	Base Occ Health
Dan Hardt	09/2010	Base Occ Health
Lisa Sweeney	09/2010	WorkCare/Concentra
Brian Wong	09/2010	WorkCare/Concentra
Michael Grimm	07/2010	WorkCare/Concentra
Angie Hulkan	07/2010	Kettering Worker's Care
Brian Sharits	07/2010	Kettering Worker's Care
Jessica Sharits	07/2010	Kettering Worker's Care
Dick Godfrey	04/2010	MedWorks
Tim Bausman	04/2010	MedWorks

MICHAEL L. GARGAS (Principal Investigator Printed Name)      Michael L. Gargas (Principal Investigator Signature)      17 FEB 2011 (Date)

## Enclosure 2: References

- Alarie, Y. (1973). Sensory Irritation of the Upper Airways by Airborne Chemicals, *Toxicology and Applied Pharmacology*, Vol 24:279–297.
- Alarie, Y. (1981). Bioassay for evaluation the potency of airborne sensory irritants and predicting acceptable levels of exposure in man. *Food Cosmet. Toxicol.* 19: 623-6.
- Armitage, P. (1971). *Statistical Methods in Medical Research*. Oxford, UK: Blackwell Scientific Publications.
- ASTM International Designation: E 981-04. (2004). Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals. 11 pp. Current edition approved April 1, Published May.
- Hays, A.M., Lantz, R.C. and Witten, M.L. 2003. Correlation between *in vivo* and *in vitro* pulmonary responses to jet propulsion fuel-8 using precision-cut lung slices and a dynamic organ culture system. *Toxicol Pathol.* 31(2):200-7.
- Mattie, D.R., J.P. Hinz, D.J. Wagner, G. Reddy, D.R. Steup, B.A. Wong and E. Zeiger. (2010). Toxicity and Health Hazard Assessment for Synthetic Paraffinic Kerosene. *The Toxicologist*, 114(1): 220.
- Sayes, C. M., Reed, K. L., Warheit, D.B. (2007). Assessing toxicity of fine and nanoparticles: Comparing *in vitro* measurements to *in vivo* pulmonary toxicity profiles. *Toxicol. Sci.* 97:163–180.
- Shirley, E.A.C. (1977). A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. *Biometrics* 33:386-389.
- Whitman, F. T. and Hinz, J. P., (2001). Sensory irritation study in mice: JP-4, JP-8, JP-8+100. AF Institute for Environment, Safety and Occupational Health Risk Analysis, Brooks AFB, TX. IERA-RS-BR-SR-2001-0005.
- Williams, D.A. (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* 27:103-117.
- Williams, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics* 28:519-531.

# WPAFB HAZARDOUS AGENT SUMMARY FORM

If the proposed study will involve the use of hazardous or potentially hazardous agents, please provide the following information. This form must be filled out for each chemical used in a study. Material Data Safety Sheets (MSDS), (if available) must be attached to each Hazardous Agent Summary Form, (HASF).

Chemical Name:(common Name) HRJ Tallow jet fuel  
Exposure Concentration: 2000 mg/m<sup>3</sup>  
Route(s) of administration: inhalation - nose-only  
Duration of treatment: 30 minutes  
Estimated maximum length of time animals will be monitored following exposure: one hour or less  
Estimated exposure time for personnel: minutes to less than one hour  
Estimated exposure concentration for personnel: less than current OEL of 200 mg/m<sup>3</sup> for other jet fuels

The following personal protective equipment (PPE) must be worn/used in the animal rooms. PPE must be removed before leaving the animal rooms.

- |                                                                             |                                           |
|-----------------------------------------------------------------------------|-------------------------------------------|
| <input type="checkbox"/> Disposable gowns                                   | <input type="checkbox"/> Scrubs           |
| <input checked="" type="checkbox"/> Lab coats                               | <input type="checkbox"/> Face Shields     |
| <input checked="" type="checkbox"/> Disposable gloves (type) <u>nitrile</u> | <input type="checkbox"/> Shoe Covers      |
| <input checked="" type="checkbox"/> Safety glasses (type) <u>safety</u>     | <input type="checkbox"/> Bouffant Cap     |
| <input checked="" type="checkbox"/> Goggles (type) <u>chem resistant</u>    | <input type="checkbox"/> Waterproof Boots |
| <input type="checkbox"/> Respirator (type) _____                            | <input type="checkbox"/> Others _____     |

PI will inform personnel on proper processes, procedure and equipment needed to work safely with this chemical. Provide information below:

Handle the same as JP-8. All personnel involved have worked on JP-8 studies and are knowledgeable regarding procedures.

Health risks associated with this chemical: slight thermal irritation of nose/skin

Exposure routes: inhalation

Other controls: used in inhalation lab w/ room ventilation & containment chambers.

Principal Investigator:

Signature David R. Matthe Print Name DAVID R. MATTIE Date 1/6/11

Environmental Safety Officer:

Signature Kathy Kincid Print Name KATHY KINCID Date 1/6/11

# WPAFB HAZARDOUS AGENT SUMMARY FORM

If the proposed study will involve the use of hazardous or potentially hazardous agents, please provide the following information. This form must be filled out for each chemical used in a study. Material Data Safety Sheets (MSDS), (if available) must be attached to each Hazardous Agent Summary Form, (HASF).

Chemical Name:(common Name) HRJ Camelina jet fuel  
 Exposure Concentration: 2000 mg/m<sup>3</sup>  
 Route(s) of administration: inhalation - nose-only  
 Duration of treatment: 30 minutes  
 Estimated maximum length of time animals will be monitored following exposure: one hour or less  
 Estimated exposure time for personnel: minutes to less than one hour  
 Estimated exposure concentration for personnel: less than current OEL of 200 mg/m<sup>3</sup> for other jet fuels

The following personal protective equipment (PPE) must be worn/used in the animal rooms. PPE must be removed before leaving the animal rooms.

- |                                                                             |                                           |
|-----------------------------------------------------------------------------|-------------------------------------------|
| <input type="checkbox"/> Disposable gowns                                   | <input type="checkbox"/> Scrubs           |
| <input checked="" type="checkbox"/> Lab coats                               | <input type="checkbox"/> Face Shields     |
| <input checked="" type="checkbox"/> Disposable gloves (type) <u>Nitrile</u> | <input type="checkbox"/> Shoe Covers      |
| <input checked="" type="checkbox"/> Safety glasses (type) <u>goggles</u>    | <input type="checkbox"/> Bouffant Cap     |
| <input checked="" type="checkbox"/> Goggles (type) <u>chem. resistant</u>   | <input type="checkbox"/> Waterproof Boots |
| <input type="checkbox"/> Respirator (type) _____                            | <input type="checkbox"/> Others _____     |

PI will inform personnel on proper processes, procedure and equipment needed to work safely with this chemical. Provide information below:

Handle the same as JP-8. All personnel involved have worked on JP-8 studies and are knowledgeable regarding procedures.

Health risks associated with this chemical: Slight to mild irritation of nose & skin

Exposure routes: inhalation

Other controls: used in inhalation lab w/ room ventilation & containment chambers.

Principal Investigator:

Signature David R. Matthe Print Name DAVID R. MATTHE Date 1/6/11

Environmental, Safety Officer:

Signature Kathy Kincaid Print Name KATHY KINCAID Date 1/6/11



# MATERIAL SAFETY DATA SHEET

## 1. CHEMICAL PRODUCT AND COMPANY INFORMATION

**Product Name:** Bio-oil Derived SPK (C100 in process)

**Product Use:** Chemical - Fuel (Experimental)

**NOTE:** This sample is for research and development purposes only. The handling and use of this material must be supervised by qualified individuals. The chemical, physical and toxicological properties of this material have not been fully investigated. Use due precaution in handling, storage and disposal.

UOP LLC  
25 E. Algonquin Road  
Des Plaines, IL 60017-5017  
USA  
Tel: +1-847-391-3189  
Fax: +1-847-391-2953

UOP Ltd.  
"Liongate", Ladymead  
Guildford, Surrey GU1 1AT  
UK  
Tel: + 44-1483-304-848  
Fax: + 44-1483-466-338

**Emergency Assistance - 24 hour Emergency Telephone Numbers:**

USA (UOP LLC) :	+ 1-847-391-2123
USA (CHEMTREC) :	+ 1-800-424-9300
Canada (CANUTEC) :	+ 1-613-996-6666
Outside USA (CHEMTREC) :	+ 1-703-527-3887

## 2. HAZARDS IDENTIFICATION

**Emergency Overview:**

The product is considered harmful via ingestion. Avoid breathing the product. Keep away from heat, sparks, and flame. The product is combustible and toxic vapors may be given off in a fire.

**Form:** Liquid  
**Color:** Colorless

**Potential Health Effects:**

**Primary Routes of Exposure:** Contact with skin, eyes and inhalation of product vapor. Product ingestion is unlikely to occur if proper safety/hygiene procedures are followed.

**Eye Contact:** Repeated or prolonged exposure may cause eye irritation.

**Skin Contact:** Causes mild skin irritation.

**Ingestion:** May be harmful if swallowed. Aspiration can be a hazard if this material is swallowed.

**Inhalation:** Inhalation of product vapors or mist may cause irritation of the respiratory system. May cause Central Nervous System effects.

**Chronic Effects:** None known.

Carcinogenicity Classification:

International Agency for Research on Cancer (IARC):  
Neither the product nor the components are classified.

U.S. National Toxicology Program (NTP):  
Neither the product nor the components are classified.

U.S. Occupational Safety and Health Administration (OSHA):  
Neither the product nor the components are classified or regulated.

American Conference of Governmental Industrial Hygienists (ACGIH):  
Neither the product nor the components are classified.

**3. COMPOSITION/INFORMATION ON INGREDIENTS**

<u>INGREDIENT &amp; CAS NO.</u>	<u>% WEIGHT</u>	<u>ACGIH TLV- TWA</u>	<u>OSHA PEL- TWA</u>	<u>UNITS</u>
C9 - C15 paraffin bio-oil derived	100% >99	200	N.E.	mg/m <sup>3</sup>

Abbreviations

N.A.	- Not Applicable	RD	- Respirable Dust	Fu	- Fume	IS	- Insoluble
N.E.	- None Established	R	- Respirable Fraction	I	- Inhalable	FuD	- Fume and Dust
STEL	- Short Term Exposure Limit	F	- Respirable Fibers	TD	- Total Dust	SC	- Soluble Compounds

#### 4. FIRST AID MEASURES

**Eye contact:** Flush immediately with plenty of water, also under the eyelids, for at least 15 minutes. Consult a physician.

**Skin contact:** REMOVE FROM SKIN IMMEDIATELY. Take off all contaminated clothing immediately. Remove adhering matter immediately. Use waterless hand cleaner. Then wash with lots of water and soap. Consult a physician.

**After inhalation:** Remove the victim into fresh air. If symptoms persist, call a physician.

**After ingestion:** Do not induce vomiting. Call a physician immediately.

**Notes to physician:** In the unlikely event that large quantities of the product are ingested, gastric lavage should be considered. Aspiration into the lungs may cause chemical pneumonia. An activated charcoal slurry taken within 30 minutes of product ingestion may reduce the toxicity of the chemical. A 5:1 ratio of charcoal to material ingested is the recommended dosage. Activated charcoal should not be considered as an antidote; normal symptomatic treatment is recommended with or without the administration of activated charcoal.

#### 5. FIRE FIGHTING MEASURES

**Suitable extinguishing media:** Water spray. Foam. Dry chemical. Carbon dioxide (CO<sub>2</sub>).

**Unsuitable extinguishing media:** Do not use a solid water stream as it may scatter and spread fire.

**Fire and explosion hazards:** In the event of fire and/or explosion do not breathe fumes. Cool containers / tanks with water spray. Heating/burning can release hazardous gases: carbon oxides (CO, CO<sub>2</sub>) and various hydrocarbons.

**Special protective equipment:** Wear protective clothing. In case of respirable dust and/or fumes, use self-contained breathing apparatus.

**Flash Point:** >100°F (>38°C)

#### 6. ACCIDENTAL RELEASE MEASURES

**Personal protection:** See Section 8.

**Environmental precautions:** Prevent product from entering drains. Do not flush into surface water or sanitary sewer system. Avoid subsoil penetration.

**Clean-up:** Remove all sources of ignition. Stop leak at source. Keep people away from and upwind of spill/leak. Contain material using temporary measures such as sand bags, booms or adsorbent socks. Soak up with inert absorbent material (e.g. sand, silica gel, universal binder, sawdust). Never use spilled product.

Spilled product should be disposed of in accordance with all applicable government regulations. (See Section 13).

Small amounts: Soak up with inert absorbent material and dispose of in accordance with applicable regulations.

#### 7. HANDLING AND STORAGE

**Handling:** Use only in well-ventilated areas. Wear personal protective equipment. In case of insufficient ventilation, wear suitable respiratory equipment (see Section 8 of MSDS). Keep away from open flames, hot surfaces and sources of ignition.

**Storage:** Keep containers tightly closed in a cool, well-ventilated place. Store in original container. Keep away from heat and sources of ignition.

## 8. EXPOSURE CONTROLS/PERSONAL PROTECTION

**Engineering measures:** Ensure adequate ventilation, especially in confined areas.

**Personal protection equipment:** Avoid contact with skin, eyes and clothing. Handle in accordance with good industrial hygiene and safety practice.

**Eye protection:** Tightly fitting safety goggles. Face-shield.

**Hand protection:** Solvent-resistant gloves.

**Skin and body protection:** Solvent-resistant apron and boots. Protective suit. Remove and wash contaminated clothing and gloves, including the inside, before re-use.

**Respiratory protection:** In case of insufficient ventilation, wear suitable respiratory equipment. Air-purifying respirator with NIOSH classification N-100 filter or P-100 (or equivalent) if oil/liquid aerosols are present (42 CFR 84).

## 9. PHYSICAL AND CHEMICAL PROPERTIES

These data do not represent technical or sales specifications.

**Form:** Liquid

**Color:** Colorless

**Odor:** Odorless to mild paraffin

**pH:** N.A.

**Boiling point/range:** 298-572°F (148-300°C)

**Melting point/range:** N.A.

**Flash point:** >100°F (>38°C)

**Autoignition temperature:** N.D.

**Bulk density:** N.D.

**Explosion limits:** N.A.

**Vapor pressure:** N.D.

**Relative density/Specific Gravity:** 0.75 - 0.80 g/ml @ 15°C

**Vapor density:** >1

**Viscosity:** N.D.

**Water solubility:** Insoluble

**Solubility:** N.D.

**Abbreviations:**

N.D. - Not Determined  
N.A. - Not Applicable

## 10. STABILITY

**Stability:** Stable at normal conditions; Decomposes on heating.

**Hazardous decomposition products:** No decomposition if used as directed. Under conditions giving incomplete combustion, hazardous gases produced may consist of carbon oxides (CO, CO<sub>2</sub>) and various hydrocarbons.

**Conditions/Materials to avoid:** Keep away from ignition sources. Oxidizing agents.

## 11. TOXICOLOGICAL INFORMATION

### Acute toxicity:

LD50/oral/rat: No data available.

LD50/dermal/rabbit: No data available.

LC50/inhalation/rat: No data available.

### Chronic toxicity: Classification of Ingredients

#### EC Carcinogenic:

Not listed.

#### Carcinogenicity (ACGIH):

Not listed.

#### EC Mutagenic:

Not listed.

#### IARC classification:

Not listed.

#### EC Toxic for Reproduction:

Not listed.

Routes of exposure: Exposure may occur via inhalation, contact with skin and eyes.

### Irritation:

Skin (rabbit): No data available.

Eye (rabbit): No data available.

### Additional product information:

Avoid repeated and prolonged exposure.

### Additional component information:

No data available.

## 12. ECOLOGICAL INFORMATION

**Mobility:**  
No data available

**Biodegradation:**  
No data available.

**Bioaccumulation:**  
No data available

**Aquatic toxicity:**  
No data available.

**Further information:**  
No information available.

## 13. DISPOSAL CONSIDERATIONS

**Provisions relating to waste:** EPA - Resource Conservation and Recovery Act (RCRA) Hazardous and Solid Waste Management Regulations.

**Disposal information:** Dispose of in compliance with all applicable regulations. Waste material may exhibit the U.S. EPA's RCRA hazardous waste characteristic of Ignitability (D001) if representative sample of the waste has a flash point of less than 140°F (60°C).

## 14. TRANSPORT INFORMATION

**UN-No.:**  
UN1863

**Proper shipping name:**  
Fuel, aviation, turbine engine

**Packing group:**  
III

<u>Transport Mode</u>	<u>Class</u>	<u>Additional Information</u>		<u>Remarks</u>
U.S. DOT:	3	Reportable Quantity:	N.A.	N.A.
		Marine Pollutant DOT:	No	
ADR/RID:	3	Danger Code:	30	N.A.
IMDG:	3	Marine pollutant:	No	N.A.
		EmS:	F-E, S-E	
IATA:	3	Instr. Passenger:	309/Y309	N.A.
		Instr. Cargo:	310	

## 15. REGULATORY INFORMATION

### United States

**Toxic Substances Control Act (TSCA):** The TSCA Inventory status has not been determined for the ingredient(s) of this product.

This material must be used in compliance with the TSCA Research and Development Exemption requirements (40 CFR 720.36). Regulations require: (1) All persons engaged in experimentation, research, or analysis on this substance, including manufacture, processing, use, transport, storage, and disposal associated with R&D activities, are notified of all health risk information; (2) The adequacy of the notification to all such persons is ensured by a technically qualified individual; (3) Activities of all such persons are supervised by a technically qualified individual; (4) All areas in which exposure may occur are conspicuously labeled; (5) Any information, test data, or indication of significant adverse reactions by persons exposed to the substance be evaluated to determine whether there is risk to health or the environment which may reasonably be associated with such exposure; (6) Prudent laboratory practices are followed, if the activity is in a laboratory.

**CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act) Reportable Quantity:** The following component(s) of this product is/are subject to release reporting under 40 CFR 302 when release exceeds the Reportable Quantity (RQ):

– None –

**CWA (Clean Water Act):**

Any spill or release of this product to navigable waters or adjoining shoreline sufficient to cause a sheen or deposit of a sludge or emulsion is subject to the Discharge of Oil Notification requirements under 40 CFR 110.6.

**SARA Title III (Superfund Amendments and Reauthorization Act of 1986):**

**Section 302 (Extremely Hazardous Substances):**

The following component(s) of this product is/are subject to the emergency planning provisions of 40 CFR 355 when there are amounts equal to or greater than the Threshold Planning Quantity (TPQ):

– None –

**Section 313 (Toxic Chemicals):**

The following component(s) have been specified as Toxic Chemicals under SARA Section 313 and may be subject to the Toxic Release Inventory (TRI) reporting requirements under 40 CFR 372:

– None –

The following components are listed in U.S. State Regulations:

<u>State Reg Reference:</u>	<u>State Reg Reference:</u>
California - Proposition 65:	None.
Massachusetts Right-To-Know:	Kerosene
New Jersey Right-To-Know:	Kerosene
Pennsylvania Right-To-Know:	Kerosene

Note: Other U.S. State Regulations may exist, check your local sources if available or contact the UOP Product Stewardship Manager (see Section 16).

Canada

Canadian Hazardous Products Act:  
Not determined.

Canadian Environmental Protection Act: Not determined.

European Union (EU)

European Inventory of Existing Commercial Chemical Substances: Not determined.

Council of European Communities Directive on Classification, Packaging and Labelling of Dangerous Substances/Preparation (67/548/EEC & 1999/45/EC, as amended):  
The following label information applies:

Caution: Substance not yet fully tested.

Symbol(s):	Xn - Harmful
Risk Phrases:	R10 Flammable. R65 Harmful: may cause lung damage if swallowed.
Safety phrases:	S23 Do not breathe spray. S24 Avoid contact with skin. S62 If swallowed, do not induce vomiting; seek medical advice immediately and show this container or label.

Additional Governmental Inventories

Australia - Inventory of Chemical Substances (AICS): Not determined.

China: Not determined.

Japan - Existing and New Chemical Substances (ENCS): Not determined.

Korea - Existing and Evaluated Chemical Substances (ECL): Not determined.

Philippines - Inventory of Chemicals and Chemical Substances (PICCS): Not determined.

## 16. OTHER INFORMATION

Summary of changes: Section 15  
Supersedes: August 2008  
Prepared by: UOP Health, Safety & Environmental Department

### HMIS™ - Hazardous Material Identification System:

HMIS™ Ratings: 0-minimal hazard, 1- slight hazard, 2- moderate hazard, 3- serious hazard, 4- severe hazard.

HEALTH : 2  
FLAMMABILITY : 2  
REACTIVITY : 0

For additional information concerning this product, contact the following:

For health, safety and environmental information,  
please contact:

Product Stewardship Manager  
UOP LLC  
25 E. Algonquin Road  
Des Plaines, IL 60017-5017  
USA  
Tel: +1-847-391-3189  
Fax: +1-847-391-2953

Product Safety Steward Europe  
UOP N.V.  
Noorderlaan 147  
B-2030 Antwerpen  
Belgium  
Tel: +32-3-5409-971  
Fax: +32-3-5417-808

For technical or purchasing  
information, please contact:

Renewable Energy and Chemicals  
UOP LLC  
25 E. Algonquin Road  
Des Plaines, IL 60017-5017 USA  
Tel: +1-847-391-2789  
Fax: +1-847-391-2253

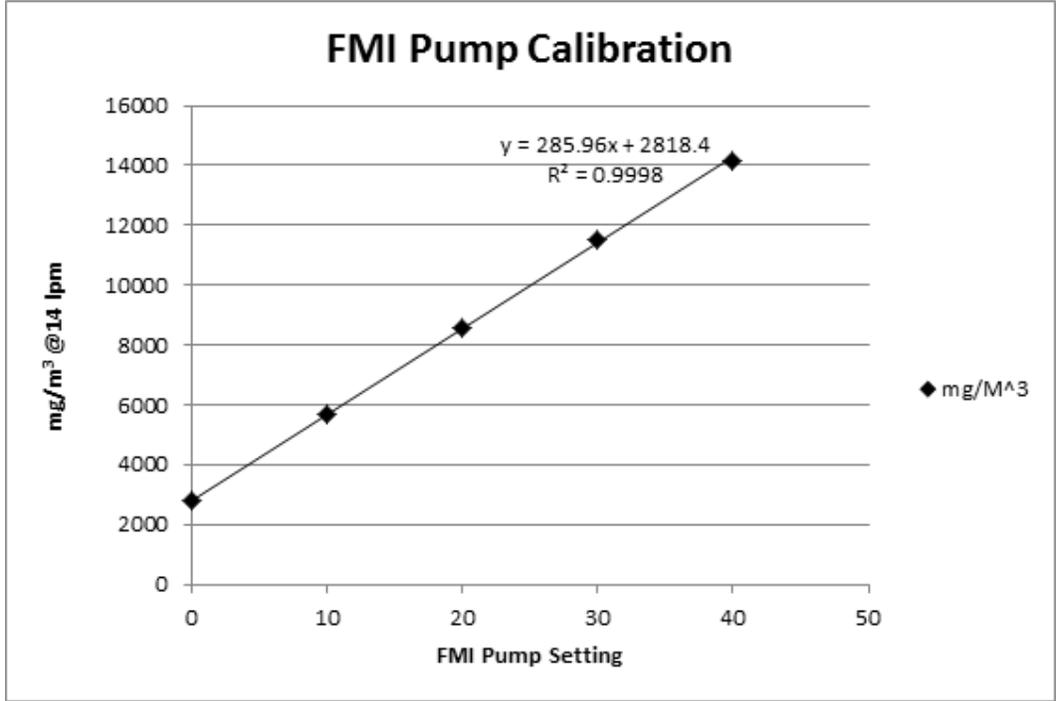
### PRODUCT EMERGENCIES

If you have a product-related emergency, resulting in an incident such as a spill or release of product or human exposure and need assistance from UOP, please contact the following number:

**24-Hour EMERGENCY NUMBER (UOP LLC) : + 1 - 847 - 391 - 2123**

The data and recommendations presented in this data sheet concerning the use of our product and the materials contained therein are believed to be accurate and are based on information which is considered reliable as of the date hereof. However, the customer should determine the suitability of such materials for his purpose before adopting them on a commercial scale. Since the use of our products by others is beyond our control, no guarantee, express or implied, is made and no responsibility assumed for the use of this material or the results to be obtained therefrom. Information on this form is furnished for the purpose of compliance with Government Health and Safety Regulations and shall not be used for any other purposes. Moreover, the recommendations contained in this data sheet are not to be construed as a license to operate under, or a recommendation to infringe, any existing patents, nor should they be confused with state, municipal or insurance requirements, or with national safety codes.

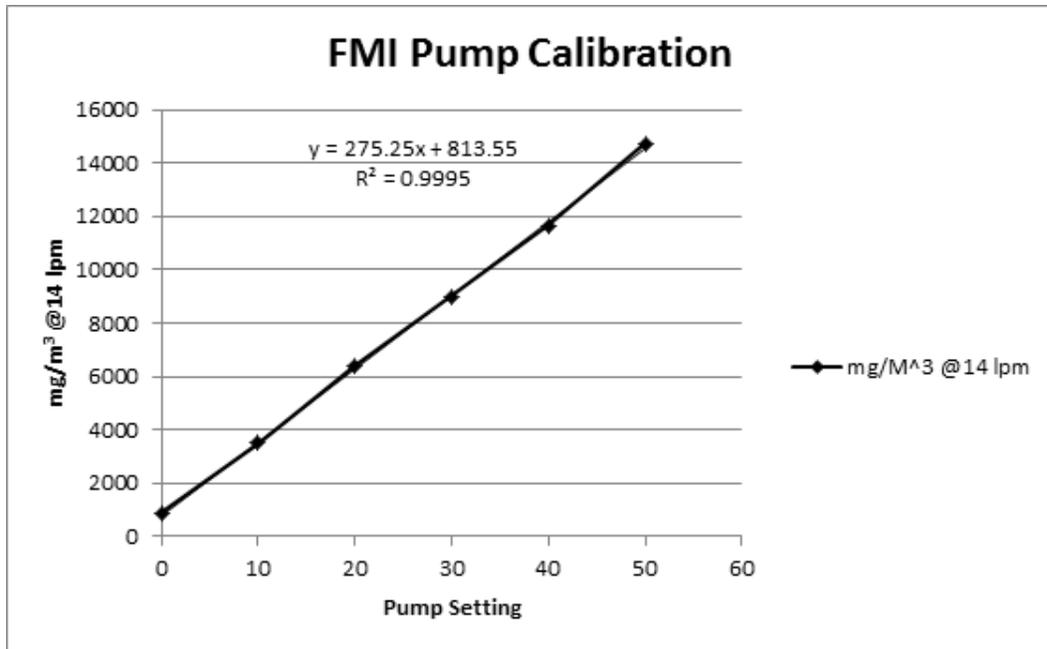
**APPENDIX B. FMI PUMP CALIBRATIONS**



FMI Pump Setting	Flow Rate (mg/min)	Flow Rate (mL/min) [a]	Concentration at 14 L/min (mg/m <sup>3</sup> )
0	39	0.050	2786
10	79.6	0.102	5686
20	119.62	0.153	8544
30	160.97	0.206	11498
40	198.49	0.254	14178

[a] Assumed density = 0.78 g/mL

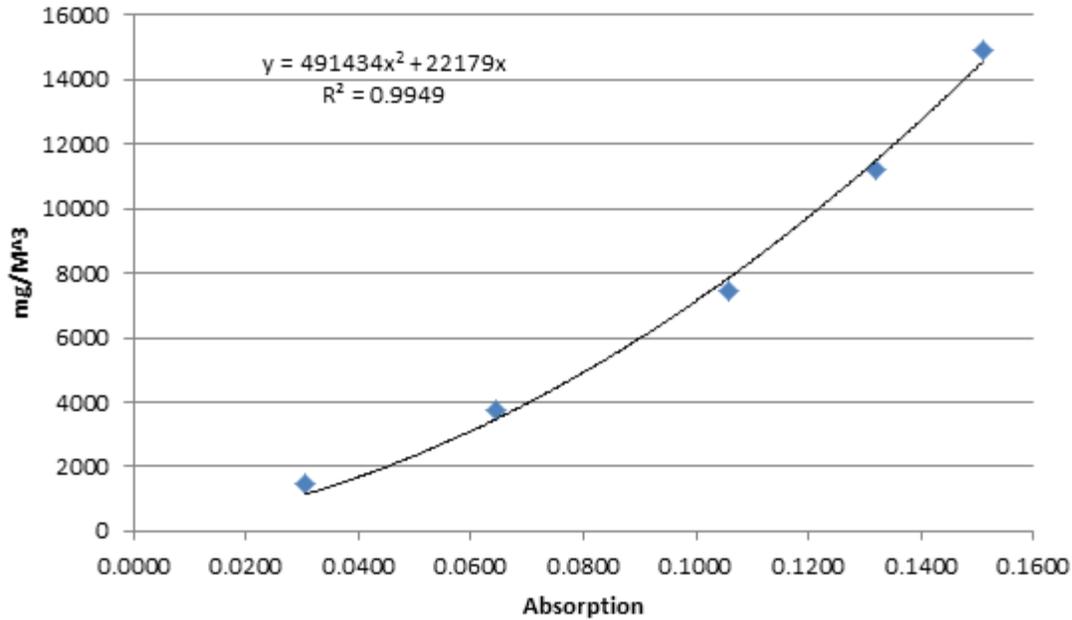
**Figure 1. FMI Pump Calibration for HEFA-C Assay: Pump Setting versus Concentration (mg/m<sup>3</sup>).** The FMI pump was set at a low flow rate and the index ring set for zero. HEFA-C was pumped into a tared, closed vial for several minutes. The vial was weighed and the total mass accumulated divided by the time gave the flow rate in mg/mL. The mg/mL was converted to mg/m<sup>3</sup> at the expected generation rate of 14 L/minute.



FMI Pump Setting	Flow Rate (mg/min)	Concentration at 14 L/min (mg/m <sup>3</sup> )
0	12.3	875
10	49.1	3504
20	89.5	6392
30	126.1	9009
40	163.3	11664
50	206.1	14724

**Figure 2. FMI Pump Calibration for HEFA-T Assay: Pump Setting versus Concentration (mg/m<sup>3</sup>).** The FMI pump was set at a low flow rate and the index ring set for zero. HEFA-T was pumped into a tared, closed vial for several minutes. The vial was weighed and the total mass accumulated divided by the time gave the flow rate in mg/mL. The mg/mL was converted to mg/m<sup>3</sup> at the expected generation rate of 14 L/minute.

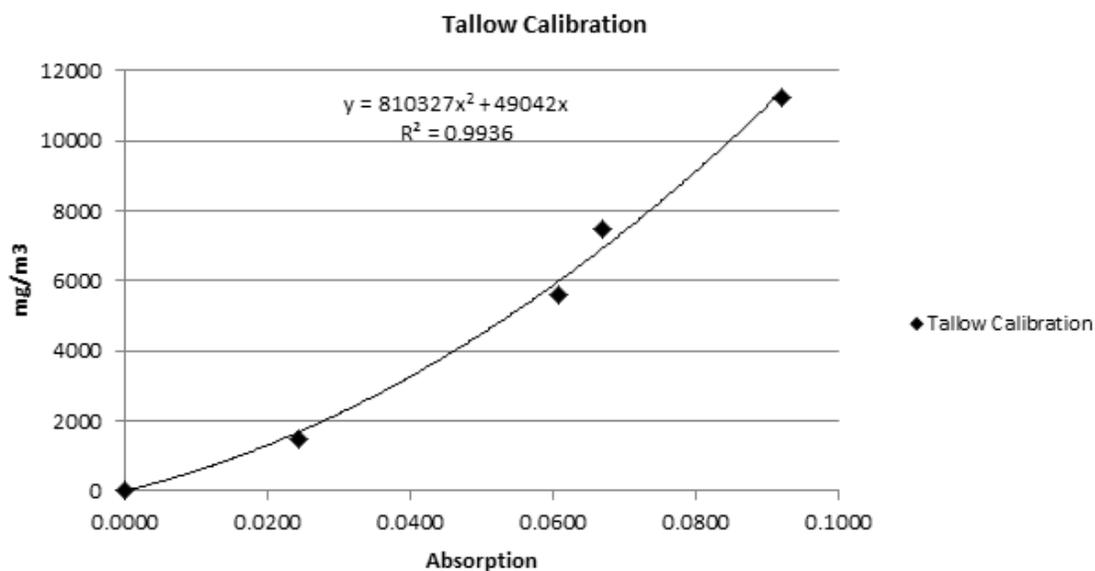
### APPENDIX C. FTIR CALIBRATION CURVES



HEFA-C Injection (uL)	FTIR Average Absorbance	Nominal Bag Concentration (mg/m <sup>3</sup> )	Estimated [a] Concentration (mg/m <sup>3</sup> )	Ratio Est:Nom (%)
20	0.0306	1492	1139	76
50	0.0644	3730	3470	93
100	0.1060	7460	7873	106
150	0.1321	11190	11503	103
200	0.1509	14920	14538	97

[a] Estimated concentration is calculated from the nominal concentration applied to the data regression equation. The comparison of the Nominal Bag Concentration to the Estimated Concentration expressed as a ratio is an indication of how well the data regression will predict real time samples.

**Figure 1. FTIR Calibration for the HEFA-C Assay.** FTIR absorption measurements were recorded approximately every 18 seconds until the maximum absorption reading was reached and maintained for several minutes. Each recording was saved as a line in a text file, which was subsequently imported into Excel for plotting. The data were plotted from the measured maximum absorption vs. nominal bag concentration (mg/m<sup>3</sup>).

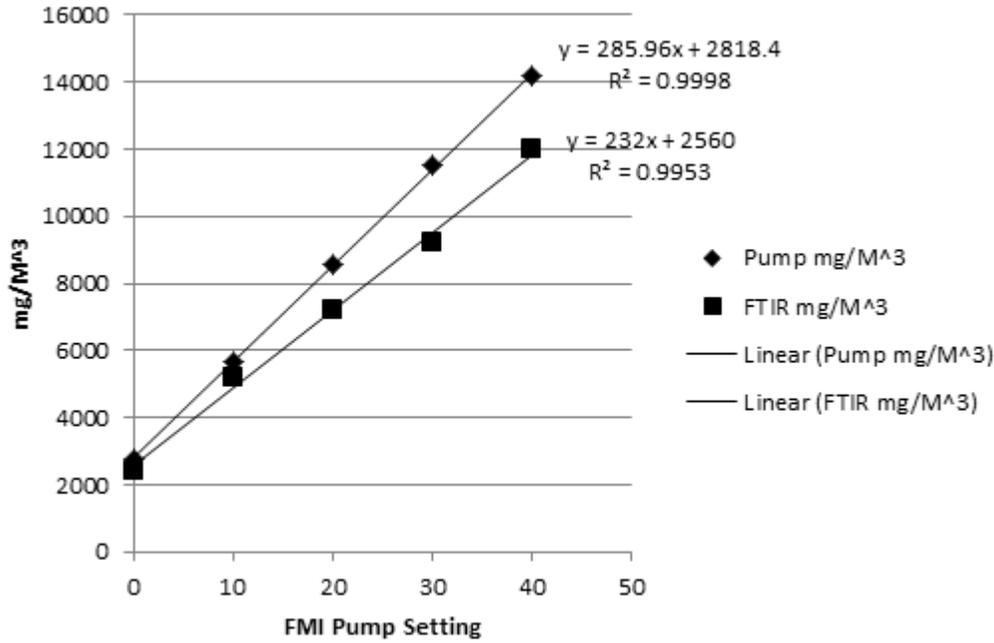


HEFA-T Injection (uL)	FTIR Average Absorbance	Nominal Bag Concentration (mg/m <sup>3</sup> )	Estimated [a] Concentration (mg/m <sup>3</sup> )	Ratio Est:Nom (%)
40	0.0244	1500	1683	112
75	0.0609	5625	5987	106
100	0.0669	7500	6912	92
150	0.0921	11250	11389	101

[a] Estimated concentration is calculated from the nominal concentration applied to the data regression equation. The comparison of the Nominal Bag Concentration to the Estimated Concentration expressed as a ratio is an indication of how well the data regression will predict real time samples.

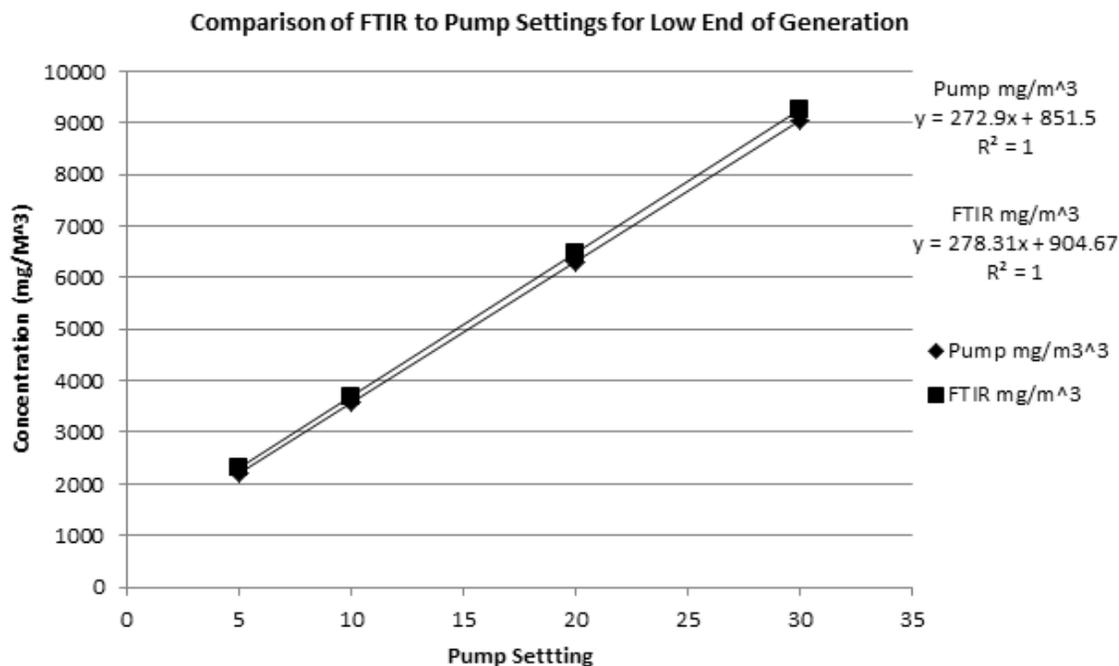
**Figure 2. FTIR Calibration for the HEFA-T Assay.** FTIR absorption measurements were recorded approximately every 18 seconds until the maximum absorption reading was reached and maintained for several minutes. Each recording was saved as a line in a text file, which was subsequently imported into Excel for plotting. The data were plotted from the measured maximum absorption vs. nominal bag concentration (mg/m<sup>3</sup>).

**APPENDIX D. TRIAL RUN DATA**



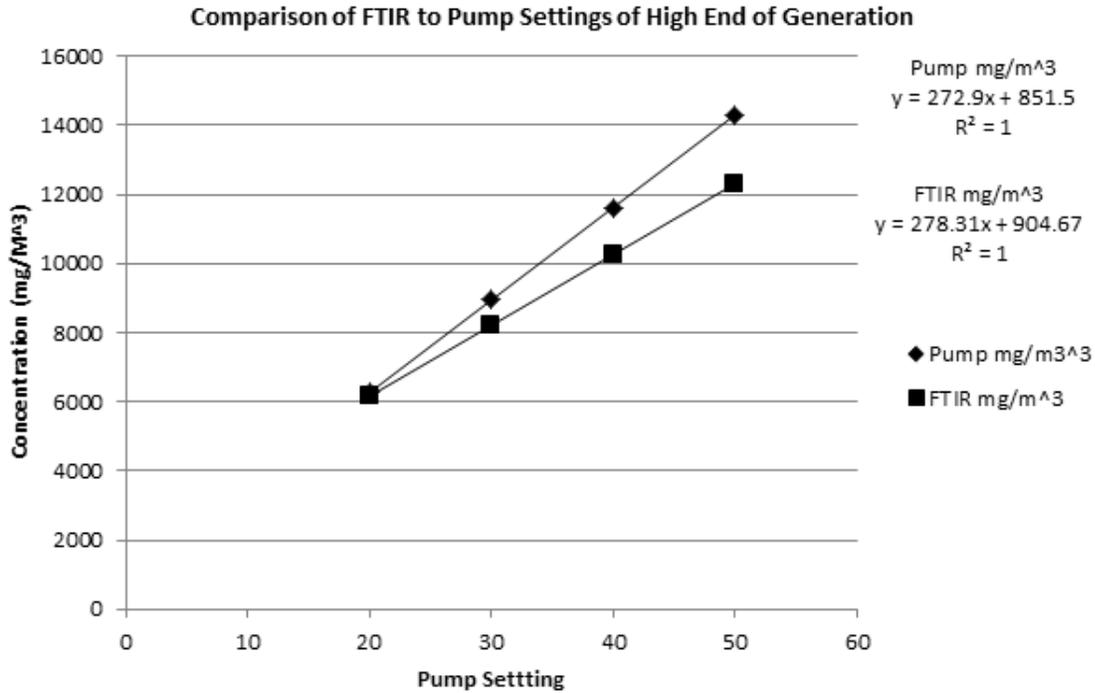
Pump Setting	Pump Flow Rate Flow Rate (mg/min)	Expected Concentration (mg/m <sup>3</sup> )	Actual FTIR Concentration (mg/m <sup>3</sup> )	Ratio of Exp:Act (%)
0	39	2785	2400	86
10	79.6	5685	5200	91
20	119.62	8544	7200	84
30	160.97	11497	9200	80
40	198.49	14177	12000	85

**Figure 1. FMI Pump Flow Rate Settings for HEFA-C Assay.** Plots depict pump setting versus concentration (mg/m<sup>3</sup>) from pumping rate and by FTIR analysis. The measured pump flow (mg/minute) at given setting is shown in the table. The calculated concentration (mg/m<sup>3</sup>) is that expected at a generator rate of 14 L/minute and the concentration found at equilibrium by the FTIR analysis system. The ratio of FTIR to expected gives the nominal.



Pump Setting	Expected Concentration (mg/m <sup>3</sup> )	FTIR Concentration (mg/m <sup>3</sup> )	Ratio of Exp:Act (%)
5	2216	2296	97
10	3581	3688	97
20	6310	6471	98
30	9039	9254	98

**Figure 2. Comparison of FTIR to Pump Settings for Low End of Generation: HEFA-T Assay.** Plots depict pump setting versus concentration (mg/m<sup>3</sup>) from pumping rate and by FTIR analysis. The measured pump flow (mg/minute) at given setting is shown in the table. The calculated concentration (mg/m<sup>3</sup>) is that expected at a generator rate of 14 L/minute and the concentration found at equilibrium by the FTIR analysis system. The ratio of FTIR to expected gives the nominal.



Pump Setting	Expected Concentration (mg/m <sup>3</sup> )	FTIR Concentration (mg/m <sup>3</sup> )	Ratio of Exp:Act (%)
20	6265	6150	98
30	8943	8209	92
40	11620	10268	88
50	14298	12327	86

**Figure 3. Comparison of FTIR to Pump Settings for High End of Generation: HEFA-T Assay.** Plots depict pump setting versus concentration (mg/m<sup>3</sup>) from pumping rate and by FTIR analysis. The measured pump flow (mg/minute) at given setting is shown in the table. The calculated concentration (mg/m<sup>3</sup>) is that expected at a generator rate of 14 L/minute and the concentration found at equilibrium by the FTIR analysis system. The ratio of FTIR to expected gives the nominal.

**APPENDIX E. ANIMAL BODY WEIGHT DATA**

**Table 1. Animal Body Weights for HEFA-C Assay**

Group	Target Exposure Level (mg/m <sup>3</sup> )	Day after receipt Weight (g) 11/30/2011	Randomization Weight (g) 12/6/2011	12/6/2011 Average Group Weight (g)	Exposure Day Weight (g)	Exposure Day Average Group Weight (g)
1	2,000	20.74	28.01	27.77	28.16	29.05
		21.18	28.26		29.83	
		22.14	27.90		29.55	
		21.63	26.91		28.67	
2	12,000	20.62	26.35	26.38	26.60	27.22
		20.71	26.25		27.53	
		20.64	26.39		27.06	
		21.70	26.52		27.68	
3	8,500	20.17	26.19	25.78	28.77	27.92
		21.03	26.05		27.96	
		21.83	25.63		27.40	
		19.56	25.24		27.55	
4	5,000	20.13	24.21	24.61	25.09	25.93
		21.97	24.94		25.87	
		20.36	25.14		26.35	
		21.80	24.15		26.40	
5	7,500	19.63	23.21	23.04	25.32	25.22
		19.85	22.66		25.03	
		20.68	22.54		24.01	
		20.89	23.76		26.50	
	Average (g)	20.86	25.52		27.07	
	Min (g)	19.56	22.54		24.01	
	Max (g)	22.14	28.26		29.83	

**Table 2. Animal Body Weights for HEFA-T Assay**

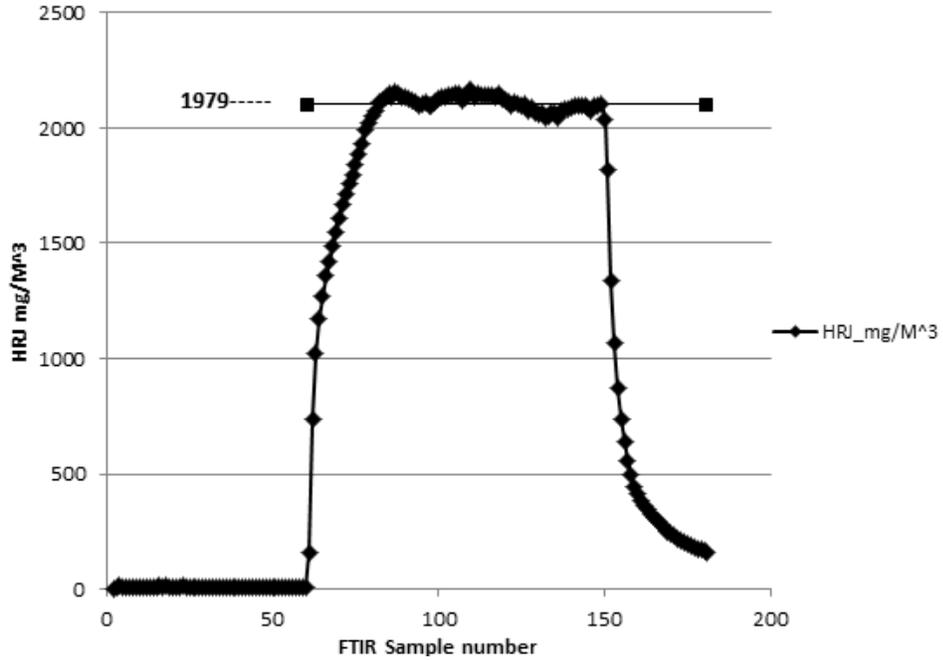
Group	Target Exposure Level (mg/m <sup>3</sup> )	Day after receipt Weight (g) 2/29/2012	Randomization Weight (g) 3/6/2012	3/6/2012 Average Group Weight (g)	Exposure Day Weight (g)	Exposure Day Average Group Weight (g)
1	2,000	19.12	26.08	26.45	26.11	26.42
		19.21	25.67		25.87	
		19.44	27.59		27.50	
		18.32	26.46		26.20	
2	14,000	16.86	24.60	24.79	26.09	25.97
		17.08	24.63		25.78	
		18.61	25.41		26.18	
		18.01	24.52		25.84	
3	10,000	17.48	24.12	24.22	24.47	24.88
		17.58	24.05		25.00	
		16.90	24.26		25.06	
		18.47	24.44		24.97	
4	6,000	17.10	23.77	23.77	25.17	25.39
		17.77	23.36		24.81	
		17.80	23.95		25.68	
		16.82	23.99		25.90	
5	6,000 [1]	17.39	23.14	23.04	24.53	24.14
		17.39	23.11		24.22	
		17.21	23.32		23.68	
		16.94	22.57		24.14	
	Average (g)	17.78	24.45		25.36	
	Min (g)	16.82	22.57		23.68	
	Max (g)	19.44	27.59		27.50	

[1] Vapor only exposure

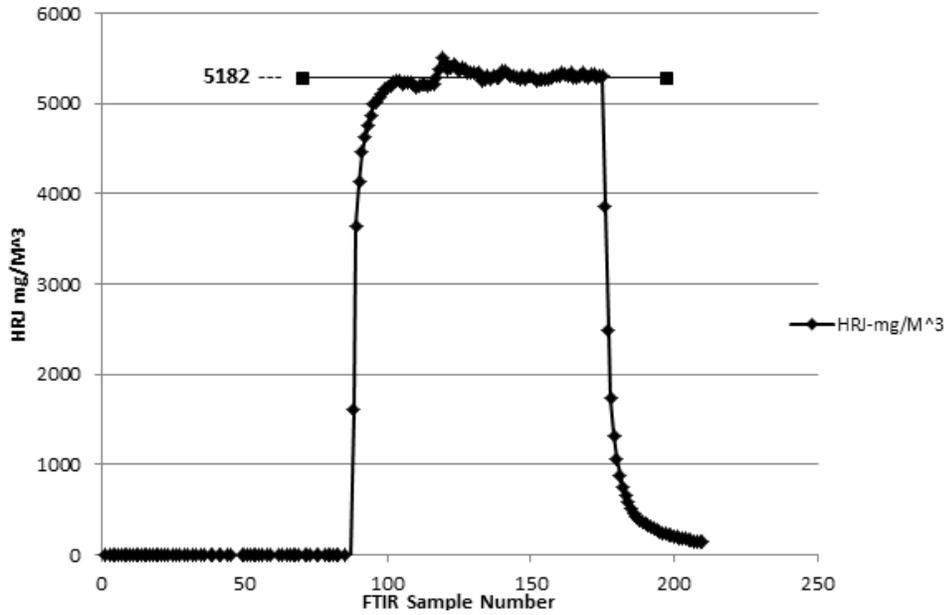
## APPENDIX F. FTIR GRAPHS

### HEFA-C

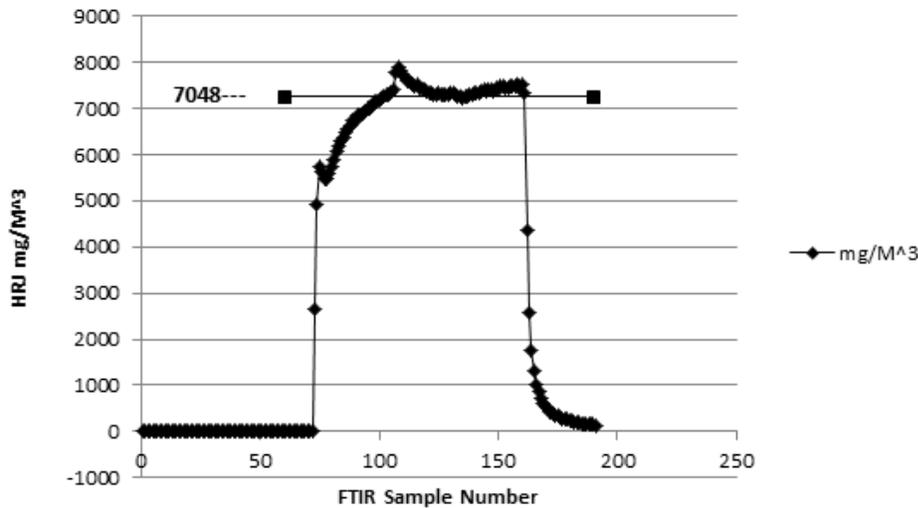
Each FTIR figure depicts the 10 minute baseline, 30 minute exposure and 10 minute recovery periods for the exposure concentration.



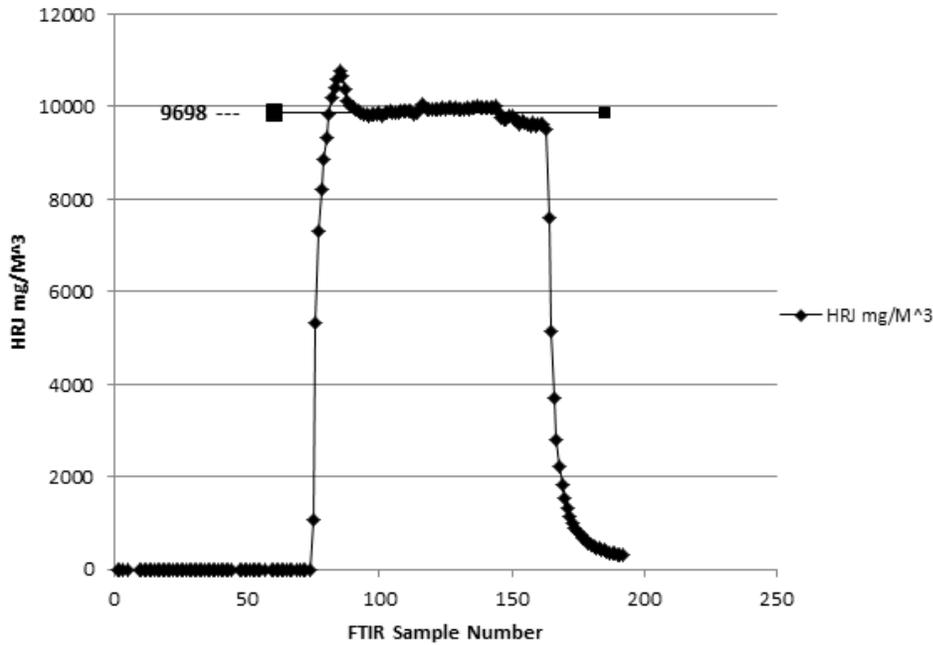
**Figure 1. FTIR Values for the 2,000 mg/m<sup>3</sup> HEFA-C Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-C exposure and 10 minute recovery periods for the target concentration of 2,000 mg/m<sup>3</sup> (actual 30 minute average: 1979 mg/m<sup>3</sup>).



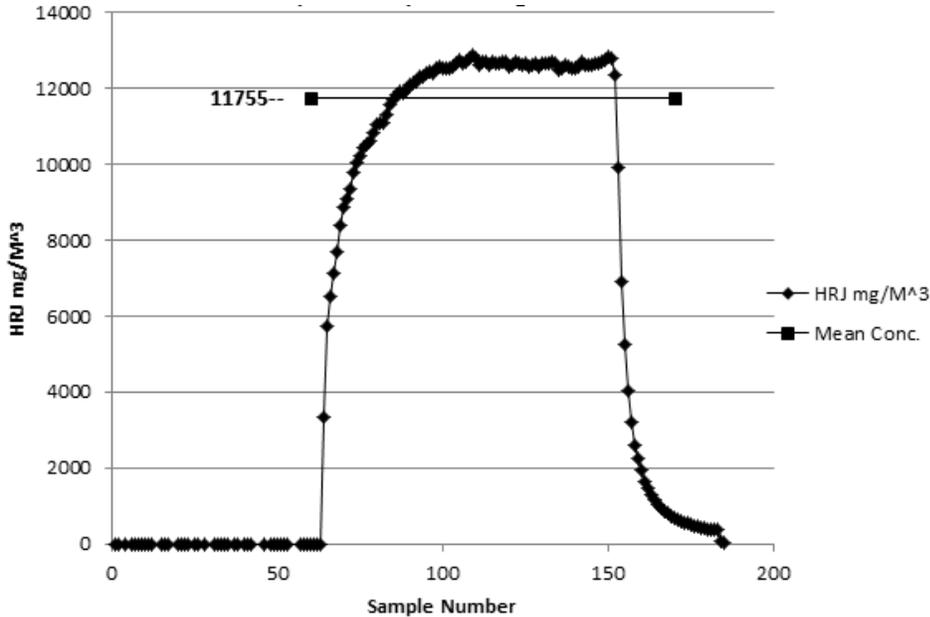
**Figure 2. FTIR Values for the 5,000 mg/m<sup>3</sup> HEFA-C Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-C exposure and 10 minute recovery periods for the target concentration of 5,000 mg/m<sup>3</sup> (actual 30 minute average: 5182 mg/m<sup>3</sup>).



**Figure 3. FTIR Values for the 7,500 mg/m<sup>3</sup> HEFA-C Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-C exposure and 10 minute recovery periods for the target concentration of 7,500 mg/m<sup>3</sup> (actual 30 minute average: 7048 mg/m<sup>3</sup>).



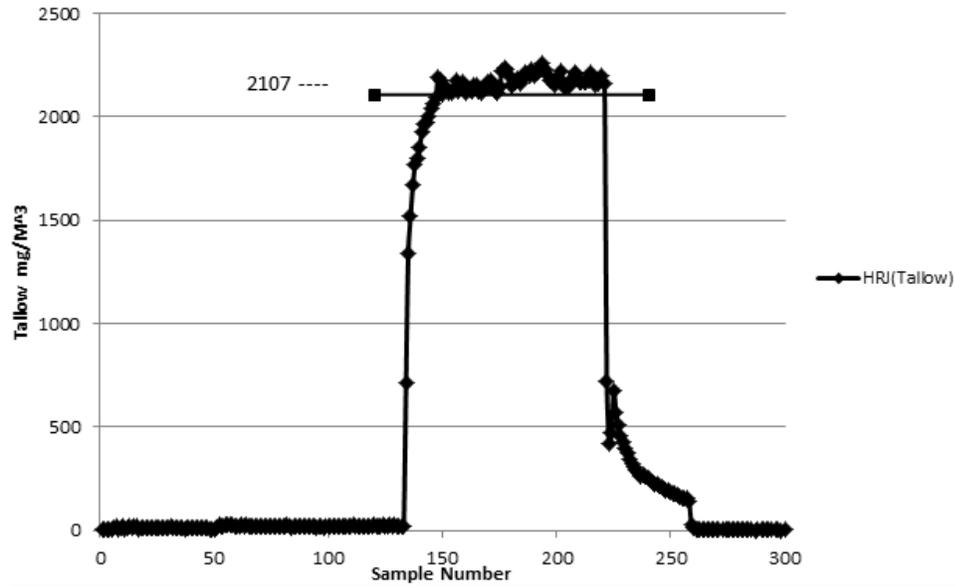
**Figure 4. FTIR Values for the 9,000 mg/m<sup>3</sup> HEFA-C Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-C exposure and 10 minute recovery periods for the target concentration of 9,000 mg/m<sup>3</sup> (actual 30 minute average: 9000 mg/m<sup>3</sup>).



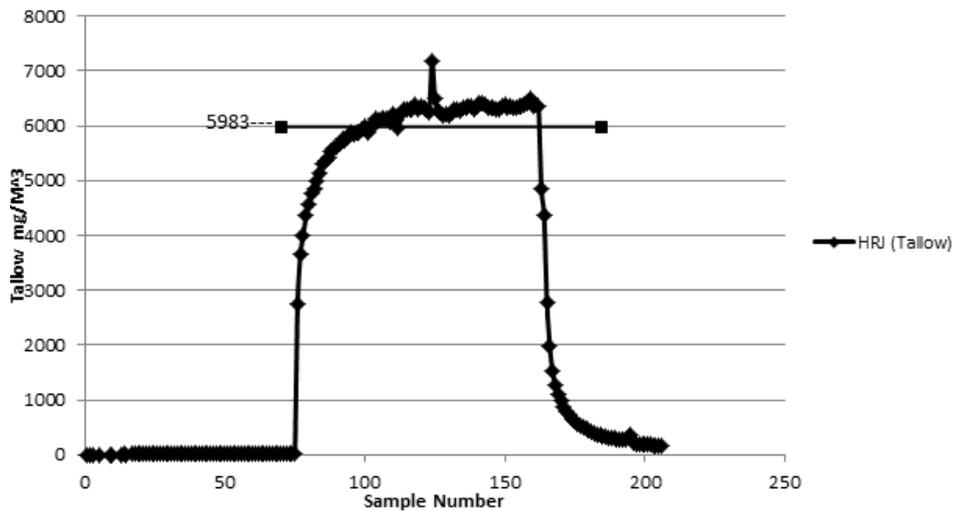
**Figure 5. FTIR Values for the 12,000 mg/m<sup>3</sup> HEFA-C Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-C exposure and 10 minute recovery periods for the target concentration of 12,000 mg/m<sup>3</sup> (actual 30 minute average: 12000 mg/m<sup>3</sup>).

## HEFA-T

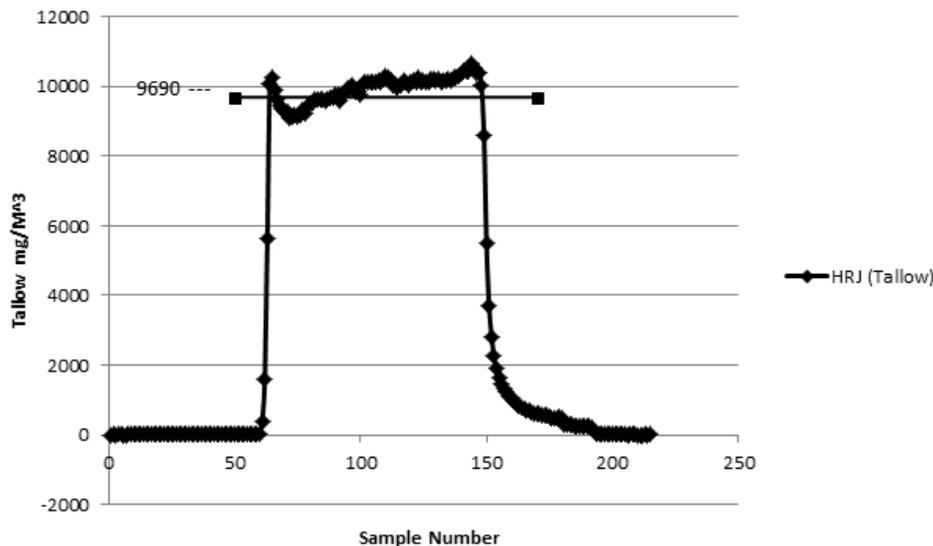
Each FTIR figure depicts the 10 minute baseline, 30 minute exposure and 10 minute recovery periods for the exposure concentration.



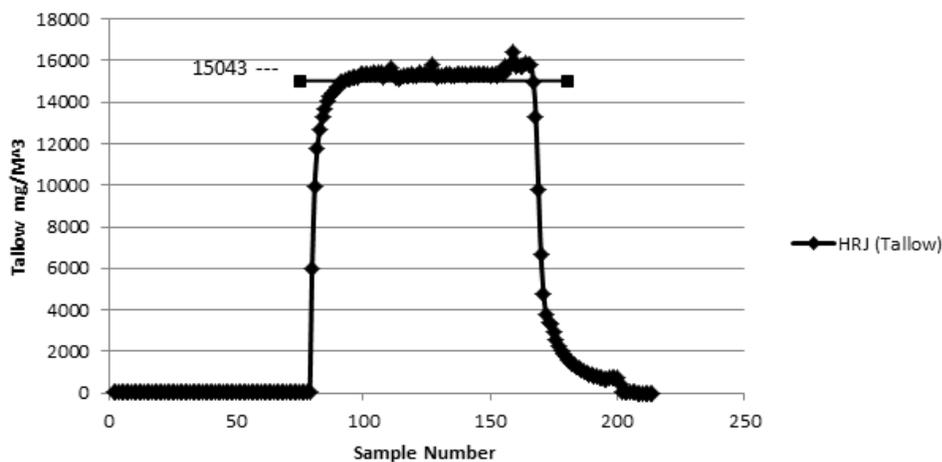
**Figure 6. FTIR Values for the 2,000 mg/m<sup>3</sup> HEFA-T Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-T exposure and 10 minute recovery periods for the target concentration of 2,000 mg/m<sup>3</sup> (actual 30 minute average: 2107 mg/m<sup>3</sup>).



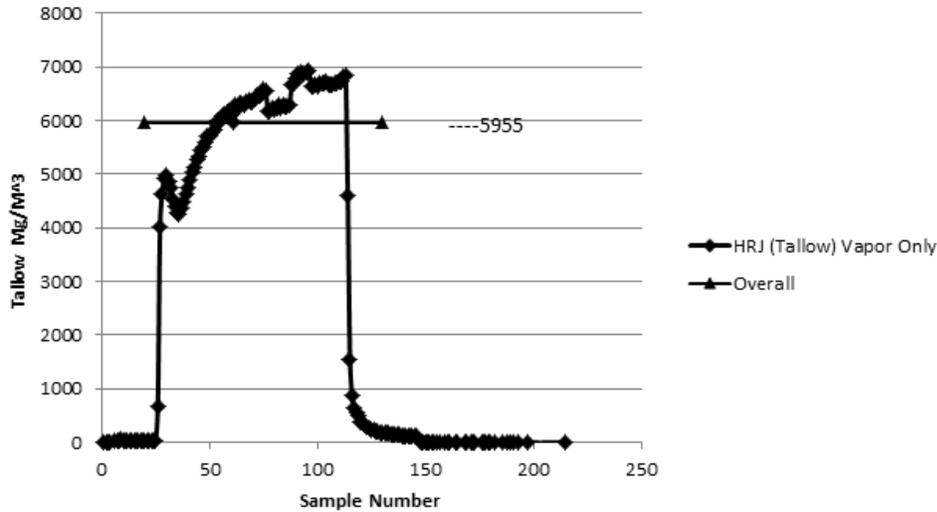
**Figure 7. FTIR Values for the 6,000 mg/m<sup>3</sup> HEFA-T Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-T exposure and 10 minute recovery periods for the target concentration of 6,000 mg/m<sup>3</sup> (actual 30 minute average: 5983 mg/m<sup>3</sup>).



**Figure 8. FTIR Values for the 10,000 mg/m<sup>3</sup> HEFA-T Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-T exposure and 10 minute recovery periods for the target concentration of 10,000 mg/m<sup>3</sup> (actual 30 minute average: 9690 mg/m<sup>3</sup>).



**Figure 9. FTIR Values for the 14,000 mg/m<sup>3</sup> HEFA-T Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-T exposure and 10 minute recovery periods for the target concentration of 14,000 mg/m<sup>3</sup> (actual 30 minute average: 15043 mg/m<sup>3</sup>).



**Figure 10. FTIR Values for the 6,000 mg/m<sup>3</sup> Vapor Only HEFA-T Exposure.** FTIR values are shown over time for the 10 minute baseline, 30 minute HEFA-T exposure and 10 minute recovery periods for the vapor only target concentration of 6,000 mg/m<sup>3</sup> (actual 30 minute average: 5955 mg/m<sup>3</sup>).

**APPENDIX G. COMPLETE ANALYSIS OF HEFA FUELS COMPOSITION AND  
COMPARISON TO JET A**

**Table 1. Aromatics**

<b>FUEL</b>	<b>Jet A</b>	<b>HEFA-C</b>	<b>HEFA-T</b>	<b>HEFA-F</b>
POSF	POSF-4658	POSF-6152	POSF-6308	POSF-5469
<b>Alkylbenzenes</b>				
benzene (C06)	<0.01	<0.01	<0.01	<0.01
toluene (C07)	0.16	<0.01	<0.01	<0.01
C2-benzene (C08)	0.78	0.02	<0.01	0.02
C3-benzene (C09)	2.24	0.08	0.04	0.07
C4-benzene (C10)	3.02	0.06	0.05	0.09
C5-benzene (C11)	2.48	0.03	0.04	0.08
C6-benzene (C12)	1.93	0.02	0.03	0.07
C7-benzene (C13)	1.19	0.03	0.02	0.07
C8-benzene (C14)	0.89	0.02	0.02	0.04
C9-benzene (C15)	0.65	<0.01	<0.01	0.01
C10+-benzene (C16+)	0.35	<0.01	<0.01	<0.01
<b>Total Alkylbenzenes</b>	<b>13.69</b>	<b>0.26</b>	<b>0.20</b>	<b>0.46</b>
<b>Diaromatics (Naphthalenes, Biphenyls, etc.)</b>				
diaromatic-C10	0.12	<0.01	<0.01	<0.01
diaromatic-C11	0.42	<0.01	<0.01	<0.01
diaromatic-C12	0.60	<0.01	0.01	<0.01
diaromatic-C13	0.40	<0.01	<0.01	<0.01
diaromatic-C14+	0.23	<0.01	<0.01	<0.01
<b>Total Alkyl-naphthalenes</b>	<b>1.76</b>	<b>&lt;0.01</b>	<b>0.02</b>	<b>&lt;0.01</b>
<b>Cycloaromatics (Indans, Tetralins, etc.)</b>				
cycloaromatic-C09	0.04	<0.01	<0.01	<0.01
cycloaromatic-C10	0.43	0.02	0.02	0.01
cycloaromatic-C11	1.13	0.02	0.03	0.03
cycloaromatic-C12	1.63	0.01	0.02	0.02
cycloaromatic-C13	1.45	0.01	0.03	0.02
cycloaromatic-C14	0.71	<0.01	<0.01	0.01
cycloaromatics-C15+	0.41	<0.01	<0.01	<0.01
<b>Total Cycloaromatics</b>	<b>5.79</b>	<b>0.06</b>	<b>0.09</b>	<b>0.10</b>
<b>Total Aromatics</b>	<b>21.25</b>	<b>0.33</b>	<b>0.31</b>	<b>0.56</b>

**Table 2. Aliphatics**

<b>FUEL</b>	<b>Jet A</b>	<b>HEFA-C</b>	<b>HEFA-T</b>	<b>HEFA-F</b>
POSF	POSF-4658	POSF-6152	POSF-6308	POSF-5469
<b>Paraffins</b>				
<b>Iso-Paraffins</b>				
C07 and lower-iso	0.23	0.09	0.02	0.14
C08-isoparaffins	0.56	0.68	0.07	1.35
C09-isoparaffins	1.08	13.44	5.87	4.97
C10-isoparaffins	3.59	18.25	12.20	9.28
C11-isoparaffins	5.12	16.92	12.71	10.72
C12-isoparaffins	5.31	9.89	13.25	11.04
C13-isoparaffins	5.25	9.01	12.43	10.60
C14-isoparaffins	4.44	6.47	11.94	9.04
C15-isoparaffins	3.10	5.72	17.65	9.44
C16-isoparaffins	1.66	3.08	1.07	10.43
C17-isoparaffins	0.69	0.60	0.02	3.66
C18-isoparaffins	0.19	0.02	<0.01	1.02
C19-isoparaffins	0.08	<0.01	<0.01	<0.01
C20-isoparaffins	0.02	<0.01	<0.01	<0.01
C21-isoparaffins	<0.01	<0.01	<0.01	<0.01
C22-isoparaffins	<0.01	<0.01	<0.01	<0.01
C23-isoparaffins	<0.01	<0.01	<0.01	<0.01
C24-isoparaffins	<0.01	<0.01	<0.01	<0.01
<b>Total iso-Paraffins</b>	<b>31.34</b>	<b>84.18</b>	<b>87.21</b>	<b>81.70</b>
<b>n-Paraffins</b>				
n-C07	0.15	0.02	<0.01	0.13
n-C08	0.54	0.75	0.11	0.83
n-C09	1.14	3.09	1.79	2.09
n-C10	2.55	2.68	1.80	2.42
n-C11	3.62	1.32	1.74	2.23
n-C12	3.70	1.13	1.71	1.90
n-C13	2.86	0.97	1.34	1.52
n-C14	2.17	0.78	2.73	1.42
n-C15	1.28	0.55	0.39	1.09
n-C16	0.61	0.10	<0.01	0.85
n-C17	0.27	0.02	<0.01	0.12
n-C18	0.05	<0.01	<0.01	0.03
n-C19	0.02	<0.01	<0.01	<0.01
n-C20	<0.01	<0.01	<0.01	<0.01
n-C21	<0.01	<0.01	<0.01	<0.01
n-C22	<0.01	<0.01	<0.01	<0.01
n-C23	<0.01	<0.01	<0.01	<0.01
<b>Total n-Paraffins</b>	<b>19.00</b>	<b>11.41</b>	<b>11.61</b>	<b>14.62</b>
<b>Cycloparaffins</b>				
<b>Monocycloparaffins</b>				
C07-monocycloparaffins	0.20	<0.01	<0.01	0.03
C08-monocycloparaffins	0.69	0.20	0.01	0.20
C09-monocycloparaffins	1.67	2.28	0.30	0.83
C10-monocycloparaffins	3.26	0.59	0.16	0.66
C11-monocycloparaffins	4.11	0.32	0.16	0.52

C12-monocycloparaffins	4.07	0.17	0.06	0.29
C13-monocycloparaffins	3.65	0.14	0.06	0.18
C14-monocycloparaffins	2.43	0.08	<0.01	0.03
C15-monocycloparaffins	1.55	<0.01	<0.01	<0.01
C16-monocycloparaffins	0.64	<0.01	<0.01	<0.01
C17-monocycloparaffins	0.28	<0.01	<0.01	<0.01
C18-monocycloparaffins	0.06	<0.01	<0.01	<0.01
C19+-monocycloparaffins	0.03	<0.01	<0.01	<0.01
<b>Total Monocycloparaffins</b>	<b>22.64</b>	<b>3.77</b>	<b>0.76</b>	<b>2.74</b>
<b>Dicycloparaffins</b>				
C08-dicycloparaffins	0.02	<0.01	<0.01	<0.01
C09-dicycloparaffins	0.29	0.02	<0.01	0.02
C10-dicycloparaffins	0.43	0.07	0.02	0.10
C11-dicycloparaffins	1.26	0.08	0.03	0.14
C12-dicycloparaffins	1.22	0.07	0.04	0.07
C13-dicycloparaffins	1.42	0.04	0.01	0.03
C14-dicycloparaffins	0.82	0.03	<0.01	0.02
C15-dicycloparaffins	0.21	<0.01	<0.01	<0.01
C16-dicycloparaffins	0.02	<0.01	<0.01	<0.01
C17+-dicycloparaffins	0.03	<0.01	<0.01	<0.01
<b>Total Dicycloparaffins</b>	<b>5.73</b>	<b>0.30</b>	<b>0.10</b>	<b>0.38</b>
<b>Tricycloparaffins</b>				
C10-tricycloparaffins	<0.01	<0.01	<0.01	<0.01
C11-tricycloparaffins	0.05	<0.01	<0.01	<0.01
C12-tricycloparaffins	<0.01	<0.01	<0.01	<0.01
<b>Total Tricycloparaffins</b>	<b>0.05</b>	<b>0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>
<b>Total Cycloparaffins</b>	<b>28.42</b>	<b>4.09</b>	<b>0.86</b>	<b>3.12</b>
<b>Total Aliphatics</b>	<b>78.75</b>	<b>99.67</b>	<b>99.69</b>	<b>99.44</b>

**Table 3. Aromatic and Aliphatic Average Molecular Formula**

<b>FUEL</b>	<b>Jet A</b>	<b>HEFA-C</b>	<b>HEFA-T</b>	<b>HEFA-F</b>
POSF	POSF-4658	POSF-6152	POSF-6308	POSF-5469
Average Molecular Formula – Carbon Atoms	11.69	11.00	12.01	12.13
Average Molecular Formula – Hydrogen Atoms	22.62	23.86	25.97	26.12

## LIST OF ACRONYMS

ACGIH	American Conference of Governmental Industrial Hygienists
AFB	Air Force Base
AFRL	Air Force Research Laboratory
BPM	breaths per minute
COT	Committee on Toxicology
DoD	Department of Defense
DTIC	Defense Technical Information Center
EPA	Environmental Protection Agency
FMI	Fluid Metering Inc.
F-T	Fischer-Tropsch
GLP	Good Laboratory Practices
GSD	geometric standard deviation
HEFA	hydroprocessed esters and fatty acids
HEFA-C	HEFA-Camelina
HEFA-F	HEFA-Animal fats and oils
HEFA-T	HEFA-Tallow
HEPA	high efficiency particulate air
HHA	health hazard assessment
HJF	Henry M. Jackson Foundation for the Advancement of Military Medicine
HRJ	hydrotreated renewable jet
IACUC	Installation Animal Care and Use Committee
MMAD	mass median aerodynamic diameter
NAC-AEGL	National Academies Council on Acute Exposure Guidelines Levels
NAMRU-D	Naval Medical Research Unit – Dayton
NOES	nose only exposure system
NRC	National Research Council
OEL	occupational exposure limit
OPPTS	Office of Prevention, Pesticides and Toxic Substances
PDII	Primary Dermal Irritation Index
RD <sub>50</sub>	concentration that produces a 50 percent decrease in respiratory rate
SD	Sprague-Dawley
sec	seconds
SOP	standard operating procedure
SPK	synthetic paraffinic kerosene
TLV-TWA	threshold limit value-time weighted average
USAF	United States Air Force
WPAFB	Wright-Patterson AFB