6:2 FTSB: DETERMINATION OF BIOSOLUBILITY USING SIMULATED EPITHELIAL LUNG FLUID

EASTON STUDY NUMBER: 264K-105
eSM PROJECT NUMBER: S23-201426

OECD Guideline for the Testing of Chemicals
OECD 105, Water Solubility

AUTHORS:

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STUDY INITIATION DATE: November 29, 2023
STUDY COMPLETION DATE: April 15, 2024

SUBMITTED TO:

American Chemistry Council
100 2nd Street, N.E.
Washington, DC 20002
USA

In fulfillment of
US Environmental Protection Agency Test Order
TO-2022-0897-XXXXXX-01-A

TESTING FACILITY:

Eurofins EAG Agroscience, LLC
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council

TITLE: 6:2 FTSB: Determination of Biosolubility Using Simulated Epithelial Lung Fluid

STUDY NUMBER: 264K-105

STUDY COMPLETION DATE: April 15, 2024

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 792) (1989) which are compatible with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); with the following exception:

The characterization of the test substance and the stability of the test substance, under the conditions of storage at the test site, were not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:

Lacey Brown — April 15, 2024

Lacey Brown, B.S.
Manager of Product Chemistry
Eurofins EAG Agroscience, LLC

Date

SPONSOR REPRESENTATIVE:

Stephen Risotto

Date

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 792) (1989) which are compatible with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17). The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Protocol	October 26, 2023	October 27, 2023	January 31, 2024
Sample Preparation	December 15, 2023	December 15, 2023	January 24, 2024
Data and Draft Report	February 21-23, 2024	February 23, 2024	April 1, 2024
Final Report	April 12, 2024	April 12, 2024	April 15, 2024

All inspections were study-based unless otherwise noted.

Troy Austin, B.S.
Quality Assurance Associate II
Eurofins EAG Agroscience, LLC

15 April, 2024
Date

REPORT APPROVAL

SPONSOR: American Chemistry Council

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STUDY NUMBER: 264K-105

STUDY DIRECTOR:

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SUMMARY

STUDY TITLE:	6:2 FTSB: Determination of Biosolubility Using Simulated Epithelial Lung Fluid
STUDY NUMBER:	264K-105
SPONSOR:	American Chemistry Council 100 2nd Street, N.E. Washington, DC 20002 USA
TESTING FACILITY:	Eurofins EAG Agroscience, LLC Easton, Maryland 21601
SPONSOR'S REPRESENTATIVE:	Steve Risotto srisotto@americanchemistry.com
LOCATION OF STUDY, RAW DATA AND THE FINAL REPORT:	Eurofins Easton, Maryland 21601
TEST SUBSTANCE:	6:2 FTSB
TEST DATES:	Experimental OECD Start – December 11, 2023 Experimental Termination – December 21, 2023

SUMMARY: The biosolubility of 6:2 FTSB in Gamble's Solution at 37ºC was determined using the shake flask method. The samples were diluted as appropriate using methanol and 50:50:0.1 Methanol: HPLC-grade water: Formic Acid. All samples were analyzed for 6:2 FTSB concentrations using liquid chromatography with tandem mass spectrometry (LC/MS/MS).

The biosolubility of 6:2 FTSB at 37°C in Gamble's Solution are presented in the table below:

Sampling Interval	Mean Measured Bisolubility of 6:2 FTSB	Percent Difference (72-192 hrs)
72 Hours	11934 ± 1299 mg/L (CV = 10.9%; N = 3)	2.16%
192 Hours	12195 ± 1317 mg/L (CV = 10.9%; N = 3)	
Overall	12064± 1179 mg/L (CV = 9.8%; N = 6)	

INTRODUCTION

A study was performed to determine the biosolubility of 6:2 FTSB at 37°C in Gamble's Solution. Eurofins EAG Agroscience, LLC conducted this study for the American Chemistry Council at the Eurofins facility in Easton, Maryland. The test was performed using Gamble's solution. The experimental portion of this study was conducted between December 11, 2023 and December 21, 2023. The raw data and final report will be filed under the Study Number 264K-105 in the archives located at the Easton site upon finalization.

OBJECTIVE

The objective of this study was to experimentally determine the water solubility of 6:2 FTSB at 37°C in reagent water using the shake flask method.

EXPERIMENTAL DESIGN

The definitive test consisted of equilibrating an excess amount of test substance with water at an elevated temperature, 45°C, followed by equilibration at 37°C.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "6:2 FTSB: Determination of Biosolubility Using Simulated Epithelial Lung Fluid" (Appendix 1). The study met the requirements of the OECD Guideline for the Testing of Chemicals, OECD 105: *Water Solubility* (2).

Test Substance

The test substance was received from Chemours for American Chemistry Council on August 17, 2023. The material, a solid, was assigned testing facility identification number 18561 and was stored under ambient conditions in darkness. The Certificate of Analysis is included in Appendix 2. The sponsor provided the following test substance information:

Test Substance Name:	Dry Capstone™ 1157 Fire Fighting Foam Fluorosurfactant
Common Name:	Dry Capstone™ 1157
Chemical Formula:	Carboxymethyldimethyl-3-[[(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)sulphonyl]amino]propylammonium hydroxide
CAS #:	34455-29-3
Lot No:	I0722
Purity:	97.7%
Expiration Date:	February 2027
Appearance:	Brown/yellow powder
Physical State:	Solid

Solvents and Reagents

Burdick & Jackson HPLC-Grade reagent water and methanol were used. The reagent water was equivalent to ASTM Type II Designation D1193-06 (1). All reagents used in the study were ACS reagent grade or better.

Stocks Preparation

A primary stock solution of 6:2 FTSB in methanol was prepared by weighing 0.0512 g of 6:2 FTSB into a 50 mL volumetric flask using an analytical balance. The material in the volumetric flask was then brought to final volume with methanol. The nominal 6:2 FTSB concentration in the primary stock solution preparation was 1.02 mg 6:2 FTSB /mL. The primary 6:2 FTSB stock solution was used to prepare a set of secondary 6:2 FTSB stock solutions in methanol using the following serial dilution scheme, volumetric flasks, and a calibrated displacement pipette.

Stock Concentration (mg 6:2 FTSB /mL)	**Aliquot (µL)**	**Final Volume (mL)**	**Secondary Stock Concentration (mg 6:2 FTSB /mL)**
1.02	500	10.0	0.0501

Standards Preparation

The 0.0501 mg 6:2 FTSB /L secondary stock solutions in methanol were used to prepare a calibration standard set in 50:50:0.1 methanol: HPLC-Grade Water: Formic Acid, for LC/MS/MS analysis, using the following dilution scheme, volumetric flasks, and a calibrated displacement pipette:

Analytical Standard ID (mg 6:2 FTSB /L)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (µg 6:2 FTSB./L)
S-001	200.0	10.0	1.00
S-002	150.0	10.0	0.750
S-003	120.0	10.0	0.600
S-004	80.0	10.0	0.400
S-005	50.0	10.0	0.250
S-006	20.0	10.0	0.100

Preliminary Test Procedure

A preliminary test was performed to provide an estimate of 6:2 FTSB biosolubility at ambient conditions. The experiment consisted of weighing approximately 0.10 g of 6:2 FTSB into a clear glass vial. Increasing amounts of Gamble's Solution were added to the vial and, following each addition, the vial

contents were mixed by vigorously shaking for approximately 10 minutes and then visually inspected for any undissolved material. The material was complete dissolved after the addition of 10 mL of Gamble's Solution, indicating an approximate solubility of 9950 mg/L.

Definitive Test Procedure and Analytical Methods

Definitive Test Procedure

A definitive test was performed to determine the solubility of 6:2 FTSB at 37°C. Five solubility samples were prepared to achieve a target concentration of 100 mg/mL. This target concentration was selected to fulfil the protocol criteria that the definitive test samples should be prepared at greater than five times the quantity of test substance required to achieve water solubility. The water solubility was estimated in the preliminary test to be approximately 9950 mg/L. Samples were prepared by weighing 1.48 – 1.51 g of the test substance directly into separate clear 20-mL glass vials on an analytical balance and adding 15 mL of Gamble's Solution pre-equilibrated to 20°C using a volumetric pipette. The vessels were sealed with foil-lined screw caps and parafilm, and placed in an agitating water bath set at 45°C.

After 24 hours of gentle shaking at 45°C, one of the five bottles was removed and re-equilibrated at 37°C for approximately 24 hours by placing it in a water bath maintained at 37°C ± 0.1°C. Following a re-equilibration period at 37°C, with occasional manual agitation, the saturation sample was removed from the 37°C water bath and was processed for determination of soluble 6:2 FTSB concentration. Triplicate aliquots were removed from the test vessel, centrifuged, and transferred into separate clear labeled vials. One replicate sample was checked for Tyndall effect. Each sample was then diluted using 50: 50: 0.1 (v/v) methanol: HPLC-grade water: formic acid dilution solvent to facilitate analysis by LC/MS/MS. A sample aliquot was taken for determination of supernatant pH prior to sample processing. A second bottle was treated in a similar manner following an initial equilibration period of 48 hours at 45°C. A third prepared bottle was treated in a similar manner following an initial equilibration period of 72 hours at 37°C, and a fourth after 192 hours.

Analytical Method

The sample preparation and instrumental determination procedures used for the analysis of 6:2 FTSB in Gamble's Solution were developed at Eurofins-Easton. A method for the sampling and analysis of 6:2 FTSB shake flask solubility samples is presented in Figure 4.

A glass Pasteur pipette was used to remove three separate aliquots of the solubility sample and transfer them to a labelled 2-mL microcentrifuge tube. The tubes were sealed and the samples were then centrifuged at 14,100 rcf for 15 minutes. Following the centrifugation, a representative sample was checked for Tyndall effect (undissolved material) and a calibrated displacement pipette was used to transfer 100 µL of the supernatant to a 10 mL volumetric flask pre-loaded with 50:50:0.1 (v/v) methanol: HPLC-grade reagent water: formic acid. The flask contents were brought to volume with 50:50:0.1 (v/v) methanol: HPLC-grade reagent water: formic acid and was inverted several times to mix.

An additional serial dilution in 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid was performed by transferring 100 µL of the diluted samples into a 25 mL volumetric flask pre-loaded with 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid. Flask contents were then brought to volume with 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid and inverted several times to mix. An aliquot of each diluted sample was submitted for LC/MS/MS analysis.

An additional aliquot of the aqueous sample was centrifuged and equilibrated to 37°C for measurement of pH using a calibrated pH meter (Thermo Orion/4 Star Plus).

Concentrations of 6:2 FTSB in aqueous samples were determined by an Applied Biosystems/MDS Sciex API 5000 LC/MS/MS system coupled with an Agilent 1200 Infinity Series High Performance Liquid Chromatograph (HPLC) system. Chromatographic separations were achieved using a Thermo Betasil C18 analytical column (50 mm × 2.1 mm, 3-µm particle size). The instrument parameters are summarized in Table 1.

Using the calculated concentrations of 6:2 FTSB in aqueous samples, the percentage inhalation bioaccessible fraction (%IBAF) using the following calculation

$$\%IBAF = (C_{ibio}/C_{total} \times 100)$$

where C_{ibio} is the concentration of the test substance which is soluble in Gamble's solution and C_{total} is the total amount of material introduced to the test vessel.

Calibration Curves and Limits of Quantitation

Five calibration standards were prepared with 6:2 FTSB in 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid dilution solvent. These standards ranged in concentration from 1.00 to 0.100 mg 6:2 FTSB /L and were analyzed concurrently with each set of solubility samples. The calibration set was injected at the beginning and end of each analytical sequence and a standard was injected after no more than five sample injections. Linear regression (1/x weighted) equations were generated using Analyst 1.7.1 software for each analytical sequence using the peak area response versus the respective 6:2 FTSB concentrations of the calibration standards. The concentrations of 6:2 FTSB in the solubility samples were determined by substituting the peak area responses of the samples into the applicable linear regression equation. A representative LC/MS/MS calibration curve is presented in Figure 2. Representative chromatograms of low-level and high-level LC/MS/MS calibration standards are presented in Figure 3.

The calculated limit of quantitation (CLOQ) was calculated as the product of the dilution factor (DF) of the matrix blank sample and the concentration of the lowest level calibration standard. The CLOQ was 1250 mg/L based on the low-level calibration standard of 0.100 mg/L and a dilution factor of 12,500.

Quality Control (QC) Samples

A matrix blank sample was prepared on the day of aqueous sample collection for each pH test condition and analyzed concurrently with the solubility samples. The matrix blank sample was processed following the same procedure as the solubility samples to identify possible interferences. No chromatographic interferences at the CLOQs were observed in the matrix blank samples (Table 2). A representative chromatogram of a matrix blank sample is presented in Figure 4.

Example Calculations

The concentration of 6:2 FTSB in the column elution solubility sample 264K-105-SF-4-A(RE) was calculated using the following equations:

$$\text{6:2 FTSB (ug/L) in sample} = \frac{\text{Peak Area - (Y-intercept)}}{\text{Slope}} \times \text{Overall Dilution Factor}$$

Peak area 6:2 FTSB = 13780000
Y-intercept = 82884.8
Slope = 15591400

Sample Volume (V_{i1}) = 0.100 mL
Final Volume (V_{f1}) = 10.0 mL
Primary Dilution Factor (V_{f1} / V_{i1}) = 100

Sample Volume (V_{i2}) = 0.200 mL
Final Volume (V_{f2}) = 25.0 mL
Secondary Dilution Factor (V_{f2} / V_{i2}) = 125

Overall Dilution Factor (V_1 x V_2) = 12,500

$$\text{6:2 FTSB (mg/L) in sample} = \frac{13780000 - (82884.8)}{15591400} \times 12{,}500$$

6:2 FTSB (mg/L) in sample = 10981.3

* Calculations were performed using Analyst Version 1.7.1 and Microsoft® Excel 2016 in full precision mode. Manual calculations may differ slightly.

RESULTS AND DISCUSSION

Preliminary Test

The solubility in water of 6:2 FTSB was estimated from a preliminary test which consisted of adding increasing amounts of Gamble's solution to a known mass of sample (approximately 0.1 g) until the sample was completely dissolved. The sample was weighed into a clear glass vial. The 6:2 FTSB sample was fully dissolved after the addition of a cumulative volume of 10.0 mL total volume. The test substance solubility under these preliminary test conditions was approximately 9950 mg/L

Definitive Test

Based on an expected solubility of 9950 mg/L, the target nominal concentration in the definitive test was set at a concentration of 100 mg/mL. This target concentration was selected to fulfill the protocol criteria that the definitive test samples be prepared at greater than five times the quantity of test substance required to achieve water solubility. Sample chromatograms for shake flask solubility samples are presented in Figure 5.

The pH of the test system and samples were evaluated concurrent with the definitive solubility test. The pH of the test system at 37°C was 7.28. The measured pH of the supernatant of Gamble's Solution saturated with the test substance at approximately 37°C was 7.36, 7.11, 7.26, and 8 for the 24, 48, 72, and 192-hour samples, respectively.

Concentrations of 6:2 FTSB in shake flask samples at 37ºC in Gamble's Solution are presented in Table 3. Mean measured biosoluble test substance concentrations for saturation samples shaken and

equilibrated at 45°C for 72 and 192 hours prior to 24-hour equilibration at 37°C were within the 15% difference criterion described in the protocol. The mean water solubility (N=6) for 6:2 FTSB at 37°C was 12064 ± 1179 mg/L (CV = 9.8%).

Data from the 48-hour equilibration at 45°C was significantly higher than solubility data at other time points. This is attributed to the samples being agitated too soon before sample collection, which would have suspended the excess material in the Gamble's solution such that it was not adequately separated during the centrifugation step. These data are not used in the final calculation of the biosolubility of the test substance and do not bias the results in any way.

Data generated during the 48 hour collection interval was noted as being much higher than the other solubility samples. This is expected to be due to variation in laboratory technique between performing technicians; agitation of the samples prior to sample collection was done too vigorously and likely created an emulsion that was not sufficiently separated during the centrifugation step.

The percentage inhalation bioaccessible fraction was 12.1% ± 0.012 (CV = 0.099).

CONCLUSIONS

The water solubility of 6:2 FTSB in Gamble's Solution at 37ºC was determined using the shake flask method. The samples were diluted using methanol and 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid dilution solvent. All samples were analyzed for 6:2 FTSB concentrations using liquid chromatography with tandem mass spectrometry (LC/MS/MS).

The biosolubility values of 6:2 FTSB at 37ºC in Gamble's Solution are presented in the table below:

Sampling Interval	Mean Measured Bisolubility of 6:2 FTSB	Percent Difference (72-192 hrs)
72 Hours	11934 ± 1299 mg/L (CV = 10.9%; N = 3)	2.16%
192 Hours	12195 ± 1317 mg/L (CV = 10.9%; N = 3)	
Overall	12064± 1179 mg/L (CV = 9.8%; N = 6)	

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8. **Sánchez-Piñero, J.; Novo-Quiza, N.; Pernas-Castaño, C.; Moreda-Piñeiro, J.; Muniategui-Lorenzo, S.; López-Mahía, P.** Inhalation Bioaccessibility of Multi-Class Organic Pollutants Associated to Atmospheric PM2.5: Correlation with PM2.5 Properties and Health Risk Assessment. Environmental Pollution 2022, 307, 119577. https://doi.org/10.1016/j.envpol.2022.119577.

Table 1

Typical LC/MS/MS Operational Parameters

INSTRUMENT:	Applied Biosystems/ MDS Sciex API 5000 LC/MS/MS and QJet Ion Guide coupled with an Agilent 1200 Infinity Series HPLC System		
ANALYTICAL COLUMN:	Thermo Betasil C18 (50.0 mm x 2.1 mm, 3-µm)		
GUARD COLUMN:	Thermo Javelin C18 (10 x 2.1 mm)		
STOP TIME:	3.00 minutes		
FLOW RATE:	0.250 mL/minute		
COLUMN OVEN TEMPERATURE:	40°C		
MOBILE PHASE:	Channel A1: 0.1% Formic Acid in HPLC-Grade Water Channel B1: 0.1% Formic Acid in Acetonitrile		
ELUTION PROFILE:	Time (min.)	Solvent A (%)	Solvent B (%)
	0.00	50.0	50.0
	3.00	75.0	50.0
VALCO VALVE SETTINGS:	Time (min.)	Position	
	0.0	A	
	0.1	B	
INJECTION VOLUME:	2.00 µL		
ION SOURCE :	Turbo Spray		
ION SOURCE CONDITIONS:	Source Temperature (TEM):	500.00	
	Collision Gas (CAD):	4.00	
	Curtain Gas (CUR):	30.00	
	Declustering Potential (DP):	141.00 V	
	Entrance Potential (EP):	10.00 V	
	Ion Spray Voltage (IS):	5500.00 V	
	Source Gas 1 (GS1):	40.00	
	Source Gas 2 (GS2):	50.00	
	Interface Heater (ihe):	On	
MS/MS CONDITIONS:	Scan Type:	Multiple Reaction Mode (MRM)	
	Polarity:	Positive	
	Dwell Time:	250.00 msec	
	MS/MS Transition (Quantitation):	571.400 → 104.100 Da	
	Collision Energy (CE):	45.00 V	
	Cell Exit Potential (CXP):	16.00 V	
APPROXIMATE 6:2 FTSB RETENTION TIME:	1.5 minutes		

Table 2

Quality Control Samples

Sample Number (264K-105-)	Concentrations of 6:2 FTSB Fortified (mg/L)	Concentrations of 6:2 FTSB Measured (µg./L) [1]	Percent Recovered
SF-MAB-1	0.0	< CLOQ [1,2]	--
SF-MAB-2	0.0	< CLOQ [1,2]	--
SF-MAB-3-RE	0.0	< CLOQ [1,2]	--
SF-MAB-4-RE	0.0	< CLOQ [1,2]	--

[1] Results were generated using Analyst Version 1.7.1.

[2] The calculated limit of quantitation (CLOQ) for determination of 6:2 FTSB (1250 mg/L) at was calculated as the product of the lowest-level calibration standard (0.100 mg/L) and the dilution factor of the matrix blank sample (12,500).

Table 3

Measured Concentrations of 6:2 FTSB in Shake Flask Samples at 45°C in Gamble's Solution

Sample ID (264K-105-SF-)	Sampling Interval (hours)	Total Material Added (g)	Initial Volume of Test Vessel (mL)	Analytical Result (mg/L)	Mean Measured Concentration (mg/L)	% IBAF	Std. Dev., CV	Percent Difference (%)	
1-A				5828.7		5.8%	1186	127.37	(24-48 hrs)
1-B	24	1.5088	15	8127.1	7148	8.1%	16.6%		
1-C				7488.8		7.4%			
2-A				31670		31.4%	731	91.88	(48-72 hrs)
2-B	48	1.5122	15	33048	32219	32.8%	2.27%		
2-C				31938		31.7%			
3-A-RE				10786		10.7%	1299	2.16	(72-192 hrs)
3-B-RE	72	1.5063	15	11672	11934	11.6%	10.9%		
3-C-RE				13344		13.3%			
4-A-RE				10981		11.1%	1317		
4-B-RE	192	1.4846	15	13595	12195	13.7%	10.8%		
4-C-RE				12008		12.1%			
				Average (mg/L)	12064	12.1%			
				SD	1179	0.012			
				CV	9.8%	0.099			

LC/MS/MS Concentrations calculated using Microsoft® Excel 2016 from values generated using Analyst Version 1.7.1.
Analytical Result(µg/L) = Calculated Concentration (µg/L) from Analyst / Dilution Factor (12,500) entered in Analyst.
Percent Difference calculated as absolute value of difference between mean results of the two sampling intervals divided by the mean of the results for the two flow rates.
CLOQ (Calculated Limit of Quantitation) (1250 mg/L) = Lowest Calibration Standard (0.100 mg/L) x Dilution factor (12,500)
%IBAF (Percentage Inhalation Bioaccessible Fraction) = (Concentration of test substance which is soluble)/(Total Material Added/Initial Volume of Test vessel x 1000mg/g)
All spreadsheet calculations performed using Microsoft® Excel 2016 in full precision mode.

1. Prepare a single matrix blank sample, consisting of the appropriate aqueous buffer, by partially filling a labeled 10-mL volumetric flask with 5mL of the Gamble's Solution.

2. Bring samples to final volume with methanol, stopper and invert repeatedly to mix.

3. Using glass Pasteur pipettes or pipette, transfer triplicate approximately 2-mL aliquots (estimated by graduations on tube) of each solubility saturation sample into labeled 2-mL microcentrifuge tubes, appending solubility sample IDs with '-A', -'B' and '-C'). Transfer an aliquot of each QC sample into a labeled 2-mL microcentrifuge tube. Seal tubes.

4. Transfer approximately 10.0 mL of the sample into a 20-mL glass vial using a displacement pipette. Append sample ID with "-pH". Cap vial and centrifuge sample for approximately 10 minutes at a setting of 872xg.

5. Remove sample from centrifuge and place into a 37ºC constant temperature water bath consisting of a clear plastic reservoir with a heated circulator and submerged copper coil connected to a recirculating chiller and allow sample to equilibrate at 37ºC (for a minimum of 15 min.).

6. Measure and record the pH of the sample.

7. Centrifuge tubes at a setting of 14,100 rcf for 15 minutes.

8. Transfer each supernatant into a separate 2-mL clear, labeled glass vials with a glass Pasteur pipette. Check at least one replicate supernatant for each water solubility sample for Tyndall effect (i.e., light scattering caused by un-dissolved material using a laser light and comparing to appropriate matrix blank).

9. Using displacement pipettes, transfer an appropriate volume of each sample supernatant into the appropriate number of glass 25-mL volumetric flasks preloaded with 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid dilution solvent and bring each volumetric flask to final volume with the dilution solvent. Cap each volumetric flask and invert several times to mix.

10. Perform an additional serial dilution in 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid dilution solvent using volumetric flasks and displacement pipettes.

11. Transfer an aliquot of each QC and test sample final dilution into separate labeled autosampler vials and submit for LC/MS/MS analysis (or store refrigerated for later analysis). If necessary, transfer remainder of original aqueous sample supernatant and an aliquot of each initial sample dilution into an appropriately sized glass vial and place vials into refrigerated storage.

Figure 1. Method for processing 6:2 FTSB shake flask biosolubility samples.

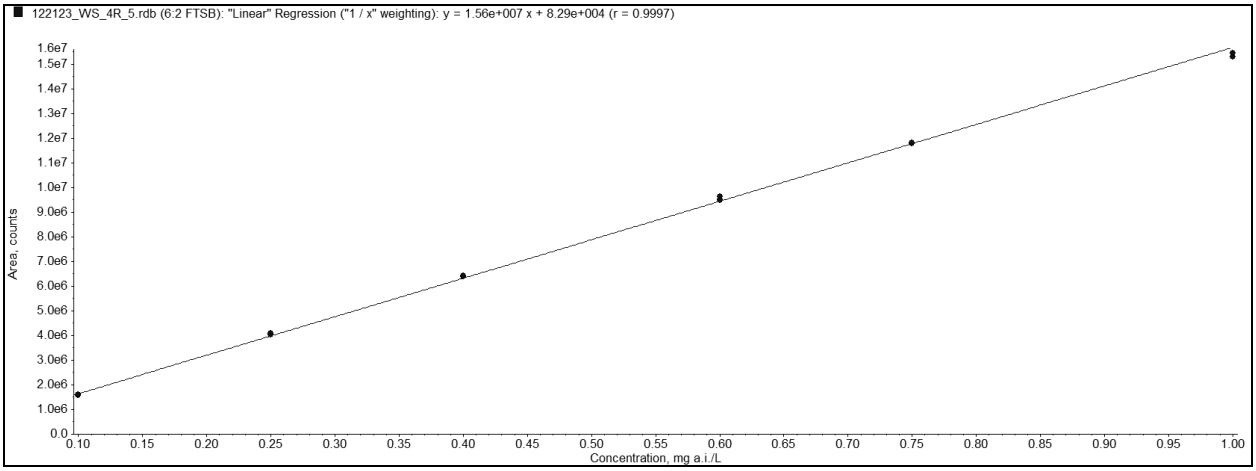
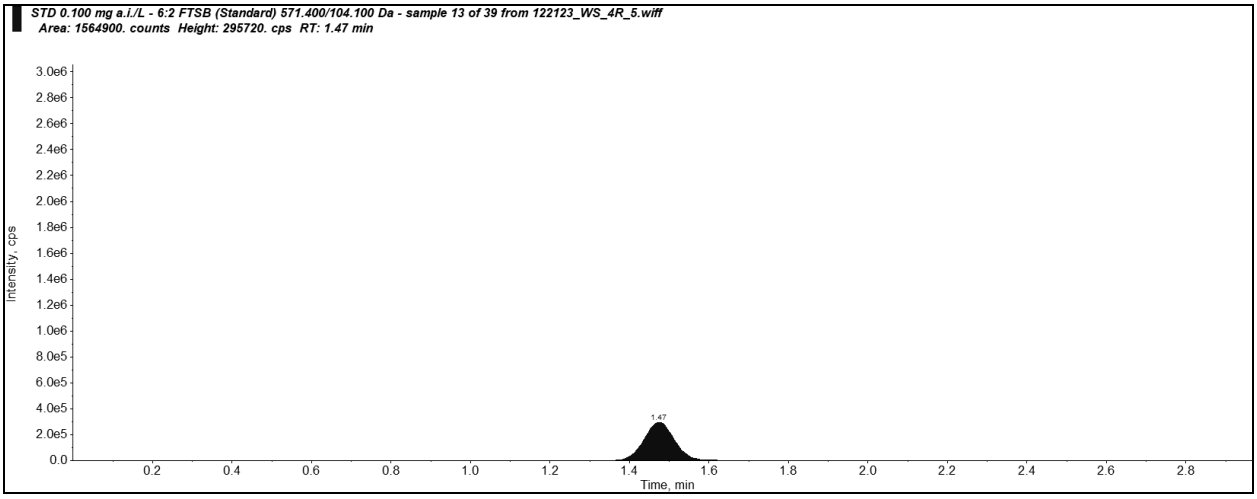
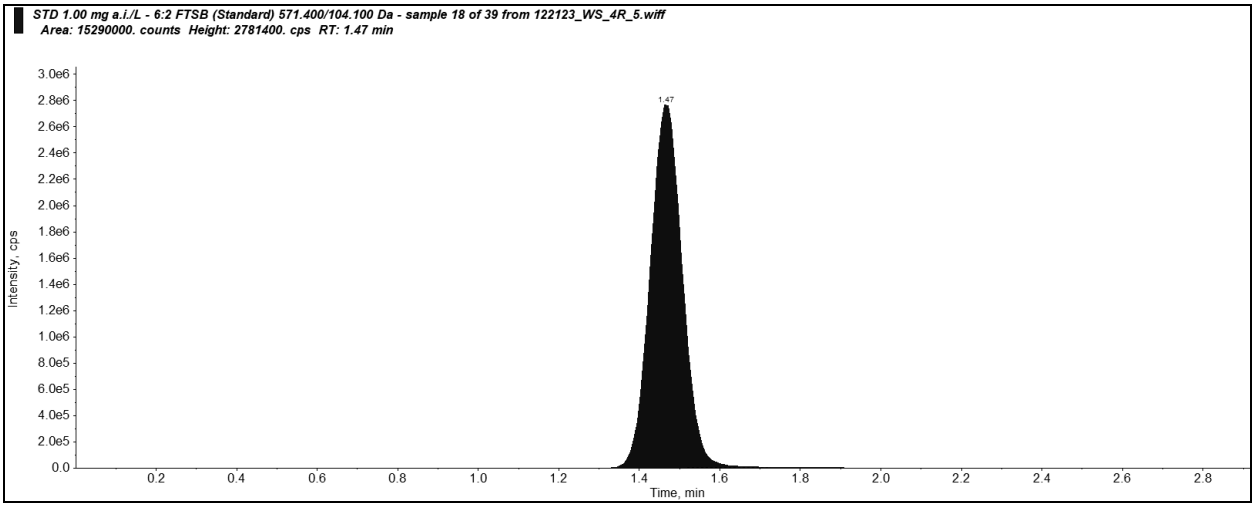


Figure 2. A representative calibration curve

Slope = 1.56 x 10^7; Y-Intercept = 82900; R = 0.9997



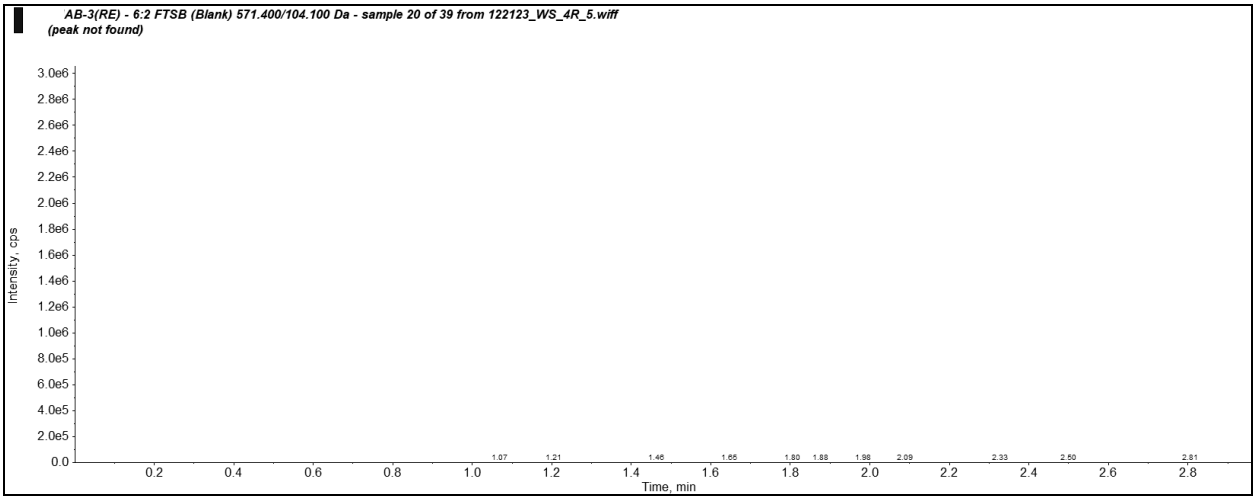
(A)



(B)

Figure 3. Representative chromatograms of low- and high-level 6:2 FTSB calibration standards.

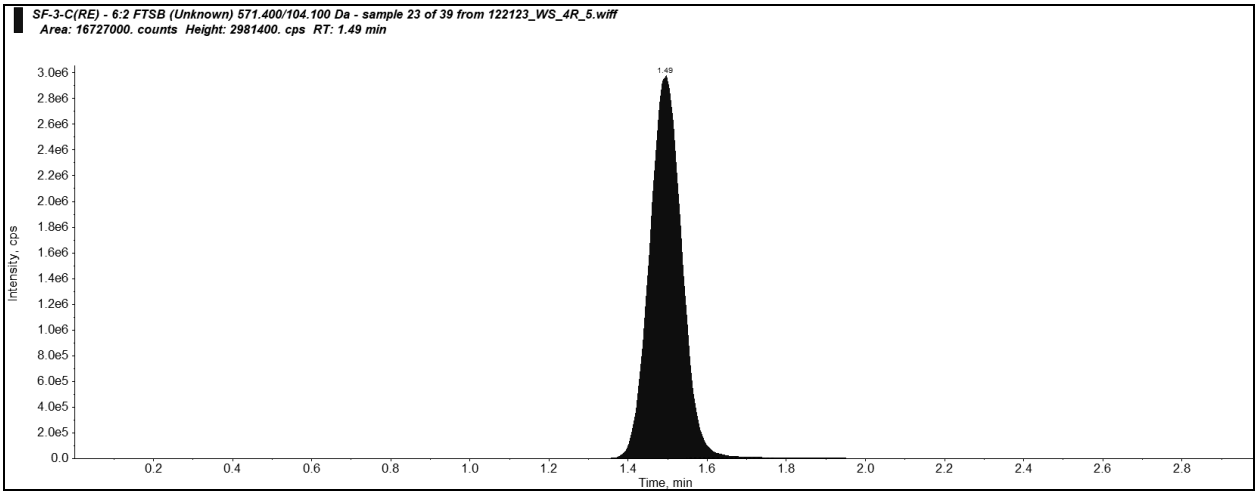
(A): 100 mg/L prepared in 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid
(B): 1.00 mg/L prepared in 50:50: 0.1 (v/v) methanol: HPLC-grade reagent water: formic acid



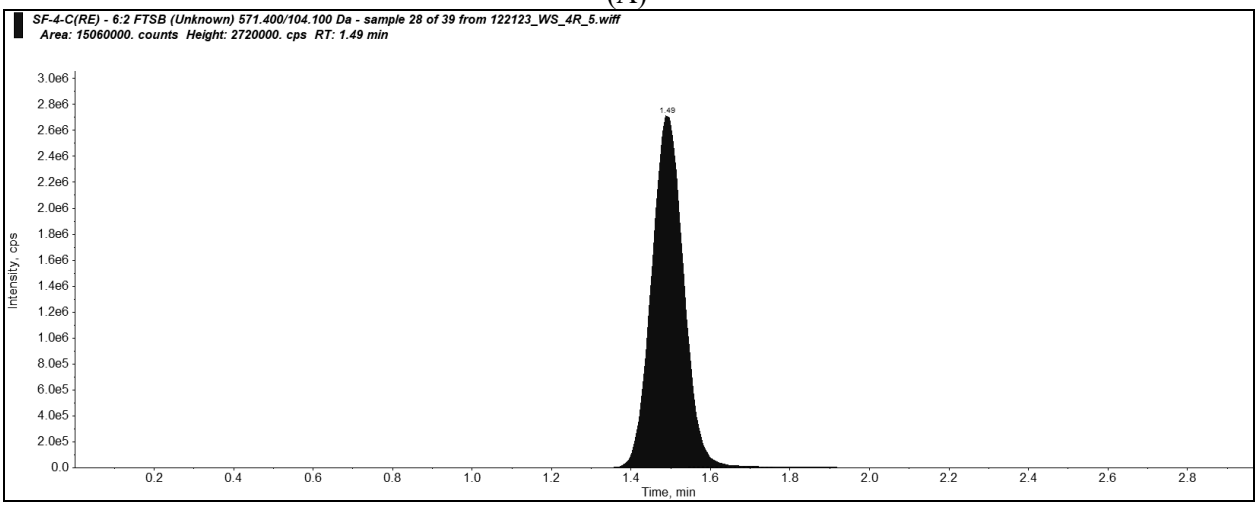
(A)

Figure 4. Representative chromatograms of the matrix blank sample.

(A): 264K-105-MAB-3(RE), Dilution Factor (DF) = 62,500



(A)



(B)

Figure 5. Representative chromatograms of biosolubility samples

(A): 264K-105-SF-3-C(RE), DF = 12,500, 72 Hour Equilibration
(B): 264K-105-SF-4-C(RE), DF = 12,500, 192 Hour Equilibration

Appendix 1

Study Protocol

PROTOCOL

6:2 FTSB: DETERMINATION OF BIOSOLUBILITY USING SIMULATED EPITHELIAL LUNG FLUID

U.S. EPA Product Properties Test Guidelines, OPPTS 830.7840
Water Solubility: Column Elution Method; Shake Flask Method

and

OECD Guideline for the Testing of Chemicals
OECD 105, Water Solubility

Consent Order ID: EPA-HQ-OPPT-2021-0897

Submitted to

American Chemistry Council
100 2nd Street, N.E.
Washington, DC 20002
USA

Testing Facility

Eurofins EAG Agroscience, LLC
8598 Commerce Drive
Easton, Maryland 21601 USA
(410) 822-8600

6:2 FTSB: DETERMINATION OF BIOSOLUBILITY USING SIMULATED EPITHELIAL LUNG FLUID

SPONSOR:	American Chemistry Council 100 2nd Street, N.E. Washington, DC 20002 USA
SPONSOR'S REPRESENTATIVE:	Steve Rissoto srisotto@americanchemistry.com
TESTING FACILITY:	Eurofins EAG Agroscience, LLC 8598 Commerce Drive Easton, Maryland 21601
STUDY DIRECTOR:	Lacey Brown, B.S. Manager of Product Chemistry Eurofins EAG Agroscience, LLC
LABORATORY MANAGEMENT:	Ling Zhang, Ph.D. Associate Director of Analytical Chemistry

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: November 30, 2023

Experimental Termination Date: December 29, 2023

Easton Study No.: 264K-105

(eSM Project No.: S23-201426)

Test Substance No.: 18561 Reference Substance No. (if applicable): n/a

PROTOCOL APPROVAL

Lacey Brown
STUDY DIRECTOR

November 29, 2023
DATE

LABORATORY MANAGEMENT

Nov 30, 2023
DATE

Sponsor Approval Date: June 26, 2023

INTRODUCTION

Eurofins will experimentally determine the biosolubility of 6:2 FTSB in Gamble's solution (a simulated epithelial lung fluid). The study will be conducted at the Eurofins analytical chemistry facility in Easton, Maryland. The study will be performed based on procedures in the U.S. EPA Product Properties Test Guidelines, OPPTS 830.7840, *Water Solubility: Column Elution Method; Shake Flask Method* (1) and U.S. EPA Product Properties Test Guidelines, OPPTS 830.7860, *Water Solubility (Generator Column Method)* (2). Additional guidance presented in OECD Guideline for the Testing of Chemicals, OECD 105: *Water Solubility* (3) may be used. These guidelines will be modified for consistency with ECETOC Technical Report 122, Section 3 (3)

OBJECTIVE

The objective of this study is to experimentally determine the biosolubility of 6:2 FTSB using the modified shake flask method.

EXPERIMENTAL DESIGN

Determination of water solubility by the shake flask method is generally applicable to test substances with water solubilities equal to or exceeding 10 mg/L although accurate determinations can be made for certain test substances at significantly lower concentrations. The shake flask methodology may not, however, be applicable to surface active or volatile test substances. Method development or preliminary trials with the test substance will indicate whether or not shake flask methodology is appropriate for determination of the test substance water solubility. If preliminary testing in aqueous solution estimates water solubility at 10% or greater on a weight-to-weight (w/w) basis, the study will be experimentally terminated and the solubility will be reported as >10% (w/w). The definitive shake flask test typically consists of equilibration of an excess amount of test substance with water at an elevated temperature, 30°C, followed by equilibration at 20°C. In this study the procedures will be modified such that the shake flask samples will be equilibrated at 45°C, followed by equilibration at 37°C and will be conducted in Gamble's solution (simulated epithelial lung fluid) to determine biosolubility. No bias is expected in this study.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing the testing

facility with verification that the test substance has been characterized according to GLPs prior to its use in the study. If verification of the GLP test substance characterization is not provided to the testing facility, it will be noted in the compliance statement of the final report.

The Sponsor is responsible for all information related to the test substance and agrees to accept, or give the testing facility authorization to dispose of, any unused test substance and/or test substance containers remaining at the end of the study.

Reagents and Buffers

All solvents used in the method will be high performance liquid chromatography (HPLC) grade or better. All reagents will be ACS reagent grade or better. Gamble's solution used in this study will be adjusted to a pH of 7.4.

Preliminary Testing

If appropriate, approximately 0.1 g of test substance will be added to a 10-mL glass stoppered graduated cylinder or other similar test vessel. Increasing amounts of Gamble's solution equilibrated to 37°C will be added. After each addition of the indicated amount of Gamble'solution is made, the mixture will be shaken vigorously, if feasible, for at least 10 minutes and will be visually inspected for any undissolved test substance. If, after the addition of 10 mL of Gamble's solution, the test substance or parts of it remain undissolved, the contents will be transferred to a larger test vessel, more Gamble's solution will be added, and the container shaken. Alternately, another aliquot of test substance may be weighed and diluted with 100 mL of Gamble's solution. The approximate solubility is listed in the following table under the amount of Gamble's solution required for complete dissolution of the test substance.

	Total Volume of Gamble's solution added (mL)						
	0.1	0.5	1	2	10	100	>100
Approximate Solubility (g/L)	>1000	1000 to 200	200 to 100	100 to 50	50 to 10	10 to 1	<1

At low biosolubilities, a period of at least 24 hours may be required to completely dissolve a test substance. If, after 24 hours, the test substance is still not dissolved, more time (up to 96 hours) can be allowed or further dilutions can be attempted.

A 24-hour shake flask test at 20°C may be utilized as an alternative to the physical dissolution method for the preliminary test.

Definitive Test Procedure

The target volume of Gamble's solution used will be 20-21 mL but may be changed depending on the analytical method and solubility range. If practical, the dose mass for the definitive test should be greater than five times the quantity of test substance required to achieve biosolubility. The test substance will be transferred to a minimum of five glass or Teflon® test vessels. The desired volume of Gamble's solution will be added to each vessel and the vessels sealed. The closed vessels will be agitated (shaken or stirred) at 45 ± 1.0°C in a water bath or temperature controlled incubator. The pH of unfortified Gamble's solution at 37°C will be measured. After 1 day, one of the vessels will be removed and re-equilibrated for 24 hours at 37 ± 1.0°C with occasional shaking or stirring. In order to minimize the possibility of Tyndall effect, a minimum of two aliquots of the vessel will then be centrifuged at an appropriate relative centrifugal force for approximately 10 minutes or longer, if necessary. The concentration of the test substance in the supernatant of these aqueous phase aliquots will be determined. After processing the replicate aliquots for concentration determination, one of the replicate supernatants will be selected for pH determination. Alternatively, an additional aliquot may be centrifuged in order to provide sufficient volume of saturated supernatant for pH measurement. Temperatures will be monitored using a min-max thermometer or with the use of a datalogger.

The remaining two (or more) vessels are treated similarly following initial equilibration periods of 2 and 3 days(and additional days for more vessels) at 30 ± 1.0°C. If the measured concentrations of the test substance in the aqueous phase of at least the last two vessels do not differ by more than 15%, the test is satisfactory. The percent difference is calculated as the difference between the mean measured concentration in two vessels divided by the overall mean measured concentration for the two vessels. If the measured concentrations in the three (or more) vessels show an increasing trend, as determined by more than 15% increase (difference) from the first to second vessels and from the second to third vessels (and likewise for additional vessels, if applicable), the test may be restarted with longer equilibration periods.

Analytical Method(s)

The analytical method(s) used for quantitation of the test substance in Gamble's solution will be based upon procedures provided by the Sponsor or developed at Eurofins. The analytical methodology employed will be documented in the raw data and summarized in the final report.

Data Analysis

The concentration of the test substance in each sample will be expressed in milligrams per liter (mg/L), micrograms per liter (µg/L), or other concentration units as appropriate. The average solubility, standard deviation and coefficient of variation will be calculated for at least five samples at each flow rate (when possible and applicable). The percent difference between the average solubility determined at each flow rate will be calculated (when possible and applicable).

The Percentage inhalation bioaccessible fraction (%IBAF) will be determined using the following calculation:

$$\%IBAF = (C_{ibio}/C_{total} \times 100)$$

Where C_{ibio} is the concentration of the test substance which is soluble in Gamble's solution and C_{total} is the total amount of material introduced to the test vessel.

Sample Handling and Safety

The Sponsor will identify any special handling or safety precautions to be used with the above referenced test substance. All normal precautions with respect to handling and storage will be taken.

Sample and Test Substance Retention

Upon completion of testing, portions of the test substance used as part of this study will be disposed of in accordance with federal, state and local regulations. Test substance and test substance containers remaining at the end of the study will be retained by the testing facility for use in other studies being conducted with this test substance and then will be returned to the Sponsor.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by the testing facility will include, but not be limited to:

1. The signed protocol and any amendments.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and completion of the study.
4. Dates of experimental start and termination.
5. Storage conditions of the test substance.
6. Test substance use log.
7. Concentration calculations and records of solution preparation, if applicable.
8. Instrument operating conditions and if applicable, chromatograms.
9. Statistical calculations.
10. Test conditions.
11. The final report.

FINAL REPORT

A final report of the results of the study will be prepared by the testing facility. A draft final report will be provided to the Sponsor's Representative for review prior to issuance of a final report. The report will include, but not be limited to the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Purpose and procedure, as stated in the approved protocol, including a copy of the study protocol and including all amendments and deviations to the protocol (if any).
5. Known test substance identification information, including name, chemical abstract number or code number, purity, composition, empirical formula, molecular formula, manufacturer's lot/batch number, and other information if provided by the Sponsor.
6. Description of the test method or reference to the method used along with any modifications made.
7. The test conditions including the amount of test substance introduced into the test vessels, volumes of Gamble's solution added to each vessel, the pH of the Gamble's solution, the pH of

the aqueous phase following equilibration, and the individual concentrations measured for each sample.

8. The mean and standard deviations of solubility determinations at each interval for test vessels with solubilities within 15% of each other.
9. Description of any problems experienced and how they were resolved.
10. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.

CHANGES TO FINAL REPORT

If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted and reported in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 792) (1989) which are compatible with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17). A statement of compliance, signed by the Study Director, will be included in the final report. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Each study conducted by the testing facility is routinely examined by the testing facility Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. Raw data for all work performed at the testing facility and the final report will be filed by study number in archives located on the Easton site or at an alternative location to be specified in the final report.

REFERENCES

1. **U.S. Environmental Protection Agency.** 1998. *Water Solubility: Column Elution Method; Shake Flask Method.* Product Properties Test Guidelines. OPPTS 830.7840.

2. **U.S. Environmental Protection Agency.** 1998. *Water Solubility (Generator Column Method).* Product Properties Test Guidelines. OPPTS 830.7860.

3. **Organisation for Economic Cooperation and Development.** 1995. Guideline for the Testing of Chemicals, OECD 105: *Water Solubility.*

4. **American Society for Testing and Materials.** 2006. Standard Specification for Reagent Water. D1193-06, ASTM Section II Water and Environmental Technology, Vol. 11.01: 45-47.

5. **ECETOC.** 2014. Technical Report 122. *Poorly Soluble Particles / Lung Overload.*

6. **Sánchez-Piñero, J.; Novo-Quiza, N.; Pernas-Castaño, C.; Moreda-Piñeiro, J.; Muniategui-Lorenzo, S.; López-Mahía, P.** Inhalation Bioaccessibility of Multi-Class Organic Pollutants Associated to Atmospheric PM2.5: Correlation with PM2.5 Properties and Health Risk Assessment. Environmental Pollution 2022, 307, 119577. https://doi.org/10.1016/j.envpol.2022.119577.

Study Number: 264K-105

Amendment to Study Protocol

Study Title: 6:2 FTSB: Determination of Biosolubility Using Simulated Epithelial Lung Fluid

Sponsor: American Chemistry Council

Amendment No.: 1

Effective Date: Date of study director signature

Amendment: Page 5

Definitive Test Procedure

Change: The remaining two (or more) vessels are treated similarly following initial equilibration periods of2 and 3 days (and additional days for more vessels) at 30 ± 1.0 °C.

To: The remaining two (or more) vessels are treated similarly following initial equilibration periods of2 and 3 days (and additional days for more vessels) at 37 ± 1.0 °C.

Reason: Typographical Error

Impact: No negative impact.

Change: After 1 day, one of the vessels will be removed and re-equilibrated for 24 hours at 37 ± 1.0 °C.

To: After 1 day, one of the vessels will be removed and re-equilibrated for approximately 24 hours at 37 ± 1.0 °C.

Reason: Typographical error.

Impact: No negative impact, times were recorded to ensure accuracy.

Amendment: Page 6

Data Analysis

Change: The Percentage inhalation bioaccessible fraction (%IBAF) will be determined using the following calculation:

To: The Percentage inhalation bioaccessible fraction (%IBAF) (5) (6) will be determined using the following calculation:

Reason: Cite appropriate references.

Study Number: 264K-105

Impact: No negative impact.

Amendment: Page 9

References

Remove: 4. **American Society for Testing and Materials.** 2006. Standard Specification for Reagent Water. D1193-06, ASTM Section II Water and Environmental Technology, Vol. 11.01: 45-47

Reason: Reagent water not applicable to this protocol.

Impact: No negative impact.

Study Director — Date: April 11, 2024

Laboratory Management — Date: April 11, 2024

QAU Review

VA / April 11, 2024

Initials/Date

STUDY NO.: 264K-105
Page 1 of 1

DEVIATION TO STUDY PROTOCOL

STUDY NUMBER: 264K-105

DEVIATION NUMBER: 1

DATE OF DE FACTO DEVIATION: 12/11/2023; 12/20/2023

DEVIATION: Protocol specifies that the Gamble's solution will be adjusted to a pH of 7.4 prior to use.

REASON: Native pH of the Gamble's Solution was 7.28. This was determined to be within the environmentally relevant range, and that preserving the composition of the Gamble's solution was of greater value than titrating to a specific pH.

IMPACT: None

DEVIATION: The 37 degree constant temperature water bath used for pH sample temperature equilibration had a recorded temperature of 34.48 °C when used for the SF-4 pH sample equilibration. When the sample was removed , the temperature was back within tolerance.

REASON: Fluctuation of water bath temperature with additions of fresh water to compensate for evaporation.

IMPACT: None

Lacey Bm
STUDY DIRECTOR

April 11, 2024
DATE

LABORATORY MANAGEMENT

April 11, 2024
DATE

QAU Review
TR / April 1, 2024
Initials/Date

Appendix 2

Certificates of Analysis

Chemours™

The Chemours Company
1007 Market Street
Wilmington, DE 19899 U.S.A.

CERTIFICATE OF ANALYSIS

This Certificate of Analysis (COA) fulfills the requirement for characterization of test substance prior to a study that is subject to GLP regulations. It documents the identity and content of the active and inactive constituents of the test substance. Where appropriate, analyses that were conducted under EPA TSCA Good Laboratory Practice Standards (40 CFR 792) are identified as such.

Identity of Test Substance

Test Substance Name: Dry Capstone™ 1157 Fire Fighting Foam Fluorosurfactant

Common Name: Dry Capstone™ 1157

Chemical Formula: Carboxymethyldimethyl-3- [[(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)sulphonyl]amino]propylammonium hydroxide

CAS #: 34455-29-3

Characterization of Specific Test Substance Sample

Identity / Code Number(s): Dry Capstone™ 1157

Batch or Lot Number: I0722

Purity: 97.7%

Composition and Concentration of Constituents (should sum to 100%):

Compound	CAS number	Concentration (%)
Carboxymethyldimethyl-3- [[(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)sulphonyl]amino]propylammonium hydroxide	34455-29-3	97.7%
Sodium chloride	7647-14-5	1.69
Water	7732-18-5	0.6
Ethanol	64-17-5	0.01

Expiration Date: February 2027

Physical Description of Sample (e.g., color): brown/yellow powder

Physical State (liquid, solid, gas): solid

Analytical method used to create COA

Description:

- ✓ Determination of Solid Content by evaporation of solvent in an oven : 3 x 1g of Dry Capstone™ 1157 in oven 105°C for 2 hours
- ✓ Determination of sodium chloride content by argentometric titration : 1g of Dry Capstone™ 1157 + 50ml demineralized water + 50ml ethanol + concentrated nitric acid, then measure by titration with silver nitrate 0.1N.
- ✓ Determination of water content by Karl-Fisher titration combined with Stromboli oven sample charger
- ✓ Determination of ethanol content with gas chromatography (internal standard)

Method conducted in accordance EPA TSCA Good Laboratory Practice Standards (40 CFR 792)?

YES☐ NO ☒

Authorship of COA

Name and signature of person conducting/preparing COA:

Claire DUQUESNOY

Date of COA creation: 14/03/2022

Appendix 3

Personnel Involved in the Study

The following key personnel were involved in the conduct or management of this study:

1. Lacey Brown, B.S.
2. Jon MacGregor, B.S.
3. Jasmine Charles, B.S.
4. Derek Oliver, B.S.
5. Ling Zhang, Ph.D.