Title:

Comparative Molecular Biology of Perfluorooctanesulfonamide (FOSA, T-7132) in Rats following four consecutive days of dosing.

Final Report
February 18, 2004

Study Number: DT15
Protocol Amendment Number*: 2.
3M Medical Department Study Number: T-7132.1
Study Director: Andrew Seacat Ph.D.

Analytical laboratories:

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   Rochester, New York 14642

Original Study Protocol Title: Comparative Molecular Biology of Peroxisome Proliferation in Rats and Guinea Pigs
Original Study Initiated:
In-Life Start Date: November 16, 1998
In-Life End Date: December 21, 1998

Purpose:

The purpose of this study was to treat three male rats treated with either no compound (vehicle control) or 40 mg/kg/day perfluorooctanesulfonamide: C8F17SO2NH2 (FOSA, PFOSA, FOSAmide, T-7132). The study was designed to evaluate the metabolism and certain toxicological effects, such as hepatic peroxisome proliferation, of this compound and to compare these effects to other compounds dosed at the same concentration..

Methods

Test Materials:

Perfluorooctanesulfonamide (FOSA, PFOSA, FOSAmide, C8F17SO2NH2 NB # 120067-108, G. Moore 10/28/00, T-7132) was investigated. The vehicle control was propylene glycol.

Dose Groups:

Three male rats received propylene glycol as the vehicle control by oral gavage at a volume of 5 ml/kg body weight.

Three male rats received a dose of 40 mg FOSA/Kg body weight, via oral gavage on days one through four of the study, and were euthanized on day five. A suspension of 8 mg/ml of FOSA in propylene glycol was prepared, and a volume of 5 ml/kg was administered. This dosing regiment achieved a cumulative dose of 160 mg/kg after four successive days of dosing. This dose was the same as the dose of PFOS administered under amendment 1 of this protocol and is comparable to effective dose levels for hepatic peroxisome proliferation in rats of PFOS found in the literature. The LD50 for FOSA is not known, but the cumulative dose of FOSA administered under this protocol is below the LD50 for PFOS 251 mg/kg for PFOS in corn oil, as a point of reference.

Method of Specimen Collection:

The liver was removed as rapidly as possible after euthanasia. The livers were divided into ~ 1 g pieces and flash-frozen directly in liquid nitrogen in tared polypropylene (Nalgene) containers. The containers were moved to dry ice and weighed after all liquid nitrogen had evaporated. The tissue was stored at -70° C and shipped on dry ice.

Sera:
Up to 10 ml of blood was collected from each animal into glass serum tubes. Following clotting, the blood was centrifuged for 10 minutes at 1100 x g at 4° C. the sera was transferred to new tubes and be centrifuged again for 10 minutes at 1100 x g at 4° C. Two aliquots of the serum sample (~ 0.75 ml) were saved for possible metabolite analysis.

Specimen Handling:
A 1-2 gram aliquot of the liver samples that were flash frozen in liquid nitrogen were shipped in dry ice to the analytical laboratories listed according to the livers sample identification chart below to:

Kendall B.Wallace, Ph.D. D.A.B.T.
Professor, Dept of Biochemistry and Molecular Biology
School of Medicine University of Minnesota
10 University Drive
Duluth, MN 55812-2496

Liver samples were analyzed by previously published methods for P450 content, Lauroyl CoA oxidase activity (Poosch and Yamazaki 1986) and protein content (Bradford 1976) in the laboratory of Ken Wallace Dept of Biochemistry and Molecular Biology at the University of MN.

Another 1-2 gram aliquot of the liver sample was sent to:

Dr. Michael Wempe
Laboratory of M. W. Anders
Department of Pharmacology and Physiology
601 Elmwood Ave, box 711
Rochester, New York 14642

Dr. Lin Xu performed the analysis of these samples and provided a brief summary of the analysis of liver samples from rats given a range of fluorocarbons. The parent and metabolites of the fluorocarbons were determined in liver samples by LC-MS/MS using previously published methods (Hansen *et al.* 2001).

Liver Sample Identification Chart for T-7132.1

Sample #	Animal #	Species	Sex	Dose Group
1	1R00742	Rat	M	control
2	1R00743	Rat	M	control
3	1R00744	Rat	M	control
4	1R00745	Rat	M	40 mg/kg/day FOSA
5	1R00746	Rat	M	40 mg/kg/day FOSA
6	1R00747	Rat	M	40 mg/kg/day FOSA

Results and Discussion

Average body weights of the FOSA treatment group were significantly lower than the control group on days four and five (Table 1). Liver weights were not significantly different between the treated and control groups, but the liver weight as a percentage of body weight was significantly increased in the FOSA treatment group

The results of P450 content (Figure 1) and Acyl CoA oxidase activity (Figure 2) for the liver samples indicated that FOSA induced the expression of these proteins and FOSA is therefore a hepatic peroxisome proliferator in rats.

The concentrations of the parent compound and metabolites in livers were measured (Table 2). The data show that PFOS was the major metabolite found in the livers of rats given FOSA and FOSA *N*-glucuronide was identified as a minor metabolite. Approximately 0.3 percent of the cumulative dose was present in the liver as either the parent compound, FOSA, or as the metabolite PFOS, one day after the last dose (Table 3). The low percentage of FOSA in the liver and the apparent low conversion of FOSA to PFOS has also been noted in in-vitro microsomal and liver slice metabolism studies (Xu *et al.* 2003) which observed that FOSA was converted to PFOS *in-vitro* by liver slices at a low rate, but not by microsomes or cytosol .

It is noteworthy that the control animals (1R00742, 1R00743, and 1R00744) contained significant concentrations of FOSA. Furthermore, no PFOS or FOSA *N*-glucuronide were found in the control samples with a high background of FOSA. A parallel analysis of livers from Fischer 344 rats maintained in the University of Rochester Vivarium did not show detectable concentrations of FOSA. These results suggest that the control liver samples for this study were contaminated at some point time ex-vivo by trace quantities of FOSA, as no metabolism to PFOS or FOSA *N*-glucuronide had occurred in the control samples.

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Signatures:

Report prepared by,

Andrew M. Seacat 2/21/07

Andrew M. Seacat, PhD, DABT — Date
Study Director

Reviewed by,

Daniel C. Hakes 2/19/07

Daniel C. Hakes — Date
Sponsor representative

References:

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.

Hansen, K. J., Clemen, L. A., Ellefson, M. E., and Johnson, H. O. (2001). Compound-specific, quantitative characterization of organic fluorochemicals in biological matrices. *Environmental Science and Technology* **35**, 766-770.

Poosch, M. S., and Yamazaki, R. K. (1986). Determination of peroxisomal fatty acyl-CoA oxidase activity using a lauroyl-CoA-based fluorometric assay. *Biochim Biophys Acta* **884**, 585-93.

Xu, L., Seacat, A. M., Butenhoff, J. L., and Anders, M. W. (2003). Biotransformation of N-Ethyl-N-(2-hydroxyethyl)perfluorooctanesulfonamide (N-EtFOSE) by rat liver microsomes, cytosol and slices. In Toxicological Sciences, Suppl., Vol. 72, p. 314.

Tables

Table 1 Biological Parameters

DT15, Amendment #2										
Comparative Molecular Biology of Perfluorooctanesulfonamide (FOSA, T-7132) in Rats following four consecutive days of dosing.										
DT15 Body Weight (BW), Liver Weight (LW), Thyroid Weight (TW)										
			Date							
			2/19/01	2/19/01	2/21/01	2/22/01	2/23/01	2/23/01		
Sample #	Animal #	Dose Group	BW day 1 (g)	BW day 2 (g)	BW day 3 (g)	BW day 4 (g)	BW day 5 (g)	LW (g)	LW/BW (%)	TW (g)
1	1R00742	control	250	259	260	264	272	10.73	3.9%	10.62
2	1R00743	control	269	278	285	292	295	10.82	3.7%	10.87
3	1R00744	control	256	260	267	268	276	10.70	3.9%	10.65
Avg			**258**	**266**	**271**	**275**	**281**	**10.75**	**3.8%**	**10.71**
SD			**10**	**11**	**13**	**15**	**12**	**0.06**	**0.14%**	**0.14**
4	1R00745	40 mg/kg/day FOSA	250	254	250	239	227	10.77	4.7%	10.78
5	1R00746	40 mg/kg/day FOSA	249	245	243	233	225	10.81	4.8%	10.66
6	1R00747	40 mg/kg/day FOSA	259	265	263	255	254	10.64	4.2%	10.84
Avg			**253**	**255**	**252**	**242**	**235**	**10.74**	**4.6%**	**10.76**
SD			**6**	**10**	**10**	**11**	**16**	**0.09**	**0.34%**	**0.09**
P-value T-Test*			**0.21**	**0.13**	**0.06**	**0.02**	**0.01**	**0.44**	**0.01**	**0.32**

*T-Test (unpaired, one tailed, equal variance). A P-value of ≤ 0.05 was considered significantly different from control.

Table 2 Liver Fluorocarbon Concentration

Hepatic Concentrations of Fluorocarbons and Fluorocarbon Metabolites in Livers of Rats Given FOSA (40 mg/kg/day)

Sample #	Animal #	Sex	Treatment	Weight of liver sample (g)	PFOS (ppm)	FOSA (ppm)	FOSAA (ppm)	FOSA *N*-glucuronide (ppm)
1	1R00742	M	Control	0.4600	0.0	154.1	0.0	0.00
2	1R00743	M	Control	0.9236	0.0	113.2	0.0	0.00
3	1R00744	M	Control	1.1287	0.0	273.2	0.0	0.00
4	1R00745	M	FOSA	0.2402	163.2	195.7	n.m.	0.44
5	1R00746	M	FOSA	0.7905	214.3	174.1	n.m.	0.39
6	1R00747	M	FOSA	0.8305	202.8	163.9	n.m.	0.34

Note: n.m. = not measured.

Table 3 Percent Fluorocarbon Dose in Liver

Hepatic Percent of Dosed Fluorocarbons and Fluorocarbon Metabolites in Livers of Rats Given FOSA (40 mg/kg/day) for Four Days

Percent Dose in Liver										
Sample #	Animal #	Dose Group	Total Dose (mg)	PFOS in dose**	Liver PFOS (ppm)	Liver PFOS (mg)	% dose as PFOS in liver (%)	Liver FOSA (ppm)	Liver FOSA (mg)	% dose as FOSA in liver (%)
1	1R00742	control	0.00		0			154.1		
2	1R00743	control	0.00		0			113.2		
3	1R00744	control	0.00		0			273.2		
Avg								**180.17**		
SD								**83.12**		
4	1R00745	FOSA	644.84	644.84	163.2	1.76	0.27	195.7	2.1	0.33
5	1R00746	FOSA	660.19	660.19	214.3	2.32	0.35	174.1	1.9	0.29
6	1R00747	FOSA	614.34	614.34	202.8	2.16	0.35	163.9	1.7	0.28
Avg					**193.43**	**2.08**	**0.32**	**177.90**	**1.91**	**0.30**
SD					**26.81**	**0.29**	**0.05**	**16.24**	**0.18**	**0.02**

Figures

Figure 1 Cytochrome P450 content in Liver

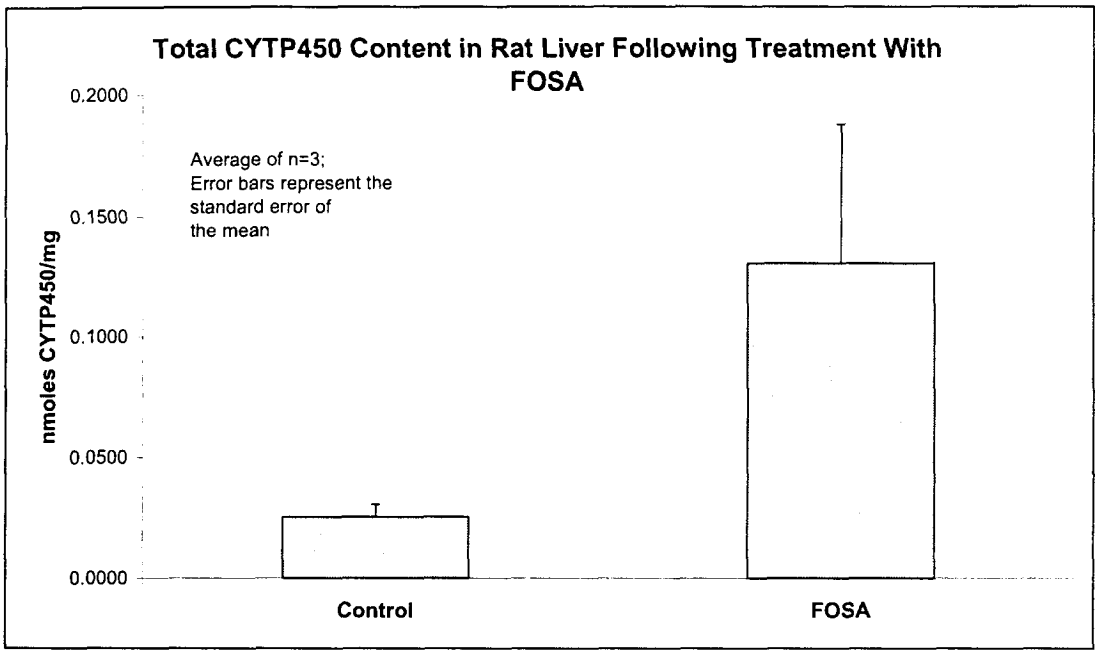
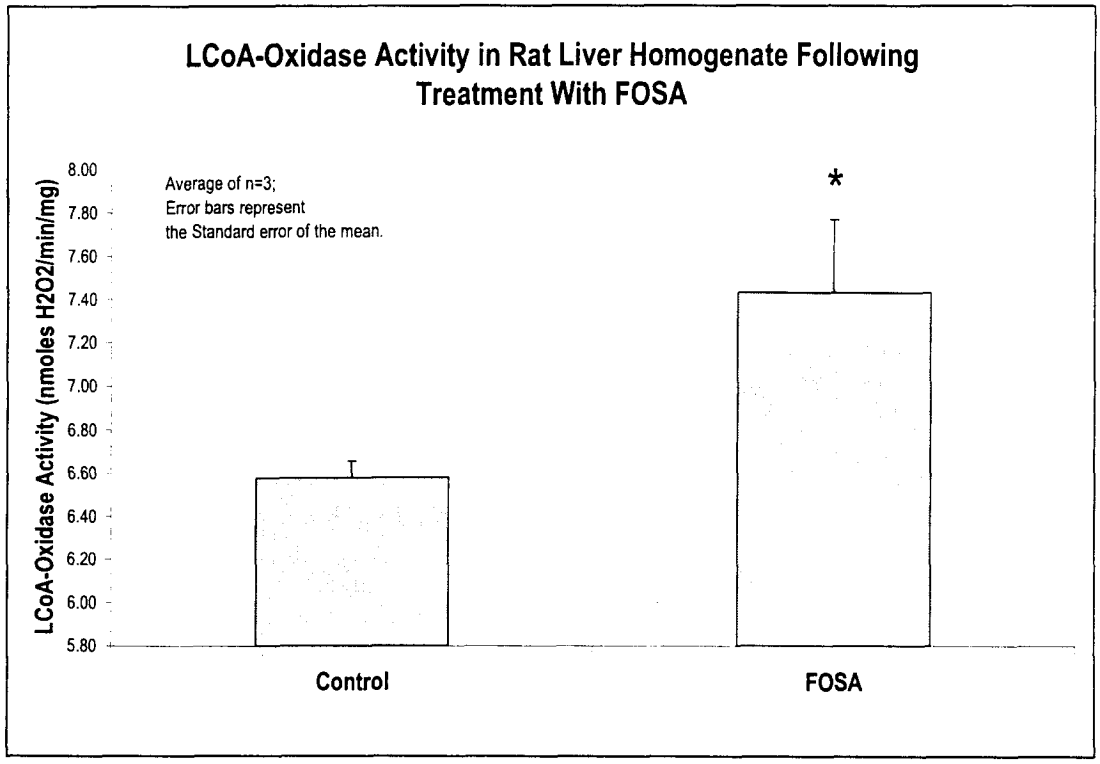


Figure 2: Lauroyl CoA oxidase activity in liver

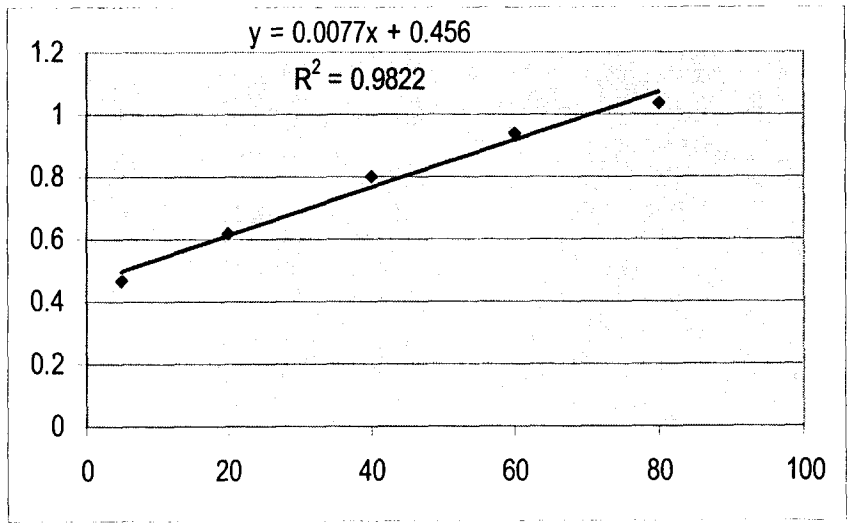


Appendix 1 Cytochrome P450 content

		Absorbance Values								
Sample #	Treatment	450	500	protein conc. (ug/ul)	mg protein added	450 - 500	uM CYTP450	volume (ml)	total nmole	nmoles CYTP450/ mg
1	Control	-0.0366	-0.0312	8.20	5	0.0054	0.059	3	0.178	0.0356
2	Control	0.02542	0.02209	8.79	5	0.00333	0.037	3	0.110	0.0220
3	Control	-0.0144	-0.0172	9.25	5	0.0028	0.031	3	0.092	0.0185
4	FOSA	-0.0404	-0.0456	9.79	5	0.0052	0.057	3	0.171	0.0343
5	FOSA	0.13601	0.10083	10.48	5	0.03518	0.387	3	1.160	0.2320
6	FOSA	0.00594	-0.013	9.86	5	0.01894	0.208	3	0.624	0.1249
					Statistics					
				Control	**FOSA**					
			n=1	0.0356	0.0343					
			n=2	0.0220	0.2320					
			n=3	0.0185	0.1249					
			Average	**0.0253**	**0.1304**					
			Stdev	0.0091	0.0989					
			Sterror	0.0052	0.0571					
			Varriance	0.0001	0.0098					
			ftest	1	0.02					
Ttest (unpaired, one tailed, unequal variance)			**p value**	0.50	0.10					

Protein Determination

	Standard Curve										
ul BSA	ul Lysis Buffer	Absorbance									
5	95	0.4645									
20	80	0.6167									
40	60	0.7992									
60	40	0.9413									
80	20	1.0379									



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sample	Treatment	ul	Absorbance	ug protein	mg/ml	average	slope	intercept				
1A	Control	5	0.7799	42.064935	8.412987	8.2	0.0077	0.456				
1B	Control	5	0.7635	39.935065	7.987013		0.0077	0.456				
2A	Control	5	0.8004	44.727273	8.945455	8.790909	0.0077	0.456				
2B	Control	5	0.7885	43.181818	8.636364		0.0077	0.456				
3A	Control	5	0.8155	46.688312	9.337662	9.248052	0.0077	0.456				
3B	Control	5	0.8086	45.792208	9.158442		0.0077	0.456				
4A	FOSA	5	0.844	50.38961	10.07792	9.788312	0.0077	0.456				
4B	FOSA	5	0.8217	47.493506	9.498701		0.0077	0.456		sample	mg/ml	ul needed to get 5 mg
5A	FOSA	5	0.8493	51.077922	10.21558	10.47792	0.0077	0.456		1	8.20	609.8
5B	FOSA	5	0.8695	53.701299	10.74026		0.0077	0.456		2	8.79	568.8
6A	FOSA	5	0.8305	48.636364	9.727273	9.855844	0.0077	0.456		3	9.25	540.7
6B	FOSA	5	0.8404	49.922078	9.984416		0.0077	0.456		4	9.79	510.8
										5	10.48	477.2
										6	9.86	507.3

Appendix 2: Lauroyl CoA Oxidase Activity

Sample #	**1**	**2**	**3**	**4**	**5**	**6**				
	206.3	205.4	209.8	216.8	227.4	218.6				
	207.5	205	216.1	218.5	229.4	218.7				
	209.6	206.9	210.3	216.5	228.9	219.5				
average	**207.8**	**205.8**	**212.1**	**217.3**	**228.6**	**218.9**				
stdev	1.7	1.0	3.5	1.1	1.0	0.5				
%dev	0.8%	0.5%	1.7%	0.5%	0.5%	0.2%				
	1	**2**	**3**	**4**	**5**	**6**				
	123	122.9	126.3	124.3	124.3	131.1				
	120.3	121.7	125.9	121.9	123	129.3				
	120.2	121.5	124.4	121.7	123.3	125.4				
average	**121.2**	**122.0**	**125.5**	**122.6**	**123.5**	**128.6**				
stdev	1.6	0.8	1.0	1.4	0.7	2.9				
%dev	1.3%	0.6%	0.8%	1.2%	0.6%	2.3%				
					Sample	**Control**	**Difference**		**slope**	**intercept**
				1	4.02	0.03	3.99		21.692	120.5
	nmoles H2O2 produced/ 30 min:			**2**	3.93	0.07	3.86		21.692	120.5
				3	4.22	0.23	3.99		21.692	120.5
				4	4.46	0.10	4.36		21.692	120.5
				5	4.98	0.14	4.84		21.692	120.5
				6	4.54	0.37	4.16		21.692	120.5

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nmoles H2O2 produced/min/mg protein:

		nmoles H2O2	time (min)	ug protein			average (n=3)
Control	1	3.99	30	20		6.66	**Control**
	2	3.86	30	20		6.43	6.6
	3	3.99	30	20		6.65	
FOSA	4	4.36	30	20		7.27	**FOSA**
	5	4.84	30	20		8.07	7.4
	6	4.16	30	20		6.94	

	Control	**FOSA**
	(nmoles H2O2 produced/min/mg protein)	
n=1	6.66	7.27
n=2	6.43	8.07
n=3	6.65	6.94
Average	**6.58**	**7.43**
Stdev	0.13	0.58
Sterror	0.07	0.34

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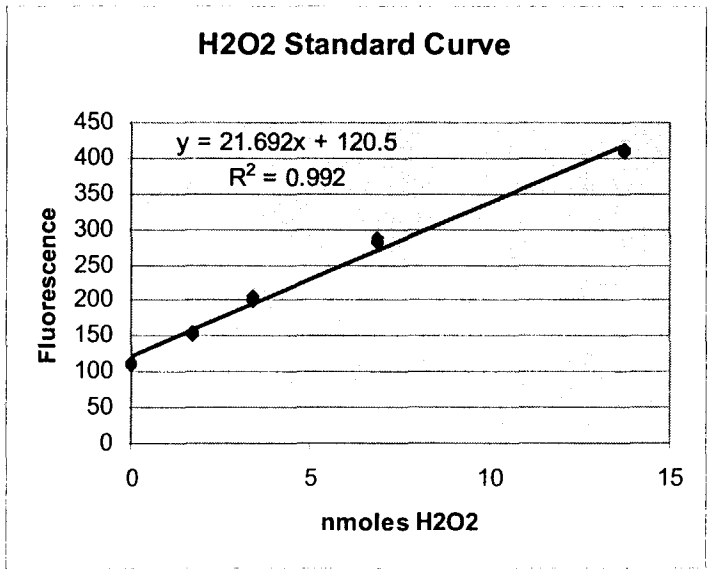
		H_2O_2 Standard Curve Data. H_2O_2 Stock was 8.6 M					
		nmnoles H2O2	**0**	**1.72**	**3.44**	**6.88**	**13.76**
			109.7	151.5	202.4	280.7	412.9
			112	153.2	206.3	288.6	410.7
			113.8	154.9	199.3	282.2	408.3
		average	111.8	153.2	202.7	283.8	410.6
		SD	2.1	1.7	3.5	4.2	2.3
		% dev	1.8%	1.1%	1.7%	1.5%	0.6%



	Protein Standard Curve									
ul BSA	ul Lysis Buffer	Absorbance								
5	95	0.4464								
20	80	0.6769								
40	60	0.854								
60	40	1.0135								
80	20	1.037								
sample	ul	Absorbance	ug protein	ug/ul	average	slope	intercept			
1	5	0.7814	34.435644	6.887129	7.054455	0.0101	0.4336			
1	5	0.7983	36.108911	7.221782		0.0101	0.4336	sample	ug/ul protein	ul needed to get 20 ug
2	5	0.7956	35.841584	7.168317	7.617822	0.0101	0.4336	1	7.05	2.84
2	5	0.841	40.336634	8.067327		0.0101	0.4336	2	7.62	2.63
3	5	0.7963	35.910891	7.182178	7.369307	0.0101	0.4336	3	7.37	2.71
3	5	0.8152	37.782178	7.556436		0.0101	0.4336	4	7.88	2.54
4	5	0.8225	38.50495	7.70099	7.882178	0.0101	0.4336	5	8.38	2.39
4	5	0.8408	40.316832	8.063366		0.0101	0.4336	6	8.61	2.32
5	5	0.8592	42.138614	8.427723	8.379208	0.0101	0.4336			
5	5	0.8543	41.653465	8.330693		0.0101	0.4336			
6	5	0.8501	41.237624	8.247525	8.610891	0.0101	0.4336			
6	5	0.8868	44.871287	8.974257		0.0101	0.4336			