Report Title

Evaluation of the Northwest Bioanalytical Method Validation Reports for PFOS and Related Analytes in Human Serum or Plasma in Meeting the Requirements of the EDA Bioanalytical Method Validation

QAI Report No. 405-503

Data Requirement
Full Validation of Bioanalytical Method

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I. SUMMARY

The validation in human serum or plasma was performed for the analytes PFOS, PFOSA, PFOSAA, POAA, PFHS, M-556, and M570. The surrogate standard IH,IH,2H,2H-perfluorooctanesulphonic acid was added before extraction, but used as an internal standard in the quantitation.

The following parameters can be considered as completely meeting the FDA requirements:

- Specificity
- Calibration in serum and plasma
- Accuracy in serum and plasma.
- Precision in serum and plasma
- Validation of Extract Dilution
- Serum sample stability to:
    - freeze-thaw. 7 cycles
    - short-term ambient storage, 6.75 hr x 7 cycles
    - freezer storage, 42 – 55 days
- Serum extract stability to:
    - ambient storage, 7 days
    - refrigerated storage, 4 days
    - frozen storage, 5 days

The following restrictions apply to these analyses:

- Because of poor analyte and internal standard extraction efficiencies, it is essential that samples, QCs and calibrations standards in a set be kept together for all analyses. Extraction efficiency within a set is consistent;
- Samples with levels of POAA above the ULOQ will show LC/MS ionization suppression of the internal standard THPFOS, resulting in a high quantitation bias on other analytes. A resolution was not demonstrated.
- For diluting samples with serum, the calibration curve must be prepared at the same dilution of serum as the diluted sample for accuracy. If the sample is at a different matrix dilution, the results will not be correct.
- When a sample must be diluted, it not only must be compared to a calibration curve prepared at the same dilution of serum, but it also must be re-extracted for that analysis, in order to keep samples, QCs, and calibration standards together.

The following parameters from the FDA requirements were not tested:

- Stock solution stability.
- Stability of the surrogate standard.

Quantitating serum samples against plasma calibration does not meet the FDA requirements for accuracy.

II. INTRODUCTION

Three reports were reviewed:

- NWBR00-122: Quantitative Determination of PFOS, PFOSA, PFOSAA, POAA, PFHS, M-556 and M570 in Human Serum by LC/MS/MS: Assay Revalidation Addendum Report, 11/20/01 (study NWBS00-040)
- NWBR00-108: Quantitative Determination of PFOS, PFOSA, PFOSAA, POAA, PFHS, M-556 and M570 in Human Serum by LC/MS/MS: Assay Revalidation Report, 1/24/01 (study NWBS00-040);
- NWBR99-005: Quantitative Determination of PFOS, PFOSA, PFOSAA, POAA, PFHS, M-556 and M570 in Human Serum by LC/MS/MS: Assay Validation Report, 1/24/01 (study NWBS98-082);

The methods used in these studies are very similar with the following differences:

	#005	#108/122
internal standard (IS)	SDS	THPFOS
partition	basified with 0.5 M tertbutylammonium hydrogen sulfate (pH 10) + 0.25 M carbonate buffer	unadjusted pH ca. 6.9
final solvent	2mM ammonium acetate: methanol (1:1)	20mM ammonium acetate in water : 20 mM ammonium acetate in MeOH (3:7)
quantitation	quadratic regression, weighted 1/x, using area ratio vs. concentration	quadratic regression, weighted $1/x^2$, using area ratio vs. concentration

This review examines which of the FDA-required tests were conducted, and which meet the standards described by FDA for a full validation of the method. This report did not consider whether additional requirements, specified by protocol, were met if they did not affect the evaluation based on the FDA guideline; however, they may be discussed in the text of this report.

III. SPECIFICITY IN SERUM AND PLASMA

This requirement is met. Endogenous levels were found in all serum and plasma samples tested, although much greater in serum.

FDA Guideline	NWB Report
≥ Six sources of blank biological matrix Measure levels of each analyte	**Requirement met.** Testing was reported on 2 lots of serum and 4 lots of plasma.

Results:

In report #005, endogenous levels of PFOS, PFOSA, PFOSAA, POAA and PFHS in human serum were determined using rabbit serum calibration; PFOSA was not detected.

	Mean ng/mL in Human Serum			
Source: report #005 (tables 5 – 6)	PFOS	PFOSAA	POAA	PFHS
Run 25 calibration standards (n=4)	16.2	1.46	3.20	0.615
Run 26/28/29 calibration standards (n=12)	30.4	1.69	4.27	1.61
Run 26/28/29 QC samples (n=12)	22.3	1.99	1.97	3.25
The LLOQ in rabbit serum for each analyte was 1.00 ng/mL.				

In report #108, serum results indicated significant endogenous levels of all analytes except PFOSA. These levels are higher than in report #005.

	Mean ng/mL in Human Serum						
Source: report #108 tables 8 - 14 & report #122 tables 8 - 14	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
endogenous levels	47.1	ND	5.0	4.76	2.15	3.3	4.6
target LLOQ	1.0	1.0	1.0	1.0	1.0	1.0	1.0
calibration LLOQ*	48.1	1.0	6.0	5.76	3.15	4.3	5.6
Calibration was with human plasma							

Pooled plasma also showed significant endogenous PFOS, PFOSAA, POAA and PFHS, but at least 5x less than in serum. The levels were determined by standards prepared in plasma at 1 – 100 ng/mL. The endogenous level is defined as the mean y-intercept of linear regressions performed on 2 separate tests.

	Mean ng/mL in Human Plasma						
Source: report# 122 tables 78 - 84	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
endogenous levels	2.94	ND	0.60	0.92	0.36	ND	ND
target LLOQ	1.00	1.00	1.00	1.00	1.00	2.50	1.00
calibration LLOQ*	3.94	1.00	1.60	1.92	1.36	2.50	1.00
Calibration was with human plasma.							

IV. CALIBRATION IN SERUM AND PLASMA

Requirement met. There is however no justification for the selection of the complicated calibration procedure (quadratic with $1/x^2$ or $1/x$ weighting).

FDA Guideline	NWB Report
Blank + 6 – 8 samples	**Requirement met.** 2 blanks and 2 blanks + IS were included in each run. No blank analyses were reported. Samples: 8 Levels for M-556; 9 levels for all others. Duplicate samples. 3 runs for serum and 2 for plasma.
LLOQ ≥ 5x blank response	**Requirement met.** Endogenous responses caused LLOQ approximately = "blank" in most cases. Summarized in table below.
(Accuracy)	**Requirement met.**
± 20% of actual @ LLOQ	All pass, except one serum POAA.
± 15% of actual >LLOQ	Passed, of 550 total: 550 for PFHS 549 for PFOS 548 for M-556, M557 547 for PFOSA, PFOSAA, POAA
≥ 4 of 6 standards meet these criteria in each curve	All runs pass.

LLOQ: An experimental determination of the analytical limits was not performed. The lowest standard injected which met the acceptance criteria was chosen as the LLOQ. In most cases, these standard concentrations were close to the endogenous levels. Since the calibration LLOQ is only slightly (1 - 4 times) higher than the endogenous (blank) level the uncertainty of sample measurements at the LLOQ will be increased by the uncertainty of measurement of the endogenous level.

Because the endogenous levels are lower in plasma than in serum, plasma blanks should be used to prepare calibration standards when an LLOQ below 5.0 ng/mL is needed.

Range:

VALIDATED RANGE (ng/mL)

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Serum (reports #108/122)							
Serum LLOQ	48	1.0	6.0	5.8	3.2	4.3	5.6
Serum ULOQ	540	500	500	500	500	500	500
Plasma (report #122)							
Plasma LLOQ	3.9	1.0	1.6	1.9	1.4	2.5	1.0
Plasma ULOQ	410	500	500	480	520	470	480

Calibration method:
Quadratic regression with $1/x^2$ weighting, of peak area ratio (sample / surrogate standard) vs. nominal concentration. (1/x weighting in report #005). There is no justification for the selection of this complicated calibration method.

In all sets, there were at least four out of six calibration standard levels that met the accuracy requirement of ±15% of actual (or ±20% of actual at the LLOQ).

V. ACCURACY IN SERUM ANALYSIS

The FDA requirements for this test were met.

FDA Guideline	NWB Report
5 replicates @ 3 concentrations in the expected range of concentrations. Levels: ≤ 3x LLOQ; at the midlevel ; near the upper boundary of the standard curve	5 replicates, each on 3 separate days Levels: LLOQ (4.0 - 51.1 ng/mL; 1.1 – 4x) midlevel (150-200 ng/mL) high (400-450 ng/mL)
(Accuracy)	**Requirement met**
Mean values ± 20% of actual @ LLOQ	All passed except PFOSAA (intraday)
Mean values ± 15% of actual >LLOQ	All passed.

Results:

Summary tables presented below, with failed results **bolded**.

In the initial test of 3 sets, PFOSA and PFOSAA failed because the fortifications were faulty. The fortifying solution was prepared from dilute stocks by evaporation, and these two analytes were partially lost. The intra-day data below was obtained from set 17 containing just these 2 analytes, added in concentrated solutions. Accuracy in this set was unacceptable at the low level for PFOSAA, and at the high level for PFOSA.

Interday QC data was obtained from a monitoring study analyzed in the weeks following this fourth set. A total of fifteen sets are reported for PFOSA and PFOSAA, which not only validate the interday accuracy requirement, but also validate the intra-day accuracy requirement (5 out of 90 results outside the limits for PFOSA, and 7 out of 90 for PFOSAA).

% Accuracy from Fortified Blank Serum Samples

Source: report #108 (tables 15-30 and App. A)

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Levels, ng/mL							
Low	51.1	4.00	9.00	8.74	6.15	5.80	8.60
Mid	197	150	155	154	152	152	155
High	446	400	405	403	402	402	405
Low level							
Run 13	92	92	73	103	111	108	97
Run 15	92	–	–	93	101	97	97
Run 16	91	–	–	90	104	105	104
Interday	92	101	108	95	105	103	99
Mid-level							
Run 13	92	99	108	102	110	108	104
Run 15	88	–	–	94	104	97	95
Run 16	85	–	–	93	100	97	94
Interday	88	99	100	96	105	101	98
High level							
Run 13	89	82	92	101	110	103	98
Run 15	88	–	–	95	111	98	99
Run 16	85	–	–	98	105	99	96
Interday	87	98	106	98	108	100	97

PFOSA / PFOSAA intra-day results are from run 17; interday results are from study NWBS00-062. They include 15 runs of 3 concentrations and 2 replicates each.

VI. ACCURACY IN PLASMA ANALYSIS

Requirement met.

FDA Guideline	NWB Report
5 replicates @ 3 concentrations in the expected range of concentrations: ≤ 3x LLOQ; at the midlevel ; near the upper boundary of the standard curve	5 replicates @ 4 concentrations levels: LLOQ low (4.0 – 6.4 ng/mL) midlevel (126 - 150 ng/mL) high (332 - 441 ng/mL)
(Accuracy)	**Requirement met**
Mean values ± 20% of actual @ LLOQ	All passed.
Mean values ± 15% of actual >LLOQ	All passed, except PFOSA and M570 at the low level.

Results:

Summary tables presented below, with failed results **bolded**. While the low level for PFOSA and M570 failed, at 116% and 118% respectively, three levels passed for all analytes. All results pass.

% Accuracy from Fortified Blank Plasma Samples

Source: report #122 (tables 85-98)

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Levels, ng/mL							
LLOQ	4.0	1.0	1.6	1.9	1.4	2.5	1.0
Low	6.4	4.0	4.6	4.8	4.5	4.0	4.0
Mid	126	150	151	145	156	150	150
High	332	400	401	385	417	441	400
LLOQ	98	101	98	91	95	98	103
Low level	112	**116**	112	104	106	106	**118**
Mid-level	106	107	103	106	101	99	107
High level	101	104	104	103	96	111	101

VII. ABSOLUTE RECOVERY IN SERUM

Requirement met with restrictions: the design of the test was different than the guideline. The QC's and calibrations in a set must be kept together for all analyses. Extraction efficiencies are poor, but are consistent within a set.

FDA Guideline	NWB Report
3 concentrations – low, medium, high – in biological matrix	2 levels (4 & 400 ng/mL) in serum; 4 replicates, in 1 set.
Compare to unextracted standards	Compared with blank extracts fortified with known concentrations.
Recovery should be consistent, precise and reproducible (limits not specified). Accuracy not required.	Extraction efficiencies are poor (15 – 70%) for analytes, worse (6%) for internal standard, but consistent within a set.

Design: Analytes and internal standards were added either to serum or to serum extract to determine losses during extraction. The loss calculations were based on comparing the peak area ratio of (the one added before extraction : the one added after extraction) to the comparable peak area ratio when both were added after extraction. The endogenous levels were included in the calculated efficiencies (levels not reported).

Results: All analytes showed losses during extraction. These were similar at low and high levels. PFOS showed considerably higher loss at the low level, but this is probably an artifact of measurement, with an endogenous level on the order of 4x the added 4 ng/mL.

Extraction losses are not a concern for this method, so long as they are consistent for samples within an extraction set. Since samples are extracted along with the calibration standards, as long as they are kept together in analysis, the relative quantitation will be unaffected. Losses in the internal standard are a greater concern, but, at least within a set, they are consistent. There is no inter-set data.

(Source: Report #122 Tables 64-70) **% MEAN EXTRACTION EFFICIENCY***

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Analyte							
low	44	69	63	17	16	38	54
high	30	70	63	14	14	40	60
overall	37	70	63	16	15	39	57
Internal Standard, THPFOS							
overall (mean 6.0%)	5.9	5.8	6.3	6.1	6.3	5.5	6.1

VIII. ACCURACY AND POAA EFFECTS ON I.S. QUANTITATION

Restriction on Accuracy:

Samples with levels of POAA above the ULOQ will show a high quantitation bias for other analytes, due to LC/MS coelution and ionization suppression of the internal standard THPFOS. The scope of this effect is presented, but not completely proven. This may affect results obtained by dilution and reanalysis.

FDA Guideline	NWB Report
None	High concentration of POAA >500 ppb depresses IS response, biasing other analytes to the high side. However, the test has too many unknowns to allow many conclusions to be drawn.

Design: The internal standard THPFOS and the analyte POAA are incompletely resolved in LC/MS. Thus, POAA at high levels in samples suppresses the THPFOS response. This causes a high bias in analyte quantitation by peak area ratio. Low level QC samples were fortified with high levels of POAA, and the analytes quantified and compared to quantitation before fortification with POAA..

Results:

The data show that when fortified to approximately the ULOQ level, the change in quantitation is slight (-2 to -11%). However, at 10 - 100x this level, the bias steadily increases, in proportion to the decrease in IS peak area.

(Source: Report #122 p. 26)

POAA Added, approx. ng/mL	% Change from QC Mean: IS Area	Analytes Found
592	+11	-2 to –11
5920	-32	+16 to +36
11800	-43	+28 to +56
59200	-75	+152 to +216

QC conc.	PFOS	PFOSA	PFOSAA	PFHS	M-556	M570
ng/mL	51.8	4.05	6.83	8.04	5.41	6.87

IX. ACCURACY OF SERUM QUANTITATION VS PLASMA CURVE

Does not meet FDA guideline for accuracy.

FDA Guideline	NWB Report
None	Serum and plasma calibration standards. Serum and plasma QC's at 3 levels, 3-4 replicates, 2 sets of each matrix Serum QC's analyzed with both calibrations

Serum QC samples were analyzed against plasma calibration, because plasma has lower endogenous levels of some analytes than does serum. The results were reported as % deviation from theoretical concentration.

Results:

This method of quantitating serum samples against plasma calibration does not meet the FDA requirements for accuracy. Most of the PFOSA, PFOSAA, POAA and PFHS results are > ±15% of theoretical.

(Source: Report 122, Tables 141) **SERUM QC *vs.* PLASMA CURVE**

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Accuracy, % Deviation from theoretical							
low	11	**57**	14	14	**26**	-19	9
mid	12	**40**	**31**	**20**	**29**	-7	15
high	6	**32**	**19**	**24**	**23**	-11	9
Precision, % CV							
low	6	14	12	10	10	10	14
mid	7	3	3	5	4	4	9
high	6	6	5	3	6	2	8
# of runs	2	1	1	2	1	1	2
Levels, ng/mL							
low	39.3	4.00	7.20	9.54	6.41	5.30	6.70
mid	161	150	153	143	132	151	153
high	370	400	403	370	345	401	403

All calibration sets passed the requirements, although occasional replicates failed. Accuracy and precision of plasma and serum QC's against the same matrix calibration met requirements, with the exception that PFOSAA / plasma precision at the low level was 16% CV and accuracy at the mid level was 17%, and POAA / serum accuracy was 16% at the low level. The low levels were above the LLOQ.

X. VALIDATION OF SERUM EXTRACT DILUTION PROCEDURE

This requirement was met, with restriction:
The calibration curve must be prepared at the same dilution of serum as the diluted sample for accuracy. If the sample is at a different matrix dilution, the results will not be correct.

FDA Guideline	NWB Report
"The ability to dilute samples originally above the upper limit of the standard curve should be demonstrated by accuracy and precision parameters in the validation." "Any required sample dilutions should use like matrix"	**Requirement met, but the method must contain a restriction on dilution analysis.** 3 levels; 4 replicates; 1 set mid; high; 10x high

Design: Serum QC samples were diluted 1:10 or 1:50 with ammonium acetate buffer, and analyzed against a calibration curve prepared in 10% serum in buffer. It is known that quantitating against a whole serum curve is not accurate.

Results:

The data show that accuracy requires that the proportion of matrix in the sample and calibration curve extracts must be similar. All 1:10 dilutions had acceptable (> ±15%) accuracy, but the 1:50 dilution was biased unacceptably low (57 – 85%). Intra-assay precision was excellent (<7% CV) in all measurements.

(Source: Report 122, Tables 113-119)

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Precision (CV)							
mid	4	4	1	3	2	4	2
high	5	6	4	3	4	4	4
10x high	2	2	2	1	2	2	2
10x high (1:50)	2	4	4	4	3	4	4
Accuracy % recovery							
mid	108	96	94	106	105	101	103
high	103	96	99	100	102	102	103
10x high	107	99	100	105	107	114	108
10x high (1:50)	**77**	**57**	**66**	85	**84**	**70**	**69**
Levels, ng/mL							
mid	193	150	153	157	154	151	157
high	443	400	403	407	404	401	407
10x high	4040	4000	4000	4010	4000	4000	4010

XI. PRECISION IN SERUM ANALYSES

The FDA requirements were met.

FDA Guideline	NWB Report
(Precision – intrabatch / interbatch)	**Requirement met**
CV ± 20% @ LLOQ	All passed, except 1 of 4 PFOSAA sets at 1.5x LLOQ, with 24 %CV.
CV ± 15% @ >LLOQ	All passed, except 1 of 4 PFOSA sets at 4x LLOQ, with 19.8 %CV.

Results:

Summary tables presented below. All analytes passed at all concentrations, with the exception of PFOSA and PFOSAA in one set each at the low level.

In the initial test of 3 sets, PFOSA and PFOSAA had low recoveries because the fortifications were faulty; a set 4 contains just these 2 analytes. Both analytes have at least three sets at each levels meeting the intra-day requirements.

Interday QC data was also obtained from a monitoring study analyzed later.

Precision (% CV) from Fortified Blank Serum Samples

Source: report #108 (tables 15-30 and app. A)

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Levels, ng/mL							
Low	51.1	4.00	9.00	8.74	6.15	5.80	8.60
Mid	197	150	155	154	152	152	155
High	446	400	405	403	402	402	405
Low level							
Set 13	4	5	6	10	4	5	4
Set 15	2	2	2	6	3	4	7
Set 16	4	**20**	11	9	5	5	9
Set 17		6	**24**				
Interday1	3	14	8	6	6	6	7
Interday2		11	10				
Mid-level							
Set 13	4	5	3	2	4	3	5
Set 15	2	5	5	5	4	4	4
Set 16	3	4	4	4	1	4	4
Set 17		9	9				
Interday1	4	12	6	6	5	6	6
Interday2		8	8				
High level							
Set 13	5	4	8	5	4	7	5
Set 15	3	6	4	3	3	3	3
Set 16	3	3	4	3	3	4	4
Set 17		2	5				
Interday1	4	7	6	4	4	5	4
Interday2		10	7				

Interday1 is the mean of sets 13,15, and 16.

Interday2 PFOSA / PFOSAA data are from study NWBS00-062, a monitoring study. This represents QC sample data from 15 runs of 3 concentrations and 2 replicates each.

XII. PRECISION IN PLASMA ANALYSES

The FDA requirements for this test were met.

FDA Guideline	NWB Report
(Precision – intrabatch / interbatch)	**Requirement met.**
CV ± 20% @ LLOQ	All passed.
CV ± 15% @ >LLOQ	All passed.

Results:

Summary tables presented below. All analytes passed at all concentrations,

Precision (% CV) from Fortified Blank Plasma Samples

Source: report #122 (tables 85 - 98)

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Levels, ng/mL							
Low	6.40	4.00	4.60	4.81	4.48	4.00	4.00
Mid	126	150	151	145	156	150	150
High	332	400	401	385	417	441	400
LLOQ	5	10	6	4	10	6	5
Low level	3	2	5	6	3	8	2
Mid-level	3	6	6	5	3	5	6
High level	2	2	3	1	2	7	2

XIII. SERUM SAMPLE STABILITY TO FREEZE-AND-THAW

All requirements were met. In serum, all analytes were stable to seven freeze / thaw cycles, except PFOSAA which was stable to three cycles. Accuracy and precision were generally acceptable.

FDA Guideline	NWB Report
Two concentrations in triplicate	**Requirement met.** 3 concentrations in serum, approx. 1x, 4-40x and 10-100x LLOQ
Analyte stability should be determined after three freeze and thaw cycles.	**Requirement met.** Analyzed after three and seven cycles.

Results: Serum samples fortified at 1x, 4x and 10x LLOQ were frozen and thawed seven times before analysis. In each thawing, the samples remained at room temperature up to 6.75 hours. Losses are measured by comparing the sample analysis results to the corresponding control analysis.

All analytes except PFOSA were stable to these conditions. PFOSA data indicate losses of 11 – 20%, considerably exceeding the precision of the sample measurement.

PFOSA was stable to 3 freeze-thaw cycles in report #005. In that report, PFOSA at approximately 1x and 20x LLOQ showed stability, as did all the other analytes.

Precision data for sample analyses are good. Accuracy of controls analyses is within guidelines except for PFOS high level , which is –15.2%.

(Source: report #122 Tables 15 - 28) **AFTER 7 FREEZE-THAW CYCLES**

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
% Deviation from Control							
low	-3	**-18**	-5	7	-6	-7	4
medium	-3	**-20**	-15	1	-1	-5	-5
high	-2	-11	-5	0.0	0.7	0.3	-5
Sample, mean ng/mL							
low	45.3	3.58	9.46	8.38	6.48	5.04	8.32
medium	164	120	136	151	162	134	142
high	369	353	392	372	406	368	366

(Source: report #005 Table 51) **PFOSA AFTER 3 FREEZE-THAW CYCLES**

PFOSA	low	high
% Deviation from Control	4	-2
Sample, mean ng/mL	21.1	369

XIV. SHORT-TERM AMBIENT STABILITY OF SERUM SAMPLES

Requirements were met. Serum samples were kept at room temperature up to 6.75 hours per cycle during seven recurring freeze-thaw cycles (discussed above). All were stable except PFOSAA, which exhibited 10 – 20% losses.

FDA Guideline	NWB Report
3 aliquots of biological matrix fortified at low and high concentrations	**Requirement met.** 3 concentrations – approx. 1x, 4-40x and 10-100x LLOQ
Store fortified samples at room temperature 4 – 24 hours.	**Requirement met.** Fortified serum samples were frozen and thawed seven times. The maximum thaw time in a cycle was 6.75 hours; the total time at room temperature is not reported.

Results: Presented in the section above.

XV. LONG-TERM FREEZER STABILITY OF SERUM SAMPLES

All requirements were met. The results demonstrate that there is no degradation over the 55-day storage period (42 days for two analytes) of extracts at freezer temperature -20° C.

FDA Guideline	NWB Report
At least 3 aliquots of biological matrix fortified at low and high concentrations	**Requirement met.** 5 replicates of serum at approximately 1x, 4x and 10x LLOQ.
Store fortified samples under the same conditions as the study samples, for a time exceeding the time between the date of first sample collection and the date of last sample analysis.	**Requirement met.** Stored at -20° C for: 42 days PFOSA and PFOSAA 55 days other analytes Stability was determined by comparison to theoretical concentration.

Results:

All analytes are stable under these storage conditions, within the accepted limits of precision of the measurements. Precision of all measurements are <11%.

(Source: report #122 Tables 57-63) **SAMPLES AFTER 42 OR 55 DAYS AT -20° C**

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
Sample, % Change from theoretical							
low	-9	8	11	-10	12	-7	-7
medium	-14	0.0	3	-3	8	-7	-4
high	-15	-1	2	-8	0.2	-9	-5
Sample, mean ng/mL							
low	46.7	4.34	9.95	7.82	6.86	5.42	8.02
medium	170	150	160	149	164	141	149
high	378	395	413	372	403	367	386

XVI. POST-PREPARATIVE AMBIENT STABILITY OF SERUM EXTRACTS

The requirements were met for all analytes. The results demonstrate that there is no degradation over the 7 day storage period of extracts at room temperature, covering at least the resident-time in the autosampler.

FDA Guideline	NWB Report
No specific requirement	**Requirement met.** Serum extracts used; approx. 1x, 4-40x and 10-100x LLOQ
The stability of the drug and the internal standard should be assessed over the anticipated run time	**Requirement met.** Serum extracts stored 3 or 7 days at room temperature. Stability was measured by comparison to previously analyzed or freshly prepared QC samples.

Results:

Two tests were conducted at different times. In the first, "standards and controls" were extracted and analyzed, then reinjected on the 4th day after extraction. Three QC levels were included. In the second, low and high QC sample extracts were stored for 7 days, then analyzed against freshly extracted QC samples.

All analytes are stable under these conditions. Recoveries were all in the range of 100% ±11. While there is no separate data for the surrogate standard, the results presented at a minimum demonstrate that, at worst, all analytes and the surrogate degrade at the same rate, if any.

Sample precision is good, <10% CV in all cases. Control precision is good in the first test, but was not reported for the second. Control accuracy is good, except for PFOSA and PFOSAA. In the first test, control values were <85% of theoretical due to a fortification error. However, the stability test is not affected by this if both were fortified with the same solution. In the second test, accuracy could not be determined because the fortification level was not reported.

(Source: report #122 Tables 29-35) **EXTRACTS STORED 3 DAYS, AMBIENT**

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
% Deviation from Control							
low	1	-3	2	4	0.3	-2	-1
mid	-3	-3	-3	4	-0.6	-1	-0.7
high	-3	-5	-4	4	-4	-4	-3
Sample, mean ng/Ml							
low	47.7	2.73	7.73	3.1	6.24	5.52	8.2
mid	168	100	110	1.6	157	145	146
high	382	286	288	4.1	427	381	390

(Source: report #122 Tables 36 - 42) **EXTRACTS STORED 7 DAYS, AMBIENT**

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
% Deviation from Control							
low	3	8	11	-2	-10	0.2	11
high	3	7	12	2	0.2	3	9
Sample, mean ng/mL							
low	48.0	3.37	8.89	7.68	6.14	5.43	8.91
high	391	334	334	378	404	379	420

XVII. POST-PREPARATIVE 1-8°C STABILITY OF SERUM EXTRACTS

All requirements were met. The results demonstrate that there is no degradation over the four day storage period of extracts at refrigerator temperature.

FDA Guideline	NWB Report
No specific requirement	**Requirement met.**
The stability should be assessed over the anticipated storage time before injection.	**Requirement met.** Stored serum extracts were refrigerated at 1-8° C for 4 days. Stability was measured by comparison to freshly prepared QC samples.

Results:

All analytes are stable under these conditions. Sample precision is good. Control accuracy is good; however, PFOSA and PFOSAA accuracy in the QC samples could not be evaluated. While there is no separate data for the surrogate standard, the results presented at a minimum demonstrate that, at worst, all analytes and the surrogate degrade at the same rate, if any.

(Source: Report #122 Tables 43 - 49) **EXTRACTS STORED 4 DAYS 1-8°C**

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
% Deviation from Control							
low	-0.2	-5	2	6	1	16	4
high	2	-2	4	4	2	10	3
Sample Precision (CV), %							
low	5	8	5	6	10	11	9
high	2	9	1	2	3	0.8	1
Sample, mean ng/mL							
low	46.6	2.96	8.22	8.31	6.95	6.29	8.36
high	384	317	310	387	411	402	397

XVIII. POST-PREPARATIVE -20°C STABILITY OF SERUM EXTRACTS

All requirements were met. The results demonstrate that there is no degradation over the five day storage period of extracts at freezer temperature (-20° C).

FDA Guideline	NWB Report
No specific requirement	**Requirement met.** Two levels in triplicate.
The stability should be assessed over the anticipated storage time before injection.	**Requirement met.** Stored serum extracts were stored at -20° C for 5 days. Stability was measured by comparison to freshly prepared QC samples.

Results:

All analytes are stable under these conditions. While there is no separate data for the surrogate standard, the results presented at a minimum demonstrate that, at worst, all analytes and the surrogate degrade at the same rate, if any.

(Source: Report #122 Tables 50-56) **EXTRACTS STORED 5 DAYS at -20° C**

	PFOS	PFOSA	PFOSAA	POAA	PFHS	M-556	M570
% Deviation from Control							
low	-5	-2	-1	6	-0.7	-0.6	4
high	-2	-5	-0.3	-0.5	2	0.8	-3
Sample Precision (CV), %							
low	7	4	5	21	12	14	4
high	2	3	3	5	2	3	3
Sample, mean ng/Ml							
low	44.5	3.08	7.91	8.30	6.81	5.39	8.30
high	371	307	296	370	409	370	374

XIX. STOCK SOLUTION STABILITY

Requirement not met. No testing reported.

FDA Guideline	NWB Report
Store at room temperature for at least 6 hours	**Requirement not met**
Store at other temperatures and time periods as required.	**Requirement not met**

Report issued by:

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Date