3 RESULTS

Like chapter 2 this chapter is divided into part 3.1 for main- and 3.2 for preliminary investigations. The preliminary investigations were the basic work necessary to understand the procedures of the test and to decide how to build up the main investigations.

3.1 MAIN INVESTIGATIONS

To remember the plan of the main investigation see Table 3-1. For more details see

.

TABLE 3-1 GENERAL SETTING OF MAIN INVESTIGATION

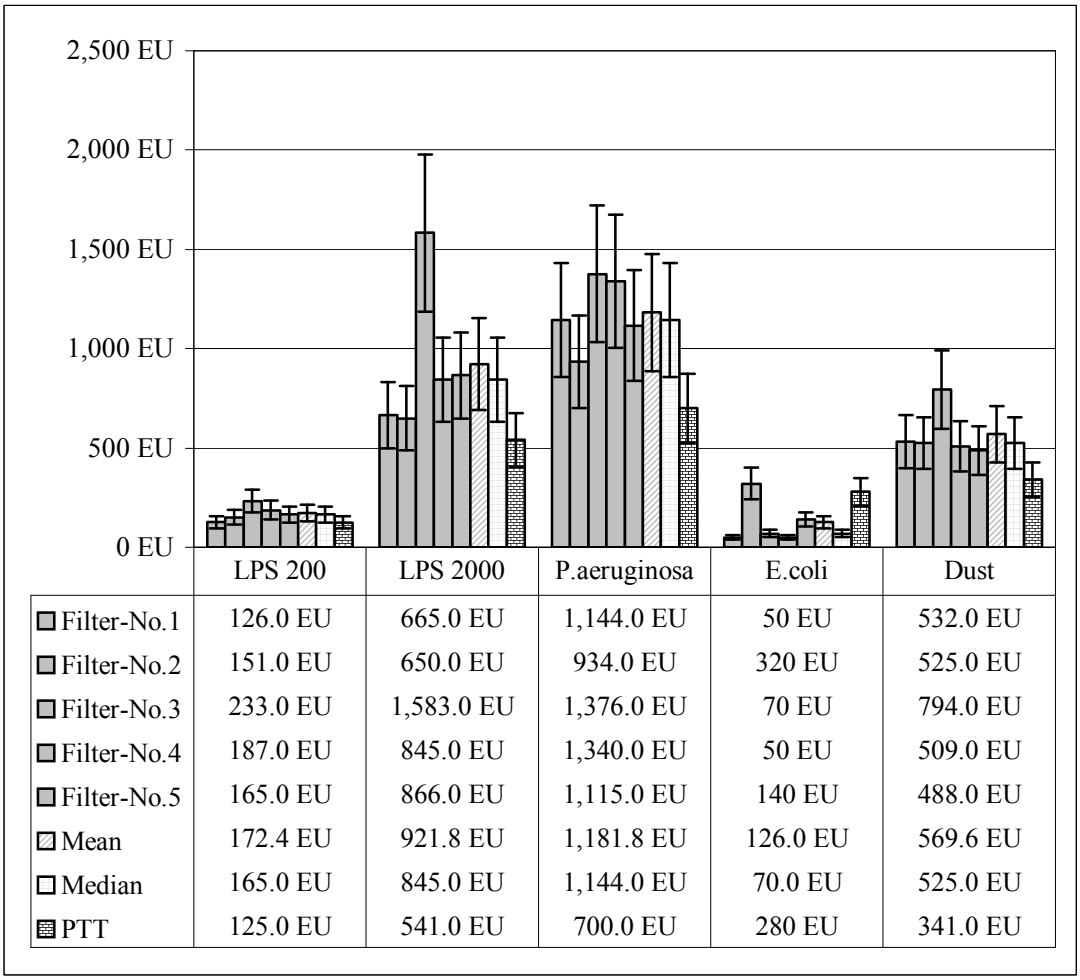
Typ of Filter	Glass fiber or Cellulose fiber or Syntetic fiber																			
Contaminant	LPS Endotoxin								*P. aeruginosa*				*E.coli*				Dust			
Concentration per punch of filter	200 EU				2000 EU				2000 EU				200 EU				200 EU			
Solvent	Tween		Buffer		Tween		Buffer		Tween		Buffer		Tween		Buffer		Tween		Buffer	
Extraction Method	sonication	shaking	sonication	shaking	sonication	shaking	sonication	shaking	sonication	shaking	sonication	shaking	sonication	shaking	sonication	shaking	sonication	shaking	sonication	shaking

The filter punches were contaminated with a certain activity and certain kind of endotoxin. Then they were extracted in a solvent. At one day 18 samples were measured, in example: all three types of filter contaminated with *Escherichia coli* and sonicated in Tween 20. There were 20 days of investigation and a total number of 360 samples.

3.1.1 MEASURED ACTIVITY ON FILTER PIECES

All levels of endotoxin activity for the different extraction procedures and filter types are shown in Figure 3-1 and Figure 6-1 to Figure 6-11. The figures show results for particular extraction methods and types of filter. In the first column are the numbers of filter pieces measured. The first line shows the type of contamination. The final three lines show mean, median and PTT for the recovered activities of endotoxin. Bar diagram shows corresponding values for better understanding.

Figure 3-1 shows the recovered activity of endotoxin for shaking glass fiber filters in Tween 20. The filter pieces were inoculated with 200 EU of LPS 200, 2000 EU of LPS 2000, 2000 EU of *Pseudomonas aeruginosa*, 200 EU of *Escherichia coli* and 200 EU of dust per filter. The ordinate shows the measured activity in EU. Five gray columns show individual activities of filter pieces. Columns with striped pattern exhibit the mean and columns with squared design show median. The columns on the most right exhibit the observed activity of the positive test tubes (PTT). Every sample activity is allowed to have an internal variation of 25% from one measurement to another which is indicated by error bars.



	LPS 200	LPS 2000	P.aeruginosa	E.coli	Dust
Filter-No.1	126.0 EU	665.0 EU	1,144.0 EU	50 EU	532.0 EU
Filter-No.2	151.0 EU	650.0 EU	934.0 EU	320 EU	525.0 EU
Filter-No.3	233.0 EU	1,583.0 EU	1,376.0 EU	70 EU	794.0 EU
Filter-No.4	187.0 EU	845.0 EU	1,340.0 EU	50 EU	509.0 EU
Filter-No.5	165.0 EU	866.0 EU	1,115.0 EU	140 EU	488.0 EU
Mean	172.4 EU	921.8 EU	1,181.8 EU	126.0 EU	569.6 EU
Median	165.0 EU	845.0 EU	1,144.0 EU	70.0 EU	525.0 EU
PTT	125.0 EU	541.0 EU	700.0 EU	280 EU	341.0 EU

error bars indicate allowed variation of 25%

FIGURE 3-1 ACTIVITY MEASURED ON ½ GLASS FIBER FILTER FOR SHAKING IT IN TWEEN

3.1.2 DESCRIPTIVE STATISTICAL ANALYSIS

This section contains calculated data (mean-SD, -difference and -square percentage difference of measured value and true amount) for measured values and true amounts as shown in section 2.1.8. The analysis with a "Generalized Linear Model" (GLM) is shown in section 3.1.3.

3.1.2.1 CLASSIFIED BY SINGLE CRITERION

Data was split in order to look at the criteria one-by-one (i.e. all data for cellulose fiber filter including both shaken and sonicated) and in section 3.1.2.2 for combined criterions (i.e. all data for glass fiber filter shaken in TAP).

Mayor effects of *Pseudomonas aeruginosa* on experimental variables and variance causes a change in statistical significance of all results. This outcome is not evident in the descriptive statistics and in Table 3-2. It was decided with the statistician to look at the data for *Pseudomonas aeruginosa* and other contaminations separately (see 4.6.3).

TABLE 3-2 CLASSIFIED BY CONTAMINATION

	SD	Difference[a]	MSFD[b]
LPS 200	38.47%	-2.4%	70.07%
LPS 2000	23.24%	-12.6%	47.96%
Pseudomonas aeruginosa	108.44%	52.8%	185.31%
Escherichia coli	2019.06%	393.7%	2336.92%
Dust	77.07%	44.1%	101.24%

[a] Difference is a fraction of measured values and true amounts (see 2.1.8.1)
[b] Mean square fractional difference (see 2.1.8.2)

3.1.2.1.1 SELECTED DATA EXCLUDING DATA FOR PSEUDOMONAS AERUGINOSA

Table 3-3 to Table 3-5 show data obtained without *Pseudomonas aeruginosa*.
Table 3-3 shows for glass fiber filter a SD of 48.18%, a difference of 2.6% and a MSFD of 71.76%.

TABLE 3-3 CLASSIFIED BY KIND OF FILTER EXCLUDING DATA FOR *PSEUDOMONAS AERUGINOSA*

	SD	Difference	MSFD
Glass Fiber	48.17%	2.6%	71.76%
Cellulose Fiber	1752.21%	267.2%	2023.64%
Synthetic Fiber	65.50%	51.0%	164.32%

Table 3-4 show a smaller value for sonication than for shaking.

TABLE 3-4 CLASSIFIED BY EXTRACTION METHOD EXCLUDING DATA FOR *PSEUDOMONAS AERUGINOSA*

	SD	Difference	MSFD
Sonication	56.83%	18.9%	138.46%
Shaking	1415.86%	190.8%	1635.44%

Shown in Table 3-5 is that TAP has a lower SD, difference and MSFD than Tween20.

TABLE 3-5 CLASSIFIED BY EXTRACTION FLUID EXCLUDING DATA FOR *PSEUDOMONAS AERUGINOSA*

	SD	Difference	MSFD
Tween 20	1432.20%	160.6%	1652.96%
TAP	94.13%	53.3%	175.30%

3.1.2.1.2 EXCLUSIVE DATA FOR PSEUDOMONAS AERUGINOSA CONTAMINATED FILTERS

Table 3-6 Table 3-8 show data only for *Pseudomonas aeruginosa* contaminated filters.
Table 3-6 exhibits results classified by type of filters. Cellulose fiber filter shows the smallest difference. Synthetic fiber filters show the smallest MSFD. Glass fiber filters present highest SD, difference and MSFD.

TABLE 3-6 EXCLUSIVE DATA FOR *PSEUDOMONAS AERUGINOSA* CLASSIFIED BY TYPE OF FILTER

	SD	Difference	MSFD
Glass Fiber	175.98%	156.4%	292.46%
Cellulose Fiber	67.82%	-44.4%	117.64%
Synthetic Fiber	41.47%	57.2%	95.03%

Sonication reveals in Table 3-7 almost same SD but higher difference and MSFD than shaking.

TABLE 3-7 EXCLUSIVE DATA FOR *PSEUDOMONAS AERUGINOSA* CLASSIFIED BY EXTRACTION METHOD

	SD	Difference	MSFD
Sonication	108.49%	63.4%	231.99%
Shaking	108.35%	41.7%	118.87%

Tween 20 as solution media presents in Table 3-8 a smaller SD, lower difference and MSFD.

TABLE 3-8 EXCLUSIVE DATA FOR *PSEUDOMONAS AERUGINOSA* CLASSIFIED BY EXTRACTION FLUID

	SD	Difference	MSFD
Tween 20	45.83%	20.2%	107.94%
TAP	150.10%	89.1%	244.21%

3.1.2.2 CLASSIFIED BY COMBINED CRITERIA

As described in section 3.1.2.1 the following tables will contain information on the combined criteria.
Selected Data Excluding Data for Pseudomonas Aeruginosa

Table 3-9 to Table 30-20 show data received without contamination of *Pseudomonas aeruginosa*. This section is subdivided for different types of filter material. Tables show SD, fractional difference and MSFD for different types and activities of endotoxin.

3.1.2.2.1.1 DATA OBTAINED FOR CELLULOSE FIBER FILTER

Data shown are received by looking only at results for cellulose fiber filter (CF).

Table 3-9 contains data for combination of Tween and sonication. From Table 3-10 to Table 3-12 data for the other possible extraction methods is shown.

TABLE 3-9 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: CF WITH TWEEN AND SONICATION

	SD	Difference	MSFD
LPS 2000	4.47%	-91.4%	91.60%
LPS 200	34.79%	-53.7%	64.03%
Escherichia coli	0.00%	-98.4%	98.39%
Dust	25.50%	-8.5%	26.83%

TABLE 3-10 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: CF WITH TAP AND SONICATION

	SD	Difference	MSFD
LPS 2000	12.65%	-6.1%	14.14%
LPS 200	93.06%	54.6%	107.89%
Escherichia coli	0.00%	-98.4%	98.39%
Dust	51.48%	40.8%	65.73%

TABLE 3-11 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: CF WITH TWEEN AND SHAKING

	SD	Difference	MSFD
LPS 2000	8.37%	-44.2%	45.06%
LPS 200	3.16%	-88.7%	88.71%
Escherichia coli	6865.82%	3,949.0%	7920.46%
Dust	56.83%	32.5%	65.50%

TABLE 3-12 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: CF WITH TAP AND SHAKING

	SD	Difference	MSFD
LPS 2000	13.78%	-24.5%	28.11%
LPS 200	32.86%	-31.1%	45.28%
Escherichia coli	319.77%	349.9%	474.01%
Dust	148.09%	164.5%	221.34%

3.1.2.2.1.2 GLASS FIBER FILTER

The data shown was obtained by looking only at results for glass fiber filter (GF). Table 3-13 to Table 2-7 contain data for combination of different extraction protocols.

TABLE 3-13 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: GF WITH TWEEN AND SONICATION

	SD	Difference	MSFD
LPS 2000	25.69%	-60.3%	65.57%
LPS 200	14.14%	-80.2%	81.49%
Escherichia coli	46.26%	-21.4%	50.89%
Dust	36.61%	50.4%	62.37%

TABLE 3-14 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: GF WITH TAP AND SONICATION

	SD	Difference	MSFD
LPS 2000	8.37%	27.9%	29.15%
LPS 200	46.04%	86.5%	97.98%
Escherichia coli	24.90%	-42.3%	48.99%
Dust	87.46%	9.1%	87.92%

TABLE 3-15 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: GF WITH TWEEN AND SHAKING

	SD	Difference	MSFD
LPS 2000	63.25%	70.4%	94.66%
LPS 200	28.98%	37.9%	47.75%
Escherichia coli	44.27%	-72.1%	84.62%
Dust	33.17%	67.0%	74.83%

TABLE 3-16 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: GF WITH TAP AND SHAKING

	SD	Difference	MSFD
LPS 2000	9.49%	-16.7%	19.24%
LPS 200	26.83%	-24.4%	36.19%
Escherichia coli	20.74%	-48.0%	52.35%
Dust	111.31%	50.2%	122.15%

3.1.2.2.1.3 SYNTHETIC FIBER FILTER

The data shown was obtained by looking only at results for synthetic fiber filters (SF). Table 3-17 exhibits lowest SD, difference and MSFD for LPS 2000. Table 3-17 up to Table 30-20 express other combinations of extraction.

TABLE 3-17 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: SF WITH TWEEN AND SONICATION

	SD	Difference	MSFD
LPS 2000	19.75%	5.5%	20.49%
LPS 200	25.10%	-34.9%	43.01%
Escherichia coli	51.48%	-14.5%	53.48%
Dust	101.34%	108.4%	148.39%

TABLE 3-18 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: SF WITH TAP AND SONICATION

	SD	Difference	MSFD
LPS 2000	4.47%	-19.8%	20.25%
LPS 200	26.65%	91.6%	95.39%
Escherichia coli	180.72%	602.2%	627.38%
Dust	64.65%	20.9%	67.97%

TABLE 3-19 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: SF WITH TWEEN AND SHAKING

	SD	Difference	MSFD
LPS 2000	17.61%	16.5%	24.08%
LPS 200	14.83%	5.0%	15.49%
Escherichia coli	55.05%	-23.1%	59.67%
Dust	58.48%	-3.5%	58.57%

TABLE 3-20 DATA EXCLUDING *PSEUDOMONAS AERUGINOSA*: SF WITH TAP AND SHAKING

	SD	Difference	MSFD
LPS 2000	9.49%	-20.1%	22.14%
LPS 200	34.06%	9.7%	35.36%
Escherichia coli	98.08%	154.7%	206.03%
Dust	50.10%	-3.2%	50.20%

3.1.2.2.2 DATA EXCLUSIVE FOR PSEUDOMONAS AERUGINOSA CONTAMINATED FILTERS

Table 3-21 to Table 3-24 show data obtained only with contamination of *Pseudomonas aeruginosa*. This section is subdivided for different extraction methods. Tables show SD, fractional difference and MSFD for different types of filter material.

Sonicating synthetic fiber filter in Tween received lowest difference (Table 3-21). Smallest MSFD shows cellulose fiber. Table 3-22 to Table 3-24 demonstrate data for the other different filter extractions.

TABLE 3-21 *PSEUDOMONAS AERUGINOSA* CONTAMINATED FILTERS SONICATED IN TWEEN

	SD	Difference	MSFD
Glass Fiber	98.08%	154.70%	206.03%
Cellulose Fiber	9.49%	-20.10%	22.14%
Synthetic Fiber	34.06%	9.70%	35.36%

TABLE 3-22 *PSEUDOMONAS AERUGINOSA* CONTAMINATED FILTERS SONICATED IN TAP

	SD	Difference	MSFD
Glass Fiber	270.87%	532.1%	586.50%
Cellulose Fiber	26.65%	-67.7%	70.71%
Synthetic Fiber	12.25%	15.1%	19.49%

TABLE 3-23 *PSEUDOMONAS AERUGINOSA* CONTAMINATED FILTERS SHAKEN IN TWEEN

	SD	Difference	MSFD
Glass Fiber	23.02%	68.8%	72.59%
Cellulose Fiber	4.47%	-97.4%	97.47%
Synthetic Fiber	72.73%	163.3%	178.80%

TABLE 3-24 *PSEUDOMONAS AERUGINOSA* CONTAMINATED FILTERS SHAKEN IN TAP

	SD	Difference	MSFD
Glass Fiber	224.48%	-24.3%	27.39%
Cellulose Fiber	144.78%	119.7%	187.86%
Synthetic Fiber	24.90%	17.5%	30.50%

3.1.3 STATISTICAL ANALYSIS WITH "GENERALIZED LINEAR MODELS" (GLM)

Because of inhomogeneous SD of *Pseudomonas aeruginosa* the results of the analysis were split into two sections. 3.1.3.1 shows the analysis excluding data for *Pseudomonas aeruginosa* and section 3.1.3.2 presents exclusive results for *Pseudomonas aeruginosa*. All tables in this section are built up the same way. The first two columns show the tested extraction method. The column „Mean" shows the mean value. 95% confidence interval (CI_{95}) is exposed in column 5 and 6 with upper and lower bound. „R-Square" shows percentage of cases explained by this extraction method and "p" shows the level of significance.

3.1.3.1 STATISTICAL ANALYSIS EXCLUDING DATA OF *PSEUDOMONAS AERUGINOSA*

Table 3-25 shows the calculated values for the analysis of single factors. From the point of statistical significance sonication is not better than shaking (p = 0.323). The mean of sonication is closer to the TA than shaking with a mean of 1.955 for sonication and 262.892 for shaking. The CI_{95} has a range from -370.005 to 373.916 for sonication and -109.069 to 634.852 for shaking. The differences between sonication and shaking explain 2.1% of the variation. TAP does not show significant better when compared to Tween but it is closer to the TA with only 2.984 deviation from the TA. The difference between Tween and TAP only explains 2.1% of the variation. The glass fiber filters show the best recovery with 0.508 for mean, synthetic fiber with 2.584 is very close too and cellulose fiber's mean is worst with 394.178. All differences are not statistical significant. The different types of filters explain only 4.3% of variation. LPS 2000 with a activity of 2000 EU per filter shows the best recovery with a mean of 0.231 followed by LPS200 with 0.483 and dust with 1.025 for mean values. *Escherichia coli* shows the worst mean value with 527.954. The differences in contamination explain 6.5% of variation and are not significant.

TABLE 3-25 UNIVARIATE ANALYSIS FOR SINGLE FACTORS OF DATA EXCLUDING CONTAMINATION WITH *PSEUDOMONAS AERUGINOSA*

				95% Confidence Interval		
Source	Type	p	Mean	Lower Bound	Upper Bound	R-Square
Extraction Method	Sonication	0.323	1.955	-370.005	373.916	0.021
	Shaking		262.892	-109.069	634.852	
Solution Media	Tween	0.327	261.863	-110.161	633.887	0.021
	TAP		2.984	-369.040	375.008	
Type of Filter	Glass fiber	0.375	0.508	-455.274	456.290	0.043
	Cellulose fiber		394.178	-61.604	849.959	
	Synthetic fiber		2.584	-453.198	458.366	
Type of Contamination	LPS 200	0.393	0.483	-525.852	526.817	0.065
	LPS 2000		0.231	-526.103	526.566	
	E.coli		527.954	1.620	1,054.289	
	Dust		1.025	-525.309	527.360	

Table 3-26 to Table 3-31 show results for analysis of combining two factors. Table 3-26 combines contamination and extraction media. Combinations are not significantly different. Ex-

cept *Escherichia coli* and Tween with a mean of 1,045.993 all combinations are close to the TA and have similar CI_{95}'s. These combinations explain 14.9% of variation.

TABLE 3-26 MULTIVARIATE ANALYSIS OF DATA EXCLUDING CONTAMINATION WITH *PSEUDOMONAS AERUGINOSA* FOR THE COMBINATION OF TYPE OF CONTAMINATION AND SOLVENT

			95% Confidence Interval	
Contamination	Solution	Mean	Lower Bound	Upper Bound
LPS 200	Tween	0.383	-746.684	747.449
	TAP	0.583	-746.484	747.649
LPS 2000	Tween	0.411	-746.655	747.478
	TAP	0.052	-747.015	747.118
E.coli	Tween	1,045.993	298.927	1,793.060
	TAP	9.915	-737.152	756.982
Dust	Tween	0.666	-746.401	747.732
	TAP	1.385	-745.682	748.451

p = 0.411
R-Square = 0.149

Table 3-27 shows combination of types of filter and type of contamination. Except for *Escherichia coli* and cellulose filter with a mean of 1,574.326 all means are close to the TA with comparable CI_{95}'s.

TABLE 3-27 MULTIVARIATE ANALYSIS OF DATA FOR COMBINATION OF TYPE OF FILTER AND TYPE OF CONTAMINATION (EXCLUDING *PSEUDOMONAS AERUGINOSA*)

			95% Confidence Interval	
Type of Filter	Contamination	Mean	Lower Bound	Upper Bound
Glass fiber	LPS 200	0.496	-916.559	917.550
	LPS 2000	0.362	-916.693	917.416
	E.coli	0.372	-916.682	917.427
	Dust	0.803	-916.251	917.858
Cellulose fiber	LPS 200	0.641	-916.413	917.696
	LPS 2000	0.285	-916.769	917.340
	E.coli	1,574.326	657.271	2,491.380
	Dust	1.458	-915.597	918.512
Synthetic fiber	LPS 200	0.311	-916.743	917.365
	LPS 2000	0.048	-917.007	917.102
	E.coli	9.164	-907.890	926.219
	Dust	0.815	-916.239	917.869

p = 0.438
R-Square = 0.236

Table 3-28 displays the means of grouping contamination and extraction method. Means are similar apart from *Escherichia coli* and shaking with a mean of 1,049.767.

TABLE 3-28 MULTIVARIATE ANALYSIS OF DATA FOR COMBINATION OF TYPE OF CONTAMINATION (EXCLUDING *PSEUDOMONAS AERUGINOSA*) AND EXTRACTION METHOD

Contamination	Extraction Method	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
LPS 200	Sonication	0.715	-745.815	747.245
	Shaking	0.250	-746.280	746.780
LPS 2000	Sonication	0.243	-746.287	746.773
	Shaking	0.220	-746.310	746.750
E.coli	Sonication	6.141	-740.389	752.671
	Shaking	1,049.767	303.237	1,796.297
Dust	Sonication	0.722	-745.808	747.252
	Shaking	1.329	-745.201	747.859

p = 0.150
R-Square = 0.404

Table 3-29 shows the combination of solution and extraction method with similar means and CI_{95}'s with the exception of shaking the filters in Tween. 6.3% of variation is explained by this combination.

TABLE 3-29 MULTIVARIATE ANALYSIS OF DATA EXCLUDING CONTAMINATION WITH *PSEUDOMONAS AERUGINOSA* FOR THE COMBINATION OF SOLUTION AND EXTRACTION METHOD

Solution	Extraction Method	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Tween	Sonication	0.562	-526.212	527.336
	Shaking	523.165	-3.610	1,049.939
TAP	Sonication	3.349	-523.426	530.123
	Shaking	2.619	-524.156	529.393

p = 0.322
R-Square = 0.063

Table 3-30 shows means and CI_{95}'s for combination of filter type and extraction method. Means and CI_{95}'s are similar not including cellulose fiber and shaking.

TABLE 3-30 MULTIVARIATE ANALYSIS OF DATA EXCLUDING CONTAMINATION WITH *PSEUDOMONAS AERUGINOSA* FOR THE COMBINATION OF FILTER TYPE AND EXTRACTION METHOD

Filter type	Extraction Method	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Glass fiber	Sonication	0.475	-645.173	646.123
	Shaking	0.542	-645.106	646.190
Cellulose fiber	Sonication	0.549	-645.099	646.197
	Shaking	787.806	142.158	1,433.454
Synthetic fiber	Sonication	4.842	-640.807	650.490
	Shaking	0.327	-645.321	645.975

p = 0.371
R-Square = 0.107

Table 3-31 shows comparable data for filter types and solution media. Only cellulose fiber filter and Tween show off much higher results for mean and a different CI_{95}.

TABLE 3-31 MULTIVARIATE ANALYSIS OF DATA EXCLUDING CONTAMINATION WITH *PSEUDOMONAS AERUGINOSA* FOR THE COMBINATION OF FILTER TYPE AND SOLUTION MEDIA

Filter type	Solution	Sig.	Mean	95% Confidence Interval		R-Square
				Lower Bound	Upper Bound	
Glass fiber	Tween		0.518	-645.504	646.539	
	TAP		0.499	-645.522	646.520	
Cellulose fiber	Tween		784.635	138.614	1,430.657	
	TAP		3.720	-642.302	649.741	
Synthetic fiber	Tween		0.437	-645.584	646.458	
	TAP		4.732	-641.290	650.753	

p = 0.377
R-Square = 0.106

3.1.3.2 STATISTICAL ANALYSIS WITH REGARD TO THE DATA OF *PSEUDOMONAS AERUGINOSA*

This section shows the GLM results obtained only from data of samples inoculated with *Pseudomonas aeruginosa*.

Table 3-32 displays the results for single factors. Tween showed a slightly better but not significant result for mean. Synthetic fiber filter shows the lowest mean followed by cellulose- and glass fiber. Shaken presents a lower mean than sonication.

TABLE 3-33 UNIVARIATE ANALYSIS FOR SINGLE FACTORS EXCLUSIVE FOR DATA OF *PSEUDOMONAS AERUGINOSA*

				95% Confidence Interval		
Source	Type	Sig.	Mean	Lower Bound	Upper Bound	R-Square
Solution Media	Tween	0.379	1.167	-5.921	8.255	0.078
	TAP		5.312	-1.776	12.401	
Type of Filter	Glass fiber	0.465	7.370	-1.517	16.257	0.157
	Cellulose fiber		1.487	-7.401	10.374	
	Synthetic fiber		0.862	-8.025	9.749	
Extraction Method	Sonication	0.441	5.064	-2.092	12.219	0.061
	Shaking		1.416	-5.740	8.572	

Table 3-34 demonstrates the analysis of combined data. All combinations show low means except glass fiber together with TAP. All differences are not significant. This combination explains 41.8% of variation.

TABLE 3-34 MULTIVARIATE ANALYSIS FOR EXCLUSIVE DATA OF *PSEUDOMONAS AERUGINOSA* FOR COMBINATION: FILTER TYPE AND SOLUTION MEDIA

			95% Confidence Interval	
Filter type	Solution	Mean	Lower Bound	Upper Bound
Glass fiber	Tween	0.874	-12.953	14.701
	TAP	13.867	0.040	27.694
Cellulose fiber	Tween	0.968	-12.859	14.795
	TAP	2.005	-11.822	15.832
Synthetic fiber	Tween	1.659	-12.168	15.486
	TAP	0.065	-13.762	13.892

p = 0.439
R-Square = 0.418

The combination of glass fiber and sonication showed the worst mean in Table 3-35. Other groupings show lower but not significant means. 46.2% of variation is explained by this setting.

TABLE 3-35 MULTIVARIATE ANALYSIS FOR EXCLUSIVE DATA OF *PSEUDOMONAS AERUGINOSA* FOR COMBINATION: FILTER TYPE AND EXTRACTION METHOD

Filter type	Extraction Method	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Glass fiber	Sonication	14.377	1.077	27.677
	Shaking	0.363	-12.937	13.663
Cellulose fiber	Sonication	0.734	-12.566	14.034
	Shaking	2.239	-11.061	15.539
Synthetic fiber	Sonication	0.080	-13.220	13.380
	Shaking	1.645	-11.655	14.944

p = 0.325
R-Square = 0.462

Tween showed the lowest mean together with sonication as expressed in Table 3-36. TAP's mean is lower with shaking. Differences are not significant. These combinations explain 22.8% of variation.

TABLE 3-36 MULTIVARIATE ANALYSIS FOR EXCLUSIVE DATA OF *PSEUDOMONAS AERUGINOSA* FOR COMBINATION: SOLUTION MEDIA AND EXTRACTION METHOD

Solution	Extraction Method	Mean	95% Confidence Interval	
			Lower Bound	Upper Bound
Tween	Sonication	0.776	-9.838	11.390
	Shaking	1.558	-9.056	12.172
TAP	Sonication	9.351	-1.263	19.965
	Shaking	1.274	-9.340	11.888

p = 0.364
R-Square = 0.228

3.2 PRELIMINARY INVESTIGATIONS

After performing preliminary investigations a decision was made to accept a basic contamination for the filters, to use a concentration of 0.005% Tween 20 and to run the standard curve with solution fluid. It was decided to improve the spiking as shown below and to choose a smaller interval for measuring the microplate.

3.2.1 DEPYROGENISATION

Dry Heat and γ-radiation with a 60Cobalt device were investigated.

3.2.1.1 DRY HEAT

In order to maintain all glassware, pairs of scissors and pairs of tweezers endotoxin free they were heated in the oven with dry heat at 180°C for a minimum of 4 hours.

3.2.1.1.1 DEPYROGENIZATION BY HEATING CHALLENGE VIALS

The oven was tested with Endotoxin Challenge Vials™ (ECV) of Co. BioWhittaker (see 2.2.4.1). The reduction in all samples was more than 10^6 (Table 3-37).

TABLE 3-37 DRY HEAT DEPYROGENISATION

Object	Recovered LAL Activity[a] (EU/ml)	Reduction [b]
ECV No. 1	< 0.005	> 10^6
ECV No. 2	< 0.005	> 10^6
ECV No. 3	< 0.005	> 10^6
ECV No. 4	0.006	> 10^6
ECV No. 5	< 0.005	> 10^6
ECV No. 6	< 0.005	> 10^6
ECV No. 7	< 0.005	> 10^6
ECV No. 8	< 0.005	> 10^6
ECV No. 9	< 0.005	> 10^6
ECV No. 10	< 0.005	> 10^6
Reference ECV No. 1	1625.93	
Reference ECV No. 2	1625.93	
Reference ECV No. 3	1625.93	

[a] lower detection limit for the assay = 0.005 EU/ml

[b] FDA recommends a factor ≤ 0.001 for depyrogenization [94].

Ten vials were placed in the oven (1 to 10) and 3 vials (reference 1 to 3) were not heated. Nine heated vials had a non detectable endotoxin activity. Only one vial was a little bit greater than the lower detectable level of 0.005 EU/ml. All three reference vials had the same activity of 1,625.93 EU/ml. Recommendations by the FDA [94] of 1000 fold reduction is exceeded by a 10^6 fold cutback. Used heating protocol is valid to remove endotoxin off ECVs.

To render filter material free of endotoxin for the investigations the use of heat and γ-radiation was studied.

3.2.1.1.2 DEPYROGENIZATION BY HEATING FILTER MATERIAL

When heating the filters at 180°C for 4 hours they changed their structure. It was investigated with an electron microscope.

3.2.1.2 RADIATION

The effect of γ-radiation with 60Cobalt to Endotoxin Challenge Vials™ was investigated. Six vials were radiated with 25 KGray. For reference three vials were not radiated (see 2.2.4.2).

3.2.1.3 DEPYROGENIZATION OF CHALLENGE VIALS WITH 60COBALT RADIATION

Table 3-38 shows no significant reduction with an arithmetic mean of 1,784 (Confidence interval $(CI)_{95}$: 1,344 – 2,224) EU/ml, for not radiated ECVs and an arithmetic mean of 1,752 (CI_{95}: 1,509 – 1,995) EU/ml for radiated ones.

TABLE 3-38 DEPYROGENIZATION OF CHALLENGE VIALS WITH γ-RADIATION

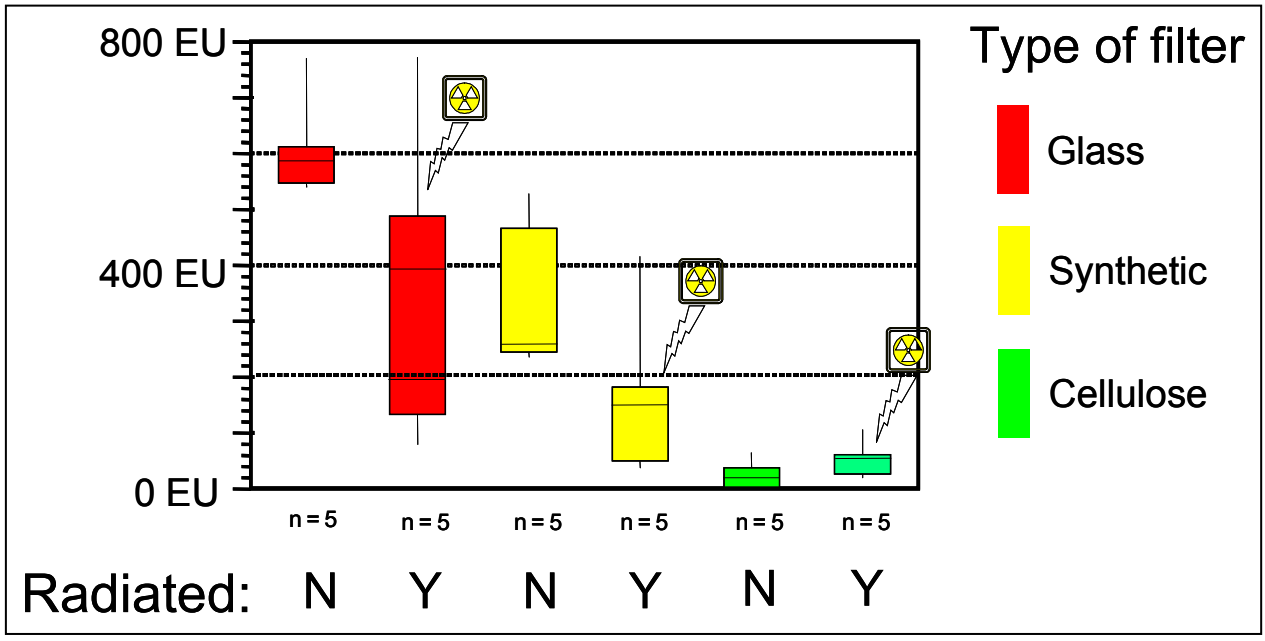
	Irradiation (EU/ml)		
	Yes	No	Reduction*
Arithmetic Mean	1,752	1,784	n.s.
Median	1,655	1,654	n.s.
CI_{95}	±243	±440	
Number of vials	n = 6	n = 3	

* Food and Drug Administration (FDA) recommends a ≥ 3 log reduction for depyrogenization [94].
n.s. = not significant

Arithmetic mean and median for radiated and not radiated are not significant different. The reduction is not enough to accomplish the recommended more than 3-log reduction [94]. Our inference in this point is that radiation with 25 KGray has no influence on endotoxin.

3.2.1.4 DEPYROGENIZATION OF FILTER MATERIAL WITH 60COBALT RADIATION

Influence of 60Cobalt γ-radiation with 25 KGray (2.2.4.4) on precontaminated filters was investigated. Five bisected filters out of every filter type were contaminated with 1000 EU of LPS. One half of was radiated. For complete data see Table 6-1.
For illustration Figure 3-2shows a part of Table 3-39's data.



N = Not radiated, Y = Radiated

FIGURE 3-2 BOX PLOT DIAGRAM FOR RADIATED AND NON RADIATED FILTERS

Cellulose filters showed a twofold increase of endotoxin activity in the treated filter pieces (Figure 3-2). No significant reduction is shown with 167.8 (CI_{95}: 34.4 – 301.2) EU/ml for radiated synthetic fiber filters and an arithmetic mean of 346.7 (CI_{95}: 224.9 – 468.5) EU/ml

for not treated ones. A log reduction of 0.32 is shown for the arithmetic mean. Glass fiber filter showed no significant reduction with 373.0 (CI_{95}: 126.4 – 619.6) EU/ml, for radiated and of 611.3 (CI_{95}: 529.2 – 693.4) EU/ml for not processed ones (Table 3-39).

TABLE 3-39 DIFFERENT TYPES OF FILTER TREATED WITH γ-RADIATION

		Treated with γ-Radiation[b] (EU/ml)					
		No	Yes	No	Yes	No	Yes
		Synthetic Fiber		Glass Fiber		Cellulose Fiber	
Arithmetic mean		346.7	167.8	611.3	373.0	25.2	54.0
Standard error mean		62.2	68.0	41.9	125.8	11.9	15.3
Median		259.2	153.0	586.8	392.4	19.8	55.8
Standard deviation		139.0	152.2	93.7	281.4	26.7	34.2
CI (95%)		±121.8	±133.4	±82.1	±246.6	±23.4	±23.0
Log reduction[a]	mean	0.32		0.21		-0.33	
	median	0.28		0.17		-0.45	

[a] Food and Drug Administration (FDA) recommends a ≥ 3 log reduction for depyrogenization [94].
[b] 25 KGray form a 60Cobalt device

None of the radiated filters meet the Food and Drug Administration (FDA) criteria [94] of 1,000 fold or more reduction of their endotoxin content. Radiation with 25 KGray is a non sufficient method to render the filters free of endotoxin.

3.2.2 BASIC ENDOTOXIN CONTAMINATION OF FILTER MATERIAL

Basic endotoxin contamination of the filter pieces was analyzed. 10 bisected filter punches of each material (cellulose fiber, glass fiber and synthetic fiber) were randomly chosen. The half pieces were sonicated in 0.005% Tween 20 for 45 minutes. All results are shown in Table 3-40.

TABLE 3-40 BASIC CONTAMINATION OF FILTERS

No. of Filter	Detected Endotoxin on ½ Filter (EU)		
	Cellulose fiber	Glass fiber	Synthetic fiber
1	0.050	0.238	0.013
2	0.018	0.011	0.014
3	0.039	0.005	0.005
4	0.020	< 0.005	0.009
5	0.017	< 0.005	0.015
6	0.031	0.006	< 0.005
7	0.042	< 0.005	0.006
8	0.050	< 0.005	< 0.005
9	0.033	0.019	0.006
10	0.241	0.215	0.005

TABLE 3-41 STATISTICAL ANALYSIS OF BASIC CONTAMINATION

	Detected Endotoxin (EU)		
	Cellulose Fiber	Glass Fiber	Synthetic Fiber
Arithmetic mean	0.054	0.049	0.007
Standard error mean	0.021	0.030	0.002
Median	0.036	0.006	0.006
Standard deviation	0.067	0.094	0.005
$CI_{(95)}$ [a]	± 0.041	± 0.058	± 0.003

[a] confidence interval (95%)

Basic endotoxin activity of filter material (Table 3-41) was investigated in order to deduct from the measured results in main investigations. Median was taken because there are outliers (i.e. cellulose fiber filter No. 10, glass fiber filter No. 1 and No. 10 in Table 3-40) which influence should be reduced. Basic endotoxin activity for cellulose fiber filters were estimated as 0.036 EU/ml, for glass fiber filters as 0.006 EU/ml and for synthetic fiber filters as 0.006 EU/ml.

3.2.3 EXTRACTION MEDIA

Extraction media used in the study were investigated.

3.2.3.1 TWEEN 20

Some research was made in the quantity of Tween 20 as a solution media (2.2.3).

The columns in Table 3-42 show the recovered activity and percentage recovering of endotoxin for four different activities (0.15 to 0.60 EU/ml) of LPS and 1.2 EU/ml of *Escherichia coli* endotoxin. (see 2.2.3)

TABLE 3-42 ENDOTOXIN ACTIVITY OF CSE + *ESCHERICHIA COLI* BACTERIA IN TWEEN RELATIVE TO CSE IN WATER

Activity [EU/ml]	Total and Percentage Endotoxin Activity [EU/ml] (%[a])				
	Concentration of Tween 20				
	0.07%	0.035%	0.007%	0.0035%	0.0007%
0.15 [b]	0.15 (98)	0.16 (107)	0.18 (123)	0.20 (135)	0.20 (136)
0.30 [b]	0.25 (82)	0.27 (88)	0.32 (107)	0.34 (114)	0.35 (116)
0.60 [b]	0.40 (66)	0.52 (86)	0.67 (111)	0.64 (107)	0.69 (116)
1.20 [b]	0.92 (77)	1.01 (84)	1.37 (114)	1.43 (119)	1.56 (130)
0.65 [c]	1.16 (179)	1.25 (192)	1.10 (169)	1.14 (176)	1.17 (180)
Arithmetic mean [d]	(100.4)	(111.4)	(124.8)	(130.2)	(135.6)
Median [d]	(82)	(88)	(114)	(119)	(130)

[a] percent activity in Tween relative to activity in water
[b] Control Standard Endotoxin
[c] *Escherichia coli* endotoxin (whole bacteria) EU/ml estimated from dilution in water
[d] mean and median of percentage endotoxin activity

Table 3-43 shows six different concentrations of Tween 20 (0.05% to 0.0005%). The columns show percentage- and total activity of endotoxin recovered for 0.41 EU/ml and 0.61 EU/ml of CSE, respectively. Last column expresses arithmetic mean of percentage recovery. Table 3-42

and Table 3-43 show similar values for percentage recovery in respect to Tween 20 concentration. A tendency of higher recovery rates with decreasing Tween 20 concentrations is visible.

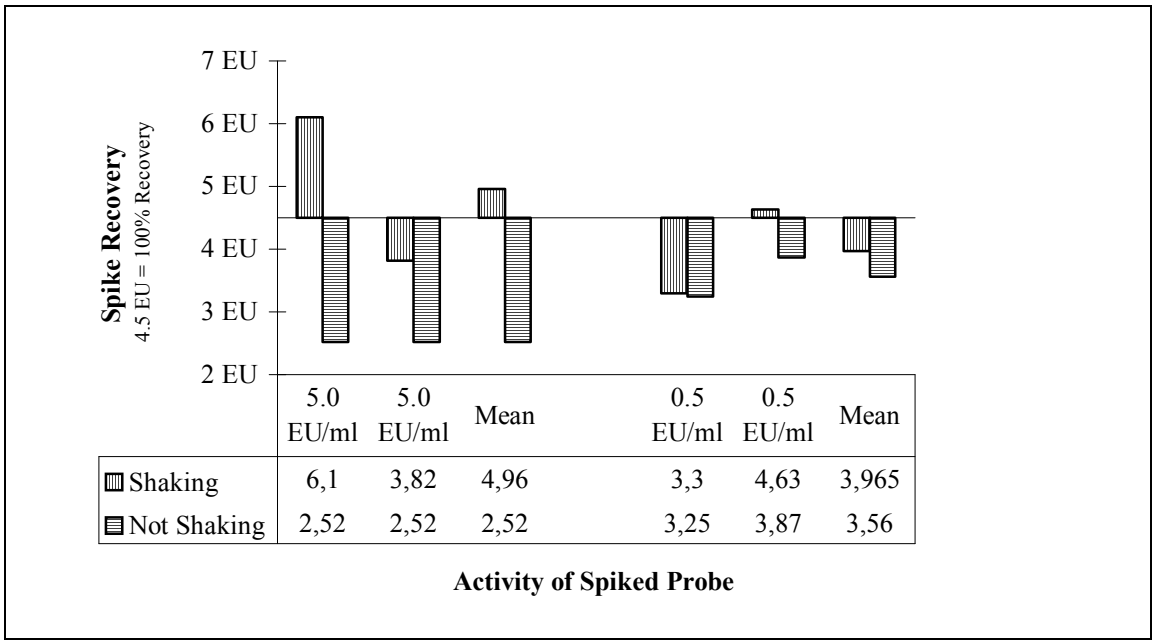
TABLE 3-43 ENDOTOXIN ACTIVITY OF CSE IN DIFFERENT TWEEN 20 CONCENTRATIONS RELATIVE TO CSE IN WATER

Concentration of Tween 20 [%]	Endotoxin Activity [EU/ml] (%[a])		
	0.41 [b]	0.61 [b]	Mean [c]
0.05	0.34 (82)	0.67 (111)	96.5
0.025	0.36 (88)	0.72 (119)	103.5
0.007	0.35 (85)	0.90 (149)	117.0
0.005	0.45 (110)	0.78 (129)	119.5
0.0025	0.49 (119)	0.74 (122)	120.5
0.0005	0.45 (109)	0.68 (112)	110.5

[a] percent activity in Tween relative to activity in water
[b] CSE activity estimated from dilution in water
[c] arithmetic mean of percentage endotoxin activity

3.2.4 SPIKING

To improve the spiking procedure two solutions of LPS (5 EU/ml and 0.5 EU/ml) were produced. Both were spiked with 5 EU. Two samples of each activity were shaken after placing the spike and two samples were not treated (2.2.1). The activities of shaken spikes had a smaller deviation from the theoretical value for the spike. However the shaken spikes had a more positive deviation than the not shaken ones. The deviation of the recovered spike from 4.5 EU is shown in Figure 3-3.



Values for shaken and not shaken spikes are in EU.

FIGURE 3-3 DEVIATION OF SPIKE RECOVERY FROM 4.5 EU FOR SPIKED 5 EU/ML AND 0.5 EU/ML SOLUTIONS

3.2.5 MODIFICATION OF THE STANDARD CURVE

Investigations of the standard curve made with LAL reagent water, Tween 20 and TAP were performed (2.2.1). Table 3-44 and Table 3-45 show in the first two columns the theoretical values and the measured activities for the standard curve made with water as solvent. Center pillar expresses the recovered endotoxin activity for standard activities diluted with Tween 20 or TAP, respectively. Last two columns indicate if the spike was reliable when measured against the standard curve made with water or diluent.

TABLE 3-44 COMPARISON OF STANDARD CURVES MADE WITH WATER OR TWEEN 20

Standard Curve[a] (EU/ml)		Standard Activitys[d] Made with Tween 20 (EU/ml)	Spike Reliable[f] ?	
Theoretical	Measured		Water[b]	Tween 20[c]
50	49.022	-[e]	-[e]	-[e]
5	4.945	3.557	yes	yes
0.5	0.515	0.428	yes	yes
0.05	0.053	0.045	yes	yes
0.005	0.005	0.004	yes	yes

[a] Endotoxin standard vial was reconstituted and dissolved with **water** as described in Table 2-1
[b] Standard activities were spiked and measured against the standard curve made with water
[c] Standard activities in Tween were spiked and measured against the standard curve made with TAP
[d] Endotoxin standard vial was reconstituted and dissolved with **Tween 20** as described in Table 2-1.
[e] Not performed
[f] Spike recovery of ± 50% [71]

TABLE 3-45 COMPARISON OF STANDARD CURVES MADE WITH WATER OR TAP

Standard Curve[a] (EU/ml)		Standard Activities[d] Made with TAP (EU/ml)	Spike Reliable[f] ?	
Theoretical	Measured		Water[b]	TAP[c]
50	56.225	22.514	No	-[e]
5	4.904	1.346	No	Yes
0.5	0.494	0.073	No	Yes
0.05	0.034	0.004	No	Yes
0.005	0.007	0.003	No	Yes

[a] Endotoxin standard vial was reconstituted and dissolved with **water** as described in Table 2-1
[b] Standard activities were spiked and measured against the standard curve made with water
[c] Standard activities in TAP were spiked and measured against the standard curve made with TAP
[d] Endotoxin standard vial was reconstituted and dissolved with **TAP** as described in Table 2-1.
[e] Not possible to retrieve activities higher than the highest standard
[f] Spike recovery of ± 50% [71]

Data show interference especially for standard activities made with TAP when calculated against a standard curve prepared with water. This interference does not exist when sample and standard curve were created with the same solvent.

3.2.6 OBSERVATIONS

The cellulose fiber filter was impregnated as it cause the fluid to form a drop-configuration on top of the filter. By the time the solution penetrated into the material and the water was evaporated the next day.