AN EVALUATION OF EXTRACTION SOLUTIONS AND FILTER TYPES FOR THE RECOVERY OF ENDOTOXIN

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Abstract

The influence of three commonly used extraction media on the recovery of endotoxin from different filter types was studied using the *Limulus* amebocyte lysate (LAL) test. Particulate and aqueous endotoxin preparations were used to spike PVC, Teflon coated glass fiber and glass fiber filters. For each filter endotoxin combination, air was sampled at 2 liter/minute for 50 minutes after which the filters were extracted with either pyrogen-free water (PFW), 0.05% Tween 20 in PFW, or a 0.05 M Potassium phosphate/0.01% triethylamine pyrogen free buffer solution. Sample extracts were assayed by the LAL. To determine the interaction between endotoxin, filter type, and extraction medium without air sampling (interaction controls), each extraction media was spiked with aqueous or particulate associated endotoxin, an unspiked filter added to the media, and the sample extracted and assayed for endotoxin. Positive controls consisted of each extraction medium spiked with the aqueous or particulate endotoxin. For particulate spiked samples, there was no difference in recovery of endotoxin from the air sampled PVC and glass fiber filters extracted with either PFW or Tween 20; however, for these extraction media, recovery efficiency from air sampled Teflon coated glass fiber was significantly less than the other two filter media. No difference was observed in particulate spiked air sample PVC and glass fiber filters and the respective positive controls and interaction controls. There was no difference in the recovery of endotoxin from any of the positive controls, including the phosphate buffer control. Regardless of the filter media, all particulate spiked samples (both air sampled and interaction controls) extracted with the phosphate buffer showed significantly lower recoveries than for the other two extraction media. An inhibition study demonstrated that in undiluted samples the phosphate buffer inhibited the recovery of endotoxin. The inhibition could be eliminated by sample dilution, however, filters extracted with the phosphate buffer, then diluted with PFW yielded lower endotoxin recoveries. These data indicate that the phosphate buffer interacts with either the filter media, the endotoxin, or both to reduce the availability of the endotoxin for the LAL analysis. These data confirm previous studies which have shown that filter type and extraction media are important variables in determining the recovery of endotoxin from environmental samples.

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Introduction

Because endotoxin is thought to be one of the main etiological agents responsible for acute pulmonary dysfunction in workers exposed to organic dusts and mists, continued research for standardizing the collection, extraction, and analysis of this compound is essential. Most of the recent methods for quantifying endotoxin have used the *Limulus* amebocyte lysate (LAL) assay as their analysis procedure. Several studies (Hollander et. al., 1993; Gordon et. al., 1992; Douwes et. al. 1995) have examined the influences of different filter types and extraction solutions on the recovery efficiency of endotoxin using the LAL assay to monitor endotoxin. The purpose of this study was to evaluate the influence of three different extraction media in combination with three different filter types on the recovery of particulate associated and aqueous preparations of endotoxin. Filter samples or the extraction solutions were spiked with either particulate or aqueous mediums containing endotoxin and assayed using the LAL assay.

Materials and Methods

Laboratory prepared particulate (30,000 EU/mg) and aqueous (1000 EU/mL) endotoxin stocks were used to spike PVC, Teflon coated glass fiber and glass fiber filters. These stocks were prepared using Snomax, a cloud seeding preparation containing 80% Pseudomonas syringae. Two conditions using filters were evaluated: First, to simulate air sampling, filters were spiked with each endotoxin preparation and air sampled at 2 liters/minute for 50 minutes. A multiple filter air sampling tree was connected inline to a vacuum pump to draw air across the 37 mm filters contained in polystyrene filter cassettes. 21 gauge hypodermic needles were used as flow orifices to obtain a critical flow of 2 liters/minute for each branch of the sampling tree. After sampling, the filters were extracted for 60 minutes using a rotating platform with either pyrogen-free water (PFW), 0/05% Tween 20 in PFW, or a 0.05 M potassium phosphate/0.01% triethylamine pyrogen-free buffer solution. A total of six filters were used for each endotoxin preparation: filter: extraction solution combination. Second, to determine the interaction between endotoxin, filter type, and extraction solution without air sampling (interaction controls), each type of extraction solution was spiked with either particulate or aqueous endotoxin, an unspiked filter and support pad added to the media, and the samples extracted and assayed for endotoxin in exactly the same manner as the air samples. Three samples were evaluated for each endotoxin solution:extraction media:filter type. Baseline controls consisted of each extraction solution spiked with particulate or aqueous endotoxin. All samples were diluted serially 10 fold in PFW and assayed by the LAL assay. To determine if the buffer or the Tween 20 extraction solutions interfered or enhanced the response of the LAL assay a positive product control assay (ppc) was done. This assay consisted of spiking each of the extraction solutions and serial 10 fold dilutions in PFW with 0.5 endotoxin units (EU) /ml and determining the recovery of the spike.

SAS statistical software (SAS Statistical Software, 1995) was used for all statistical analyses. An ANOVA was performed on the extraction solution, filter type, and their interaction using a general linear model to account for imbalances in the experimental design. As part of the analysis, the least square mean, standard deviation and coefficient of variation were computed as a function of diluent and filter type. Due to considerations for generalizability, computations for the air samples, interaction controls and baseline controls were conducted separately for the particulate and aqueous experiments.

RESULTS AND DISCUSSION

The results for the positive product control (PPC) experiments are shown in Table 1. Recovery of both the particulate associated and aqueous endotoxin preparations was inhibited in the undiluted buffer samples. For both the particulate and aqueous preparations the inhibition was overcome, according the U.S. Food and Drug Administration criteria (BioWhittaker), by a 10 fold dilution of the buffer with PFW. For all studies using filters in which the buffer was the extraction solution, samples were diluted a minimum of 10 fold prior to assay so the inhibition in the undiluted buffer was not a factor. No inhibition was observed for either the particulate or aqueous preparations in the Tween 20 extraction solutions. Milton et. al. has recommended the use of this buffer as the extraction medium of choice, however, based on these results environmental samples extracted with the 0.05 M potassium phosphate/0.01% triethylamine pyrogen-free buffer solution should be diluted a minimum of 10 fold prior to assay.

The recovery of endotoxin from the baseline control extraction solutions was compared for both the particulate and aqueous preparations. For particulate spiked extraction solutions there was no significant difference in the endotoxin concentration for the Tween and buffer solutions (Table II). However, the recovery from PFW was statistically lower than the either the Tween or buffer solutions. These data would suggest that PFW extracts endotoxin less efficiently from the particulate material than either of the other two solutions. Tween 20 is a surfactant and may enhance the solubilization of the particulate associated endotoxin and allow a higher recovery than that observed with PFW. The buffer solution contains charged ions and triethylamine both of which may also enhance the solubility of particulate associated endotoxin. For aqueous spiked extraction solutions, the means for the baseline controls were all statistically different (Table III). These differences, while small, are likely to reflect a combination of experimental error and differences in the interaction of the aqueous endotoxin with the extraction solutions. For subsequent analysis, each extraction solution baseline control, for both particulate and aqueous spiked samples, was used independently to calculate recovery efficiency for air samples and interaction controls for that extraction solution.

Particulate Air Samples

The mean recovery for particulate spiked air samples are given in Table IV and Figure 1. Recovery efficiencies using PFW range from 53-154 % with the highest recoveries obtained using glass fiber filters. Recoveries for glass fiber were statistically higher than those for PVC. Recoveries from both the glass fiber and PVC were significantly higher than for the Teflon coated glass fiber filters. For Tween 20, recoveries ranged from 25-110% with the highest recoveries again observed for glass fiber, but there was no statistical difference in the recovery using PVC filters. Recoveries from TCGF extracted with Tween 20 were statistically lower than the other two filters. It appears that recoveries of particulate associated endotoxin is greater from GF filter that from PVC filters and that PFW yields a higher extraction efficiency regardless of the filter type. Regardless of the filter type the recovery of endotoxin from filters extracted with the phosphate triethylamine buffer were significantly less than for the other extraction media. These results suggest either an inhibition of endotoxin by the buffer or an enhanced interaction (adsorption) of endotoxin with the filter media. Because samples were diluted at least 10-fold prior to assay it is unlikely that the buffer is inhibiting the response of the LAL assay.

Particulate Interaction Controls

The mean recovery for particulate interaction controls are given in Table V and Figure 2. The trend in recoveries was similar to those observed for the particulate spiked air samples. For samples extracted with either PFW or Tween 20, recoveries were highest from glass fiber filters. For these two extraction media there was no difference between recoveries from PVC and TCGF, however recoveries from these filters was less than obtained from glass fiber. As with the air samples, the phosphate triethylamine buffer significantly inhibited the interaction controls, regardless of filter type. The recovery efficiency for the buffer extracted samples ranged from 16-22%. Comparison of the interaction controls with the spiked air samples (Figures I and II) suggest that air sampling reduced the recoverable endotoxin from the TCGF. A similar trend was not observed for either glass fiber or PVC.

Aqueous Air samples

The mean recovery for aqueous spiked air samples are given in Table VI and Figure 3. For both aqueous spiked air samples and interaction controls recoveries, regardless of filter type, from phosphate triethylamine buffer were significantly lower that from other extraction media. As for the particulate spiked samples, extracted samples were diluted 10 fold in PFW so the buffer did not interfere with the assay. These data support the postulate that the buffer enhances the interaction of endotoxin with the filter media making it unavailable for the LAL assay. In contrast to the particulate spiked air samples, the highest recoveries of the aqueous spike were from PVC for both PFW (225%) and Tween 20 (132%). The high recovery for the PVC filter extracted with PFW cannot be explained, but reflect a combination of filter interactive effects and non-random experimental error. Teflon coated glass fiber and glass fiber filters were comparable (not statistically different)when extracted with PFW (38-56%) and Tween 20 (75-80%). These data suggest that PVC may be the media of choice for the collection of airborne aqueous samples for endotoxin analysis.

Aqueous Interaction Controls

The mean recovery for aqueous spiked interaction controls are given in Table VII and Figure 4. The spiked interaction controls showed a different pattern of recovery than the spiked air samples. Recoveries from PFW or Tween 20 extracted filters were similar for PVC (162 and 101%), TCGF (97 and 89%), and GF 158 and 211%) whereas in the spiked air samples recoveries were highest for PVC. As with several of the other spiked samples some of the recoveries were unexplainably high. These levels likely reflect the variability in sample preparation for the LAL assay, experimental error, and undefined interactions of the endotoxin with the different filter media and extraction solutions.

Conclusions

1. Regardless of the type of filter or endotoxin preparation, the potassium phosphate triethylene buffer recommended by Milton et al (Milton et. al., 1990) sample extraction inhibited the recovery of endotoxin.
2. For particulate samples recoveries appear to be better with GF filters and for aqueous samples recoveries appear to be better using PVC.
3. For particulate samples, there was no difference recovery trends, within a filter type, between air sampling and interactive controls, but for aqueous samples, air sampling significantly reduced the recovery efficiency of endotoxin from GF and TCGF.
4. This study confirms previous studies which have shown that filter type, extraction media and the makeup of the endotoxin are important variables in determining the recovery of endotoxin from environmental samples.
5. These data suggest that the LAL assay is a highly variable analysis procedure and that influences caused by the variables studied in this research project must be addressed and international standardization and validation are necessary in order to compare results from different laboratories.

References

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Table I. Recovery of Endotoxin Spiked into Serial Dilutions of Buffer Solution

Potassium Phosphate Buffer (pH = 7.5)	Recovery of Spike
Dilution Factor = 1	36 %
Dilution Factor = 10	91 %
Dilution Factor = 100	100 %

Table II. Mean & P-Value Matrices for Particulate Spiked Baseline Controls

Ext. Sol.	Mean (EU/mg)	PFW	Buffer	Tween
PFW	10879	*	0.0013	0.0162
Buffer	16756	0.0013	*	0.3071
Tween	15066	0.0162	0.3071	*

Table III. Mean & P-Value Matrices for Aqueous Spiked Baseline Controls

Ext. Sol.	Mean (EU/mL)	PFW	Buffer	Tween
PFW	214.6	*	0.0004	0.0001
Buffer	172.9	0.0004	*	0.0001
Tween	287.9	0.0001	0.0001	*

Table IV. Recovery Efficiency (%) for Particulate Spiked Air Samples

	Filter Type					
	PVC		TCGF		GF	
Ext. Sol	Mean	CV	Mean	CV	Mean	CV
PFW	102	20	53	30	154	14
Buffer	9	32	6	43	8	40
Tween	76	20	25	34	110	26

PVC-Polyvinyl chloride Ext. Sol.-Extraction solution
TCGf -Teflon coated glass fiber PFW-Pyrogen-free water
GF-Glass fiber CV-Coefficient of variation

Table V. Recovery Efficiency (%) for Particulate Spiked Interaction Controls

	Filter Type					
	PVC		TCGF		GF	
Ext. Sol	Mean	CV	Mean	CV	Mean	CV
PFW	78	9	104	12	184	7
Buffer	17	4	16	14	22	9
Tween	105	9	85	10	123	26

PVC-Polyvinyl chloride Ext. Sol.-Extraction solution
TCGf -Teflon coated glass fiber PFW-Pyrogen-free water
GF-Glass fiber CV-Coefficient of variation

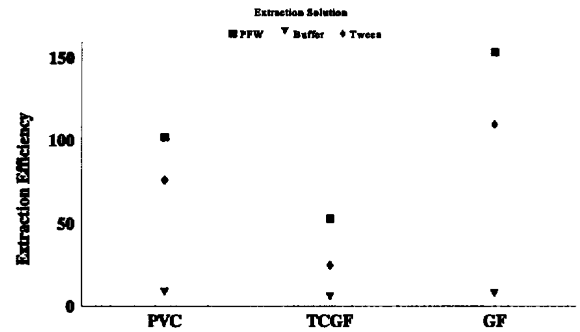

Figure 1. Recovery efficiency for Particulate Spiked Samples.

Table VI. Recovery Efficiency for Aqueous Spiked Air Samples

	Filter Type					
	PVC		TCGF		GF	
Ext. Sol	Mean	CV	Mean	CV	Mean	CV
PFW	225	26	38	42	56	27
Buffer	4	65	2	71	1	17
Tween	132	46	75	11	80	22

PVC-Polyvinyl chloride Ext. Sol.-Extraction solution
TCGf -Teflon coated glass fiber PFW-Pyrogen-free water
GF-Glass fiber CV-Coefficient of variation

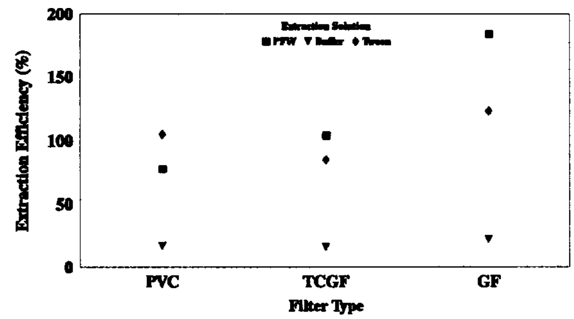

Figure 2. Recovery Efficiency for Particulate Spiked Interaction Controls

Table VII. Recovery Efficiency (%) for Aqueous Spiked Interaction Controls

	Filter Type					
	PVC		TCGF		GF	
Ext. Sol.	Mean	CV	Mean	CV	Mean	CV
PFW	162	22	97	31	158	2
Buffer	7	65	2	47	7	51
Tween	101	18	89	2	211	9

PVC-Polyvinyl chloride Ext. Sol.-Extraction solution
TCGf -Teflon coated glass fiber PFW-Pyrogen-free water
GF-Glass fiber CV-Coefficient of variation

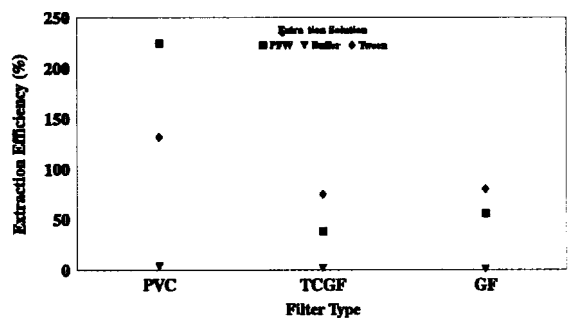

Figure 3. Recovery Efficiency for Aqueous Spiked Air Samples

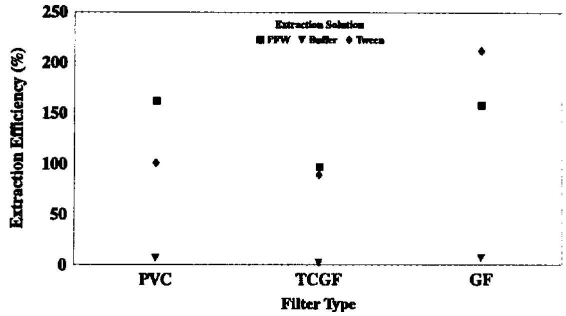

Figure 4. Recovery Efficiency for Aqueous Spiked Interaction Controls

<u>Hollander, A., D. Heederik, P., P. Versloot, and J. Douwes</u> : Extraction-rotating platform, 60 min, **not centrifuged** Measurements dependent on choice of dilution factor **boundary effects** of standard curve
interlaboratory comparisons can not be made when samples have different dilution levels.

<u>Douwes et. al.</u> **-** More than **50 % of the variation in endotoxin concentration was explained by extraction media (PFW,Tween)**, 6% was filter type.
Samples were parallel samples of potato dust, and house dust

this study looked at log transformed values
glass fiber, Teflon, and polycarbonate filters - two fold increase in detectable endotoxin compared to cellulose ester. **No significant differences in Teflon, glass fiber and polycarbonate filters.**

Both the ***Gordon study and the Douwes study,*** *the glass fiber filters yielded the highest extractable endotoxin.*

Both the Gordon study and the Douwes study ***concluded that different interactions exist for the different matrices of endotoxin with filter type and extraction solution and that comparisons may be hard to make between different dusts.***

Olenchock (1989)**-** bulk samples particulate oat dusts and spring wheat dusts
PFW higher recovery than albumin, bovine serum and other diluents. **Tween** higher than **PFW**

Milton (1990)**-** animal cage cleaning,
Extraction **Buffer , sonication**, instead of rotating platform
Inactivation of LPS by filter type. PVC, Polyflon, Teflon, Cellulose mixed ester did not specifically address support pad.