Endotoxin Epidemiology and Exposure Assessment

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Technical Report on Airborne Endotoxin Measurement Validation Studies

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List of Abbreviations

3OHFA: 3-hydroxy fatty acid

ATCC: American Type Culture Collection

C: Celcius

cfm: cubic feet per minute

CSE: control standard endotoxin

EU: endotoxin unit

GC-MS: gas chromatography – mass spectrometry

GSD: geometric standard deviation

h: hour

HEPA: high efficiency particle arrestance

KLARE: kinetic Limulus assay with resistant-parallel-line estimation

LAL: Limulus amebocyte lysate

LPS: lipopolysaccharide

M: Molar

MEAD: median endotoxin activity aerodynamic diameter

Min: minute

ml: milliliter

MMAD: mass median aerodynamic diameter

MOI: microoriface impactor

ng: nanogram

OD: optical density

RAM: real-time aerosol monitor

RSE: reference standard endotoxin

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Significant Findings

Endotoxin measurements are indicators of biological activity, not the amount of LPS in an air sample. The biological activity detected by the Limulus assay is dependent on the filter media and the extraction method. Some extraction methods result in higher estimates of endotoxin biological activity – without increasing the amount of LPS extracted from the filter. Thus, accurate comparison on the endotoxin methods requires that external methods of validating the analysis be included in the experimental design. When that was done, we found that collection on polycarbonate filters and extraction with a phosphate-triethylamine buffer produced the highest yields of LPS and endotoxin.

Usefulness of Findings

Endotoxin is an important respiratory toxin. Occupational exposures to high levels of endotoxin occur in agriculture, machining, and wherever contaminated water becomes airborne. Increasing evidence suggests that even low levels of endotoxin encountered in the home environment may be problematic for susceptible individuals. Yet, because measurement of endotoxin is not standardized, no standards for endotoxin exposure have been established. This leads the way to establishing valid methods that will allow consistent measurements to be obtained across a wide range of environments. It is an essential step toward understanding and controlling endotoxin exposures.

Abstract

The optimal and standardized methods for measuring endotoxin have not been established. Several publications have suggested that one or another combination of collection and extraction methods are superior. However, the results of these studies give mostly contradictory signals. The discrepancy between studies and the need for better understanding and standardization of the methods is nowhere clearer than in the area of indoor air quality where different laboratories have reported more than 100 fold differences in the "normal" levels of airborne endotoxin.

In this project, we developed an aerosol chamber capable of achieving mass balance for both gravimetric measurements, and to a lesser extent, endotoxin and LPS measurements. By using gravimetric measurements and LPS measurements, we established two internal standards for comparison of the levels of endotoxin activity recovered from the aerosol chamber. We found that changes in the extraction media used for the endotoxin samples caused large shifts in the apparent potency of the starting materials. These shifts would have suggested that a nonionic detergent (Tween-20) was a superior means of extracting endotoxin from filters. However, by comparison with the internal standards, and by computing yields based on a mass balance, we were able to show that this appearance was merely an artifact of Tween-20's interference with the Limulus assay. The highest valid yields of both endotoxin activity and LPS were achieved by collection of aerosol on capillary-pore polycarbonate filters and extraction in a phosphate triethylamine buffer.

Methods

Aerosol Chamber Design and Operation

We designed and built an aerosol calibration chamber to allow simultaneous examination of various devices for measuring endotoxin (Figure 1). The chamber has multiple sampling ports enabling up to 12 simultaneous samples to be collected. The chamber volume is approximately 350 liters. Air entering the chamber is filtered through two roughing filters and a high efficiency particulate air filter (HEPA). The aerosol is introduced at the centerline of a straight 6-inch clear plastic duct immediately upstream of the chamber. To assure smooth flow streamlines, there are no flow obstructions within 10 diameters upstream and downstream of the aerosol charging point. We generated aerosols with a McGraw Solo-Sphere Nebulizer operated to deliver from 30 to 50 grams (or ml) of aerosol per hour with airflow to the nebulizer of 10 to 30 liters/minute. The chamber was operated under slightly negative pressure conditions (0.1 to 0.5 inches of water) by adjusting the exhaust and flow dampers. Negative pressure in the chamber and return side of the loop pulls room air into the system prior to the HEPA filter. This excess air is exhausted prior to injection of aerosol so that recirculation in the loop is only partial, avoiding buildup of water vapor during experimental runs. Airflow was measured at regular intervals during chamber operation in both the inlet and outlet ducts using in-line venturi meters with draft gauges (manometers).

Chamber airflow between 150 to 400 cubic feet of air per minute (cfm) {4,300 to 11,300 liters/minute} was achieved with constant particle concentrations of 1 to 4 mg/m^3 using saline aerosols demonstrated by use of a continuous particle counting MiniRAM. Under these conditions, approximately 12 to 32 air changes per minute ventilate the chamber. The air flow pathway in the chamber, and turbulent conditions assure excellent mixing and maintain the aerosol suspension. Rapid drying of the aerosol after injection into the air stream results in small particle sizes (MMAD 0.4 µm, GSD 2.6, endotoxin MEAD 0.5 µm, GSD 2.5) in the chamber. Wall deposition is minimal at these particle dimensions because of insufficient time for settling or diffusion and low probability of impaction. The sample collection devices (up to 12 – cassettes plus a microorifice impactor for these experiments) were placed in the upward flow section of the chamber. The sampling devices were immersed directly in the airflow carrying the aerosol particles. Thus, there is no pre-device particle loss (such as losses to the walls of the sampling tubing, etc.). Each sampling device directly samples the aerosol simulating field conditions.

The maximum sampling rate summed over the multiple sampling ports is less than 100 liters/minute or less than 1% to 2% of the total chamber flow. Therefore, sampling has a negligible impact on aerosol concentrations. Air from the chamber is pulled through the sampling devices using personal sampling pumps typically used for worker personal air monitoring. The chamber was run from two to four hours at steady-state conditions. This length of operation assured smoothing of any momentary operational or sampling abnormalities.

The air supply to the nebulizer was filtered though sintered metal in a liquid knockout trap and a final HEPA filter. Testing for endotoxin levels in the chamber between runs

including the addition of air through the nebulizer using pyrogen free water found endotoxin levels five to six orders of magnitude less than those in the chamber during experiments. These results compared favorably with background and blank analysis which ranged from "limit of quantification" to 0.25 EU/m^3 endotoxin. Therefore, the design of each recovery experiment provides sufficiently elevated concentrations of each component, to make background and blank concentration negligible with respect to experimental concentrations. The room air where the chamber is located was also sampled during operation of chamber. Background endotoxin concentrations in the lab air were low (at normal indoor background levels) when experiments were performed using concentrated endotoxin in the chamber, indicating that the chamber did not leak into the room air. In the experiments described here, the 12 samples collected from each experimental run were usually divided among several tests of two or more test factors.

Saline, LPS and Bacterial Aerosol Solutions and Expected Air Concentrations

Two types of aerosols were generated: a) pure LPS and b) washed whole gram negative bacteria. The LPS was *E. coli* O55:B5 (Difco), and the bacteria were from the same species (obtained from ATCC) grown in trypticase-soy broth and washed twice with normal saline. The nebulizer solution for LPS experiments was prepared by mixing 20 gm NaCl, 12.5 ml rhodamine dye, 15 mg of LPS, and bring up to 500 ml with Baxter pyrogen free sterile water. The nebulizer solution for aerosolization of washed bacteria was made by replacing the LPS with a pellet of washed bacteria with an approximate volume of 80 ml.

The expected aerosol concentrations in the chamber were computed for each experimental run as follows. The dry nebulizer was weighed and then weighed again loaded with the nebulizer solution both before and after the run. Three 10-ml samples of the nebulizer contents were placed in preweighed vials. The samples were taken before the preweighing and after the postweighing of the loaded nebulizer. The three samples were each weighed and used to calculate density of the nebulizer fluid. Each of the samples was then dried in an 80°C oven for 24 hours and weighed again to determine the residual mass of solute. The amount of particle mass introduced into the air stream was then computed as:

$$M_a = r_1(N_1 - N_0)/d_1) - r_2(N_2 - N_0)/d_2)\,, \qquad (1)$$

where r_i is the residual mass, d_i is the density, and N_i is the nebulizer weight, and $i = 0$ for the empty nebulizer, 1 for the start of the run, and 2 for the end of the run. The amount of endotoxin or LPS introduced into the air stream was computed by replacing the residual mass by the average amount of endotoxin (or LPS) measured in samples of the before and after nebulizing fluid. The average was used because the before and after endotoxin concentrations did not differ significantly for any of the experiments. The air concentration in the chamber was computed by dividing the aerosolized amount by the total airflow during the experiment.

Endotoxin Measurement

To measure the endotoxin potency of nebulizer solutions and filter extracts, we used the kinetic Limulus assay with resistant-parallel-line estimation (KLARE) method, reported in detail by Milton and colleagues[1, 2]. Control standard endotoxin and samples were serially diluted in the same medium, either a standard buffer (0.05 M potassium phosphate, 0.01% triethylamine pH 7.5), water, or Tween-20. Duplicate 50 µl aliquots of five serial dilutions of sample and standard were placed in a 96-well, flat-bottomed polystyrene microplate with 50µl of LAL and agitated. The optical density (OD) of each well was recorded at 405 nm every 30 sec for 90 to 120 min. Endotoxin potency was computed by the parallel-line bioassay method[3]. The response parameter for each well of the microplate was the maximum rate of OD change computed as the maximum of the first derivative of a smoothing spline[4] fit to the OD data. The log potency, and its variance was computed using resistant regression. Possible interference from substances in each sample, such as detergents, was detected by analysis of covariance and a standard algorithm will be used to eliminate such effects during data analysis[1]. All results are reported in endotoxin units with reference to EC6 reference standard endotoxin (U.S. Pharmacopoeia, Inc., 1 ng EC6 = 10 endotoxin units, EU).

LPS Measurement

To measure the amount of LPS in nebulizer fluids and filter samples, we measured the amount of 3-hydroxy myristic acid in the samples. The *E. coli* used in these experiments produces LPS containing only this one 3-hydroxy fatty acid (3OHFA).

Tridecanoic acid hydroxylated in position 3 (3-OH 13:0) was purchased from Larodan Lipids (Malmo, Sweden). Bis(trimethylsilyl)trifluoroacetamide (BSTFA, 98%), dichloromethane (p.a. stabilized with 50 ppm amylene), diethyl ether (p.a.), and acetonitrile (99%) were from Janssen Chimica (Geel, Belgium). Methanol (p.a.) and n-hexane (99%) were from Lab Scan (Dublin, Ireland), acetyl chloride (p.a.) and pyridine(p.a.) from Merck (Darmsatadt, Germany) and triethylamine (TEA) from Sigma (St. Louis, MO). All chemicals were used without further purification. The glass test tubes (equipped with PTFE-lined screw-caps) were heated at 350°C overnight before use.

Methanolic HCl (1.3 and 3.6M) was prepared by adding acetyl chloride (1 to 3 ml) dropwise to methanol (9 or 7.5 ml) at 0°C, and 2% TEA solution was prepared by mixing 980 µl of acetonitrile with 20 µl TEA. All reagents were stored at 4°C in glass tubes with PTFE-lined screw caps and used within one week of preparation.

Details of the GC-MS analysis were previously described[5]. Briefly, a VG Trio-1S GC-MS system (Manchester, UK) was used. The gas chromatograph was a Hewlett-Packard model 5890 (Avondale, PA) equipped with a fused-silica capillary column (25 m x 0.25 mm I.D.) containing cross-linked OV-1 as the stationary phase. Injections were made using a Hewlett-Packard model 7673 autosampler in the splitless mode. Helium was used as the carrier gas, at an inlet pressure of 7 psi, and a temperature of the column was programmed from 120 to 260°C at 20°C/min. Both the injector and the interface (between GC and MS) were kept at 260°C. TMS derivatives were analyzed in the EI mode (ion source temperature 220°C) Isobutane (1 psi) was used as the reagent gas;

ionization was performed at 70 eV. Results of 3OHFA analyses were converted to ng of LPS by assuming that each mole of LPS contained 4 moles of 3-OHFA, and that the average molecular weight of the LPS was 8000 daltons.

Air Sample Collection

Air samples were collected in closed face, polystyrene cassettes with either 0.4-µm pore polycarbonate filters (Nuclepore), or GFA glass fiber filters (Whatman). Filters were pre and postweighed on a Cann 21 electrobalance after 24 h equilibration at 50% relative humidity, 20°C. Each sampling train was pre and post calibrated with a bubble meter (Gillibrator). Gilian 513 and Gilair pumps were used most frequently at sampling rates of 2 liters/min.

Filter Extraction

Filters for Limulus assay were extracted by bath sonication for 1 h with vortexing at 15 min intervals, or by agitation for 1 h in a wrist action shaker. The extraction medium was either the standard phosphate-triethylamine buffer, water, or 0.05% Tween-20.

Extracts for GC-MS analysis of fatty acids were made by two methods. First, 1 ml of buffer extracts prepared for the Limulus assay were lyophilized then subjected to methanolysis. Alternatively, filters were directly subjected to methanolysis without prior buffer extraction. The procedure consisted of adding 50 ng of the internal standard 3-OHFA: 13:0, to the sample (lyophilized extract or filter) in a glass reaction vessel. Ten ml of 3.6 M methanolic HCl was added and the sample was heated to 100°C for 18 hours. After methanolysis was complete the methyl esters were extracted and washed as described by Z. Mielniczuk et al. [5]. Trimethylsilyl (TMS) methyl ester derivatives were formed by adding 50 µl of BSTFA and 5 µl of pyridine and heating to 80°C for 15 minutes.

Data Analysis

Yield for gravimetric, endotoxin, and GC-MS analyses of filters was computed by dividing the observed air concentration by the expected concentrations. Yields were Gaussian distributed, and analyzed in linear models controlling for effects of experiment and sampling port using SAS Proc Mixed.

Results

Analysis of 13 chamber experiments found that the sampling ports provided equivalent mass sampling results (range for mass yields from each sampling port were 80% to 83%) with small differences in gravimetric yield that were not statistically significant. Additionally, real-time, port-to-port particle mass measurements using a modified MIE MiniRAM with active sampling rate of 1.5 liter/min, found all ports to agree within ± 5%. There were no significant port-to-port differences in endotoxin yields. Particle size distribution was measured using a Particle Measuring System Active Scattering Aerosol Spectrometer Probe as well as using a MOI impactor [6].

Tables

Table I shows the yields from gravimetric and Limulus assay for experimental aerosols collected on 0.4-μm capillary-pore polycarbonate membranes and extracting with 0.05 M potassium phosphate, 0.01% triethylamine buffer pH 7.5.

Comparison of different Limulus assay methods required a further control. It was observed that the endotoxin activity in the nebulizer at the start of the experiment was a function of the diluent used in the Limulus assay (Table II). Both the phosphate-triethylamine buffer and Tween-20 inhibited the reaction of Limulus amebocyte lysate with the reference standard LPS, but did not inhibit the reaction with the LPS used in the nebulizer solution. As a result, the nebulizer appeared to contain much higher amounts of endotoxin activity when assayed in a dilutent other than water.

To correct for this effect of the extraction and dilution media on the Limulus assay, we computed the amount of endotoxin activity expected in the aerosol separately for each of the extraction media. Thus, because Tween-20 made the endotoxin appear more potent, we expected to obtain higher values for endotoxin activity from the aerosol when analyzed in Tween-20. As shown in Table 3, the observed endotoxin activity in the aerosol samples was highest when Tween-20 was used. However, when yield was computed based on the expected amounts, the yield was significantly higher for samples extracted and analyzed in buffer than in either water or Tween-20.

Similar methods were used to compare the yields from aerosols of whole bacteria (*E. coli* O55:B5, Tables 1 and 4). The experiment shown in Table 4 examined endotoxin activity and LPS mass yields from polycarbonate membranes and glass fiber filters comparing water and buffer extraction media. The yields were similar for buffer and water extractions, however, yields from polycarbonate membranes were greater than from glass fiber filters. It should be noted, however, that crude endotoxin bioactivity of the aerosol was greater with water extraction, due to the effect of buffer on potency of the nebulized bacteria. Therefore, had we not used these well controlled methods, we would not have identified the method with highest yield.

An additional finding of the experiments with washed bacteria was that the yield of biological activity was higher than the yield of LPS mass from the aerosol samples. LPS mass yield was similar for pure LPS and whole bacterial aerosols. A comparison of EU/ng of LPS in the aerosol samples and nebulizer solutions suggested that the bacteria were rendered of higher potency by the process of nebulization and collection, while pure LPS was not.

Conclusions

Measurement of endotoxin in aerosol samples is not a measurement of an amount of substance, but a measure of the degree or level of biological activity. The biological activity depends on factors other than the amount of LPS present in the sampled aerosol. Therefore, it is essential that the conditions of the assay be strictly controlled. Among the conditions that must be controlled are the lot of the LAL reagent, and the collection and extraction media. Without external means of identifying the amounts of substance recovered, as in these experiments, comparisons of levels of biological activity between

different methods are meaningless. Thus, much of the literature published thus far comparing endotoxin measurements using different filter media and extraction methods cannot be considered reliable indicators of the optimal methods for airborne endotoxin measurement. This study provides methods for establishing definitively the optimal methods. The results of these experiments indicate that collection on polycarbonate filters and extraction in a phosphate-triethylamine buffer is the best available method.

Acknowledgements

The design and execution of the aerosol chamber experiments was made possible by the collaboration of several students and colleagues: Michael Walters, ScD; Daryl Johnson, MS; Ju-Hyeong Park, MS; and Yi-Jen, Tseng, MS.

Tables

Table I

Summary of Aerosol Experiments Using Polycarbonate Capillary Pore Membranes and Buffer Extraction

Aerosolized Material	Runs	Samples	LAL Activity* % Yield	Gravimetric Mass* % Yield
Pure LPS	10	68	20 (16-25)	
Washed Bacteria	4	27	44 (38-49)	
Saline	13	128		81 (75-86)

Mean % yield (95% Confidence Interval)

Table II

Endotoxin Potency of Nebulized Solution: Effect of Assay Diluent*

	Water	Buffer	0.05% Tween-20
Before	305	436	948
After	356	494	990

* EU/ml $\times 10^3$

Table III

Recovery of Endotoxin Activity from Aerosolized LPS

Extraction Media	Expected*	Observed†	O/E‡ %
Water	21.6	3.9 (0.6)	18 (12-24)
Buffer	30.4	8.3 (1.0)	27 (20-34)
Tween-20	62.0	12.4 (0.2)	20 (19-21)

* EU/m^3 $\times 10^3$; † Mean of 4 replicate samples EU/m^3 $\times 10^3$ (SD); ‡ Mean % yield (95% CI)

Table IV

Washed Bacteria-Saline Aerosols:
Endotoxin yields for various filter and extraction media

Filter type	Extraction solution	Bioactivity*	LPS Mass*	n§
PC†	Buffer	39 (37-40)	26 (15-36)	3
	Water	38 (29-47)	24 (18-30)	3
GF/A‡	Buffer	18 (16-21)	11 (2.0-20)	3
	Water	24 (21-26)	13 (11-15)	3

* Mean % yield (95% Confidence Interval), bioactivity by KLARE method, LPS mass by GC-MS for 3-hydroxy fatty acids in the extraction media; † Polycarbonate filter; ‡ Whatman Glass fiber filter/A; § number of samples for each filter extraction codition.

Figure Legends

Figure 1: Schematic for aerosol chamber.

Figure 2: Effect of Dilutent on Limulus Reaction Rate: Reference standard endotoxin (RSE, USP EC5), and control standard endotoxin (CSE, Associates of Cape Cod) were serially diluted in Tween-20, water, or buffer and assayed with Limulus amebocyte lysate. Panels A and B show assays performed in one lot of LAL, while panels C and D show assays in a second lot of LAL from the same manufacturer.

Figures

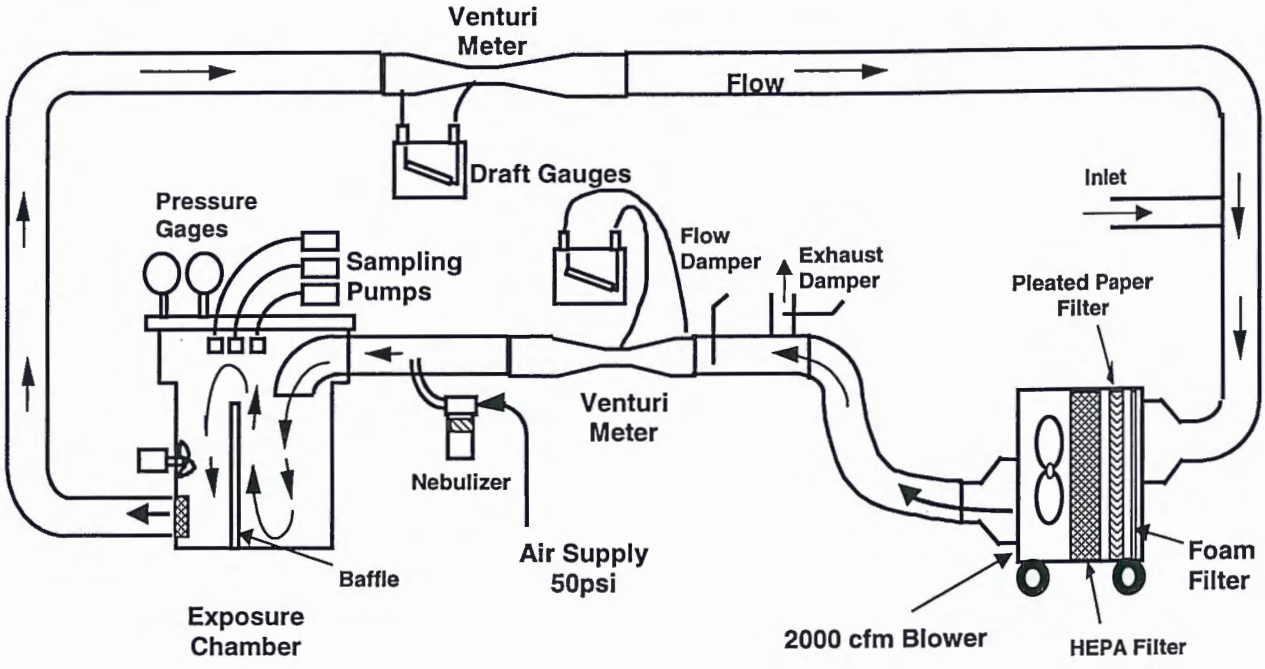

Figure 1

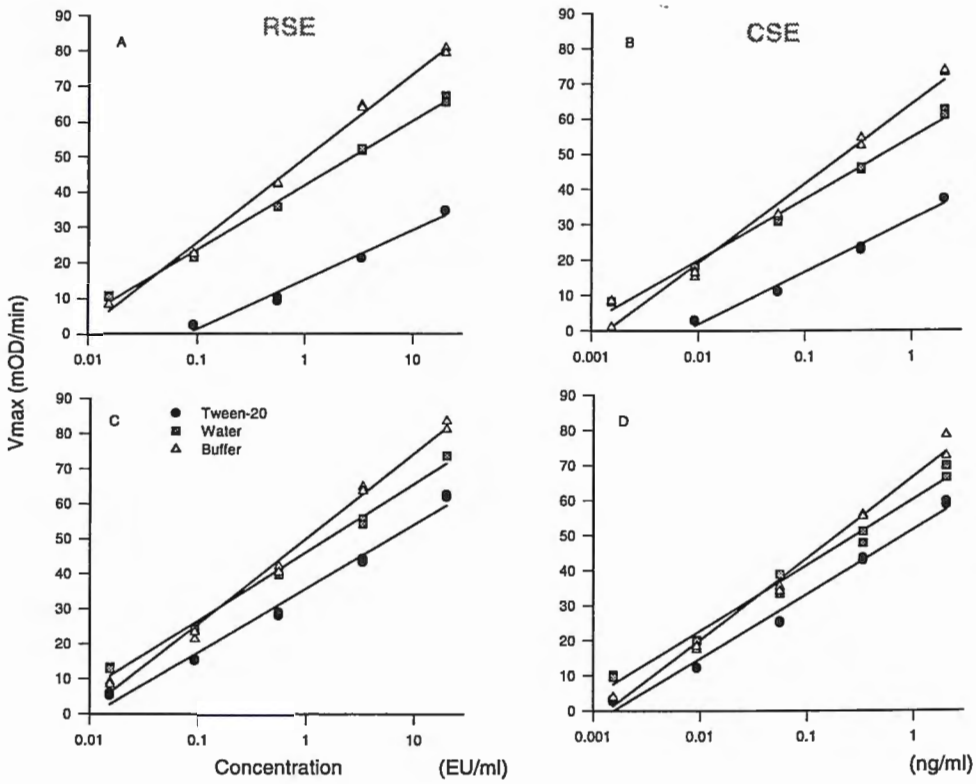
RSE
CSE
A
B
C
D
Tween-20
Water
Buffer
Vmax (mOD/min)
Concentration
(EU/ml)
(ng/ml)

Figure 2

Literature Cited

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