# Optimization and Influence Mechanism of Sampling and Analysis of Airborne Endotoxin Based on Limulus Amebocyte Lysate Assay

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## ABSTRACT

Airborne endotoxin, a bioaerosol component of Gram-negative bacterial cell walls, is a considerable risk to human health. In this study, a systematic optimization and the analysis of corresponding influence mechanism based on the *Limulus* amebocyte lysate assay were operated by changing sampling duration, sonication pretreatment, extraction solution, and impinger types. Moreover, the corresponding influential mechanisms of these four factors were identified. Experimental results showed that endotoxin concentration tended to increase initially and then declined over time, and that the extraction solution reached saturation after 15 min of sampling. The majority of the cells were disrupted by an ultrasonication pretreatment of less than 800 W, allowing the detection of free endotoxins by the *Limulus* amebocyte lysate assay. However, a sonication power greater than 800 W could destroy endotoxin structure. Furthermore, the lipophilic and hydrophilic groups in the molecular structure of Tween 20 promoted endotoxin dissolution. Three samplers with different pore sizes and aperture numbers were compared. The results showed that collection efficiency was directly proportional to nozzle aperture size. Small pore sizes and high aperture numbers enhanced airborne endotoxin absorption because they could generating more bubbles with small specific surface area, thereby increasing the interaction between the endotoxins and extraction solution and improving absorption efficiency. Therefore, an optimized sampling method was proposed that collecting air with an AGI-30 impinger and pyrogen-free, sterile purified water (PFW) containing 0.05% Tween 20 at a sampling duration of 10 min. The sample was then sonicated at 800 W for 10 min.

*Keywords:* Airborne endotoxin; *Limulus* amebocyte lysate (LAL) assay; Sampling optimization; Ultrasonication pretreatment; Influence mechanism.

## INTRODUCTION

Endotoxin, a hazardous biological substance, is an essential component of biological aerosols (Boehlecke *et al.*, 2003; Hsu *et al.*, 2012; Kallawicha *et al.*, 2015). The purified endotoxin structure is a complex of lipopolysaccharides (LPS) and proteins and is widely distributed in the outer cell wall membranes of Gram-negative bacteria and other microorganisms (e.g., chlamydia, rickettsia, and spirochetes) (Nilsson *et al.*, 2011). After the death of a bacterium, embedded LPS is released into the air to combine with other biological and non-biological particles, thus forming airborne, stable endotoxins that accumulates in the air (Oldenburg *et al.*, 2007l Tianjia *et al.*, 2014). Accumulated airborne endotoxins adversely affect human health because LPSs are composed of lipid A, core oligosaccharides, and

O-side chains (Oppliger *et al.*, 2005) (Fig. 1). The toxicity of lipid A and the antigenicity of the core polysaccharide is recognized by the immune system, resulting in fever, leukocyte reaction, Shwarztman response, and disseminated intravascular coagulation (Bin *et al.*, 2007; Sean *et al.*, 2010). Chronic exposure to endotoxins can induce and exacerbate various respiratory symptoms (Angelico *et al.*, 2016), such as asthma, cough, and impaired lung function, leading to chronic obstructive pulmonary diseases and to lung diseases caused by organic dust (Reiman *et al.*, 2000; John *et al.*, 2006).

An effective detection method is needed to understand the content and nature of airborne endotoxins. Some common endotoxin detection methods include the rabbit pyrogen test method, *Limulus* amebocyte lysate (LAL) assay (Oppliger *et al.*, 2005; Cristiane *et al.*, 20113; Frederic *et al.*, 2013; Lin *et al.*, 201), polymerase chain reaction (PCR) (Mafu *et al.*, 2009), gas chromatography coupled with mass spectrometry (GC/MS) (Saito *et al.*, 2009), recombinant factor C (rFC) method and enzyme-linked immunosorbent assays (ELISA) (Xu *et al.*, 2002; Mohanan *et al.*, 2011). Novel techniques include biomarker detection and biosensor methods. 3-hydroxy fatty acids are usually considered as the biomarkers

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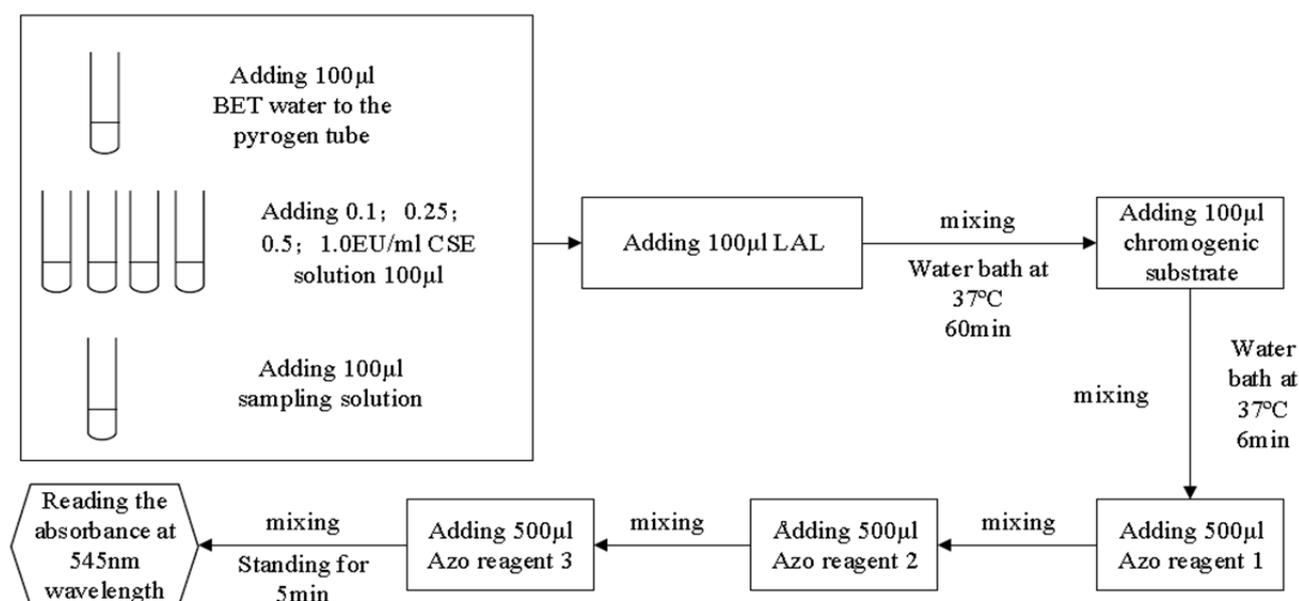  *E-mail address:* wangcan@tju.edu.cn

**Fig. 1.** Specific steps of the LAL method.

for the quantification and characterization of endotoxins using HPLC-MS (High Performance Liquid Chromatography - Mass Spectrometry) (Zhou *et al.*, 2010; Uhlig *et al.*, 2016). Biomarker detection is frequently used with LAL or ELISA. The development of biosensor is based on optical sensors, mass-based sensors, magnetic aptasensors and electrochemical techniques (Das *et al.*, 2014; Mingyan *et al.*, 2014). The characteristics of the detection methods are summarized in Table 1. The LAL assay has several advantages, such as simple operation, short reaction time, high sensitivity, and easy standardization (Kumar, 2006). Therefore, the LAL assay is extensively used to detect bacterial endotoxins in various settings, such as hospitals (Huntington *et al.*, 2007; Miao *et al.*, 2007; Dutil *et al.*, 2009), agriculture (Frederic *et al.*, 2010; Singh *et al.*, 2011; Chow *et al.*, 2014), wastewater treatment (Gwangpyo *et al.*, 2010; Schlosser *et al.*, 2011), and living environments (Milton *et al.*, 1997; Adhikari *et al.*, 2010; Aynul *et al.*, 2011; Sung *et al.*, 2016).

The operating protocols for field sampling and analysis of endotoxins are different, leaving room for individual interpretation. The sampling parameters for quantifying airborne endotoxins with AGI-30 (All Glass Impinger) are summarized in Table 2. The most frequently used set of parameters are selected based on methods and data derived from literatures (Spaan *et al.*, 2007; Duquenne *et al.*, 2013) as the template for our study's sampling method. This study aims to perform the LAL assay to analyse airborne endotoxin concentration levels by modifying four factors and to investigate their influence on the detection of airborne endotoxins.

**METHODS**

***Endotoxin Collection and Sampling***

A series of pretreatment steps was performed to remove the interference of exogenous pyrogens and improve the accuracy of experimental results. Three types of impingers (AGI-30, straight-hole gas-washing bottom, and porous-hole gas-washing bottom) were washed first with distilled water and then with ultrapure water prior to wrapping with aluminum foil. To completely remove any endotoxins, all glassware were heated at 250°C for at least 2 h in a muffle furnace. The water bath was pre-warmed to and maintained at $37 \pm 1°C$. The sampler outlet was connected to the main engine inlet by a rubber tube. The sampling medium was 50 mL of pyrogen-free sterile purified water (PFW). The impinger was located 102 cm aboveground and operated for 30 min at an air flow rate of $12.5 \ L \ min^{-1}$. The sample solution was designated as the test solution and stored in a sealed pyrogen-free flask at 4°C. A similar collection procedure was reported by previous studies (Zhang *et al.*, 2007; Yu *et al.*, 2016).

***Endotoxin Analysis Using the LAL Assay***
*LAL Reaction Mechanism*

Gel-clot technique and spectrophotometry are two types of LAL assays. Gel-clot technique can be further divided into the turbid metric assay and chromogenic assay. The endpoint chromogenic assay, which uses synthetic peptides as cleavage substrates for the clotting enzyme and causes a color change in the reaction mixture (Lin *et al.*, 2013), is conducted in our study. The reaction mechanism of the endpoint chromogenic assay is described in the following.

The LAL reagent is extracted from the multifunctional white amebocytes of horseshoe crab blood. Bacterial endotoxins activate the pro-clotting enzyme, coagulogen, factor C, and factor B of the horseshoe crab blood (Sandle, 2012). LPS activates factor C under conducive temperature and pH and in the absence of unfavorable external conditions. Factor C then initiates the sequential activation of factor B, proclotting enzyme, and coagulogen to form the clotting

**Table 1.** The characteristics of some common endotoxin detection methods.

| Detection methods | Assessment | |
|---|---|---|
| | Advantage | Disadvantage |
| Rabbit pyrogen test method | Reflect the situation of each source of pyrogen directly | Cannot be quantified and standardized, poor reproducible results; Not suitable for large-scale testing; Rabbit response has individual differences; Low sensitivity; Longtime |
| LAL assay | Quickandeasy operation; High sensitivity; Easy to promote and standardize | Sampleswith color or high turbidity will affect the results; Preparation of reagent required to kill a large number of horseshoe crabs; Reagents are expensive and consumed quickly |
| Enzyme-linked immunosorbent assay (ELISA) | Avoid the color interference; Easy operation; Cheap | Lower detection accuracy compared to LAL method |
| Biosensor assay | Adapt to complex environment; Fast detection; High sensitivity and low detection limit | Immature technology, Not popular |
| Biomarker detection analysis | Types of LPS in the sample can be determined; High sensitivity | Endotoxin with biological effects cannot be detected accurately |

**Table 2.** Methods used in the literatures to measure airborne endotoxins by AGI-30.

| Aerosol type | Solution | Volume (mL) | Flow rate (L min$^{-1}$) | Time (min) | LAL Assay | References |
|---|---|---|---|---|---|---|
| Poultry House | PFW | 50 | 12.5 | 30 | End point | Yu *et al.*, 2016 |
| Mink Breeding Houses | PFW | 50 | 12.5 | 30 | End point | Zhong *et al.*, 2015 |
| Deer houses | PFW | 50 | 12.5 | 30 | End point | Meng *et al.*, 2015 |
| Swine houses | PFW | 50 | 12.5 | 30 | End point | Li *et al.*, 2014 |
| Campus | 0.05% Tween 20 | NI | 12.5 | 15 | Kinetic chromogenic | Cristiane *et al.*, 2011 |
| Animal barns | PFW | 50 | 12.5 | 20 | End point | Eckardt *et al.*, 2010 |
| Dental offices | 0.09% NS | 20 | 12.5 | 16 | End point | Dutil *et al.*, 2009 |
| Dental office | NI | NI | NI | NI | Kinetic chromogenic | Huntington *et al.*, 2007 |
| Duck houses | PFW | 50 | 12.5 | 30 | End point | Tian *et al.*, 2007 |
| Fox houses | PFW | 50 | 12.5 | 30 | End point | Zhang *et al.*, 2007 |
| Rabbit houses | PFW | 50 | 12.5 | 20 | End point | Duan *et al.*, 2006 |
| Dental rooms | PFW | 50 | 12.5 | 30 | End point | Miao *et al.*, 2006 |
| Sawmills | 0.09% NS | 20 | 12.5 | 15 | Kinetic chromogenic | Oppliger *et al.*, 2005 |

AGI-30: All Glass Impinger; PFW: pyrogen-free sterile purified water; NS: normal saline.

enzyme. The clotting enzyme decomposes coagulogens, thus facilitating gel production (Peter *et al.*, 2010).

*Endotoxin Analysis*

Endotoxin concentration was determined by a Chromogenic Endpoint Tachypleus Amebocyte Lysate assay reagent (Xiamen Limulus Reagent Demonstration Plant Co., Ltd., China). 100 µL of test sample or bacterial endotoxin test (BET) water was add to a pyrogen-free test tube. BET water was used as the blank sample. Afterwards, 100 µL LAL solution was added. The solution was mixed and incubated at 37°C for 60 min. After incubation, 100 µL of the chromogenic substrate solution was added. The resulting solution was mixed and incubated at 37°C for 6 min. Then,

500 µL of 1; 2; 3 azo reagent solution was added successively. The reacted solution was mixed and allowed to stand for 5 min. Absorbance was read at 545 nm after the color change stabilized.

The standard curve of absorbance versus endotoxin concentration was generated by diluting the Control Standard Endotoxin (CSE) solution with BET water provided with the Chromogenic Endpoint LAL reagent. The standard curve ranged from 0.01 to 1.00 endotoxin units (EU). In this study, the CSE solution, negative control, and samples were tested in triplicate. All the reagents used to evaluate endotoxin concentrations under different conditions were obtained from a single supplier.

Endotoxin concentration per cubic meter of air (Y) was

calculated as Eq. (1).

$$Y = \frac{cV}{QT} \tag{1}$$

where *c* was the endotoxin concentration of the extraction solution (EU mL$^{-1}$),
*V* was the volume of the extraction solution (mL),
*Q* was the air flow rate (mL min$^{-1}$), and
*T* was the sampling duration (min).

*Interference Test*

Approximately 1.0 EU mL$^{-1}$ CSE solution was added into the extraction solution, which was previously diluted twice. All air samples did not exceed the detection limit (0.005 EU mL$^{-1}$) relative to the negative control. Recovery rates within 50%–200% were considered acceptable, as suggested by the supplier of the LAL assay reagent.

*Optimization Methods*

The study aimed to determine the influence of sampling duration, ultrasonication, extraction solution, and impinger types on endotoxin detection. The sampling duration using AGI-30 to detect airborne endotoxins varied per individual study (Table 2). To explore the biological efficiency of the sampling method, this experiment implemented gradient sampling durations ranging from 5 min to 30 min. The specific parameters are listed in Table 3. To analyze endotoxin solubility over time, the parameter gas/liquid volume ratio was introduced and defined as the air volume flowing through the solution during sampling. The gas/liquid volume ratio was calculated by Eq. (2).

$$\frac{G}{L} = \frac{12.5 \times T}{V} \times 1000 \tag{2}$$

where *G/L* was the gas/liquid volume ratio,
*T* was the sampling duration (min),

*V* was the volume of extraction solution (mL).

Endotoxin forms were also considered. Theoretically, three main endotoxin forms (LPS) were presented in the air: LPSs existed on the membrane of bacteria cells, LPSs associated with other biological or non-biological aerosol particles, and free LPS molecules (Duquenne *et al.*, 2013). LPS molecules embedded in the cell membrane were not toxic and could not activate the enzymes that triggered the LAL reaction (Reynolds *et al.*, 2005; Spaan *et al.*, 2008). Ultrasonication not only broke cells to release LPS, but also separated LPS and fine particles. Given that endotoxin concentrations were designed to be monitored under different ultrasonication conditions, the test solutions were successively sonicated for 10 min under 500, 600, 700, 800, 900, and 1000 W.

PFW with Tween 20 and normal saline (NS) were frequently recommended as extraction solutions (Oppliger *et al.*, 2005; Madsen *et al.*, 2006; Dutil *et al.*, 2009; Thorne *et al.*, 2010). In this study, endotoxin concentrations were detected and evaluated under different types and different concentration gradient solution conditions.

To explore the influence of the sampler's nozzle aperture size, straight-hole and porous-hole gas bottles with structures similar to AGI-30 were selected. The specific physical parameters (bottle size, inlet/outlet diameter, and nozzle aperture size and length) of the different samplers were listed in Table 4. Only the nozzle aperture sizes of the three impingers were different, and the other parameters had relatively small differences. The details of nozzle apertures were illustrated in Fig. 2. The endotoxin concentrations of three samplers were compared with the same other conditions.

*Statistical Analysis*

The arithmetic mean, standard deviation, and range of each endotoxin measurement group were calculated. These statistical parameters are suitable for small samples and objectively reflect the situation float samples. The factorial effects on airborne endotoxin concentrations were determined

**Table 3.** Four primary influence factors on the measurement of endotoxin concentration: sampling time, ultra sonication, sampling solution, and impinger types.

| Sampling time (min) | Ultra sonication (W) | Sampling solution | Impinger type |
|---|---|---|---|
| 5 | 500 | NS | AGI-30 |
| 10 | 600 | PFW | straight hole gas washing bottom |
| 15 | 700 | PFW with 0.010% Tween-20 | |
| 20 | 800 | PFW with 0.025% Tween-20 | porous holes gas washing bottom |
| 25 | 900 | PFW with 0.050% Tween-20 | |
| 30 | 1000 | | |

PFW: pyrogen-free sterile purified water; NS: normal saline.

**Table 4.** Physical parameters of different types of samplers.

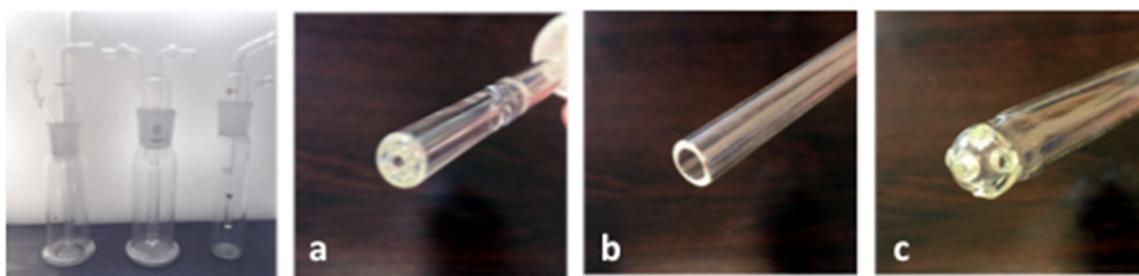| No. | Impinger type | Bottle size (mm × mm) | In/outlet diameter (mm) | Nozzle aperture size (mm) | Length (mm) |
|---|---|---|---|---|---|
| I | AGI-30 impinger | Φ36 × 233 | 10 | 1.55 | 30 |
| II | Straight hole gas bottle | Φ42 × 210 | 12 | 3.4 | 36 |
| III | Porous hole gas bottle | Φ42 × 210 | 12 | 1.8 | 36 |

**Fig. 2.** Different types of samplers and their nozzle apertures (a: AGI-30 impinger; b: straight hole gas bottle; c: porous hole gas bottle).

by one-way ANOVA (Natcha *et al.*, 2010). Each controllable factor was changed ($\alpha = 0.005$) in this step. The correlation between influential factors and variables were determined by linear regression analysis to explain the observed variability.

## RESULTS

A total of 69 airborne endotoxin samples were collected in the laboratory. Endotoxin concentrations ranged from 47 EU·m$^{-3}$ to 712 EU·m$^{-3}$. Most of the samples exceeded the recommended occupational exposure limit of 90 EU·m$^{-3}$ (DECOS, 2010). The average blank concentration of airborne endotoxins was 0.203 EU m$^{-3}$, which was considerably lower than the measured values. The standard deviation per factor was relatively small and ranged from 0.003 to 0.177 under different optimization conditions.

### Collection and Sampling Optimization

Fig. 3 showed the relationship between endotoxin concentration and sampling duration. Endotoxin concentration initially increased and then declined as sampling duration increased. Increased air flow into the extraction solution indicated that the gas/liquid volume ratio increased at a constant rate.

The gas/liquid volume ratio increased from 2083 to 4167 as sampling duration was extended from 5 min to 10 min. The airborne endotoxin concentration increased from 187 EU m$^{-3}$ to a maximum value of 209 EU m$^{-3}$, suggesting that more endotoxins were collected in the extraction solution. However, according to Eq. (1), a negative correlation existed between endotoxin concentrations and sampling duration. Endotoxin concentration decreased to 77 EU m$^{-3}$ when the sampling duration was further extended to 30 min. Therefore, longer sampling durations translated to lower endotoxin concentration.

### Pretreatment Using Sonication

The effects of ultrasonic power on endotoxin concentration were presented in Fig. 4. Endotoxin concentration initially increased and then decreased as sonication power was amplified. Airborne endotoxin concentration was 485 EU m$^{-3}$ under a minimum ultrasonic power of 500 W. Increasing the ultrasonic power to 800 W increased endotoxin concentration to the highest value of 753 EU m$^{-3}$, which was 3.6 times greater than 209 EU m$^{-3}$ measured for the untreated sample. Increasing ultrasonic power from 900 W
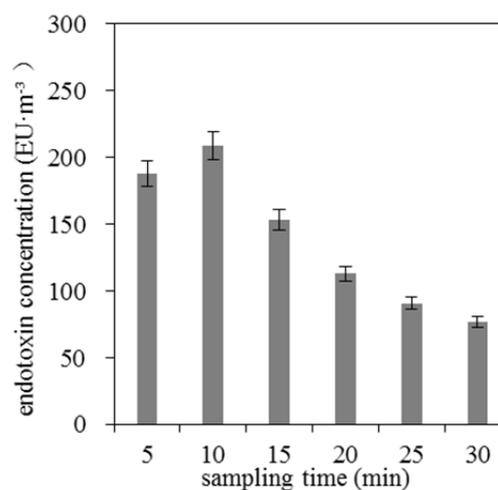


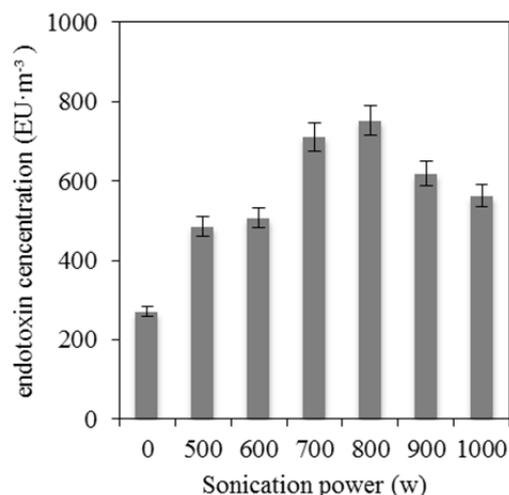**Fig. 3.** The relationship of endotoxin concentration and sampling time.



**Fig. 4.** Endotoxin concentration using different ultrasonic power pretreatment.

to 1000 W decreased endotoxin concentrations from 619 EU m$^{-3}$ to 562 EU m$^{-3}$. These results indicated that the airborne endotoxin concentration levels of the pretreated extraction solution are higher than the untreated solution. Furthermore, 800 W was the optimal ultrasonication power because it positively affected ultrasonic lysing.

## Absorption and Extraction

Different extraction solutions had different effects on detected endotoxin concentrations. NS (0.85% NaCl) could provide a suitable environment for microbial survival because its concentration was equal to the plasma osmolality in a human or animal. Tween 20 was an organic surfactant containing several hydrophilic groups in its molecular structure. It could dissolve more LPS molecules because of its compatibility and similitude. As shown in Fig. 5, NS had the lowest absorption efficiency (159 EU m$^{-3}$), which was approximately 24% lower than that of the PFW solution. Adding Tween 20 to the extraction solution significantly increased the detected endotoxin concentration from 521 EU m$^{-3}$ to 584 EU m$^{-3}$. Further investigation of the Tween 20 dosage tests revealed that the absorption of airborne endotoxin was improved with increasing Tween 20 concentration. The PFW with 0.050% Tween 20 showed the best performance, which was 2.8 times higher than that of the standard PFW solution.

## Impinger Type and Transport Condition

Fig. 6 showed the endotoxin concentrations detected with various impinger types. The average concentrations collected by AGI-30 impinger were 208 EU m$^{-3}$, 269 EU m$^{-3}$, and 287 EU m$^{-3}$, separately. The straight-hole gas bottle did not seem to have any profound effect on airborne endotoxin collection. The other two impingers showed similarly high collection efficiencies. These results indicated that the number and size of holes considerably influence collection efficiency for airborne endotoxins. Thus, smaller pore sizes can improve collection efficiency.

## DISCUSSION

### Influence of Sampling Time

Sampling duration affected endotoxin concentration in terms of capture efficiency. The amount of absorbed gas and gas/liquid volume ratio increased as sampling duration increased. Fig. 7 showed the relationship between the total absorbance of the extraction solution, which represented the total amount of endotoxins, and the gas/liquid volume ratio, which represented sampling duration. When the gas/liquid volume ratio increased from 2083 to 6250, the total absorbance of the extraction solution increased from 1.18 to 2.15. This result indicated that more endotoxins were captured in the extraction solution. However, further increases in the gas/liquid volume ratio resulted in a stable absorbance phase, suggesting the saturation of the solution.

This trend also explained the decreased endotoxin concentrations after 10 min, as shown in Fig. 3. In addition, air flow can carry out partially dissolved endotoxins, thereby decreasing concentration. Sampling duration must

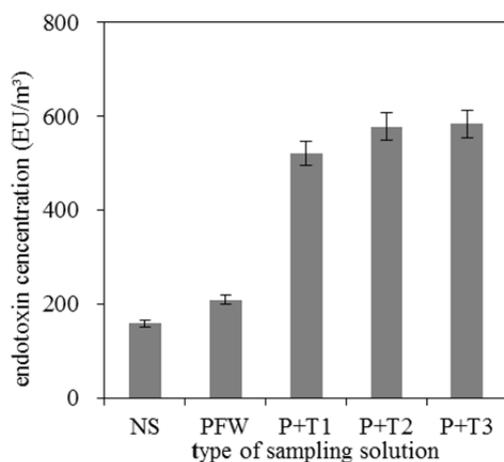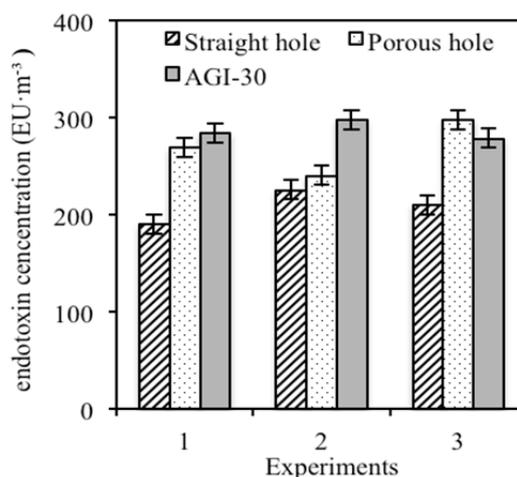**Fig. 6.** The influence of impingers to the endotoxin concentration.

**Fig. 5.** The relationship of endotoxin concentration and types of sampling solution. PFW: pyrogen-free sterile purified water; NS: normal saline; P+T1: 0.010%Tween 20 with PFW; P+T2: 0.025%Tween 20 with PFW; P+T3: 0.050%Tween 20 with PFW.
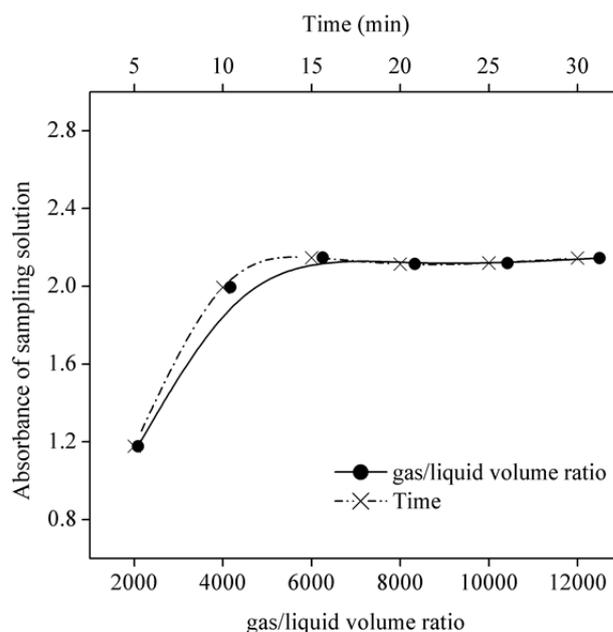
**Fig. 7.** The relationship of total absorbed endotoxin and gas/liquid ratio.

be selected such that the maximum time before the solution saturated by the method was compatible with the expected concentration for the investigated environment. Therefore, we recommend a sampling duration of 15 min. This same sampling duration was also used to assess the exposure risk of wood workers to endotoxins (Oppliger *et al.*, 2005). However, no explanation was provided for this adjustment.

### *Endotoxin Release and Degradation by Ultra Sonication*

The degradation of endotoxins via ultrasonication was shown in Fig. 8. According to Cui *et al.* (2011), *Escherichia coli* cells were severely damaged after 35 min of ultrasonic disinfection (20 kHz, 64 kJ L$^{-1}$), indicating that ultrasonication could undermine cell structure. In our study, Gram-negative bacterial cells were broken by low-power sonication at 500 W to 800 W. LPS were liberated from broken cells and detected by LAL. LPS chemical bonds were disrupted as ultrasonication intensity continuously increased from 800 W to 1000 W. LPS molecules were disintegrated into lipid, protein, and carbohydrate fragments. The fragments lost their antigenicity and could not be detected in the solution by LAL.

Sonication could disrupt cells by generating microbubbles and introducing extreme power into the solution (Paola *et al.*, 2007). The formation and collapse of microbubbles occured in milliseconds, producing high-pressure gradients leading to cavity expansion and shattering Gram-negative bacterial cells (Reiman *et al.*, 2000). Endotoxins associated with cell walls dispersed after cell damage and expressed their toxicity in the solution. Moreover, the microbubbles generated by sonication vibrated and imploded, producing mechanical shear stress and turmoil (Ruifang *et al.*, 2012; Sandle, 2012). Shear stress agitated the solution, preventing zoogloea formation and endotoxin adsorption on glassware walls (Duquenne *et al.*, 2013). Therefore, the endotoxin concentration detected after sonication was equivalent to the total airborne endotoxin content.

Further increasing ultrasonication power may disintegrate LPS molecules into lipid and carbohydrate fragments. Furthermore, atomic rearrangement may occur, inducing changes in the composition and molar ratio of LPS monosacchraides, thereby altering physical and chemical properties and biological activity (Nanü *et al.*, 2011).

### *Effects of Tween-20 on Enhancing Endotoxin Absorption*

Normal saline could maintain cell shape and create a favorable condition for bacterial survival. Gram-negative bacteria could remain active in NS solution. Therefore, major bioactive endotoxins were not detected by LAL. Only particle-associated and free were detected. Therefore, the detected endotoxin concentration was lower than that of the standard control (209 EU m$^{-3}$).

Adding Tween-20 to PFW could significantly improve extraction efficiency, as proven by previous studies (Liebers *et al.*, 2007; Spaan *et al.*, 2007). However, the effects of Tween-20 on extraction efficiency were still unclear. Tween 20 ($C_{58}H_{114}O_{26}$), also called polyoxyethylene (20) sorbaitanmonolaurate, was a non-ionic surfactant. Tween 20 was extensively used as a solubilizer because its hydrophilic and lipophilic groups were regularly oriented in solution. Tween 20 had a hydrophilic polyoxyethylene (($OCH_2CH_2)_xOH$) residue that could easily combine with water molecules, as well as lipophilic molecules that bind to LPS. These characteristics accelerated LPS dissolution and prevented the precipitation of poorly dissolved LPS, as shown in Fig. 9. Therefore, more free endotoxins were absorbed in the Tween solution compared with the PFW solution. Given that different Tween 20 concentrations minimally affected the results, a Tween 20 dose of 0.05% was recommended.

### *Relationship of Endotoxin Concentration and Nozzle Aperture Size*

Linear regression analysis (Fig. 10) showed that the collection efficiencies of Gram-negative bacteria were closely related to the size of nozzle aperture at the end of the gas inlet ($R^2 = 0.800$). Smaller nozzle apertures resulted in higher collection efficiency.

Three explanations could support this result. First, microbial aerosols flowed faster through a small aperture
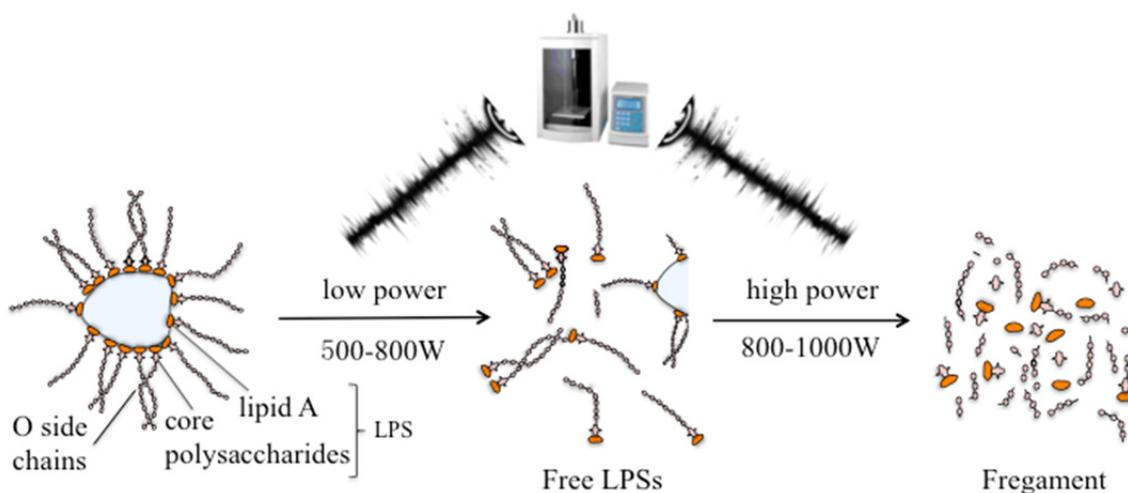


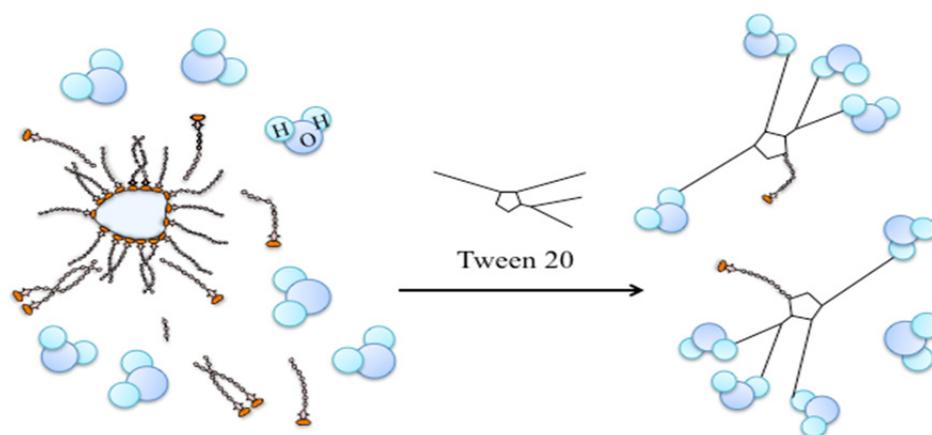**Fig. 8.** The degradation process of cells and endotoxins by sonication.

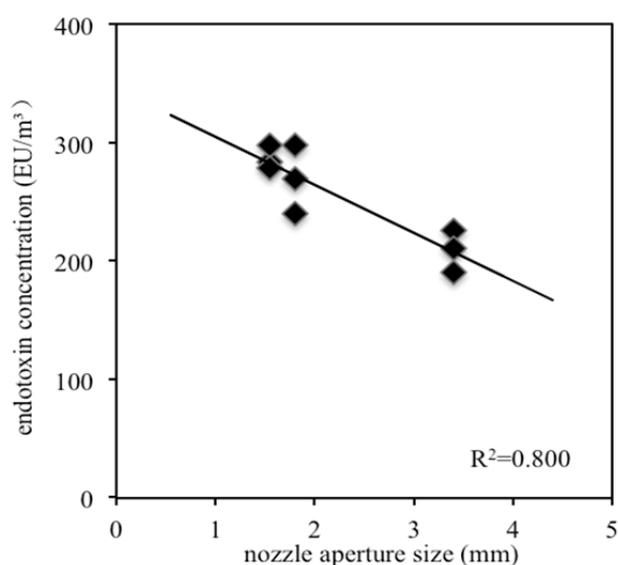**Fig. 9.** Accelerational dissolution mechanism of tween 20.



**Fig. 10.** The relationship of endotoxin concentration and nozzle aperture size.

size, such that the airborne microorganisms were shocked into the sample solution. Microbial particles were captured through liquid adhesion. Second, accelerated airflow produced massive shear forces, which extensively dispersed Gram-negative bacteria to prevent zoogloea formation. Endotoxins were more likely to dissolve in the absorption solution. Finally, small bubbles were produced when air passed through the small aperture. Small bubbles have small diameters and large specific surface areas, improving airborne endotoxin absorption by facilitating endotoxin interaction with the absorption liquid. The AGI-30 impinger and porous-hole gas bottle had similar nozzle aperture sizes and absorption efficiencies.

### *Overview through One-Way ANOVA Method*

One-way ANOVA was conducted to analyze the overall data and evaluate the influence of the four factors. Calculated results were shown in Table 5. A factor with a $F$ value greater than $F_{0.995}$ (2, j) had significant effects on detected endotoxin concentration.

As listed in Table 5, sampling duration had an $F$ value of 5.25. The standard $F_{0.995}(2, 30)$ was 3.32. Similarly, ultrasonication and extraction solution had $F$ values higher than their standard values. These results indicated that the three factors significantly affect detected endotoxin concentration. However, impinger types had an $F$ value of 2.45, which was lower than the standard value ($F_{0.995}(2, 6)$ = 5.14), suggesting that impinger type minimally affected endotoxin detection.

The individual effects of different factors were analyzed as above. Fig. 11 illustrated the integrated results of various interfering factors. S-nU-P was relatively lower than the standard reference value, indicating poor absorption efficiency. Endotoxin concentration increased when Tween 20 was added. A-nU-PT3 is the combination of AGI-30 and 0.05% Tween 20 without sonication pretreatment. When sonication was applied, the detected endotoxin concentration increased, especially under sonication at 800 W. A-U4-P indicateed sampling with AGI-30 impinger and sterile purified PFW and sonication at 800 W. This group had the highest collection efficiency in our study. Based on the above analysis, we recommend a sampling method for airborne endotoxin collection that utilizes an AGI-30 sampler and PFW with 0.05% Tween 20 for a sampling duration of 10 minutes, followed by sonication at 800 W for 10 min. Then, the appropriate amount of the treated solution should be collected and reacted with LAL.

### CONCLUSIONS

The experimental results showed that the extraction solution was saturated at a gas/liquid volume ratio of 6250, at which the absorption efficiency of airborne endotoxins was ideal. Ultrasonication increased endotoxin concentration by destroying cell structures to release LPS. Tween 20 positively affected endotoxin adsorption by expanding the compatibility limitation between endotoxins and PFW. The polyoxyethylene residue of Tween 20 could combine with $H_2O$ molecules and its lipophilic group could combine with LPS to facilitate endotoxin dissolution in PFW. The AGI-30 impinger and the porous-hole gas bottle had similar working mechanisms, wherein a small nozzle aperture size

**Table 5.** One-way ANOVA analyze of four factors: sampling time; ultra sonication; sampling solution and impinger types.

| Factors | No. of samples | $F$ | $F_{0.995}(2,j)$[*] |
|---|---|---|---|
| Sampling time | 33 | 5.25 | 3.32 |
| Ultra sonication | 12 | 10.28 | 4.96 |
| Sampling solution | 15 | 4.94 | 3.89 |
| Impinger types | 9 | 2.45 | 5.14 |

[*] $\alpha = 0.005$; The j values of four controllable factors are 30; 9; 12; 6 respectively.
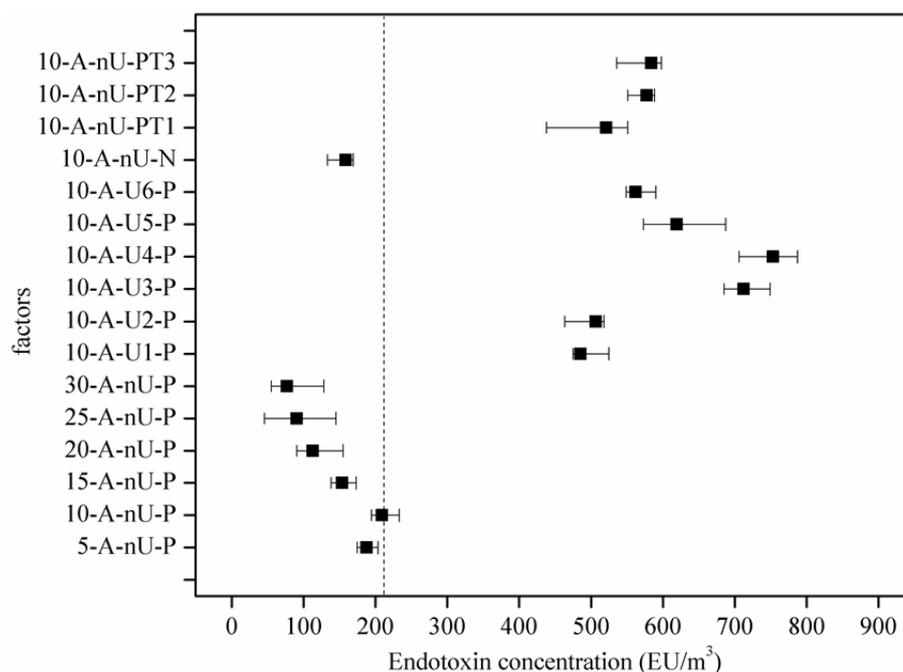


**Fig. 11.** Combination of factors compared to a standard reference with 95% confidence interval. A: AGI-30 impinger; nU: no ultra sonication; U1–U6: ultra sonication 500 to 1000w; P: pyrogen-free sterile purified water; N: normal saline; T1: 0.010%Tween 20; T2: 0.025%Tween 20; T3: 0.050%Tween 20.

generated high-speed airflow and small bubbles with large specific surface areas to accelerate endotoxin adsorption, therefore facilitating interaction among LPS molecules. To improve collection efficiency, airborne endotoxins could be collected in PFW with Tween for 10 minutes using an AGI-30 sampler. The sample solution could then be sonicated at 800 W for 10 min. Finally, the appropriate amount of the treated solution can be collected and reacted with LAL.

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**REFERENCES**

Adhikari, A., Lewis, J.S., Reponen, T., Grasse, E.C., Grimsley, L.F., Chew, G.L., Iossifova, Y. and Grinshpun, S.A. (2010). Exposure matrices of endotoxin, (1→3)-β-d-glucan, fungi, and dust mite allergens in flood-affected homes of New Orleans. *Sci. Total Environ.* 408: 5489–5498.

Angelico, M., Richard, D.C. and Peter, S.T. (2016). Endotoxin exposure, serum vitamin D, asthma and wheeze outcomes. *Respir. Med.* 114: 61–68.

Aynul, B., Morgan, M., Warren, B.K., Lance, W., Marie, E.H. and Amanda, J.W. (2011). Predictors of coarse particulate matter and associated endotoxin concentrations in residential environments. *Atmos. Environ.* 92: 221–230.

Bin, Z., Xiaoxia, Y. and Tongjie, C. (2007). Detection of airborne endotoxin and bacterial aerosol in fox farm. *J. Agro-Environ. Sci.* 26: 1585–1590.

Boehlecke, B., Hazucha, M., Alexis, N.E., Jacobs, R., Reist, P., Bromberg, P.A. and Peden, D.B. (2003). Low-dose airborne endotoxin exposure enhances bronchial responsiveness to inhaled allergen in atopic asthmatics. *J. Allergy Clin. Immunol.* 112: 1241–1243.

Chow, J.C., Yang, X.F., Wang, X.L., Kohl, S.D., Hurbain, P.R. Chen, L.W.A. and Watson, J.G. (2014). Characterization of ambient PM$_{10}$ bioaerosols in a California agricultural town. *Aerosol Air Qual. Res.* 15: 1433–1437.

Cristiane, D., Fernanda, L., Regiani, O., Julian, M. and Paulo, S. (2011). Correlation of fungi and endotoxin with PM$_{2.5}$ and meteorological parameters in atmosphere

of Sao Paulo, Brazil. *Atmos. Environ.* 45: 2277–2283.

Cui, X., Talley, J.W., Liu, G. and Larson, S.L. (2011). Effects of primary sludge particulate (PSP) entrapment on ultrasonic (20 kHz) disinfection of Escherichia coli. *Water Res.* 45: 3300–3308.

Das, A.P., Kumar, P. and Swain, S. (2014). Recent advances in biosensor based endotoxin detection. *Biosens. Bioelectron.* 51: 62–75.

Duan, H.Y., Ma, B.C., Wang, L. and Chai T.J. (2007). Detection of airborne endotoxin in rabbit houses. *J. Domestic Anim. Ecol.* 2: 80–84.

Duquenne, P., Marchand, G. and Duchaine, C. (2013). Measurement of endotoxins in bioaerosols at workplace: A critical review of literature and a standardization issue. *Ann. Occup. Hyg.* 57: 137–172.

Dutch Expert Committee on Occupational Standards. (2010). Endotoxins: health-based recommended occupational limit. Publication No. 2010/04OSH. The Hague: The Health Council of the Netherlands.

Dutil, S., Meriaux, A., Latremoille, M.C., Lazure, L., Barbeau, J. and Duchaine, C. (2009). Measurement of airborne bacteria and endotoxin generated during dental cleaning. *J. Occup. Environ. Hyg.* 6: 121–130.

Eckardt, K., Hinz, S. and Zucker, B. (2010). Endotoxins-the dominant pro-inflammatory substance in bioaerosols from animal barns. *Gefahrstoffe - Reinhalt. Luft* 70: 11–12.

Frederic, G.M., Olga, S., Nicole, C., Eulalia, S. and Anne, O. (2013). Concentration of airborne Staphylococcus aureus (MRSA and MSSA), total bacteria, and endotoxins in pig farms. *Ann. Occup. Hyg.* 57: 550–557.

Gwangpyo, K., Otto, D. and Christina, A. (2010). Endotoxin levels at swine farms using different waste treatment and management technologies. *Environ. Sci. Technol.* 44: 3442–3448.

Hsu, Y.C., Kung, P.Y., Wu, T.N. and Shen, Y.H. (2012). Characterization of indoor-air bioaerosols in Southern Taiwan. *Aerosol Air Qual. Res.* 12: 651–661.

Huntington, M.K., Williams, J.F. and Mackenzie, C.D. (2007). Endotoxin contamination in the dental surgery. *J. Med. Microbiol.* 56: 1230–1234.

Jihong, B., Gu, Y., Min, P., MinJung, K., Joachim, J. and Jaechul, P. (2014). Ultrasonic isolation of the outer membrane of Escherichia coli with autodisplayed Z-domains. *Enzyme Microb. Technol.* 66: 42–47.

John, S.S., William, A.W., Janette, L.W., Joel, N.K. and David, A.S. (2006). Safety of incremental inhaled lipopolysaccharide challenge in humans. *J. Endotoxin Res.* 12: 113–119.

Kallawicha, K., Lung, S.C.C., Chuang, Y.C., Wu, C.D. Chen, T.H., Tsai, Y.J. and Chao, H.J. (2015). Spatiotemporal distributions and land-use regression models of ambient bacteria and endotoxins in the greater Taipei area. *Aerosol Air Qual. Res.* 15: 1448.

Kumar, H. (2006). Variability in the bacterical endotoxins test or LAL (Limulus Amebocyte Lysata) Test. *EndoSafe Times.* 12: 1–7.

Lei, W., Ming, L. and Jianhui, H. (2002). Studies of ultrasonic disruption process to release inclusion body in recombinant E. Coli. *Chem. Ind. Chem. Eng. Q.* 19: 422–425.

Li, C., Hao, H.Y., Shun, L.Y., Chai, T.J. and Wang, H.R. (2014). Airborne microbiological of swine houses monitoring. *Chin. J. Anim. Vet. Sci.* 45: 1684–1692.

Liebers, V., Raulf-Heimsoth, M., Linsel, G., Goldscheid, N., Dueser, M., Stubel, H. and Bruening, T. (2007). Evaluation of quantification methods of occupational endotoxin exposure. *J. Toxicol. Environ. Health Part A* 70: 1798–1805.

Lin, C. and Ned, M. (2013). Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions. *J. Pharm. Biomed. Anal.* 80: 180–185.

Madsen, A.M. (2006). Airborne endotoxin in different background environments and seasons. *Ann. Agric. Environ. Med.* 13: 81–86.

Mafu, A.A., Pitre, M. and Sirois, S. (2009). Real-time PCR as a tool for detection of pathogenic bacteria on contaminated food contact surfaces by using a single enrichment medium. *J. Food Prot.* 72: 1310–1314.

Marchand, G., Lalonde, M., Beaudet, Y., Boivin, G., Villeneuve, S. and Pepin, C. (2007). Documentation of the endotoxins present in the ambient air of cotton fiber textile mills in Québec. *J. Environ. Monit.* 9: 869–876.

Meng, K., Sun, W.J., Chai, T.J., Liu, J.Z., and Ma, Z.F. (2015). Detection of airborne endotoxin and Gram negative bacteria in deer houses. *Prog. Veterinary Med.* 36: 108–112.

Miao, Z.M., Li, S., Zhang, B. and Chai, T.J. (2006). Detection of airborne endotoxin in sickroom environment. *J. Jining Med. Coll.* 3: 7–49.

Milton, D.K., Johnson, D.K. and Park, J.H. (1997). Environmental endotoxin measurement: Interference and sources of variation in the limulus assay of house dust. *Am. Ind. Hyg. Assoc. J.* 58: 861–867.

Mingyan, Z., Lijuan, C., Hao, J., Lin, T., Zhaofeng, L., and Yanmei, W. (2014). Detecting endotoxin with a flow cytometry-based magnetic aptasensor. *Anal. Biochem.* 466: 38–43.

Mohanan, P.V., Siddharth, B. and Geetha, C.S. (2011). Detection of pyrogenicity on medical grade polymer materials using rabbit pyrogen, LAL and ELISA method. *J. Pharm. Biomed. Anal.* 55: 1170–1174.

Nanü, L., Jing, Z., Runguang, S., Pingping, X., Ke, D. and Peijuan, D. (2011). The Effect Mechanism of ultrasound on the structure and biological activity of polysaccharide isolated from *Coprinus comatus*. *Acta Chim. Sinica* 69: 1824–1832.

Natcha, M., Tharadeth, M. and Nussara, P. (2010). Power of the test of One-Way Anova after trans- forming with large sample size data. *Procedia. Social Behav. Sci.* 9: 933–937.

Nilsson, S., Merritt, A.S. and Bellander, T. (2011). Endotoxins in urban air in Stockholm, Sweden. *Atmos. Environ.* 45: 266–270.

Oldenburg, M., Latza, U. and Baur, X. (2007). Exposure–response relationship between endotoxin exposure and lung function impairment in cotton textile workers. *Int.*

*Arch. Occup. Environ. Health* 80: 388–395.

Oppliger, A., Rusca, S., Charriere, N., Duc, T.V. and Droz, P.O. (2005). Assessment of bioaerosols and inhalable dust exposure in Swiss sawmills. *Ann. Occup. Hyg.* 49: 385–391.

Paola, F., Bruni, L., Andreittola, G. and Ziglio, G. (2007). Effects of sonication on bacteria viability in wastewater treatment plants evaluated by flow cytometry-Fecal indicators, wastewater and activated sludge. *Water Res.* 41: 235–243.

Reiman, M. and Uitti, J. (2000). Exposure to microbes, endotoxins and total dust in cigarette and cigar manufacturing: An evaluation of health hazards. *Ann. Occup. Hyg.* 44: 467–473.

Reynolds, S.J., Milton, D.K., Heederik, D., Thorne, P.S., Donham, K.J., Croteau, E.A., Donham, K.J., Croteau, E.A., Kelly, K.M., Douwes, J., Lewis, D. and Whitmer, M. (2005). Interlaboratory evaluation of endotoxin analyses in agricultural dusts–comparison of LAL assay and mass spectrometry. *J. Environ. Monit.* 7: 1371–1377.

Ruifang, L., Long, X. and Hongzhen, W. (2012). Influence of ultrasonic on broken effect of foodborne polluting strains. *J. Anhui Agri. Sci.* 40: 6180–6182.

Saito, R., Cranmer, B.K., Tessari, J.D., Larsson, L., Mehaffy, J.M., Keefe, T.J. and Reynolds, S.J. (2009). Recombinant factor C (rFC) assay and gas chromatography/mass spectrometry (GC/MS) analysis of endotoxin variability in four agricultural dusts. *Ann. Occup. Hyg.* 53: 713–22.

Sandle, T. (2012). Pyrogens, endotoxin and the LAL test: An introduction in relation to pharmaceutical processing. In *Global BioPharmaceutical Resources Newsletter*, Heitman, E. (Ed.), 173: 263–271.

Schlosser, O., Huyard, A. and Catalan, V. (2011). Exposure to dust, endotoxin and airborne microorganisms in wastewater sludge thermal drying units. *Water Sci. Technol.* 64: 1073–1080.

Sean, S., Delan, D., Fullerton, D.G., Peter, S.T., Nervana, M., Anthony, C., Gordon, S.B., Manandhar, D.S. and Ayres, J.G. (2010). Airborne endotoxin concentrations in homes burning biomass fuel. *Environ. Health Perspect.* 118: 988–991.

Singh, U., Reponen, T., Cho, K.J., Grinshpun, S.A., Adhikari, A., Levin, L., Indugula, R. and Green, B.J. (2011). Airborne endotoxin and beta-D-glucan in PM1 in agricultural and homeenvironments. *Aerosol Air Qual. Res.* 11: 376–386.

Spaan, S., Heederik, D.J., Thorne, P.S. and Wouters, I.M. (2007). Optimization of airborne endotoxin exposure assessment: effects of filter type, transport conditions, extraction solutions, and storage of samples and extracts. *Appl. Environ. Microbiol.* 73: 6134–6143.

Spaan, S., Smit, L.A., Eduard, W.; Larsson, L., Arts, H.M., Wouters, I.M. and Heederik, D.J.J. (2008). Endotoxin exposure in sewage treatment workers: Investigation of exposure variability and comparison of analytical techniques. *Ann. Agric. Environ. Med.* 15: 251–261.

Sung, H.H., Dong, J.P., Wha, M.P., Dong, U.P., Jae, K.A. and Chung, S.Y. (2016). Seasonal variation in airborne endotoxin levels in indoor environments with different micro-environmental factors in Seoul, South Korea. *Environ. Res.* 145: 101–108.

Thorne, P.S., Sarah S.P., Rena, S., Patrick, T.O., John, M., Nervana, M., Thomas, K., Kelley, J.D. and Stephen, J.R. (2010). Evaluation of the limulus amebocyte lysate and recombinant factor C assays for assessment of airborne endotoxin. *Appl. Environ. Microbiol.* 76: 4988–4995.

Tian, Y., Chen, B.L. and Chai, T.J. (2007). Detection of airborne endotoxin in duck stables. *J. Domestic Anim. Ecol.* 1: 66–69.

Tianjia, G., Maosheng, Y., Junxia, W., Yanhua, F., Songhe, H., Yan, W., Anindita, D., Junnan, Y., Yusheng, W., Min, H. and Tong, Z. (2014). Airborne endotoxin in fine particulate matter in Beijing. *Atmos. Environ.* 97: 35–42.

Uhlig, S., Negård, M., Heldal, K.K., Straumfors, A., Madsø, L., Bakke, B. and Eduard, W. (2016). Profiling of 3-hydroxy fatty acids as environmental markers of endotoxin using liquid chromatography coupled to tandem mass spectrometry. *J. Chromatogr. A* 1434: 119–126.

Xu, X.L., Xue, C.F., Yu, W.B., Zhang, J.F., Ding, Z.R. and Zhang, H. (2002). Study on application of a double-mAb sandwich ELISA to quantitatively detecting endotoxin. *Chin. J. Cell. Mol. Immunol.* 18: 175–176, 183.

Yu, G.L., Wang, Y., Wang, S.G., Duan, C.M., Wei, L.M., Gao, J., Chai, T.J. and Cai, Y.M. (2016). Effects of microbial aerosol in poultry house on meat ducks' immune function. *Front. Microbiol.* 7: 247–251.

Zhang, B., Yu, X.X., and Chai, T.J. (2007). Detection of airborne endotoxin and bacterial aerosol in fox farm. *J. Agro-Environ. Sci.* 4: 1585–1590.

Zhong, Z.B. (2015). Detection of bacterial aerosol and airborne endotoxin in mink breeding houses. *Chin. Anim. Health Inspect.* 32: 22–26.