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., 2007; Tianjia et al., 2014). Accumulated airborne endotoxins adversely affect human health because LPSs are composed of lipid A, core oligosaccharides, and O-side chains (Oppliager 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, Shwartzman 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 (Oppliager et al., 2005; Cristiane et al., 2013; 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...
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 ± 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.

<table>
<thead>
<tr>
<th>Detection methods</th>
<th>Assessment</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit pyrogen test method</td>
<td>Reflect the situation of each source of pyrogen directly</td>
<td>Cannot be quantified and standardized, poor reproducible results; Not suitable for large-scale testing; Rabbit response has individual differences; Low sensitivity; Longtime</td>
</tr>
<tr>
<td>LAL assay</td>
<td>Quick and easy operation; High sensitivity; Easy to promote and standardize</td>
<td>Samples with 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</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>Avoid the color interference; Easy operation; Cheap</td>
<td>Lower detection accuracy compared to LAL method</td>
</tr>
<tr>
<td>Biosensor assay</td>
<td>Adapt to complex environment; Fast detection; High sensitivity and low detection limit</td>
<td>Immature technology, Not popular</td>
</tr>
<tr>
<td>Biomarker detection analysis</td>
<td>Types of LPS in the sample can be determined; High sensitivity</td>
<td>Endotoxin with biological effects cannot be detected accurately</td>
</tr>
</tbody>
</table>

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

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

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

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 added 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).

\[
G / L = \frac{12.5 \times T}{V} \times 1000 \tag{2}
\]

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

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Impinger type</th>
<th>Bottle size (mm × mm)</th>
<th>In/outlet diameter (mm)</th>
<th>Nozzle aperture size (mm)</th>
<th>Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>AGI-30 impinger</td>
<td>Φ36 × 233</td>
<td>10</td>
<td>1.55</td>
<td>30</td>
</tr>
<tr>
<td>II</td>
<td>Straight hole gas bottle</td>
<td>Φ42 × 210</td>
<td>12</td>
<td>3.4</td>
<td>36</td>
</tr>
<tr>
<td>III</td>
<td>Porous hole gas bottle</td>
<td>Φ42 × 210</td>
<td>12</td>
<td>1.8</td>
<td>36</td>
</tr>
</tbody>
</table>
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 soniconation 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 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
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 occurred 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 monosaccharaides, 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 (C₃₈H₇₆O₂₆), also called polyoxyethylene (20) sorbitanmonolaurate, 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 ((OCH₂CH₂)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
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 indicated 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$_2$O 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.

<table>
<thead>
<tr>
<th>Factors</th>
<th>No. of samples</th>
<th>$F$</th>
<th>$F_{0.005}(2, j)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling time</td>
<td>33</td>
<td>5.25</td>
<td>3.32</td>
</tr>
<tr>
<td>Ultra sonication</td>
<td>12</td>
<td>10.28</td>
<td>4.96</td>
</tr>
<tr>
<td>Sampling solution</td>
<td>15</td>
<td>4.94</td>
<td>3.89</td>
</tr>
<tr>
<td>Impinger types</td>
<td>9</td>
<td>2.45</td>
<td>5.14</td>
</tr>
</tbody>
</table>

$\alpha = 0.005$; 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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