AN ANALYTICAL METHOD TO QUANTIFY 1,3- β -D-GLUCAN USING THE LAL ASSAY

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A thesis submitted to the Faculty of Graduate Studies in partial
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An Analytical Method to Quantify 1,3- β -D-Glucan Using the LAL Assay

in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The use of 1,3- β -D-glucan for the determination of fungi in indoor air and dust in research studies has been increasing steadily over the last decade. A very sensitive glucan-specific assay exists, however its cost makes the determination of 1,3- β -D-glucan in large-scale studies financially difficult. Hence, 1,3- β -D-glucan was quantitatively measured by developing chemical procedures to adapt the relatively inexpensive *Limulus* amebocyte lysate assay (commonly used for LPS endotoxin) to 1,3- β -D-glucan.

0.5 N NaOH showed best results as an extracting solvent for samples containing both endotoxin and 1,3- β -D-glucan, with no significant difference in calculated LAL concentration of a known amount of 1,3- β -D-glucan (curdlan) when present with concentrations of endotoxin below 5ng/mL. The modified LAL method was also useful in analyzing other materials. The glucan content of ragweed pollen (*Ambrosia artemisiifolia*) was found to be comparable to values obtained by others with the glucan-specific test (Rylander *et al*, 1999a).

Size-Exclusion Chromatography was explored with curdlan standards to confirm the size of insoluble 1,3- β -D-glucans. The correlation between the LAL assay and SEC method (based on concentration and area, respectively) was 0.517 ($p < 0.000$) over a full year of field samples. However, this correlation increased to 0.822 ($p < 0.006$) at 180-190 kDa when SEC samples were grouped in 10 kDa ranges. Strong correlations were also seen between 1,3- β -D-glucan/ergosterol, and 1,3- β -D-glucan/mold area (0.873, $p < 0.000$ and 0.763, $p < 0.000$, respectively). A moderate correlation was observed between 1,3- β -D-glucan/spore counts (0.501, $p < 0.011$).

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II INTRODUCTION

II-1 The Indoor Environment and Exposure to Allergens

Most people spend a great deal of their time indoors; be it at home, at work or in transit. Canadians and Americans can spend up to 90% of their time indoors (Leech *et al.*, 1996). A common belief is that the indoor environment protects us from outdoor pollutants, when in fact the indoor environment can contain many air pollutants 2-5 times and as high as 100 times that of outdoor levels. Of particular concern are the biological and chemical agents that can be directly linked to allergic diseases (Pope *et al.* 1993). An allergen, typically a protein, is an antigenic substance capable of producing hypersensitivity. Allergens cause, or are thought to cause asthma. Common sources of indoor allergens are house dust mites, animal dander, pollen and molds.

II-1.A Dust and Dust Mites

House dust is made up of a varied mixture of materials, some of which are potentially antigenic. Dust is composed of food particles, human skin scales, sand particles, fibres from fabrics, soot, and allergens from pets, insects, plants and fungi. In Canada, there are two important species of dust mite found within the indoor environment, *Dermatophagoides farinae* and *D. pteronyssinus*. They live in bedding, furniture and carpets. Dust mites live on human skin scales and other debris as they absorb water through a hygroscopic substance extruded from their leg joints (Pope *et al.* 1993). The absorption of water from their environment makes the mite dependent on ambient humidity, and the temperature for human comfort is close to optimal for the growth of the dust mite. The typical size of the dust mite is approximately 250-300 µm in length.

At humidity levels above 50%, house dust mites thrive and produce large amounts of fecal pellets. In a typical mattress, there may be between 100,000 and 10 million dust mites. The waste pellets of these mites contain partially digested food and digestive enzymes that are thought to be the principle allergens in house dust. The principal mite allergens, *D. pteronyssinus* allergen I (*Der p* I) and *D. farinae* allergen I (*Der f* I), are found in large quantities in mite fecal pellets. These are mainly digestive enzymes as they are present in glands surrounding the gut (Tovey 1982). Mite fecal pellets are similar in size (10-35 μm diameter) and in the amount of allergen they contain (on average about 0.2 ng) with that of pollen.

II-1.B Plants and Pollen

Except during snow cover, trees, weeds and grasses release pollen into the air which can be carried long distances by the wind. These particles often enter the human body through the nose and throat, triggering a type of seasonal allergic rhinitis called pollen allergy as well as asthma. Of all agents that cause allergies, including foods, drugs and molds, pollen is perhaps the hardest to avoid (NIAID 1998).

Pollen grains can be found as far as 600 kilometres out at sea and as high as 3.5 km in the sky. However, it is the chemical makeup of the grain, either small and light or large and heavy, which really determines whether the type of pollen is likely to cause an allergic reaction. For indoor environments, small pollen grains are usually introduced by the wind through open windows or doors, or they may be transported on people and their clothing. Indoor pollen concentrations of up to 5.5 million pollen grains per gram of settled house dust have been reported (O'Rourke *et al.* 1984) and 30 pollen grains per cubic meter of air are considered typical.

II-1.C Bacteria

Bacteria are the oldest and most abundant life forms on earth and can live almost anywhere that there is sufficient water. Bacteria are prokaryotic (no membrane which separates nuclear material from other cell compartments), single-celled organisms which range in size from 0.5-5 μm . Certain bacteria (i.e. actinomycetes) produce antibiotics, such as streptomycin and nocardicin, whereas others are essential in the guts of animals (i.e. humans) where they live symbiotically. However, certain bacteria are associated with causes of human and animal diseases in the indoor environment. *Legionella pneumophila* growing in cooling towers or domestic hot-water systems can cause Legionnaires' Disease or Pontiac Fever. Sick Building Syndrome (SBS) symptoms can arise from Gram-negative bacteria (i.e. *Micrococcus*) present in the respiratory tract of an occupant in such an environment. The endotoxin-producing bacteria *Pseudomonas* are now also thought to be important in the development of sick building syndrome.

The quantitation of gram negative bacteria has been the focus of many indoor air investigations (Michel *et al.* 1996). This is partly due to endotoxins which are outer cell wall constituents of gram-negative bacteria. Endotoxins are lipopolysaccharides (LPS) which have been shown to be potent, proinflammatory substances present in domestic environments leading to the development and severity of asthma (Michel *et al.* 1996). Although concentrations of harmful bacterial species may be low in the indoor environment, their presence may be an indication that conditions for microbial growth or accumulation exist. Area measurements of airborne endotoxin indoors are usually very similar or slightly above that of normal outdoor levels, except in cases where contamination of humidifiers or recent building water damage is present. High exposures

to endotoxin are common in agricultural environments, composting facilities and in sewage treatment plants, and can result in severe disease (Rylander, 1997).

II-1.D Airborne Fungi

Fungi are eukaryotic organisms, which means their cells have a membrane separating the nuclear material from the rest of the cell protoplasm. The cell walls are defined structures consisting mainly of polysaccharides, and sometimes polypeptides and chitin. Fungi are ubiquitous in the outdoor environment where most species are involved in the decay of various materials, primarily of plant origin. Fungi enter buildings from the outdoors through open windows and doors mainly during summer months. In North America, regardless of geographical location, similar species of filamentous fungi can contaminate buildings. Building-associated fungi can grow on most organic material, provided that sufficient water is present. In indoor environments, this means that fungal growth is typically due to condensation or water leaks as a result of environmental conditions (i.e. humidity) and envelope failure (Miller 2001). Building elements that allow growth of fungi include wallboard, vapor barriers and insulating materials.

Fungi may be unicellular (e.g. yeasts) or multicellular, as indicated by the formation of microscopic, branched filaments known as hyphae. When hyphae become interwoven they form a mycelium and it is this form, referred to colloquially as mold, that is found most commonly in the indoor environment. Inhalation of both the reproductive propagules, or spores, and mycelia fragments cause allergic reactions. Both in the outdoor and indoor environments, fungi can be measured by counting the number of spores per cubic meter of air.

II-2 Gram-Negative Bacteria and Endotoxins in Indoor Air

Classification of bacteria relies on several things, of which cell wall composition and reaction to dyes are a few. A difference between gram-negative and gram-positive bacteria is the construction of the cell wall (Figure 1). Gram-positive bacteria consist of a thick peptidoglycan layer followed by the plasma membrane. Gram-negative bacteria have an outer membrane, consisting of lipoproteins and lipopolysaccharides (LPS), followed by a thin peptidoglycan layer and a plasma membrane.

When lipopolysaccharides are chemically isolated from the cell wall, it is called LPS and is considered "pure" lipopolysaccharide. However, when lipopolysaccharides are present in bacterial cells in the natural form along with other proteins and cell wall constituents, they are collectively known as endotoxin. The lipopolysaccharide structure (Figure 2) consists of lipid A linked to a polysaccharide made up of many different repeating structures (the O-antigen). The O-antigen chains vary among serotypes within species of bacteria, and it is the resulting unique units that vary the antigenicity of bacterial strains. The lipid A portion consists of a 1,4'-diphosphoryl β -(1,6)-D-glucosamine disaccharide backbone with covalently linked hydroxy fatty acids (Milton 1999). It is the lipid portion of LPS which makes up the characteristic toxicity of the molecule, as the number of non-hydroxylated fatty acids esterified to the 3-OH varies.

Figure 1. Cell wall construction of both Gram-positive and Gram-negative bacteria. Key notes are the thickness of the peptidoglycan layer of each, as well as the additional outer membrane of the Gram-negative bacteria. (Adapted from McKenna and Mondal, 1997).

Figure 2. Structure of Lipopolysaccharide which is an integral component of the outer membrane of Gram-negative bacteria (Adapted from: Kiselica *et al.* 1999)

II-2.A Health Effects Associated with Endotoxins

A large number of investigations have focussed on the health effects associated with exposure to endotoxins (Michel *et al.* 1996, Michel *et al.* 1991, Burrell 1990, Burrell 1994, Thorn and Rylander 1998a). Rylander (1997) and Douwes (1997) have shown endotoxins to be potent inducers of non-immune mediated airway inflammation and respiratory disease. During continuous or repeated exposures to endotoxins, a gradual increase in inflammatory cells (neutrophils in early or acute stages, or eosinophils and lymphocytes in chronic conditions) occurs. A strong dose-response relationship between endotoxin and acute airflow obstruction was demonstrated by experimental cotton dust exposures in humans (Rylander *et al.* 1985, Castellan *et al.* 1987). Thresholds of 90-330 Endotoxin Units/m³ were seen in a similar study of animal feed handlers by Smid *et al.* (1994) in which exposures of 300-400 EU/m³ were obtained.

These industrial and agricultural environments are known, however, to have high endotoxin exposures. When endotoxin exposures are slightly above that of normal outdoor air, as is the case in most indoor environments, there is an association with increased severity of asthma and building related symptoms (Michel *et al.* 1996).

II-2.B Chemical Markers of Gram-negative Bacteria

The most common chemical method for the quantification of endotoxins, and Gram-Negative bacteria, is analysis of 3-hydroxy fatty acids (3-OH FAs) by HPLC (Morris *et al.*, 1988) or more commonly gas chromatography-mass spectrometry (Saraf and Larsson 1996, Fox *et al.* 1993, Mielniczuk *et al.* 1993). As noted earlier, 3-OH fatty acids are distinctly characteristic to LPS, in particular with the Lipid A portion. Using GC-MS, it is possible to identify the organisms present by their unique combination of 3-

OH fatty acids, or fatty acid profiles. Through this identification, the potential to estimate biological potency is achieved (Saraf *et al.* 1997). Another advantage of this chemical method is that it measures both biologically and non-biologically active LPS with a low likelihood of interference.

For GC-MS analysis of the 3-OH fatty acids, a methyl ester-trimethylsilyl derivatization is first performed, after which specific ion monitoring (m/z -175) is measured. Common 3-OH fatty acids contain between 10-18 carbon atoms. Unfortunately, the sensitivity of the GC-MS method is approximately 1000-fold less sensitive than the biological LAL assay for endotoxins to be discussed later.

A less common method for the measurement of Gram-negative bacteria is that of muramic acid. Like 3-OH fatty acids, muramic acid is characteristic to bacteria, specifically peptidoglycan found in the cell wall structure. Muramic acid is an ideal chemical marker for peptidoglycan as it is found nowhere else in nature. It is more commonly used for the measurement of Gram-positive bacteria, as peptidoglycan accounts for ~50% of the cell wall mass, while Gram-negative bacteria contains limited quantities of the polymer. Like 3-OH fatty acids, muramic acid undergoes a methyl ester/TMS derivatization prior to analysis by GC-MS/MS. Single ion monitoring of m/z -185 is typical (Saraf and Larsson, 1996).

II-2.C Biological Assays

The determination of endotoxins most commonly relies on a biological assay derived from a crude lysate of blood cells from the horseshoe crab (*Limulus polyphemus*). The assay is called the *Limulus* amoebocyte lysate (LAL), which will be discussed in

greater detail in following chapters. Briefly, the lysate contains a serine protease specifically activated by endotoxin, which in turn activates a cascade of serine proteases (Myatt and Milton 2001). In this pathway, the activation of 'factor C' is directly related to the concentration of endotoxin (See Figure 4). This assay has been chosen as the method of choice in the field due to its ease, speed and sensitivity towards endotoxins, with a range of 1 pg/ml to 10 ng/ml. Unfortunately, there are also disadvantages associated with the assay when compared to the chemical methods. The assay produces relative rather than analytical data (Milton, 1999), meaning there are factors other than LPS concentration that can change the measured endotoxin activity level. Ikemura *et al* (1989) and others (Tanaka *et al.* 1991, Roslansky and Novitsky 1991, Cooper *et al.* 1997) have all shown that the LAL assay is not specific to endotoxin, and that activation of 'factor G' can be initiated by the fungal cell wall constituent 1,3- β -glucan if present in sufficient concentrations (nanogram to microgram).

II-3 Fungi in Indoor Air and Fungal Biomarkers

II-3.A Health Effects Associated with Fungi

Mold spores can survive in a dormant state for years. When adequate moisture is present these fungi can grow rapidly. In the indoor environment, moisture problems occur in poorly ventilated or water-damaged buildings. Due to leaks or flood, molds can have an impact on indoor air quality, as well as the comfort and health of the occupants. Several studies have reported that children living in damp and moldy homes show a greater prevalence of respiratory symptoms (cough, phlegm, wheeze, sore throat and

runny nose) and asthma (Hu *et al.* 1997, Cuijpers *et al.* 1995). At this time, associations between fungal exposures and human health are primarily based on questionnaire surveys. Absent objective measurements, and questionnaire-based data, cannot produce accurate information on a relationship between indoor fungal growth and illness (Dales *et al.* 1999). Additionally, molds produce many agents that co-exist within the colony and with other agents such as bacteria and dust mites that might affect human health (Rylander 1999a).

Mold spores contain a variety of biologically-active compounds with varying health effects. Identifying these agents within the building environment is of importance when evaluating health hazards in a building (Andersson *et al.* 1997). The cell-wall constituent 1,3- β -D-glucan, low-molecular weight compounds (toxins) and allergens are of greatest interest when investigating mold in indoor air. "Low-molecular weight compounds" are sometimes known as mycotoxins. They are secondary metabolites that can cause disease in humans and/or animals. Some mycotoxins interfere with the cells metabolic processes, with carcinogenic, neurotoxic, and teratogenic activity (Rylander 1999a, Samson 1992).

II-3.B Sampling Methods for Fungi

When obtaining samples for analysis of fungal contamination, it is essential to identify the agents to be sampled and their particle sizes. Care must also be taken so that the collection process does not damage the sample (Burge 2001).

II-3.B.1 Spore Collection on Sticky Surface

Total spore concentrations can be determined by drawing air into a sampling device for impaction on a sticky surface. The most commonly used device is the Burkhard spore trap. Briefly, air is drawn into the instrument and fungal propagules and other particles are impacted on a glass slide prepared with a suitable adhesive. Typical sampling times are 10-15 minutes at ~10 Litres/minute. The slide is then examined by light microscopy to identify pollen, some inorganic debris and genera and possibly species of fungi. A disadvantage of this method is that most spores found indoors are from genera with small conidia (i.e. *Aspergillus*, *Penicillium*) which are difficult to see compared to darker large spores. This method is also dependent on the skill and experience of the microscopist. Another disadvantage of this method is that the slide can be overloaded with debris. This leads to difficulty in differentiating and counting the spores and hyphael fragments present.

II-3.B.2 Culturing Fungi

Culturing of microorganisms from air or surface samples is a common method. The most widely used air sampler is the Anderson bioaerosol impactor which draws air in via a vacuum pump. As the air passes through the impactor, multiple jets of air direct airborne particles toward the agar collection surface where they are retained for analysis (SKC Technologies). The collection times are short (often less than 5 minutes) and the environmental variability of spore concentrations can be orders of magnitude over just a few minutes (Miller 1992b). For surface samples such as dust, aqueous suspensions are

made, diluted and applied to an agar surface. For both methods, fungi present in the samples are allowed to grow under carefully controlled conditions. At this point, a trained mycologist may be able to identify the many fungi present to the genus level, and after isolation perhaps to the species level. Disadvantages of this method include the fact that growing conditions (i.e. substrate, temperature, and pH) dictate which mycoflora will be obtained. Also, there is concern for the competition between species present in large numbers which are rapid growers (*Aspergillus* and *Penicillium* species) and those fungi which are not able to grow well on the medium selected. Finally, this method has the potential of identifying only culturable or "viable" spores, thereby giving no information on total fungal biomass but rather only the taxa present in a sample that are living. In the case of *Stachybotrys chartarum*, the half life of the spores can be as short as one month and perhaps only 1 of 10 conidia found in the air would be viable (Miller 1992).

II-3.B.3 Collection of Fungal Spores on Filters

Fungal spores are typically collected on microporous filters because they are easy to use and allow larger volumes of air to be sampled as desired. Air can be drawn at a rate of 1-4 L/minute via a vacuum pump through a cassette holder that contains the microporous filter. There are various types of filters that can be employed, ranging from cellulose ester, Teflon and polycarbonate membranes. The pore size of filters utilized in fungal analyses is typically 0.47 μm , which is sufficiently small enough to capture fungal spores which range from 3-4 μm to 60 μm (Miller 1992). After the desired amount of air has been sampled, the filter may be examined by microscope, as described earlier, or it may be extracted for specific agents indicative of fungal biomass. An extensively studied

fungus biomarker is ergosterol, the primary membrane sterol found mainly in filamentous fungi (Newell 1992). Recently, the new method of determining 1,3- β -D-glucans for evaluating fungal biomass has been receiving recognition. Both of these biomarkers can determine viable and non-viable spore counts. Species of fungi present cannot be determined using either of the agents.

II-3.C Ergosterol as an Indicator of Fungal Biomass

Ergosterol has been used as a marker for determining fungal biomass for over 20 years. Since its introduction by Seitz *et al* (1977) as an indicator of fungal invasion in grains, it has been used in more than 1,500 studies. Ergosterol is the primary membrane sterol of most fungi and yeasts. The fact that ergosterol is essentially unique to higher fungi makes it a useful chemical marker for the indication of fungal growth. Miller and Young (1997) have measured the spore ergosterol contents of many common species of molds found in Canadian indoor environments, and there are also data on airborne ergosterol concentrations in homes. The range of ergosterol content for the various species of molds was determined to be 1.71-5.11 pg/spore. The difference in ergosterol content between species was attributed to the size of the spores, as data was normalized against either volume or area (based on a prolate spheroid) of the spore.

II-3.C.1 Extraction Methods

There have been many extraction techniques presented (Seitz *et al.* 1977, Lee *et al.* 1980, Griffiths *et al.* 1985), however, the most widely used extraction method for ergosterol has been reported by Newell *et al* (1988). The majority of methods presented

use either methanol or ethanol in the initial extraction step. Samples are refluxed for 80 minutes before being saponified with an alcoholic base (KOH or NaOH). Due to the greater affinity of ergosterol for pentane than for methanol or ethanol, the extracts are subsequently partitioned into pentane and dried under nitrogen for analysis. As an alternative to the long reflux times and large solvent volumes, Young (1995) developed a microwave-assisted extraction (MAE) technique which addressed the above concerns. In this technique, samples are suspended in methanolic base, microwaved for 25-30 seconds, neutralized and extracted with pentane. Nielson and Madsen (2000) showed that the extraction efficiencies between the MAE and classical methods did not differ significantly.

II-3.C.2 Analysis of Ergosterol

Perhaps the most common analytical technique for ergosterol has been separation on HPLC with UV-detection at 282 nm. Like the extraction techniques, various mobile and stationary phases have been reported. The most common of these mobile phases has been either isocratic methanol (Newell *et al* 1988, Padgett and Posey 1993, Bermingham *et al* 1995), or an isocratic acetonitrile-methanol mixture ranging from 80:20 to 98:2 v/v (Miller and Young 1997, Montgomery *et al* 2000). Separation is achieved on reverse-phase C8 columns or C18 reverse-phase columns, respectively.

A more sensitive alternative to the quantitation of ergosterol by HPLC is gas chromatography-mass spectrometry (GC-MS). With added sensitivity, down to 0.44 pg (Miller and Young 1997), and confirmation of the sample peak by MS, GC-MS is an

ideal substitute for HPLC. Notably, there is no real variation in protocol for this analytical technique, as is the case for HPLC. Methods involve the use of a general 5%-phenyl column and temperature programming up to, or just over, 300°C.

II-3.D *1,3- β -D-glucans as an Indicator of Fungal Biomass*

β -glucans are a class of naturally occurring polysaccharides found in the outer cell membrane of fungi, higher plants, and some bacteria. They are homopolymers of D-glucose linked in a β configuration that may be present in a variety of forms. These polymers vary in molecular weight, solubility, degree of branching and linkage type. Higher molecular weight 1,3- β -glucans can take on three different conformations: single chain, single helix and triple helix, the latter appearing to be the preferred form in fungi (Williams 1997). 1,3- β -D-glucans produced by micro-organisms and plants probably contain all three forms in various proportions. Although the specific function of glucans in the physiology of fungi is not quite fully understood, it has been suggested that these polymers help maintain the rigidity and integrity of the fungal cell wall (Kolar *et al.* 1997)

Within the last decade, the use of 1,3- β -D-glucan as an indicator of fungal biomass in indoor air has gained attention. This is in part due to its toxicity. 1,3- β -D-glucan retains its biological activity even when the mold spore is dead.

II-3.D.1 Health Effects Associated with 1,3- β -glucans

There have been numerous studies from various countries in which the relation between moist or damp housing and the health of its occupants have been evaluated. In these studies, various species of molds, along with their respective metabolites/compounds have been linked to a variety of health effects. The major symptoms are irritation in the eyes, nose and throat, dry cough, fatigue/headache, and skin problems, all of which can be caused by nonspecific airway inflammation. More recently, 1,3- β -D-glucan (Figure 3) has been considered the possible causative agent of the aforementioned respiratory diseases, especially in children (Rylander 1999b).

Figure 3. Structure of linear 1,3- β -D-glucan showing linkage of glucose molecules

Rylander *et al* (1992) have demonstrated a dose-response relationship between levels of airborne 1,3- β -D-glucan and eye, nose and throat irritation, dry cough, itching skin, hoarseness and tiredness. Subsequently, Thorn and Rylander (1998b) found that 1,3- β -D-glucans were also related to an increased prevalence of atopy and a decrease in forced expiratory volume (FEV₁) over a number of years living in a building.

As 1,3- β -D-glucans are present in many environments, and in varying concentrations, the effects of inhalation are of particular interest. Acute inhalation

studies on β -D-glucan have focussed on the effects associated with curdlan, a linear, water-insoluble, unbranched glucan obtained from the yeast *Saccharomyces cerevisiae*. In a guinea pig model, Rylander and Holt (1998) have shown that curdlan produces only small changes in the number of inflammatory cells in the airways, with no neutrophil invasion. This is opposite to what was observed with endotoxin, where a massive neutrophil invasion in airways was seen. However, when exposed to 1,3- β -D-glucan prior to endotoxin exposure or simultaneously, the endotoxin-induced neutrophil invasion was depressed by the 1,3- β -D-glucan, suggesting a synergistic effect with regards to pulmonary symptomology. The depression of the neutrophil influx was dose-dependent on the dose of curdlan.

Guinea pigs chronically exposed to curdlan or endotoxin resulted in small changes in inflammatory cell density in the airways. Once again, when exposed to both simultaneously, the adaptation to endotoxin which normally occurred, was reduced by concurrent 1,3- β -D-glucan exposure. This resulted in the persistence of increased numbers of neutrophils in the lung and the formation of granulomas (Fogelmark *et al.* 1994).

Sakurai *et al* (1995) and Ohno *et al* (1995) have shown that different conformation, branching and molecular weights of 1,3- β -D-glucan have different biological activities. For example, a high branching ratio (e.g. a 1,6- β -glucan branch for every three 1,3-linked glucose molecules in the backbone represents a 33 % branching ratio) or a high molecular weight glucan induced cytokine production. Also, glucans configured in a single helix conformation resulted in *in vitro* production of tumor

necrosis factor- α by mouse peritoneal macrophages, whereas a triple helix configuration produced the strongest 1,3- β -D-glucan receptor binding.

II-3.D.2 1,3- β -D-glucans in Alkaline Solutions

Many studies have been performed on the characteristics of pure 1,3- β -D-glucan in aqueous sodium hydroxide, particularly curdlan. Curdlan possesses some very interesting characteristics in alkaline solution, ranging from changes in conformation to the formation of various sets of gels.

Based on the results of these experiments, it has been suggested that a reversible conformational transition of curdlan from an ordered (triple helix structure) to the disordered (random coil) form occurs in the range of 0.19-0.24 M NaOH (Ogawa *et al.* 1972). Others have speculated that curdlan conformation does not change to a random coil, but rather to single helices in alkali solution. In this critical range, the elimination of hydrogen bonds between each of the strands making up the triple-helix structure causes a random-coil/single helix to be produced. Also, in these alkali solutions, chain length, and thus average molecular weight, is decreased over time, as alkali decomposition at the reducing end of the glucan occurs (Futatsuyama *et al.* 1999). Many of the above experiments relied on the use of such techniques as viscometry measurements, light-scattering measurements, solid state ^{13}C NMR and x-ray crystallography (Tada *et al.* 1997, Ogawa *et al.* 1972, Nakata *et al.* 1998). Young *et al.* (2000) have recently used fluorescence resonance energy transfer (FRET) and verified that curdlan does indeed take on a random-coil, or partially opened, conformation. Upon neutralization of the alkali solution, the random coil slowly reverts back to its original linear triple-helix form.

II-3.D.3 Various Extraction Methods for 1,3- β -D-glucans

Over the years, many modifications have been made to the extraction procedures of 1,3- β -D-glucans from fungi and plants. As various conformations exist with differing degrees of branching and molecular weight, not all can be extracted with a single method. 1,3- β -D-glucans that exist with a high degree of branching tend to be water-soluble. This leads to their extraction in water. Glucans with a relatively low average molecular weight, namely with a degree of polymerization of less than 20, also tend to be water soluble (Aketegawa *et al.* 1993). However, approximately 60% of the fungal cell wall consists of glucans (Kliss *et al.* 1994), for which linear 1,3- β -D-glucans with a much higher average molecular weight leading to a water-insoluble molecule is a major portion (Douwes *et al.* 1996). This is why extraction of the glucan in an alkaline solution is necessary. Other polysaccharides which make up the fungal cell wall are (1,3)- β -glucans with (1,6) branching, galactomannan, 1,6- β -glucan and chitin.

For insoluble 1,3- β -D-glucans, Douwes *et al.* (1996) and Aketagawa *et al.* (1993) heated an aqueous suspension of glucan above 120°C, making the molecule water-soluble. Temperatures of 120°C (1hr), 150°C (1hr), 160°C (1hr) and 180°C (10min) have been used. With these varying temperatures, it was determined that depolymerization of curdlan increased as temperatures increased, leading to lower reactivity with the LAL assay. Perhaps the most common method of extracting insoluble 1,3- β -D-glucans from environmental samples is the extraction in alkaline solution. Rylander *et al.* (1999a, 1999b) have commonly used 0.3 N NaOH on ice to extract the triple-helix structure of common 1,3- β -D-glucans. This method has been widely used by the Rylander group to analyze various environmental samples.

II-3.D.4 Biological Methods Used for Analysis of 1,3- β -D-glucans

During an experiment involving horseshoe crabs in the mid-1950s, Dr. Fred Bang noticed that one of his crabs died of a *Vibrio* bacterial infection. He also noticed that Gram-negative bacteria caused the *Limulus* blood to clot (Bang 1953). During the following years, Bang and another scientist, Jack Levin, jointly determined that this clot was a result of an enzymatic reaction, and that the enzymes were located in the granules of the amebocyte. These observations have been proven correct. As a defense mechanism, this exquisitely sensitive reaction is very sensible in that any invading bacteria are quickly engulfed and stopped within the clot. As well, if any cracks in the shell, major injuries, or loss of appendages has occurred, then this clotting reaction will effectively serve as a barrier against bacterial entry and infection.

Within the last decade, this clotting reaction has also been observed with 1,3- β -D-glucans. The current understanding of the clotting reaction consists of a proteolytic cascade of enzyme activation steps terminating in the cleavage of the peptide, coagulogen (Figure 4). While the complete reaction is not well understood, the final step of the activated clotting enzyme cleaving coagulogen to insoluble coagulin (Associates of Cape Cod) is well described in the literature. Briefly, the cascade activated by LPS alone consists of 3 protease zymogens: Factor C, Factor B and proclotting enzyme, and coagulogen. Factor C is the first to be autocatalytically activated, which in turn converts Factor B to its active form. If 1,3- β -D-glucans are also present, it is at this point where activated Factor G and/or activated Factor B would in turn convert proclotting enzyme to clotting enzyme. In recent years, scientists in Japan have isolated Factor G, which is now used as a specific LAL test for 1,3- β -D-glucans.

Figure 4. LAL coagulation schemes of both factor C (endotoxin specific) and factor G (glucan specific) involved in the A. chromogenic endpoint method, and B. gel-clot method.

Coagulogen has a calculated molecular weight of 19 600, consisting of a 175-amino acid single basic polypeptide chain. This protein is then divided into 3 separate segments: A chain, peptide C, and B chain. In the presence of either LPS or 1,3- β -D-glucan, the clotting enzyme cleaves two bonds in the coagulogen, the Arg-Gly and Arg-Thr linkages, to yield an insoluble coagulin gel. Upon gelation of the coagulogens

by this clotting enzyme, peptide C is released. Cleavage of the peptide C from within the inner portion of the molecule exposes an extended hydrophobic cove (Iwanaga and Kawabata 1998). The resulting gel now consists of two chains of A and B bridged by two disulfide linkages.

Various modifications have been made to the original gel-clot method since it was first introduced in the mid-1970's. To date, there are three different LAL tests utilized: gel-clot, endpoint chromogenic, and kinetic. Both the endpoint chromogenic and kinetic tests use an artificial colorless peptide substrate, which is cleaved by the clotting enzyme. Upon cleaving, p-nitroaniline (pNA) is liberated, producing a yellow color that can be measured at 405 nm. The greater the endotoxin/glucan concentration in the sample, the faster the pNA will be produced. In the endpoint method, a standard curve is constructed to which the sample absorbance is compared. For the kinetic method, the amount of pNA released is measured against a fixed incubation time. Both methods are built upon the same theory and chemistry.

To obtain the lysate used in all LAL test methods, the horseshoe crab is first caught and bled. Approximately 30% of the animals blood is extracted before it is returned to the water where they completely recover. As the blood is a mixture of both the amebocytes and liquid plasma, the extracted blood must be centrifuged to separate the amebocytes from the serum. The pellet is then washed with saline, followed by re-suspension in distilled water. This causes the cells to absorb the fresh water and rupture (lyse). The absorption of the fresh water is caused by the high concentration of salt contained in the amebocytes versus the absence of salt in the distilled water (Novitsky,

2001). The lysed cells then release coagulogen into solution, which is ultimately filtered and freeze-dried to form the white powder that is distributed.

The physical requirements for 1,3- β -D-glucan in the activation of factor G are relatively well understood. Both linear and branched 1,3- β -D-glucans, as well as mixed linkages of (1,3),(1,4)- β -D-glucans, have been demonstrated to activate factor G at concentrations of 10^{-8} to 10^{-10} g/ml. The activating ability increased with increasing number-average molecular weight (6.8-216 kDa) of linear 1,3- β -D-glucan, while increasing the degree of branching progressively decreased the LAL activation (Tanaka *et al.*, 1991). The apparent critical value for molecular weight necessary to be effective in the LAL assay was determined to be 6800. This molecular weight dependency of the potency was later found not to be the dominant factor in LAL activation, provided that the molecular weight was sufficient to allow the formation of a single helix. Aketagawa *et al* (1993) demonstrated that the single helical conformation of 1,3- β -D-glucans was clearly the dominant contributor to the activation of factor G, based on various 1,3- β -D-glucans consisting of chains of the same molecular weight in single and triple helical forms.

Douwes *et al* (1996) developed an inhibition enzyme immunoassay (EIA) as an alternative to the *Limulus* assay to quantify 1,3- β -D-glucans. The inhibition immunoassay was initially applied to organic dust samples from residential and industrial environments. The purpose of developing the assay was in response to the non-specific classical *Limulus* test, and to the cost of the newly developed glucan-specific *Limulus* test. The laminarin-based calibration curve in the inhibition EIA ranged from 40-3000 ng/ml (15 to 85% inhibition). The assay is specific to the 1,3-glycosidic linkage and to

water-insoluble glucans. The inhibition EIA developed by Douwes *et al* is more sensitive than the original (1 ug/ml) described by Adachi *et al* (1994), but less sensitive than the *Limulus* test (1-10 pg/ml). An enzyme-linked immunosorbent assay (ELISA) originally developed for systemic fungemia (Fisette *et al.* 1998) was modified by Milton *et al.* (2001) to be highly specific for (1→6) branched, 1,3-β-D-glucans. The detection level for the ELISA was 0.8 ng/ml for baker's yeast and Betafectin (a yeast cell wall derived β-glucan). These issues of sensitivity may play a crucial role in the analysis of indoor airborne 1,3-β-D-glucan, as levels can be much lower than 40 ng/ml in the majority of residential environments.

II-4 *Project Aim*

The use of 1,3- β -D-glucan in air and dust samples from both residential and occupational environments has become a common method of determining fungal exposures in research studies. In this project, the biological assay used for the determination of bacterial endotoxins was used in combination with an extraction procedure in order to make the test specific to 1,3- β -D-glucans. In order for the test to be competitive with the recently available glucan-specific assay, the chemical conditions used must also be appropriate for small quantities of 1,3- β -D-glucan. An inexpensive and sensitive alternative to the glucan-specific assays is necessary for large-scale epidemiological studies, such as those currently underway by Health Canada in Prince Edward Island. Adaptation of the LAL assay to 1,3- β -D-glucan is a more cost efficient alternative to competing methods (Factor G specific assay is approximately 4x more expensive), and is more sensitive than other ELISA-type tests (which are currently at the ng/mL range compared to 1-10 pg/mL for the LAL). Availability of the glucan-specific assay to the North American market is only recent and its supply in large-scale epidemiological studies, such as the one undertaken by Health Canada, is questionable. Modifying the LAL protocols to achieve an efficient, non-destructive and sensitive test towards 1,3- β -D-glucans is a useful approach.

III MATERIALS

The “Pyrochrome” *Limulus* ameocyte lysate assay (LAL), Control Standard Endotoxin (CSE) and Lysate Reagent Water (LRW) were all obtained from Associates of Cape Cod (Falmouth, MA). All reagents and solvents used for extraction and chromatography were of HPLC grade. Sodium hydroxide (2N solution), Hydrochloric Acid (1N solution) and n-Pentane were obtained from Fisher Scientific (Nepean, ON). Polycarbonate Nuclear Track-Etch Filters (0.4µm, 37mm) used for air sampling were obtained from Omega Specialty Instrument Company (Chelmsford, MA). Acrodisc filters (0.45 µm, 13 mm) were obtained from Gelman Sciences (Ann Arbor, MI). Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and Sodium Periodate (100.2%) were obtained from Sigma Chemical Company (St. Louis, MO). All standards such as curdlan (lot #89H4032, 1,3-β-D-glucan from *Alcaligenes faecalis*), ergosterol (>90%) and cholesterol (99+%) were also obtained from Sigma Chemical Company (St. Louis, MO).

IV EXPERIMENTAL

IV-1 Controlled Test Experiments Using LAL Test

IV-1.A.1 Destruction of Endotoxin Using Various Concentrations of NaOH

To a 10 ng/vial of Control Standard Endotoxin (CSE), various concentrations of sodium hydroxide were added in 1 ml volumes. The concentrations of sodium hydroxide utilized were 0.5 N, 0.3 N, 0.15 N as well as a control containing only lysate reagent water (LRW). All tests were performed in triplicate. The solutions were allowed to

stand at room temperature for 2.5 hours after which all vials were neutralized with equal volumes of Tris-HCl (1.0, 0.6, 0.3 and 0 M, respectively). All LPS test solutions were serially diluted to give concentrations of 5.0, 2.5, 1.0, 0.5 and 0.1 ng/ml. To a 96-well microplate, 50 µl of each dilution was added followed by 50µl of reconstituted LAL reagent (3.2 ml of Pyrochrome reconstitution buffer added to lyophilized Pyrochrome). The plate was incubated at 37°C in a block heater (Associates of Cape Cod, Falmouth, MA) for 15 minutes after which the absorbance was read using a Dynatech MR5000, microplate reader equipped with a 405 nm filter (Dynatech Laboratory, Chantilly, VA.). Absorbance values obtained were referenced against a standard curve made with the control endotoxin dissolved in only LRW. The data output from the microplate reader was viewed and manipulated using Biolinx V.2.01 software.

1.A.2 Destruction of Endotoxin using 0.15 N NaOH vs Time

To 5 groups of 10 ng/vial CSE, each group in triplicate, 0.15 N NaOH was added to a volume of 1 ml. Another group of 10 ng/vial CSE was dissolved in 1 ml of LRW and this was used as a control. Test solutions were allowed to stand for various times at room temperature (group 1: 0.5 hrs, group 2: 1.0 hr, group 3: 1.5 hrs, group 4: 2.0 hrs, group 5: 2.5 hrs). At each time interval, the test samples were neutralized with an equal amount of 0.3 M Tris-HCl and serially diluted to concentrations of 2.5, 1.25, 0.625 and 0.3125 ng/ml using LRW. Samples were then analysed as above, using the control as a reference.

IV-1.B Effect of 0.15 N NaOH on curdlan, a 1,3- β -D-glucan

Using the optimal endotoxin hydrolysis time of 2.5 hours obtained from the above experiments, the effect of 0.15 N NaOH on 1,3- β -D-glucan was investigated. Various amounts of curdlan, a water insoluble 1,3- β -D-glucan, ranging from 50-0.3125 ng/ml, were allowed to stand for 2.5, 12 and 25 hours at room temperature. After each incubation period, a 100 μ l aliquot of each sample was neutralized with an equal volume of 0.3 M Tris-HCl. As a control, curdlan was dissolved in 0.15 N NaOH and immediately neutralized with Tris-HCl. At each time interval, the samples were analysed as above.

Anticipating that the triple helix conformation of curdlan would become partially opened when dissolved in sodium hydroxide, the reactivity of 1,3- β -D-glucans with the LAL reagent was also investigated with respect to the stability of the open-stranded conformation after neutralization with Tris-HCl. The conversion back to the triple helix form was observed on the neutralized 2.5 hour sample at times $t=10$ and $t=23$ hrs after neutralization.

This experiment was repeated once more following the same experimental procedure except for the following changes: 1. using a lower concentration of 1,3- β -D-glucans (10-0.375 ng/ml), 2. 1,3- β -D-glucan in 0.15 N NaOH for 2.5 hrs, and 3. without observing the conversion of the open stranded 1,3- β -D-glucan back to the triple helix over time after neutralization. The purpose was to obtain results in an adequate range where the LAL reagent would not be saturated.

IV-1.C Sodium Periodate Oxidation of 1,3- β -D-glucan

The purity of the curdlan used was checked by NaIO₄ oxidation, which eliminates any reaction of 1,6- β -glucans with the LAL reagent, using a modified version of the method employed by Douwes *et al* (1996). 1,3- β -D-glucans should not be oxidized by this agent, and thus any decrease in reactivity with the reagent would most likely be as a result of impurities in the standard.

Due to the insolubility of 1,3- β -D-glucans in NaIO₄, the curdlan sample was first dissolved in 0.15 N NaOH, and then a small aliquot of this was immediately put into a volume of 0.25 M NaIO₄ so not to affect the concentration of the periodate. Additionally, from the 0.15 N NaOH dissolved 1,3- β -D-glucan standard, an aliquot was taken and immediately neutralized with 0.3 M Tris-HCl for a control. Oxidized 1,3- β -D-glucan concentrations ranged from 50-3.125 ng/ml. All sample solutions were allowed to stand for 2.5 hours after which the test sample reactions were stopped by adding 1 M ethanolamine (300 μ l sample + 35 μ l ethanolamine + 265 μ l LRW) leading to a 1:1 dilution of the test samples. Each sample and control standard were added (50 μ l) to a microplate where 50 μ l of reconstituted LAL reagent followed. The plate was incubated at 37°C and analyzed as previously described.

IV-1.D Effect of Adding Tween to Extraction of 1,3- β -D-glucan

Since spores of many mold species are hydrophobic, they tend to clump together when suspended in aqueous solutions. It is common practice to add a non-ionic detergent (~0.01%) to the water in order to combat this problem. Therefore the effect of adding Tween 20 to 1,3- β -D-glucan standards was investigated. Vials containing 10 ng of

curdlan were dissolved in 0.15 N NaOH and either 0.1%, 0.05%, 0.02%, 0.005% or 0% (control 1,3- β -D-glucan) Tween was added. All tests were performed in triplicate. The final concentration of each vial was 10 ng/ml. All sample solutions were shaken on a rotary shaker (220 rpm) for 15 minutes at which time they were removed and allowed to stand for an additional 2.25 hours. After a total of 2.5 hours, all vials were neutralized with 0.3 M Tris-HCl. All sample solutions were serially diluted to 5, 2.5, 1.25, 0.625, 0.3125 ng/ml. Samples were then analyzed using the LAL test as previously described. Absorbance values were compared to that of the control 1,3- β -D-glucan, which was dissolved in 0.15 N NaOH without any Tween.

IV-1.E.1 LAL Reactivity of Various Glucan Concentrations With Addition of Endotoxin

Experiments were done combining various concentrations of curdlan (10, 5 and 2 ng/ml) with a constant amount of endotoxin (10 ng/vial, lyophilized). All samples had a final volume of 1 ml 0.15 N NaOH, and all were done in triplicate. Samples, as well as a curdlan standard, were placed on a shaker (220-rpm) for 2.5 hours after which they were all neutralized with 0.3 M Tris-HCl. The curdlan standard was serially diluted to give a concentration range of 5-0.5 ng/ml. Samples and standards were all analyzed as above using the LAL test.

The same experiment was repeated using a smaller amount of endotoxin (0.4 ng) and concentrations of curdlan ranging from 10-1 ng/ml. The range of endotoxin concentrations (10-0.4 ng/ml) correspond to those which are commonly seen in residential air samples. Analysis using the LAL test was performed as described above.

1.E.2 LAL Reactivity of Glucan With Addition of Various Amounts of Endotoxin

A constant amount of curdlan (5 ng) had various amounts of LPS added (10, 7.5, 3.75, 1.875, 0.9375 ng and 0 ng/ml). All vials contained a final volume of 1.0 ml of 0.15 N NaOH, and were performed in triplicate. Samples, as well as the control curdlan standard (0 ng LPS added), were placed on a rotary shaker (220 rpm) for 2.5 hours at which time they were all neutralized with 0.3 M Tris-HCl. The control 1,3- β -D-glucan standard was serially diluted to give a concentration range of 5-0.3125 ng/ml. Sample solutions were analysed as above.

The same experiment was repeated with 5 ng/ml 1,3- β -D-glucan control standard using different endotoxin concentrations (10, 5, 2.5, 1.25, 0.625 ng/ml) added.

The experiment was repeated for a third time with further changes. All concentrations of 1,3- β -D-glucan, endotoxin and NaOH were used as above, however the ratio of sample to reconstituted LAL reagent was changed. Instead of the traditional 1:1 mixture, a 2:1 mixture was used in favour of the reagent (ie. 45 μ l of sample to 90 μ l of reconstituted LAL reagent).

1.E.3 Glucan Reactivity With Addition of Various Amounts of Endotoxin vs Time

The above experiment was repeated once again using the same concentrations of curdlan (5 ng/ml) and endotoxin (10, 5, 2.5, 1.25, and 0.625 ng/ml). However, to determine if longer periods of time were necessary to decrease the over-estimate of 1,3- β -D-glucan in the LAL test, periods of 2.5, 3.5, 5.0 and 25.0 hours were used in the experiment. Briefly, triplicate vials containing each test sample, as well as the standard control 1,3- β -D-glucan, were shaken (220 rpm) in 0.15 N NaOH for the aforementioned

times. At each time period, a 400 μ l aliquot was removed from the vial and neutralized with an equal amount of 0.3 M Tris-HCl. To a 96-well microplate, 45 μ l of each sample was added followed by 90 μ l of reconstituted LAL reagent. The plate was incubated at 37°C for 12 minutes at which time the absorbance was read at 405 nm using a microplate reader.

IV-1.F.1 Extraction of 1,3- β -D-glucans From Fungal Spores

Spores obtained from a culture of *Cladosporium cladosporioides* were gently removed from the agar, weighed and suspended in deionized distilled water. The suspension was briefly homogenized in a 45 ml Potter Elvehjem Tissue Grinder. The total number of spores were then counted in a hemocytometer using a light microscope (400x, WILD, Heerbrugg, Switzerland). From the spore suspension, a known volume was quantitatively transferred to a polycarbonate membrane filter (0.4 μ m, 37 mm) under suction. Amounts of spores added, in triplicate, were 2.0, 1.0, 0.5, 0.25 and 0.125 mg.

Each filter was placed in a depyrogenated 125 ml Erlenmeyer flask with 10 ml of 0.15 N NaOH. The flasks were placed on a rotary shaker (220 rpm) for 2.5 hours to extract. The sample solutions were then neutralized and serially diluted 10X, 100X and 1000X with LRW. A control standard of curdlan was used to compare the results with concentrations ranging from 5-0.3125 ng/ml. The samples were analysed as above. Making the assumption that the 1,3- β -D-glucan content per spore is similar to that of the ergosterol content per spore, the total number of spores were estimated from the results. This experiment was repeated 3 times, each time with a new spore suspension.

1.F.2 Extraction of 1,3- β -D-Glucan from Outdoor Air Samples

Outdoor air samples were obtained from outside the Steacie Building (Carleton University, Ottawa) in April 2001. The samples were obtained using a Zefon Analytical air pump and a polycarbonate membrane filter (0.4 μ m, 37 mm) for a time of 15 minutes per sample at 10.0 L/minute. The total volume sampled was 0.15 m². The filters were added to depyrogenated 125 ml Erlenmeyer flasks followed by 10 ml of 0.15 N NaOH. Curdlan standards were made in the range of 5-0.3125 ng/ml for comparison. The flasks were then allowed to shake (220 rpm) for 2.5 hours. Each flask was then neutralized with an equal volume of 0.3 M Tris-HCl and serially diluted 10X, 100X and 1000X with LRW. Each sample was analysed as above. Using the amount of 1,3- β -D-glucan per spore obtained from the *C. cladosporioides* data above, an average number of spores on the filters was obtained for the 3 air samples taken.

This experiment was repeated once more on a different day, using sampling times of 15 and 30 minutes for 1,3- β -D-glucan analysis (0.15 m² and 0.3 m² of air sampled, respectively), and 15 minutes for endotoxin analysis. Standards of each compound (curdlan and endotoxin) were used for comparison purposes.

IV-1.G.1 LAL Reactivity of Fungal Glucan With Addition of Endotoxin

To determine whether endotoxin above 5 ng affected the calculated 1,3- β -D-glucan concentration from fungal spores in the LAL assay, tests were done involving spores from *C. cladosporioides*. Once again, spores were gently scraped from an agar culture, suspended, homogenized and counted in a hemocytometer, as done previously. The necessary dilutions required to get approximately 16,500 spores/ml were done (3.09

pg/spore x 16,500 spores = ~51 ng). To polycarbonate membrane filters, 1.0 ml aliquots of the suspension were added and filtered under suction. The filters were placed in depyrogenated 125 ml Erlenmeyer flasks. Appropriate standards of endotoxin were made up in 0.15 N NaOH which were then immediately added to the extraction volume in 6 different concentrations: 10, 5, 2.5, 1.25, 0.625 and 0 ng/ml. All tests were performed in triplicate. All flasks then had appropriate volumes of 0.15 N NaOH added, to give a total volume of 10 ml. All flasks were shaken (220 rpm) for 2.5 hours, at which time all flasks were neutralized and further diluted 1:1 with LRW to achieve a final expected 1,3- β -D-glucan concentration of 1.25 ng/ml.

The '0 ng/ml endotoxin added' was used as a control 1,3- β -D-glucan from spores to ensure that the correct amount of spores, and thus 1,3- β -D-glucan, were added to the filters. Also, curdlan standards were made up in the concentration range of 5-0.3125 ng/ml for comparison. Samples were analysed as above.

This experiment was repeated two more times using 0.3 N NaOH and 0.5 N NaOH for the extraction, and 0.6 M and 1.0 M Tris-HCl, respectively, to neutralize. All other concentrations were kept the same.

IV-1.G.2 Reactivity of Fungal Glucan with Added Endotoxin vs Time in 0.5 N NaOH

Suspension and addition of *C. cladosporioides* spores was carried out as above. Briefly, approximately 16,500 spores were added to a series of polycarbonate filters. There were 6 groups of filters, each group being tested in triplicate, to which varying concentrations of endotoxin were added (10, 5, 2.5, 1.25, 0.625, and 0 ng/ml) within a depyrogenated flask. After the addition of the endotoxin to each flask, the volume was

adjusted accordingly with 0.5 N NaOH to a volume of 10 ml. All flasks were placed on a rotary shaker (220 rpm) for various times (0.5, 1.0, 1.75 and 2.5 hours). At each time interval, a 500 μ l aliquot was removed, neutralized, diluted 1:1 with lysate reagent water, and analysed as above.

IV-1.H Effect on 1,3- β -D-glucan in 0.5 N NaOH

Curdlan standards were made in the range of 10-0.625 ng/ml in 0.5 N NaOH. A control set of curdlan standards were also made using 0.15 N NaOH in the same concentration range. All vials were allowed to stand for 2.5 hours at which time they were neutralized with an equal amount of 1.0 M and 0.3 M Tris-HCl, respectively. Each sample was analysed as above. To determine the total glucose eliminated during the hydrolysis of 1,3- β -D-glucan, a glucose-specific enzymatic assay (lot #061K6132, Sigma Diagnostics, St. Louis) was used. Curdlan standards used in the above experiment were used for this purpose. Briefly, the enzyme solution was prepared by adding the contents of one capsule of PGO enzymes supplied in the assay kit to 100 ml of deionized distilled water in an amber bottle. The bottle was inverted several times with gentle shaking to dissolve. The color reagent solution was prepared by reconstituting one vial of o-Dianisidine Dihydrochloride, also supplied in the assay kit, with 20 ml of water. The combined enzyme-color reagent solution was prepared by combining 100 ml of the enzyme solution and 1.6 ml of the color reagent solution. The mixture was inverted several times with mild shaking. Glucose standard solution (100 mg/dL) was supplied.

To perform the glucose assay, a blank vial containing 0.5 ml of water, three vials containing 0.5 ml of a 40, 80 and 160-fold dilution of the glucose standard solution, and three vials containing 0.5 ml of the 1 mg/ml, 100 ng/ml and 10 ng/ml curdlan standards, were made. To each vial, 5.0 ml of the combined enzyme-color reagent solution was added followed by thorough mixing, and incubation at 37°C for 30 minutes. At the end of the incubation, an aliquot of each vial was added to a microplate and the absorbance was read at 465 nm in a microplate reader using the blank as a reference. This test is believed to be sufficiently accurate to a level of 25 mg/dL of glucose.

To observe the time requirement to achieve the increase in LAL reactivity, curdlan standards were made in the same concentration range as above. Vials were allowed to stand for 0, 0.5, 1.0, 1.75, 2.5 and 24 hours in 0.5 N NaOH. At each time interval, a 100 µl aliquot was removed, neutralized, diluted and analyzed using the LAL test above.

IV-1.I.1 Extraction of Glucan From Ragweed Pollen

A common pollen found in outdoor air in North America, *Ambrosia artemisiifolia*, or ragweed, was tested using the above method employing 0.5 N NaOH as the extraction solvent. Briefly, triplicate trials of 1 and 2 mg of pollen were added to 125 ml Erlenmeyer flasks followed by 5 ml of 0.5 N NaOH. All flasks were shaken for 2.5 hours. Due to the yellow colour of the suspended pollen granules, the NaOH extract of each sample was transferred to a sterile 17 ml centrifuge tube. Each sample tube was centrifuged at 2000 rpm for 10 minutes, at which time a 500 µl aliquot of the supernatant was removed. This aliquot was neutralized with 1 M Tris-HCl and further diluted 1:1

and 10X with LRW. Analysis of 1,3- β -D-glucan was performed as above using curdlan in the range of 5-0.3125 ng/ml as a standard.

IV-1.1.2 Extraction of Glucan From Yeast

Baker's yeast was obtained from a local grocery store and ground in a mortar and pestle. To a 500 ml Erlenmeyer flask, 100 ml of tap water and 1 g of sucrose were added, autoclaved for 20 minutes and cooled in a laminar flow hood. Once cool, a small amount of the ground yeast was added to the flask, which was then allowed to stand in a dark growth room for 1 week at 25°C.

After 1 week, the yeast was centrifuged in two 17 ml sterile centrifuge tubes. From each tube, the supernatant was decanted and discarded. The yeast pellet was kept in the tube while more of the initial yeast suspension was added. This procedure was repeated until the whole yeast suspension had been centrifuged and decanted. The combined yeast pellets were then re-suspended in 50 ml of LRW and qualitatively transferred to a 125 ml Erlenmeyer flask. Using a hemocytometer, the number of yeast spores was measured to be 725,000 spores/ml. This suspension was further diluted to give a concentration of 140,000 spores/ml.

From the above diluted suspension, three different volumes were added to a depyrogenated 125 ml Erlenmeyer flask. The number of spores added were 350 000, 140 000 and 70 000 spores, all in triplicate. Appropriate volumes of LRW and 1.0 N NaOH were added to give a final volume of 10 ml and a final concentration of sodium hydroxide of 0.5 N. Each flask was then shaken for 2.5 hours at which time sample solutions were neutralized and analysed using the LAL method above.

IV-1.J Effect of a Freeze-Thaw Cycle on LAL Reactivity of 1,3- β -D-Glucan

A series of three 1 mg/ml solutions of curdlan in 0.5 N NaOH were prepared. To one vial, the contents were neutralized with an equal amount of 1.0 M Tris-HCl. To the remaining two, no neutralization of the contents took place. All three vials were placed in a freezer for 1 month at -8°C. One of the vials which had not been neutralized prior to freezing was removed from the freezer after two weeks and allowed to completely thaw. Upon complete thawing of the sample, the vial was placed back into the freezer for the remaining two weeks. This created a set of samples consisting of a single freeze-thaw cycle for a neutralized and basic alkaline sample, as well as a double freeze-thaw cycle for a basic alkaline sample. After the one month freezing period, all vials were removed from the freezer and allowed to thaw completely. At this time, the samples were serially diluted with LRW to a concentration range of 5-0.3125 ng/ml. All samples were analyzed using the LAL test as done previously.

IV-2 LAL Analysis Method for 1,3- β -D-glucan From Field Samples

The final method used for analysing field samples was deemed as an appropriate procedure based upon all of the above experiments. Briefly, field samples were collected during the winter of 2000 and 2001. The homes of approximately 60 families per year were sampled, focusing on the bedroom and livingroom areas. Air was pumped through a three-piece cartridge equipped with a polycarbonate filter for approximately 7 days. The total volume of air sampled ranged from 6.0 to 23.9 m³. Once collected, the cartridges and filters were sealed in plastic bags and stored at room temperature in a dry area until analysis. Prior to analysis, filters were removed from their three-piece

cartridge using sterile tweezers and weighed. The weighed filters were then folded into four and carefully placed into separate depyrogenated 125 ml Erlenmeyer flasks. To each flask, 5 ml of 0.5 N NaOH was added and each was shaken for 2.5 hours on a shaker set at 220 rpm. After the 2.5 hour extraction period, a 500 μ l aliquot of each sample solution was removed and neutralized with an equal amount of 1.0 M Tris-HCl. The neutralized sample was further serially diluted with LRW.

To a 96-well microplate, 40 μ l of each sample and its respective dilutions, was added, followed by 80 μ l of reconstituted Pyrochrome LAL reagent. For comparison purposes, a standard curve was constructed using curdlan in the range of 5 to 0.3125 ng/ml. The plate was incubated for 16-20 minutes at 37°C and then placed into a microplate reader where the absorbance was read at 405 nm. The reaction was not deemed necessary to be stopped with acetic acid (as the manufacturer recommends), as the microplate reader can read the whole plate within 3 seconds.

All data obtained was then recorded in Appendix 1, where the concentration of 1,3- β -D-glucan per cubic meter of air sampled was calculated for each sample.

All samples were frozen after being neutralized to be re-analyzed using the LAL test at a future date. These data provided statistics on the reproducibility of the modified LAL method.

IV-3 Size-Exclusion Chromatography of 1,3- β -D-Glucan

Size-exclusion chromatography (SEC) coupled to refractive index (RI) and diode array detectors (DAD) was employed on filter extracts previously tested using the LAL method. A TSK-Gel G4000P_wXL (7.8 x 300 mm, 10 μ , 1-750 kDa cutoff) column with a

TSK-Gel P_wXL Guard column (6.0 x 40 mm, 12 μ), both supplied by Tosoh Biosep (Montgomeryville, PA), were connected in series to an Agilent 1100 series quaternary HPLC pump. Fractionated pullulan standards (5.8-788 kDa, Showa Denko KK, Tokyo) were prepared by suspending 1 mg of each molecular weight standard in 1 ml of deionized distilled water (ddH₂O). Vials were not shaken or disturbed for 24 hours to allow the polymer to go into solution on its own, at which time the vial was gently shaken. The mobile phase consisted of ddH₂O run isocratically at 1.0 ml/min for 15 minutes. Detection of all standards and samples was performed using a RI detector (Waters Differential Refractometer RR401, Waters Inc.) and a DAD (Agilent 1100 series, Agilent Technologies) with a full spectrum range of 190 nm to 900 nm. For each standard, 20 μ l was injected onto the column. A curdlan standard (1.0 mg/ml) was also injected onto the column to determine the average molecular weight of the standard used throughout the LAL experiments. The UV spectrum obtained from the DAD showed that curdlan had a maximum absorbance at 216 nm, and therefore the DAD was set to focus on this particular wavelength, as well as the full spectrum, for the remainder of the analyses. After the calibration curve was completed, and curdlan was confirmed using the above conditions, the limit of detection was determined using serially diluted curdlan standards in the range of 1 mg/ml to 0.3125 ng/ml. Immediately after performing the LOD experiment, the same curdlan standards were used to determine the calculated LAL concentration of each of them. Frozen neutralized extracts used in the LAL analysis were then thawed each day for SEC analysis.

IV-4 Ergosterol Field Samples

IV-4.A Extraction of Ergosterol from Filters

Field samples for ergosterol analysis were obtained concurrently with those of 1,3- β -D-glucan, and were collected and stored using the same protocol. The total volume of air sampled ranged from 10.7 to 25.4 m³. The microwave assisted extraction method developed by Young (1995) was employed for the extraction of ergosterol from the filters. Briefly, filters were removed from the plastic cassettes and weighed. Filters were then folded and placed into separate 17 ml culture tubes to which 2.0 ml of methanol and 0.5 ml of 2 M NaOH were added. The tubes were sealed tightly with Teflon-lined screw caps which themselves were sealed with parafilm M. The sealed tubes were then placed into a 500 ml plastic bottle which were individually arranged in the centre of a domestic microwave oven (Citizen 0.7 cu. Ft., Model JM5224, 700W output power, 2450 MHz frequency). Each filter was digested for 20 seconds at 50% power, allowed to stand for 1 minute, and then digested again for 15 seconds at 50% power. After allowing the culture tube to cool (~15 minutes), the tube was removed from the plastic bottle and its contents were neutralized with 1 ml of 1 M HCl. The neutral solution was then extracted with 3 X 2 ml of pentane. The combined extracts were transferred to a 10 ml glass vial where they were dried down under nitrogen gas. The dried extracts were then re-dissolved in 3 X 0.5 ml of pentane, from which each aliquot was put through a 0.45 μ m Acrodisc filter (13 mm, Gelman Sciences) into a 1 dram vial. The combined filtered extracts were once again dried down under nitrogen gas and placed into the freezer until time of analysis.

IV-4.B Recrystallization of Ergosterol

Commercial ergosterol (Sigma Chemical Company) was recrystallized in the dark prior to the analysis. Briefly, 2 g of the crude standard was placed in a 250 ml beaker. In a separate beaker, ethanol was gently heated to an almost boil using a steam bath. A minimal amount of the hot ethanol was added to the crude ergosterol in order for it to completely dissolve. Upon complete dissolution, the solution was allowed to cool for 4 hours at room temperature at which time the beaker was placed in an ice bath for 30 minutes. The crystals formed during this period were then filtered through a Whatman No.1 filter paper under suction. The purified ergosterol crystals were transferred to a 3 dram amber vial where it was freeze-dried overnight. The dried ergosterol crystals were stored at 4°C prior to the preparation of standard solutions.

IV-4.C Analysis of Ergosterol by Gas-Chromatography / Mass Spectrometry

The dried samples were dissolved in 75 µl of pentane at the time of analysis. The presence of ergosterol in the extracted air samples was confirmed using an Agilent 5973 quadrupole mass spectrometer (Agilent Technologies), operating in the electron ionization (EI) mode at 70 eV. The concentration of ergosterol in each sample was compared against an external pure standard in the selective ion mode (SIM) using m/z 363 and 396. Compounds were separated on an Agilent 6890 Series gas chromatograph equipped with a 30m x 0.25mm ID x 0.25µm ZB-5 capillary column (5% Phenyl-95% dimethyl-polysiloxane, Phenomenex, Torrance, Calif.) using the pulsed-splitless mode

(35 psi pulse for 1 min.). The column was temperature programmed from 150 to 300°C at 20°C minute⁻¹ under constant flow.

Cholesterol was also analysed at the same time as ergosterol simply by adding ions at m/z 368 and 386, in the SIM mode. All data, both ergosterol and cholesterol, were recorded in Appendix 2 where the amount of each compound was calculated per cubic meter of air sampled.

V. RESULTS

During preliminary tests involving endotoxin in lysate reagent water, and curdlan in 0.1 N NaOH, it was observed that the sensitivity of the *Limulus* amoebocyte lysate test was approximately 1000 times more sensitive towards endotoxin than to 1,3- β -D-glucan. Initial tests involving the hydrolysis of pure endotoxin showed that 0.15 N NaOH eliminated the biological activity of 10 ng to a greater extent than other concentrations tested (Figure 5). As concentrations of sodium hydroxide decreased from 0.5 N to 0.15 N, the LAL activity of endotoxin also decreased. Using the highest concentration of sodium hydroxide, the activity of 10 ng of pure endotoxin was lowered to an equivalence of 4.7 ± 0.3 ng, or 53% biological inactivation. This was lowered further to only 1.5 ± 0.2 ng (85%) and 0.0 ± 0.1 ng (100%) for both 0.3 N and 0.15 N NaOH, respectively.

Figure 5. Graph depicting the elimination of endotoxin LAL activity in various concentrations of sodium hydroxide for a time of 2.5 hours. Control endotoxin was dissolved in lysate reagent water (LRW). Note: Standard deviations of Absorbance Units are too small to be observed on graph.

Using the optimal concentration of 0.15 N sodium hydroxide, the above experiment was carried out again with respect to time. Results indicated that during the first hour of incubation, only 20% of the 10 ng of endotoxin had been inactivated by the sodium hydroxide. A gradual decrease in activity was observed every half-hour thereafter. Almost complete elimination of the endotoxin activity was not observed until 2.5 hours (Figure 6)

Figure 6. Elimination of endotoxin activity by hydrolysis in 0.15 N NaOH at various times, ranging from 0.5 hours to 2.5 hours.

Note: Standard deviations of Absorbance Units are too small to be observed on graph.

When curdlan, a 1,3- β -D-glucan, was dissolved in 0.15 N NaOH, the addition of the base had the opposite effect to that for endotoxin. When the 1,3- β -D-glucan was immediately neutralized, its reactivity with the LAL reagent was lower than that of standards that were allowed to hydrolyze for 2.5 hours or more. Although the reactivity was greatly enhanced from the initial time to 2.5 hours, no significant change in absorbance was observed for times greater than 2.5 hours ($t < 25$ hours) (Figure 7).

Figure 7. Effect of 0.15 N NaOH hydrolysis on curdlan with respect to time, over a period of 25 hours. Control curdlan was immediately neutralized with an equal amount of 0.3 M Tris-HCl. Note: Standard deviations are too small to be observed on graph.

Anticipating the partially-opened triple helix would revert to its original conformation upon neutralization, an experiment was performed to confirm the assumptions and to establish a useful operating range of time for the test. The results obtained showed that after the extract was neutralized, a decrease in curdlan reactivity with the LAL reagent occurred at 10 hours and 23 hours (Figure 8).

Figure 8. Curdlans conversion of partially-opened triple helix back to original triple helix conformation after neutralization with respect to time.

Note: Standard deviations of Absorbance Units are too small to be observed on graph.

Upon oxidation of the curdlan standard with NaIO_4 , no significant change in absorbance was observed in the concentration range of 50-3.125 ng/ml (Figure **9a**). Since the oxidation is specific to the 1,6- β linkage of glucans, the relatively consistent LAL reactivity of the 1,3- β -D-glucan indicates that the curdlan standard was pure within the limits of detection. Upon addition of Tween to curdlan standards (10 ng/ml) in sodium hydroxide, similar results were obtained, as no significant change ($p < 0.05$) in 1,3- β -D-glucan reactivity was observed over the various amounts of Tween added. The concentrations of Tween tested ranged from 0.1% to 0.005% (Figure **9b**).

Figure 9. A. Effect of 0.25M periodate oxidation on curdlan, a 1,3- β -D-glucan, in the concentration range of 50-3.125 ng/ml for a time period of 2.5 hours. B. Effect of various concentrations of Tween added to 0.15 N NaOH on a constant concentration of curdlan (10 ng/ml).

The appropriateness of all the above conditions was tested prior to their application towards fungal spores. The experiments conducted fixed the concentration of either 1,3- β -D-glucan or endotoxin, respectively, across a range of amounts of the other compound. These experiments revealed that when pure endotoxin was tested in combination with 1,3- β -D-glucan, the endotoxin was not destroyed within the 2.5 hour hydrolysis time. This meant that the amount of 1,3- β -D-glucan measured by the LAL method was higher than the correct value. In some cases, the calculated amount was as high as 350% that of the expected control 1,3- β -D-glucan standards when 10 ng of endotoxin had been added to 5 ng of curdlan. This value decreased as the amount of endotoxin decreased, reaching a low of around 190% when only 0.625 ng of endotoxin was added (Figure 10).

Figure 10. Calculated LAL reactivity of curdlan, a 1,3- β -D-glucan, by the addition of various amounts of endotoxin using a 1:1 sample to reagent ratio.

When the same experiment was repeated using a 1:2 ratio of sample and reagent, the "levelling effect seen in Figure 10 at moderate to high concentrations of endotoxin was eliminated (Figure 11). To deal with the problem of the over-estimation of 1,3- β -D-glucan by the LAL test when present with endotoxin, the combined curdlan/endotoxin solutions were hydrolysed for longer periods of time (2.5, 3.5, 5.0 and 25 hours). The longer hydrolysis times did not affect the results.

Figure 11. Calculated LAL activity of curdlan by the addition of various amounts of endotoxin using a 1:2 sample to reagent ratio. The impact of endotoxin above 5 ng was reduced when more LAL reagent was present. An expected 1,3- β -D-glucan concentration of 1.25 ng/ml rather than 2.5 ng/ml as in Figure 10.

Prior to testing the effect of pure endotoxin on 1,3- β -D-glucan from fungal spores, a pure culture of *C. cladosporioides* was tested for its 1,3- β -D-glucan content. Four independent replications of this experiment were done, with a mean value of 3.1 ± 0.6 pg 1,3- β -D-glucan/spore. Based on the mean 1,3- β -D-glucan content in outdoor air samples, and the measured 1,3- β -D-glucan content per spore, a value of 2400 spores/m³ was obtained. This compared favorably to direct measurements taken using spore counting

with a microscope. No endotoxin was detected in concurrent outdoor air samples. This removes endotoxin as a possible variable in the estimation of spores from these outdoor samples as determined by this method.

Again, based on the data obtained above for *C. cladosporioides*, a known number of spores were added to filters, which were later combined with various amounts of endotoxin and extracted for 2.5 hours. The concentration of spores in the aqueous suspension was determined using a hemocytometer prior to adding the desired number of spores to the filter (16,500 spores). The 1,3- β -D-glucan content from spores added to the control filters was not statistically different ($p < 0.05$) from the expected value of 1.25 ng/ml.

The above results led to experiments involving greater concentrations of sodium hydroxide in order to eliminate the effect of endotoxin. Concentrations of base increased from the initial 0.15 N NaOH to 0.3 N and 0.5 N NaOH. These increases in base concentration resulted in much more accurately estimated 1,3- β -D-glucan concentrations, however 1,3- β -D-glucan was still being over-estimated. For both the 0.3 and 0.5 N NaOH tests, there was not a significant change ($p < 0.02$) in LAL calculated 1,3- β -D-glucan concentration in the low range of endotoxin, namely below 2.5 ng. However, when the endotoxin concentration was increased to greater than 2.5 ng, the 0.3 N NaOH test showed a significant increase in apparent 1,3- β -D-glucan concentration. The 0.5 N NaOH test still continued to show no change. However, when the endotoxin concentration exceeded the 5 ng range, all sodium hydroxide concentrations indicated an over-estimate of 1,3- β -D-glucan (Figure 12). These results are satisfactory for indoor and outdoor air samples as endotoxin concentrations rarely exceed the 5 ng range (Health

Canada, unpublished data). A subsequent test involving similar conditions of fungal 1,3- β -D-glucan and endotoxin in 0.5 N NaOH with respect to time indicated that a full 2.5 hours was needed to eliminate endotoxin activity. Once again, there was no significant difference between the control fungal 1,3- β -D-glucan and that seen below the 5 ng/ml of endotoxin added.

Figure 12. Calculated 1,3- β -D-glucan concentration from *C. cladosporioides* in three different concentrations of sodium hydroxide (0.15, 0.3 and 0.5 N) when combined with various amounts of pure endotoxin.

(* indicates statistically different from control, $P < 0.02$, ANOVA, Tukey-HSD)

When curdlan was tested by itself in 0.5 N NaOH, a significant increase in sensitivity was achieved over that of curdlan in 0.15 N NaOH. At high concentrations of curdlan, an increase of approximately 340% in absorbance was observed. At lower concentrations of curdlan, an even larger increase in absorbance was seen leading to an increase in sensitivity of almost 500%.

As the reactivity of curdlan with the LAL reagent increased with sodium hydroxide concentrations, it was worthwhile to investigate the time at which the 1,3- β -D-glucan test was most sensitive. Curdlan was immediately hydrolyzed by the higher concentration of sodium hydroxide. After the immediate increase of reactivity, curdlan showed no further rise in absorbance over time, up to a period of 24 hours.

Other experiments carried out which demonstrated the LAL applicability for the measurement of 1,3- β -D-glucan were performed on pollen and yeast. The 1,3- β -D-glucan content obtained from an extract of ragweed pollen, 83.0 ± 5.6 ng/mg, is comparable to values for related species obtained by Rylander *et al* (1999a), using the glucan-specific LAL test. The 1,3- β -D-glucan content of yeast was comparable to fungal spores, with an average value of three independent tests being 0.321 pg/cell.

The above experimental conditions for the extraction of 1,3- β -D-glucan in 0.5 N NaOH for 2.5 hours was then used on field samples obtained from the livingroom of approximately 110 homes. Values for the years 2000 and 2001 ranged from 20.55-0.04 ng/m³ and 11.59-0.02 ng/m³, respectively. The means of each year were 4.39 and 3.93 ng/m³. The reproducibility of individual samples performed within the same test and on different days yielded coefficients of variation of <6% (n = 113) and <10% (n=8), respectively. Out of 113 samples analyzed, only one did not contain detectable 1,3- β -D-

glucan. The limit of detection for the field samples was determined to be approximately 0.01 ng/m^3 , or about 15 spores based on 1,3- β -D-glucan content per spore from 11 common indoor fungal species determined in our laboratory (data not shown).

To relate the data obtained from the biological assay to a physical measurement, size-exclusion chromatography was employed. Based on the calibration curve of pullulan standards (Figure 13), curdlan was first examined using SEC to confirm that the 1,3- β -D-glucan analysis was appropriate for the water-insoluble glucan. After dissolving the 1,3- β -D-glucan in 0.15 N NaOH to a concentration of 2 mg/ml, the standard was neutralized with Tris-HCl, as done previously, and 20 μ l was injected. The average molecular weight of the curdlan sample was determined to be 179 kDa. Both a RI detector and a diode-array detector, set in series, confirmed the curdlan peak (UV absorbance maximum at 216 nm). This procedure was repeated with a new curdlan standard dissolved in 0.5 N NaOH, neutralized, and then serially diluted to achieve a concentration range of 1 mg/ml to 0.3125 ng/ml. The limit of detection of curdlan using the DAD was lower (10 ng/ml) than that of the RI detector (50 μ g/ml). A more accurate estimate of the average molecular weight for curdlan in 0.5 N NaOH was also obtained, $169 \pm 10 \text{ kDa}$. An average molecular weight of $186 \text{ kDa} \pm 16\%$ for 1,3- β -D-glucan field samples was determined by the SEC analysis of sample extracts collected in the homes during the winter of 2001. The strongest correlation (0.822, $p < 0.006$) between calculated 1,3- β -D-glucan from the LAL assay and the SEC area was achieved at an average molecular weight range of 180-190 kDa, when samples were grouped in ranges of 10 kDa (Table 1). The correlation between the LAL assay and SEC area over the whole molecular weight range of field samples was 0.517 ($p < 0.000$).

Table 1. Correlation coefficients of 1,3- β -D-glucan concentration (determined by LAL) vs Area of various molecular weight ranges (determined by size-exclusion chromatography)

Average-molecular weight range, kDa	Correlation of [1,3- β -D-glucan] vs SEC Area (<i>p</i> value)
160-170 (n= 4)	0.229 (1.000)
171-179 (n= 5)	0.483 (0.790)
180-190 (n= 11)	0.822 (0.006)
191-200 (n= 6)	0.781 (0.200)
201-210 (n= 10)	0.646 (0.131)
211-220 (n= 10)	0.573 (0.250)

Figure 13. Pullulan standard curve using SEC over a wide molecular weight range. The log(molecular weight) gives a linear plot.

Correlation statistics between 1,3- β -D-glucan and each of ergosterol, mold area, microscope spore counts and endotoxin were performed individually. Statistics were calculated using SYSTAT v.8.0 statistical software. Pearson correlation coefficients for field samples obtained during the year 2000 were 0.758, 0.694, 0.417 and 0.501, respectively, all of which were significant. Likewise, for field samples obtained during the year 2001, Pearson correlation coefficients were 0.873, 0.763, 0.501 and 0.114, respectively (Table 2). Once again, all correlations were significant except for 1,3- β -D-glucan and endotoxin.

Table 2. Correlation coefficients of 1,3- β -D-glucan with respect to ergosterol, mold area, spore counts, and endotoxin. Statistics were calculated in SYSTAT v.8.0 statistical software. Included are Pearson Correlation Coefficients (r^2), number of samples involved in statistics (n), the number of outliers removed from original sampling as a percentage (%), and probabilities ($p <$).

1,3- β -D-glucan vs.	Year 2000				Year 2001			
	r^2	n =	% outliers removed	$p <$	r^2	n =	% outliers removed	$p <$
Ergosterol	0.758	42	$\pm 8\%$	0.000	0.873	48	$\pm 10\%$	0.000
Mold Area	0.694	29	$\pm 21\%$	0.000	0.763	29	$\pm 17\%$	0.006
Spore counts	0.417	36	$\pm 14\%$	0.011	0.501	50	$\pm 10\%$	0.001
Endotoxin	0.501	31	$\pm 19\%$	0.004	0.114	44	$\pm 12\%$	0.461

VI. DISCUSSION

Fungal biomass in indoor air quality studies has primarily been determined by ergosterol concentrations. For the current study, ergosterol was extracted using the microwave-assisted extraction (MAE) procedure developed by Young (1995) followed by GC-MS analysis. Although recoveries from the extraction procedure have been validated recently (Nielson and Madsen 2000), the microwave itself was tested in our own laboratory. Due to the fact that power equivalencies between each microwave may be different, the parameters outlined by Young (1995) were first investigated. Through a preliminary test involving the recovery of ergosterol using the MAE method over a range of standard concentrations, $>92 \pm 2.6 \%$ recovery was obtained. Therefore, the microwave parameters were appropriate for the quantitative extraction of ergosterol from field samples.

After microwave extraction, samples were analyzed by GC-MS. Based on GC-MS conditions outlined by Miller and Young (1997), the retention time of ergosterol was approximately 12 minutes (depending on length of column on any given day). The necessity of peak confirmation achieved with mass spectrometry during ergosterol analysis has been previously published. Using HPLC, many groups have had the problem of an unresolved interference peak believed to be a co-extractive from the filter (Young 1995, Miller and Young 1997). With the ability of confirming a peaks identity by its mass spectrum, as well as the ability to selectively choose which ions the mass spectrometer detects, the peak purity was never in question using GC-MS. Using the selective-ion mode (SIM) of the mass spectrometer, ergosterol as well as cholesterol, were easily identified with a much greater sensitivity than that obtained using the Total

Ion mode, or of that possible using HPLC. The limit of detection for the field samples was estimated to be 0.03 ng/m^3 for GC-MS.

Figure 14. GC chromatogram of ergosterol field sample (223-1-ERG-F-LR-01) extracted using MAE method. GC-MS parameters included using the selective ion mode (SIM), focussing on m/z 363 and 396 for ergosterol and m/z 368 and 386 for cholesterol. Confirmation of ergosterol by retention time and ion ratio was employed.

The preliminary tests involving the LAL sensitivity towards endotoxin and 1,3- β -D-glucan were consistent with those obtained by Roslansky and Novitsky (1991) who found similar results for LAL-reactive glucans (LRG's), in particular curdlan and laminarin. These results indicate the importance of reducing the activity of endotoxin in field samples, while at the same time increasing the reactivity, and hence the sensitivity, of 1,3- β -D-glucans in the LAL assay. The exquisite sensitivity towards endotoxin reflects the nature of the horseshoe crabs immune system, as the horseshoe crab is in constant danger of bacterial infection within its environment. Roslansky and Novitsky (1991) had also observed that there was a 200-fold difference in the sensitivity of the LAL reagent obtained from Associates of Cape Cod to these reactive glucans when compared to other LAL reagents obtained from Mallinkrodt, Inc. or Whittaker Bioproducts. For this reason, LAL reagents were obtained from Associates of Cape Cod throughout the remainder of the experiments, as these reagents would give the optimal reactivity with 1,3- β -D-glucan.

During initial tests involving the hydrolysis of 10 ng of pure endotoxin with sodium hydroxide, a decrease in calculated concentration was observed using the LAL assay. Generally, polysaccharides are resistant to alkali treatment. However, when O-acyl groups like that found in the O-antigen portion of lipopolysaccharide are in the presence of alkali solutions, they tend to be hydrolysed, thereby degrading the polysaccharide, and hence diminishing the LAL activity markedly (Mikami *et al* 1982).

In addition to the possible alkali degradation of the polysaccharide chain mentioned above, it may also be assumed that the decrease in LAL activity is a result of the alkaline degradation over time of the Lipid A moiety, as possibly shown in tests involving various hydrolysis times. This is in part due to the biological activity of

endotoxin being found in the Lipid A portion of lipopolysaccharide. Therefore, the optimal base concentration and reaction time for the elimination of pure endotoxin was determined to be 0.15 N NaOH and 2.5 hours, respectively (Figure 6).

When curdlan was dissolved in 0.15 N NaOH and mixed for 2.5 hours or more in the base solution, the 1,3- β -D-glucan reactivity with the LAL reagent was greatly increased when compared to the same curdlan standard which was immediately neutralized. Futatsuyama *et al* (1999) reported that in alkaline solution, the chain length of curdlan was gradually shortened because of the decomposition of the reducing end of the 1,3- β -D-glucan. There was some evidence of this occurring within the 25 hour time period of the present experiment. Curdlan did not show any significant decrease in LAL reactivity up to 10 hours of hydrolysis in 0.15 N NaOH, indicating that the 'peeling effect' of glucose molecules at the reducing end of the polymer had not yet occurred. However, between 10 hours and 25 hours, there was a slight decrease in 1,3- β -D-glucan reactivity in the range of 0.31-25 ng/ml (Figure 7). This decrease is most likely due to the polymer degrading in the alkaline solution, as previously described by Futatsuyama *et al* (1999). With respect to the elimination of glucose molecules at the reducing end, the polymer reduces in molecular weight and hence the reactivity decreased. Tanaka *et al* (1991) had observed a molecular weight requirement for the activation of the LAL test, indicating that larger M_w had a greater potency than low M_w . The possible decrease in molecular weight would be an explanation to the decrease in reactivity with the LAL reagent seen in this experiment. However, the decrease in reactivity was, as noted above, minimal, and not great enough to lower the reactivity to that of the control below concentrations of 5 ng/ml 1,3- β -D-glucan. As seen in Figure 7, the LAL reagent was completely used up by

the 1,3- β -D-glucan at concentrations above 5 ng/ml, causing the response to level off when hydrolysis was allowed to occur beyond the initial time (ie. beyond an immediate neutralization). For this reason, the experiment was repeated in the range of 5-0.3125 ng/ml, producing similar results. The triple helix conformation of curdlan was also expected to change to a partially-opened triple helix in sodium hydroxide (Young *et al* 2000, Nakata *et al* 1998). This change in conformation is due to the elimination of intermolecular hydrogen bonds. Although it is assumed that the conformation does not completely convert to a single-helix, it is expected to take on a random coil (Figure 15). Hence, an increase in reactivity was expected, as LAL is 100 to 1000 times more sensitive towards the single helical conformation than it is towards the triple helix form (Aketagawa *et al* 1993, Nagi *et al* 1993, Sakurai *et al* 1995, Ohno *et al* 1995).

Figure 15. Conformational change of triple-helical curdlan to a partially opened triple helix due to the elimination of intermolecular hydrogen bonds in alkaline solution (Adapted from: Young *et al.* 2000)

Upon neutralization of curdlan standards after 2.5 hours of base hydrolysis, a decrease in the LAL calculated 1,3- β -D-glucan concentration was observed at 10 and 23 hours. The decreased reactivity is an indication of the renaturation of the triple helix from its partially opened state possibly due to the reconstitution of conditions that favour the triple helix form. At this time, the reactivity of curdlan with the LAL reagent was still greater than the initial control 1,3- β -D-glucan at concentrations greater than 2.5 ng/ml. However, at concentrations below 2.5 ng/ml, the LAL reactivity of neutralized samples had already decreased to that of the control after only 10 hours (Figure 8). Young *et al* (2000) observed a complete conversion back to the original conformation over a period of 8 days for an 8 mg/ml sample.

The next step was to determine the appropriateness of the LAL method with the addition of pure endotoxin to the curdlan standard, since endotoxin and 1,3- β -D-glucan often occur together in outdoor air. With fixed concentrations of either curdlan or endotoxin, experiments revealed that endotoxin was not being destroyed within the 2.5 hours of base hydrolysis. The LAL reagent was being consumed by the endotoxin/curdlan mixture to the point where the reaction plateaued (Figure 7). At this point, the ratio of sample:reagent used in the LAL method was modified from the traditional 1:1 ratio to a 1:2 ratio. This eliminated the plateau observed due to the lack of reagent present in the mixture, thereby not allowing all the endotoxin and/or 1,3- β -D-glucan to react with the LAL test. With calculated LAL values of 1,3- β -D-glucan still higher than those expected, it was assumed that the endotoxin activity was not being eliminated in the base. One explanation for this is that with the colloidal shape of the 1,3- β -D-glucan triple helix and the linear shape of endotoxin, the endotoxin was being shielded within the structure of curdlan (having an inner diameter of approximately 16 Å, Cheeseman and Brown, 1995) from the sodium hydroxide. The diameter of endotoxin at its widest point (the lipid A portion of the molecule) was estimated to be approximately 14.7 Å, according to molecular modeling in PC Spartan2002 software.

When hydrolysis times were increased (up to 25 hours) to allow the elimination of endotoxin to take place, no change in results were observed. This indicated that the over-estimate of curdlan resulting from the presence of endotoxin occurred rapidly and that longer periods of incubation in sodium hydroxide were ineffective in preventing this problem. The concentration of the base may not have been great enough to result in sufficient strand-opening of the triple helix structure of curdlan. One speculation is that

at higher sodium hydroxide concentrations, there would be greater elimination of the intermolecular hydrogen bonds resulting in greater strand separation. The increased strand opening would ultimately release the shielded endotoxin into alkaline solution where its hydrolysis would take place (Figure 16).

Figure 16. Proposed theory of the release of the lipopolysaccharide (endotoxin) from the triple-helix structure of 1,3-β-D-glucan as a result of the strand opening of the helix in high concentrations of sodium hydroxide.

The modified LAL test was applied to actual fungal spores from a pure culture of *C. cladosporioides*. The value obtained was 3.1 ± 0.6 pg/spore (4 separate experiments in triplicate). *C. cladosporioides* spores have a large surface area (56.88 ± 26.66 μm) compared to common indoor air fungal spores. Using the 1,3-β-D-glucan content of *C. cladosporioides* spores obtained above, the applicability of the test was determined on

outdoor air samples. The mean of 2400 spores/m³ compares well with direct measurements of concurrent samples analyzed for total spores by microscopy. A key point is that *C. cladosporioides* represents approximately 55% of the spores in outdoor air during growing season (Roland, 1997).

When 1,3- β -D-glucan was extracted from fungal spores which had been spiked with 10 ng of pure endotoxin, similar results were obtained as previously seen on pure chemicals with respect to the over-estimation of the 1,3- β -D-glucan concentration by the LAL assay. This indicates that pure 1,3- β -D-glucan acts similarly in alkaline solution to that of fungal extracted 1,3- β -D-glucan in the presence of endotoxin. If the proposed theory (ie. endotoxin being shielded within the colloidal triple helix structure of curdlan) is correct, and based on the time trials for the destruction of endotoxin activity, then the extraction of 1,3- β -D-glucan from fungal spores must be relatively fast. This is explained by the fact that in order for the endotoxin to be shielded by the 1,3- β -D-glucan before it can be hydrolyzed, the 1,3- β -D-glucan from the fungal spores must already be in solution. The time range for extraction can be estimated to be most likely less than 0.5 hours, and no more than 1 hour. Therefore, the experiment was repeated with two more concentrations of sodium hydroxide, 0.3 and 0.5 N. The logic for this was that the higher concentrations of sodium hydroxide would result in a greater proportion of the open-stranded triple helix structure compared to that of the closed triple helix. The increased open-stranded structure would release any endotoxin within the helix, thereby allowing it to be hydrolyzed in the alkaline solution (See Figure 16). Contrary to the first experiment performed on pure endotoxin where the lower concentration of NaOH resulted in the greatest elimination of endotoxin LAL reactivity, the present experiment showed that as

NaOH concentration increased, the endotoxin concentration appeared to gradually decrease. Upon closer investigation, the calculated 1,3- β -D-glucan LAL concentration in 0.5 N NaOH was over-estimated by approximately 4.5 ng by the addition of 10 ng of endotoxin, relating to an inactivation of 55%. When compared to the initial experiment where 10 ng of pure endotoxin was exposed to 0.5 N NaOH leading to an inactivation of 53%, then it is assumed that all available endotoxin had been released from the inner core of the triple helix and hydrolyzed to its full extent.

When curdlan was tested by itself (with no endotoxin) in 0.5 N NaOH, a significant increase in sensitivity was observed with respect to curdlan in 0.15 N NaOH. The increase in sensitivity for curdlan in 0.5 N NaOH reached a maximum of 500% at the low end concentrations when compared to 0.15 N NaOH. Possibly, the higher ratio of sodium hydroxide ions present with respect to 1,3- β -D-glucan in the lower concentrations allowed a much greater elimination of hydrogen bonding between the 1,3- β -D-glucan strands. Ultimately, the increased sensitivity may once again be attributed to the degree of the partially-opened conformation of curdlan in the higher concentration of sodium hydroxide. The increase in absorbance for curdlan standards in 0.5 N NaOH, when compared to the same standard concentrations in 0.15 N NaOH, shows the capability of applying the LAL test to the measurement of 1,3- β -D-glucan with great sensitivity. Although the test is most sensitive towards endotoxin, these experiments show that the LAL test can still be very sensitive towards 1,3- β -D-glucan. The glucose-specific assay, which is based on the following coupled enzymatic reactions:

did not detect any glucose, even in the concentrated 1 mg/ml standard of curdlan, within the limit of detection of this test (25 mg/dL). Therefore, the increase in sodium hydroxide concentration is not damaging to the 1,3- β -D-glucan polymer to a measurable degree, further emphasizing that this base concentration is functional in the LAL assay.

During time trials investigating the point at which 1,3- β -D-glucan achieves its maximum sensitivity with the LAL reagent (e.g. at which point the greatest amount of strand opening of the triple helix structure has occurred), results showed that curdlan immediately was hydrolyzed by the larger concentration of sodium hydroxide. After the immediate increase of reactivity, the 1,3- β -D-glucan showed no further enhancement over time, up to a period of 24 hours. The fact that the reactivity of curdlan with the LAL reagent did not deviate significantly from its original enhancement indicates that upon extraction of 1,3- β -D-glucan from fungal spores, it was immediately converted to the partially-opened triple helix. Thus, any endotoxin present in the samples could not be trapped within the colloidal shape of the closed triple-helix.

With the above LAL experimental conditions believed to be appropriate for the analysis of 1,3- β -D-glucan, field samples obtained from the livingroom of approximately 110 homes was undertaken. Approximately 4 samples were added to each microplate, in duplicate, along with their respective dilutions. For each plate, a new standard curve was performed using curdlan in the concentration range of 5-0.3125 ng/ml. The parameters of approximately 25 standard curves yielded r^2 values consistently above 0.99 with the following linear equation:

$$y = 0.096x + 0.013 \quad (\pm 0.011x + 0.009)$$

The slight deviation in the equations is most likely due to the lot to lot variation in LAL activity noted in the product literature (Pyrochrome chromogenic LAL assay, Associates of Cape Cod Inc.). To a lesser extent, the incubation time of each plate, which varied slightly (± 2 minutes), may have caused deviations.

The data obtained for samples taken in the Health Canada PEI experiment (average 1,3- β -D-glucan = 4.392 and 3.932 ng/m³, respectively) compare very well with results obtained with a glucan-specific LAL test, which is based on isolated factor G, used by Thorn and Rylander (1998) in a study involving 75 rowhouses. Data on 1,3- β -D-glucan concentrations in that study ranged from 0-19 ng/m³, with approximately 27% of the homes having concentrations less than 1 ng/m³, and only 17% above 6 ng/m³. Similar values were obtained in the present study.

In comparison of results from the biological assay to a physical measurement, size-exclusion chromatography was employed. Using both RI and UV detection, the average molecular weight of curdlan standards was determined to be 169 ± 10 kDa. This value is in the correct range according to literature which states that the molecular weight of curdlan is >136 kDa (Tanaka *et al.* 1991, Aketagawa *et al.* 1993). Exact values cannot be achieved for this type of structure as the degree of polymerization changes, and thus average molecular weights are more suitable. Although there was a slight decrease in average molecular weight of the standard dissolved in 0.5 N NaOH with that of the standard in 0.15 N NaOH, the change was not significant. Also, as shown previously in the control experiments, a potential decrease in molecular weight due to the loss of glucose has little or no effect on LAL reactivity. In fact, the resulting increase in strand opening of the triple-helix in 0.5 N NaOH is of greater benefit to the reaction of 1,3- β -D-

glucan with the LAL reagent. To confirm the curdlan concentrations used in SEC analysis, the standards were diluted accordingly and subjected to the LAL test immediately following SEC. All standards used in SEC analysis of curdlan fit well within a separate standard curve made for the LAL assay (equivalence of approximately 98%).

As can be seen in Figure 13a and 17, due to the maximum molecular weight cut-off of the column, the larger standards elute out of the column immediately following the void volume. Since SEC is based on the molecular weight, or size of the molecule rather than compound interactions with the column as in HPLC, the resolution of peaks at larger M_w is sacrificed using this type of column. This led to a logarithmically shaped standard curve. However, when the y-axis (M_w) underwent a logarithmic manipulation, a linear standard curve resulted. The linear curve provided a simple equation that could be used to determine the unknown average molecular weights of the field samples.

Figure 17. Pullulan standard chromatograms over a wide range of molecular weights. Larger molecular weight standards elute off of the column at, or close to the void volume (V_o). The red peak represents the average molecular weight of 1,3- β -D-glucan extracted from field samples from the year 2001 ($186 \text{ kDa} \pm 10\%$)

Figure 18. Sample chromatogram of 1,3- β -D-glucan extract (obtained from a field sample) analyzed by SEC.

The average molecular weight of $186 \text{ kDa} \pm 16\%$ for 1,3- β -D-glucan field samples was based on the SEC analysis of sample extracts collected in the PEI homes during the winter of 2001. The chromatographic peaks of the samples were not all symmetrical, as each peak gradually increased to a maxima followed by a sharp decline in intensity. This 'fronting' of peaks is suggestive that there is a range of molecular weights for 1,3- β -D-glucan (with most being in the M_w range of 186 kDa), or that there are other minor constituents with larger M_w which are not resolved from the 1,3- β -D-glucan. To test the latter, the mobile phase was adjusted with either a phosphate buffer containing 100mM NaCl (ph 6.9) or with 0.1 N NaOH. The pH range capability of the column used was pH 2-12.

Based on the LAL biological assay and the SEC analysis for Health Canada PEI samples taken during the year 2001, the correlation of LAL concentration and SEC area,

as determined by UV detection, for all samples was 0.517 ($p < 0.000$). As there are many factors that may influence the reactivity of 1,3- β -D-glucan with the LAL assay (ie. molecular weight, conformation, and branching), the moderate correlation may be due to one, or a number of these factors. In addition, small numbers of pollen and yeasts were seen in the samples analyzed by microscopy. As seen in Figure 19, the average molecular weight was spread over a range (90-230 kDa) indicating a moderate polydispersity of 1,3- β -D-glucans, possibly between different origins (bacteria, fungi, yeast, pollen, etc.), different fungal species or between spores and hyphal fragments. As β -glucanases are predominantly produced by fungi, the 1,3- β -D-glucan which makes up the majority of the cell wall can be used as a nutritional source when external nutrients are exhausted or as a building block for further growth of the cell. Depending on the glucanase present, glucose may consequently be sequentially cleaved from the non-reducing end of the polymer, thereby decreasing its molar mass. In Figure 19, the lowest observed average molecular weight was 90 kDa, which indicates that approximately half of the original molecule was hydrolyzed, one glucose chain at a time. This is highly unlikely, which leads to the belief that the original molecule was randomly cleaved, at approximately the middle, by an endo- β -glucanase producing two individual oligosaccharides. Therefore, depending on the environment in which the spore was present or even in the stage of hyphal morphogenesis, these glucanases may ultimately produce a variety of 1,3- β -D-glucan chain lengths. However, the majority of the field samples provided a consistent molecular mass between 180-210 kDa, minimizing to an extent the possible effect of M_w on LAL reactivity. Size-exclusion chromatography of extracts of 12 different building-associated fungi showed that 1,3- β -D-glucans from these

tested species have similar molecular weight profiles, with an average of 191 ± 11 kDa (unpublished data). These data suggest that the 1,3- β -D-glucans measured in the PEI study were likely to be of fungal origin.

Samples containing molecular weights in the range of 160-220 kDa underwent statistical analysis correlating SEC area with 1,3- β -D-glucan concentration determined by LAL. This range was further divided into equal groups of 10 kDa. Pearson correlation statistics showed that from 160-179 kDa, the correlation did not exceed 0.621 ($n = 4,5$). However, the correlation (0.822, $p < 0.006$) was highest for the molecular weight range 180-190. The best correlations were observed in the range of 180-220 kDa, where approximately 60% of the samples fall. The average-molecular weight of the 1,3- β -D-glucans in the field samples was determined to be 186 kDa, where a significant correlation of 1,3- β -D-glucan LAL concentration and SEC area was observed. This indicates the importance of molecular weight in the activation of factor G in the LAL assay, as previously described by Tanaka *et al* (1991) and Aketagawa *et al* (1993).

Figure 19. Histogram of average molecular weights for 1,3-β-D-glucan of 60 field samples determined by SEC analysis for the year 2001.

All data obtained from 1,3-β-D-glucan analysis and ergosterol analysis was combined with external data received on concurrent field samples for spore counts (collected using sticky surface method and analysed by microscope), endotoxin, as well as mold area. Strong correlations were observed for each year when 1,3-β-D-glucan was compared to ergosterol ($r^2 = 0.758$ and 0.873) or mold area ($r^2 = 0.694$ and 0.763). As each set of data contained outliers, statistics were performed on those data from which the outliers, identified by the statistical program, had been removed. The correlation between 1,3-β-D-glucan and ergosterol was expected to be high if the chemical modifications to the LAL test provided accurate 1,3-β-D-glucan concentrations. Ergosterol is only found in fungi and thus is proportional to fungi in the sample.

However, there may be some variables commonly found in the indoor environment which may cause the 1,3- β -D-glucan results to be over-estimated. As shown in experiments involving the extraction and analysis of 1,3- β -D-glucan from yeast and pollen, a substantial portion of the pollen cell (~100 ng/mg or 3 pg/cell) consists of 1,3- β -D-glucan. This also is true for yeast where up to 0.3 pg/spore can be observed. The LAL assay cannot differentiate between fungal, yeast and pollen 1,3- β -D-glucan, and thus the 1,3- β -D-glucan concentration measured reflects all three.

The correlation between 1,3- β -D-glucan and the spore counts was observed to be positive but moderate (0.417 and 0.501). Depending on the length of sampling for each sample (which stayed constant throughout each year of sampling), the microscope slide may become too dense to accurately determine spore numbers and fungal fragments. If a long sampling time is used (as in the field samples taken during the year 2000, 60 minutes), excess debris such as skin scales, particulate matter and even other fungal fragments may overlap each other, making the slide very difficult to differentiate and read. Shorter sampling times (as for field samples taken during the year 2001, 15 minutes) allow the microscopist to differentiate between each fragment much more easily as the slide is less dense. The shorter sampling time showed a slightly better correlation with respect to 1,3- β -D-glucan when compared to the longer sampling time.

Endotoxin, which is a cell wall constituent of gram-negative bacteria, and 1,3- β -D-glucan were not expected to give a strong correlation (0.501 and 0.114) as two different microorganisms were being compared. However, it is worthwhile to compare these sets of data in indoor air exposure studies as both agents are related to human health problems, mainly respiratory disease.

From a health perspective, 1,3- β -D-glucan is toxic regardless of its source. These measurements are critical for their primary purpose, health analyses, and useful for their secondary purpose, evaluating fungal burdens in the home environment.

VII. GOING FORWARD

The LAL assay used throughout this study is not necessarily specific to the 1,3-glucosidic linkage, although it is understood that the assay is more sensitive to this linkage type over others. The LAL assay may react, to a lesser extent, with other forms of glucans, including 1,6-glucosidic linkages, 1,6-branched glucans as well as (1,3)(1,6)- β -D-glucans, which are composed of a mixture of 1,3 and 1,6 linkages in the backbone. This is important because fungi, and pollen and yeasts, may contain all these various forms of glucans, albeit in minor amounts. The chemical structure of 1,3-D-glucan is dependent on the source. Our lab is currently investigating the molecular weight profiles of many forms of glucans from various origins. The many forms being studied include (1,6) backbones with (1,3),(1,4) branches (dextran, from bacteria), a (1,3),(1,6) backbone (laminarin from brown algae), an α -1,4 mannose polymer (mannan from fungi), as well as (1,4),(1,3) backbones (barley and wheat glucans). Also, our lab has recently determined the 1,3- β -D-glucan content and molecular weight profiles of 12 common indoor fungal species. The molecular weights of each of these species may help suggest the origin of 1,3- β -D-glucans obtained in the PEI study.

In health studies, for which 1,3- β -D-glucan is the main focus, these other conformations may lead to an increased result of the target compound. Therefore, it is important to continue working with the oxidation of glucan extracts with sodium periodate. Sodium periodate specifically destroys the 1,6- β -D-glucan conformational structure, while leaving the 1,3-linkage intact. By eliminating the possible reactivity of any form of 1,6- β -D-glucans to that of the LAL assay, a more accurate estimation to the true 1,3- β -D-glucan concentration can be achieved.

Other experiments which may be of interest to this study is to determine the conformation of the 1,3- β -D-glucans present in the field samples. As noted above, other linkage forms of glucans are present in fungi, ultimately leading to different conformations of the oligosaccharides. Single stranded and single helix 1,3- β -D-glucans may be present with the more common triple helix conformation. As observed by many groups, the conformation of the 1,3- β -D-glucan has a large influence on the LAL activity (Aketegawa *et al* 1993, Young *et al* 2000, Tanaka *et al* 1991). Such experiments to determine the actual conformation of 1,3- β -D-glucans in field samples may involve high resolution solid state ^{13}C NMR or x-ray crystallography, depending on the availability of each of the instruments.

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APPENDIX

Appendix 1: Year 2000 and 2001 field samples. 1,3- β -D-glucan extraction and quantification performed by using the modified-LAL method.

Sample ID (Year 2000)	Area (m ³)	1,3- β -D-glucan (ng/m ³)
112-2-GLU-F-LR-01	22.8	0.29
138-1-GLU-F-LR-01	16.8	6.97
141-1-GLU-F-LR-01	19.7	0.63
144-1-GLU-F-LR-01	19.7	2.21
145-1-GLU-F-LR-01	17.5	8.78
146-1-GLU-F-LR-01	20.6	1.79
147-1-GLU-F-LR-01	17.15	0.19
148-1-GLU-F-LR-01	17.9	1.18
149-1-GLU-F-LR-01	19.9	5.77
150-1-GLU-F-LR-01	17.3	11.03
152-1-GLU-F-LR-01	17.2	6.37
153-1-GLU-F-LR-01	17.4	2.76
154-1-GLU-F-LR-01	16.9	3.66
155-1-GLU-F-LR-01	16.9	7.32
156-1-GLU-F-LR-01	17.2	4.96
157-1-GLU-F-LR-01	16.9	20.21
158-1-GLU-F-LR-01	20.2	0.27
159-1-GLU-F-LR-01	17.4	11.66
160-1-GLU-F-LR-01	15.9	8.59
161-1-GLU-F-LR-01	16.3	1.44
162-1-GLU-F-LR-01	17.2	9.8
163-1-GLU-F-LR-01	17.4	8.14
164-1-GLU-F-LR-01	17.0	0.64
165-1-GLU-F-LR-01	17.5	4.46
166-1-GLU-F-LR-01	17.4	5.86
167-1-GLU-F-LR-01	17.4	1.71
168-1-GLU-F-LR-01	20.9	4.25
169-1-GLU-F-LR-01	17.4	6.48
170-1-GLU-F-LR-01	11.8	7.6
171-1-GLU-F-LR-01	23.1	1.29
172-1-GLU-F-LR-01	17.8	1.03
173-1-GLU-F-LR-01	17.3	0.35
174-1-GLU-F-LR-01	14.5	0.04
175-1-GLU-F-LR-01	17.6	20.55
176-1-GLU-F-LR-01	17.9	4.06
177-1-GLU-F-LR-01	19.5	10.76
178-1-GLU-F-LR-01	8.9	3.17
179-1-GLU-F-LR-01	14.9	0.14
180-1-GLU-F-LR-01	17.2	1.91
181-1-GLU-F-LR-01	17.7	0.28
182-1-GLU-F-LR-01	17.2	0.02
183-1-GLU-F-LR-01	20.7	0.73
184-1-GLU-F-LR-01	23.9	1.23
185-1-GLU-F-LR-01	20.2	0.03
186-1-GLU-F-LR-01	9.6	10.23
187-1-GLU-F-LR-01	14.3	3.23
188-1-GLU-F-LR-01	20.3	6.14
189-1-GLU-F-LR-01	22.8	2.72
190-1-GLU-F-LR-01	21.1	0.15
191-1-GLU-F-LR-01	20.1	ND

Sample ID (Year 2001)	Area (m ³)	1,3- β -D-glucan (ng/m ³)	SEC Area (units)
193-1-GLU-F-LR-01	22.5	58.4	9659.04
194-1-GLU-F-LR-01	16.9	15.97	10180.5
195-1-GLU-F-LR-01	20.6	7.63	9521.9
196-1-GLU-F-LR-01	20.8	3.59	9112.4
197-1-GLU-F-LR-01	20	0.65	7149.5
198-1-GLU-F-LR-01	20.3	8.49	9710.9
199-1-GLU-F-LR-01	17.93	2.43	8550.3
200-1-GLU-F-LR-01	17.4	11.59	9755.3
201-1-GLU-F-LR-01	16.8	3.43	8835.9
202-1-GLU-F-LR-01	21	4.78	911.31
203-1-GLU-F-LR-01	17.9	0.61	5314.00
204-1-GLU-F-LR-01	14.3	6.65	9335.12
206-1-GLU-F-LR-01	14.3	0.12	ND
207-1-GLU-F-LR-01	17.1	3.73	4748.1
208-1-GLU-F-LR-01	23.2	3.84	7952.6
209-1-GLU-F-LR-01	20	0.86	5871.2
210-1-GLU-F-LR-01	20	8.78	9202.1
211-1-GLU-F-LR-01	19.6	1.9	7662.2
212-1-GLU-F-LR-01	19.7	2.8	8971.2
213-1-GLU-F-LR-01	19.3	0.6	6778.02
214-1-GLU-F-LR-01	18.3	4.62	9750.3
215-1-GLU-F-LR-01	20	0.36	4122.4
216-1-GLU-F-LR-01	14.3	0.56	2798.99
217-1-GLU-F-LR-01	17.8	1.69	6338.02
218-1-GLU-F-LR-01	16.6	4.26	9492.07
219-1-GLU-F-LR-01	17.6	0.12	ND
220-1-GLU-F-LR-01	17.8	0.57	5887.9
221-1-GLU-F-LR-01	17.5	1.67	8211.5
222-1-GLU-F-LR-01	15.4	0.39	1608.5
223-1-GLU-F-LR-01	20.1	5.37	8804.9
224-1-GLU-F-LR-01	17.5	4.05	9200.6
225-1-GLU-F-LR-01	20	0.52	6490.0
226-1-GLU-F-LR-01	17.9	4.23	7418.03
227-1-GLU-F-LR-01	17.2	2.28	8987.9
228-1-GLU-F-LR-01	14.6	11.45	8699.6
229-1-GLU-F-LR-01	19.7	2.48	9348.01
230-1-GLU-F-LR-01	20.3	1.38	8235.3
231-1-GLU-F-LR-01	17.4	2.05	9012.1
232-1-GLU-F-LR-01	20.7	0.61	6798.9
233-1-GLU-F-LR-01	17.5	0.46	8891.04
234-1-GLU-F-LR-01	22.4	0.99	6770.4
236-1-GLU-F-LR-01	17.4	0.64	3074.8
237-1-GLU-F-LR-01	19.6	4.45	7821.96
238-1-GLU-F-LR-01	20.9	0.2	ND
239-1-GLU-F-LR-01	22.6	3.18	8913.13
240-1-GLU-F-LR-01	22.8	1.45	7412.0
241-1-GLU-F-LR-01	14.4	0.93	8170.5
242-1-GLU-F-LR-01	16.8	1.05	8784.99
243-1-GLU-F-LR-01	20	2.75	8658.03
244-1-GLU-F-LR-01	20	2.52	7210.4
245-1-GLU-F-LR-01	22.7	1.63	7578.4
246-1-GLU-F-LR-01	17.4	1.14	7856.34
247-1-GLU-F-LR-01	20.1	0.04	ND
249-1-GLU-F-LR-01	17.1	1.41	8852.24
250-1-GLU-F-LR-01	23.17	1.03	8310.0
251-1-GLU-F-LR-01	17.5	1.11	7660.4
252-1-GLU-F-LR-01	20.6	8.58	9793.02
254-1-GLU-F-LR-01	16.6	2.51	9155.52
255-1-GLU-F-LR-01	20.2	1.74	8427.96
256-1-GLU-F-LR-01	6	2.62	9155.72

Appendix 2 a: Year 2000 field samples. Ergosterol and cholesterol obtained by the microwave-assisted extraction followed by GC-MS analysis.

Sample ID	Area (m ³)	Ergosterol MS (ng/m ³)	Cholesterol MS (ng/m ³)
112-2-ERG-F-BR-01	21.1	0.08	4.06
112-2-ERG-F-LR-01	22.8	0.09	5.59
138-1-ERG-F-LR-01	15.8	0.11	2.11
141-1-ERG-F-BR-01	19.9	0.21	5.64
141-1-ERG-F-LR-01	19.8	0.13	1.93
144-1-ERG-F-BR-01	19.7	0.26	12.47
144-1-ERG-F-LR-01	19.7	ND	0.6
145-1-ERG-F-BR-01	17.4	1	11.17
145-1-ERG-F-LR-01	17.5	0.11	7.5
146-1-ERG-F-BR-01	20.6	0.16	77.06
146-1-ERG-F-LR-01	20.6	ND	33
147-1-ERG-F-BR-01	20.8	0.1	1.71
147-1-ERG-F-LR-01	17.2	ND	ND
148-1-ERG-F-BR-01	17.4	0.26	4.07
148-1-ERG-F-LR-01	17.9	0.2	2.09
149-1-ERG-F-BR-01	19.9	0.15	2.95
149-1-ERG-F-LR-01	19.9	0.28	6.25
150-1-ERG-F-BR-01	14.7	0.65	8.71
150-1-ERG-F-LR-01	14.8	0.45	13.06
152-1-ERG-F-BR-01	17.2	0.24	12.99
152-1-ERG-F-LR-01	17.3	0.27	2.65
153-1-ERG-F-BR-01	17.4	0.09	1.65
153-1-ERG-F-LR-01	17.4	0.11	62.09
154-1-ERG-F-BR-01	16.7	0.28	32.14
154-1-ERG-F-LR-01	16.7	0.56	10.84
155-1-ERG-F-BR-01	16.9	0.47	16.9
155-1-ERG-F-LR-01	16.7	ND	0.29
156-1-ERG-F-BR-01	17.2	0.29	11.55
156-1-ERG-F-LR-01	17.2	0.14	1.71
157-1-ERG-F-BR-01	16.9	1.21	32.27
157-1-ERG-F-LR-01	16.9	2.07	59.83
158-1-ERG-F-BR-01	20.2	0.08	5.32
158-1-ERG-F-LR-01	20.2	0.08	1.59
159-1-ERG-F-BR-01	17.5	0.22	1.21
159-1-ERG-F-LR-01	17.4	ND	0.88
160-1-ERG-F-BR-01	21.8	0.35	24.92
160-1-ERG-F-LR-01	16.8	1.32	14.52
161-1-ERG-F-BR-01	16.9	0.38	20.88
161-1-ERG-F-LR-01	16.5	0.11	0.93
162-1-ERG-F-BR-01	17.2	0.92	40.5
162-1-ERG-F-LR-01	17.2	0.61	6.4
163-1-ERG-F-BR-01	17.5	0.1	133.69
163-1-ERG-F-LR-01	17.3	0.53	5.7
164-1-ERG-F-BR-01	17.1	0.14	2.41
164-1-ERG-F-LR-01	17.0	0.21	ND
165-1-ERG-F-BR-01	17.5	ND	12.44
165-1-ERG-F-LR-01	17.6	ND	2.68
166-1-ERG-F-BR-01	17.4	0.49	46.08
166-1-ERG-F-LR-01	16.5	0.61	12.7
167-1-ERG-F-BR-01	17.5	0.12	25.35

Sample ID	Area (m ³)	Ergosterol MS (ng/m ³)	Cholesterol MS (ng/m ³)
167-1-ERG-F-LR-01	17.4	0.61	50.58
168-1-ERG-F-BR-01	20.9	0.57	15.61
168-1-ERG-F-LR-01	20.9	0.14	10.45
169-1-ERG-F-BR-01	17.4	0.39	31.93
169-1-ERG-F-LR-01	17.4	0.35	6.58
170-1-ERG-F-BR-01	14.9	0.51	3.68
170-1-ERG-F-LR-01	16.7	0.38	37.24
171-1-ERG-F-BR-01	23.1	0.07	44.75
171-1-ERG-F-LR-01	23.1	0.07	1.86
172-1-ERG-F-BR-01	17.8	0.1	34.76
172-1-ERG-F-LR-01	17.8	0.47	17.07
173-1-ERG-F-BR-01	16.9	0.09	2.5
173-1-ERG-F-LR-01	16.9	ND	1.27
174-1-ERG-F-BR-01	13.7	ND	17.01
174-1-ERG-F-LR-01	14.4	0.17	1.03
175-1-ERG-F-BR-01	17.7	2.03	38.31
175-1-ERG-F-LR-01	17.6	1.64	35.92
176-1-ERG-F-BR-01	17.9	0.16	10.29
176-1-ERG-F-LR-01	19.6	0.09	3.38
177-1-ERG-F-BR-01	19.6	0.17	1.15
177-1-ERG-F-LR-01	19.5	0.67	56.2
178-1-ERG-F-BR-01	14.9	0.13	23.21
178-1-ERG-F-LR-01	14.9	0.28	27.21
179-1-ERG-F-BR-01	14.8	0.37	12.17
179-1-ERG-F-LR-01	14.9	ND	7.55
180-1-ERG-F-BR-01	17.2	0.12	24.89
180-1-ERG-F-LR-01	17.2	0.31	49.76
181-1-ERG-F-BR-01	16.8	ND	ND
181-1-ERG-F-LR-01	16.8	0.17	4.33
182-1-ERG-F-BR-01	17.2	ND	3.1
182-1-ERG-F-LR-01	17.2	ND	4.67
183-1-ERG-F-BR-01	20.7	0.08	9.03
183-1-ERG-F-LR-01	20.6	0.09	3.7
184-1-ERG-F-BR-01	23.9	0.1	1.86
184-1-ERG-F-LR-01	23.9	ND	1.13
185-1-ERG-F-BR-01	20.3	ND	1.61
185-1-ERG-F-LR-01	20.3	0.17	18.23
186-1-ERG-F-BR-01	10.7	1.9	76.09
186-1-ERG-F-LR-01	25.4	0.39	12.7
187-1-ERG-F-BR-01	14.5	0.23	14.56
187-1-ERG-F-LR-01	14.4	ND	7.56
188-1-ERG-F-BR-01	20.4	0.19	16.16
188-1-ERG-F-LR-01	20.3	0.16	13.27
189-1-ERG-F-BR-01	16.4	0.34	6.28
189-1-ERG-F-LR-01	16.4	0.11	2.53
190-1-ERG-F-BR-01	21.1	0.09	2.12
190-1-ERG-F-LR-01	21.1	0.5	6.51
191-1-ERG-F-BR-01	20.1	0.08	7.02
191-1-ERG-F-LR-01	20.1	0.78	3.34

Appendix 2 b: Year 2001 field samples. Ergosterol and cholesterol obtained by the microwave-assisted extraction followed by GC-MS analysis.

Sample ID	Area (m ²)	Ergosterol (ng/m ³)	Cholesterol (ng/m ³)
193-1-ERG-F-BR-01	22.8	1.46	27.01
193-1-ERG-F-LR-01	3.7	1.3	10.16
194-1-ERG-F-BR-01	15.8	0.73	29.88
194-1-ERG-F-LR-01	16.9	0.49	9.91
195-1-ERG-F-LR-01	20.3	0.5	49.38
195-1-ERG-F-BR-01	20.75	0.36	2.99
196-1-ERG-F-LR-01	19.3	0.1	43.1
196-1-ERG-F-BR-01	20.1	0.08	3.25
197-1-ERG-F-LR-01	20	0.04	32.3
197-1-ERG-F-BR-01	20	0.16	13.65
198-1-ERG-F-LR-01	20.4	ND	63.12
198-1-ERG-F-BR-01	20.1	0.11	86.24
199-1-ERG-F-BR-01	17.96	0.06	7.83
199-1-ERG-F-LR-01	17.65	0.06	18.41
200-1-ERG-F-BR-01	5.9	2.08	25.57
200-1-ERG-F-LR-01	17.3	1.08	23.35
201-1-ERG-F-LR-01	16.9	0.39	8.33
201-1-ERG-F-BR-01	16.8	0.39	7.61
202-1-ERG-F-LR-01	20.2	0.1	9.1
202-1-ERG-F-BR-01	20.5	0.08	28.51
203-1-ERG-F-LR-01	17.7	0.25	25.52
203-1-ERG-F-BR-01	17.7	0.15	42.48
204-1-ERG-F-BR-01	13.9	0.55	32.53
204-1-ERG-F-LR-01	13.3	0.22	16.03
206-1-ERG-F-LR-01	13.9	0.13	6.34
206-1-ERG-F-BR-01	14.2	0.09	9.18
207-1-ERG-F-BR-01	17.2	0.36	31.54
207-1-ERG-F-LR-01	17.1	0.32	36.36
208-1-ERG-F-LR-01	23.3	0.11	33.27
208-1-ERG-F-BR-01	2.7	0.29	11.72
209-1-ERG-F-BR-01	7.9	0.23	113.29
209-1-ERG-F-LR-01	20	0.12	91.84
210-1-ERG-F-LR-01	20.2	0.62	25.09
210-1-ERG-F-BR-01	20.1	0.53	4.58
211-1-ERG-F-BR-01	16.8	ND	6.76
211-1-ERG-F-LR-01	19.4	ND	ND
212-1-ERG-F-LR-01	18.2	0.15	63.78
212-1-ERG-F-BR-01	19.7	0.21	235.06
213-1-ERG-F-BR-01	17.5	0.34	10.23
213-1-ERG-F-LR-01	19.6	0.14	1.81
214-1-ERG-F-BR-01	7.8	1.02	240.57
214-1-ERG-F-LR-01	17.6	1.18	269.27
215-1-ERG-F-BR-01	17.9	0.12	45.56
215-1-ERG-F-LR-01	18.5	0.35	14.54
216-1-ERG-F-LR-01	14.1	0.12	21.05
216-1-ERG-F-BR-01	14.3	0.19	20.03
217-1-ERG-F-LR-01	17.8	ND	24.04
217-1-ERG-F-BR-01	17.8	ND	34.83
218-1-ERG-F-LR-01	16.1	0.09	23.87
218-1-ERG-F-BR-01	15.5	ND	22.33
219-1-ERG-F-LR-01	16.5	0.05	14.93
219-1-ERG-F-BR-01	16.7	ND	3.37
220-1-ERG-F-BR-01	17.3	0.07	46.99
220-1-ERG-F-LR-01	17.8	0.06	53.21
221-1-ERG-F-BR-01	17.4	0.47	20.1
221-1-ERG-F-LR-01	17.3	0.44	8.08
222-1-ERG-F-BR-01	14.8	0.27	83.47
222-1-ERG-F-LR-01	14.8	0.15	19.98

Sample ID	Area (m ²)	Ergosterol (ng/m ³)	Cholesterol (ng/m ³)
223-1-ERG-F-BR-01	20	1.4	13.93
223-1-ERG-F-LR-01	20.1	1.34	19.4
224-1-ERG-F-BR-01	17.6	0.1	99.19
224-1-ERG-F-LR-01	17	0.1	42.29
225-1-ERG-F-BR-01	19.3	0.06	7.94
225-1-ERG-F-LR-01	20	0.06	4.72
226-1-ERG-F-BR-01	5.3	0.19	124.22
226-1-ERG-F-LR-01	17.5	0.18	17.63
227-1-ERG-F-BR-01	16.6	0.16	33.78
227-1-ERG-F-LR-01	16.9	0.09	5.03
228-1-ERG-F-LR-01	14.6	0.24	6.5
229-1-ERG-F-LR-01	19.1	0.1	1.88
229-1-ERG-F-BR-01	19.2	ND	9.35
230-1-ERG-F-LR-01	18.8	0.13	12.71
231-1-ERG-F-LR-01	16.8	0.15	18.49
231-1-ERG-F-BR-01	16.8	0.12	22.66
232-1-ERG-F-LR-01	20.7	0.06	3.63
232-1-ERG-F-BR-01	20.1	0.05	18.67
233-1-ERG-F-LR-01	17	0.16	37.25
233-1-ERG-F-BR-01	16.7	0.16	10.68
234-1-ERG-F-BR-01	22.3	0.08	2.25
234-1-ERG-F-LR-01	22.4	0.05	12.93
236-1-ERG-F-BR-01	17.2	0.31	24.07
236-1-ERG-F-LR-01	15.1	0.14	11.43
237-1-ERG-F-BR-01	19.1	0.27	23.87
237-1-ERG-F-LR-01	19.7	0.21	15.91
238-1-ERG-F-LR-01	20.9	0.11	17.91
238-1-ERG-F-BR-01	20.8	0.25	6.51
239-1-ERG-F-LR-01	22.6	0.12	41.35
239-1-ERG-F-BR-01	20.9	0.15	77.39
240-1-ERG-F-LR-01	22.6	0.09	36.04
240-1-ERG-F-BR-01	22.8	0.07	12.06
241-1-ERG-F-BR-01	14.4	ND	57.15
241-1-ERG-F-LR-01	13.9	0.2	189.11
242-1-ERG-F-LR-01	16.4	0.12	6.05
242-1-ERG-F-BR-01	14.9	0.14	11.7
243-1-ERG-F-BR-01	20.1	0.39	88.27
243-1-ERG-F-LR-01	20	0.23	130.41
244-1-ERG-F-LR-01	21.3	0.1	12.32
244-1-ERG-F-BR-01	20.1	ND	41.71
245-1-ERG-F-LR-01	22.7	0.15	18.49
245-1-ERG-F-BR-01	22.7	0.24	43.52
246-1-ERG-F-LR-01	17.4	0.1	7.89
246-1-ERG-F-BR-01	16.8	0.08	76.53
247-1-ERG-F-LR-01	18.5	0.12	15.43
247-1-ERG-F-BR-01	19.5	0.08	11.94
249-1-ERG-F-LR-01	2.7	0.46	11.69
249-1-ERG-F-BR-01	16.3	0.07	40.66
250-1-ERG-F-LR-01	22.5	0.05	0.37
250-1-ERG-F-BR-01	22.6	ND	2.8
251-1-ERG-F-LR-01	16.3	0.14	11.22
251-1-ERG-F-BR-01	15.3	0.13	4.24
252-1-ERG-F-LR-01	19.1	1.73	87.74
252-1-ERG-F-BR-01	19	1.57	86.01
254-1-ERG-F-LR-01	16.1	0.1	0.31
255-1-ERG-F-LR-01	20	0.08	0.2
255-1-ERG-F-BR-01	20.3	no filter	no filter
256-1-ERG-F-LR-01	6	0.28	9.29
256-1-ERG-F-BR-01	8.1	0.21	17.3