DISSERTATION

ANALYSES AND EXPOSURE ASSESSMENT OF BACTERIAL ENDOTOXIN IN AGRICULTURAL ENVIRONMENTS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RENA SAITO ENTITLED ANALYSES AND EXPOSURE ASSESSMENT OF BACTERIAL ENDOTOXIN IN AGRICULTURAL ENVIRONMENTS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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Endotoxins, or lipopolysaccharides (LPS), found in organic dust are a component of the cell membrane of Gram-negative bacteria that play an important role in respiratory disease. However, accurate measurements of endotoxin exposures are difficult in agricultural environments since agricultural dusts contain a complex mixture of biological and chemical agents. This dissertation research was designed to improve the understanding of the variability in endotoxin measurements in agricultural environments.

The first study determined patterns of 3-hydroxy fatty acid (3-OHFA) distribution in dusts from dairy farms, cattle feedlots, grain elevators, and farms, and evaluated correlations between the gas chromatography/ mass spectrometry (GC/EI-MS) and the biological recombinant Factor C (rFC) assay results. Patterns of 3-OHFA distribution varied by dust type; livestock dusts contained approximately two times higher concentrations of 3-OHFAs than grain dusts. Grain dust contained a higher proportion of shorter chain 3-OHFAs (< C9:0) than livestock dusts. Pearson correlations and multiple linear regressions showed higher correlations between GC/EI-MS and rFC results for livestock dusts than for grain dusts. Odd-chain length 3-OHFAs were found to correlate with rFC results, as well as with even-chain length 3-OHFAs.

The second study evaluated traditional Limulus amebocyte lysate (LAL) and novel rFC assay responses to endotoxins in chicken, dairy, horse, swine, and turkey dusts, and investigated potential interference with assays using GC/EI-MS analyses. Strong positive correlations existed between LAL and rFC results, but responses to assays varied by dust type. The LAL overestimated (or the rFC underestimated) endotoxin exposures in chicken and horse dusts, and the LAL underestimated (or the rFC overestimated) endotoxin concentrations in dairy, swine, and turkey dusts. Ergosterol was not a major factor of interference overall, but the magnitude of interference varied by dust type. The variability in assay responses might be explained by differences in bacterial composition and other dust components; the rFC assay may react positively with Actinobacteria.

The goals of the third study were to characterize agricultural tasks and to apply empirical modeling to evaluate determinants of personal dust and endotoxin exposures in dairy farms, cattle feedlots, grain elevators, and farms. Dust and endotoxin exposures differed by agricultural environment and combinations of tasks varied by environment. Based on multiple regression analysis, hours at running legs in grain elevators was the major determinant of dust. Hours at running legs in grain elevator and hours at feeding livestock in cattle feedlots were two major determinants in endotoxin measurements.

This dissertation addressed the need for understanding differences in agricultural environments for endotoxin exposure assessment, and identified specific tasks and factors associated with high exposures.

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DEDICATION

This dissertation is dedicated to my mother Yoshiko Saito and to the memory of my father Shigeru Saito. Without their encouragement and trust, this dissertation would not be completed.

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LIST OF UNITS

EU	endotoxin unit
m^3	cubic meters
mg	milli-gram
ng	nano-gram
pmol	pico-mole

LIST OF ABBREVIATIONS

3-OHFA	3-hydroxy fatty acid
3-OHFAME	3-hydroxy fatty acid methyl ester
ACGIH	American Conference of Governmental Industrial Hygienists
AM	Arithmetic mean
ANOVA	Analysis of variance
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
cDNA	Complementary DNA
COPD	Chronic obstructive pulmonary diseases
CV	Coefficient of variation
DCM	Dichloromethane
FEV	Forced expiratory volume
GC/MS	Gas chromatography/mass spectrometry
GC/CI-MS	Gas chromatography/chemical ionization mass spectrometry
GC/EI-MS	Gas chromatography/electron impact mass spectrometry
GC/MS-MS	Gas chromatography/tandem mass spectrometry
GLC	Gas-liquid chromatography
GLM	General linear model
GM	Geometric mean
GSD	Geometric standard deviation
HLB	Hydrophilic lipophilic balance
IL	Interleukin
IOM	Institute of Occupational Medicine
LAL	Limulus amebocyte lysate
LBP	LPS-binding protein
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LPS	Lipopolysaccharide
m/z	Mass to charge ratio
ODTS	Organic dust toxic syndrome
OSHA	Occupational Safety and Health Administration
PAMPs	Pathogen-associated molecular patterns
PEL	Permissible Exposure Limit
pf	Pyrogen-free
PVC	Polyvinyl chloride
rFC	Recombinant Factor C

S/N	Signal-to-noise ratio
SIM	Selected ion monitoring
SPE	Solid-phase extraction
TLR	Toll-like receptor
TLV	Threshold Limit Value
TNF	Tumor necrosis factor
TMS	Trimethylsilyl
TWA	Time weighted average

CHAPTER 1

INTRODUCTION

Agricultural dusts are complex mixtures of chemical and biological agents including fecal components, urine, bacterial endotoxin, and glucan.[1] A recent study has indicated that present day animal feeds also contain a wide range of biological and chemical substances, such as rendered animals and antibiotics, in addition to grains.[2] Endotoxins, or lipopolysaccharides (LPS), found in organic dust are a component of the cell envelope (outer membrane) of Gram-negative bacteria that play an important role in the causation of respiratory disease. However, there are significant gaps in our understanding of exposure-response relationships, and universal occupational standards or guidelines do not yet exist. Development of an accurate measurement protocol for endotoxin exposure is critical for understanding the relationship between endotoxin exposures and development of diseases, such as chronic bronchitis and asthma, and for establishing appropriate occupational guidelines and controls.

Structure of bacterial endotoxin

Bacteria, single cell microorganisms, can be separated into two groups by structure of the cell-wall. Gram-positive bacteria contain thick multiple peptidoglycan layers and Gram-negative bacteria have thin peptidoglycan layers surrounded by a complex outer-

membrane. The outer-membrane is unique to Gram-negative bacteria and contains pathogen-related endotoxin (LPS).

Endotoxin is composed of three parts: O-specific chain, core oligosaccharide, and lipid A components (Figure 1-1).[3] The O-specific chain, constituents of multiple repeating polysaccharide units, is the outermost part of endotoxin. The inner part of the O-specific chain is attached to the core oligosaccharide region, and then to the lipid A. The lipid A component is the innermost part of endotoxin and is the most important portion for activating innate immune responses.[4] Although lipid A from any Gram-negative bacteria contains two D-gluco-configurated pyranosidic hexosamine residues with ester and amide linked fatty acids, the structure and length of carbon chain of fatty acids vary by bacterial group.[3-7] For example, lipid A of the well known Gram-negative bacteria *Escherichia coli* has C14:0 3-hydroxy fatty acids (3-OHFA) whereas another well studied bacteria *Pseudomonas aeruginosa* contains C10:0 3-OHFA.[4] Recent studiess have indicated that these structural and conformational differences relate to the intensity of immune responses and differences in disease outcomes.[4, 8]

Endotoxin activity

During bacterial cell lysis or bacterial growth, endotoxins are released from the outer membrane of the bacterial cell. Endotoxins are pathogen-associated molecular patterns (PAMPs) of Gram-negative bacteria; when endotoxins enter into the body, they are recognized by the innate immune system through various pathways.[4, 8-10] The principal mechanism is through LPS-binding protein (LBP) and toll-like receptor 4 (TLR4) complex found on the surface of macrophages.[4, 8] Free endotoxin, specifically the lipid

A portion, is bound to LBP and then to a receptor molecule called CD14. The binding of this complex and TLR4-MD2 complex stimulates the macrophage to release pro-inflammatory cytokines including tumor necrosis factor α (TNF- α), interleukin-1β (IL-1β), IL-6, and IL-8.[9, 11] Non-membrane bound soluble CD14 molecules can also be found, allowing endotoxins to be circulated in blood and serum.[4, 9] In addition, several bacteria, such as *Legionella pneumophilia*, may activate TLR2 receptors instead of TLR4 complex.[8]

Endotoxin is also recognized by other molecules including the macrophage scavenger receptor, ion channels found on the cell surface, and plasma proteins;[8-10, 12] some of molecules, such as lipoprotein, may be involved in detoxification of endotoxin.[13] However, the sensitivity of host-endotoxin interaction and the immune-response pathways and the mechanisms relating to endotoxin exposures are still unclear.[8, 9, 13]

Health effects

Respiratory diseases

Bacteria can enter the human body through different routes, including oral ingestion (food poisoning) and inhalation. Inhalation is the largest concern and the major route of airborne endotoxin exposure in occupational settings. The association between airborne endotoxin exposures and respiratory disease has been studied in a wide variety of agricultural and industrial environments. Exposure to endotoxins in agricultural dusts including swine, poultry, and grain is associated with asthma, chronic bronchitis, hypersensitivity pneumonitis, chronic obstructive pulmonary disease (COPD) and

decrease in pulmonary function especially FEVs.[14-21] Acute pulmonary effects associated with endotoxin exposures in grain dust include flu-like symptoms (also known as organic dust toxic syndrome, ODTS), occupational asthma, chest tightness, wheezing, and short-term reduction in pulmonary function.[22] Workers in cotton mills,[23, 24] fiberglass manufacturing,[25] and metal working [26] have reported similar associations. Low level endotoxin exposure has been associated with non-specific building-related symptoms, also known as "Sick Building Syndrome," among office workers.[27-29] Exposure to house dusts containing low levels of endotoxins has been associated with an increase in asthma prevalence or severity,[30-32] frequency of asthmatic symptoms,[33] and risk of wheezing in infancy.[34, 35]

In vitro and *in vivo* animal and human inhalation studies provide evidence that endotoxins are potent inflammatory agents producing systematic effects and lung obstruction.[36-46] Inhaled endotoxin induces biological responses by activating airway macrophages and causing the release of pro-inflammatory cytokines and metabolites of arachidonic acids.[41, 42] Lambert et al.[47] found significant association between hyper-responsiveness of one cytokine, TNF- α, and chronic bronchitis among Iowa farmers. Similarly, Zhang et al.[48] found that the genotype of TNF polymorphism was significantly associated with the magnitude of change in chronic lung function among textile workers. In addition, LeVan et al.[49] suggested that variations in the human CD14 gene are associated with the development of airway obstruction among agricultural workers. Inhalation of agricultural dust causes similar respiratory problems;[39, 46, 50] Jagielo et al.[51] and George et al.,[52] using mice as an animal model, explained that endotoxin in grain dust is the principal agent for the causation of airway inflammation

and chronic airway disease. The concentration of endotoxins in dust is an important factor in the acute biologic effects of agricultural dust.[38, 39, 44] Some studies have reported that the responsiveness of endotoxin was greater in atopic subjects than in non-atopic subjects.[53] Eldridge et al.[54] investigated the interaction between endotoxin and allergen in the endotoxin-induced inflammation; this study indicated that presence of allergen may enhance endotoxin-induced inflammation.

Animal models, mainly mice [13, 36, 38, 44, 51, 52, 55-57] and rats,[58, 59] were used to investigate the mechanism and pathway relating to endotoxin exposure and lung injuries. Although inhalation studies have shown that inhaled grain dust or endotoxin produce similar physiologic (*in vivo*) and biologic (*in vitro*) inflammatory responses in humans [39, 40] and mice,[36, 38, 44, 60] specific mechanisms and susceptibility of endotoxin should vary by specie.[4] For example, humans have more selective TLR4 receptors than mice; TLR4 of mice recognizes a wide variety of lipid A structures and other chemicals while TLR4 of human does not recognize certain types of lipid A and non-lipid A chemicals.[4] In addition, there are large physiological differences between rodents and humans.[61] Differences in airway structures and breathing patterns may lead to difference in sensitivity to endotoxins and in disease outcomes.[4] Therefore, it is important to use animal models for explaining human disease with caution.

Several studies have attempted to establish dose-response relationships between endotoxins and respiratory diseases.[14, 17, 20, 25, 62] However, a universal dose-response relationship has not been developed since some studies have reported high exposure without symptoms [63] or low exposure with a possible dose-response relationship.[64] Several studies using cotton dust showed no effect levels for inhalable endotoxin

exposure in a broad range of 90 – 1,700 EU/m^3.[23, 24, 65] In addition, very high [66] or very low [27] endotoxin exposures have been reported with similar symptoms. Even among agricultural workers, dose-response relationships were not consistent. Several epidemiological studies found no association between endotoxin exposure and respiratory symptoms [19, 63] while many studies found mild to strong associations among agricultural workers.[14-16, 20-22, 67, 68] This difference could be explained by inconsistent sampling and analytical methods for endotoxin exposure, as well as significant genetic variability among subjects. Lack of consistent protocols for exposure assessment complicates the task of determining exposure-response relationships.

Hygiene hypothesis

Several studies have found that endotoxin exposure in early life may be protective against asthma and atopic sensitization.[34, 69-73] In addition, this protective effect was also observed in adult farmers.[74-76] These studies found that endotoxin exposures have protective effects against atopic asthma and atopic sensitization, but endotoxin exposure increases the risk of non-atopic asthma and airway hyper-responsiveness.[74-76] Differences in response may depend on immunological reaction (balance between T-helper 1 and T-helper 2 stimulated cytokines) and the timing of exposure in the life stage appears to be an important factor;[70, 71, 73, 77] however, the specific mechanisms are still unclear.

Cancer

Some epidemiological studies have investigated the carcinogenicity of organic dust and endotoxins in agricultural environments. Interestingly, organic dust or endotoxin exposure were related to reduction in lung cancer mortality.[78-82] Mastrangelo et al.[81] found that an increase in the number of cattle, which may relate to increase in endotoxin exposure, decreases the lung cancer risk. The authors explained that this trend could not be explained by healthy workers' effects or lighter smoking habits in dairy workers.[82] This anti-cancer effect could be explained by immunological or pharmacological effects of endotoxin.[79, 81]

Mastrangelo et al.[81] found an increased risk of brain cancer in dairy farmers. In addition, Laakkonen et al.[78] found an association between grain dust exposure and increased risk of laryngeal cancer. Immunological effects of endotoxin may relate to causation of these cancers, but the actual mechanism is unknown.[81] For all above mentioned studies, results were adjusted for age and smoking habits.

Other health effects

Gastrointestinal symptoms, such as diarrhea and stomach pain, were reported in some occupational settings. These symptoms were especially common in waste and sewage plants,[83, 84] but also found in seed-handling plants.[85] Exposure to endotoxin also causes septic shock and organ failure.[86] However, these symptoms are not commonly related to occupational endotoxin exposure.

Endotoxin analysis

There are two approaches to measuring endotoxin in dusts: biological assay and chemical analysis. Bioassays measure the relative reactivity of endotoxins with enzymes and chemical analyses focus on quantification of biomarkers of endotoxins. Each method is explained below and summarized in Table I-I.

Bioassay

The Limulus amebocyte lysate (LAL) assay is the most commonly used biological assay to measure endotoxin exposure and depends on the relative reactivity of endotoxins with Limulus lysate, an agent extracted from horseshoe crabs.[87-89] This assay was first reported by Levin and Bang in 1968; prior to this report, Bang had discovered that horseshoe crab blood clots when endotoxins are present (when LPS binds to LPS receptors) and the blood clotting agent is Limulus amebocyte lysate.[90] The reaction cascade of the LAL assay is shown in Figure 1-2.[88, 91] Although the LAL assay is exquisitely sensitive, it neither detects cell-bound endotoxins that may be associated with respiratory symptoms nor provides any specific information on chemical structure.[92-94] Furthermore, the LAL assay may experience interference from non-endotoxin agents, such as (1→3)-β-D-Glucans from fungi.[95-99] The LAL assay may also be interfered with polynucleotide and proteins.[100] These interferences are extremely concerning in agricultural dusts since these dusts contain a complex mixture of biological and chemical agents. In addition, the LAL assay exhibits some lack of specificity due to high variability in laboratory methods for sample collection, sample handling and storage, sample analysis, and variation in the reporting of results.[101-110] Moreover, since the LAL

assay uses a reagent extracted from horseshoe crabs, there is lot-to-lot variation.[111] To support this, Liebers et al.[112] compared LAL assay kits from different manufacturers; the results indicated there were 2.7 to 5-fold differences.

The new recombinant Factor C (rFC) assay offers some improvement in specificity and reproducibility. This assay is developed based on the same reaction mechanism of the LAL assay, but the rFC assay uses the genetically modified reagent produced from the cDNA of *Cacinoscorpius rotundicauda*. The rFC assay requires shorter reaction cascade compared to the LAL assay; reaction cascade of the rFC assay is shown in Figure 1-2. Since the rFC assay does not use a reagent extracted from living animals, the reactivity of reagent to endotoxins does not vary significantly by the lot. In addition, use of this genetically modified reagent eliminates Factor G reaction step, which can cause interference from (1→3)-β-D-Glucans; thus, the rFC assay eliminates false-positive response to glucan. The rFC assay still measures the overall response to the mixture of endotoxins in a sample, which does not provide any information on chemical structure, and fails to detect cell-bound residues.[113] Moreover, potential interference from non-endotoxin agents have not been studied in detail.

Chemical analysis

Chemical analyses using gas chromatography / mass spectrometry (GC/MS) have focused on quantification of 3-OHFAs in lipid A of endotoxin [94, 114-116] that serve as indirect biomarkers of endotoxin levels. Unlike bioassays, GC/MS analysis of 3-OHFAs allows determination of total amounts of both cell-bound and non-cell-bound endotoxins.[94, 107] This method has not been widely adapted and has been applied mainly

to dust from indoor house or office environments.[28, 116-121] Table I-II summarizes studies adopting the chemical analysis methods, mainly GC/MS, for endotoxin measurements in agricultural dusts. As seen from the table, only even-numbered carbon chain 3-OHFAs were monitored in most studies since predominant gram-negative bacteria in house dust contain even-numbered 3-OHFAs.[121] However, odd-numbered 3-OHFAs, such as C13:0 3-OHFA, are also found in agricultural dusts.[121] In addition, some longer-chain 3-OHFAs may associate to Gram-positive bacteria.[122] Monitoring a wide variety of 3-OHFAs is extremely useful to identify potential interference with bioassays in heterogeneous agricultural dusts and is important for accurate endotoxin measurement.

Gas-liquid chromatography (GLC) was also used in several earlier studies,[123] detecting the same chemical markers of endotoxin as GC/MS. However, GLC has low selectivity comparing to GC/MS since GLC identifies chemicals only by their retention times while GC/MS identifies molecular weights (mass to charge ratios, m/z) of analytes in addition to their retention times. Thus, GC/MS is used in most studies now (Table I-II).

Dust and endotoxin exposures

Agricultural environments

Dust and endotoxin exposures vary by workplace, and high dust concentration does not necessarily mean high endotoxin concentration. Compared to non-agricultural workers, agricultural workers, in general, are exposed to high concentrations of endotoxin but are not always exposed to high dust concentrations.[91] In addition, the agricultural industry includes a wide variety of environments from grain handling storage to animal-

processing facilities; not all agricultural environments and tasks have the same level of dust and endotoxin concentrations.[124, 125] Studies of dust and endotoxin exposures in agricultural environments are summarized in Table I-III. All selected studies used personal samplers for dust and endotoxin collection and the chromogenic (kinetic) LAL assays for endotoxin measurements. As seen from Table I-III, dust and endotoxin exposures vary by agricultural environments; among animal production and farms, dairy farms might have lower dust and endotoxin concentrations than swine or poultry houses. In addition, results shown in the table indicate that a wide range of dust and endotoxin exposures was observed even in the same agricultural environment. For example, the lowest endotoxin value in swine environment was 58 EU/m^3,[126] and the highest value was 3,690 EU/m^3.[127] Although in the same agricultural environment (swine) from the same country (the Netherlands), an approximately 16-fold difference was observed.[125, 128] These differences could relate to differences in sampling method and study site, as well as operational differences. This result further supports the need for a standard, accurate exposure assessment method.

Endotoxin exposure assessment using GC/MS

Several studies suggest that the presence of house pets, including dogs and cats, significantly increases the concentration of endotoxins in indoor household air.[34, 62] Similarly, studies conducted in agricultural environments show that livestock dusts contain much higher levels and more variable 3-OHFA distributions than grain dusts.[107, 121, 129] Species differences in livestock (cow, pig, and chicken) may contribute to

differences in 3-OHFA distributions, but few studies have compared 3-OHFA compositions among various agricultural dusts.[107, 129]

Saraf et al.[118, 119] and Hines et al.[28] have determined correlations between LAL bioassay and GC/MS determination of 3-OHFAs in house dusts; they found positive correlations between bioassay and C10:0, C12:0, and C14:0 3-OHFAs but low or negative correlations for C16:0 and C18:0 3-OHFAs. However, this tendency may be different in agricultural environments. A previous study using chicken, swine, and corn dusts found weak correlations of C12:0 and C14:0 3-OHFAs with biological LAL assay response.[107] Haack et al.[130] and Zelles [122] have conducted studies to identify fatty acid profiles in soil bacteria. These studies have suggested that some straight-chain (with >14 carbon chain lengths) and also some branched-chain (iso, anteiso) 3-OHFAs may be signatures of certain Gram-positive bacteria.[122, 130] Sebastian et al.[120] observed that 3-OHFAs with straight carbon chain lengths of 16 and 18, and branched-chains of C17 were present in several species of Actinobacteria, one or Gram-positive bacteria, from house dusts. These data suggest that longer straight chain 3-OHFAs may not originate from Gram-negative bacteria but from Actinobacteria.[120] In addition, few studies indicated that C12:0, C14:0 and C16:0 3-OHFAs were also present in the yeast and C14:0 and C18:0 were present in some *Hypericum* herbs.[131,132]

Occupational standards and guidelines

Dust

Agricultural dust is a heterogeneous mixture of different types of dust, such as grain and soil dusts. There are no established occupational exposure standards for agricultural

dusts except for grain dusts. The Occupational Safety and Health Administration (OSHA) and the American Conference of Governmental Industrial Hygienists (ACGIH®) set 8-hour time weighted averaged (TWA) exposure limits for total and respirable dusts not otherwise specified.[133-135] The OSHA Permissible Exposure Limit (PEL) for total dust is 15 mg/m^3, and the ACGIH Threshold Limit Value (TLV®) is 10 mg/m^3.[132, 133] The exposure limits of the OSHA and the ACGIH for respirable dust are 15 mg/m^3 and 10 mg/m^3, respectively.[134, 135] However, these total and respirable dust exposures apply to particles with low toxicity,[134] which may not be relevant for agricultural dust since agricultural dust exposure is related to respiratory diseases. For such concerns, the OSHA and the ACGIH also established exposure limits for grain dust, particularly for oat, barley, and wheat. The OSHA PEL for grain dust is 10 mg/m^3, and the ACGIH TLV is 4 mg/m^3.[133, 135] Since not all agricultural dusts contain oat, barley, or wheat dusts, applying exposure limits of grain dust may not be appropriate in specific agricultural environments.

Donham et al.[14, 136] and Reynolds et al.[15] recommended occupational exposure limits of 2.4 to 2.5 mg/m^3 for total organic dust and 0.16 to 0.23 mg/m^3 for respirable organic dust in swine and poultry environments based on epidemiological studies of pulmonary function.

Endotoxins

There are no established occupational standards or guidelines for endotoxin exposures. In the Netherlands, the Dutch Expert Committee on Occupational Standards has recommended the limit of 50 EU/m^3 for 8-hour TWA personal inhalable endotoxin

exposure.[137] This value was based on a no-effect level of 90 EU/m^3 reported by Castellan et al. for cotton textile workers.[91] Since a wide range of no-effect levels was reported depending on industry, this exposure limit may not be suitable in all working environments. For the same reason, Germany decided to not establish occupational threshold limits or recommended limits for endotoxin.[138]

Based on epidemiological studies in swine and poultry environments, Donham et al.[14] suggested the occupational limits of 900 EU/m^3 and 614 EU/m^3, respectively, for total endotoxin exposures. The recommended limits for respirable endotoxin were 100 EU/m^3 (swine) and 0.35 EU/m^3 (poultry).[14]

Goals of the dissertation research

To understand the exposure-response relationship between endotoxin and occupational diseases, development of an accurate measurement protocol for endotoxin exposure is critical. However, accurate measurements of endotoxin exposures are difficult in agricultural environments since agricultural dusts contain a complex mixture of biological and chemical agents, which could vary by facility type, environment, location, and agricultural operation. The overall goal of this dissertation research was to enhance understanding of variability in endotoxin exposures and measurement methods in various agricultural environments.

The specific aims were:

1. To determine patterns of 3-OHFA distribution and proportion in four types of agricultural dusts (dairy farms, cattle feedlots, grain elevators, and farms), and to

evaluate correlations between the results of GC/EI-MS analysis and biological rFC assay;

2. To compare the traditional chromogenic LAL and the novel fluorometric rFC assay responses to endotoxin in five livestock dusts (chicken, dairy, horse, swine, and turkey), and to investigate potential interference with assays using GC/EI-MS analyses; and
3. To characterize agricultural tasks and to identify determinants of personal dust and endotoxin exposures in four agricultural environments (dairy farms, cattle feedlots, grain elevators, and farms) using empirical modeling.

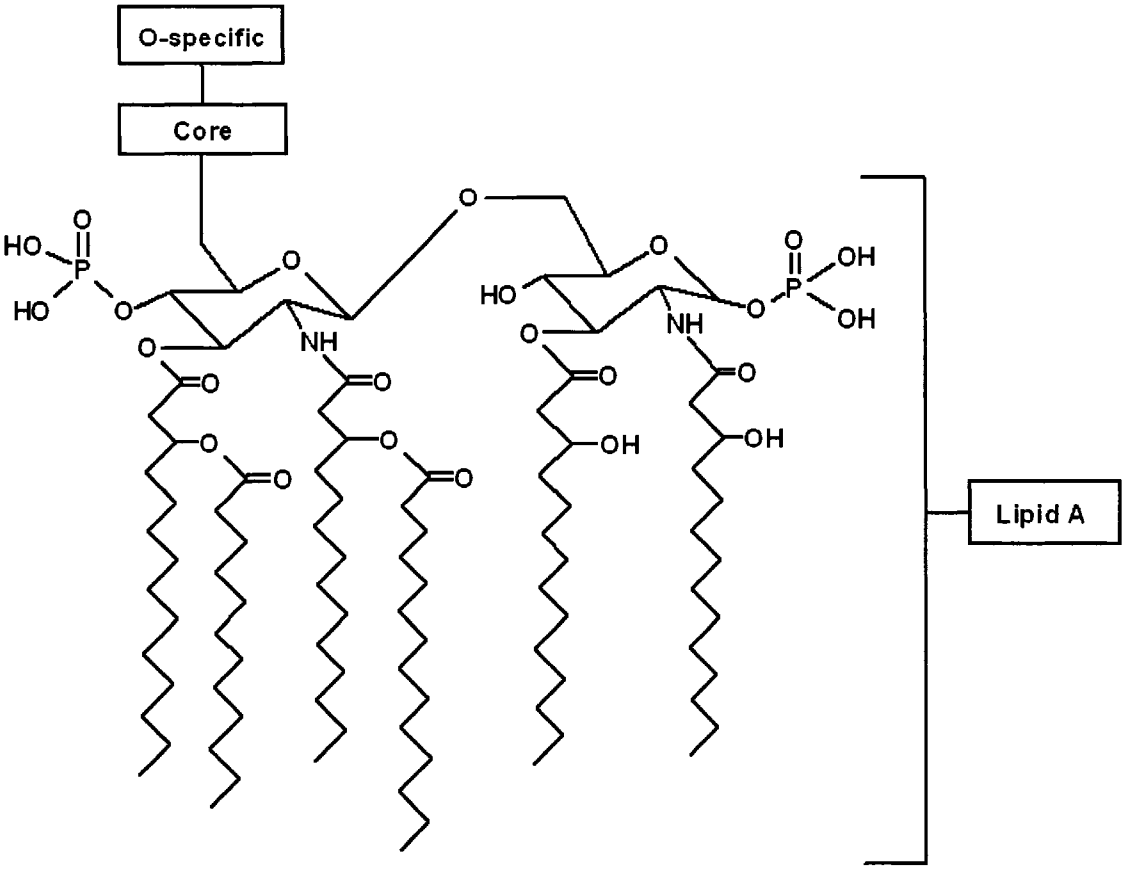

FIGURE 1-1. Chemical structure of *Escherichia coli* endotoxin. Actual length and structure of carbon chain depends on bacterial specie.[4]

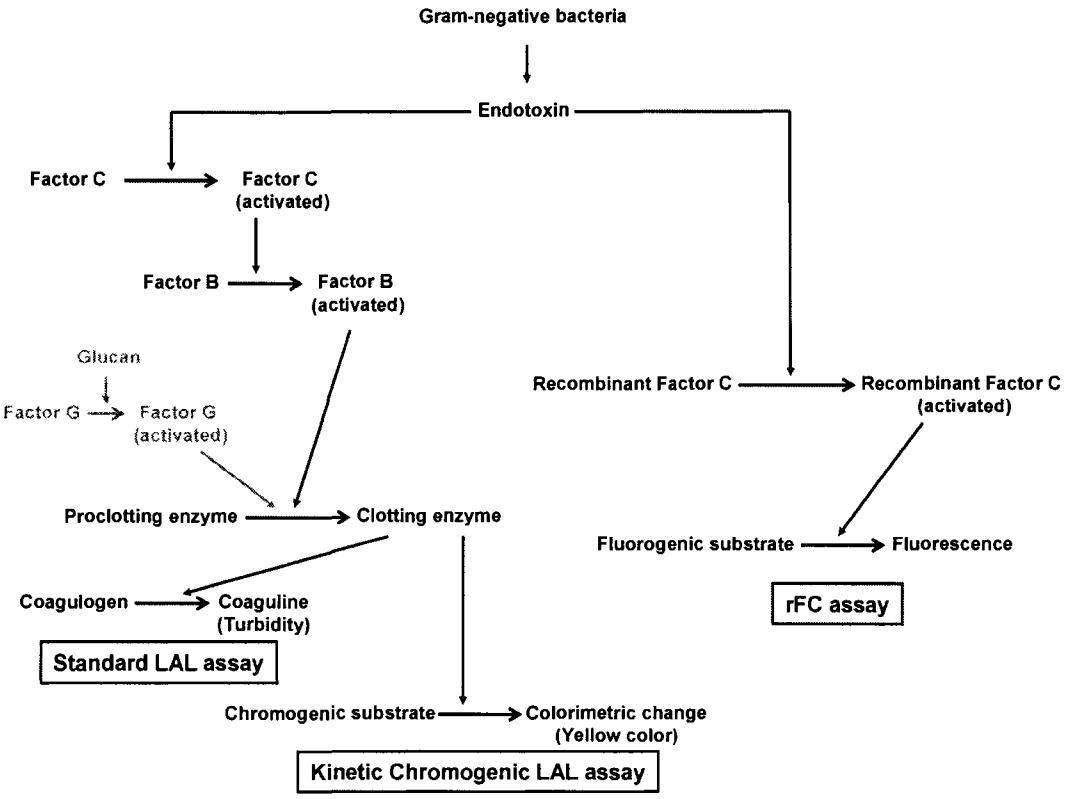

FIGURE 1-2. Reaction cascade of LAL and rFC endotoxin assays.[88, 91]

TABLE I-I. Summary of bioassay and GC/MS methods

	Bioassays	**GC/MS**
Measurement	Endotoxin activity	3-OHFAs in lipid A
Unit	EU	ng or pmol
Endotoxin type	Free	Free and cell-bound (total)
Experiment time	< 1 day	2 days
Labor intensity	Low	Medium
Sensitivity	High	Medium - High
Specificity	Low	High
Reproducibility	Medium [A]	Medium [B]

[A] Low intra-lot variability, high inter-lot variability; [B] Low intra-set (run) variability, high inter-set variability

TABLE I-II. GC/MS endotoxin analysis of agricultural dusts

Author	Type of GC[A]	Monitored 3-OHFAs	Internal standard	Dust type(s)
Andersson et al. [1999][(129)]	GLC	14[B]	C17:0 acid	Cow and pig shed / compared with school and day care
Chun et al. [1999-2004][(139-142)]	GC/MS	n.i.	n.i.	Cotton
Helander et al. [1982][(123)]	GLC	14,16[C]	D-mannoheptulose	Bacteria isolated from cotton and swine air
Krahmer et al. [1998][(143)]	GC/MS-MS	10,12,14, 16,18	^{13}C-labelled muramic acid	Dairy and equipment storage
Laitinen et al. [2001][(144)]	GC/EI-MS	10,12,14,16	C13:0 3-OHFA	Slaughterhouses, grain/vegetable storage and animal-feed industry
Martensson et al. [1997][(145)]	GC/EI-MS	12,14,16,18	C13:0 3-OHFA	Swine
Pomorska et al. [2007][(146)]	GC/MS-MS	10,12,14, 16,18	^{13}C-labelled cyanobacterial cells	Animal houses (cow, pig, sheep, poultry, horse) and hay storages
Reynolds et al. [2005][(107)]	GC/MS-MS	10,12,14,16	n.i.	Chicken, swine and corn
Sonesson et al. [1990][(94)]	GC/CI-MS	10,12,14, 16,18	C9:0 3-OHFA	Poultry processing
Szponar et al. [2001][(121)]	GC/MS-MS	10,12,14, 15,16,17,18	Denuterated C13:0 3-OHFA	Swine / compared with house dust
Wang et al. [1997][(46)]	n.i.	n.i.	n.i.	Swine
Zhiping et al. [1996][(21)]	GC/EI-MS	n.i.	n.i.	Swine

Note: n.i. = not indicated; [A] GLC = gas-liquid chromatography, GC/EI-MS = gas chromatography/electron impact mass spectrometry, GC/CI-MS = gas chromatography/chemical ionization mass spectrometry, GC/MS-MS = gas chromatography/tandem mass spectrometry; [B] Identified comprehensive 3-OHFAs (10 - 17 including iso) but only C14:0 3-OHFA was used for quantification; [C] Also monitored non-3-OHFAs (non-hydroxy fatty acids)

TABLE I-III. Summary of studies of dust and endotoxin exposures in agricultural environments using personal samplers

Dust type	Author(s)	Place of study	Sampling methods	Dust concentration (mg/m^3)		Endotoxin concentration (EU/m^3)	
				n	GM or AM*	n	GM or AM*
Animal production/ livestock farms							
Poultry	Sonesson et al. [1990][94]	Sweden	37-mm samplers with membrane cellulose acetate filters; sampled for 2 - 3 hours	61	1.94 - 7.94 [A]	61	290 - 7700 [A]
Poultry	Radon et al. [2001][126]	Denmark and Switzerland	Threaded holders with glass fiber filters; median sampling time of 118 minutes	36	7 [B]	36	257.6 [B]
Poultry	Donham et al. [2000][14, 147]	Iowa, USA	37-mm cassettes with PVC filters	238	6.5*	236	1589.1*
Chicken	Schierl et al. [2007][148]	Germany	Samplers with glass fiber filters; sampled for 1 - 6 hours	-	-	18	463.2
Chicken	Spaan et al. [2006][125]	The Netherlands	Conical inhalable samplers with glass fiber filters; mean sampling time of 7.3 hours	9	3.6 - 9.5	9	880 - 2140

TABLE I-III. (Continued)

Dust type	Author(s)	Place of study	Sampling methods	Dust concentration (mg/m^3)		Endotoxin concentration (EU/m^3)	
				n	GM or AM*	n	GM or AM*
Turkey	Schierl et al. [2007][148]	Germany	Samplers with glass fiber filters; sampled for 1 - 6 hours	-	-	6	1902
Turkey	Reynolds and Milton [1993][108]	Minnesota, USA	37-mm cassettes with teflon filters; sampled over the workshift	-	-	28	1130 - 5720
Turkey	Reynolds et al. [1994][16, 149]	Minnesota, USA	37-mm cassettes with teflon filters; sampled over the workshift	26	1.2 - 7.6	26	208 - 10960
Swine	Vogelzang et al. [2000][19]	The Netherlands	Samplers with 6-mm diameter inlet opening; PTFE filters; average of 8.3 hours of sampling time on 2 days	171	2.63	171	105
Swine	Schierl et al. [2007][148]	Germany	Samplers with glass fiber filters; sampled for 1 - 6 hours	-	-	18	668.7

TABLE I-III. (Continued)

Dust type	Author(s)	Place of study	Sampling methods	Dust concentration (mg/m^3)		Endotoxin concentration (EU/m^3)	
				n	GM or AM*	n	GM or AM*
Swine	Dosman et al. [2006][127]	Canada	Samplers with glass fiber filters	20	0.15 - 2.48*	20	430.7 - 3689.9*
Swine	Radon et al. [2001][126]	Denmark and Switzerland	Threaded holders with glass fiber filters; median sampling time of 118 minutes	40	4 [B]	40	58 [B]
Swine	Cormier et al. [2000][150]	Canada	Closed-face cassettes with PVC filters; sampled for 4 hours	8	2.53 - 4.31	8	281 - 596
Swine	Reynolds et al. [1996][15]	Iowa, USA	Closed-face cassettes with cellulose acetate filters; sampled over the workshift	151	3.45 - 4.55	151	176.12 - 202.67
Swine	Donham et al. [1995][136]	Iowa, USA	Closed-face cassettes with cellulose acetate filters; sampled over the workshift	108	4.53	108	202.35

TABLE I-III. (Continued)

Dust type	Author(s)	Place of study	Sampling methods	Dust concentration (mg/m^3)		Endotoxin concentration (EU/m^3)	
				n	GM or AM*	n	GM or AM*
Swine	Preller et al. [1995][128]	The Netherlands	Samplers with 6-mm diameter inlet opening; PTFE filters; average of 8.3 hours of sampling time on 2 days	360	2.4	350	92
Swine	Spaan et al. [2006][125]	The Netherlands	Conical inhalable samplers with glass fiber filters; mean sampling time of 7.3 hours	6	2.6	6	1510
Dairy	Kullman et al. [1998][151]	Wisconsin, USA	15-mm cassettes with DM-800 filters; sampled for 4 - 6 hours	159	1.78	194	647
Dairy	Schierl et al. [2007][148]	Germany	Samplers with glass fiber filters; sampled for 1 - 6 hours	-	-	22	16.9 [B]

TABLE I-III. (Continued)

Dust type	Author(s)	Place of study	Sampling methods	Dust concentration (mg/m^3)		Endotoxin concentration (EU/m^3)	
				n	GM or AM*	n	GM or AM*
Dairy	Spaan et al. [2006][125]	The Netherlands	Conical inhalable samplers with glass fiber filters; mean sampling time of 7.3 hours	12	1.3 - 1.5	12	560 - 1570
Beef cattle	Schierl et al. [2007][148]	Germany	Samplers with glass fiber filters; sampled for 1-6 hours	-	-	6	557.9
Grain, seed, and legimen							
Grain	Schwartz et al. [1995][17]	Iowa, USA	37-mm cassettes with PVC filters; sampled over the workshift	410	4.8*	410	2858.7*
Grain	Halstensen et al. [2007][152]	Norway	Cassettes with polycarbonate and glass fiber filters; sampled for 10-60 minutes	104	4.4	104	5.9
Grain	Spaan et al. [2006][125]	The Netherlands	Conical inhalable samplers with glass fiber filters; mean sampling time of 7.3 hours	190	1.5	188	580

TABLE I-III. (Continued)

Dust type	Author(s)	Place of study	Sampling methods	Dust concentration (mg/m^3)		Endotoxin concentration (EU/m^3)	
				n	GM or AM*	n	GM or AM*
Corn	Buchan et al. [2002][153]	Colorado, USA	37-mm cassettes with mixed cellulose ester filters	29	3.3 - 3.4	29	983 - 3175
Wheat	Viet et al. [2001][22]	Colorado, USA	37-mm cassettes with glass-fiber filters	98	0.83	98	54.24
Seed	Smit et al. [2006][85]	The Netherlands	Samplers with glass fiber filters; averaged sampling time of 324 minutes	101	1.6	101	1800
Soybean	Roy and Thorne [2003][154]	Iowa, USA	37-mm cassettes with glass fiber and PTFE filters; sampled for 3 hours	15	1.1	32	8 - 310
Other							
Farm	Nieuwenhuijsen et al. [1999][155]	California, USA	IOM inhalable samplers with PVC filters; not specified sampling time	142	0.30 - 45.14	142	4.74 - 1861.18

TABLE I-III. (Continued)

Dust type	Author(s)	Place of study	Sampling methods	Dust concentration (mg/m^3)		Endotoxin concentration (EU/m^3)	
				n	GM or AM*	n	GM or AM*
Farm	Eduard et al. [2001][156]	Norway	25-mm closed face aerosol monitors with polycarbonate filters	106	0.31	105	1200
Citrus	Lee et al. [2004][157]	California, USA	37-mm cassettes with polycarbonate filters; sampled for 6 - 8 hours	21	39.7	11	201
Grape	Lee et al. [2004][157]	California, USA	37-mm cassettes with polycarbonate filters; sampled for 6 - 8 hours	20	3.5	10	11
Potato	Zock et al. [1995][158]	The Netherlands	Samplers with 6-mm diameter inlet opening; glass fiber filters	81	0.64	68	279

Note: GM = geometric mean, AM = arithmetic mean, EU = endotoxin unit; [A] Originally reported in ng/m^3, 10 EU = 1 ng; [B] Median

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CHAPTER 2

RECOMBINANT FACTOR C (rFC) ASSAY AND GAS CHROMATOGRAPHY / MASS SPECTROMETRY (GC/MS) ANALYSIS OF ENDOTOXINS IN FOUR AGRICULTURAL DUSTS

Abstract

Bacterial endotoxins play an important role in occupational lung disease. Endotoxin exposure is a huge issue in agricultural environments due to relatively high exposure levels. However, a poor understanding of their role in disease pathogenesis has complicated establishment of universal guidelines for agricultural endotoxin exposure. Two techniques are commonly used to measure endotoxin: bioassay responses to the lipopolysaccharide (LPS) component of the Gram-negative cell wall, and chemical analysis of signature 3-hydroxy fatty acids (3-OHFAs) of LPS. The goals of this study were to determine patterns of 3-OHFA distribution in dusts from four types of agricultural environments (grain elevator, cattle feedlot, dairy, and farm), and to evaluate correlations between the results of GC/MS analysis (total endotoxin) and biological recombinant Factor (rFC) assay (free bioactive endotoxin). An existing GC/MS-MS method (for house dust) was modified and optimized for agricultural dusts using GC/EI-MS. A total of 134 breathing zone samples using IOM inhalable samplers were collected from agricultural workers in Colorado and Nebraska. Livestock dusts contained

approximately two times higher concentrations of 3-OHFAs than grain dusts. Patterns of 3-OHFA distribution and proportion of each individual 3-OHFA varied by dust type. Grain dust contained a higher proportion of shorter chain 3-OHFAs (C8:0 and C9:0) than livestock dusts. Livestock dusts contained more variable 3-OHFAs than grain dust. Pearson correlations and multiple linear regressions showed higher correlations between GC/EI-MS and rFC results for livestock dusts than for grain dusts. The rank order of Pearson correlations was feedlot (0.72) > dairy (0.53) > farm (0.33) > grain elevator (0.11). Odd chain length 3-OHFAs were found to correlate with rFC assay response as well as even chain length 3-OHFAs. In general, good correlations were found between the biological assay and the modified GC/EI-MS method. The GC/EI-MS method should be especially useful for identification of specific 3-OHFAs for endotoxins from various agricultural environments and may provide useful information for evaluating the relationship between bacterial exposure and respiratory disease among agricultural workers.

Introduction

Endotoxins (or lipopolysaccharides, LPS) are cell membrane components of Gram-negative bacteria and play an important role in occupational lung diseases. There is a huge concern in endotoxin exposures in agricultural environments due to relatively high exposure levels. Several studies have found that endotoxin exposures are associated with a high prevalence of respiratory disease in agricultural environments.[1-10] Effects include decreases in pulmonary function and increases in severity of asthma and asthma-like diseases.[2, 4-6, 8, 9, 11, 12] In addition to agricultural workers, this endotoxin-disease

association is found in other environments, such as cotton mills,[13, 14] fiberglass manufacturing,[15] and more broadly, in general indoor air quality in office buildings.[16-18] In contrast, recent studies indicate that endotoxin exposure in early life may be protective against asthma and atopic sensitization.[19-25] The role of endotoxin in disease pathogenesis is not clear; thus, universal guidelines or standards for endotoxin exposure do not yet exist. Development of an accurate measurement protocol for endotoxin exposure is critical for understanding the relationship between endotoxin exposure and development of diseases.

There are two approaches to measuring endotoxin: biological assay and chemical analysis. The most commonly used Limulus amebocyte lysate (LAL) biological assays measure the relative reactivity of endotoxins with Limulus lysate, providing rapid and sensitive results.[26, 27] However, bioassay may underestimate endotoxin exposure because it does not detect cell-bound endotoxins that may be associated with respiratory disease.[28-31] Furthermore, traditional bioassay technology may experience interference from non-endotoxin agents, such as glucans, and this lack of specificity may yield misleading data. The newly developed recombinant Factor C (rFC) assay operates on the same basic principle as the previous LAL assay, but provides greater sensitivity and specificity (is not prone to glucan interference) and less variability. However, like its predecessor, the rFC assay detects only biologically active free (released from the bacterial cell-wall) endotoxins and still does not offer structural information for specific chemical components of endotoxins, which may be vital in understanding disease mechanisms.[28-31]

Gas chromatography/mass spectrometry (GC/MS) analysis focuses on quantification of biomarkers of endotoxins, 3-hydroxy fatty acids (3-OHFAs) in lipid A of LPS. One mole of LPS contains approximately 4 moles of 3-OHFA. Unlike bioassay, GC/MS analysis of 3-OHFAs allows determination of both cell-bound and non-cell-bound endotoxins.[31, 32] In addition, GC/MS provides information about the chemical composition of endotoxins. Laitinen et al.[33] have indicated that the specific chemical structure of endotoxin, such as C14:0 3-OHFA is associated with respiratory symptoms; however, involvement of specific components of LPS has not been studied in detail for agricultural exposures. In addition, Helander et al.[34] found that differences in chemical structures of lipid A portion, specifically fatty acid components, of LPS related to differences in acute pulmonary toxicity in guinea pigs. Understanding the chemistry of endotoxins may be important for explaining disease pathology, and ultimately, for interventions. Therefore, GC/MS may offer an advantage over traditional endotoxin bioassay in predicting respiratory disease, especially if specific 3-OHFAs are associated with disease pathogenesis.

A number of studies have applied GC/MS for endotoxin analysis;[35-39] however, many were not directed at agricultural dusts. The original chemical analysis method was developed as a tool for assessing indoor air quality in a poultry house by using chemical ionization mass spectrometry (GC/CI-MS)[31] and then modified for tandem mass spectrometry (GC/MS-MS) using house dusts.[36-39] GC/MS-MS uses ion-trap or triplequadrupole technology to reduce background signal, providing more sensitive results than widely available electron impact mass spectrometry (GC/EI-MS); however, GC/MS-MS may not be available in most facilities. In this study, we modified the

GC/MS-MS method developed for house dusts for GC/EI-MS analysis of agricultural dusts. Unlike house dusts, agricultural dusts often contain a wide variety of 3-OHFAs – the range studied here was C8-C18. Moreover, our preliminary studies with agricultural dusts involved use of personal air sampling devices and often yielded very small dust samples and subsequently very low residues of 3-OHFAs. This finding necessitated a sensitive methodology for GC/EI-MS to assess occupational exposures accurately. Our modified method provides a simple, reliable sample preparation procedure compared to the existing method.

As part of a larger study of agricultural exposures and respiratory diseases, the goals of this study were to determine patterns of 3-OHFA distribution and proportion in four types of agricultural dusts and to evaluate correlations between the results of GC/EI-MS analysis and the biological rFC assay. This study is the first to report the comparison of GC/EI-MS results to the rFC assay in various agricultural dusts.

Methods

Dust sample collection and preparation of samples

A total of 134 personal breathing zone samples using IOM inhalable samplers were collected in four agricultural environments in Colorado and Nebraska: dairy farms (n = 17), cattle feedlots (n = 48), grain elevators (n = 58), and corn farm (n = 11). IOM inhalable samplers used 5 μm pore size PVC filters (SKC Inc., Eighty Four, PA), at a flow rate of 2 l/min over 6 to 8 hours during typical work shifts. Samples were collected in 2004 – 2006, during all four seasons. This study was approved by the Colorado State University's institutional review board for human subject protection.

Collected dust samples were extracted in sterile, pyrogen-free (pf) water containing 0.05% Tween-20 for 1 hour at room temperature, 22 °C, with continuous shaking. A portion of each extract was analyzed by the rFC assay, and another portion was lyophilized (at -50 °C) for GC/EI-MS determination of 3-OHFAs. Lyophilized samples were stored at -70 °C until analysis.

Materials

Acetyl chloride (99.8% purity) and pyridine (99.9% purity) were purchased from Fluka (St. Louis, MO); N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Regis Technologies (Morton Grove, IL); C8:0 to C18:0 3-OHFAs were purchased from Matreya (Pleasant Gap, PA); pentadecanol (99% purity) was purchased from Acros Organics (Geel, Belgium); diethyl ether and methanol (99.8% purity) were purchased from Sigma Aldrich (St. Louis, MO); and heptane (pesticide grade) was purchased from Fisher Scientific (Pittsburgh, PA). Strata-X polymeric reversed phase chromatography cartridges (P/N 8B-S100-UBJ) were purchased from Phenomenex (Torrance, CA). Glassware was baked at 250 °C and rinsed with acetone and hexane prior to use. All test tubes had PTFE-lined screw caps.

GC/EI-MS analysis

Samples and external 3-OHFA standards of 8 to 18 carbon chain lengths (except C11:0, method surrogate) were digested and methylated with 0.5 ml of 3 M methanolic HCl (2.5 ml of acetyl chloride added to 11 ml of methanol) for 16 to 18 hours at 80 °C and cooled to room temperature. Samples and standards were amended with 10 µl pentadecanol

(100 µl per ml in heptane) as a keeper solvent and diluted with 1 ml deionized water for solid-phase extraction (SPE) clean up. For SPE clean up, samples were applied to a 60 mg/ 3 ml Strata-X polymeric reversed phase cartridge. Cartridges were conditioned with 1 ml diethyl ether and 1 ml deionized water prior to sample loading. Following 20 min aspiration, 3-OHFA methyl esters were eluted with 2 ml diethyl ether and evaporated to dryness with a Nitrogen stream. No volatilization of any 3-OHFAs was observed. Samples were converted to trimethylsilyl (TMS) analogs for GC/EI-MS analysis by adding 50 µl BSTFA and 5 µl pyridine and heating for 20 min at 80 °C. Derivatized samples and standards were diluted with 50 µl heptane and a 2 µl aliquot of each was analyzed by GC/EI-MS using a HP 5890 Series II Plus GC equipped with HP-5MS column (0.25 mm × 30 m, 0.25 µm film thickness, Hewlett-Packard, Palo Alto, CA) with split/splitless inlet, electronic pressure control, 7673 automatic liquid sampler, and a HP 5972 Mass Selective Detector. Selected Ion Monitoring (SIM) for individual 3-OHFA was used for endotoxins, and the concentration calculated in picomole (pmol). For each 3-OHFA, monitored ions were: C8:0, *m/z* 175 and 231; C9:0, *m/z* 175 and 245; C10:0, *m/z* 175 and 259; C11:0, *m/z* 175 and 273; C12:0, *m/z* 175 and 287; C13:0, *m/z* 175 and 301; C14:0, *m/z* 175 and 315; C15:0, *m/z* 175 and 329; C16:0, *m/z* 175 and 343; C17:0, *m/z* 175 and 357; C18:0, *m/z* 175 and 371. Selected ions represented the M-15 ion and *m/z* 175, the acid portion of the fatty acid cleaved between C3 and C4. Ion ratios were monitored to identify interference from 2-OHFAs, which have the same M-15 ion as the corresponding 3-OHFA but lack the *m/z* 175 fragment.

Calibration curves and method performance

The 3-OHFAs of C8:0 to C18:0, except C11:0, were processed identically to samples, from the first step of sample preparation to the end. The 3-OHFA of C11:0 was added as surrogate to each sample prior to digestion and methylation. Since agricultural dusts contain a wide range of endotoxin concentration levels, spike levels of 2, 6, 20, 100, and 500 ng of individual 3-OHFAs (in ethanol, stored at -20 °C) were used for creating the calibration curve.

Limit of detection (LOD) and limit of quantitation (LOQ) were determined by signal-to-noise (S/N) ratio based on the chromatograms of blank controls and 0.5 and 1 ng spikes (S/N > 3 for LOD and > 10 for LOQ).[40] The coefficient of variation (CV) of C11:0 3-OHFA surrogate peaks on the chromatogram was calculated to evaluate precision and reproducibility of the modified method.

rFC assay

Extracted samples were analyzed using the rFC endotoxin assay (Cambrex, East Rutherford, NJ). The rFC assay method for endotoxin detection uses recombinant factor C (rFC), the first component of the cascade.[28] The activation of rFC was determined by fluorescence generated by the enzymatic cleavage of a peptide-coumarin substrate. Fluorescence was measured after 1 hour incubation with endotoxin standards at 37 °C. Log fluorescence was proportional to log endotoxin concentration and was linear in the 0.01 to 10 EU/ml range.

Two-fold serial dilutions of endotoxin standards (*Escherichia coli* O55:B5) and sample extracts were prepared using sterile, pf water with 0.05% Tween-20. Use of

0.05% Tween-20 resulted in the high spike recovery and reproducibility. The samples were added to a 96-well plate followed by 100 µl of a mixture of enzyme, buffer and fluorogenic substrate. The plates were incubated at 37 °C for one hour and read in a fluorescence microtiter plate reader (Biotek Instruments, Winooski, VT; FLX800TBIE) at excitation/emission 380/440 nm. Background (0 EU/ml) fluorescence was subtracted and log change in fluorescence plotted against log endotoxin concentration. Endotoxin concentrations of samples were calculated according to the standard curve. Four assay reagent blank wells served as reference and control for the pf status of the reagent water, centrifuge tubes, pipette tips and microplates. Quality assurance spiking assays were performed to assess matrix interference or enhancement.

Statistical analysis

Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). The UNIVARIATE procedure in SAS was used to evaluate the distributions of data. Based on the box-cox test and histograms of sample distribution, data were log-normally distributed; therefore, data were natural log transformed before proceeding with analysis. Any 3-OHFA concentrations below the LOD were assigned a value of LOD divided by square-root of two,[41] which was 0.5 to 2 pmol depending on the specific 3-OHFA.

Geometric mean (GM) and geometric standard deviation (GSD) of GC/EI-MS and rFC results in each agricultural environment were calculated. Analysis of variance (ANOVA) via GLM procedure of SAS followed by Bonferroni multiple-comparison tests of means was used to test for differences by dust type. Pearson correlations were calculated between GC/EI-MS results and rFC results, between individual 3-OHFAs, and

between odd and even carbon length 3-OHFAs. Multiple regressions were performed to evaluate the relationship between rFC assay and GC/EI-MS results accounting for effects of individual 3-OHFAs. In addition to the correlations and regression analyses, proportions of C8:0 to C18:0 3-OHFAs in each dust type were calculated.

Results

GC/EI-MS Calibration curves and method performance

Two calibration curves were created for each experimental set: one for lower concentrations (2, 6, and 20 ng) and one for higher concentrations (20, 100, and 500 ng). The calibration curves yielded R^2 of 0.99. The S/N ratios of the chromatograms showed that the method provided an LOD (S/N > 3) of 1 to 3 pmol (equivalent to 0.5 ng spike) and an LOQ (S/N > 10) of 3 to 6 pmol (equivalent to 1 ng spike), depending on carbon chain length of 3-OHFAs measured. Original values were used for samples that have less than LOQ but greater than LOD and any 3-OHFA concentrations below the LOD were assigned a value of LOD divided by square-root of two.[41] Proportion of < LOD and < LOQ samples for each 3-OHFA is summarized in Table II-I.

The reproducibility of C11:0 3-OHFAs surrogate recovery was satisfied based on a CV less than 12% CV (n = 24) since this method contained two derivatization steps including one overnight reaction. Correlation between 3-OHFAs taken through the entire sample preparation process and 3-OHFA methyl esters (3-OHFAMEs) converted directly to trimethylsilyl analogs was poor, probably due to the structural difference in 3-OHFAs and 3-OHFAMEs (data not shown). Since 3-OHFAME standards were already methylated, use of 3-OHFAMEs would not allow monitoring the methylation step. Thus,

spike recovery was not evaluated in a traditional manner. Instead, C11:0 surrogate coupled with calibration linearity was used to ensure strong quality control of data reporting. As stated above, fatty acid standards were submitted to the entire sample workup and covered the analytical working range (2 to 500 ng spikes of individual 3-OHFA). This method effectively assessed relative spike recovery at 5 levels for each compound and yielded excellent calibration linearity.

Analysis of agricultural dusts

Table II-II presents the GM and GSD of total 3-OHFAs per mg dust (pmol/mg) and per cubic meter air (pmol/m^3) obtained by GC/EI-MS, and endotoxin activity per mg of dust (EU/mg) and per cubic meter air (EU/m^3) obtained by the rFC assay. Total 3-OHFAs was calculated as the sum of concentrations of each 3-OHFA with chain lengths of 8 to 18 except 11 and 16. C11:0 was not included in the total because it was used as a surrogate due to its absence in agricultural dusts. C16:0 experienced interference, probably resulting from contamination, in 41% of total 134 dust samples. Based on the subset analysis of 79 samples with C16:0, C16:0 was not significantly correlated with the bioassay ($p > 0.15$); therefore, C16:0 was eliminated from the analyses. Results are presented separately for each agricultural environment. A clear difference between agricultural environments was discovered. The general linear model ANOVA confirmed that the variation in 3-OHFA and endotoxin concentrations among four agricultural environments was statistically significant ($p < 0.01$). Among all, cattle feedlots had the highest concentration of total 3-OHFAs and endotoxin activity, followed by dairies. Livestock dusts contained approximately 1.5 to 2.5 times higher concentrations of 3-

OHFAs than grain dust. A similar trend was found for endotoxin activity per mg dust, but 3-OHFAs and endotoxin activity per m^3 of air did not exhibit as large a difference. There was a statistically significant difference in means of total 3-OHFAs and endotoxin activity between cattle feedlots and grain elevator dusts ($p < 0.05$).

Pearson correlations between GC/EI-MS (total 3-OHFAs) and the rFC assay (endotoxin activity) are shown in Table II-III. GC/EI-MS and rFC assay results were strongly positively correlated ($p < 0.01$) in livestock dusts [feedlot (0.72) and dairy (0.53)] but were not statistically significant for grain dust and farm dust.

A more detailed analysis for each individual carbon chain length of 3-OHFA is summarized in Table II-IV. More variable chain lengths of 3-OHFAs, C9:0 to C18:0, were more significantly correlated with endotoxin activity in feedlot dust than in other dusts. The C8:0 3-OHFA was not correlated with endotoxin activity in any environments in this study. C10:0 3-OHFA was correlated with endotoxin activity in only livestock dusts. All statistically significant correlations in livestock dusts were strongly positive, whereas in grain dusts, both statistically significant correlations and non-significant correlations were positive or negative. No correlations were statistically significant in farm dust.

Multiple linear regression analysis, based on stepwise selection, was performed to evaluate the relationship between GC/EI-MS and rFC assay results accounting for effects of individual 3-OHFAs at the same time. The results of the regression analyses for the combination of 3-OHFAs with the rFC assay are shown in Table II-V. Feedlot dust had the highest association ($R^2 = 0.73$) with rFC assay results. The combination of C9:0, C10:0, and C17:0 yielded the highest correlation with rFC assay results for grain

elevators; however, the correlation was weaker than that for livestock dusts. C18:0 3-OHFA was retained only in the models for livestock dusts.

The correlations between individual 3-OHFA was calculated to evaluate the potential interactions between each individual 3-OHFA. The correlations between single 3-OHFAs varied by lengths of carbon chain and agricultural environments. In general, 3-OHFAs with longer carbon chain (C12:0 to C18:0) correlated with other 3-OHFAs significantly. Among shorter chain 3-OHFAs (C8:0 to C10:0), correlations were nonexistent or weak. Except for the correlation between C9:0 and C17:0 in grain elevator, all statistically significant correlations were positive. No interactions were entered to the regression models.

In Table II-VI, the correlations between odd-numbered (the sum of C9:0, C13:0, C15:0, and C17:0) and even-numbered (the sum of C8:0, C10:0, C12:0, C14:0, and C18:0) carbon chain 3-OHFAs and endotoxin activity (the rFC assay) are presented. Total even-numbered length 3-OHFAs correlated more strongly with endotoxin activity than odd-numbered length 3-OHFAs overall, but odd-numbered length 3-OHFAs correlated more strongly than even-numbered 3-OHFAs in livestock dusts. The odd-numbered length 3-OHFAs of grain and farm dusts were not significantly correlated with endotoxin activity ($p = 0.28$ and 0.35, respectively). However, odd-numbered length 3-OHFAs and endotoxin activity were strongly positively correlated in livestock dusts. The correlations between odd and even-numbered carbon length 3-OHFAs and total 3-OHFAs were strongly positive in all environments.

As seen from Figure 2-1, the relative proportion of each 3-OHFA varied by dust type. Overall, C12:0 and C14:0 were dominant in all environments, and C13:0 was least

prevalent. Grain dust contained a higher proportion of shorter chain 3-OHFAs (C8:0 and C9:0) than livestock dusts.

Discussion

Figure 2-2 shows the flowchart of the modified GC/EI-MS method and the existing method GC/MS-MS.[36-39] The major changes in the modified method are elimination of liquid-liquid extraction, use of polymeric solid-phase extraction (Strata-X or equivalent Oasis HLB, Water Corp, Milford, MA) instead of silica cartridge for sample clean-up, and use of deionized water instead of 1:1 pentane: dichloromethane (DCM) mixture for sample loading to SPE.

In addition, the modified method is calibrated by running 3-OHFA standards through the entire digestion/sample clean-up process instead of introducing 3-OHFA methyl esters at the silylation step. For our study, this approach provides better method performance information for the 16 to 18 hours methylation step by monitoring individual 3-OHFAs at several concentrations throughout analysis rather than relying only on the recovery of standards. To support the above statement, we found poor correlation between standards prepared this way and 3-OHFAMEs introduced at the final step, trimethylsilylation. Ideally, isotope dilution would provide sample-specific method performance information and make this step unnecessary. However, isotopically labeled standards for each 3-OHFA were unavailable; our compromise was this method of calibration coupled with addition of a single surrogate, C11:0 3-OHFA, to each sample. This modified method has advantages over the existing method. Due to the elimination

of liquid-liquid extraction, needs for pentane and highly toxic dichloromethane are eliminated. This modification reduces sample handling and cost of analysis.

The distribution of 3-OHFAs varied by dust type. Among all dust types analyzed in this study, cattle feedlot dust showed the highest correlation between 3-OHFAs and endotoxin activity, followed by dairy dust. Farm and grain dusts showed the lowest correlation to endotoxin activity. A recent study conducted by Pomorska et al.[42] also reported a low correlation in grain (hay storage) dust and high correlations in sheep and poultry dusts. Differences in dust composition, including the bacterial distribution, may explain differences in the correlations between 3-OHFAs and endotoxin activity since the major source of dust in livestock environments might be fecal components while the major source of dust in grain elevator and farm might be plants. The bacterial flora must be different in fecal and plant components. In addition, the proportion of free and cell-bound endotoxin might be different in livestock and grain environments. In livestock environments, bacteria may actively grow and die since fecal component is a nutrient-rich medium compared to plants. Endotoxins are released to environment as free form when bacteria grew or died; thus, a high proportion of endotoxins in livestock environments could be in free, non-cell bound form. As stated previously, the rFC assay only detects free endotoxins while GC/EI-MS detects total (both free and cell-bound) endotoxins. However, the mechanisms relating to the differences in the correlations are still unclear.

For the four dust types, endotoxin activity (the rFC assay) showed moderate or weak correlations with C8:0 to C14:0 3-OHFAs. Longer chain (C15:0 to C18:0) 3-OHFAs were strongly positively correlated with endotoxin activity in livestock dusts but

were not correlated in grain dust. Several studies reported that the C10:0 to C14:0 3-OHFAs had strong positive correlations and longer-chain 3-OHFAs had lower or negative correlations with endotoxin activity in house dusts;[38, 43] however, agricultural dusts, especially livestock dusts, had an opposite tendency. This relationship could be explained by differences in biological assay response, as well as the difference in microbial community. For example, C18:0 3-OHFA may be derived from Actinobacteria rather than from Gram-negative bacteria; [44, 45] the rFC assay may positively react with this Actinobacteria. Moreover, C10:0, C12:0, and C14:0 3-OHFAs were thought to be biologically active since their presence has been confirmed in lipid A of Gram-negative bacteria.[37, 46, 47] However, our results showed that the correlation between rFC assay results and C10:0 3-OHFA was significant in only livestock dusts, C12:0 3-OHFA was only significant in cattle feedlot, and C14:0 3-OHFA was significant in only cattle feedlot and grain elevator dusts. Similar to this study, a previous study using chicken, swine, and corn dusts found weak correlations of C12:0 and C14:0 3-OHFAs with biological LAL assay response.[32] This tendency may be unique to agricultural dusts. These results illustrate that chemical compositions of agricultural dusts differ from house dusts. In addition, Pomorska et al.[42] also found that the correlations between individual 3-OHFA and LAL results varied by type of animal farm; dusts from sheep sheds had statistically significant correlations between C12:0 to C18:0 3-OHFAs and LAL results, while none of 3-OHFA significantly correlated with LAL results in dusts from hay storage. This finding agreed with our results. Thus, the chemical composition of endotoxins also varied by agricultural dust type. Helander et al.[34] have explained that LPS chemical composition could cause differences in acute pulmonary toxicity of LPS in guinea pigs;

two bacterial species that had similar chemical composition and structure caused similar toxicity. Laitinen et al.[33] found that C14:0 3-OHFA was related to self-reported respiratory and eye symptoms. The same study did not use quantitative measurement of respiratory symptoms; however, their results indicate the importance of specific 3-OHFAs in workers' respiratory problems. Thus, understanding the chemical structure of endotoxin can provide better modeling of dose-response relationships between endotoxin exposure and respiratory disease.

Our study used the rFC assay to determine endotoxin activity instead of the traditional LAL assay. Recent studies found results from the LAL and the rFC assays were strongly correlated, though the LAL assay yielded higher endotoxin levels than the rFC assay.[28] This finding further supports the need for a better understanding of bioassay data in assessing endotoxin exposure. Since this paper is the first to report a correlation between GC/EI-MS and rFC assay results in agricultural dusts, additional analyses in future studies are expected.

All four dust types contained a wide variety of 3-OHFAs including odd-numbered carbon chain 3-OHFAs, which are not significant in house dusts.[48] In agricultural dusts, odd-numbered carbon chain 3-OHFAs contributed significant portion of the total 3-OHFA. For example, C17:0 3-OHFA was significantly positively correlated to rFC assay results in livestock dusts ($r = 0.74$, $p < 0.01$ for dairy and $r = 0.60$, $p < 0.01$ for cattle feedlot) but slightly negatively correlated in grain dust ($r = -0.23$, $p = 0.08$).

Most studies to date have only investigated the even-numbered carbon chain 3-OHFAs for endotoxin exposure assessment in dusts;[17, 32, 34, 42, 43, 49, 50] however, our findings showed that the odd-numbered carbon chain 3-OHFAs might also be important.

Thus, excluding the odd-numbered carbon chain 3-OHFAs may underestimate the total 3-OHFAs in agricultural environments. Including odd-numbered 3-OHFAs may provide better understanding of the bacterial sources. Future studies using 3-OHFAs for endotoxin exposure should monitor odd-numbered carbon chain 3-OHFAs, as well as even-numbered carbon chain 3-OHFAs.

Limitations

Seasonal variations in bacterial distributions likely exist. However, because sample size was unevenly distributed among the four seasons with small sample size in one or more seasons, seasonal variability could not be evaluated in this study. In addition, although geographical differences between Colorado and Nebraska could cause difference in bacterial distribution, not enough samples were collected in Nebraska for geographical comparison in this study.

Since the biological assays only measure the response of lipid A to the enzyme, bioassay results do not necessarily relate to the toxic effects of endotoxins. Therefore, measuring the total (both free and cell-bound) endotoxin and identifying chemical composition of endotoxin using the GC/MS method may provide better understanding of exposure-response relationships. However, there are several limitations on the GC/MS method. Although our modified GC/EI-MS method significantly reduced sample handling compared to the parent GC/MS-MS method, the GC/MS methods, in general, require a longer sample preparation time and higher labor intensity than the bioassays. In addition, the GC/MS is relatively expensive compared to the bioassays. Thus, the GC/MS may be available in the research facilities, but not in general industries.

Conclusions

A GC/EI-MS method for endotoxin analysis has been successfully applied to assessment of 3-OHFA distribution in several agricultural environments. Compared to the parent GC/MS-MS method, it reduces use of toxic chemicals and sample handling, allows sensitive monitoring of the experimental process, and can be used for analysis of very small samples, typical of personal air samples.

Evaluating personal exposure to endotoxin using chemical and biological analyses in various agricultural environments is very important for developing accurate assessment of endotoxin exposure in agriculture. Understanding differences in 3-OHFA distributions in various agricultural environments may provide better explanations of the relationship between endotoxin exposure and development of respiratory diseases. This study evaluated the rFC assay and GC/EI-MS results in four agricultural environments. The distribution of 3-OHFAs and the correlation coefficients varied by agricultural environment. Overall, livestock dusts had more variable 3-OHFAs and stronger correlations between GC/EI-MS and rFC assay results than grain dusts, probably due to the possible difference in bacterial distribution. Quantification of 3-OHFAs may provide useful information for evaluating the relationship between bacterial exposure and respiratory disease among agricultural workers. In future applications, it will be important to: increase sample size, especially in dairy and farm environments; analyze dust samples from different agricultural environments; evaluate seasonal and geographical variability; and investigate the roles of specific 3-OHFAs (including both even and odd chain length 3-OHFAs) in human respiratory diseases.

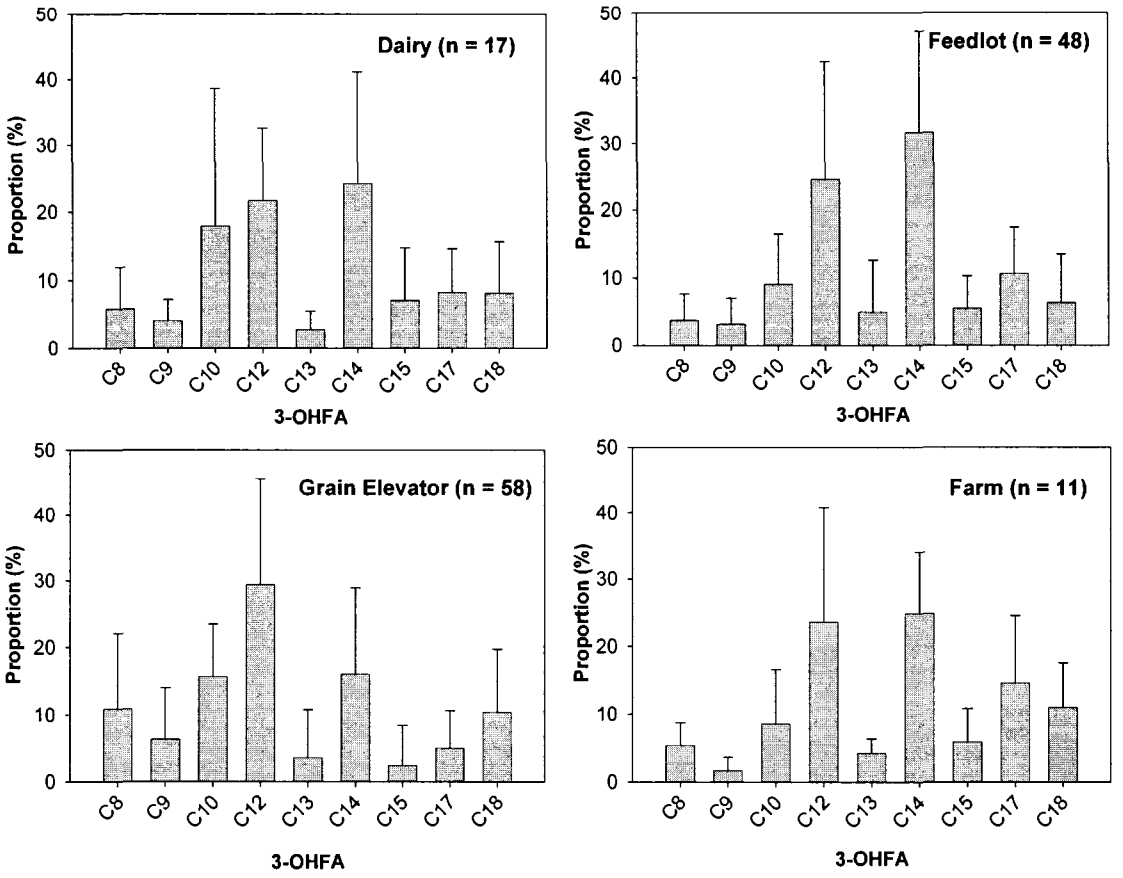
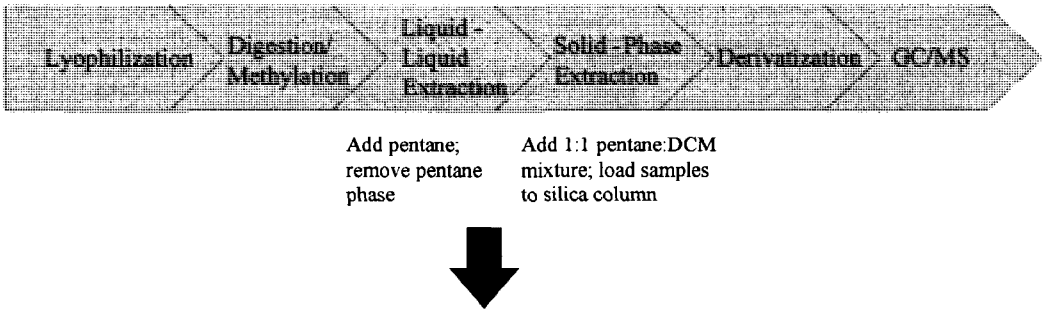


FIGURE 2-1. Mean proportion of 3-OHFA with standard deviation (error bar) found in each dust type.

The Existing Method



The Modified Method

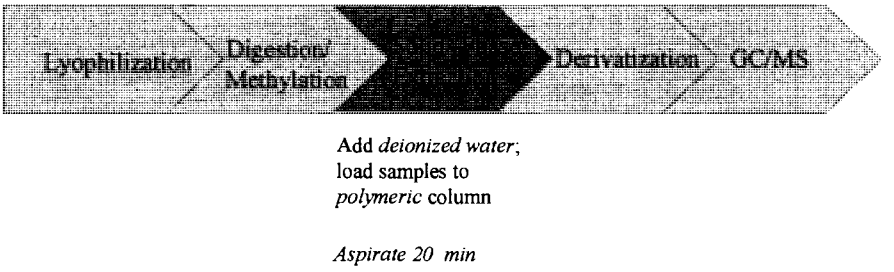


FIGURE 2-2. Changes in experimental procedures for GC/EI-MS analysis.

TABLE II-I. Proportion of samples with <LOD and <LOQ

	C8:0 (%)	C9:0 (%)	C10:0 (%)	C12:0 (%)	C13:0 (%)	C14:0 (%)	C15:0 (%)	C17:0 (%)	C18:0 (%)
> LOQ[A]	79.9	86.6	90.3	99.3	58.2	94.8	60.4	80.6	80.6
LOD < x < LOQ	4.5	5.2	3.7	0.0	8.2	1.5	0.7	3.0	1.5
< LOD[B]	15.7	8.2	6.0	0.7	33.6	3.7	38.8	16.4	17.9

[A] LOQ = 1 ng, [B] LOD = 0.5 ng

TABLE II-II. GM and GSD of 3-OHFAs (GC/EI-MS) and endotoxin activities (rFC) per dust and air

		3-OHFAs per mg dust (pmol/mg)		3-OHFAs per m^3 air (pmol/m^3)		Endotoxin activity per mg dust (EU/mg)[A]		Endotoxin activity per m^3 air (EU/m^3)[A]	
	n	GM	GSD	GM	GSD	GM	GSD	GM	GSD
Dairy	17	620	2.6	1333	3.5	350	2.8	752	3.9
Cattle Feedlot	48	1062	2.9	2778	3.7	419	5.4	1097	6.6
Grain Elevator	58	421	4.2	1968	5.6	143	3.9	669	8.8
Farm	11	825	2.5	2176	3.0	179	3.0	473	4.3

Note: GLM ANOVA for endotoxin activity per mg dust and 3-OHFA per mg dust by environment yielded p-value < 0.01. [A] EU = endotoxin unit

TABLE II-III. Pearson correlation between GC/EI-MS (total 3-OHFAs) and rFC assay

	n	R	p-value
Overall [A]	134	0.43	< 0.01
Dairy	17	0.53	0.02
Cattle Feedlot	48	0.72	< 0.01
Grain Elevator	58	0.11	0.39
Farm	11	0.33	0.32

[A] Combining all four dust types

TABLE II-IV. Pearson correlation between individual 3-OHFA and rFC assay result

	n	C8:0	C9:0	C10:0	C12:0	C13:0	C14:0	C15:0	C17:0	C18:0
Overall [A]	134			++	++	++	++	++	++	++
Dairy	17			++					++	
Cattle Feedlot	48		++	++	++	++	++	++	++	++
Grain Elevator	58		++[B]				++		+[B]	
Farm	11									

Note: "++" if p-value is <0.05 and "+" if p-value is 0.05<p<0.10. [A] Combining all four dust types. [B] Negative correlation.

TABLE II-V. Stepwise forward multiple regression analyses for 3-OHFAs and rFC assay

	n	3-OHFA combination [B]	R^2	P-value
Overall [A]	134	14, 8, 10	0.33	< 0.01
Dairy	17	17, 10	0.65	0.06
Cattle Feedlot	48	14, 18, 13	0.73	0.04
Grain Elevator	58	9, 10, 17	0.25	0.05
Farm	11	None at the 0.10 level	-	-

[A] Combining all four dust types. [B] In order of entrance to the model

TABLE II-VI. Pearson correlation of odd and even-numbered carbon chain 3-OHFAs with the rFC assay and total 3-OHFAs

	Endotoxin activity (EU/mg)	Total 3-OHFAs (pmol/mg)	Even carbon chain 3-OHFA (pmol/mg)
Dairy (n = 17)			
Odd 3-OHFAs	0.60 [A]	0.51 [A]	0.35
Even 3-OHFAs	0.50 [A]	0.98 [A]	
Cattle Feedlot (n = 48)			
Odd 3-OHFAs	0.70 [A]	0.82 [A]	0.74 [A]
Even 3-OHFAs	0.68 [A]	0.99 [A]	
Grain Elevator (n = 58)			
Odd 3-OHFAs	-0.24 [B]	0.56 [A]	0.47 [A]
Even 3-OHFAs	0.14	0.99 [A]	
Farm (n = 11)			
Odd 3-OHFAs	0.15	0.82 [A]	0.68 [A]
Even 3-OHFAs	0.31	0.97 [A]	

Note: Odd = sum of C9:0, C13:0, C15:0, and C17:0 3-OHFAs. Even = sum of C8:0, C10:0, C12:0, C14:0, and C18:0 3-OHFAs. Total = sum of C8:0, C9:0, C10:0, C12:0. C13:0, C15:0, C17:0, and C18:0. [A] p-value < 0.05. [B] 0.05 < p-value < 0.10.

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CHAPTER 3

COMPARISON OF RECOMBINANT FACTOR C (rFC) AND LIMULUS AMEBOCYTE LYSATE (LAL) ASSAYS FOR ENDOTOXIN EXPOSURE IN FIVE LIVESTOCK DUSTS

Abstract

The goal of this study was to compare the traditional chromogenic LAL and the novel fluorometric rFC assay responses to endotoxin in five livestock dusts (chicken, dairy, horse, swine, and turkey). A total of 713 samples was analyzed by rFC assay and 689 samples were analyzed by LAL assay (689 matched samples). Gas chromatography/ electron impact mass spectrometry (GC/EI-MS) analyses of chemical markers of endotoxin and fungi were used to investigate potential interference with assays. In general, strong positive correlations ($r > 0.91$) exist between results from the LAL and rFC assays. However, responses to assays vary by agricultural environment or dust type. LAL may overestimate (or rFC may underestimate) endotoxin exposures in chicken and horse dusts and LAL may underestimate (or rFC may overestimate) endotoxin concentrations in dairy, swine, and turkey dusts. Our findings showed that the ergosterol concentration may not be the major source of interference in the LAL assay overall, but the interference may vary by dust type. Other than ergosterol contribution, this variability could be explained by differences in bacterial composition and other dust

components; the rFC assay may react positively with Actinobacteria. Future applications will be expected to investigate presence of potential interference of assays including Actinobacteria and proteins in agricultural dusts.

Introduction

Gram-negative bacterial endotoxins (lipopolysaccharides, LPS) are a major component of organic dusts and are clearly associated with respiratory symptoms in humans. Exposure to endotoxins in agricultural dusts including swine, poultry, and grain has been associated with asthma, chronic bronchitis, hypersensitivity pneumonitis, chronic obstructive pulmonary disease (COPD) and decrease in pulmonary function especially FEVs.[1-8] In addition to agricultural workers, this association is found in other occupational environments, such as fiberglass manufacturing, cotton mills, and in general indoor air quality in office buildings.[9-13] Endotoxins impact the immune system, and recent studies indicate that timing of exposure is important, with endotoxin exposure in early life protecting against asthma and atopic sensitization.[14-20]

The Limulus amebocyte lysate (LAL) assay is the most commonly used bioassay for endotoxin measurement. This assay measures relative reactivity of endotoxins with Limulus lysate, an enzyme extracted from horseshoe crabs.[21, 22] Although the LAL assay is exquisitely sensitive, the LAL assay exhibits some lack of specificity due to high variability in laboratory methods for sample collection, sample handling and storage, sample analysis, and variation in the reporting of results.[23-33] In addition, the LAL assay may experience interference from non-endotoxin agents, such as (1→3)-β-D-Glucans from fungi.[34-36] The novel recombinant Factor C (rFC) assay was developed on the

same basic principle as the LAL assay, but use of the genetically engineered rFC prevents interference from (1→3)-β-D-Glucans by eliminating glucan response Factor G from the assay cascade, and provides greater sensitivity and specificity and less variability.[37-41] Both bioassays do not detect cell-bound endotoxins that may be associated with respiratory symptoms, or provide any specific information on chemical structure.[42-44]

A recent study by Alwis and Milton found that the LAL and rFC assay responses were strongly correlated and that (1→3)-β-D-Glucans was not a major source of interference in house dust.[37] Agricultural dust contains complex mixtures of organic and non-organic sources including urine, fecal material, grain, bacteria, and fungi.[45] Therefore, higher interference in LAL and rFC assays can be expected for agricultural dusts. Conducted within the context of a study of aerosol sampling devices in multiple agricultural environments, the goal of this study was to compare the traditional chromogenic LAL and the novel fluorometric rFC assay responses to endotoxin in five livestock dusts. Gas chromatography/ electron impact mass spectrometry (GC/EI-MS) analyses of chemical markers of endotoxin and fungi were used to investigate potential sources of interference with these endotoxin assays.

Methods

Sample collection

The sampling strategy and methods have been previously described but are summarized here.[46] Area samples were collected using four personal samplers: the 37-mm closed-face cassette (CFC), the SKC aluminum respirable cyclone, the IOM inhalable sampler, and the Button inhalable sampler (all available from SKC Inc., Eighty Four, PA). Flow

rates for each sampler were adjusted with a needle valve and calibrated to within 5% of the suggested flow rate before each trial with an electronic soap bubble flow meter (Gilibrator®, Sensidyne, Clearwater, FL). Flow rates of 2.0, 4.0, and 2.5 L/min were set according to manufacturers' instructions for the IOM, Button, and cyclone, respectively. A flow rate of 2.0 L/min for the CFC was chosen from the suggested range of 1.0 – 2.0 L/min. Polyvinyl chloride filters with 5 μm pore size were weighed to the nearest microgram using a 6-place balance (Model MT5, Mettler-Toledo Inc., Columbus, OH). Laboratory samples were collected in a still air chamber (in Iowa) and in a wind tunnel (in Colorado) operated at 0.2 m/s, and 1.0 m/s wind velocities using three agricultural dusts (chicken, swine, and turkey). Field samples were collected in five agricultural environments (chicken, dairy, horse, swine, and turkey). Chicken, dairy, swine, and turkey dusts were collected in Colorado and Iowa, and horse dust was collected only in Colorado. Pairs of each sampler were attached to a rotating mannequin.[46] A total of ten trials were conducted for each wind velocity and each field visit (total of 720 samples). Sample sizes are summarized in Table III-I. One set of samples was shipped to Colorado State University for rFC assay and GC/EI-MS analyses, and a duplicate set was shipped to the University of Iowa for LAL assay analysis.

rFC assay - Colorado

Collected dust samples were extracted in sterile, pyrogen-free (pf) water containing 0.05% Tween-20 for 1 hr at room temperature, 22 ˚C, with continuous shaking. A portion of each extract was analyzed by rFC assay and another portion was lyophilized

(at -50 °C) for GC/EI-MS determination of 3-OHFAs and ergosterol. Lyophilized samples were stored at -70 °C until analysis.

Extracted samples were analyzed using the rFC endotoxin assay (Cambrex, East Rutherford, NJ). The rFC assay method for endotoxin detection uses recombinant factor C (rFC), the first component of the cascade.[37] The activation of rFC was determined by fluorescence generated by the enzymatic cleavage of a peptide-coumarin substrate. Fluorescence was measured after 1 hr incubation with endotoxin standards (*Escherichia coli* O55:B5) at 37 °C. Log fluorescence was proportional to log endotoxin concentration and was linear in the 0.01 to 10 EU/ml range.

Two-fold serial dilutions of endotoxin standards and sample extracts were prepared using sterile, pf water with Tween-20. The samples were added to a 96-well plate followed by 100 µl of a mixture of enzyme, buffer and fluorogenic substrate. The plates were incubated at 37 °C for one hour and read in a fluorescence microtiter plate reader (Biotek Instruments, Winooski, VT; FLX800TBIE) at Excitation/Emission 380/440 nm. Background (0 EU/ml) fluorescence was subtracted and log change in fluorescence plotted against log-endotoxin concentration. Endotoxin concentrations of samples were calculated according to the standard curve. Four assay reagent blank wells served as reference and control for the pf status of the reagent water, centrifuge tubes, pipette tips and microplates. Quality assurance spiking assays were performed to assess matrix interference or enhancement.

LAL assay - Iowa

Samples were extracted in sterile, pf water containing 0.05% Tween-20 for 1 hr at 22 °C with continuous shaking. Extracts were centrifuged and supernatants were transferred into pf cryotubes. Samples were then analyzed using the kinetic chromogenic LAL assay. Two-fold serial dilutions of endotoxin standards (*Escherichia coli* O111:B4) and sample extracts were prepared using sterile, pf water with Tween-20 in borosilicate glass tubes that had been heated for 4 hr at 200 °C to remove endotoxin activity. A twelve-point calibration curve and four point endotoxin determination was performed. The standard curve ranged from 0.05 to 100 EU/ml of standard endotoxin. Aliquots (100 ml) of the serial dilutions of endotoxin standards and extracts were pipetted into a pf polystyrene microplate and assayed via the addition of the LAL reagent and substrate. The absorbance in each well was measured at 405 nm every 30 sec for 90 min. Endotoxin determinations were based upon the maximum slope of the absorbance versus time plot for each well. Four assay reagent blank wells served as reference and control for the pf status of the reagent water, centrifuge tubes, pipette tips and microplates. The endotoxin value for a sample was calculated from the arithmetic mean of those dilutions that fall in the middle two-thirds of the standard curve. Quality assurance spiking assays were performed to assess matrix interference or enhancement.

GC/EI-MS determination of 3-OHFAs (chemical marker of endotoxins) - Colorado

GC/MS analysis focuses on quantification of biomarkers of endotoxins, 3-hydroxy fatty acids (3-OHFAs) in lipid A of LPS. GC/MS analysis of 3-OHFAs allows determination

of both cell-bound and non-cell-bound endotoxins.[44, 47] This method has been described in Chapter 2 in detail, but is summarized here.

Samples and external 3-OHFA standards of 8 to 18 carbon chain lengths (except C11:0, method surrogate) were digested and methylated with 0.5 ml of 3 M methanolic HCl (2.5 ml of acetyl chloride added to 11 ml of methanol) for 16 to 18 hrs at 80 °C and cooled to room temperature. Samples and standards were amended with 10 µl pentadecanol (100 µl per ml in heptane) as a keeper solvent and diluted with 1 ml deionized water for solid-phase extraction (SPE) clean up. For SPE clean up, samples were applied to a 60 mg/ 3 ml Strata-X polymeric reversed phase cartridge (Phonemenex, Torrance, CA). Cartridges were conditioned with 1 ml diethyl ether and 1 ml deionized water prior to sample loading. Following 20 min aspiration, 3-OHFA methyl esters were eluted with 2 ml diethyl ether and evaporated to dryness with a Nitrogen stream. Samples were converted to trimethylsilyl (TMS) analogs for GC/EI-MS analysis by adding 50 µl BSTFA and 5 µl pyridine and heating for 20 min at 80 °C. Derivatized samples and standards were diluted with 50 µl heptane and a 2 µl aliquot of each was analyzed by GC/EI-MS using a HP 5890 Series II Plus GC equipped with HP-5MS column (0.25 mm × 30 m, 0.25 µm film thickness, Hewlett-Packard, Palo Alto, CA) with split/splitless inlet, electronic pressure control, 7673 automatic liquid sampler, and a HP 5972 Mass Selective Detector. Selected Ion Monitoring (SIM) for individual 3-OHFA was used for endotoxins, and the concentration calculated in picomole (pmol). The M-15 ion and *m/z* 175, the acid portion of the fatty acid cleaved between C3 and C4, were monitored. The 3-OHFAs of C8:0 to C18:0 except C11:0 were processed identically. The 3-OHFA of C11:0 was added as surrogate to each sample prior to digestion and

methylation. Since agricultural dusts contain a wide range of endotoxin concentration levels, spike levels of 2, 6, 20, 100, and 500 ng of individual 3-OHFAs (in ethanol, stored at -20 °C) were used for creating the calibration curve.

GC/EI-MS determination of ergosterol (chemical marker of fungi) - Colorado

To each sample, 3 ml 10% methanolic KOH and 10 µl of 1 µg/ml D2-ergosterol in acetone was added and sealed with a threaded PTFE closure. Samples were placed in an 80 °C sand bath for 90 minutes and then cooled down to room temperature. SPE cartridges (Strata-X 60 mg / 3 ml, Phenomenex, Torrance, CA) were conditioned with 2 ml methanol, followed by 2 ml water. Sample tubes were rinsed with 1 ml water and added to the cartridge. When water was no longer dripping from the cartridge, the vacuum was increased to 20 psi and the column was aspirated for 20 minutes to ensure dryness. Samples were then eluted into a clean 10 ml conical tube with 2 ml 10% methanol in MTBE. Samples were blown dry using a stream of nitrogen on an N-Evap evaporator after 20 µl 0.1% paraffin oil in acetone was added to each sample. Standards prepared in keeper solvent were dried similarly. Samples were reconstituted in 50 µl of 1:1 hexane: BSTFA (N,O-bis-(trimethylsilyl)trifluoroacetamide) and heated at 80 °C for 30 minutes. Samples (2 µl) were injected into the GC/EI-MS (described in GC/EI-MS determination of 3-OHFAs section). The method was calibrated via isotope dilution using D2-ergosterol (provitamin D2). Standards were prepared at 4 levels in 50 µl final volume (1, 4, 10, 40 ng) with 10 ng D2-ergosterol added to each standard. Native ergosterol contributed to D2-ergosterol signal at 3.7% of the intensity of the ergosterol signal, and this was accounted for in construction of the calibration curve and during

sample analysis. Each sample set contained no more than 12 samples, including quality control, and a complete set of calibration standards was run at the beginning and at mid sequence. For every 10 unknown samples, two quality control samples were processed, introduced at digestion in methanolic KOH. These included a control with only methanolic KOH and a fortified sample containing 10 ng ergosterol and 10 ng D2-ergosterol. All standards and unknown samples also contained 10 ng D2-ergosterol added prior to methanolic KOH digestion.

Statistical analysis

Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). The UNIVARIATE procedure in SAS was used to evaluate the distributions of data. Since dust concentration, rFC results, LAL results, an rFC/LAL ratio, and GC/EI-MS results were log-normally distributed, they were natural log transformed before proceeding with analysis.

The geometric mean (GM) and geometric standard deviation (GSD) of dust concentration, endotoxin concentrations, and the rFC/LAL ratio in each agricultural environment were calculated. Analysis of variance (ANOVA) based on SAS's GLM procedure followed by Bonferroni multiple-comparison tests of means was used to test for differences by dust type, sampling location, and sampling device. Pearson correlations were calculated between dust concentration and rFC results, dust concentration and LAL results, rFC results and LAL results, and ergosterol concentration and rFC and LAL responses. Multiple regression analyses were performed to evaluate

the relationship between assays and GC/EI-MS results accounting for effects of individual 3-OHFAs for each environment and each sampling device.

Results

Of the total of 720 samples collected in this study (Table III-I), 713 samples were analyzed by the rFC assay and 689 samples were analyzed by the LAL assay (689 matched pairs). A subset of 405 samples were analyzed by GC/EI-MS for 3-OHFAs (chemical marker of endotoxins), and 194 samples were analyzed for ergosterol (chemical marker of fungi). Unanalyzed samples were eliminated due to the breakage of tubes during shipping or for samples contained insufficient amount dust for GC/EI-MS analysis.

Figure 3-1 shows the box-plot (median, 10%, and 90%) of dust and endotoxin levels and the ratio of rFC/LAL responses in five types of agricultural dusts. A significant difference among agricultural dust types was discovered ($p < 0.01$) for all variables. Among the five livestock environments, swine had the highest endotoxin levels per mg dust in both the rFC and the LAL assays, followed by chicken, turkey, and horse. The lowest endotoxin level per mg dust was found in dairy dust. A similar trend was observed for endotoxin concentrations in the air with a slight difference in rank orders between LAL and rFC results. For LAL, endotoxin concentration in chicken dust was the second highest followed by turkey dust; this order is opposite in rFC. The rFC/LAL ratio results indicate that the LAL assay provided approximately 1.3 to 1.5 times higher responses to endotoxin than the rFC assay in chicken and horse dusts; rFC

response was 1.1 to 1.4 times higher than LAL response in turkey, swine, and dairy dusts. The rank order of the rFC/LAL ratio was dairy > swine > turkey > chicken > horse.

Geographical difference was evaluated in the study. Based on ANOVA via the GLM procedure, no significant difference in the rFC/LAL ratio was found between Colorado and Iowa samples ($p = 0.13$), but there was a significant difference in dust and endotoxin concentrations between two sampling sites ($p < 0.02$). Overall, Iowa samples contained higher geometric mean endotoxin concentrations with both the LAL and the rFC assays, but the geometric mean rFC/LAL ratio was only slightly higher for Colorado samples (1.05) than for Iowa samples (0.93). Because the difference between laboratory and field samples was not statistically significant ($p = 0.14$), laboratory (samples collected in the wind tunnel and the still chamber) and field samples were combined for each agricultural dust type.

The correlations between dust and endotoxin concentrations are shown in Figure 3-2 and Table III-II. Both LAL and rFC measurements were significantly positively correlated to dust concentrations in all five environments ($p < 0.01$). The slope of the regression line between LAL and rFC measurements was 0.99 with the intercept of 0.09. Horse dust had the highest correlation between dust concentration and LAL results ($r = 0.98$), whereas chicken dust was the highest for rFC results ($r = 0.94$). Dairy dust showed the lowest correlations between dust and endotoxin exposures in LAL and rFC assay results ($r = 0.54$ and 0.55, respectively). Correlations between results from the LAL and the rFC assays were highly positive and significant ($r = 0.92$, $p < 0.01$). However, the magnitude of correlations between LAL and rFC results varied by

environment. The rank order of LAL and rFC correlations was chicken (0.96) > horse (0.92) > dairy (0.84) > turkey (0.84) > swine (0.82).

The correlations between LAL and rFC results in each sampling device were evaluated (Figure 3-3). Significant correlations were found in all devices ($r > 0.89$). In general, cyclone had the highest correlation between the LAL and the rFC assays ($r = 0.92$) while button was the lowest ($r = 0.89$). Based on ANOVA (GLM), a significance difference among sampling devices was found for the rFC/LAL ratio ($p < 0.01$). Multiple comparison test of means found significant differences between cyclone and each of the other devices ($p < 0.05$), but no differences among the other three sampling devices.

Multiple linear regression analyses using the stepwise selecting method were performed to evaluate the relationship between endotoxin assay results and GC/EI-MS accounting for effects of individual 3-OHFAs, chemical markers of endotoxins simultaneously. The results of regression analyses are shown in Table III-III. Both LAL and rFC assay results had the same combination of 3-OHFAs (C9:0, C12:0, C13:0, and C14:0), but R^2 was lower for the LAL assay (0.21) than for the rFC assay (0.45). In addition, total 3-OHFAs (sum of all 3-OHFAs) was significantly correlated with LAL and rFC responses in all environments, but correlation coefficients were consistently higher for the rFC assay (Table III-IV).

Correlations between the chemical marker of fungi (ergosterol) and rFC result, LAL result, and the rFC/LAL ratio were calculated to evaluate the magnitude of potential interference from fungi (Figure 3-4). Ergosterol was moderately correlated with both LAL and rFC results ($p < 0.01$). There was no significant correlation between ergosterol and the rFC/LAL ratio ($p = 0.25$).

Discussion

The endotoxin concentrations vary by type of agricultural dust. Both LAL and rFC results showed that the swine dust contained the highest and dairy dust contained the lowest levels of endotoxins. However, correlations between dust concentration and endotoxin concentrations were different for the LAL and the rFC assays. Although correlations were significant in all five livestock dusts, horse dust showed the highest correlation for the LAL assay, and chicken dust was the highest for the rFC assay.

High correlation between the LAL and the rFC assay results was observed in all five livestock dusts with correlation coefficients greater than 0.81. This result agreed with Alwis and Milton's study[37] conducted on house dust. Correlations were statistically significant in all sampling devices. However, there were statistically significant differences in sampling devices for the rFC/LAL ratio. Thus, differences in sampling device and aerosol size distribution may contribute to the differences in LAL and rFC responses in each environment. In addition, the difference between Colorado and Iowa samples for the rFC/LAL ratio was not statistically significant, but there was a geographical difference for dust and endotoxin concentrations. Samples collected in Iowa provided consistently higher endotoxin concentrations (both the LAL and the rFC assays) than Colorado samples. Freshness of samples or shipping time may contribute to the difference in measured endotoxin concentrations; however, since samples were shipped between both institutions, this effect would be negated.

Alwis and Milton[37] reported that the LAL assay gave higher endotoxin concentrations than the rFC assay in all of their house dust samples. They explained that

this result could be due to the differences in the reagent source and in responsiveness to endotoxins (the LAL assay used the reagent extracted from *Limulus polyphemus*, while the rFC assay was produced from the cDNA of *Cacinoscorpius rotundicauda*). Unlike house dust, LAL results were not consistently higher than rFC results in livestock dusts. The LAL assay responded more in chicken and horse dusts while rFC responses were higher in dairy, swine, and turkey dusts. Our previous study found that individual C18:0 3-OHFA was significantly positively correlated with rFC results in personal breathing zone samples collected in dairy farms and cattle feedlots (Chapter 2). In this study, we found that individual C18:0 3-OHFA was significantly positively correlated with rFC results in dairy, swine, and turkey dusts (which had higher rFC responsiveness than LAL), but only positively correlated with LAL results in dairy and not or negatively correlated in other dusts. Several studies have indicated that C18:0 3-OHFA may be derived from Actinobacteria rather than from Gram-negative bacteria.[48, 49] Our findings showed that the difference in responsiveness to endotoxins by two endotoxin assays may be explained not only by the reagent source difference in agricultural dusts, but also by the difference in dust composition and existence of Actinobacteria.

Stepwise regression models for the LAL and rFC assays contained the same combination of 3-OHFAs (C9:0, C12:0, C13:0, and C14:0) but yielded lower multiple correlation coefficients for the LAL results than the rFC results. Based on R^2 values for each model, only 21% of the variation in endotoxin measurements was explained by the combination of 3-OHFAs for the LAL assay responses while 45% of the variation in endotoxin measurement could be explained by the same combination for the rFC responses. Multiple regression models for each individual type of agricultural dust

contained different combination of 3-OHFAs for the LAL and the rFC assays, but correlation coefficients for the LAL assay was lower than the rFC assay in all dust types. The LAL assay may have higher variability than the rFC assay and may also respond to additional dust components.

The LAL assay is known to be interfered with by non-endotoxin agents, such as (1→3)-β-D-Glucans of fungi.[34-36] The LAL assay may also be interfered by polynucleotide and proteins.[40] We analyzed for ergosterol, a chemical marker of fungi, in this study. Ergosterol can be found in the membrane of most fungi; thus, it is very useful to evaluate the magnitude of interference of fungi in endotoxin measurements. The overall results showed that the rFC/LAL ratio was not significantly correlated with ergosterol. However, since turkey dusts showed a weak but statistically significant positive correlation ($r = 0.29$) and horse dust showed a moderate negative correlation ($r = -0.66$) between ergosterol and the rFC/LAL ratio, fungi could be a potential interfering factor in specific agricultural environment. Since the number of ergosterol measurements in horse dust was small, further study is needed to confirm this hypothesis.

Limitations

Due to the small sample size for LAL results in horse dust, variability of sampling devices in correlations between LAL and rFC results could not be evaluated in this study. In addition, detailed evaluation of the performance of sampling devices for endotoxin measurements was not conducted in this study but is the subject of another paper. Ergosterol measurement as a chemical marker of fungi was performed in a limited number of samples in this study due to detection limits of the GC/MS method; thus,

detailed investigation of ergosterol concentrations in each dust type was not possible. Moreover, although several studies indicated that ergosterol is likely to be a reliable indicator of fungi,[50-52] ergosterol measurement is not yet widely adopted for fungi exposure assessment in agricultural dusts. Further study is needed.

Conclusion

In general, strong positive correlations exist between the LAL and the rFC assays. However, responses to assays vary by agricultural environment or dust type. The LAL assay may overestimate (or the rFC assay may underestimate) endotoxin exposures in chicken and horse dusts, and the LAL assay may underestimate (or the rFC assay may overestimate) endotoxin concentrations in dairy, swine, and turkey dusts. Our finding showed that ergosterol concentration may not be a major factor of interference in the LAL assay overall, but the magnitude of interference may vary by dust type. Other than ergosterol contribution, this variability could be explained by differences in bacterial composition and other dust components; the rFC assay may react positively with Actinobacteria. Future studies will be expected to increase sample size for ergosterol measurements, to analyze dusts from different agricultural environments, and to investigate presence of potential interference of assays including Actinobacteria and proteins in agricultural dusts.

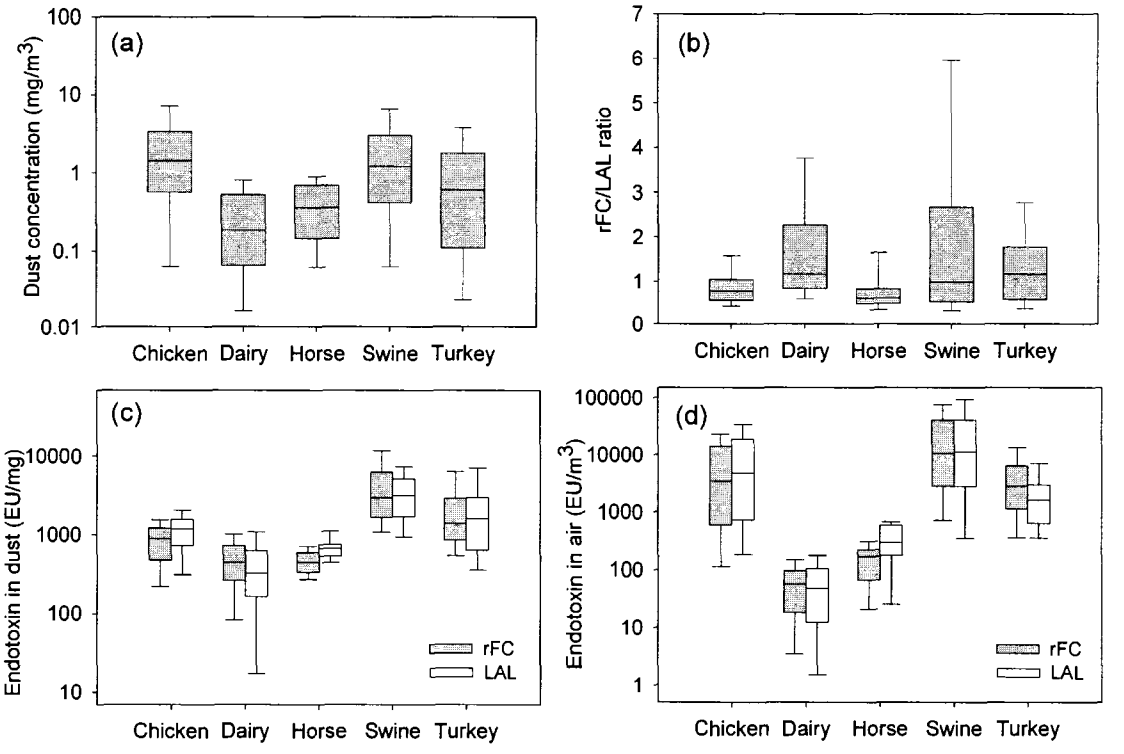

FIGURE 3-1. Box-plot graphs of (a) dust concentration (mg/m^3), (b) rFC/LAL ratio, (c) endotoxin level in dust (EU/mg), and (d) endotoxin concentration in air (EU/m^3) in each dust type. Error bars indicate 10% and 90% and lines in the box indicate median.

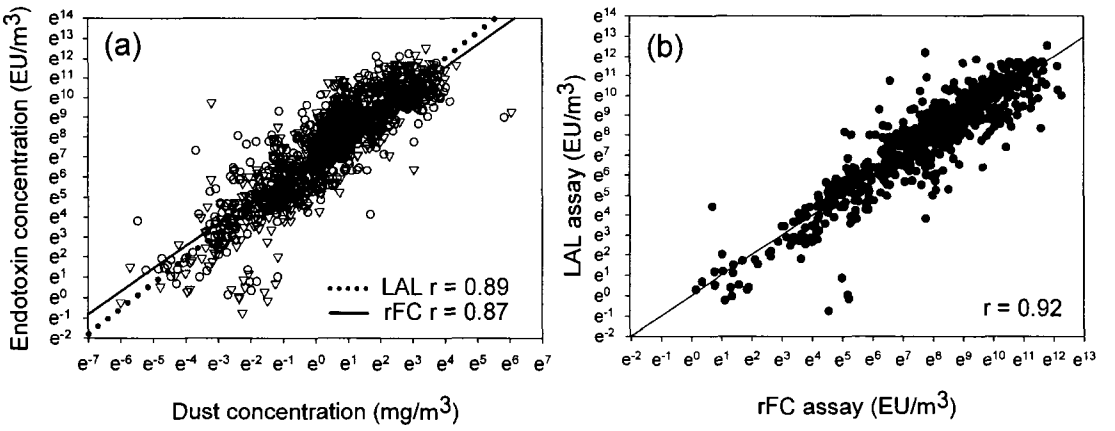

FIGURE 3-2. Correlation (a) between dust and endotoxin concentrations and (b) between the LAL and the rFC assays. Lines indicate regression lines (intercept not forced to zero).

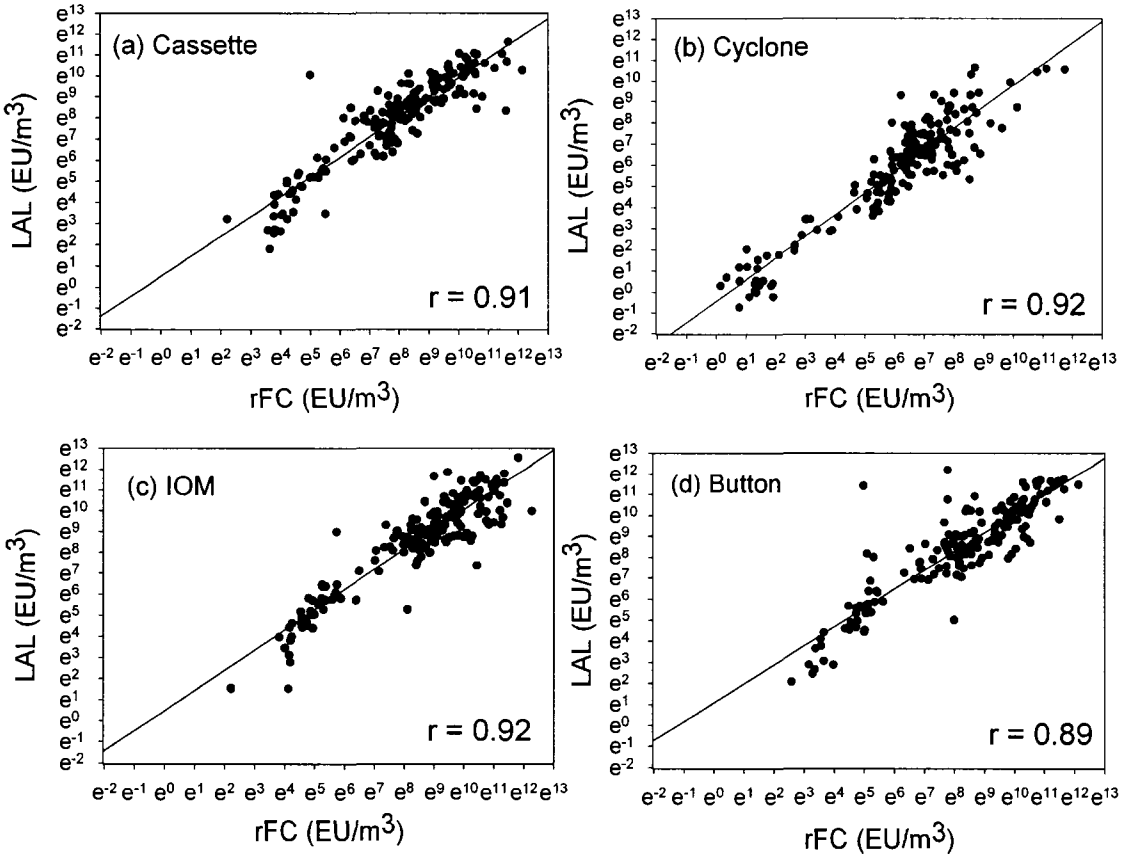

FIGURE 3-3. Correlations between the LAL and the rFC assays for each sampling device: (a) cassette (n = 182), (b) cyclone (n = 181), (c) IOM (n = 181), and (d) button (n = 177). Lines indicate regression lines.

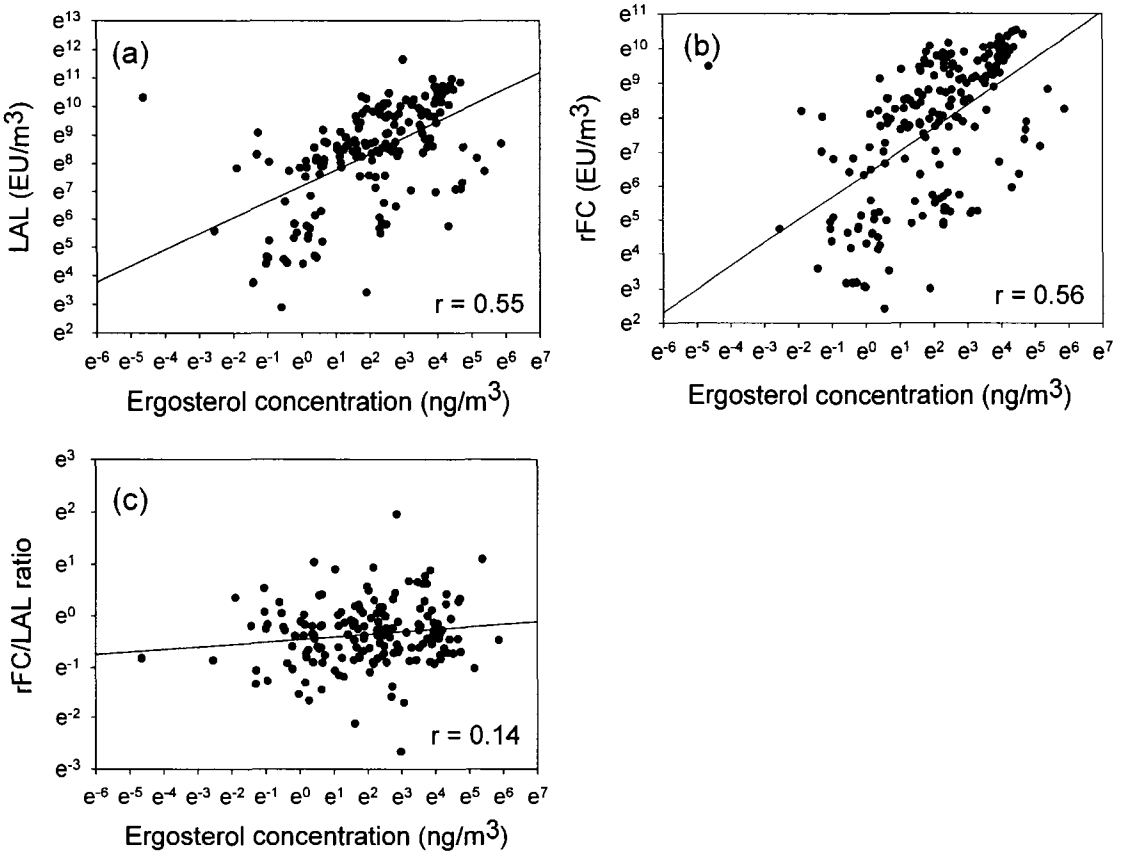

FIGURE 3-4. Correlations between ergosterol and (a) LAL, (b) rFC, and (c) rFC/LAL. Lines indicate regression lines.

TABLE III-I. Summary of sample sizes for each sample type

	Colorado		Iowa	
	Field	Wind tunnel (0.2 and 1.0 m/s)	Field	Still air chamber (0 m/s)
Chicken	40	80	40	40
Dairy	40	-	40	-
Horse	40	-	-	-
Swine	40	80	40	40
Turkey	40	80	40	40

TABLE III-II. Correlation between dust (mg/m^3) and endotoxin concentrations (EU/m^3) with p-values (bold = $p < 0.01$)

	Dust concentration vs. Endotoxin Concentration		
	rFC	LAL	rFC vs. LAL
Chicken	0.94	0.94	0.96
(n = 204/202)[A]	**<0.01**	**<0.01**	**<0.01**
Dairy	0.55	0.54	0.84
(n = 78/76)[A]	**<0.01**	**<0.01**	**<0.01**
Horse	0.93	0.98	0.92
(n = 40/15)[A]	**<0.01**	**<0.01**	**<0.01**
Swine	0.84	0.89	0.81
(n = 198/197)[A]	**<0.01**	**<0.01**	**<0.01**
Turkey	0.69	0.66	0.84
(n = 193/199)[A]	**<0.01**	**<0.01**	**<0.01**

[A] First number shows rFC sample size and second number shows LAL sample size

TABLE III-III. Stepwise forward multiple linear regression analyses for 3-OHFAs and each endotoxin assay

		n	3-OHFA combination [A]	R^2	P-value
rFC	Overall	405	13,9,14,12	0.45	<0.01
	Chicken	148	13,8,9,14,12	0.46	<0.01
	Dairy	35	17,14,9	0.68	<0.01
	Horse	37	None	-	-
	Swine	35	13,12,8	0.46	0.02
	Turkey	150	13,15,14,18	0.46	<0.01
LAL	Overall	376	13,12,9,14	0.21	<0.01
	Chicken	145	15,8,13	0.25	<0.01
	Dairy	33	15	0.28	<0.01
	Horse	14	None	-	-
	Swine	35	None	-	-
	Turkey	149	15,13,12	0.19	<0.01

[A] In order of entrance to the model

TABLE III-IV. Pearson correlations of total 3-OHFAs (pmol/m^3) with each endotoxin assay (EU/m^3) with p-values (bold = $p < 0.05$)

	Total 3-OHFAs and rFC	Total 3-OHFAs and LAL
Overall	0.86	0.80
(n = 405/376)[A]	**<0.01**	**<0.01**
Chicken	0.93	0.90
(n = 148/145)[A]	**<0.01**	**<0.01**
Dairy	0.39	0.33
(n = 35/33)[A]	**0.02**	0.05
Horse	0.79	0.74
(n = 37/14)[A]	**<0.01**	**<0.01**
Swine	0.75	0.72
(n = 35/35)[A]	**<0.01**	**<0.01**
Turkey	0.36	0.18
(n = 150/149)[A]	**<0.01**	**0.02**

[A] First number shows n for rFC and second number shows n for LAL

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CHAPTER 4

DETERMINANTS OF PERSONAL EXPOSURES TO DUST AND ENDOTOXIN IN FOUR AGRICULTURAL ENVIRONMENTS

Abstract

Organic dusts, especially endotoxin components, play an important role in occupational lung disease among agricultural workers. The goals of this study were to characterize operational tasks and to estimate their contribution to personal dust and endotoxin exposures in four agricultural environments: dairy, cattle feedlot, grain elevator, and corn farm. Work shift personal breathing zone samples were collected from 145 agricultural workers in Colorado and Nebraska using IOM inhalable samplers. The samples were analyzed for endotoxin using recombinant Factor C (rFC) assay and Gas Chromatography/ Mass Spectrometry (GC/MS). Geometric mean dust levels were highest among grain elevator operators (4.50 mg/m^3) and lowest among farm workers (2.49 mg/m^3), whereas geometric mean endotoxin exposure level was highest among feedlot workers (1,093 EU/m^3 for rFC). As confirmed via analysis of variance (ANOVA), dust and endotoxin exposures differed by agricultural environment ($p < 0.05$). Multiple regression analyses were applied to identify the contribution of each task in dust and endotoxin exposures. Hours at running legs in grain elevators was the major determinant of dust concentration followed by hours at housekeeping in grain elevator

operations and hours at feeding livestock in cattle feedlots. Hours at running legs in grain elevator and hours at feeding livestock in cattle feedlots were two major determinants in endotoxin measurements, followed by hours at feed storage in grain elevator operation and hours at loading and unloading feed truck in cattle feedlots. In general, a high percentage of dust and endotoxin measurements exceeded suggested occupational health guidelines. The characterization of dust and endotoxin exposure determinants is important for developing and prioritizing exposure control strategies and for the prevention of organic dust lung disease among agricultural workers.

Introduction

Agricultural organic dusts contain complex mixtures of chemical and biological agents including fecal components, urine, bacterial endotoxin, and glucan.[1] Organic dusts, especially endotoxin components, play a key role in occupational lung disease. Organic dust and endotoxin exposures are especially concerning among agricultural workers due to relatively high exposure levels of dust and endotoxin. Several studies have indicated that exposure to endotoxins in agricultural dusts, including livestock and grain dusts, is clearly associated with asthma, chronic bronchitis, hypersensitivity pneumonitis, chronic obstructive pulmonary disease and decrease in pulmonary function.[2-9] Recent studies have shown that timing of exposure to endotoxin is also important and that exposure in infancy may also be protective against asthma and atopic sensitization.[10-16]

Occupational exposure limits of 2.4 mg/m^3 for total dust have been suggested based on epidemiological studies of swine and poultry workers.[2, 3, 17, 18] Several studies have also attempted to establish dose-response relationships between endotoxins and

respiratory diseases,[2, 5, 8, 19, 20] but a universal dose-response relationship has not been developed since some studies have reported high exposures without symptoms[21] or low exposure with a possible dose-response relationship.[22] Development of an accurate exposure assessment method is critical for understanding the relationship between endotoxin exposures and development of diseases, such as chronic bronchitis and asthma. However, exposure assessment in agricultural settings is relatively difficult since a number of factors may influence exposure levels and constituents of exposure.

One of the important factors is agricultural task. A wide variety of operational tasks can be found in agricultural environments, and dust and endotoxin exposure levels may vary by working pattern. Commonly observed agricultural tasks include harvesting, feeding, and maintenance, and tasks vary by type of agriculture.[23] Several studies have been conducted to evaluate task-specific dust or endotoxin exposures in agricultural environments;[6, 23-28] however, few studies have used the empirical modeling (regression) technique.[24, 26, 27] Empirical modeling is especially useful for estimating the magnitude of influence of each factor to increase or decrease exposure levels. The identification of factors associated with dust and endotoxin exposures is critical for establishing appropriate occupational guidelines and controls.

As part of a larger study of agricultural exposures and respiratory diseases, the goals of this study were to characterize agricultural tasks and to estimate their contributions to personal dust and endotoxin exposures in four agricultural environments: dairy, cattle feedlot, grain elevator, and corn farm. In this study, empirical modeling was applied to evaluate determinants of exposures.

Method

Sample collection

A total of 145 personal breathing zone samples using IOM inhalable samplers were collected in four agricultural environments in Colorado and Nebraska: dairy farms (n = 21), cattle feedlots (n = 55), grain elevators (n = 58), and corn farm (n = 11). Prior to the study, lists of owners and operators of agricultural facilities were obtained from producer organizations. Once owners agreed to participate in the study, workers were recruited at each facility. The overall response and participation rate was approximately 50%. IOM inhalable samplers used 5 μm pore size polyvinyl chloride (PVC) filters (SKC Inc., Eighty Four, PA) at a flow rate of 2 l/min over 6 to 8 hrs during typical work shifts. Pre- and post-shift surveys were conducted on each worker in English or Spanish. The pre-shift survey included questions on the worker's demographic data and working experience (Table IV-I), and the post-shift survey included hours spent in each task during the sampling shift (Table IV-II for description of each task). Samples were collected in 2004 – 2006 during all four seasons. Morning (6 – 9 am) and late afternoon (2 – 5 pm) ambient temperature and humidity were measured in selected facilities (58% of all samples). This study was approved by the Colorado State University's and the University of Nebraska Medical School's institutional review boards for human subject protection.

Sample preparation

Collected dust samples were placed in desiccators for 24 hrs before weighing using a 6-place balance (Model MT5, Mettler-Toledo Inc., Columbus, OH). Filters were then

extracted in sterile, pyrogen-free (pf) water containing 0.05 % Tween-20 for 1 hr at room temperature, 22 °C, with continuous shaking for endotoxin analysis. A portion of each extract was analyzed by recombinant Factor C (rFC) endotoxin assay and another portion was lyophilized (at -50 °C) for gas chromatography/ mass spectrometry (GC/EI-MS) determination of chemical markers of endotoxin (3-OHFAs). Lyophilized samples were stored at -70 °C until analysis. Field and laboratory blanks were handled in the same manner.

rFC assay for endotoxin measurement

The rFC assay method for endotoxin detection uses recombinant factor C (rFC), the first component of the cascade.[29] The activation of rFC was determined by fluorescence generated by the enzymatic cleavage of a peptide-coumarin substrate. Fluorescence was measured after 1 hr incubation with endotoxin standard (*Escherichia coli* O55:B5) at 37 °C. Log-fluorescence was proportional to log-endotoxin concentration and was linear in the 0.01 to 10 EU/ml range. Two-fold serial dilutions of endotoxin standards and sample extracts were prepared using sterile, pf water with Tween-20. The samples were added to a 96-well plate followed by 100 µl of a mixture of enzyme, buffer and fluorogenic substrate. The plates were incubated at 37 °C for one hour and read in a fluorescence microtiter plate reader (Biotek Instruments, Winooski, VT; FLX800TBIE) at Excitation/Emission 380/440 nm. Background (0 EU/ml) fluorescence was subtracted and log change in fluorescence plotted against log endotoxin concentration. Endotoxin concentrations of samples were calculated according to the standard curve. Four assay reagent blank wells served as reference and control for the pf status of the reagent water,

centrifuge tubes, pipette tips and microplates. Quality assurance spiking assays were performed to assess matrix interference or enhancement.

GC/EI-MS determination of 3-OHFAs (chemical marker of endotoxins)

GC/MS analysis focuses on quantification of biomarkers of endotoxins, 3-hydroxy fatty acids (3-OHFAs) in lipid A of LPS. This method has been described in detail previously (Chapter 2), but is summarized here.

Samples and external 3-OHFA standards of 8 to 18 carbon chain lengths (except C11:0, method surrogate) were digested and methylated with 0.5 ml of 3 M methanolic HCl (2.5 ml of acetyl chloride added to 11 ml of methanol) for 16 to 18 hrs at 80 °C and cooled to room temperature. Samples and standards were amended with 10 µl pentadecanol (100 µl per ml in heptane) as a keeper solvent and diluted with 1 ml deionized water for solid-phase extraction (SPE) clean up. For SPE clean up, samples were applied to a 60 mg/ 3 ml Strata-X polymeric reversed phase cartridge (Phonemenex, Torrance, CA). Cartridges were conditioned with 1 ml diethyl ether and 1 ml deionized water prior to sample loading. Following 20 min aspiration, 3-OHFA methyl esters were eluted with 2 ml diethyl ether and evaporated to dryness with a Nitrogen stream. Samples were converted to trimethylsilyl (TMS) analogs for GC/EI-MS analysis by adding 50 µl BSTFA and 5 µl pyridine and heating for 20 min at 80 °C. Derivatized samples and standards were diluted with 50 µl heptane and a 2 µl aliquot of each was analyzed by GC/EI-MS using a HP 5890 Series II Plus GC equipped with an HP-5MS column (0.25 mm × 30 m, 0.25 µm film thickness, Hewlett-Packard, Palo Alto, CA) with split/splitless inlet, electronic pressure control, 7673 automatic liquid sampler, and a HP

5972 Mass Selective Detector. Selected Ion Monitoring (SIM) for individual 3-OHFA was used for endotoxins, and the concentration calculated in picomole (pmol). The M-15 ion and *m/z* 175, the acid portion of the fatty acid cleaved between C3 and C4, were monitored. The 3-OHFAs of C8:0 to C18:0 except C11:0 were processed identically. The 3-OHFA of C11:0 was added as surrogate to each sample prior to digestion and methylation. Since agricultural dusts contain a wide range of endotoxin concentration levels, spike levels of 2, 6, 20, 100, and 500 ng of individual 3-OHFAs (in ethanol, stored at -20 °C) were used to create the calibration curve.

Statistical analysis

Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). The UNIVARIATE procedure in SAS was used to evaluate the distributions of data. Since dust concentration, rFC results, GC/EI-MS results, and years in job were log-normally distributed (based on the results of the Box-Cox test and histograms of the data), these variables were log-transformed (specifically, the natural logarithm) before proceeding with analysis.

Demographic differences in each agricultural environment were evaluated via χ^2 analysis, which was also used to test differences in the number of single and multi-task workers among agricultural environments. The geometric mean (GM) and geometric standard deviation (GSD) of dust and endotoxin measurements in each agricultural environment were calculated. The average hours at each task among workers who performed the task were also evaluated. Pearson correlations were calculated between dust concentration, rFC result, GC/EI-MS result, number of years in job, and hours at

each task. Ambient air temperatures and humidity were categorized into four levels by quartiles (< 25%, 25 – 50%, 50 – 75%, and > 75%). Analysis of variance based on the GLM procedure in SAS, followed by Bonferroni multiple-comparison tests of means, was used to test for differences by agricultural environment, education level, morning and afternoon ambient air temperatures, and humidity.

Since many agricultural workers performed multi-task activities and it was not practical to monitor each worker's exposure in each task for the entire workshift, creating the empirical models were needed to predict the workers' exposures by task. The empirical models were constructed for dust concentration, rFC result, and GC/EI-MS result using multiple linear regression analysis. Factors evaluated in the regression models were agricultural environment (1/0 yes/no variable for each environment) and hours at each task (14 tasks); cross-products of these variables were used because each agricultural environment had different combinations of tasks. In addition, regression analyses were also performed only for tasks without environment factors. Multiple regression analyses based on stepwise selection were also performed to find the major contributor (factor) of dust and endotoxin exposures. The general equation of the empirical model in shown below as:

$$\text{Ln(dependent variable)} = \beta_1 X_1 + \ldots + \beta_k X_k \qquad (1)$$

In the equation, β_j represents the regression coefficient and X_j represents the corresponding independent variable measurements. The 8-hour exposure level for each task in each environment was predicted using the regression models; predicted values and actual values of single-task workers were compared. Dust (mg/m^3), endotoxin (EU/m^3), and 3-OHFA ($pmol/m^3$) concentrations were adjusted to 8-hr time weighted average

(TWA) for the GM and GSD calculations, but original, non-TWA concentrations were used in regression models.

Results

The demographic data of participating workers are summarized in Table IV-I. Proportions of male and female workers were not significantly different among the four agricultural environments; more than 90% of workers were male in all environments. The majority of workers was Hispanic or Latino in dairy farms, which was opposite to other environments. A higher percent of workers had higher education (college or above) in cattle feedlot (61%) and farm environments (45%) compared to dairy farms (15%) and grain elevators (27%). In general, a very small proportion of workers used respirators; grain elevator workers had the highest proportion of respirator use (25%), and the lowest proportion was found in dairy farm workers (0%). Ethnicity, job status, education, and respirator use were statistically different among agricultural environments ($p < 0.05$).

Figure 4-1 shows GM and GSD of dust and endotoxin concentrations for the four agricultural environments. A significant difference was found for dust concentration ($p = 0.03$) and endotoxin concentration per mg dust ($p < 0.01$) among the agricultural environments and specifically between cattle feedlots and grain elevators ($p < 0.05$). Among the four agricultural environments, grain elevators had the highest geometric mean dust concentration (4.50 mg/m^3), followed by dairies (3.02 mg/m^3), cattle feedlots (2.54 mg/m^3), and farms (2.49 mg/m^3). Endotoxin concentrations were the highest among cattle feedlot workers (450 EU/mg and 1,093 EU/m^3). Farms contained the lowest mean endotoxin concentration per m^3 air (447 EU/m^3) while grain elevators had

the lowest endotoxin concentration per mg dust (143 EU/mg). Total 3-OHFAs (sum of C8:0, C9:0, C10:0, C12:0, C13:0, C14:0, C15:0, C17:0, and C18:0 3-OHFAs) concentration per m^3 air was the highest in farm (2,266 pmol/m^3) and the lowest among grain elevator operators (1,345 pmol/m^3), although the difference among agricultural environments for total 3-OHFAs concentrations was not statistically significant based on ANOVA ($p = 0.31$).

The frequencies of self-reported tasks in each agricultural environment are summarized in Figure 4-2. Each worker could choose one or more answers. In livestock environments (dairy and cattle feedlots), the largest number of workers identified feeding livestock, followed by loading and unloading feed and mechanical maintenance for their typical jobs. Running legs in the grain elevators was the most common task among grain elevator operators, and harvest activity and mechanical maintenance were the highest in farm workers. The average number of hours spent in each task among workers who performed the task is presented in Table IV-III by agricultural environments, and the numbers of single- and multi-task workers in each environment are summarized in Table IV-IV. Workers spent approximately 1 to 4 hours in a single task and moved to other tasks. Our study found 82 different combinations of tasks overall (10 combinations in dairy, 28 combinations in cattle feedlots, 46 combinations in grain elevator and 6 combinations in farm). The percentage of single- and multi-task (more than or equal to 2 tasks) workers varied by environment (p-value < 0.01). Farm and dairy workers had the lowest percentage of multi-task workers (20% and 33%, respectively) while high percentages of workers performed multi-tasks in cattle feedlot (75%) and grain elevator (79%).

Pearson correlations between dust and endotoxin concentrations are presented in Figure 4-3. Significant but moderate correlations in overall samples (combined for all environments) were found between dust and endotoxin concentrations ($r > 0.50$). The correlation coefficients were positive for endotoxin and 3-OHFAs concentration in air both overall and in each agricultural environment ($p < 0.05$) except for the correlation between dust and 3-OHFAs levels among farm workers ($p = 0.34$). The correlation between dust and endotoxin concentrations was the highest among grain elevator operators ($r = 0.80$) and the lowest in cattle feedlots ($r = 0.37$). The correlation between dust and 3-OHFA concentration was the highest in grain elevators ($r = 0.74$) and the lowest among farm workers ($r = 0.42$).

As noted preciously, ANOVA based on SAS's GLM procedure was performed to evaluate differences in dust and endotoxin concentrations due to difference in ambient air temperatures and humidity. Each morning temperature, afternoon temperature, morning humidity, and afternoon humidity were categorized by quartile. Endotoxin concentration was found to differ between the lowest and highest afternoon temperatures ($p = 0.01$), but no significant difference in endotoxin concentrations was found among the morning temperatures. Dust and 3-OHFA exposures were not affected by temperature differences, but afternoon temperature was weakly positively correlated with overall 3-OHFA concentration ($r = 0.22$, $p = 0.03$). Endotoxin concentrations varied with respect to morning humidity but not significantly ($p = 0.06$); endotoxin exposures vary significantly with respect to afternoon humidity ($p = 0.05$). Dust concentration also varied significantly with respect to afternoon humidity ($p < 0.01$) and was weakly positively correlated with afternoon humidity ($r = 0.23$, $p = 0.03$).

Multiple linear regression models were constructed for dust concentration, endotoxin concentration per m^3 air, and total 3-OHFA level per m^3 air. Task combinations were different in each agricultural environment, and several tasks, such as running legs in grain elevators, were not observed in some environments. Thus, the cross-products of agricultural environment (yes/no 1/0 variables for each environment) and hours at each task were used in each regression model. In all, 41 cross-product variables were included, and 8-hour predicted values were calculated using the regression models for dust exposure (Table IV-V), endotoxin exposure (Table IV-VI), and 3-OHFA concentration (Table IV-VII). The 8-hour predicted dust exposure was found to be the highest in working at storage in farms (114.80 mg/m^3), followed by housekeeping and cleaning in grain elevator environment (110.81 mg/m^3). The highest 8-hour predicted exposures to endotoxin was found in combine harvesting in dairies (3.35×10^{14} EU/m^3), followed by combine harvesting in cattle feedlots (7.47×10^{14} EU/m^3). Weighing grain or feed in cattle feedlots had the highest 8-hour predicted value for 3-OHFA concentration (3.75×10^{19} $pmol/m^3$) while the second highest was in combine harvesting in dairies (1.90×10^{12} $pmol/m^3$). It is important to note that the actual average hours spent at these high 8-hour predicted exposure tasks were 1 to 3 hours, and no worker had performed 8-hour work at these tasks. The lowest 8-hour predicted level was observed for sampling grain or feed in dairy for all dust, endotoxin, and 3-OHFA concentrations (0.01 mg/m^3, < 0.01 EU/m^3, and < 0.01 $pmol/m^3$, respectively). The rank order of 8-hour predicted values calculated based on the regression models only containing tasks (ignoring environment factors) are summarized in Table IV-VIII. As seen from Table IV-VIII, the rank orders using the task-only regression models, ignoring environment

factors, were different from the rank orders using the regression models with cross-products of environment and task.

Multiple linear regression analysis, based on stepwise selection ($\alpha = 0.05$), was performed to identify the major contributing factors in dust, endotoxin, and 3-OHFA exposures. Variables included in the models were labeled in Table IV-V, Table IV-VI, and Table IV-VIII. Based on the stepwise regression, hours at running legs in grain elevators accounted for the most variability (partial $R^2 = 0.21$) in dust exposures, followed by hours at housekeeping and cleaning in grain elevator operations (partial $R^2 = 0.07$) and hours at feeding livestock in cattle feedlots (partial $R^2 = 0.04$). Similar to dust exposure, hours at running legs in grain elevators was the major contributor in the model for endotoxin exposure (partial $R^2 = 0.15$), as well as hours at feeding livestock in cattle feedlots (partial $R^2 = 0.14$). In addition, hours working in feed storage in grain elevator environments (partial $R^2 = 0.05$) and hours at loading and unloading feed truck in cattle feedlots (partial $R^2 = 0.04$) were major factors in endotoxin exposure. For 3-OHFA level, hours at feeding livestock in cattle feedlots (partial $R^2 = 0.15$) and hours at running legs in grain elevators (partial $R^2 = 0.14$) contributed the most, followed by hours at loading and unloading feed trucks in cattle feedlots (partial $R^2 = 0.05$), hours working in feed storage in grain elevator environments (partial $R^2 = 0.05$), and hours at loading and unloading feed trucks in grain elevator environments (partial $R^2 = 0.04$).

Comparisons between actual and predicted values for single-task workers (7 to 10 hours) are summarized in Table IV-IX. No workers performed full shift works at working in feed storage, sampling grain or seed, weighing grain or seed, running legs in grain elevators, milling, and housekeeping and cleaning. The percent difference between

actual and predicted values varied by task. Truck harvest, combine harvest, and mixing had the best prediction for all dust, endotoxin, and 3-OHFA exposures. Good prediction was observed for feeding livestock in dust and 3-OHFA exposures (< 5% difference) and for loading and unloading feed truck in dust and endotoxin exposures (< 20% difference). A large difference between actual and predicted values was observed in mechanical maintenance and supervising.

Pearson correlations were computed between the numbers of years in job, hours at each task, and dust, endotoxin, and 3-OHFA concentrations (Table IV-X). Significant negative but weak correlations were found between hours at supervising and dust, endotoxin, and 3-OHFA concentrations ($r = -0.27$, -0.23, and -0.18, respectively, with $p < 0.05$). The number of years in job was also negatively correlated with dust and endotoxin concentrations ($r = -0.18$ and -0.19, respectively, with $p < 0.05$). However, there were no statistically significant correlations between the numbers of years in job and any other tasks. Moreover, no strong correlations were observed among tasks. Similar to job experience, educational level may also contribute to the difference in exposures. Thus, ANOVA was performed to evaluate differences in dust and endotoxin concentrations due to difference in educational levels. There was a significant difference in dust and endotoxin exposures between high school or lower education and college or above education levels ($p < 0.01$).

Discussion

Although the dust and endotoxin concentrations vary by agricultural environment, agricultural workers are exposed to high concentrations of dust in all four agricultural

environments. The American Conference of Governmental Industrial Hygienists (ACGIH®) set the recommended Threshold Limit Value (TLV®) for total inhalable dust (10 mg/m^3) and grain dust (4.0 mg/m^3).[30, 31] Geometric mean dust concentrations did not exceed the total inhalable dust TLV, but the geometric mean for grain elevator exceeded the ACGIH TLV of 4.0 mg/m^3 for grain dust.[30] The inhalable TLV does not account for the biological activity of these agricultural dusts. The 2.4 mg/m^3 for total dust was recommended for agricultural environments based on several exposure-response studies to prevent work-related health effects in swine and poultry production facilities.[2, 3, 17, 18] This recommended value was based on measurements with the 37-mm cassette total dust sampler; thus, we cannot compare our results collected by IOM samplers with this value directly. Our previous study[32] recommended to increase this value by a factor of 2 to produce an occupational exposure limit of 4.8 mg/m^3 for IOM samplers. In general, high dust concentrations were observed in all tasks. Based on the results, 16% of workers exceeded 10 mg/m^3, 37% exceeded 4.0 mg/m^3, and 33% exceeded 4.8 mg/m^3 values. The Dutch Expert Committee on Occupational Standards has recommended the limit of 50 EU/m^3 for 8-hour TWA personal inhalable endotoxin exposure.[33] Geometric mean 8-hr TWA endotoxin concentrations in this study exceeded seven times the limit in all agricultural environments and in all tasks. Among all samples, 92% of workers exceeded the 50 EU/m^3 recommended exposure limit. Previous epidemiological studies suggested occupational limit values of 614 EU/m^3 for poultry and 900 EU/m^3 for swine environments (both for total endotoxin);[2, 3, 18] although neither of these environments was studied in this present paper, 59% of workers exceeded 614 EU/m^3 and 52% of workers exceeded 900 EU/m^3.

Workers performed different tasks in each agricultural environment and a large number of workers performed multi-task works. The largest number of workers reported feeding livestock and loading and unloading feed tasks in livestock farms whereas the largest number of responses was found at running legs in elevator and housekeeping among grain elevator operators. Predicted dust and endotoxin exposures varied among tasks. The source of dust or dust composition may be different among agricultural environments. Bacterial composition in livestock fecal matter and grain or plant dust may cause the difference in endotoxin levels in dust.

There was a significant difference in endotoxin concentration between the lowest and the highest afternoon outside temperature. This may be related to more bacteria growth at higher temperature environments. The afternoon humidity also significantly positively correlated with dust concentrations ($r = 0.22$). This is opposite from the finding of Niewenhuijsen and Schenker.[26] This difference could be explained by the geographical difference. We observed relatively low mean humidity (25%) with only 5% of all measurements exceeding 50% humidity, whereas Niewenhuijsen and Schenker reported mean humidity of 41 – 45% in their study.[26] Thus, the influence of humidity may differ between dry and wet seasons or environments. In addition, only outside temperature and humidity were used in this study. Therefore, these measurements may not represent the working condition indoors.

Based on the empirical models, dust and endotoxin exposures were associated with the type of agricultural environment and hours at each task. Running legs in grain elevators (in grain elevator environments) contributed the most to dust and endotoxin exposures. Running legs was also reported in cattle feedlots where grain elevators were

used to store feed for livestock. The 8-hour predicted dust exposure for running legs in grain elevators was approximately 11 times higher in grain elevator operations than in cattle feedlots; however, the 8-hour predicted value of endotoxin exposure was approximately 3 times higher in cattle feedlots than in grain elevator operations. Thus, it is important to realize that high dust exposures do not necessarily mean high endotoxin exposures. Based on stepwise regression analyses, although running legs in grain elevators among grain elevator operators was included in all three models as one of the major contributors, different combinations of agricultural environment and task were included in dust, endotoxin, and 3-OHFA exposure models. The difference between dust and endotoxin exposures may be explained by the difference in dust composition. The rank orders of 8-hour predicted values the regression models, ignoring agricultural environments, were different from the rank orders of 8-hour predicted values using the regression models with cross-products of environment and task. Thus, ignoring agricultural environments misleads the contributions of tasks to dust and endotoxin exposures.

A large difference between actual and predicted values was observed in mechanical maintenance and supervising. A large variability of works in mechanical maintenance and supervising job may be present and dust and endotoxin exposures may vary by each day. For example, many different types of equipments were used in agricultural environments, from combine trucks to grain elevators, and mechanical maintenance workers may work in multiple locations within the facility. Similar to mechanical maintenance, workers may supervise other workers in the field, but also from a distance.

Hours supervising and years in the job negatively correlated with dust and endotoxin concentrations. The more experience a worker had and the more supervising or management they did, the lower dust and endotoxin exposures they had. In addition to the job experience, there was a significant difference in dust and endotoxin exposures between high school or lower education and college or above education levels ($p < 0.01$). Lower dust and endotoxin exposures were found more among highly educated (college or above) workers than among lower educated (high school or below) workers. Similar to the job experience, workers with higher education do cleaner jobs, such as supervising.

Based on our results, dust and endotoxin controls are very important in agricultural environments and operations; controlling major determinant tasks, working in feed storage and running legs in grain elevators may greatly reduce overall dust and endotoxin exposures. Dust and endotoxin exposures in these areas may be reduced by installing proper ventilation systems with filter bags or dust collectors. However, such engineering controls are relatively expensive.

Although not the preferred control strategy by industrial hygienists, use of personal protective equipment may be one of the easiest and least expensive control methods to reduce personal dust and endotoxin exposures in agricultural operations. Even though a higher percentage of workers used any type of respirators in grain elevators (25%) compared to livestock environments (< 2%), use of respirators was still low in all agricultural environments probably due to the lack of recognition of potential health effects and a low comfort level in the use of respirators. Thus, education on dust and endotoxin exposures and their potential health effects and training on respirator use in agricultural operations is very important.

Limitations

Although there was only limited data available, we found that ambient temperature and humidity may affect dust and endotoxin exposures. Stepwise regression analyses found that both temperature and humidity were not statistically significant in all models, but since including temperature and humidity data reduced the total sample size in the models, further investigation with larger sample size is recommended to investigate this finding. Temperature and humidity differences may relate to seasonal differences; however, due to the non-uniformly distributed sample size in four seasons and the small sample size in one or more seasons, seasonal variability could not be evaluated in this study. Agricultural tasks may be different in each season in each agricultural environment. In addition, geographical differences between Colorado and Nebraska could cause differences in bacterial distribution, dust composition, or agricultural operations. However, not enough samples were collected in Nebraska for geographical comparison in this study.

Conclusion

In this study, dust and endotoxin exposures were evaluated for each agricultural environment and for each agricultural task. In addition, determinants of personal dust and endotoxin exposures were identified in four agricultural environments: dairy, cattle feedlot, grain elevator, and farm. A high proportion of workers had exposures to dust and endotoxin exceeding the recommended occupational guidelines. There is a need to improve control methods to reduce exposures. However, exposure assessment and

control are very difficult in agricultural environments since agricultural operations are a combination of multiple short-time tasks in a variety of locations and seasons. In the same agricultural facility, each worker may have a different combination of tasks. The multiple linear regression models (empirical models) were successfully applied for dust and endotoxin exposures in agricultural environments. The characterization of dust and endotoxin exposure determinants is important for developing and prioritizing exposure control strategies and for the prevention of organic dust lung disease among agricultural workers. Overall, workers weighing grain or feed, housekeeping, and working in feed storage have high dust and endotoxin exposure levels. Results suggest that dust exposure control is especially important in grain elevator environments, particularly during work in running legs in grain elevators and in housekeeping. Working in feed storage in grain elevator environment, running legs in grain elevator, and feeding livestock in cattle feedlots should have a high priority for control strategies to reduce endotoxin exposure. Future studies should analyze additional determinants, including dust and bacterial composition in various agricultural environments, to further evaluate the effects of seasonal and geographic variation, and to conduct task-specific monitoring and compare results with the statistical empirical models in detail.

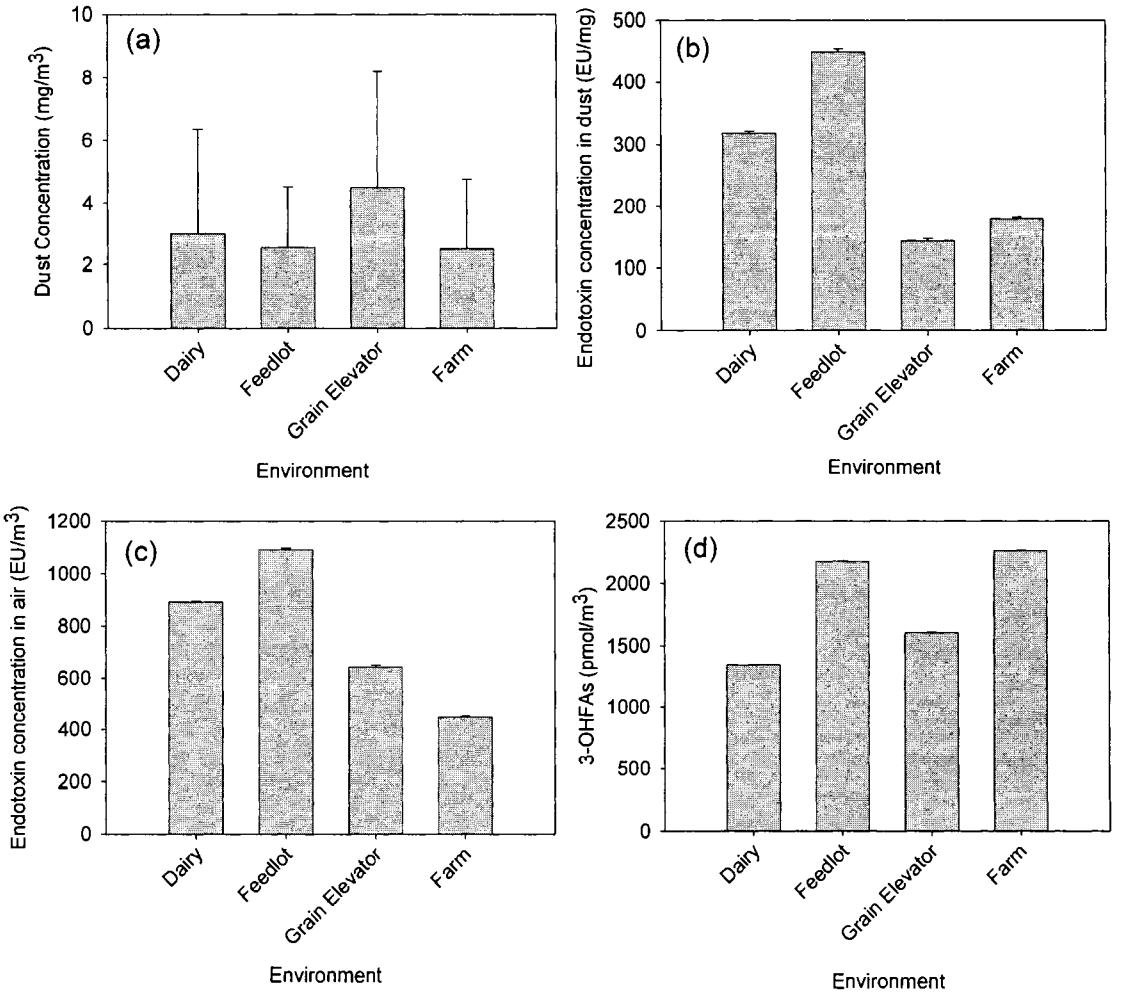

FIGURE 4-1. GM with error bar (GSD) of (a) dust concentration, (b) endotoxin concentration per mg dust, (c) endotoxin concentration per m^3 air, and (d) GC/EI-MS endotoxin result (total 3-OHFAs) per m^3 air.

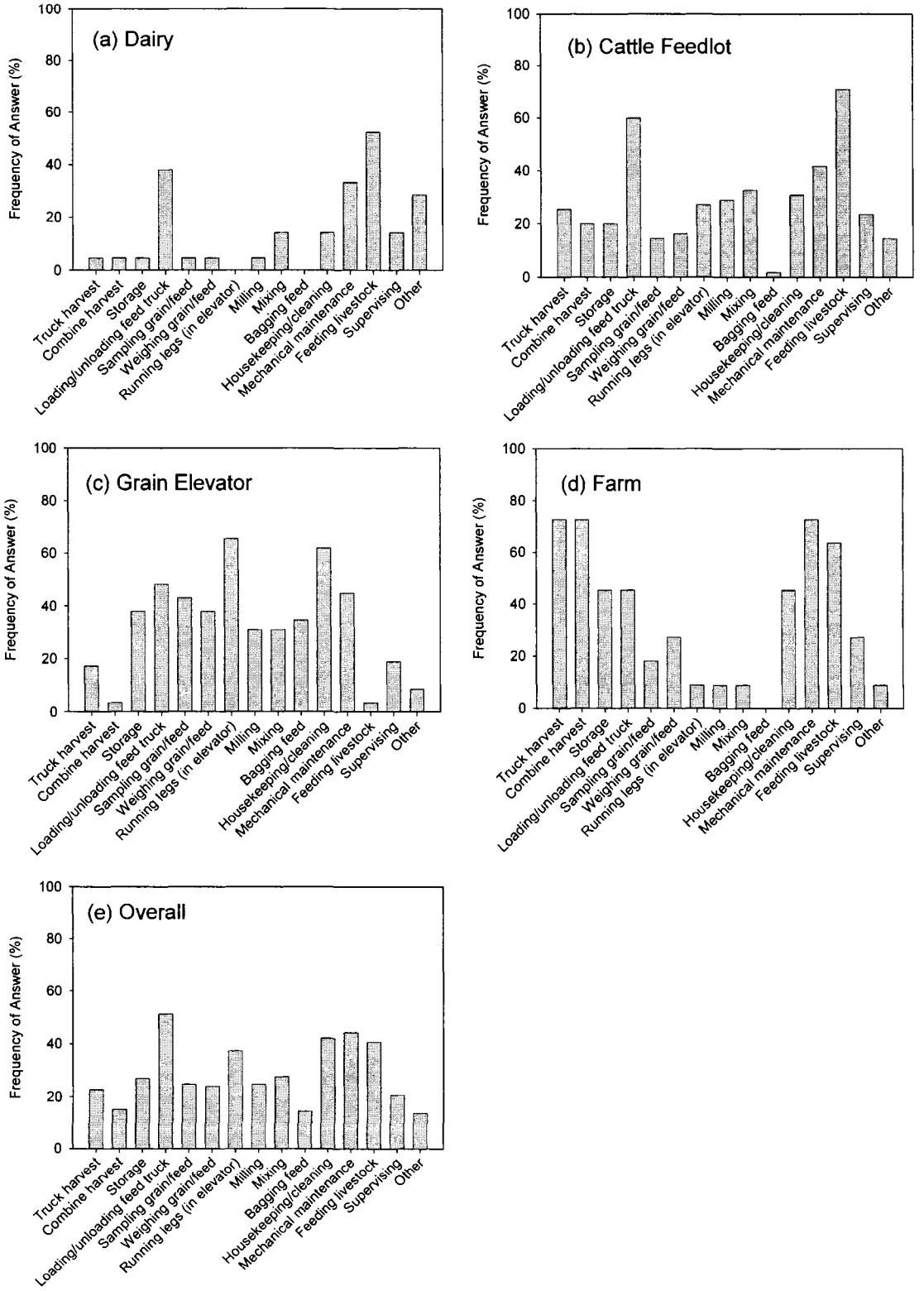

FIGURE 4-2. Frequency of self-reported tasks in each agricultural environment (a-d) and overall (e).

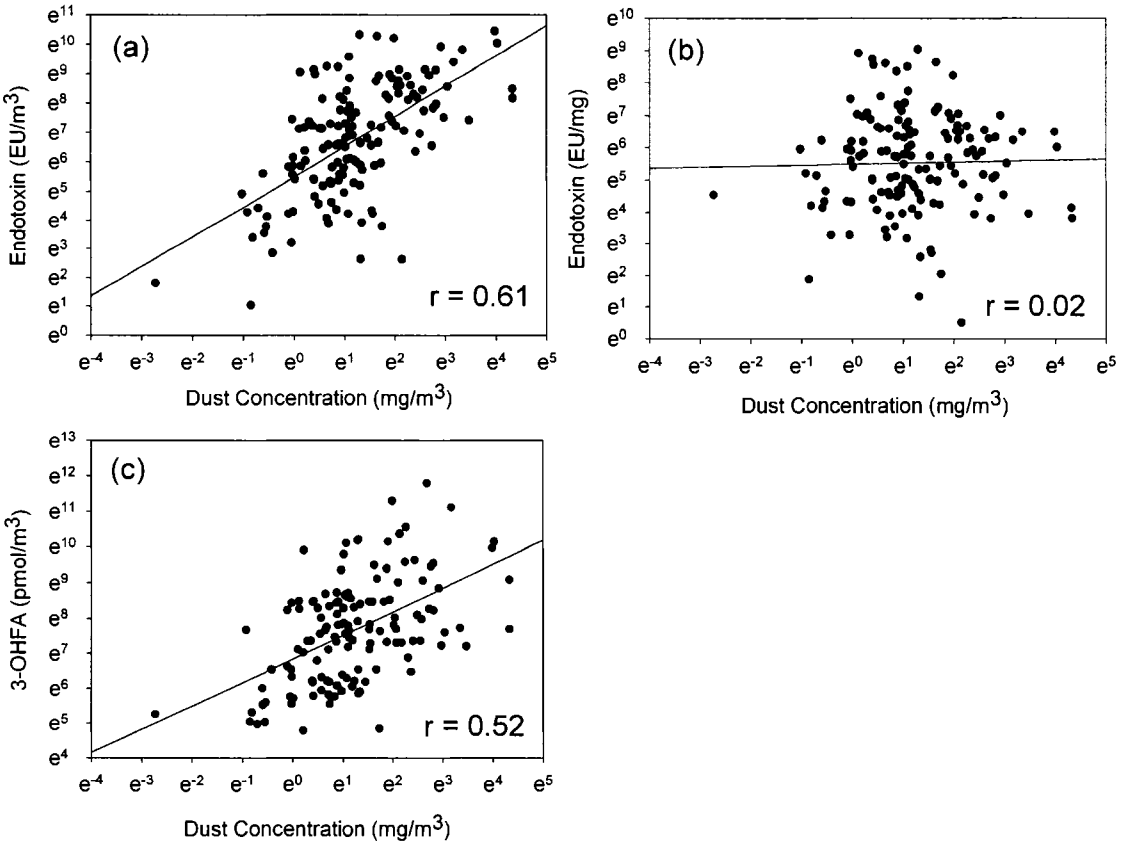

FIGURE 4-3. Correlations between dust concentration and (a) endotoxin concentration per m^3 air, (b) endotoxin concentration per mg dust, and (c) GC/EI-MS endotoxin result (total 3-OHFAs) per m^3 air.

TABLE IV-I. Demographic data of 145 workers

	Overall		Dairy		Feedlot		Grain Elevator		Farm	
	M	**(SD)**	**M**	**(SD)**	**M**	**(SD)**	**M**	**(SD)**	**M**	**(SD)**
Age	36.8	(12.5)	31.2	(8.6)	33.1	(10.2)	37.7	(12.9)	31.7	(10.5)
Years in job [A]	3.92	(3.89)	3.25	(3.77)	3.17	(3.61)	3.61	(3.56)	6.44	(3.00)
	n	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**
Gender										
Male	139	(96)	19	(91)	53	(96)	56	(97)	11	(100)
Female	6	(4)	2	(9)	2	(4)	2	(3)	0	(0)
Hispanic*										
Yes	46	(32)	16	(76)	15	(27)	12	(21)	3	(27)
No	89	(61)	4	(19)	37	(67)	41	(71)	7	(64)
Unknown	10	(7)	1	(5)	3	(6)	5	(9)	1	(9)
Race*										
White	105	(72)	8	(38)	41	(75)	46	(79)	10	(91)
Native American	3	(2)	0	(0)	1	(2)	2	(3)	0	(0)
Other	29	(20)	11	(52)	10	(18)	8	(14)	0	(0)
Unknown	8	(5)	2	(10)	3	(7)	2	(3)	1	(9)
Job status*										
Owner	16	(11)	4	(19)	9	(16)	1	(2)	2	(18)
Family	9	(6)	1	(5)	6	(11)	0	(0)	2	(18)
Local hire	100	(69)	10	(48)	29	(53)	55	(95)	6	(55)
Seasonal	15	(10)	5	(24)	9	(16)	0	(0)	1	(9)
Other	4	(3)	0	(0)	2	(4)	2	(3)	0	(0)
Unknown	1	(1)	1	(5)	0	(0)	0	(0)	0	(0)
Education* [B]										
< High school	20	(14)	6	(29)	10	(18)	3	(5)	1	(9)
High school	67	(46)	11	(52)	15	(27)	38	(66)	3	(27)
College	47	(32)	1	(5)	27	(49)	14	(24)	5	(45)
> College	7	(5)	2	(10)	3	(12)	2	(3)	0	(0)
Unknown	4	(3)	1	(5)	0	(0)	1	(2)	2	(18)
Respirator*										
Respirator with filter	4	(3)	0	(0)	0	(0)	3	(5)	1	(9)
Dust/surgeon's mask	14	(10)	0	(0)	1	(2)	12	(20)	1	(9)
None	121	(83)	19	(90)	52	(95)	41	(71)	9	(82)
Unknown	6	(4)	2	(10)	2	(3)	2	(3)	0	(0)

Note: M = mean, SD = standard deviation; * indicates significance difference (χ^2, p-value < 0.05); [A] Years in job shows geometric mean and geometric standard deviation; [B] Included 32 workers educated in Spanish.

TABLE IV-II. Description of agricultural tasks

Task	Description
Truck harvest	Harvesting vegetables or grain using trucks
Combine harvest	Harvesting vegetables or grain with integrated machine / equipments
Working in storage	Working in feed storage
Loading/unloading feed truck	Loading and unloading feed from trucks
Sampling grain/feed	Sampling grain or feed for quality check
Weighing grain/feed	Weighing grain or feed on scales
Running legs (in elevator)	Operating conveyor belts in grain elevators
Milling	Grinding grain or feed
Mixing	Mixing grain or feed (formulation)
Bagging feed	Bagging feed or grain
Housekeeping/cleaning	Sweeping and cleaning facilities
Mechanical maintenance	Mechanical maintenance of machine and equipments
Feeding livestock	Feeding livestock
Supervising	Supervising other workers

TABLE IV-III. Average hours and (standard deviations) in each task among workers who performed the task

	Overall		Dairy		Cattle feedlot		Grain elevator		Farm	
	n	AM	n	AM	n	AM	n	AM	n	AM
Truck harvest	11	3.30 (2.14)	2	5.38 (3.02)	3	2.60 (2.17)	6	2.96 (1.80)		
Combine harvest	8	2.77 (2.96)	2	1.08 (0.43)	2	2.07 (2.66)	3	2.21 (1.61)	1	9.18 (-)
Storage	21	2.64 (1.50)	2	2.93 (2.18)	4	1.83 (1.04)	12	2.72 (1.67)	3	3.22 (1.01)
Loading/unloading feed truck	57	3.04 (2.34)	4	5.18 (4.18)	26	3.11 (1.84)	27	2.65 (2.37)		
Sampling grain/feed	18	1.10 (1.03)	1	1.39 (-)	4	1.08 (0.94)	13	1.09 (1.13)		
Weighing grain/feed	13	1.27 (1.11)			4	0.71 (0.68)	9	1.52 (1.20)		
Running legs (in elevator)	32	2.40 (1.34)			9	1.82 (1.32)	23	2.63 (1.31)		
Milling	18	2.47 (1.60)	1	4.47 (-)	7	2.40 (2.21)	10	2.32 (1.06)		
Mixing	17	2.48 (2.10)	1	9.20 (-)	5	1.95 (1.27)	11	2.11 (1.25)		
Bagging feed	12	3.16 (3.07)					12	3.16 (3.07)		
Housekeeping/cleaning	29	0.93 (0.76)			8	1.09 (1.13)	21	0.86 (0.59)		
Mechanical maintenance	28	3.43 (2.64)	2	6.58 (4.91)	14	2.69 (2.35)	9	3.61 (2.78)	3	4.26 (0.72)
Feeding livestock	40	3.95 (3.03)	4	7.86 (4.32)	29	3.57 (2.55)	3	1.86 (2.05)	4	4.37 (3.34)
Supervising	23	3.73 (2.43)	5	5.30 (2.41)	7	3.26 (2.82)	9	3.38 (2.14)	2	3.01 (2.52)

TABLE IV-IV. Numbers of single- and multiple-task workers

Numbers of tasks	Overall	Dairy	Cattle feedlot	Grain elevator	Farm
1	40	10	11	11	8
2	26	3	9	13	1
3	18	1	9	7	1
4	18	0	7	11	0
5	6	1	1	4	0
6	5	0	2	3	0
7	7	0	3	4	0
11	2	0	2	0	0

Note: 67% of workers performed multi-task (> 1 task) overall, 33% in dairy, 75% in cattle feedlot, 79% in grain elevator, and 20% in farm. Significant differences were observed in numbers of single- and multi-task workers in environments (χ^2, p-value < 0.01).

TABLE IV-V. 8-hour predicted dust exposures (mg/m^3) using the multiple regression model

	Dairy	Cattle feedlot	Grain elevator	Farm
Truck harvest	3.69	2.07	1.45	
Combine harvest	0.61	7.83	0.37	1.13
Storage	39.28	49.13	15.23 [AB]	114.80 [AB]
Loading/unloading feed truck	1.65	2.37	5.24 [AB]	
Sampling grain/feed	0.01	0.12	0.18	
Weighing grain/feed		10.21	63.09	
Running legs (in elevator)		1.09	11.38 [AB]	
Milling		8.55 [B]	6.84	
Mixing	2.07	1.13	5.82	
Bagging feed			4.86 [AB]	
Housekeeping/cleaning		2.40	110.81	
Mechanical maintenance	2.20	1.46	4.63 [B]	12.96
Feeding livestock	2.42	2.60 [AB]	0.64	3.03
Supervising	1.32	1.47	0.58	0.35

Note: $R^2 = 0.59$, $p < 0.01$; [A] $p < 0.05$; [B] included in stepwise regression, $p < 0.05$, stepwise regression model $R^2 = 0.48$ and model $p < 0.01$.

TABLE IV-VI. 8-hour predicted rFC endotoxin measurements (EU/m^3) using the multiple regression model

	Dairy	Cattle feedlot	Grain elevator	Farm
Truck harvest	3589.26	4769.52	500.00	
Combine harvest	3.35E+14	7.47E+10 [B]	1.91	165.75
Storage	4.59E+06 [AB]	4.93	3.81E+04 [AB]	3.59E+07 [AB]
Loading/unloading feed truck	238.72 [AB]	162.63 [AB]	391.00 [AB]	
Sampling grain/feed	0.00	3.25	6.59	
Weighing grain/feed		9.18E+06	6.15E+04 [B]	
Running legs (in elevator)		5.37E+04 [B]	1.81E+04 [AB]	
Milling		8238.72 [AB]	94.02	
Mixing	300.82	0.00	79.11	
Bagging feed			199.12 [AB]	
Housekeeping/cleaning		1.35E+04	7980.85	
Mechanical maintenance	51.83	1025.16 [AB]	1055.43 [AB]	1.23E+04 [B]
Feeding livestock	392.80 [AB]	1849.18 [AB]	1010.40	2189.22 [AB]
Supervising	551.48 [AB]	41.36	118.07 [B]	1.15

Note: $R^2 = 0.75$, $p < 0.01$; [A] $p < 0.05$; [B] included in stepwise regression, $p < 0.05$, stepwise regression model $R^2 = 0.67$ and model $p < 0.01$.

TABLE IV-VII. 8-hour predicted GC/MS 3-OHFA concentrations (pmol/m^3) using the multiple regression model

	Dairy	Cattle feedlot	Grain elevator	Farm
Truck harvest	6983.46 [B]	4187.67	238.32	
Combine harvest	1.90E+12	8.19E+07	2.67E+04	1339.70
Storage	1.75E+06	61.05	1.08E+04 [AB]	5.60E+07 [AB]
Loading/unloading feed truck	968.84 [AB]	424.62 [AB]	962.95 [AB]	
Sampling grain/feed	0.00	2.13	88.63	
Weighing grain/feed		3.75E+19	3.48E+05 [B]	
Running legs (in elevator)		1877.69 [B]	1.13E+04 [B]	
Milling		1.07E+04	1376.09 [AB]	
Mixing	660.96	0.43	3077.58	
Bagging feed			1302.45 [AB]	
Housekeeping/cleaning		5.85E+04	2616.26	
Mechanical maintenance	162.86	2202.39 [AB]	3484.22 [AB]	1.86E+06 [AB]
Feeding livestock	520.56 [AB]	5648.81 [AB]	1.31E+05	7172.43 [AB]
Supervising	884.75 [AB]	584.12	516.36 [AB]	4.14

Note: $R^2 = 0.78$, $p < 0.01$; [A] $p < 0.05$; [B] included in stepwise regression, $p < 0.05$, stepwise regression model $R^2 = 0.70$ and model $p < 0.01$.

TABLE IV-VIII. Rank-order of 8-hour predicted values based on the task-only regression models

	Dust (mg/m^3)	rFC (EU/m^3)	GC/MS ($Pmol/m^3$)
Low	Sampling grain/feed	Sampling grain/feed	Sampling grain/feed
	Combine harvest	Supervising	Loading/unloading feed truck
	Supervising	Bagging feed	Supervising
	Truck harvest	Mixing	Milling
	Feeding livestock	Combine harvest	Truck harvest
	Loading/unloading feed truck	Loading/unloading feed truck	Bagging feed
	Mechanical maintenance	Milling	Mixing
	Mixing	Mechanical maintenance	Combine harvest
	Bagging feed	Feeding livestock	Mechanical maintenance
	Milling	Truck harvest	Feeding livestock
	Running legs (in elevator)	Running legs (in elevator)	Running legs (in elevator)
	Storage	Housekeeping/cleaning	Housekeeping/cleaning
	Housekeeping/cleaning	Storage	Storage
High	Weighing grain/feed	Weighing grain/feed	Weighing grain/feed

TABLE IV-IX. Comparison of actual and predicted values of exposures of single-task workers using the regression models

	Dust (mg/m^3)			rFC (EU/m^3)			GC/MS (pmol/m^3)		
	Actual	Predicted	Diff. (%)	Actual	Predicted	Diff. (%)	Actual	Predicted	Diff. (%)
Truck harvest	3.41	3.41	0.0	2182.66	2182.66	0.0	4078.16	4078.16	0.0
Combine harvest	1.15	1.15	0.0	353.01	353.01	0.0	3886.25	3886.25	0.0
Storage									
Loading/unloading feed truck	2.86	3.35	17.0	493.37	403.19	18.3	595.62	1245.82	109.2
Sampling grain/feed									
Weighing grain/feed									
Running legs (in elevator)									
Milling									
Mixing	2.31	2.31	0.0	707.97	707.97	0.0	1750.75	1750.75	0.0
Bagging feed	11.13	6.18	44.5	575.19	445.41	22.6	1570.89	3876.93	146.8
Housekeeping/cleaning									
Mechanical maintenance	8.86	3.43	61.3	1161.31	269.99	76.8	1500.12	705.42	53.0
Feeding livestock	2.78	2.85	2.3	3649.77	2268.15	37.9	5077.22	5253.55	3.5
Supervising	0.17	0.89	419.2	13.54	135.00	896.8	197.24	320.15	62.3

Note: Diff. % (difference between actual and predicted values) = absolute[100 × (actual value – predicted value)/actual value]

TABLE IV-X. Correlations between dust and endotoxin concentrations and task

	Dust (mg/m^3)	rFC (EU/m^3)	GC/MS ($pmol/m^3$)
Hours			
Truck harvest	-0.08	0.04	-0.05
Combine harvest	-0.11	-0.02	0.07
Storage	**0.23**	**0.19**	0.09
Loading/unloading feed truck	-0.02	-0.10	-0.10
Sampling grain/feed	-0.08	-0.12	-0.10
Weighing grain/feed	0.12	0.04	0.09
Running legs (in elevator)	**0.26**	**0.17**	0.05
Milling	0.15	0.04	0.05
Mixing	0.06	-0.06	-0.01
Bagging feed	0.09	-0.11	-0.05
Housekeeping/cleaning	**0.18**	0.09	0.01
Mechanical maintenance	-0.06	-0.10	-0.07
Feeding livestock	-0.13	0.06	0.10
Supervising	**-0.27**	**-0.23**	**-0.18**
Experience			
Years in job	**-0.17**	**-0.19**	-0.03

Note: bold = $p < 0.05$

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CHAPTER 5

CONCLUSION

This dissertation was designed to aid in understanding the variability in endotoxin measurements in agricultural environments. In chapter 2, a GC/MS chemical analysis method was optimized for endotoxins in agricultural dusts and applied in various agricultural environments. Chapter 3 compared responses of two bioassays, the LAL and rFC, in livestock dusts. Then, chapter 4 evaluated the task-specific exposures and identified contributions of determinants in personal dust and endotoxin exposures in agricultural environments using empirical modeling. Each chapter was important for the future development of accurate endotoxin exposure assessment methods in agricultural settings.

Summary and significance of each chapter

Chemical analysis of endotoxins

In this chapter, a GC/MS method was optimized for agricultural dusts. A modified GC/EI-MS method reduced the use of toxic chemicals and sample handling, and allowed sensitive monitoring of the experimental process. This method was especially useful for analysis of very small samples, typical of personal air samples, in agricultural environments.

This chapter also evaluated rFC assay and GC/EI-MS results in four agricultural environments. Overall, livestock dusts had more variable 3-OHFAs and stronger correlations between GC/EI-MS and rFC assay results than grain dusts, probably due to the difference in bacterial distribution. Understanding differences in 3-OHFA distributions in various agricultural environments may provide better explanations of the relationship between endotoxin exposure and development of respiratory diseases.

The LAL and rFC assays for endotoxin measurements

In this chapter, strong positive correlations were observed between the LAL and rFC assays in all agricultural dusts. However, assay responses varied by agricultural environment or dust type. The LAL overestimated (or rFC underestimated) endotoxin exposures in chicken and horse dusts and the LAL underestimated (or rFC overestimated) endotoxin concentrations in dairy, swine, and turkey dusts. On the basis of results from this chapter, ergosterol concentration does not appear to be a major factor of interference in the LAL assay overall, but the magnitude of interference may vary by dust type. Other than ergosterol contribution, between-method variability may be explained by differences in bacterial composition and other dust components; the rFC assay may react positively with Actinobacteria. Investigation of the LAL and rFC responses to various dust types provides better understanding of each bioassay and is an important step toward developing accurate endotoxin measurement protocols.

Task-specific dust and endotoxin exposure assessments

This chapter described that a high proportion of workers had exposures to dust and endotoxin exceeding the recommended occupational guidelines, which suggests the need to improve control methods to reduce exposures. Multiple linear regression models were successfully applied to evaluate determinants for dust and endotoxin exposures in agricultural environments. Results suggest that dust exposure control is especially important in grain elevator environments, particularly during work in running legs in grain elevators and housekeeping. Working in feed storage, running legs in grain elevators, and feeding livestock in cattle feedlots should be highly prioritized operations for control to reduce endotoxin exposure. The characterization of dust and endotoxin exposure determinants was useful for developing and prioritizing exposure control strategies, and for the prevention of organic dust lung disease among agricultural workers.

Conclusion and future research

This dissertation addressed the need for understanding differences in agricultural environments with respect to endotoxin exposure assessment. Since all chemical compositions, bioassay responses, and tasks varied by agricultural operation and environment, assessment of endotoxin exposures and the conduct of epidemiological studies in agricultural environments should proceed with caution. Findings of this dissertation also raised several questions for future investigation:

1. *Investigation of seasonal and geographical variability.* Due to small sample sizes in one or more seasons, seasonal variability could not be studied in this dissertation. In addition, samples in this dissertation were collected in two

geographical locations. Sampling in multiple geographical locations with different climate conditions is recommended to study geographic variation in endotoxin exposures.

2. *Investigation of potential interference of bioassays using agricultural dusts.* Bioassay responses (both the LAL and rFC response) were found to vary by agricultural dust type. This finding could be explained by interference, but the sources of interference were not identified in this dissertation. Use of the combination of chemical, biological, and microbiological approaches would provide detailed information on compositions of agricultural dusts, which may lead to the identification of the sources of interference.
3. *Investigation of dust and bacterial composition.* There was a high variation in combinations of 3-OHFA and in the contribution of different 3-OHFAs to assays in agricultural environments. The mechanism related to this variability was unclear but the variability may be explained by differences in microbial communities present. Thus, identifying both Gram-negative and Gram-positive bacterial distributions in various agricultural environments is recommended. Identifying bacterial compositions may lead to better control methods to prevent and reduce diseases.
4. *Investigation of specific 3-OHFA roles in human diseases.* The 3-OHFA profiles varied by agricultural dusts. Thus, if a specific 3-OHFA was associated with the prevalence of human respiratory diseases, monitoring the specific 3-OHFA in the air would be advantageous, not only in agricultural settings, but also in other occupational and general public settings.

APPENDICES

APPENDIX A

CORRELATIONS BETWEEN EACH INDIVIDUAL 3-OHFA

(PERSONAL BREATH ZONE SAMPLES)

TABLE A-I. (a) Correlations between each individual 3-OHFA with p-value: Overall (n = 134)

	rFC	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	-0.14	0.03	0.27	0.29	0.37	0.49	0.39	0.35	0.23
		0.11	0.73	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01
C8	-0.14	1.00	0.42	0.54	0.64	-0.01	0.22	0.05	0.19	0.51
	0.11		<0.01	<0.01	<0.01	0.94	0.01	0.57	0.03	<0.01
C9	0.03	0.42	1.00	0.34	0.40	0.26	0.31	0.20	0.40	0.30
	0.73	<0.01		<0.01	<0.01	0.00	0.00	0.02	<0.01	<0.01
C10	0.27	0.54	0.34	1.00	0.76	0.05	0.40	0.05	0.27	0.53
	<0.01	<0.01	<0.01		<0.01	0.58	<0.01	0.59	<0.01	<0.01
C12	0.29	0.64	0.40	0.76	1.00	0.16	0.57	0.23	0.38	0.70
	<0.01	<0.01	<0.01	<0.01		0.06	<0.01	0.01	<0.01	<0.01
C13	0.37	-0.01	0.26	0.05	0.16	1.00	0.53	0.77	0.64	0.23
	<0.01	0.94	<0.01	0.58	0.06		<0.01	<0.01	<0.01	0.01
C14	0.49	0.22	0.31	0.40	0.57	0.53	1.00	0.59	0.61	0.52
	<0.01	0.01	0.00	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01
C15	0.39	0.05	0.20	0.05	0.23	0.77	0.59	1.00	0.74	0.28
	<0.01	0.57	0.02	0.59	0.01	<0.01	<0.01		<0.01	<0.01
C17	0.35	0.19	0.40	0.27	0.38	0.64	0.61	0.74	1.00	0.40
	<0.01	0.03	<0.01	0.00	<0.01	<0.01	<0.01	<0.01		<0.01
C18	0.23	0.51	0.30	0.53	0.70	0.23	0.52	0.28	0.40	1.00
	0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	

TABLE A-I. (b) Correlations between each individual 3-OHFA with p-value: Dairy (n = 17)

	rFC	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	-0.04	0.40	0.55	0.19	0.26	0.00	0.23	0.74	0.42
		0.88	0.11	0.02	0.46	0.31	0.99	0.38	<0.01	0.09
C8	-0.04	1.00	0.07	0.40	0.73	0.35	0.32	0.50	0.05	0.54
	0.88		0.78	0.11	0.00	0.17	0.21	0.04	0.85	0.03
C9	0.40	0.07	1.00	0.05	0.09	-0.02	0.22	0.27	0.67	0.07
	0.11	0.78		0.85	0.72	0.95	0.40	0.30	<0.01	0.79
C10	0.55	0.40	0.05	1.00	0.43	-0.01	-0.01	-0.09	0.33	0.24
	0.02	0.11	0.85		0.09	0.96	0.97	0.74	0.20	0.35
C12	0.19	0.73	0.09	0.43	1.00	0.44	0.39	0.43	0.31	0.52
	0.46	0.00	0.72	0.09		0.08	0.12	0.08	0.23	0.03
C13	0.26	0.35	-0.02	-0.01	0.44	1.00	-0.05	0.82	0.44	0.65
	0.31	0.17	0.95	0.96	0.08		0.85	<0.01	0.08	0.01
C14	0.00	0.32	0.22	-0.01	0.39	-0.05	1.00	0.27	0.09	0.30
	0.99	0.21	0.40	0.97	0.12	0.85		0.30	0.74	0.24
C15	0.23	0.50	0.27	-0.09	0.43	0.82	0.27	1.00	0.53	0.68
	0.38	0.04	0.30	0.74	0.08	<0.01	0.30		0.03	0.00
C17	0.74	0.05	0.67	0.33	0.31	0.44	0.09	0.53	1.00	0.32
	<0.01	0.85	<0.01	0.20	0.23	0.08	0.74	0.03		0.20
C18	0.42	0.54	0.07	0.24	0.52	0.65	0.30	0.68	0.32	1.00
	0.09	0.03	0.79	0.35	0.03	0.01	0.24	0.00	0.20	

TABLE A-I. (c) Correlations between each individual 3-OHFA with p-value: Cattle feedlot (n = 48)

	rFC	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	-0.12	0.29	0.30	0.41	0.63	0.83	0.61	0.60	0.33
		0.43	0.05	0.04	0.00	<0.01	<0.01	<0.01	<0.01	0.02
C8	-0.12	1.00	0.02	0.41	0.47	-0.06	0.03	0.15	0.30	0.24
	0.43		0.91	0.00	0.00	0.71	0.83	0.32	0.04	0.10
C9	0.29	0.02	1.00	0.13	0.18	0.51	0.34	0.36	0.38	0.11
	0.05	0.91		0.38	0.23	<0.01	0.02	0.01	0.01	0.45
C10	0.30	0.41	0.13	1.00	0.65	0.14	0.37	0.22	0.40	0.29
	0.04	<0.01	0.38		<0.01	0.36	0.01	0.14	<0.01	0.05
C12	0.41	0.47	0.18	0.65	1.00	0.26	0.50	0.38	0.56	0.54
	<0.01	<0.01	0.23	<0.01		0.08	<0.01	0.01	<0.01	<0.01
C13	0.63	-0.06	0.51	0.14	0.26	1.00	0.63	0.66	0.53	0.43
	<0.01	0.71	<0.01	0.36	0.08		<0.01	<0.01	<0.01	<0.01
C14	0.83	0.03	0.34	0.37	0.50	0.63	1.00	0.79	0.84	0.57
	<0.01	0.83	0.02	0.01	<0.01	<0.01		<0.01	<0.01	<0.01
C15	0.61	0.15	0.36	0.22	0.38	0.66	0.79	1.00	0.82	0.50
	<0.01	0.32	0.01	0.14	0.01	<0.01	<0.01		<0.01	<0.01
C17	0.60	0.30	0.38	0.40	0.56	0.53	0.84	0.82	1.00	0.64
	<0.01	0.04	0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01
C18	0.33	0.24	0.11	0.29	0.54	0.43	0.57	0.50	0.64	1.00
	0.02	0.10	0.45	0.05	<0.01	<0.01	<0.01	<0.01	<0.01	

TABLE A-I. (d) Correlations between each individual 3-OHFA with p-value: Grain elevator (n = 58)

	rFC	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	-0.13	-0.28	0.22	0.17	-0.15	0.27	-0.14	-0.23	0.10
		0.33	0.03	0.10	0.20	0.25	0.04	0.29	0.08	0.44
C8	-0.13	1.00	0.72	0.65	0.75	0.03	0.45	0.03	0.39	0.70
	0.33		<0.01	<0.01	<0.01	0.80	<0.01	0.81	<0.01	<0.01
C9	-0.28	0.72	1.00	0.47	0.51	0.11	0.26	-0.01	0.42	0.43
	0.03	<0.01		<0.01	<0.01	0.43	0.05	0.97	<0.01	<0.01
C10	0.22	0.65	0.47	1.00	0.90	-0.03	0.67	-0.08	0.29	0.81
	0.10	<0.01	<0.01		<0.01	0.84	<0.01	0.54	0.03	<0.01
C12	0.17	0.75	0.51	0.90	1.00	-0.11	0.67	-0.05	0.27	0.86
	0.20	<0.01	<0.01	<0.01		0.39	<0.01	0.73	0.04	<0.01
C13	-0.15	0.03	0.11	-0.03	-0.11	1.00	0.20	0.62	0.39	-0.12
	0.25	0.80	0.43	0.84	0.39		0.14	<0.01	<0.01	0.38
C14	0.27	0.45	0.26	0.67	0.67	0.20	1.00	0.15	0.26	0.60
	0.04	<0.01	0.05	<0.01	<0.01	0.14		0.25	0.05	<0.01
C15	-0.14	0.03	-0.01	-0.08	-0.05	0.62	0.15	1.00	0.29	-0.07
	0.29	0.81	0.97	0.54	0.73	<0.01	0.25		0.03	0.59
C17	-0.23	0.39	0.42	0.29	0.27	0.39	0.26	0.29	1.00	0.22
	0.08	<0.01	<0.01	0.03	0.04	<0.01	0.05	0.03		0.09
C18	0.10	0.70	0.43	0.81	0.86	-0.12	0.60	-0.07	0.22	1.00
	0.44	<0.01	<0.01	<0.01	<0.01	0.38	<0.01	0.59	0.09	

TABLE A-I. (e) Correlations between each individual 3-OHFA with p-value: Farm (n = 11)

	rFC	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	0.07	-0.25	0.14	0.13	0.39	0.17	0.16	0.01	0.44
		0.83	0.46	0.68	0.70	0.23	0.62	0.64	0.98	0.18
C8	0.07	1.00	0.76	0.92	0.89	0.34	0.59	0.20	0.23	0.34
	0.83		0.01	<0.01	<0.01	0.30	0.05	0.55	0.50	0.30
C9	-0.25	0.76	1.00	0.58	0.57	0.56	0.71	0.44	0.48	0.54
	0.46	0.01		0.06	0.07	0.07	0.01	0.17	0.14	0.09
C10	0.14	0.92	0.58	1.00	0.95	0.20	0.41	0.03	0.01	0.17
	0.68	<0.01	0.06		<0.01	0.55	0.21	0.94	0.97	0.62
C12	0.13	0.89	0.57	0.95	1.00	0.31	0.51	0.18	0.18	0.21
	0.70	<0.01	0.07	<0.01		0.36	0.11	0.59	0.61	0.54
C13	0.39	0.34	0.56	0.20	0.31	1.00	0.88	0.88	0.79	0.96
	0.23	0.30	0.07	0.55	0.36		<0.01	<0.01	<0.01	<0.01
C14	0.17	0.59	0.71	0.41	0.51	0.88	1.00	0.90	0.86	0.85
	0.62	0.05	0.01	0.21	0.11	<0.01		<0.01	<0.01	<0.01
C15	0.16	0.20	0.44	0.03	0.18	0.88	0.90	1.00	0.94	0.83
	0.64	0.55	0.17	0.94	0.59	<0.01	<0.01		<0.01	<0.01
C17	0.01	0.23	0.48	0.01	0.18	0.79	0.86	0.94	1.00	0.75
	0.98	0.50	0.14	0.97	0.61	<0.01	<0.01	<0.01		0.01
C18	0.44	0.34	0.54	0.17	0.21	0.96	0.85	0.83	0.75	1.00
	0.18	0.30	0.09	0.62	0.54	<0.01	<0.01	<0.01	0.01	

APPENDIX B

GM AND GSD OF DUST AND ENDOTOXIN CONCENTRATIONS

(AREA SAMPLES USING PERSONAL SAMPLERS)

TABLE B-I. GM and GSD dust and endotoxin concentrations (dust type)

		Dust Concentration (mg/m^3)		Endotoxin per dust (EU/mg)				Endotoxin per air (EU/m^3)				Ratio (rFC/LAL)	
				rFC		LAL		rFC		LAL			
	n	GM	GSD	GM	GSD	GM	GSD	GM	GSD	GM	GSD	GM	GSD
Chicken	204 (202)	3.156	6.244	733.97	2.142	973.53	2.198	2334.46	8.317	3119.39	9.155	0.758	1.715
Dairy	78 (76)	0.101	3.379	353.92	3.503	234.97	4.389	34.59	4.017	27.38	5.687	1.334	2.087
Horse	40 (15)	0.258	2.461	446.55	1.452	663.84	1.358	115.23	2.759	212.16	3.270	0.642	1.555
Swine	198 (197)	2.965	5.281	3162.19	2.636	2849.90	2.619	9375.33	5.828	8248.44	7.236	1.097	3.723
Turkey	193 (199)	1.616	2.811	1610.10	2.630	1479.89	3.304	2625.84	3.739	2597.38	4.775	1.135	2.421
GLM p-value:		*<0.01*		*<0.01*		*<0.01*		*<0.01*		*<0.01*		*<0.01*	

Note: n (n) = rFC (LAL)

TABLE B-II. GM and GSD endotoxin concentrations (sampling site)

Sampling site		n [A]	Endotoxin per dust (EU/mg)				Endotoxin per air (EU/m^3)				Ratio (rFC/LAL)	
			rFC		LAL		rFC		LAL			
			GM	GSD	GM	GSD	GM	GSD	GM	GSD	GM	GSD
Chicken	Colorado	116/118	536.87	2.117	743.91	2.381	1690.22	12.986	2169.88	14.747	0.709	1.707
	Iowa	88/84	1108.42	1.742	1420.57	1.551	3573.16	3.346	5194.05	3.011	0.829	1.708
Dairy	Colorado	38/31	509.27	2.994	367.31	2.360	23.27	3.129	18.70	3.803	1.758	2.235
	Iowa	40/44	250.48	3.698	171.52	5.661	50.90	4.541	36.12	7.018	1.077	1.832
Horse	Colorado	40/15	446.55	1.452	663.84	1.358	115.23	2.759	212.16	3.270	0.642	1.555
	Iowa	-	-	-	-	-	-	-	-	-	-	-
Swine	Colorado	159/159	2788.74	2.550	2587.41	3.042	7964.35	6.362	6776.48	9.110	1.059	3.850
	Iowa	79/79	3821.24	2.701	3292.39	1.936	11986.41	4.963	11063.24	4.668	1.156	3.557
Turkey	Colorado	117/118	1575.90	2.061	1085.77	2.720	2077.98	3.536	1639.51	5.049	1.420	2.393
	Iowa	76/80	1664.21	3.520	2336.72	3.717	3764.59	3.781	5120.02	3.306	0.807	2.209

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE B-III. GM and GSD dust and endotoxin concentrations (sampling device)

Device	n [A]	Dust Concentration (mg/m3)		Endotoxin per dust (EU/mg)				Endotoxin per air (EU/m3)				Ratio (rFC/LAL)	
				rFC		LAL		rFC		LAL			
		GM	GSD	GM	GSD	GM	GSD	GM	GSD	GM	GSD	GM	GSD
Cassette	180/172	1.818	4.617	1304.17	2.898	1420.63	2.994	2374.73	8.426	2698.98	8.592	0.959	2.129
Cyclone	176/167	0.325	4.588	1142.26	4.113	760.19	4.930	366.60	10.651	315.98	12.304	1.442	2.817
IOM	181/173	3.947	6.000	1120.43	3.340	1372.11	3.129	4316.87	8.714	5680.37	9.004	0.890	2.912
Button	177/175	2.354	5.005	1340.40	2.897	1715.11	2.947	3204.34	9.256	4346.73	9.157	0.837	2.327

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE B-IV. GM and GSD dust and endotoxin concentrations for each sampling device in each dust type

		Dust Concentration (mg/m^3)		Endotoxin per dust (EU/mg)				Endotoxin per air (EU/m^3)				Ratio (rFC/LAL)	
				rFC		LAL		rFC		LAL			
	n [A]	GM	GSD	GM	GSD	GM	GSD	GM	GSD	GM	GSD	GM	GSD
Chicken													
Cassette	52/50	4.002	3.786	696.37	2.062	1024.47	1.875	2783.99	5.717	4395.74	5.316	0.703	1.552
Cyclone	52/51	0.547	5.054	814.37	1.859	844.18	2.217	444.77	7.985	450.22	9.304	0.966	1.730
IOM	52/51	8.367	5.224	652.90	2.514	955.87	2.442	5463.17	6.290	8072.63	6.581	0.697	1.655
Button	49/50	5.594	4.012	788.64	2.114	1090.09	2.240	4411.60	6.505	6044.46	6.007	0.694	1.818
Dairy													
Cassette	21/20	0.093	1.984	564.20	1.741	401.31	2.036	52.64	1.659	38.06	2.537	1.430	1.898
Cyclone	16/15	0.045	3.654	102.76	5.313	21.87	3.746	3.35	1.725	1.51	1.901	2.047	2.675
IOM	20/19	0.202	4.321	406.93	3.914	410.97	2.528	80.48	1.880	70.79	2.610	1.259	2.031
Button	21/21	0.124	2.264	498.74	1.708	463.91	1.764	66.76	2.281	64.22	3.078	1.075	1.947
Horse													
Cassette	10/4	0.325	1.379	597.62	1.253	778.46	1.416	194.19	1.324	274.78	1.934	0.733	1.387
Cyclone	10/3	0.064	1.359	343.18	1.428	458.34	1.331	22.11	1.469	25.46	1.402	1.076	1.575
IOM	10/4	0.570	1.459	442.71	1.394	622.68	1.134	252.16	1.268	481.31	1.345	0.554	1.289
Button	10/4	0.372	1.436	437.94	1.460	796.80	1.170	162.85	1.202	354.11	1.417	0.443	1.371
Swine													
Cassette	49/49	3.671	2.518	3500.83	2.372	3771.62	2.317	12850.81	3.998	12005.43	3.313	0.918	2.625
Cyclone	49/48	0.410	4.183	4502.73	2.765	1736.52	2.803	1848.25	5.403	892.37	6.753	2.510	3.536
IOM	50/50	9.702	3.188	1999.74	2.454	2436.15	2.929	19402.03	3.781	19312.13	4.156	0.821	4.492
Button	50/50	5.102	3.069	3201.00	2.552	4076.03	1.832	16332.07	4.806	20623.51	4.335	0.785	3.120
Turkey													
Cassette	48/49	1.979	1.497	1594.32	2.590	1314.39	2.962	3180.68	2.733	2530.76	3.125	1.199	2.053
Cyclone	49/50	0.460	2.038	1156.42	2.227	923.82	3.921	537.99	2.195	469.92	4.350	1.196	2.654
IOM	49/49	3.549	2.659	2010.93	3.194	1894.12	2.875	7137.76	2.459	8279.81	2.599	1.130	2.924
Button	47/50	2.177	1.737	1821.49	2.333	2090.83	2.996	3974.25	2.420	4728.11	2.454	1.022	2.072

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

APPENDIX C

CORRELATIONS BETWEEN EACH INDIVIDUAL 3-OHFA

(AREA SAMPLES USING PERSONAL SAMPLERS)

TABLE C-I. (a) Correlations between each individual 3-OHFA with p-value: Overall (n = 406/377) [A]

	rFC	LAL	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	0.62	0.08	-0.10	0.10	0.25	0.61	0.50	0.34	0.32	0.29
		<0.01	0.10	0.05	0.06	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
LAL	0.62	1.00	-0.11	-0.22	-0.16	-0.11	0.27	0.12	0.06	0.07	0.06
	<0.01		0.03	<0.01	<0.01	0.04	<0.01	0.02	0.21	0.16	0.23
C8	0.08	-0.11	1.00	0.62	0.51	0.48	0.31	0.46	0.37	0.29	0.38
	0.10	0.03		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C9	-0.10	-0.22	0.62	1.00	0.40	0.34	0.11	0.23	0.33	0.15	0.17
	0.05	<0.01	<0.01		<0.01	<0.01	0.03	<0.01	<0.01	<0.01	<0.01
C10	0.10	-0.16	0.51	0.40	1.00	0.63	0.28	0.43	0.21	0.25	0.36
	0.06	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C12	0.25	-0.11	0.48	0.34	0.63	1.00	0.52	0.72	0.36	0.29	0.55
	<0.01	0.04	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01
C13	0.61	0.27	0.31	0.11	0.28	0.52	1.00	0.66	0.49	0.42	0.47
	<0.01	<0.01	<0.01	0.03	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01
C14	0.50	0.12	0.46	0.23	0.43	0.72	0.66	1.00	0.61	0.54	0.68
	<0.01	0.02	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01
C15	0.34	0.06	0.37	0.33	0.21	0.36	0.49	0.61	1.00	0.64	0.52
	<0.01	0.21	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01
C17	0.32	0.07	0.29	0.15	0.25	0.29	0.42	0.54	0.64	1.00	0.74
	<0.01	0.16	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01
C18	0.29	0.06	0.38	0.17	0.36	0.55	0.47	0.68	0.52	0.74	1.00
	<0.01	0.23	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE C-I. (b) Correlations between each individual 3-OHFA with p-value: Chicken (n = 155/148) [A]

	rFC	LAL	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	0.76	-0.03	0.27	-0.02	0.09	0.52	0.46	0.52	0.44	0.13
		<0.01	0.74	<0.01	0.76	0.30	<0.01	<0.01	<0.01	<0.01	0.10
LAL	0.76	1.00	-0.23	-0.01	-0.17	-0.05	0.32	0.21	0.36	0.22	0.01
	<0.01		0.01	0.91	0.04	0.57	<0.01	0.01	<0.01	0.01	0.89
C8	-0.03	-0.23	1.00	0.63	0.40	0.50	0.19	0.35	0.15	0.18	0.37
	0.74	0.01		<0.01	<0.01	<0.01	0.02	<0.01	0.08	0.03	<0.01
C9	0.27	-0.01	0.63	1.00	0.38	0.38	0.32	0.30	0.27	0.19	0.20
	<0.01	0.91	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01	0.02	0.02
C10	-0.02	-0.17	0.40	0.38	1.00	0.48	0.04	0.15	-0.14	0.03	0.22
	0.76	0.04	<0.01	<0.01		<0.01	0.66	0.06	0.08	0.75	0.01
C12	0.09	-0.05	0.50	0.38	0.48	1.00	0.40	0.59	0.08	0.19	0.60
	0.30	0.57	<0.01	<0.01	<0.01		<0.01	<0.01	0.34	0.02	<0.01
C13	0.52	0.32	0.19	0.32	0.04	0.40	1.00	0.53	0.49	0.45	0.34
	<0.01	<0.01	0.02	<0.01	0.66	<0.01		<0.01	<0.01	<0.01	<0.01
C14	0.46	0.21	0.35	0.30	0.15	0.59	0.53	1.00	0.57	0.63	0.60
	<0.01	0.01	<0.01	<0.01	0.06	<0.01	<0.01		<0.01	<0.01	<0.01
C15	0.52	0.36	0.15	0.27	-0.14	0.08	0.49	0.57	1.00	0.73	0.35
	<0.01	<0.01	0.08	<0.01	0.08	0.34	<0.01	<0.01		<0.01	<0.01
C17	0.44	0.22	0.18	0.19	0.03	0.19	0.45	0.63	0.73	1.00	0.59
	<0.01	0.01	0.03	0.02	0.75	0.02	<0.01	<0.01	<0.01		<0.01
C18	0.13	0.01	0.37	0.20	0.22	0.60	0.34	0.60	0.35	0.59	1.00
	0.10	0.89	<0.01	0.02	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE C-I. (c) Correlations between each individual 3-OHFA with p-value: Dairy (n = 46/35) [A]

	rFC	LAL	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	0.75	0.38	0.12	0.54	0.62	0.60	0.67	0.70	0.73	0.58
		<0.01	0.03	0.48	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
LAL	0.75	1.00	0.28	-0.04	0.33	0.37	0.44	0.43	0.53	0.47	0.41
	<0.01		0.12	0.83	0.06	0.03	0.01	0.01	<0.01	0.01	0.02
C8	0.38	0.28	1.00	0.64	0.74	0.75	0.78	0.75	0.50	0.46	0.65
	0.03	0.12		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
C9	0.12	-0.04	0.64	1.00	0.62	0.65	0.59	0.61	0.41	0.21	0.43
	0.48	0.83	<0.01		<0.01	<0.01	<0.01	<0.01	0.01	0.22	0.01
C10	0.54	0.33	0.74	0.62	1.00	0.87	0.87	0.89	0.63	0.54	0.88
	<0.01	0.06	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C12	0.62	0.37	0.75	0.65	0.87	1.00	0.84	0.90	0.80	0.70	0.87
	<0.01	0.03	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01
C13	0.60	0.44	0.78	0.59	0.87	0.84	1.00	0.88	0.64	0.55	0.80
	<0.01	0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01
C14	0.67	0.43	0.75	0.61	0.89	0.90	0.88	1.00	0.65	0.62	0.82
	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01
C15	0.70	0.53	0.50	0.41	0.63	0.80	0.64	0.65	1.00	0.85	0.76
	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01
C17	0.73	0.47	0.46	0.21	0.54	0.70	0.55	0.62	0.85	1.00	0.74
	<0.01	0.01	0.01	0.22	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01
C18	0.58	0.41	0.65	0.43	0.88	0.87	0.80	0.82	0.76	0.74	1.00
	<0.01	0.02	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE C-I. (d) Correlations between each individual 3-OHFA with p-value: Horse (n = 40/15) [A]

	rFC	LAL	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	-0.16	-0.18	-0.21	-0.12	0.23	-0.25	-0.09	0.00	-0.12	-0.10
		0.58	0.29	0.21	0.47	0.18	0.13	0.61	1.00	0.48	0.54
LAL	-0.16	1.00	0.16	-0.29	-0.36	-0.43	0.06	-0.16	-0.39	-0.32	-0.48
	0.58		0.58	0.32	0.20	0.12	0.84	0.57	0.17	0.26	0.08
C8	-0.18	0.16	1.00	0.59	-0.07	0.06	0.29	0.47	0.14	-0.21	-0.03
	0.29	0.58		<0.01	0.68	0.74	0.08	<0.01	0.40	0.22	0.87
C9	-0.21	-0.29	0.59	1.00	0.04	0.24	0.54	0.51	0.38	0.08	0.25
	0.21	0.32	0.00		0.83	0.15	<0.01	<0.01	0.02	0.65	0.13
C10	-0.12	-0.36	-0.07	0.04	1.00	0.01	-0.15	-0.17	-0.06	0.23	0.25
	0.47	0.20	0.68	0.83		0.97	0.37	0.31	0.71	0.16	0.14
C12	0.23	-0.43	0.06	0.24	0.01	1.00	0.09	0.61	0.32	-0.01	0.48
	0.18	0.12	0.74	0.15	0.97		0.58	<0.01	0.06	0.97	<0.01
C13	-0.25	0.06	0.29	0.54	-0.15	0.09	1.00	0.62	0.54	0.16	0.16
	0.13	0.84	0.08	<0.01	0.37	0.58		<0.01	<0.01	0.34	0.35
C14	-0.09	-0.16	0.47	0.51	-0.17	0.61	0.62	1.00	0.50	0.04	0.39
	0.61	0.57	<0.01	<0.01	0.31	<0.01	<0.01		<0.01	0.79	0.02
C15	0.00	-0.39	0.14	0.38	-0.06	0.32	0.54	0.50	1.00	0.28	0.58
	1.00	0.17	0.40	0.02	0.71	0.06	<0.01	<0.01		0.09	<0.01
C17	-0.12	-0.32	-0.21	0.08	0.23	-0.01	0.16	0.04	0.28	1.00	0.64
	0.48	0.26	0.22	0.65	0.16	0.97	0.34	0.79	0.09		<0.01
C18	-0.10	-0.48	-0.03	0.25	0.25	0.48	0.16	0.39	0.58	0.64	1.00
	0.54	0.08	0.87	0.13	0.14	<0.01	0.35	0.02	<0.01	<0.01	

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE C-I. (e) Correlations between each individual 3-OHFA with p-value: Swine (n = 40/35) [A]

	rFC	LAL	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	0.11	0.20	0.26	0.05	0.00	0.54	0.27	0.45	0.34	0.36
		0.48	0.25	0.13	0.76	1.00	<0.01	0.12	0.01	0.05	0.03
LAL	0.11	1.00	-0.21	0.11	-0.15	-0.21	0.09	-0.06	-0.07	-0.02	-0.04
	0.48		0.23	0.55	0.39	0.23	0.59	0.73	0.67	0.92	0.84
C8	0.20	-0.21	1.00	0.46	0.73	0.72	0.34	0.48	0.32	0.08	0.14
	0.25	0.23		0.01	<0.01	<0.01	0.05	<0.01	0.07	0.64	0.42
C9	0.26	0.11	0.46	1.00	0.46	0.25	0.09	0.18	0.06	-0.15	-0.13
	0.13	0.55	0.01		0.01	0.14	0.60	0.31	0.74	0.38	0.47
C10	0.05	-0.15	0.73	0.46	1.00	0.88	0.38	0.73	0.39	0.29	0.36
	0.76	0.39	<0.01	0.01		<0.01	0.02	<0.01	0.02	0.10	0.04
C12	0.00	-0.21	0.72	0.25	0.88	1.00	0.49	0.81	0.51	0.43	0.47
	1.00	0.23	<0.01	0.14	<0.01		<0.01	<0.01	<0.01	0.01	<0.01
C13	0.54	0.09	0.34	0.09	0.38	0.49	1.00	0.79	0.92	0.83	0.84
	<0.01	0.59	0.05	0.60	0.02	<0.01		<0.01	<0.01	<0.01	<0.01
C14	0.27	-0.06	0.48	0.18	0.73	0.81	0.79	1.00	0.78	0.79	0.84
	0.12	0.73	<0.01	0.31	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01
C15	0.45	-0.07	0.32	0.06	0.39	0.51	0.92	0.78	1.00	0.87	0.87
	0.01	0.67	0.07	0.74	0.02	<0.01	<0.01	<0.01		<0.01	<0.01
C17	0.34	-0.02	0.08	-0.15	0.29	0.43	0.83	0.79	0.87	1.00	0.96
	0.05	0.92	0.64	0.38	0.10	0.01	<0.01	<0.01	<0.01		<0.01
C18	0.36	-0.04	0.14	-0.13	0.36	0.47	0.84	0.84	0.87	0.96	1.00
	0.03	0.84	0.42	0.47	0.04	<0.01	<0.01	<0.01	<0.01	<0.01	

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE C-I. (f) Correlations between each individual 3-OHFA with p-value: Turkey (n = 157/158) [A]

	rFC	LAL	C8	C9	C10	C12	C13	C14	C15	C17	C18
rFC	1.00	0.47	0.28	0.15	0.39	0.42	0.59	0.49	0.10	0.08	0.19
		<0.01	<0.01	0.07	<0.01	<0.01	<0.01	<0.01	0.24	0.31	0.02
LAL	0.47	1.00	-0.07	-0.14	-0.07	-0.13	0.15	-0.04	-0.23	-0.18	-0.13
	<0.01		0.37	0.09	0.38	0.13	0.06	0.67	<0.01	0.02	0.10
C8	0.28	-0.07	1.00	0.62	0.52	0.49	0.50	0.58	0.52	0.42	0.46
	<0.01	0.37		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C9	0.15	-0.14	0.62	1.00	0.32	0.37	0.33	0.33	0.46	0.22	0.25
	0.07	0.09	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
C10	0.39	-0.07	0.52	0.32	1.00	0.68	0.60	0.62	0.43	0.46	0.52
	<0.01	0.38	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C12	0.42	-0.13	0.49	0.37	0.68	1.00	0.68	0.78	0.50	0.41	0.55
	<0.01	0.13	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01
C13	0.59	0.15	0.50	0.33	0.60	0.68	1.00	0.71	0.46	0.42	0.50
	<0.01	0.06	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01
C14	0.49	-0.04	0.58	0.33	0.62	0.78	0.71	1.00	0.62	0.49	0.64
	<0.01	0.67	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01
C15	0.10	-0.23	0.52	0.46	0.43	0.50	0.46	0.62	1.00	0.49	0.48
	0.24	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01
C17	0.08	-0.18	0.42	0.22	0.46	0.41	0.42	0.49	0.49	1.00	0.82
	0.31	0.02	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01
C18	0.19	-0.13	0.46	0.25	0.52	0.55	0.50	0.64	0.48	0.82	1.00
	0.02	0.10	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

APPENDIX D

ERGOSTEROL

(AREA SAMPLES USING PERSONAL SAMPLERS)

TABLE D-I. GM and GSD of ergosterol concentration for each dust type

	n	Ergosterol (ng/m^3)	
		GM	GSD
Chicken	91	11.66	5.59
Dairy	7	0.58	2.04
Horse	26	5.58	2.99
Swine	10	1.18	2.21
Turkey	60	7.83	5.02

TABLE D-II. Correlations between ergosterol concentration and dust or endotoxin concentrations by dust type with p-value

	Ergosterol vs. dust conc	Ergosterol vs. rFC/LAL ratio	Ergosterol vs. EU air		Ergosterol vs. EU dust	
			rFC	LAL	rFC	LAL
Overall	0.57	0.09	0.56	0.54	0.09	-0.06
(n=194/175)[A]	<0.01	0.25	<0.01	<0.01	0.02	0.44
Chicken	0.69	0.06	0.75	0.74	0.31	0.26
(n=91)	<0.01	0.54	<0.01	<0.01	<0.01	0.01
Dairy	0.67	-0.28	-0.01	0.23	0.72	0.22
(n=7/6)[A]	0.10	0.58	0.97	0.67	0.06	0.66
Horse	0.71	-0.66	0.77	0.47	-0.18	0.27
(n=26)	<0.01	0.05	<0.01	0.20	0.37	0.49
Swine	0.07	-0.33	-0.14	0.13	-0.26	-0.19
(n=10)	0.84	0.34	0.69	0.71	0.46	0.58
Turkey	0.38	0.29	0.12	-0.13	-0.02	-0.36
(n=60/59)[A]	<0.01	0.02	0.37	0.31	0.86	<0.01

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

TABLE D-III. Correlations between ergosterol concentration and dust or endotoxin concentrations by sampling device with p-value

	Ergosterol vs. dust conc	Ergosterol vs. rFC/LAL ratio	Ergosterol vs. EU air		Ergosterol vs. EU dust	
			rFC	LAL	rFC	LAL
Cassette	0.61	0.10	0.51	0.51	-0.09	-0.22
(n=49)	<0.01	0.48	<0.01	0.01	0.52	0.12
Cyclone	0.61	0.18	0.56	-0.05	-0.39	-0.47
(n=24/18)[A]	<0.01	0.52	<0.01	0.84	0.08	0.07
IOM	0.51	0.02	0.58	0.60	0.27	0.24
(n=64/57)[A]	<0.01	0.89	<0.01	<0.01	0.03	0.08
Button	0.67	0.16	0.64	0.66	-0.10	-0.25
(n=57/54)[A]	<0.01	0.25	<0.01	<0.01	0.47	0.06

[A] First n indicates rFC and second n indicates LAL sample sizes (n1/n2=rFC/LAL)

APPENDIX E

SUPPLEMENTAL INFORMATION FOR TASK-SPECIFIC EXPOSURE ASSESSMENT

(PERSONAL BREATH ZONE SAMPLES)

TABLE E-I. Combinations of tasks in each environment

Dairy		Feedlot		Grain Elevator		Farm	
Combination	**n**	**Combination**	**n**	**Combination**	**n**	**Combination**	**n**
1	1	4	3	3	1	2	1
4	2	12	2	4	2	3	2
9	1	13	7	10	3	12	2
12	1	1,13	1	11	1	13	3
13	3	2,9	1	12	3	12,14	1
14	2	4,13	3	14	1	3,13,14	1
3,8	1	7,8	1	1,4	1		
4,14	2	8,11	1	2,3	1		
2,12,14	1	11,12	1	3,10	1		
1,2,3,5,13	1	13,14	1	4,5	1		
		3,4,7	1	4,7	1		
		4,5,13	1	4,11	1		
		4,12,13	5	5,6	1		
		4,13,14	1	6,11	1		
		1,4,11,13	1	7,10	1		
		3,4,5,7	1	7,11	2		
		4,7,11,14	1	8,9	1		
		4,7,12,13	1	11,12	1		
		4,8,9,13	1	1,2,7	1		
		4,8,12,13	1	3,4,11	1		
		4,11,12,13	1	4,5,8	1		
		4,7,8,9,13	1	4,5,13	1		
		4,7,11,12,13,14	1	4,7,11	1		
		4,9,11,12,13,14	1	4,11,12	1		
		1,2,4,6,8,9,13	1	7,8,9	1		
		3,4,5,6,7,8,11	1	1,3,4,7	1		
		3,5,4,7,12,13,14	1	3,5,7,10	1		
		1,4,5,6,7,8,9, 10,11,13,14	2	3,6,7,11	1		
				3,7,11,12	1		
				4,5,7,11	1		
				4,5,12,14	1		
				4,5,13,14	1		
				4,7,9,10	1		
				4,7,11,12	1		
				4,8,9,10	1		
				7,8,11,12	1		
				1,5,6,11,14	1		
				3,4,5,9,11	1		
				4,6,7,8,9	1		
				7,8,9,10,14	1		
				1,2,3,4,11,14	1		
				3,4,7,8,9,10	1		
				4,6,7,9,10,11	1		
				1,4,5,6,7,11,14	1		
				3,4,5,7,8,9,14	1		
				4,5,6,7,11,12,14	1		
				4,6,7,8,9,10,11	1		

1. Truck harvest; 2. Combine harvest; 3. Storage; 4. Loading/unloading feed truck; 5. Sampling grain/feed; 6. Weighing grain/feed; 7. Running legs (in elevator); 8. Milling; 9. Mixing; 10. Bagging feed; 11. Housekeeping/cleaning; 12. Mechanical maintenance; 13. Feeding livestock; 14. Supervising.

TABLE E-II. (a) Regression model for dust (mg/m^3): multiple regression

	Regression coefficient (β)	Standard error	P-value
Dairy			
Truck harvest	0.163	0.167	0.33
Combine harvest	-0.061	1.738	0.97
Storage	0.459	0.281	0.11
Loading/unloading feed truck	0.063	0.100	0.53
Sampling grain/feed	-0.905	2.018	0.65
Mixing	0.091	0.136	0.51
Mechanical maintenance	0.099	0.125	0.43
Feeding livestock	0.111	0.072	0.13
Supervising	0.035	0.102	0.73
Feedlot			
Truck harvest	0.091	0.236	0.70
Combine harvest	0.257	0.596	0.67
Storage	0.487	0.499	0.33
Loading/unloading feed truck	0.108	0.081	0.18
Sampling grain/feed	-0.261	0.557	0.64
Weighing grain/feed	0.290	0.787	0.71
Running legs (in elevator)	0.011	0.290	0.97
Milling	0.268	0.164	0.10
Mixing	0.015	0.504	0.98
Housekeeping/cleaning	0.109	0.313	0.73
Mechanical maintenance	0.047	0.101	0.64
Feeding livestock	0.120	0.060	0.05
Supervising	0.048	0.116	0.68
GE			
Truck harvest	0.047	0.158	0.77
Combine harvest	-0.124	0.305	0.69
Storage	0.340	0.129	0.01
Loading/unloading feed truck	0.207	0.077	0.01
Sampling grain/feed	-0.213	0.318	0.51
Weighing grain/feed	0.518	0.300	0.09
Running legs (in elevator)	0.304	0.107	0.01
Milling	0.240	0.192	0.21
Mixing	0.220	0.187	0.24
Bagging feed	0.198	0.086	0.02
Housekeeping/cleaning	0.588	0.334	0.08
Mechanical maintenance	0.192	0.101	0.06
Feeding livestock	-0.056	0.291	0.85
Supervising	-0.069	0.111	0.54
Farm			
Combine harvest	0.015	0.137	0.91
Storage	0.593	0.219	0.01
Mechanical maintenance	0.320	0.216	0.14
Feeding livestock	0.139	0.120	0.25
Supervising	-0.131	0.327	0.69

Note: variables = environment×task; $\ln(mg/m^3) = \beta_1 X_1 + ... + \beta_k X_k$

TABLE E-II. (b) Regression model for dust (mg/m^3): stepwise regression

Step	Variables		Regression coefficient (β)	Standard error	p-value	Partial R^2	Model R^2
1	GE	Running legs (in elevator)	0.336	0.101	<0.01	0.206	0.206
2	GE	Housekeeping/cleaning	0.908	0.299	<0.01	0.069	0.275
3	Feedlot	Feeding livestock	0.161	0.053	<0.01	0.042	0.317
4	GE	Storage	0.305	0.119	0.01	0.038	0.355
5	Farm	Storage	0.601	0.214	0.01	0.031	0.386
6	GE	Loading/unloading feed truck	0.182	0.071	0.01	0.027	0.414
7	GE	Bagging feed	0.206	0.084	0.02	0.026	0.440
8	Feedlot	Milling	0.337	0.148	0.02	0.020	0.460
9	GE	Mixing	0.356	0.157	0.03	0.020	0.480

Note: variables = environment×task; $\ln(mg/m^3) = \beta_1 X_1 + ... + \beta_k X_k$

TABLE E-III. (a) Regression model for rFC results (EU/m^3): multiple regression

	Regression coefficient (β)	Standard error	P-value
Dairy			
Truck harvest	1.023	0.557	0.07
Combine harvest	4.181	5.802	0.47
Storage	1.917	0.938	0.04
Loading/unloading feed truck	0.684	0.335	0.04
Sampling grain/feed	-5.006	6.736	0.46
Mixing	0.713	0.455	0.12
Mechanical maintenance	0.494	0.417	0.24
Feeding livestock	0.747	0.241	0.00
Supervising	0.789	0.340	0.02
Feedlot			
Truck harvest	1.059	0.787	0.18
Combine harvest	3.130	1.991	0.12
Storage	0.200	1.665	0.90
Loading/unloading feed truck	0.636	0.270	0.02
Sampling grain/feed	0.147	1.859	0.94
Weighing grain/feed	2.004	2.627	0.45
Running legs (in elevator)	1.361	0.970	0.16
Milling	1.127	0.547	0.04
Mixing	-0.713	1.682	0.67
Housekeeping/cleaning	1.189	1.044	0.26
Mechanical maintenance	0.867	0.336	0.01
Feeding livestock	0.940	0.200	<.0001
Supervising	0.465	0.389	0.23
GE			
Truck harvest	0.777	0.527	0.14
Combine harvest	0.081	1.017	0.94
Storage	1.319	0.429	0.00
Loading/unloading feed truck	0.746	0.256	0.00
Sampling grain/feed	0.236	1.061	0.82
Weighing grain/feed	1.378	1.001	0.17
Running legs (in elevator)	1.225	0.358	0.00
Milling	0.568	0.643	0.38
Mixing	0.546	0.625	0.38
Bagging feed	0.662	0.286	0.02
Housekeeping/cleaning	1.123	1.114	0.32
Mechanical maintenance	0.870	0.338	0.01
Feeding livestock	0.865	0.971	0.38
Supervising	0.596	0.371	0.11
Farm			
Combine harvest	0.639	0.456	0.16
Storage	2.175	0.733	0.00
Mechanical maintenance	1.178	0.722	0.11
Feeding livestock	0.961	0.401	0.02
Supervising	0.017	1.091	0.99

Note: variables = environment×task; $\ln(EU/m^3) = \beta_1 X_1 + ... + \beta_k X_k$

TABLE E-III. (b) Regression model for rFC results (EU/m^3): stepwise regression

Step	Variables		Regression coefficient (β)	Standard error	p-value	Partial R^2	Model R^2
1	GE	Running legs (in elevator)	1.521	0.324	<0.01	0.154	0.154
2	Feedlot	Feeding livestock	0.993	0.197	<0.01	0.141	0.295
3	GE	Storage	1.483	0.395	<0.01	0.052	0.346
4	Feedlot	Loading/unloading feed truck	0.660	0.266	<0.01	0.044	0.390
5	GE	Loading/unloading feed truck	0.848	0.241	<0.01	0.036	0.426
6	GE	Mechanical maintenance	0.986	0.317	<0.01	0.026	0.453
7	Dairy	Feeding livestock	0.756	0.243	<0.01	0.026	0.479
8	Farm	Storage	2.176	0.734	<0.01	0.026	0.504
9	Dairy	Supervising	0.881	0.333	0.01	0.022	0.526
10	Feedlot	Milling	1.247	0.515	0.02	0.020	0.547
11	Feedlot	Mechanical maintenance	0.942	0.328	<0.01	0.020	0.567
12	Feedlot	Running legs (in elevator)	1.665	0.675	0.02	0.016	0.583
13	GE	Weighing grain/feed	1.741	0.759	0.02	0.015	0.598
14	Farm	Feeding livestock	0.962	0.404	0.02	0.015	0.613
15	Feedlot	Combine harvest	2.444	1.066	0.02	0.014	0.626
16	GE	Bagging feed	0.656	0.287	0.02	0.014	0.640
17	Dairy	Storage	2.054	0.903	0.02	0.013	0.653
18	Farm	Mechanical maintenance	1.185	0.567	0.04	0.011	0.665
19	GE	Supervising	0.744	0.360	0.04	0.011	0.676
20	Dairy	Loading/unloading feed truck	0.672	0.337	0.05	0.010	0.686

Note: variables = environment×task; $\ln(EU/m^3) = \beta_1 X_1 + ... + \beta_k X_k$

TABLE E-IV. (a) Regression model for GC/MS results (pmol/m^3): multiple regression

	Regression coefficient (β)	Standard error	P-value
Dairy			
Truck harvest	1.106	0.585	0.06
Combine harvest	3.534	6.094	0.56
Storage	1.797	0.985	0.07
Loading/unloading feed truck	0.860	0.351	0.02
Sampling grain/feed	-4.691	7.075	0.51
Mixing	0.812	0.478	0.09
Mechanical maintenance	0.637	0.438	0.15
Feeding livestock	0.782	0.254	0.00
Supervising	0.848	0.358	0.02
Feedlot			
Truck harvest	1.042	0.828	0.21
Combine harvest	2.278	2.832	0.42
Storage	0.514	1.795	0.78
Loading/unloading feed truck	0.756	0.292	0.01
Sampling grain/feed	0.094	1.958	0.96
Weighing grain/feed	5.634	7.606	0.46
Running legs (in elevator)	0.942	1.123	0.40
Milling	1.160	2.180	0.60
Mixing	-0.105	2.605	0.97
Housekeeping/cleaning	1.372	1.149	0.24
Mechanical maintenance	0.962	0.353	0.01
Feeding livestock	1.080	0.212	<.0001
Supervising	0.796	0.446	0.08
GE			
Truck harvest	0.684	0.553	0.22
Combine harvest	1.274	1.068	0.24
Storage	1.161	0.451	0.01
Loading/unloading feed truck	0.859	0.269	0.00
Sampling grain/feed	0.561	1.115	0.62
Weighing grain/feed	1.595	1.052	0.13
Running legs (in elevator)	1.167	0.376	0.00
Milling	0.903	0.675	0.18
Mixing	1.004	0.657	0.13
Bagging feed	0.897	0.300	0.00
Housekeeping/cleaning	0.984	1.170	0.40
Mechanical maintenance	1.020	0.356	0.01
Feeding livestock	1.473	1.020	0.15
Supervising	0.781	0.389	0.05
Farm			
Combine harvest	0.900	0.479	0.06
Storage	2.230	0.770	0.00
Mechanical maintenance	1.804	0.758	0.02
Feeding livestock	1.110	0.421	0.01
Supervising	0.178	1.146	0.88

Note: variables = environment×task; $\ln(\text{pmol/m}^3) = \beta_1 X_1 + \ldots + \beta_k X_k$

TABLE E-IV. (b) Regression model for GC/MS results (pmol/m^3): stepwise regression

Step	Variables		Regression coefficient (β)	Standard error	p-value	Partial R^2	Model R^2
1	Feedlot	Feeding livestock	1.158	0.216	<0.01	0.147	0.147
2	GE	Running legs (in elevator)	1.321	0.362	<0.01	0.144	0.291
3	Feedlot	Loading/unloading feed truck	0.863	0.296	<0.01	0.049	0.340
4	GE	Storage	1.475	0.431	<0.01	0.048	0.387
5	GE	Loading/unloading feed truck	0.971	0.263	<0.01	0.042	0.429
6	GE	Mechanical maintenance	1.104	0.345	<0.01	0.028	0.457
7	Farm	Mechanical maintenance	1.878	0.619	<0.01	0.024	0.481
8	Dairy	Feeding livestock	0.785	0.265	<0.01	0.024	0.505
9	Farm	Storage	2.243	0.801	0.01	0.023	0.528
10	Dairy	Supervising	0.939	0.363	0.01	0.022	0.550
11	GE	Bagging feed	0.870	0.313	0.01	0.022	0.572
12	Feedlot	Mechanical maintenance	1.066	0.358	<0.01	0.022	0.594
13	GE	Weighing grain/feed	2.108	0.828	0.01	0.020	0.614
14	GE	Supervising	0.981	0.393	0.01	0.017	0.631
15	Farm	Feeding livestock	1.113	0.441	0.01	0.017	0.648
16	GE	Milling	1.418	0.598	0.02	0.015	0.662
17	Dairy	Loading/unloading feed truck	0.847	0.368	0.02	0.014	0.676
18	Feedlot	Running legs (in elevator)	1.646	0.754	0.03	0.012	0.688
19	Dairy	Truck harvest	1.149	0.563	0.04	0.011	0.699

Note: variables = environment×task; $\ln(\text{pmol/m}^3) = \beta_1 X_1 + \ldots + \beta_k X_k$

TABLE E-V. (a) 8-hour predicted values: dust (mg/m^3)

		Predicted value	95% LL	95% UL
Dairy	Truck harvest	3.69	0.26	52.18
	Combine harvest	0.61	0.00	5.83E+11
	Storage	39.28	0.46	3387.00
	Loading/unloading feed truck	1.65	0.34	8.11
	Sampling grain/feed	0.00	0.00	5.79E+10
	Mixing	2.07	0.24	18.04
	Mechanical maintenance	2.20	0.30	15.94
	Feeding livestock	2.42	0.77	7.63
	Supervising	1.32	0.26	6.67
Feedlot	Truck harvest	2.07	0.05	87.22
	Combine harvest	7.83	0.00	1.01E+05
	Storage	49.13	0.02	1.34E+05
	Loading/unloading feed truck	2.37	0.66	8.54
	Sampling grain/feed	0.12	0.00	856.97
	Weighing grain/feed	10.21	0.00	2.71E+06
	Running legs (in elevator)	1.09	0.01	109.88
	Milling	8.55	0.64	115.13
	Mixing	1.13	0.00	3365.06
	Housekeeping/cleaning	2.40	0.02	342.58
	Mechanical maintenance	1.46	0.30	7.21
	Feeding livestock	2.60	1.01	6.73
	Supervising	1.47	0.23	9.31
GE	Truck harvest	1.45	0.12	17.73
	Combine harvest	0.37	0.00	46.86
	Storage	15.23	1.98	117.26
	Loading/unloading feed truck	5.24	1.55	17.73
	Sampling grain/feed	0.18	0.00	28.33
	Weighing grain/feed	63.09	0.54	7362.10
	Running legs (in elevator)	11.38	2.08	62.25
	Milling	6.84	0.32	145.15
	Mixing	5.82	0.30	113.70
	Bagging feed	4.86	1.25	18.89
	Housekeeping/cleaning	110.81	0.56	2.21E+04
	Mechanical maintenance	4.63	0.93	23.14
	Feeding livestock	0.64	0.01	64.41
	Supervising	0.58	0.10	3.35
Farm	Combine harvest	1.13	0.13	9.88
	Storage	114.80	3.53	3736.48
	Mechanical maintenance	12.96	0.42	400.17
	Feeding livestock	3.03	0.45	20.44
	Supervising	0.35	0.00	62.68

TABLE E-V. (b) 8-hour predicted values: rFC (EU/m^3)

		Predicted value	95% LL	95% UL
Dairy	Truck harvest	3589.26	0.52	2.49E+07
	Combine harvest	3.35E+14	0.00	3.31E+54
	Storage	4.59E+06	1.58	1.33E+13
	Loading/unloading feed truck	238.72	1.18	4.83E+04
	Sampling grain/feed	0.00	0.00	1.10E+29
	Mixing	300.82	0.22	4.13E+05
	Mechanical maintenance	51.83	0.07	3.86E+04
	Feeding livestock	392.80	8.51	1.81E+04
	Supervising	551.48	2.48	1.23E+05
Feedlot	Truck harvest	4769.52	0.02	1.27E+09
	Combine harvest	7.47E+10	0.00	3.94E+24
	Storage	4.93	0.00	1.48E+12
	Loading/unloading feed truck	162.63	2.25	1.17E+04
	Sampling grain/feed	3.25	0.00	2.13E+13
	Weighing grain/feed	9.18E+06	0.00	1.18E+25
	Running legs (in elevator)	5.37E+04	0.01	2.59E+11
	Milling	8238.72	1.40	4.85E+07
	Mixing	0.00	0.00	1.32E+09
	Housekeeping/cleaning	1.35E+04	0.00	2.12E+11
	Mechanical maintenance	1025.16	4.97	2.12E+05
	Feeding livestock	1849.18	77.62	4.41E+04
	Supervising	41.36	0.09	1.98E+04
GE	Truck harvest	500.00	0.12	2.13E+06
	Combine harvest	1.91	0.00	1.96E+07
	Storage	3.81E+04	41.79	3.48E+07
	Loading/unloading feed truck	391.00	6.67	2.29E+04
	Sampling grain/feed	6.59	0.00	1.37E+08
	Weighing grain/feed	6.15E+04	0.01	4.90E+11
	Running legs (in elevator)	1.81E+04	62.01	5.27E+06
	Milling	94.02	0.00	2.53E+06
	Mixing	79.11	0.00	1.61E+06
	Bagging feed	199.12	2.14	1.85E+04
	Housekeeping/cleaning	7980.85	0.00	3.82E+11
	Mechanical maintenance	1055.43	4.90	2.27E+05
	Feeding livestock	1010.40	0.00	4.96E+09
	Supervising	118.07	0.33	4.24E+04
Farm	Combine harvest	165.75	0.12	2.31E+05
	Storage	3.59E+07	320.15	4.03E+12
	Mechanical maintenance	1.23E+04	0.13	1.16E+09
	Feeding livestock	2189.22	3.75	1.28E+06
	Supervising	1.15	0.00	3.80E+07

TABLE E-V. (c) 8-hour predicted values: GC/MS (pmol/m^3)

		Predicted value	95% LL	95% UL
Dairy	Truck harvest	6983.46	0.64	7.63E+07
	Combine harvest	1.90E+12	0.00	2.08E+54
	Storage	1.75E+06	0.28	1.09E+13
	Loading/unloading feed truck	968.84	3.65	2.57E+05
	Sampling grain/feed	0.00	0.00	3.25E+32
	Mixing	660.96	0.33	1.31E+06
	Mechanical maintenance	162.86	0.16	1.70E+05
	Feeding livestock	520.56	9.27	2.92E+04
	Supervising	884.75	3.02	2.59E+05
Feedlot	Truck harvest	4187.67	0.01	2.14E+09
	Combine harvest	8.19E+07	0.00	2.82E+27
	Storage	61.05	0.00	1.47E+14
	Loading/unloading feed truck	424.62	4.08	4.42E+04
	Sampling grain/feed	2.13	0.00	6.86E+13
	Weighing grain/feed	3.75E+19	0.00	1.11E+72
	Running legs (in elevator)	1877.69	0.00	1.05E+11
	Milling	1.07E+04	0.00	1.18E+19
	Mixing	0.43	0.00	4.03E+17
	Housekeeping/cleaning	5.85E+04	0.00	4.91E+12
	Mechanical maintenance	2202.39	8.03	6.04E+05
	Feeding livestock	5648.81	194.47	1.64E+05
	Supervising	584.12	0.49	6.98E+05
GE	Truck harvest	238.32	0.04	1.56E+06
	Combine harvest	2.67E+04	0.00	6.25E+11
	Storage	1.08E+04	8.33	1.39E+07
	Loading/unloading feed truck	962.95	13.35	6.95E+04
	Sampling grain/feed	88.63	0.00	4.35E+09
	Weighing grain/feed	3.48E+05	0.02	6.26E+12
	Running legs (in elevator)	1.13E+04	29.03	4.41E+06
	Milling	1376.09	0.03	6.24E+07
	Mixing	3077.58	0.09	1.04E+08
	Bagging feed	1302.45	11.11	1.53E+05
	Housekeeping/cleaning	2616.26	0.00	3.09E+11
	Mechanical maintenance	3484.22	12.29	9.88E+05
	Feeding livestock	1.31E+05	0.01	1.42E+12
	Supervising	516.36	1.06	2.50E+05
Farm	Combine harvest	1339.70	0.66	2.70E+06
	Storage	5.60E+07	275.12	1.14E+13
	Mechanical maintenance	1.86E+06	10.97	3.14E+11
	Feeding livestock	7172.43	8.88	5.79E+06
	Supervising	4.14	0.00	3.33E+08

TABLE E-VI. 8-hour predicted values for task (overall)

	Dust (mg/m^3)	rFC (EU/m^3)	GC/MS ($Pmol/m^3$)
Truck harvest	1.92	1303.23	1297.64
Combine harvest	0.94	243.28	2238.81
Storage	18.23	3.78E+04	2.68E+04
Loading/unloading feed truck	2.83	267.71	831.14
Sampling grain/feed	0.13	0.71	2.60
Weighing grain/feed	78.99	1.68E+05	4.26E+06
Running legs (in elevator)	11.33	6905.68	4251.38
Milling	5.98	427.09	980.73
Mixing	3.42	227.35	1464.98
Bagging feed	4.95	196.96	1314.09
Housekeeping/cleaning	21.99	1.84E+04	9306.79
Mechanical maintenance	2.87	604.98	2537.67
Feeding livestock	2.30	1033.70	2558.56
Supervising	0.99	172.26	903.88

TABLE E-VII. 8-hour predicted values for each environment (no task)

	Dust (mg/m^3)	rFC (EU/m^3)	GC/MS ($Pmol/m^3$)
Dairy	2.97	946.53	1384.58
Feedlot	2.52	1094.33	2666.31
GE	4.68	668.81	1967.86
Farm	2.64	473.05	2176.49

TABLE E-VIII. Rank order of 8-hour predicted value for each environment (no task)

	Dust (mg/m^3)	rFC (EU/m^3)	GC/MS ($Pmol/m^3$)
Lowest	Feedlot	Farm	Dairy
	Farm	GE	GE
	Dairy	Dairy	Farm
Highest	GE	Feedlot	Feedlot

TABLE E-IX. Correlations between tasks and years in job (n = 169)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Truck harvest	1.00	0.04 0.63	-0.03 0.67	-0.07 0.37	0.02 0.82	-0.03 0.73	-0.01 0.93	-0.08 0.30	-0.06 0.41	-0.05 0.52	-0.04 0.62	-0.10 0.21	-0.03 0.65	-0.02 0.80	0.01 0.88
2. Combine harvest		1.00	0.06 0.44	-0.10 0.21	-0.02 0.79	-0.03 0.65	-0.06 0.41	-0.05 0.51	0.06 0.41	-0.03 0.67	-0.04 0.56	-0.05 0.51	-0.08 0.33	-0.02 0.76	0.02 0.85
3. Storage			1.00	-0.13 0.09	0.00 0.98	-0.03 0.66	0.03 0.72	0.00 0.95	-0.06 0.45	0.02 0.78	-0.02 0.81	-0.10 0.22	-0.15 0.05	-0.09 0.24	0.06 0.46
4. Loading/unloading feed truck				1.00	0.04 0.62	-0.07 0.38	0.01 0.90	-0.10 0.18	-0.11 0.16	-0.12 0.12	-0.08 0.32	-0.13 0.08	-0.11 0.15	-0.14 0.07	0.07 0.36
5. Sampling grain/feed					1.00	0.33 <0.01	0.02 0.80	0.04 0.57	-0.02 0.82	-0.05 0.50	-0.05 0.52	-0.09 0.24	-0.10 0.18	0.01 0.88	0.03 0.68
6. Weighing grain/feed						1.00	0.06 0.43	0.00 0.98	0.01 0.89	-0.02 0.76	0.12 0.12	-0.08 0.31	-0.10 0.18	-0.05 0.55	-0.03 0.67
7. Running legs (in elevator)							1.00	0.06 0.43	0.02 0.83	0.03 0.71	0.14 0.07	-0.14 0.07	-0.23 <0.01	-0.09 0.25	0.03 0.67
8. Milling								1.00	0.20 0.01	-0.03 0.65	-0.02 0.84	-0.11 0.17	-0.13 0.09	-0.08 0.30	0.05 0.51
9. Mixing									1.00	-0.02 0.78	-0.02 0.81	-0.12 0.13	-0.11 0.15	-0.08 0.31	0.00 0.96
10. Bagging feed										1.00	-0.07 0.39	-0.08 0.31	-0.10 0.17	-0.07 0.38	-0.12 0.14
11. Housekeeping /cleaning											1.00	0.12 0.11	-0.13 0.08	-0.07 0.33	-0.06 0.41
12. Mechanical maintenance												1.00	-0.13 0.09	-0.05 0.49	0.08 0.30
13. Feeding livestock													1.00	-0.14 0.08	-0.17 0.03
14. Supervising														1.00	0.12 0.11
15. Years in job															1.00

APPENDIX F

FLOWCHARTS OF EXPERIMENTAL PROCEDURE

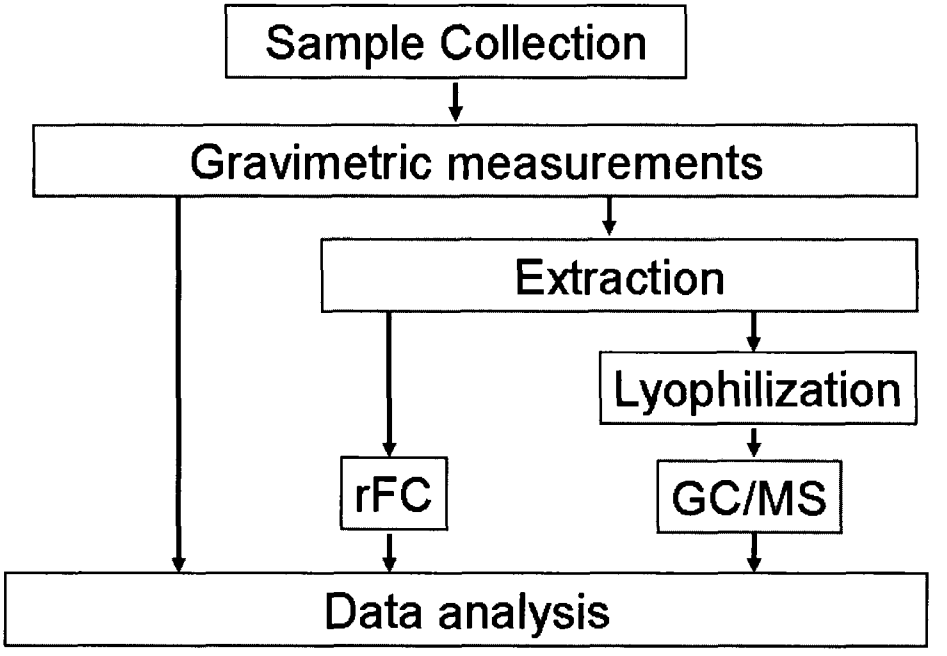


Figure F-1. Flowchart of sampling and experimental procedure for chapter 2 and 4.

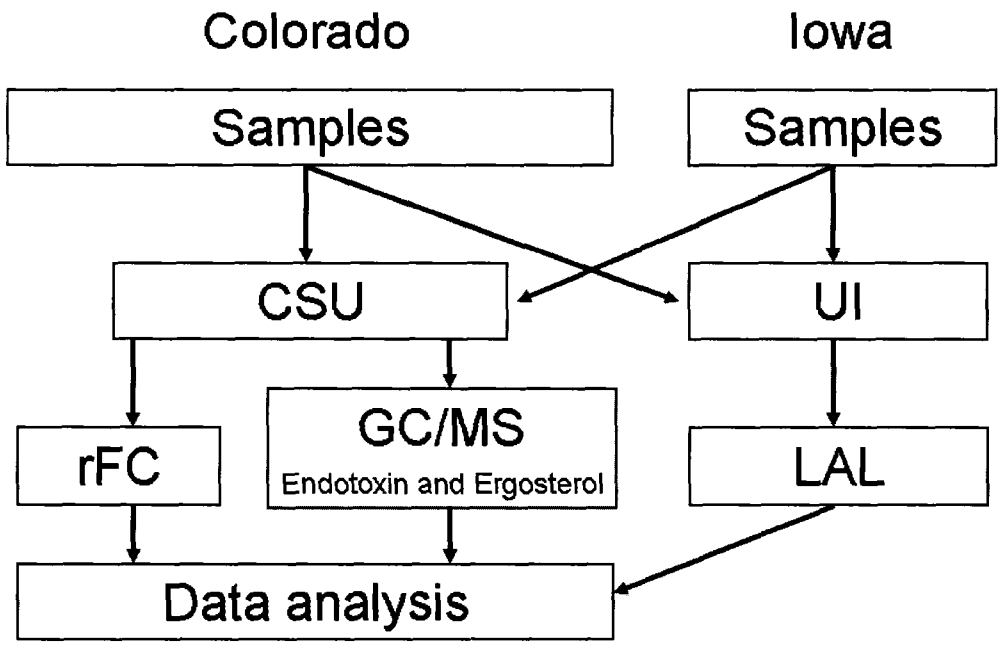


Figure F-2. Flowchart of sampling and experimental procedure for chapter 3.

APPENDIX G

GAS CHROMATOGRAPHY/ MASS SPECTROMETRY FOR ENDOTOXIN:

SUPPORTING INFORMATION

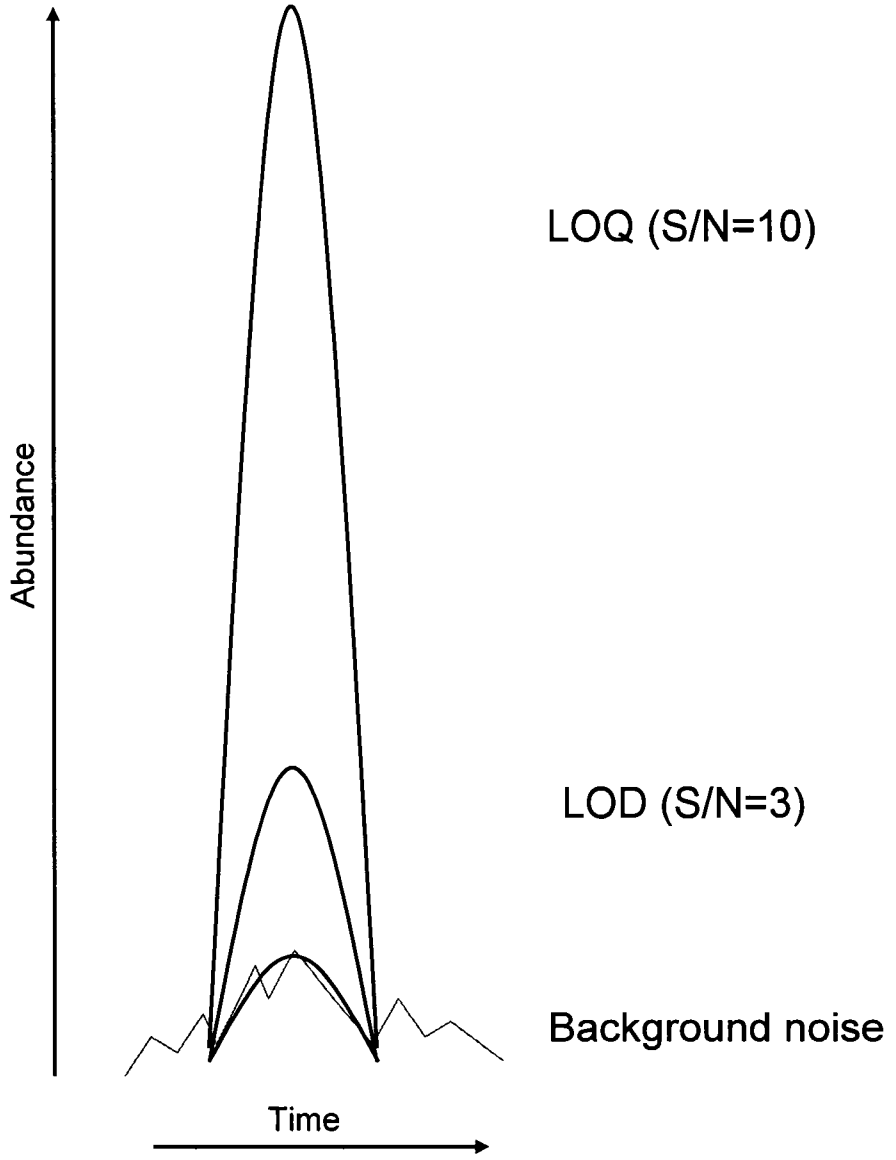
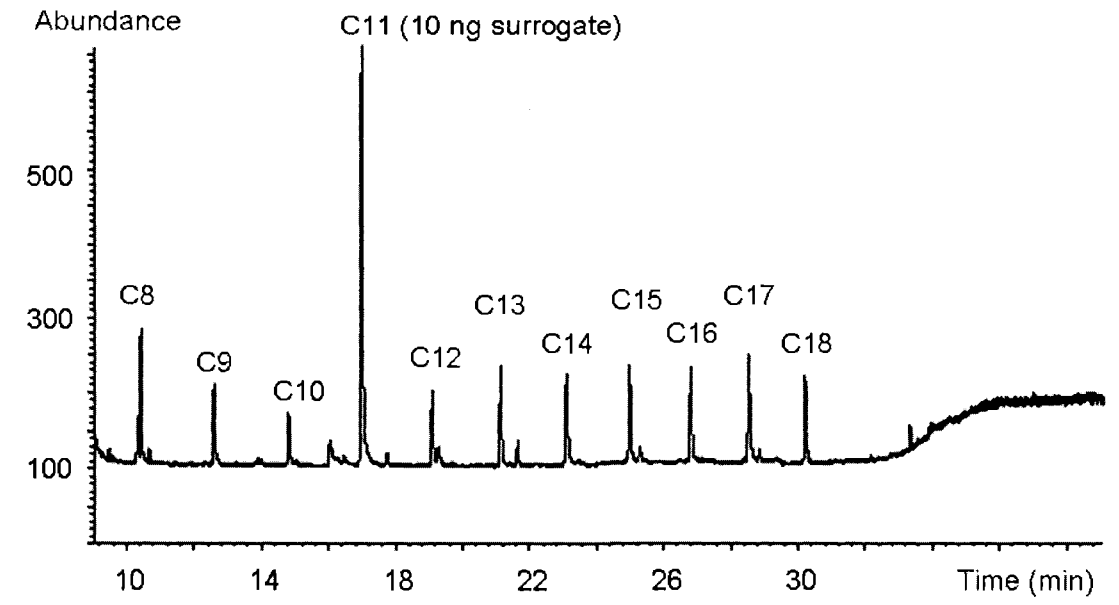


Figure G-1. Limit of detection (LOD) and limit of quantification (LOQ) by signal-to-noise ratio (S/N). In our study, 1 ng spike level was equivalent to LOQ (S/N > 10) based on the chromatogram; thus, 0.33 ng spike level thought to be LOD. However, spiking 0.33 ng standard was practically difficult; therefore, 0.5 ng spike level was used. LOD was confirmed at 0.5 ng spike level.

(a) 2 ng standard



(b) Field sample

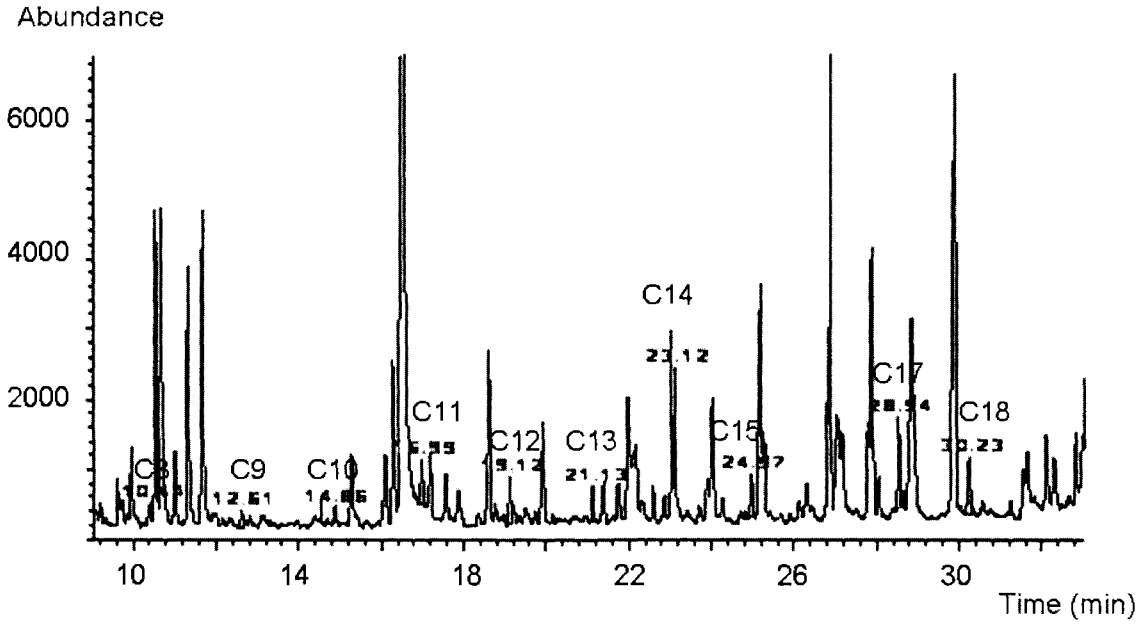


Figure G-2. Sample chromatograms of (a) 2 ng standard and (b) field sample. Peaks in samples were identified by using retention time and *m/z* of each 3-OHFA.

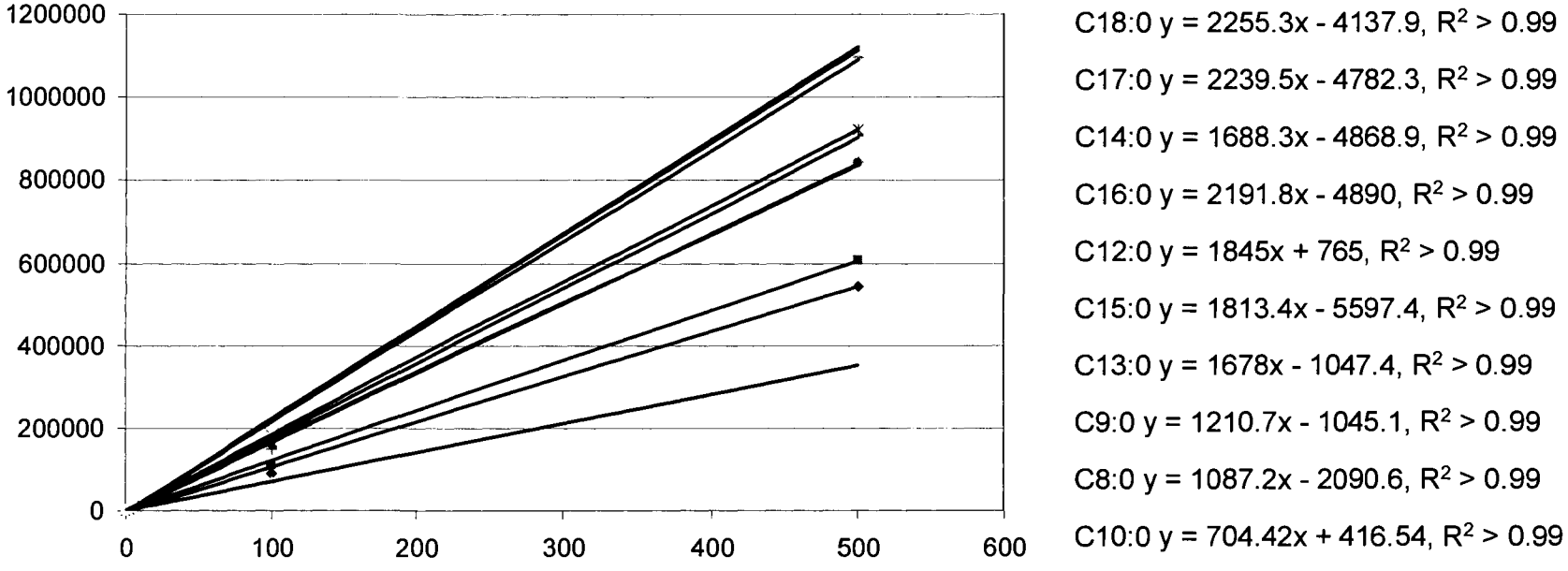


Figure G-3. Sample calibration curves for each 3-OHFA. Calibration curves were constructed using 2, 6, 20, 100, and 500 ng of individual 3-OHFAs (mixture of C8:0 to C18:0 3-OHFAs except C11:0 3-OHFA in ethanol and stored at -20 °C) for each run.

TABLE G-I. List of 3-OHFAs

Name	Molecular formula	Molecular weight
C8:0 3-OHFA	$C_8H_{16}O_3$	160.0
C9:0 3-OHFA	$C_9H_{18}O_3$	174.3
C10:0 3-OHFA	$C_{10}H_{20}O_3$	188.2
C11:0 3-OHFA	$C_{11}H_{22}O_3$	202.3
C12:0 3-OHFA	$C_{12}H_{24}O_3$	216.3
C13:0 3-OHFA	$C_{13}H_{26}O_3$	230.3
C14:0 3-OHFA	$C_{14}H_{28}O_3$	244.4
C15:0 3-OHFA	$C_{15}H_{30}O_3$	258.4
C16:0 3-OHFA	$C_{16}H_{32}O_3$	272.4
C17:0 3-OHFA	$C_{17}H_{34}O_3$	286.4
C18:0 3-OHFA	$C_{18}H_{36}O_3$	300.5

TABLE G-II. Summary of GC/MS method for endotoxin measurements

Original GC/MS-MS sample preparation method	**Modified GC/EI-MS sample preparation method**
Method	
- Require liquid-liquid extraction prior to solid-phase extraction (SPE)	- Eliminated liquid-liquid extraction due to the change in SPE cartridges
- Use silica cartridge for SPE and must avoid water (too polar for silica cartridge)	- Use polymeric reversed-phase SPE cartridge, which allow using water (handle a wide range of polarity and pH)
- 1:1 Pentane:Dichloromethane for sample load	- Deionized water for sample load
Results	
- Comparable results between two methods (peak area) - The modified method yielded better intra-day reproducibility (CV = 6%, n = 4) than the original method (CV = 16%, n = 3)	

TABLE G-III. GC parameters

Oven program	90 °C initial temperature, 5 °C/min to 250 °C, and then 20 °C/min to 290 °C. Hold for 5 min.
Inlet temperature	280 °C
Ion source temperature	180 °C
Transfer line temperature	300 °C
Total retention time	39 min

TABLE G-IV. Equipment models

Equipment	**Model**	**Manufacturer**
Lyophilizer	Benchtop 6.6 Freeze Dryer (S/N 211563)	VisTis Company
Scale	BP210D (S/N 51008616)	Sercom
Nitrogen evaporator	N-Evap Model 105	Organomation Association
Block heater	Multi-blok heater No.2093	Lab-line Instrument
Pump	GE MODEL 5KH36KNA5 10X (HP1/4 RPM 1725/1425)	General Electric Motor
GC	GC5890 Series II Plus (S/N 3336A51216)	Hewlett-Packard
MSD	MSD5972 (S/N 3626A03684)	Hewlett-Packard
Column (for GC/MS)	HP-5MS (Part number 19091A-433)	Hewlett-Packard