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Recombinant Factor C (rFC) Assay and Gas Chromatography/Mass Spectrometry (GC/MS) Analysis of Endotoxin Variability in Four Agricultural Dusts

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Endotoxin exposure is a significant concern in agricultural environments due to relatively high exposure levels. The goals of this study were to determine patterns of 3-hydroxy fatty acid (3-OHFA) distribution in dusts from four types of agricultural environments (dairy, cattle feedlot, grain elevator, and corn farm) and to evaluate correlations between the results of gas chromatography/mass spectrometry (GC/MS) analysis (total endotoxin) and biological recombinant factor C (rFC) assay (free bioactive endotoxin). An existing GC/MS-MS method (for house dust) was modified to reduce sample handling and optimized for small amount (<1 mg) of agricultural dusts using GC/EI-MS. A total of 134 breathing zone samples using Institute of Occupational Medicine (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. The rank order of Pearson correlations between the biological rFC assay and the modified GC/EI-MS results was feedlot (0.72) > dairy (0.53) > corn farm (0.33) > grain elevator (0.11). In livestock environments, both odd- and even-numbered carbon chain length 3-OHFAs correlated with rFC assay response. 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.

Keywords: agriculture; endotoxins; lipopolysaccharides; organic dust

INTRODUCTION

Endotoxins (or lipopolysaccharides, LPS) are cell membrane components of Gram-negative bacteria and play an important role in occupational lung diseases. There is significant concern with 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 (Donham *et al.*, 1995, 2000; Reynolds *et al.*, 1996; Liebers *et al.*, 2008).

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There are two approaches to measuring endotoxin: biological assay and chemical analysis. The most commonly used biological assay, Limulus amebocyte lysate (LAL), measures the relative reactivity of endotoxins with Limulus lysate, providing rapid and sensitive results (Morrison *et al.*, 1987; Takada *et al.*, 1988). However, bioassays may underestimate endotoxin exposure because cell-bound endotoxins that may be associated with respiratory disease are not detected (Alwis and Milton, 2006). The newly developed recombinant factor C (rFC) bioassay operates on the same basic principle as the previous LAL assay, but may provide greater sensitivity and specificity and less variability.

Gas chromatography/mass spectrometry (GC/MS) analysis focuses on quantification of biomarkers of endotoxins, 3-hydroxy fatty acids (3-OHFAs) in the lipid A portion of LPS. Unlike bioassays, GC/MS analysis of 3-OHFAs allows determination of both cell-bound and non-cell-bound endotoxins since the GC/MS methods chemically isolate 3-OHFAs from lipid A (Sonesson *et al.*, 1990; Reynolds *et al.*, 2005). In addition, GC/MS provides information about the chemical composition of endotoxins. GC/MS methods require a longer sample preparation time with smaller sample series, are more labor intensive, and are relatively expensive comparing with bioassays, but understanding the chemistry of endotoxins may be important in explaining disease pathology, and ultimately, for interventions.

A number of studies have applied GC/MS for endotoxin analysis (Saraf *et al.*, 1997, 1999; Sebastian and Larsson, 2003); however, many were not directed at agricultural dusts. The original chemical analysis method was developed as a tool for assessing air quality in a poultry house by using chemical ionization mass spectrometry (GC/CI-MS) and then modified for tandem mass spectrometry (GC/MS-MS) using house dusts (Sonesson *et al.*, 1990; Saraf and Larsson, 1996). GC/MS-MS uses ion-trap or triplequadrupole technology to reduce background signal, providing more sensitive results than widely available electron ionization mass spectrometry (GC/EI-MS); however, GC/MS-MS may not be available in most facilities.

Our preliminary studies with agricultural dusts involved the 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. In this study, we modified a GC/MS-MS method developed for house dusts for GC/EI-MS analysis of small amount (<1 mg) of agricultural dusts, collected by personal air sampling. 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 and the first to evaluate endotoxin in cattle feedlots.

METHODS

Dust sample collection and preparation of samples

A total of 134 unique personal breathing zone samples using Institute of Occupational Medicine (IOM) inhalable samplers were collected in four agricultural environments in Colorado and Nebraska: dairy farms ($n = 17$ workers), cattle feedlots ($n = 48$ workers), grain elevators ($n = 58$ workers), and corn farms ($n = 11$ workers). This study was approved by the Colorado State University's institutional review board for human subject protection. Samples were collected in 2004–2006, during all four seasons. IOM inhalable samplers used 5 μm pore size polyvinyl chloride filters (SKC Inc., Eighty Four, PA, USA), at a flow rate of 2 l min^{-1} over 6–8 h during typical work shifts. All samples and blanks were weighed using a Mettler MT balance (Mettler Toledo Inc., Columbus, OH, USA). Collected dust samples were extracted in sterile, pyrogen-free (pf) water containing 0.05% Tween 20 for 1 h 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. Endotoxin and 3-OHFA results per mg dust were calculated based on the personal air sampling.

Materials for GC/EI-MS analysis

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

GC/EI-MS analysis

In this study, the GC/MS-MS method developed by Saraf and Larsson (1996) for house dust was modified for our small amount (<1 mg) of agricultural dust, collected by personal air sampling, using GC/EI-MS (see Fig. 1 for modification). The geometric mean (GM) of dust amount used for GC/EI-MS analysis was 0.60 mg with geometric standard deviation (GSD) of 0.60. C8:0 to C18:0 3-OHFA standards were dissolved in ethanol (1 μg ml^{-1}) and stored at −20°C. Samples and external 3-OHFA standards of 8–18 carbon chain lengths (except C11:0, method surrogate—see below) 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 16–18 h at

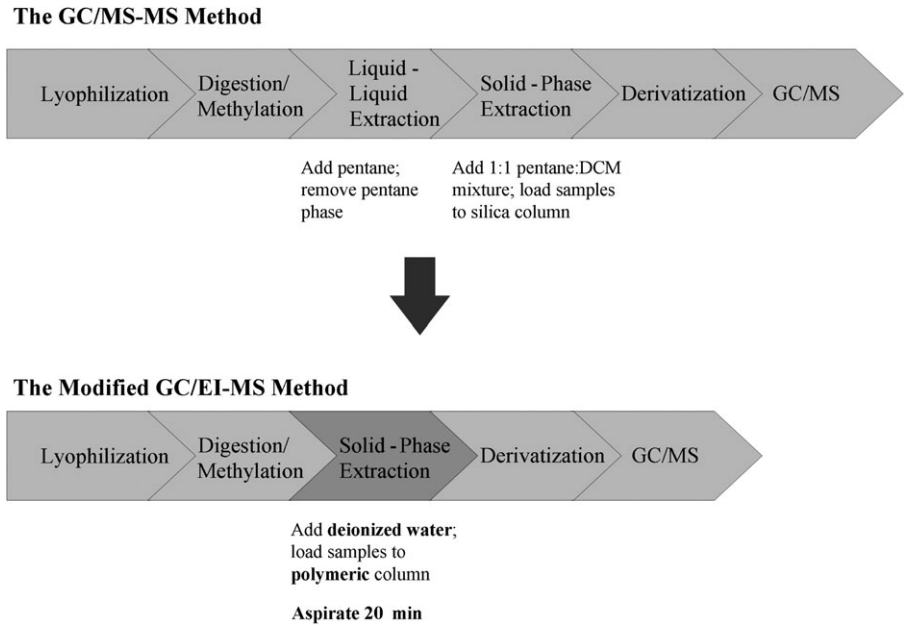

Fig. 1. Changes in experimental procedures for GC/EI-MS analysis.

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) cleanup. For SPE cleanup, samples were applied to a 60 mg per 3 ml Strata-X polymeric reversed phase cartridge. Cartridges were conditioned with 1 ml diethyl ether and then with 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, USA) with split/splitless inlet, electronic pressure control, 7673 automatic liquid sampler, and a HP 5972 Mass Selective Detector. Selected ion monitoring for individual 3-OHFA was used for endotoxins and the result 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.

Figure 1 shows the flowchart of the modified GC/EI-MS method and the existing GC/MS-MS method (Saraf and Larsson, 1996). The major changes in the modified method are elimination of liquid–liquid extraction, use of polymeric SPE instead of silica cartridge for sample cleanup, and use of deionized water instead of 1:1 pentane: dichloromethane (DCM) mixture for sample loading to SPE. The elimination of liquid–liquid extraction reduces sample handling and cost and obviates the need for pentane and DCM.

Calibration curves and method performance for GC/EI-MS

Quality control was implemented at two levels in this study; calibration and surrogate recovery. Calibration was accomplished via laboratory fortified matrix blanks covering 3-OHFA levels anticipated in samples. This method allowed assessment of recovery as a function of fortification level for each 3-OHFA standard. C11:0 3-OHFA was used as a surrogate as defined by the International Union of Pure and Applied Chemistry in Harmonized Guidelines for the use of Recovery Information in Analytical Measurement (Thompson *et al.*, 1999). While chemically similar to investigated analytes, it was not found in the agricultural dust samples analyzed in this study. The C11:0 3-OHFA surrogate, which served to monitor analyte recovery variability in the lengthy sample processing procedure, was added to all standards and samples. The reproducibility of

C11:0 3-OHFA surrogate recovery was satisfied based on a <12% coefficient of variation ($n = 24$) since this method contained two derivatization steps including one overnight reaction. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined by signal-to-noise (S/N) ratio based on the chromatograms of controls and 0.5 and 1 ng spikes (S/N > 3 for LOD and >10 for LOQ) (ACS Committee on Environmental Improvement, 1980).

rFC assay

Extracted samples were analyzed using the rFC endotoxin assay (Cambrex, East Rutherford, NJ, USA). The rFC assay method for endotoxin detection uses rFC, the first component of the cascade to quantify potency in endotoxin units (EU) relative to a standard endotoxin (Alwis and Milton, 2006). The activation of rFC was determined by fluorescence generated by the enzymatic cleavage of a peptide-coumarin substrate. Fluorescence was measured after 1 h incubation with endotoxin standards at 37°C. Log fluorescence was proportional to log endotoxin concentration and was linear in the 0.01–10 EU ml^{-1} 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. Our previous study indicated that use of 0.05% Tween 20 resulted in optimal spike recovery and reproducibility for the rFC assay (Reynolds *et al.*, 2005 and S. Reynolds, unpublished data). 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 1 h and read in a fluorescence microtiter plate reader (Biotek Instruments, Winooski, VT, USA; FLX800TBIE) at excitation/emission 380/440 nm. Background (0 EU ml^{-1}) 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, USA). The UNIVARIATE procedure in SAS was used to evaluate the distributions of data. Based on the Box–Cox test and histograms of the sample distribution, data were approximately log-normally distributed; therefore, data were natural log transformed before proceeding with analysis. Any 3-OHFA measurements below the LOD were assigned a value of LOD divided by square root of two, which was 0.5–2 pmol depending on the specific 3-OHFA.

The GM and the GSD of the GC/EI-MS and rFC results of each agricultural environment were calculated. Analysis of variance (ANOVA) via the general linear model (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 and rFC results, between individual 3-OHFAs, and between the sum of odd (C9:0, C13:0, C15:0, and C17:0) and even (C8:0, C10:0, C12:0, C14:0, and C18:0) carbon length 3-OHFAs. Multiple regressions, based on stepwise selection (inclusion/exclusion at $P = 0.05$), were performed to evaluate the relationship between the rFC assay and GC/EI-MS results accounting for effects of multiple 3-OHFAs. The rFC result (EU) was the dependent variable and the individual 3-OHFAs were the independent variables. 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

Calibration was established with 3-OHFAs at 2, 6, 20, 100, and 500 ng matrix blank fortifications that were processed identically alongside samples. Because of the wide range of endotoxin concentrations observed in the agricultural dusts, these data were split into two overlapped calibration curves; low calibration at 2–20 ng fortification and high calibration at 20–500 ng fortification. Both calibration curves yielded an R^2 of 0.99. Based on S/N > 3, the LOD was established at 0.5 ng fortification. Similarly, the LOQ was based on S/N > 10 and established at 1 ng for each analyte. The proportions of <LOD and <LOQ samples for each 3-OHFA (in most cases small) are summarized in Table 1.

The use of calibration standards submitted to the entire extraction process, coupled with C11:0 surrogate ensured strong quality control. This method effectively assessed relative spike recovery at five levels for each compound and yielded excellent calibration linearity. Though more labor intensive, we felt our method, processing 3-OHFA standards identically to samples from the first step of sample preparation to the end, provided more reliable data for our GC/MS method. Ideally, individual samples would be fortified with isotopically labeled analogs of target analytes; however, this option is not yet available.

Analysis of agricultural dusts

Table 2 presents the GM and GSD of total 3-OHFAs per mg dust (pmol mg^{-1}) obtained by GC/EI-MS and endotoxin activity per mg of dust (EU mg^{-1}) obtained by the rFC assay. Total 3-OHFAs were calculated as the sum of concentrations of each

Table 1. Proportion of samples with detectable and non-detectable (<LOD and >LOQ) 3OHFAs

	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 2. GM and GSD of total 3-OHFAs (GC/EI-MS) and endotoxin activities (rFC) per mg of dust

	n	3-OHFAs per mg dust (pmol mg^{-1})[a]		3-OHFAs per m^3 air (pmol m^{-3})		Endotoxin activity per mg dust (EU mg^{-1})[a,b]		Endotoxin activity per m^3 air (EU m^{-3})[b]	
		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
Corn farm	11	825	2.5	2176	3.0	179	3.0	473	4.3

Total 3-OHFAs = sum of both odd and even 3-OHFAs except C11:0 and C16:0.
[a]GLM ANOVA confirmed the difference between cattle feedlot and grain elevator at *P*-value < 0.05.
[b]EU = endotoxin unit.

3-OHFA with chain lengths of 8–18 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 (unreasonably high *m/z* 343 ion), 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 evident. The general linear model ANOVA confirmed that the type of dust has a significant effect on 3-OHFA measurements and endotoxin activity per mg dust ($P < 0.01$). Among all personal samples, cattle feedlots had the highest amount of total 3-OHFAs (pmol mg^{-1}) and endotoxin activity (EU mg^{-1}). Grain elevators had the lowest endotoxin activity and total 3-OHFA per mg. Levels of 3-OHFAs in livestock dusts (pmol mg^{-1}) were 1.5 (cattle feedlot) to 2.5 (dairy) times higher than that in grain elevator dust; endotoxin activity in livestock dusts (EU mg^{-1}) were ~3 (cattle feedlot) to 2.5 (dairy) times higher than that in grain elevator dust. Mean amounts of total 3-OHFAs (pmol mg^{-1}) and endotoxin activity (EU mg^{-1}) were significantly higher in dust from cattle feedlots than from grain elevators ($P < 0.05$).

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

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

	n	*R*	*P*-value
Dairy	17	0.53	0.02
Cattle feedlot	48	0.72	<0.01
Grain elevator	58	0.11	0.39
Corn farm	11	0.33	0.32

Total 3-OHFAs = sum of both odd and even 3-OHFAs except C11:0 and C16:0.

More detailed analyses for the individual carbon chain length of 3-OHFA are summarized in Table 4. Eight of the individual 3-OHFA (C9:0 to C18:0) were significantly correlated with endotoxin activity in feedlot dust ($P < 0.05$), while only two were significant for dairy and grain elevator dusts, and none for corn farm dust. The C8:0 3-OHFA was not correlated with endotoxin activity in any environment in this study. C10:0 3-OHFA was significantly correlated with endotoxin activity in only livestock dusts. All statistically significant correlations in livestock dusts were positive, whereas in grain dusts, statistically significant correlations were positive except for C9:0.

The correlations between individual 3-OHFA were calculated to evaluate the potential relationships between each individual 3-OHFA (Table 5). The correlations between single 3-OHFAs varied by lengths of carbon chain and agricultural environments. In general, 3-OHFAs with longer carbon chain (C17:0 and C18:0) correlated with other 3-OHFAs significantly. Correlations between C8:0 and C12:0, C13:0 and C15:0, and C15:0 and C17:0 were

Table 4. Pearson correlations between each 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
Dairy	17	*r*	−0.04	0.40	**0.55**	0.19	0.26	0.01	0.23	**0.74**	0.42
		P	0.88	0.11	**0.02**	0.46	0.31	0.99	0.38	**<0.01**	0.09
Cattle feedlot	48	*r*	−0.12	**0.29**	**0.30**	**0.41**	**0.63**	**0.83**	**0.61**	**0.60**	**0.33**
		P	0.43	**0.05**	**0.04**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**
Grain elevator	58	*r*	−0.13	**−0.28**	0.22	0.17	−0.15	**0.27**	−0.14	−0.23	0.10
		P	0.33	**0.03**	0.10	0.20	0.25	**0.04**	0.29	0.08	0.44
Corn farm	11	*r*	0.07	−0.25	0.14	0.13	0.39	0.17	0.16	0.01	0.44
		P	0.83	0.46	0.68	0.70	0.23	0.62	0.64	0.98	0.18

Bold indicates $P < 0.05$.

Table 5. Correlations between individual 3-OHFA: values indicate *r* with $P < 0.05$

		C9:0	C10:0	C12:0	C13:0	C14:0	C15:0	C17:0	C18:0
C8:0	Dairy			0.73			0.50		0.54
	Feedlot		0.41	0.47				0.30	
	GE	0.72	0.65	0.75		0.45		0.39	0.70
	Farm	0.76	0.92	0.89					
C9:0	Dairy	—						0.67	
	Feedlot	—			0.51	0.34	0.36	0.38	
	GE	—	0.47	0.51				0.42	0.43
	Farm	—				0.71			
C10:0	Dairy		—	0.65					0.29
	Feedlot		—			0.37		0.40	
	GE		—	0.90		0.67		0.29	0.81
	Farm		—	0.95					
C12:0	Dairy			—					0.52
	Feedlot			—		0.50	0.38	0.56	0.54
	GE			—		0.67		0.27	0.86
	Farm			—					
C13:0	Dairy				—		0.82		0.65
	Feedlot				—	0.63	0.66	0.53	0.43
	GE				—		0.62	0.39	
	Farm				—	0.88	0.88	0.79	0.96
C14:0	Dairy					—			
	Feedlot					—	0.79	0.84	0.57
	GE					—			0.60
	Farm					—	0.90	0.86	0.85
C15:0	Dairy						—	0.53	0.68
	Feedlot						—	0.82	0.50
	GE						—	0.29	
	Farm						—	0.94	0.83
C17:0	Dairy							—	
	Feedlot							—	0.64
	GE							—	
	Farm							—	0.75

Dairy = dairy ($n = 17$), feedlot = cattle feedlot ($n = 48$), GE = grain elevator ($n = 58$), and farm = corn farm ($n = 11$); no values = not significant at $P = 0.05$ with $r < 0.25$.

significant in all four dust types while correlations between C8:0 and C13:0 and C10:0 and C15:0 were non-existent.

Since the predominant Gram-negative bacteria in house dust contain even-numbered 3-OHFAs, most studies have focused only on the even-numbered

carbon chain 3-OHFAs for endotoxin exposure assessment in dusts. However, the chemical composition of endotoxins in agricultural dusts could be different from house dust. To investigate if odd and even 3-OHFAs are derived from the same source, 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 (rFC assay) were conducted (Table 6). Both odd-numbered and even-numbered 3-OHFAs were significantly correlated with endotoxin activity in livestock dusts; odd-numbered 3-OHFAs, but not even-numbered 3-OHFAs, were negatively correlated with endotoxin activity in grain elevator dusts. Neither odd-numbered nor even-numbered 3-OHFAs were correlated with endotoxin activity in corn farm dusts. Total 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 corn 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 correlation between odd- and even-numbered carbon length 3-OHFAs was positive in cattle feedlots and corn farms ($r = 0.74$ and 0.68, respectively) but was weak or not significant in grain elevators and dairies.

Table 6. Pearson correlation of odd- and even-numbered carbon chain 3-OHFAs with the rFC assay

	Endotoxin activity (EU mg^{-1})	Even-numbered carbon chain 3-OHFAs (pmol mg^{-1})
Dairy ($n = 17$)		
Odd-numbered carbon chain 3-OHFAs	0.60[a]	0.35
Even-numbered carbon chain 3-OHFAs	0.50[a]	
Cattle feedlot ($n = 48$)		
Odd-numbered carbon chain 3-OHFAs	0.70[a]	0.74[a]
Even-numbered carbon chain 3-OHFAs	0.68[a]	
Grain elevator ($n = 58$)		
Odd-numbered carbon chain 3-OHFAs	−0.24[b]	0.47[a]
Even-numbered carbon chain 3-OHFAs	0.14	
Corn farm ($n = 11$)		
Odd-numbered carbon chain 3-OHFAs	0.15	0.68[a]
Even-numbered carbon chain 3-OHFAs	0.31	

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.
[a] P-value < 0.05.
[b] $0.05 <$ P-value < 0.10.

As seen from Fig. 2, the relative proportion of each 3-OHFA varied by dust type. Overall, C12:0 and C14:0 (each comprised 25–30% of total 3-OHFAs) were dominant in all environments, together comprising 40–60% of total 3-OHFAs, and C13:0 was least prevalent (<5%). Grain dust contained a higher proportion of shorter chain 3-OHFAs (10% for C8:0 and 7% for C9:0) than livestock dusts (<5% of total 3-OHFAs) and corn farm dust (5%).

DISCUSSION

Personal air samplings often yielded very small dust masses and subsequently very low residues of 3-OHFAs. This finding necessitated a sensitive methodology for GC/EI-MS to assess agricultural occupational exposures accurately. In general, GC/MS-MS provides more sensitive results than GC/EI-MS; however, GC/MS-MS may not be available in most facilities, including our facility. In this study, we modified the GC/MS-MS method developed for house dusts for GC/EI-MS analysis of small sample masses (<1 mg) of agricultural dusts, collected by personal air sampling. The modified method significantly reduced analyst exposure to solvents.

In addition, the modified method is calibrated by running 3-OHFA standards through the entire digestion/sample cleanup process instead of introducing 3-OHFAMEs (methyl esters) at the silylation step. Though more labor intensive, processing 3-OHFA standards identically to samples from the first step of sample preparation to the end provided more reliable data. Ideally, individual samples would be fortified with isotopically labeled analogs of target analytes; however, this option is not yet available.

A previous multi-laboratory study (Reynolds *et al.*, 2005) indicated that extraction protocol method may affect the fatty acid distribution recovered, although the effect of this factor was not as pronounced as the source of the dust. In this study, we used the extraction media (pf water with 0.05% Tween 20) that yielded the optimal recovery for the rFC assay (highest recovery with least variability). The previous study also indicated that centrifugation may remove 3-OHFAs associated with particles. We did not centrifuge extracts. Further research on extraction protocols and GC/MS analysis would be useful.

Concentrations of endotoxin and 3-OHFAs were highest in cattle feedlots and dairies. In addition, the distribution of 3-OHFAs varied by dust type. Cattle feedlots and dairies contained greater proportions of larger carbon chain 3-OHFAs compared to grain handling facilities. Among all dust types analyzed in this study, cattle feedlots showed the highest correlation between total 3-OHFAs and endotoxin

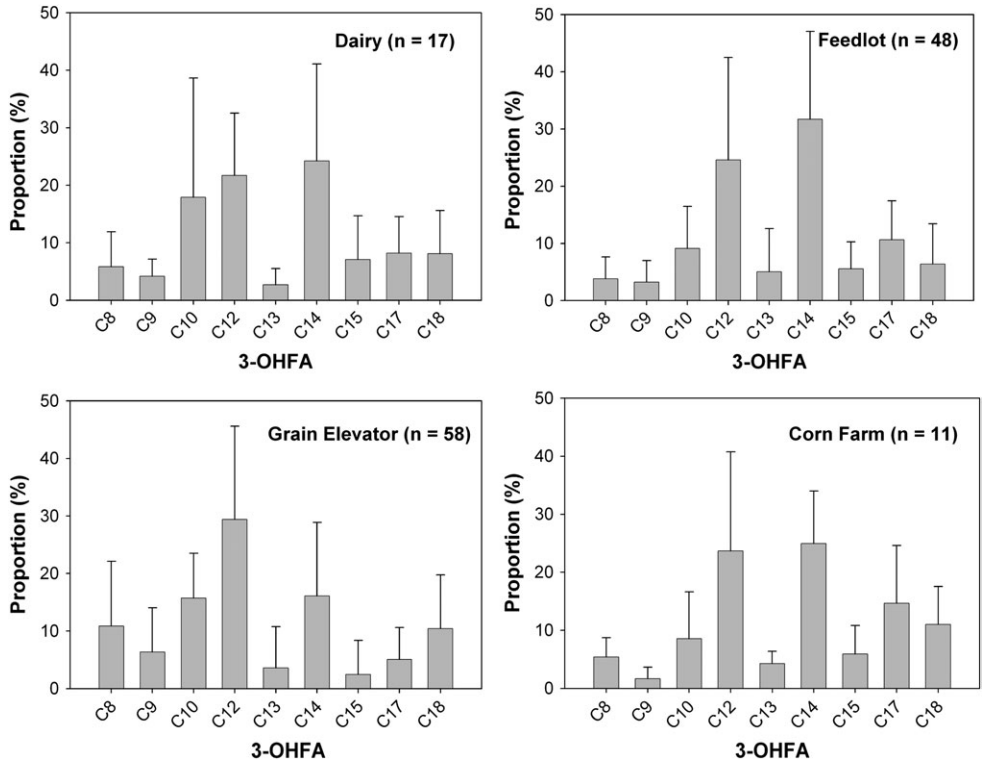

Fig. 2. Mean relative proportion of 3-OHFA with standard deviation (error bar) found in each dust type by GC/EI-MS analysis.

activity. Corn farm and grain dusts showed the lowest correlation to endotoxin activity. A recent study conducted by Pomorska *et al.* (2007) also reported a low correlation between the LAL and GC/MS total 3-OHFAs in grain (hay storage) dust and high correlations in sheep and poultry dusts. Dust from grain and livestock environments may differ in several respects. The major endotoxin source in livestock dust may be fecal material while the major source in grain dust is likely plant material. Fecal and plant materials are likely colonized with different bacterial species. In addition, the proportion of free and cell-bound endotoxin might be different in livestock and grain dust. However, the mechanisms relating to this difference are still unclear.

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 (Saraf *et al.*, 1999; Park *et al.*, 2004); however, agricultural dusts had a different tendency. For the four dust types in this study, 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. Although recent studies using house dust found results from the LAL and the rFC assays were strongly correlated ($r = 0.86$) (Alwis and Milton, 2006), this relationship could be explained by differences in biological assay response (rFC versus LAL), as well as the difference in microbial community. To support this, our recent study using livestock dusts found strong correlations between rFC and LAL assay responses (overall $r = 0.91$), but the magnitude of assay responses varied by livestock type (R. Saito, P. Thorne, J. Mehaffy, B. Cranmer, T. Keefe, N. Metwali, K. Donham, P. O'Shaughnessy, S. Reynolds, submitted). For example, C18:0 3-OHFA may be derived from Actinobacteria (Gram-positive bacteria) rather than from Gram-negative bacteria (Sebastian *et al.*, 2005); 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 (Rietschel, 1976). 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 (Reynolds *et al.*, 2005). In addition, Pomorska *et al.* (2007) 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 no 3-OHFA significantly correlated with LAL results in dusts from hay storage. This finding agreed with our results.

Most studies to date have only investigated the even-numbered carbon chain 3-OHFAs. All four dust types in this study contained a wide variety of 3-OHFAs including odd-numbered carbon chain 3-OHFAs, which are not significant in house dusts (Szponar and Larsson, 2001). In agricultural dusts, odd-numbered carbon chain 3-OHFAs made up a significant portion of the total 3-OHFA. Our findings showed that the odd-numbered carbon chain 3-OHFAs also correlated with rFC assay response. Excluding the odd-numbered carbon chain, 3-OHFAs may underestimate the total 3-OHFAs in agricultural environments. Including odd-numbered 3-OHFAs may also provide better understanding of the bacterial sources. Helander *et al.* (1982) have explained that LPS chemical composition could cause differences in acute pulmonary toxicity of LPS in guinea pigs. Laitinen *et al.* (2001) found that C14:0 3-OHFA was related to self-reported respiratory and eye symptoms. Thus, an important question is whether these different numbered carbon chain lengths are related to differences in human responses. Our recent study does indicate that specific (even carbon chain length) 3-OHFAs are important in eliciting immune responses (Burch *et al.*, 2009).

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. 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

Although we had enough power to detect the difference among agricultural environments, small sample sizes in the dairy and corn farm environments limited our ability to evaluate these environments (including the choice of ANOVA model applied). 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 measure only the response of the enzyme to lipid A, 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 to 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 are more labor intensive than bioassays. In addition, the GC/MS is relatively expensive compared to bioassays.

CONCLUSIONS

A GC/EI-MS method for endotoxin analysis has been applied to the assessment of 3-OHFA distribution in four agricultural environments. Compared to the parent GC/MS-MS method, this modified method reduces use of toxic chemicals and sample handling, allows sensitive monitoring of the experimental process, and can be used for analysis of small samples ($<$1 mg dust), typical of personal air samples.

This was the first study to evaluate endotoxin exposure in cattle feedlot environments. Overall, personal samples from cattle feedlots and dairies had the highest concentrations of endotoxin and 3-OHFAs. Livestock dusts also contained more variable chain length 3-OHFAs and had stronger correlations between GC/EI-MS and rFC assay results than grain dusts. Odd-numbered chain length 3-OHFAs correlated with rFC assay results as well as even-numbered chain length 3-OHFAs. In future investigations, it will be important to evaluate the roles of specific 3-OHFAs (including both even and odd chain length 3-OHFAs) in human respiratory diseases.

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REFERENCES

ACS Committee on Environmental Improvement. (1980) Guidelines for data acquisition and data quality evaluation in environmental chemistry. Anal Chem; 52: 2242–9.

Alwis KU, Milton DK. (2006) Recombinant Factor C assay for measuring endotoxin in house dust: comparison with LAL, and (1-3)-b-D-Glucans. Am J Ind Med; 49: 296–300.

Burch JB, Svendsen E, Siegel PD *et al*. Endotoxin exposure and inflammation markers among agricultural workers in Colorado and Nebraska. J Toxicol Environ Health; in press.

Donham KJ, Cumro D, Reynolds SJ *et al*. (2000) Dose-response relationships between occupational aerosol exposures and cross-shift declines of lung function in poultry workers: recommendations for exposure limits. J Occup Environ Med; 42: 260–9.

Donham KJ, Reynolds SJ, Whitten P *et al*. (1995) Respiratory dysfunction in swine production facility workers: dose-response relationships of environmental exposures and pulmonary function. Am J Ind Med; 27: 405–18.

Helander I, Saxen H, Salkinoja-Salonen MS *et al*. (1982) Pulmonary toxicity of endotoxins: comparison of lipopolysaccharides from various bacterial species. Infect Immun; 35: 528–32.

Laitinen S, Kangas J, Husman K *et al*. (2001) Evaluation of exposure to airborne bacterial endotoxins and peptidoglycans in selected work environments. Ann Agric Environ Med; 8: 213–9.

Liebers V, Raulf-Heimsoth M, Brüning T. (2008) Health effects due to endotoxin inhalation (review). Arch Toxicol; 82: 203–10.

Morrison DC, Vulajlovich SW, Ryan JL *et al*. (1987) Structural requirements for gelation of the Limulus amebocyte lysate by endotoxin. Prog Clin Biol Res; 231: 55–73.

Park J-H, Szponar B, Larsson L *et al*. (2004) Characterization of lipopolysaccharides present in settled house dust. Appl Environ Microbiol; 70: 262–7.

Pomorska D, Larsson L, Skorska C *et al*. (2007) Levels of bacterial endotoxin in air of animal houses determined with the use of gas chromatography-mass spectrometry and Limulus test. Ann Agric Environ Med; 14: 291–8.

Reynolds SJ, Donham KJ, Whitten P *et al*. (1996) Longitudinal evaluation of dose-response relationships for environmental exposures and pulmonary function in swine production workers. Am J Ind Med; 29: 33–40.

Reynolds SJ, Milton DK, Heederik D *et al*. (2005) Interlaboratory evaluation of endotoxin analyses in agricultural dust—comparison of LAL assay and mass spectrometry. J Environ Monit; 7: 1371–7.

Rietschel ET. (1976) Absolute configuration of 3-hydroxy fatty acids present in lipopolysaccharides from various bacterial group. Eur J Biochem; 64: 423–8.

Saraf A, Larsson L. (1996) Use of gas chromatography/ion-trap tandem mass spectrometry for the determination of chemical markers of microorganisms in organic dust. J Mass Spectrom; 31: 389–96.

Saraf A, Larsson L, Burge H *et al*. (1997) Quantification of ergosterol and 3-hydroxy fatty acids in settled house dust by gas chromatography-mass spectrometry: comparison with fungal culture and determination of endotoxin by a Limulus amebocyte lysate assay. Appl Environ Microbiol; 63: 2554–9.

Saraf A, Park J-H, Milton DK *et al*. (1999) Use of quadrupole GC-MS and ion trap GC-MS-MS for determining 3-hydroxy fatty acids in settled house dust: relation to endotoxin activity. J Environ Monit; 1: 163–8.

Sebastian A, Larsson L. (2003) Characterization of the microbial community in indoor environments: a chemical-analytical approach. Appl Environ Microbiol; 69: 3103–9.

Sebastian A, Szponar B, Larsson L. (2005) Characterization of the microbial community in indoor environments by chemical marker analysis: an update and critical evaluation. Indoor Air; 15: 20–6.

Sonesson A, Larsson L, Schutz A *et al*. (1990) Comparison of the Limulus amebocyte lysate test and gas chromatography-mass spectrometry for measuring lipopolysaccharide (endotoxins) in airborne dust from poultry-processing industries. Appl Environ Microbiol; 56: 1271–8.

Szponar B, Larsson L. (2001) Use of mass spectrometry for characterising microbial communities in bioaerosols. Ann Agric Environ Med; 8: 111–7.

Takada H, Kotani S, Tanaka S *et al*. (1988) Structural requirements of lipid A species in activation of clotting enzymes from the horseshoe crab, and the human complement cascade. Eur J Biochem; 175: 573–80.

Thompson M, Ellison SLR, Fajgelj A *et al*. (1999) Harmonized guidelines for the use of recovery information in analytical measurement. Pure Appl Chem; 71: 337–48.