

MODIFICATION AND APPLICATION OF A GC/MS METHOD FOR ENDOTOXIN ANALYSIS IN AGRICULTURAL DUSTS

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ABSTRACT

Bacterial endotoxins play an important role in occupational lung disease. GC/MS may offer advantages over traditional endotoxin assays, leading to improved exposure assessment. GC/MS analyses focus on quantification of biomarkers of endotoxins; 3-hydroxy fatty acids (3-OHFA's) in lipid A of lipopolysaccharide. The goal of this study is to modify the GC/MS method for endotoxin analysis and determine the correlations between the results of this modified GC/MS analysis and biological recombinant Factor C (rFC) assays for endotoxins in personal samples from three types of agricultural dusts (grain elevator, feedlot, and dairy). The existing method provides slightly more sensitive results (limit of quantitation = 2 picomol) than the modified method (validated limit of quantitation = 2 – 4 picomol); however, the modified method is amenable to GC/EI-MS. The modification to the existing method includes change in materials and chemicals and change in the experimental procedures. The major changes in the modified method are elimination of the liquid-liquid extraction step, use of polymeric media in the place of silica for sample clean up and use of 3-OHFA's to construct the calibration curves instead of 3-OHFA methyl esters. This approach allows detecting small changes in the experimental process that is critical for agricultural dusts containing very low concentration of 3-OHFA's. The calibration curve yields $R^2 > 0.99$ with standard deviation of 0.008. The modified method reduces sample handling, use of organic chemicals and the analyst's exposure. Results from the modified method and the rFC assay show that livestock dusts have a better correlation (0.9450 feedlot; 0.4185 dairy) than grain dusts (0.1723). Feedlot dusts contain more variable 3-OHFA's than grain dusts. In general, good correlations exist between the biological assay and our modified GC/MS method. The modified GC/MS method is especially useful for identification of specific 3-OHFA's for endotoxins from various agricultural environments.

INTRODUCTION

Endotoxins (or lipopolysaccharides) are cell wall components of Gram-negative bacteria and play an important role in occupational lung diseases. Several previous studies found that endotoxin exposures are associated with a high prevalence of respiratory symptoms in

agricultural environments (Beard, 1996, Donham, 1995, 2000, Reynolds, 1993, 1996, Smid, 1994, Viet, 2001, Vogelzang, 1998, 2000). Symptoms include decreases in pulmonary function and increases in asthma severity. In addition to agricultural workers, this association is found in different environments such as cotton mills (Castellan, 1987, Rylander, 1985), fiberglass manufacturing (Milton, 1996) and more broadly, in general indoor air quality in office buildings (Reynolds, 2001). However, several studies have indicated that endotoxin exposure in early life may be protective against asthma and atopic sensitization (Gereda, 2000, Liu, 2002, Singh, 2005) but endotoxin roles in disease pathogenesis are not clear. Thus, universal guidelines or standards for endotoxins do not yet exist.

There are two approaches to measuring endotoxin activity: biological assay and chemical analysis. Bioassay measures the relative reactivity of endotoxins with an enzyme, providing rapid and sensitive results (Morrison, 1987, Takada, 1988). The detection limit of bioassay is approximately 0.01 EU. However, bioassay may underestimate endotoxin exposure since it does not detect cell-bound endotoxins that may be associated with respiratory disease (Jorgensen, 1974, Rylander, 1989, Sonesson, 1990). In addition, current bioassay technology experiences interference from non-endotoxin agents and this lack of specificity may yield misleading data (Reynolds, 2005). An example of the bioassay procedure is shown in Figure 1.

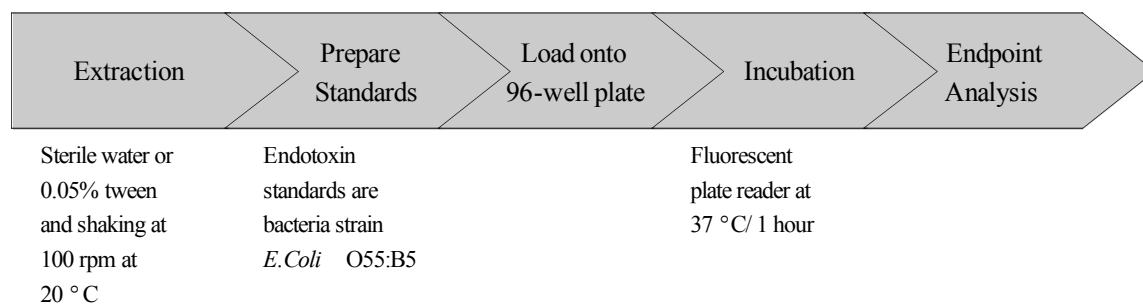


Figure 1. Flow Chart of Bioassay (rFC Assay)

Gas Chromatography/Mass Spectrometry (GC/MS) analysis focuses on quantification of indirect biomarkers of endotoxins, 3-hydroxy fatty acids (3-OHFA's) in lipid A of lipopolysaccharide (LPS). One mole of LPS carries approximately 4 moles of 3-OHFA. Unlike bioassay, GC/MS measures free and cell-bound endotoxins (Reynolds, 2005, Sonesson, 1990). Therefore, GC/MS offers advantages over traditional endotoxin bioassay in predicting respiratory disease, especially if specific 3-OHFA's are associated with disease pathogenesis. The existing method was developed as a tool for assessing indoor air quality with GC/MS-MS (Saraf, 1996, 1997, 1999). GC/MS-MS provides more sensitive results than GC/EI-MS; however, GC/MS-MS may not be available in the most facilities. Our modified method was developed for GC/EI-MS, especially for agricultural dusts containing wide variety of 3-OHFA's.

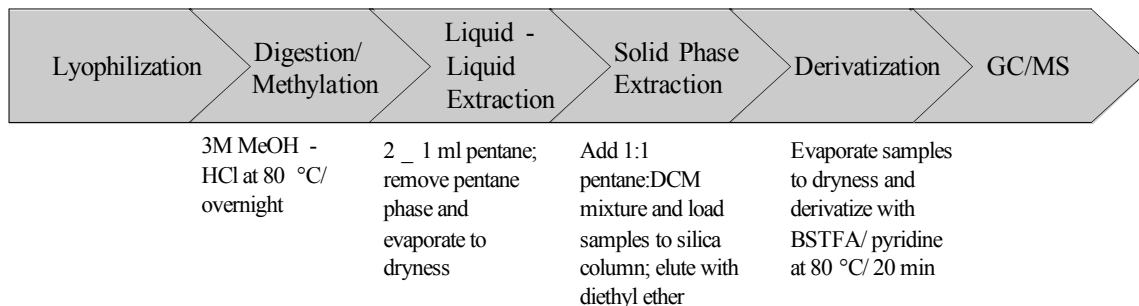
The goals of this study were:

1. To modify an existing GC/MS-MS method for endotoxin analysis by readily available GC/EI-MS.
2. To determine the correlations between the results of the modified GC/MS analysis and biological recombinant Factor (rFC) assay in agricultural dusts.

MODIFICATION OF EXISTING METHODOLOGY

The summary of the experimental procedure for existing GC/MS-MS methodology and the modified GC/EI-MS method are shown in Figure 2.

The Existing Method



The Modified Method

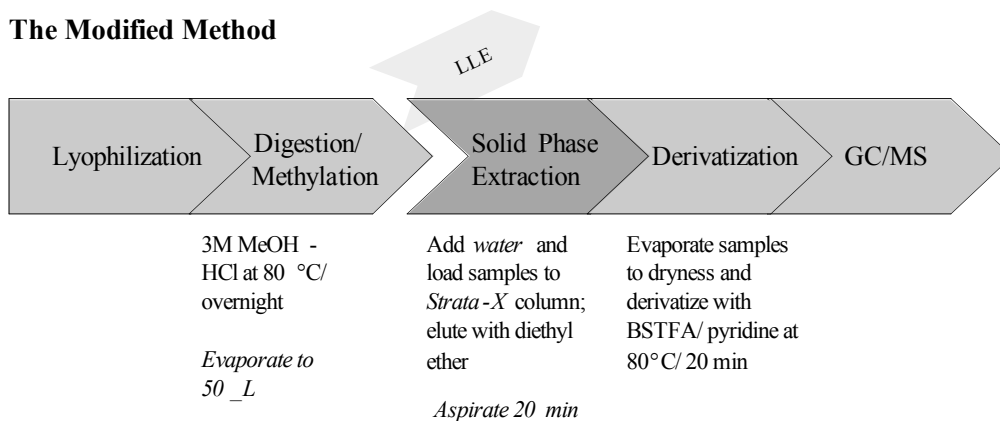


Figure 2. Flow Chart of the Existing and Modification GC/MS Methods

The major changes in the modified method are elimination of the liquid-liquid extraction, use of polymeric solid-phase extraction (Strata-X, Phenomex or equivalent Oasis HLB, Water) instead of silica cartridge for sample clean-up and use deionized water instead of 1:1 Pentane: Dichloromethane mixture for sample load.

In addition, the modified method uses a different approach to create calibration curves. In the modified method, 3-OHFA's (C8 to C18 except C11) are processed identically to samples instead of introducing 3-OHFA-methyl esters only at the final derivatization step. Both methods rely on 3-OH C11 as surrogate added to each sample. However, by constructing calibration curves in this manner, we achieve better quality control of the methylation step by collecting method performance data for each fatty acid. Since agricultural dusts contain a wide range of endotoxin concentration levels, spike levels of 0, 2, 6, 20, 100 and 500 ng are used for creating

the calibration curve. The calibration curves yields R^2 of greater than 0.99 with standard deviation of 0.008.

This modified method has advantages over the existing method. Due to the elimination of the liquid-liquid extraction step, the modified method reduces sample handling and needs for organic chemicals. This modification reduces overall sample loss and provides economic benefits. Moreover, changing solid-phase cartridge allows us to discontinue use of the highly toxic chemical, dichloromethane, in favor of deionized water. This change significantly reduces the analyst exposures to hazardous chemicals.

APPLICATION OF THE METHOD

Personal samples using IOM inhalable samplers were collected in three agricultural environments in Colorado: grain elevator, feedlot, and dairy farm. The modified GC/MS method and biological rFC assay (shown in Figure 1) were used for endotoxin analysis. Results were log transformed. Pearson's correlations and multiple linear regressions between the GC/MS method and rFC assay results for each dust type were calculated by SAS v.9.1.

Among all dust types studied in this project, grain elevator has the lowest concentration of endotoxins per quantity of dust. The grain dust contains less endotoxins than the livestock dusts in both GC/MS (in picomol) and biological rFC assay (in EU) results (Table 1). The Dutch Expert Committee on Occupational Standards recommended an exposure limit of 50 EU/m³ for airborne endotoxins (Heederik, 1997). All individuals sampled in the project exceeded this standard, indicating a potential for respiratory problems to be found among these workers.

Table 1. Descriptive Statistics

	Overall (n=55)	Grain elevator (n=20)	Feedlot (n=24)	Dairy (n=11)
EU/mg	1058 (1708)	253 (276)	1891 (2319)	705 (382)
Pmol/mg	871 (978)	201 (117)	1311 (1181)	1130 (657)
EU/m ³	3844 (5902)	2803 (4559)	5646 (7576)	1807 (1445)
	mean (stdev)			

Correlations between GC/MS and rFC assay results vary by environments. Livestock dusts have higher correlations between the GC/MS method and rFC assay than grain dusts (Table 2).

Table 2. Pearson's Correlations

	R	p-value
OVERALL	0.8176	< 0.0001
Grain elevator	0.1723	0.4676
Feedlot	0.9450	< 0.0001
Dairy	0.4185	0.2002

Correlations between single 3-OHFA's vary by length of carbon chains. In general, 3-OHFA's with longer carbon chain length (C17 and C18) correlate with other 3-OHFA's and shorter chain

3-OHFA's (C8 to C10) do not correlate with other shorter chain 3-OHFA's (Table 3).

Table 3. Correlations between Individual 3-OHFA's at 0.10 significant level

	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
C8			-	D	DF		D	-	D	DF
C9			-	OF	FG	OFG	OFG	-	OF	OFG
C10			-	FG		G		-	O	F
C11				-	-	-	-	-	-	-
C12					O	OFG	ODF	-	ODF	OD
C13						ODG	ODFG	-	ODF	ODF
C14	O=Overall						OF	-	OF	OF
C15	D=Dairy							-	ODF	ODF
C16	F=Feedlot								-	-
C17	G=Grain									ODF
C18										

For different dust types, different patterns of 3-OHFA's exist (Table 4). Based on Table 4, feedlot dust contains more variable 3-OHFA's than other dusts.

Table 4. Correlation between Single 3-OHFA's (GC/MS) and EU (rFC)

	C8	C9	C10	C12	C13	C14	C15	C17	C18
OVERALL		++	++	++	++	++	++	++	++
Grain Elevator				+		++			
Feedlot		++		++	++	++	++	++	+
Dairy									+

"++" If p<0.05; "+" If p<0.10

Multiple regressions were performed to evaluate the relationship between assay and GC/MS results accounting for effects of individual 3-OHFA's at the same time. Dairy dust has the lowest correlation in multiple regression analysis (Table 5).

Table 5. Regression Analyses

	Combinations of 3-OHFA	R ²
OVERALL	14, 17	0.6177
Grain elevator	13, 14	0.4047
Feedlot	13, 14, 18	0.9604
Dairy	18	0.2969

with 90% significance level

DISCUSSION/CONCLUSIONS

The modified GC/MS method reduces the use of chemicals, sample handling and uses less toxic chemicals than the existing GC/MS method. In addition, the modified method is calibrated by running 3-OHFA's standards through the entire digestion/sample clean-up process instead of introducing 3-OHFA-methyl esters at the silylation step. This provides better method

performance information for the 18 hour methylation step by monitoring individual 3-OHFA's at several concentrations throughout analysis. 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 and our compromise was this method of calibration coupled with addition of a single surrogate, 3-OH C11 fatty acid, to each sample. Additionally, we found poor correlation between 3-OHFA-methyl esters and 3-OHFA's – likely due to relative instability of 3-OHFA-methyl esters during storage. This modification allows detection of small changes or losses in the experimental process critical for agricultural dusts containing very low concentrations of 3-OHFA's.

The existing method provides slightly more sensitive results with limit of quantitation of 2 picomol (Park, 2004) than the modified method with limit of quantitation of 2 to 4 picomol; however, the modified method is amenable to GC/EI-MS.

In general, good correlations exist between the biological assay and the modified GC/MS analysis method. The modified GC/MS method is especially useful for identification of specific 3-OHFA's for endotoxins from various agricultural environments.

Future applications include: to increase numbers of sample size, to analyze dust samples from different agricultural environments and to evaluate specific 3-OHFA's role in human respiratory diseases.

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