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3-Hydroxy-Tetradecanoic Acid: A Chemicalmarker of Endotoxin and Gram-Negative Bacteria in Occupational Health Monitoring

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Abstract: LAL assay is the most widely used method for endotoxin detection in occupational health monitoring but critical aspects have been reported.

This paper reports the results of a field study, in which the concentration of 3-hydroxy-dodecanoic and 3-hydroxy-tetradecanoicacidswere measured by HPLC-MS/MS on air samples collected on filters in workplaces in order to verify if a chemical approach can be used for the assessment of the environmental contamination from endotoxins and/or Gram-negative bacteria.

At each site, two sampling lines were used simultaneously: onefilter was analysed for endotoxin detection by LAL test, while the other was extracted for determining its content of fatty acids.All Gram-negative bacterial strains were identified to genera/species level in each sample.

Endotoxin contamination measured by LAL test was detected in all samples (range 0.04-210.7 EU/m^3) as well as 3-OH C_{14} (range: 8.72-224.20 ng/m^3) while 3-OH C_{12} levels were below the analytical detection limit in 23 out of 25 samples.

Principal Component Analysis shows that endotoxin levels positively correlate with the content of fatty acids. However, the main component of lipopolysaccharide detected in our samples is 3-hydroxytetradecanoicacid, and therefore it can be considered as a good marker of endotoxin presence.

Key words: 3-hydroxy fatty acids, LAL assay, HPLC/MS-MS, PCA analysis

1. Introduction

Endotoxins, also known as pyrogen or fever-causing toxin, are the major component of the outer membrane of Gram-negative bacteria and are essential to the integrity and function of their cell wall. Small amounts of endotoxins may be released in a soluble form by young cultures grown in the laboratory but, for the most part, they remain associated with the cell wall until disintegration of the organisms. In vivo, this results from autolysis, external lysis mediated by complement and lysozyme, and phagocytic digestion of bacterial cells [1].

Chemically, endotoxins are lipopolysaccharides (LPSs) composed of three regions: Lipid A, an R polysaccharide and an O polysaccharide. The endotoxic principle of LPS resides in the lipid A domain, since polysaccharide-deprived free lipid A appears to exhibit similar endotoxic activities as intact LPS [2]. It consists of a phosphorylated N-acetylglucosamine (NAG) dimer with six or seven fatty acids (FA) attached, all saturated. Some are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. These hydrophobic fatty

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acid chains anchor the LPS into the bacterial membrane while the rest of the LPS projects from the cell surface.

Endotoxins can be found in high concentrations in all occupational settings where organic dust is present. What was initially considered to be a problem in only few activities turned out to also affect workers in the livestock industry, scientists working with rodents, workers in waste and sewage treatment and even indoor workers [3].

Several studies have demonstrated that inhalation of endotoxin-contaminated air induces acute or chronic inflammatory airway reactions that phenotypically resemble asthma (reversible airflow obstruction, persistent airway hyper-reactivity, wheeze, etc.) [4, 5]. Research in recent years has revealed major clinical effects of endotoxins ranging from organic dust toxic syndrome (ODTS), chronic bronchitis to lethal effects such as septic shock, organ failure and death. In contrast, some authors suggest that exposure to endotoxins, even at a low level, reduces the risk of lung cancer [6].

For these reasons, in the last years, the attention of the international scientific community to these components is strongly increased and it focused to the standardization of sampling and analytical methods. In fact, to date there are no generally accepted standards for the detection of endotoxin in occupational settings [7, 8]. The most widely used analytical method is the *Limulus Amebocyte lysate* (LAL) assay, in its kinetic Chromogenic version but alternative techniques in GC/MS and HPLC have been proposed [9-11].

LAL assay detects endotoxins based on their biological activity, while chemical methods are able to measure 3-OH fatty acids in lipid A, after hydrolysis of the lipopolysaccharide. Furthermore, while LAL test fails to detect cell-bound endotoxin, the chemical approach has the advantage of estimating the total amount of endotoxin (both cell-bound and non-cell-bound) present in a sample. Besides, it overcomes critical issues of commercial and widely used *Limulus Amebocyte Lysate* (LAL) assay due to phenomena of inhibition and/or activation of the enzymatic reaction due to substances of non-bacterial origin (β-glucans) or to the presence of peptidoglycans [12]. Chemical methods can also detect the water-insoluble fraction of the bioactive endotoxin [13].

Among chemical methods, GC-MS requires a long sample preparation time, including the need for derivatization of the acid in order to obtain a volatile substance.

In a previous study, we developed and validated a method using HPLC coupled to triple quadrupole mass spectrometer (HPLC-MS/MS) for the quantitative determination of 3-hydroxy-dodecanoic (3-OH C_{12}) and 3-hydroxy-tetradecanoic (3-OH C_{14}) acids as chemical markers of lipopolysaccharide in Gram-negative strains from *American Type Culture Collection* (ATCC): the analysis showed that samples contained3-OH $C_{12}$and 3-OH C_{14}acids in variable ratio and that the sum of their concentrations positively correlated significantly ($R^2 = 0.82$) with the results of LAL test [14]. The choice of the two fatty acids is due to the fact that previous reports indicated that shorter-chain (C_{10}-C_{14}) are positively correlated with endotoxin activity in the Limulus bioassay, while longer-chain (C_{16}-C_{18}) 3-OHFAs tend to have lower or even no correlation [15, 16]. It was also suggested that C_{12}-C_{14} 3-OHFA may elicit more significant potent immunological effects in humans [17].

The objective of the present study was to measure the concentration of the two fatty acids in samples of airborne particulate matter collected on filters in different occupational settings in order to verify if this chemical approach can be used for measuring the environmental contamination from endotoxins and/or Gram-negative bacteria in workplaces as alternative method to LAL assay.

2. Material and Methods

2.1 Bioaerosol Sampling

Stationary inhalable dust samples were collected

with airChek 2000 pumps (SKC, Inc., Eighty Four, PA, USA), at a flow rate of 2 l min-1, equipped with IOM sampler and fiberglass filters (Whatman, GF/A, diameter 25 mm, pore size of 1.6 μm). Stationary samples were located in the middle at 1.5 m above the floor to simulate human breathing zone. The sampling time was 4 hours.

At each sampling site, two sampling lines were used simultaneously: once in laboratory, one filter was analysed for the detection of endotoxin by LAL test, while the other was extracted for the determination of its content of the two 3-hydroxy fatty acids.

All Gram-negative bacterial strains contained in each sample were identified to genera/species level using biochemical methods by API 20E and NE tests (bioMerieux, Marcy l'Etoile, France) and Biolog Microstation System (Biolog, Inc. Hayward, CA, USA).

2.2 Kinetic Chromogenic LAL Assay

In order to carry out endotoxin analysis, filter samples were extracted with 5 ml of 0.05% Tween 20 in Pyrogen-Free Water (PFW) using analternative horizontal mixer (Promax 1020), set to 160 rpm at room temperature for 1 hour, centrifuged at 1000 rpm for 20 min. Surnatants were analysed in duplicate with Kinetic LAL method (QCL-LAL assay Lonza Walkersville, MD USA) and were interpreted against a5-point (concentration range from 0.005 to 50 EU/ml) standard curves of Escherichia coli lipopolysaccharide (CSE).

A replicate of each sample was spiked with a CSE standard (5 EU/ml final activity). If the recovery of spiked samples was out of the range 50-200%, the measurement was repeated. The LLOD was 0.005 EU/ml. Results are reported as EU/m^3, converting millilitres into cubic meter of aspirated air.

All procedures were carried out using pyrogen-free reagents and all glassware was baked at 200°C for 4 hours.

2.3 HPLC/MS-MS Analysis

The analytical method used in this paper has been already described elsewhere [14].

Briefly, sample filters were extracted with purified water (Milli Q) and hydrolyzed by addition of sodium hydroxide solution (Sigma-Aldrich, Milan) and heating at 90°C for 40 minutes. After the hydrolysis40 μl of a 10 mg/l solution in acetonitrile (Sigma-Aldrich, Milan) of 3-hydroxy-tridecanoic acid (Larodan Fine Chemicals, Malmo Sweden), as internal standard, were added.

Hydrolyzed fatty acids were extracted with n-hexane, in three subsequent steps, each one with 1 ml; the 3mln-hexane extracts were finally dried in evaporator (MIVAC Quattro concentrator, Genevac, UK) for 30 minutes at 60°C. The residue was dissolved in 0.5 ml of acetonitrile, and after filtration with 0.2 μm Anotop10 IC membrane filters, was injected into the HPLC-MS/MS system.

Standard solutions of 3-OH C_{12} and 3-OH C_{14} and 3-hydroxy-tridecanoic acid were prepared by dissolving in acetonitrile suitable amount of pure standards, which were stored at 4°C; from this solution, by further dilutions, the calibration and quality control (QC) samples were prepared.

The HPLC device was a PerkinElmerseries200, equipped with a SupelcoHypersylBDSC8 (150×4.6, 5 μm) chromatographic column, maintained at 40°C,withmobilephaseconsisting of a gradient of acetonitrile (A) and acetic acid 1% v/v in water (B) for a total time of analysis of 10 minutes, at a flow rate of 1000 μl/min.

The detector was an AB/Sciex API 4000, triple quadrupole mass spectrometer, equipped with a Turbo Ion Spray (TIS) probe working in the negative ion, multiple reaction monitoring (MRM) mode; the *m/z* ion transitions (precursor→product), corresponding to the loss of a fragment of 46 u.m.a, typical of β-hydroxyfatty acids, were monitored for both the qualitative and the quantitative analysis. The 1.4 Analyst† software was used to process the quantitative

data. The method was validated in the range 10-1000 µg/L for both analytes. Intraday accuracy is never lower than 95% and interday than 85%. Intraday variability is never higher than 7% and interday than 15%. LOD was 2.5 µg/L for 3-OH $C_{12}$and 1 µg/L for 3-OH $C_{14,}$ while LLOQ was 10 µg/L and 5 µg/L respectively.

Results, expressed in µg/l of fatty acids, were converted in µg/m^3, taking into account the volume of sampled air.

2.4 Chemometric Analysis

Principal Component Analysis (PCA) was used to examine the relationships among all the variables studied and to evidence the correlation between the experimental indices and the predominant bacterial strains identified.

3. Results

A total of 50 filters was collected. The Fig. 1 shows the species of Gram-negative bacteria identified in our air samples. The predominant species were *Acinetobacter shindleri* (28%) and *Flavimonashorizihabitans* (18%), followed by *Pseudomonas stutzeri* and *Moraxella spp.* (7%).

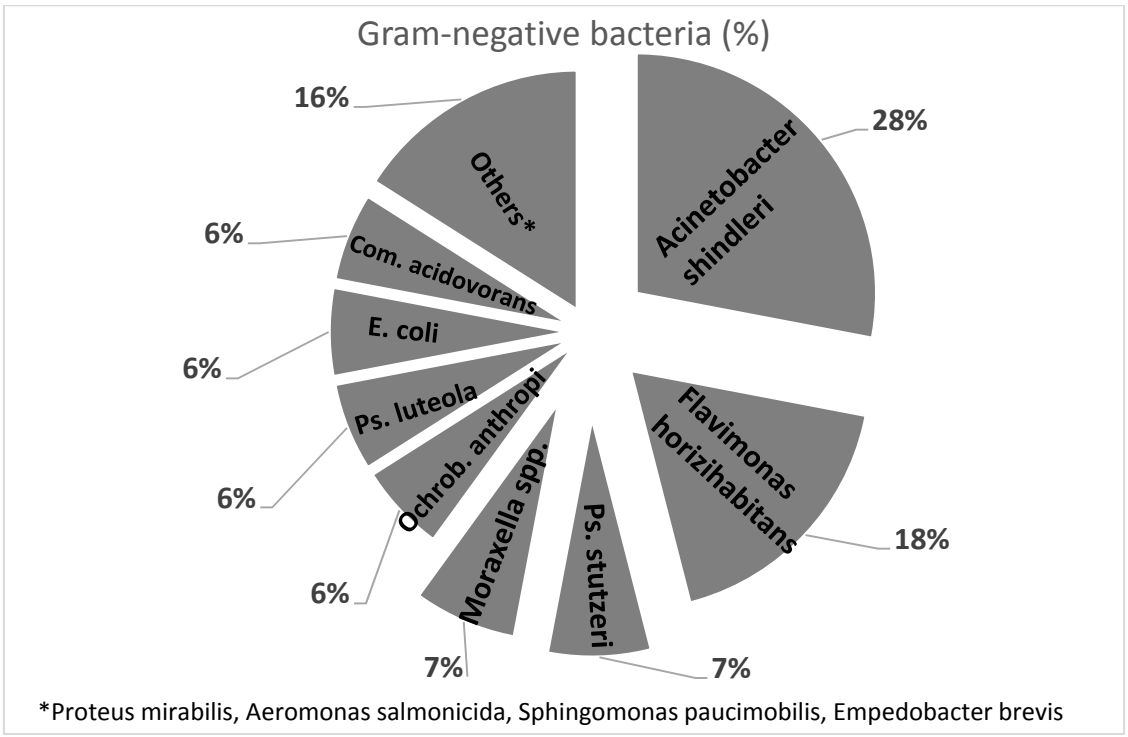

Fig. 1 Gram-negative bacteria identified in filter samples.

Concentrations of 3-hydroxy-dodecanoic and 3-hydroxy-tetradecanoic fatty acids and endotoxins using both LAL test and HPLC-MS/MS are reported in Table 1. Endotoxins measured by LAL test was detected in all air samples with a range of 0.04-210.7 EU/m^3.

Also3-OH FA with chain lengths of 14 carbons was measurable in all samples (range: 8.72-224.20 ng/m^3) while 3-hydroxy-dodecanoic acid levels were below the analytical detection limit (LOD: 2.5 µg/L) in 23 out of 25 samples.

Fig. 2 shows the chromatogram obtained from the analysis of one of the filters, spiked with the internal standard.

Loadings plot (Fig. 3), used to interpretate relations among variables confirms our previous results: moving along PC1 (First Principal Component), the concentration of endotoxin positively correlates with

the sum of the two fatty acids measured.In fact, along PC1 direction the contribution of endotoxin and fatty acids concentrations is comparable, being around 0.3 and 0.4 respectively.

Table 1 Concentrations of chemical and biological markers in the samples.

3-hydroxy-dodecanoic ($\mu g/m^3$)	3-hydroxy-tetradecanoic ($\mu g/m^3$)	Endotoxin concentration (EU/m^3)
< LOD	224.20	0.04
< LOD	92.79	7.60
< LOD	74.11	0.05
< LOD	52.05	7.54
< LOD	52.06	0.04
< LOD	46.78	6.90
< LOD	79.78	7.50
< LOD	83.60	11.04
< LOD	1646	8.73
< LOD	42.69	11.81
< LOD	75.91	4.67
< LOD	30.87	10.49
< LOD	82.37	37.74
8.13	117.23	210.70
< LOD	23.53	2.46
< LOD	45.80	2.57
< LOD	8.80	10.57
< LOD	11.77	5.29
< LOD	12.09	6.73
< LOD	51.48	1.64
< LOD	52.18	1.82
< LOD	8.72	5.30
< LOD	14.83	2.67
< LOD	15.80	7.53
28.74	93.85	1600.00

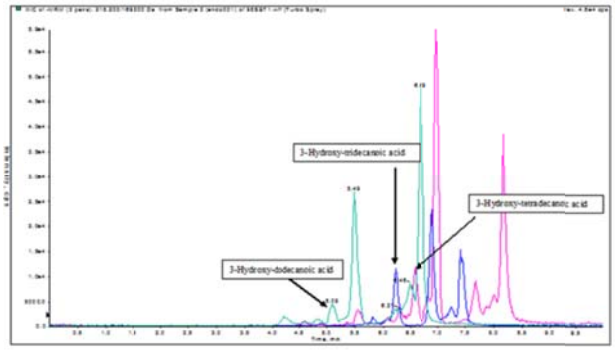

Fig. 2 HPLC-MS/MS chromatogram obtained from one filter sample.

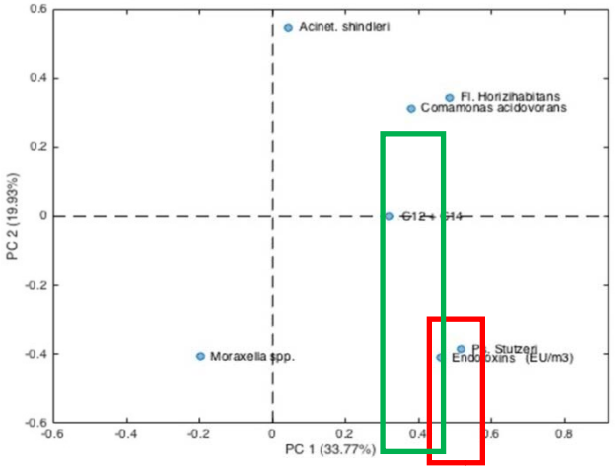

Fig. 3 Loading plot.

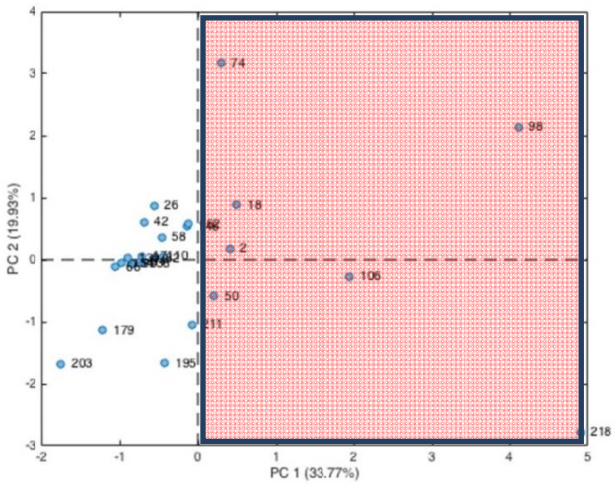

Fig. 4 Score plot.

The PCA results provided additional information: *Pseudomonas stutzeri* and endotoxins are strongly correlated and, depending on the samples, different correlations with other groups of bacterial strains were evidenced.

In order to interpret the relations among environmental samples, a score plot (Fig. 4) has been built. It shows that samples with positive scores along PC1 have higher values of Gram-negative bacteria, endotoxins and 3-hydroxy fatty acids, according to loading plot reported above.

4. Conclusion

At present, the LAL assay is the most widely used method for endotoxin detection in occupational health monitoring. However, although this testis highly

sensitive, some critical aspects have been reported in the literature. As this method is designed to detect the water-soluble bioactive endotoxin fraction it is less suited to study the true recovery of endotoxin from different types of environmental matrixes, and therefore it may produce false positive or false negative results; the determination of LPS markers instead of LAL in occupational exposure studies can be particularly useful when determining endotoxin in samples rich in molds, which are known to activate the Limulus reaction leading to amplification of the response, and therefore to an overestimation of the results.

Here we present a chemical approach for profiling of 3-hydroxydodecanoic and 3-hydroxytetradecanoic fatty acids from filter samples collected in occupational settings, which results are compared to LAL test.

In accordance with our previous results observed on ATCC Gram-negative bacterial strains, the concentration of endotoxin positively correlates with the content of the two fatty acidsalso in environmental samples, confirming that they can be considered chemical markers of endotoxin contamination. The main component of lipopolysaccharide detected in our samples is 3-hydroxytetradecanoicacid that can be considered as a good marker of endotoxin presence. On the other side, as 3-hydroxydodecanoicis detected only when endotoxin levels are very high (detected by LAL, 210.70 and 1600 EU/m^3), it could be used for preliminary screening of workplaces highly contaminated by Gram-negative bacteria, but this hypothesis requires to be confirmed by further experimental data.

These results are in line with those recently reported by Uhlig et al. [17] who have highlighted a correlation between endotoxin activity measured by the LAL assay and the sum of 3-hydroxydecanoic, 3-hydroxydodecanoic and 3-hydroxytetradecanoic acids in drilling mud recycling plants.

The strong correlation between *Pseudomonas stutzeri* and endotoxin concentration is difficult to explain; interestingly, the presence of this specie was always observed in correspondence of the highest levels of endotoxin. Besides, chemometrics seems to be a suitable statistical tool for analyzing this type of experimental data.

Few papers have investigated this subject until now, rendering these issues worth of further investigations.

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