Comparison of Metal Oxide Catalysts for Pyrolytic MOLI-MS Bacterial Identification

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Abstract

Six metal oxide catalysts were investigated for effective cleavage and laser ionization of bacterial cell wall phospholipid extracts. Calcium oxide has been utilized in the past for this application, but suffered from degradation of catalytic activity. Following evaluation of the six catalysts, CeO₂ was found to be the most stable over time and produced fatty acid profiles of bacterial pathogens similar to freshly activated CaO. A suite of ten bacteria previously studied using CaO catalysis provided cross validation results of analysis of 97% correct correlation for negative-ion data. Cerium oxide catalysis studies yielded cross validation results of 100%. Using analysis of variance-principal component analysis (ANOVA-PCA), four sample sets collected with stored catalyst at 0, 8, 24, and 504 h showed no effect based on long-term catalyst degradation. Supervised learning by a fuzzy rule-building expert system (FuRES) that was validated with training and prediction set partitions independent of CeO₂ age and unsupervised data analysis using a dendrogram of Euclidean distance confirmed that the CeO₂ catalyst age had no effect on the fatty acid mass spectral profiles. **Key words:** MALDI-MS, MOLI-MS catalytic pyrolysis, chemometrics, bacteria, fatty acids

1. Introduction

Fatty acid profiling has been used for bacterial identification since the 1960s [1]. The original work utilized an *ex situ* method involving saponification followed by derivatization of cell wall fatty acids to form fatty acid methyl esters (FAMES) followed by gas chromatography analysis [2]. Later, an *in situ* thermochemical process using tetramethyl ammonium hydroxide (TMAH) was developed, which significantly reduced sample analysis time to less than three min for Curie-point pyrolysis-mass spectrometry (PyMS) [3,4].

It has been observed during lipid analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), that sufficient heat was generated to pyrolyze the sample [5]. During an investigation of TMAH as a methylating agent for MALDI-TOF MS with CaO as a matrix, lipid cleavage was observed. Tandem MALDI analysis identified the resulting cleavage products as calcium fatty acid adducts [5]. It was later determined that a strong base, acid, or heat was necessary to activate CaO [5,6]. Using activated CaO, monoacylglycerides, diacylglycerides, triacylglycerides, phospholipids, as well as bacterial and algal extracts, produced peaks for their constituent fatty acids [7]. In addition, spectra from bacterial and algal extracts were found to be reproducible, which suggested that this technique could be used for microbial fatty acid profiling. In a follow up publication, five Gram-positive and five Gram-negative bacteria were analyzed using CaO in positive- and negative-ion MALDI-TOF MS modes followed by principal component analysis (PCA) [8]. PCA showed distinct clusters for five replicates of each bacterial species with 94% cross validation

correlation of positive-ion data. Negative-ion data yielded a higher classification rate of 97% and exhibited less within group variation than the positive-ion data.

The current state-of-the-art for MALDI MS-based clinical microbial identification centers on protein profiling. Three commercial protein databases [9] have been developed for this application [10]. Two companies, Bruker and BioMérieux, have received European ‘CE’ mark and US Food and Drug Administration approval for use in clinical laboratories. These databases contain multiple profiles of over 3000 bacteria and claim over 98% correct identification according to company literature. In an analysis of 1,371 clinical isolates, a peer-reviewed comparison of the Bruker Biotyper to conventional typing methods found 93.2 % were putatively identified to the species level [11]. A second study focused specifically on Gram positive rods, which typically have lower Biotyper identification rates compared to Gram negatives or Gram positive cocci [12], reported 79.1% of 215 isolates were correctly identified at the species level [13].

The most important criterion for a diagnostic technique centers on the fact that each bacterial protein profile is unique and reproducible, which depends on bacterial growth conditions and instrument stability [14]. In the case of a catalytic process such as metal oxide lipid cleavage, catalyst stability must also be considered. The following paper describes the degradation associated with CaO stability and the screening of five additional metal oxide catalysts.

2. Experimental

2.1 Bacterial strains and culture conditions

Table 1 summarizes the bacteria used in this study [8]. All bacterial strains were cultured overnight in brain heart infusion (BHI) medium (BD-Difco, Franklin Lakes, NJ) at 37°C

[8]. Following incubation of broth cultures, bacteria were streaked onto agar plates and incubated for 22 h. Extracts of single colonies were immediately prepared upon removal from the incubator. To minimize culture conditions as a variable in fatty acid analysis, these parameters were used as a carefully controlled, standardized method for all experiments.

2.2 Lipid extraction

Individual colonies were suspended in 100 μ L of a 33/66 v/v% methanol/chloroform mixture (Pharmco-AAPER, Shelbyville, KY and Fisher, Pittsburgh PA, respectively) and vortexed for 2 min at 3200 rpm. Phase separation was achieved by addition of 100 μ L of phosphate buffer saline (PBS, pH 7.4) followed by an additional 60 s of vortexing and cell debris removal by centrifugation for 30 s at 1400 x g. The resulting organic fraction was then removed for MALDI-TOF MS analysis.

2.3 Catalysts

The following metal oxides were investigated for their combined catalytic properties and temporal stability during bacterial phospholipid cleavage: calcium oxide, purity 99.4% (Nano-Active Inc. Manhattan KS), magnesium oxide, purity 99.6% (Nano-Active Inc.), nickel oxide, purity 99.8% (Sigma Aldrich, St. Louis, MO), cerium (IV) oxide, purity 99.9% (Cerac, Milwaukee, WI), barium oxide, purity 99.5% (Cerac), and strontium oxide, purity 99.5% (Alfa Aesar, Ward Hill, MA). Thermal activation was performed on CaO, BaO, and SrO in an open-air tube furnace at 800 °C for 3 h while NiO and MgO were heated to 350 °C to remove surface water. Activation of CeO₂ was not required. All catalysts were stored in a desiccator under vacuum.

An investigation of the stability of the CaO, BaO, and SrO was conducted by taking MALDI-TOF MS measurements termed metal oxide laser ionization-MS (MOLI-MS) of

the same bacterial phospholipid extracts at 0, 8, and 24 h post activation. Cerium oxide spectra were generated at 0, 8, 24, and 504 h, where the catalyst for t=0 was obtained directly from the manufacturer's bottle. This same catalyst was stored in a vacuum dessicator and removed for the 8, 24, and 504 h time points.

2.4 Mass spectrometry

Samples were prepared for MOLI-MS as previously described [7]. Briefly, 100 mg catalyst was added to one mL of *n*-hexane (Sigma-Aldrich). One μ L was then removed from the bottom of the resulting slurry and spotted on a stainless steel MALDI sample plate followed by 2 μ L of lipid extract. Other sample preparation methods such as overlay and sandwich methods, did not improve the data quality.

Mass spectrometric measurements were acquired in positive- and negative-ion modes with a Bruker Ultraflexreme MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a 355 nm Nd:YAG laser. Spectra were collected in reflector mode at 4000 resolution with a grid voltage of 50.3%, a delayed extraction time of 120 ns, and a low-mass cutoff of 150 Da. Five replicate spectra were collected for each bacterium as 500 shot composites at a sampling frequency of 1 kHz using automated laser rastering.

2.5 Data analysis

Selected fatty peaks (Table 2) from each spectrum were exported from the instrument in ASCII format and processed by two procedures. In the first, an in-house program centroided and normalized the spectra to total intensity, and selected fatty acid peaks prior to PCA. Processed data were recorded in a tab-delimited format and imported directly into R Version 3.0.2 (The R Foundation for Statistical Computing, Vienna,

Austria) as a data frame. PCA was performed using the *prcomp()* function with an argument passed to return rotated variables; data were automatically mean-centered by *prcomp()*. Data were plotted using the built-in *plot()* function in R. Leave-one-out cross validation was performed on the input data by passing the appropriate argument to the linear discriminate analysis function *lda()*, which is part of the Modern Applied Statistics with S package [15]. Results from *lda()* returned class assignment based on supervised learning.

In supervised analysis, the data were imported into MATLAB R14a using the 150 unprocessed spectra as text files. The spectra were normalized to unit vector length and analyzed using a fuzzy rule-building expert system (FuRES) [16] with customized MATLAB scripts and functions. The FuRES was validated with 100 bootstraps with 3 Latin partitions [17,18]. The data were bootstrapped individually and also by removing all the replicates that corresponded to the same catalyst age. The normalized data were also used to construct an unsupervised dendrogram using average centroid linkages and minimum Euclidean distances. The normalized mass spectra also were subjected to analysis of variance-principal components analysis (ANOVA-PCA) with bacterial species, catalyst age, and interaction as factors. The statistical significance was measured by using the pooled sums of squares method that provides a conservative estimate of significance.

3. Results and Discussion

MOLI-MS using CaO as a catalyst is known to cleave phospholipids into their fatty acids [5,7]. Although cross-validation results were above 97% correct classification for the previously referenced 10 bacteria, for the technique to be useful in the clinical laboratory,

the catalyst must be stable over time and capable of producing highly reproducible fatty acid profiles. CaO is known to be susceptible to moisture and carbon dioxide poisoning and must be activated prior to use for catalytic pyrolysis of lipids. It was unclear if the catalyst could be stored under vacuum and maintain its original activity. Fig. 1 is a PCA score plot of the first 2 principal components (PCs) for negative-ion CaO-catalyzed MOLI mass spectra of *Escherichia coli*, *Salmonella typhimurium*, and *Shigella boydii* extracts. Measurements of a single extract from each bacterial type were taken over three time points from 0, 8, and 24 h post-thermal catalytic activation. Although unique spatial groupings corresponding to each time point were observed, the relative position of distinct groupings for each bacterial species changed with time, and clearly showed a temporal effect on catalyst deactivation. The overlapping alignment observed for the *S. boydii* and *S. typhimurium* groupings suggests that these genera may be difficult to differentiate using supervised learning techniques. Table 3 summarizes the variance associated with the first six PCs. Group separation was observed at a cumulative variance of 85.6% using PCs 1 and 2.

Because CaO appears to be deactivated even in short time periods, five additional prospective catalysts were evaluated for generation of fatty acids from glyceride standards and their overall stability investigated over a 24-504 h timeframe. Nickel oxide and MgO failed to cleave glyceride standards to any degree regardless of instrument parameters, rather, molecular species of intact glycerides in both positive- and negative-ion modes were observed. Cerium oxide, BaO, and SrO cleaved the standards (data not shown) and BaO and SrO produced both positive- and negative-ion peaks in the same manner as previously reported for CaO. However, only negative-ions were observed for

CeO₂. This result was not surprising because the +4 oxidation state of cerium produces a +3 charge on resulting single fatty acid complexes. The low-mass, non-integer peaks of these complexes places them in a spectral region that complicates data analysis. Based on reactivity and adverse health effects, BaO and SrO were not investigated beyond our initial screening. Because it's observed reactivity, CeO₂ was subjected to further in-depth studies.

To determine whether CeO₂ demonstrated improved stability over CaO, a time study was conducted with the same 10 bacteria listed in Table 1. Fig. 2 contains selected negative ion spectra of *Salmonella* from 0, 8, and 24 h for the two catalysts. The intensities of the three spectra in Fig. 2B obtained using CeO₂ were consistent with each other as a function of time and comparable to the CaO-derived spectrum at time zero in Fig. 2A. Calcium oxide spectra (Fig. 2B) obtained at 8 and 24 h showed changes in intensity of the individual fatty acids and a prominent, unidentified peak at m/z 473, indicating degradation of the catalyst. Fig. 3 shows PCA score plots of CeO₂ derived bacterial data and previously published CaO data for the same bacteria [8]. Table 4 summarizes the variance associated with the first six PCs for each catalyst. The cumulative variance for the CeO₂ data was 92.1% for the first 2 PCs. In contrast, the cumulative variance for CaO was only 73% encompassing 6 PCs. In the case of the CaO data, all combinations (PCs 1 and 2, PCs 1 and 3, etc.) were plotted, however, PCs 1 and 2 gave the best separation. Comparison of 3D plots of the first 3 PCs for CaO (Fig. 3A) and CeO₂ (Fig. 3B) had similar separations (between group variation), however the precision (within group variation) was better using CeO₂. Cross-validation using LDA of CeO₂-derived fatty acid profiles gave 100% correct classification.

Analysis of variance-principal components analysis [19] was used to determine the significant factors responsible for differentiation. The p -value for the mathematical means between species was below 10^{-4} . The storage time of CeO₂ and interaction among species factors were highly insignificant with p -values close to 1.0. [19]

Cluster analysis of the data produced the dendrogram of the Euclidean distances between pairs of objects and the cluster averages represented in Fig. 4. It is interesting that two distinct clusters were observed that almost corresponds to Gram type, which was previously observed by FAME analysis using PyMS [20]. If *E. faecalis* and *F. tularensis* were interchanged, separation of Gram type would be complete. Note that the separation that occurs among the species in the dendrogram is natural, and not because of unsupervised clustering of the data.

A principal component transform (PCT) was used to compress the spectra before FuRES analysis. The classification tree in Fig. 5 is similar in structure to the dendrogram in Fig. 4, although FuRES [19] is supervised and the cluster analysis was unsupervised. For the PCT during the bootstrap procedures, principal components were calculated from the training data set and prediction data were projected onto these components for each bootstrap-partition.

The results were 100% accurate for classification at the species level. Further analysis used a partition procedure so that each group of spectra collected for a specific catalyst age was removed from the model-building step using the same procedure as before of 100 bootstraps and 3 partitions. Replicate spectra from the same catalyst age and species were either in the prediction set or training set, but never both at the same time.

Classification accuracy was not affected by CeO₂ post-activation time and remained at 100%.

4. Conclusions

In total, six metal oxide catalysts were evaluated for their capacity to reproducibly cleave bacterial cell wall phospholipid extracts to their constituent fatty acids followed by negative-ion MOLI MS. CaO was found to partially degrade in less than one-hour post-activation. Of the six catalysts evaluated, CeO₂ provided superior stability and reproducibility and required no activation. An extended time study up to 504 h using the same fatty acid extract and stored catalyst showed that no difference in fatty acid distribution occurred. A comparison of CaO data collected immediately after activation to CeO₂-derived data collected over a one-week period showed that similar fatty acid profiles were observed and clearly established the stability of the CeO₂. Both supervised and unsupervised classifiers gave 100% accurate species assignment, demonstrating the future potential as a clinical technique to enhance current MALDI protein profiling.

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Figure captions

Figure 1. CaO reproducibility as a function of time. Square, *E. coli*; circle, *S.*

typhimurium; triangle, *S. boydii*. Red, 0 hrs; green, 8 hrs; blue, 24 hrs.

Figure 2. Comparison of catalyst stability as a function of time. *S. typhimurium* spectra obtained over 24 hrs using A) CaO and B) CeO₂.

Figure 3. PCA plots of 10 bacteria from A) freshly activated CaO and B) Three CeO₂

time points. (●) *A. baumannii*, (●) *B. anthracis*, (●) *C. putrefaciens*, (●) *E. coli*,

(●) *E. faecalis*, (●) *F. tularensis*, (●) *L. monocytogenes*, (●) *S. aureus*, (●) *S. typhimurium*,

(●) *Y. pestis*.

Figure 4. Dendrogram of the Euclidean distances among the normalized spectra and the centroid averages.

Figure 5. FuRES classification tree built from the entire set of normalized spectra where H=entropy and Nc=number of objects in class.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1. Bacterial phylotypes used.

Gram positive	Strain	Source
<i>Bacillus anthracis</i>	Sterne	Armed Forces Institute of Pathology
<i>Clostridium putrefaciens</i>	ATCC 25786	ATCC
<i>Enterococcus faecalis</i>	V583	University of Oklahoma Health Sciences Center
<i>Listeria monocytogenes</i>	ATCC 19112	ATCC
<i>Staphylococcus aureus</i>	ATCC 27660	ATCC
Gram negative		
<i>Acinetobacter baumannii</i>	AC54	Felix d'herelle Reference Center Viruses
<i>Escherichia coli</i>	ATCC 15597	ATCC
<i>Francisella tularensis</i>	LVS	Colorado State University
<i>Salmonella typhimurium</i>	ATCC 13311	ATCC
<i>Yersinia pestis</i>	A1122	CDC Division of Vector-borne Diseases

Table 2. Fatty Acids used in Principal Component Analysis

Fatty Acid	[M-H] ⁻
C13:1	211
C13:0	213
C14:1	225
C14:0	227
C15:0	241
C16:1	253
C16:0	255
C17:2	265
C17:1	267
C17:0	269
C18:2	279
C18:1	281
C18:0	283
C19:1	295
C19:0	297
C20:1	309
C20:0	311
C21:1	323
C21:0	325
C22:1	337
C22:0	339
C23:1	351
C23:0	353
C24:1	365
C24:0	367
C25:1	379
C25:0	381
C26:1	393
C26:0	395

Table 3. Percentage of total variance for CaO reproducibility

Principal component	% individual variance	% cumulative variance
1	46.4	46.4
2	39.2	85.6
3	6.3	91.9
4	5.9	97.9
5	.9	98.8
6	.7	99.5

Table 4. Comparison of percentage of total variance

CeO₂			CaO		
Principal component	% individual variance	% cumulative variance	Principal component	% individual variance	% cumulative variance
1	82.7	82.7	1	20.7	20.7
2	9.4	92.1	2	18.9	39.6
3	5.7	97.8	3	11.3	50.6
4	1.3	99.0	4	9.4	60.1
5	.6	99.6	5	7.9	68.2
6	.2	99.9	6	4.8	73.0