

Identification of Whole Cells with Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry and Protein Database Inquiries.

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Abstract: Matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry was used to analyze whole cells. Two strains of *Escherichia coli* were used as models for evaluation of sample treatment methodologies. Mass spectral peaks were identified for one *E. coli* (ATCC No. 23724), and protein searches in the Swiss-Prot/TrEMBL database were conducted in order to attempt identification of the sample as *E. coli*. Two types of cancerous cells, K562 and Jurkat, were also analyzed in the MALDI using the same basic methodology that was used to sample the two strains of *E. coli*. Mass spectra with identifiable peaks were observed for these cells. This work represents a preliminary step toward the possibility of differentiating between cancerous and non-cancerous cells.

Introduction

In recent reports, the employment of matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry to detect high-mass protein biomarkers has been suggested as a means by which to obtain proteomic “fingerprints” of microorganisms.^{1,2,3} The advantages of using MALDI mass spectrometry include its capability of detecting molecules of high masses, soft ionization properties, rapidness of sampling, and relatively simple sample preparation techniques, despite its lack of reproducibility due to environmental factors in the cells such as age and ongoing biological processes.⁴ Coupled with MALDI, the employment of protein databases can identify the presence of proteins in spectra and indicate differences among protein expression in biological samples. The use of protein databases is desirable for a host of reasons. Databases contain enormous amounts of information about the types of proteins found in a variety of organisms, including whole proteomes of several prokaryotes.¹ Protein searches can be performed based on a variety of criteria, including molecular weight. The speed in which proteomic information can be acquired from databases makes such searches very convenient. Databases can also resolve the problem of identifying organisms which lack reproducibility among mass spectra. If varying mass spectra of the same sample contain peaks that represent a distinctive set of ions that are listed within the protein database, such a search should identify these ions as part of the bacterial proteome.⁴

The ability to rapidly identify whole cells can provide numerous advantages and capabilities in a diversity of fields, from microbiology to medical diagnosis of diseases.

In terms of accurate recognition of cancerous cells, the development of such a direct method could allow the medical community to quickly locate such cells and to perhaps diagnose at an earlier stage. Although a host of “biomarkers” could be used to identify cells (e.g. lipids, DNA), proteins seem to be the primary candidate for cell categorization since they constitute 60-70% dry mass of *Escherichia coli*, suggesting the feasibility for high detection sensitivity for these bacterial cells.⁵ Concerning human cancer cells, this method would be dependent upon the criteria that the expressed proteomes in both normal and cancerous cells would differ by both the amount of proteins commonly expressed by each cell type and differences in the types of proteins expressed by each.⁶ In all cases of cancer, the level of expression of specific proteins (i.e. oncoproteins, tumor suppressors) will vary between the two cell types,^{7,8} and such an analysis of the varied protein expression may allow for detection and differentiation between malignant and benign cells.

In this project, two strains of *E. coli* (ATCC Nos. 23724 and 11775) were analyzed on the MALDI mass spectrometer (PerSeptive Biosystems), with a host of various sample preparation methods explored. A protein database investigation was conducted on one of the bacterial samples in attempt to confirm its identity. Finally, two samples of human cancer cells, K562 and Jurkats, were analyzed on the MALDI in order to evaluate the possibility of obtaining quality spectra using methods similar to those for the *E. coli*.

Procedural

***Escherichia coli* culture growth.** A sample of *E. coli* (ATCC No. 23724) was used initially for mass spectral analysis. A microgram of this strain was grown overnight in 100 mL of LB media and maltose. A microgram of this newly grown bacteria was spread over a plate of 100 mL media and allowed to grow for six hours. Cells were removed from this media and centrifuged for 10 minutes at 4000 g and 12°C. Cells were then washed three times with sterile water. Finally, they were frozen with liquid nitrogen and lyophilized for 24 hours prior to mass spectrometry. A second sample of *E. coli* (ATCC No. 11775) was purchased. These cells had already been harvested and lyophilized. No culture from this cell line was harvested upon purchase, and these cells were taken directly from their vial for MALDI analysis.

Sample preparation for MALDI. Both strains of *E. coli* were suspended in a solution of 70:30 acetonitrile/0.1% trifluoroacetic acid (TFA) at a concentration of 5 mg of lyophilized *E. coli* per 1 mL of prepared acetonitrile/0.1% TFA solution. This solution, combined with a MALDI matrix, provided the generic sample for analysis by mass spectrometry. Sinapinic acid (SA, 100 mM in acetonitrile/methanol/water) was the primary matrix used, though trials with α -cyano-4-hydroxycinnamic acid (α -CHCA, 33 mM in acetonitrile/methanol) and 2,5-dihydroxybenzoic acid (DHB, 100 mM in methanol/water) were also performed. All matrixes were from Hewlett Packard. A gold 100 well MALDI sampling plate was used. As an instrumental calibrant, a standard mix consisting of insulin and apomyoglobin, with SA as a matrix, was used, and peaks were matched with those from a reference file. Calibration files were created in order to assign accurate mass/charges to spectral peaks prior to protein assignment via a protein database. The first sample of *E. coli* aggregated when introduced to the prepared solvent,

settling to the bottom of the microfuge tube. Prior to sampling the *E. coli* stock, the suspension of *E. coli* was vortexed to get as consistent samples as possible. Various factors were explored in order to optimize the signals of proteins, including the order in which *E. coli* and matrix were applied, amounts of each, treatment by corona plasma discharge (CPD), addition of acetic acid, and washes with trifluoroacetic acid. Similar processes and variables were carried out and explored with the second *E. coli* sample (ATCC No. 11775). When the method of sample preparation for the first *E. coli* sample was optimized, the spectral peaks were compared with molecular weights of proteins from the SwissPROT/TrEMBL database (www.expasy.org). The limitations for the search were that only bacterial proteins were eligible for selection and that the molecular weight window around each peak observed was ± 3 Da.¹ All bacteria with proteins of molecular weights falling in these ranges around the peaks were recorded, and a comparison of protein "hits" by several bacteria was made.

Cancerous cells. Cancerous K562 (chronic myeloid leukemia cell lines) and Jurkat (human T cell leukemia cell lines) cells were grown and cultured in the Tissue Culture Growth Facility in the Department of Chemistry and Biochemistry at the University of Colorado in Boulder. Cells were suspended in a 10 mM magnesium sulfate solution. These two solutions were centrifuged and the magnesium sulfate solution was discarded, leaving the cancerous cells, which were immediately resuspended in the 70:30 solution of acetonitrile/0.1% TFA before MALDI analysis. Methodology criteria that were investigated included different matrixes and order of matrix/cell application. Spectra were taken from each sample, but no quantitative analyses were performed from this data.

Results and Discussion

Escherichia coli. The sample preparation method was optimized for intensity of peaks (i.e. best signal-to-noise). The order of preparation of samples, the order in which matrix and sample were applied, was studied. Three methods of preparing MALDI samples were examined: application and drying of the sample followed by application of the matrix, application and drying of the matrix followed by application of the sample, and a sample prepared in the manner previously stated followed by another matrix application creating a "sandwich" of matrix-sample-matrix. The matrix used was sinapinic acid, as initial trials displayed a single small signal with sinapinic acid while no signal was observed with alpha-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid as matrixes. The procedure of applying matrix then sample easily gave the best spectra, with the other two methods not producing any signals of value. A representative mass spectrum of *E. coli* (ATCC No. 23724) can be seen in **Figure 1**. With this criterion established, the amounts of matrix and sample, both total and relative amounts, were altered. The effects of corona plasma discharge with a tesla coil (Fisher Scientific) were examined as a potential releaser of sought after biomarkers.⁹ As final tests for this sample of *E. coli*, acetic acid (0.5%) was added to bacteria samples in amounts between 0.3 and 0.6 microliters, and sample washes with 0.1% TFA, in 1.0 microliter allotments, were conducted on top of bacteria samples for the same purpose that acetic acid was added. Adding acid to sample or washing sample with acid prior to MALDI analysis should remove some of the undesirable alkali cations which may bind to the proteins,

giving them the positive charge for mass spectrometry instead of a hydrogen ion. This can weaken peak intensity and widen the base of the peak, resulting in poorer resolution.

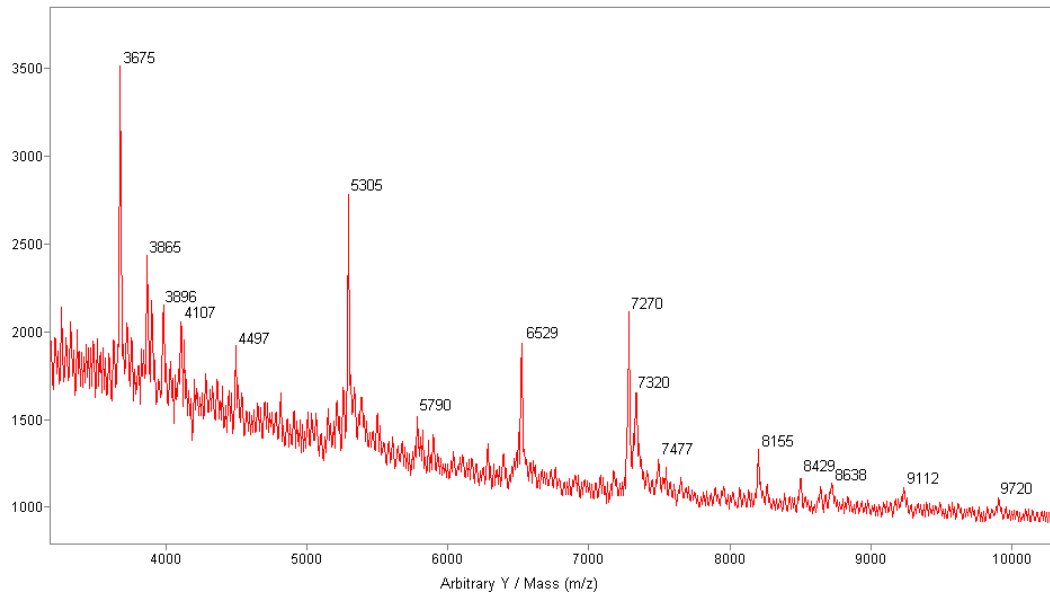


Figure 1: *E. coli* (ATCC No. 23724). 0.2 μL SA was applied to the MALDI plate. After drying, the SA was covered with 0.6 μL 0.5% acetic acid. When this layer dried, it was covered with 2.0 μL *E. coli*.

Table 1 lists all the different processes to which the first strain of *E. coli* was subjected, and **Figure 2** compares individual peaks from all these tested conditions with one another. The 3 individual peaks measured for this figure (approximate mass/charges 3680, 5310, and 7280 before mass calibration) were added together from each spectrum, and their totals are found in **Figure 3**. Rather than comparing averages of the signal-to-noise ratios of peaks, the best spectrum, or highest signal-to-noise ratio, from each treatment was used in the comparison of all treatments. There are several reasons for this. Because bacteria in suspension were studied rather than a solution with a single protein, samples applied to the MALDI plate were not homogeneous. It was very visible that some regions of the sample contained large amounts of bacteria, some had moderate amounts, and others had no bacteria at all. Clearly samples from each of these regions would differ greatly with one another. Multiple samples from the same region would be inconceivable since the region is good usually for a single analysis due to the laser blasting sample off the plate. In some instances, matrix and sample may interact more directly than in others, producing more ions in one spot than in another. Also, in some areas the desired ions found at the interface of the matrix and bacteria could have been buried under bacteria that did not interact with the matrix. In such cases, “mining” for ions, i.e. blasting the top layer of bacteria to get to the interface, would be necessary, thus producing unmeritedly poor spectra and giving an inadequate average of spectral peaks.

Table 1: Variations of *E. coli* (ATCC No. 23724) sample preparation methodology

| | | | | | |
|-------------|-----------------------------|-------------|---|--------------|---|
| Treatment A | 0.2µL SA→0.2µL E. coli | Treatment J | 0.4µL SA→0.4µL E. coli→CPD | Treatment S | 0.2µL SA→2.0µL E. coli→0.6µL 0.5% acetic acid |
| Treatment B | 0.4µL SA→0.4µL E. coli | Treatment K | 0.2µL SA→0.4µL E. coli→CPD | Treatment T | 0.1µL SA→0.3µL 0.5% acetic acid→ 1.0µL E. coli |
| Treatment C | 0.5µL SA→0.5µL E. coli | Treatment L | 0.2µL SA→0.4µL E. coli→CPD (5 second treatment) | Treatment U | 0.1µL SA→0.6µL 0.5% acetic acid→ 1.0µL E. coli |
| Treatment D | 0.2µL SA→0.4µL E. coli | Treatment M | 0.2µL E. coli→ 0.2µL SA→CPD | Treatment V | 0.2µL SA→0.6µL 0.5% acetic acid→ 2.0µL E. coli |
| Treatment E | 0.1µL SA→0.5µL E. coli | Treatment N | 0.4µL E. coli→ 0.4µL SA→CPD | Treatment W | 0.1µL SA→1.0µL E. coli→1 wash w/ 1.0µL 0.1% TFA |
| Treatment F | 0.1µL SA→1.0µL E. coli | Treatment O | 1.0µL E. coli→ CPD→0.1µL SA | Treatment X | 0.1µL SA→1.0µL E. coli→2 wash w/ 1.0µL 0.1% TFA |
| Treatment G | 0.2µL SA→2.0µL E. coli | Treatment P | 0.1µL SA→1.0µL E. coli→0.3µL 0.5% acetic acid | Treatment Y | 0.1µL SA→1.0µL E. coli→3 wash w/ 1.0µL 0.1% TFA |
| Treatment H | 0.4µL SA→4.0µL E. coli | Treatment Q | 0.1µL SA→1.0µL E. coli→0.5µL 0.5% acetic acid | Treatment Z | 0.2µL SA→2.0µL E. coli→1 wash w/ 1.0µL 0.1% TFA |
| Treatment I | 0.05µL SA →5.0µL E. coli | Treatment R | 0.1µL SA→1.0µL E. coli→0.6µL 0.5% acetic acid | Treatment AA | 0.2µL SA→2.0µL E. coli→2 wash w/ 1.0µL 0.1% TFA |

*All samples treated with CPD were done so for 15 seconds unless otherwise noted.

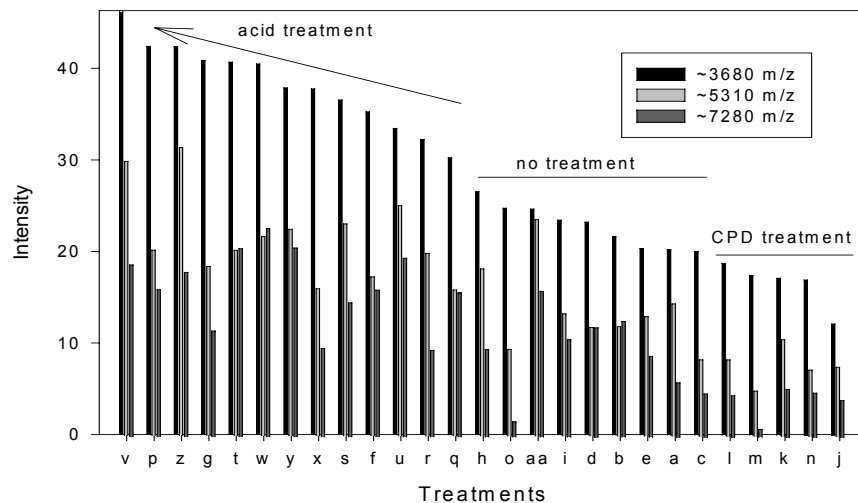


Figure 2
E. coli (ATCC No. 23724). This chart is a comparison of the signal-to-noise ratios of 3 peaks common to all *E. coli* spectra. General descriptions of treatments are listed above their respective bars.

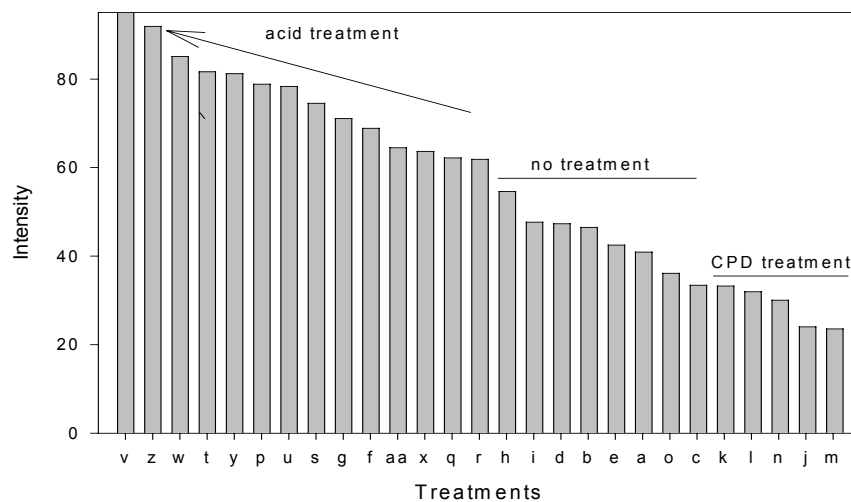


Figure 3
E. coli (ATCC No. 23724). This chart is a comparison of the combination of the 3 peak heights from Figure 2. General descriptions of treatments are listed above their respective bars.

It is apparent that a greater amount of bacteria sample than matrix produces higher-quality spectra. The sample consisting of 0.1 μ L SA matrix, dried, and covered with 1.0 μ L *E. coli* produced excellent spectra, as did the sample of 0.2 μ L SA matrix, dried, and followed by 2.0 μ L *E. coli*. The 2 other matrices, α -cyano-4-hydroxycinnamic acid (α -CHCA) and 2,5-dihydroxybenzoic acid (DHB), were tested with the same procedure, 0.1 μ L matrix followed by 1.0 μ L *E. coli*. As initial experiments indicated, neither of these samples produced spectra remotely comparable to that of SA. None of the samples employing CPD showed an improvement in signal, but rather they reveal that the use of the tesla coil to “zap” the cell sample actually decreases acquired signal from spectra.

Finally, treatments with a weak and a strong acid, acetic acid and trifluoroacetic acid respectively, were carried out on the *E. coli*. It appeared as though the acids did in fact improve samples, yielding slightly higher signal-to-noise results than the same samples without either the acetic acid or TFA acid washes. From data of the best spectra from each treatment, the most intense peaks were found in a spectrum containing acetic acid (**Figure 1**), though samples washed with TFA produced spectra with peaks more intense than other samples with acetic acid.

Unlike the first sample of *E. coli*, the second sample (ATCC No. 11775) did not aggregate when the prepared solvent was added. Similar experiments, such as amounts of matrix/bacteria and types of matrix, were preformed, and these treatments are listed in **Table 2**. Spectra from this sample of bacteria (**Figure 4**) were much poorer

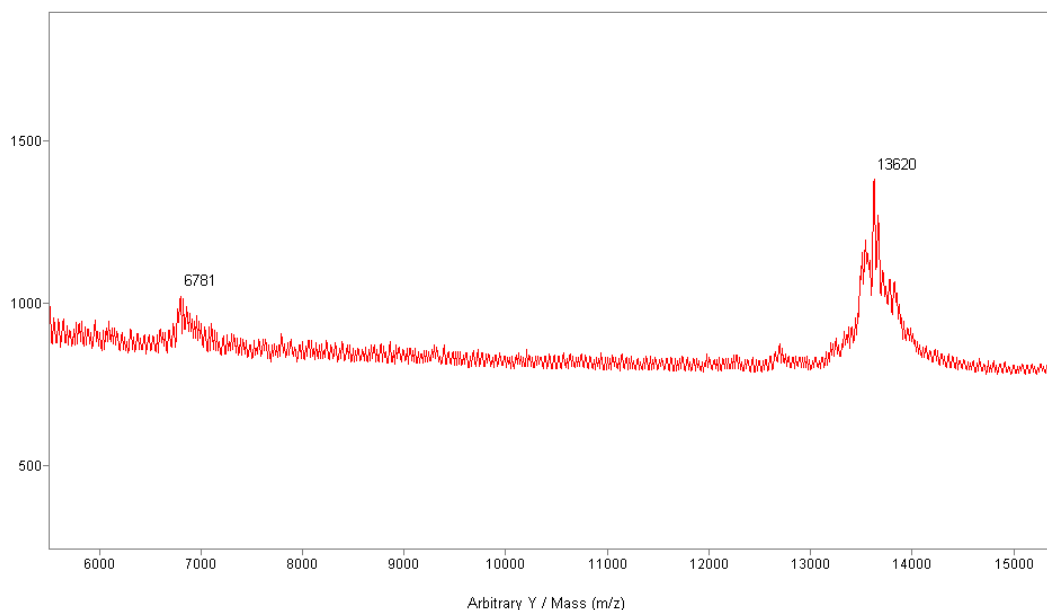


Figure 4: *E. coli* (ATCC No. 11775). 0.1 μ L SA was applied to the MALDI plate. After drying, the SA was covered with 1.0 μ L *E. coli*. When this layer was dried, it was covered with 0.5 μ L 0.1% TFA.

Table 2: Variations of *E. coli* (ATCC No. 11775) sample preparation methodology

| | | | | | |
|-------------|--|-------------|--|-------------|--|
| Treatment A | 0.2 μ L SA \rightarrow 0.2 μ L <i>E. coli</i> | Treatment G | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrow0.6μL 0.5% acetic acid</i> | Treatment M | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrow2 wash w/ 1.0μL 0.1% TFA</i> |
| Treatment B | 0.2 μ L SA \rightarrow 0.4 μ L <i>E. coli</i> | Treatment H | 0.1 μ L SA \rightarrow 0.3 μ L 0.5% acetic acid \rightarrow 1.0 μ L <i>E. coli</i> | Treatment N | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrow3 wash w/ 1.0μL 0.1% TFA</i> |
| Treatment C | 0.2 μ L SA \rightarrow 1.0 μ L <i>E. coli</i> | Treatment I | 0.1 μ L SA \rightarrow 0.6 μ L 0.5% acetic acid \rightarrow 1.0 μ L <i>E. coli</i> | Treatment O | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrowCPD (15 seconds)</i> |
| Treatment D | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli</i> | Treatment J | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrow0.3μL 0.1% TFA</i> | Treatment P | 0.2 μ L SA \rightarrow 0.4 μ L <i>E. coli\rightarrow1 wash w/ 1.0μL 0.1% TFA</i> |
| Treatment E | 1.0 μ L <i>E. coli\rightarrow0.1μL SA</i> | Treatment K | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrow0.5μL 0.1% TFA</i> | Treatment Q | 0.2 μ L SA \rightarrow 0.4 μ L <i>E. coli\rightarrow2 wash w/ 1.0μL 0.1% TFA</i> |
| Treatment F | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrow0.3μL 0.5% acetic acid</i> | Treatment L | 0.1 μ L SA \rightarrow 1.0 μ L <i>E. coli\rightarrow1 wash w/ 1.0μL 0.1% TFA</i> | Treatment R | 0.2 μ L SA \rightarrow 0.4 μ L <i>E. coli\rightarrow3 wash w/ 1.0μL 0.1% TFA</i> |

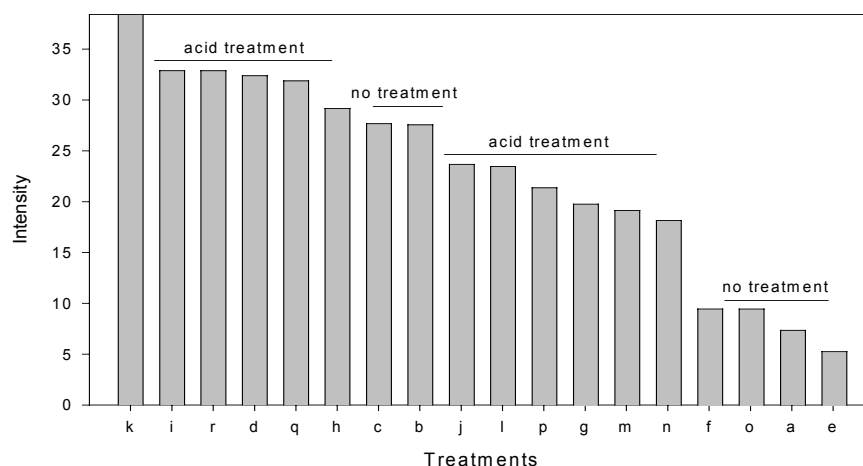


Figure 5
E. coli (ATCC No. 11775). This chart represents a signal-to-noise comparison of treatments. General descriptions of treatments are listed above their respective bars.

than spectra from the previous sample. Consequently, only one peak from this sample of bacteria near m/z 13620 was used to assign signal-to-noise ratios for comparison (**Figure 5**). Like the previous sample of *E. coli*, this sample produced better spectra as the ratio of *E. coli* to SA increased. It appeared as though nothing conclusive could be said about the effects of either acetic acid applications or TFA washes on these samples, as some samples of both treatments gave spectra better than the same bacteria/matrix samples without the treatment, while others gave worse spectra than the standard bacteria/matrix sample. Interestingly enough, the most intense peak came from a sample on which TFA was applied, not used as a washing agent. This attempt to quantitate the peak intensities from this sample would have been more accurate and reliable if more consistent spectral

peaks would have been observed, rather than ranking sample preparation methods based on a single peak.

Table 3: Organismal identification ranking according to the *E. coli* spectrum from Figure 3

| | 3674 | 3864 | 3895 | 4106 | 4496 | 5304 | 5789 | 6528 | 7269 | 7319 | 7476 | 8154 | 8428 | 8637 | 9111 | 9719 | 15817 |
|---|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|-------|
| <i>E. coli</i> | 1 | 2 | 1 | 1 | | 3 | 3 | | 7 | | 1 | | 3 | 6 | 3 | 2 | 1 |
| <i>N. meningitidis</i> (serogroup B) | | 1 | 1 | | | | | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| <i>M. leprae</i> | | | | | | 1 | 1 | | | 1 | 1 | 1 | 2 | 1 | 1 | 1 | |
| <i>B. subtilis</i> | | | | 1 | | | 1 | 1 | 2 | 1 | 2 | | | 1 | 1 | 2 | |
| <i>V. cholerae</i> | 3 | | 1 | 1 | 1 | 1 | | 1 | | | | | | 1 | 1 | | |
| <i>B. burgdorferi</i> | 1 | 2 | 1 | | | | | 1 | | | 1 | | | 1 | 1 | | |
| <i>H. pylori</i> | 1 | 1 | | | | 1 | | | | | | | 2 | 1 | 3 | | 2 |
| <i>M. tuberculosis</i> | | | | 2 | | 1 | 1 | 1 | 2 | | | 1 | | 1 | 2 | | |
| <i>S. coelicolor</i> | | 1 | | | 1 | | | 1 | | | 1 | | 1 | 2 | | | 1 |
| <i>X. fastidiosa</i> | | | | 3 | | | | | 1 | | 1 | | 2 | 1 | 1 | 1 | |

*All hits were within a ± 3 Da window.

Database searches. Protein database searches were carried out for the first sample of *E. coli* (ATCC No. 23724), as the lack of signal for the other *E. coli* sample was a hindrance in accurately determining its identity. These protein databases acquire vast amounts of information of proteins from paper submissions containing various pieces of data about a particular protein. Databases contain several criteria on which to base a protein search, including organism identification, strain, author of protein-describing paper, molecular weight, sequence length, and a host of others. When performing the search, the different criteria on which the search should be based are selected, and the boundaries of each criterion are inserted into the database. In identifying the mass/charge ratios of peaks, it was assumed that a hydronium ion gave each protein its positive charge, and 1 Da was subtracted from each mass/charge for database analysis. The spectrum from **Figure 1** was used to identify masses for the database searches. The results from these searches, both number of peaks hit by each bacterium and number of hits under each mass/charge, are in **Table 3**. *E. coli* had the most hits with 13, followed by *Neisseria meningitidis* serogroup B with 10 and *Mycobacterium leprae* and *Bacillus subtilis* with 9. Furthermore, *E. coli* had 7 hits at 7266, the most common and recurring peak in all spectra taken of this bacterium going back to the generally peakless spectra of initial samples.

Cancerous cells. Analyzed using techniques similar to those incorporated for *E. coli*, both Jurkat and K562 leukemia cells were examined on the MALDI merely for qualitative purposes. Spectra from each of these cell lines are displayed in **Figures 6** and **7**. In contrast with *E. coli*, the MALDI sample of Jurkat cells that yielded the most spectral peaks included α -CHCA as the MALDI matrix (both orders of matrix/cell application generating similar spectra), while the other two matrixes, SA and DHB,

provided spectra with fewer, less intense peaks. K562 cell line spectra produced high numbers of peaks when combined with any of the three matrixes. The sample that seemed to produce the largest peaks both in height and width was DHB matrix applied over the K562 cells. The identities of the spectral peaks from both Jurkat and K562 cell lines are somewhat ambiguous. The large width of these peaks suggests salt contamination of cells. Treating these cells with various sample purification methods may produce more resolved peaks. While there is more certainty that bacterial peaks are in fact released proteins,⁵ this may not be the case with more complex eukaryotic cells. These peaks could be the result of a number of different biomolecules, including lipids, carbohydrates, or DNA. Further research into this hypothesis could suggest if not confirm their identities.

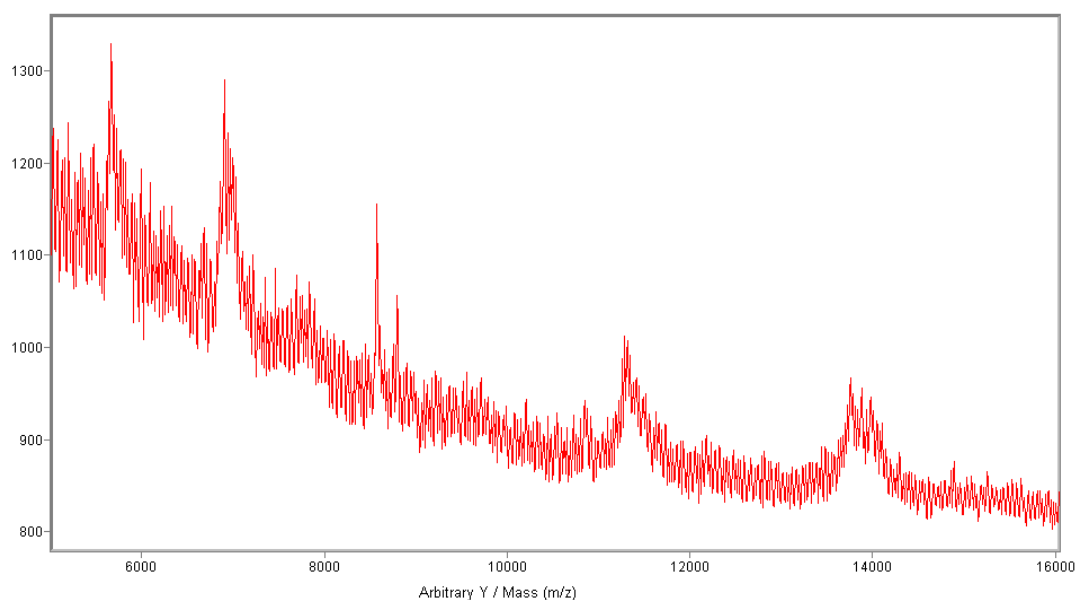


Figure 6: Jurkat leukemia cells. 0.4 μ L Jurkats suspension was applied to the MALDI plate, dried, and covered with 0.4 μ L α -CHCA.

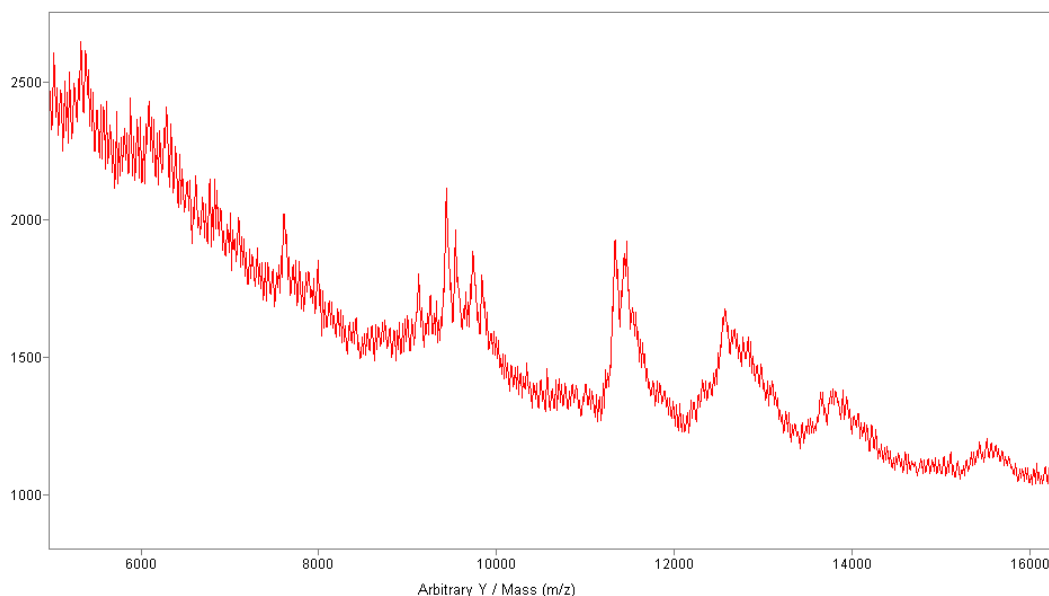


Figure 7: K562 leukemia cells. 0.4 μ L K562 suspension was applied to the MALDI plate, dried, and covered with 0.4 μ L DHB.

Conclusion:

Bacterial samples of *E. coli* have been analyzed on MALDI-TOF mass spectrometry, and the identity of *E. coli* sample ATCC No. 23724 has been suggested. Various sample preparation methods have been carried out on both strains of *E. coli*, and the results of these treatments suggest that treatment with acid, either addition of acid to sample or washing of sample with acid, improves peak intensity within spectra. Two leukemia cell lines, Jurkat and K562, have been sampled on MALDI, though this study is still in its infant stages. Ideally, this work could lead to the development of a similar procedure to incorporate protein database searches to identify human carcinoma cell lines and to distinguish them from those of healthy cells.

References:

- (1) Demirev, P. A.; Ho, Y.; Ryzhov, V.; Fenselau, C. *Anal. Chem.* **1999**, *71*, 2732-2738.
- (2) Madonna, A. J.; Basile, F.; Ferrer, I.; Meetani, M. A.; Rees, J. C.; Voorhees, K. J. *Rapid Comm. Mass Spec.* **2000**, *14*, 2220-2229.
- (3) Madonna, A. J.; Basile, F.; Furlong, E.; Voorhees, K. J. *Rapid Comm. Mass Spec.* **2001**, *15*, 1068-1074.
- (4) Lay, J. O. *Trends in Anal. Chem.* **2000**, *19*, 507-516.
- (5) Xiang, F.; Anderson, G. A.; Veenstra, T. D.; Lipton, M. S.; Smith, R. D. *Anal. Chem.* **2000**, *72*, 2475-2481.
- (6) Guyton, A. C.; *Textbook of Med. Phys.* **1986**, Ch. 3, 38.

- (7) Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D. *Mol. Bio. of the Cell*, **1994**, Ch. 24, 1279.
- (8) Milani, G.; Kowaltowski, A. J.; Saad, S. T. O.; Metze, K.; Vercesi, A. E. *Drug Dev. Research* **2001**, 52, 508-514.
- (9) Birmingham, J.; Demirev, P.; Ho, Y.; Thomas, J.; Bryden, W.; Fenselau, C. *Rapid Comm. Mass Spec.* **1999**, 13, 604-606.