Development of Ion Mobility Spectrometry (IMS) Applications for *Listeria* detection and monitoring in Food Processing Plants

Final Report

AMIF grant/USDA contract to the University of Connecticut

Robert Vinopal, Department of Molecular and Cell Biology
Claudia Koerting, Department of Marine Science

August 26, 2005

Summary of Results

The main objective of the project was to collect and distinguish Listeria species by employing a ramped heating system. The temperature ramp device was developed and shown to improve resolution of peaks allowing us to distinguish between strains. IMS detection of Listeria on meat, collection of aerosolized bacteria with subsequent analysis by IonScan was demonstrated. Selectivity of bacterial detection was further improved by combining whole cell desorption and analysis with short incubation times in the presence of Listeria diagnostic media.

The temperature ramp development was delayed early in the project because the first attempt at a wire array heating source did not provide sufficiently uniform heating across the entire sample area to allow adequate temporal separation of different bacterial components during sample desorption. Looking for an alternative approach, we found that placing glass fiber filters between the sample on a Teflon filter and the standard heater on the unmodified IonScan instrument allowed for a more gradual increase in sample temperature. While the resulting temporal ion peak separation was similar to separations first observed by moving samples between different instruments set at increasing fixed desorber temperatures, it could not replace a temperature ramp. However, as discussed in the previous report, (Attachment A) we could distinguish unequivocally between *Listeria innocua* and *L. monocytogenes* using the glass fiber filters.

While the use of glass fiber filters was preferred for collection of aerosol, direct swipes of meat and surfaces were best done with the Teflon filters, yielding the cleanest results as reported previously (Attachment A). When the temperature ramping was not available, Teflon filters were preferred for direct assays of diagnostic media, from which species-specific enzyme substrate products could also be identified. Using the glass fiber filter approach, we tested aerosols with subsequent incubation on diagnostic media for refinement of identifications. In another study, *Bacillus cereus* was used as a surrogate target during aerosol collection (Attachment B). Aerosol containing Bacillus was bubbled through esculin-containing broth. After 2 hours or more of incubation the broth was analyzed on Teflon and the esculetin product identified. Aerosol collected on glass fiber filters of different species of *Listeria* and *Staphylococcus* could be distinguished after incubation in diagnostic media in less time than standard tests, and can serve as a first step rapid presumptive test.

The temperature ramping device was redesigned. Results from tests with the redesigned device demonstrated clearly that the only way to use the IonScan with complex samples is by ramping (stepped thermal desorption) or using some other upfront separation technique such as gas or liquid chromatography, and not the rapid, fixed high temperature desorption that was used. The use of the Teflon and glass fiber pads initially was the correct way to go to achieve a limited thermal ramping capability by increasing the thickness of the pads to slow down the heat transfer from the heated desorber to the bacterial cells.

1. Ramp design

Previously we reported that a low thermal mass heater was designed and mounted in a modified Teflon sample holder, with the purpose of regulating temperature ramping of the sample. At that time, the wire array failed to provide sufficiently uniform heating to allow temporal separation of different bacterial components during sample desorption. Since the submission of the previous report, Dr. Philip Best has redesigned the heater with improved ramping capabilities. (Figures 8-11)

A. Design of the apparatus.

This apparatus consisted of three parts: the mechanical structure, the control electronics and the over-reaching computer control and measurement. The Teflon pad support is a standard card for the IonScan and ensured compatibility with that instrument. Two 0.02" diameter tantalum wires led current to the nichrome ribbon heater. The chromel-alumel thermocouple wires meet at a point near the center of the heater.

Several challenges were encountered while moving from the design to the working model. The first challenge was working with the small components required to obtain the near ideal steps in temperature ramp. While 0.001" thick nichrome ribbons and 0.0005" thermo-couple (TC) wires were preferred, the difficulty in assembling the apparatus with these led to the physicists settling for 0.002" wire and 0.003" ribbon. The wires were joined welded with the aid of a low-power microscope. Figure 9 shows the ribbon and location of sample loading.

An electronic controller was chosen over a programmed PID system because of the expected low response time of the original design. The PID system would have been adequate. Standard design principles were used for both the hardware PID system and the power supply. The control and measurement functions of the computer were programmed in DELPHI. A graphical interface enables the operator to choose the size of the temperature steps, the measurement interval and the step hold time. The TC signal is returned to the A/D interface of the PC to give a record of the actual temperature history (of the TC junction). We did not have time to consider the temperature uniformity across the region of the ribbon covered by sample. Given time, we would analyze the temperature field of the ribbon in the vicinity of the TC. It may also be possible to test many samples of the one target, each loaded at a different location relative to the TC junction.

In the runs that were made, sample was applied onto the ribbon in the vicinity of the TC junction. A temperature sampling time of 25 ms was chosen. The step time was about 75 ms, much larger than originally planned, but much less than the step duration.

B. Heat Ramp Results and Discussion

In a typical IMS analysis of bacterial cells, A small colony of bacterial cells are transferred from a plate to the surface of a Teflon® membrane using a disposal Difco inoculating needle. The Teflon® membrane carrying bacterial cells is placed on a desorber with the spotted bacterial cells on the upper side of the membrane facing the inlet region of the IMS system. The desorber is at a fixed temperature of 270°C or less. The Teflon® membrane, containing the bacterial cells, is not in direct contact with the desorber at this time, but is within a few millimeters of the heated desorber surface. In this position the temperature of the membrane containing the bacterial cells begins to increase. During analysis the desorber is raised and is in direct contact with the membrane. The bacterial cell components are subjected to intense heat and volatilization of components occurs rapidly, within a few seconds. The sample is then thermally desorbed at fixed temperature of 270° C for a period of time up to 120 seconds. Data acquisition begins 0.025 seconds after the desorber is raised and is in contact with the Teflon® membrane.

In the Ramp technique, the bacterial cells are transferred to a metal strip (ribbon) after which the temperature of the metal surface is raised to 95° C and is held at this temperature for a period of 30 seconds. After 30 seconds the metal ribbon is ramped in a series of incremental steps to a final maximum temperature of 325° C. IMS data acquisition in the ramp mode starts before the temperature ramp is started.

Each scan of the IMS spectrum starts when the gating grid opens briefly to admit a burst of ions into the drift tube, and it ends just before the gating grid opens again. This interval is called the scan period. The data from several scans are co-added together to improve the signal-to-noise ratio. This summed data is called a segment. A series of segments with characteristic ion peak patterns for the strain are obtained, and can be displayed either as a series of individual segments versus desorption time in seconds (a 3-D plasmagram) or as an average of all segments obtained during the analysis (a 2-D plasmagram).

A combined negative ion mode 3D and 2D plasmagram obtained from the analysis of *Listeria innocua* cells obtained using the normal and ramped desorption technique are shown in Fig. 1 and 1A respectively. The x-axis shows the drift time of the detected ions in milliseconds. The y-axis displays the amplitude of the detected ions reaching the collector electrode in digital units (digitized analog output voltage from the pre-amplifier). The z-axis displays each individual segment (co-added scans showing the drift time of each detected ion in milliseconds and its amplitude during that segment) obtained at one-second intervals during the 120 second thermal desorption of the sample. Ion patterns change over the course of heating, with a tendency for smaller molecules, and those having a high vapor pressure, to be released and detected first. Within the drift tube region of the IMS, ions travel at different rates, based on their size, charge to mass ratio, and shape, and are identified by measuring the time required for them to reach the collector electrode (ion drift time), relative to that of the internal calibrant. Identification of compounds is based on the calculation of their characteristic reduced ion mobility K_0 (cm^2/Vsec) values. K_0 values of observed peaks are determined using the following equation:

$$\text{(I)} \qquad K_0 \text{ observed} = K_0 \text{ internal calibrant} \times (t_{cal}/t_{obs})$$

where t_{cal} is the drift time of the internal calibrant and t_{obs} is that of the observed peak. The K_0 value of a detected ion peak can be used to estimate the mass of the detected ion based on correlation tables provided by the instrument manufacture.

In the 2D plasmagrams shown in Figures 1 and 1A, two ion peaks having the same K_0 values rounded to two decimals (1.83 and 1.59) are detected in both the normal and ramp desorption techniques. These ion peaks are detected in the normal mode within 3 seconds and appear much later (55 seconds) in the ramp technique as would be expected. What is significant is that there is a major ion peak detected, having a K_0 value of 1.57 in the ramp technique as shown in Figure 1A that is absent in the normal mode of analysis as shown in Figure 1. Several possibilities exists that can be used to account for the absence of this ion peak in the normal mode; - (1) ion competition in the reaction chamber of the IMS system masking the ion formation of this component, and - (2) chemical reactions occurring on the high temperature surface of the Teflon or within the gas phase during the desorption process at 270° C; - (3) thermal instability of the component and fragmentation of this component occurring on the Teflon surface and/or in the heated inlet of the IMS system.

Figures 2 and 2A compares the 2D ion plasmagrams of the normal mode at 10-seconds and the ramp mode at 70-seconds. What is significant in Figures 2 and 2A is that the ion peak having a K_0 value of 1.59 in both the normal and ramp mode in Figures 1 and 1A is still present in the normal mode at 10 seconds but not detected in the ramp mode at 70 seconds. Inspection of the ramp mode 3D plasmagram in Figure 2A indicates that the initial 1.59 ion peak seen in Figure 1A has grown into a much larger ion peak having a K_0 value 1.57 as seen in Figure 2A. The normal mode contains two ion peaks having K_0 values of 1.49 and 1.44 which are not seen in the ramp mode 70-secods into the desorption cycle, and the ramp mode shows an ion peak having a K_0 value of 1.48 which is absent in the normal mode as seen when comparing Figures 2 and 2A. This peak, however, appears to be present in the normal mode as seen in Figure 1 (3 second desorption) in the 11.5 to 12.0 millisecond drift time. An unresolved ion peak is present that has a K_0 value of 1.48. The ramp mode at 70 seconds shows a series of 3 strong ion peaks in the 9.5 to 10.5 millisecond range that are essentially absent, or barely detectable, in the normal mode during the first 10 seconds of desorption when the bacterial cells are rapidly heated and volatilized at 270° C form the Teflon surface as seen in Figures 1 and 2.

Figures 3 (26-second) and 4 (45 and 56 second) are selected 2D plasmagrams using the normal mode of desorption. Figure 3 shows that the ion peak having a K_0 value of 1.44 is absent at 26 seconds into the desorption cycle and is replaced by an apparent "double" ion peak having at least two components with K_0 values of 1.49 and 1.45 as seen in Figure 4 during the 45 to 56 second period of desorption. It is suspected that the 1.45 is actually the 1.44 ion peak, but the resolution of this ion peak has been impacted by the presence of multiple ions in the drift tube at during the rapid volatilization of many compounds at 270° C. Figure 4 also shows that the 1.59 ion peak at 45 seconds is now possibly composed of two components as indicated by the shape of the 1.59 peak. Figure 4 also shows the appearance of a series of ion peaks in the 13.5 to 17 millisecond range with apparent growth in the amplitude of these ion peaks as the desorption time increases as seen in the 3D Plasmagram in Figure 4.

Figure 5 shows selected 2D plasmagrams at 101 seconds using the ramp mode. The ion peaks in the 13.5 to 17 millisecond range in the normal mode are essentially absent in the ramp mode and none detectable based on their amplitudes. There are three possible explanations for this occurrence - (1) the 13.5 to 17 millisecond ion peaks seen in the normal mode are products of combination reactions occurring either on the heated Teflon surface (270° C) or within the gas phase; – (2) they are dimmers formed by the coupling of ions in the gas phase; - or (3) the ramp did not achieve its final temperature of 325° C.

Figure 5 indicates that the ion peak having a K_0 value of 1.44 detected in the 10-second 2D plasmagram in the normal mode (Figure 2) is now detected in the Ramp mode at 101 seconds (Figure 5). The 2D plasmagram in Figure 5 identifies apparently a "single" ion peak ion peak with a K_0 value of 1.36. This ion peak was also detected at 88 seconds in the Ramp mode, but it was one of three ion peaks that is clearly resolved in the Ramp mode as seen in Figure 7. This cluster of three ion peaks seen in Figure 7 transitions only a single peak at 101 seconds as seen in the 101 second Ramp 2D desorption plasmagram (Figure 5).

Figure 6 shows the 2D plasmagram at 26 seconds in the normal desorption mode using Teflon. The amplitude scale has been expanded to show the initial presence of an ion peak having a K_0 value of 1.37. This ion peak transitions into the 1.38 ion peak shown in the 56 second 2D plasmagram shown in Figure 4. It is suspected that the 1.37 or 1.38 ion peak seen in the normal desorption mode is the same as the 1.36 ion peak shown in the 101 second Ramp desorption 2D plasmagram shown in Figure 5, and the reason for the difference in K_0 values is that the resolution of this ion peak in the normal mode has been impacted by the presence of multiple ions in the drift tube at during the rapid volatilization of many compounds at 270° C. This suggests that the Ramp mode at 101 seconds is directly relatable to the normal mode using Teflon at a desorption time of 56 seconds in which the presence of the ion peaks in the 13.5 to 17 millisecond range in the normal mode are clearly detectable. Their absence in the Ramp mode 2D and 3D plasmagrams cannot be explained without further studies. Based on other studies these ion peaks may be the result of reactions occurring on the rapid heating of the Teflon to 270° C, and reactions within the gas phase and/or dimmer formation during IMS analysis.

Conclusion

However, the data clearly shows:

1. that ion peak resolution and detection is enhanced using the stepped Ramp mode; and
2. that the Ramp mode allows for the detection of ions that are essentially masked by the rapid desorption of cells at a fixed temperature of 270° C

A major benefit of using the stepped desorption is that two approaches to IMS detection of bacteria can be combined. At low temperatures, after a short incubation with diagnostic media, enzyme products are released and detected, that would otherwise be degraded at the higher temperatures. Followed by increasing temperatures, bacterial cell components could be distinguished.

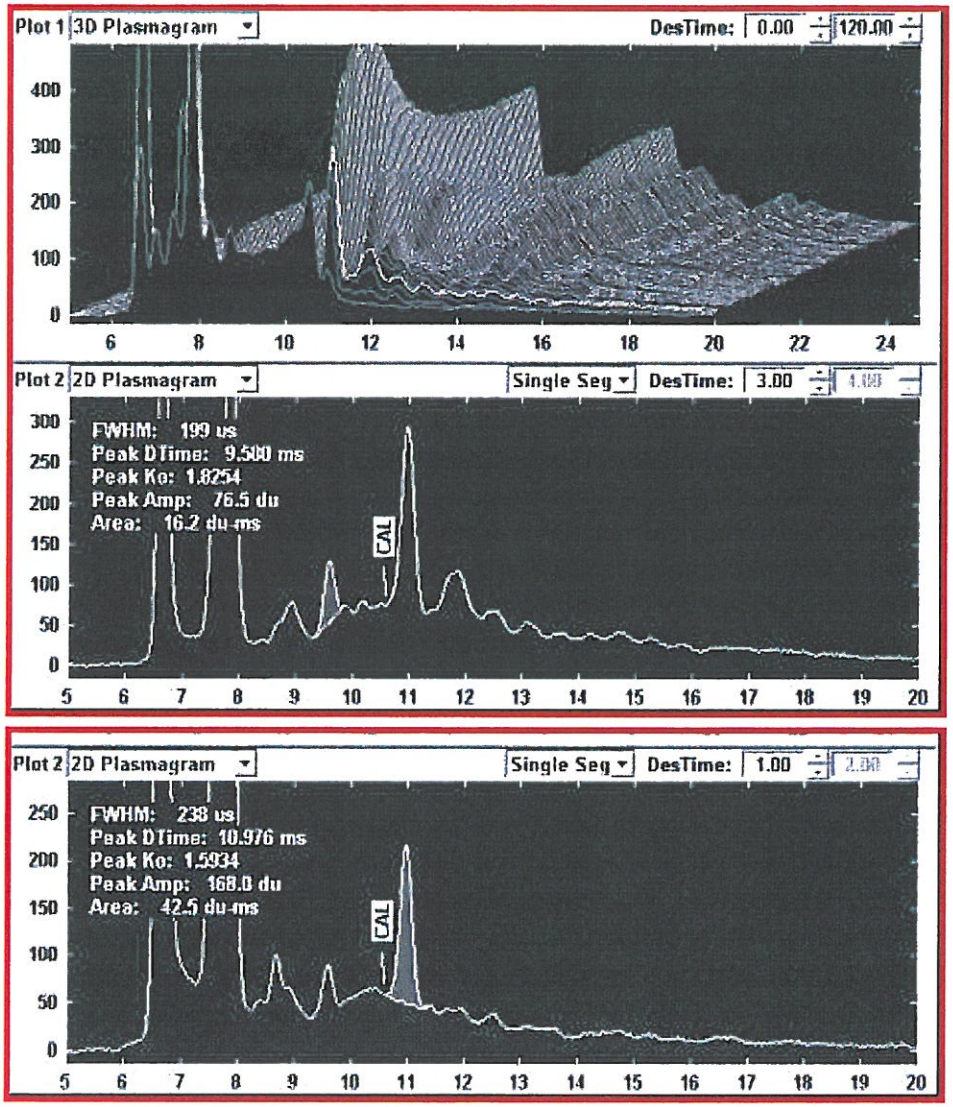


Figure 1: 1 and 3 Second 2D Plasmagrams, Normal IMS desorption analysis mode using Teflon - rapid thermal desorption of bacterial cells at a fixed temperature of 270°C.

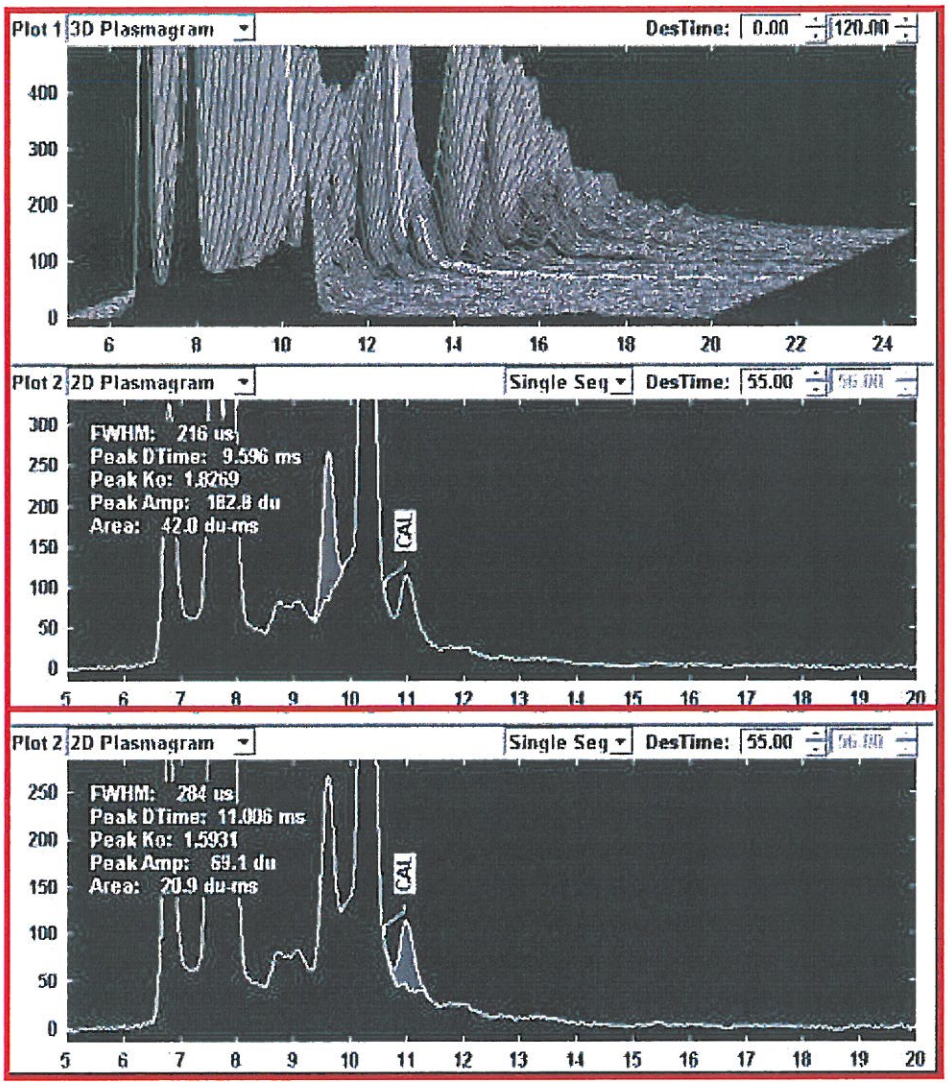


Figure 1A: 55 Second 2D Plasmagrams using the Stepped Ramp Desorption Technique

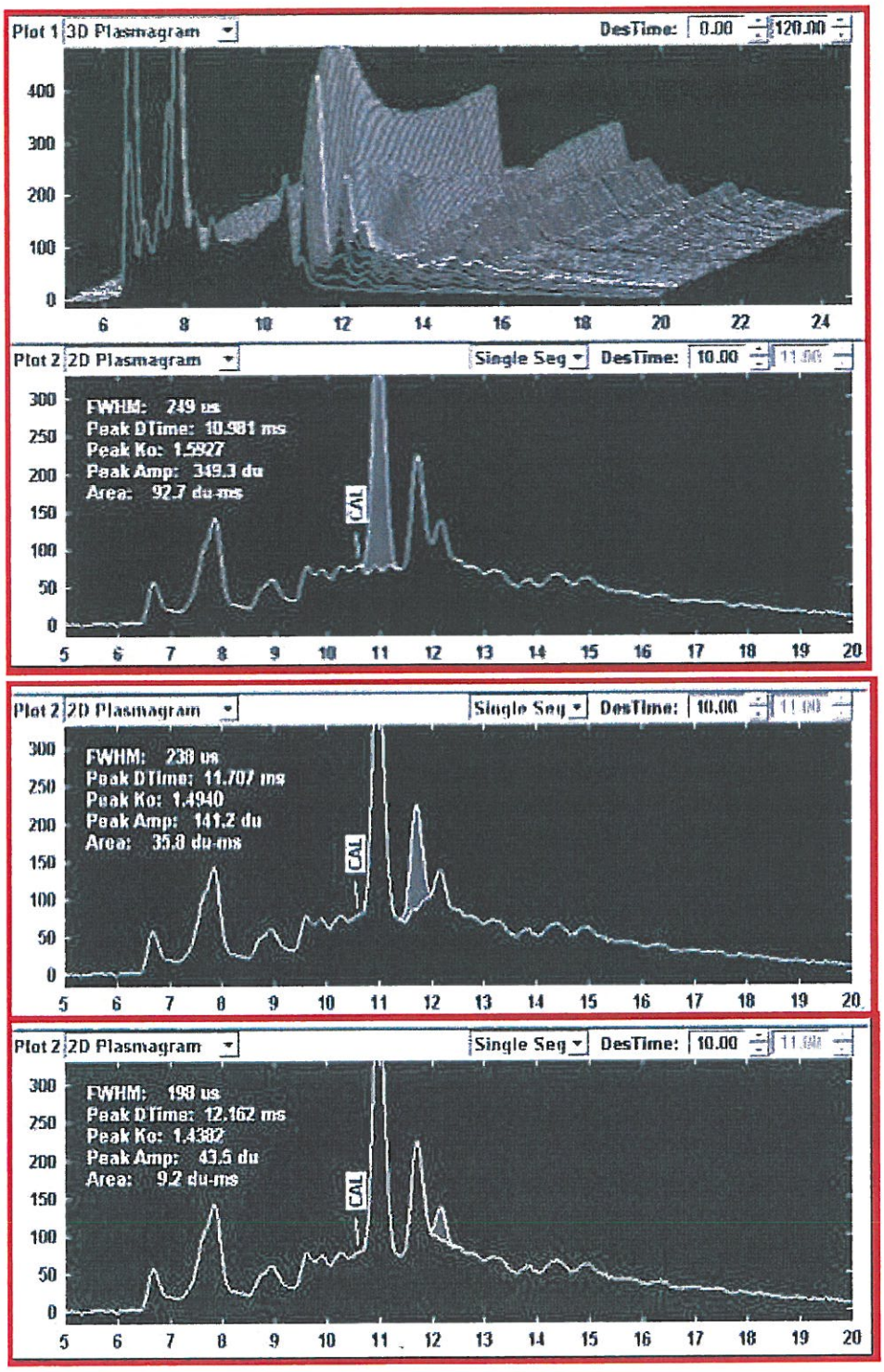


Figure 2: 10 Second 2D Plasmagrams Normal Mode

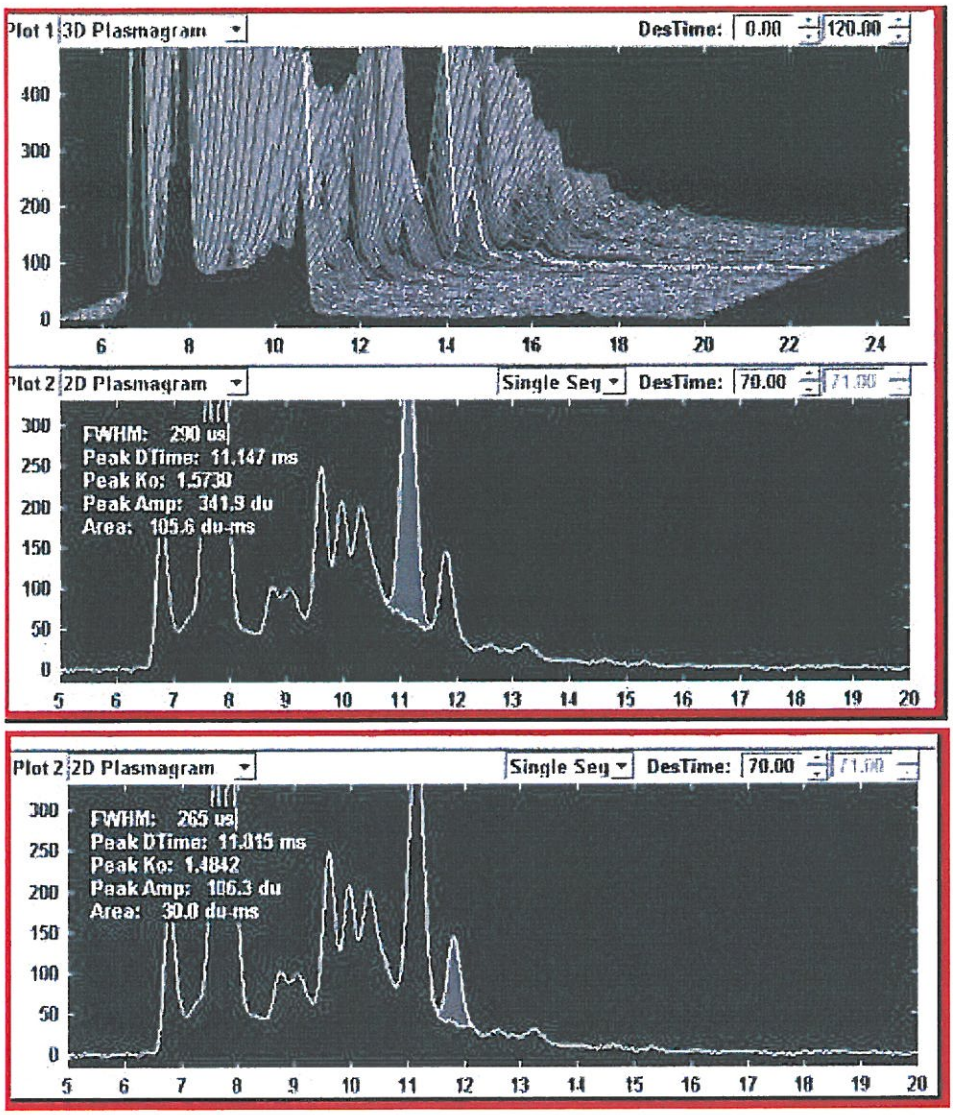


Figure 2A: 70 Second 2D Plasmagrams Ramp Mode

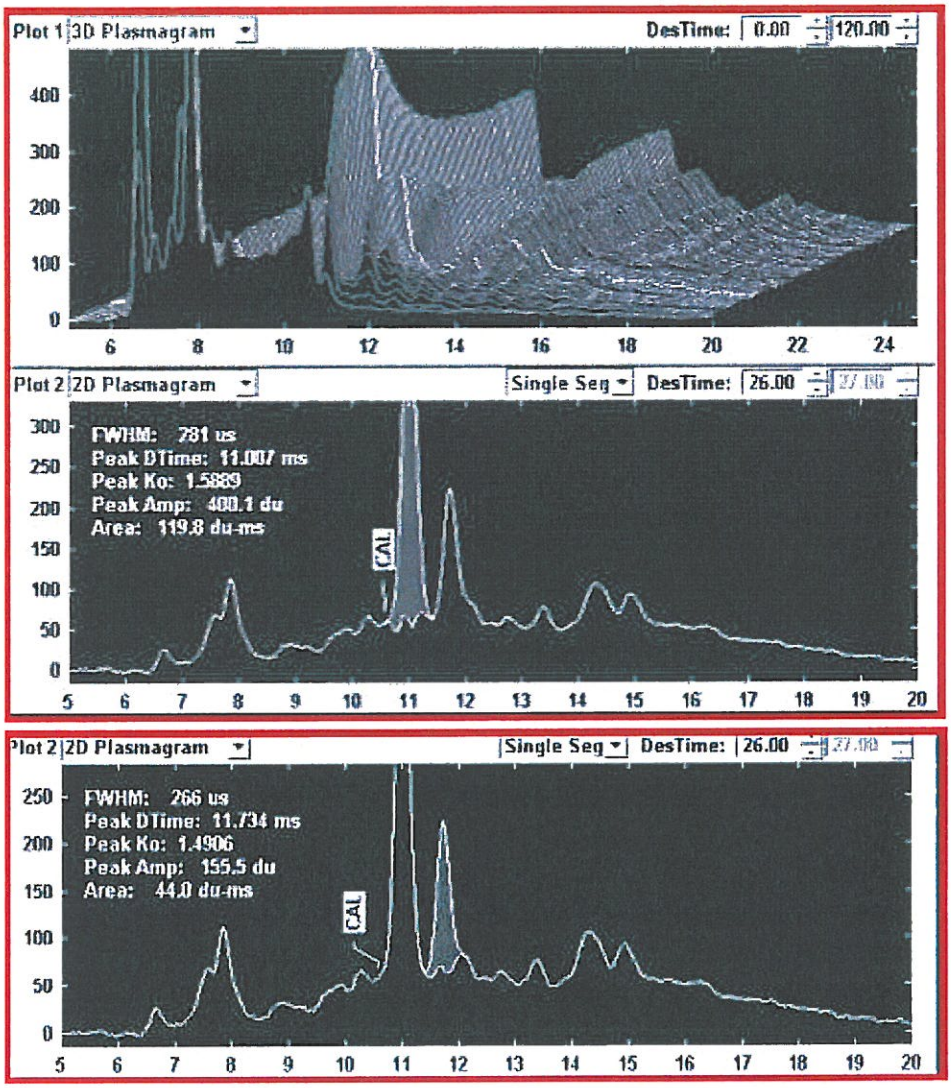


Figure 3: 26 Second 2D Plasmagrams, Normal Mode desorption using Teflon.

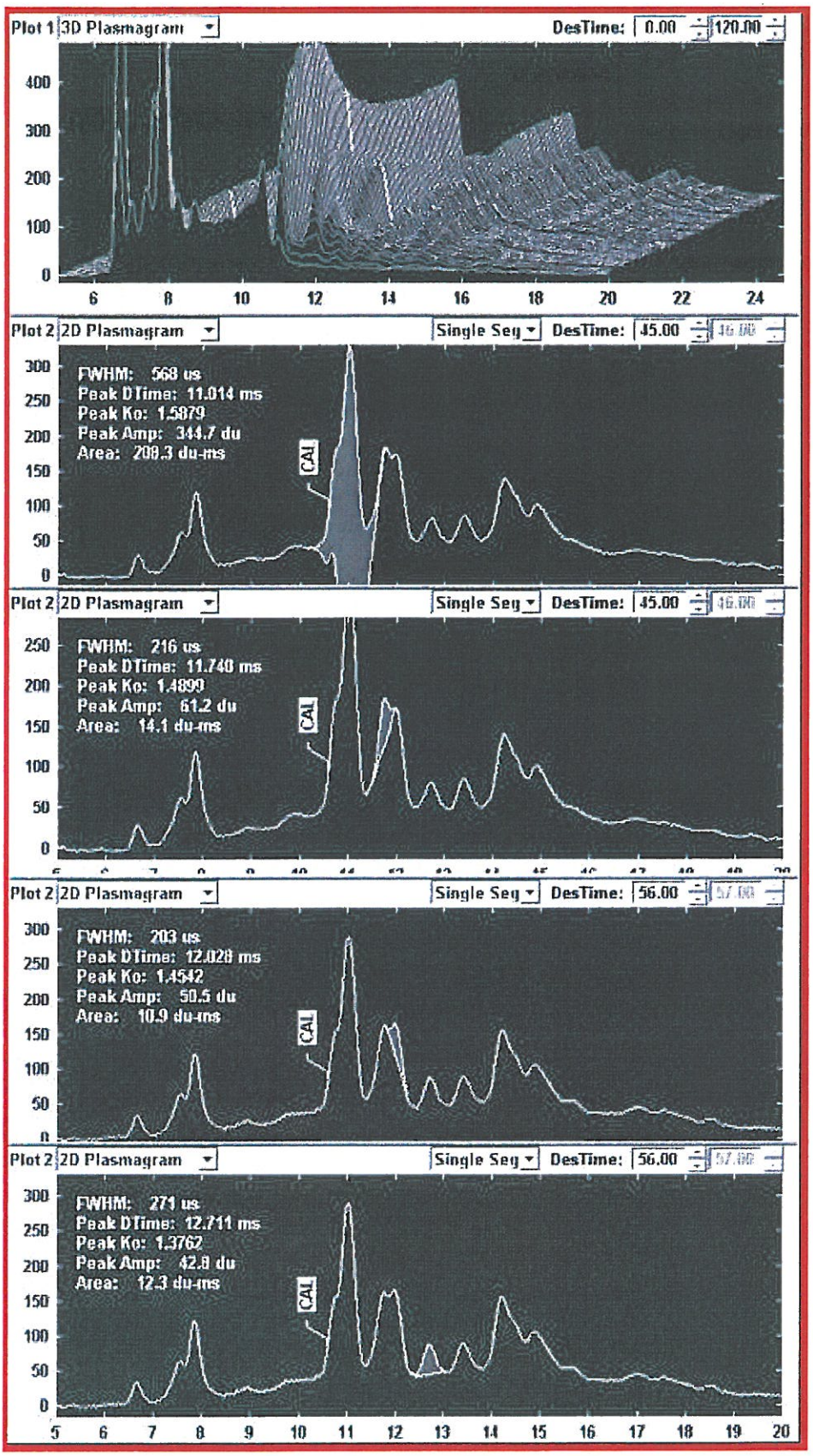


Figure 4: 45 and 56 Second 2D Plasmagrams, Normal Mode using Teflon

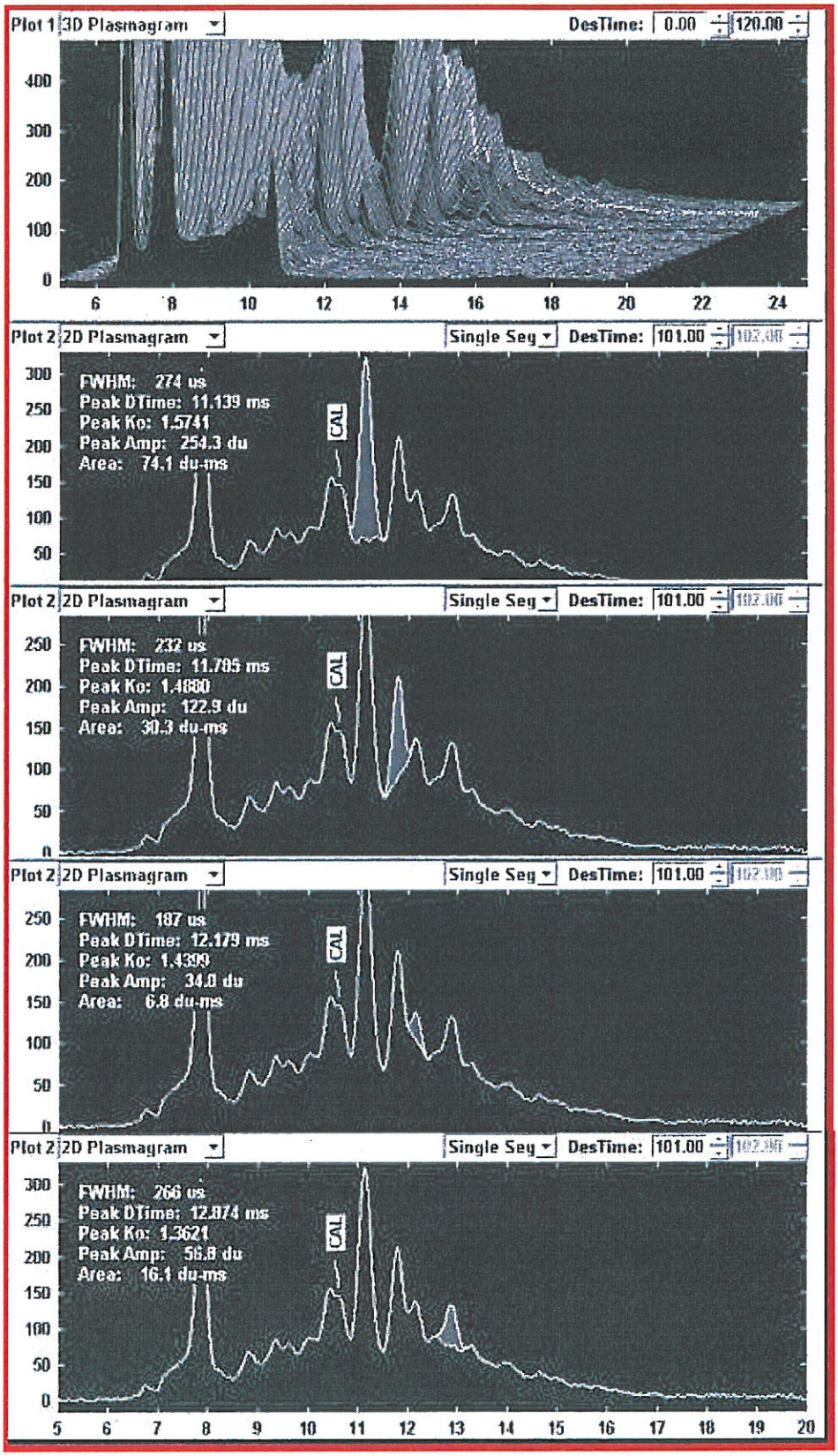


Figure 5: 101 Second 2D Plasmagrams Ramp Mode

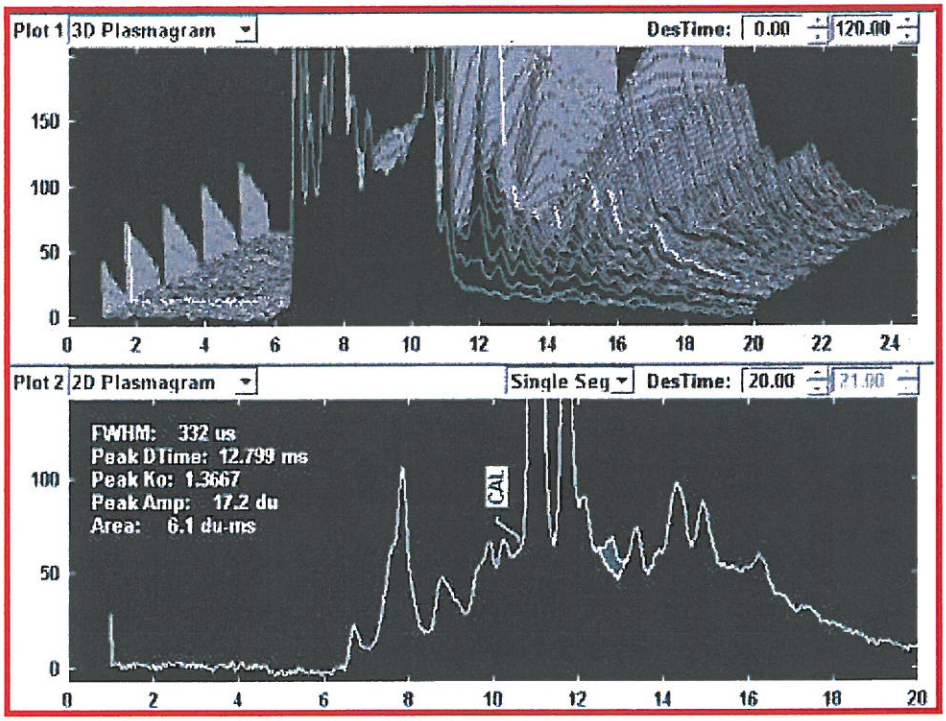


Figure 6: 20 Second 2D Plasmagram Normal Mode using Teflon.

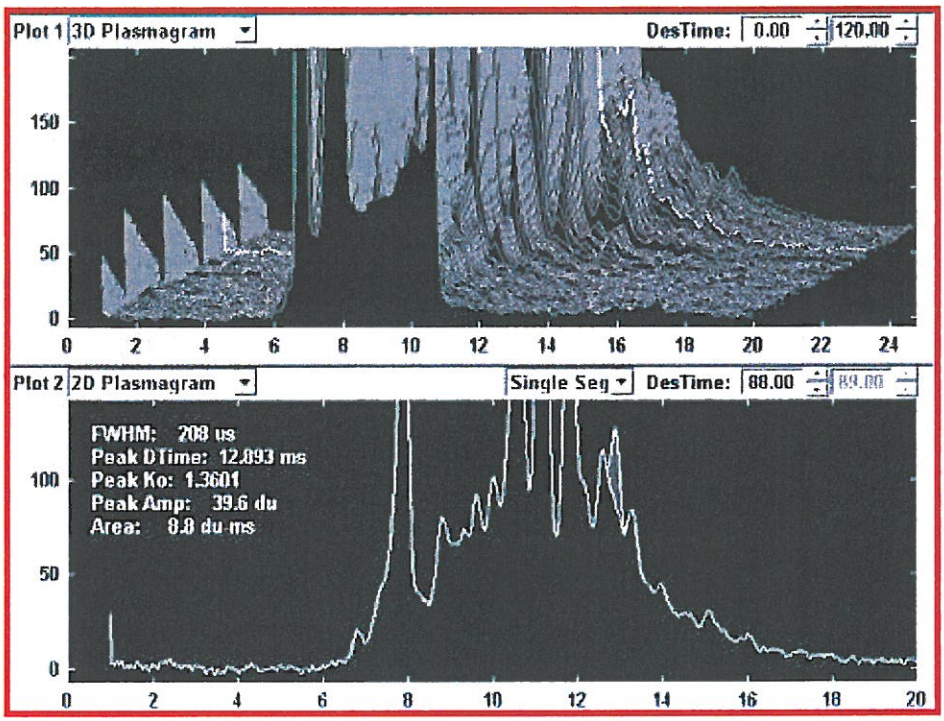


Figure 7: 88 Second 2D Ramp Plasmagram

2. Selective media

Several types of media were tested for increasing the selectivity and sensitivity of the IMS method: Frazer's broth, ALOA and BioRad Rapid. After collecting cells on filters, the filters were placed on the solid medium (the only form commercially available.) Using this method, unconverted substrate and solid medium were also transferred on to the filter and interfered with sampling and contaminated the sample holder. A solution to this, since very little medium was required, was to elute the diagnostic components from the commercial medium. The glass filters were then dampened with the medium extract, incubated 4 – 6 hours, and then analyzed. Figures 12 – 16 compare the results for a simulated stepped desorption on glass fiber filters to distinguish between *L. innocua* and *L. monocytogenes* incubated on selective media after capture on filters. For comparison purposes figures 12 and 13 demonstrate direct analysis of the bacteria on Teflon pads using different desorption temperatures (low and high) and consecutive burns (simulating a ramping effect). Figures 14-16 compare plasmagrams of *L. innocua* and *L. monocytogenes* after incubation on diagnostic media for 6 hours under a variety of desorption conditions. The results indicate that the selective medium contributes somewhat to discernment of the different species when limited to using pad thickness as a substitute for a heat ramp. However, a heat ramp will improve resolution of the species-specific peaks.

3. Aerosol collection devices

The design for our aerosol tests is shown in figures 17- 19. An aerosol can was used to create a Listeria mist, the pumps then ran for five minutes. At the end of aerosol collection, filters were removed and placed on medium or buffer or analyzed directly by IMS. No benefit was found by adding octanol to these samples. We estimated that the filters collected 10^4 bacteria, which was close to the detection limit for *L. innocua*, S. *aureus* and the *L. seeligeri*. Interestingly, *L. monocytogenes* 4b seemed to be more difficult to detect, concentrations on the filter required about 10^6 cells, even with repeated samples. Incubation for 6 hrs on selective media enhanced the detection of the lower concentrations. The ALOA gave a more distinctive plasmagram compared to the BioRad rapid medium.

how much?

Analysis takes place with the glass filters laid over a clean glass filter, to simulate heat ramping. Low inlet and desorption temps (220/230) were used because undigested metabolites are degraded at the higher temperatures (masking the selective nature of the medium and yielding false positives). Bacteria collected on filters and directly analyzed without prior incubation and media were burned at elevated temps of 260/270. Increasing temps to 270/280 did not appear to improve the resolution or yield new peaks. *L. monocytogenes* type 4b was compared to *L. seeligeri*, *L. innocua* and *Staphylococcus aureus* using the aerosol collection method and the simulated temperature ramp with layered filters. Small distinctions could be made, but would require a true temperature ramp to enhance and improve resolution.

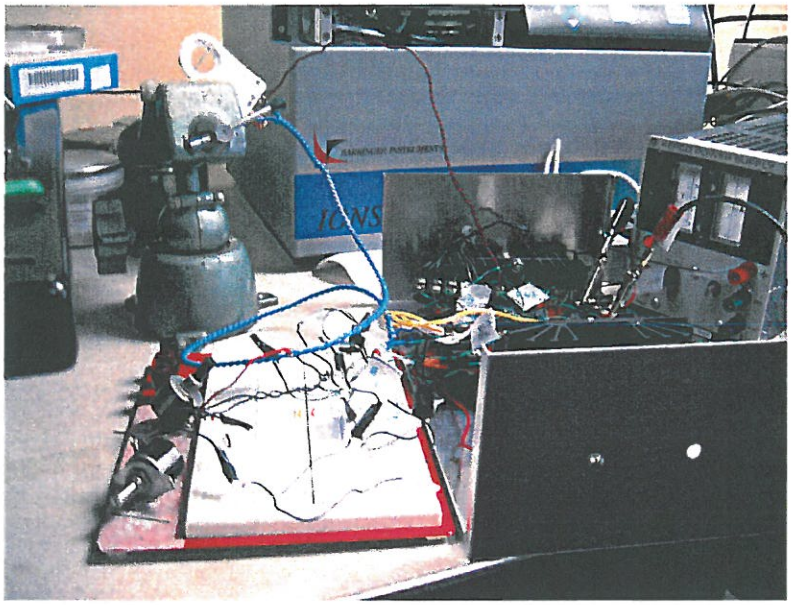


Figure 8. Ramp apparatus connected to IonScan

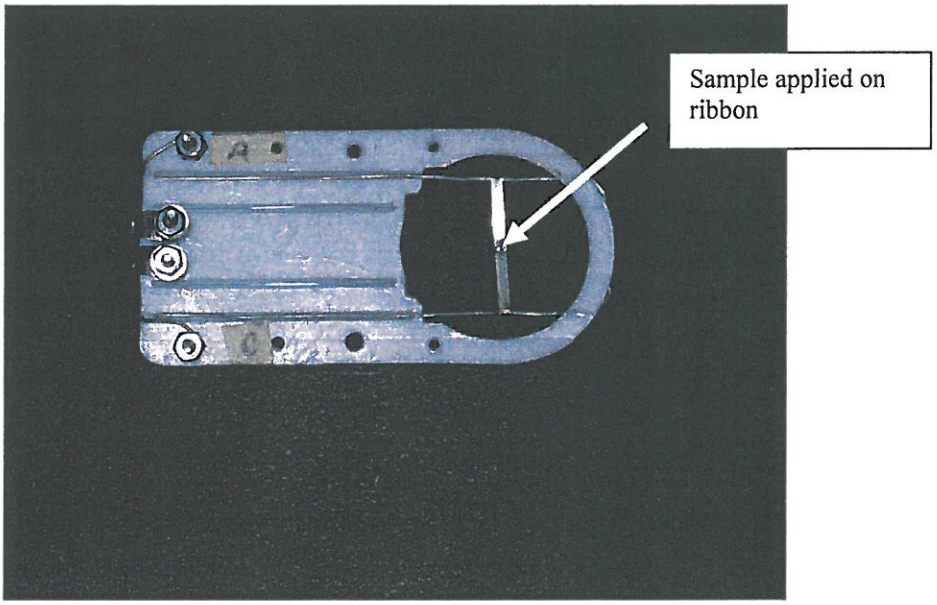


Figure 9. Sample platform, sample is applied to ribbon where indicated. Temperature ramp is controlled by the wires leading from the ribbon to the external ramping device.

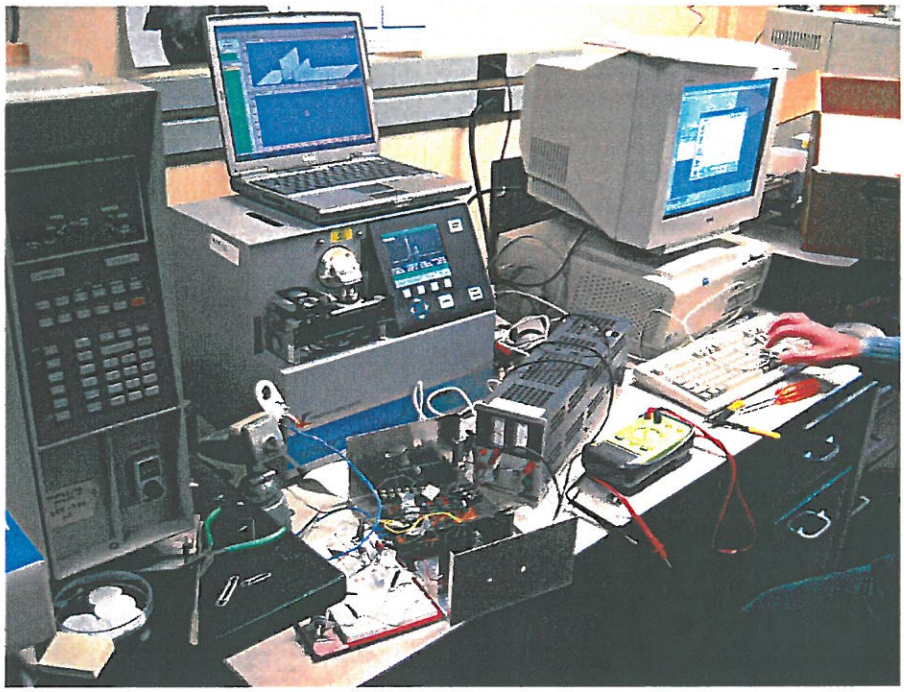


Figure 10. Final IonScan assisted by externally controlled sample heat ramp.

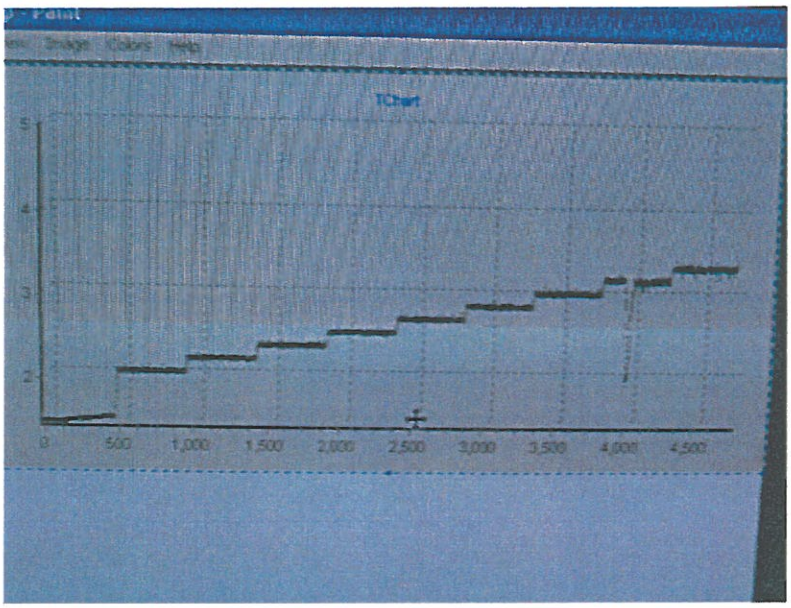
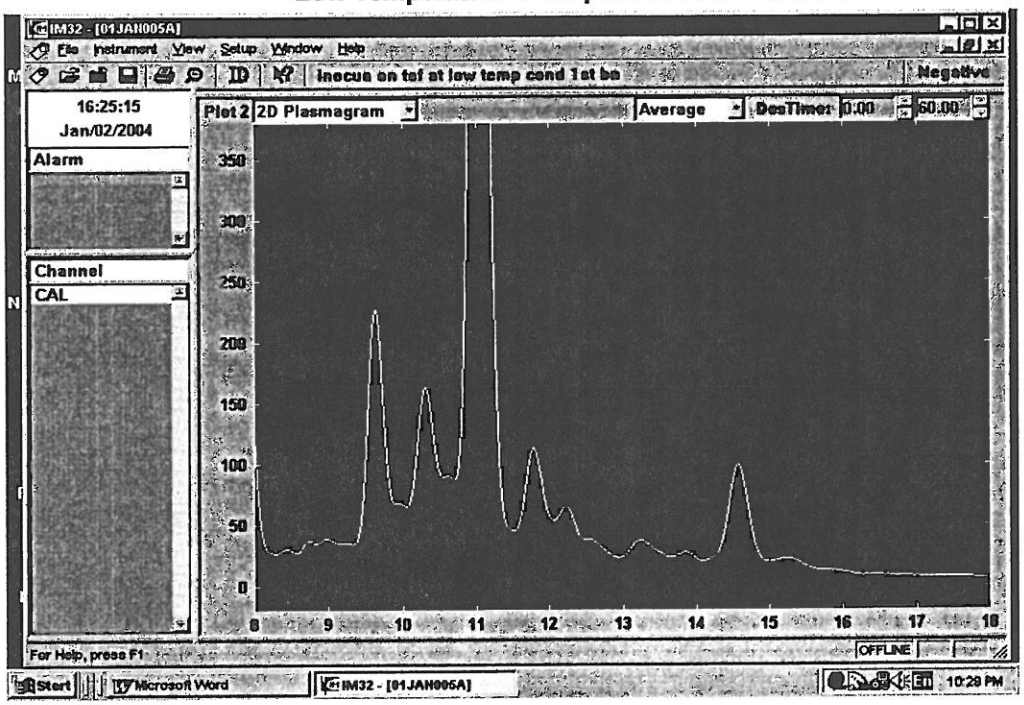
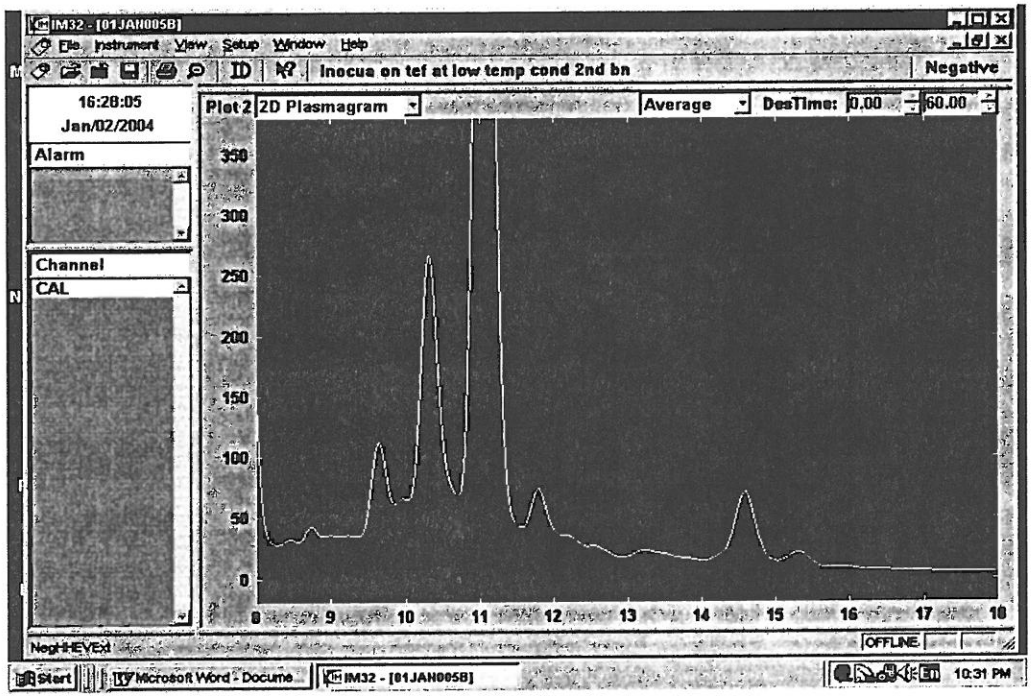


Figure 11. Ramp Chart

Simulated Temperature Ramping
Low Temperature Desorption of *Listeria inocua*



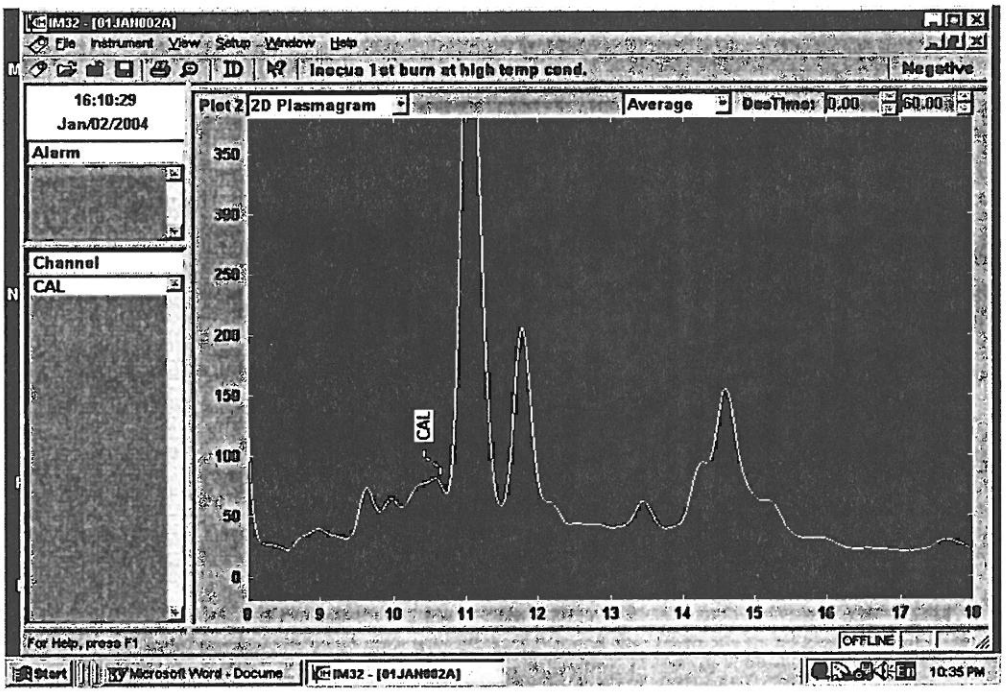
Desorption Step 1



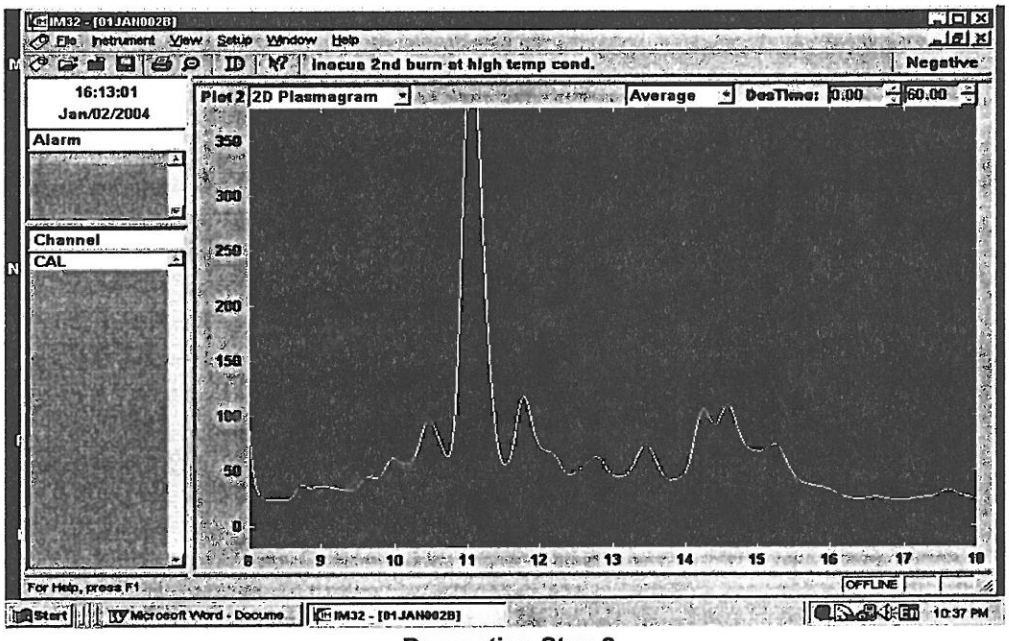
Desorption Step 2

Figure 12. Consecutive burns of *Listeria innocua* using standard heating method on Teflon pads. *L. innocua* was picked up from a colony and directly applied to the pad using a disposable loop.

High Temperature Desorption of *Listeria inocua*



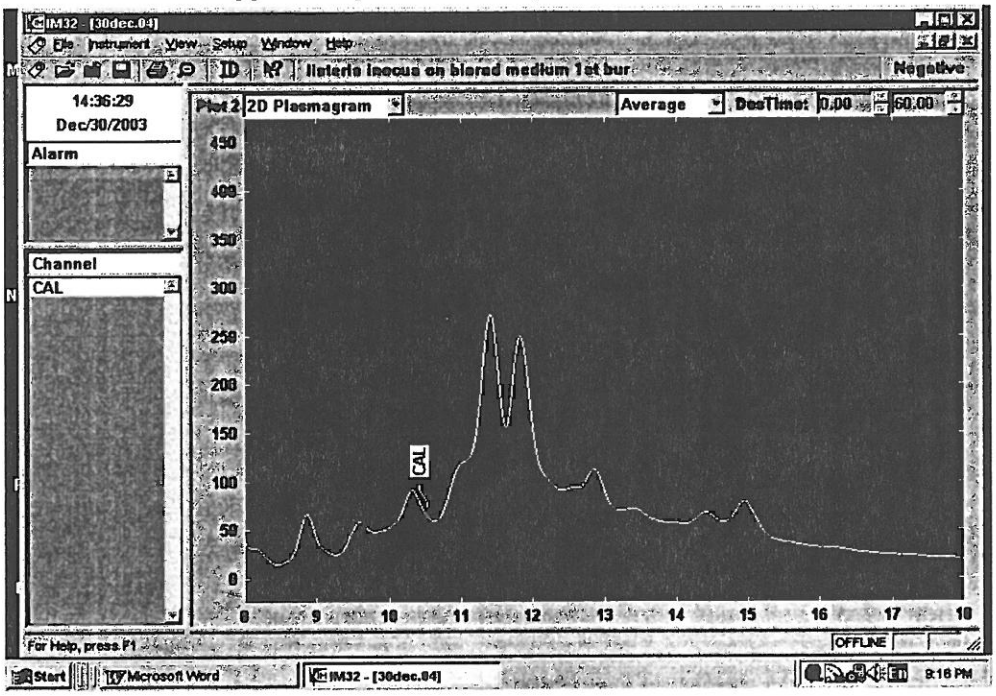
Desorption Step 1



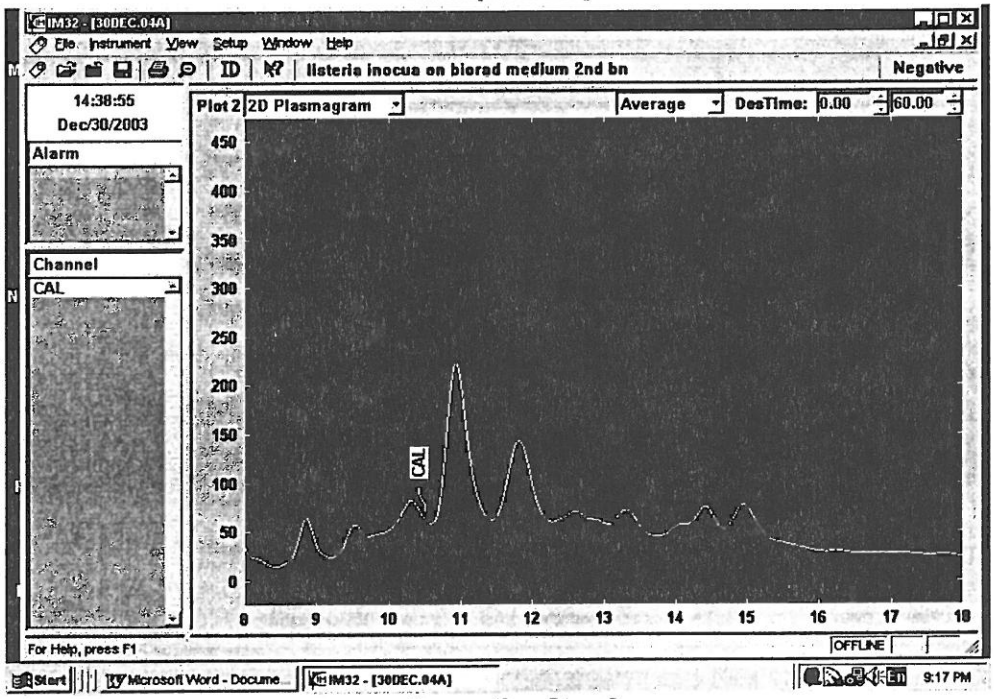
Desorption Step 2

Figure 13. High temperature desorption of *L. innocua* on Teflon compared over 2 burns, simulating a heat ramp approach.

Listeria Cultured on Biorad L mono medium
Stepped Temperature Desorption of *Listeria inoccua*



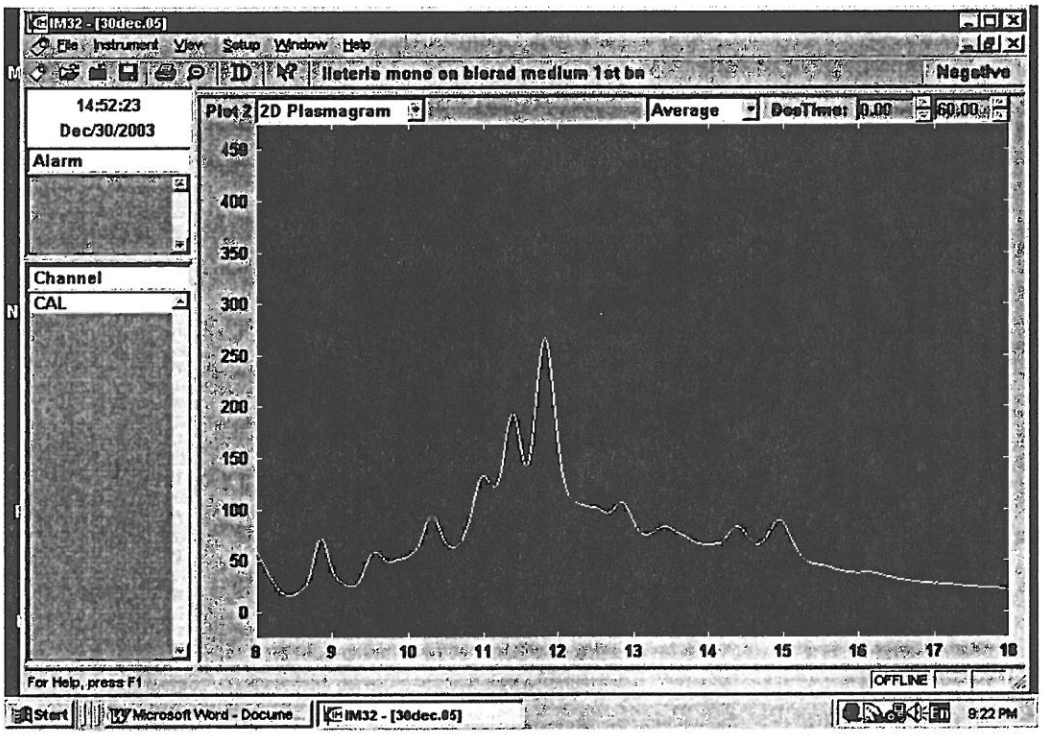
Desorption Step 1



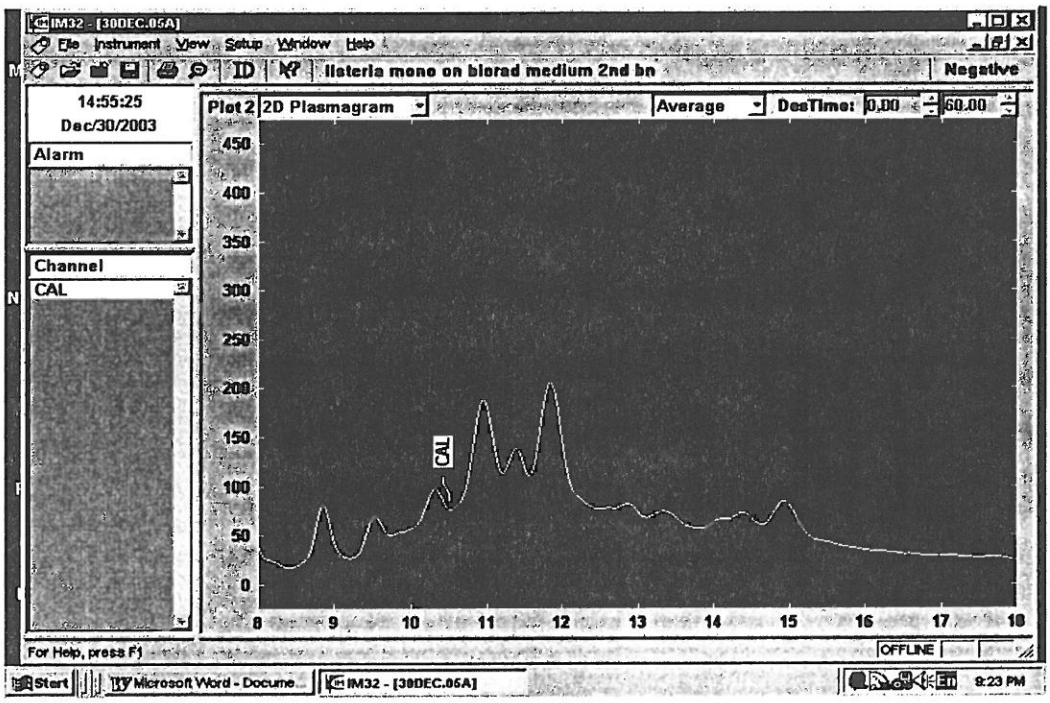
Desorption Step 2

Figure 14. *L. innocua* desorption after incubation on BioRad L mono Rapid medium.

Stepped Temperature Desorption of *Listeria monocytrogenes*



Desorption Step 1

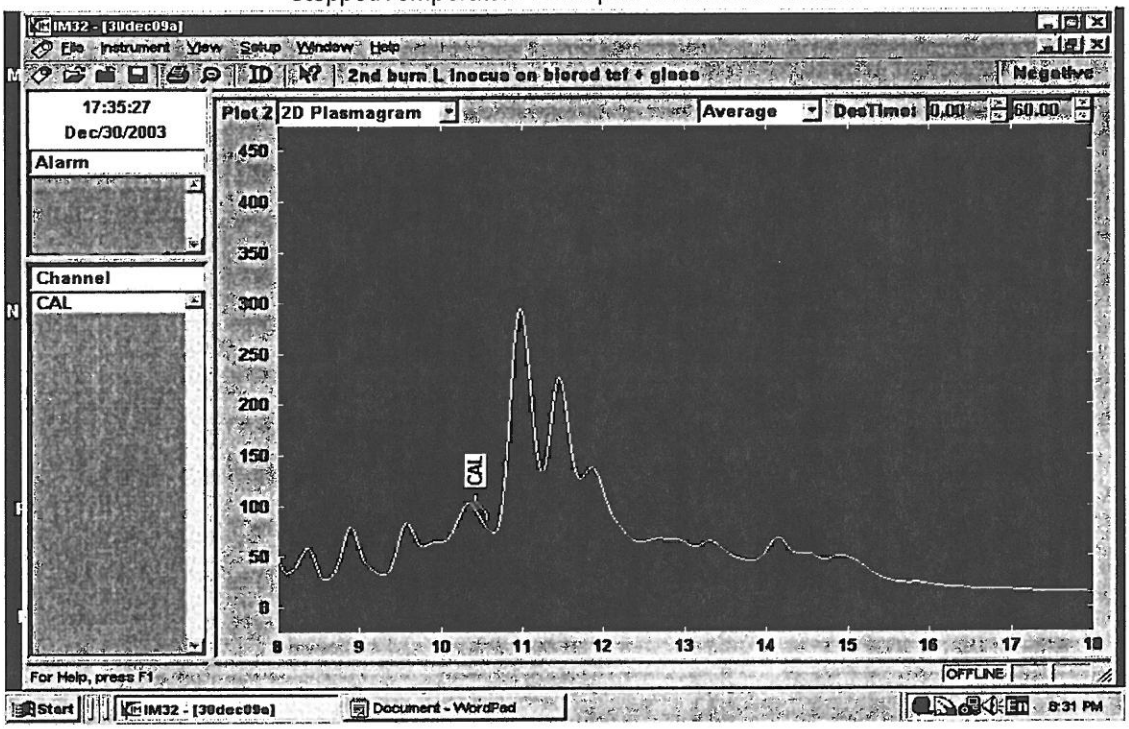


Desorption Step 2

Figure 15. *L. monocytogenes* detection after incubation on BioRad L mono Rapid medium.

Effect of Desorption Membrane on IMS Spectra of Listeria

SteppedTemperature Desorption of *Listeria inoccua*



Stepped Temperature Desorption of *Listeria monocytrogenes*

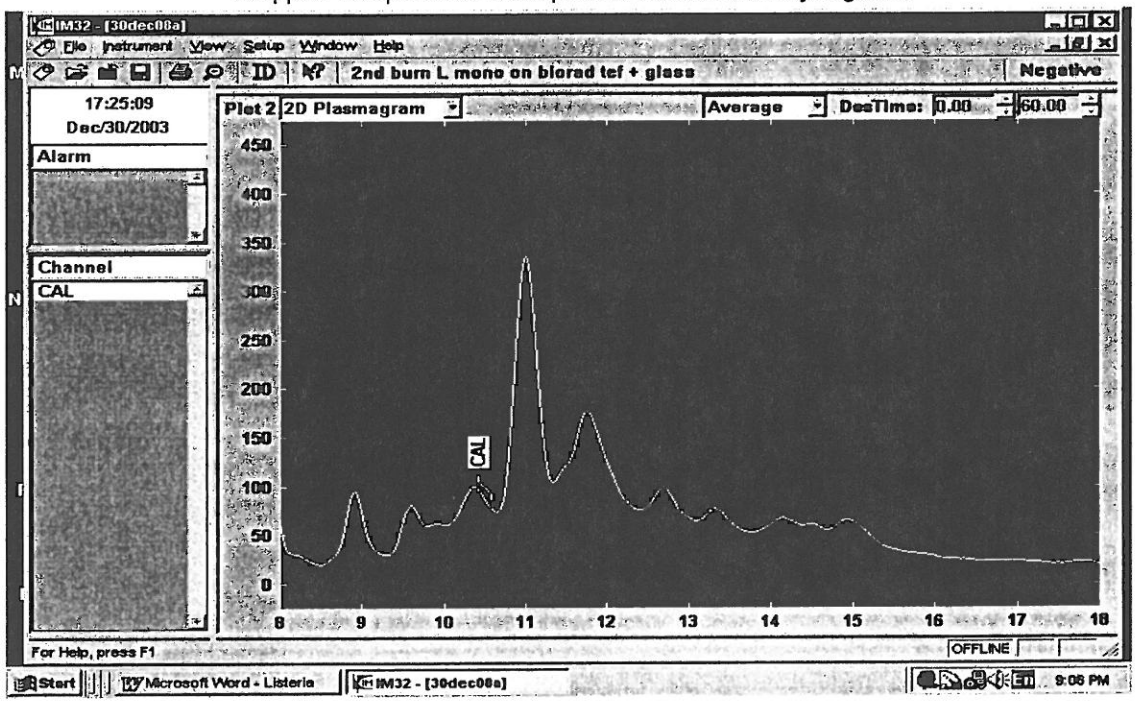


Figure 16. Simulated ramping using stepped desorption on layers of pads after incubation of *L. monocytogenes* on BioRad diagnostic medium.

Figure 17. Aerosol sampling unit in biological safety cabinet.

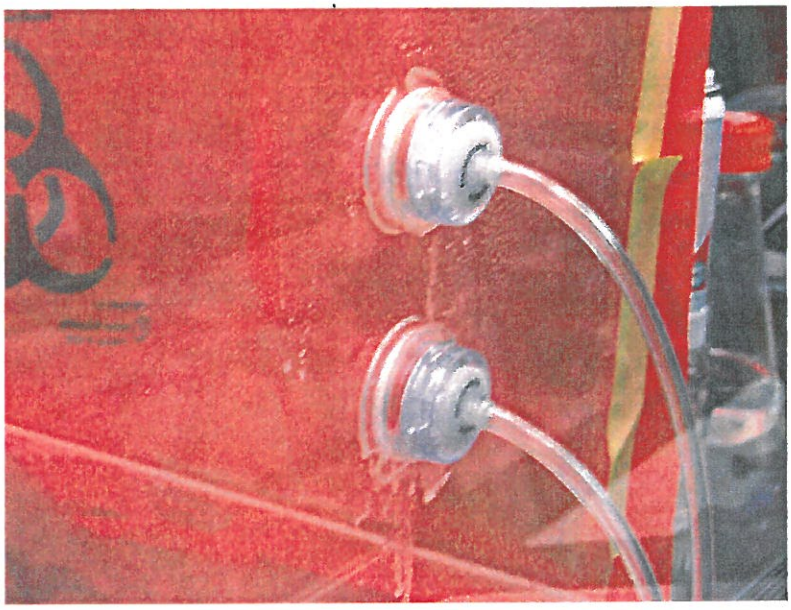


Figure 18. Aerosol collection filters in holders connected to unit backed with 0.2 micron filter traps and connected to vacuum pump.

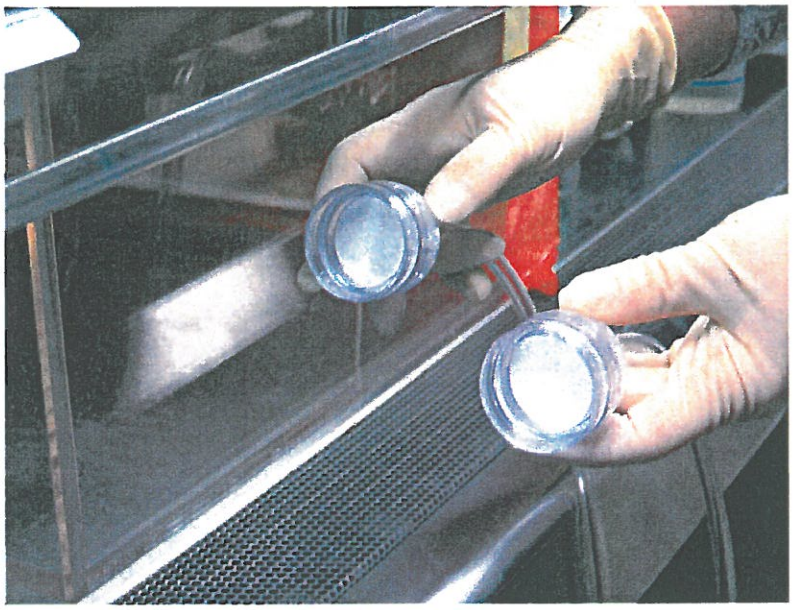


Figure 19. Shown are glass fiber filters used to trap Listeria from aerosol for analysis.

Attachment A

"Development of Ion Mobility Spectrometry (IMS) Applications for *Listeria* detection and monitoring in Food Processing Plants"

AMIF grant/USDA contract to the University of Connecticut

Progress report, February 27, 2004

1. Design and fabrication of an accessory for the Barringer (now Smiths Detection, Ltd.) IonScan ion mobility spectrometry (IMS) instrument allowing stepped-temperature desorption of samples.

As described in our initial proposal and in Vinopal et al. 2002. Anal. Chim. Acta 457:83-95 (see Fig. 1 and Fig. 8 in the article and explanatory text), the key instrument modification needed for greater sensitivity of detection in complex matrices is programmable temperature increases during sample volatilization. Dr. Philip Best and technicians in the Physics Department designed a unit to provide this capability, to be used on unmodified IonScan IMS instruments. The approach was to use an electronic control module to regulate current flow through a wire array heating source, and thus regulate the temperature. This low thermal mass heater was mounted in a modified IonScan Teflon sample holder, which fitted into the IonScan instrument just as the unmodified holder does, thus requiring no changes in the instrument. The heater was powered independently, and computer-controlled. However, and despite several design changes, the wire array heating source did not provide sufficiently uniform heating across the entire sample area to allow adequate temporal separation of different volatility classes of bacterial components during sample desorption. Looking for an alternative approach, we found that placing glass fiber filters between the sample on a Teflon filter and the standard heater on the unmodified IonScan instrument allowed for a more gradual increase in sample temperature, and thus resulted in temporal ion peak separation of the kind we had first observed by moving samples between separate instruments set at increasing fixed desorber temperatures. With glass fiber filters we can now distinguish unequivocally between *Listeria innocua* and *L. monocytogenes* for all the strains of each we have tested. Smiths Detection, Ltd., manufacturers of the IMS instruments we use, plans to engineer a prototype modified IonScan instrument with built-in stepped-temperature desorption to see if this would provide significant benefits over the fixed desorption temperature and fiberglass filter approach. If nothing else, it could make the use of the glass filters unnecessary.

Investigations in three other areas were underway while programmable desorption was being tested and the use of fiberglass filter spacers was being developed. These studies have been continued and extended with spacer-extended thermal desorption.

2. IMS analysis of meat and meat/*Listeria* mixtures.

a. Evaluation of materials for sampling/swiping of meats and subsequent desorption. A number of sampling materials with enough thermal stability to permit thermal desorption have been developed for use with the IonScan instrument.. We evaluated all of these materials, supplied by Smiths Detection, for detection of *Listeria innocua* from contaminated hot dogs. The materials were Teflon, fiberglass (GFF glass fiber), filter paper (S&S #404), cotton cloth, and stainless steel mesh. Hot dogs (Ball Park beef singles) were dipped into a suspension of *L. innocua* and allowed to drip and dry for about an hour. Duplicate samplings/swipes with each of the five sampling materials were made from two sides of a hot dog. *Listeria*-free control hot dogs were sampled

with each material as background controls. Cotton cloth and filter paper gave many background peaks in analysis, interfering with detection of target *Listeria* peaks, at least with fixed-temperature desorption. Glass fiber as a sampling material gave few and small ion peaks from samples. Stainless steel mesh gave very low background and thus clean spectra, but peaks were low, we think because of slow heating due to incomplete contact of the stiff mesh with the desorber heater. A more flexible metal mesh might have useful applications once the IonScan instrument is modified internally by Smiths Detection. Teflon gave the best recovery of *Listeria* and picked up only small amounts of peak-yielding material from uninoculated hot dogs. We plan eventually to test other materials for recovery of *Listeria* from meat surfaces. Obtaining target *Listeria* ion peaks from contaminated meat surfaces requires two things of a sampling material, first the efficient removal of *Listeria* cells from the surface, and then good performance during thermal desorption, which requires stability and the lack of production of competing ion peaks. Our food microbiology consultants, California Microbiological Consulting, have suggested additional sampling materials to try.

b. Evaluation of production of ion peaks from meat samples that interfere with detection of target ion peaks from *Listeria*. In complex mixtures of materials, such as samples of meat products were anticipated to be, competition for charge acquisition and ion detection can impair target peak detection. Stepped-temperature desorption should go a long way toward solving the problem, but we wanted to have some background information with fixed-temperature analysis. While swiping uninoculated hot dog surfaces gave relatively low backgrounds (a., above), not all meat surfaces are as smooth. We compared ion spectra obtained from hot dog paste (ground to a consistency like that of *Listeria* colonies on agar) by itself with spectra from *L. innocua* growth from colonies on agar by itself and with spectra from equal mixtures of hot dog paste and *L. innocua* growth. The hot dog paste did produce ion peaks and resulted in competition with some target *Listeria* peaks, but two characteristic *Listeria* peaks could be detected from the mixtures, with k_0 values of 1.59 and 1.49 (at 11.2 and 12.2 millisecond drift times in Figs. 2 and 3). (Our early work with *Listeria* strains used growth from MOX agar, reasonable for strain typing in a laboratory setting. We find that some of the target peaks we identified are not present in *Listeria* grown on other lab media, brain-heart infusion, etc., or grown on cold cuts, thus we have been identifying new target peaks.)

One reassuring finding was noted with the analysis of meat samples. A basic question existed initially: when meat is thermally desorbed, do the ion peaks that are produced result from thermal degradation of large molecules or from mobilization of small, volatile molecules already present - free - in the meat interstices? If the first, production of huge amounts of material competing with *Listeria* target peak detection might result. For bacterial cells we have found that most or all ion peaks arise from low molecular weight species (up to perhaps 800-900 MW) already there, not from the degradation of large molecules. With hot dogs we get the same ion peaks from juice squeezed from the meat as from meat paste, suggesting that small and more or less soluble molecules give rise to the peaks. It's possible that food additives as well as natural meat constituent small molecules would give rise to peaks. We plan to investigate the natural IMS signatures of various kinds of cold cuts and preserved fish - smoking might introduce specific peaks in salmon, for example. Each natural background would present possible problems for *Listeria* peak detection, but stepped-temperature desorption should remove most or all of these.

3. Detection of *Listeria* in aerosols.

a. We have tested materials for the filtration of air for recovery of *Listeria* cells from aerosols. Membrane filters have too much backpressure for efficient recovery, and some membrane materials are degraded by desorption, nitrocellulose for example. Depth filters, however, work

well. The depth filters we have tested with excellent results are made of Teflon, and are commercially available in a wide range of thicknesses and pore sizes from Fluorotechniques, Castleton-on-Hudson, NY. A potential problem with thick Teflon (although thinner Teflon is the default sampling material for use in detection of explosives and narcotics, and in most of our bacterial work) was that Teflon can adsorb volatiles and depth filters might thus release masking substances upon heating that would interfere with ion peak formation (charge acquisition) and/or detection. We have found that Teflon depth filters, if kept in closed containers for protection against exposure to ambient volatiles, produce little more background than thin Teflon.

b. Quantization of sensitivity of detection of *Listeria* in aerosols requires production of test aerosols of known *Listeria* content and their filtration. We have fabricated an aerosol chamber, used within a microbiological air-flow cabinet, for work with aerosols. Using *L. innocua* we have developed methods for generating uniform aerosols. Our chamber allows the taking of parallel, dual samples by simultaneous filtration of aerosols through two depth filters for a fixed time, one sample for desorption for IMS analysis, the other for making viable counts for assessment of the bacterial load. Recovery of filtered bacterial cells from depth filters for quantification by viable counts isn't efficient enough for accuracy if the number of cells is small. Accordingly, we generate aerosols and collect dual samples for time spans of up to 15 minutes and more, relating ion peak formation and viable counts to the known volume of air passed through the filters. We find that a short time of collection of aerosols followed by the provision of growth medium to support multiplication of bacteria right on/in the depth filter eventually desorbed for IMS detection enhances the sensitivity of detection. Simply pressing the underside of the depth filter onto a nutrient agar surface can provide growth medium. Use of an agar formulation that is selective and differential for *Listeria* or for *L. monocytogenes* specifically should increase the specificity of detection greatly, and we are about to test this by producing and sampling mixed aerosols with cells of *Listeria* mixed with other food bacteria. Using one of the newly introduced agar media for specific detection of *L. monocytogenes* might permit "one-peak" detection, as explained in the next paragraph.

4. Detection of *Listeria* in general and *L. monocytogenes* specifically by one- and two-peak detection of chemical moieties released from diagnostic enzyme substrates.

In standard laboratory practice, presence of *Listeria* in food samples is revealed by the cleavage of esculin, a beta-glucoside with esculetin as aglycone, to release esculetin, which forms a black complex with iron. In selective media such as Fraser broth, Vermont agar, and MOX agar, cleavage of esculin indicates presence of *Listeria*, although not specifically *L. monocytogenes*. Detection of free esculetin by IMS in these media could give very sensitive and early indication of a potential contamination problem. This would be possible only if IMS analysis procedures - desorption procedures - could be found that would volatilize and detect free esculetin and yet not cause breakdown of uncleaved esculin to release esculetin non-enzymatically. In earlier work we have shown that this can indeed be done, by using low desorption temperatures, sufficient to volatilize esculetin without causing esculin breakdown. A number of beta-glucosidase substrates other than esculin have been used recently in diagnostic media (e.g. James, et al. 1996. Appl. Environ. Microbiol. 62:3868-3870). One of these, CHE-glucoside, is used in the new Lab M Harlequin *Listeria* detection agar (IDG, Ltd., UK), and we can detect CHE very sensitively with IMS. This "single peak" detection of the presence of *Listeria* should be very sensitive - we don't have quantitation - but would be of somewhat limited usefulness, because it detects the presence of all species of *Listeria*, not just *L. monocytogenes*. Still, it could be used in a high-throughput cheap and rapid initial test for the presence of *Listeria*, to be followed (for positive samples) by more definitive, but slower and more expensive tests, e.g. PCR detection of *L. monocytogenes* DNA sequences. Beta-glucosidase, the target enzyme, is present in all *Listeria* species, and is

unrelated to virulence. A recently developed diagnostic agar medium for detection of *L. monocytogenes* specifically, ALOA (agar for *Listeria* of Ottaviani and Agosti, Vlaemynck et al. 2000. J. Appl. Microbiol. 88:430-441), allows separation of *L. monocytogenes* from other species on the basis of a halo around *L. monocytogenes* colonies resulting from the action of a phosphatidyl inositol-specific phospholipase C that is coded for by one of 6 genes in the *L. monocytogenes* virulence gene cluster, and last year a chromogenic substrate for this enzyme was reported (Jinneman et al. 2003. J. Food Protection 66:441-445), allowing identification of colonies of *L. monocytogenes* by color production. Selective and differential agar incorporating this test is now commercially available (BioRad L mono medium). Cleavage of the chromogenic substrate was expected to be detectable by IMS - the chromophore in the substrate is "X," the same as that in the chromogenic substrate X-galactose, and we can detect the presence of *Escherichia coli* by IMS detection of X released by cleavage of that substrate. Thus a selective and differential medium containing both beta-glucosidase and phospholipase C substrates, cleaved by *Listeria* to release moieties detectable by IMS, might allow very rapid and sensitive detection of both *Listeria* generally and *L. monocytogenes* specifically. There may be a few false positive considerations (Jinneman et al., see above), but a fast, cheap, and sensitive initial indication could, as above, be followed by a more expensive but definitive test for *L. monocytogenes*, such as polymerase chain reaction (PCR). We have found that *L. monocytogenes* cells cultured on BioRad L mono agar show an ion peak, not seen in cells grown on other media, and not seen with *L. innocua* cells from L mono agar, that has the mobility expected for X.

Attachement B
Analysis of *Bacillus cereus* by Ion Mobility Spectrometry

The following is a report examining detection of *Bacillus cereus* in a simulated room environment using IMS technology. The feasibility of detecting *B. cereus* via IMS by siphoning air from the normal air circulation of a contaminated room, through an impinger and filter.

Because no known "library" (known data reference points used to identify *B. cereus*) existed prior to testing a set of pure reference points were established to identify *B. cereus*. One reference point selected is a chemical marker for *B. cereus*, dipicolinic acid, a chemical unique to bacterial spores including *Bacillus anthracis* and makes up approximately 10% of the spores dry weight. As a second indicator I used esculin (this was used as a visual aid). Esculin is a glycoside composed of glucose and esculetin. Enzymatic hydrolysis of esculin releases the esculetin which subsequently form a dark brown phenolic iron complex in the presence of ferric citrate. Although this test is not specific for *B. cereus* it does provide a visual indicator of the presence of esculin positive bacteria.

3. SCOPE AND OBJECTIVES: Develop new applications for the IonScan with respect to bacterial detection, specifically Bacillus species. To this end, the following methods were developed:

a. Detection of Dipicolinic acid
b. Detection of *B. cereus* spores to include but not limited to those cultured in regular agar, nutrient broth, and esculin.
c. Final detection of *B. cereus* in test atmosphere. See figures 1 through 3.

4. EXPERIMENTAL METHOD:

4.1 Standards Preparation

Drift time for dipicolinic acid (Sigma) was identified in several redundant steps using various solution mixtures and concentrations. The dipicolinic acid was tested in powder form by placing a minute amount, using a disposable inoculating needle, on Teflon filter paper. The Powder form was mixed to form a 1.0 % mixture (100mg in 100ml of purified and sterile DI-water) and .01% mixture. 10 micro liters was placed on Teflon paper using a Hamilton syringe needle. Dipicolinic acid was also mixed with MEOH in the same proportions and tested. All steps were conducted several times to ensure accuracy.

B. cereus was cultured and grown on various mediums, nutrient broth, regular agar plate, and esculin plate. Prior to testing, spore verification was conducted by conducting a gram stain and verifying the presence of spore formation under a microscope. This took approximately two to three weeks to occur. This spore formation was induced by nutrient deprivation. Once a satisfactory stock solution

of *B. cereus* in spore form was obtained all test were conducted from this original stock.

Spore and culture samples for testing and establishing instrument parameters were made. This was done by collecting all growth of off the plate using DI water and placing it in a small sterile container. These samples were then equally transferred to centrifuge tubes. The tubes were then placed in a centrifuge machine for two minutes at 3000 rpm. Upon removal the DI-water above the concentrated portion of *B. cereus* was removed and discarded. The mixture in the tube was then mixed again with DI-water and placed back in the centrifuge machine. This was done three times, thus concentrating the *B. cereus* into a pellet form. This was removed and processed through four serial dilutions. Tube 1: 100 micro liters of *B. cereus* into 9.9 ml of DI-water, tube 2: contained .1 ml of tube one suspension and 9.9 ml of DI-water, tube 3: contained 1 ml of tube 2 suspension and 9 ml of DI-Water, tube 4: contained 1ml of tube 3 and 9 ml of DI-water. 0.1 ml samples of tubes 3 and 4 were each placed into four Petri dishes (two of each sample) containing nutrient agar. This was to ascertain a spore count. Tube #3 dilution resulted in approximately 250 colonies; tube #4 resulted in 30 colonies. Dilutions 1 through 4 were tested via IMS. See 5.1f for dilution two results. Dilution 3 or 4 did not yield quantifiable results on the IMS.

A stock sterile solution of esculin broth was prepared. Per 100ml of nutrient broth used 0.1g of esculin, 0.05g Ferric ammonium citrate. The esculin was sterilized and subsequently tested via IMS, both by itself to provide background data and in the presence of *B. cereus*. See results section for data.

A container serving as the simulated room environment was constructed of Plexiglas purchased from a local vendor. It was constructed to the following dimensions of 18 x 18 x 18 inches (cube geometry) and water tight for the exception of the 6 x 6 inch opening at the top. Two compression fittings served as the inlet and outlet valve, which contained one-way check valves in the direction of the impinger. A 1500 cc/min pump with two outlets were used; one to pump air into the box (which discharged air directly 3 inches above Petri dish containing sample to assist in forced circulation. The other discharge lead to an impinger that served as a control to ensure the air/area around the pump was free of gross contamination. A Plexiglas cover with sealing foam was placed above the opening to reduce outside contamination upon introduction of a sample. The outlet of the box was affixed with both a globe valve and check valve to reduce outside contamination. Sample air, assisted by a vacuum machine from the outlet side (was protected by a .05 micron filter) this proceeded from the box to a filter chamber (filter chamber was air tight and contained Teflon which was periodically tested) to the impinger, which contained esculin (see figures 2 and 3). Prior to testing the box was left to sit for two weeks after construction to ensure materials/and vapor fumes (from adhesive) did not effect final testing. Two day prior to testing the box was purged with the same pump and vacuum used for final testing. The day prior to testing background testing was conducted.

Operating Parameters

Table 2. Ionscan Operating Conditions

Operational Parameters	Negative Dual Mode
Drift heater	233
Inlet heater	215
Desorber Temperature (°C)	200
Calibrant block heater	71
Bake out drift heater	275
Bake out inlet heater	280
Drift flow cc/min	300
Stand by flow cc/min	75
Bake out drift flow cc/min	200
Bake out cal. time (min.)	120
Bake out cal. Threshold (d.u.)	1000
Timed auto bake off	Varied
Purge on time (ms.)	1000

Sample Analysis

All testing was conducted with the Ionscan in negative mode. System calibration and maintenance was conducted I.A.W. the operator's manual. Prior to running a sample the Teflon pad was burned and sampled for contaminants a minimum of three times. Samples were than deposited on the on the Teflon pad with an Eppendorf micropipette, unless otherwise specified. The mass of a sample was calculated by multiplying the concentration of the standard solution and the volume applied, unless otherwise specified sample size was 10 micro liters. The sample trap was inserted into the sample desorber for analysis. Upon the completion of the Ion scan's testing cycle (approx. 60 seconds) the sample would be slid out of the sample desorber and a concurrent test would be performed, this was done without disturbing or contaminating the sample. The sample would be analyzed up to six times to assure that all compounds within a sample would

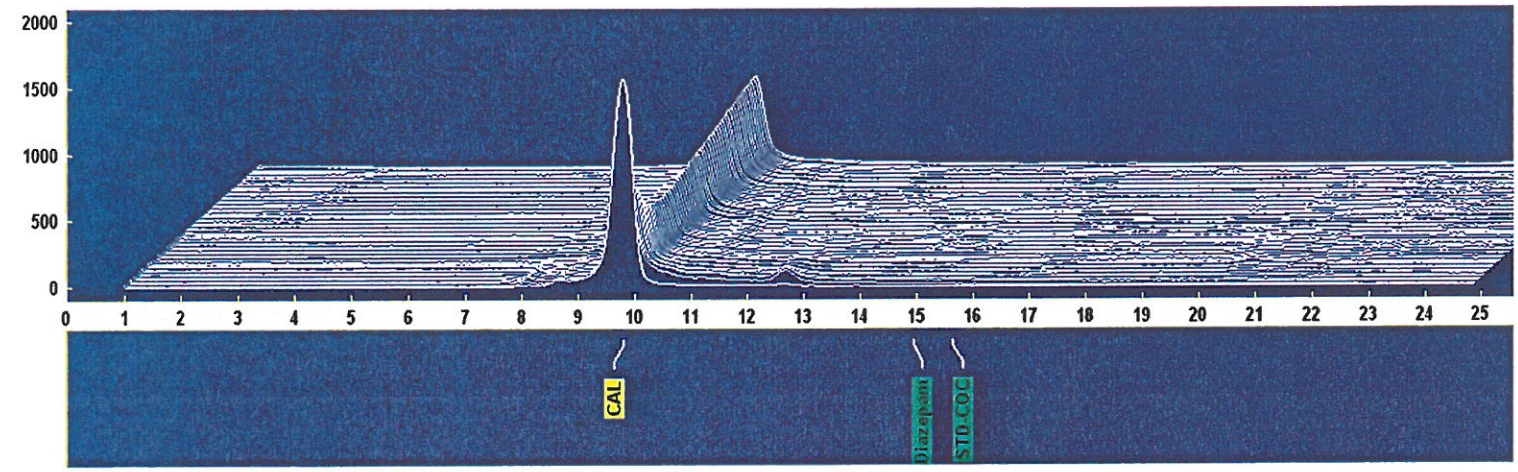
evaporate and be detected by the IMS. A blank would be conducted after each sample group to ensure no contaminants would affect subsequent test. In those instances were contaminants could not be immediately removed, a purge cycle would be initiated. In the event the fault was not corrected the machine would be sent into a burn cycle and testing would resume on the following day.

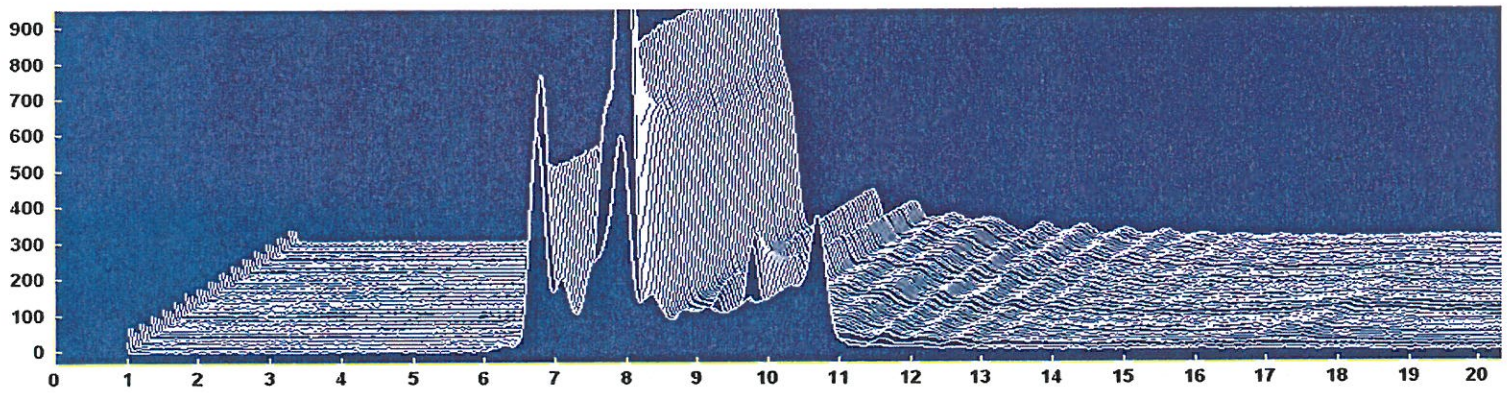
Samples were compared through the use of Gaussian curve analysis (a peak fitting function which determines peak position and half width), K_o (the reduced mobility of a channel or of the calibrant it is a characteristic parameter in the calibration and is used in the calculation of expected drift times) and drift times in milliseconds (ms).

5. EXPERIMENTAL RESULTS AND DISCUSSIONS

Where *B. cereus* was tested, the bacteria were first grown in a specific medium. Specimens were scraped off of the medium on the Petri dish and concentrated using the centrifuge as stated above. Samples were directly taken from the appropriate centrifuge tube. By comparing identified K_O's, comparing Gaussian curve data, and drift times subsequent data could be analyzed.

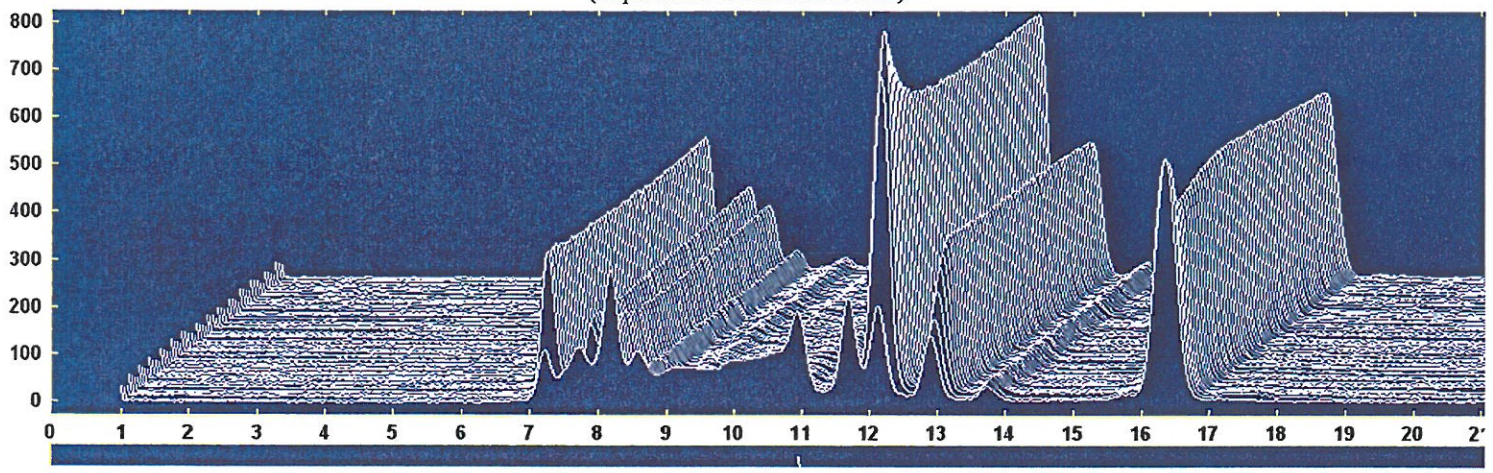
5.1a several blanks were run at the beginning of the day to establish background data. Notice no peaks in the range of 15 to 20 milli-seconds (ms). This established a base background after the machine went through a burn cycle. More traditional blanks have small peaks around the 6 to 13 ms range (this is well outside the target tested range of intended research subjects). Significantly more purges, burns, and blanks were conducted in the first phase of testing to ensure good background data.



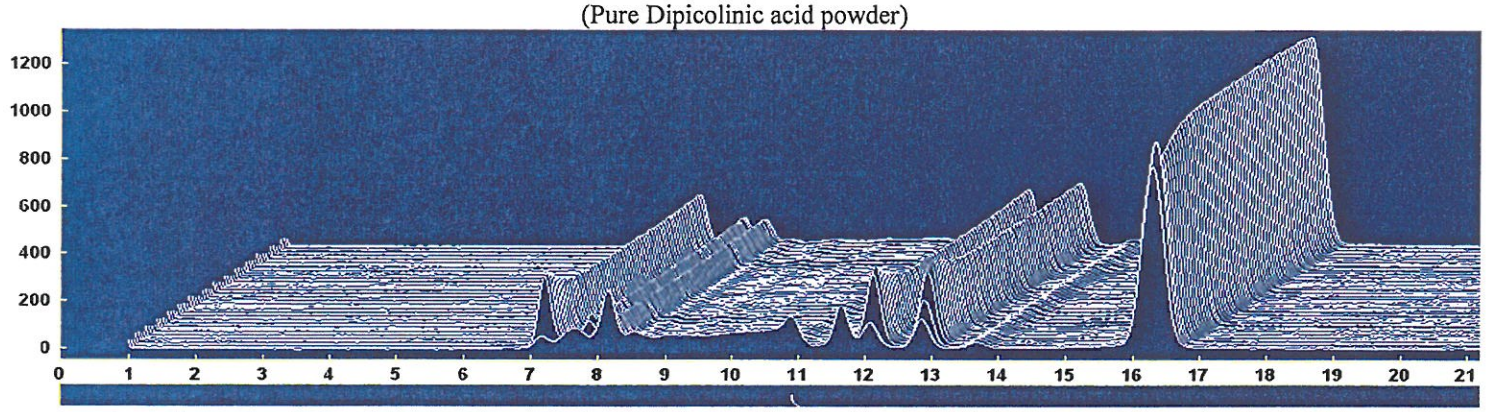


5.1b the average drift time of all Dipicolinic acid mixtures and the pure form of Dipicolinic acid were approximately 16.44 ms (±0.35)

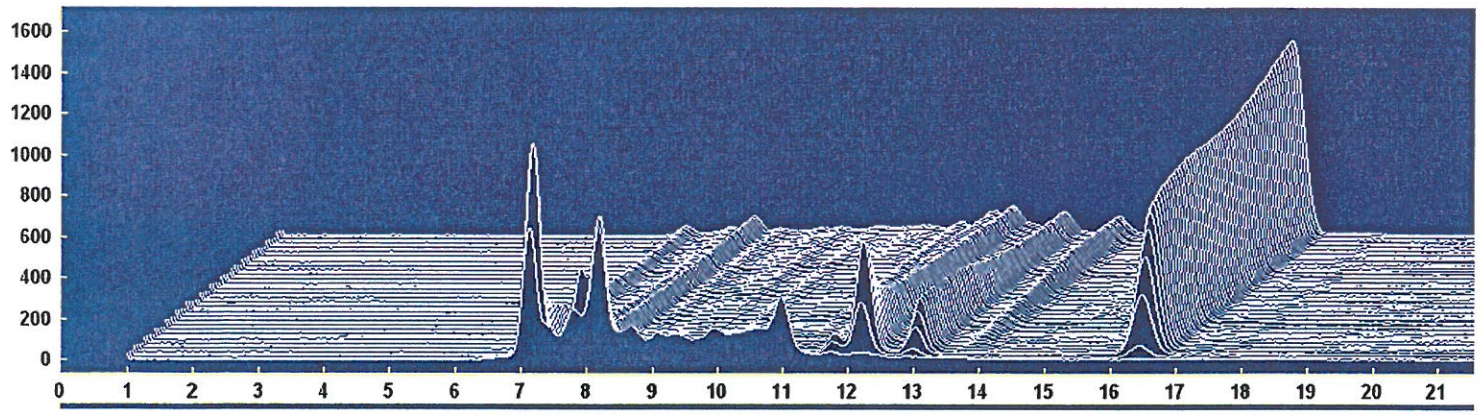
(Dipicolinic acid/MEOH mix)



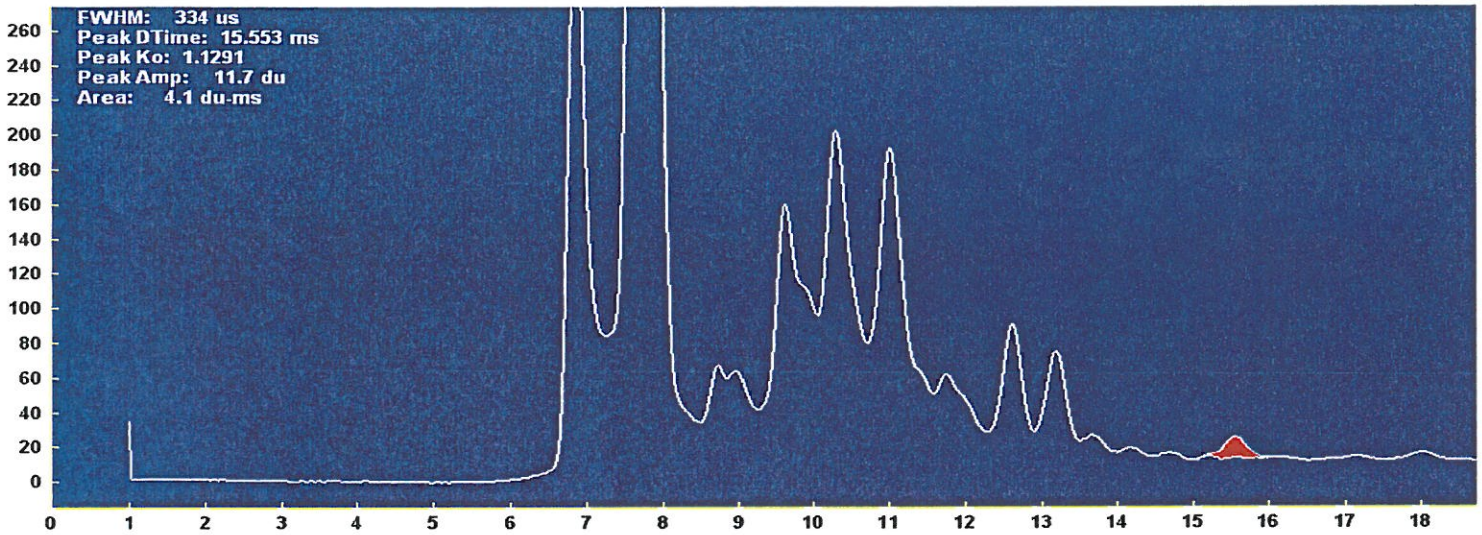
(Pure Dipicolinic acid powder)



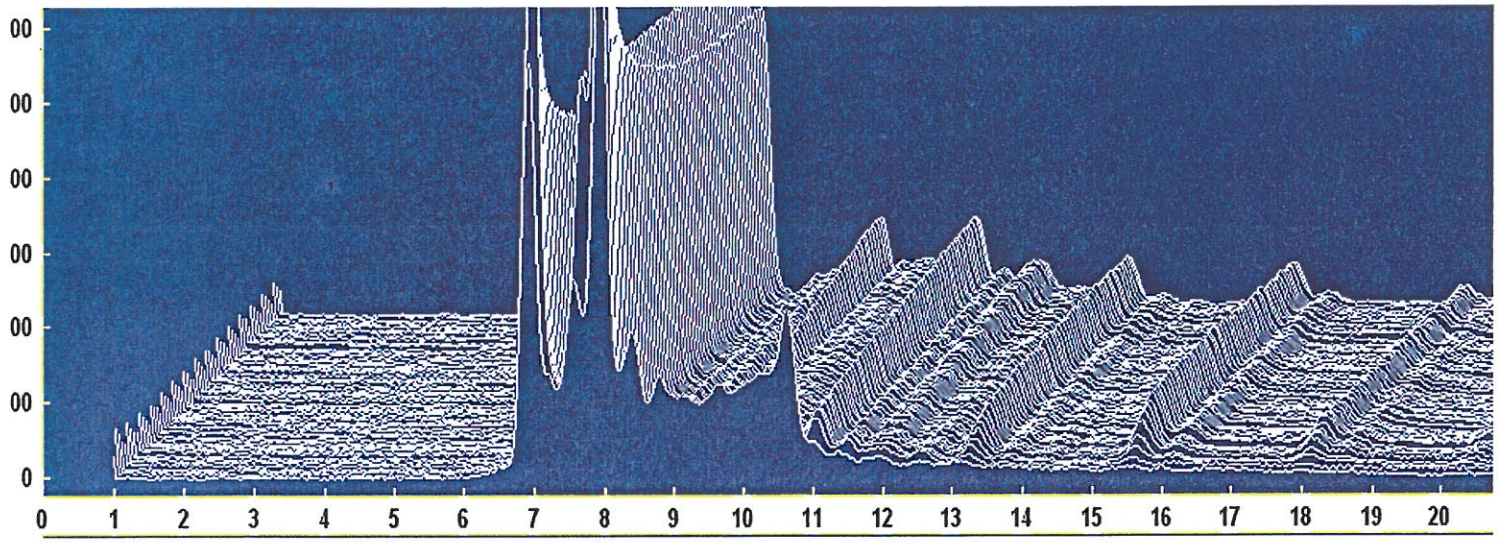
(Dipicolinic acid/DI-water mix)



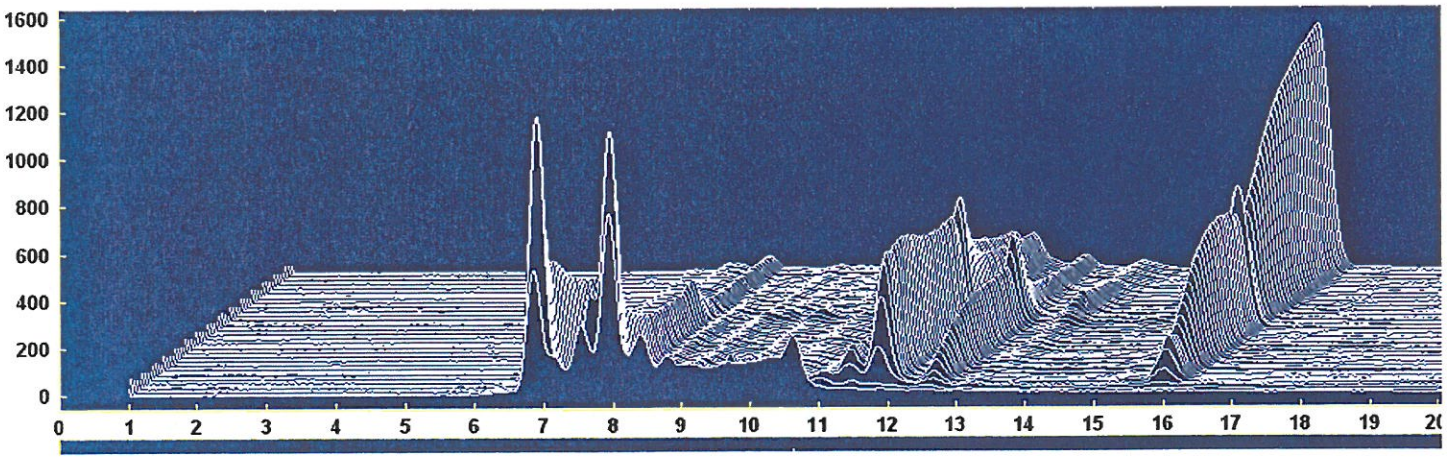
5.1c *B. cereus* grown in regular agar and tested via tube dilution method. This and other samples in this category place the average drift time at 15.72 (± .36). This is close to the established data of Dipicolinic acid.



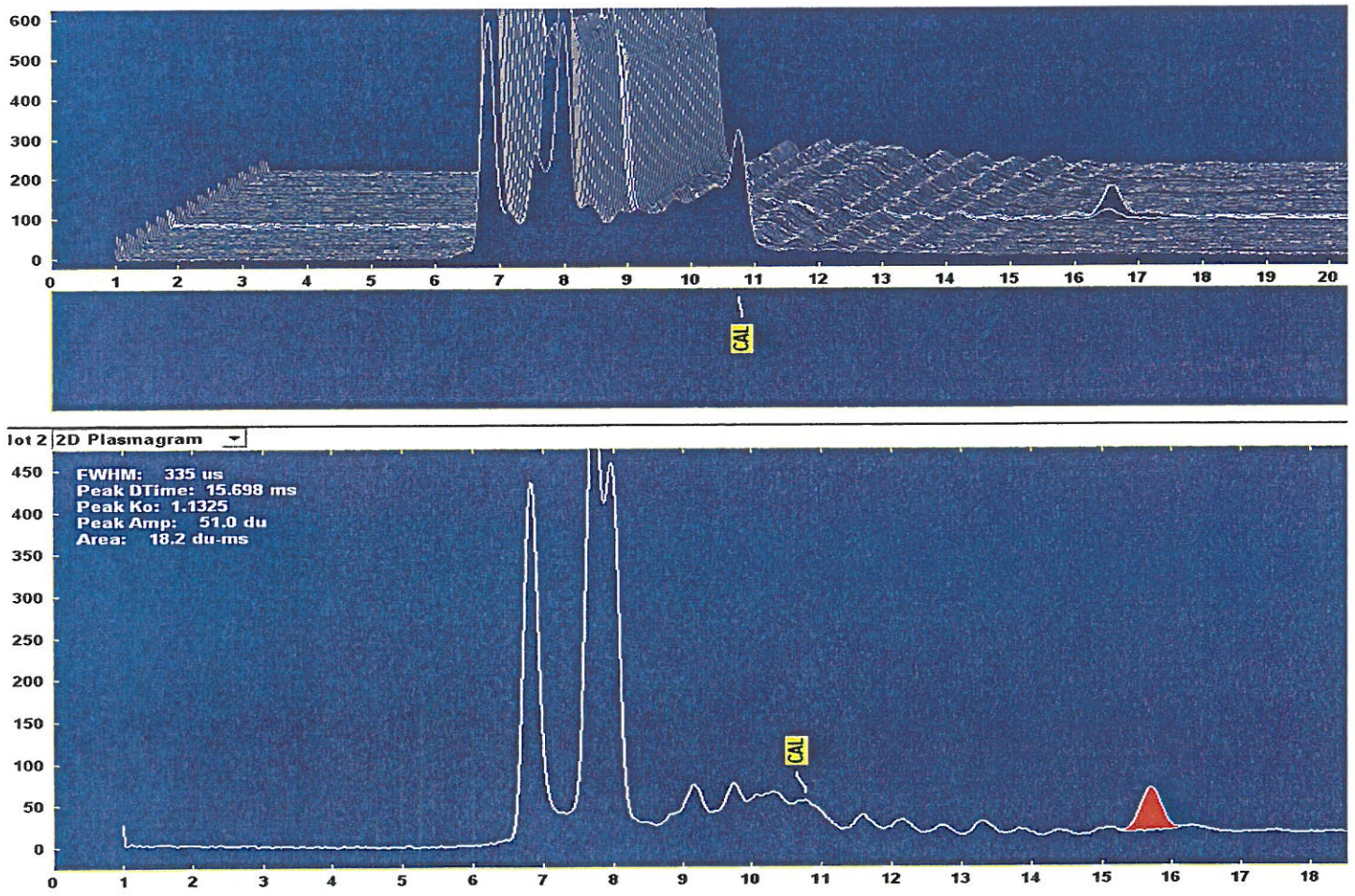
5.1d *B. cereus* grown on an esculin medium in a Petri dish. When *B. cereus* was grown in this medium several peaks of interest were observed. There are two peaks that border each other around sixteen. The larger peek is similar to the one above or representative of *B. cereus* grown in regular agar. The smaller peak share identical K_O's with that of dipicolinic acid. Another peak around 18 cannot be explained.



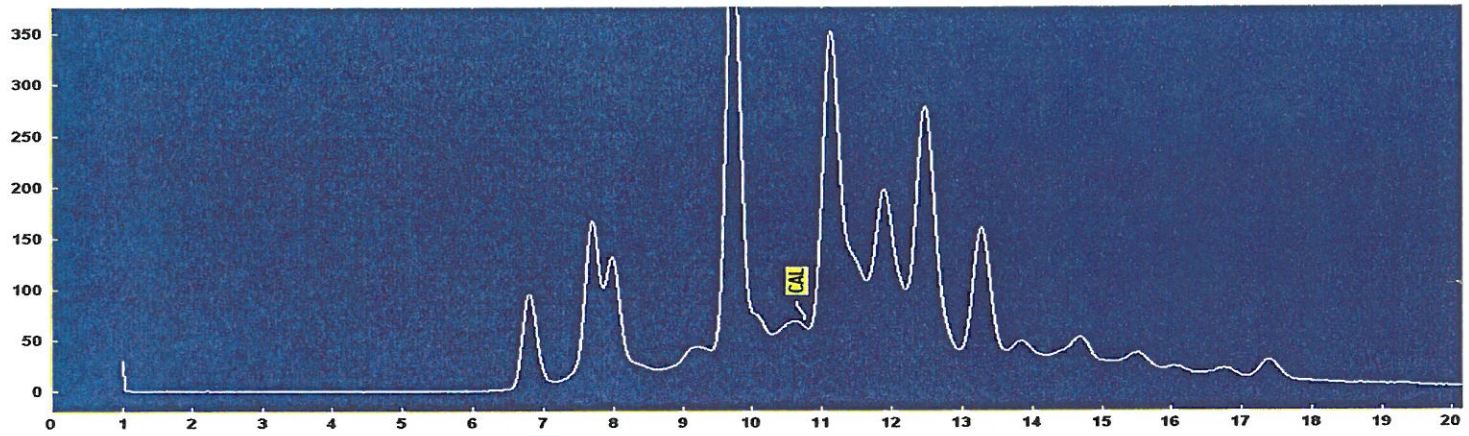
5.1e to ensure the Dipicolinic acid was similar to the peak produced by the *B. cereus* (as shown above) several samples were run of *B. cereus* spiked with 10 µl of a 0.01% Dipicolinic acid solution in DI-water. This sample and others like it resulted in the same peak location. Even when observing this data in real time (a feature of the machine which allows the operator to observe data in real time as it occurs) did not give any indication of a separate peak. This points to the possibility of using dipicolinic acid standard as a bench mark for detecting spore-forming bacteria. However, further study is required.



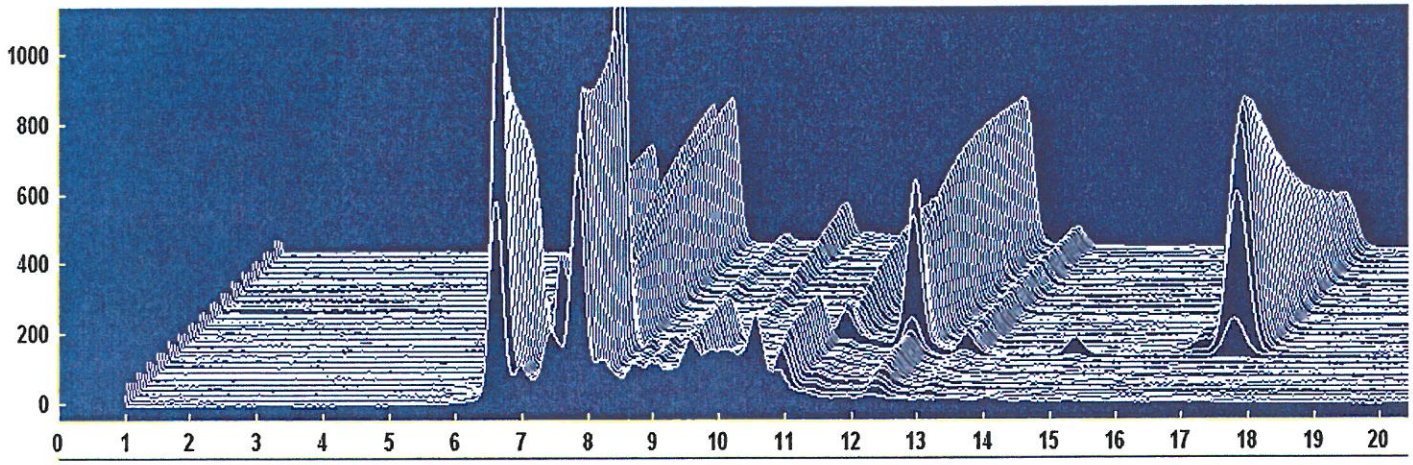
5.1f *B. cereus* was identified from tube # 2 dilution, which contains approximately 2.7 x 10^6 CFU/ml. This was done by isolating peak data between the 21 and 24-second analysis range. This is consistent with peaks identified from both dipicolinic acid and *B. cereus* spiked with dipicolinic acid.



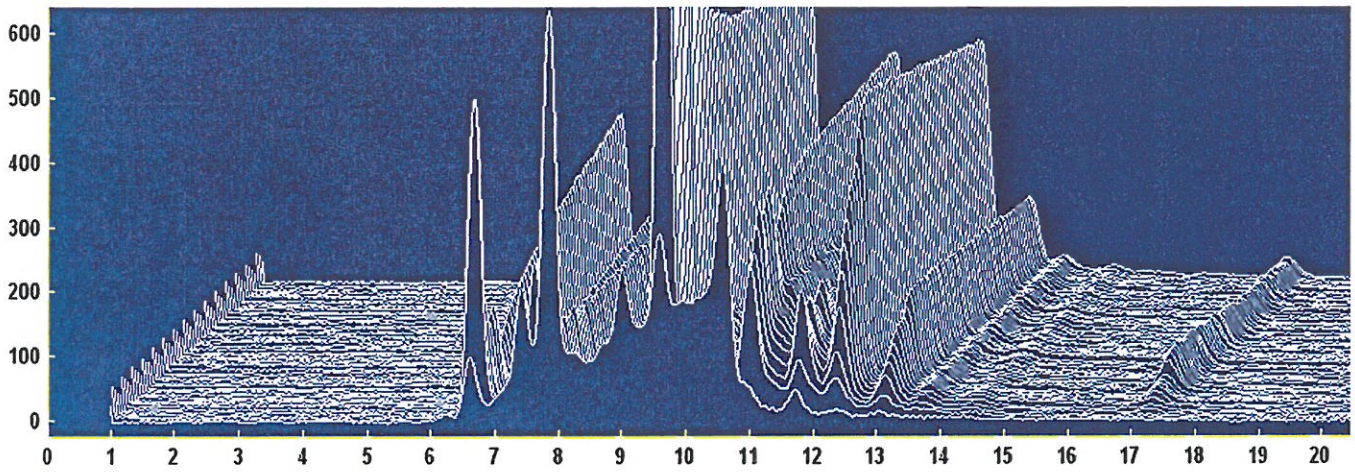
5.1g esculin sample, made by afore-mentioned procedure, produced a minor peak at an average drift time of 17.32 ms (± 0.21). Most times esculin was tested either a very small peak or no peak was observed.



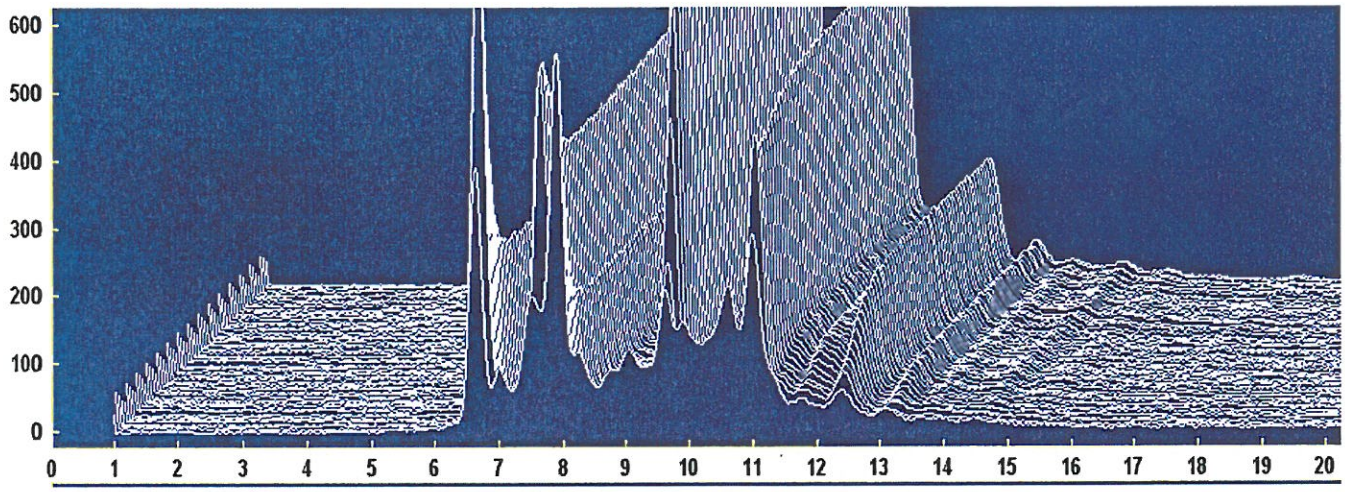
5.1h Esculetin plasmagram. Drift time is 17.122 ms. is consistent with the esculetin made as a by product of *B. cereus*.



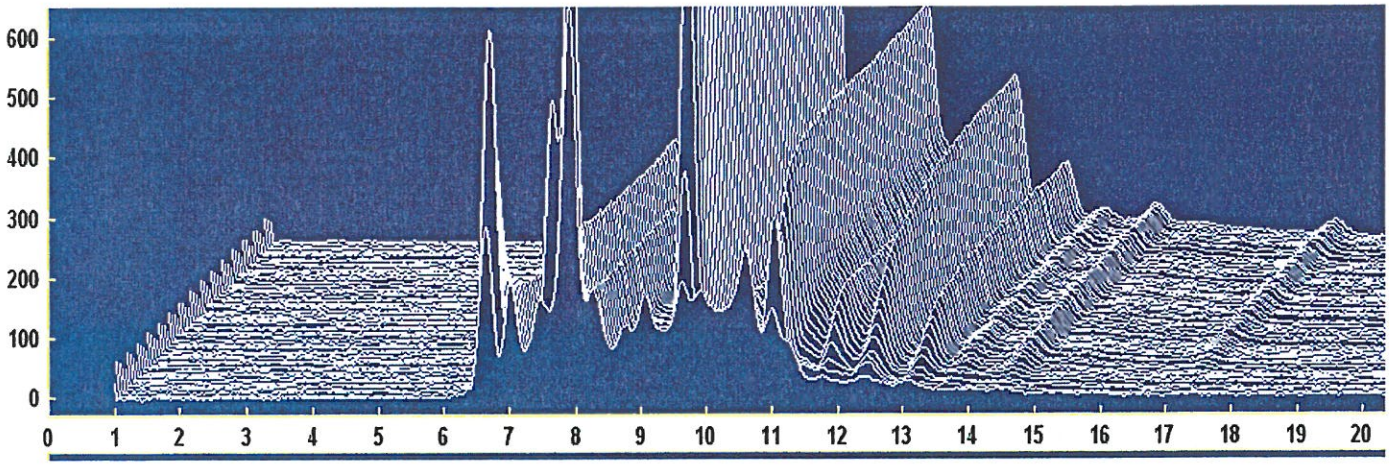
5.1i a swipe of the inside of the box was taken approximately two hours after commencement of the test. [1 ml of Dilution tube #1 or approximately 2.7 x 10^7 CFU/ml was placed inside of a Petri dish inside of the box, this was done to both contain the sample and to facilitate circulation, which was facilitated by the pump discharge which was approximately 3 inches above this Petri dish]. The filter paper was swabbed across a random side once and tested. Drift time was 17.103, which is consistent with Dipicolinic acid. It should be mentioned that esculin has a drift time that is close to this range. However due to the check valves in place I would estimate that this sample is consistent with *B. cereus*.



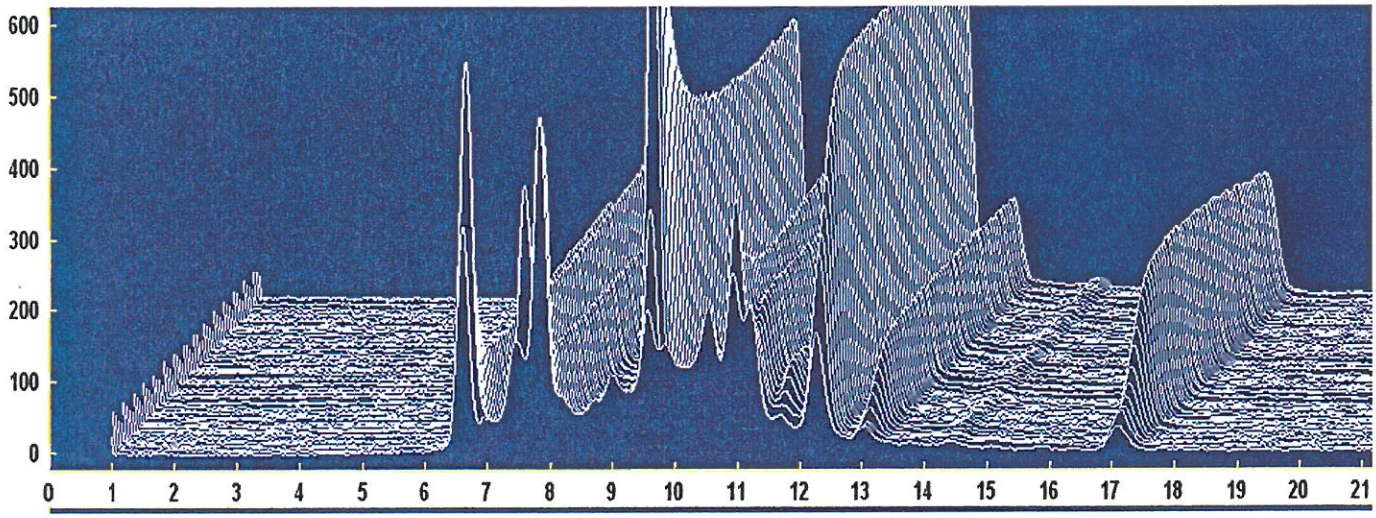
5.1j impinger one shows no sign of *B. cereus* or esculetin 2 hours after the commencement of testing.



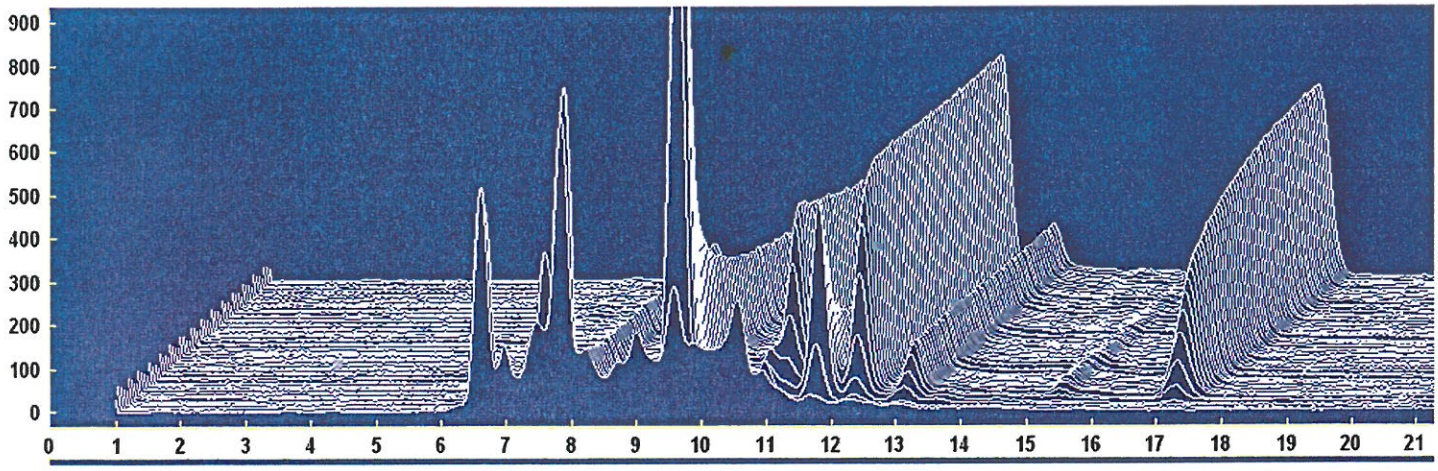
5.1k impinger 2 at the 2-hour mark does show a minor peak at 17.2, which may be indicative of either *B. cereus* or esculetin produced by the bacteria. This is constant with the filter swipe from inside the box, which was conducted on the second hour.



5.1l a swipe of the inside of the box was taken at the 2 hour point and placed in a sterile esculin broth (esculin broth in all portions of final experiment are from the same batch) and incubated for 24 hours at 27C(unfortunately I did not record the temperature of the incubator used in the laboratory at the time of the experiment, an error on my part, however I believe it was 27C). No separate peak exist other than the one observed at 17.130 ms. This is consistent with drift times associated with *B. cereus* and/or the production of esculetin.



5.1m impinger two after 6 hours. This sample was spiked with 10 µl of pure esculetin to see if there was a difference between the esculetin produced and the pure form. In addition a minor peak is also observed around 15.76, which may be indicative of Dipicolinic acid.



6. CONCLUSIONS

Dipicolinic acid standards could be used to identify *B. cereus* and possibly other spore forming bacteria. When using esculetin as a component of diagnostic media for esculin positive bacteria, such as Listeria, Staphylococcus, Enterococcus and Bacillus, additional IMS targets can help distinguish among the different bacterial groups and identify contaminations. While the esculetin peak dominates at an average drift time of 17.13 for *B. cereus* grown in an esculin media small peaks closer to that of dipicolinic acid could also be observed. It should be pointed out that at the beginning of testing both impinger 1 and 2, which contained sterile esculin, produced no quantifiable peaks in the 16 to 18 range. However within 6 hours impinger 2 test (the impinger which was receiving airflow from the test box) resulted in observable peaks around 17.2. It appears that when *B. cereus* is tested in large amounts ($>10^7$) in the presence of esculetin, *dipicolinic* acid like peaks can be detected around 16 in addition to esculetin peaks around 17. It is possible to detect *B. cereus* at lower quantities (approximately 10^5 MDL testing was not established, further test would have to confirm the MDL) when esculetin is not present.

Illustration 1

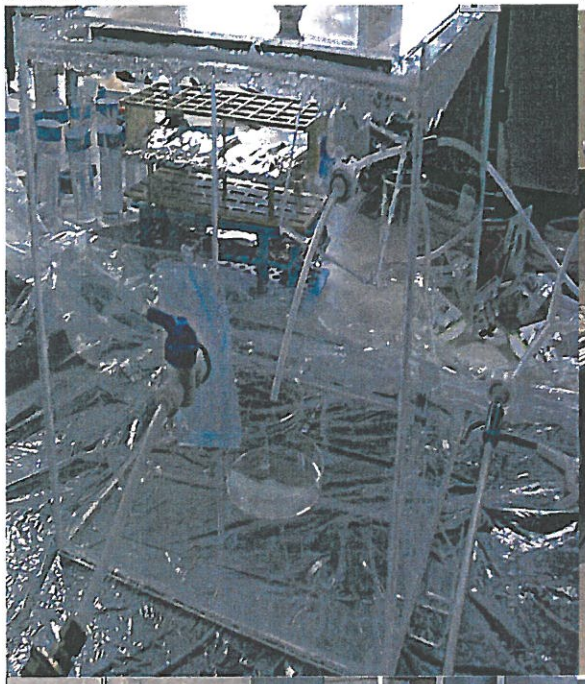


Illustration 2

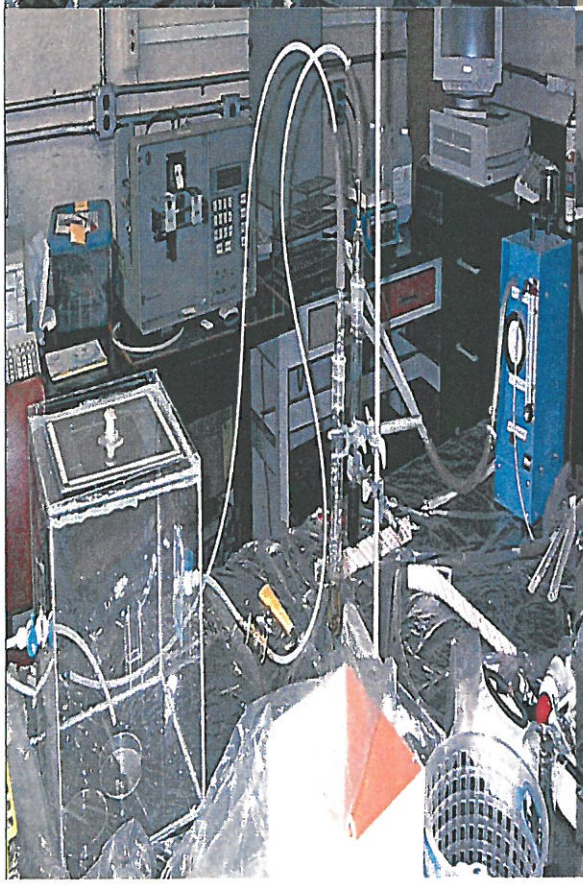


Illustration 3

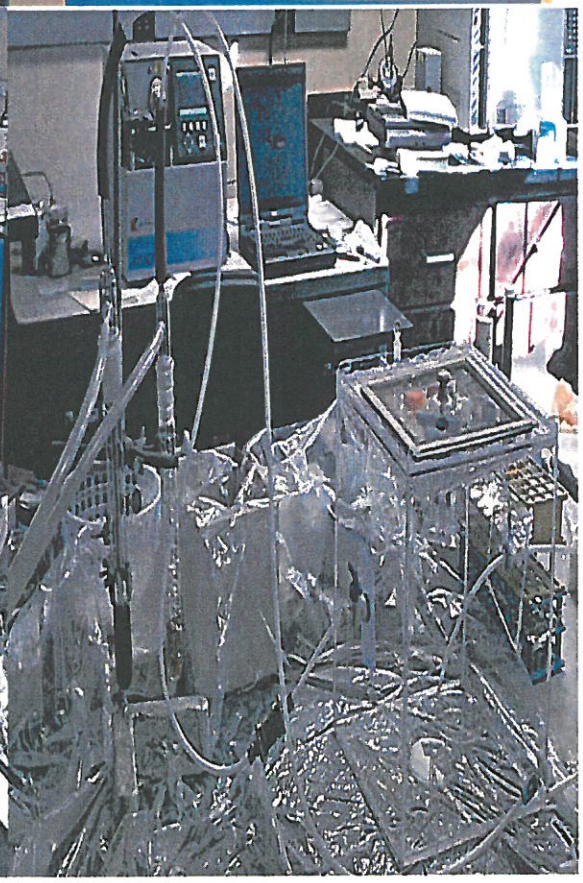


Illustration 4