UV Raman detection of micro-organisms and their toxins in fish tissue.

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Abstract

It has been determined that domoic acid(DA) can be quantitated from homogenized shellfish tissue by means of resonance Raman spectra excited by 251 nm light. Detection limits have been found to be substantially below the 20 micrograms DA per gram tissue deemed by regulators to be unfit for human consumption. Clam tissue obtained from a supermarket has been prepared for analysis by direct homogenization for 2 minutes in a Waring blender. The homogenized samples were placed in a flow system and subjected to a 5-10 mw 251 nm excitation. Back-scattering collected for 20-30 seconds provided sufficient information for analysis. The method is extremely simple to use since the DA produces a single intense peak at 1652 cm^{-1}. Because relatively-weak protein, nucleic acid and lipid spectra are excited from the tissue, background interference is surprisingly low. Bacteria can be detected using the same approach, but sensitivities are much lower primarily due to spectral interference from tissue nucleic acids.

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Introduction

Domoic acid(DA) is produced[1-8] by plankton diatoms in the species *Pseudonitzschia.* Special attention[9,10] has been drawn to this neurotoxin, DA, since the serious poisoning outbreak in P.E.I., Canada in 1987 which killed several persons and hospitalized dozens of others. The Canadian victims of DA(amnesic shellfish) poisoning had eaten shellfish that in turn had fed upon toxic phytoplankton. It was determined[11] that symptoms of poisoning occurred in those who had eaten clams having greater than 20 ppm DA in tissue. Concentrations as high as 100 ppm DA in clam tissue[12] and 20 pg/cell in diatoms[13] have been reported.

Domoic acid poisoning has been determined to be the cause of massive deaths of birds[14] and even aquatic mammals[15,16] which feed upon toxic clams and fish such as anchovies. Large numbers[14-16] have been known to perish in a single episode off the coast of California.

Incidents have been reported worldwide. These have implicated algae[17] from New Zealand waters and the European North Atlantic coast. DA at toxic levels has been found in dungenous crabs in the Pacific Northwest as well as scallops in Scottish waters.

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Separations methods[10,18-24] have been developed to detect DA in algae and in food. These methods are very sensitive and can detect DA at levels well below those of concern in food. However, separations methods require sample preparation to remove protein and other potential interferences from tissue prior to analysis. Methods tend to be costly to apply and can take hours to complete. Bioassays[25-31] require the sacrifice of animals or the use of expensive reagents. Consequently, tests typically are not performed routinely on shellfish sent to market. The shelf-life of live shellfish is especially short. Shellfish must be marketed promptly after harvesting and results of tests must be available before the shellfish are sold to reduce significantly the public health threat of DA poisoning..

Recent success in direct analysis of water and in phytoplankton cells by means of UV resonance Raman has been reported. The toxin can be detected[32] easily at 1ppm levels in water with 242 nm and 257 nm excitation. The most sensitive detection, 80 ppb, occurs[33] with 251 nm excitation which gives rise to the largest Raman spectral cross-sections for DA. DA concentrations determined by UV Raman spectral methods[33] correlate closely with those obtained by HPLC both in water and in intact phytoplankton cells. The UV Raman method is especially rapid since spectra can be taken from intact cells in the presence of growth media and salt water without significant spectral interference from any of the cell or media constituents.

In this study data will be presented which demonstrate that DA can be determined directly from homogenized shellfish tissue excited by 251 nm laser light. DA concentrations in the shellfish tissue are produced by direct addition of DA followed by homogenization in a blender. The DA spectra obtained from the slurries can be checked against standard sulfate spectra to determine if the cross-section of the DA peak from slurries is affected significantly due to the presence of the minced tissue.

A number of successful efforts have been made to detect bacteria using UV resonance Raman spectroscopy. These have been reviewed[34]. It is possible to detect very small numbers[35] of bacterial cells using UV resonance Raman spectroscopy if this is done in the absence of spectral interference. Significant spectral differences can be observed which allow differentiation at least to the genus level[36,37]. Still, it is not clear if it will be possible to detect bacteria from complex matrices such as seafood due to spectral interference. In this study efforts have been made to detect bacteria directly from clam tissue without recourse to prior separations.

Experimental Section

Instrumentation and Spectral Measurement. The instrumentation[38] has been described. Basically, the output of a Lexel Model 95 CW argon ion laser was doubled by means of a BBO crystal to allow output at 251 nm. The sample was flowed through a 1 ml quartz cell in a closed system by means of a Masterflex peristaltic pump. The laser power varied between 3-10 mw at the sample. The scattered light was collected and depolarized by quartz lenses and a prism located along the path to the spectrograph slits perpendicular to the cell surface. The entrance slit was set to 150 μ, which corresponded to a spectral slit of 8 cm^{-1}. Collection times ranged from 20-30 s. Multiple spectra were obtained from each sample. Typically, at least three duplicate spectra were taken from each sample. A Spex 1 meter, single-grating spectrograph was equipped with a 2400

grids/mm grating. This produced a dispersion of 0.4 nm/mm across a 1100B Princeton Instruments CCD camera which was cooled by liquid nitrogen. A solid edge filter (Barr Associates) designed to eliminate 251 nm light removed most Rayleigh scattering in the fingerprint region and provided 90-95 % throughput for the Raman lines. The laser spot size ranged from 10X20 to 50X50 μm at the sample cell. The Raman shift axis was calibrated with an ethanol-acetonitrile mixture. Tissue samples were either used directly or filtered through a porous paper coffee filter prior to excitation. All data were collected digitally by means of Winspec software and imported into GRAMS 32 software(Galactic Industries, Inc, Salem, N.H.) for display and processing. Peak heights, areas and baselines were determined with the aid of GRAMS 32 for all spectra. Since the spectral bandwidths were not entirely dominated by the instrument band-pass, spectral peak areas were used throughout in calculations of DA concentrations from Raman spectra. Background subtraction was used to remove contributions from water, the quartz cell, and the clam tissue. Care was taken to use the same cell position to obtain both the background and the sample spectra.

Raman Spectral Cross-Section Calculations. A sulfate internal standard was used . the direct comparison of sulfate peak areas with DA peak areas allowed DA concentrations to be calculated assuming that the cross-section of the DA peak can be taken as proportional to the integrated intensity of the sulfate 981 cm^{-1} peak. The relation between the Raman cross-section of sulfate solutions and the integrated Raman peak intensity of DA is defined by the equation:

$$\sigma_{DA} = \sigma_{STD}(I_{DA}/I_{STD})(C_{STD}/C_{DA})[(\nu_o-\nu_{STD}/(\nu_o-\nu_{DA})]^4$$

The Raman cross-section of the DA band, σ_{DA}, at the frequency, $\nu_{DA,}$ is determined by a comparison if its peak area, I_{DA}, with the peak area of the sulfate internal standard, I_{STD}, at frequency, ν_{STD}. C_{DA} and C_{STD} are the molar concentrations while σ_{DA} and σ_{STD} are the molar cross-sections of the DA 1652 cm^{-1} and the sulfate 981 cm^{-1} peaks, respectively. The laser frequency is ν_o. The absolute Raman cross-section of the sulfate peak has been determined[39,40] previously as a function of the UV excitation wavelength.

Reagents. Domoic acid and sodium sulfate were purchased from Sigma Chemical Co, St. Louis, MO, and used as received. Sodium sulfate could be used in all cross-section determinations, since the sulfate 981 cm^{-1} peaks did not overlap the DA or interfering tissue peaks.

Sample Preparation. The clam tissue had been separated from the shells at the supermarket. The tissue was weighed and mixed with an amount of DA required to prepare mixtures with DA concentrations ranging from 1-100 ppm. Water was added to samples to reduce the viscosity of the mixture to allow easy circulation of the sample through the flow cell.

RESULTS

Figure 1 shows the UV resonance Raman spectra of whole clam tissue, homogenized for 2 minutes in a blender and diluted 20:1 with distilled water, compared with spectra of the same sample containing 20 ppm(parts per million) DA.

Figure 2 shows spectra of homogenized clams suspended in 0.1 **M** sodium sulfate. This sample was prepared adding 9 parts water to 1 part clam tissue plus enough sodium sulfate and domoic acid to produce the desired concentrations. The concentration of DA in the sample was 0.900 ppm. Collection times were 30 seconds.

Figure 3 consists of three difference spectra involving the clam homogenate used to obtain spectra of Figure 2. This data was obtained from suspensions having enough DA added to make the liquids 4.0 ppm, 2.1 ppm and 0.48 ppm, respectively, in DA.

Figure 4 is composed of 6 superimposed spectra. Homogenized clam suspensions were prepared by adding 9 parts water to one part tissue. A sodium sulfate concentration of 0.100 M was achieved by adding weighed amounts of sulfate. DA amounts ranged from 0 to 4.8 ppm. Filtrate was passed through a course paper coffee filter before spectra were excited.

Figure 5 compares the clam supernate spectra of Figure 4 with spectra taken from comparable supernate possessing *E.coli* as a constituent. Concentrations of the bacteria were high, 10^9/mL. Subtraction spectra are presented as well to indicate the extent of the contribution of the bacteria to the spectra.

DISCUSSION

Figure 1 reflects the very-large Raman spectral cross-section of DA excited at 251 nm. The homogenized clam spectra show surprisingly weak interference. The interference is weak because the large amounts of protein present in the tissue show very low Raman spectral cross-sections. Compared to the DA peak at 1652 cm^{-1} even the tissue nucleic acid components are relatively feeble in intensity. Only at very low DA concentrations(below 1 ppm) will the tissue spectral interference become a problem.

Figure 2 compares the same clam tissue spectra as Figure 1 over a smaller wavelength range with spectra of suspensions having relatively low DA concentrations. Even the solutions having 0.90 ppm DA concentration have a DA peak which is easily detected against the tissue background. Corrected for dilution of the clams 10:1 the experiment corresponds to the spectra which should belong to clams having 9ppm DA concentration. This value of 9 ppm is significantly lower than the 20 ppm DA concentration recognized as too high for safe human consumption. Spectra are relatively noisy in part because collection time was limited to 30 sec. However, additional contributions to noise were present due light scattered elastically irregularly from the rapidly circulating tissue fragments. This light - scattering contribution was excluded in the main by the edge filter, but because of the great intensity of elastic scattering from the unfiltered suspensions some noise resulted from scattering which was not excluded entirely.

Figure 3 shows subtraction spectra. The resultant constitutes the DA spectra . The clam spectra have been removed. The clam tissue was diluted 10:1 by distilled water prior to homogenization. DA was added to the mixture prior to homogenation in

sufficient amounts to provide DA concentration of 0.48, 2.1 and 4.0 ppm, respectively, by adding known volumes of standard DA to the mixture.

Figure 4 contains spectra taken from solutions prepared by passing the liquid from clam tissue homogenate through a course paper coffee filter. The spectra show in addition to the DA spectra well-resolved nucleic acid spectra attributed to the clam nucleic acids. The most intense of these occurs at 1480 cm^{-1} and is attributed to adenine and guanine. Most significantly, the enhancements in the intensity of all 1652 cm^{-1} peaks are clearly resolved even for the suspensions containing the DA concentration of 0.96 ppm. Increments in the peak areas of the DA mode are proportion to the DA concentrations. Error at this low DA concentration range is nearly 20 %. Cross-sections for DA calculated using known sulfate 981 cm^{-1} mode cross- sections agree with those measured from the spectra to the same 20 % degree of reliability. Calculations can be made with or without subtraction of the water 1648 cm^{-1} peak contributions.

Figure 5 compares spectra of clam homogenate in water made 0.100 M in sodium sulfate with a comparable suspension having 10^9 cells/mL *E. coli* in addition. The difference spectra clearly indicate a significant contribution from bacteria. Just as clearly, the sensitivity of bacterial detection appears to be significantly lower than that of domoic acid. This can be understood as due in part to the lesser cross-sections of the nucleic acid modes as opposed to DA. However, the direct interference from the tissue nucleic acid spectra seems to present the greatest challenge in the direct spectral detection of bacteria from tissue with 251 nm excitation.

CONCLUSION

The neurotoxin DA can be detected by UV resonance Raman spectroscopy in clam tissue at levels well below those deemed toxic. This can be done directly with samples prepared by simple homogenization of the clam tissue in a Waring blender. No other modification of the clam sample was necessary. Since spectra of fish and octopus tissue are very similar to those of clams when excited at 251 nm, it appears likely that UV Raman can be used to detect DA even at sub-toxic levels in most sea foods. Detection of DA in tissue at levels deemed toxic will be especially rapid and easy.

In contrast, the Raman spectral detection of bacteria directly from the same samples is more difficult. The lesser sensitivity of bacterial detection is attributed to two factors: 1., the lower Raman cross sections of bacteria compared with DA values, and 2., direct spectral interference by tissue nucleic acid spectra. With 251 nm excitation bacterial spectral excitation is neither as sensitive or as specific as DA excitation. Bacteria can be detected directly from tissue using Raman spectroscopy with 251 nm excitation, but this appears possible only at relatively high concentrations of bacteria. It is suggested that if bacteria are to be detected sensitively in tissue by Raman methods the spectral analysis must be preceded by a separations step which can effectively remove the nucleic acid interference due to tissue.

Fluorescence interference was negligible for all spectra excited at 251 nm in this study. Fluorescence generated is very strongly Stokes-shifted and does not overlap the Raman spectra in the fingerprint region.

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RAW SPECTRAL DATA: MINCED CLAM TISSUE:BOTTOM SPECTRA: MINCED CLAM TISSUE PLUS 20 PPM DOMOIC ACID, TOP SPECTRA. LASER POWER 12 MW: COLLECTION TIME 20 SECONDS.

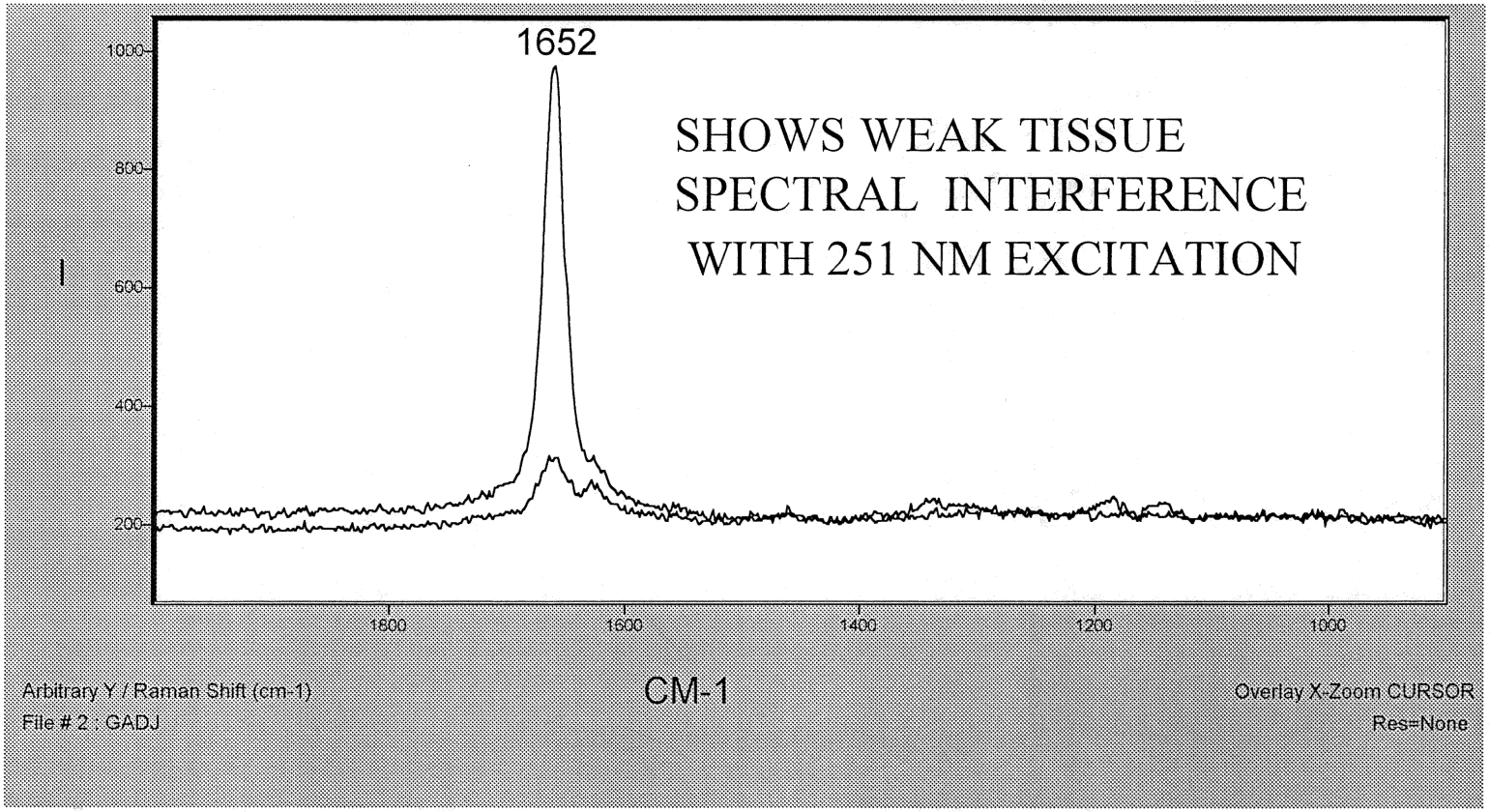
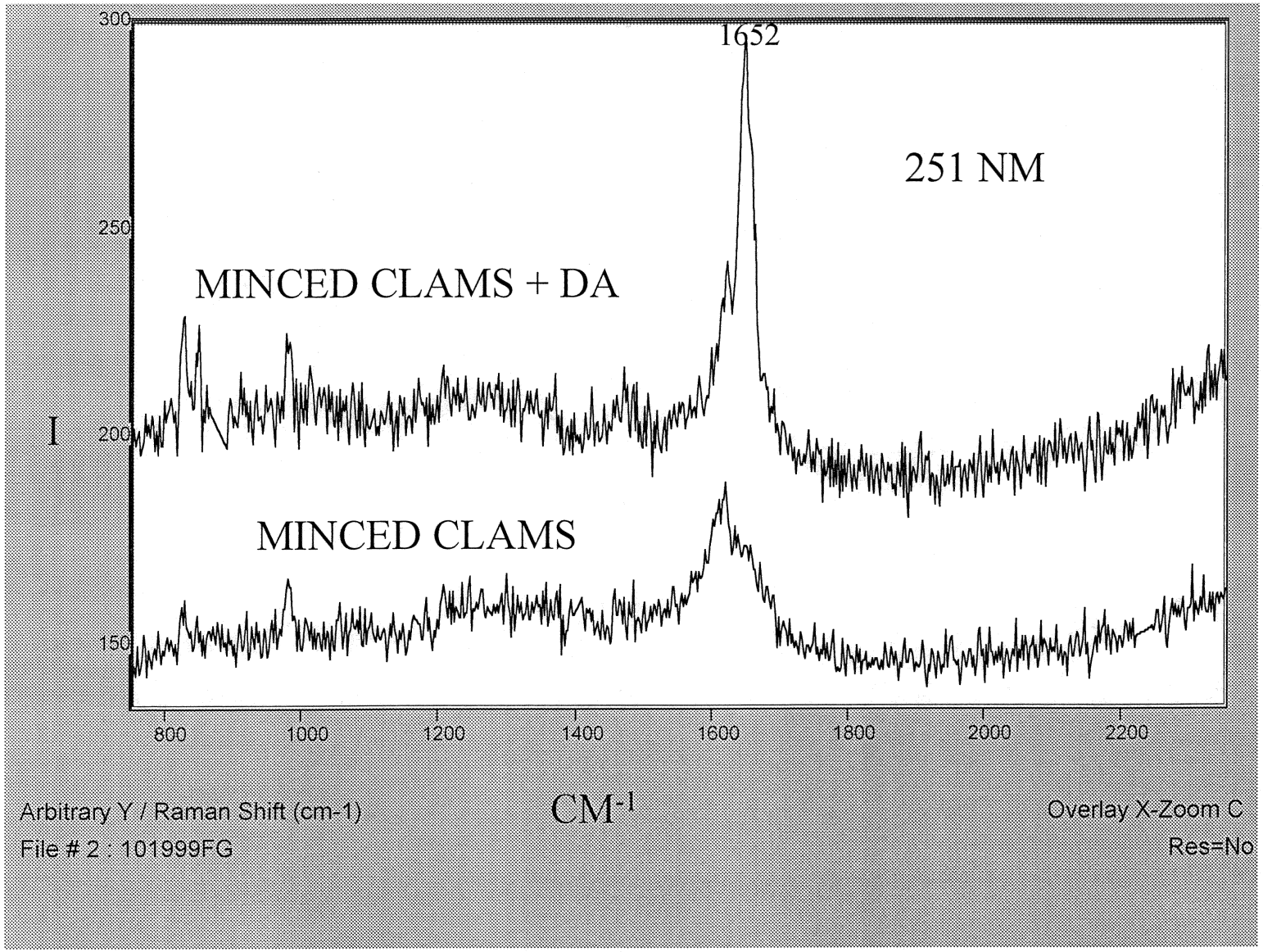


Figure 1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251 NM
MINCED CLAMS + DA
MINCED CLAMS
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150
800
1000
1200
1400
1600
1800
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2200
Arbitrary Y / Raman Shift (cm-1)
CM-1
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Figure 2

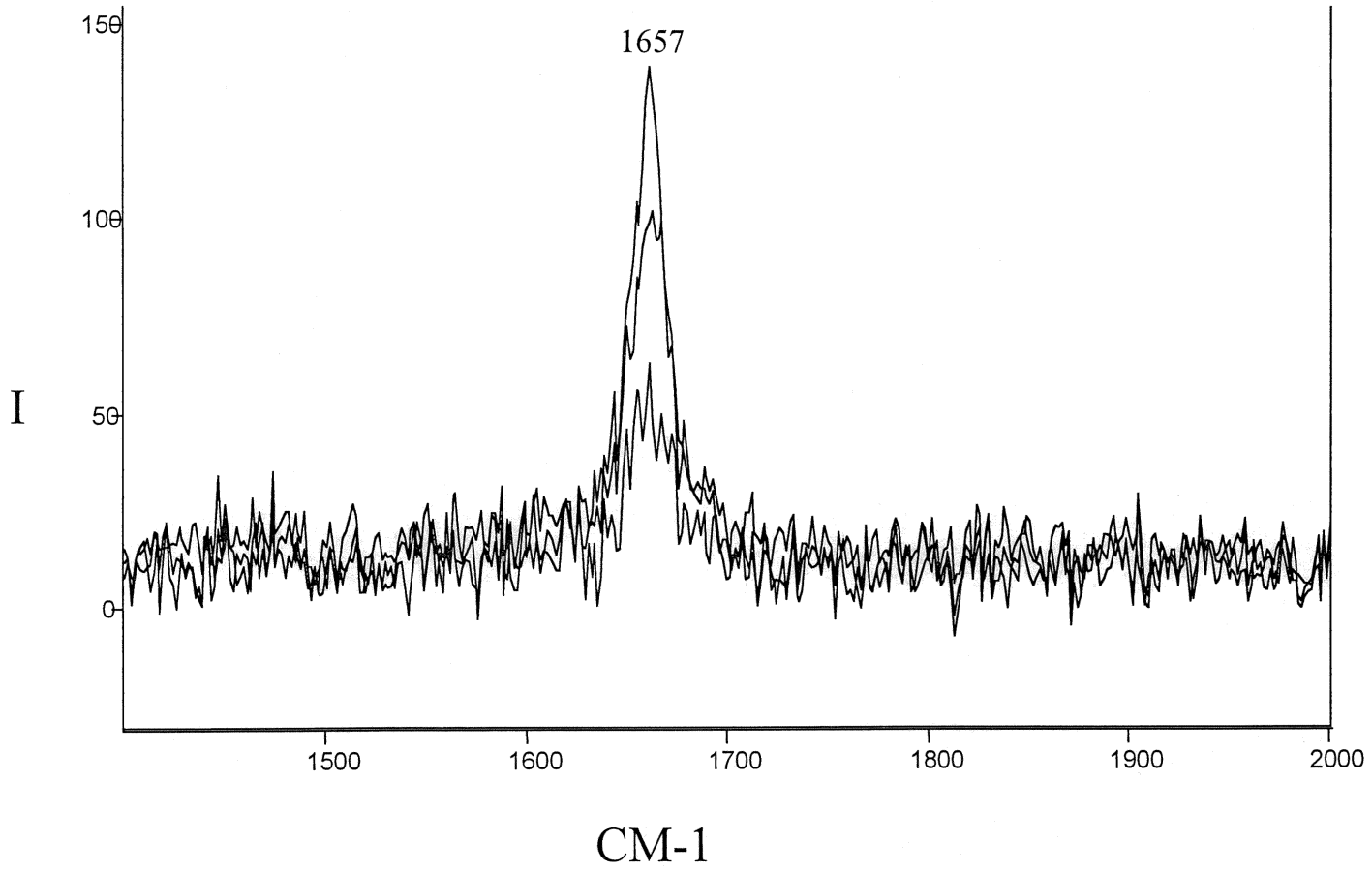
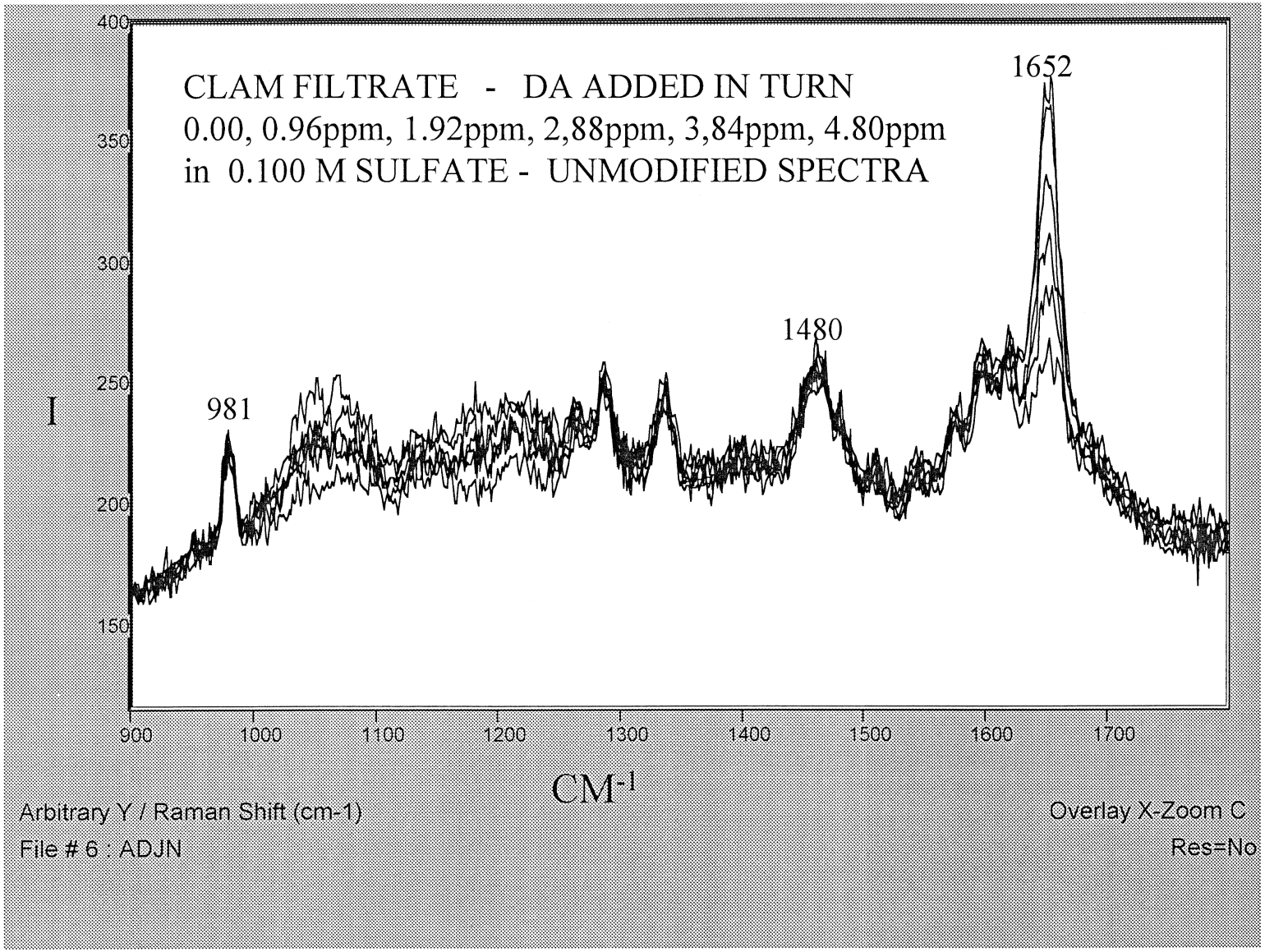


Figure 3


CLAM FILTRATE - DA ADDED IN TURN
0.00, 0.96ppm, 1.92ppm, 2,88ppm, 3,84ppm, 4.80ppm
in 0.100 M SULFATE - UNMODIFIED SPECTRA
1652
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CM-1
Arbitrary Y / Raman Shift (cm-1)
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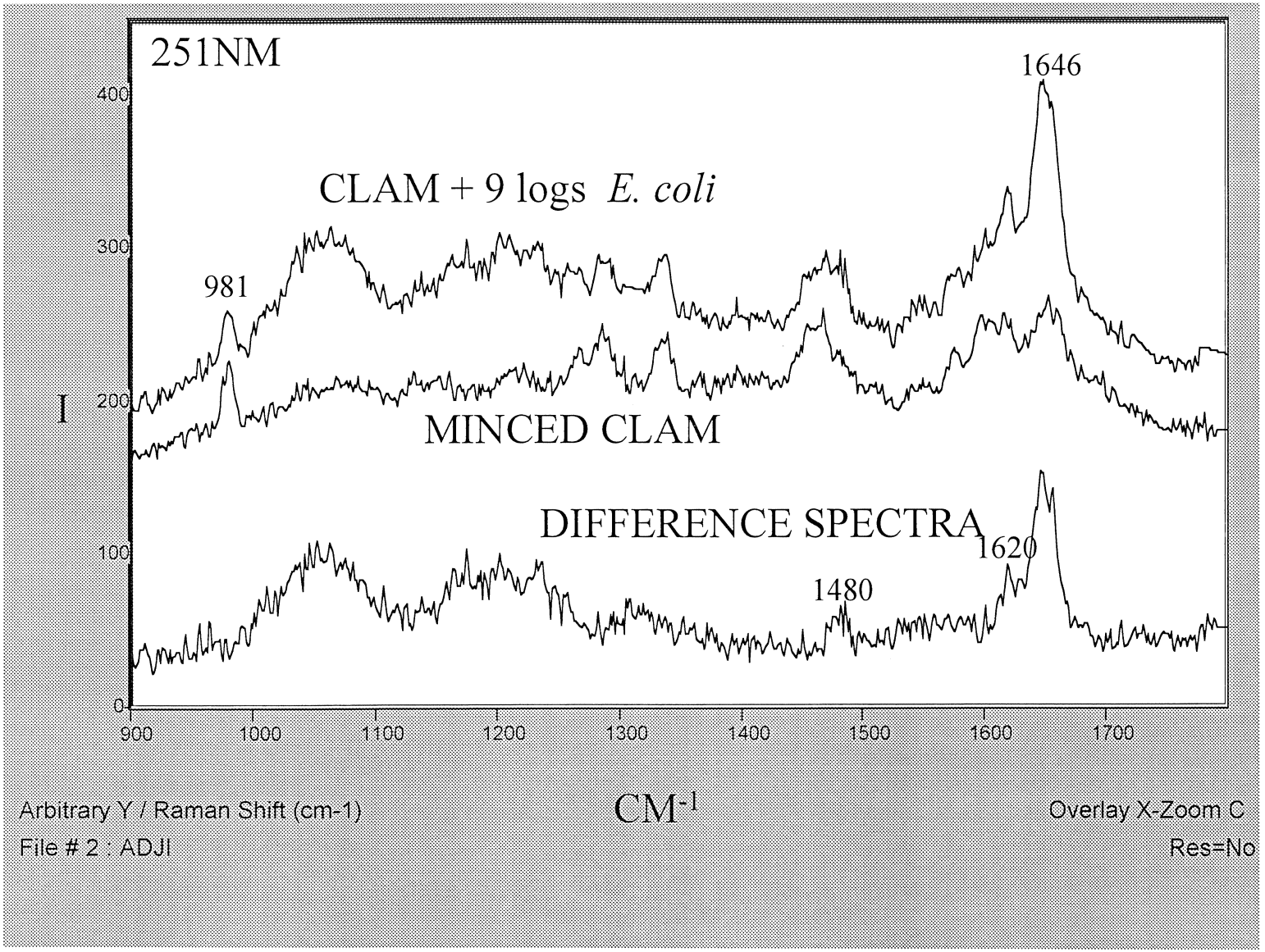
Figure 4


251NM
1646
CLAM + 9 logs E. coli
981
MINCED CLAM
DIFFERENCE SPECTRA
1620
1480
I
400
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CM-1
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Figure 5