Addendum to the NIDS Report on a Mutilated Cow in Dupuyer Montana, June 2001

National Institute for Discovery Science
Las Vegas, NV
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SUMMARY

A mutilated Red Angus cow was found in Montana with no obvious tracks from vehicles, people, or predators around the animal. The mutilations consisted of very clean excisions of the left eye and eyelid, rectum, genitalia, and tongue. The previous NIDS Report (http://198.63.56.18/pdf/montana_cattlemutilation.pdf) documented the analyses of green colored tissue from underneath the left jaw-bone, vitreous fluid, and a maggot mass. A cursory examination of vitreous fluid from a control heifer was also done. This addendum report summarizes the results obtained when additional brain tissues samples were submitted for analysis from the Montana cow, as well as the control heifer. The assignment was to look for any components that should not be normally present in the mutilated animal. Secondly, an in-depth evaluation of the MS data previously obtained in November 2001 from vitreous fluid of the control heifer was completed as an aid to accomplish this objective.

The results in this addendum report support those reported previously. Oxindole was found at low levels in the brain of the mutilated animal but not in the brain of the control. Oxindole was found at high concentrations in the eye-fluid of the Montana animal and was completely absent from the eye-fluid of the control animal. This addendum report (three photos, one table and eight figures) documents the complete GCMS comparative analysis of the eye fluids from both mutilated and control animal. No other unusual molecules were found in the brain of the mutilated animal.

PROCEDURE

Samples. The following samples were submitted Frontier Analysis Ltd Chagrin Falls, Ohio and to it's GCMS subcontractor Richard L Wilson.

Brain Tissue. Two samples were received in plastic vials surrounded by cold packs.

- A brain tissue sampling from the mutilated cow received February 27,2002. On receipt the tissue appeared to be in almost a liquid consistency. As shown in photos 1–3, the removal of the brain was accomplished by sawing through the skull to create a flap of bone. The flap of bone was removed from the top of the skull and the gray matter carefully removed. The consistency of the brain was unexpectedly good (see photo#3) considering the animal's head had been exposed to several hours of summer Montana heat and humidity, followed by freezing and transport to Las Vegas,

followed by freezing and subsequent thawing in the Las Vegas laboratory. Brain tissue is known to be especially sensitive to rapid decomposition.

- A brain tissue from a control heifer, which was not mutilated, was submitted for reference. It was also received on February 27,2002. The control animal, obtained from a slaughterhouse, was exposed to environmental conditions expected for mutilated animal carcass. It was laid out for 4 days, and protected from predators and scavengers.

The above samples were extracted with methylene chloride. Solvent was added to the "as received" sample, and it was allowed to soak for 8 days in the refrigerator. The sample was subjected to ultrasonic agitation for approximately one hour a day. The solvent was not completely removed and reduced to 2 mls. Both GC/MS and infrared analyses were then done to characterize the extracts.

Photo 1. A bone flap from the top of the skull was removed using a Black and Decker rotary saw.

Photo 2. The top of the skull was removed in two sections.

Vitreous Fluid

- Vitreous fluids from the left and right eye were additionally submitted from a control heifer for reference on 11/13/2001. (See background on control animal above.) Both had been examined “as received” by GC/MS. A more in-depth examination/ interpretation was completed on this previously acquired data and is reported in this report. It is to be used as a “benchmark” to possible future samples.

The detailed information regarding the instrumental data acquisition conditions can be found in the appendix.

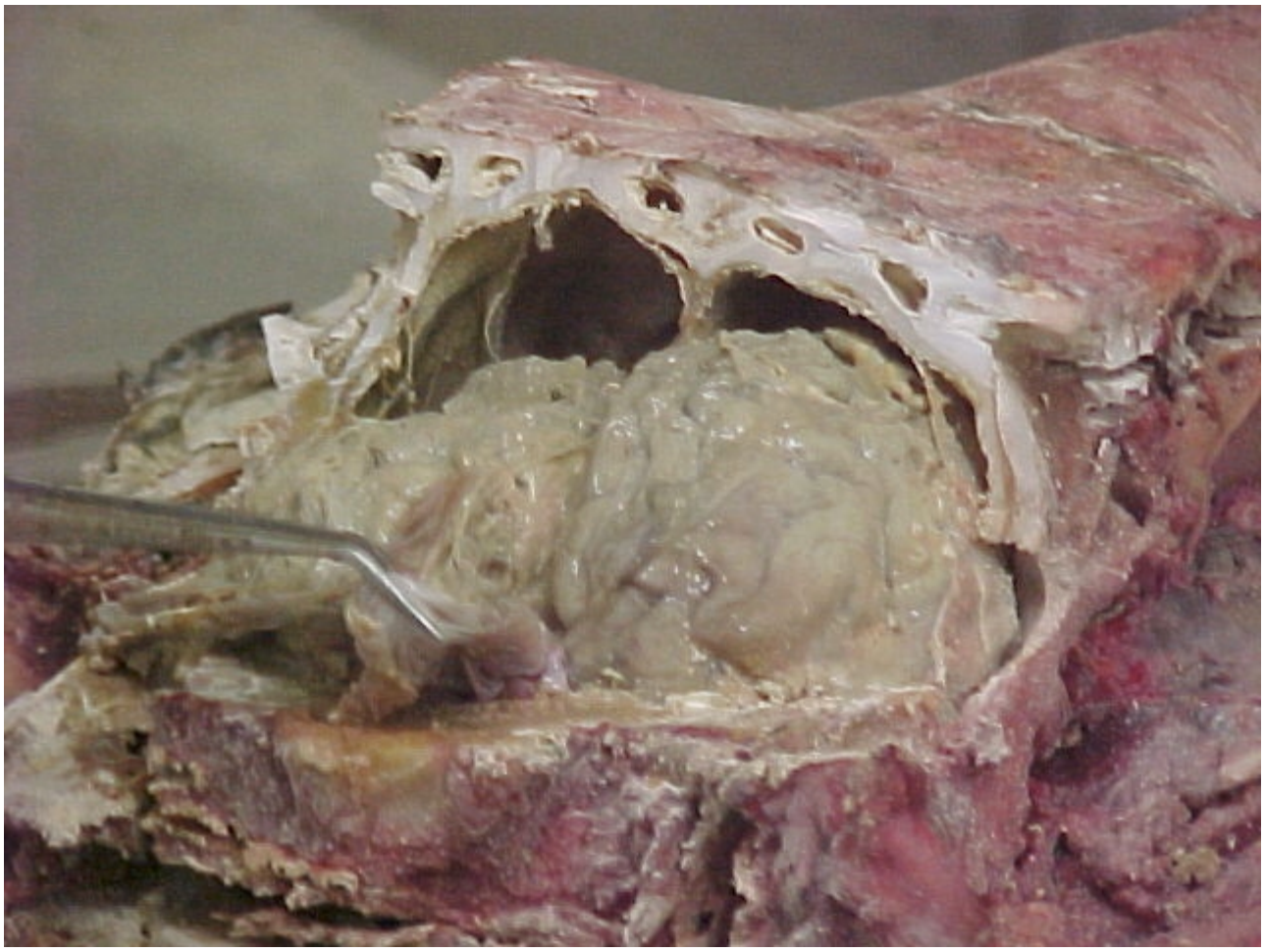

Photo 3. The brain of the animal was in remarkably good condition.

RESULTS

The results of the individual tests done on the brain tissues and control vitreous fluids follow. These results are summarized in the conclusions section of this report. All tables and figures referenced in this report can be found in an appendix.

Brain Tissue

GC/MS Analysis. The methylene chloride extraction removed a large amount of soluble material from the brain tissue of the mutilated cow and the control heifer. Expectedly, this analysis shows natural and degradation products predominate. Cholesterol and its derivatives are most abundant. However, a very small amount of unusual compound is uniquely observed in the extract from the mutilated cow when comparing the data from the mutilated animal and the control. This is oxindole. This molecular structure, as well as some derivatives of this structure, is known to possess a sedative property. It has a GC retention time of 17.89 minutes and is positively identified in the mass spectrum. The characteristic

masses of oxindole are all present (51, 63, 78, 89, 104 and 133). Masses 104 and 133 are the strongest. The GC chromatograms of the extracts from the tissues of the mutilated cow and the control heifer are shown in figures 1 and 2. The mass spectrum along with a reference of oxindole is shown in figure 3. There are other extraneous peaks in the spectrum, which are probably due to interfering noise. These peaks appear intense because of the very low concentration of the oxindole (<1 ppm). Table I displays the MS identifications of the GC peaks for the tissue extracts from both the mutilated and control animal.

Infrared Analysis. Infrared analysis of the methylene extracts from the Montana and control animal are identical. The spectra can be found in figure 4. Detected are major amounts of ester and cholesterol components. A smaller amount of protein matter is also indicated. The ester is a glycerol derivative, which compares to references of L-α-phosphatidylinositol, 4,5-diphosphate sodium salt and L-α-phosphatidylinositol, 4-monophosphate sodium salt from bovine brain.[1] This material, as well as the protein, would not be detected by the above GC/MS analysis, because it " probably does not pass through a GC column." References of cholesterol, L-α-phosphatidylinositol, 4,5-diphosphate sodium salt and L-α-phosphatidylinositol, 4-monophosphate sodium salt are shown for comparison in figures 5 and 6.

Vitreous Fluid (Mutilated Montana Cow Versus Control Heifer)

GC/MS Analysis. GC chromatograms of "as received" vitreous fluids from the right and left eyes of the control heifer expectedly match each other. The graphs show a plethora of components. The MS identifications are a good reference to natural and putrefaction products existing in the animal four days after euthanasia. As noted in the original NIDS report on the Dupuyer animal mutilation, no oxindole was found in the control eye fluids. The ion chromatogram scans for masses of 104 and 133 from GC retention times 17:00 to 20:00 min. of the vitreous fluid from the right eye of the control animal would be expected at an identical retention times if oxindole is present. This is not observed. Oxindole was uniquely identified at 50–100 ppm in the vitreous fluid from the mutilated cow. Table II lists the MS identifications of the control heifer GC peaks along with those of the eye fluid from the mutilated animal, which are reported in the previous NIDS report to conveniently compare the data. The GC chromatograms are displayed in figures 7 and 8.

Instrumental Data Acquisitions Conditions

Infrared. Both transmittance and reflectance infrared spectra were obtained from the samples using a Nicolet Avatar 360 spectrometer. Transmittance spectra were obtained from smears on KBr crystals. Reflectance spectra were acquired using the Harrick SplitPea® sampling accessory.

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[1] Roger J. Keller, "The Sigma Library of FT-IR Spectra," Edition I, Volume 1, Sigma Chemical Company, Inc., 1986, References 1542A, 1542B.

GC/MS. A Hewlett-Packard GC/MS (DOS-MSD/ChemStation) employing a 6890 gas chromatography, 5973 Mass selective detector and capillary injection system was used for analysis. Chromatographic separation was accomplished by using a 60m x 0.32mm i.d., 1.0 mm film thickness DB-1 capillary column from J&W Scientific (sn 0433924; Cat # 123-1063). The following GC/MS conditions were used:

Instrument:	GC/MS-4
Injector Temp:	Inj. 300°C
GC Oven Program:	50°C (0.0 min.) to 290°C @ 10.0°C/min. (36.0 min.)
Injection Volume:	1.0 µl, splitless
Run Time:	60.6 min.
MS Run Type:	Scan
Mass Range:	25-600 Da; Scan threshold: 100
Scan Start Time:	0 min.
Sampling:	No.=5
Multiplier Volt.:	Emv offset=200; resulting volt.=1490
Method File:	RWSVM.M
Tune File:	ATUNE.U

APPENDIX

TABLE 1. GC/MS Data from Methylene Chloride Extractions of Brain Samples from the Mutilated Cow and from a Control Animal

Brain Extract of Montana Mutilated Cow			Brain Extract of Control Heifer		
Compound	**Match**	**GC Retention Time (min.)**	**Compound**	**Match**	**GC Retention Time (min.)**
•Butanoic Acid	80	6.686	-	-	-
•Possible C6 Nitrile or Protein Fragment (4-Methyl-3-pentenitrile)	37	7.533	•M/Z Possible C6 Nitrile or Protein Fragment (4-Methyl-3-pentenitrile)	38	7.533
•Benzeneacetaldehyde, .alpha.-ethylene- (<5ppm)	91	15.150	-	-	-
•Octanoic Acid	50	16.345	-	-	-
•M/Z 104, 133 Oxindole (<1 ppm)	*	17.75	-	-	-
•MW=256 Amino Derivative (2-Amino-3,9-Dimethyl-5-dimethylamino-3H-1,3,4,6 tetrazacyclopent(E)azulene)	78	18.933	•MW=256 Amino Derivative (2-Amino-3,9-Dimethyl-5-dimethylamino-3H-1,3,4,6-tetrazacyclopent(E)azulene)	78	933
-	-	-	•MW=195 Nitrogen Compound (H-Carbazole, 9-ethyl-)	35	19.182
•MW=195 (Methyl 3-ethylamino-5-Hydroxybenzoate)	43	19.232	-	-	-
•C16-C18 Aldehyde (Tetradecanal)	96	22.070	•C16-C18 Aldehyde (Hexadecanal)	94	22.070
-	-	-	•Benzene, dodecyl-	91	22.966
•Fatty Acid (<215 ppm) (Hexadecanoic acid)	99	23.414	•C16 Acid (Hexadecanoic acid)	93	23.414
•~C18 Aldehyde (9-Octadecenal)	95	23.962	•~C18 Aldehyde (9-Octadecenal)	90	23.961
•~C18 Aldehyde (17-Octadecenal)	93	24.161	•~C18 Aldehyde (17-Octadecenal)	91	24.161
•~C18 Fatty Acid (Heptadecene-(8)-carbonic acid-(1))	97	25.306	•~C18 Fatty Acid (9-Octadecenoic acid (Z)-	43	25.306
•~C18 Fatty Acid (Octadecanoic acid)	99	25.505	•~C18 Fatty Acid (Octadecanoic acid)	99	25.505
-	-	-	•Cholesterol Derivative [(22S,25S)-22,26-Epiminocholest-5-ene-3beta, 16 alpha-diol 16- acetate) (Muldamine)]	27	27.496
•Nitrogen Compound (Piperidine, 1,1'-methylene bis-)	18	27.546	-	-	-
•Nitrogen Compound (Decanamide, N-(2-hydroxy ethyl)-)	56	27.845	•M/Z 85 Amide (Decanamide, N-(2-hydroxy ethyl)-)	56	27.845
-	-	-	•M/Z 97 Thiophene Derivative (Thiophene, 2-decyl-)	23	30.185
•M/Z 131 (Naphthalene, 1,2,3,4-tetra hydro-1-methyl-)	36	33.570	•M/Z 131 Naphthalene Deriv. (Naphthalene, 1,2,3,4-tetra hydro-1-methyl-)	47	33.620
-	-	-	•M/Z 97 Thiophene Derivative (Thiophene, 2-butyl-)	25	34.167
•Cholesterol Derivative (Cholest-5-en-3-ol (3.beta.)-)	64	38.748	•Cholesterol Derivative (Cholest-5-en-3-ol (3.beta.)-)	12	38.748
•Cholesterol Derivative (Cholesta-3,5-diene)	78	42.083	•Cholesterol Derivative (Cholesta-7,14-diene)	42	42.083
•Cholesterol Derivative (Cholesta-3,5-diene)	99	43.477	•Cholesterol Derivative (Cholesta-3,5-diene)	99	43.477
•Cholesterol	99	53.733	•Cholesterol	99	53.783

*Oxindole was detected in ion chromatogram scans of ions 104 and 133 between GC retention times of 6.00 - 18.40 minutes.

TABLE 2. GC/MS Data from the Vitreous Fluid of the Mutilated Cow and the Control Heifer.

Mutilated Montana Cow			Control Heifer		
Compound	**Match**	**GC Retention Time (min.)**	**Compound**	**Match**	**GC Retention Time (min.)**
•Acetaldehyde	91	3.380	•Acetaldehyde	39	3.191
•Trimethylamine	86	3.589	•Methanamine, N,N-dimethyl- (Trimethylamine)	72	3.480
•Butane C4H10	4	4.077	-	-	-
•1-Propanol	72	4.326	-	-	-
•Acetic Acid	91	4.824	-	-	-
•Methyl Butanal	45	5.421	-	-	-
•Propionic Acid	93	5.969	-	-	-
•Butanoic Acid	90	7.263	-	-	-
•C6 Acid		8.159	-	-	-
Hexanoic Acid	12				
•Dimethyl Sulfone	59	9.055	-	-	-
•Butyrolctone (GBL)	83	9.254	-	-	-
-	-	-	•MW=97 C4H3NO3		10.039
			1H-Pyrrole-2.5-dione (Maleimide)	78	
•Phenol	91	10.698	•Phenol (~15 ppm)	64	10.369
•Urea	86	10.848 & 10.997 & 11.196	-	-	-
•C8H16 Hydrocarbon		12.142	-	-	-
1-Ethyl-3-methyl-cyclopentane	83				
-	-	-	•MW=99 C4H4NO2		12.143
			Succinimide (~21 ppm)	80	
•4-Methyl-phenol	95	12.341	-	-	-
•Amine?		12.441	-	-	-
1-Piperazineethanamine	12				
-	-	-	•M/Z 44, 98 Nitrogen Compound		12.597
			2-Pentanamine, 4-methyl-	37	
•MW=99		13.735	-	-	-
2-Piperidinone	35				
-	-	-	•M/Z 112, 56 (MW=112)		13.793
			1,4-Cyclohexanedione	38	
•MW=99		13.835	-	-	-
2-Piperidinone	50				
•MW=114		14.581	-	-	-
5-Methylhydantoin	50				
•N-Butyl-1-hexanamine	42	14.731			

TABLE 2 (Continued)
GC/MS Data from the Vitreous Fluid of the Mutilated Cow and the Control Heifer.

Mutilated Montana Cow			Control Heifer		
Compound	**Match**	**GC Retention Time (min.)**	**Compound**	**Match**	**GC Retention Time (min.)**
-	-	-	•M/Z 70		14.742
			L-Proline	35	
•Amine	37	15.030	-	-	-
N-Ethyl-cyclopentanamine					
-	-	-	•MW=114		15.154
			Parabanic acid	47	
•MW=98 C3H6N4 Amine		15.278	-	-	-
4-Methyl-1,2,4-triazol-3-amine	72				
•MW=114 C4H6N2O2		15.577	-	-	-
5-Methylhydantoin	83				
•1H-Indole	93	15.926	•1H-Indole	94	15.608
-	-	-	•M/Z 98		15.732
			Mepivacaine	43	
•MW=112		16.324	-	-	-
4,5-Dihydro-6-methyl-3(2H)-pyridazinone)	32				
			•MW=138		16.474
•2-Methoxy-5-methyl-2,5-cyclohexadiene-1,4-dione	40	16.573	-	-	-
-	-	-	•MW=152		16.763
			4(3H)-Pyrimidinone, 2-ethyl-3,6-dimethyl-	38	
			2-Methyl-3-(2-thienyl)-2-propenal	64	
•M/Z 42, 98, 111		16.772 to 16.822	-	-	-
1,1'-Methylenebis-piperidine	47				
-	-	-	•M/Z 100		17.052
			4-Morpholinebutyric acid, .beta.-methyl-.alpha.,.alpha.-diphenyl	42	
			4,9-Decadien-2-amine, N-butyl-	42	
•MW= 152 Aromatic Oxygenate		17.120	-	-	-
2-Hydroxy-5-methoxy-benzaldehyde)	43				
•M/Z 100 Nitrogen Compound		17.419	-	-	-
2,4-Imidazolidinedione	64				
-	-	-	•M/Z 98 Ketone		17.423
			3-n-Butylcyclohexanone	32	
•Tyramine	72	17.469	-	-	-
•MW=152 ?Oxygenate		17.817	-	-	-
3-Hydroxy-2-isobut-1-enylcyclopent-2-en-1-one	90				

TABLE 2 (Continued)
GC/MS Data from the Vitreous Fluid of the Mutilated Cow and the Control Heifer.

Mutilated Montana Cow			Control Heifer		
Compound	**Match**	**GC Retention Time (min.)**	**Compound**	**Match**	**GC Retention Time (min.)**
-	-	-	•MW=166		17.959
			Phenol, 3-methoxy-2,4,6-trimethyl-	30	
•Oxindole (50-100 ppm)	93	18.216	-	-	-
			•M/Z 100, 166		18.496
			Hexahydropyrimidin-2-one	40	
•4-Hydroxy-3-methoxy-benzaldehyde	23	18.365	-	-	-
•M/Z 165		18.465	-	-	-
2-Amino-1,7-dihydro-7-methyl-6H-purine-6-one	38				
•MW=166		18.614	-	-	-
3-(1-Amino ethylidine)-6-methyl-1H, 3H-2, 4- pyridinedione	35				
•M/Z 100		18.813	-	-	-
2-Methyl-2-butenoic Acid	49				
1-Nitroso-pyrrolidine	45				
-	-	-	•M/Z 138, 180		19.032
			Acetamide, N-(2-nitrophenyl)-	38	
			3-Methoxy-2-methylphenol	38	
•Thymin	87	19.211	-	-	-
•MW=180		19.361	-	-	-
4-(Acetyloxy)-benzoic Acid	49				
•Glutamic Acid	72	19.709 to 19.759	•M/Z 84 Glutamic Acid or Derivative		19.321
			L-Glutamic Acid	72	
•MW=194 C12H18O2 Lactone Type		19.958	-	-	-
5-Acetyl-1,3,3,4,5-pentamethylbicyclo[2.1.0] pentan-2-one	27				
•M/Z 120 Phenylalanine Derivative		20.307	-	-	-
L-Phenylalanine-4-nitroanilide	50				
-	-	-	•M/Z 138, 70		20.558
			Bicyclo [2.2.1]heptane-2-one, 3,3-dimethyl-	53	
			Endo-6-methylbicyclo[2.2.2]octan-2-one	47	
•M/Z 168		20.954			
Imidazo[2,1-a]isoquinoline	11				
-	-	-	•MW=154		20.971
			6,8-Diazabicyclo[3.2.2]nonane-7,9-dione	35	
			2,4(1H,3H)-Pyrimidinedione, 1,3,5-trimethyl-	14	
•M/Z 123, 165 Acetanilide Derivative		21.153	-	-	-
3-Methoxyacetanilide	25				

TABLE 2 (Continued)
GC/MS Data from the Vitreous Fluid of the Mutilated Cow and the Control Heifer.

Mutilated Montana Cow			Control Heifer		
Compound	**Match**	**GC Retention Time (min.)**	**Compound**	**Match**	**GC Retention Time (min.)**
-	-	-	•M/Z 116, 61		21.177
			Hexanoic, 2-methylpropyl ester	12	
•M/Z 114, 41, 83 Amine?		21.302	-	-	-
3-(Hexylamine)-propanenitrile	25				
•M/Z 116 Glutaminic Acid Derivative		21.551	-	-	-
Glutaminic acid dimethyl ester	32				
•M/Z 154, 70		22.298	-	-	-
Benzene, 2-chloro-1,3,5-trimethyl-	45				
•M/Z 154, 70		22.547	-	-	-
2-Thiatricyclo[3.3.1.1.(3,7)]decane	43				
Phenol, 3,5-dimethoxy-	40				
-	-	-	•MW=154		23.157
			2,4(1H,3H)-Pyrimidinedione, 1,3,6-trimethyl-	38	
-	-	-	•MW=154		23.322
			2,4(1H,3H)-Pyrimidinedione, 1,3,5-trimethyl-	17	
			Phenol, 3,4-dimethoxy-	27	
•M/Z 154, 70		23.493	-	-	-
•M/Z 154, 70		23.642	-	-	-
•C18 Fatty Acid		23.891	-	-	-
Octadecanoic Acid	91				
-	-	-	•M/Z 186, 117 Indole Derivative Probable		24.188
			1H-Indole	50	
			4-fluoro-2', methylbiphenyl	83	
•M/Z 186 Indole Derivative		24.538			
Fragments for Indole itself +186	49				
-	-	-	•M/Z 200, 117 Indole Derivative		24.890
			1H-Indole	43	
•M/Z 200 Indole Derivative		25.285	-	-	-
Fragments for Indole + 200					
-	-	-	•M/Z 91 Aromatic (Phenyl Group)		25.467
			Benzene, 1-nitro-4-(2-phenylethyl)-	35	
			Benzaldehyde, 2-hydroxy-6-methyl-4-(phenol?)	35	
•M/Z 91 Phenyl Component		25.883	-	-	-
Nonylbenzene	25				

TABLE 2 (Continued)
GC/MS Data from the Vitreous Fluid of the Mutilated Cow and the Control Heifer.

Mutilated Montana Cow			Control Heifer		
Compound	**Match**	**GC Retention Time (min.)**	**Compound**	**Match**	**GC Retention Time (min.)**
•Phenyl Component		26.082	-	-	-
Methyl(4.alpha.)-2.alpha.,3.beta.-dihydrozy-5,5-dimethyl-11-oxatricyclo(7.2.1.0)dodecane-1.alpha.-carboxylate	27				
-	-	-	•M/Z 70		26.334
			Tetramethyl-1,2-cyclopentanedione	50	
•M/Z 186 Phenoxy Component		26.530	-	-	-
Phenol, 3-phenoxy-	43				
(1,1'-Biphenyl) 2,5-diol	38				
-	-	-	•M/Z 186 Phenoxy Group		27.736
			Phenol, 3-phenoxy-	59	
-	-	-	•Phenylalanine derivative		27.860
			Phenylalanine-proline diketopiperazine	39	
•M/Z 186 Phenoxy Component		28.222	-	-	-
Similar to 26.53. Probably oligomer of some large aromatic compound					
•Cholest-5-en-3-ol	89	56.948			

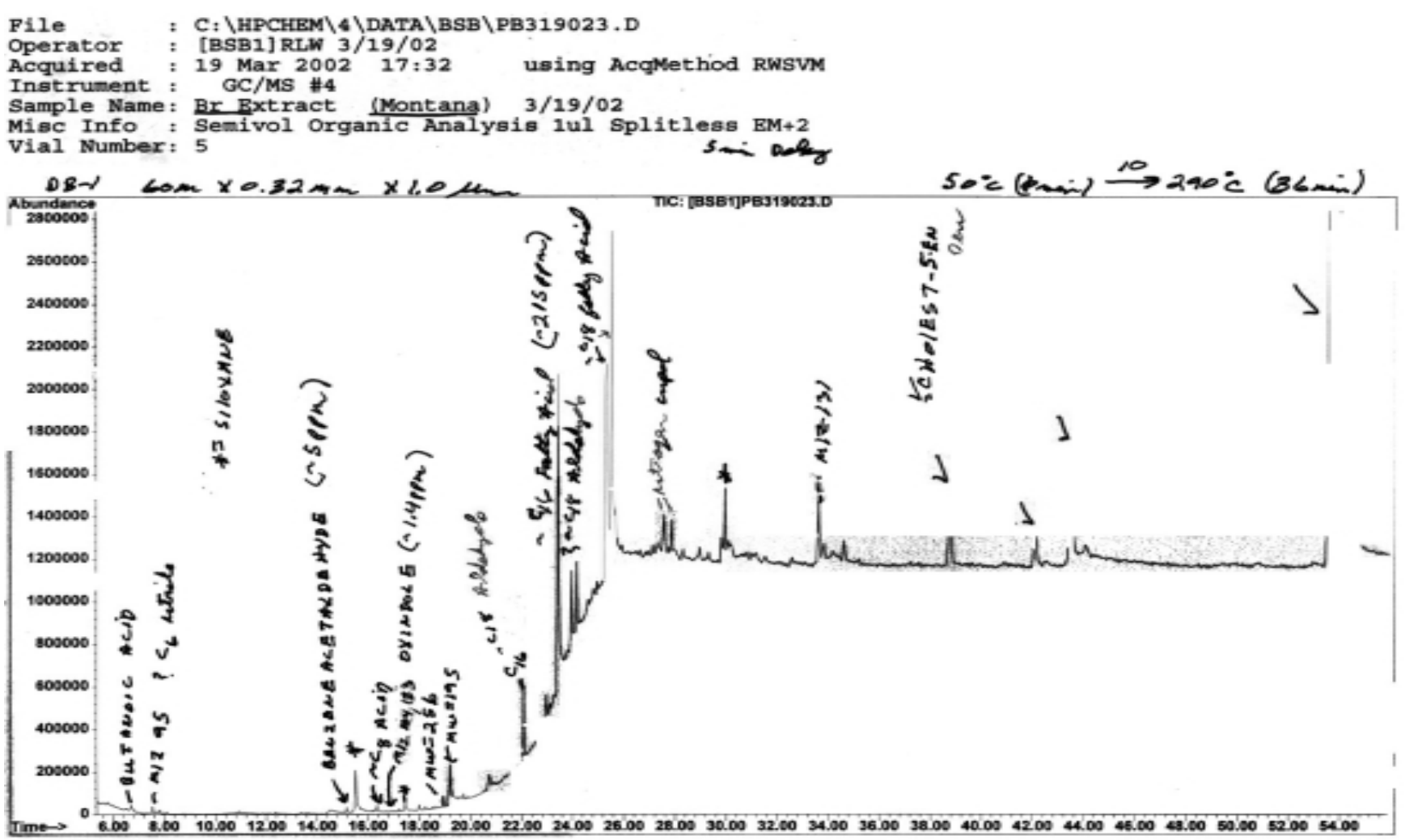

Figure 1. GC chromatogram of the methylene chloride extract from the brain tissue of the mutilated cow.

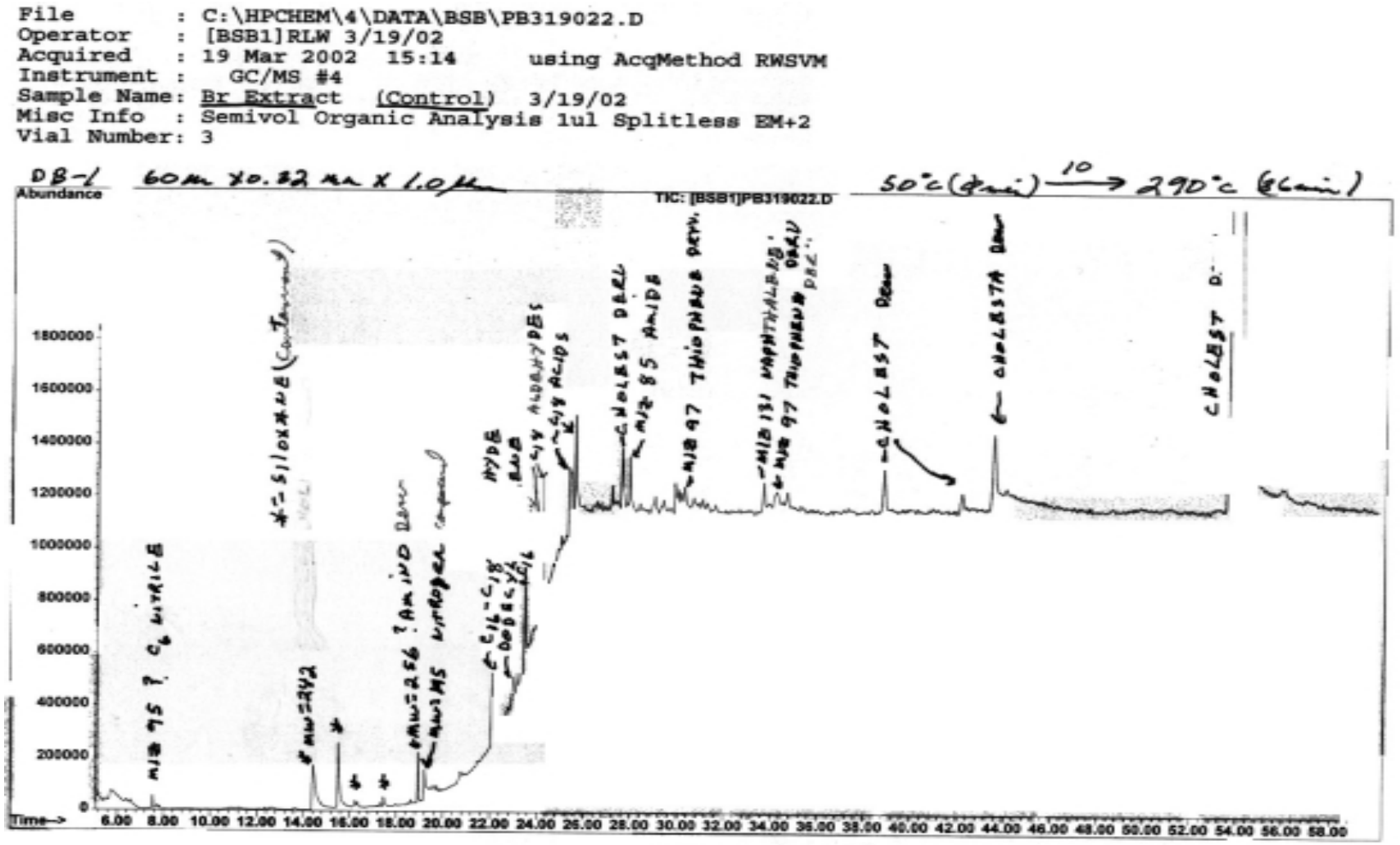

Figure 2. GC Chromatogram of the methylene chloride extract from the brain tissue of the control heifer.

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Operator    : RLW 3/19/02
Acquired    : 19 Mar 2002  17:32     using AcqMethod RWSVM
Instrument  :    GC/MS #4
Sample Name: Br Extract  (Montana)  3/19/02
Misc Info   : Semivol Organic Analysis 1ul Splitless EM+2
Vial Number: 5
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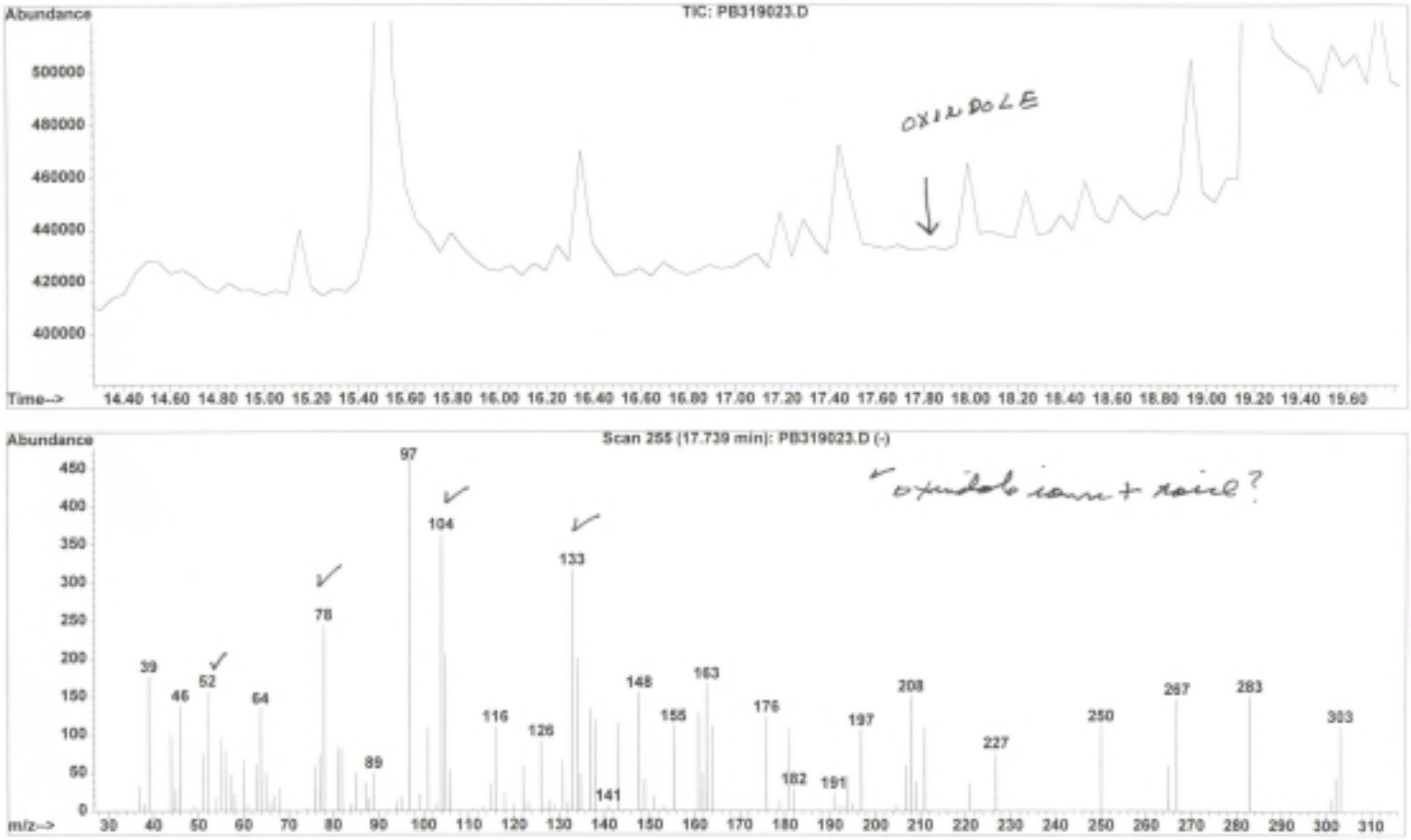

Figure 3. Expanded GC chromatogram and MS spectrum of oxindole from the brain tissue of the mutilated cow.

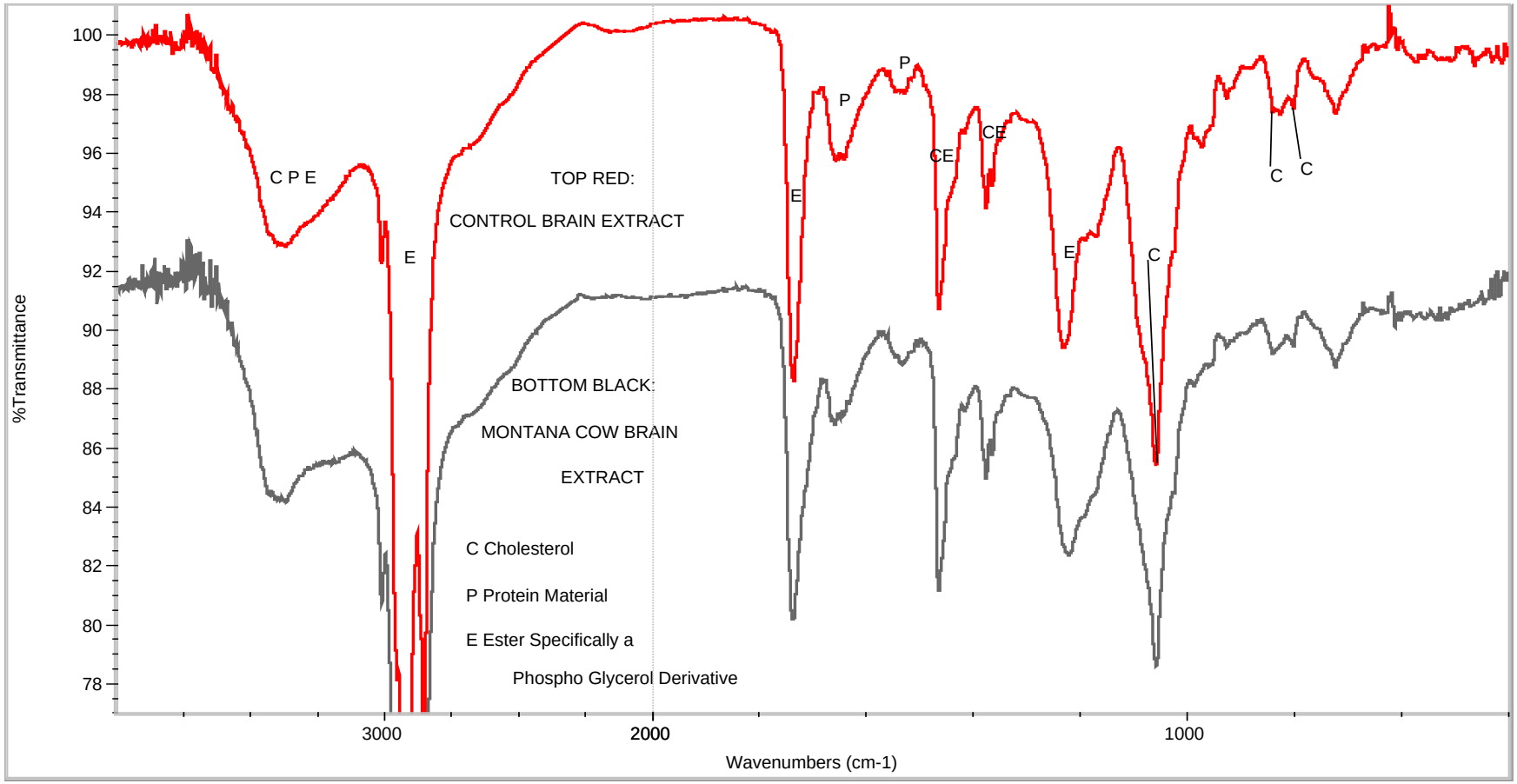

Figure 4. Infrared spectra of the methylene chloride extracts from the brains of the mutilated animal (top, red) and the control heifer (bottom, black).

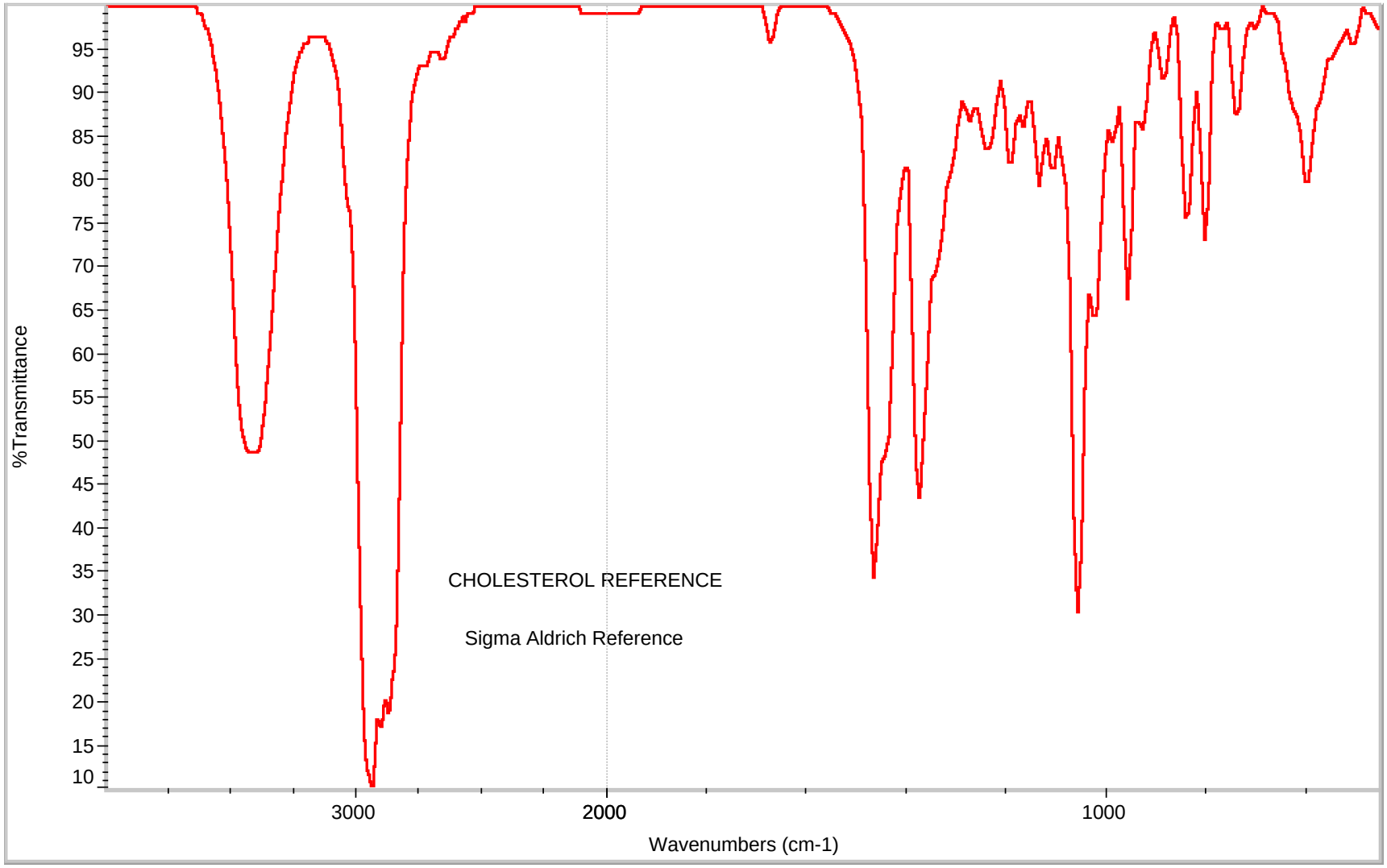

Figure 5. Infrared reference spectrum of cholesterol (Sigma-Aldrich).

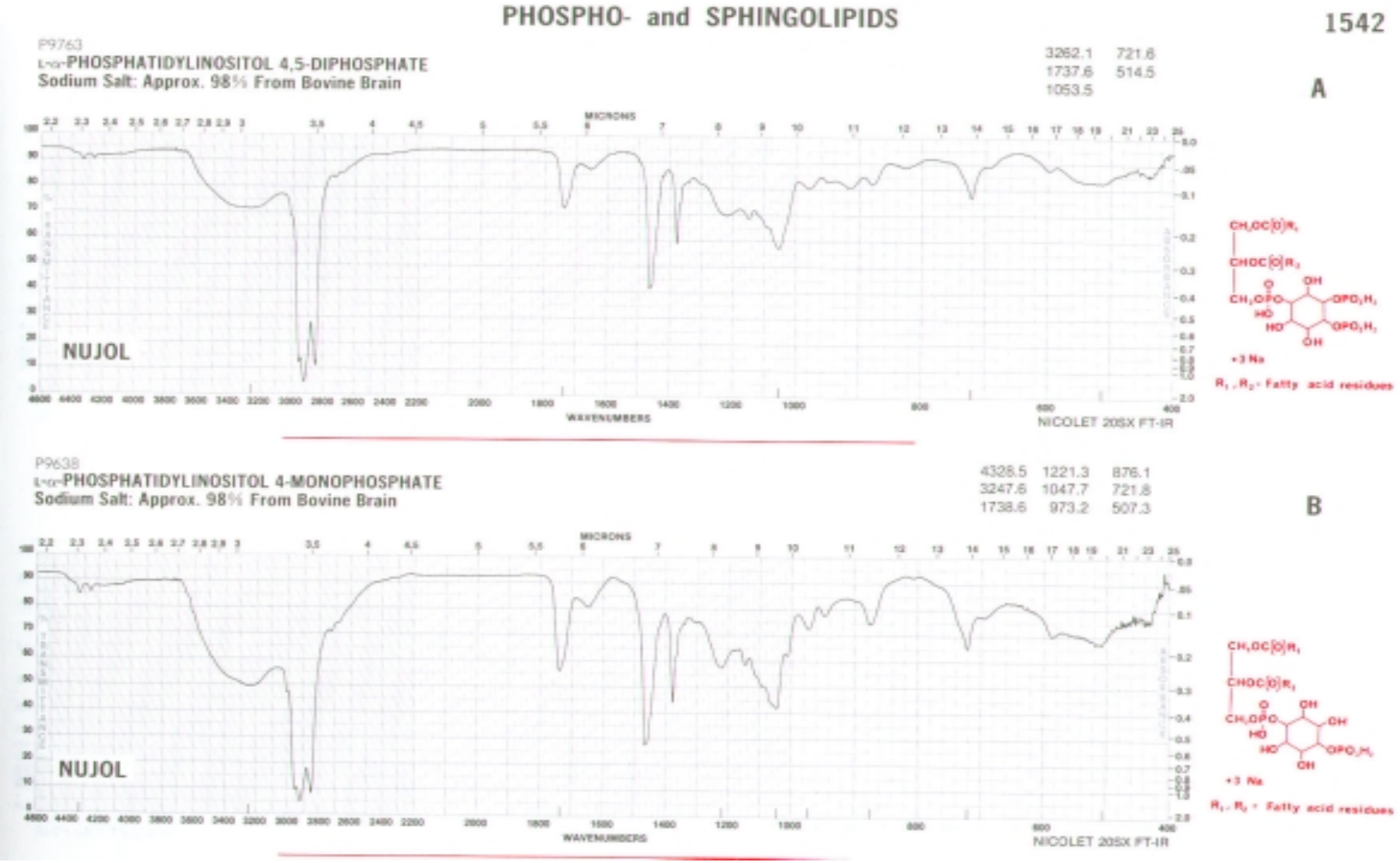

Figure 6. Infrared reference spectra of L-α-phosphatidylinositol, 4,5-diphosphate sodium salt and L-α-phosphatidylinositol, 4-monophosphate sodium salt (Sigma Aldrich).

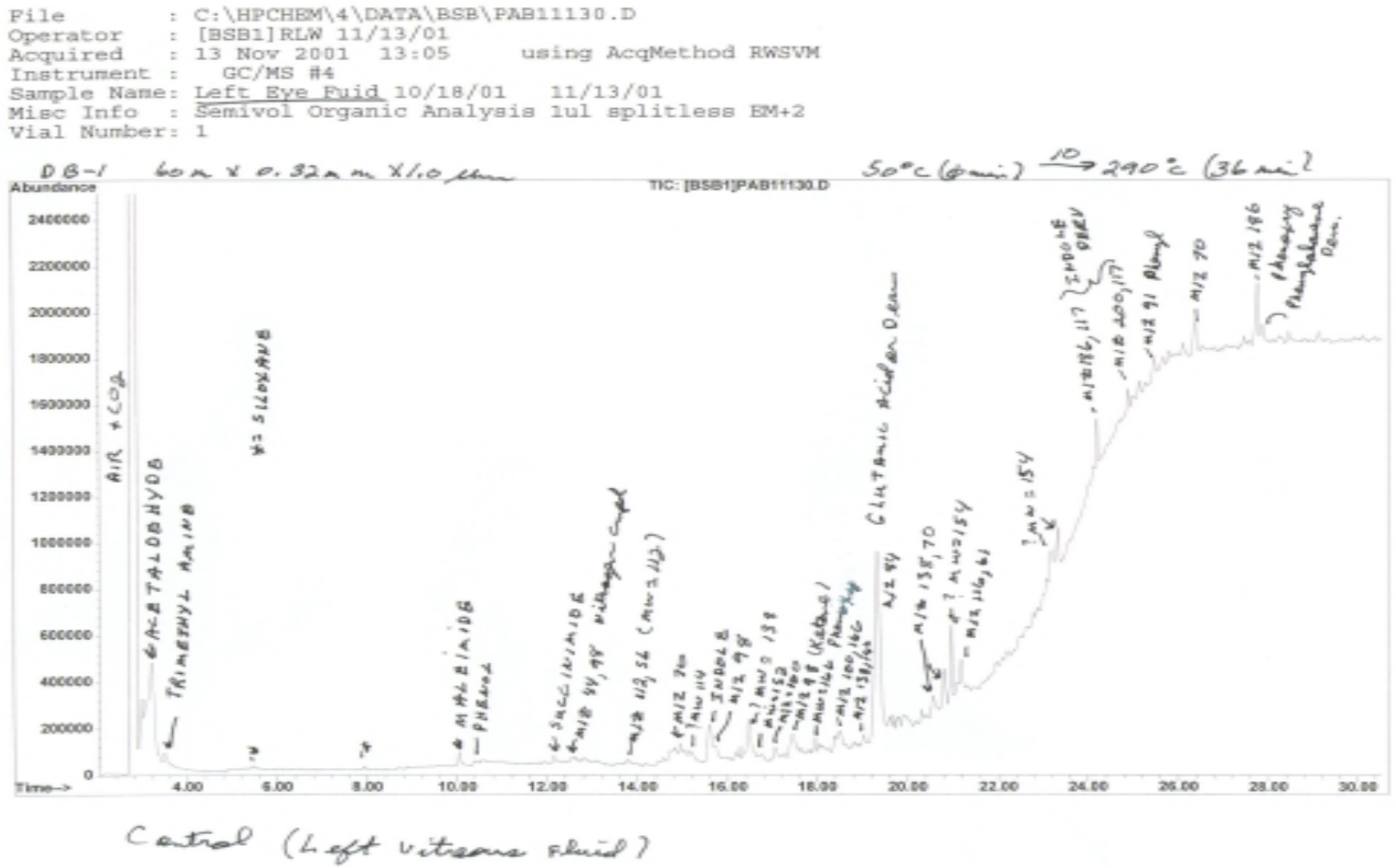

Figure 7. GC chromatogram of the vitreous fluid from the control heifer.

Figure 8. GC chromatogram of the vitreous fluid from the mutilated cow.