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Digestible Fat and Digestive Disorders in Beef Cattle

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INTRODUCTION

This study was involved with the investigation of the possible existence of toxic agents in certain fats used in animal diets. Based on previous field observations such fats in animal diets exhibit animal dietary disorders.

The research aims were:

- to use the Microtox® testing system for identifying “bad” fats
- to develop a method for the extraction and separation of components found in fat samples
- to extract all fat samples received from study sponsor and test both by extraction/separation technique and by Microtox® and investigate correlation between components found and field observations of “Bad Fat”
- to investigate the possibility to “screen” fats for acceptability
- to attempt the identification of the “bad” fat components

Background Information:

It is known that fat exposed to extreme conditions such as very high temperatures and oxidation for long time periods can cause severe irritation to the gastrointestinal tract, growth retardation, and death (1). Other studies have shown increased liver and kidney weights, cellular damage to various organs, and a change in fatty acid composition of tissue lipids with use of deteriorated fat (2). According to Dr. Del Miles the fat component of beef diets are critical. Dr. Miles has shown as much as 0.3% deaths may be related to added fat to the diet other than tallow. He has shown that when yellow grease was incorporated into beef diets a death loss or poor performance of animals occurred. However when this form of fat was removed from the diet and switched to bleached tallow death loss and poor animal performance subsided. Thereby a relationship between loss of animals/poor animal performance with a type of fat “bad fat” was established. This investigation involves correlation of 30+ feed lots with 100,000’s head of cattle (3).

In working with Dr. Miles, Dr. Henry M. Stahr from the Veterinary Diagnostic Lab at Iowa State University, investigated the use of the Mutatox® system to develop a test for “bad fat”. These are fats which are correlated to animal death loss/poor performance as stated earlier as opposed to ‘good or normal fats’ which consist of bleached tallow. The Mutatox® test when used with the “bad fats” showed a toxic effect on the microbes. This test uses a marine bacteria *Vibrio fischeri* but a dark strain or low light producer species of microbes. When this type of microbe is exposed to genotoxic substances, the microbe can be mutated from a microbe of a dark strain (low light producer) to a microbe produces more light than normal. The light is measured using the photomultiplier tube of a scintillation counter. This test may be performed in two ways: 1) by exposing the agent directly to the microbes or 2) by first exposing the agent to S-9 enzymes. The S-9 enzymes may be used to assimilate how an agent can be turned into a toxic substance by being metabolized by a mammalian. There are several types of mutations that may occur

to cause the toxic effect to the microbes. The types of mutation can be base substitution, frameshift, DNA synthesis inhibitors, DNA damaging agents, and intercalation agents. Due to the high cost of the Mutatox® test approximately \$300/sample and the numerous samples needing to be tested the Microtox® test, a similar test, was investigated as a lower cost substitute (4,5).

The Microtox® test is a non-specific toxicity test, which was developed for water monitoring for toxic wastes. This test is similar to the Mutatox® test in that it uses light producing microbes. However this test measures a reduction of light as an indicator of toxicity. The Microtox® test is accepted by both FDA and EPA to screen for toxicity. This test uses *Photobacterium phosphoreum* or *Vibrio fischeri* which are bioluminescent salt-water bacteria. These bacteria produce light as a byproduct of cellular respiration. Thereby if some agent interrupts their growth or this process a reduction of light production is seen. The bacteria are freeze dried and stored at -20°C and have shelf life of one year. The organisms are rehydrated or reconstituted to perform the bioassay. The bioassay measures the light output produced by the bacteria as a byproduct of cellular respiration. The toxic agents in varying concentrations are exposed to the bacteria and if a toxic effect is seen the light levels produced by the bacteria are decreased as compared to a control. There is a direct correlation between toxicity and light production. The more toxic a substance the more reduction of light (6,7). The Microtox® test was found to have fairly good correlation between 'bad fats' and the reduction of light. Whereas the 'good or normal fat' was shown not to effect the light production in the bacteria. Using fat samples provided by Dr. Miles, which were known to have caused death loss or poor performance in animals' correlation's between the Microtox® test and "bad fats" were noted. Seventy percent has been shown as the level at which concern for toxic agent is suggested. Fats which were less than 70% of light output were shown to be slightly toxic and those fats to register less than 50% were shown to be very toxic by the Microtox® test. With this tool we propose to investigate the toxic principle/s found in the "bad fats". This type of testing in earlier work was also used by Tom Sun, a graduate student of Dr. Henry M. Stahr. He looked at the toxic substances produced by heated, air-oxidized fats from cooking oil. These products were oxygenated, epoxides, peroxides, and hydroperoxides (8). These products could be fractionated using silica gel column chromatography and/or preparative normal phase TLC to investigate the toxic substances. These same techniques may be useful in determining the toxic properties of the "bad fats".

SIGNIFICANCE OF THE WORK

With beef producer deriving the least amount of profit possible, the death loss/poor performance in cattle from contaminated fat in the ration is unacceptable. The availability of knowledge of what causes the toxicity will allow detecting the contaminants and eliminating problems of beef cattle deaths.

The potential food safety problem is more a function of the animals which do not die but survive and may become food for unsuspecting humans. Once the compounds are identified and tests are developed for them, meat can be checked for them so that significant residues are eliminated from the food of animal origin.

METHODS

Collection of samples:

Fat samples were obtained from beef cattle feedlot operations experiencing digestive fat disorder. The samples were stored at -10°C.

Microtox testing:

Fat samples are melted to allow complete mixing so that aliquots may be removed. Fat sample is weighed and dissolved in a solvent. The fat sample is then added to vials in three concentrations with five replicates at each concentration. The solvent is removed. The bacteria is reconstituted and exposed to the fat in its growing media. The light production is measured at zero time and then at 15 minutes of exposure. A blank sample is used as 100% light produced and fat samples are compared to this to determine the percentage of light produced. A control sample of tallow is also analyzed for quality assurance of the test.

MICROTOX® M500 METHOD

The M500 Microtox® system is used to determine acute toxicity in aqueous samples by use of freeze-dried luminous living bacteria. This is a bioassay system designed to determine the toxicity of water, sediment, or soil samples.

Reagents and Apparatus

M500 Microtox® apparatus

Microtox® Cuvettes

Reconstitution solution: use 1 ml per vial of reagent.

Reagent: freeze-dried living bacteria stored at -20°C.(Azur Environmental)

Control: Diluent, use 1 ml for each vial of sample, store in refrigerator.

Pipetman 200 µl and 1000 µl with appropriate tips

water.

Prepare fat sample: weigh 10 mg of fat into a 10 ml volumetric flask on an analytical balance. Bring up volume with reagent grade methylene chloride (Fisher Scientific). The fat concentration is 1 $\mu\text{g}/\mu\text{l}$.

To the first row of cuvettes add 1 ml diluent, to subsequent rows add 1, 10, and 100 μl diluted fat sample and control fat sample. Remove methylene chloride solvent by blowing nitrogen into all cuvettes containing fat samples to evaporate solvent. Add 1 ml diluent to cuvettes after removing solvent and vortex. Then to each cuvette add 20 μl of bacteria and record time. Mix each cuvette using pasteur pipette. Read absorbance after 15 minute time for all cuvettes and record. Calculate averages for each group of samples read. Calculate a percent for each group based on control diluent being 100%.

Silica Gel Column Chromatography:

Silica gel 35-60 mesh was obtained from Fisher Scientific Co. The activity of the silica gel was controlled by storing the silica gel in a 125°C oven. A 2x15 cm glass fritted column was used. Approximately 4 inches of silica gel was used with a 2-inch upper layer of sodium sulfate. A 2 gm of fat was dissolved in 5 ml of diethyl ether (Fisher Scientific) and this was fractionated on the silica gel column into 5 fractions. The elution was done with increasing diethyl ether amounts in hexane (Fisher Scientific), such as 1:9, 3:7, 7:3, and 10:0 (diethyl ether:hexane, v:v). All elutions were 100 ml except for 1:9 was 200ml.

Thin Layer Chromatography:

A 0.12 gram fat sample was weighed and dissolved in 1.5 ml of methylene chloride. This was done for all fat samples. A 40 μl volume of each fat sample was chromatographed on 20x20 cm normal phase TLC plates with fluorescent indicator. The plates were developed in diethyl ether:hexane:acetic acid, 50:50:1, v:v:v. After development the plates were dried and viewed by UV long wave and the Rf values for the fluorescent bands were marked and recorded.

Gas Chromatography:

Gas chromatography (GC) was performed on a Varian 3600 equipped with a 30-meter DB 5 megabore column and a FID detector. The injector temperature was set at 240°C and the detector temperature was set at 300°C. The column initial temperature was set at 50°C and was held for 3 minutes and then ramped to 100°C at a rate of 5°C/min. and held for 5 minutes. The final temperature program 250°C at a rate of 20°C/min. and then held for 5.5 minutes. Samples and standards were injected manually in 1 μl amounts.

RESULTS AND DISCUSSION

Initially fat samples were fractionated by silica gel chromatography and four fractions were obtained. These were fraction 1 = diethyl ether:hexane, 1:9, fraction 2 = diethyl ether:hexane, 3:7, fraction 3 = diethyl ether:hexane, 7:3, and fraction 4 = diethyl ether:hexane, 10:0. All the fractions (approx. 10 µg/µl) were from silica gel were analyzed by the Microtox® test.

Table 1. Microtox® analysis of fraction 1- 4 from silica gel column.

**Amount of fat 10633
(10µg/µl)**

	Blank	Control	fraction 1	fraction 2	fraction 3	fraction 4
10 µl	100%	91%	91%	30%	2%	2%
5 µl	100%	88%	77%	44%	12%	20%
3 µl	100%	91%	88%	54%	30%	34%

Fraction 1 gave a value above 70% indicating no toxicity. However fractions 2, 3, and 4 were all below 50% indicating a toxic response. Fractions 3 and 4 seem to be the most toxic giving values at the 2% level for approximately 10 µg. These fractions were also developed on TLC and the Rf values are shown in Table 2.

Table 2. Rf values from a TLC plate of fractions 1, 2, 3, and 4 from silica gel chromatography, developed in diethyl ether:hexane:acetic acid, 50:50:1, (v:v:v).

Sample Rf Value

fraction 1	0.91
fraction 2	0.77 to 0.46
fraction 3	0.63 to 0.20
fraction 4	0.57 to 0.11

Fractions 2, 3, and 4 were diffuse broad bands which may indicate a mixture a compounds. Due to this finding all remaining fats were chromatographed directly on TLC after dissolved in methylene chloride. The results of the Microtox® testing and TLC work of all the fat samples are listed in Table 3., indicating the Microtox® response and the TLC Rf values for compound elucidated. Dependent on the Rf value the TLC band was given a letter designation from A through E to identify the TLC band. With band A having a Rf value of 0.93, band B approx. 0.61-0.71 Rf values, band C approx. 0.50-0.57 Rf values, band D approx. 0.43-0.46 Rf values, and band E approx. 0.32-0.39 Rf values.

Table 3. Results of Microtox® analysis of fat samples including the results of compounds found by TLC according to Rf values with letter designations.

SAMPLE ID	MICROTOX® RESULTS	Rf VALUES				
		A	B	C	D	E
34575	89%	0.93				
10633	56%	0.93		0.57	0.46	0.32
30238	41%	0.93	0.68		0.46	0.32
34313	63%	0.93	0.61		0.46	0.32
34314	67%	0.93			0.46	
34315	64%	0.93	0.64	0.54	0.46	
34316	62%	0.93	0.64	0.54	0.46	
34318	62%	0.93	0.64		0.46	
34319	59%	0.93	0.64	0.54	0.43	
22729	77%	0.93	0.61		0.46	0.32
22735	78%	0.93	0.64	0.54		0.39
23190	60%	0.93		0.57	0.46	0.32
23195	103%	0.93				
23200	100%	0.93				
23572	64%	0.93		0.57	0.46	0.32
23613	73%	0.93		0.57	0.46	0.32
22618	58%	0.89	0.61	0.54		0.39
22721	61%	0.89	0.61			0.39
23158	68%	0.89	0.61	0.54		0.39
23825	78%	0.89	0.64	0.54		0.39
15531	42%	0.96	0.64		0.46	
TALLOW	100%	0.93				
15494	54%	0.96	0.64		0.46	
GREASE	65%	0.93	0.61			0.32
39266	69%	0.93	0.64			0.36
94202	77%	0.93	0.61		0.46	
93622	75%	0.96	0.64	0.54	0.46	
94326	61%	0.96	0.64	0.54	0.46	
31055	58%	0.93		0.57	0.46	0.32
31056	64%	0.93	0.61		0.46	
11271	85%	0.93	0.64	0.54		0.39
11272	86%	0.93	0.64	0.54		0.39
11273	90%	0.93	0.71	0.54		0.39
11274	93%	0.89	0.68	0.50		0.36
12275	93%	0.89		0.50		0.36
12276	94%	0.89	0.68	0.50		0.36
10101	71%	0.93	0.68	0.54	0.43	
10631	61%	0.93	0.68	0.54	0.43	
11086	63%	0.93	0.68	0.54	0.43	

Continuation of **Table 3.**

SAMPLE ID	MICROTOX RESULTS	Rf VALUES				
		A	B	C	D	E
11219	59%	0.93				0.32
11221	82%	0.93				0.32
11222	60%	0.93	0.68			0.32
10620	103%	0.93	0.68	0.54		0.32
10737	97%	0.93	0.68	0.54		0.32
10814	102%	0.93	0.68	0.54		0.32
11084	73%	0.93		0.57	0.46	
11085	72%	0.93		0.57	0.46	
TRUCK	75%	0.93	0.68	0.57	0.46	
MIXER	73%	0.93	0.68	0.57	0.46	
GREASE	75%	0.93	0.61	0.54		0.36
NPB						
NPB38765	94%	0.93				0.36
NPB38791	70%	0.93	0.61			0.36

Figure 1. below shows a typical TLC plate development showing Bands A-E.

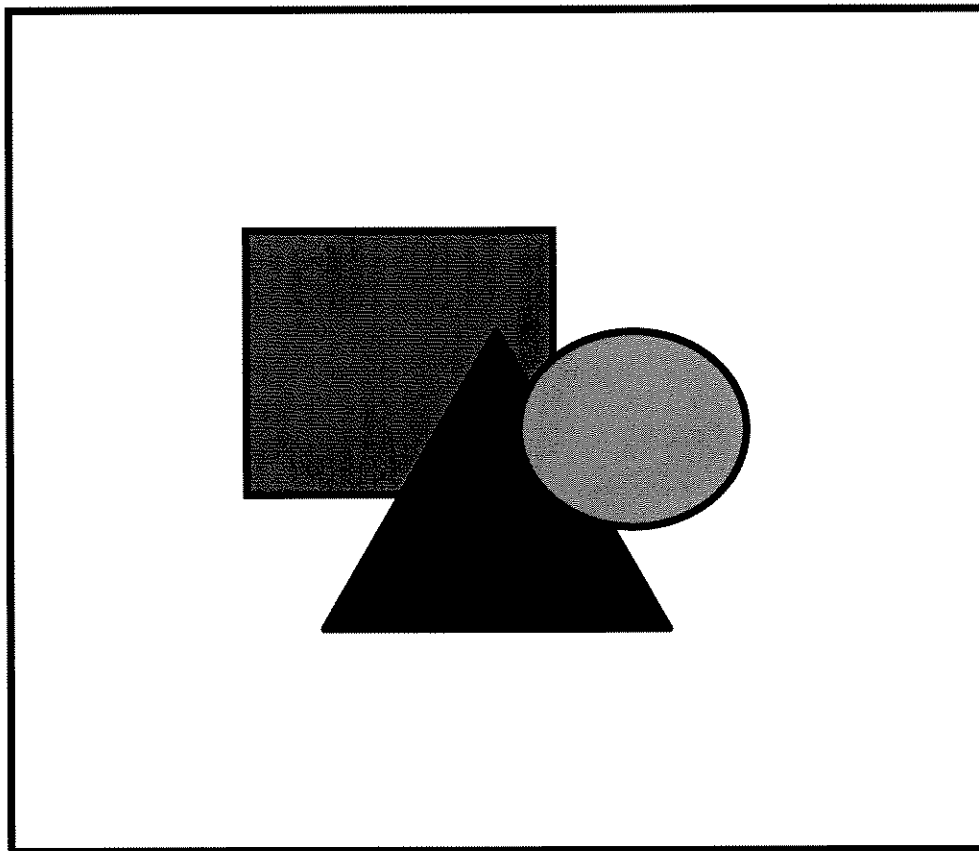


Figure 1. A picture of a typical TLC plate of fat samples developed in diethyl ether: hexane:acetic acid, 50:50:1 (v:v:v).

All fats including the tallow contain the TLC band at Rf value 0.93 labeled band A, this band would not be considered as contributing to the toxicity of the fat. Fats 30238 and 15531 gave a Microtox values of 41% and 42% respectively and were the most toxic by this analysis. These fats both contained TLC bands labeled B and D. Further investigation into TLC bands B and D is needed to determine if they contain the toxic factor. Fats 10633, 34319, 22618, 15494, 31055, 11219, and TCP were all below 60% on the Microtox® test. These may indicate that TLC bands labeled C and E may also show some toxicity but at a lesser degree.

Fats 30238 and 10633 were further investigated by spotting a 50 µg fraction on TLC and developed. The corresponding bands A, B, C, D, and E were scraped off TLC and eluted from the silica gel in methylene chloride. The solvent was removed under nitrogen and the bands were analyzed by the Microtox® test. The results are shown in Table 4.

Table 4. Bands A-E eluted from TLC and analyzed by Microtox® test.

Fat ID	Blank	TLC Blank	Band A	Band B	Band C	Band D	Band E
30238	100%	93%	93%	35%		59%	44%
10633	100%	100%	92%		30%	58%	64%

This data shows that Band A is definitely not toxic based on the Microtox® test. However Bands B and C indicate the most toxicity with Bands D and E being slightly less toxic based on the Microtox® findings.

In an effort to identify the TLC bands another set (Bands A-E) were prepared as above and removed from the TLC plate and then these bands were diluted into a small volume of methylene chloride and analyzed by gas chromatography. The gas chromatograph retention time data from this analysis is listed in Table 5.

Table 5. Retention times and area counts of TLC bands A-E from two fat samples along with several fatty acid standards.

Sample ID	Amount injected (micrograms)	Retention time (minutes)	Area (counts)
linoleic acid	3	6.8	422
oleic acid	3	6.84	373
stearic acid	2	6.91	81
30238 - A	15	5.84	368
		6.63	975
30238 - B	25	6.97	922
30238 - C			
30238 - D	22	6.78	135
		8.22	180
		9.1	462
30238 - E	11.5	6.8	55
		8.21	63
		9.04	219
10633 - A	25	5.81	462
		6.61	1459
10633 - B			
10633 - C	25	6.65	25
		8.38	10
		9.21	44
10633 - D	13	6.92	32
		8.32	24
		9.21	231
10633 - E	18	6.91	67
		8.34	116
		9.26	205

Preliminary investigation using gas chromatography revealed that the major peaks present in TLC bands did not match the known fatty acids such as stearic, linoleic, or oleic, which are commonly found in fats and oils used in the restaurant business. Several peaks were noted resulting from the individual bands being injected onto GC. This may be due to the fact that the individual TLC bands represent a mixture of compounds and may not be pure compounds. Another reason for the many peaks by gas chromatography could be due to a breakdown of compounds caused by introduction into the gas chromatograph due to thermal lability.

CONCLUSION

The Microtox® test was used on a number of fat samples collected that were related to death loss or poor animal performance. Initially these fat samples were fractionated on silica gel using column chromatography to obtain the polar fractions which from the literature were shown to be toxic.(9,10) The separation of the fat components by column chromatography was shown by TLC to be not acceptable and abandoned for another approach. The fat components were separated more favorably by TLC and therefore all the fat samples were applied to this method. The results of the TLC revealed that five different TLC bands were observed with Rf values of approximately 0.93 called band A, 0.64 called band B, 0.54 called band C, 0.46 called band D, and 0.32 called band E. These subcomponents of several of the fat samples were isolated from TLC and subjected to the Microtox® test. The results of this showed band A not to be toxic by the Microtox® test. However bands B and C were considered to be very toxic by this test giving values below a 50% reading. Bands D and E were less toxic giving values less than 70% but greater than 50%. Below 70% of light output was correlated with the Microtox® test and the 'bad fats' from the study of Dr. Miles.

Further investigation of these TLC bands using gas chromatography was done. Several known fatty acid standards (oleic, linoleic, and stearic acids) were injected along with the isolated TLC bands A-E. The retention times of the unknowns did not match those of the known standards, thereby eliminating but not identifying the bands. For each TLC band injected several chromatographic peaks resulted. This result would indicate that the TLC bands are not pure entities or thermal lability occurred in the injection port causing the compounds to breakdown before elution off the GC column. Suggested future work should include the isolation of larger quantities of these separated toxic fat components. Then further investigation of identification by mass spectrometry or other techniques can be used to further determine what type of compounds are in toxic components of fat.

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