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Enzymic and Nonenzymic Factors Affecting Lipid Peroxidation in Raw Beef Muscles

Ki Soon Rhee,* Steven C. Seideman, and H. Russell Cross

The longissimus dorsi, psoas major, semimembranosus, and semitendinosus beef muscles from steers and bulls were analyzed for ether-extractable fat, myoglobin, and nonheme iron content, microsomal enzymic lipid peroxidation activity, fiber type profile, and other properties. Each muscle was also ground and stored at 4 °C for 0 and 7 days to monitor lipid peroxidation by the thiobarbituric acid (TBA) test. For muscles from steers, microsomal enzymic lipid peroxidation activity was positively correlated with intermediate fiber number, but inversely related to ether-extractable fat content and red fiber number; for muscles from bulls, it was positively correlated with nonheme iron content, but inversely related to white fiber number. While TBA values of refrigerated, ground muscles were correlated with microsomal lipid peroxidation activity for muscles from steers, they were correlated with total pigment and myoglobin content for muscles from bulls.

INTRODUCTION

The quality deterioration of meat and meat products through lipid peroxidation is of major concern at the present time because of the increased use of precooked or convenience meat items by the food service industry and in the home. Although cooked meat is more susceptible to lipid peroxidation than uncooked meat, oxidative changes in lipids can become a serious problem for uncooked meat when it is subjected to size reduction (grinding, flaking, chunking), freeze-thawing, temperature abuses in handling and distribution, and/or prolonged storage. Also, due to the free-radical chain reaction nature of lipid peroxidation, any degree of the oxidation occurring in raw meat materials can accelerate the development of "warmed-over" flavor (the oxidized flavor) in stored, cooked meat.

The mechanisms of lipid peroxidation in cooked meat have been studied in different laboratories, with no consensus in regard to the relative role of heme iron vs. nonheme iron as the catalyst most responsible for the oxidation (Igene et al., 1979; Kwoh, 1971; Love and Pearson, 1974; Younathan and Watts, 1959). Less effort has been directed toward investigating the nature of lipid peroxidation in raw meat. However, there have been some studies implicating the meat pigment myoglobin (heme iron) as playing a direct role in lipid peroxidation in raw meat (Govindarajan et al., 1977; Greene, 1969; Hutchins et al., 1967; Verma et al., 1984). Moreover, because the oxidized meat pigment (metmyoglobin) can catalyze the peroxidation of unsaturated fatty acids in model systems (Kendrick and Watts, 1969; Kwoh, 1971; Lee et al., 1975; Rhee, 1978a) and because the extent of lipid peroxidation is highly correlated with the degree of discoloration in raw meat products (Rhee et al., 1983, 1985b), one may readily assume that heme iron catalysis can indeed play an important role in lipid peroxidation occurring in uncooked meat and meat products. In spite of the observed correlation between the two oxidative changes, it has not been directly proven that the oxidation of heme pigments causes or initiates lipid peroxidation in raw meats. Liu (1970a,b) determined the effects of pH and additives on linoleate oxidation catalyzed by metmyoglobin, a nonheme iron chelate (Fe^{2+}–EDTA), and beef homogenate and concluded, on the basis of responses to additives and pH, that the catalytic activity of beef homogenate was due to both heme iron and nonheme iron.

While lipid peroxidation in red meats generally has been regarded as a nonenzymic reaction (i.e., the reaction primarily catalyzed by nonheme iron or heme iron, or by both), our recent studies have shown the presence of an enzymic lipid peroxidation system associated with beef muscle microsomes (Rhee et al., 1984), with beef (steer) trapezius muscles having a higher activity per milligram of microsomal protein than beef (steer) longissimus dorsi muscles (Rhee et al., 1985a). The present study was conducted to determine nonenzymic and enzymic factors influencing lipid peroxidation in several different beef muscles from steers and bulls and possible interrelations among them. The factors determined in this study include

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microsomal enzymic lipid peroxidation activity, muscle fiber type profile, and levels of total pigment, myoglobin, total iron, nonheme iron and ether-extractable fat. Muscle fiber profile was determined because lipid metabolism, oxidative metabolism, and the level of myoglobin are higher in red fibers of mammals than in white fibers (Briskey et al., 1970).

MATERIALS AND METHODS

Materials. Four Angus bulls (275–332 kg hot carcass weight) and four Angus steers (259–289 kg hot carcass weight) were slaughtered at the Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE. The carcasses were of Low Choice or Good quality grade. Animals were approximately 15 months of age and had been on an 84% total digestible nutrient corn (International Feed Number, IFN, 4-02-931) –corn silage (IFN 3-08-153) diet supplemented with soybean meal (IFN 5-04-604) and minerals for 5 months prior to slaughter. The longissimus dorsi (4–6th lumbar region: LD), psoas major (center portion: PM), semimembranosus (center portion: SM), and semitendinosus (center portion: ST) were removed from each carcass at 24-h postmortem. All muscles were trimmed of outside fat and epimysium. Each muscle sample weighed approximately 250 g. Two 1 × 1 × 10 cm samples were removed from the center of each muscle sample, frozen in liquid nitrogen, and stored at −40 °C for use in muscle fiber typing. The remainder of each muscle sample (>200 g) was divided into half, frozen in liquid nitrogen, packaged with dry ice, transported to the Texas A&M University Meats and Muscle Biology Laboratory, College Station, TX, and then stored at −20 °C for a maximum period of 1 month for microsomal enzymic lipid peroxidation assay and up to 4 months for assays of nonenzymic factors. Since we previously found that microsomal enzymic lipid peroxidation activity decreased in beef muscles even during the frozen storage (Rhee et al., 1984), experiments were so scheduled that any effect due to different storage lengths would be reflected in differences between animals, not in differences between muscles of an animal. On a given day for an assay, subsamples of the four different muscles (LD, PM, SM, ST) from each animal were simultaneously taken out of the freezer and used immediately.

Muscle Fiber Typing. Transverse frozen (tempered to −20 °C) sections (10 μm) were mounted on glass slides and stained for alkaline (pH 9.4) ATPase (Guth and Samaha, 1970) and succinate dehydrogenase (Troyer, 1980). Fibers possessing ATPase activity were classified according to the nomenclature of Ashmore and Doerr (1971)—dark-staining fibers as white (α-white), intermediate-staining fibers as intermediate (α-red), and light-staining fibers as red (β-red). Fiber type classification by ATPase staining was confirmed by succinate dehydrogenase staining.

Photomicrographs were taken with a Zeiss inverted microscope (Model IM 35) with a built-in camera using a 4× objective. Photomicrographs were enlarged to allow a minimum of 100 fibers to be counted. Fiber types, as differentiated by stains, were traced with a Bioquant particle sizer to measure the cross-sectional area of each fiber.

Microsomal Lipid Peroxidation Activity. Procedures used for preparation of microsomes and determination of microsomal protein and lipid peroxidation were those described in a previous study (Rhee et al., 1984), except that pH of the reaction mixture for microsomal lipid peroxidation was 5.5, a common ultimate pH for postmortem beef muscles. The procedure for preparation of microsomes (Rhee et al., 1984) included a step of washing the initial crude microsomal fraction to remove contaminating myofibrillar proteins, which increased lipid peroxidation activity per milligram of protein by more than 50% for beef muscle microsomes. The reaction mixtures (4 mL) contained 0.27–0.58 mg of microsomal protein/mL, 0.20 mM NADPH, 0.2 mM ADP, and 0.015 mM $FeCl_3$ in 0.12 M KCl–5 mM histidine buffer (pH 5.5), and were incubated at 36 °C for 30 min. Microsomal lipid peroxidation activity was expressed as nanomoles of malonaldehyde/milligram of microsomal protein.

Total Pigment, Myoglobin, Total Iron, and Nonheme Iron Assays. Total pigment and myoglobin concentrations were determined by the procedures described by Rickansrud and Henrickson (1967).

For total iron determination, 10-g samples were oven-dried and wet-ashed with concentrated HNO_3 and HCl, and iron concentrations of the samples were determined with a Varian Model AA6 atomic absorption spectrophotometer. Nonheme iron content was determined by the method of Schricker et al. (1982).

Determination of Overall Lipid Peroxidation. Subsamples of the four different muscles from each of the eight animals were separately ground through a plate with 4.76-mm (diameter) holes and mixed with 30 ppm chlortetracycline to inhibit microbial growth. Twenty-gram portions of each ground muscle sample were placed in sterile Petri dishes (8.75-cm diameter), and the meat surface was made even by pressing with the hand with a sterile glove. The Petri dishes were then covered with Saran wrap and stored in a household refrigerator (4 °C) for 0 or 7 days (one Petri dish/muscle sample per storage period).

The extent of lipid peroxidation in stored, ground muscle sample was determined by the thiobarbituric acid (TBA) procedure as described by Rhee (1978b) using antioxidants (propyl gallate, EDTA) during the blending step to protect the meat from further lipid peroxidation that might occur during the assay. The antibiotic chlortetracycline present in meat samples and the antioxidants used in blending of samples did not interfere with the chromophore development of the distillates of meat samples with the TBA reagent. Results were expressed as TBA number (milligrams of malonaldehyde/kilogram of sample).

Fat, Moisture, and pH Determinations. Immediately before a muscle subsample was used for preparation of microsomes, three pH readings per sample were made on an Orion needle combination electrode (Model No. 916300) and an Orion pH meter (Model No. 611), and the mean was computed for each sample.

Fat and moisture contents were determined by the AOAC (1980) procedures, using the ground muscles prepared for determination of overall lipid peroxidation.

Statistical Analysis. The statistical model used was a split plot design. Main plots were represented by sex classes (steer, bull) and subplots were represented by muscles (LD, PM, SM, ST). Analysis of variance was used to test main effects (sex class, muscle) and interaction between sex class and muscle (SAS Institute, Inc., 1982). Means for main effects were separated by the Newman–Keuls' test (Steel and Torrie, 1980). When interaction between sex class and muscle was significant, the interaction mean square was used in test for main effects.

The correlation coefficients among variables were computed for each sex class.

RESULTS AND DISCUSSION

Various muscle properties for each of the different

Table I. Physicochemical, Biochemical, and Histological Properties of Beef Muscles according to Muscle Groups

variable	muscle[a] LD	PM	SM	ST
pH	5.40[e]	5.48[d]	5.41[e]	5.44[d,e]
ether-extractable fat, %	5.24[d]	4.95[d,e]	3.82[e,f]	3.01[f]
moisture, %	72.50[e]	73.89[d]	74.03[d]	74.58[d]
total pigment, mg/g	3.27[d,e]	3.25[d,e]	3.65[d]	2.82[e]
myoglobin, mg/g	2.83[d,e]	2.74[d,e]	3.18[d]	2.35[e]
total iron, μg/g	18.64[d]	21.83[d]	20.59[d]	18.99[d]
nonheme iron, μg/g	5.57[d]	5.91[d]	6.18[d]	5.79[d]
microsomal lipid peroxidation activity[b]	4.61[d]	7.49[d]	10.21[d]	7.76[d]
percentage of fiber number				
red fiber	21.4[e]	33.5[d]	18.2[e]	17.5[e]
intermediate fiber	18.5[e]	18.0[e]	26.9[d]	22.1[e]
white fiber	60.5[d]	48.6[e]	54.0[d,e]	60.6[d]
fiber areas (m^2)				
red fiber area, area/fiber	2161[d]	902[e]	2147[d]	2538[d]
intermediate fiber area, area/fiber	2235[d,e]	862[f]	1808[e]	2603[d]
white fiber area, area/fiber	3726[d,e]	1729[f]	3543[e]	4437[d]
percentage of fiber area				
total red fiber area[c]	14.5[e]	23.9[d]	13.6[e]	11.9[e]
total intermediate fiber area[b]	13.9[d]	12.1[d]	18.4[d]	15.8[d]
total white fiber area[b]	72.2[d]	64.5[d]	68.0[d]	72.3[d]
TBA no. of ground muscle stored at 4 °C				
day 0	1.12[e]	1.34[d,e]	1.75[d]	1.28[d,e]
day 7	11.43[e]	13.66[d]	14.43[d]	11.88[e]

[a] LD, longissimus dorsi; PM, psoas major; SM, semimembranosus; ST; semitendinosus. [b] Interaction between sex class and muscle was significant for the variable, and therefore, the interaction mean square was used in test for the muscle effect. [c] [(Red fiber no. × red fiber area)/((red fiber no. × red fiber area) + (intermed fiber no. × intermed fiber area) + (white fiber no. × white fiber area))] × 100. [d-f] Means within the same row followed by a common superscript letter are not different ($P > 0.05$).

Table II. Physicochemical, Biochemical, and Histological Properties of Beef Muscles according to Sex Classes

variable	sex class bull	steer
pH	5.42[c]	5.45[c]
ether-extractable fat, %	4.00[c]	4.50[c]
moisture, %	73.90[c]	73.60[c]
total pigment, mg/g	3.24[c]	3.25[c]
myoglobin, mg/g	2.64[c]	2.92[c]
total iron, μg/g	20.32[c]	19.70[c]
nonheme iron, μg/g	5.81[c]	5.92[c]
microsomal lipid peroxidation activity[a]	8.75[c]	6.28[c]
percentage of fiber number		
red fiber	20.4[d]	24.9[c]
intermediate fiber	24.0[c]	18.5[d]
white fiber	55.7[c]	56.2[c]
fiber areas (m^2)		
red fiber area, area/fiber	2036[c]	1838[c]
intermediate fiber area, area/fiber	2001[c]	1754[c]
white fiber area, area/fiber	3375[c]	3338[c]
percentage of fiber area		
total red fiber area[b]	14.8[c]	17.2[c]
total intermediate fiber area[a]	17.4[c]	12.4[c]
total white fiber area[a]	67.9[c]	70.4[c]
TBA no. of ground muscle stored at 4 °C		
day 0	1.38[c]	1.37[c]
day 7	12.12[d]	13.57[c]

[a] Interaction between sex class and muscle was significant for the variable, and therefore, the interaction mean square was used in test for the sex class effect. [b] [(Red fiber no. × red fiber area)/((red fiber no. × red fiber area) + (intermed fiber no. × intermed fiber area) + (white fiber no. × white fiber area))] × 100. [c,d] Means within the same row followed by the same superscript letter are not different ($P > 0.05$).

muscles are presented in Table I. The PM tended to have higher pH values than other muscles. It has been reported that lipid peroxidation of meat animal tissues decreases with increasing pH (Chen and Waimaleongora-Ek, 1981; Watts and Peng, 1947; Yasosky et al., 1984). In the present study, any inhibitory effect of a slightly higher pH (at most, a pH difference of 0.08) for PM muscles might have been marginal and overshadowed by other prooxidative factors for this muscle, as will be discussed later. The ether-extractable fat content was higher for the LD and PM than for the ST (the SM fat content was intermediate between the PM and ST); the order among LD, PM, and ST muscles was similar to that of the total lipid content for these beef muscles as reported by Hornstein et al. (1967). The lipid extracted by ether from beef muscles is largely marbling fat (mostly triglycerides); phospholipids, which are more unsaturated than triglycerides and thus are primarily responsible for oxidative rancidity development in meats, require a more polar solvent or solvent system. The percentage of phospholipids has been reported to be higher for PM muscles than for the LD (Hornstein et al., 1967).

Total pigment and myoglobin content tended to be highest for SM muscles and lowest for the ST. In terms of the relative order among different muscles, the total pigment and myoglobin data (Table I) are similar to the findings of Hunt and Hedrick (1977), but much different from the results reported by Rickansrud and Henrickson (1967) who found the LD having much more myoglobin than the PM (3.18 mg/g for LD vs. 2.40 mg/g for PM).

Neither total iron nor nonheme iron content was significantly different among different muscles. The amounts of total iron and nonheme iron in beef skeletal muscle have been determined in many studies, but no report has dealt with differences in nonheme iron content among different beef muscles. Reported mean values for the nonheme iron content of beef LD muscles (raw) include 8.4 μg/g or 35.9% of total iron (Schricker et al., 1982) and 1.80 μg/g or 8.7% of total iron (Igene et al., 1979). In the present study, the mean nonheme iron content of LD muscles was 5.57 μg/g or 29.9% of total iron. Reported nonheme iron values for other beef muscles are 1.00 μg/g or 4.2% of total iron for the SM (Sato and Hegarty, 1971) vs. 6.18 μg/g or 30.0% of total iron for the same muscle in the present study and 1.31 μg/g or 6.2% of total iron for the ST (Chen et al., 1984) vs. 5.79 μg/g or 30.5% of total iron for the same muscle in the present study. Examination of the various studies revealed that differences between the two groups of nonheme iron data (i.e., less than 2 μg/g vs. more than 5 μg/g) were due to different assay methods used.

Microsomal enzymic lipid peroxidation activity was not statistically different among the four beef muscles in spite of a rather large numerical difference between some muscles. A significant interaction between sex class and muscle was found in analysis of variance for this variable. When data were analyzed according to sex classes, the SM had the highest activity and the LD had the lowest activity for steers whereas differences among muscles were not significant for bulls (data not shown). Rhee et al. (1985a) reported that microsomes from beef steer trapezius muscles had a higher lipid peroxidation activity than microsomes from beef steer LD muscles.

The number of red fibers was highest for the PM, which also had the lowest number of white fibers (Table I). This is in agreement with the findings of Hunt and Hedrick (1977). The number of intermediate fibers was higher for SM and ST muscles than for LD and PM muscles; Hunt and Hedrick (1977) reported the PM had the lowest percentage of intermediate fibers among the four beef muscles. The percentage of total area (computed from information

Table III. Correlation Coefficients among Variables

variables showing signif correln ($P < 0.10$)			steer muscles: correln coeff[a]	steer muscles: statistical signif	bull muscles: correln coeff[a]	bull muscles: statistical signif
microsomal lipid peroxidation activity	vs.	ether-extractable fat	−0.57	<0.05	NS	
		nonheme iron content	NS		0.57	<0.05
		red fiber number	−0.42	0.10	NS	
		intermediate fiber number	0.62	0.01	NS	
		white fiber number	NS		−0.45	<0.10
ether-extractable fat	vs.	total pigment content	0.54	<0.05	NS	
		myoglobin content	0.56	<0.05	NS	
		red fiber number	0.45	<0.10	0.45	<0.10
		intermediate fiber number	−0.42	0.10	−0.43	0.10
		total red fiber area	NS		0.48	<0.10
TBA value of refrigerated, ground muscle at day 0	vs.	total pigment content	NS		0.54	<0.05
		myoglobin content	NS		0.47	<0.10
		microsomal lipid perox act.	0.47	<0.10	NS	
TBA value of refrigerated, ground muscle at day 7	vs.	total pigment content	NS		0.64	<0.01
		myoglobin content	NS		0.52	<0.05
		microsomal lipid perox act.	0.51	<0.05	NS	

[a] NS: no significant correlation ($P > 0.10$).

on the number and area; see the footnote in Table I) of red fibers was higher for the PM than for the other three muscles; that of intermediate or white fibers was not significantly different among the four muscles. When muscles were ground and stored at 4 °C, TBA values at day 0 tended to be higher for the SM compared with the other three muscles (Table I). However, at day 7, TBA values of both SM and PM muscles were significantly higher than those of LD and ST muscles. Cursory observations indicated that SM and PM muscles were much more discolored than the LD and ST on the 7th day of storage at 4 °C.

Properties of the beef muscles according to sex classes (bulls vs. steers) are shown in Table II. The muscle properties that were different between bulls and steers were the number of red fibers (greater for steers), the number of intermediate fibers (greater for bulls), and TBA values at day 7 of ground muscles stored at 4 °C (greater for steers). Since only four animals were used for each sex class, any differences found in this study between bulls and steers need to be confirmed by further research.

The correlation coefficients among variables showing significant correlation with each other are shown in Table III for each sex class. For muscles from steers, microsomal lipid peroxidation activity was most highly correlated with intermediate fiber number; an inverse relationship was found between microsomal lipid peroxidation activity and red fiber number or ether-extractable fat content. For muscles from bulls, microsomal lipid peroxidation activity was correlated with nonheme iron content and inversely related to white fiber number. Ether-extractable fat content was correlated with total pigment content, myoglobin content, and red fiber number for the steer muscles; however, it was correlated with red fiber number and total red fiber area for the bull muscles. For both steer and bull muscles, ether-extractable fat content was inversely related to intermediate fiber number. While TBA values at day 0 or day 7 were correlated with microsomal lipid peroxidation activity for muscles from steers, they were correlated with total pigment and myoglobin content for muscles from bulls.

The finding that, for the steer muscles, microsomal lipid peroxidation activity was positively correlated with the intermediate fiber number and negatively correlated with the red fiber number was rather unexpected. It was anticipated that the red fiber number would be positively related to the lipid peroxidation activity because lipid metabolism and oxidative metabolism are known to be higher in red fibers than in white fibers, and intermediate fibers are intermediate between red and white fibers in terms of lipid and oxidative metabolism (Briskey et al., 1970). It is possible that lipid peroxidation, which is not one of the typical biochemical reactions involved in lipid metabolism, may have a different association in regard to different fiber types. Further research is needed on this subject.

Although differences were found between the steer and bull muscles, the correlation data seem to indicate that microsomal enzymic lipid peroxidation activity (as shown by the steer muscle correlation) and heme pigments (as shown by the bull muscle correlation) may play important roles in production of TBA-reactive substances in beef muscles in general. Further studies are in progress for assessment of the roles of microsomal lipid peroxidation system, meat pigment (heme iron), and nonheme iron in beef muscle lipid peroxidation.

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Rapid Separation and Identification of Urinary Metabolites of Zeranol by HPLC–UV Spectrophotometry

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Bovine urinary metabolites of zeranol (1), a growth stimulant, have been separated and identified by HPLC–UV spectrophotometry. Both free and conjugated zeranol (1) and taleranol (3), a C-7 epimer of zeranol, were readily separated and identified in the urine samples collected from a bull that received zeranol intramuscularly or intravenously. Only a trace amount of zearalanone (2), an oxidation product of zeranol (1) and reportedly a major metabolite in many animal species, has been found. On the other hand, none was found in the urine samples collected from a bull that received zeranol intraperitoneally. Ethyl acetate extracts of urine samples, following with or without β-glucuronidase treatment and silica gel column or TLC cleanup, were analyzed by GC–MS and HPLC. Zeranol (1) and taleranol (3) were indistinguishable by GC–MS when analyzed as trimethylsilylates. These two epimers were readily separated and identified by HPLC–UV spectrophotometry.

INTRODUCTION

Zeranol (1,7α-zearalanol or [35-(3*R**,7*S**)]-3,4,5,6,7,8,9,10,11,12-decahydro-7,14,16-trihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1-one) has been adopted as a growth stimulant and also employed to reduce stress in cattle (Bennet et al., 1974; Cole et al., 1984; Ingerowski et al., 1976). The radioactivity of subcutaneously implanted tritium-labeled zeranol pellets in cattle is excreted in feces (45%) and urine (10%) and remained at the site of implant (10%) (Sharp and Dyer, 1972). The metabolism of zeranol has been studied in vitro as well as in vivo, and zearalanone (2), an oxidation product, was reported to be the major metabolite. Ingerowski and Stan (1979) confirmed the presence of zearalanone (2) in the incubation medium by GC–MS when zeranol was incubated with microsomal preparations of bovine liver, uterus, and muscle. Migdalof et al. (1983) reported zearalanone in the urine and feces of the female rat, dog, rabbit, and Rhesus monkey by reverse isotope dilution analyses following oral administration of radioactive zeranol. The presence of taleranol (3), the C-7 epimer of zeranol, also has been reported in rabbit urine as a minor metabolite.

In order to investigate the biotransformation of zeranol (1), a rapid HPLC–UV spectrophotometric method was developed for the analysis and identification of urinary metabolites.

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MATERIALS AND METHODS

Zeranol (1), taleranol (3), and zearalanone (2) were gifts from the International Minerals and Chemical Corp., Terre Haute, IN. β-Glucuronidase (Type H-2) containing approximately 100 000 units/mL of β-glucuronidase and 1000–5000 units/mL of sulfatase activity and Amberlite XAD-4 were purchased from Sigma Chemical Co., St. Louis, MO, and Regisil, a trimethylsilylating reagent, was purchased from Regis Chemical Co., Morton Grove, IL.

Treatment of Animals. Zeranol (4.0 g) in dimethyl sulfoxide (Me_2SO), total volume 15.0 mL, was administered intraperitoneally to a bull (mixed breed) weighing 100 kg. The urine was collected via a urine bag for 48 h following treatment (urine sample 1).

Zeranol (4.0 g) in Me_2SO, total volume 19.0 mL, was administered intramuscularly (im, injected at four different sites) to a Holstein bull weighing 270 kg, and the urine was collected for 4 days following treatment. The bull was kept in a metabolic crate, and the urine samples collected were slightly contaminated with feces (urine sample 2).

The same Holstein bull was given zeranol (1.0 g) in propylene glycol, total volume 220 mL, intravenously (40 mL/h) 51 days after the previous im treatment. The urine was collected via a urine bag for 3 days following treatment (urine sample 3).

All urine samples were collected at room temperature and kept frozen in polyethylene bottles until further study.

Extraction of Urinary Metabolites. The first 24-h collection of urine samples 1 and 2 was filtered with the

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