Meat Science **33** (1993) 265–275

PURCHASED BY THE UNITED STATES
DEPARTMENT OF AGRICULTURE FOR
OFFICIAL USE.

Calcium Chloride Marination Effects on Beef Steak Tenderness and Calpain Proteolytic Activity

G. Whipple & M. Koohmaraie*

US Department of Agriculture,‡ Agricultural Research Service,
Roman L. Hruska US Meat Animal Research Center,
Clay Center, NE 68933, USA

(Received 13 September 1991; revised version received 18 February 1992;
accepted 22 February 1992)

ABSTRACT

A study was conducted in three phases to examine the effect of calcium chloride marination on tenderness. Steaks obtained 5 days postmortem were marinated in a 150 mM calcium chloride solution for 24 h and 48 h in phase 1, and for 48 h in phases 2 and 3. The steaks utilized were obtained from mature cows 8–11 years of age—phase 1; four control and four β*-agonist fed steers—phase 2; and three Peidmontese and two Nelore 18-month-old steers—phase 3. Data were analyzed by analysis of variance for a split-plot design. In phase 1, marination failed to improve (*$P > 0{\cdot}05$*) shear force values. However, shear force values were less than 5 kg which was uncommonly low for mature cows. In phase 2, marination improved (*$P < 0{\cdot}05$*) meat tenderness regardless of diet. Yet, the steaks from the* β*-agonist fed steers remained less tender, even after marination, than the steaks from the control steers. In phase 3, shear force requirements were decreased (*$P < 0{\cdot}01$*) with marination. Also, the activities of* m*-calpain and calpastatin decreased (*$P < 0{\cdot}05$*) with calcium marination. It appeared that the improvement in tenderness was through the activation of* m*-calpain.*

* To whom correspondence should be addressed.

‡ Mention of a trade name, proprietary product or specific equipment is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

© 1992 US Government

INTRODUCTION

Meat tenderness is one of the main attributes of meat quality. However, variation in tenderness often occurs among cattle breeds (Koch *et al.*, 1982; Crouse *et al.*, 1989) and different muscles (Cross *et al.*, 1973). Currently, marination is widely used by consumers to improve meat tenderness and flavour. The use of calcium in marinades may improve tenderness by activating the natural endogenous calpain proteinases. The calpain proteolytic system consists of μ-calpain, *m*-calpain and calpastatin (a specific inhibitor of both calpains). Sufficient calcium concentrations are reached postmortem to activate μ-calpain, but not *m*-calpain. Therefore, after a few days *postmortem*, only minimal amounts of detectable μ-calpain activity remain, but virtually all of *m*-calpain activity is present (Koohmaraie *et al.*, 1987). Infusing or injecting calcium chloride into the muscle within 1 h post-exsanguination dramatically improves longissimus muscle tenderness (Koohmaraie *et al.*, 1988, 1989, 1990; Koohmaraie & Shackelford, 1991) by way of activating the calpain proteinase system, thus increasing the rate of proteolysis. Tenderness of less desirable cuts can be improved by this same method (Morgan *et al.*, 1991; Wheeler *et al.*, 1991). However, the application of this method immediately postmortem would require several modifications in current slaughter techniques. Therefore, a different calcium application method may be of greater benefit if it proves successful in improving tenderness. Alarcon-Rojo & Dransfield (1989) reported that soaking beef strips (taken at 24 h postmortem) in a calcium chloride solution increased tenderness by 40%. Yet, for a marinade to be beneficial for consumers, it must improve tenderness in later postmortem, at which time *m*-calpain would be one of the primary endogenous calcium-dependent proteinases present. The objective of this study was to determine if calcium chloride marination of beef steaks, 5 days postmortem, could improve tenderness.

MATERIALS AND METHODS

Phase 1

Calcium chloride marination of longissimus and semimembranosus steaks from mature beef carcasses

Longissimus and semimembranosus muscles were removed 5 days *postmortem* from carcasses of six mature cows ranging in age from 8 to 11 years with several breed crosses represented. Steaks were cut 2·54 cm thick from both muscles. Each steak was marinated in approximately

600 ml of a cold, 150 mM $CaCl_2$ solution for 24 and 48 h at 4°C. Control, non-marinated steaks, were vacuum packaged and stored at 4°C for the same duration. After each marination time, steaks were immediately cooked on Farberware Open-Hearth Broilers (Farberware Co., Bronx, NY) to an endpoint temperature of 70°C monitored with iron constantan thermocouple wires attached to a Honeywell potentiometer (Honeywell, Inc., Scarborough, Ontario). After cooking, steaks were tempered at 4°C for 24 h. Six, 1·27-cm diameter cores were removed from each steak parallel to muscle fiber direction with a hand coring device. Cores were sheared with an Instron model 1132/Microcon II using a Warner–Bratzler shear attachment. Data were analyzed using the General Linear Models of SAS (1985) for a split plot design by muscle type. The whole plot was marination treatment and the split plot was length of marination time. The whole plot error term was animal × treatment. The split plot error term was the residual error.

Phase 2

Calcium chloride marination on longissimus steaks from steers fed either a control diet or a diet supplemented with $L_{644,969}$ (Merck, Sharpe and Dohme Research Laboratories, Rahway, NJ), a β-adrenergic agonist

Four steers were fed $L_{644,969}$, a β-adrenergic agonist, at 3 ppm and the other four steers were fed a control diet. The management of these animals is explained in detail by Wheeler & Koohmaraie (1992). The longissimus muscle was removed 1 day *postmortem* from eight steer carcasses. Steaks, 2·54 cm thick, were vacuum packaged and stored at 4°C for an additional 4 days, at which time half of the steaks were marinated in a cold 150 mM $CaCl_2$ solution for 48 h at 4°C with steaks from both the control and β-agonist fed steers being equally represented. Each steak was placed in approximately 600 ml of marinade. The remaining steaks served as controls, remaining vacuum packaged for the same duration. Steaks were cooked and Warner–Bratzler shear values were obtained as in phase 1. Data were analyzed as a split plot design with animal feeding treatment as the whole plot and marination treatment as the split plot. Animal × feeding treatment was the whole plot error term.

Phase 3

Calcium chloride marination of longissimus beef steaks and effects on activities of the calpain proteolytic system

The longissimus muscle was removed 1 day *postmortem* from five steer

carcasses (three Peidmontese and two Nelore). Steaks, 2·54 cm thick, were vacuum packaged and stored at 4°C for an additional 4 days. At 5 days *postmortem*, a 5 g sample was removed from a steak representing each animal, in order to determine calpain and calpastatin activity. In addition, half of the steaks were marinated at 5 days *postmortem* in 600 ml of a cold 150 mM $CaCl_2$ solution at 4°C for 48 h with each animal being represented. The remaining steaks remained vacuum packaged to serve as controls. After marination was completed, 6 g were removed from each steak (marinated and nonmarinated), then the steaks were cooked and Warner–Bratzler shear force values were determined as in the other two phases.

For calpain and calpastatin activity determinations, 5 g samples were extracted in 150 mM Tris-HCl, pH 8·3, 50 mM ethyleneglycol-bis-*N,N,N',N'*-tetraacetic acid (EGTA), 7 mM β-mercaptoethanol (MCE), 2 mM phenylmethylsulfonyl fluoride (PMSF) and 30 μg/ml leupeptin. Homogenates were centrifuged and supernatants were filtered, pH adjusted to 7·5 and dialyzed against 20 mM Tris-HCl, pH 7·5, 5 mM ethylenediaminetetraacetic acid (EDTA) and 7 mM MCE. After dialysis, supernatants were centrifuged, filtered and loaded on 1·5 × 20 cm DEAE-Sephacel columns that were equilibrated with 20 mM Tris-HCl, pH 7·35, 0·5 mM EDTA and 7 mM MCE. Columns were washed with at least six column volumes of equilibrating buffer + 25 mM NaCl to remove unbound proteins. The bound proteins were eluted with a 375 ml continuous gradient of 25 mM to 375 mM NaCl in equilibrating buffer (flow rate, 20 ml/h; fraction volume, 3 ml). All these procedures were conducted at 4°C. Fractions were assayed for calpain and calpastatin activity according to Koohmaraie (1990), except that 1 ml of each fraction was added to 1 ml of casein media with 5 mM $CaCl_2$ in the assay. One unit of calpain activity was defined as the amount of enzyme that catalyzed an increase of 1·0 absorbance unit at 278 nm in 1 h at 25°C. One unit of calpastatin activity was defined as the amount that inhibited one unit of *m*-calpain activity.

For immunoblotting, a 1 g sample from each steak (marinated and nonmarinated) was extracted in 10 volumes of the same extraction solution used for activity determinations, except *N*-[M-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E-64, 5 μg/ml), which is an irreversible inhibitor of cysteine proteinases and inhibits calpain only in the presence of calcium, replaced leupeptin and PMSF. Homogenants were centrifuged (34 000g_{max}) for 60 min. Supernatants were filtered and P_{0-30} and P_{30-65} ammonium sulfate cuts were performed. Pellets were dissolved in 5 ml of equilibrating buffer. Protein concentrations were determined using Bio-Rad protein dye reagent (Bio-Rad, Richmond, CA) with bovine

serum albumin as the standard. Proteins (200 μg) were separated by gel electrophoresis using 12% acrylamide gels (37·5:1, acrylamide to bis ratio) and then transferred to nitrocellulose. Free-binding sites were blocked with 5% nonfat dry milk (NFM) in Tris buffered saline + 0·05% Tween 20 (TTBS; 20 mM Tris, 500 mM NaCl, pH 7·5 at 25°C). The primary antibody (to *m*-calpain 80 K subunit) was diluted in TTBS + 1% NFM. Rabbit alkaline phosphatase IgG (Sigma Chemical Co., St Louis, MO) diluted in TTBS served as the secondary antibody, and for protein detection and color development an alkaline phosphatase substrate kit (Bio-Rad, Richmond, CA) was utilized.

Data were analyzed for Warner–Bratzler shear force values, calpain and calpastatin activities using General Linear Models of SAS (1985) for a split plot design (animal as whole plot and marination treatment as split plot).

RESULTS AND DISCUSSION

Marination is frequently applied in households and restaurants to improve meat flavor and tenderness. Because calcium chloride is already approved by the United States Food and Drug Administration at levels of 3% of an 0·8 M solution (reg #318.7(c) (4); FSIS, 1973), its application for improving meat tenderness is conceivable. Infusion or injection of calcium chloride into carcasses immediately after slaughter improved meat tenderness such that aging of meat to enhance tenderness was not necessary (Koohmaraie *et al.*, 1988, 1989, 1990; Koohmaraie & Shackelford, 1991; Morgan *et al.*, 1991; Wheeler *et al.*, 1991). Alarcon-Rojo & Dransfield (1989) demonstrated that soaking beef strips obtained 24 h *postmortem* in a 30 mM $CaCl_2$ solution improved tenderness by 40%. Yet, only a 6% tenderness improvement was observed for strips obtained 48 h *postmortem*. Therefore, application of $CaCl_2$ in later *postmortem* appears to improve meat tenderness.

In phase 1, steaks from mature cows (8–11 years) were utilized because steaks from older animals are usually less tender (Cross *et al.*, 1973; Reagan *et al.*, 1976). However, shear force values were much lower (<5·0 kg) than expected for steaks obtained from mature cows; and marination failed to improve ($P > 0·05$) shear force values regardless of length of marination (24 h *vs* 48 h) for either muscle (Table 1). Factors such as connective tissue probably contributed to the shear force requirements, because the proportion of thermally stable intermolecular collagen crosslinks increases with age (Shimokomaki *et al.*, 1972). This could possibly explain why improvements in shear force values were not

TABLE 1
Warner–Bratzler Shear Force Values of Control and Calcium Chloride Marinated (24 and 48 h) Longissimus and Semimembranosus Mature-Beef Steaks

Marination time	*Warner–Bratzler Shear Least-Squares Means (kg)*	
	Longissimus	*Semimembranosus*
24 h		
Control	4·79	4·35
Marinated	4·44	4·49
48 h		
Control	4·29	4·57
Marinated	4·36	4·23
SE	0·16	0·24
P-value		
Time	0·13	0·94
Treatment	0·40	0·69
Treatment × time	0·25	0·37

achieved with $CaCl_2$ marination. Myofibrillar tenderness actually may have been improved but was masked by the connective tissue present. Morgan *et al.* (1991) reported less of an improvement in shear force values for beef steaks from top round subprimals than steaks from top sirloin and strip loin subprimals when injected with 0·3M $CaCl_2$. Also in that study, the top round contained the greatest amount of insoluble collagen with no differences between the sirloin and loin samples. Therefore, connective tissue may have contributed to the differences in the amount of response to $CaCl_2$ injection reflected by shear force values.

Steaks from β-agonist fed steers were selected for marination in phase 2, because reports indicate that meat is less tender when a β-agonist is fed to lambs (Hamby *et al.*, 1986; Kretchmar *et al.*, 1990; Koohmaraie & Shackelford, 1991), pigs (Aalhus *et al.*, 1990) and cattle (Miller *et al.*, 1988; Fiems *et al.*, 1990; Wheeler & Koohmaraie, 1992). Calcium chloride marination decreased ($P < 0·05$) shear force values of steaks from both the control and β-agonist fed steers (Table 2). An approximate 2 kg improvement in shear force requirements was observed regardless of diet treatment, but the steaks from the β-agonist fed steers were less tender than steaks from the control fed steers. Koohmaraie & Shackelford (1991) found that infusion of a 0·3M $CaCl_2$ solution into carcasses

TABLE 2
Warner-Bratzler Shear Force Values of Control and Calcium Chloride Marinated Longissimus Steaks from β-Agonist Supplemented and Regular Diet Fed Steers

	Warner–Bratzler shear (kg)		
	Least-squares means	*Min.*	*Max.*
β-Agonist fed			
Control	9·01	7·64	10·40
Marinated	7·29	5·93	9·02
Regular diet			
Control	6·58	4·05	8·70
Marinated	4·23	3·01	5·87
S.E.	0·85		
P-value			
Diet	0·01		
Treatment	0·05		
Diet × treatment	0·73		

immediately post-exsanguination equally improves tenderness of chops from β-agonist and control fed lambs. However, in that study, final shear force values after infusion remained greater for chops from β-agonist fed lambs than for those from the control diet fed lambs as in the present study.

An explanation for the greater shear force values after infusion was that muscle fiber size possibly could have been greater in the β-agonist fed lambs, which may have contributed to the shear force requirements, especially since no collagen characteristic differences were found (Koohmaraie & Shackelford, 1991). In support, Wheeler & Koohmaraie (1992) reported large muscle fiber areas for intermediate and white fibers in the longissimus muscle of β-agonist fed steers, from which the steaks for phase 2 were obtained. Also, calpastatin activity was greater at 0 h and 7 days *postmortem* in the β-agonist fed steers than in the control steers (Wheeler & Koohmaraie, 1992). Therefore, the greater calpastatin activity possibly could have regulated the capacity of *m*-calpain to hydrolyze myofibrillar proteins during marination, such that shear force requirements did not decline further. Others have reported an association between greater *postmortem* calpastatin activity and less tender meat (Shackelford *et al.*, 1991; Whipple *et al.*, 1990).

Phase 3 was conducted to investigate the role that the calpain proteolytic system may have in improving shear force requirements of $CaCl_2$

TABLE 3.
Least-Squares Means for Warner–Bratzler Shear Force Values and Activities of *m*-Calpain and Calpastatin for Control and Marinated Longissimus Beef Steaks

Treatment	*Warner–Bratzler shear (kg)*	*m-Calpain*[a]	*Calpastatin*[b]
Control	4·80	1·50	0·62
Marinated	3·94	0·45	0·13
SE	0·10	0·07	0·19
P-value	0·01	0·01	0·14

[a] One unit of activity = amount of enzyme that catalyzed an increase of 1·0 absorbance unit at 278 nm in 1 h at 25°C; units expressed/g of muscle.
[b] One unit of activity = amount that inhibited one unit of *m*-calpain activity; units expressed/g of muscle.

marinated steaks. Again, calcium marination improved ($P < 0{\cdot}01$) shear force values (Table 3). Results at 5 days *postmortem* indicated no detectable μ-calpain activity, whereas *m*-calpain and calpastatin mean activities were 1·56 and 0·82 units/g of muscle, respectively (data not shown). In agreement, Koohmaraie *et al.* (1987) reported that the activities of μ-calpain and calpastatin decrease with postmortem aging relative to their initial activities with no change in *m*-calpain activity at 6 days *postmortem*. With $CaCl_2$ marination, *m*-calpain ($P < 0{\cdot}01$) and calpastatin ($P < 0{\cdot}15$) activities declined (Table 3). Thus, it appears that calcium was able to penetrate through the meat surface to activate *m*-calpain. In the presence of sufficient calcium, autolysis of the calpains reduces their calcium requirement for activation (Hathaway *et al.*, 1982; DeMartino *et al.*, 1986; Cong *et al.*, 1989). The autolysis of *m*-calpain in the marinated steaks was confirmed by immunoblotting for the 80 K subunit of *m*-calpain (Fig. 1), in which the 80 K subunit was no longer detectable. The calcium concentration needed for autolysis in the presence of phosphatidylinosital, a phospholipid, is less than the calcium concentration needed for catalytic activity (Cong *et al.*, 1989); but once autolysis occurs, the calcium requirement for catalytic activity decreases to a concentration comparable to that needed for autolysis (DeMartino *et al.*, 1986; Cong *et al.*, 1989). In support of *m*-calpain being catalytically active, calpastatin activity declined, which is possibly due to it being hydrolyzed by *m*-calpain (Mellgren *et al.*, 1986). Furthermore, since it appears than *m*-calpain was catalytically active, degradation of myofibrillar proteins could explain the decrease in shear force values for $CaCl_2$ marinated steaks.

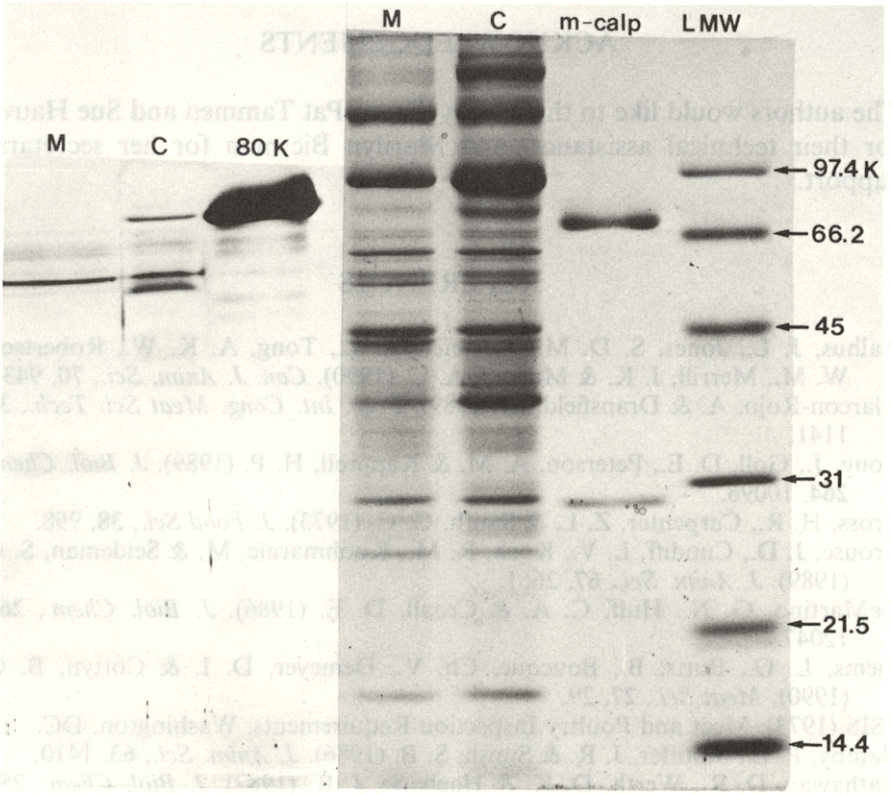

Fig. 1. SDS-PAGE (65 μg) and *m*-calpain 80 K subunit immunoblot of sample from $CaCl_2$ marinated steak (M), sample from control steak (C) and purified *m*-calpain (*m*-calp). Low molecular weight standards = LMW.

Although sensory panel evaluation was not conducted in the present study, it should be mentioned that St. Angelo *et al.* (1991) found that infusing lamb carcasses with 0·3 M $CaCl_2$ had no effect on desirable flavor attributes, but slightly increased salty and bitter flavors. Morgan *et al.* (1991) reported that sensory panel ratings improved with 0·3M $CaCl_2$ injection when compared to noninjected meat. Therefore, marination of steaks with a calcium-containing marinade may prove beneficial in improving meat tenderness and simultaneously calcium-fortify the meat without affecting its flavor.

In conclusion, calcium marination of steaks, at a time when they are normally consumed, may be beneficial in improving meat tenderness. The improvement in tenderness appears to be through the activation of *m*-calpain, an endogenous proteinase. If adapted, tenderness could be improved with the use of substances already present in the meat and, in turn, introduce a type of calcium fortification.

ACKNOWLEDGEMENTS

The authors would like to thank Kay Theer, Pat Tammen and Sue Hauver for their technical assistance, and Marilyn Bierman for her secretarial support.

REFERENCES

Aalhus, J. L., Jones, S. D. M., Schaefer, A. L., Tong, A. K. W., Robertson, W. M., Merrill, J. K. & Murray, A. C. (1990). *Can. J. Anim. Sci.*, **70**, 943.

Alarcon-Rojo, A. & Dransfield, E. (1989). *Proc. Int. Cong. Meat Sci. Tech.*, **35**, 1141.

Cong, J., Goll, D. E., Peterson, A. M. & Kapprell, H. P. (1989). *J. Biol. Chem.*, **264**, 10096.

Cross, H. R., Carpenter, Z. L. & Smith, G. C. (1973). *J. Food Sci.*, **38**, 998.

Crouse, J. D., Cundiff, L. V., Koch, R. M., Koohmaraie, M. & Seideman, S. C. (1989). *J. Anim. Sci.*, **67**, 2661.

DeMartino, G. N., Huff, C. A. & Croall, D. E. (1986). *J. Biol. Chem.*, **261**, 12047.

Fiems, L. O., Butts, B., Boucque, Ch. V., Demeyer, D. I. & Cottyn, B. G. (1990). *Meat Sci.*, **27**, 29.

FSIS (1973). Meat and Poultry Inspection Requirements. Washington, DC.

Hamby, P. L., Stouffer, J. R. & Smith, S. B. (1986). *J. Anim. Sci.*, **63**, 1410.

Hathaway, D. R., Werth, D. K. & Haeberle, J. R. (1982). *J. Biol. Chem.*, **257**, 9072.

Koch, R. M., Dikeman, M. E. & Seideman, S. C. (1982). *J. Anim. Sci.*, **54**, 35.

Koohmaraie, M. (1990). *J. Anim. Sci.*, **68**, 659.

Koohmaraie, M. & Shackelford, S. D. (1991). *J. Anim. Sci.*, **69**, 2463.

Koohmaraie, M., Babiker, A. S., Schroeder, A. L., Merkel, R. A. & Dutson, T. R. (1988). *J. Food Sci.*, **53**, 1638.

Koohmaraie, M., Crouse, J. D. & Mersmann, H. J. (1989). *J. Anim. Sci.*, **67**, 934.

Koohmaraie, M., Whipple, G. & Crouse, J. D. (1990). *J. Anim. Sci.*, **68**, 1278.

Koohmaraie, M., Seideman, S. C., Schollmeyer, J. E., Dutson, T. R. & Crouse, J. D. (1987). *Meat Sci.*, **19**, 187.

Kretchmar, D. H., Hathaway, M. R., Epley, R. J. & Dayton, W. R. (1990). *J. Anim. Sci.*, **68**, 1760.

Mellgren, R. L., Mericle, M. T. & Lane, R. D. (1986). *Arch. Biochem. Biophys.*, **246**, 233.

Miller, M. F., Garcia, D. K., Coleman, M. E., Ekeren, P. A., Lunt, D. K., Wagner, K. A., Procknor, M., Welsh, Jr., T. H. & Smith, S. B. (1988). *J. Anim. Sci.*, **66**, 12.

Morgan, J. B., Miller, R. K., Mendez, F. M., Hale, D. S. & Savell, J. W. (1991). *J. Anim. Sci.*, **69**, 4469.

Reagan, J. O., Carpenter, Z. L. & Smith, G. C. (1976). *J. Anim. Sci.*, **43**, 1198.

SAS (1985). *SAS User's Guide: Statistics.* Statistical Analysis Systems Inst. Inc., Cary, NC.

Shackelford, S. D., Koohmaraie, M., Miller, M. F., Crouse, J. D. & Reagan, J. O. (1991). *J. Anim. Sci.*, **69**, 171.
Shimokomaki, M., Elsden, D. F. & Bailey, A. J. (1972). *J. Food Sci.*, **37**, 892.
St. Angelo, A. J., Koohmaraie, M., Crippen, K. L. & Crouse, J. D. (1991). *J. Food Sci.*, **56**, 359.
Wheeler, T. L. & Koohmaraie, M. (1992). *J. Anim. Sci.* (In press).
Wheeler, T. L., Koohmaraie, M. & Crouse, J. D. (1991). *J. Anim. Sci.*, **69**, 4871.
Whipple, G., Koohmaraie, M., Dikeman, M. E., Crouse, J. D., Hunt, M. C. & Klemm, R. D. (1990). *J. Anim. Sci.*, **68**, 2716.