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The Detection of Central Nervous System Tissue on Beef Carcasses and in Comminuted Beef

G. R. SCHMIDT,[1]* R. S. YEMM,[1] K. D. CHILDS,[1] J. P. O'CALLAGHAN,[2] AND K. L. HOSSNER[1]

[1]*Department of Animal Sciences, Colorado State University, Fort Collins, Colorado 80523-1171; and* [2]*Center for Disease Control and Prevention, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, West Virginia 26505, USA*

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ABSTRACT

We report the development and validation of a fluorescent enzyme-linked immunosorbent assay (ELISA) for glial fibrillary acidic protein (GFAP), which can be used as a rapid and sensitive method to detect CNS tissue in meat products. The fluorometric assay is sensitive to 0.2 ng GFAP and has an intra-assay coefficient of variation (CV) of 2.0% and an interassay CV of 14.1%. Bovine spinal cord and brain demonstrate dose-response curves that are parallel to GFAP standards, whereas peripheral sciatic nerve and cervical ganglia also cross-react at high tissue levels. The use of another central nervous system marker, syntaxin 1-B, was not effective for neural tissue detection. Less than 1.0 ng GFAP per mg tissue was found on most beef subprimals and advanced meat recovery (AMR) product. Occasional samples contained higher levels of GFAP, probably because of contamination by the carcass-splitting saw, incomplete removal of the spinal cord, or a chance sampling of a major nerve. Further reduction of CNS content was facilitated by removal of the cervical vertebrae and the spinal canal prior to processing beef chuck bones through AMR equipment. The presence of GFAP was very low (0.037 ng/mg) in beef patties collected from major processors throughout the USA. The presence of normal sausage ingredients or heating the product to 80°C for 60 min did not affect the detection of GFAP. Heating the product to 115°C for 100 min eliminated the detectability of GFAP.

The presence of brain or spinal cord material as an inadvertent contaminant of meat may result from stunning livestock, splitting the carcass, or preparing advanced meat recovery (AMR) products from the vertebral column *(2).* In light of current consumer concern about bovine spongiform encephalitis (BSE), a disease transmitted by consumption of central nervous system (CNS) tissue, a reliable analytical test for CNS tissue in meat products is essential to ensure consumer confidence and allay consumer fears of BSE in meat products. A method to detect the presence of CNS tissue in meat products has been developed in our laboratory *(8).* The method uses an enzyme-linked immunosorbent assay (ELISA) for the detection of glial fibrillary acidic protein (GFAP), an antigen that is highly, but not completely, restricted to astrocytes in the CNS. This assay provides a simple, cost-effective, and efficient method to detect small amounts of CNS tissue in meat products. The assay is capable of detecting 1 ng (10^{-9} g) of GFAP and can be used on product samples that have been stored at 4°C for up to 8 days.

The ELISA we used to detect GFAP is a nonradioactive, color-based assay. The sensitivity of this assay is about 1 ng and can be increased approximately 10- to 30-fold by using a fluorescent label *(5).* In the current study, we developed a fluorescent system to further increase the sensitivity of GFAP detection in meat. Since our publication of the GFAP ELISA, others have reported the use of an ELISA for the presynaptic neural protein syntaxin 1-B for the detection of CNS in beef blood *(1, 3).* We felt that the syntaxin 1-B assay could be used to detect CNS tissue in meat products. Thus, we compared the syntaxin 1-B ELISA to the fluorescent GFAP ELISA for detection of bovine neural tissues.

The fluorescent GFAP ELISA was used to examine the incidence and level of CNS contamination on beef subprimal cuts, in commercial hamburger patties, and in AMR products. Product from a modified AMR procedure, in which the bone surrounding the spinal canal was removed, was also examined. The effects of heating meat products containing known amounts of neural tissue was examined to determine if GFAP could be detected in cooked meat products in the presence and absence of sausage additives.

MATERIALS AND METHODS

Fluorescent GFAP ELISA reagents. The following reagents were used. Reagent A was phosphate-buffered saline (PBS): one packet of PBS (Pierce Chemical, Rockford, Ill.) was dissolved in 500 ml of deionized water (final concentration: 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, pH 7.4). Reagent B consisted of PBS plus 0.5% Triton X-100 (Bio-Rad, Hercules, Calif.). Reagent C was PBS plus 5% Carnation nonfat dry milk. Reagent D was PBS plus 5% powdered milk plus 0.5% Triton X-100. Reagent E was 1/400 polyclonal anti-GFAP in PBS (Dako, Carpinteria, Calif.). Reagent F was 1/500 monoclonal anti-porcine GFAP (Boehringer Mannheim, Indianapolis, Ind.) prepared in reagent D. Reagent G was peroxidase-labeled goat anti-mouse IgG (Pierce #31434; Pierce Chemical) in reagent D (1/10,000). Re-

* Author for correspondence. Tel: (970) 491-6527; Fax: (970) 491-0278; E-mail: gschmidt@ceres.agsci.colostate.edu.

agent H was Quantablu Fluorogenic Peroxidase Substrate (Pierce #15169; Pierce Chemical) prepared according to the manufacturer's instructions.

Preparation of standards and samples for ELISA. Bovine GFAP standard (American Research Products, Boston, Mass.) was dissolved in distilled water, and serial dilutions of the standards were prepared in reagent B. The final concentrations of each standard were 0.075, 0.15, 0.30, 0.60, 1.2, 2.4, and 4.8 ng/100 μl. For each ELISA plate (Immulon-2, Dynex Technologies, Chantilly, Va.), reagent B (blank) and each of the seven standards, in duplicate, were added to the first 16 wells of the plate. Diluted standards could be stored at 4°C (not frozen) for up to 10 days.

Samples were homogenized in 10 volumes of 1% sodium dodecylsulfate (SDS) at 90°C. Samples were diluted in reagent B for protein analysis and ELISA testing. Muscle samples were diluted at least 1/12 to avoid interference of SDS in the ELISA, whereas neural tissue was diluted at least 1/3,000. Two 10-fold serial dilutions of the minimal dilutions provided three samples that were within the range of ELISA sensitivity. Samples were analyzed in duplicate at each dilution.

Assay procedure. Reagent E (100 μl) was added to each microplate well, and the plate was incubated at 37°C for 1 h. Plates were then washed four times with 200 μl/well reagent A and blotted after each wash. Reagent C (100 μl) was added, and the plate was incubated for 1 h at room temperature; the plate was emptied and blotted, and standards and samples were added in a volume of 100 μl/well and incubated for 1 h at room temperature. The plates were washed four times with 200 μl/well reagent B before the addition of 100 μl/well reagent F and incubated for 1 h at room temperature. The plates were washed four times with 200 μl/well reagent B and incubated for 30 min at room temperature with 100 μl/well reagent G. After four washes with 200 μl/well reagent B, plates were incubated for 30 min at room temperature with reagent H (100 μl/well). The reaction was stopped by the addition of 100 μl/well of the reagent provided with Reagent H (Pierce #15169), and fluorescence at 460 nm (360 nm excitation) was recorded using a Bio-Tek FLX800B plate reader (Biotek Instruments, Winooski, Vt.).

Syntaxin 1-B ELISA protocol. Meat and neural tissue samples for the Syntaxin 1-B colorimetric ELISA were prepared by homogenizing the samples in 10 volumes of 0.5% Triton X-100 at room temperature. Extracted samples were diluted as necessary in PBS–0.05% Tween 20 before addition to the ELISA. The procedure described by Anil et al. *(1)* was followed exactly. Purified recombinant syntaxin 1-B produced in *Escherichia coli* (Department of Clinical Veterinary Science, Bristol University, Bristol, UK) was used as the standard in this assay.

Meat product sampling: subprimals. Surface tissue from beef subprimals, bone sawdust, blood clots from beef hearts, and spinal cord samples were collected from 10 fed beef slaughter plants located in Colorado, Kansas, Nebraska, and Texas. Samples of approximately 5 by 5 by 0.3 cm were collected from three sites on different subprimals by plant employees during normal carcass fabrication and placed in plastic bags for transport to the laboratory. A strip (1 by 5 cm) was cut from the center of each sample for GFAP analysis. The subprimals and specific locations sampled were (i) medial surface of the cervical–dorsal region of the boneless beef chuck, (ii) medial surface of the cervical–ventral surface of the beef chuck (contained blood from the stick wound), (iii) cranial surface of the longissimus dorsi at the 12/13 rib separation, (iv) medial surface of the boneless beef rib between the sites of removed vertical processes of the thoracic vertebrae, (v) medial surface of the boneless beef strip loin between the sites of removed lumbar split spine, (vi) medial–dorsal surface of the boneless beef top sirloin butt, (vii) medial–ventral surface of the boneless beef top sirloin butt, (viii) sagittal surface of the beef inside round, (ix) sagittal surface of the beef gooseneck round, (x) medial–ventral surface of the boneless beef chuck in the area between the sites of removed vertical processes of the 2 through 5 thoracic vertebrae, (xi) medial–dorsal surface of the boneless beef brisket, (xii) sagittal surface of the beef chuck shoulder clod. Samples of bone dust were collected from the saw where the chine bone was cut from the beef rib. Samples of blood clots from the left ventricle of beef hearts were collected at the viscera inspection table by plant employees. In addition, samples of spinal cord were collected at each plant.

Meat product sampling: AMR. Meat produced by advanced meat and bone separation machinery and recovery systems (AMR) were sampled at six of the fed beef slaughter plants. All plants were processing beef chuck neck bones with tandem piston pressure recovery systems followed by drum type mechanical desinewers. As the equipment was operating, 50 separate samples (approximately 20 g each) were collected and bagged over a 1-h time period at each of the six plants.

Meat product sampling: modified AMR procedure. Vertebrae from beef chucks were modified by eliminating the cervical vertebrae and sawing the chine and associated vertebrae foramen from the first five thoracic vertebrae. This material was collected at the start of the production shift as the first material processed in the clean AMR machine. Approximately 13 kg of material was recovered and analyzed as 50 subsamples. Later in the production shift, a 13 kg sample was collected from the normal chuck bone production and was similarly subsampled and analyzed for GFAP.

Meat product sampling: ground beef patties. Seven different ground beef production facilities in California, Georgia, Illinois, Minnesota, Ohio, Pennsylvania, and Utah supplied 10 samples of 100-g beef patties. Each patty was identified from a different lot of raw materials. Patties were sampled raw and analyzed for GFAP.

Meat product sampling: cooked product preparation. Two samples of approximately 9 kg of AMR, 9 kg beef chuck shoulder clod, and 200 g spinal cord were obtained from a fed cattle slaughter plant at approximately 48 h postmortem. The AMR product was produced as described above. The beef chuck shoulder clod (3.5 kg) and the AMR (3.5 kg) were separately chopped in a six-blade, 35-liter bowl chopper (Meissner/RMF, Kansas City, Mo.) at 4,000 rpm for 15 revolutions of the bowl. A second 3.5-kg batch of each meat source was chopped similarly with typical sausage ingredients according to the following formulation: 78.96% meat, 15% water, 1.6% salt, 0.15% alkaline phosphate, 0.20% cure salt (6.25% sodium nitrite), 0.04% sodium erythorbate, 2.0% dextrose, 1.0% ground mustard, 0.1% coriander, 0.2% nutmeg, 0.1% cardamom, 0.35% paprika, and 0.3% ground black pepper. Spinal cord was chopped for 30 s in a food processor. Portions (500 g) of each of the four treatments were mixed in a Kitchen Aid mixer at high speed for 30 s with 0, 0.2, 0.4, 0.6, 0.8, or 1.0% chopped spinal cord. Six 60-g samples were removed from the mixtures, individually vacuum packaged, and cooked for 1 h in an agitated water cooker at either 60, 66, 71, 77, or 82°C or left raw. Samples were chilled in ice water and stored at 4°C until extracted for analysis of GFAP. Approximately 120 g of each of the treatments was placed in a 303 by 400 steel can, vacuum sealed, and retorted at 115°C for 100 min. The cans were cooled in ice water and stored at 4°C until analyses.

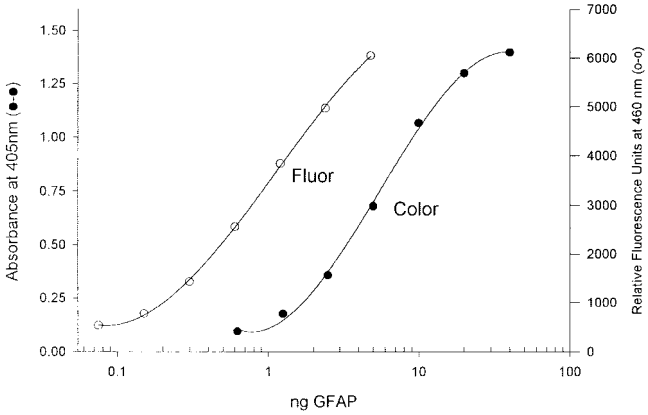

FIGURE 1. *Comparison of colorimetric and F-ELISA standard curves for GFAP. Each data point represents the average of duplicate samples from a single representative ELISA.*

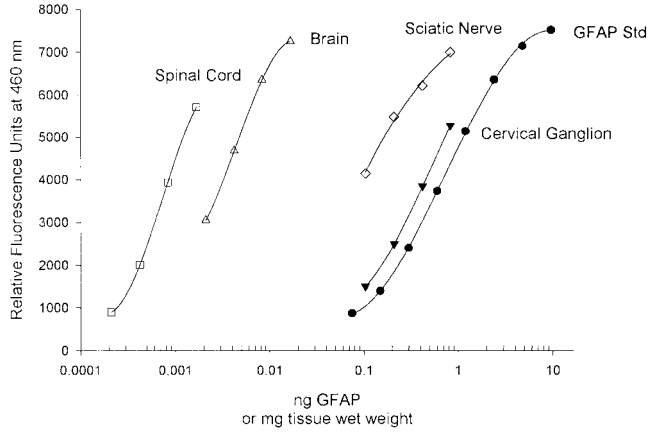

FIGURE 2. *Neural tissue dose-response curves compared to GFAP standard in the F-ELISA. Each data point represents the average of duplicate samples from a single representative ELISA.*

Statistical analyses. Data collected was subject to analysis of variance, whereas mean differences between plant site, sample site and replicate were compared by using Tukey's multiple range test *(6)*. The cooked meat data were evaluated by regression analyses with 95% confidence intervals generated by Sigma Plot version 4.0 *(9)*.

RESULTS AND DISCUSSION

Fluorometric GFAP ELISA validation. Figure 1 shows a comparison of the fluorometric GFAP ELISA (GFAP F-ELISA) standard curve with the colorimetric standard curve. Sensitivity of the assays, defined as the lowest measurable value different by two standard deviations from the blank wells, was 0.3 and 0.075 ng for the colorimetric and fluorescent assays, respectively. Although this statistical definition is valid, we use a practical working sensitivity above the lower 20% of the standard curve. This provides values of 2.0 ng (color) and 0.20 ng (fluorescent) for the minimal limits of detection. Interassay coefficient of variation, assessed in four GFAP F-ELISAs was 14.1%. When samples were assessed in sextuplicate, the intra-assay coefficient of variation was 5.1% for the color ELISA and 2.0% for the fluorometric ELISA

Figure 2 shows the dose-response curves of various tissues in the F-ELISA. Curves generated from bovine CNS tissue, brain, and spinal cord were readily identified at very low tissue concentrations. Brain and spinal cord dose-response curves were parallel to the GFAP standard curve, whereas high levels of sciatic nerve cross-reacted with a nonparallel dose-response curve. Cervical ganglia also cross-reacted in a parallel manner at high tissue concentrations in the GFAP F-ELISA. These results suggest that the presence of very high levels of peripheral nerve (e.g., sciatic) and cervical ganglia in meat products will indicate the presence of CNS tissue.

Table 1 shows the GFAP concentrations in various bovine neural tissues and compares the results of color and fluorescent GFAP ELISAs when performed simultaneously (same day) on the same tissues. Generally, on a wet tissue weight basis, the fluorometric ELISA yielded 25 to 30% lower absolute tissue GFAP concentrations than the colorimetric ELISA. However, when the results are presented relative to spinal cord levels, essentially identical rank values are observed with both colorimetric and fluorometric assays. Spinal cord contains the highest levels of GFAP, whereas brain has about one-third of the GFAP levels observed in spinal cord. Peripheral sources of neural tissue, such as the sciatic nerve and cervical ganglia, contain less than 1% of the GFAP present in spinal cord. Because sciatic nerve contains approximately 0.5% of the GFAP present in spinal cord, a great deal of peripheral nerve (>1%) would have to be present to invoke a false positive in meat products.

Evaluation of bovine neural tissues for levels of syntaxin 1-B indicated that very low levels of this antigen were present. We were unable to detect syntaxin 1-B in samples of spinal cord, sciatic nerve, and cervical ganglia at the dilutions employed for detection of GFAP. Low levels of syntaxin 1-B (37.1 ng/mg wet weight) were detected in bovine brain. We concluded that the syntaxin 1-B ELISA was not an effective method for the detection of CNS tissue in meat products because the low concentrations of this antigen preclude efficient detection of small amounts of CNS contamination in meat products.

All ELISAs tested were affected by SDS, the detergent used to solubilize tissues for analysis. The presence of 0.01% SDS in the syntaxin ELISA inhibited color development by 20%, and 0.02% SDS inhibited color by 55%.

TABLE 1. *Summary of GFAP concentrations in different bovine neural tissues as quantified by the colorimetric and fluorometric ELISAs*

	GFAP–colorimetric		GFAP–fluorometric	
Tissue	ng/mg wet wt	% spinal cord[a]	ng/mg wet wt	% spinal cord[a]
Spinal cord	2,220	100	1,724	100
Brain	710	32.0	574	33.3
Sciatic nerve	12.1	0.55	11.6	0.67
Cervical ganglia	1.7	0.08	1.4	0.08

[a] Represents the percentage of GFAP in each tissue relative to spinal cord GFAP concentrations.

TABLE 2. *GFAP levels on cut surfaces of beef subprimals, in bone sawdust, and in heart blood clots*

Carcass site of sample	GFAP level			
	Mean[a]	SD	Minimum	Maximum
Medial neck	0.541	0.690	0.013	2.933
Ventral neck	0.390	0.554	0.000[b]	2.827
Ribeye	0.140	0.163	0.000	0.721
Medial rib	0.495	1.578	0.000	8.700
Medial lumbar	0.227	0.337	0.000	1.384
Dorsal top butt	0.478	1.120	0.000	6.041
Medial top butt	0.306	1.096	0.000	6.080
Sagittal inside round	0.264	0.394	0.000	1.837
Sagittal outside round	1.464	3.843	0.000	17.759
Medial chuck	1.482	0.797	0.005	4.083
Medial brisket	0.349	0.578	0.000	2.373
Sagittal clod	0.135	0.165	0.000	0.861
Bone sawdust	1.349	2.529	0.000	11.284
Heart blood clot	0.718	1.712	0.000	7.072

[a] GFAP ng/mg tissue, average of 30 samples (three from each of 10 plants).
[b] All 0.000 values were less than the lower limit of detection.

Higher levels (0.04 to 0.16%) essentially abolished color development. The GFAP F-ELISA was less sensitive to SDS, but 0.16 and 0.33% SDS inhibited fluorescence by 40 and 97%, respectively. However, lower SDS concentrations (0.04 and 0.08%) had no effect in the F-ELISA. These results indicate that SDS should not be used in the syntaxin ELISA and should not exceed 0.08% in the GFAP F-ELISA. Homogenized samples prepared for analysis in the GFAP F-ELISA were diluted at least 12-fold to avoid interference from SDS.

These results confirm and extend the validity of our published observations on GFAP levels in these tissues using the colorimetric ELISA *(8).* Although the F-ELISA results in 25 to 30% lower absolute GFAP values than the colorimetric assay, it provides the same rank order of neural tissues while displaying a 10-fold increase in sensitivity. The syntaxin 1-B ELISA was inferior to the GFAP ELISA because tissue concentrations of syntaxin were much lower then GFAP. The lower tissue levels of syntaxin mean that much higher (at least 10-fold) levels of tissue must be used in the assay. This increases the possibility of assay interference from nonspecific sources (e.g., detergents, non-CNS neural tissue) and negates the use of syntaxin analysis for neural tissue contamination of meat products.

Table 2 gives the GFAP levels on select surfaces of common beef subprimals. Generally, the samples contained less than 1 ng/mg wet weight GFAP. However, samples occasionally contained higher levels of GFAP, especially the sagittal surface of the outside round (gooseneck). For example, 5 of 30 samples contained >1 ng/mg GFAP, and only two of those contained >10 ng/mg. When these samples were removed, a section of a major nerve, such as the sciatic or the ishiatic nerve, may have been collected. Likewise, 7 of 30 bone sawdust samples had elevated levels of GFAP. Three of these had 1.5 to 3.0 ng/mg GFAP, whereas the highest had >10 ng/mg tissue GFAP. This may have been the result of repeatedly cutting the spinal canal and associated nervous tissue. Four of the 30 blood clot samples had levels of GFAP >2.0 ng/mg (2.0 to 7.0 ng/mg GFAP), which could be the result of dislodging neural tissue during captive bolt stunning *(7).*

Table 3 and Figure 3 show the effect of heating and sausage spices on the detection of GFAP in meat containing known amounts of added spinal cord. Regardless of whether the product was raw or heated to 60 to 82°C for 1 h, the spinal cord was detectable in product manufactured from beef chuck clod or beef AMR (Table 3). Figure 3 shows that the presence of spices and other sausage ingredients reduced apparent GFAP concentration ($P < 0.0001$). The reduction was the result of less water released on cooking from the spice–salt–phosphate–water treatment which had a cook yield of 99 versus 75% for the nonspice treatments. The variation at a given level of spinal cord (Fig. 3) was probably due to the inability to uniformly homogenize the

TABLE 3. *Effects of cooking for 1 h and the presence of spices on the detection of GFAP in ground chuck and AMR*

Temperature (°C)	GFAP concentration[a,b]							
	Ground chuck				AMR			
	0		1		0		1	
	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)
Raw	0.60	0.02	25.85	19.80	0.38	0.30	32.25	17.30
60	0.08	0.03	31.25	17.13	1.28	0.59	38.90	19.25
66	0.00[c]	0.00	11.95	21.60	1.82	0.48	22.28	15.10
71	0.14	0.07	17.50	22.25	0.86	0.52	34.25	21.98
77	0.10	0.05	22.00	14.83	1.47	1.09	34.50	24.48
82	0.04	0.00	23.75	18.63	1.19	0.97	22.75	15.65
Canned[d]	0.00	0.00	0.00	0.00	0.38	0.00	3.80	2.35

[a] GFAP ng/mg tissue, average of two replicates.
[b] (−), ground chuck or AMR with no additives; (+), ground chuck or AMR with added water, cure, and other ingredients; 0, 0% added spinal cord; 1, 1% added spinal cord.
[c] All 0.00 values were less than the lower limit of detection.
[d] Product heated at 115°C for 100 min.

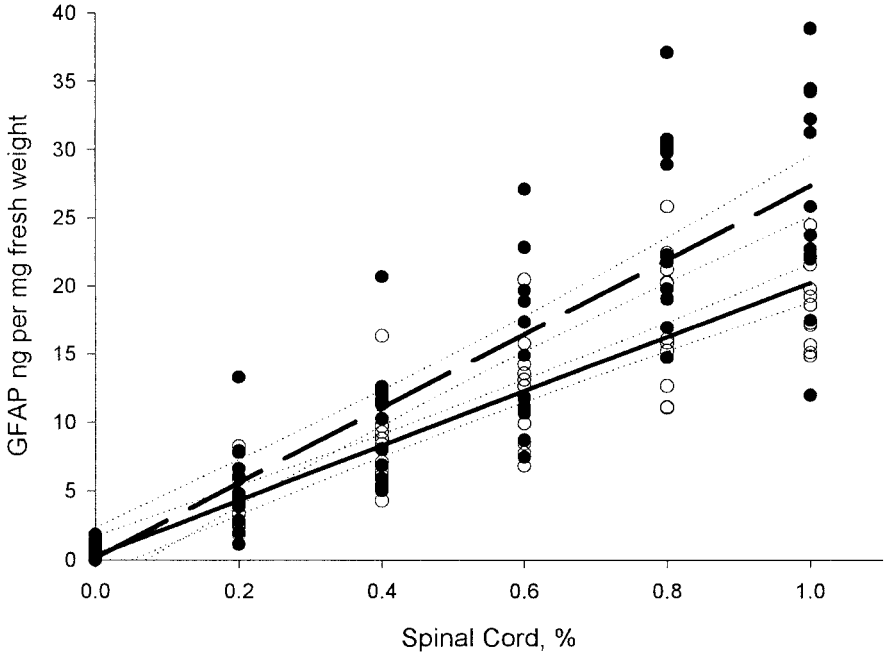

FIGURE 3. *Detection of GFAP in beef (●—●) or sausage (○—○) containing 0 to 1% added spinal cord by the F-ELISA. Each data point represents an average value derived from six samples of ground chuck and six samples of AMR product, which were sampled raw or after heating for 60 min at 60 to 82°C. Dotted lines represent the 95% confidence interval.*

spinal cord into the mix. When the product was canned and heated at 115°C for 100 min, GFAP was no longer detectable (Table 3).

Table 4 presents the GFAP levels in AMR produced from beef chuck vertebrae collected at six fed cattle plants. AMR was resampled from plant A from chuck bones that were intact (AA) or had been modified (AA-modified AMR) by eliminating the cervical vertebrae and sawing the chine and associated spinal canal from the first five thoracic vertebrae. Two of the six plants surveyed had higher levels of GFAP in the AMR. This may have been the result of carcass splitting, incomplete spinal cord removal, or the carcass washing technique. It was clear that removal of the spinal canal and remaining spinal cord remnants greatly reduced the level of GFAP in the resulting AMR from 0.61 ng/mg (Plant AA) to 0.015 ng/mg (AA-modified AMR).

TABLE 4. *GFAP levels in AMR product prepared from unmodified and modified beef chuck vertebrae collected at seven fed cattle slaughter plants and in ground beef from seven plants*

	GFAP (ng/mg tissue)			
Plant	Mean	SD	Minimum	Maximum
AMR				
A	1.308[a]	0.886	0.138	3.642
B	2.461	3.506	0.096	14.359
D	0.433	0.610	0.000[b]	3.597
F	0.238	0.233	0.012	1.430
J	0.067	0.098	0.000	0.520
K	0.692	1.006	0.000	6.018
AA	0.610	0.578	0.126	3.215
Modified AMR				
AA	0.015	0.021	0.000	0.085
Ground beef				
A	0.015[c]	0.033	0.000	0.098
B	0.029	0.035	0.000	0.086
C	0.023	0.030	0.000	0.079
D	0.025	0.017	0.001	0.050
E	0.049	0.029	0.000	0.088
F	0.121	0.275	0.000	0.900
G	0.000	0.000	0.000	0.000
Mean, all patties	0.037	0.109	0.000	0.900

[a] Least square means of plant site ($n = 50$ samples).
[b] All 0.000 values were less than the lower limit of detection.
[c] Least square means of plant site ($n = 10$ samples).

The GFAP levels in ground beef patties from 70 commercial production lots at seven different plants located throughout the United States are also shown in Table 4. All but one value was less than 0.1 ng/mg. We have no immediate explanation for the one high value at 0.9 ng/mg (plant F), other than it may have contained a section of a major nerve.

An F-ELISA for GFAP was shown to be a rapid and sensitive method to detect the presence of CNS tissue in meat products. The syntaxin 1-B ELISA was not an effective method of detecting CNS in meat products.

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