Determination of Pyridinolines in Bovine Collagenous Tissues

Hans Steinhart,* Andreas Bosselmann, and Carsten Möller

Institute of Biochemistry and Food Chemistry, University of Hamburg, Grindelallee 117, D-21046 Hamburg, Germany

A new and sensitive method is described for the determination of the trivalent collagen cross-linkers hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) in intramuscular and other connective tissues. The procedure includes hydrolysis of the isolated connective tissue, filtration and phosphocellulose chromatography of the crude hydrolysates, and reversed-phase ion-paired high-performance liquid chromatography (RP-HPLC), using sodium octanesulfonic acid as ion-pairing agent. Detection is done by measuring the natural fluorescence of pyridinolines with a spectrofluorometer. The method has been tested by determining pyridinolines in several bovine connective tissues varying in collagen content.

Keywords: *Meat tenderness; collagen cross-linking; pyridinolines; HPLC*

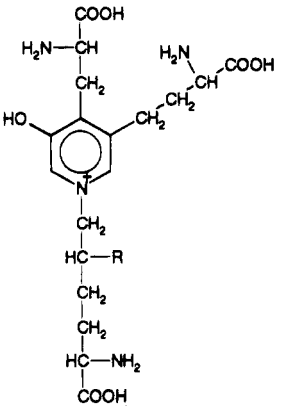
INTRODUCTION

Collagen is a major constituent in connective tissues of muscles, and it is an important parameter concerning the quality of meat. In general, meat with large amounts of connective tissue is regarded to be of lower quality because its tenderness and biological value are reduced by its higher collagen content. This is mainly due to the high mechanical stability of collagen fibers and their resistance to proteolytic enzymes. It is, however, known that differences in the stability of collagen occur. With advancing age of the animal, collagen content remains nearly constant, whereas its mechanical stability increases.

Therefore, the chemical characterization of collagen is considered to be a decisive aspect in describing the quality of meat. The differences in stability of collagen are caused by maturation processes. Tropocollagen molecules are stabilized by inter- and intramolecular covalent cross-links. These cross-links increase the mechanical and thermal stability of collagen fibers as well as their tensile strength, which is correlated to the toughness of meat (Bailey, 1984).

Amino acids, especially lysine and hydroxylysine, are the fundamental structures for cross-linking. Several collagen cross-links have been identified. Most of them appear as unstable Schiff bases, which are accessible for analysis only after reduction with sodium borohydride. The amounts of these so-called reducible cross-links are correlated to the stability of collagen only in young tissues. During growth, the content of these cross-linking compounds decreases. Simultaneously, the amount of more stable nonreducible cross-links increases and is accompanied by a rise in collagen stability (Shimokomaki et al., 1972; Robins et al., 1973; Fujii and Tanzer, 1974). Light and Bailey (1982) suggested that the reducible compounds may be regarded as initial cross-links that are later converted to nonreducible, more stable forms. Nevertheless, there is only little knowledge about these nonreducible mature cross-links. Several mechanisms have been discussed to explain the stabilization of cross-links during growth (Eyre et al., 1984a; Bailey, 1984).

Fujimoto et al. (1977) discovered a nonreducible collagen cross-link, pyridinoline, which is a 3-hydroxypyridinium derivative with three carboxyl and three amino groups. Eyre (1981) identified two different



R = H Lysylpyridinoline (LP)

R = OH Hydroxylysylpyridinoline (HP)

Figure 1. Structures of the 3-hydroxypyridinium cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).

forms of this fluorophore: hydroxylysylpyridinoline (HP), consisting of three hydroxylysyl residues, and its nonhydroxylated analogue lysylpyridinoline (LP), which embodies one lysyl and two hydroxylysyl residues (Figure 1). These trivalent cross-links connect three tropocollagen molecules. The high mechanical stability of collagen is mainly caused by the formation of these cross-links.

Pyridinolines can be isolated after acid hydrolysis of the connective tissue. Alkali treatment, however, destroys HP and LP (Robins, 1982). The fluorescence of pyridinolines, caused by the hydroxylated pyridinium ring, enables their quantitative determination. These have been quantified in many sources with abundant collagen including bone, dentine, cartilage, tendon, and other sources (Eyre, 1987; Eyre et al., 1984a). In contrast, in cornea, which is rich in connective tissue, pyridinolines could not be detected. Their absence has been explained as being due to their lability against ultraviolet irradiation (Robins, 1982).

A high analytical expense is required for the determination of pyridinolines in intramuscular connective tissue because of their sparse occurrence in collagen. Therefore, few data are available concerning the content of pyridinolines in skeletal muscle collagen. In this paper we describe a new method for the determination

0021-8561/94/1442-1943$04.50/0

of pyridinolines in intramuscular collagen and ligament using ion-paired reversed-phase high-performance liquid chromatography (RP-HPLC).

MATERIALS AND METHODS

Animal Tissues. It was necessary first to obtain samples of pure HP to be used as standard for subsequent experiments determining pyridinolines in bovine sternomandibularis muscle (m.stern) and neck ligament (ligamentum nuchae). Pure HP was prepared from bovine knee joints (articulus femotibialis) obtained immediately after the animals' death from the slaughterhouse in Hamburg, Germany. M.stern and ligamentum nuchae were chosen as samples typical of exhibiting increased toughness with advancing age of the animal and differing in collagen content. The samples were gathered from the carcasses of 2–11.5-year-old milk cows immediately after slaughter.

Reagents. Acetonitrile, HPLC grade, was from Merck, Darmstadt, Germany. House-distilled deionized water, filtered through a 0.2-μm pore, was used. Cellulose acetate membrane was obtained from Schleicher & Schüll, Dassel, Germany. Bovine serum albumin and sodium octanesulfonic acid were of HPLC grade (Fluka, Neu-Ulm, Germany). Bio-Gel P2 (200–400 mesh) and phosphocellulose Cellex P were from Bio-Rad, Munich, Germany. All other chemicals were of analytical grade (Merck).

HPLC eluents were filtered through a 0.2-μm-pore membrane of regenerated cellulose (Schleicher & Schüll). The gradient system consisted of two eluents. Eluent A was a solution of 5 g of sodium dihydrogen phosphate monohydrate, 1.17 g of sodium octanesulfonic acid monohydrate, and 1 mL of 85% phosphoric acid per liter. Eluent B was a mixture of 75% eluent A and 25% acetonitrile (v/v).

Preparation of HP Standards. LP was a kind gift from S. P. Robins (Aberdeen, Scotland). HP was prepared from bovine articular cartilage using a modified method described by Eyre et al. (1984b). In bovine articular cartilage, they found large amounts of HP and negligible shares of LP (ratio HP/LP $\cong$ 1:0.02). Thus, this connective tissue is a suitable source for the preparation of pure HP. In our experiment, cartilage substance was isolated with a scalpel from 50 bovine knee joints with a yield of 60 g (dry weight). Collagen was freed from proteoglycans by extraction (48 h, 4 °C) with 3 L of a solution of 4 M guanidine hydrochloride and 0.05 M tris(hydroxymethylamino)methane hydrochloride. After hydrochloric acid (HCl) hydrolysis of the collagen fraction (48 h, 105 °C, 3.5 L of 3 M HCl), pyridinolines were enriched by repeated precipitation using 1.2 L of a mixture containing HCl, 1-butanol, and glacial acetic acid (1:4:1 v/v/v). They were separated from the main part of amino acids by gel filtration using polyacrylamide gel (Bio-Gel P2, column size 2.6 × 80 cm). Elution was performed with 1 M acetic acid with a flow rate of 2 mL/min. Pyridinolines were detected by measuring their natural fluorescence (λ_{ex} = 295 nm, λ_{em} = 395 nm). The pyridinoline-containing fraction was pooled, evaporated to dryness, and taken up in 0.01 M HCl. After Bio-Gel size exclusion chromatography, the pyridinoline fraction was further purified by cation-exchange chromatography on phosphocellulose to remove some fluorophores and amino acids that remained in the pyridinoline fraction (Fujimoto and Moriguchi, 1978). The fraction in 0.01 M HCl was applied to a 2.6 × 45 cm phosphocellulose column (H^+-form) that had been equilibrated with 0.1 M HCl. A linear gradient from 0.05 to 0.5 M HCl was used; first the fluorophores and amino acids and then the HP were eluted. The eluent was monitored by fluorescence spectroscopy; the excitation wavelength was 295 nm and the emission wavelength 395 nm (Figure 2). The pyridinoline fraction was dried, taken up in deionized water, and freeze-dried to yield about 16 mg of the hydrochloric salt. Structural identity was verified by ^{1}H- and ^{13}C-NMR spectroscopy. For measurement, the isolated HP was taken up in D_2O. The pH value of the solution was 2.0. The NMR was conducted at 400.13 MHz. The internal standards were HOD (4.8 ppm) for ^{1}H measuring and acetonitrile (1.7 ppm) for ^{13}C measuring.

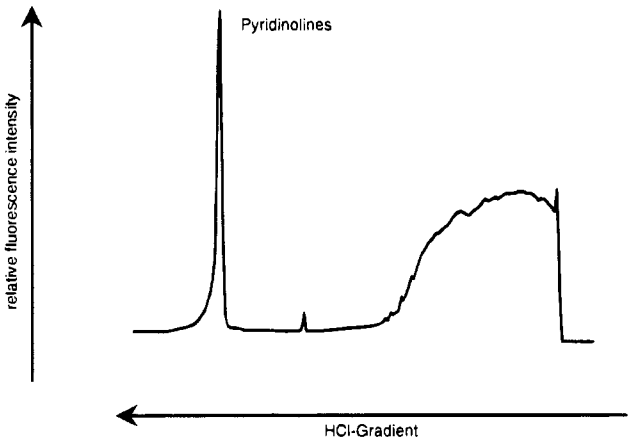


Figure 2. Phosphocellulose cleanup of a cartilage collagen hydrolysate using fluorescence to assay pyridinolines (for conditions see text).

Table 1. NMR Data of Hydroxylysylpyridinoline

^{1}H NMR	δ 8.27/8.24 (2 s, 2 H, H-7/H-11), 4.67 (m, 1 H, H-6), 4.35 (m, 1 H, H-6^1), 4.25 (m, 1 H, H-17), 4.15–3.97 (m, 3 H, H-2, 5, 14), 3.48–3.34 (d, 2 H, H-16), 3.04 (d, 1 H, H-12), 2.93 (d, 1 H, H-12^1), 2.33–2.09 (m, 3 H, H-3, 13, 13^1), 2.02 (m, 1 H, H-3^1), 1.76 (m, 1 H, H-4), 1.66 (m, 1 H, H-4^1)
^{13}C NMR	δ 172.4/172.0/171.3 (C-1, 15, 18), 155.9 (C-10), 141.7 (C-8), 141.3 (C-9), 137.4 (C-11), 130.3 (C-7), 70.0 (C-6), 66.6 (C-5), 53.1/52.9/52.0 (C-2, 14, 17), 30.6/29/7/28.0/26.7/26.0 (C-3, 4, 12, 13, 16)

The results were interpreted by H,H and H,C correlation. The NMR data (Table 1) corresponded to those described by Fujimoto and Moriguchi (1978) and Robins (1982). The purity of HP was assessed by comparing the fluorescence intensity to that of known amounts of pure HP kindly provided by D. Fujimoto (Hamamatsu, Japan). The purity of the preparation was 98.9% HP. It was used as a standard for developing the determination method.

Instrumentation. The high-performance liquid chromatograph was equipped with a 655-A13 pump, an L 5000 LC gradient controller (Merck/Hitachi, Darmstadt), and a Rheodyne 7125 injection valve with 20-μL sample loop. A variable-wavelength SFM 23 fluorescence detector (Kontron, Eching, Germany) was coupled to a D 2000 integrator (Merck/Hitachi). A reversed-phase Nucleosil 120 $3C_{18}$ column (3 μm, 12.4 cm × 4 mm) protected by a guard cartridge (3 cm × 4 mm) packed with Nucleosil 120 $3C_{18}$ (5 μm), both Macherey & Nagel (Düren, Germany), was used. A WM 400 NMR spectrometer (Bruker, Rheinstetten, Germany) was used for NMR measurements.

Analysis of Sternomandibularis Muscle and Ligamentum Nuchae. *Preparation of Muscles.* Samples were prepared by removing the outer fat layer with a scalpel while avoiding cutting off adhering myofibrillar material. Epimysium was dissected from muscle samples. The material then was freeze-dried and blended. Ligamentum nuchae samples could be hydrolyzed without further handling, whereas connective tissue had to be isolated from the intramuscular material.

Isolation. Isolation of connective tissue from m.stern muscle was done as described by Möller et al. (1993). Connective tissue was separated by passing the freeze-dried and blended muscle sample through a sieve with 160-μm square holes. The material left on the sieve was washed and sieved in acetone. After drying at 40 °C, the material was powdered and sieved again. The intramuscular connective tissue residue remaining on the sieve was ready for hydrolysis.

Hydrolysis. Under conditions different from those used for the preparation of the HP standard from the bovine articular cartilage, all samples were hydrolyzed in 6 M HCl for 4 h at 145 °C in Pyrex glass tubes with Teflon-lined screw caps. The

Table 2. Hydroxylysylpyridinoline (HP) Recoveries after Phosphocellulose Cleanup with Bovine Serum Albumin (BSA) as a Protein Matrix

HP added, nmol of HP/mg of BSA	recovery, nmol of HP/mg of BSA	recovery rate, %
0.014	0.013	94.0
0.069	0.064	92.5
0.138	0.134	96.5
0.277	0.263	95.1
0.830	0.781	94.1

hydrolysis conditions are described by Roach and Gehrke (1970). For the preparation of the HP standard, these conditions were not suitable because of the large volumes that have to be handled.

Phosphocellulose Cleanup. The crude hydrolysates were precleaned and filtered through a cellulose acetate membrane filter and then transferred to a phosphocellulose column (H^+-form, 2.6 × 30 cm). After the column was washed with 0.1 M HCl (450 mL) to remove the bulk of amino acids, pyridinolines were eluted with 0.5 M HCl (100 mL). The flow rate was 2 mL/min. The pyridinoline fraction was used for further investigation; the other fractions were discarded. The pyridinoline fraction was evaporated and the residue taken up in 0.01 M HCl and diluted with the same volume of eluent B.

HPLC Conditions. Different eluent compositions described under Results and Discussion were tested to determine optimal conditions for rapid elution and complete separation of HP and LP from a reversed-phase HPLC column. Finally, pyridinolines were determined by ion-paired reversed-phase HPLC with sodium octanesulfonic acid as the ion-pairing agent. Elution of pyridinolines was performed with two eluents using a linear gradient program starting at 60% eluent A/40% eluent B time 0, followed by linear gradients, to 40% A/60% B for 1 min and then to 15% A/85% B in 14 min. A flow rate of 1 mL/min was used. For the compositions of eluents A and B, see Reagents.

RESULTS AND DISCUSSION

Phosphocellulose Purification. Recovery of HP was determined by spiking hydrolysates of bovine serum albumin (BSA) as a protein matrix with standard solutions of 0.01−0.83 nmol of HP/mg of BSA. The HP content in these samples was determined by HPLC before and after cleanup using the HPLC conditions described for analysis of m.stern and ligamentum nuchae. Recovery rates after cleanup ranged between 92 and 97%. These results indicate that HP was not bound irreversibly to phosphocellulose. Results of the recovery experiments are summarized in Table 2.

Reversed-Phase HPLC. Because of their ionic character, determination of pyridinolines is only possible using ion-pairing agents. Different ion-pairing agents and buffers were tested for their suitability for the separation of pyridinolines. The use of quaternary amines as ion-pairing agents (tetramethylammonium bromide, tetrabutylammonium bromide, and *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide) led to insufficient retention of the pyridinolines at pH 7.2. At higher pH value retention is increased; however, at these pH values the alkali-labile pyridinolines are destroyed.

Eyre et al. (1984b) described an HPLC method for the determination of HP and LP using heptafluorobutyric acid (HFBA) as the ion-pairing agent. HFBA is reactive and volatile, which in our experience affects buffer stability and reproducibility of separation. Moreover, HFBA obtained from several manufacturers contained fluorescent contaminations which gave rise to wide peaks in the pyridinoline area. Black et al. (1988) have described the same phenomenon.

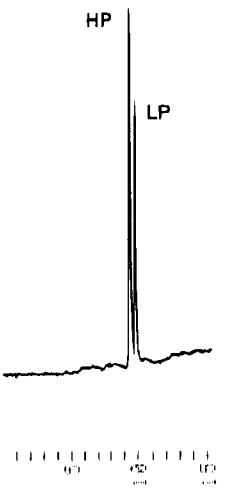


Figure 3. Reversed-phase HPLC chromatogram of a standard solution containing hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).

Acetate and citrate buffers were initially applied to adjust a pH value to 2.5. This led to interfering signals and unstable baselines. These problems were overcome by using a phosphate buffer. Similar problems with acetate buffers were reported by Black et al. (1988). These authors recommended the use of ammonium chloride for eluent pH adjustment to 3.5. The buffer capacity of this system is too low. For this reason, they used it in combination with octanesulfonic acid as the ion-pairing agent and an acetate buffer. The pH value of 3.5 and the use of octanesulfonic acid as ion-pairing agent led to complete separation of HP and LP with retention times of pyridinolines of about 20 min.

In our experiment, the best separation of HP and LP was obtained with the gradient system described under Materials and Methods combining phosphate buffer and octanesulfonic acid. The resulting pH value of 2.5 led to shorter retention times. HP and LP eluted after 9.5 and 10.5 min, respectively. A representative chromatogram of a HP (49.8 pmol injected) and LP (33.5 pmol injected) standard solution is given in Figure 3.

Fluorescence detection of the pyridinolines gave linear correlations (each $r = 0.99$) between 2 and 83 pmol of HP and 2 and 55 pmol of LP injected. The limit of detection was 2 pmol injected for HP as well as for LP. The fluorescence signal per mole of HP is nearly the same as that per mole of LP. The variation coefficient calculated from 10 successive analyses was 3.7%.

A number of interfering peaks appeared in chromatograms of hydrolysates of muscles with low collagen content, so that identification and quantitation of pyridinolines was impossible in such samples (Figure 4A). Therefore, it was necessary to extract some of the noncollagenous tissue substances from these samples as described under Materials and Methods before they could be accurately assayed for pyridinoline content. Hydrolysates of tissues with high amounts of collagen containing abundant pyridinolines such as tendon, ligament, or epimysium can be assayed directly, however, without a preceding cleanup step. A cleanup procedure of hydrolysates is recommended to ensure accurate determination of the pyridinolines. Figure 4B shows a chromatogram of the same sample as in Figure 4A but including the cleanup step.

Pyridoxine was applied as an internal standard at first. It shows fluorescence properties similar to those of the pyridinolines and elutes after 13.5 min. However, reproducibility was not improved using this internal

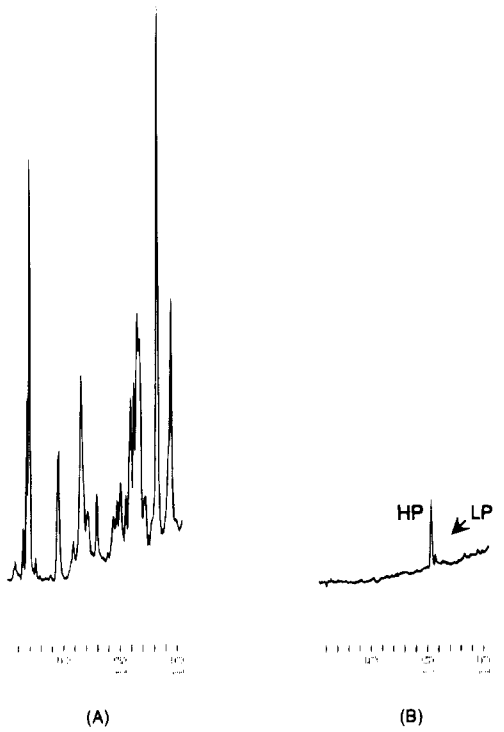


Figure 4. Reversed-phase HPLC chromatogram of an intramuscular collagen hydrolysate before (A) and after (B) phosphocellulose cleanup.

Table 3. Collagen and Pyridinoline Contents in Bovine Sternomandibularis Muscle Depending on the Age of the Animals

age of animals, years	collagen, % of dry mass: muscle	collagen, % of dry mass: isolate after sieving	pyridinoline content, mol/mol of collagen: HP	LP	total
1.9	5.3	47.4	0.193		0.193
3.2	5.4	34.0	0.175	0.023	0.198
3.4	3.5	36.1	0.216	0.010	0.226
4.9	7.0	49.4	0.207	0.034	0.241
5.5	6.0	26.5	0.221	0.026	0.246
7.1	7.5	43.2	0.211	0.028	0.239
9.8	4.2	50.0	0.235	0.026	0.261
11.5	4.9	48.5	0.275	0.008	0.284

standard. In fact, there is a disadvantage when this internal standard is applied. Interfering peaks occur in the region where pyridoxine is eluted when muscle samples are analyzed. In accordance with the study of Black et al. (1988), an internal standard was not applied in our experiment.

Analysis of Sternomandibularis and Ligamentum Nuchae Samples. Collagen contents of m.stern samples determined by hydroxyproline analysis varied from 4 to 7% (related to dry mass), whereas their connective tissue isolates had collagen contents approximately 10 times higher after sieving (Table 3). Pyridinolines were detected with the HPLC method described. The concentrations of HP and LP in the samples were calculated by interpolating the peak areas using the appropriate standard curves (Figure 5). Amounts of HP and LP found in the samples are summarized in Table 3. The results shown in this table are based on two determinations of each sample. As in other collagenous connective tissues, in bovine m.stern HP is the dominant component of intramuscular connective tissue pyridinolines. LP contents are smaller. LP comprises about 5–10% of the total pyridinolines. In muscle collagen from m.stern, the sum of HP and

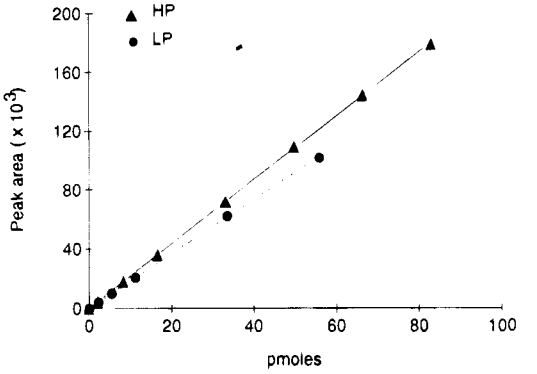


Figure 5. Calibration curves for hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) standards.

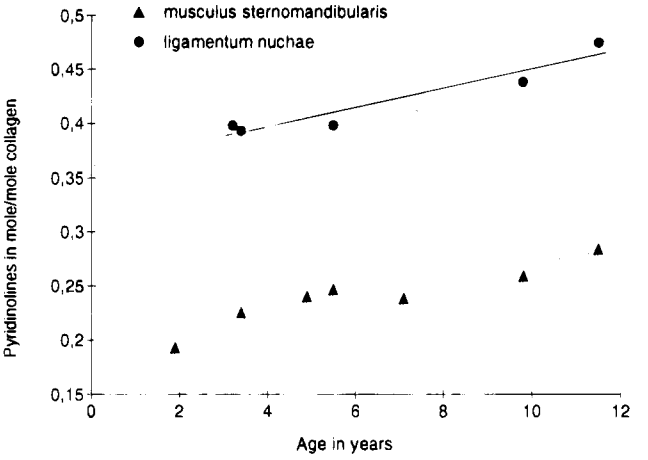


Figure 6. Dependence of pyridinoline content on age in bovine musculus sternomandibularis and ligamentum nuchae.

LP contents ranged from 0.19 to 0.28 mol of pyridinoline/mol of collagen. Pyridinoline amounts increase with advancing age of the animals. This correlation is shown in Figure 6.

Ligamentum nuchae exhibited pyridinoline contents of 0.39–0.47 mol/mol of collagen, whereas LP could not be detected in this tissue. The pyridinoline content results completely from HP. Collagen cross-linkage of ligamentum nuchae increases upon aging of the animal (Figure 6).

Collagen of ligamentum nuchae is a component of a tissue which is heavily stressed by tensile force. Its pyridinoline content is nearly twice that of the less stressed intramuscular collagen of m.stern.

These results indicate a marked correlation between the degree of cross-linkage and the physical function of the connective tissue. This has also been reported by Eyre et al. (1984a), who examined cross-linkage in tendon and ligament. They correlated differing pyridinoline amounts with the physical strain of the tissue.

We may conclude that pyridinolines seem to be one possible parameter in describing meat quality. A systematic investigation is underway to test this hypothesis.

ABBREVIATIONS USED

BSA, bovine serum albumin; HCl, hydrochloric acid; HFBA, heptafluorobutyric acid; HP, hydroxylysylpyridinoline; HPLC, high-performance liquid chromatography; LP, lysylpyridinoline; m.stern, sternomandibularis muscle; RP-HPLC, reversed-phase high-performance liquid chromatography; NMR, nuclear magnetic resonance.

ACKNOWLEDGMENT

We thank the Deutsche Forschungsgemeinschaft for financial support and the Schaumann Foundation for HPLC equipment. We also thank D. Fujimoto, Department of Chemistry, Hamamatsu University School of Medicine, Hamamatsu, Japan, and S. P. Robins, Biochemistry Division, Rowett Research Institute, Aberdeen, Scotland, for providing pure HP and LP. This paper is dedicated to Dr. h.c. mult. M. Kirchgessner, Technical University of Munich, on the occasion of his 65th birthday.

LITERATURE CITED

Bailey, A. J. In *Recent Advances in the Chemistry of Meat*; Bailey, A. J., Ed.; The Royal Society of Chemistry: London, 1984; pp 22–40.

Black, D.; Duncan, A.; Robins, S. P. Quantitative Analysis of the Pyridinium Crosslinks of Collagen in Urine using Ion-paired Reversed-Phase High-performance Liquid Chromatography. *Anal. Biochem.* **1988,** *169*, 197–203.

Eyre, D. R. In *The Chemistry and Biology of Mineralized Connective Tissues*; Veis, A., Ed.; Elsevier/North Holland: Amsterdam, 1981; pp 51–55.

Eyre, D. R. Collagen Crosslinking Amino Acids. *Methods Enzymol.* **1987,** *144*, 115–139.

Eyre, D. R.; Paz, M. A.; Gallop, P. M. Cross-linking in Collagen and Elastin. *Annu. Rev. Biochem.* **1984a,** *53*, 717–748.

Eyre, D. R.; Koob, T. J.; van Ness, K. P. Quantitation of Hydroxypyridinium Crosslinks in Collagen by High-Performance Liquid Chromatography. *Anal. Biochem.* **1984b,** *137*, 380–388.

Fujii, K.; Tanzer, M. L. Age Related Changes in the Reducible Crosslinks of Human Tendon Collagen. *FEBS Lett.* **1974,** *43*, 300–303.

Fujimoto, D.; Moriguchi, T. Pyridinoline, a Non-reducible Crosslink of Collagen. *J. Biochem.* **1978**, *83*, 863–867.

Fujimoto, D.; Akiba, K.; Nakamura, N. The Structure of Pyridinoline, a Collagen Crosslink. *Biochem. Biophys. Res. Commun.* **1977**, *76*, 1124–1129.

Light, N. D.; Bailey, A. J. Covalent Cross-links in Collagen. *Methods Enzymol.* **1982**, *84*, 360–372.

Möller, C.; Steinhart, H.; Bosselmann, A. Isolation of Bovine Intramuscular Connective Tissue. *Z. Lebensm. Unters. Forsch.* **1993**, *196*, 415–416.

Roach, D.; Gehrke, C. W. The Hydrolysis of Proteins. *J. Chromatogr.* **1970**, *52*, 393–404.

Robins, S. P. Analysis of the Crosslinking Components in Collagen and Elastin. *Methods Biochem. Anal.* **1982**, *28*, 329–379.

Robins, S. P.; Shimokomaki, M.; Bailey, A. J. The Chemistry of the Collagen Cross-links. Age-related Changes in the Reducible Components of Intact Bovine Collagen Fibres. *Biochem. J.* **1973**, *131*, 771–780.

Shimokomaki, M.; Elsden, D. F.; Bailey, A. J. Meat Tenderness: Age related Changes in Bovine Intramuscular Collagen. *J. Food Sci.* **1972**, *37*, 892–896.

Received for review January 24, 1994. Accepted June 8, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1994.