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The Fate of Intramuscular Connective Tissue in Aged Beef

D.W. Stanley and R.G. Brown
Departments of Food Science and Animal and Poultry Science
University of Guelph, Guelph, Ontario, Canada

Summary

The objective of this research was to investigate the effect of aging on intramuscular connective tissue in beef muscle. Intramuscular connective tissue was isolated from bovine psoas and semitendinosus muscles aged 0,3,6 and 13 days using salt, acid and guanidine hydrochloride as extractants. This material was heat denatured and separated on carboxymethylcellulose columns. Total hydroxyproline and hexosamines were determined on freeze-dried samples. Post-mortem aging of muscle seems to produce differences in intramolecular cross-linking of the collagen molecule as evidenced by changes in elution patterns. Also, the total amount of extractable collagen increased from 59% of that in the muscles at day 0 to 88% by day 13. As the severity of the extraction process increased, differences in elution patterns were noticed earlier in the aging period. For example, significant changes were noted in guanidine extracts between days 0 and 3. Although the collagen fractions have not been positively identified by amino acid analysis or molecular weight determinations, aging appears to result in the disappearance of larger, less charged molecules and the appearance of smaller, higher charged molecules. These results may be attributed to a post-mortem breakdown of higher molecular weight components (dimers and trimers) or to selective changes in extractability, although the former seems more likely. It is possible that this phenomenon may prove a significant factor in meat tenderness.

Le maintien des tissus connectifs intramusculaires dnas le boeuf agé.

D.W. Stanley et R.G. Brown

Département de Science Alimentaire et Département de Science Aviculture
Université de Guelph, Guelph, Ontario, Canada

EXTRAIT

L'objectif de cette recherche est de sonder les effets de vieillissements des tissus connectifs intramusculaires dans le muscle du boeuf. Les tissus connectifs intramusculaires sont isolés des muscles du psoas bovin et du muscle semi-tendineux vieillis de 0,3,6 et 13 jours en utilisant du sel, de l'acide et du guanidine hydrochlorine comme extractibles. Ce matériel a subi une action de chaleur dénaturé et a été dissocié par colonnes de celluloses carboxy-methyl. Les totaux hydroxyprolines et hexosamines ont été déterminés sur des échantillons séchés à froid. Le vieillissement post-mortem du muscle semble produire des différences dans le lien croisé intra-moléculaire de la molécule collagène et porte à évidence le changement du système d'épuration. Aussi le montant total de collagène extractible a augmenté de 59% de celui des muscles par jour 0 à 88% par jour 13. Comme la rigueur du processus d'extraction augmente, des différences dans le processus d'épuration ont été remarquée durant la période de vieilissement. Par exemple, des changements significatifs ont été remarqués dans les extraits de gianidines entre 0 et 3 jours. Quoique des fractions de collagène n'ont pas été identifiées positivement par l'analyse amino acide ou en déterminant les poids moléculaire, le vieillissement paraît résulter par la disparition des molécules plus grandes et moins chargés et par l'apparition des molécules plus petites et moins chargés. Ces résultats peuvent être attribués à une rupture post-mortem du poids des parties constituantes des molécules plus élevées(dimères & trimères)ou des changements sélectifs extractibles, malgré que le premier soit plus vraisemblable. Il est possible que ce phénomène prouvera des facteurs significatifs en ce qui concerne la production de viande tendre.

Das Verhalten des intramuskulären Bindegewebes bei abgelagertem Rindfleisch.

Zusammenfassung.

Das Ziel dieser vorliegenden Untersuchung war die Erforschung des Alterungseffekts auf das intramuskuläre Bindegewebe bei Rinder-Muskeln. Intramuskuläres Bindegewebe wurde von Rinder-Lendenmuskeln und von semitendinösen Muskeln, die 0, 3, 6 bzw. 13 Tage lang gealtert hatten, mit Hilfe von Salz, Säure und Guanidin-Hydrochlorid als Extraktionsmitteln isoliert.
Das gewonnene Material wurde mit Hitze denaturiert und auf Karboxymethylzellulose-Säulen abgeschieden. Hydroxyprolin und Hexosamin wurden an gefrier-getrockneten Proben bestimmt.
Der Postmortem-Alterungsprozeß des Muskels scheint Unterschiede in den intramolekularen Querverbindungen des Kollagen-Moleküls zu erzeugen, wie aus den Veränderungen des Elutions-Verhaltens hervorgeht. Auch stieg die Gesamtmenge des aus den Muskeln extrahierbaren Kollagen von 59 % am Tage 0 auf 88 % am 13. Tag. Bei gesteigerter Effektivität des Extraktions-Vorganges wurden Unterschiede im Elutionsverhalten schon früher während des Alterungsprozesses festgestellt. So wurden bei Guanidin-Extrakten schon zwischen 0 und 3 Tagen erhebliche Unterschiede bemerkbar. Obgleich die Kollagen-Fraktionen nicht durch Aminosäuren-Analyse oder Molekular-gewichts-Bestimmung eindeutig identifiziert wurden, scheint der Alterungsprozeß ein Verschwinden größerer Moleküle mit geringer Ladung und ein Auftauchen kleinerer Moleküle mit höherer Ladung zur Folge zu haben.

Dieses Resultat könnte einem Postmortem-Zerfall von Komponenten mit hohem Molekular-Gewicht (Dimere und Trimere) oder einer selektiven Veränderung in der Extrahierbarkeit zugeschrieben werden. Die erste Möglichkeit scheint die wahrscheinlichere zu sein. Es ist möglich, daß sich dieses Phänomen als ein wichtiger Faktor für die Zartheit von Fleisch erweist.

История внутримышечной соединительной ткани в выдержанной говядине

Д.В.Стенли и Р.Г.Браун

Отделы пищевой, животноводческой и птицеводческой наук
Гуэлфский Университет, Гуэлф, Онтарио, Канада

Р е з ю м е

Целью этого исследования было изучение влияния выдерживания на соединительную ткань в бычачьей мышце. Внутримышечная соединительная ткань была выделена из поясничной и полужилистой бычачьей мышцы, выдержанной 0, 3, 6 и 13 дней, употребляя соль, кислоту и хлористоводородный гуанидин , как вещества, при помощи которых была сделана вытяжка. Вытяжка была денатурирована теплом и разделена на углероднометилцеллюлозных столбиках. Весь гидроксипролин и все гексозамины были определены на образцах, приготовленных сушкой сублимацией. Послеубойное выдерживание мышцы, казалось, создает различия во внутримолекулярной поперечной связи молекул коллагена, как показывают изменения в образцах вымывания. Увеличилось также общее количество извлекаемого коллагена с 59 проц. из мышцы, выдержанной 0 дня, до 88 проц. к 13 дням. С увеличением суровости процесса извлечения различия в образцах вымывания в период выдерживания были замечены ранее. Например, важные перемены были замечены в вытяжках гуанидином. между 0 и 3 днями. Хотя коллагеновые фракции не были определенно опознаны амино кислотным анализом или измерениями молекулярного веса, выглядит, что в результате выдерживания происходит исчезновение больших менее заряженных молекул и появление меньших более заряженных молекул. Эти результаты можно объяснить послеубойным разложением компонентов более высокого молекулярного веса (состоящих из двух и трех частей) или выборочных изменениях в извлекаемости, хотя первое кажется более выроятным. Возможно, что это явление может стать важным фактором в мягкости мяса.

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Department of Food Science and Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada

Introduction:

It is widely known that post-mortem aging of beef tissue results in cooked meat that is more tender whether measured objectively or subjectively. Three factors which may influence this phenomenon are microbial action, catheptic activity and breakdown of connective tissue. The latter is the subject of this paper. Previous work in our laboratory and elsewhere has led to the conclusion that knowing the total hydroxyproline content of muscle is of little value in predicting tenderness when comparing the same muscle from different animals with similar backgrounds, although one can distinguish different muscles within an animal by this method.

The degree of cross-linking in intramuscular connective tissue has been postulated to affect tenderness (Shimokomki et al., 1972). Attempts have been made to determine if post-mortem aging produces changes in cross-linking characteristics and the differences found were suggestive of collagen breakdown (Pfeiffer et al., 1972; Kruggel and Field, 1971). The purpose of the present experiments was to investigate the effect of post-mortem aging on the chemical composition of intramuscular connective tissue.

Methods and Materials:

The semitendinosus and psoas major muscles were removed from five Angus steers slaughtered at the University abattoir. The

muscles were maintained at 0-5°C in sealed plastic bags and samples of approximately 200g removed 0,3,6 and 13 days post-mortem. These samples were trimmed and immediately freeze-dried and then ground with dry ice in a Waring blendor. All subsequent chemical analyses were done in duplicate.

A portion of the tissue was trimmed and defatted with cold acetone. Approximately 20 mg. samples were accurately weighed for total hydroxyproline and elastin determinations. Another 100 mg was taken for total hexosamine. These measurements were done by the Auto-Analyzer method of Grant (1964).

A 2 g sample of total tissue was serially extracted by mechanical shaking with 10 volumes of 1M NaCl followed by 1N Hac. Another 2 g sample was extracted with 1N Hac followed by saturated guanidine HCl (pH approximately 12). Guanidine extractions were performed at room temperature; salt and acid extractions at 5°C. Extractions were carried out 4 times with each extractant for 24h. At the end of each 24h period the sample was centrifuged for 30 min at 13,000 x g. Supernatants for each extraction were pooled and the residue was re-extracted.

The above procedure resulted in 4 final solutions per sample: 1) initial 1M NaCl extraction, 2) initial 1N HAc extraction, 3) secondary 1N HAc extraction of 1) and 4) secondary guanidine extraction of 2. Solutions 1, 3 and 4 were dialized first against H_2O and then 0.5N HAc (guanidine extracts were dialyzed against H_2O only) for 24h at 5°C. Following both treatments the solutions were centrifuged at 43,000 x g for 2h and the sediment discarded. The final supernatant was reduced in volume by freeze-drying to 2-3 ml. All of this material was applied to a carboxymethyl-

cellulose column maintained at 40°C and subsequently eluted with an acetate buffer gradient at pH 4.8 using the method of Bornstein and Piez (1964).

Five ml fractions were collected and read at 230 nm in a Unicam SP800 recording spectrophotometer fitted with a 40°C constant temperature block. It was observed that the effluent did not absorb at 230 nm if read at 20°C, but did absorb strongly at 40°C. This indicates that any contaminating muscle proteins not previously removed did not interfere at the wavelength employed. Fractions were tentatively identified by retention volumes using the nomenclature of Bornstein and Piez (1964). Aliquots were taken from the effluents for total hydroxyproline measurement using the Auto-Analyzer method.

Results and Discussion:

Total hydroxyproline and hexosamine in freeze-dried muscle.

The variation in these substances with post-mortem aging is shown in Figs. 1 and 2. Analysis of variance of these data (Table 1) indicates that for total hydroxyproline a significant effect was found for muscles and animals as was expected. A more surprising result was the appearance of variations due to aging. This was not expected and since no trend is visable and the two muscles are not consistent in their response it seems likely this may be a random sampling effect with no biological significance.

The hexosamine data are more consistent with respect to muscles and animals but again the result of aging is seen. It seems unlikely, however, that a real change in ground substance would occur. No elastin was detected in any of the samples.

Initial salt extraction. Two major fractions occur in this extract,

α1 and α2 monomers. Other smaller peaks were occasionally found between the α peaks, probably β dimers, but these were not consistently observed in our elution patterns. Using acrylamide disc gel electrophoresis, McClain et al. (1971) have reported a α1 and 2 ratio of 2:1; our results are on the basis of peak height rather than area but do show α1 as the major component.

Analysis of variance (Table 2) showed a very significant decrease in the α2 component at the expense of the α1 component during aging, the largest decline occurring between days 6 and 13. Examination of the results on the basis of peak height showed that α1 was more predominant in psoas at all time periods while α2 was higher in semitendinosus. McClain et al. (1970) have observed variations in collagen components for different muscles. No change in α1 level was observed with either muscle during aging but α2 increased significantly through day 6. The data for α2 as a percentage of the total extractable material is shown in Fig. 3.

When the total hydroxyproline of the salt extracted collagen was expressed as a percentage of the total extractable collagen (Fig. 4) the former was found to decrease significantly at the expense of the guanidine fraction (Fig. 10) which extracted from 2 to 5 times as much material as salt. An indication that a greater total extractability results from aging is given by summing the total hydroxyproline recovered from the serial extraction steps and comparing this with values for the freeze-dried material. It was found that while at day 0, 59% of the intramuscular hydroxyproline was recovered, this figure had increased significantly to 88% by day 13. McClain et al. (1971) found they

could isolate 60-70% of the total collagen from unaged muscle.

Subsequent acid extraction. Again two major fractions were found, β11 and β12. Figure 5 shows these results in terms of β12 as a percent of the total. Most of the α material seemed to have been extracted by the prior salt treatment and these peaks were not routinely present in the column effluent. Although the influence of aging proved non-significant (Table 2) this may be misleading in that it is a single figure for grouped data. Examining the two muscles separately it may be seen that with semitendinosus there is a steady decrease from 31% at day 0 to 12% at day 13. The major decline occurs between day 3 and 6. The same is true for psoas with the exception that no β12 was found in the day 0 samples.

Comparison of peak heights showed that in both muscles the two β components increased very significantly with time post-mortem. This difference was significant by day 3. At this point it is difficult to determine if this was due to breakdown of a higher molecular weight compound (γ trimer) or to increased extractability.

The level of total hydroxyproline in the sample was so small it could not be measured accurately but it undoubtedly represented the difference between the sum of the salt and guanidine extractions and the total (about 10%).

Guanidine extraction. This extraction proved the most complex and the hardest to interpret. Extraction of fresh muscle (Fig.6) gave three major peaks which were identified, judging from retention volumes and subsequent behaviour, as two undifferentiated peaks (α1 and β11 followed by β12 and α2) coming before

a large, uncharged group of molecules tentatively identified as 'γ' collagen. The effect of post-mortem aging on this system was very evident. A typical elution pattern of semitendinosus muscle aged 13 days (Fig. 7) shows that the larger heavier peak has declined while the smaller collagen molecules have increased and a better separation is seen. We have deciphered these results as a function of aging time in Figs. 8 and 9.

Statistical analyses were difficult to perform on these data because of the emergence of peaks through aging. An analysis of variance was conducted on the 'γ' peak since this was the only component present at all time periods. Table 2 shows the decrease in 'γ' as a per cent of the total with time was very highly significant while no difference was noted between the two muscles. A subsequent Duncan's test indicated that for psoas muscle a significant reduction of 'γ' collagen occured between days 0 and 3 and also between 6 and 13. A simple correlation of the two muscles from the same animals over the entire aging period gave a correlation coefficient of + 0.87 ($P<.01$).

Analysis of the guanidine extracted collagen as a per cent of the total extractable collagen showed that aging resulted in an increased extraction by this solution as mentioned previously (Fig. 10).

General discussion: The major findings of this series of experiments may be summarized as follows:

1. Intramuscular connective tissue was isolated from bovine psoas and semitendinosus muscles with various extractants, heat denatured and separated on carboxymethylcellulose columns.

2. Post-mortem aging of these tissues seems to produce changes in intramolecular cross-linking of the collagen molecules as evidenced by changes in elution patterns. Also the total amount of extractable collagen increases with time.
3. In general, the two muscles behaved similarly during aging but some specific differences were noted.
4. As the severity of the extraction processes increased, differences in elution patterns were noted earlier in the aging period. Significant differences were noted between days 0 and 3 in guanidine extracts.
5. Although the collagen fractions have not been positively identified by amino acid analysis or molecular weight determination aging appears to result in the disappearance of large, less charged molecules and the appearance of smaller, higher charged molecules.

These results may be attributed to a post-mortem breakdown of higher molecular weight components or to selective changes in extractability. Guanidine extracted collagen showed an orderly decrease in larger molecules and an increase in smalller molecules over the aging period which suggests a causal relationship. The reason for these changes is not yet clear but it has been suggested that there are proteolytic enzymes present in meat capable of solubilizing collagen (Kruggel and Field, 1971).

Since it has been implied that meat tenderness may be influenced by the degree of cross-links in the intramuscular connective tissue (Shimokomaki et al., 1972) the results of our experiments may be important from the aspect of meat organoleptic quality. We have evidence for beef psoas muscle that certain tensile properties,

uncooked shear force and two taste panel parameters chew count) and tenderness) all decreased significantly by day 3-4 post-mortem (Eino and Stanley, 1973; unpublished data). Collagen has a very great tensile strength as compared to muscle proteins and any factor affecting collagen structure may prove significantly in meat tenderness. At present it is difficult to relate behaviour of intramuscular connective tissue in a guanidine extract to eating quality but more must be known about how the former affects the latter.

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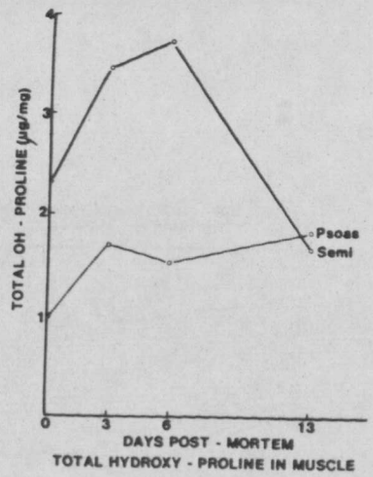
TABLE 1. Analysis of variance for collagen fractions in unextracted muscle. Data given are probabilities.

Source	d.f.	Total Hydroxyproline (μg/mg)	Hexosamine (μg/mg)
Days	3	.0007	.0214
Muscles	1	.0000	.1720
Animals	4	.0242	.7975

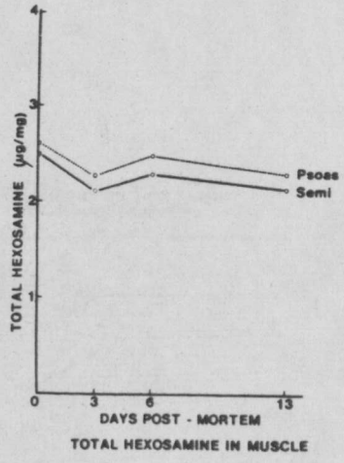
Table 2. Analysis of variance for extracted collagen fractions. Data given are probabilities.

Source	d.f.	Initial Salt Extraction		Subsequent Acid Extraction	Guanidine Extraction (% of total)	
		$\alpha 2$ as % of total	total OH Proline as % of total	$\beta 12$ as % of total	'γ'	Total OH Proline as % of total
Days	3	.0003	.0172	.2708	.0000	.0255
Muscles	1	.0596	.8238	.1507	.4026	.0462
Animals	4	.0006	.3533	.5032	.2936	.2781



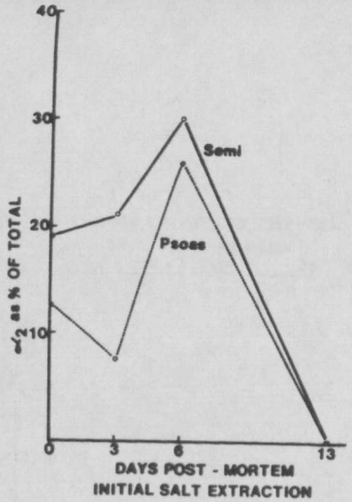
TOTAL HYDROXY - PROLINE IN MUSCLE

1



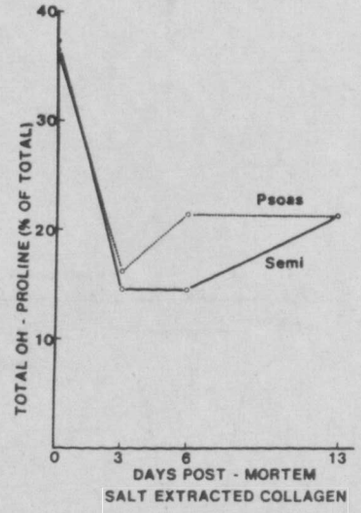
TOTAL HEXOSAMINE IN MUSCLE

2



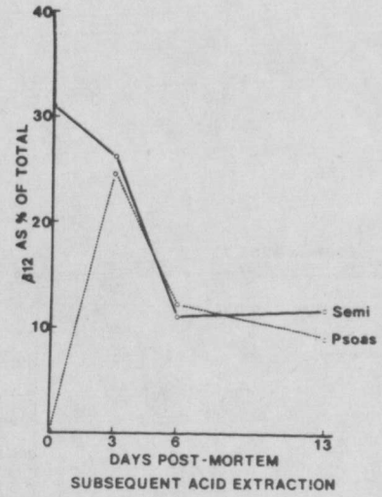
INITIAL SALT EXTRACTION

3



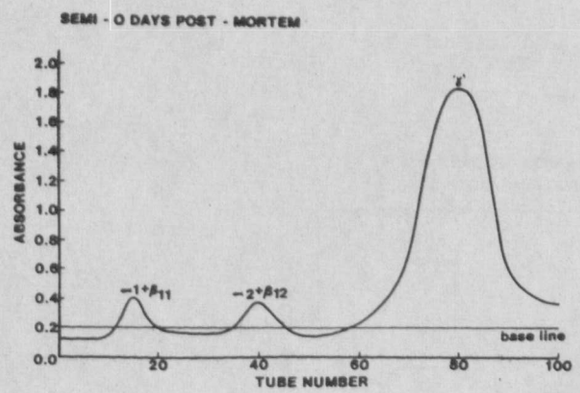
SALT EXTRACTED COLLAGEN

4

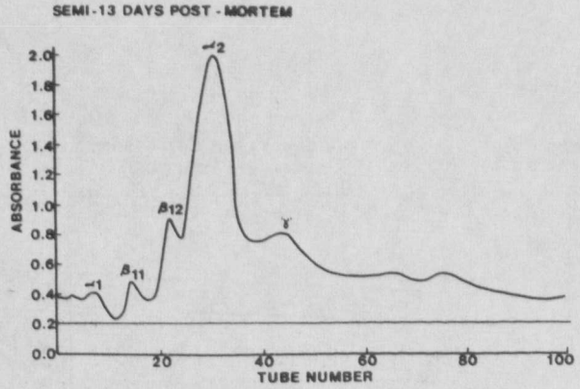


SUBSEQUENT ACID EXTRACTION

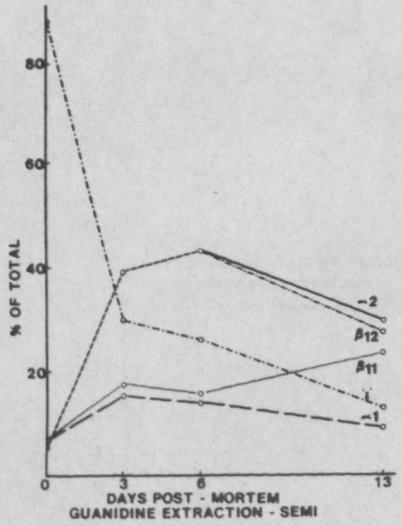
5


SEMI - O DAYS POST - MORTEM
ABSORBANCE
TUBE NUMBER
base line

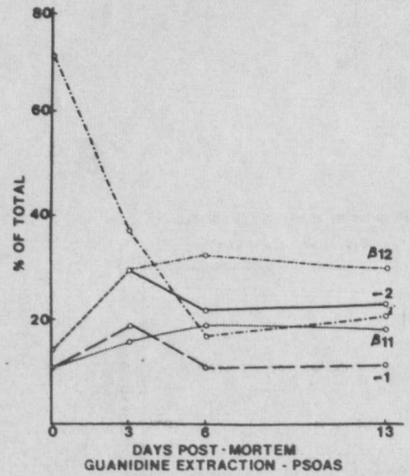
6


SEMI - 13 DAYS POST - MORTEM
ABSORBANCE
TUBE NUMBER

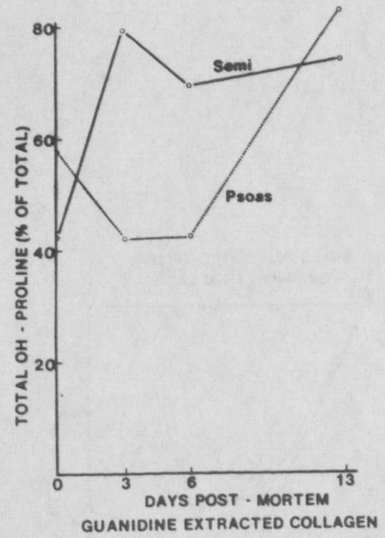
7


% OF TOTAL
DAYS POST - MORTEM
GUANIDINE EXTRACTION - SEMI

8


% OF TOTAL
DAYS POST - MORTEM
GUANIDINE EXTRACTION - PSOAS

9


TOTAL OH - PROLINE (% OF TOTAL)
Semi
Psoas
DAYS POST - MORTEM
GUANIDINE EXTRACTED COLLAGEN

10