IMPLICATIONS OF 3-METHYLHISTIDINE TITRES OF ACTIN AND MYOSIN IN DETERMINING MEAT PROTEIN.

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ABSTRACT.

The widespread use of non-meat protein in meat products necessitates a method for the robust, unequivocal quantification of meat in foods. Protein-bound levels of the co- or post-translationally modified amino acid, 3-methylhistidine, virtually unique to the myofibrillar proteins actin and myosin, have previously been proposed as such an index, determination being by high-performance liquid chromatography of acid stable fluorescamine derivatives.

Although robust to the severest processing conditions, variations in the titres of 3-methylhistidine in certain manufacturing cuts have been reported. The present study has revealed that such variations can be attributed to the low level of 3-methylhistidine in the myosin of muscles high in "red" (oxidative) fibres, such as ruminant Masseter; constant levels of 3-methylhistidine being found in all actins investigated.

Methods for the determination of actin-bound 3-methylhistidine have therefore been developed. Electrophoretic separation of actin with 3-methylhistidine determination of the resulting actin band was found to be only semi-quantitative. The isolation of actin by conventional SDS-gel filtration was time consuming and resulted in low yields of 3-methylhistidine. SDS-gel filtration using the Pharmacia fast protein liquid chromatography (FPLC®) system, allowed rapid, reproducible and quantitative isolation of actin-bound 3-methlhistidine. Using the latter method, constant levels of actin-bound 3-methylhistidine have been found for all muscles investigated. A new unequivocal definition of "meat", is proposed as that which has an actin-bound 3-methylhistidine content of 3mg/g non-connective tissue nitrogen. This is expected to be robust to all but the severest processing conditions.

Such an index, based on connective tissue free units, requires the accurate determination of hydroxyproline, for which a sensitive method using gas chromatography-mass spectrometry has been developed. The use of an assumed average "factor" for the conversion of hydroxyproline to connective tissue appears valid, since the hydroxyproline contents of the connective tissue of all muscles investigated were similar.

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To my parents

and Astrid.

1. INTRODUCTION.

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Meat is generally accepted as the main source of high quality protein in the human diet, and as such, demands a high price. The recent advances in food technology and processing, now enable the replacement of meat protein by less expensive protein, such as that of vegetable and microbial origin, without reducing the palatability and apparent "quality" of the product. This manufacture of "traditional" products from "novel" ingredients gives the opportunity for consumer deception if methods are not established for the unequivocal identification of meat in food.

Ever since 1919, when Stubbs and More, devised their classical formula for the determination of the meat content of sausages, a search has been made for a parameter that is a robust, unequivocal and specific index of meat content; that is, robust to all of the processing conditions encountered in food manufacture, unequivocal regardless of the intracarcass and interspecies origin of the meat and, specific to meat protein, being absent from all other protein sources likely to be encountered in meat products.

Before the use of non-meat protein sources in meat products (typified by soya protein), a reasonable estimation of meat content could be obtained by methods based on nitrogen content, using formulae originating from that of Stubbs and More. Adequate correction for nitrogen due to carbohydrate fillers and added connective tissue could be made by modifications to such formulae. The onset of the use of non-meat proteins in meat products precluded the use of such methods, since no correction

for such protein sources could be made. A method was required that relied on a parameter that was specific to meat and absent from all other proteins.

The "rare" amino acid, 3-methylhistidine, was first proposed as a meat specific index by Hibbert and Lawrie (1972). On initial investigation, levels of this amino acid appeared to be constant in a range of meats, unaffected by even the severest processing conditions, and absent from non-meat proteins. 3-Methylhistidine in fact appeared to fulfill the requirements of an ideal index for the determination of meat in food.

Developments in the methodology of 3-methylhistidine determination, allowed the more rapid and accurate quantification of the very low levels of this amino acid in meat, using the high performance liquid chromatography of acid stable fluorescamine derivatives (Jones, Shorley and Hitchcock, 1982a,b). The robust nature of the total protein-bound 3-methylhistidine titres, was not refuted; a dried soup powder, which had previously defied analysis, being sucessfully analysed (Jones, Shorley and Hitchcock, 1982a,b), although the unequivocal nature of such titres was in doubt. Analysis of a wide range of muscles and "cuts", commonly used as meat, has revealed a significant level of intracarcass variation in the total protein-bound 3-methylhistidine titres, specifically a very low titre for the bovine cheek muscles, Masseter and Malaris (Jones et al., 1985; White and Lawrie, 1985b), an inexpensive "cut" commonly used in comminuted meat products.

Actin and myosin are the only muscle proteins known to contain significant levels of 3-methylhistidine. Assuming that the ratios of the various (fat free, connective tissue free) muscle proteins are constant in striated muscle, regardless of its origin, variations in the total protein-bound 3-methylhistidine titres would be expected to be due to different levels of histidine methylation in actin and/or myosin. Histidine methylation in myosin has been shown to be under developmental control, and to vary depending on the fibre-type composition of the muscle. White and Lawrie (1985b) have reported that the low levels of total protein-bound 3-methylhistidine in bovine Masseter and Malaris (cheek) correlated with the low levels of this amino acid in the myosin purified from this muscle.

The major purpose of the present investigation, was to investigate the biochemical basis of the reported variation in the total protein-bound 3-methylhistidine titres of certain muscles used as meat. The aim of the research was then, on the basis of the results of the above investigations, to develop a method of meat determination based on 3-methylhistidine that was truly unequivocal. Considering the reported variations in the level of histidine methylation of myosin, and the highly conserved nature of actin, actin-bound 3-methylhistidine appeared to be a parameter of meat content suitable for investigation.

All 3-methylhistidine titres of meat are expressed in terms of connective tissue free units, since connective tissue does not contain 3-methylhistidine and the level of connective tissue in meat varies. Determination of connective tissue is

usually carried out by measuring hydroxyproline, a secondary amino (imino) acid virtually unique to this class of proteins, conversion to connective tissue being by an average "factor". The accurate determination of hydroxyproline and its conversion to connective tissue is therefore very important in preventing false assumptions being made when calculating the 3-methylhistidine titres of connective tissue free samples.

Another aspect of the present study has been to determine the validity of using a single average "factor" for the conversion of hydroxyproline to connective tissue. The development of an alternative method to the standard colorimetric method (BSI, 1979), for the determination of hydroxyproline in meat and meat products was also a subject of research. With the recent developments in gas chromatography-mass spectrometry, making the method more available and data interpretation simpler and more rapid, this method appeared suitable for investigation.

2. LITERATURE REVIEW.

2.1. BIOCHEMISTRY OF 3-METHYLHISTIDINE.

2.1.1. THE DISCOVERY OF 3-METHYLHISTIDINE.

Naturally occuring 3-methylhistidine (N^{τ} - methylhistidine, 3MeHis) was first identified in urine. Filter paper chromatography was used by Dent (1948), to resolve the amino acids in normal and pathological urine, using ninhydrin to visualise the resulting "map" of spots. A spot was identified as "methylhistidine" (in fact 1-methylhistidine), by comparison with the amino acids of the dipeptide anserine. An adjacent spot of unknown composition named the "green spot", was also reported. This was identified as an amino acid, since it survived hydrolysis. Searle (1951) carried out similar work on urine and also noted the occurance of an unknown spot, believed to be the "green spot" of Dent. Searle concluded on the evidence of physiochemical data obtained from the isolated "green spot" material, that it was in fact 1-methylhistidine, even though they (and Dent), had shown that authentic 1-methyl-histidine migrated in a neighbouring position. It seems more likely that the two spots were in fact 1 and 3-methylhistidine.

Work on the amino acid constituents of urine was continued by Stein (1953), using ion-exchange chromatography as the mode of separation, with detection by ninydrin. A peak of "methylhistidine" (1-methylhistidine) was identified in normal urine, as well as an unidentified peak "X" seen on the leading edge of the histidine peak. "Methylhistidine" was also reported in the chromatographic fractionation of the nitrogenous extracts

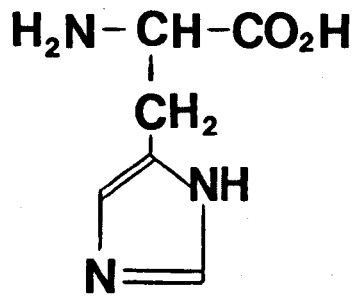
from gadoid, but not elasmobranch fish muscle (Shewan, 1953). At this time methylhistidines had not been linked with protein metabolism and were assumed to originate solely from dietary intake of histidine dipeptides from meat, Datta and Harris (1951), having shown that urinary excretion of "methylhistidine" was greatest in carnivorous species. Stein's work was continued by Tallan, Stein and Moore (1954), who identified the peak "X" as an amino acid, it being unaffected by hydrolysis. The component "X" was isolated by ion-exchange chromatography and tentatively identified as 3-methylhistidine ([S]-1-methylimidazole-4-alanine), the structure of which can be seen in figure 2.1, along with those of histidine, 1-methylhistidine and the dipeptide balenine (β -alanyl-3-methylhistidine). Identification of 3-methylhistidine was accomplished by elementary analysis and infra-red spectroscopy, along with physiochemical comparisons with the synthetic molecule. Virtually complete ion-exchange separation of 3-methylhistidine from histidine was accomplished, scaling-up of the separation, using litres of urine, permitting the isolation of the amino acid.

2.1.2. OCCURENCE OF 3-METHYLHISTIDINE.

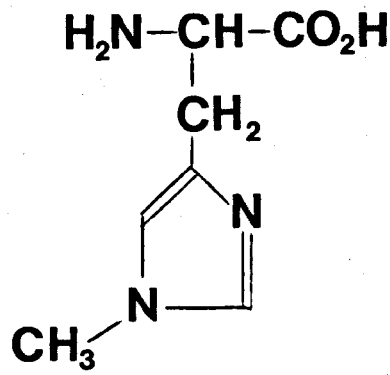
Positive identification of 3-methylhistidine in urine by Tallan, Stein and Moore (1954), and the discovery of the free amino acid in human blood plasma (Stein and Moore, 1954) and in feline kidney and blood plasma by Tallan, Moore and Stein (1954), did not give any clues to the source of this amino acid. The possibility that 3-methylhistidine was the product of the

Figure 2.1. Chemical structure of histidine, 3-methylhistidine, 1-methylhistidine and balenine (β-alanyl-3-methylhistidine).

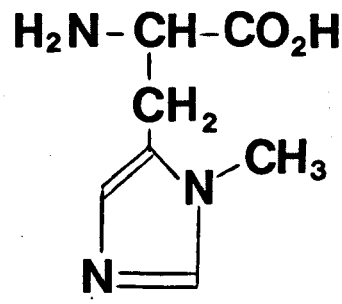
HISTIDINE.



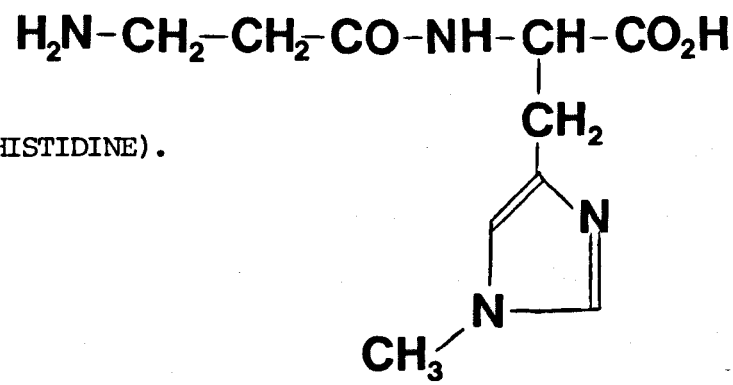
3-METHYLHISTIDINE.



1-METHYLHISTIDINE.



BALENINE
(β-ALANYL-3-METHYLHISTIDINE).



breakdown of protein-containing tissues was proposed by Leibholz (1968), who noted the increasing concentration of this amino acid in the blood plasma of sheep on starvation diets.

The presence of an "uncommon" amino acid, believed to be 1-methylhistidine from contaminating anserine (β-alanyl-1-methylhistidine), was reported by Kominz et al (1962), at a level of approximately 1 mole/60000g protein, in rabbit actin preparations (currently assumed molecular weight of actin, 41785 daltons; Elzinga et al., 1973) and 2 moles/60000g protein in Pharmoria (blowfly) actin preparations. The contamination of protein preparations by 1-methylhistidine from anserine was unlikely as it is now known that there are far higher levels of 3-methylhistidine than 1-methylhistidine, even in crude muscle preparations.

Asatoor and Armstrong (1967) confirmed the endogenous origin of 3-methylhistidine, when they administered labelled histidine and methionine to rats. The specific activity of the 1 and 3-methylhistidine in fasting rats was measured, the results indicating that histidine and the methyl group of methionine were the precursors of 3-methylhistidine. Highest levels of 3-methylhistidine were found in striated muscle. On purification 3-methylhistidine was found to be absent from tropomyosin, at low levels in myosin (currently assumed molecular weight, 480000 daltons; White, 1986) and at the level of slightly over 1 mole/60000g protein in purified actin. The identity of 3-methylhistidine in crude protein precipitates and purified actin was confirmed by its physiochemical behaviour compared with the

authentic amino acid. That it was part of the primary structure of actin was shown by subjecting the protein to conditions expected to dissociate any non-covalently bound constituents under which conditions the 3-methylhistidine content of the protein did not change.

Much important work on the biochemical occurence of 3-methylhistidine was carried out by Johnson, Harris and Perry (1967a,b). Routine amino acid analysis of actin was found to give a peak not corresponding to any known amino acid. This peak was identified as 3-methylhistidine by isolation, using paper electrophoresis, of actin hydrolysates, and like the authentic amino acid, it gave a distinctive colour with ninhydrin. Treatment to remove peptides from the actin preparation left the 3-methylhistidine content unchanged. This, along with the absence of β-alanine in amino acid elution profiles, was evidence against 3-methylhistidine being present in the form of an isomer of anserine. The possibility of methylation by the organic solvents, such as acetone (used during actin preparation) was dismissed since 3-methylhistidine levels were unchanged in actin prepared without the use of such solvents. The 3-methylhistidine contents of actins prepared from skeletal muscles of various species are shown in table 2.1, the actin preparations having a relatively constant 3-methylhistidine content. It was suggested that this "rare" amino acid might be a constituent of actins of all species. The 3-methylhistidine content of adult rabbit skeletal muscle myosin was found to be 1.8 - 1.9 moles / mole protein.

TABLE 2.1. 3-METHYLHISTIDINE CONTENT OF ACTIN FROM SKELETAL MUSCLE OF VARIOUS SPECIES.

SPECIES	3MEHIS:HIS RATIO	AMOUNT OF 3MEHIS (moles/10^5g actin)
RABBIT	1 : 7.60	2.10
HUMAN	1 : 8.62	
CHICKEN	1 : 7.46	
TROUT	1 : 10.1	
RABBIT FOETUS (28 day old)	1 : 11.8	

(From Johnson, Harris and Perry, 1967b).

The occurence of 3-methylhistidine in fish muscle actin at a level of 1 mole/mole actin, has been reported by Bridgen (1971), though Shenouda and Pigott (1975), failed to detect this amino acid in fish actin, and made the unlikely proposal that 3-methylhistidine may be absent from the actins of some fish species.

Smooth muscle proteins are known to contain 3-methylhistidine. Gosselin-Rey et al. (1969), purified bovine carotoid actin, and found it to be physically and chemically similar to that of skeletal muscle, with a 3-methylhistidine

content of 1.22 moles / 60000g protein. Both the myosin and actin of the smooth muscle of bovine uterus were found to contain levels of 3-methylhistidine, similar to those of mixed adult skeletal muscle (Johnson and Perry, 1970).

The occurance of 3-methylhistidine in Invertebrate (Lobster) muscle actin was reported at a level of 1.8 moles / mole actin by Kuehl and Adelstein (1970).

A number of proteins of non-muscle origin contain 3-methylhistidine. Weihing and Korn (1969,1971,1972), purified actin from the soil amoeba, Acanthamoeba castellanii. This actin was found to closely resemble muscle actin both physically and chemically, including the presence of 1 mole 3-methylhistidine / mole protein. Wooley (1972), found similar results when investigating the actin-like protein of the amoeba Dictyostelium discoidium and 1 residue of 3-methylhistidine was found in the amino acid sequence of actin from the slime mold Physarum polycephalum (Vandekerkhove and Weber, 1978).

Actomyosin-like protein (neurostenin) and actin-like protein (neurin) were isolated from bovine brain by Puszkin and Berl (1972). Neurin had many actin-like properties and contained 3-methylhistidine at a level of 0.33moles/50000g protein. An actin-like protein has also been isolated from the ganglion cells of the goldfish retina (Giulian, Des Ruisseaux and Cowburn, 1980),this protein containing a 3-methylhistidine to histidine ratio similar to that of muscle actin. Another retinal protein, rhodopsin has also been reported to contain 3-methylhistidine (Reporter and Reed, 1972).

Reports are scarce on the occurence of 3-methylhistidine in proteins unrelated to myosin or actin. Gershey et al. (1969), detected this amino acid in avian erythrocyte histones, tentatively concluding that 3-methylhistidine was a normal constituent of such histones. Similar reports for rat histones were made by Byvoet (1971).

Protein-bound 3-methylhistidine would appear to be a constituent of only muscle actin and myosin, certain related actin-like proteins from other sources, and a few unrelated obscure occurences. The virtually unique occurence of 3-methylhistidine in myosin and actin and the fact that the free amino acid is not re-utilised for protein synthesis (section 2.1.3) means that the intracellular breakdown of these myofibrillar proteins to give excretion of 3-methylhistidine in the urine is a reliable indicator of the rate of muscle protein breakdown. Young and Munro (1978) give an overview of the use of 3-methylhistidine in this context.

Non protein-bound 3-methylhistidine is found in the free amino acid pool of muscle, is present in blood plasma and is excreted in the urine in the unmodified and acetylated forms (Young et al., 1972). The occurence of the dipeptide balenine (β-alanyl-3-methylhistidine) has been reported from whale meat (Carisano, 1964; Cocks, Dennis, and Nelson, 1964), and from pork (Rangely and Lawrie, 1976). Balenine is isomeric with the predominant histidine dipeptides in bovine muscle, namely carnosine (β-alanyl-histidine), and anserine (β -alanyl-1-methylhistidine).

2.1.3. METHYLATION OF PROTEIN-BOUND HISTIDINE.

The nature of radioactive urinary excretion products, following the administration of a physiological dose of a radioactive compound, can usually throw some light on its normal metabolic pathway in the intact animal. Wolf, Wu and Heck (1956), found that, on administration of radioactive histidine to rats, methylhistidines were major radioactive urinary constituents. From this it was concluded that histidine can act as a methyl group acceptor. Confirmation of this proposition was made by Asatoor and Armstrong (1967), who showed that histidine and methionine were the precursors of 3-methyhistidine, by administering labelled histidine and methionine to rats, collecting their urine whilst fasting and measuring the specific activity of the 1 and 3-methylhistidine present. In agreement with earlier findings by Cowgill and Freeburg (1957), it was found that no radioactivity was incorporated into proteins of animals fed 3-^{14}C-methylhistidine, indicating that methylation occured co- or post-translationally. Long *et al.* (1975) has since reported similar quantitative excretion of administered 3-methylhistidine in man.

In vitro methylation studies were carried out by Hardy and Perry (1969), who incubated rabbit muscle homogenates with S-adenosylmethionine, labelled with ^{14}C in the methyl group. Amino acid analysis of myofibrillar protein hydrolysates, by ion-exchange chromatography and electrophoresis, detected radioactivity corresponding to 3-methylhistidine (and N^{ϵ}-methyllysine), suggesting that *in vitro* methylation had occurred

with S-adenosylmethionine as the methyl group donor. Further *in vitro* work was carried out by Reporter (1969), with cultured rat leg muscle cells. [^{14}C] histidine and [^{14}C-methyl]-methionine could be converted to protein-bound ^{14}C-3-methylhistidine. Incorporation of the methyl group of methionine into 3-methylhistidine in histones was reported by Gershey *et al.* (1969) and Byvoet (1971).

Though the precursor of 3-methylhistidine had been established as histidine, with (S-adenosyl) methionine as the methyl donor by radioactve studies, it had not been unequivocally established whether methylation was pre-, co- or post-translational. Young *et al* (1970), carried out investigations to determine if methylation occurred before or after binding of the amino acid to transfer RNA (tRNA). Circumstantial evidence, such as the lack of incorporation of administered radioactive 3-methylhistidine, indicated that the amino acid could not be used directly for the synthesis of fibrillar proteins. Using an amino-acyl ligase preparation from rat muscle, the *in vitro* binding of various radioactive amino acids to t-RNA was compared with the binding of tRNA to 3-methyl[^{3}H]histidine. No evidence of the attatchment of the latter to t-RNA was found, although considerable incorporation of other amino acids occured. *In vivo* studies also failed to reveal binding of radioactive 3-methylhistidine to tRNA. These findings all indicated that methylation of histidine occurs after the formation of histidyl-tRNA.

Krzysik, Vergnes and McManus (1971), studied the incorporation of methyl groups from L-methionine-methyl ^{14}C into

3-methylhistidine of actin from embryonic and post-hatched chicks. Incorporation was found to be deficient in 9-16 day embryonic chicks, reaching normal levels in 18 day embryos. This indicated developmental control of the methylation of histidine in actin. In vitro studies showed that actin isolated from a myofibrillar protein fraction, after incubation with S-adenosyl-L-methionine ^{14}C contained radioactive 3-methyl-histidine indicating that the myofibrillar protein fraction catalysed the methyl group transfer to histidine residues in the completed polypeptide chain. This provided more evidence for post-translational methylation.

The lack of incorporation of administered 3-methylhistidine was reported by Young et al. (1972), orally and parenterally administered 3-[methyl-^{14}C]methylhistidine being 100% quantitatively recovered in the urine and faeces as the free or N-acetylated amino acid. More evidence was also found to support the absence of a 3-methylhistidyl-tRNA.

Reporter (1973) investigated protein synthesis in cultured rat leg muscle cells, concentrating on the methylation of nascent proteins. Morse and McManus (1973) and Morse et al (1975), investigated the site of biological methylation of proteins in cultured chick muscle cells. The general conclusions of all these investigations were as follows- studies using cycloheximide and puromycin indicated that N-methylation of basic amino acid residues occurred whilst the polypeptide was still being synthesised on the ribosome and could occur on the nascent polypeptide in the absence of protein synthesis. The uniqueness

and specificity of methylation sites suggests that recognition of the site by the appropriate methylase, and access to the site by the enzyme and by S-adenosylmethionine, must be due to more than just the primary sequence. Methylation could be obligatory before the folding of the protein since the resulting tertiary structure may cause steric hindrance to the enzyme system, though Reporter (1973), believed that methylation of accessible residues could be completed in the cytosol.

In summary, there is no evidence of a 3-methylhistidine charged tRNA. Methionine is believed to donate the methyl group, via S-adenosylmethionine, to histidine residues on the nascent polypeptides of actin and myosin, or possibly after the release of the polypeptide from the ribosome (Young and Munro, 1978).

2.1.4 METHYLATION OF HISTIDINE: ITS DEPENDENCE ON AGE AND TISSUE OF ORIGIN.

Methylation of imidazole amino acids, as exemplified by anserine formation, is known to be more active in post-natal life, though Johnson, Harris and Perry (1967a) found that the 3-methylhistidine levels in foetal skeletal muscle actin were only slightly lower than in the adult.

The possibility that the methylation of histidine in myofibrillar proteins is under developmental control was reported by Trayer, Harris and Perry (1968), who detected 3-methylhistidine at a level of 1.63 residues / mole in adult skeletal muscle myosin, but found none in the myosin of foetal muscle, the actins from both these muscles having similar titres

of 3-methylhistidine. A comparison between the 3-methylhistidine titres of myosins from cardiac and white skeletal muscle, showed a lower titre for the former. It was concluded that myosin may exist in the form of isoenzymes, some of which contain 3-methylhistidine, and that the complement of which in any myosin preparation will be characteristic of the type of muscle from which the protein was isolated. Johnson, Lobley and Perry (1969), confirmed that 3-methylhistidine was present in actin from both adult and foetal skeletal muscle of the rabbit, but was only present in myosin from shortly after birth. The 3-methylhistidine contents of myosins prepared from red and white skeletal, and cardiac muscle were compared, far lower titres being found in the red skeletal and cardiac myosins, than that from white skeletal muscle, though the 3-methylhistidine titres of all of the actins were similar. In white skeletal muscle from vitamin E deficient, dystrophic rabbits, the 3-methylhistidine:histidine ratio in myosin was significantly lower than for normal animals, though the ratio of these amino acids in actin was unchanged.

Work on cultured mammalian muscle cells carried out by Reporter (1969), indicated that there was a gradual, age dependent histidine methylation of actin, methylation of myosin being found to be limited; but it occured in both early and late cultures. These findings were contradictory to the earlier findings of Trayer, Harris and Perry (1968) and Johnson, Lobley and Perry (1969), which indicated that complete methyation of histidine in actin occured early in foetal development, whereas myosin histidine methylation only reached adult levels

after birth; but the results agreed with those of Krzysik, Vergnes and McManus (1971), ie. that actin histidine methylation was under developmental control. Further reports were made of low levels of 3-methylhistidine in 28 day foetal and 1 day old rabbit myosin, whereas 28 day foetal rabbit and foetal sheep skeletal muscle actin had adult levels of the amino acid (Kuehl and Adelstein, 1970). Both 2 day old chick myosin and late embryonic chick actin being fully methylated (Kuehl and Adelstein, 1970; Krzysic, Vergnes and McManus, 1971).

Most actins so far analysed have been shown to contain 1 residue of 3-methylhistidine/mole actin. One exception to this is from the work of Krzysic, Vergnes and McManus (1971), which indicated that chicken muscle actin, prior to day 11 of embryonic life, was extremely undermethylated, the 3-methylhistidine content not reaching adult levels until day 18. These unusual findings have been contradicted by Cass, Clark and Rubenstein (1983), who found that cardiac muscle actin from day 2 of embryonic life to hatching, and brain actin and skeletal muscle actin from as early as day 9 and 11 respectively, was fully methylated. Thus it now seems more likely that actin methylation is not under developmental control in the chick embryo.

Haverberg, Munro and Young (1974) showed that there was no age dependent changes in the extent of methylation of histidine in whole rat muscle, indicating that the methylation system is fully developed in the young growing animals.

A relationship between muscle type and the 3-methylhistidine content of the actin and myosin, has been established. Trayer, Harris and Perry (1968), found that cardiac myosin had a lower 3-methylhistidine titre than that of white skeletal muscle. Kuehl and Adelstein (1970), reported that cat soleus (98% red fibre) and cardiac myosins contained no 3-methylhistidine, that of mixed fibre (mouse hind limb), containing significantly less than the 2 moles 3-methylhistidine / mole myosin assumed for full methylation. A summary of the results are given in tables 2.2, 2.3, and 2.4.

TABLE 2.2. 3-METHYLHISTIDINE CONTENTS OF ADULT SKELETAL MUSCLE MYOSINS.

MUSCLE	FIBRE TYPE	3-METHYLHISTIDINE CONTENT OF MYOSIN (moles / 500000g protein)
Rabbit *Psoas*	Predominantly white and intermediate	1.5
Chicken *Pectoralis*	ditto	1.5
Sheep thigh	ditto	1.7
Cat *Flexor Hallucis Longus*	ditto	1.3
Cat *Soleus*	Predominantely red	<0.05

(From Kuehl and Adelstein, 1970).

TABLE 2.3. 3-METHYLHISTIDINE CONTENT OF CARDIAC MYOSINS.

SPECIES	3-METHYLHISTIDINE CONTENT OF MYOSIN (moles / 500000g protein)
Rabbit	< 0.10
Sheep	nil
Cat	< 0.15
Ox	nil

(From Kuehl and Adelstein 1970).

TABLE 2.4. 3-METHYLHISTIDINE CONTENT OF SKELETAL MUSCLE MYOSINS FROM FOETAL AND POST-NATAL ANIMALS.

MUSCLE	AGE	3-METHYLHISTIDINE CONTENT OF MYOSIN (moles / 500000g protein)
Sheep hind leg and back	1-30 days gestation	< 0.10
Rabbit hind leg	1 day old	nil
	10 day old	0.4
	Adult	1.8
Chicken leg	2 day old	1.5

(From Kuehl and Adelstein, 1970).

It was proposed that white fibre myosin contained 2 moles / mole of protein and red fibre myosin none, the latter being an intermediate between foetal and white fibre myosin. Actins from various sources were all found to contain 0.7-0.8 moles /mole protein regardless of tissue of origin.

Other examples of variations in the 3-methylhistidine titres of myosins were reported by White and Lawrie (1985b). A comparison of the 3-methylhistidine levels in the myosins prepared from a range of bovine muscles, revealed the abnormally low titre in the combined cheek muscles (Masseter and Malaris) of 0.24 moles / mole myosin, compared with levels of 1.25 - 1.53 moles/mole for the other muscles investigated.

Masaki et al. (1986) reported on the 3-methylhistidine content of adult and embryonic chicken cardiac ventricular myosins (table 2.5). A suprising difference was found between chicken and mammalian cardiac myosins. Adult chicken red, white and cardiac as well as embryonic cardiac myosin all had similar 3-methylhistidine titres compared with the absence of this amino acid in the myosins of mammalian cardiac muscle and muscles containing predominantly "red" fibres, showing differences exist in the histidine methylation of myosin between mammals and birds.

TABLE 2.5. 3-METHYLHISTIDINE CONTENTS OF CHICKEN, BOVINE AND RABBIT MUSCLE MYOSINS.

MUSCLE			3-METHYLHISTIDINE CONTENT OF MYOSIN (moles / 500000g protein)
Chicken	adult	fast white	2.20
		slow red	2.25
		cardiac	2.00
	embryonic	cardiac	1.50
Bovine	adult	cardiac	0
Rabbit	adult	cardiac	0

(From Masaki et al., 1986).

2.1.5. THE RELATIONSHIP BETWEEN PRIMARY STRUCTURE AND LEVEL OF HISTIDINE METHYLATION OF MYOSIN AND ACTIN.

The absence of histidine methylation could be due to a failure to methylate, or the absence of the appropriate histidine residue. Investigations have been carried out to determine the nature of any differences in the primary structure of actins and myosins from different sources.

Actin consists of a single polypeptide chain, G-actin of assumed molecular weight, 41785 daltons (Elzinga et al.,

1973), which on polymerisation to F-actin, forms part of the thin filament of muscle. Initially its primary structure was studied by amino acid analysis and peptide mapping of the enzymatically degraded molecule, using thin-layer chromatography, in an attempt to elucidate any differences between smooth and skeletal muscle actins (Gerday, Robbins and Gosselin-Rey, 1968; Gosselin-Rey et al., 1969). No apparent differences were detected, and the amino acid sequence of smooth muscle (chicken gizzard) actin has now been shown to be more like that of striated muscle actin than of non-muscle actins such as found in the brain (Elzinga and Kolega, 1978).

Johnson, Harris and Perry (1967a) reported that 3-methylhistidine was restricted to one peptide of a tryptic digest of rabbit actin, this being confirmed by Adelstein and Kuehl (1970) and Elzinga (1970), who carried out structural studies on rabbit skeletal muscle actin using cyanogen bromide cleavage. It was found that only one highly purified peptide, "CB10", contained significant levels of 3-methyl-histidine, with no detectable histidine. This was consistent with the unique position of a single residue of 3-methylhistidine in actin. The amino acid sequence of the "CB10" peptide of rabbit skeletal muscle actin which can be seen in figure 2.2, was determined by Elzinga (1971). Bridgen (1971) determined the amino acid sequences of peptides from tryptic and chymotryptic peptides of trout muscle actin, and concluded that there was a high degree of homology with the sequences of rabbit muscle actin, incuding the presence of 1 mole 3-methylhistidine / 45000g actin.

Figure 2.2. Amino acid sequence around the single 3-methylhistidine residue, in adult skeletal muscle actin and myosin.

ACTIN:

-Leu-Leu-Gly-Ser-Ile-Asp-Val-Asp-3MeHis-Gln-Thr-Tyr-Lys-

MYOSIN:

-Leu-Thr-Leu-Lys-Tyr-Pro-Ile-Glu-3MeHis-Trp-Gly-Ile-Ile-

(From Elzinga, 1971; Huszar and Elzinga, 1971)

The similarity of actins from widely different sources was illustrated by Weihing and Korn (1972), who found a cyanogen bromide peptide equivalent to "CB10" in the actin of the soil micro-organism, Acanthamoeba.

Elzinga et al. (1973), went on to determine the complete amino acid sequence of rabbit skeletal muscle actin, by sequencing isolated cyanogen bromide peptides, then establishing their order within the molecule by comparison with the results of digests usig other enzymes. 374 residues , including one 3-methylhistidine were found, giving a total molecular weight of 41785 daltons. By comparison of sequences of actins from different sources it was concluded that actin had a highly conserved sequence. The similarity of primary structure from philogenically distant species could be due to the transduction of the structural gene from one organism to another via viruses, or from the evolution of a primordial precursor molecule.

Myosin, which forms the thick filaments of muscle, consists of two identical heavy chains (molecular weight 200000 daltons) forming the tail of the molecule, and four light chains (molecular weight in the range of 20000 daltons) (Lowey and Risby, 1971), which together with part of the heavy chains, form two globular heads. The total assumed molecular weight of myosin is 480000 daltons (White, 1986). The location of the 3-methylhistidine within the myosin molecule was investigated by Johnson, Harris and Perry (1967a). From controlled tryptic digests of myosin the 3-methylhistidine

residue was localised in the heavy meromyosin, which consists of part of the rod attached to the globular head region. Further digests showed that each of the two globular head (subfragment 1) regions contained 1 mole 3-methylhistidine / mole protein. Huszar and Elzinga (1971) isolated the 3-methylhistidine-containing peptide from the cyanogen bromide/tryptic digests of subfragment 1. The residue was localised in the heavy chain portion of the subfragment, 3-methylhistidine being found to be absent from the light chains. This was later confirmed by Elzinga and Collins (1977), who found that the 3-methylhistidine-containing cyanogen bromide peptide also contained proline, and concluded that it was therefore likely to have come from the folded globular head region of the heavy chain. The sequence around the 3-methylhistidine residue in adult skeletal muscle myosin, in comparison with the analogous sequence in actin, is given in figure 2.2. Although in both cases, there is a methylated histidine residue in a single position, there are few other similarities in the sequences. This lack of homology indicates that different methylating systems may be involved, although the acidic residues present in both sequences make it likely that the 3-methylhistidine is on the surface of the molecule.

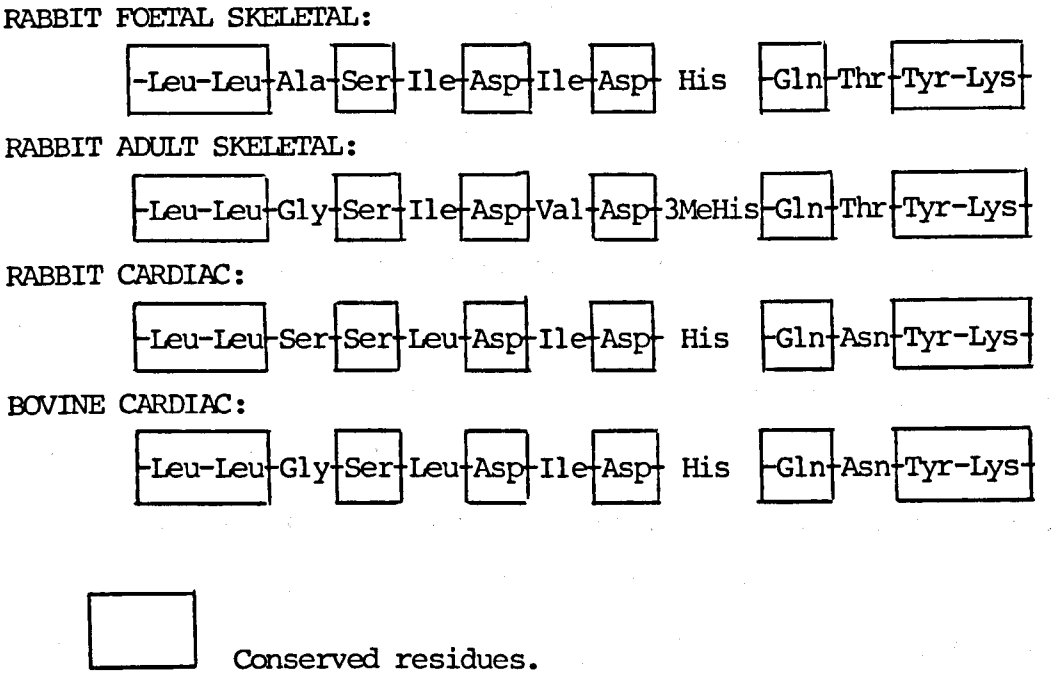
The absence of 3-methylhistidine in red, cardiac and foetal muscle, could be a manifestation of either a reversible regulatory process or an irreversible developmental process, being due to a lack of methylating enzymes, a different amino acid sequence, or different folding of the molecule, making the histidine residue unavailable for methylation.

Huszar and Elzinga (1972) isolated tryptic peptides from rabbit and bovine cardiac myosins containing no 3-methylhistidine, which were homologous with the 3-methylhistidine containing peptides from rabbit skeletal muscle myosin. The sequence of the peptides is given in Figure 2.3, from which it can be seen that there is more homology between the same tissue of different species, than different tissues of the same species, all differences being attributable to single base changes. It was proposed that cardiac and skeletal muscle myosins arose from different genes.

Sequencing studies by Huszar (1972), on rabbit foetal myosin, identified, by its chromatographic behaviour, a non-methylated histidine peptide analogous to the methylated peptide of adult myosin, the sequence of which can also be seen in figure 2.3. A comparison of the sequences around the 3-methylhistidine (or histidine) in adult skeletal, foetal and cardiac myosins indicates that there are three genes involved in the synthesis of myosin in rabbits.

In summary, actin has a highly conserved amino acid sequence with theoretically one residue of 3-methylhistidine, regardless of age or origin of tissue. On the other hand myosin is a more heterogeneous molecule, that from "white" skeletal muscle theoretically containing one residue / heavy chain (2 per myosin molecule), whereas foetal and cardiac myosins have no methylated histidine residues. The low levels of 3-methylhistidine found in the myosin of predominantly "red" tissues is likely to be due to a predominance of the foetal or

Figure 2.3. Amino acid sequence of methylated and non-methylated histidine peptides of foetal skeletal, adult skeletal and cardiac muscle myosins.



(From Huszar and Elzinga, 1972; Huszar, 1972)

cardiac type myosin or a specific non-methylated "red" type myosin, as yet uncharacterised.

2.1.6. THE BIOCHEMICAL ROLE OF 3-METHYLHISTIDINE.

The specific function of 3-methylhistidine in proteins has yet to be determined, though histidine itself is often directly involved in biological activity. The stability of the 3-methylhistidine, histidine and arginine ratios of actins from sources as different as muscle and amoeba, may have significance regarding the active sites of the protein (Wooley, 1972). Methylation of histidine is significant structurally, as it confers non-polar characteristics to the imidazole ring thus destroying its hydrogen-bonding potential.

Whilst comparing foetal and adult myosin, Trayer, Harris and Perry (1968) concluded that 3-methylhistidine was not required for the combination with actin, or for ATPase activity, although foetal myosin has a lower ATPase activity than that of the adult. Johnson, Lobley and Perry (1969) and Johnson and Perry (1970) confirmed this. They reported that photo-oxidation, which specifically destroys histidine and 3-methylhistidine residues, did not affect the ATPase activity or actin-combining properties of myosin. On the other hand photo-oxidation totally destroyed the polymerisation properties of G-actin, even when only half of the 3-methylhistidine had been eliminated. It was reported that the methylation of histidine in myosin increases with the speed of the muscle, as does the ATPase activity, the significance of which is unknown. Huszar (1972), suggested that

methylation of histidine could just be one of the many structural differences between fast and slow myosin. Structural and enzymatic properties of myosin are characteristic of the activity pattern of the muscle from which it is derived. In their work on vitamin E-dystrophic rabbits, Lobley, Perry and Stone (1971), concluded that there had been a reversal of normal development- to isoenzymes typical of foetal tissue, giving myosins with reduced 3-methylhistidine contents and lowered Ca^{2+} ATPase activity, although no change in the actin was apparent. Krzysik, Vergnes and McManus (1971) found that though early embryonic chick actin appeared to lack 3-methylhistidine, it still polymerised successfully.

The sequence around the 3-methylhistidine residue in actin and myosin gives no clue regarding its purpose. If directly involved in one of the characteristic function of actin (polymerisation, nucleotide or metal binding, actin/myosin interaction or interaction with other myofibrillar proteins), the residue would be expected to be located on the outside surface of the molecule. Major segments of the primary structure around 3-methylhistidine are rather hydrophobic suggesting that a large part of the region is not in contact with the solvent (Elzinga, 1971). On the other hand the fact that histidine may be available for methylation post-translationally indicates the contrary. Elzinga and Collins (1977) found evidence that the 3-methylhistidine residue in myosin was near to the binding site of actin, though the specific involvement of this amino acid was not suggested.

The absence of 3-methylhistidine from the actin of

the amoeba Naegleria gruberi (Sussman et al., 1984), is unique among characterised actins, Naegleria actin being the most divergent of all known actins, being only about 70% homologous with skeletal muscle actin. It has generally been considered that post-translational modification at residue 73, the site of 3-methylhistidine, is a conserved property of actin, suggestive of an important biological function. But in the case of Naegleria actin, the methylated residue is not essential for the actin-actin or actin-myosin interactions.

Masaki et al. (1986), when investigating the 3-methylhistidine content and pH dependency of ATPase activity of adult and embryonic chicken cardiac myosin, concluded that the 3-methylhistidine content of the myosin heavy chain was closely correlated with the activation of its ATPase activity at alkaline pH. The presence of myosins with differing enzymatic properties, - one which can undergo histidine methylation and one which cannot - was indicated, though proof of the direct involvement of 3-methylhistidine in these enzymatic differences was not given.

Recent work on the role of actin's 3-methylhistidine has been carried out by Solomon and Rubenstein (1987), using site specific mutagenisis of actin to change the 3-methylhistidine residue at position 73 to arginine or tyrosine. Using these mutant actins, the requirement for 3-methylhistidine in areas in which its role has been implicated (viz; amino-terminal processing of actin, the binding of DNase I and polymerisation) was critically investigated. The results showed that 3-methylhistidine is not absolutely required for any of

these processes. The biological function of 3-methylhistidine in both actin and myosin thus remains unclear. Actin, however, is known to be controlled by a large number of binding proteins and it is possible that 3-methylhistidine may play a role in their interaction.

The biochemical role of the 3-methylhistidine dipeptide balenine is also uncertain, though the possibility that imidazole dipeptides play a role in glycolysis, muscle contraction or as physiological buffers has been suggested (Waley, 1966).

2.2. FLUORESCAMINE: A VERSATILE REAGENT FOR THE DETERMINATION OF AMINO ACIDS, PEPTIDES AND PROTEINS.

2.2.1. THE DEVELOPMENT OF FLUORESCAMINE.

Fluorescamine is a powerful fluorogenic reagent. It was synthesised after elucidation of the structures of fluorophores derived from ninhydrin, phenylacetaldehyde and primary amines.

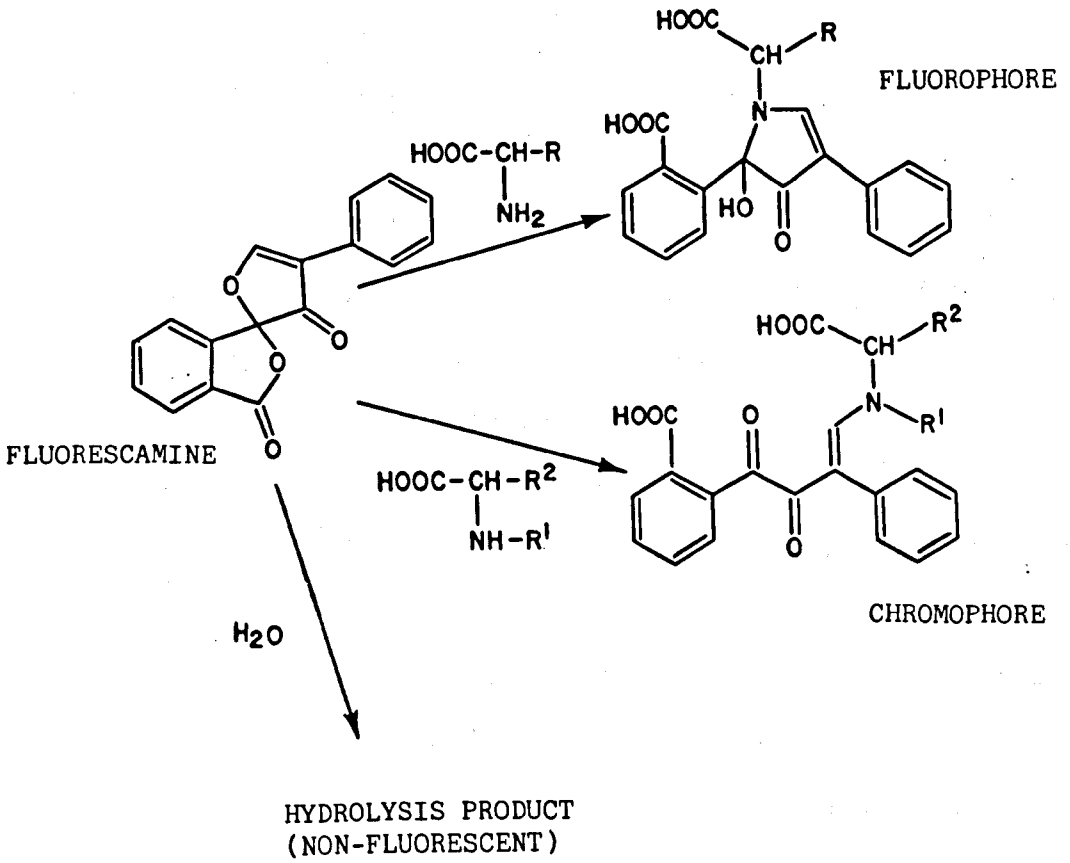
The most widely used colorimetric method for the determination of amino acids has involved ninhydrin, this reagent detecting amino acids in the nanomole range. Reports that ninhydrin could form highly fluorescent products with amines was exemplified by the assay developed for serum phenylalanine by McCamen and Robbins (1962). It was found that phenylalanine itself yielded fluorescence with ninhydrin; but the addition of small peptides, such as leucylalanine, increased the response over fifty fold. Close (1969) reported on the fluorescence of amino acids and amines when incubated with ninhydrin and n-butyraldeyde. Samejima, Dairman and Udenfriend (1971), investigated the mechanism of these fluorogenic ninhydrin reactions. It was found that phenylalanine reacted with ninhydrin to form phenylacetaldeyde which, on reaction with peptide, formed fluorescent products. A primary amino group was required in the peptide, secondary or tertiary amino groups giving no fluorescence. The fluorescent ternary product was purified and characterised by its mass spectrum. The interconversion between fluorescent and non-fluorescent forms at different pH's, suggested a lactone structure. Samejima et al (1971)., went on

to apply the fluorescent ninhydrin reaction to the detection and assay of peptides, amino acids and amino sugars. It was found that this assay was one or two orders of magnitude more sensitive than the colorimetric method. The greatest increase in sensitivity was found with basic amino acids and peptides, no fluorescence being seen for proline, hydroxyproline and ammonia. The fluorescent ninhydrin reaction did not suffer from contamination due to side reactions and excess reagent. Such problems had previously been encountered in another fluorogenic assay, that using dansyl chloride (Gray and Hartley, 1963).

The structure of the fluorescent principal in the fluorogenic ninhydrin reaction was elucidated by Weigele et al. (1972a). This led to the single reagent, fluorescamine, being developed by Weigele et al. (1972b), to replace ninhydrin and phenylacetaldehyde. Its structure can be seen in figure 2.4. Fluorescamine was synthesised by a series of reactions from 1-dimethylamino-2,4-diphenyl-1-butene-3,4-dione. Intensely fluorescent products were formed with primary amines of the type found using the fluorescent ninhydrin reaction. The reaction being pH dependent, with an optimum at pH 8-9.

Udenfriend et al. (1972), investigated the fluorescamine reaction with primary amines and found that the reaction had a $t\frac{1}{2}$ of 100-500 milliseconds, concomitant hydrolysis of fluorescamine occuring to give non-fluorescent products at a $t\frac{1}{2}$ of 5-10 seconds. An outline of these reactions is given in figure 2.4., along with the reaction of fluorescamine with secondary amines (Felix et al, 1975), which gives non-fluorescent chromophores. It was obvious that the fluorescamine reagent

Figure 2.4. The reaction of fluorescamine with primary and secondary amino acids, and the hydrolysis of the reagent.



(From Udenfriend et al, 1972; Felix et al, 1975).

needed to be prepared in a non-hydroxylic, water miscible solvent, to prevent hydrolysis. Control of pH was found to be important, derivatisation of amino acids being low at pH 7 and high at pH 9. The reverse was found for peptides, allowing an extra dimension of specificiy. Further investigations were carried out by DeBernardo et al. (1974), who reported that the fluorescent pyrollinones produced by the reaction of fluorescamine with primary amines, had an excitation maximum of 390nm and an emission maximum of 475-490nm. Fluorescamine was shown to react with other nucleophiles such as alcohols and secondary amines, but with the production of non-interfering, non-fluorescent products. Kinetic studies by Stein, Böhlen and Udenfriend (1974), indicated that the fluorescamine reaction proceeded by the rapid addition of the primary amine across the double bond, followed by a multi-step rearrangement to the fluorophore.

Fluorescamine as a reagent for assaying primary amines was thus established. The problem of assaying secondary amines such as proline and hydroxyproline using fluorescamine was solved by Weigele, DeBernardo and Leimgruber (1973), who subjected secondary amines to oxidative decarboxylation, using halogenating agents such as N-chlorosuccinimide. This converted the secondary amino acid to imine, which on hydrolysis yielded primary amines capable of giving fluorescent products with fluorescamine. With this modification it was possible to assay all natural amino acids using fluorescamine.

The most important modification of the fluorescamine assay in terms of the detection of 3-

methylhistidine, was the discovery of the unique fluorescent derivatives of histidine, histamine and certain related imidazoles, after heating in acid (Nakamura and Pisano, 1976b). Similar acid-stable fluorophores were found for tryptophan, tryptamine and some related indoles (Nakamura and Pisano, 1976a). Considering the acid stable fluorescence of imidazoles, it was found that an unsubstituted -NH- in position 1 of the imidazole ring, and a free $-NH_2$ in the side chain were required. For example histidine, esters of histidine, 3-methylhistidine, histamine and peptides containing amino terminal histidine, form acid-stable fluorophores with an excitation maximum at approximately 390nm and an emission maximum at approximately 490nm. 1-Methylhistidine, N-acetyl or other N-protected histidines do not form fluorescent derivatives. The acid-stable fluorophore (the structure of whch is unknown), is believed to be due to the thermal rearrangement of non-fluorescent lactones, as the acid-stable fluorescence appears after the initial fluorescence has completely decayed (Nakamura,1977).

Murray, Ballard and Tomas (1981) reported a modification to the acid-stable fluorescamine reaction, which made it specific for 3-methylhistidine alone. Interference due to histidine and other acid stable fluorophores was removed by prior treatment with aldehydes. This method was found to be directly applicable to blood and intracellular fluid samples, but not to protein hydrolysates, due to the vastly greater amounts of histidine present in the latter.

2.2.2. APPLICATIONS OF FLUORESCAMINE.

Fluorescamine has found many applications for the assaying of amino acids, proteins and peptides, particularly after their separation by chromatographic methods.

The visualisation of histidine, histamine and histidyl peptides on thin layer chromatograms, using acid stable fluorescamine derivatives (Nakamura, 1977), allowed detection in the picomole range, which was ten times as sensitive as previous methods, such as the Pauly reagent (a diazonium coupling reaction) or O-phthalaldehyde.

Traditional, ion-exchange, chromatographic amino acid analysis, but using post-column derivatisation with fluorescamine instead of ninhydrin, allowed the amino acid composition of as little as 1 microgram (μg) of protein to be determined (Stein et al., 1973a,b). Such an amount would be too little for ninydrin detection. Felix and Terkelson (1973b), developed an automated method of measuring the full range of amino acids in protein hydrolysates using ion-exchange chromatography and post-column fluorescamine derivatisation, secondary amino acids being detectable only after the specific introduction of N-chlorosuccinimide into the flow system. Parallel work was carried out by Georgiadis and Coffey (1973), using higher pressure chromatography to reduce the time of analysis. The need for all reagents to be free of primary amine contaminants was paramount, since the fluorescamine assay was so sensitive. Schwabe and Catlin (1974), found that some sources of hydrochloric acid used during analysis contained interfering compounds assumed to be primary amines, which could

be removed by distillation with appropriate reagents. The use of purer hydrochloric acid increased the sensitivity of post-column amino acid detection from 100 to 1 nanomole (nmole), due to reduction in base-line noise and removal of extraneous peaks.

Post-column detection of amino acids using fluorescamine can also be carried out colorimetrically, by absorbance at 380nm (Felix et al., 1975). The reaction products of both primary and secondary amino acids absorb at this wavelength, allowing the determination of all amino acids without the conversion of secondary amino acids using N-chlorosuccinimide.

With the development of high performance liquid chromatography (HPLC), which gave high speed and excellent resolution in amino acid analysis, there was a requirement for a rapid sensitive detection method. Fluorescamine fulfilled these requirements and has been used successfully for the determination of 3-methylhistidine by pre-column derivatisation. Wassner, Schlitzer and Li (1980) developed a rapid and sensitive method for the determination of 3-methylhistidine in urine and plasma, using pre-column acid-stable fluorescamine derivatisation, with reverse-phase HPLC separation. The separation time for the 3-methylhistidine derivative was less than ten minutes, with a limit of sensitivity of less than 600 femtomoles (600×10^{-15} moles), giving a rapid and sensitive assay. A similar method was employed by Jones, Shorley and Hitchcock (1982a,b) for the fluorometric determination of 3-methylhistidine in meat and meat products (section 2.4.6).

Fluorescamine has found a role in the staining of

protein bands of electrophoretic gels. Eng and Parkes (1974) pre-labelled proteins with a fluorescamine tag prior to electrophoresis and detected the resultant bands under UV light. The sensitivity of detection allowed very small initial loadings to be made. Superior results were found compared with fluorescent pre-labelling with dansyl chloride, where fluorescent by-products were produced. Similar investigations, carried out by Ragland, Pace and Kempner (1974), illustrated the sensitivity of pre-labelling with fluorescamine; 6 nanograms (ng) of myoglobin could be accurately measured. The electrophoretic mobility of some proteins was changed by pre-labelling, though it was still reproducible. Post-electrophoretic labelling was also possible by the immersion of the gel in a fluorescamine / dimethyl sulphoxide solution.

An elegant method utilizing the properties of fluorescamine was reported by Stein et al. (1974), who carried out amino acid analysis with fluorescamine on the stained protein bands of polyacrylamide gels. After sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and staining, bovine serum albumin (BSA) bands were excised, hydrolysed and the amino acid composition determined by ion-exchange chromatography with post-column fluorescamine derivatisation, the results agreeing with previously published data on the amino acid composition of BSA. This was an improvement on the work of Houston (1971), who carried out similar investigations but found that the post column ninydrin detection system used for the amino acid analysis was inadequate due to the high levels of ammonia present.

The diverse applications of fluorescamine are apparent from the wide variety of other uses to which it has been put. Böhlen et al. (1973) developed a method for the fluorometric assay of proteins in the nanogram range, the assay being based on the reaction of the free amino groups of the N-terminus and the N^{ϵ}-amino groups of lysine. The use of BSA as a standard was therefore rather arbitary due to the variation in the number of free amine groups from protein to protein; but no more so than the Lowry procedure (Lowry et al., 1951), which is based on tyrosine. It was suggested that this assay could be applied to proteins extracted from electrophoretic bands; after the separation of interfering material by small, disposable gel filtration columns (Bohlen et al., 1974). Nakai, Li and Horecker (1974) applied fluorescamine to the detection of peptides after chromatographic separation, finding a five-fold increase in sensitivity compared with ninhydrin detection. The sensitivity was increased fifty fold after alkaline hydrolysis.

A fluorescent assay for proteolytic enzymes was developed by Schwabe (1973), proteolytic activity being based on the amino acids released. No interference by the protein substrate occurred since it was succinylated to cap free amino groups. An application of fluorescamine in peptide synthesis was reported by Felix and Jiminez (1973), who used this reagent to detect trace amounts of uncoupled produts in solid phase synthsis. The mass spectra of the reaction products of primary amines and fluorescamine has been found to be useful in the identification and characterisation of biologically important amines (Narasimhachari, 1973).

2.2.3. ALTERNATIVE FLUOROGENIC AMINO ACID DETECTION METHODS.

A number of fluorogenic reagents other than fluorescamine have been used for amino acid determination.

O-phthalaldehyde was reported as a chromogenic and fluorogenic reagent by Turner and Wightman (1968), for a wide variety of biologically important molecules. Histidine was found to give intensely fluorescent spots after alkaline overspray on thin layer chromatograms. Benson and Hare (1975) made a comparative study of the sensitivity of detection of primary amines by o-phthalaldeyde, fluorescamine and ninydrin. It was found that, in the presence of 2-mercaptoethanol, o-phthal-aldehyde gives highly fluorescent derivatives of primary amines, so that picomole levels of amino acids, peptides and proteins could be detected. The increased sensitivity obtained by the addition of 2-mercaptoethanol gave a system which was reportedly more sensitive than ninydrin or fluorescamine, although it could not be used for secondary amines since the N-chlorosuccinimide required to oxidise the amine, also appeared to oxidise the reagent. Roth and Hampai (1973), found that the use of o-phthal-aldehyde and 2-mercaptoethanol for post-column detection of amino acids was fifty times as sensitive as ninydrin, being of the same order of sensitivity as fluorescamine but more specific.

Amino acid analysis (both primary and secondary), at the picomole level, was found to be possible by reaction with pyridoxal followed by reduction to the pyridoxal amino acid (Lustenberger, Lange and Hempel, 1972). After column chromatography of the derivative, as little as 500 picomoles

(pmoles) could be detected fluorometrically, or as little as 1 picomole by reduction with radioactive sodium tetrahydridoborate, and determination by scintillation measurement.

The 1-N,N'-dimethylaminonaphthalene-5-sulphonyl derivatives of amino groups (dansyl derivatives), show very strong fluorescence and have been used for the separation and detection of amino acids by HPLC. As little as 68 femtomoles of derivative is detectable, with a linear response over five orders of magnitude (Bayer et al., 1976).

Colorimetric determination, using 1-fluoro-2,4-dinitrobenzene (FDNB), has been used for the determination of 3-methylhistidine in foods by Skurray and Lysaght (1978). Hydrolysates were reacted with FDNB, followed by extraction with ether to remove the dervatives of non-basic amino acids, the remainder being chromatographically separated. A similar method was employed by Poulter and Lawrie (1980a).

Ward (1978), reported the use of a ninhydrin / o-phthalaldehde reagent for the determination of 3-methylhistidine. Since most amino acids are not detected by this reagent the determination of 3-methylhistidine can be accomplished in the presence of high concentrations of other amino acids. The method yields a chromophore with maximum absorbance at 490nm.

The requirement for oxidative degradation of secondary amino acids, prior to determination using fluorescamine or o-phthalaldehyde, was overcome by Yoshida et al. (1982), who reported the post-column detection of amino acids with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), a reagent

introduced by Ghosh and Whitehouse (1968). This reagent detected picomole levels of both primary and secondary amino acids.

2.3. REGULATIONS FOR MEAT AND MEAT PRODUCTS.

2.3.1. THE HISTORY OF FOOD LEGISLATION.

The human weakness for trying to obtain something for nothing by tricking and defrauding others, has meant that measures for the protection of consumers against the adulteration of food are amongst the earliest examples of social legislation (Giles, 1976). In the United Kingdom, an act passed in 1266 to protect the purchaser against short weight in bread and the sale of unsound meat appears to be the earliest food legislation. This act, like the many passed between the thirteenth and nineteenth centuries was ineffective, it being the duty of the Guilds to control the commodities with which their members dealt. Targets of adulteration included spices, such as pepper, highly prized as a meat preservative, beverages, flour, beer and milk, in which were substituted inexpensive, often hazardous materials. Without reliable methods of analysis it was extremely difficult to identify and prevent malpractices, significant developments in analytical chemistry not being made until the late eighteenth and early nineteenth centuries.

During the nineteenth century there was increased public awareness of food adulteration, with the publication of books such as "Treatise on Adulteration of Foods and Culinary Poisons", by Frederick Acum, in 1820, along with articles in the press and popular literature of the day. The Lancet in 1851 and 1854, reporting that 65% of food examined had been adulterated (Dalley, 1983). Public outrage led to the setting up of a "Select Committee of Parliament" in 1855. This resulted in the first

"Adulteration of food and Drink Act" in 1860, which empowered (but did not require) local authorities to appoint analysts (later known as public analysts). This act was virtually ineffective, an attempt being made to strengthen it by the "Adulteration of Food and Drink and Drugs Act" of 1872, which made the appointment of public analysts mandatory. This act also empowered inspectors to take samples for analysis, and required analysts to make quarterly reports to local authorities.

Unfortunately, enforcement of the 1872 act was patchy, making it necessary to set up a select committee in 1874 to study its operation. The result of this was the 1875 "Sale of Food and Drugs Act", which is the basis of our present law. This Act had great impact on the quality of basic foods, heavy penalties being imposed for adulteration.

The 1875 act remained in force until 1928, when it was repealed by the consolidating "Food and Drugs (Adulteration) Act". The latter ended a decade in which regulations dealing with composition and labelling of specific products appeared, such as in 1925, when the use of preservatives was prohibited except in certain foods in which their presence had to be declared on the label.

Food and Drug legislation and all public health legislation relating to food stuffs were combined in the 1938 "Food and Drugs Act", which remained in force during the war when Government control of food supply and advice on nutrition were at a high level, many early compositional regulations being made

under the "Defence (Sale of Food) Regulations" of 1943.

The "Food and Drugs Act" of 1955 had the aims of ensuring that the consumer could buy safe, wholesome food and would not be misled about its character and quality, and of protecting the honest trader from less honest competition.

Currently the primary legislative powers are contained in the "Food Act 1984", which was a consolidation of the 1955 Act. The Act states that food should not be injurious to health, and should be of the nature or substance or quality demanded by the purchaser (Jukes, 1987). Food composition, labelling and hygiene, are controlled by the issuing of regulations known as Statutory Instruments.

2.3.2 THE NEED FOR MEAT PRODUCT REGULATION.

The high cost of prime meat has made it economically advantageous to use other cheaper protein sources in meat products, such as soya protein, whey, mechanically recovered meat protein and blood protein isolate. These changes in protein composition could be a health concern. There is therefore a need to control protein nutritive quality, whilst not hindering the development of food technology (FSIS, 1984).

Meat is traditionally seen as the main source of high quality protein, B vitamins and trace elements. Products fabricated from processed meat by-products or non-meat ingredients could lead to a decrease in protein nutritional value and change levels of other nutrients as well as affecting food consumption patterns, all which might lead to consumer deception. Though the use of novel ingredients may not

necessarily affect the quality of the food concerned, technological advances are allowing the use of alternative protein sources in traditional products. This includes such processes as the pumping of isolated soya protein into whole cuts of meat, which yields a product of traditional appearance but of possibly different nutritional value.

The Food Standards Committee "Report on Sausages" (1956), concluded that some type of control over the composition of sausages was necessary in order to protect the consumer. Freedom from compositional control offered the public no assurance that sausages with lower meat content were proportionately cheaper. Types of control recommended included standards for meat content, types of permitted meats and control of meat quality and inclusion of other (filler) ingredients. Similar recommendations for meat pies were made in the Food Standards Committee "Report on Meat Pies" (1963).

The inclusion of offals in meat products was the subject of a Food Standards Committee Report in 1972. This report considered to what extent offals should be regarded as meat in both cooked and uncooked products. The majority of offals have a nutritive value similar to meat, some being richer in specific nutrients (such as liver, which is rich in vitamin A). To completely exclude the use of offals in meat products would be a nutritional loss. The effective biological value of the protein of skin and of offal with a high connective tissue content is often regarded as low owing to deficiencies in essential amino acids.

The increased tendency to include non-traditional protein sources such as textured vegetable protein and protein from micro-organisms, prompted the Food Standards Committee "Report on Novel Protein Foods" (1975). This report reviewed the use and control of unconventional protein foods and recognised the urgent need for established methodology, which would allow analysts to distinguish between proteins of different sources and to make quantitative assessment of their individual levels in commercial meat products. "Novel" proteins were classified as soya products, protein-rich materials from oil-seeds and legumes other than soya bean, proteins from other plant sources, proteins from animal sources and cereal isolates, protein-rich materials from micro-organisms, micro-fungal materials, yeast materials, bacterial materials and algal materials. It was proposed that specific care needed to be taken in supplying nutritionally vulnerable population groups such as old people, members of large families on low incomes and institutionalised groups. Where rising food costs have created financial difficulties, a greater substitution of meat and fish by cheaper "novel" protein sources might occur, with the possibility of nutritional effects.

Recommendations put forward by the "Report on Offals in Meat Products" (1972) and the "Report on Novel Protein Foods" (1974) culminated in the Food Standards Committee "Report on Meat Products" (1980). This report considered the whole range of meat products and their regulations, particularly in the light of the technological changes which had taken place in the food industry. The importance of added water was considered and it was concluded that added water used in texturising agents and

mechanical manipulation often made cheaper cuts of meat more palatable and therefore provided useful products for the consumer as long as an adequate system of labelling was employed.

Current regulations prescribe no limits for the connective tissue content of meat products, although the Food Standards Committee recommended in 1980 that new regulations for meat content should be based on the content of trimmed lean meat as sold by the butcher, and containing no more than 10% fat and 10% connective tissue. This has yet to be embodied in British regulations, though EEC regulations do limit the level of connective tissue in British pork sausages intended for export.

The 1980 report also concluded that significant growth in consumer awareness concerning the importance to health of the composition of foods had occured, as well as an increased knowedge of food composition and labelling. The trade responding by pressing for less rigid forms of control, allowing more freedom for the development of new products.

2.3.3. CURRENT REGULATIONS GOVERNING MEAT AND MEAT PRODUCTS.

Currently meat and meat products are controlled by the "Meat Products and Spreadable Fish Products Regulations 1984" (Statutory Instruments, 1984), along with the "Meat Products and Spreadable Fish Products (Amendment) Regulations 1986" (Statutory Instruments, 1986). A number of important points in terms of (lean) meat content and its determination are included in the regulations, particularly definitions of "meat", "lean meat" and offal.

"Meat" is defined as "the flesh, including the fat, and the skin, rind, gristle and sinew in amounts naturally associated with the flesh used of any animal (the term "animal", one would assume, specifically refers to mammals) or bird which is normally used for human consumption". This includes the offals listed in part a) of the definition of offal given below, but does not include any other part of the carcass.

"Lean meat content" is defined as the "total weight of lean meat free when raw of visible fat".

Offals are defined as any of the following parts of the carcass:

a) for Mammalian species: diaphragm, head meat (muscle meat and associated fatty tissue only), heart, kidney, liver, pancreas, tail meat, thymus and tongue;

for Avian species: gizzard, heart, liver and neck.

b) for Mammalian species: brains, feet, large intestines, small intestines, lung, oesophagus, rectum, spinal cord, spleen, stomach, testicles and udder.

The regulations give minimum "meat" contents (as percentage of food), minimum "lean meat" contents (as percentage of required meat content) and minimum level of named component such as beef in beefburgers (as a percentage of food), for the entire range of meat products, as well as specific labelling requirements, including the declaration of minimum meat content for meat products. An outcome of this legislation was to

increase the responsibility of meat processers in formulating, monitoring and verifying specifications of products (Newman, 1986).

2.4. THE DETERMINATION OF MEAT IN FOOD.

2.4.1. METHODS BASED ON NITROGEN CONTENT.

Most standard methods for the determination of the meat content of foods are based on the determination of organic nitrogen, with conversion to protein using the appropriate factors.

The determination of nitrogen by the Kjeldahl method (Egan, Kirk and Sawyer, 1981) has been in use since the late nineteenth century, since when it has been highly modified, and developed into a rapid automated procedure. The basic assay still depends on the quantitative conversion of organic nitrogen to ammonium sulphate with the aid of a metal catalyst (mercury or selenium). On addition of sodium hydroxide, with heating, ammonia is released from the ammonium sulphate, this being collected in a fixed volume of a standard acid solution (typically 5% boric acid), titration to an indicator end-point being used to enable the calculation of nitrogen emanating from the original sample (Zapsalis and Beck, 1985). An alternative to distillation for the measurement of ammonia nitrogen produced by Kjeldahl digestion, which is eminently suitable for automation, is by reaction of the ammonia with hypochlorite and phenate ion in alkali to produce indophenol, which can be measured spectrophotometrically at 630nm (Benedict, 1987). This colorimetric method was the subject of a collaborative study by Gantenbein (1973), who concluded that it was an accurate screening procedure for the analysis of meat products for protein content.

When Stubbs and More (1919), devised their classical formula for calculating the proportion of meat in fresh sausages, using nitrogen content, the only sources of protein considered were those from the meat and the bread filler, the contribution from the latter being substracted from the total to give that due to meat. With the addition of the extracted fat a value for the total meat was obtained.

To conform with the "Sausage and Other Meat Products Regulations, 1967" (Statutory Instruments, 1967), which stated that lean meat is that free of visible fat, allowances needed to be made for intramuscular fat, which has been shown to vary considerably, and for the nitrogen present in the outside fat. Pearson (1968), proposed formulae for the calculation of the lean meat content of pork sausages. The formula assumed an average nitrogen (fat free) content of pork including that contributed by outside fat of 3.45%, a fat content of the outside fatty tissue of 90%, and an allowance of 10% fat in lean meat.

The general formula for pork sausage was:

$$LM(\%) = \frac{112.5\ N_T - 0.4312^{*}\ F_{EX} - 2.25C}{3.45} \qquad (1)$$

* = 0.4437 for beef.

Where: LM = % Added lean meat.
N_T = % Total nitrogen in sausage sample (by analysis).
F_{EX} = % Total extracted fat in sausage sample.
C = % Dry carbohydrate + cellulose (by difference).

In addition to factors for nitrogen in fat-free meat, it was proposed that there was a requirement for an accepted maximum fat content of lean meat and an accepted average or limiting fat content for added fat.

Modifications to the formula for the calculation of lean meat content (1), were made to allow for the natural variation in the nitrogen content of fat free meat, and in the "permissible" proportions of fat in lean and fatty tissue by Pearson (1970).

The modified formula was:

$$LM = \frac{100N_T - F_{EX} \cdot N_M \left[\frac{100}{F_F} - 1\right] - 2C}{N_M \left[1 - \frac{F_L}{F_F}\right]} \qquad (2)$$

Where : LM = % of lean meat in product (containing invisible fat).
N_M = Mean nitrogen content of meat used (expressed as a percentage of the fat free meat).
F_F = % fat contributed by outside fat in the product.
C = % [carbohydrate + cellulose] present (by difference) associated with 2% of its weight of nitrogen.
F_L = % fat in the lean meat.

The formula (2) is applicable to any meat product with the introduction of appropriate values for N_M, F_L and F_F.

With the widespread use of fillers other than wheat starch, such as other cereals and milk powder in meat products, formulae were derived for the calculation of lean meat content, with special reference to nitrogen derived from other sources

(Pearson and Gardiner, 1971). Formula (2), can be applied more generally if - 2C is replace by - K_FC, where K_F is the percentage of nitrogen in the filler used, calculated on the dry carbohydrate. For wheat rusk K_F is 2.0 (AMC, 1965b), for pearl barley 1.8 (AMC,1968a), but for starch fillers such as cornflour and potato starch K_F is zero.

If dried skimmed milk is present in the product - K_FC becomes - K_F (C-0.5 S) - 5.8 S, where S is the percentage of dried milk in the product and C is the percentage of total carbohydrate (by difference), including lactose, in the product.

The formulae so far described still assumed that the only sources of protein were meat, cereal fillers and milk. They would not apply if other nitrogen containing materials such as soya flour, sodium caseinate, purified proteins or seasonings were present.

Using the formulae derived by Pearson (1968, 1970), it is possible to reasonably accurately assess the lean meat content of sausages and other meat products, the method of calculation developed by Stubbs and More (1919) being similarly applicable to the assessment of total meat content. These methods of calculation are inadequate if the product contains added gristle, sinew or loose connective tissue. Meat by definition includes rind and connective tissue "in amounts naturally associated with the flesh used". Excessive amounts can be present as part of the filler, but should not count towards the meat content. Correction for excessive connective tissue was thus required, analogous to the correction for carbohydrate fillers.

If no analytical control was excercised over the replacement of muscle meat by connective tissue protein, there would be a drop in the nutritive quality with respect to essential amino acids. Coomaraswamy (1972) proposed formulae for the determination of meat content in the presence of excess rind and/or connective tissue. Hydroxyproline was used to determine connective tissue content, it being assumed that 10% wet connective tissue satisfied the "amounts naturally associated with the flesh used".

An important consideration when calculating meat content by the formula presented here is the use of appropriate nitrogen factors. A history of the recommended average nitrogen percentage in the fat-free meat for pork and beef is given in table 2.6.

TABLE 2.6. HISTORY OF AVERAGE RECOMMENDED NITROGEN FACTORS (PERCENTAGE IN FAT-FREE MEAT) FOR PORK AND BEEF.

	Stubbs and More	Jackson and Jones	SPA*	AMC**	AMC	AMC
YEAR	1919	1932	1940, 1952		1961	1963a
Pork	4.00	3.55	3.60		3.45	-
Beef	3.75	3.42	3.40		-	3.55

* Society of Public Analysts.
** Analytical Methods Committee.

(From Pearson, 1975).

The gradual reduction in the factors was most probably due to the fact that the Stubbs and More method used relatively small (and therefore partially dried) samples. Moreover, in modern manufacturing plants, pork (for instance) is used soon after slaughter of the pig, before significant moisture loss can occur (Pearson, 1975). Nitrogen (fat free) factors have been recommended for other meat and poultry; viz, chicken (AMC, 1963b), liver (AMC, 1964), veal (AMC, 1965a), turkey (AMC,1965c), kidney (AMC,1966), tongue (AMC, 1967), and blood (AMC,1968b). All of these mean factors were derived from a statistical survey of several samples, which showed a wide biological variation. This variation can be illustrated by a recent investigation into the nitrogen factors for pork (AMC, 1986). It was found that although the proposed mean nitrogen (fat free) figure of 3.45 (AMC, 1961) was still applicable to the comminuted raw meat of a whole side, significantly different factors were found for individual joints; factors for these being recommended.

The determination of meat content based on nitrogen presents several difficulties. Apart from the wide variation in the nitrogen content observed in raw meat, there is a lack of agreement on the amount of fat and connective tissue that can be allowed in lean meat, the calculation of lean meat content also having to take into consideration the possible presence of materials containing non-meat protein.

2.4.2. ELECTROPHORETIC METHODS.

The applications of electrophoresis in the analysis of meat products can be broadly divided into those methods based on the determination of proteins specific to muscle, or on proteins specific to non-meat fillers, such as soya which have been added to a product.

Separation of proteins by electrophoresis is on the basis of charge, upon the application of an electric field, stabilised on a supporting medium, which can range from paper to polyacrylamide gel. In "native" electrophoresis, separation is due to the differences in the net charge of the proteins at the pH chosen for separation. In addition, the supporting medium (depending on its form), may have some molecular sieving effect. On the other hand, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) depends solely on molecular sieving, since, on binding with SDS all proteins carry the same net charge to mass ratio.

The detection of the non-meat proteins, casein and soya protein, in meat products using urea-starch gel electrophoresis was carried out by Olsman (1969). In luncheon meat heated up to 115°C or liver paste heated to 105°C, levels as low as 0.25% casein and 0.5% soya protein could still be detected. The addition of polyphosphates, starch or connective tissue to the products did not affect the results.

Mattey, Parsons and Lawrie (1970), used laser densitometry of stained starch gel electrophoretograms of proteins extractable in 8M urea to quantitatively identify ox

and rabbit in mixtures of these meats, even when heated to 120°C for 3-6 minutes.

Qualitative detection of soya protein in cooked meat products by polyacrylamide gel electrophoresis was reported by Fischer and Berlitz (1971), who found that the non-meat proteins could still be detected in sausages heated to 116°C. The quantitative identification of soya protein in fresh and heated meat products was reported by Parsons and Lawrie (1972). Laser densitometry of stained polyacrylamide electrophoretograms of proteins extractable in 10M urea / 2% 2-mercaptoethanol was used to quantitatively identify soya by the intensity of a unique protein band. Quantitation was possible in samples treated at up to 100°C for one hour, only qualitative assessment being possible after commercial sterilizing (124°C for 24 minutes). Unique electrophoretic bands were also identified in electrophoretograms of field bean and egg proteins, indicating that they too could be quantitatively assessed in a similar manner to soya, when admixed with meat.

Spell (1972) and Hoffman and Penny (1973) reported on the possibilities of using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) for the identification and quantitative determination of meat and foreign proteins. This method was used by Lee et al. (1975), for the quantitative determination of soya bean protein in fresh and cooked meat / soy blends. Electrophoretic bands unique to soya protein and to meat were identified and used for quantitation. In the case of meat, the band used was that corresponding to actin. The use of an internal standard, bovine serum albumin (BSA), to

counteract the variations due to non-reproducibility of gel staining, and the assessment being based on the ratio of the intensity of the soya specific band compared with the meat specific band (actin), resulted in accurate quantitation.

Lee et al. (1976), went on to use SDS PAGE for the detection of various non-meat extenders in meat products. The possibility of the inclusion of plant proteins such as cotton-seed, peanut, sunflower seed, safflower seed and grain protein, as well as other non-meat proteins such as non-fat milk powder, casein, whey protein and egg protein in meat products necessitates methods for their identification. It was found that all of these proteins have unique SDS PAGE patterns distinct from those of meat and soya. In complex mixtures of meat and extenders, such as a meat/soy/cotton-seed/peanut blend, SDS PAGE bands unique to each component were identifiable.

The analysis of commercial soya additives in meat products using urea polyacrylamide gel electrophoresis was investigated by Guy, Jayaram and Wilcox (1973). Densitograms of the stained protein bands were found to give quantitative estimation of the soya protein content, based on the peak height of soya specific bands. Quantification was reportedly easier in heated products since this resulted in a decrease in the meat protein bands without affecting the more heat resistant soya specific bands. Armstrong, Richert and Riemann (1982) used urea SDS PAGE to develop a refined method for soya protein determination in raw and pasteurised meat products. Electrophoresis was performed on proteins extractable by urea/

SDS/2-mercaptoethanol, from raw, pasteurised (70°C) and retorted (121°C), commercial and simulated meat products. Quantification was performed on a soy specific band between muscle actin and myosin, no muscle, organ, or other vegetable proteins having bands which would interfere. The protein haemocyanin was used as the internal standard to correct for variations in staining of the gels. Known amounts of pure soya protein standard were run, and after staining and densitometry, the ratio of the peak height of the soy specific band to the haemocyanin peak height was plotted against the amount of soy protein loaded to produce a calibration curve. The method was found to be applicable to raw and pasteurised meat products with a detection limit of 0.5% soy protein, though retorting caused the loss of the soy specific band thus precluding quantification.

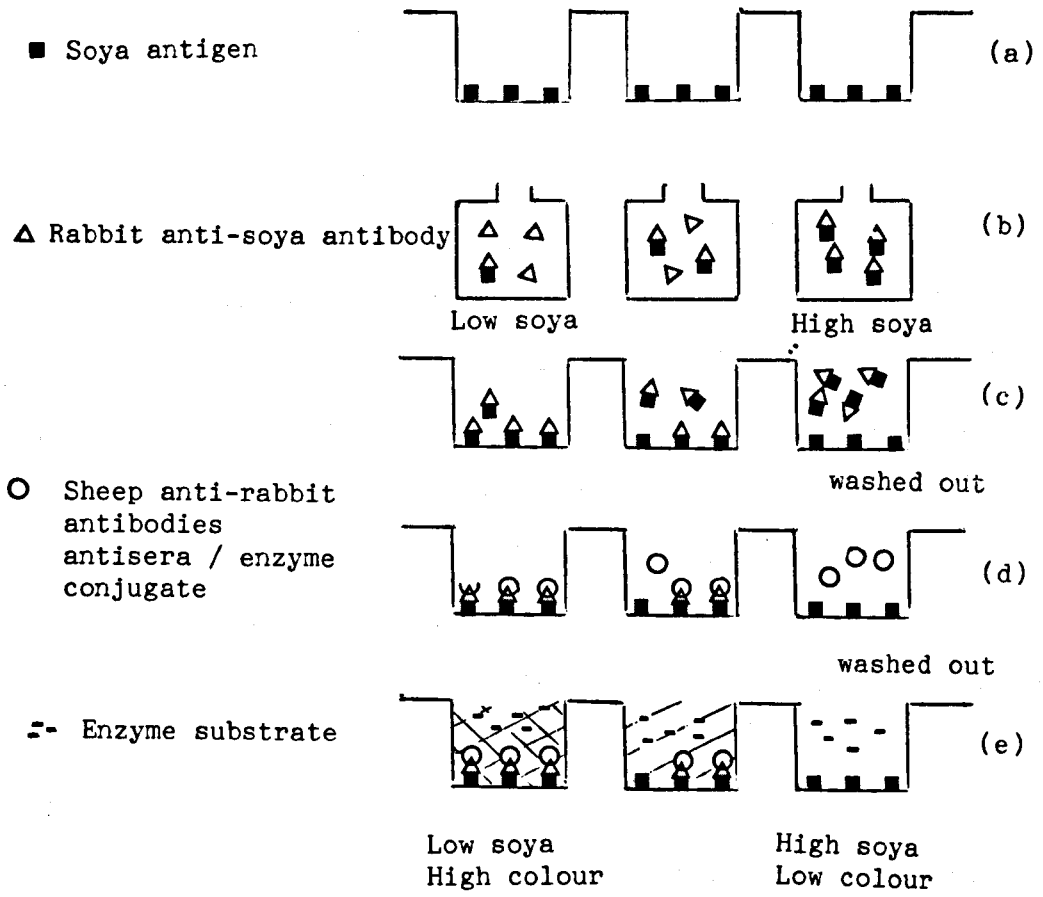
Isoelectric focusing, a method based on the separation of proteins by their isoelectric points, has been used by Flaherty (1975) and Llewellyn and Flaherty (1976) for the detection and estimation of soya proteins in food products using polyacrylamide gels. It was concluded that the limitations of the method were those of electrophoretic methods in general; viz, problems of extraction, staining and densitometric scanning. Isoelectric focusing was not applicable to extensively heated samples, though as a qualitative technique the superior resolution of the protein species, compared with conventional electrophoretic methods could have useful applications. King (1978), found that isoelectric variants of actin in raw compared with cooked meats, could be used to determine the severity of heat treatment that a meat or meat product had been exposed to.

An alternative to gel electrophoresis is electrophoresis on cellulose acetate membranes. This inexpensive and less cumbersome method was used by van Gils and Hidskes (1973) for the detection of casein, soya protein and coagulated egg white protein in meat products. Levels as low as 0.1% casein and soya protein and 0.5% egg white protein could be detected. The method was proposed as suitable for quantitative evaluation of meat products, but with the rapid development of gel electrophoretic methods, never became popular.

2.4.3. IMMUNOCHEMICAL METHODS.

Food immunoassays have been developed within the last two decades, to the extent that they are now available in kit form for routine food analysis (Allen and Smith, 1987). Initially conservatism, high cost, difficulties in using solid samples and problems with using radio-isotopes near food meant that immunoassays were slow to gain acceptance (Morris and Clifford, 1985). The replacement of radio-labels with enzyme labels was an important step forward and led to the development of the enzyme-linked immunosorbent assay (ELISA), which is currently widely used in the food industry. The term ELISA can be applied to all solid-phase immunoassays using enzyme labelled reagents, a diagrammatic representation of one such method can be seen in figure 2.5. Unlike classical immunochemical methods, ELISA does not rely on the precipitation of the antigen-antibody complex, instead the presence of the complex is quantitatively assessed by colorimetric measurement of the activity of the

Figure 2.5. Enzyme-linked immunosorbent assay procedure.



(a) PVC microtitration plates sensitised with antigen.
(b) Samples and standard solutions incubated with excess antibody, in separate glass vials.
(c) Reaction mixture (b), transferred to sensitised wells (a), followed by incubation and washing out of the unbound antibody-antigen complexes.
(d) Excess anti-antibody labelled with enzyme added, followed by incubation and washing out.
(e) Enzyme substrate added and incubated. Colour intensity measured spectrophotometrically.

(From Hitchcock et al, 1981).

enzyme linked to it. Commonly used enzyme conjugates include, horseradish peroxidase and alkaline phosphatase.

ELISA has found applications for determining the species of origin of raw meat based on the measurement of serum albumins, though the antisera generated for raw meat is not applicable to cooked meat.

Possibly the most important application of immunoassays in the analysis of meat products is the determination of their soya content, based on the two principal storage proteins of soya; viz, glycinin (11-S protein) and β-conglycinin (7-S protein). These proteins are attractive immunologically as they are phylogenically distant from any animal proteins (Berkowitz and Webert, 1987). On heating glycinin loses much of its immunoreactivity, β-conglycinin being rather more resilient, the latter being responsible for most of the immunoreactive response in soya immunoassays (Hitchcock et al., 1981).

The problem of loss of immunoreactivity on heating can be partially overcome in a number of ways. Polyclonal antibodies can be generated which give immunoreactivity to as many sites as possible, changes in soya on heating only affecting some of the sites. Alternatively soya, processed in different ways to give immunologically different proteins, can be chemically denatured by urea/2-mercaptoethanol. On renaturation, by the removal of the denaturants, all samples however previously processed, will return to immunologically similar "nearly native" states. "Nearly native" soya has been shown to react with native antibodies and must therefore be very similar, but unfortunately

"nearly native" soyas, previously processed differently, still have slightly different immunoreactivities, making accurate quantification of processed soya by a general method difficult.

The qualitative detection of soya protein in heated meat products was investigated by Poli et al. (1979), using cross-over electrophoresis with immunofluorescence detection. The sample proteins were extracted with sodium chloride and electrophoresed against soya-specific rabbit antisera. The resulting antibody/antigen precipitate was incubated with sheep anti-rabbit γ-globulin fluorescent sera, the complex being visualised under ultra-violet light. The use of fluorescent tagging allows the detection of the very low levels of immuno-precipitate which occur with heated soya. Using native soya antibody, qualitative detection was possible in a 2.5% soya in meat mixture, heated to commercial sterility (125°C, 25min).

An ELISA procedure for the determination of denatured soya in processed products, using antibodies generated against a "nearly native" soya protein preparation, was reported by Hitchcock et al. (1981). Unfortunately the response of different soya products to the ELISA was rather variable, but still suitable for quantitative analysis, especially when the type of soya product was known. Using methods based on those of Hitchcock et al. (1981), but using the type of commercially available reagents that routine analytical laboratories could easily obtain, Griffiths et al. (1984), obtained satisfactory data. This indicated the suitability of this ELISA method for the routine screening of soya in foods.

The performance of this ELISA method for the determination of soya protein in meat products in comparison with the SDS PAGE method of Armstrong, Richert and Riemann (1982) (section 2.4.2) was compared in an international collaborative study, involving 26 laboratories in 10 European countries (Olsman, Dobbelaere and Hitchcock, 1985). It was concluded that both methods give zero blanks and similar inter-laboratory variances, SDS PAGE giving more repeatable intra-laboratory data, whilst ELISA gives more accurate determinations. Both methods appeared to be at an interim stage of method development, being useful, but requiring further refinements to make them suitable as standard analytical methods.

Rittenberg et al. (1987) reported an improved ELISA for the determination of soya protein in food products. This involved the extraction of standards and samples with a denaturing solution consisting of urea and dithiothreitol. Renaturation was accomplished using cystine and sodium chloride, the renatured samples being used in an ELISA type assay. Autoclaved samples (121°C, 15 psi, 20min) still gave a much reduced immunological response, making quantification difficult.

The principle of ELISA on "nearly native" protein, could have more general applications. For instance, ELISA procedures could be developed to estimate other individual components of meat products, such as muscle protein (lean meat), collagen (connective tissue), and non-meat protein additives.

A further method for the quantitative immunological detection of soya in heat treated meat products was proposed by Yasumoto, Hiroshi and Suzuki (1985) and Yasumoto et al. (1986),

based on an ELISA of a heat stable pentapeptide "SP-1" from trypsin digested glycinin. Purified "SP-1", gave a quantitative response, allowing the generation of a calibration curve. Using this method autoclaved, trypsin digested soya bean isolate and flakes could be quantitated with satisfactory results. It was concluded that an ELISA method based on the "SP-1" peptide could be a sensitive specific tool for the quantification of glycinin in heat processed products.

A recently reported, rapid immunological method for the detection of non-meat proteins in heat treated meat products, was developed by Janssen, Vootman and de Baaij (1987), using the method of dot blotting. The sample of interest was extracted with a buffer containing SDS, diluted, and applied as 0.5µl drops on a nitrocellulose film. This film is incubated with a primary antiserum (rabbit) against the protein of interest, detection of the resulting antibody-antigen complex being by a secondary anti-rabbit antiserum, coupled to colloidal gold or peroxidase enzyme. Both systems made it possible to detect non-meat proteins at the 0.1% level.

2.4.4. HISTOCHEMICAL METHODS.

The histochemistry of meat products has mainly been focused on the identification and quantification of their non-meat components.

Coomaraswarmy and Flint (1973) reported on the detection of soya "novel proteins" in comminuted meat products, by the identification of the carbohydrate material of the

cellular fraction of soya using histochemical methods involving controlled oxidation and staining. Positively stained areas were easily identifiable in extruded soyas and meat/soya blends, in which soya cells could be observed. This method, being only applicable to identification by morphology, is of no use in samples where no cellular material is present, such as those containing spun soya protein (Flint and Lewin, 1976). To overcome this, the development of a specific stain for a unique soya protein has been proposed.

Quantitative histochemical determination of meat product constituents was performed by Flint and Meech (1978), who developed a stereological method for the measurement of toluidine blue stained soya in meat/soya mixtures. Stereology is the term given to a collection of methods that enable information about 3-dimensional qualities (including volume ratios), to be obtained from the study of 2-dimensional sections. The method chosen by Flint and Meech (1978), being the 2-dimensional systematic point count. A 42 point graticule was used on 10μm thick sections of the sample, the results being expressed as volume in volume fractions. This method is applicable to the quantification of any constituent of a comminuted meat product, such as rind or rusk in which the constituents are sufficiently contrasted. It has been proposed that the method could be used to determine the proportion of total nitrogen due to gluten , soya flour or excess connective tissue in both raw and heat treated meat products. The determination of lean meat content, by counting muscle fibres, is also a possibility.

2.4.5. OTHER CHEMICAL AND PHYSICAL METHODS.

Colorimetric and dye binding methods have important applications in the protein determination of meat products. As early as 1951, Lowry et al., used Folin-phenol reagent to determine the apparent protein content of whole rabbit tissues. When compared with the Kjeldahl method the colorimetric method was reportedly more reproducible.

Torten and Whittaker (1964), made a comparison of the Biuret with various dye-binding methods for protein determination in meat. In comparison with the Kjeldahl method, the Biuret method, based on the reaction of cupric ion with peptide bonds, was reported to be more accurate and reproducible, as well as being quick, simple and suitable for automation. Dye binding using the dye Orange-G, was found only to be applicable to meat protein determination if sample preparation and experimental conditions were carefully standardized, the amount of dye bound / g protein being highly variable. The dye Amido Black was found to have similar drawbacks, due to non-specific binding to vessel walls. Amido Black was further investigated as a method for protein determination in meat by Moss and Kielsmeier (1967), who obtained satisfactory results, though the dye concentration was found to be critical for reproducibility. A similar method using the dye Cochineal Red A was reportedly cumbersome and required equally great care for reproducibility.

Udy (1971) reported an improved method for the estimation of protein using the monosulphonic azo dye, Acid Orange 12 (AO-12), which reacts with the basic groups of

histidine, arginine and lysine. Proteins of natural products bind the dye in regular but individually characteristic manners, therefore it is difficult to apply this assay to irregular systems such as meat products whose components are not known.

Peptide analysis has been suggested for the determination of composition in meat/soya blends by Bailey (1976), who identified a soya specific peptide "SP-1", in the tryptic digests of meat/soya blends after fractionation by ion-exchange chromatography. This peptide could be used to quantify soya, even in severely heat treated samples, as although heating alters the physiochemical properties of proteins, it rarely disrupts their primary structure. The "SP-1" peptide was found to originate from the 11-S (glycinin) fraction of soya, having similar chromatographic properties regardless of the source of soya protein isolate (Bailey et al., 1978). Llewellyn et al. (1978), used a similar approach to identify soya specific peaks, "SP-2A" and "SP-2B", as well as a meat specific peak, "MP-1", in the tryptic digests of meat/soya blends. By comparison of the size of these specific peaks in unknown mixtures with those in standard mixtures, meat content could be estimated. Peptide analysis gave reasonable estimates of meat content for cooked meat products such as pies and canned products, and was proposed as being suitable for use as a lean meat index, all species investigated giving similar peptide profiles. Peptide analysis, utilising the advantages of HPLC, was compared with two other methods for the determination of soya protein in meat products, namely, stereology by microscopy and ELISA, by Griffiths,

Billington and Griffiths (1981). It was concluded that a brief examination by microscopy to determine what kind of soya material was present could be followed by an ELISA screening procedure, samples of interest being taken for more extensive investigation by peptide analysis. All of these methods lend themselves to the analysis of almost any proteins in food and could therefore have important applications to the determination of meat content.

HPLC was suggested as a method of separating complex, high molecular weight, mixed protein systems by Flaherty (1975). A procedure for separating mixtures of meat and soya protein, using weak anionic exchange HPLC on an acetate buffer extract of the sample was developed. Meat and soya specific protein peaks were identified, linear correlation between soya content and the area of a soya specific peak being found. It was suggested that HPLC of the proteins of both processed and unprocessed meat products, containing soya in all its various forms, could provide a quick and quantitative analytical procedure.

When considering soya determination in foods, the use of chemical markers as an index of soya content, could be useful. The search for a soya index has included insoluble polysaccharides, oligosaccharides, protein-bound sugars, free amino acids, free peptides, phytate, saponins, sterols, and metal ions. The statutory addition of an artificial marker (titanium dioxide), has been suggested, but is not widely favoured (Hitchcock et al., 1981).

Potassium content, as determined by gamma-ray emission of the naturally radioactive potassium-40 isotope

(Kulwich et al., 1961), or by flame photometry (Kirton and Pearson, 1963), has been proposed as a method of estimating the composition of meat. The possibility of using potassium content of a meat mixture as an indicator of lean meat content was suggested by Kreuzer, Ring and Shröder (1968), when it was found that pork and beef muscle of similar composition (water, fat and nitrogen contents) had similar potassium contents. Similarly the use of creatine content for the rapid estimation of muscle protein in beef/vegetable protein mixtures has been suggested (Dahl, 1963), the level of this metabolite being relatively constant in lean beef but absent from vegetable protein, though little was known about the fate of creatine on meat storage, freezing and processing.

Histidine dipeptides can be rapidly quantified by HPLC methods (Carnegie et al., 1983). Approximate levels of lean meat in a product can be determined by the levels of these dipeptides, though the method is most applicable to the determination of the level of pork in a product (Carnegie, collins and Ilic, 1984), since pork is the only commonly used meat that contains high levels of balenine (β-alanyl-3-methylhistidine).

Herrmann, Thoma and Kotta (1976) developed a simple method using buffer extraction, which reportedly removed foreign proteins, blood plasma, collagen and non-protein nitrogen sources from a meat sample, leaving the undissolved muscle protein which could be determined directly. A method involving the use of potassium iodide/phosphate buffer

extractable proteins for estimating meat content of products was suggested by Khan and Cowan (1977), meat myofibrillar proteins being highly soluble in this buffer, proteins of vegetable origin having low solubility.

A fluorometric technique for the quantitative determination of soya flour in meat/soya blends was evaluated by Eldridge and Holmes (1979). Using the difference in the natural fluorescence of soya flour and beef dispersed in guanidine hydrochloride (soya; excitation maximum 360nm / emmission maximum 440 and 740nm. Beef, excitation maximum 360nm / emmission maximum 740nm), it was possible to determine the amount of soya at the 30% level to within ±2.4%.

Near infra-red (NIR) reflectance spectroscopy, uses very small differences in absorption of NIR radiation, corresponding to overtones and combinations of fundemental infra-red frequencies of chemical functional groups that are characteristic of particular analytes. It has found numerous applications in the analysis of agricultural and food products, including the determination of protein, moisture, oil, starch, sucrose and fibre (Osbourne, 1981). Though NIR reflectance spectroscopy requires complex mathematics to convert absorbtion to analytical results, the use of improved sampling techniques and the most capable computer programming means that it can offer a real alternative technique for the rapid determination of selected meat components (Lee, 1985).

2.4.6. 3-METHYLHISTIDINE AS AN INDEX OF MEAT IN FOODS.

The occurence of 3-methylhistidine as part of the primary structure of contractile protein was established by Asatoor and Armstrong (1967), who proposed that the content of this amino acid in a tissue hydrolysate would be an estimation of the contractile protein content. On this basis, Hibbert and Lawrie (1972) applied the methods previously used by Johnson, Harris and Perry (1967a) for the determination of 3-methylhistidine in purified myofibrillar proteins, to the determination of this amino acid in meat/soya hydrolysates. The method involved lengthy ion-exchange chromatography, with ninhydrin assay of the resulting fractions, based on the methods originally developed by Spackmann, Stein and Moore (1958). Relatively constant levels of 3-methylhistidine of 5-6 milligrams (mg)/g nitrogen were found in beef, lamb and pork, none being detectable in wheat protein, soya protein, casein and gelatin. When the method was applied to mixtures of beef and textured soya protein, which had been subjected to commercial sterilising procedures in cans, a satisfactory correlation was found between the 3-methylhistidine titre and the percentage meat in the mixture. These results indicated the potential of 3-methylhistidine as an index of meat in foods, and the robustness of this amino acid to extreme processing conditions.

Haverberg *et al.* (1974) developed a method for isolation and quantification of 3-methylhistidine, using ion-exchange chromatography with pyridine elution, which allowed pre-concentration of the low levels of 3-methylhistidine present,

before conventional amino acid analysis. This method was used to determine the content of this amino acid in various rat tissues (Haverberg et al., 1975) (table 2.7). Such levels would be expected to be typical of those found in other mammalian species, including food species.

TABLE 2.7. 3-METHYLHISTIDINE LEVELS IN VARIOUS RAT TISSUES.

TISSUE	3-METHYLHISTIDINE CONTENT (moles/g protein)
Skeletal muscle	3.85
Diaphragm	2.61
Heart	1.54
Liver	0.31
Stomach	1.98
Kidney	0.51
Lung	0.90
Spleen	1.22
Testis	0.43
Brain	0.56

(From Haverberg et al., 1975).

The high levels of 3-methylhistidine in the tissues of the gastrointestinal tract are due to the presence of smooth muscle, the 3-methylhistidine content of which has since been reported to be close to that of mixed skeletal muscle (Holbrook, Gross and Irving, 1979).

Rangeley and Lawrie (1976) and Poulter, Rangeley and Lawrie (1977) developed and validated the analytical method

for the determination of methylamino acids as indices of meat in meat products. Levels of 3-methylhistidine and N-methyllysine were determined for beef, lamb, pork and whale meats using ion-exchange chromatography of meat hydrolysates, with ninhydrin detection of the resulting fractions, a single separation taking as long as eight hours. 3-methylhistidine titres showed far less variation than those of N-methyllysine, levels of the former being approximately 6.0 mg/g nitrogen in beef and lamb but from 5-56 mg/g nitrogen in pork, an increase being seen with age (table 2.8).

In whale meat 3-methylhistidine levels of over 300 mg/g nitrogen were found (table 2.8). The source of these high levels of 3-methylhistidine in pork and whale meat was found to be the dipeptide balenine (β-alanyl-3-methylhistidine), this dipeptide replacing its isomer anserine (β-alanyl-1-methylhistidine) with age in pigs. Balenine was found to be readily removable from both pork and whale meat by an aqueous washing procedure, the levels of residual protein-bound 3-methylhistidine being similar to those found for beef and lamb. 3-methylhistidine was absent from all non-meat/organ proteins analysed, enabling this amino acid to be tentatively suggested as an index of meat content of food.

TABLE 2.8. 3-METHYLHISTIDINE TITRES OF MEAT.

SAMPLE		3MEHIS TITRE (mg/g nitrogen)
Lamb:	Chump chop	5.40
	L.dorsi	6.15
	Leg	6.13
Beef:	L.dorsi	5.83
	Leg	5.95
	Shin	5.42
	Steak	6.48
Pork:	L.dorsi	19.95
	L.dorsi (washed 3x H_2O)	5.21
Whale:	Unwashed	368.36
	Washed 3x H_2O	9.65
	Washed 3x sarcoplasmic buffer	6.07

(From Rangeley and Lawrie, 1976).

The 3-methylhistidine levels in a variety of other protein sources were investigated by Rangeley and Lawrie (1977), who also discussed the practicalities of applying methylamino acid titres to predicting meat content. In comparison with the titres previously found for beef and lamb (Rangeley and Lawrie, 1976), chicken and turkey were found to have a higher titre, whereas clupeine and elasmoblanch fish and offals had lower titres (table 2.9. and 2.10), the amino acid being absent from vegetable proteins. It was proposed that 6mg 3-methylhistidine / g nitrogen could represent 100% lean meat and be the basis of a lean meat index. This premise was applied to samples of meat products including commercial sausages and meat pies, the percentages calculated being in terms of "lean meat" (not including intramuscular fat and connective tissue), with respect to protein content, not to the whole product.

Low titres of 3-methylhistidine, due to large amounts of added connective tissue (connective tissue being devoid of 3-methylhistidine) were avoided by expressing the 3-methylhistidine titres in terms of mg/g of non-connective tissue nitrogen, the connective tissue level being determined by its hydroxyproline content. More consistent results were obtained after the removal of interfering compounds from the meat products. Interfering constituents of fillers and binders were removed by alcohol/acetone extraction prior to sample hydrolysis. This washing procedure also removed soluble 3-methylhistidine giving a titre in terms of protein-bound 3-methylhistidine as mg/g protein (non-connective tissue) nitrogen.

TABLE 2.9. 3-METHYLHISTIDINE TITRES OF MEAT AND FISH.

SAMPLE		3MEHIS TITRE (mg/g nitrogen)
Rabbit:	Thigh	5.59
Chicken:	Leg	8.22
	Breast	8.44
Turkey:	Breast	7.80
Cod	(gadoid)	6.11
Mackerel	(clupeine)	4.81
Skate	(elasmobranch)	3.90

(From Rangeley and Lawrie, 1977).

TABLE 2.10. 3-METHYLHISTIDINE TITRES OF MEAT OFFALS.

SAMPLE		3MEHIS TITRE (mg/g nitrogen)
Kidney	(lamb)	None detected
Liver	(pig)	0.80
Lung	(pig)	None detected
Stomach	(pig)	2.00
Tripe	(ox)	4.77
Stomach	(sheep)	2.73
Heart	(ox)	2.82
Blood serum protein (fibres)		None detected

(From Rangeley and Lawrie, 1977).

The reported absence of 3-methylhistidine in proteins of plant origin, was contradicted by Nishizawa et al (1978), who whilst investigating the quantitative isolation of 3-methylhistidine by pyridine elution ion-exchange chromatography, reported significant levels of this amino acid in maize, soyabean meal, oats, wheat and rice brans, and lucerne meal. These results seem questionable, especially since the elution characteristics of 3-methylhistidine, which they reported were different than those previously reported by Haverberg, Munro and Young, 1974, using the same method, indicating that mis-identification of the chromatographic peaks had occured.

Alhough the relatively constant titres of 3-methylhistidine in meat and its absence in vegetable proteins had been established, the technique of ion-exchange chromatography used was time consuming (run time of hours), prone to experimental error (up to 10%) and non-specific, which may have accounted for some of the inconsistencies of published results. Automated ion-exchange methods, using post-column ninydrin derivatisation have been employed by Neuhäuser and Fürst (1979) and Žunić, Stanimirović and Savić (1984) for the determination of 3-methylhistidine. Methods that were more rapid and specific were required to ensure that the method of 3-methylhistidine quantification was not the limiting factor in the practicability of utilising 3-methylhistidine as an index of meat content in food.

In 1978 Skurray and Lysaght reported on a method utilising high performance liquid chromatography (HPLC) for the

determination of 3-methylhistidine. 1-fluoro-2,4-dinitrobenzene (FDNB) derivatives of sample hydrolysates were prepared, the non-basic amino acid derivatives being separated prior to chromatography by ether extraction. Time of chromatographic analysis was reduced to 10 min with an error of 1.9%, compared with the run time of 7 h and error of 11.9%, by the method of Rangeley and Lawrie (1976).

FDNB derivatives were used by Poulter and Lawrie (1980a). Pre-column derivatisation of sample hydrolysates was followed by ether extraction to remove non-basic derivatives, the remainder being separated by a short ion-exchange column. Slightly lower titres of 3-methylhistidine for lean meat (5.52 mg/g non-connective tissue nitrogen) were found by this method compared with traditional ion-exchange chromatography with post-column ninydrin detection.

An account of the practical applications of 3-methylhistidine in determining the lean meat content of food products was given by Poulter and Lawrie (1980b). Determination using 3-methylhistidine was contrasted with standard methods based on nitrogen content (section 2.4.1). It was concluded that "lean meat" as determined by 3-methylhistidine content in terms of mg/g nitrogen, being uncorrected for fat and connective tissue, was not equivalent to "lean meat" as determined by the regulations in force at the time (Statutory Instruments, 1967), which included intramuscular fat (1-18%) and connective tissue, skin, rind, sinew and gristle "in amounts naturally associated with the flesh used". To allow comparisons with determinations made by the public analyst to be made, the lean

meat content as determined by 3-methylhistidine was corrected so as to include the total amount of connective tissue present. No limits were placed on the connective tissue contents as there were no statutory limits. Total meat was then "lean meat" plus the total fat present. "Lean meat" contents as determined by 3-methylhistidine were generally lower than those determined by the standard method as there was no consideration of the "invisible" fat in meat.

The fact that offals were allowed in sausages would give low lean meat values as determined by 3-methylhistidine compared with the determination by the standard method, since offals contain low levels of this amino acid (Rangeley and Lawrie, 1977), but are indistinguishable from muscle protein by total nitrogen determination. Another point of contrast is the fact that 3-methylhistidine titres are based on protein nitrogen, water soluble nitrogen (including non-protein nitrogen) being removed in the wash process required to eliminate balenine. In comparison nitrogen factors used in the standard method include nitrogen from water soluble protein and non-protein components, the levels of which are variable.

The most important advancement in the methodology of 3-methylhistidine determination in relation to meat analysis was made by Jones, Shorley and Hitchcock (1982a,ab), who developed a method using reverse-phase HPLC of acid stable fluorescamine derivatives (Nakamura and Pisano, 1976b). Separation of components by reverse-phase HPLC depends on the relative retention of the species in question by the non-polar

phase bonded to the column matrix, in the presence of the more polar solvent. In meat and meat products, only histidine and 3-methylhistidine give significant levels of acid stable derivatives, these being easily and rapidly separated by HPLC. The method employed consisted of the preparation of an acetone powder which included washing the sample with aqueous ethanol to remove balenine and other interfering material. Acid stable fluorescamine derivatives were prepared from the acetone powder hydrolysates, these being separated on a 20cm x 0.4cm i.d. reverse-phase column of octadecyldimethylmonochlorosilane bonded to 5μm Lichrosorb SI 100, in no more than a few minutes, with isocratic elution using methanol/acetate buffer. Detection was by an Aminco Bowman (V.A.Howe, London) filter fluoromonitor, with a Corning 760 primary and a Wratten no.3 secondary filter. Both the histidine and 3-methylhistidine fluorophores were found to have a maximum absorbtion at 370nm and maximum emission at 453nm, 3-methylhistidine having a linearity of response from 15ng/ml to 3.7 μg/ml, the limit of sensitivity being 10ng/ml (equivalent to a peak of 16pg = 95 femtomoles). By this method slightly lower results were obtained than those previously reported probably due to its more discriminating nature, successful analysis of a complex dried soup powder, which had previously defied analysis being possible. Rapid, routine 3-methylhistidine analysis was now possible, lean meat of all species investigated giving levels of around 5 mg/g non-connective tissue nitrogen, no protein-bound 3-methylhistidine being detectable in collagen, blood, sarcoplasmic proteins, milk, egg, vegetable and other

foreign proteins.

Jones et al. (1985) applied the method developed by Jones, Shorley and Hitchcock (1982 a,b) to an investigation of the levels of 3-methylhistidine in a range of beef cuts and offals. New units, expressing 3-methylhistidine titres in terms of μg/g fat free, connective tissue free (fresh) sample (μg/g ff,cf,sam) were used, since this amino acid is absent from both fat and connective tissue. The titres found for beef cuts and offals are given in table 2.11. The variations between prime cuts were found not to be statistically significant due to intra-cut variations. Flank was found to have a significantly higher titre, whereas cheek and mechanically recovered meat (MRM), had only around 70% of the prime cut level. Offals had lower titres than prime cuts, 3-methylhistidine being almost absent from liver and kidney. It was proposed that the differences between prime and manufacturing cuts could have been due to differing protein/H_2O or actin/myosin ratios, differences in the proportions of myosin types or even invalid assumptions made in deriving the 3-methylhistidine based index.

TABLE 2.11. MEAN LEVELS OF 3-METHYLHISTIDINE FOR PRIME AND MANUFACTURING BEEF CUTS AND OFFALS.

CUT/OFFAL	3MEHIS LEVEL (μg/g ff,cf,sam)
Topside	132
Chuck	117
Shin	111
Silverside	120
Leg	119
Brisket	123
Flank	133
Cheek	67
Tongue	65
Heart	48
Mechanically recovered meat	73
Kidney	<1
Liver	<1

(From Jones et al., 1985).

Further development of the methodology for 3-methylhistidine determination was carried out by White and Lawrie (1985a), who obtained more complete sample homogeneity by milling of acetone powders, which allowed the use of smaller sample size. As little as 60mg acetone powder / 15ml 6M HCl was used for sealed bottle hydrolysis, compared with the 100ml volumes used for reflux hydrolysis by Jones, Shorley and Hitchcock (1982a,b). This reduced the cost of analysis and allowed rapid throughput of samples at the hydrolysis stage which was the rate limiting

step. Using their adapted method, White and Lawrie (1985b) determined the variations in the level of 3-methylhistidine in whole bovine muscles and their isolated myosins (table 2.12). The low titre of bovine cheek reported by Jones et al.(1985) was confirmed and reflected in a low level of histidine methylation of the myosin prepared from this muscle, though the high titre previously reported for flank muscle was not found.

TABLE 2.12. 3-METHYLHISTIDINE TITRES OF BOVINE MUSCLES AND THEIR RESPECTIVE MYOSINS.

MUSCLE	3MEHIS TITRE	
	Whole muscle (μg/g ff,cf,sam)	Myosin (moles/mole)
Masseter/Malaris (cheek)	83.0	0.24
Sternomandibularis	119.5	1.25
L.dorsi	139.1	1.53
Semimembranosus	123.8	1.35
Aponeurosis of the Obliquus externus abdominus (flank)	134.6	1.51

(From White and Lawrie, 1985b).

The low values for cheek muscle and its myosin are indicative of the observation that muscles of predominantly "red" fibre type are low in 3-methylhistidine, apparently due to the lack of methylation of "red" muscle myosin. This, when considered along with the reportedly constant titres of this amino acid in actins (section 2.1.4), indicates that the principle cause of variation in the total 3-methylhistidine titres of meat is due to variations in the level of histidine methylation in the myosin.

The 3-methylhistidine levels in a range of pork and chicken meats was determined by Jones, Homan and Favell (1987). Important adaptations to the method of 3-methylhistidine determination had been made, viz: increasing the number of acidified ethanol and acetone washes in the acetone powder preparation from one to three, to ensure the complete removal of balenine and other interfering compounds and the use of a smaller, 5cm x 0.49cm i.d. reverse-phase HPLC column for more rapid separation. Low 3-methylhistidine titres were found for manufacturing compared with prime pork cuts, and chicken leg along with mechanically recovered chicken, had a lower 3-methylhistidine titre than breast (table 2.13). This variation amongst legitimate meat sources was a serious drawback for the use of 3-methylhistidine as a quantitative index of lean meat.

TABLE 2.13. MEAN LEVELS OF 3-METHYLHISTIDINE IN PRIME AND MANUFACTURING PORK CUTS AND CHICKEN.

CUT		3MEHIS LEVEL (μg/g ff,cf sam)
Pork:	Belly	116
	Loin	130
	Spare rib	106
	Chump chop	115
	Shoulder	112
	Skirt	86
	Sinewy pork	99
	Lean head	97
	Chaps	79
	Semi-lean pork	118
Chicken:	Breast	116
	Leg	129
	Mechanically recovered	92

(From Jones, Homan and Favell, 1987).

The method of 3-methylhistidine determination developed by Jones, Shorley and Hitchcock (1982a,b), later modified by White and Lawrie (1985a) and Jones, Homan and Favell (1987), represents the currently used method for the determination of this amino acid in meat and meat products. Various other analytical approaches have recently been reported for the measurement of 3-methylhistidine in other biological tissues and fluids, that have not yet been applied to food analysis. These include colorimetry, amino acid analysis, thin

layer chromatography, gas chromatography, gas chromatography-mass spectrometry and HPLC methods (Minkler et al., 1987).

3-methylhistidine is virtually unique to meat, being found only in the actin and myosin of the contractile tissue. Certain problems were identified in its use as an unequivocal index of lean meat in foods, namely the lack of this amino acid in mammalian myosins from cardiac, foetal and red muscle and its reported absence from certain fish actins. On the other hand, evidence suggests that 3-methylhistidine is present at a constant level in most, if not all actins, holding promise for further refinement of the methodology.

2.4.7. ACTIN AS A MEASURE OF MEAT CONTENT.

The highly conserved nature of actin makes it potentially an ideal indicator of the amount of meat, regardless of the species, in a food product. Actins from mammalian and avian striated muscle have been shown to be very similar as regards sedimentation coefficients, electrophoretic patterns, amino acid composition and peptide "maps" (Carsten and Katz, 1964).

Actin is an integral part of all classes of muscle tissue, as well as being present in small amounts in many non-muscle tissues such as the brain, platelets, and liver, and in micro-organisms such as Acanthamoeba castellani (Gordon, Boyer and Korn, 1977), and in the alga Chara corallina (Williamson, 1974); in fact almost every tissue in the body has been shown to contain actin-like proteins, but the levels of actin in these tissues is so low that interference to a meat assay based on actin would be negligible.

Actin had previously been used along with soya-specific proteins to quantify soya protein in fresh and cooked meat/soya blends using densitometry of stained SDS PAGE electrophoretograms (Lee et al., 1975). The heat resistance of this protein, in comparison with myosin, was noted by Hofmann and Penny (1973), when using SDS PAGE for the identification and quantitative determination of meat and foreign proteins in food.

An electrophoretic procedure for the determination of actin was used by Bray and Thomas (1975). The amount of protein having the same electrophoretic mobility as standard actin by SDS PAGE was measured by densitometric methods. Like

all electrophoretic methods, this approach is subject to the inaccuracies introduced by the variability of staining by Coomassie brilliant blue (Fishbein, 1972).

Actin was proposed as a target protein for the estimation of muscle content of meat products by den Hartog (1980). It was found by SDS PAGE that actin was particularly resistant to heat treatmeant at up to 90°C for 30 minutes, indicating the possibilities of using this protein as a meat content indicator in cooked products.

Jonker, den Hartog and van Roon (1982) and Jonker, van Roon and den Hartog (1985) proposed that the determination of muscle content in foods could be carried out by determining the actin content based on 3-methylhistidine. Actin needed to be quantitatively isolated from the other 3-methylhistidine containing muscle components; viz, myosin heavy chain and any balenine and free 3-methylhistidine present. If this could be accomplished the 3-methylhistidine content of the actin containing fraction would be a good indicator of the actin content of the sample since the 3-methylhistidine titres of all muscle actins had been shown to be relatively constant (section 2.1.4). The actin content, in turn would be a good indicator of the meat content of a sample since striated muscle from whatever source can be assumed to contain similar ratios of actin to other myofibrillar proteins, though smooth muscle is known to contain a far higher ratio of actin to myosin than striated muscle (Lawrie, 1985). Actin content was proposed as a better, more direct method of meat determination than collagen-free muscle protein

(CFMP) (CFMP = 6.25 x percentage nitrogen - 8 x percentage hydroxyproline, or total protein - collagen protein), for the determination of quality in cured meat products; which was the method used at the time in Holland. It was found that actin monomers could be well separated from the components of much higher and much lower molecular weight containing 3-methylhistidine using sodium dodecyl sulphate (SDS) gel filtration of samples dispersed in a sample buffer containing SDS and dithioerythritol (an isomer of dithiothreitol). Nevertheless SDS/2-mercaptoethanol has since been shown to give more complete extraction of the muscle proteins: Jonker, van Roon and den Hartog, (1987). Separation of proteins by gel filtration depends on the different abilities of the various sample molecules to enter pores within the stationary phase. Molecules which are too large to enter the stationary phase move through the chromatographic bed faster. Smaller molecules, which can enter the gel pores, move more slowly through the column, since they spend a proportion of the time in the stationary phase. Molecules are therefore eluted in order of decreasing molecular size. Separation required a large (90cm bed height) column a using buffers containing SDS, run at low flow rates, meaning that separation took many hours and required the collection of many fractions. The effluent was monitored spectrometrically, the protein composition of the absorbtion peaks being determined by SDS PAGE of the approriate fractions. The 3-methylhistidine content of fractions was determined by conventional ion-exchange amino acid analysis of the hydrolysed samples. When this method was applied to a smoked,

pasteurised meat product, the Guelders ring sausage, actin could be well separated from myosin heavy chain and the low molecular weight components (Jonker, van Roon and den Hartog, 1985). The actin and myosin containing components were separately pooled and, after hydrolysis, their 3-methylhistidine content was determined. Only 39% of the amino acid found in the sample prior to gel filtration, was detected after separation into the constituent proteins. This was thought to be due to the high salt concentration of the hydrolysed enriched samples: these could not be de-salted due to their very small volumes. High performance gel filtration was recommended for further investigations to allow a much reduced separation time of from 40 h to minutes.

An alternative method of actin determination was proposed by Anderson (1976), who isolated and quantified the 18 residue peptide from the N-terminal region of chicken actin by chromatographic and electrophoretic methods. Isotopic methods were used for quantification, the method being applied to the determination of the actin content of acetone dried powders of chicken breast muscle. A similar approach was used to calculate the meat content of meat products, by Anderson (1981), using double isotope labelling and peptide isolation. Double isotope labelling and peptide isolation consists of dissolving samples in tris/urea/dithiothreitol buffer and labelling with iodo [2-^{3}H] acetic acid. Pure actin was similarly labelled, but with iodo [2-^{14}C] acetic acid. Aliquots of labelled actin were mixed with labelled sample, the mixture being separated by SDS PAGE. The electrophoretic band corresponding to actin was excised,

extracted and digested with chymotrypsin, the resulting peptides being separated by paper chromatography or electrophoresis. By its chromatographic/electrophoretic behaviour, the C-terminal carboxymethylated peptide was identified, eluted, and its $^{3}H/^{14}C$ ratio determined by scintillation counting. Various animal muscle samples gave similar proportions of material co-eluting with the actin derived peptide, soya giving very low $^{3}H/^{14}C$ ratios. By this method all muscle samples investigated had similar actin contents, the two non-meat protein sources examined, namely soya and milk, contained none. Processing affected the results, curing reducing the apparent actin content of muscle. Significant correlation was found between ground beef content and actin content per unit protein, of soya/beef mixtures.

Double labelling and peptide isolation depends on the availability of cysteine residues for chemical modification and on intact protein molecules for electrophoretic separation. The curing process can result in chemical alteration of proteins, such as oxidation, or in proteolysis, both changes resulting in a reduction of the apparent actin content. It is therefore necessary to compare processed products with an unknown meat content, with 100% meat samples processed in the same manner.

2.4.8. APPLICATIONS OF GAS CHROMATOGRAPHY TO MEAT ANALYSIS.

Gas chromatography (GC) as a mode of separation, relies on the partition of a substance between a mobile gas phase and a stationary phase, bonded to a solid support.

The non-volatile nature of amino acids means that their separation by GC requires their conversion to volatile derivatives. This derivatisation usually involves silylation (eg. Gehrke and Leimer, 1971) or acetylation/esterification (eg. Gamerith, 1983a,b) of the molecules' functional groups.

Meat hydrolysates, like many biological samples, are a complex mixture of substances including the amino acids of interest. Their complexity provides special problems for their analysis by GC in which interfering substances can reduce the response of compounds of interest to an unacceptably low level.

Zumuralt, Roach and Gehrke (1970) reported on the GC of amino acids in biological substances. The amino acids of blood plasma and urine were quantitatively isolated by ion-exchange resins, which separated out substances which would interfere with the GC analysis. The amino acids isolated were derivatised to their N-trifluoroacetyl n-butyl esters, quantitative analysis being possible on as little as 20μg of total amino acids, the results agreeing well with those from traditional ion exchange chromatographic analyses. Of the common amino acids, histidine was found to be difficult to chromatograph and quantitate successfully.

Separation of a mixture of twenty amino acids was performed by Moss and Lambert (1971) using N-heptafluorobutyryl

n-propyl derivatives, histidine again being found difficult to chromatograph reproducibly.

Zanetta and Vincendon (1973) separated and quantified the constituent amino acids of proteins and glycoproteins by GC using N(O)-heptafluorobutyryl isoamyl ester derivatives. As little as 0.1ng of the constituent amino acids could be detected, all amino acids being quantified in a single run. The problem of contaminating substances in protein hydrolysates was investigated. High concentrations of salts were found to interfere with the derivatisation process, these being eliminated by dialysis or gel filtration prior to hydrolysis. Lipids gave interfering fatty acid peaks which could be eliminated by exhaustive extraction with chloroform/methanol. Nucleic acids and SDS could interfere with analysis, the latter being eliminated prior to hydrolysis by acidification and ether extraction of an aqueous suspension of the protein.

Developments in the determination of amino acid profiles of biological samples were reported by Adams (1974), who, using N-acetyl n-propyl derivatives, successfully generated amino acid profiles of protein hydrolysates, plant tissue extracts, urines and sera. Pure protein hydrolysates could be derivatised directly, complex samples usually requiring pre-treatment to separate the amino acids from the matrix, the treatment method employed consisting of small cation exchange columns. These columns removed anions, large molecules and non-ionic material, although some amines and small peptides (if originally present) may not be removed.

The amino acid profile of a meat or meat product can give valuable information about its nutritional value, based on the levels of essential amino acids present, though only amino acids unique to meat and associated tissue, such as 3-methylhistidine and hydroxyproline can be used in the determination of the meat content of a product. Histidines are notoriously difficult to analyse quantitatively by GC (Moodie, 1974), due to the polar and unstable nature of the derivatives formed and absorbtion onto the stationary phase due to free imidazole (Hušek, 1979). Although the imidazole-ring nitrogen in 3-methylhistidine is already substituted with a methyl group, this amino acid is still quite polar, even when derivatised for gas chromatography, and can undergo absorptive losses on gas chromatography columns. Hydroxyproline on the other hand is not a problem to derivatise and chromatograph, the derivatives giving excellent detector responses (Adams, 1974), quantitative GC responses being obtained by Moss and Lambert (1974) and MacKensie and Tenaschuk (1974, 1975).

Gas chromatographic separation of 3-methylhistidine from 1-methylhistidine and histidine was accomplished by Vielma and Mendez (1980), using trimethylsilyl derivatives. Quantitative detection in the range of 5-50ng of derivative was possible using peak height, after flame ionisation detection. For biological samples a pre-derivatisation, ion-exchange clean-up step (Nishizawa et al., 1978) was found to be necessary for quantitative results.

Cotellessa et al. (1980), detected urinary 3-

methylhistidine by isolating the amino acid by ion-exchange or charcoal/celite column chromatography, then carrying out GC on the N-trifluoroacetyl n-propyl derivatives of the isolated sample. Flame ionization detection, which is a general (universal) GC detection method (Gordon and McCrae, 1987), could detect 3-methylhistidine down to a level of 12 nmoles/ml. More specialised detection methods, such as electron capture (Gordon and McCrae, 1987), increased the sensitivity one-hundred fold. Although this level of sensitivity was not required for the determination of the relatively high levels of free 3-methylhistidine in urine it was proposed that it might be neccessary for the detection of the lower levels found in muscle proteins.

Of the GC procedures for the determination of 3-methylhistidine, only a few can be applied to samples other than those such as urine, with relatively high levels of this amino acid. A highly sensitive GC method for the determination of both 1- and 3-methylhistidine in biological samples was developed by Rogoskin, Krylov and Khlebnikova (1987). The amino acid fraction containing these amino acids was isolated using ion-exchange chromatography, N-trifluoroacetyl isobutyl esters being used for chromatography. The use of ion resonance detection (analogous to electron capture detection, Gordon and McCrae, 1987), allowed a detection limit of as little as 0.3 picomoles.

A specific method for the gas chromatographic determination of proline and hydroxyproline was reported by Mussini and Marcucci (1965). Gelatin and serum albumin were

hydrolysed, the dried hydrolysate residue being treated with nitrous acid, to destroy the amino acids, but not the imino acids (proline and its derivatives). The resulting imino acid N-nitroso derivatives were hydrolysed with HCl, then converted to acetylated/esterified derivatives prior to chromatography. As expected albumin gave only a proline peak, whilst gelatin gave both proline and hydroxyproline.

Mee (1973) developed a GC method for assaying hydroxyproline in normal and pathological blood sera and urines using N-trifluoroacetyl n-butyl derivatives, without laborious sample clean-up. Specificity was obtained using a nitrogen detector system.

Collagen amino acids in biopsy tissue were determined using gas chromatography of their N-trifluoroacetyl n-butyl derivatives, by Perier et al. (1980). The hydroxyproline peak, within the complex elution patterns, was identified by its mass spectrum. Compared with traditional ion-exchange chromatographic determination of amino acids, this GC method, which gives comparable results, has the advantages of increased sensitivity, accuracy and speed, enabling the quantitative determination of the amino acid composition of a sample in a single run.

Little work has been published on the application of GC to the quantification of meat protein amino acids. Berg (1982), using flame ionisation detection of N-heptafluorobutyryl isobutyl derivatives, found that the low levels of 3-methylhistidine in meat, along with the uncertainty surrounding its derivatisation, made the GC determination of this

amino acid difficult. The levels of hydroxyproline present meant that this amino acid was easily detectable and that hydroxyproline levels determined by GC could be used to indicate the quality of a meat or meat product. The non-protein amino acid β-alanine, found in the histidine dipeptides, was detected in the gas chromatograms of meat samples at relatively constant levels of approximately 2mg/g fesh meat, but was not detected in non-meat protein sources. β-Alanine was proposed as an indicator of meat content in beef and pork products, the level of this amino acid being shown to be a good indicator of the meat content of meat/soya mixes, although no account was made for the possibility of losses of the water soluble dipeptides which contain this amino acid acid, on product preparation, storage and proccessing, or during the sample preparation.

Mass spectrometry (MS), as a detection method after GC separation of food components by GC (and HPLC) has great prospects as a specific and unequivocal means of identification and quantification. The basis of mass spectrometry is the separation of chemical elements into their isotopes, based on the principle that ions accelerated to a certain kinetic energy, describe in a subsequent magnetic field, paths that differ according to their mass to charge ratio. In the ionisation process, not only molecular ions are formed, but also a large number of fragments, the fragmentation pattern found being characteristic of the organic molecule ionised (Ten Noever de Brauw, 1979).

Matthews et al. (1981), developed a picomole assay

for 3-methylhistidine using GC-MS. The amino acids in biological fluids were isolated using micro ion-exchange chromatography columns and N-acetyl n-propyl derivatives of the isolate were prepared for GC. Detection was by chemical ionisation mass spectrometry, on the basis of the abundant M+1 ions which are unique to 3-methylhistidine and its deuterated analogue. The deuterated amino acid was used as an "ideal" internal standard, to counteract losses of 3-methylhistidine due to absorption onto the column and to act as a carrier for the low levels of the natural amino acid present. By this method nanomole levels of 3-methylhistidine could be rapidly determined with a precision of 0.5%, picomole levels with a precision of 10%.

The combination of gas chromatography with mass spectrometry (GC-MS) is one of the most powerful tools available in analytical chemistry, allowing the unequivocal detection of very small amounts of organic compounds in complex mixtures. These characteristics make it a promising method for the quantification of the low levels of meat-specific compounds in meat products.

2.5. ANALYTICAL ASPECTS OF CONNECTIVE TISSUE DETERMINATION IN FOODS.

2.5.1. THE COMPOSITION OF CONNECTIVE TISSUE.

Connective tissue accounts for about 1% of the wet weight of a typical adult mammalian muscle (Lawrie, 1985), connective tissue consisting of the proteins collagen (approximately 95% of total) and elastin (approximately 5% of total).

Collagen is the most abundant protein in the body, being found in bone, skin, tendon and muscle. In muscle it contributes 1-9% of the dry, fat free mass (Etherington and Sims, 1981), and is found in the form of a network of fibres which allow the effective and efficient transmission of contraction through muscle to bone (Light and Champion, 1984) and which prevents the over extension of the muscle and resulting tissue damage. Collagen forms the endomysium which encloses each muscle fibre, the perimysium which surrounds bundles of fibres and the epimysium which surrounds the whole muscle, the perimysium and epimysium making attachments to the tendons.

The sub-unit of collagen is tropocollagen which is a triple helix of molecular weight of appoximately 300000 daltons, the helices being aligned in parallel and held together by covalent internal cross-links in the collagen fibril. Repeating sequences of glycine, proline and 4-hydroxyproline are present, prolines accounting for over 20% of the total amino acids, over a half of these being hyroxylated to 4-hydroxyproline, on the nascent collagen peptide, immediately

after its synthesis (Lazarides, Lukens and Infante, 1971. Bornstein, 1974). Hydroxyproline accounts for around 14% of the dry weight of collagen, some variation occuring in genetically distinct forms, the relative abundance of which varies with the age and type of tissue. Analytical methods for the quantification of collagen are most commonly based on the detection of this amino acid, which is virtually unique to the connective tissue proteins, although it has been found in the lung protein, alveolyn (Bisker et al., 1982) and the collagen-like sequence of acetylcholinesterase. Glycosylated hydroxyprolines are found in plant cell walls (Wold, 1981).

An alternative approach to the determination of collagen in comminuted meat products is by histochemical methods. Flint and Firth (1983) have demonstrated that intramuscular connective tissue and added rind contrasted dramatically with muscle fibres and starch granules, when stained with Sirius red systems, as well as being distinguishable from each other. These differences could be the basis of a stereological method (Flint and Meech, 1978) for the determination of muscle associated connective tissue and added rind in meat products.

A number of other approaches have been proposed for connective tissue determination. These include physical (nuclear magnetic resonance), and immunochemical methods (Etherington and Sims, 1981), although they have not gained widespread use as methods for the determination of connective tissue in food.

The other major connective tissue protein, elastin, contains 1-5% hydroxyproline and is found mainly in ligaments and arterial cell walls, with low levels in lungs and skin. The low

ratio of elastin to collagen in muscle means that it does not usually interfere with the collagen hydroxyproline assay. The standard conversion factor of 7.25 (Goll, Bray and Hoekstra, 1963) is based on a collagen to elastin ratio of 3 : 1. The hydroxyproline content of the collagen is taken as 13.3% by weight, eight times that of elastin. Problems due to the low hydroxyproline content of elastin can arise in comminuted meat products with high levels of added connective tissue, in this case, elastin can be analysed separately, since unlike collagen it is insoluble in dilute alkali.

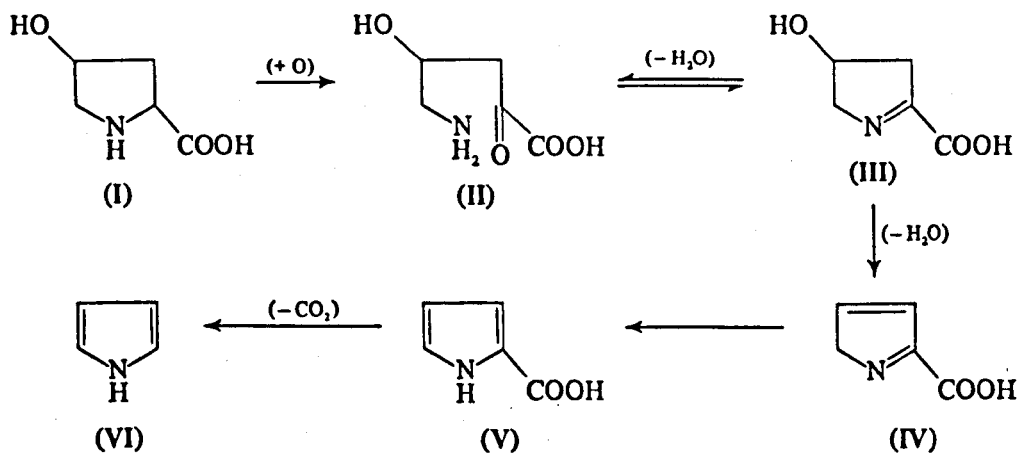
2.5.2. THE DETERMINATION OF HYDROXYPROLINE.

There are two position isomers of hydroxyproline, 4-hydroxyproline (which from here on will be referred to as simply, hydroxyproline) and 3-hydroxyproline, which is found at only very low levels in certain forms of collagens (Bisker et al., 1982).

Hydroxyproline is most widely assayed by colorimetric methods, although recently other chromatographic, chemical and physical methods have been introduced.

The standard method of hydroxyproline determination in meat and meat products (BSI, 1979) is based on the oxidation of the free amino acid to a pyrrole, which is then reacted with p-dimethylaminobenzaldehyde (Ehrlich's reagent) to produce a red-brown colour which can be measured spectrometrically. The postulated mechanism of the oxidation is seen in figure 2.6. Hydroxyproline (I) is oxidised to a linear α-keto-γ-hydroxy-5-

Figure 2.6. Postulated mechanism of oxidation of hydroxyproline (I) to pyrolle (VI), in the colorimetric determination of hydroxyproline.



(From Etherington and Sims, 1981).

amino-valeric acid (II), which is in equilibrium with the cyclic Δ'-pyrroline-4-hydroxy-2-carboxylic acid (III). Loss of water gives the unstable structure (IV), which spontaneously rearranges to pyrrole-2-carboxylic acid (V). The final decarboxylation step to pyrrole (VI) occurs during heating, after the addition of p-dimethylaminobenzaldehyde.

Neuman (1950) reviewed the early developments in colorimetric hydroxyproline determination. Various oxidising agents have been employed for the conversion of hydroxyproline to pyrrole including sodium hypochlorite, sodium peroxide and copper sulphate/sodium hydroxide. Colour has been generated by isatin (2,3-indolinedione), isatin / copper or p-dimethylamino-benzaldehyde. The colorimetric method was generally regarded as being the best available although problems of incomplete oxidation and interference from other compounds did occur. Microbiological assays of the type used for other amino acids, such as phenylalanine (van Steirteghem and Young, 1978) were not possible since no micro-organism had been found whose growth was affected by the level of hydroxyproline. Chemical isolation procedures were impractical since large amounts of sample would be required to isolate sufficient amounts of amino acid for classical chemical analysis.

There were reports that proline and tyrosine gave positive responses in the colorimeric hydroxyproline assay. In the case of proline the response was found to be due to hydroxyproline impurities. Tyrosine gave a response but the wavelength of maximum absorbance was 500mn compared with 550nm for hydroxyproline. Tryptophan was also found to give a response,

but this amino acid is destroyed during hydrolysis and is therefore not a problem in the analysis of meat protein amino acids.

Adaptations to the methods described by Neuman (1950) were made by Martin and Axelrod (1953), who obtained better linear correlation between absorbance and hydroxyproline concentration by removal of the excess peroxide from the oxidation step, using ferrous sulphate, although interference from tyrosine was still found to be a problem.

A method of colorimetric hydroxyproline determination that has found widespread use in the analysis of food products is that of Woessner (1961), the method being developed for the analysis of samples with low levels of hydroxyproline. To increase the sensitivity and stability of the chromogen, perchloric acid was used to destroy excess oxidant. Interference from other amino acids, in samples with very low hydroxyproline content, could be removed by extracting the chromogen with benzene, then measuring the absorbance of the aqueous layer.

The colorimetric hydroxyproline assay in one of its standard forms was developed by Stegemann and Stalder (1967) (appendix I). Chloramine-T (sodium chloro-p-toluenesulphonamide) was used as the oxidant, with p-dimethyl-aminobenzaldeyhde/perchloric acid for colour development. With this combination of reagents, compounds closely related to hydroxyproline interfered very little, tyrosine giving no response. Excess oxidation reagent will bleach the chromophore,

but is easily removed in the case of chloromine-T, by the inclusion of perchloric acid in the final colour reagent. A similar method has been fully automated (Grant, 1964).

The precision of the colorimetric method of hydroxyproline determination was investigated in a collaborative study, using the British Standards Institute method for the determination of L(-)-hydroxyproline in meat products (BSI, 1979). The results of the study were reported by Lord and Swan (1986). Previously, Jonas and Wood (1983), had carried out a similar trial, but of the four samples analysed, only one had a hydroxyproline content in the range commonly found in meat and meat products. The repeatability of the 1983 trial was found to be disappointingly lower than the internal repeatability achieved by public analysts within their own laboratories. The 1986 trial analysed pet-foods with 0.1-1.0% hydroxyproline contents. The samples were carefully selected and prepared to ensure complete sample homogeneity. Data of adequate precision compared with the statutory requirements (BSI, 1979) were obtained; viz, "the difference between the two calculated values obtained simultaneously, or in rapid succession from the duplicate test portions by the same analyst shall not exceed 5% of the arithmetic mean value".

Conventional ion-exchange chromatography with ninhydrin detection, has rarely been used for the determination of hydroxyproline, due to the availability of the simple, more rapid colorimetric methods, although the analysis of hydroxyproline by post column derivatisation by fluorescamine, after conversion of the secondary amino acid to a fluorescamine sensitive form using

N-chlorosuccinimide, was reported by Felix and Terkelson (1973a) (section 2.2.2). Further developments in the fluorometric determination of secondary amines, based on their reaction with fluorescamine, were made by Nakamura and Tamura (1980), who found that the non-fluorescent aminoenones produced by the reaction of secondary amines with fluorescamine, at pH 12 (at which pH, the reaction with primary amines is supressed), could readily be converted to fluorescent pyrollinones using primary amines such as L-leucyl-L-alanine. This method is more sensitive than measuring the non-fluorescent aminoenones colorimetrically, or by fluorescamine derivatisation after conversion of the secondary amines using N-chlorosuccinimide. A reagent, 2-methoxy-2,4-diphenyl-3(2H)-furanone, analogous to fluorescamine, has been similarly applied to the determination of secondary amines (Nakamura et al., 1982).

Yoshida et al. (1982) found that post column detection using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was a sensitive method for the detection of proline and hydroxyproline with a limit of sensitivity of 1 picomole. Thin layer chromatography of NBD-Cl derivatives, has been used by Bisker et al. (1982) in a sensitive method for the quantitative evaluation of the hydroxyproline isomers.

The recent advances in the use of high performance liquid chromatography (HPLC) in food analysis has included the development of useful techniques for the determination of hydroxyproline. Casini et al. (1982) determined hydroxyproline in biological samples using gradient elution reverse-phase HPLC of

4-dimethylaminobenzene-4′-sulphonyl chloride (dabsyl) derivatives. This method was found to be rapid (6 minutes for derivatisation and 20 minutes for chromatography) and reliable, requiring no extraction step before chromatography, and having a limit of detection of 458 picomoles. A reverse-phase HPLC method for the separation of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) derivatives was developed by Ahnoff et al. (1981), for the determination of hydroxyproline in collagen hydrolysates. Linblad and Diegelmann (1984) used reverse-phase HPLC of NBD-Cl derivatives, with colorimetric detection at 495nm, to separate the 3-hydroxy and 4-hydroxy isomers of proline. The advantages of NBD-Cl for hydroxyproline detection were its sensitivity (40 picomoles being easily detected, compared with the 7.6 nanomole detection limit of standard colorimetric methods), as well as its specificity for secondary amines, since it has a far greater sensitivity for secondary than for primary amines.

Pre-column derivatisation using NBD-Cl was employed by Jones et al. (1986), who developed a method using reverse-phase HPLC with column switching for the determination of hydroxyproline in meat and meat products. Column switching avoids the chromatography of amino acids other than hydroxyproline, thereby shortening analysis time and allowing the determination of very low levels of this amino acid, where previously interference from other far more abundant constituents made quantification difficult. This method has been shown to give good recovery of hydroxyproline, good agreement with standard colorimetric methods and has been used routinely for the entire

range of hydroxyproline levels normally encountered in meat and meat products (0.05-12.5%).

As previously discussed (section 2.4.8), the determination of hydroxyproline by gas chromatography has been proposed as a specific, rapid, sensitive and simple method of determining hydroxyproline in biological samples (Moss, 1974; MacKensie and Tenaschuk, 1974,1975), and as an indicator of meat quality (Berg, 1982).

Micro-scale analysis, when only very small amounts of material are present, have been developed. Airhart et al. (1979) described an ultramicro method, in which a known quantity of (^{3}H)-hydroxyproline is mixed with the sample hydrolysate and then reacted with (^{14}C)-dansyl chloride. The dansyl-amino acid mixture is resolved using thin-layer chromatography and the dansyl-hydroxyproline spot eluted. The ratio of (^{3}H) to (^{14}C) in the recovered fraction is then determined by scintillation counting and from this value the amount of hydroxyproline in the original sample calculated using appropriate standards. The method is very sensitive, detecting as little as 2 picomoles of the amino acid.

Accurate determination of collagen levels is not always possible by standard colorimetric assays, due to interfering substances, the levels of which may be high, especially in processed food products. Chromatographic methods may be more suitable for these determinations, especially if more stringent legislation controlling the inclusion of added connective tissue in food is introduced. This will require

routine methods of analysis which are simple, reliable and sufficiently rapid to avoid undue delays during processing.

2.5.3. THE IMPORTANCE OF CONNECTIVE TISSUE IN MEAT DETERMINATION.

The determination of connective tissue content of meat and meat products is important from a legislative and nutritive point of view. Connective tissue added to products above the amount naturally associated with the flesh used, does not constitute "meat" (Statutory Instruments, 1984, 1986), the addition of such protein sources reducing the nutritive value of a product as connective tissue is deficient in essential amino acids (Coomaraswamy, 1972), almost completely lacking tryptophan and being deficient in methionine, cystine and tyrosine. There is also a correlation between gross collagen content and toughness in meat (Light, Voyle and Champion, 1984). The hydroxyproline content of a meat or meat product is indicative of its nutritional "quality" and palatability.

Standard methods of meat determination, based on nitrogen content, can be corrected for excess added connective tissue using the formula derived by Coomaraswamy (1972) (section 2.4.1). Methods based on 3-methylhistidine (section 2.4.6) are also corrected for connective tissue (which does not contain 3-methylhistidine), the level of connective tissues being variable, and depending on the age and origin of the muscle. Jones et al. (1985) suggested that the calculation of connective tissue content, based on hydroxyproline, with conversion using an appropriate factor, could be the root cause of the unnaccountable

variation of 3-methylhistidine titres, expressed in terms of fat free, connective tissue free sample. This means that any method involving 3-methylhistidine as an index of meat on a connective tissue free basis requires the accurate determination of connective tissue (as hydroxyproline), to prevent the introduction of errors or false assumptions in the calculation.

The Food Standards Committee, in its Report on Meat Products (1980), states that:

> "For most cuts of meat, generally trimming should reduce the connective tissue content to less than 10 per cent. of the lean meat content. However slightly higher levels might occasionally be found even after trimming in cuts such as shoulder, flank, shin and brisket, which are the main cuts used for manufacturing."

The Food Standards Committee therefore recommended that lean meat should contain (in most cases) not more than 10% of connective tissue. It was obvious that some of the inexpensive bovine cuts used in the manufacture of comminuted meat products often have atypically high levels of connective tissue. This is particularly true for Masseter (cheek), which (along with diaphragm) was formerly simply classed as offal but now is classed as "part of the carcass to be regarded as meat" (Statutory Instruments, 1984,1986). Lord and Swan (1985) compared the connective tissue content of four bovine manufacturing cuts; shin, flank, diaphragm and Masseter (cheek). The lean portion of shin, flank and diaphragm had wet, fat-free, connective tissue contents of approximately 10%, whereas

Masseter contained approximately 19%. Recommendations were made that products such as beefburgers, traditionally made of high grade cuts, should have connective tissue contents (wet, fat-free) of 10-20% (Lord and Swan, 1984, 1985). This was morally reasonable, as such products should not really contain more than a small percentage of Masseter, even though it is classified as meat. The measure of product "quality" in, for example, beefburgers could be based on connective tissue content as determined by hydroxyproline.

Sample homogeneity in connective tissue determination (as in any analysis of samples of the heterogeneous nature of most meat products), is of utmost importance. Difficulties in obtaining homogeneity were reported in the comparison between the hydroxyproline contents of various porcine joints (AMC, 1987). This study found that hydroxyproline contents of the component tissues of meat were as expected, being lowest in the lean sample, intermediate in subcutaneous fat (which is held in a framework of connective tissue), and highest in rind. Individual joints varied in their hydroxyproline content but there was no significant difference in the content of this amino acid in the subcutaneous fat of any joint. On the other hand, though there were some significant differences in the hydroxyproline contents of rinds of certain joints, there was no systematic anatomical pattern.

Connective tissue determination of meat and meat products requires representative sampling, sample homogeneity and a reproducible method of hydroxyproline determination which can

be applied to a whole range of products, regardless of interfering substances present and which is applicable to the entire range of hydroxyproline levels encountered.

2.6. RESUMÉ.

The virtually unique occurance of the "unusual" amino acid, 3-methylhistidine, in the myofibrillar proteins, myosin and actin has been established, although the role of this amino acid in the functioning of these muscle proteins is not understood. Methylation of the histidine residues has been shown to be co- or post-translational.

Apart from a few questionable reports of the absence of 3-methylhistidine in certain actins, the level of 3-methylhistidine in this protein from muscle and non-muscle sources (except that from the alga Naegleria) appears to be 1 residue / G-actin molecule. On the other hand, histidine methylation in myosin is variable, under developmental control and depends on the ratio of muscle fibre types present.

Methods for the determiation of 3-methylhistidine have now been developed to a routine level, using the unique acid stable fluorescamine fluorophores of this amino acid and histidine, with separation of the two, by HPLC.

Quantitative determination of meat protein in food products is necessary to protect the consumer and manufacturer against unscrupulous traders. Food legislation has been enforced so that manufacturers have guide-lines to follow, and levels of "quality" of meat products, to which they must adhere.

The occurence of 3-methylhistidine in myofibrillar proteins (the essence of meat), but not in protein of non-meat and vegetable origin, and the robustness of this amino acid to the severe processing conditions in food manufacture, has led to the development of methods of meat determination based on a 3-

methylhistidine index.

Although the levels of this amino acid in prime meat cuts have been found to be relatively constant, when corrected for their variable fat and connective tissue contents , levels in certain manufacturing cuts show more variation. These intracarcass variations in 3-methylhistidine titres required more investigation, to determine their extent, and their fundamental biochemical cause, since the variations questioned the suitability of an index based on 3-methylhistidine as an unequivocal determinant of meat content.

The possibility of using the evolutionarily conserved muscle protein, actin, as a measure of meat content of foods has been proposed. Methods of actin quantification have been based on the physico-chemical properties of the entire actin molecule, its fragments, or its unvariable 3-methylhistidine content. Since no method has been reported that can accurately and simply quantify actin in food, this area of research held promise for further development.

Methods of meat determination based on actin or 3-methylhistidine contents need to be based on connective tissue free units, the connective tissue being determined separately, by its hydroxyproline content. Methods for the determination of hydroxyproline, such as the standard colorimetric method, are susceptible to error, due to interference from other chemical species. The recent developments in computer integrated gas chromatography-mass spectrometry, making it more freely available

and "user-friendly", suggested that investigations into the possibilities of using this method for the routine determination of hydroxyproline (and possibly 3-methylhistidine) in food would be worthy of investigation. Assumptions made in using an average factor for the conversion of hydroxyproline to connective tissue, for all muscles, of all species, may introduce errors into the calculation of 3-methylhistidine titres on a connective tissue free basis. The intracarcass and interspecies variation in the hydroxyproline contents of intramuscular connective tissues therefore required investigation.

EXPERIMENTAL.

3. INTRACARCASS AND INTERSPECIES VARIATION IN TOTAL PROTEIN-BOUND 3-METHYLHISTIDINE TITRES.

3.1. INTRODUCTION.

The possibility of using protein-bound 3-methylhistidine as an unequivocal index of meat in food (Hibbert and Lawrie, 1972) was based on the presumptions that it was present at a more or less constant level in the tissues accepted as "meat" (Statutory Instruments, 1984, 1986); and that it was absent from all other proteins in foods. Titres of 5-6 mg 3-methylhistidine / g nitrogen appeared characteristic of skeletal muscle (Hibbert and Lawrie, 1972); the amino acid being absent from all vegetable proteins examined (Hibbert and Lawrie, 1972; Rangeley and Lawrie, 1976a,b). Abnormally high values for whale-meat and the flesh of older pigs were found to be due to the dipeptide balenine. When the methodology was amended to correct for the presence of balenine, the titres of 3-methylhistidine were found to conform with those of other skeletal muscles.

The first reports of intracarcass variation in the 3-methylhistidine titres was by Jones *et al.* (1985) and White and Lawrie (1985b), who found relatively constant titres in prime bovine cuts and muscles, but abnormally low levels in the *Masseter* and *Malaris* (cheek). Jones *et al.* (1985) found abnormally high levels in the flank, but these were not confirmed by White and Lawrie (1985b), Jones *et al.* (unpublished), went on to show that only certain muscle of the flank exhibited these high values. To investigate these reported anomalies, the total protein-bound 3-methylhistidine titres of a range of

bovine muscles, including both prime and manufacturing cuts was determined. A number of regions of the ovine and porcine carcass were also analysed to determine any interspecies differences.

Fish is not classed as "meat" (Statutory Instruments, 1984) but has been shown to contain levels of 3-methylhistidine similar to those of prime bovine cuts, with some species variation (Rangely and Lawrie, 1977). The report by Shenouda and Pigott (1975) of the absence of 3-methylhistidine in fish actin should be mirrored in the total protein-bound titre for fish muscle, which was determined for a number of species. A tropical species was included since the physical properties of tropical fish muscle proteins are different to those of temperate species (Poulter et al., 1985), and may reflect their amino acid composition.

Various levels of 3-methylhistidine have been reported in non-skeletal muscle protein sources, some of which are often incorporated into comminuted meat products (Rangely and Lawrie, 1977; Jones et al., 1985). Various offals, some of which are classed as meat (Statutory Instruments, 1984, 1986), were analysed to determine if they would make a significant contribution to the 3-methylhistidine content of a product into which they were incorporated.

The absence of 3-methylhistidine in proteins of vegetable origin has been extensively reported (Hibbert and Lawrie, 1972; Rangeley and Lawrie, 1977). A widely available vegetable protein, textured soya protein (TVP), commonly used as a meat substitute, was thus analysed.

3.2. MATERIALS AND METHODS.

3.2.1. SAMPLE DESCRIPTION.

The bovine muscles analysed were obtained from a local slaughter house from a single carcass. They were Longissimus dorsi (L.dorsi) (loin), semimembranosus (round), Sternomandibularis (neck), Masseter and Malaris (cheek) and Aponeurosis of the Obliquus externus abdominus (Aponeurosis) (flank). These muscles were chosen as they are representative of various cuts. L.dorsi represents a standard muscle, being low in connective tissue and high in lean meat, it represents an ideal "model" of meat. Semimembranosus is equivalent to the round cuts, topside and silverside, Masseter and Malaris together corresponding to cheek meat. Sternomandibularis and Aponeurosis are easily accessible and of low commercial value, the removal of Aponeurosis not damaging the carcass on dissection.

Three regions of the porcine carcass were analysed, namely the neck, shoulder and cheek regions, along with ovine cheek. These samples were obtained from local butchers.

Diaphragm of the bovine, ovine and porcine carcass were analysed, the samples being obtained from local slaughter houses.

The fish species used were cod (Gadus murhua), redfish (Sebastes marinus), a close relative of the rockfish (Sebastes auriculatus) used by Shenouda and Pigott (1975), and, an important tropical species red snapper (Lutjanus sebae). Fish was obtained from various markets and stored on ice.

The non-skeletal muscle protein sources used were as follows: bovine heart, "tripe", kidney, liver and tongue; porcine "melts" (spleen); and textured soy protein. These were obtained from local retailers.

The total protein-bound 3-methylhistidine titres of these tissues was determined by the standard method described in section 3.2.2.

3.2.2. FLUOROMETRIC DETERMINATION OF 3-METHYLHISTIDINE.

Unless otherwise stated, all of the reagents used were of "analytical reagent" (AR) grade, obtained from Fisons (Loughborough, UK).

The determination of 3-methylhistidine was carried out by a method based on that of Jones, Shorley and Hitchcock (1982 a,b), as adapted by White and Lawrie (1985a). This method involved the production of acid stable fluorophores of histidine and 3-methylhistidine by reaction with fluorescamine (Sigma). The resulting derivatives were separated by reverse-phase high performance liquid chromatography (rp-HPLC), and quantified by fluorescence detection.

3.2.2.1 ACETONE POWDER PREPARATION.

Samples were minced through a 4mm plate. In the case of muscle samples, all of the visible fat and connective tissue was first removed.

10g of the minced sample was homogenised with 200ml chloroform : methanol (2:1 v/v) using an Ultraturrax TP 18/10 (Janke & Kunkel GmbH & Co, W.Germany). The homogenate

was filtered through a Whatman (Maidstone, UK) no. 541 paper using a Hartley Funnel over a low vacuum. The residue was then washed with 2 X 200ml 80% ethanol, followed by 200ml acetone and dried overnight at 30°C.

The residue was weighed and then ground to a fine homogeneous powder using a Retsch ZM-1 ultracentrifugal mill (distributed by Glen Creston Ltd, UK), with a 0.5mm screen. The acetone powder was stored in a desiccator.

3.2.2.2. HYDROLYSIS.

60mg of acetone powder was oven hydrolysed with 15ml 6M HCl in 14ml McCartney bottles with polypropylene caps (Gallenkamp, Loughborough, UK), at 110°C for 16h. The hydrolysate was filtered through Whatman no. 541 paper before derivatisation. 0.1 ml samples were used for fluorescamine derivatisation.

3.2.2.3. PREPARATION OF 3-METHYLHISTIDINE STANDARDS.

1g 3-Methylhistidine (Sigma) was dissolved in 1000ml, 0.1M hydrochloric acid. The variable water content of the 3-methylhistidine (Jones et al., 1985; White, 1986) meant it was necessary to determine the exact concentration of the solution by Kjeldahl nitrogen determination (appendix I). Four 100ml aliquots of the 3-methylhistidine solution were freeze-dried in Kjeldahl tubes and their nitrogen content determined by Kjeldahl distillation. There are 3 moles nitrogen/ mole 3-methylhistidine, therefore the mass of nitrogen determined can

be converted to the actual 3-methylhistidine concentration of the solution. The stock solution was stored as 5ml aliquots at -20°C in 14ml McCartney bottles with polypropylene caps (Gallenkamp).

A fresh aliquot of standard was used for each batch of fluorescamine derivatisations to minimise loss of 3-methylhistidine by decomposition or contamination. Before fluorescamine derivatisation the standard solution was diluted to a range of concentrations between 0.02 - 2.0 µg/ml depending on the expected 3-methylhistidine content of the samples being analysed. 0.2ml of these dilute standards were used for fluorescamine derivatisation.

3.2.2.4. PREPARATION OF FLUORESCENT DERIVATIVES.

0.1 ml of the hydrolysate to be analysed was pipetted into a 150mm X 15mm pyrex test tube. Neutralisation was carried out by the addition of 0.1ml of 6M sodium hydroxide. All pipetting was carried out using Gilson (Villiers le Bel, France) Pipeteman automatic pipettes. 2.3ml of 0.1M di-sodium tetraborate buffer pH 9.0 was added, followed by 2.5ml of freshly made fluorescamine reagent (2mg/ml fluorescamine (Sigma) in acetonitrile), while vortexing the tube contents. The tube contents must be thoroughly mixed to ensure complete reaction with the sample as fluorescamine also reacts, if relatively slowly, with water (Udenfriend et al., 1972). After 1-2 min. the sample was acidified by adding 2.5ml of 2M HCl. It was then mixed by vortexing and transferred to 14ml McCartney bottles sealed with polypropylene caps

(Gallenkamp). These were heated at 80°C in a thermostatically controlled water bath for 1 h. This heating, in the presence of acid, destroys all the fluorescamine derivatives of the amino acids except those of histidine and 3-methylhistidine (Nakamura, 1977b).

Standard 3-methylhistidine solutions were derivatised similarly, except that 0.2ml of the standard solutions was used and neutralisation by sodium hydroxide was not necessary.

3.2.2.5. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FLUORESCENT DERIVATIVES.

The acid-stable fluorescent derivatives of histidine and 3-methylhistidine were separated by reverse-phase high - performance liquid chromatography. Separation was found to be successful using the 5cm X 0.49cm column of 5 μm Lichrosorb O.D.S (C_{18}), prepared on site (White, 1986), a suitable commercially available material being Waters (Millipore UK Ltd, Harrow) C_{18}- μBondapak. Isocratic elution by 49% methanol (HPLC grade; Fisons) / 49% acetate buffer pH.4 / 2% tetrahydrofuran (Sigma) was employed, the acetate buffer being prepared from 1g sodium acetate (anhydrous) and 2.5g glacial acetic acid made to 1000ml with distilled water. The solvent pump used was a Beckman (California, USA) 112 solvent delivery module, consisting of a single reciprocating piston pump. With this pump it was possible to maintain a constant back-pressure or a constant flow rate. A constant flow rate of 1.4ml/minute was found to be satisfactory. Sample injection was by a Talbot (Alderly Edge, UK) ASI-3

autosampler. The samples were put into septum capped 2ml Wheeton vials (supplied by Chromacol Ltd, London, UK) which were placed on the autosampler turntable (capacity of 60 vials). Sample injection was by a peristaltic pump via a Rheodyne (California, USA) 7010 injection valve with a 20 μl sample loop. After separation by the column, fluorescence detection was performed by an Aminco Fluoromonitor (American Instrument Company, Maryland, USA). This fluorescence detector employed a mercury blacklight lamp with maximum emission at 360nm. The excitaion filter was a Corning 760 (narrow band pass 360nm), and the emission filter a Wratten 2A (405nm cut off), the 3-methylhistidine/fluorescamine derivative having an excitation maximum of 380nm and an emission maximum of 470nm (Nakamuro and Pisano 1976).

3.2.2.6. QUANTIFICATION.

Quantification was originally carried out by measuring peak height using a Servoscribe (Supplied by Lucas, Birmingham, UK) 511.20 Potentiometric recorder. To increase the ease, speed and accuracy of analysis a Hewlett Packard (Pensylvania, USA) 3392A Integrator was purchased. Initially it had been planned to use peak area instead of height for quantification to reduce errors due to variation in peak widths which were apparent during the analysis of large numbers of samples. After some investigation it was found that peak height was more reproducible than area if standards were run between every few samples. An example of a chromatogram generated

by the method described can be seen in figure 3.1.

The peak heights due to the fluorescence of the standards were used to construct a standard curve of peak height (arbitary units) against 3-methylhistidine concentration (μg/ml), from which the 3-methylhistidine concentration of the samples could be determined. Initially standard curves were drawn and interpolated by hand. Later a BBC Statistics "Linear regression" computer programme was used to increase the speed and accuracy of the data handling. It was possible to use this programme as there was a linear relationship between peak height and concentration at all of the 3-methylhistidine concentrations commonly encountered.

3-Methylhistidine titres were expressed as μg / g fat free, connective tissue free, fresh sample (μg/g ff,cf,fsam) (appendix II) and as mg / g non-connective tissue nitrogen (mg/g nc N) (appendix II). Connective tissue was determined as hydroxyproline by the standard method of Stegeman and Stalder (1967)(appendix I). Moisture, lipid and nitrogen were determined by standard methods (Egan, Kirk and Sawyer, 1981) (appendix I). The results of the compositonal analyses are given in appendix III. Corrections for the variable levels of fat and connective tissue found in meat, (neither of which contain 3-methylhistidine) were necessary to obtain comparable 3-methylhistidine titres. The correction for connective tissue was based on "dry connective tissue", calculated as:

Figure 3.1. HPLC separation of 3-methylhistidine (3MeHis), from histidine (His), in beef L.dorsi.

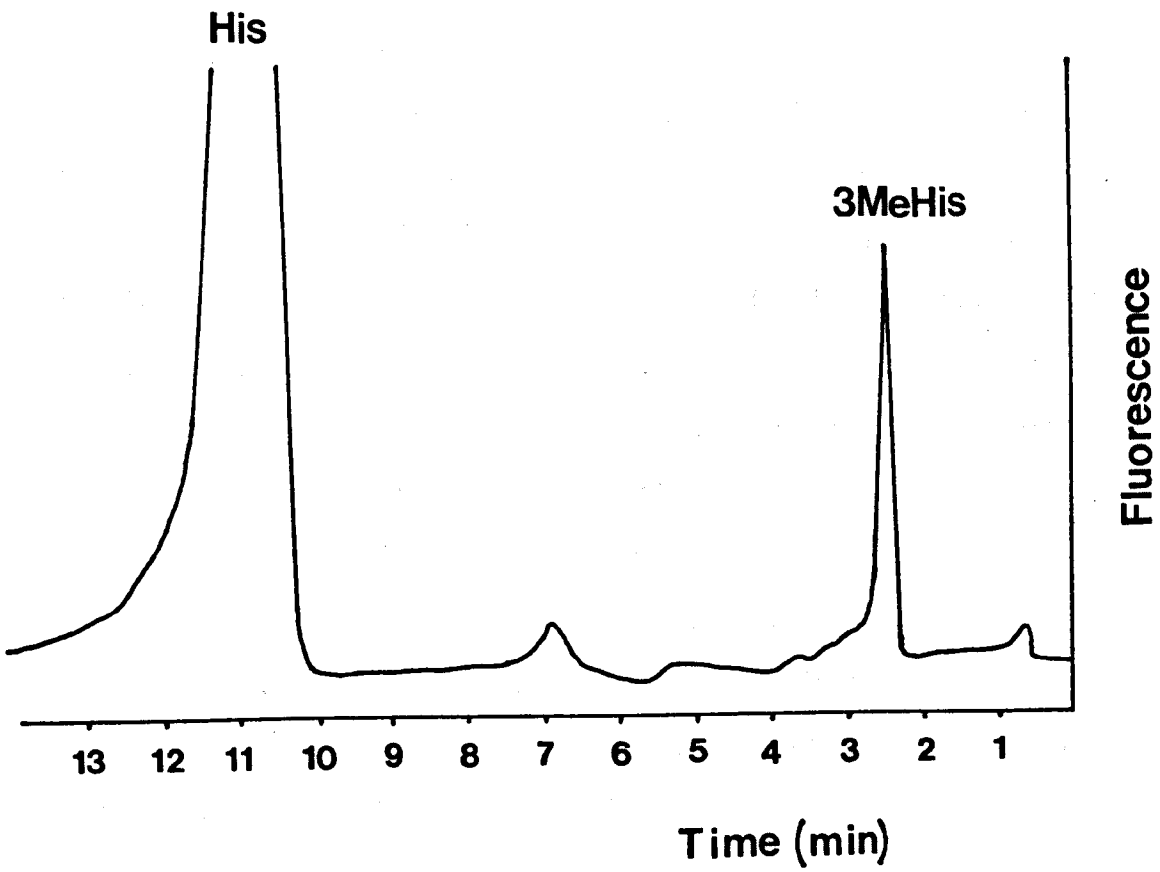
COLUMN: 5µm Lichrosorb ODS, 5cm x 0.49cm.

INJECTION VOLUME: 20µl.

ELUENT: 49% Methanol, 49% acetate buffer pH 4.0, 2% THF.

FLOW RATE: 1.4ml/min.

DETECTOR: Aminco filter fluoromonitor.
Primary filter- Corning 760.
Secondary filter- Wratten no. 2A.



% hydroxyproline x 7.25 = % "dry connective tissue"

(Goll, Bray and Hoekstra, 1973),

so that the results could be compared with those of White (1986), the dry protein being the essence of connective tissue. Jones et al. (1985) and Jones, Homan and Favell (1987) used a "wet connective tissue" value calculated as:

% hydroxyproline x 37 = % "wet connective tissue".

3.3. RESULTS AND DISCUSSION.

Table 3.1 shows the total protein-bound 3-methylhistidine titres of the bovine muscles investigated. Regardless of the units used to express the results there is a significant variation between the titres for the muscles. Masseter and Malaris has an abnormally low titre, confirming previous reports. Aponeurosis (flank) does not have a particularly abnormal titre, contradictory to the findings of Jones et al. (1985). This result could be due to the reported variations between the individual flank muscles (Jones et al., unpublished).

The titres found indicate that an index for meat content based on total protein-bound 3-methylhistidine would have to assume a mean titre for "meat", similar to the assumption made with nitrogen factors.

TABLE 3.1. TOTAL PROTEIN-BOUND 3-METHYLHISTIDINE TITRES OF BOVINE MUSCLES.

MUSCLE	PROTEIN-BOUND 3MEHIS TITRE			
	µg/g ff,cf,fsam.		mg/g nc	
	MEAN*	SD(±)	MEAN*	N SD(±)
L.dorsi	145.46	8.18	5.16	0.35
Semimembranosus	127.42	8.54	4.54	0.28
Sternomandibularis	125.99	7.85	5.30	0.35
Masseter/Malaris	75.80	8.40	3.46	0.42
Aponeurosis	110.83	2.35	4.24	0.14

* Means of 4 determinations.

The total protein-bound 3-methylhistidine titres for the porcine and ovine cuts investigated are given in table 3.2. All of the porcine regions have similar "normal" titres, even the cheek, whereas the ovine cheek, like that of the bovine has a low titre. This is indicative of the fact that ruminant Masseter and Malaris (cheek) have an abnormally low titre compared with the "normal" titre of non-ruminant cheek.

TABLE 3.2. TOTAL PROTEIN-BOUND 3-METHYLHISTIDINE TITRES OF PORCINE AND OVINE CUTS.

SPECIES	CUT	PROTEIN-BOUND 3MEHIS TITRE			
		µg/g ff,cf,fsam		mg/g nc N	
		MEAN*	SD(±)	MEAN*	SD(±)
Porcine	Neck	123.76	-	5.03	-
	Shoulder	125.59	-	5.08	-
	Cheek	115.43	17.29	4.49	0.48
Ovine	Cheek	72.92	5.95	2.82	0.27

* Porcine neck, shoulder; Mean of 2 determinations.
Porcine cheek, ovine cheek; Mean of 8 determinations.

The diaphragm of the three species investigated can be seen from table 3.3, to have similar 3-methylhistidine titres, of an intermediate value between that of ruminant cheek and "prime cuts".

TABLE 3.3. TOTAL PROTEIN-BOUND 3-METHYLHISTIDINE TITRES OF BOVINE, OVINE AND PORCINE DIAPHRAGM.

SPECIES	PROTEIN-BOUND 3MEHIS TITRE			
	μg/g ff,cf,fsam		mg/g nc N	
	MEAN*	SD(±)	MEAN*	SD(±)
Bovine	98.39	4.60	3.58	0.19
Ovine	93.39	4.28	3.79	0.17
Porcine	81.47	8.34	3.19	0.33

* All values are means of 4 determinations.

Table 3.4 shows the total protein-bound 3-methylhistidine titres for the fish species investigated. This data indicates that the titres for fish muscle are "normal", being in the range found for prime bovine muscles. On this basis fish actin is unlikely to be devoid of 3-methylhistidine, as previously reported (Shenouda and Pigott, 1975). The use of protein-bound 3-methylhistidine as a measure of meat content would include fish muscle as "meat", if incorporated into a product, contrary to the present regulations.

TABLE 3.4. TOTAL PROTEIN-BOUND 3-METHYLHISTIDINE TITRES OF FISH.

SPECIES	PROTEIN-BOUND 3MEHIS TITRE			
	μg/g ff,cf,fsam		mg/g/nc N	
	MEAN*	SD(±)	MEAN*	SD(±)
Cod	110.18	6.55	4.21	0.31
Redfish	112.57	14.83	4.41	0.43
Red snapper	112.16	10.28	4.26	0.45

* Cod and red snapper; Mean of 4 samples.
Redfish; Mean of 8 samples.

The 3-methylhistidine titres of the non-skeletal muscle sources investigated are given in table 3.5.

TABLE 3.5. TOTAL PROTEIN-BOUND 3-METHYLHISTIDINE TITRES OF NON-SKELETAL MUSCLE PROTEIN SOURCES.

SPECIES	SAMPLE	PROTEIN-BOUND 3MEHIS TITRE*			
		μg/g ff,cf,fsam		mg/g nc N	
		MEAN*	SD(±)	MEAN*	SD(±)
Bovine	Heart	53.60	8.84	2.30	0.29
	"Tripe"	30.30	3.59	(10.36	1.73)
	Kidney	15.18	4.71	0.73	0.23
	Liver	8.39	1.04	0.37	0.08
	Tongue	84.99	7.88	3.48	0.39
Porcine	"Melts"	35.31	3.42	1.61	0.16
Soybean	TVP	Non detectable		Non detectable	

* All values are means of 4 determinations.

All of the samples have low titres (it is difficult to give adequate explanation for the high 3-methylhistidine titre of "tripe" when expressed as mg/g nc N, though the reportedly higher ratio of actin:myosin in smooth muscle, of which tripe consists, compared with striated muscle Lawrie (1985), could provide some explanation). The data supports that of Rangeley and Lawrie (1977) and Jones et al. (1985), 3-methylhistidine being undetectable in soy and only at low levels in kidney and liver. Only in the case of heart, tongue, "tripe" and "melts" would their inclusion in a product make a significant contribution to the "meat" content, if determined by the protein-bound level of this amino acid. This is contrary to current regulations (Statutory Instruments, 1984, 1986), which does not class "tripe" and "melts" as meat, but does class kidney and liver as such, even though they contain very low levels of 3-methylhistidine.

Other compositional data (moisture, lipid, dry connective tissue and nitrogen contents) for the samples analysed are listed in appendix III.

4. 3-METHYLHISTIDINE TITRES OF PURIFIED ACTINS AND MYOSINS IN RELATION TO MUSCLE FIBRE TYPE.

4.1. INTRODUCTION.

Myofibrillar proteins are the essence of meat. The major constituents of the myofibrillar proteins are actin and myosin, the only proteins to contain 3-methylhistidine.

The variation in the total protein-bound 3-methylhistidine titres of skeletal muscles (section 3), all of which consist essentially of the same group of components, may be due to differences in the extent of histidine methylation of the actin and/or myosin. Inherent errors in the assumptions made when calculating the values (Jones et al. 1985) may also make a contribution to the variation.

Previously the conserved nature of actin structure has been extensively reported (section 2.1.4). Actins purified from sources as different as rabbit skeletal muscle and Acanthamoeba micro-organisms have in the region of 1 mole 3-methylhistidine/ mole actin monomer. The only reported example of an actin deficient in this amino acid being that from the alga Naegleria (Sussman et al., 1984).

The variation in the 3-methylhistidine content of myosins has been well documented (section 2.1.4), the 3-methylhistidine content being dependent on the age of the animal and the tissue concerned. Reports of a relationship between a muscle's ATPase activity (related to speed of contraction) and the 3-methylhistidine content of the muscle's myosin has been made (Johnson et al., 1970). This indicates

that there may be a relationship between the fibre type of a muscle and the 3-methylhistidine content of its myosin.

The 3-methylhistidine titres of myosin and actin purified from selected bovine, ovine and porcine muscles were determined to demonstrate the extent of the variation in these individual titres. Muscles were then examined histologically to determine any relationship between the fibre type, the 3-methylhistidine titre of actin, the 3-methylhistidine titre of myosin and the total protein-bound 3-methylhistidine content of muscle concerned.

4.2. 3-METHYLHISTIDINE CONTENT OF ACTINS.

4.2.1. MATERIALS AND METHODS.

4.2.1.1. PURIFICATION OF MUSCLE ACTIN.

The 3-methylhistidine content of actin purified from the following muscles was determined: (i) Bovine L.dorsi, Semimembranosus, Sternomandibularis, Masseter and Malaris, Aponeurosis and "tripe" (smooth muscle). (ii) Ovine L.dorsi, Masseter and Malaris, and Psoas. (iii) Porcine L.dorsi, Masseter and Malaris, and Psoas. Each set of muscles were obtained from a single carcass.

The purification of muscle actin was based on the method of Pardee and Spudich (1982), as modified by White (1986). The method involves the extraction of actin in the monomer form (G-actin) from a muscle acetone powder using a low ionic strength buffer. Polymerisation is performed by increasing the

ionic strength of the actin solution. A series of polymerisation/depolymerisation and high speed centrifugation steps eliminates all other actomyosin-associated proteins to give an actin preparation of high purity.

(a) PREPARATION OF ACETONE POWDER- All experimental work was carried out at 4°C in a cold room. Disposable latex gloves were used throughout the purification to minimise contamination of the protein preparation, actin being susceptible to proteolysis by bacteria. Sodium azide was included in some buffers to destroy any such contamination. Muscle samples (500g) were obtained from local slaughter-houses, excised from the carcass immediately after slaughter and chilled on ice. Acetone powder preparation, was carried out within several hours after slaughter, since, on completion of rigor mortis, the actin becomes far less extractable from the actomyosin complex. The muscle was trimmed of visible fat and connective tissue, washed with distilled water and minced at 4°C, in a precooled domestic mincer with a 4mm plate.

350g mince was extracted whilst stirring for 10min with 1000ml ice-cold 0.1M KCl / 0.15M potassium phosphate buffer pH. 6.5. All extracts were filtered by squeezing through muslin (previously boiled in distilled water for 20min, rinsed in distilled water and cooled to 4°C).

The residue was stirred for 10min, at 4°C, in 2 l 0.05M $NaHCO_3$, and filtered, followed by 1000ml 1mM EDTA (Sigma), pH 7.0, for 10min, at 4°C. Two extractions by 2 l of distilled water for 5min at 4°C, were carried out on the resulting residue,

followed by five extractions with 1000ml acetone, for 10min each at 20-25°C. The resulting residue was dried overnight in a fume-cupboard, then stored at -20°C, at which temperature it is stable for several months.

(b) ISOLATION OF ACTIN- Typically, 10g of acetone powder was used , the expected yield being 10-30mg actin/g acetone powder.

Acetone powder was extracted at 0-0.5°C for 30min with 20ml "buffer A"/g acetone powder. "Buffer A" consists of: 2mM "Tris buffer" / 0.2mM Na_2ATP (Sigma) / 0.5mM 2-mercaptoethanol (Sigma) / 0.2mM $CaCl_2$ / 0.005% sodium azide (Sigma), final pH 8.0, at 25°C (2-mercaptoethanol was added after the final pH adjustment as it interferes with the measurement). ATP was added to the buffer as a solid just prior to use, as it is unstable in solution. The extract was separated by squeezig through sterile muslin, the residue being re-extracted for 10min, at 0-0.5°C, with 20ml "Buffer A" / g acetone powder originally used. After filtration the extracts were combined.

Centrifugation of the extracts was carried out at 15000g (11500 rpm, MSE 18 High speed centrifuge, 6 X 100ml angle rotor, polypropylene tubes with stainless steel caps. MSE Scientific Instruments, Crawley, UK), for 1h, at 4°C. The clear G-actin containing supernatant was removed using a 50ml disposable syringe.

The [KCl], $[Mg^{2+}]$ and [ATP] of the supernatant was raised to 50mM, 2mM and 1mM, using 2.5M KCl, 1M $MgCl_2$ and

solid Na_2ATP (Sigma), respectively. Polymerisation was allowed to proceed at 4°C for 2h (when the viscosity should visibly increase).

The [KCl] was raised to 0.6M by the slow addition of solid KCl, whilst stirring for 30min at 4°C, dissolving any contaminating tropomyosin. The sample was then centrifuged at 160000 g (47000 rpm in a Beckman - Beckman Instruments Co., California, USA - L8 55M Ultra-centrifuge, using a 70 Ti rotor) with Beckman "Ultra-clear", "Quick-seal" 25 X 89mm centrifuge tubes of 39ml capacity. These tubes were used to minimise bacterial contamination of the sample, and for ease and speed of handling.

The pellets of polymerised actin (F-actin) were resuspended, by gentle homogenisation, with 2ml "Buffer A"/g acetone powder originally used, using a glass rod. The pooled suspensions were depolymerised by dialysis in 18/32" Visking tubing (Medicell International Ltd, London, UK), previously boiled in distilled water for 15min then cooled to 4 °C. Dialysis was against 1000ml "Buffer A", at 4°C for 3 days, with a change of buffer every 24hrs. The resulting G-actin solution was centrifuged at 160000 g (47500 rpm, Beckman L8 55M Ultratra-centrifuge, with a 70 Ti rotor), using uncapped Beckman thick walled polycarbonate (25 X 89mm) tubes (maximum volume uncapped; 16.5ml). The clarified G-actin solution was then stable for several days, when stored air-tight at 4°C. Long term storage was possible by freeze-dehydration, using a Vickers freeze-drier (Vickers Armstrong Ltd, Swindon, UK), with storage at -20°C.

4.2.1.2. DETERMINATION OF PROTEIN CONCENTRATION OF ACTIN PREPARATIONS.

The protein concentration of the G-actin solutions was determined by dye-binding using Coomassie Brilliant Blue G-250 (CBB) (Sigma), based on the method of Bradford (1976).

Standard bovine serum albumin (BSA) (Sigma) was prepared at concentrations of 0-1000 μg/ml in "Buffer A" (section 4.2.1.1(a). To 0.1ml standard or purified actin sample was added 5ml "protein reagent" ("protein reagent" consisted of 100mg CBB dissolved in 50ml 95% ethanol, to which was added 100ml 85% (w/v) phosphoric acid, the mixture being made up to 1000ml with distilled water). Before use the reagent was filtered through Whatman no.1 paper over a low vacuum. The tube contents were mixed by gentle vortexing and after 3-5 min, the absorbance was read at 595nm, against a reagent blank. The protein concentration of the G-actin solution could be interpolated from the standard curve of protein (μg) vs absorbance (595nm).

4.2.1.3. ASSESSING THE PURITY OF ACTIN.

The purity of the G-actin solutions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), based on the method of Pollard (1982). The apparatus employed was the Biorad "Protean", dual vertical slab gel electrophoresis cell (Biorad Laboratories, California, USA), which was set up according to the manufacturer's instructions.

A 1.5mm thick, 7.5% slab gel was prepared as outlined in table 4.1.

TABLE 4.1. PREPARATION OF SDS PAGE GEL.

STOCK SOLUTION	COMPOSITION	VOLUME* (ml)
A	30% acrylamide (Electrophoresis grade; Fisons) / 0.8% N,N´- methylenebisacrylamide (BDH).	12.5
B	10% SDS (Primar grade; Fisons).	0.5
C	250mM Tris/glycine buffer pH 8.6, consisting of 30.3g/l "Tris buffer" / 144g/l glycine.	5.0
D	N,N,N´,N´-tetramethylethylenediamine (TEMED).	0.025
E	Ammonium persulphate (Sigma) 0.1g/ml, freshly made.	0.375
	Distilled water	31.75

* All of the components, except the ammonium persulphate, were mixed gently. Polymerisation was initiated by adding the ammonium persulphate. After gentle mixing the solution was poured between the prepared plates, the appropriate well-forming comb was inserted, and the gel was left to polymerise for 2h.

Sample preparation was carried out by mixing the sample with an equal volume of sample buffer to give a final protein concentration of 1mg/ml, followed by heating at 100°C for 2-3min. The sample buffer consisted of 20ml stock solution "B" (SDS) (see table 4.1), 2ml "C" (Tris / glycine), 2ml 2-mercaptoethanol (Sigma), 20ml glycerol and 10mg bromophenol blue (BDH), made up to 100ml with distilled water. The electrode buffer consisted of 50ml stock solution "B" (SDS) and 500ml "C" (Tris/glycine), made up to 5 l with distilled water.

The sample was loaded into the appropriate wells using a Hamilton syringe (Hamilton Bonaduz A.G., Bonaduz, Switzerland). For most purposes a loading of a volume equivalent to 10-20μg protein/well was adequate; this causes overloading of the actin bands, to enable visualisation of the low levels of contaminants present. The molecular weight markers used throughout this work were Sigma SDS 6H and SDS 7. SDS 6H consisted of myosin heavy chain (molecular weight; 205000 daltons), β-galactosidase (116000), phosphorylase B (97400), bovine albumin (66000), egg albumin (45000). SDS 7 consisted of bovine albumin (66000), egg albumin (44000), glyceraldehyde-3-phosphate dehydrogenase (36100), carbonic anhydrase (29000), trypsin pmfs treated (24000), trypsin inhibitor (20100) and α-lactalbumin (14200). Sample bands on electrophoretograms were identified by comparing the known molecular weights of myofibrillar proteins with the molecular weights of the markers. The molecular weight markers were prepared at a concentration of 2mg/ml, and 10μl was loaded onto the gel.

The gel was run at a maximum of 200V and a maximum of 50 mA using a Pharmacia electrophoresis power supply EPS 500/400, until the dye front was within 0.5cm from the bottom of the gel (approximately 3h).

Fixing and staining was carried out by immersing the gel for 30min in 0.2% CBB (Sigma)/ 50% methanol (SLR; Fisons)/ 12% acetic acid. Destaining was carried out initially with 10% acetic acid / 50% propan-2-ol (SLR; Fisons), for several hours, followed by 10% acetic acid (SLR; Fisons), overnight.

The gels were photographed then stored in sealed polyethylene bags.

4.2.1.4. 3-METHYLHISTIDINE DETERMINATION OF PURIFIED ACTIN.

100 μl of the G-actin solutions were freeze dried in 2ml glass ampoules (Supplied by BDH, Poole, UK) using a Vickers freeze drier. 50μl 6M HCl was added to each ampoule, which was then flame sealed. Oven hydrolysis was carried out at 110°C for 16h. The 3-methylhistidine content of the hydrolysates was determined by the standard method (section 3.2.2.).

Data was expressed as moles of 3-methylhistidine / mole G-actin. The molecular weight of 3-methylhistidine is 169.2 (Sigma), and that of G-actin was assumed to be 41785 (Elzinga et al., 1973).

4.2.2. RESULTS AND DISCUSSION.

Actin preparation by the original method of Pardee and Spudich (1982), as modified by White (1986), included a

further polymerisation and high speed centrifugation step. Pardee and Spudich (1982), also suggested that further purification by ion-exchange chromatography might be necessary to obtain pure actin preparations. These steps were found not to be necessary to obtain the level of purity required.

Protein determination of the actin preparations by CBB dye binding (Bradford, 1976; Sedmark and Grossberg, 1977) had significant advantages over other methods of protein determination routinely used. White (1986) used Kjeldahl distillation (appendix I) of the actin hydrolysates, but this had the disadvantage of consuming large quantities of actin. Colorimetric methods for the determination of the concentration of actin (or myosin) preparations, such as the Lowry method (Lowry et al., 1951), as used by Carsten (1963), with lyophilised actin as the standard, and the Biuret method (Gornall, Bardawill and David, 1949) as used by Bridgen (1971), standardised against dried trout actin, often have the disadvantage of interference from various ions, and large differences in the response depending on the protein. Dye binding assays appeared suitable since they are generally rapid, sensitive and less susceptible to interference from solutes, particularly as the standards are prepared in the same buffer as the samples, thus minimising error due to interference from buffer components. The CBB assay gave excellent results, but the absorbance of the solution had to be read from 3-5min after the addition of the "protein reagent" as time-dependent precipitation occasionally occurred. The use of bovine muscle actin (Sigma) as the standard was investigated, but the high cost and small amounts of actin available out-weighed

the error due to the small difference in the dye-binding characteristics of actin and BSA. Other suitable dye-binding assays are those using bromophenol blue (Flores, 1978) and Orange-G (Udy, 1971).

The "continuous" method of SDS PAGE used (Pollard, 1982), employing an electrode buffer and gel of the same pH and buffer composition was found to be simpler and more rapid than the more commonly used "discontinuous" method of Laemmli (1970). The "dicontinuous" method requires a different pH and buffer composition for the electrode buffer and gel, as well as the preparation of a separate "stacking gel". Although the resolution was not quite as good as that using a "Laemmli" gel, all of the important myofibrillar proteins could be well resolved on the 7.5% "Pollard" gel.

The purity of the actin preparations was high. Figure 4.1 shows that the actin preparation gave a single major electrophoretic band with a molecular weight approximating to that assumed for actin. Contamination was present at only very low levels, non-actin bands being barely visible even at the very high loadings used. Contaminants of a molecular weight coinciding with that assumed for myosin heavy chain were rarely seen in any of the actin preparations. A common contaminant corresponded to the 38000 dalton actin protease-resistant core (Pardee and Spudich, 1982). The method of staining, using CBB, is adequately sensitive; but if contaminants present at very low levels need to be detected, such as in studies on the association of the cytoskeletal proteins, silver staining (eg. Sigma silver stain

Figure 4.1. SDS PAGE electrophoretogram of actin preparations from ovine L.dorsi, Masseter and Malaris and Psoas.

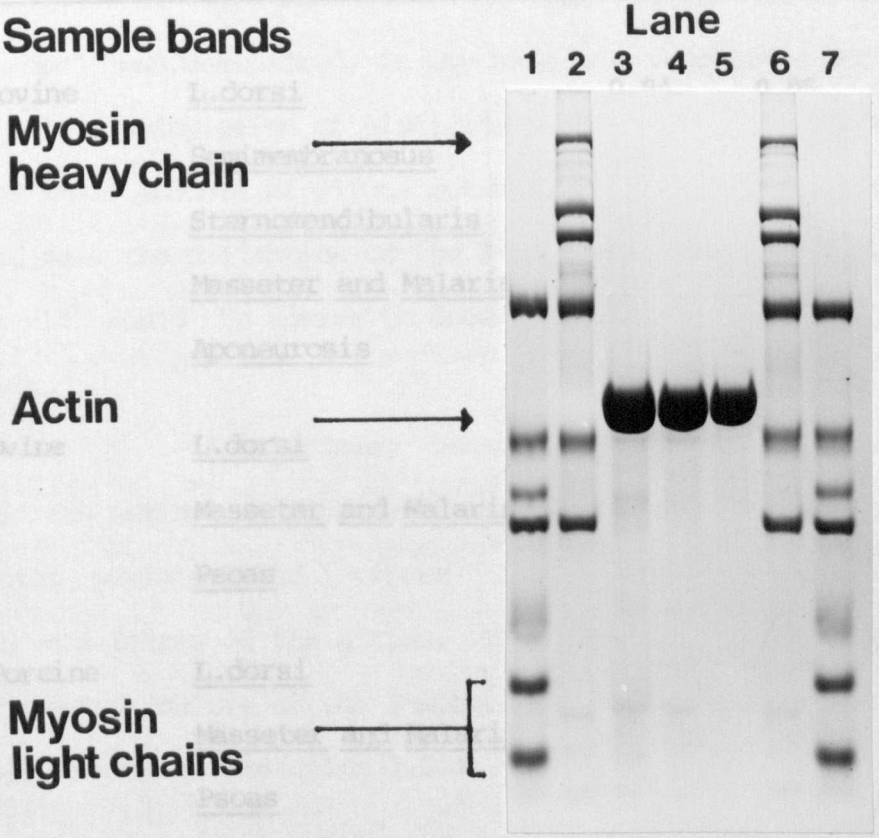
Lane 1,7: SDS 7 molecular weight marker.

2,6: SDS 6H molecular weight marker.

3: Ovine Masseter and Malaris actin.

4: Ovine L.dorsi actin.

5: Ovine Psoas actin.



kit for polyacrylamide gels), could be used. This staining method is 50-100 times as sensitive as CBB.

Table 4.2 gives the 3-methylhistidine titres of the purified actins. Considering each species individually there is good agreement between the titres of all of the skeletal muscles investigated.

TABLE 4.2. 3-METHYLHISTIDINE TITRES OF ACTINS OF VARIOUS MUSCLES.

SPECIES	MUSCLE	3-METHYLHISTIDINE TITRE OF ACTIN (moles/mole) MEAN*	SD(±)
Bovine	L.dorsi	0.84	0.05
	Semimembranosus	0.85	0.07
	Sternomandibularis	0.77	0.02
	Masseter and Malaris	0.76	0.08
	Aponeurosis	0.84	0.09
Ovine	L.dorsi	0.67	0.00
	Masseter and Malaris	0.69	0.00
	Psoas	0.65	0.03
Porcine	L.dorsi	0.65	0.01
	Masseter and Malaris	0.62	0.04
	Psoas	0.64	0.10
Bovine	"Tripe"	0.45	0.09

* Bovine samples: Mean of 4 determinations.
Ovine and porcine samples: Mean of 2 determinations.

It is notable that ruminant Masseter and Malaris, actin has a similar titre to the prime cut actins within a species, unlike the total protein-bound 3-methylhistidine (section 3.3). The titres for bovine actins agree well with literature values (section 2.1.4), those for ovine and porcine actins are rather low. This may be attributed to losses of sample during freeze drying prior to hydrolysis in a rather unreliable freeze dryer. The freeze drying step could be eliminated by adding an equal volume of 12M (1.18 SG.) HCl to the actin solutions prior to hydrolysis.

The low titre of the smooth muscle actin from "tripe" was unexpected, it may be a true representation of a low level of methylation of histidine in smooth muscle actin, but the small amounts of dilute actin solution prepared from "tripe" did make the estimation of the 3-methylhistidine titre difficult, so it would be unwise to draw any firm conclusions from this data.

Similarities between the 3-methylhistidine titres of the actins indicates that the intracarcass variation in the total protein-bound titres is not due to variations in the titres of the actins. This is not unexpected since the conserved nature of the 3-methylhistidine content of actins is well documented (section 2.1.4).

4.3. 3-METHYLHISTIDINE CONTENT OF MYOSINS.

4.3.1. MATERIALS AND METHODS.

4.3.1.1. PURIFICATION OF MUSCLE MYOSIN.

The 3-methylhistidine titres of myosins purified from the following muscles was determined: Ovine and porcine L.dorsi, Masseter and Malaris and Psoas.

Preparation of skeletal muscle myosin was carried out by a method based on that of Margossian and Lowey (1982), as modified by White (1986).

All preparative work was carried out at 4°C in a cold room. The muscles (100g) were excised from the newly slaughtered animal and stored on ice. Myosin preparation was commenced within 1h after slaughter, since with the development of rigor mortis, the myosin becomes increasingly inseparable from associated myofibrillar proteins.

Firstly the muscle was trimmed of all external fat and connective tissue, and minced in a precooled domestic mincer with a 4mm plate. 50g of mince was extracted by stirring with 3 volumes of "myosin extraction buffer" for 15min at 4°C. This buffer consisted of 0.3M KCl / 0.15M Potassium phosphate pH 6.5. The sample was then centrifuged at 5000g (6500rpm, MSE 18 High speed centrifuge, 6 X 100ml angle rotor, polypropylene tubes with stainless steel caps), for 45min at 4°C, to stop the extraction by pelleting the solid. The supernatant was filtered through pulped Whatman no.1 paper, soaked in "myosin extraction buffer", the clarified filtrate being poured into 10 times its

volume of distilled water at 4°C, swirled and left overnight to allow the myosin to precipitate and settle. Excess supernatant was syphoned off and the precipitate centrifuged at 5000g (6750rpm , Beckman Model J2-21 centrifuge, JA-10 6 X 500ml rotor, polypropylene bottles with screw tops :filling capacity 360ml), for 45min , at 4°C. The myosin pellets obtained were dissolved in an equal volume of "buffer B". "Buffer B" consisted of 1.2M KCl / 10mM potassium phosphate, pH 6.5. The resulting myosin solution was clarified by filtration through glass wool and was stable for several days if stored airtight at 4°C with a crystal of thymol (BDH). Long term storage was possible at -20°C, in an equal volume of glycerol.

4.3.1.2. DETERMINATION OF PROTEIN CONCENTRATION OF MYOSIN PREPARATIONS.

Protein determination was carried out using the CBB dye binding assay (section 4.2.1.2), using BSA in "buffer B" as the standard.

4.3.1.3. ASSESSING THE PURITY OF MYOSIN.

The purity of the myosin preparations was assessed by SDS PAGE based on the method of Pollard (1982) (section 4.2.1.3), using 1.5mm thick, 3-18% acrylamide gradient gels. All details were as outlined in section 4.2.1.3 except for the preparation of the gel itself which was carried out as in table 4.3 and figure 4.2. The pump used for the gradient preparation was an Eyela micro-tube pump MP-3 (Tokyo Rikakikai Co Ltd, Japan).

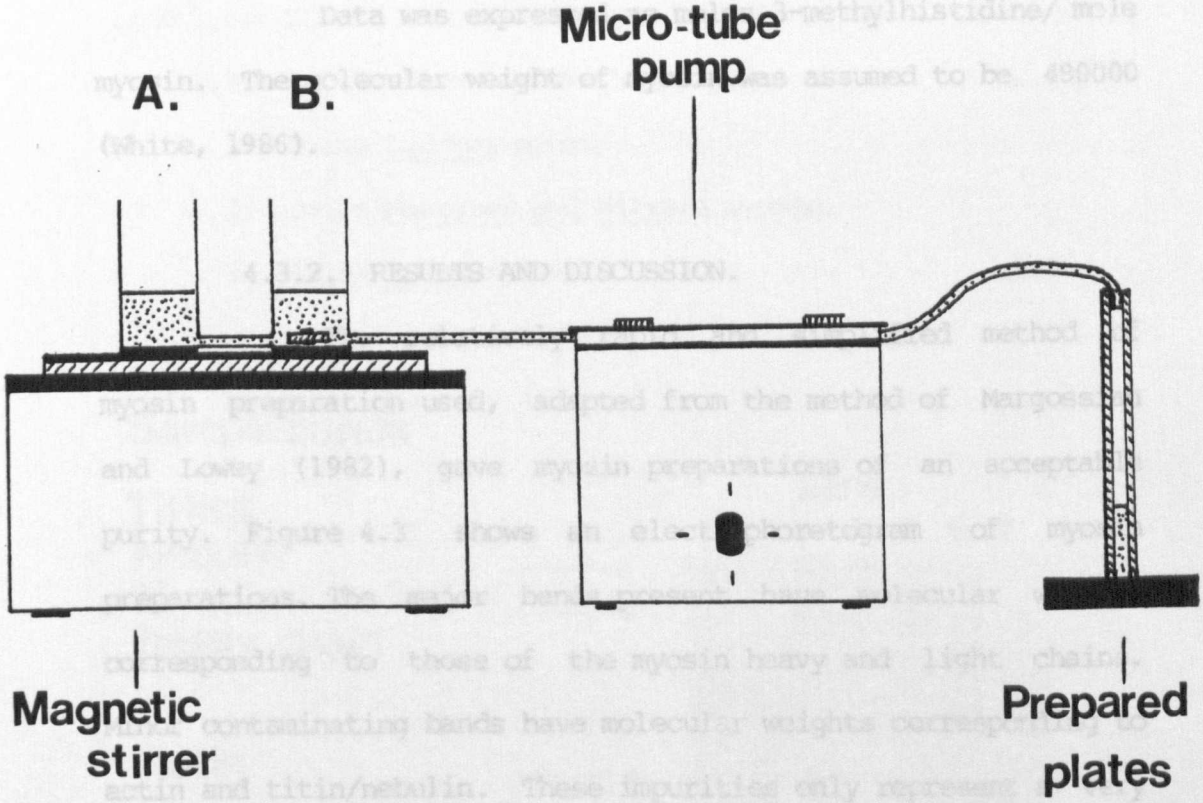
TABLE 4.3. PREPARATION OF 3-18% GRADIENT SDS PAGE GEL.

STOCK SOLUTION*	VOLUME (ml)**	
	3%	18%
A : Acrylamide	1.60	9.60
B : SDS	0.16	0.16
C : Tris/glycine	1.60	1.60
D : TEMED	0.008	0.008
E : Ammonium persulphate	0.12	0.12
Distilled water	12.51	4.51

* See table 4.1.

** The solutions required for the 3% and 18% gels (exept the ammonium persulphate), are mixed in two separate vessels, the gradient being cast as described in figure 4.2.

Figure 4.2. Schematic representation of gradient forming apparatus for SDS PAGE electrophoresis.



The apparatus was based on the Biorad (model 385) gradient former.

The 3% acylamide solution was put in vessel A, the 18% in vessel B, with the connecting tube closed with a tube clip. With the magnetic stirrer running, the ammonium persulphate was added to each, vessel A being stirred with a glass rod. The clip was removed and the pump started. Pumping was continued until the gradient was complete, it was then overlaid with distilled water and allowed to set for 2h. The water was then poured off the polymerised gradient, a freshly prepared 3% acrylamide solution was cast on top with the insertion of the appropriate well forming comb, the gel being left to set for a further 2h.

4.3.1.4. 3-METHYLHISTIDINE DETERMINATION OF PURIFIED MYOSIN.

Myosin hydrolysates were prepared and analysed as for actin (section 4.2.1.4).

Data was expressed as moles 3-methylhistidine/ mole myosin. The molecular weight of myosin was assumed to be 480000 (White, 1986).

4.3.2. RESULTS AND DISCUSSION.

The relatively rapid and simplified method of myosin preparation used, adapted from the method of Margossian and Lowey (1982), gave myosin preparations of an acceptable purity. Figure 4.3 shows an electrophoretogram of myosin preparations. The major bands present have molecular weights corresponding to those of the myosin heavy and light chains. Minor contaminating bands have molecular weights corresponding to actin and titin/nebulin. These impurities only represent a very low percentage of the total protein present and should not significantly affect the estimated 3-methylhistidine titre of the myosin.

Carraro and Catani (1983) reported that the myosins of "fast" and "slow" contracting muscles could be distinguished by their SDS PAGE pattern of the light chains as well as the mobility of the heavy chain, and Young and Davely (1981) reported the occurence of many different polymorphs of the myofibrillar proteins, including the myosin heavy chain. The biochemical differences between "fast" and "slow" muscle myosins, in terms of their ATPase activity and the release of

Figure 4.3. SDS PAGE electrophoretogram of myosin preparations from ovine L.dorsi, Masseter and Malaris and Psoas.

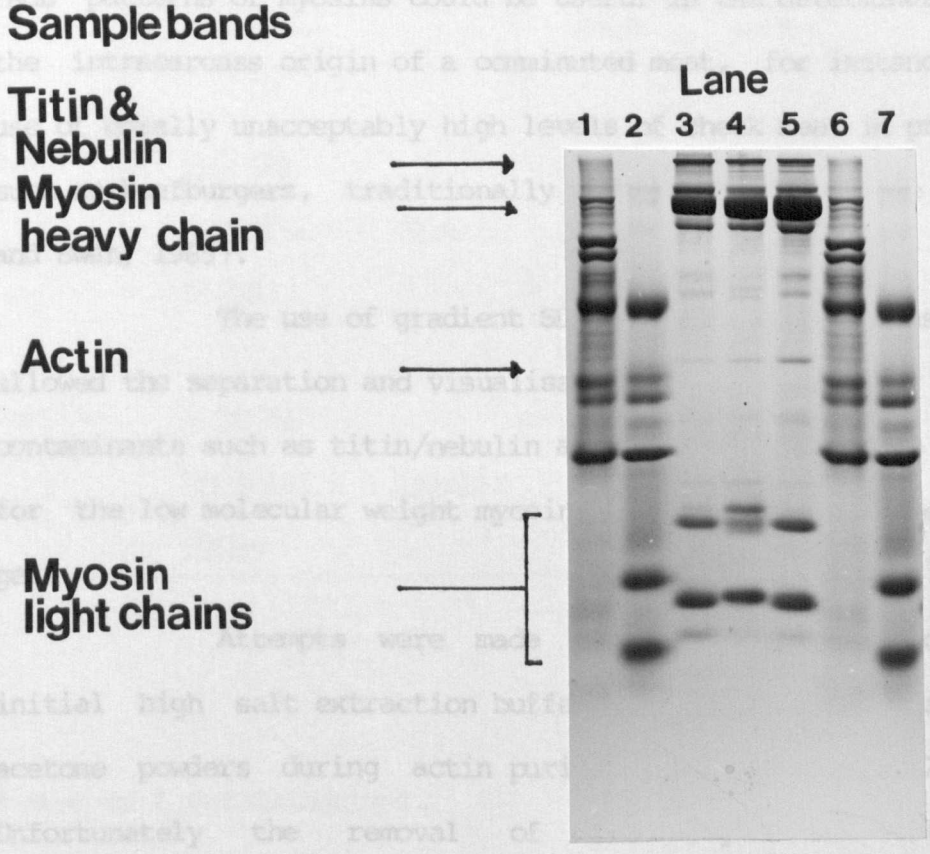
Lane 1,6: SDS 6H molecular weight marker.

2,7: SDS 7 molecular weight marker.

3: Ovine L.dorsi myosin.

4: Ovine Masseter and Malaris myosin.

5: Ovine Psoas myosin.



associated proteins on chemical treatment, were reported by Samaha, Guth and Albers (1970).

Differences in the myosin light chain composition can be clearly seen between the ovine Masseter and Malaris and the "prime" muscle samples (similar variation was also seen between the light chain patterns of the different porcine myosins), although no difference in the electrophoretic mobility of the heavy chain is apparent (possibly due to the overloading of the gel with respect to myosin, to enable the detection of contaminants). These apparent differences in the SDS PAGE patterns of myosins could be useful in the determination of the intracarcass origin of a comminuted meat, for instance the use of morally unacceptably high levels of cheek meat in products such as beefburgers, traditionally made of "prime" muscle (Lord and Swan, 1985).

The use of gradient SDS PAGE was advantageous as it allowed the separation and visualisation of high molecular weight contaminants such as titin/nebulin as well as giving sharp bands for the low molecular weight myosin light chains, on the same gel.

Attempts were made to purify myosin from the initial high salt extraction buffer used in the preparation of acetone powders during actin purification (section 4.2.1.1). Unfortunately the removal of the significant levels of actomyosin-associated contaminants present, could not be accomplished.

Table 4.4 gives the 3-methylhistidine titres of the prepared myosins.

TABLE 4.4 3-METHYLHISTIDINE TITRES OF MYOSINS OF VARIOUS MUSCLES.

SPECIES	MUSCLE	3-METHYLHISTIDINE TITRE OF MYOSIN (moles/mole) MEAN*	SD(±)
Ovine	L.dorsi	1.52	0.19
	Masseter and Malaris	0.29	0.09
	Psoas	1.42	0.08
Porcine	L.dorsi	1.27	0.16
	Masseter and Malaris	0.96	0.17
	Psoas	1.06	0.14

* Mean of 6 determinations.

The titres show the very low level of 3-methylhistidine in the myosin of ovine Masseter and Malaris (some of which may arise from actin contamination), compared with the higher value for the porcine Masseter and Malaris. These results when considered with those of White and Lawrie (1985b) and White (1986), who reported the low 3-methylhistidine titre of bovine Masseter and Malaris myosin, indicate the unusual nature of ruminant cheek muscle. The low level of histidine methylation in the myosin of ruminant cheek appears to be the origin of the low total protein-bound 3-methylhistidine titres of these muscles.

4.4. HISTOCHEMICAL FIBRE TYPING OF MUSCLE.

4.4.1. MATERIALS AND METHODS.

1g samples of bovine, ovine and porcine; L.dorsi and Masseter were excised from the carcass within 6h after slaughter. The samples were wrapped in sterile muslin dampened with physiological saline (0.9% NaCl; Humanson, 1972) and kept at 4°C for transfer to the Pathology Department, Medical School, University of Nottingham, where histochemical fibre typing was performed by Dr.J. Lowe. The muscle samples were stained for oxidative enzymes, at pH 4.2, to distinguish type I (oxidative) fibres, characteristic of "red", "slow" muscle, from type II (glycolytic) fibres, characteristic of "white", "fast" muscle.

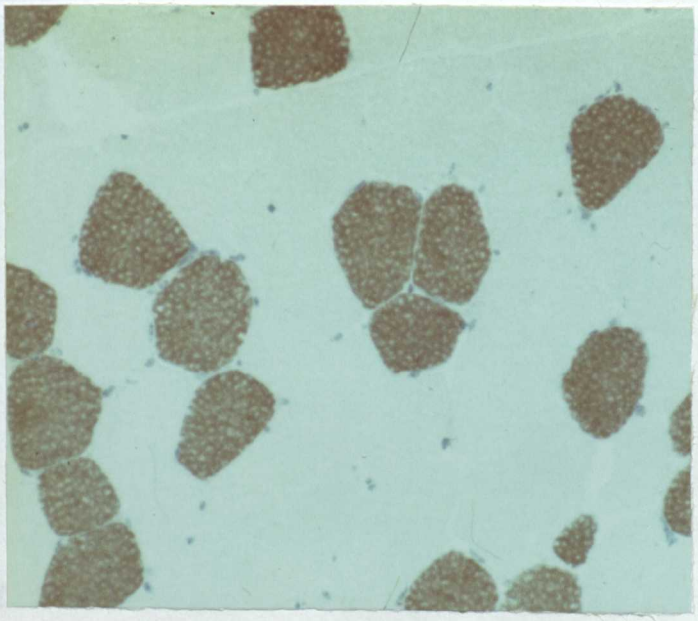
Although the fibre typing was carried out by clinical techniques, methods for the simultaneous histochemical determination of fibre types in single sections of ovine, bovine and porcine skeletal muscle has been recently reported (Solomon and Dunn, 1988).

4.4.2. RESULTS AND DISCUSSION.

Figures 4.4 a,b, and c, show the results of the fibre typing, the oxidative fibres being positively stained. Bovine and ovine Masseter can be seen to consist almost entirely of type I (oxidative) fibres, agreeing with Young and Davey (1981), who noted that bovine cheek muscle could be a reference for "slow twitch" fibres. Porcine Masseter, and the L.dorsi of all three species contain a majority of type II (glycolytic)

Figure 4.4a. Histochemical fibre typing of BOVINE L.dorsi and Masseter muscles, staining for oxidative enzymes at pH 4.2.

L.dorsi



Masseter

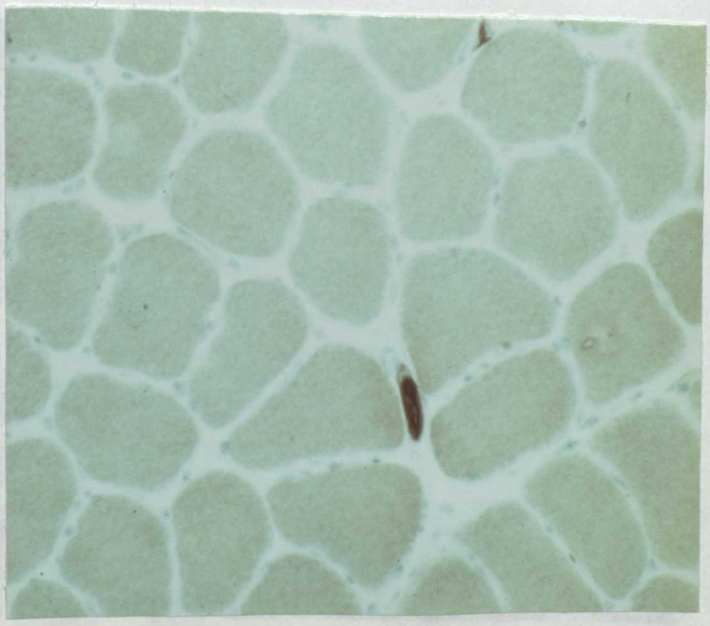
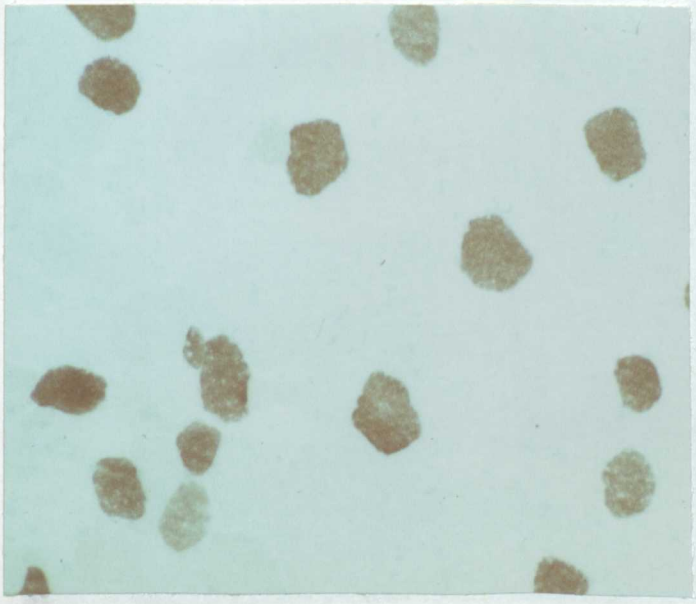


Figure 4.4b. Histochemical fibre typing of OVINE L.dorsi and Masseter muscles, staining for oxidative enzymes at pH 4.2.

L.dorsi



Masseter

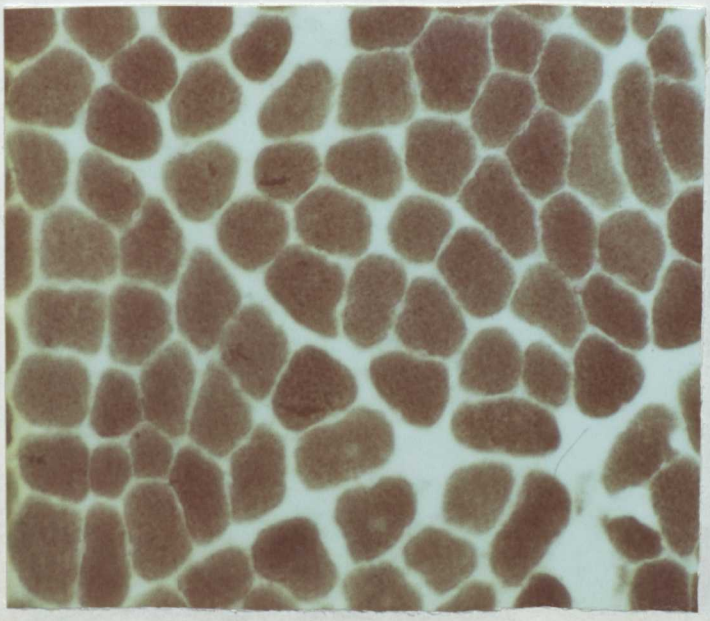
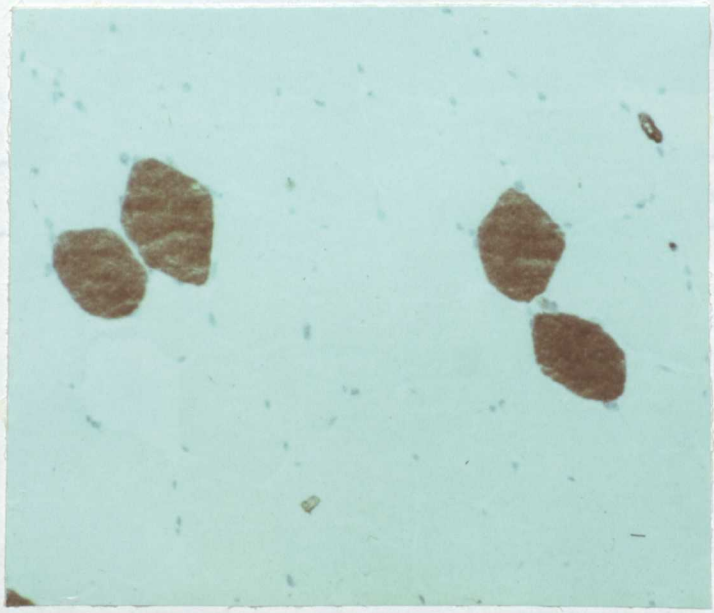
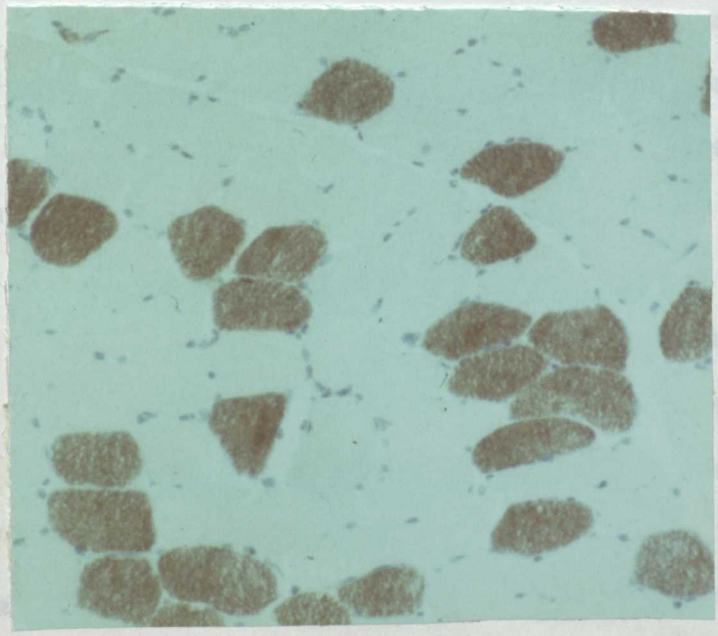


Figure 4.4c. Histochemical fibre typing of PORCINE L.dorsi and Masseter muscles, staining for oxidative enzymes at pH 4.2.

L.dorsi



Masseter



fibres. The areas occupied by the two fibre types in bovine (ruminant) and porcine (non-ruminant) L.dorsi and Masseter are given in table 4.5.

TABLE 4.5. RELATIVE AREA OCCUPIED BY FIBRE TYPES I AND II IN BOVINE AND PORCINE MUSCLES.

SPECIES	MUSCLE	% TYPE I	% TYPE II
Bovine	L.dorsi	22	78
	Masseter	100	0
Porcine	L.dorsi	13	87
	Masseter	25	75

Type I fibres are myoglobin-rich and highly oxidative in their metabolism, their biochemical constitution equiping them for slow continuous action, energised by respiration, rather than for the swift intermittent, anaerobic metabolism, typical of "white" muscles. It is presumed therefore that the mode of mastication of the ruminant is especially dependent on powerful continuous muscular action. The situation in the ruminant Masseter represents another extreme example of the tendency of the myosin of "red" muscles to have a lower 3-methylhistidine titre than that of "white". These

findings agree with those of Johnson et al. (1970) who previously noted that the extent of methylation of histidine in myosin increased along with the speed of the muscle and the ATPase activity. Similarly, the intermediate levels of total protein-bound 3-methylhistidine found for bovine, ovine and porcine diaphragm (section 3.3), indicates an intermediate "red" fibre content, between that of ruminant Masseter and that of both ruminant and non-ruminant L.dorsi.

The results in section 4 have established that the higher the type I (oxidative) fibre content of a muscle the lower the total protein-bound 3-methylhistidine titre and the lower the level of this amino acid in the myosin, although the 3-methyl-histidine titre of the actin is independent of the fibre type composition, being more constant. When histological / electrophoretic evidence suggests that muscle high in type I (oxidative) fibres has been incorporated into a food product, a more accurate assessment of the meat content could be obtained, by methods based on the 3-methylhistidine of the actin present rather than on the overall protein-bound titre of this amino acid.

5. DEVELOPMENT OF A METHOD FOR THE DETERMINATION OF ACTIN-BOUND 3-METHYLHISTIDINE.

5.1. 3-METHYLHISTIDINE CONTENT OF ACTIN SEPARATED ELECTROPHORETICALLY.

5.1.1. INTRODUCTION.

Houston (1971) reported on the amino acid analysis of stained protein bands from polyacrylamide gels. Stein et al. (1974) developed the method to determine the complete amino acid composition using fluorescamine, of as little as 1μg of protein after separation by SDS PAGE (section 2.2.2). Based on these reports, investigations into the determination of the 3-methylhistidine content of stained actin bands from SDS PAGE gels were carried out, with a view to using actin-bound 3-methylhistidine as an index of meat protein.

5.1.2. MATERIALS AND METHODS.

To investigate the possibility of determining the 3-methylhistidine content of stained actin bands from SDS PAGE gels, those prepared to determine the purity of actin preparations (sections 4.2.1.3 and 4.2.2) were initially used. From such gels the actin band (containing approximately 40μg protein) was excised, along with control samples consisting of similarly sized portions of gel containing no protein and stained bands of protein from standard molecular weight markers (eg. BSA) containing no 3-methylhistidine. Freeze-drying was carried out in 2ml glass ampoules (BDH), to avoid diluting the acid which was to be used for hydrolysis. A second set of samples was prepared

similarly, each containing 0.15 μg added 3-methylhistidine (Sigma). To the freeze-dried contents of each ampoule was added 0.5ml 6M HCl, 1% with respect to thioglycollic acid (Sigma). The thioglycollic acid was included as it was known to prevent the destruction of histidine during hydrolysis (Houston, 1971), and could therefore be expected to help maintain the integrity of the 3-methylhistidine in the harsh hydrolysis conditions. The ampoules were flame-sealed and oven hydrolysis was carried out at 110°C for 16h. During hydrolysis the gel dissolves, but on cooling a loose gel structure reforms. The gel piece was removed after rinsing with 0.2ml 6M HCl, the hydrolysate being concentrated by freeze-drying. The residue was dissolved in 125μl 6M HCl, 100μl of this solution being taken for 3-methylhistidine determination by the standard method (section 3.2.2).

A method of determining the actin-bound 3-methylhistidine content of meat by the analysis of the actin band of myofibrillar proteins separated by SDS PAGE was then developed.

5.1.2.1. SAMPLE PREPARATION.

Standard actin samples were prepared by mixing bovine L.dorsi actin solution (section 4.2.1.1) with an equal volume of electrophoresis sample buffer (section 4.2.1.3) to a final actin concentration of 1 mg/ml. The sample was heated at 70°C for 1h in a water bath. Samples of bovine L.dorsi acetone powder (section 3.2.2.1) at a concentration of 5 mg/ml were incubated

similarly in 14ml McCartney bottles (Gallenkamp), after vortexing for 1min. During the incubation occasional shaking was perfomed. The insoluble connective tissue (Jonker, van Roon and den Hartog, 1987) was pelleted by centrifuging at approximately 5000g (5000 rpm: MSE Multex bench centrifuge) in 12ml Pyrex centrifuge tubes (Fisons), sealed with Nescofilm (Nippon Shoji Kaisha Ltd) for 10 min at room temperature. The supernatant was taken for electrophoresis.

5.1.2.2. SDS PAGE GEL PREPARATION AND RUNNING.

A Shandon disc gel apparatus (Shandon Scientific Company Ltd, London, UK) was used in which eight 0.5cm X 7.5cm rod gels could be run at once. The apparatus was set up according to the manufacturer's instuctions. The 7.5% gel mixture was prepared by the method of Pollard (1982) (section 4.2.1.3) and cast into the glass cylinders provided. A few drops of water were layered onto the unpolymerised gel to ensure that on polymerisation, a flat meniscus was obtained.

For the preparation of the calibration curve 20-80 μl of actin standard was loaded onto the gel rods. For the muscle protein sample 70μl was loaded, the latter being the maximum loading possible for good resolution without blocking the gel pores. Molecular weight markers were also used as in section 4.2.1.3. The gels were run for approximately 45 min at a maximum of 200V and a maximum of 50mA, using a Pharmacia EPS 500/400 electrpophoresis power supply, until the dye front had reached the bottom of the gel. After removal from the glass cylinders (by rimming if necessary; Hames, 1981), the gel rods

were stained and destained as in section 4.2.1.3.

5.1.2.3. HYDROLYSIS.

The actin bands were identified by their mobility compared with the molecular weight markers and purified actin standard. The actin bands were sliced from the gel with a scalpel. Duplicate bands were freeze-dried in 14ml McCartney bottles (Gallenkamp), after which was added 2ml 6M HCl, 1% with respect to thioglycollic acid (Sigma). The bottles were sealed with polypropylene caps (Gallenkamp) and oven hydrolysis was carried out at 110°C for 16h. On cooling a loose gel structure reforms and a known volume of hydrolysate (the maximum possible) was transferred to a 150mm X 15mm pyrex test tube, and freeze-dried. It was reasonable to assume that there was no preferential binding of 3-methylhistidine to the gel (Stein *et al.*, 1974).

5.1.2.4. FLUORESCAMINE DERIVATISATION AND 3-METHYLHISTIDINE/ACTIN QUANTIFICATION.

3-Methylhistidine determination was carried out by the standard method (section 3.2.2), with minor modifications. The freeze-dried hydrolysate was derivatised without neutralisation. Prior to separation by HPLC, the derivatised sample was cooled to 4°C for 30min to allow the complete precipitation of the insoluble material present, the sample then being centrifuged at approximately 5000g (5000*rpm*: MSE Multex bench centrifuge) in 12ml pyrex centrifuge tubes (Fisons), for 10min at room temperature. The clear supernatant was used for

analysis.

From the equivalent weight of actin loaded onto the gel, and the 3-methylhistidine concentration of the gel hydrolysate, a calibration graph of G-actin loaded (μg) against 3-methylhistidine detected (μg) can be plotted. From this graph the 3-methylhistidine in actin bands from muscle acetone powder samples can be converted to actin, giving a measure of the actin and, therefore, of the myofibrillar protein content of the sample.

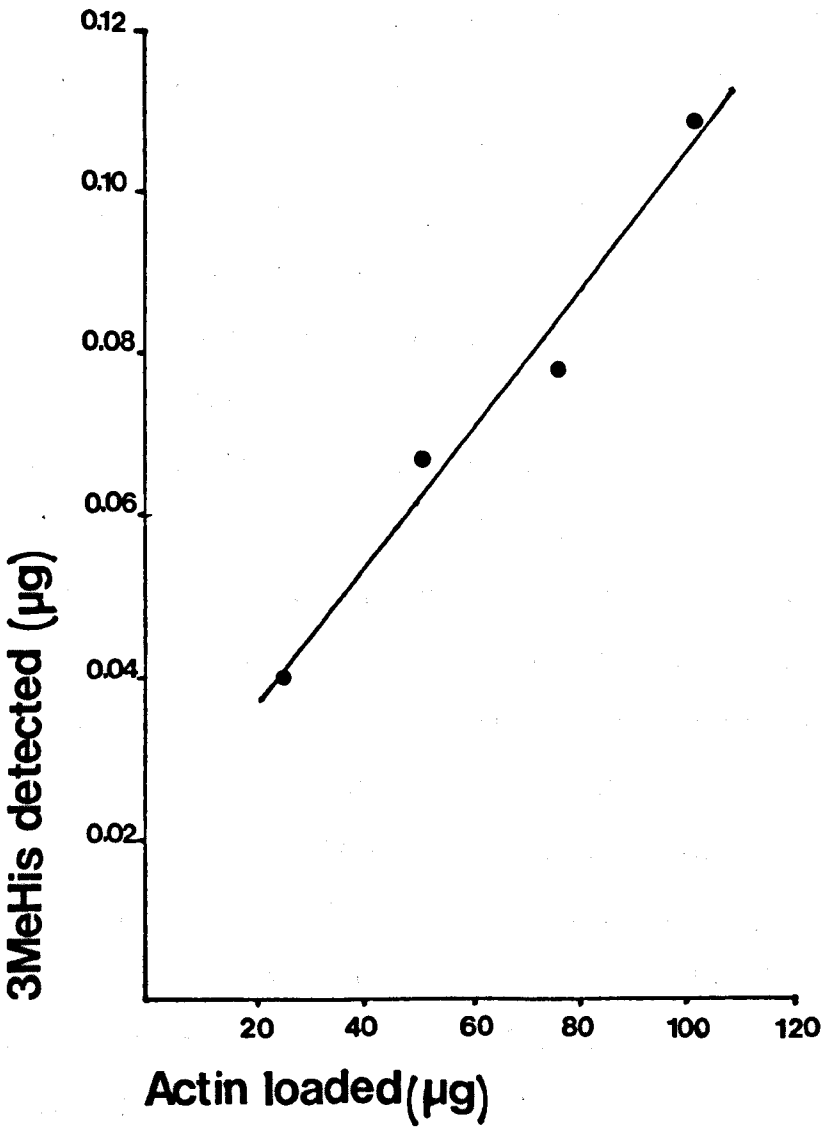
5.1.3. RESULTS AND DISCUSSION.

The initial investigation to determine if it was possible to detect 3-methylhistidine in actin bands excised from SDS PAGE gels showed that on HPLC of the hydrolysate, 3-methylhistidine could be detected as a small peak, which in samples with added standard amino acid, increased in size, confirming its identity. The use of SDS PAGE to separate myofibrillar proteins prior to the 3-methylhistidine analysis of the actin band was thus shown to be plausible and worthy of further investigation.

Disc gel in preference to slab gel as the mode of electrophoresis, was chosen for ease and speed of gel preparation, economical use of reagents, speed of separation, and high sample loading, all characteristics required if a method is to be developed for routine use.

Figure 5.1 shows the calibration curve for the 3-methylhistidine titres of the purified actin detected after electrophoresis, expressed as μg 3-methylhistidine detected

Figure 5.1. Calibration plot for the determination of actin separated by electrophoresis.



against μg actin loaded (means of duplicate analyses). Taking the molecular weight of 3-methylhistidine as 169.2 (Sigma) and assuming that of actin to be 41785 (Elzinga et al., 1973) the average 3-methylhistidine titre of the actin was only 0.34 moles/mole compared with a value of approximately 0.8 moles/mole determined prior to electrophoresis (section 4.2.2). This deficiency was most likely to be due to a loss of actin during electrophoresis and inaccuracies in the 3-methylhistidine determination due to chemical reaction during hydrolysis in the presence of polyacrylamide; and to difficulty in quantification in the presence of contaminating fluorescence peaks. The data suggested that the 3-methylhistidine titre determined for the actin band of bovine L.dorsi cannot be converted directly to actin by assuming the previously determined 0.8 moles 3-methylhistidine / mole actin. The use of a calibration curve using known amounts of standard actin is necessary to correct for losses of the protein and of 3-methylhistidine between loading the sample onto the gel and the fluorometric detection of the amino acid.

Using the calibration curve in figure 5.1, the actin titre determined for bovine L.dorsi from the 3-methylhistidine content of its actin band is 0.099 (±0.043) μg actin/μg acetone powder (mean of four determinations standard deviation). By conversion using the appropriate acetone powder ratio (appendix II), an actin content of 19.36 (±8.18) mg actin/g fresh sample was obtained. The actin content as determined by this method is similar to those previously reported

(Lawrie, 1985). The high standard deviation is a reflection of the difficulties in accurately quantifying the low levels of 3-methylhistidine present.

The use of alternative SDS PAGE methods such as that of Shapiro, Viñuela and Maizel (1967) and the use of urea and agarose gels was investigated, with similar results, indicating that the method was only semi-quantitative and in its present form, little better than more traditional methods of quantifying electrophoretic bands, such as laser densitometry (section 2.4.2). With this in mind no futher development of this procedure was carried out.

5.2. SEPARATION OF MYOFIBRILLAR PROTEINS BY CONVENTIONAL GEL FILTRATION, PRIOR TO DETERMINATION OF ACTIN-BOUND 3-METHYLHISTIDINE.

5.2.1. INTRODUCTION.

Actin was suggested as the target protein for the estimation of meat content of food products by den Hartog (1980), after numerous references to the use of the stained actin band in SDS PAGE gels for the semi-quantitative estimation of meat in products containing meat / vegetable protein mixtures (Parsons and Lawrie, 1972; Lee et al., 1975, 1976). A method was developed by Jonker, den Hartog and van Roon (1982) to measure actin-bound 3-methylhistidine after isolation of actin by SDS gel filtration. This method was then applied to a smoked, pasteurised meat product, "Guelders ring sausage", by Jonker, van Roon and Den Hartog (1985), who, although successfully separating the myosin heavy-chain (the location of the 3-methylhistidine residue in myosin; Kuehl and Adelstein, 1970) from actin, obtained only low yields of the expected amino acid in the pooled actin fractions, when determined by conventional amino acid analysis. The use of large columns, and slow flow rates, meant that separation was time-consuming, necessitating the collection of large numbers of fractions.

Against this background, the possibility of coupling gel filtration, based on the method of Jonker, den Hartog and van Roon (1982), with the standard fluorescamine / HPLC method of 3-methylhistidine determination (section 3.2.2.) was investigated. The sensitivity of the 3-methylhistidine assay was such that only small amounts of sample needed to be separated

allowing the possibility of shorter columns, with the collection of fewer fractions.

5.2.2. MATERIALS AND METHODS.

The column used throughout these investigations was a Pharmacia K9/60 (60cm X 0.9cm, bed volume 38ml). This was packed with Sephacryl S-400 (Pharmacia), according to the manufacturer's instructions. All solutions were degassed prior to use and deionised water was used throughout. Control of flow rate was accomplished using an Eyela micro-tube pump MP-3, and fractions were collected using a Gilson model 203 fraction collector (Gilson Medical Electronics Inc. Wisconsin, USA).

Samples were prepared by stirring acetone powders of whole muscles (prepared as in section 3.2.2.1) at a concentration of 5mg/ml, with gel filtration sample buffer, consisting of 8M urea / 3% SDS (Primar grade; Fisons) / 2% 2-mercaptoethanol (Sigma) / 0.1M ammonium acetate pH 6.5, for 2h at 60°C. This was followed by centrifugation at approximately 5000g (5000rpm, MSE Multex bench centrifuge) in 12ml Pyrex centrifuge tubes (Fisons). The clear supernatant was taken for chromatography. The eluent was 0.1M ammonium acetate pH 6.5 / 0.1% SDS (Primar grade; Fisons). Sample volume was 0.25-0.50ml, loading being manually via a short piece of tubing from the top of the column. Flow rate was maintained at 3ml / cm^2 / hr, near to the manufacturers recommended optimum (Pharmacia, 1985-6). 35 X 1ml fractions were collected from each separation.

Initially calibration of the column was carried out using bovine L.dorsi acetone powder, to determine the elution volume of the actin and myosin containing fractions, and to ensure that complete separation of myosin heavy-chain and actin had been accomplished. The protein composititon of the resulting fractions of the calibration was determined by SDS PAGE electrophoresis (section 4.2.1.2).

On the basis of the calibration, samples of muscle acetone powders could be separated with the aim of determining their actin-bound and myosin-bound 3-methylhistidine contents.

Bovine L.dorsi, Masseter and Malaris and Semimembranosus were chromatographed as described. The myosin and actin containing fractions (as determined by the calibration run) were separately pooled, rinsing the tubes with distilled water to reduce losses. The protein composition of the fractions (i) immediately prior to the myosin heavy-chain fractions, (ii) between the myosin and actin fractions, and (iii) immediately after the actin fraction, were determined by SDS PAGE electrophoresis (section 4.2.1.2), to ensure that the total myosin heavy-chain, and actin had been pooled. The pooled fractions were freeze-dried in 14ml McCartney bottles (Gallenkamp), then oven hydrolysed in 1ml 6M HCl / bottle (sealed with polypropylene caps ; Gallenkamp) for 16h at 110 C. The myosin heavy chain and the actin hydrolysates were each washed into single 24mm X 200mm Pyrex boiling tubes (Fisons), using distilled water, then freeze-dried. 0.2ml Distilled water was added to each tube, and the 3-methylhistidine content of the samples was determined by the

standard method (section 3.2.2), modified, in so far as neutralisation prior to fluorescamine derivatisation was not necessary. The results were expressed as actin-bound and myosin-bound 3-methylhistidine in units of μg/g ff,cf,fsam using compositional data previously determined (section 3.2.2.6, and appendix I and III).

The percentage of the total protein-bound 3-methylhistidine solubilised by the gel filtration sample buffer was determined by dialysing 1ml aliquots of the sample prepared from bovine L.dosi acetone powder, against distilled water, in 18/32" Visking tubing which had previously been boiled in distilled water for 20min. Dialysis was for 24hr at room temperature, with frequent changes of water. The removal of urea from the sample by dialysis was required as urea interferes with the fluorescent determination of 3-methylhistidine. The urea in the samples subjected to gel filtration is not a problem as the molecule's small size means that it is eluted near the total volume of the column, ie. later than myosin heavy-chain and actin. The dialysed sample was washed into a 14ml McCartney bottle (Gallenkamp) with distilled water, and freeze-dried, the residue being oven hydrolysed in 2ml 6M HCl, for 16h at 110 °C, in the bottles sealed with polypropylene caps (Gallenkamp). The 3-methylhistidine content of the hydrolysate was determined by the standard method (section 3.2.2), and the data was expressed as percentage extracted compared with the total protein-bound titres of this amino acid previously determined (section 3.3).

5.2.3. RESULTS AND DISCUSSION.

The low solubility of myofibrillar proteins required the use of SDS gel filtration. Urea was included in the sample buffer to ensure the maximum possible solubility of the proteins. Urea has been extensively used to solubilise proteins for SDS PAGE electrophoresis (Mattey, Parsons and Lawrie, 1970; Parsons and Lawrie, 1972; Armstrong, Richert and Riemann, 1982). Ammonium acetate buffer was the choice for the eluent due to its volatility, which meant that, on freeze-drying, any buffer salts present which might interfere with the 3-methylhistidine assay would be removed. The SDS in the eluent ensured that the dissolved proteins remained in solution. To minimise the chance of protein loss due to precipitation during chromatography, ideally the composition of the sample buffer and the eluent should be the same. This was not possible due to the high levels of reagents needed to solublise the sample which, if included in the eluent, would have interfered with the 3-methylhistidine assay.

Complexation of SDS with a protein increases its apparent molecular weight by a factor of approximately 2.4 (Jonker, den Hartog and van Roon, 1982). This has to be borne in mind when choosing a gel with the appropriate fractionation range.

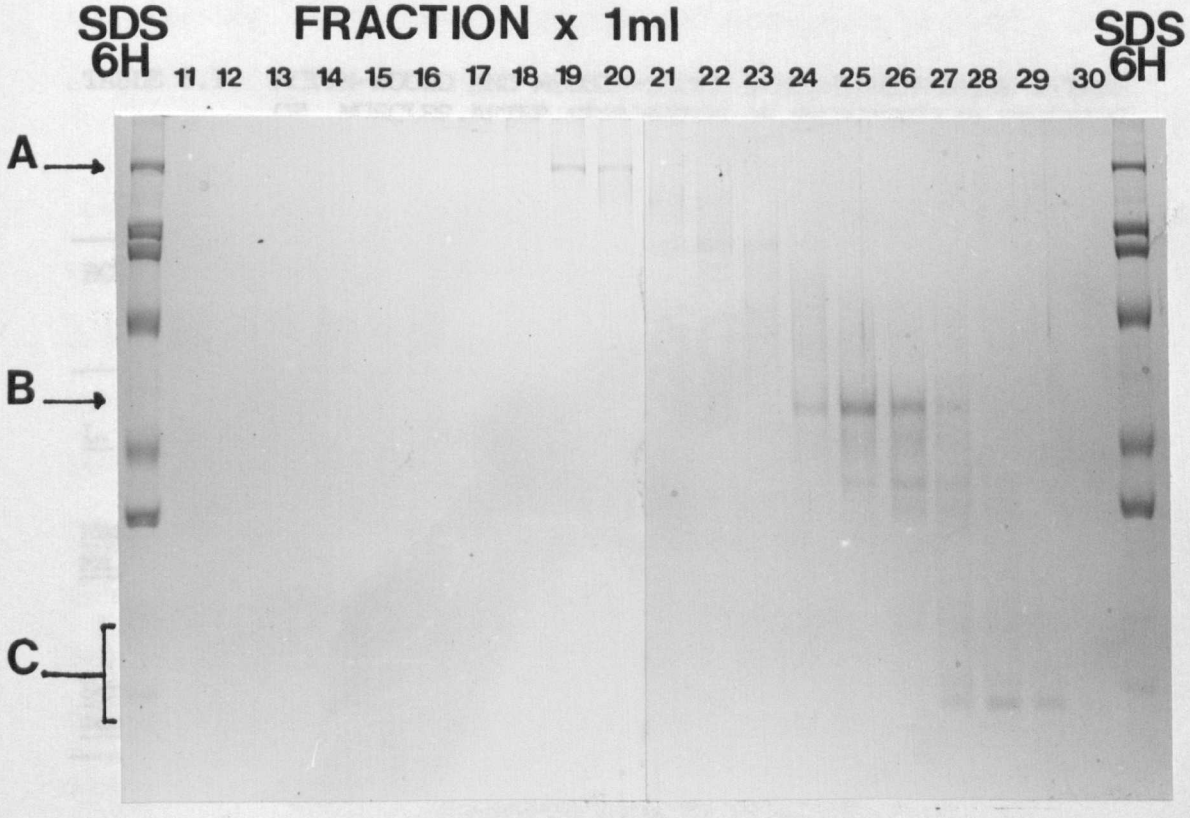
Figure 5.2 shows the electrophoretogram of the fractions from a calibration chromatographic run. The two components of interest, myosin heavy chain and actin, are completely separated, myosin heavy chain being eluted in

Figure 5.2. Electrophoretogram of fractions from a calibration chromatogram by conventional gel filtration of bovine L.dorsi muscle.

Sample band A: Myosin heavy chain.

B: Actin.

c: Myosin light chains.



fractions (18), 19, 20, (21), actin in fractions (23), 24, 25, 26, 27, (28). From these findings the volumes in which the total myosin heavy chain and total actin are eluted can be predicted. In chromatographic runs used for 3-methylhistidine determination, myosin heavy chain and actin were always absent from the electrophoretograms of the fractions adjacent to the pooled fractions, indicating that all of the proteins of interest had been pooled.

Actin-bound and myosin-bound 3-methylhistidine titres obtained by the method described are given in table 5.1.

TABLE 5.1. ACTIN-BOUND AND MYOSIN-BOUND 3-METHYLHISTIDINE TITRES OF MUSCLES AFTER SEPARATION OF MYOFIBRILLAR PROTEINS BY CONVENTIONAL GEL FILTRATION.

BOVINE MUSCLE	3-METHYLHISTIDINE TITRE (μg/g ff,cf,fsam)*			
	Actin-bound	Myosin-bound	Total	Yield (%)**
L. dorsi	73.39	14.34	87.73	58
Masseter and Malaris	28.32	3.07	31.39	42
Semi-membranosus	54.63	8.55	62.85	49

* L.dorsi and Masseter and Malaris; mean of 2 determinations, Semimembranosus; mean of three determinations.
** Compared with total protein-bound titre (section 3.3).

These results show the far greater contribution to the total protein-bound 3-methylhistidine titre, by the actin-bound titre; but the low yields, compared with those expected, and the high variability of the data meant that no conclusions about the extent of variation in the actin-bound titres between the different muscles could be drawn.

The percentage of the total protein-bound 3-methylhistidine extracted by the gel filtration sample buffer was found to be 77.39 % (mean of 3 determinations), indicating that the low yields of the amino acid detected were not solely due to incomplete extraction. Other losses could have occurred due to precipitation and binding on the column, during transferring and freeze-drying of samples, as well as interference and quenching of the 3-methylhistidine assay.

The results obtained indicated that the use of conventional gel filtration had serious drawbacks for the determination of actin-bound 3-methylhistidine in meat. Disadvantages of this method were: low recovery of total protein-bound 3-methylhistidine, the long separation times (approximately 16h), and the large numbers of fractions which had to be laboriously pooled and concentrated prior to 3-methylhistidine determination. A faster, more efficient separation technique was required. As described in the following section, this proved to be fast protein liquid chromatography (FPLC) - gel filtration.

5.3 SEPARATION OF MYOFIBRILLAR PROTEINS BY FAST PROTEIN LIQUID CHROMATOGRAPHY PRIOR TO DETERMINATION OF ACTIN-BOUND 3-METHYLHISTIDINE.

5.3.1 INTRODUCTION.

Although separation of myosin heavy chain and actin could be successfully accomplished by conventional gel filtration (section 5.2), accurate quantification of the resulting actin-bound 3-methylhistidine was not possible.

The use of the Pharmacia fast protein liquid chromatography (FPLC©) system was investigated in view of the manufacterer's claims of "fast, high resolution separation and maximum recovery of proteins" (Pharmacia, 1986), along with the possibility of using a highly automated system. The gel filtration medium recommended was Superose 6HR, consisting of highly cross-linked agarose beads suitable for use in extreme conditions, being stable at relatively high back pressures, and in the presence of high concentrations of SDS (Pharmacia, 1987).

The development of a method of determining actin-bound 3-methylhistidine after separation of the myofibrillar proteins by FPLC-gel filtration was investigated. Both raw and heat-treated muscle were analysed, in view of the fact that, although it was known that total protein-bound 3-methylhistidine titres are robust to processing temperatures (Hibbert and Lawrie, 1972), it was uncertain whether or not actin <u>per se</u> was.

5.3.2 MATERIALS AND METHODS.

5.3.2.1 GENERAL PROCEDURE FOR DETERMINATION OF ACTIN-BOUND 3-METHYLHISTIDINE AFTER SEPARATION OF MYOFIBRILLAR PROTEINS BY FPLC-GEL FILTRATION.

(a) FPLC-GEL FILTRATION APPARATUS- FPLC-gel filtration was carried out using the Pharmacia FPLC system comprising the following: P500 pump, V-7 injection valve (with 200μl sample loop), UV-1 flow through UV detector, FRAC-100 fraction collector, REC-482 chart recorder, and a GP-250 control unit. The high resolution prepacked gel filtration column used was a Superose 6HR 10/30 (Pharmacia) (10mm internal diameter X 30cm length). A photograph of the apparatus used can be seen in figure 5.3.

(b) SAMPLE PREPARATION- Distilled deionised water was used throughout this investigation. Actin and myosin were prepared from bovine L.dorsi as previously described (sections 4.2.1.1 and 4.3.1.1). Whole muscle acetone powders were prepared as in section 3.2.2.1. The samples were dissolved by rapid stirring for 1h at 20°C in the FPLC sample buffer consisting of 2% SDS (Primar grade; Fisons) / 2% 2-mercaptoethanol (Sigma) / 0.1M ammonium acetate pH 5.0. Muscle acetone powders were prepared at a concentration of 20mg/ml, purified actin and myosin at 0.5 mg/ml. The samples were then centrifuged at approximately 5000g (5000rpm, MSE Multex bench centrifuge) in 12ml Pyrex centrifuge tubes (Fisons), for 10min at room

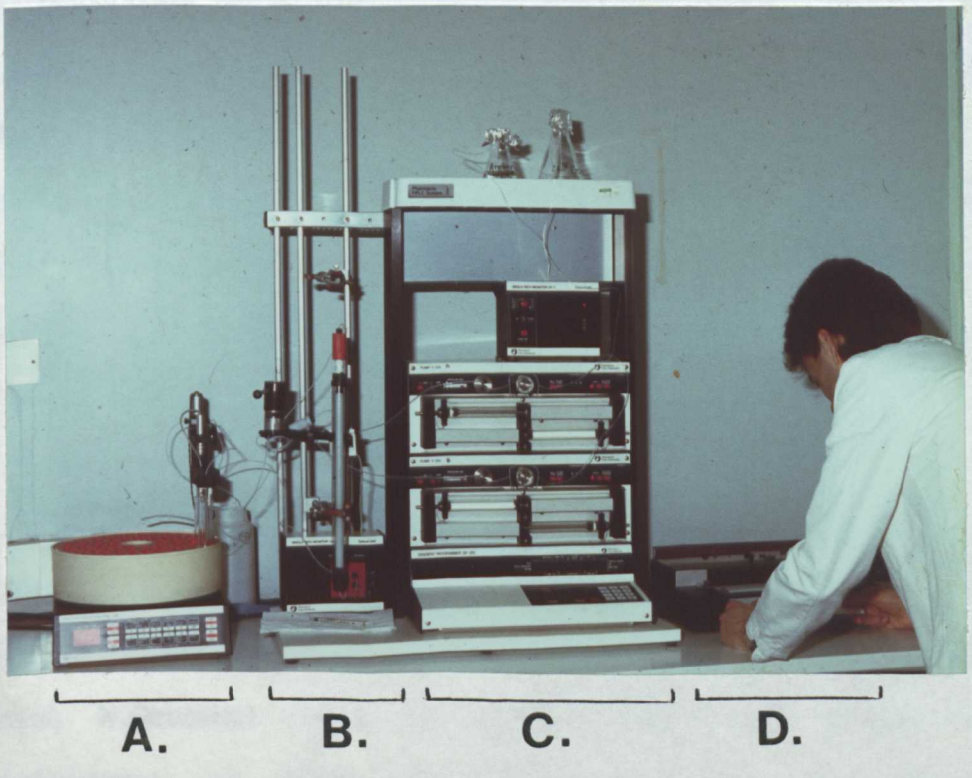
Figure 5.3. Pharmacia fast protein liquid chromatography (FPLC) apparatus.

A: Frac-100 fraction collector.

B: V-7 injection valve, Superose 6HR 10/30 column, UV-1 detector.

C: P-500 pumps, GP-250 control unit.

D: REC-482 chart recorder.



temperature. The clear supernatant was taken for chromatography.

(c) GEL FILTRATION CONDITIONS- FPLC was carried out at 20°C with an eluent consisting of 0.1M ammonium acetate pH 5.0 / 2% SDS (Primar grade; Fisons) filtered and degassed using a 0.22μm Millipore filter (Molsheim, France). Prior to chromatography the prepared sample was re-centrifuged at approximately 15000g (13000rpm, Heraeus Christ Biofuge A: Heraeus Christ GmbH, W.Germany) for 10min at room temperature, in Eppendorph vials, the supernatant being used for FPLC. A 0.2ml injection volume was used, with isocratic elution at 0.3ml/min. The fraction collector could be programmed to collect the required fraction size, the eluent being monitored by UV absorbtion at 280nm. The protein composition of the resulting fractions was determined by gradient SDS PAGE electrophoresis (section 4.3.1.3).

(d) CONCENTRATING THE FRACTIONS- Individual fractions from the FPLC could be concentrated using Centrisart I, "centrifugal filtration" tubes (cut off 10000 daltons) (Sartorius GmbH, W.Germany) spun at 2000g (3400rpm, MSE Multex bench centrifuge) at 20°C, for a total of 90min. This gave an approximately four fold increase in the protein concentration of the fraction.

(e) HYDROLYSIS- The fractions were hydrolysed by adding an equal volume of 1.18SG (12M) HCl, with oven hydrolysis

for 16h at 110°C in 14ml or 7.5ml McCartney bottles with polypropylene caps (Gallenkamp).

(f) 3-METHYLHISTIDINE DETERMINATION- 3-Methylhistidine determination was carried out by the standard method (section 3.2.2), with minor modifications to compensate for the low levels of 3-methylhistidine being detected. It proved necessary to increase the sample loop size on the HPLC apparatus used for 3-methylhistidine determination, from 0.02ml to 0.1ml.

5.3.2.2 APPLICATION OF FPLC TO DETERMINATION OF ACTIN-BOUND 3-METHYLHISTIDINE IN MEAT.

Initially samples of purified actin and myosin were chromatographed by FPLC-gel filtration to determine the elution characteristics of these proteins. 15 X 2.0ml fractions were collected, and their protein composition determined by gradient SDS PAGE electrophoresis.

To determine how effectively myosin heavy chain and actin were separated, a sample of bovine L.dorsi acetone powder was chromatographed with the collection of 30 X 1.0ml fractions, their composition being determined by SDS PAGE electrophoresis.

From the results of the preliminary investigations the volumes in which the total myosin heavy chain and total actin were eluted could be determined, and the fraction collector programmed to collect all of the myosin heavy chain and all of the actin in two separate fractions.

Bovine L.dorsi, Masseter and Malaris and Semimembranosus acetone powders were chromatographed, with the

collection of 6 X 4.25ml fractions, the composition of which were determined by gradient SDS PAGE. The fraction containing the total actin was concentrated by centrifugal filtration, hydrolysed and the 3-methylhistidine content determined.

The method was applied to a heat treated sample of bovine L.dorsi, which was sterilised at 121 C for 5min as follows: the muscle was obtained from a local slaughter house, trimmed free of visible fat and connective tissue, then minced twice through a 2.5mm plate using a domestic mincer. 125g of meat and 50g added water (to ensure adequate heat transfer) were thoroughly mixed and sealed in 307mm X 204mm cans (Metal Box Ltd, UK). Control cans were made by inserting a thermocouple into the centre of the sample through the side of the can, sealing the point of entry with Araldite "Rapid" epoxy resin (Ciba-Geigy Plastics, Cambridge, UK). The thermocouple was connected to an electronic thermometer (Comark, UK), so that the actual internal temperature of the sample could be monitored. Sterilisation was by the use of a Millwall static retort (J. Frazer and son Ltd, London) at 121°C internal temperature for 5mins. After cooling at room temperature the content of each can was thoroughly mixed and samples were taken for whole muscle acetone powder preparation (section 3.2.2.1) and compositional analysis (Appendix I) (Hydroxyproline values were those previously determined on unheated samples: section 3.2.2.6, since the severe heat treatment resulted in a loss of the amino acid, most probably on acetone powder preparation). The acetone powders were subjected to FPLC-gel filtration and the actin-bound 3-methylhistidine content determined as for unheated samples.

The actin-bound 3-methylhistidine values determined for unheated and heat treated samples were expressed in terms of actin-bound 3-methylhistidine with respect to "non connective tissue nitrogen" (mg/g nc N) and with respect to "fat-free, connective tissue-free fresh sample" (μg/g ff,cf,fsam). The results were also expressed as actin with respect to fresh sample (mg/g fresh sample), with respect to "fat-free, connective tissue-free fresh sample" (mg/g ff,cf,fsam) and with respect to "non-connective tissue nitrogen" (g/g nc N). The molecular weight of 3-methylhistidine is 169.2 (Sigma), that of actin was assumed to be 41785 (Elzinga et al., 1973). The 3-methylhistidine titres of the actins from the muscles investigated were based on the results of section 4.2., namely, 0.845, 0.763 and 0.854 moles/mole for bovine L.dorsi, Masseter and Malaris and Semimembranosus respectively.

5.3.2.3 THERMAL STABILITY OF ACTIN.

The thermal stability of actin was investigated as follows:

A sample of bovine L.dorsi, obtained from a local slaughter house was trimmed of all visible fat and connective tissue and minced twice through a 4mm plate, using a domestic mincer. The mince was mixed with distilled water at a ratio (w/v) of 2:1, then homogenised using an Ultraturrax TP 18/10 (Janker and Kunkel GmbH & Co).

5g samples of the muscle homogenate (in duplicate) were heated at an internal temperature of 60, 80 and 100°C, in

14ml McCartney bottles (Gallenkamp) sealed with Nescofilm (Nippon Shoji Kaisha Ltd), by immersion in thermostatically controlled water baths. Internal temperature was monitored by the use of thermocouples inserted into the sample through the Nescofilm and connected to an electronic thermometer (Coman, UK). After heat treatment, the samples were cooled under a running tap, then stored at 4 C until required.

The treated samples were stirred to re-incorporate the separated water; 20mg of each sample was dissolved in 1ml electrophoresis sample buffer (section 4.2.1.3) plus 1ml distilled water by heating at 50°C for 30min , with occasional vortexing. The clear supernatant was taken for electrophoresis, which was carried out by the gradient method (Pollard, 1982) (section 4.3.1.3), with a loading of 20μl / track (equivalent to 20 g protein).

Semi-quantitative analysis of the stained myofibrillar protein bands was carried out using an LKB Ultroscan XL Enhanced Laser Densitometer (LKB Producter AB, Bromma, Sweden).

5.3.3 RESULTS AND DISCUSSION.

The separation of purified actin by FPLC-gel filtration gave a single peak of absorbance at 280nm (disregarding the peak eluting near the total volume of the column, which corresponded to low molecular weight compounds found in all samples), which was identified as actin by electrophoresis, eluting after approximately 15ml. Purified myosin gave a single major peak, identified by electrophoresis as

the heavy chain, which eluted after approximately 10.5ml. The several minor peaks present corresponded to contamination by myosin light chains and actin. The FPLC chromatograms of purified actin and myosin can be seen in figure 5.4. The large difference in the elution volume between the two peaks of interest indicated that it should be possible for myosin heavy chain and actin to be separated in a meat sample.

The electrophoretogram of 1ml fractions of eluent from the separation of a sample of bovine L.dorsi is shown in figure 5.5. It can be seen that the myosin heavy chain and actin were completely resolved. This allowed the fraction collector to be programmed to collect all of the myosin heavy chain in one fraction and all of the actin in another. Fractions of 4.25ml were found suitable, myosin heavy chain eluting in fraction 3, actin in fraction 4.

Other parameters for the FPLC separation were determined by experimentation. The sample concentration, and time and temperature of acetone powder extraction were optimised to extract the maximum possible protein as simply as possible. Jonker, van Roon and den Hartog (1987) found that buffers containing SDS and dithioerythritol, which they had previously used to dissolve the collagen free muscle proteins of meat (Jonker, van Roon and den Hartog, 1985), were far less efficient than those containing SDS and 2-mercaptoethanol; the latter was the type of sample buffer used in these investigations. Stirring for 1h at 20°C gave an extraction of 73.5% of the total protein-bound titre expected (determined by hydrolysis

Figure 5.4. FPLC chromatograms of purified actin and myosin.

A. Actin.

B. Myosin.

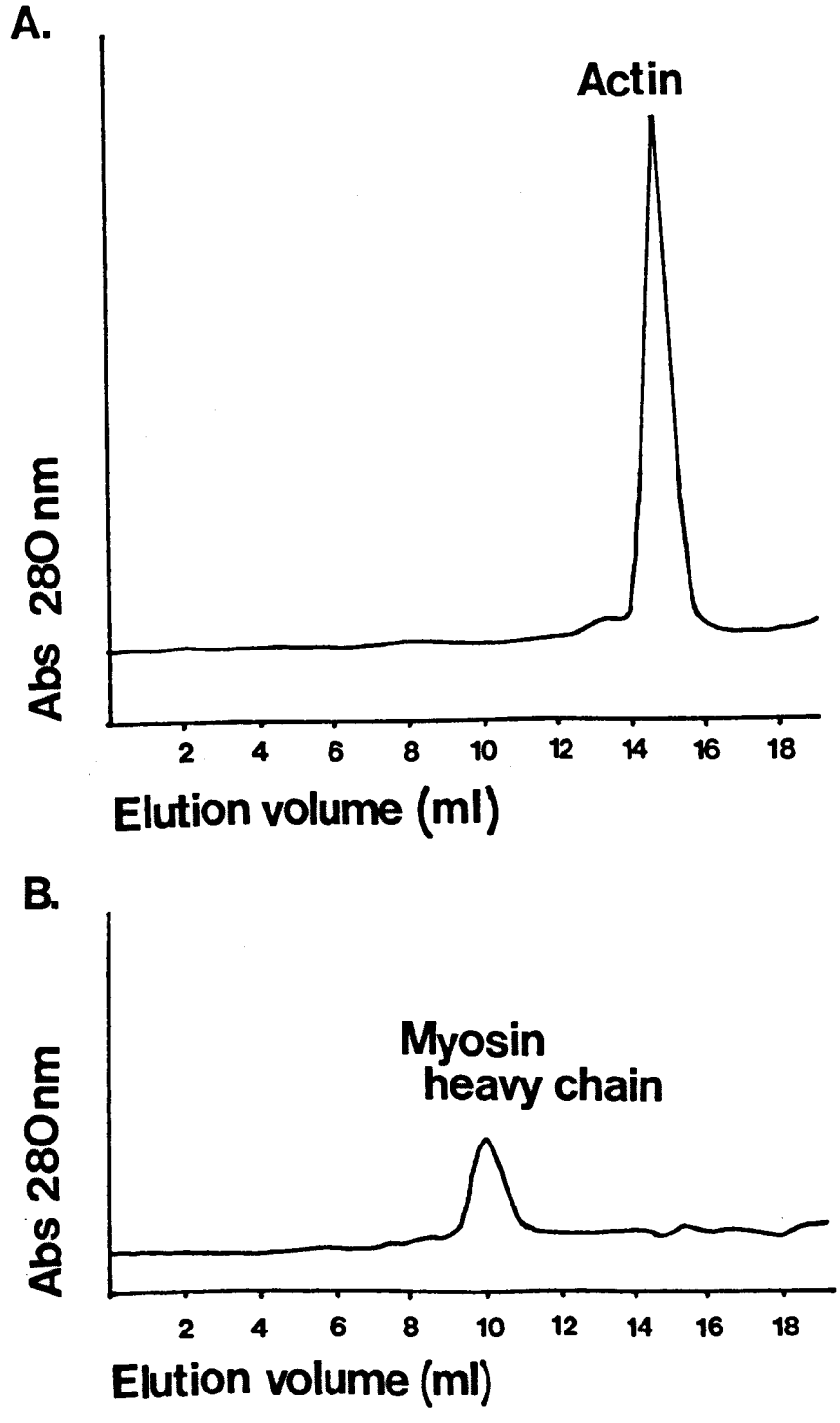
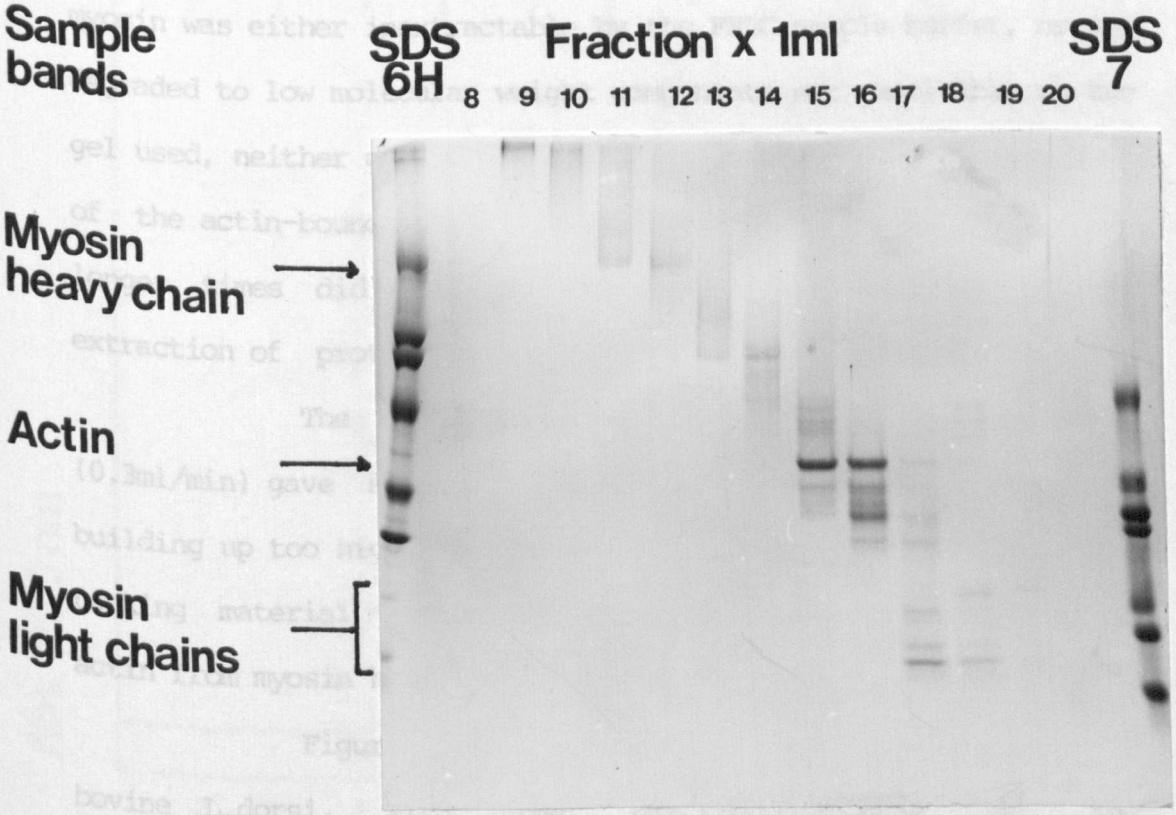


Figure 5.5. Electrophoretogram of fractions from a calibration chromatogram of bovine L.dorsi muscle, by FPLC-gel filtration.



and 3-methylhistidine analysis of the prepared sample prior to FPLC). The percentage of the actin-bound titre extracted was assumed to be far closer to the ideally required 100%, since the ratio of the intensities of the myosin heavy chain : actin bands on the electrophoretogram of the extracted acetone powder was far lower than that of acetone powder proteins before extraction. This indicated that some of the myosin was either inextractable by the FPLC sample buffer, or was degraded to low molecular weight components not resolvable on the gel used, neither of which would interfere with the determination of the actin-bound titre. Extraction at higher temperatures for longer times did not give an appreciable increase in the extraction of protein-bound 3-methylhistidine.

The flow rate used during FPLC-gel filtration (0.3ml/min) gave suitable resolution without the danger of building up too high a back-pressure so that compression of the packing material occurred. This allowed complete separation of actin from myosin heavy chain in approximately 1h.

Figure 5.6a shows the FPLC chromatogram of bovine L.dorsi, with the electrophoretogram of the corresponding 4.25ml fractions shown in figure 5.6b. The successful isolation of the two components of interest, myosin heavy chain and actin, can be clearly seen.

The sterilised meat sample gave an FPLC chromatogram, seen in figure 5.7, that was greatly different from that of raw sample (figure 5.6a), the only major peak corresponding to that of a protein of slightly lower molecular

Figure 5.6a. FPLC chromatogram of bovine L.dorsi muscle.

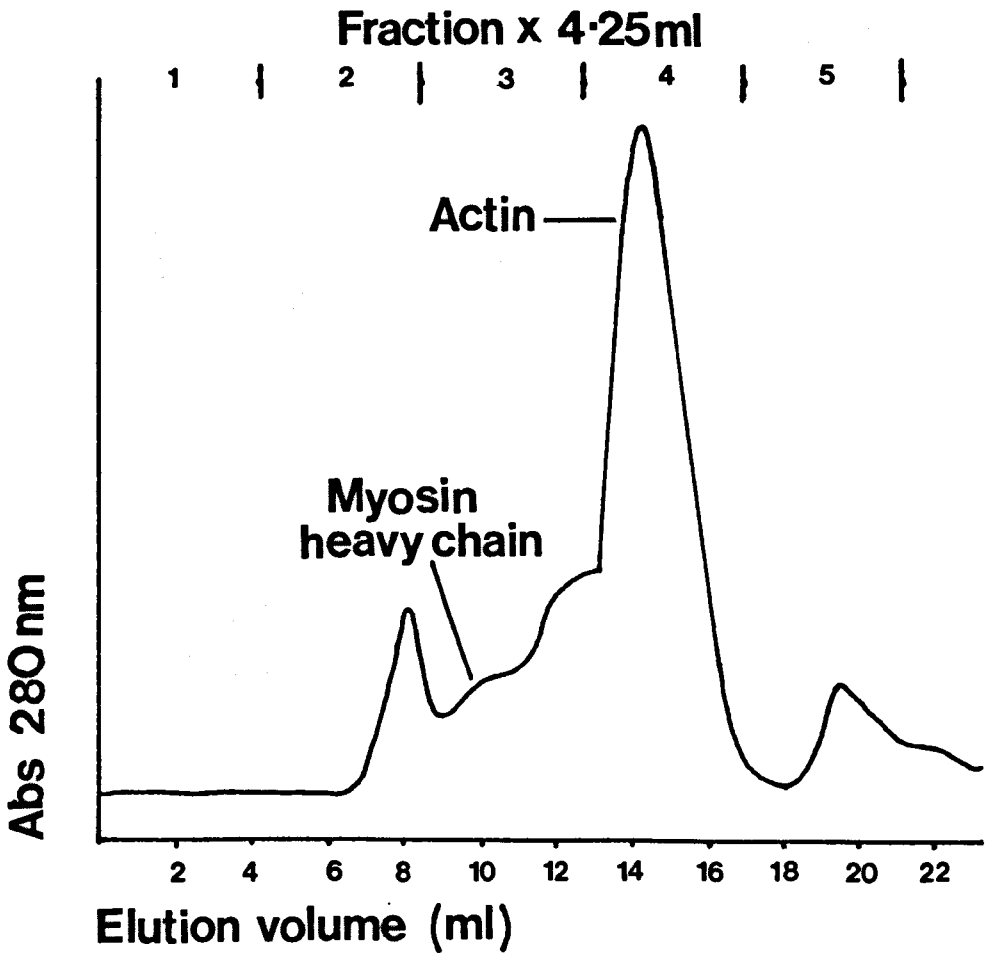


Figure 5.6b. Electrophoretogram of 4.25ml fractions from the FPLC separation of bovine L.dorsi muscle.

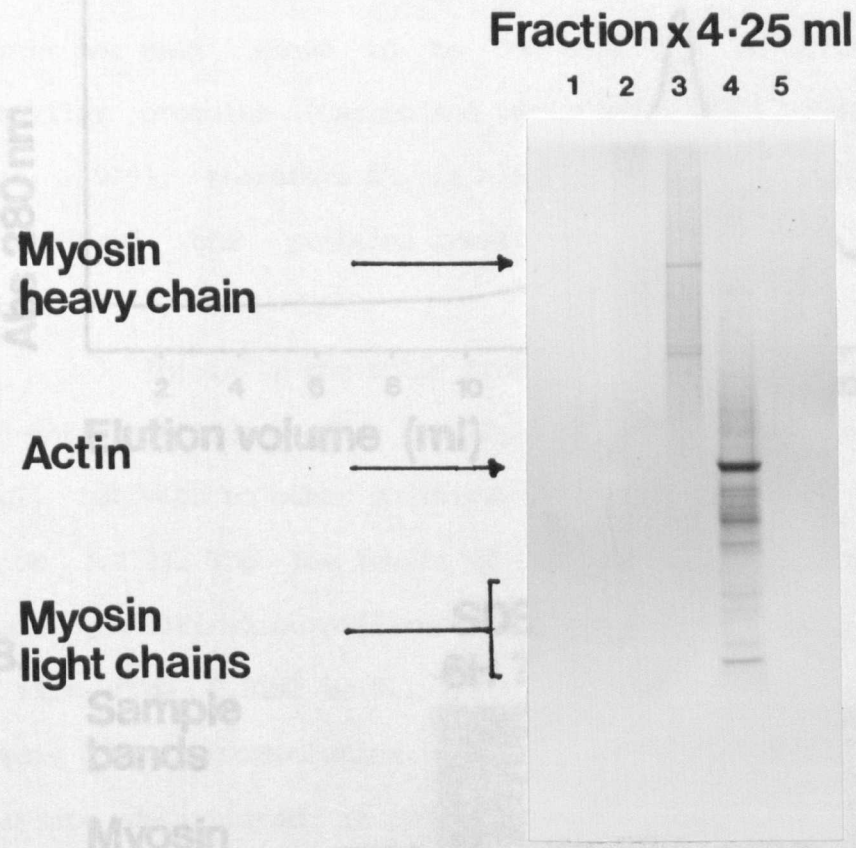
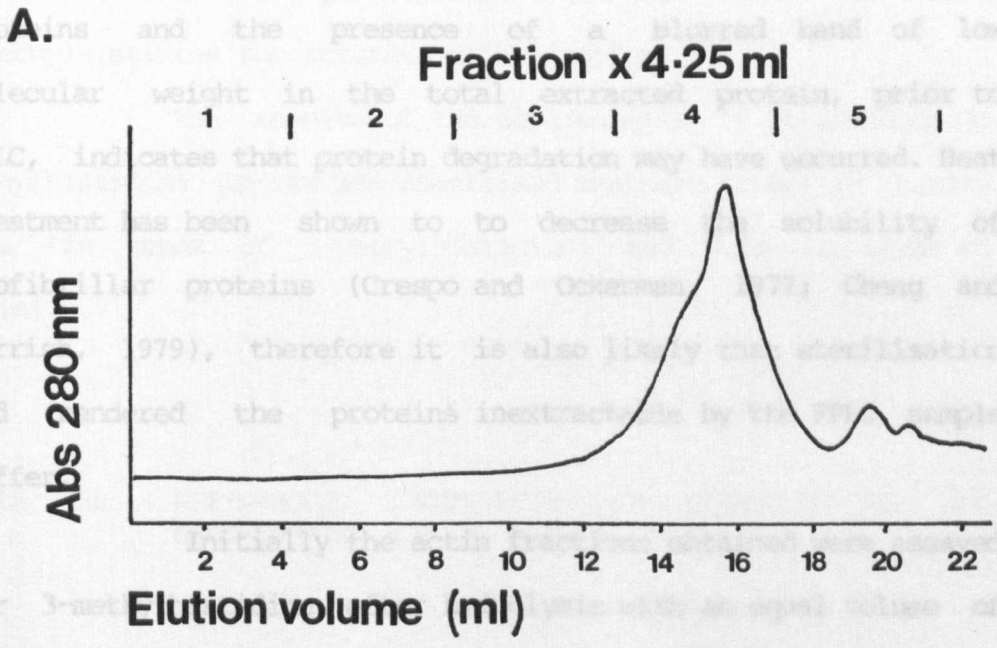
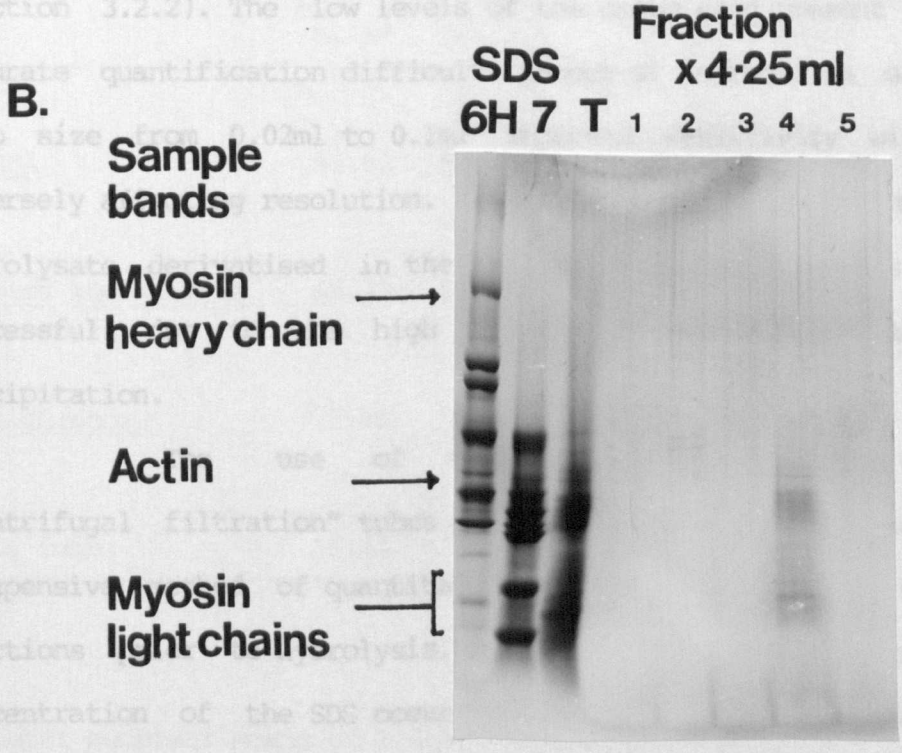


Figure 5.7. A. FPLC chromatogram of sterilised bovine L.dorsi muscle.

B. Electrophoretogram of the total extracted protein prior to FPLC (lane T), and of the corresponding 4.25ml fractions collected.





weight than actin (possibly a stable actin degredation product). The corresponding electrophoretogram in figure 5.7 confirms this. The absence of higher molecular weight proteins and the presence of a blurred band of low molecular weight in the total extracted protein, prior to FPLC, indicates that protein degradation may have occurred. Heat treatment has been shown to to decrease the solubility of myofibrillar proteins (Crespo and Ockerman, 1977; Cheng and Parrish, 1979), therefore it is also likely that sterilisation had rendered the proteins inextractable by the FPLC sample buffer.

Initially the actin fractions obtained were assayed for 3-methylhistidine after hydrolysis with an equal volume of 12M HCl, but with no other modifications to the standard method (section 3.2.2). The low levels of the amino acid present made accurate quantification difficult, though an increase in sample loop size from 0.02ml to 0.1ml improved sensitivity without adversely affecting resolution. Increasing the relative volume of hydrolysate derivatised in the fluorescamine reaction was not successful due to the high levels of SDS present, causing precipitation.

The use of Centrisart I (Sartorius GmbH) "centrifugal filtration" tubes proved to be a rapid, simple and inexpensive method of quantitatively concentrating the actin fractions prior to hydrolysis. By this method no undesirable concentration of the SDS occurs, since this molecule passes through the 10000 dalton cut off of the filter. The use of this

protein concentrating step is very important as without it, lean meat samples gave actin-bound 3-methylhistidine titres close to the limits of sensitivity of the method, therefore products containing only a few percent meat would not contain sufficient 3-methylhistidine for accurate determination.

The results of the determination of actin-bound 3-methylhistidine in raw and sterilised meat are given in tables 5.2a (in terms of 3-methylhistidine) and 5.2b (in terms of actin).

TABLE 5.2a. ACTIN-BOUND 3-METHYLHISTIDINE TITRES OF RAW AND HEAT-TREATED MUSCLE.

BOVINE MUSCLE	ACTIN-BOUND 3-METHYLHISTIDINE TITRE			
	μg/g ff,cf,fsam		mg/g nc N	
	MEAN*	SD(±)	MEAN*	SD(±)
L.dorsi	85.63	6.52	2.99	0.23
Masseter and Malaris	66.43	5.93	2.97	0.27
Semimembranosus	79.10	9.64	2.74	0.33
L.dorsi 121°C, 5min.	15.44	2.27	0.48	0.06

* L.dorsi samples: means of 4 determinations.
Masseter and Malaris and Semimembranosus: means of 5 determinations.

TABLE 5.2b. ACTIN CONTENTS OF RAW AND HEAT-TREATED MUSCLES.

BOVINE MUSCLE	ACTIN CONTENT OF MUSCLE					
	mg/g fresh sample		mg/g ff,cf,fsam		g/g nc N	
	MEAN*	SD(±)	MEAN*	SD(±)	MEAN*	SD(±)
L.dorsi	23.01	1.75	25.03	1.91	0.88	0.05
Masseter and Malaris	20.50	1.83	21.50	1.92	0.97	0.09
Semimembranosus	21.94	2.67	23.36	2.42	0.79	0.10
L.dorsi 121°C, 5min.	4.05	0.58	4.51	0.66	0.14	0.02

* Means of at least 4 determinations.

Considering the unheated samples first, table 5.2a shows the similarity between the actin-bound 3-methylhistidine titres of the muscles, particularly when expressed as mg/gnc N. This is a result of eliminating the variation caused by the low level of methylation in "red" muscle fibre myosin (section 4.4.2). On conversion to actin (table 5.2b), the content of this protein in the different muscles is also similar, agreeing with actin contents previously reported (Lawrie, 1985), and with that determined after electrophoretic separation of myofibrillar proteins (section 5.1.3). The contribution of actin-bound 3-methylhistidine to the total protein-bound titre (section 3.3) when expressed as mg/g nc N is 58.0%, 86.3% and 60.4% for L.dorsi, Masseter and Malaris and Semimembranosus respectively, the high contribution in Masseter and Malaris being a consequence of their high "red" fibre content and therefore low level of histidine methylation in the myosin. Jonker, van Roon and den Hartog (1985) estimated from molar ratios of 3-methylhistidine that, depending on the muscle type, 70-82% of this amino acid in meat should come from the actin, although the results presented here indicate far greater variation in the contribution by actin-bound 3-methylhistidine.

The results of the FPLC chromatography and electrophoresis of the sterilised samples (figure 5.7) indicated that the integrity of the actin had been lost. This was mirrored in the low actin-bound 3-methylhistidine titres, and therefore actin content of the sterilised sample (table 5.2a, b).

The investigation into the thermal stability of actin gave the electrophoretogram shown in figure 5.8. The densitometer traces of the control (no heat treatment) and the sample heated at 100°C for 10min are given in figure 5.9, the scale of the "density" axis on the two traces being equivalent. The integrated densitometer results for the myosin heavy chain and actin bands, for all of the heat-treatments, are given in table 5.3.

TABLE 5.3. DENSITOMETER RESULTS OF THE ELECTROPHORETOGRAM (figure 5.8) OF HEAT-TREATED BOVINE L.DORSI MUSCLE.

TREATMENT		PEAK AREA* (arbitary units)	
Temp (°C)	Time (min)	Actin	Myosin
Control (no treatment)		1.81	2.46
60	5	1.47	1.72
80	5	1.31	1.76
100	5	1.26	1.84
100	1	1.24	1.92
100	3	1.08	1.82
100	5	1.23	1.78
100	10	1.20	1.67

* Means of 2 determinations.

Figure 5.8. Electrophoretogram of heat-treated bovine L.dorsi muscle.

C= Control (no heat-treatment).

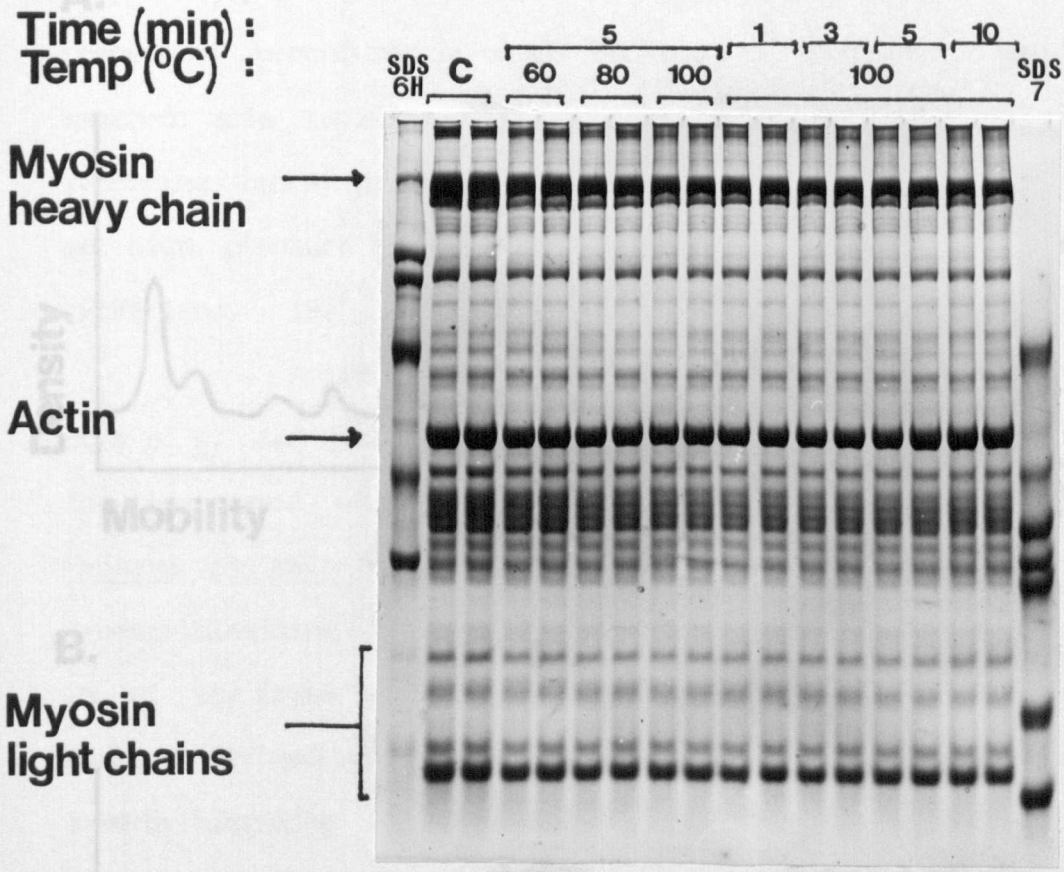
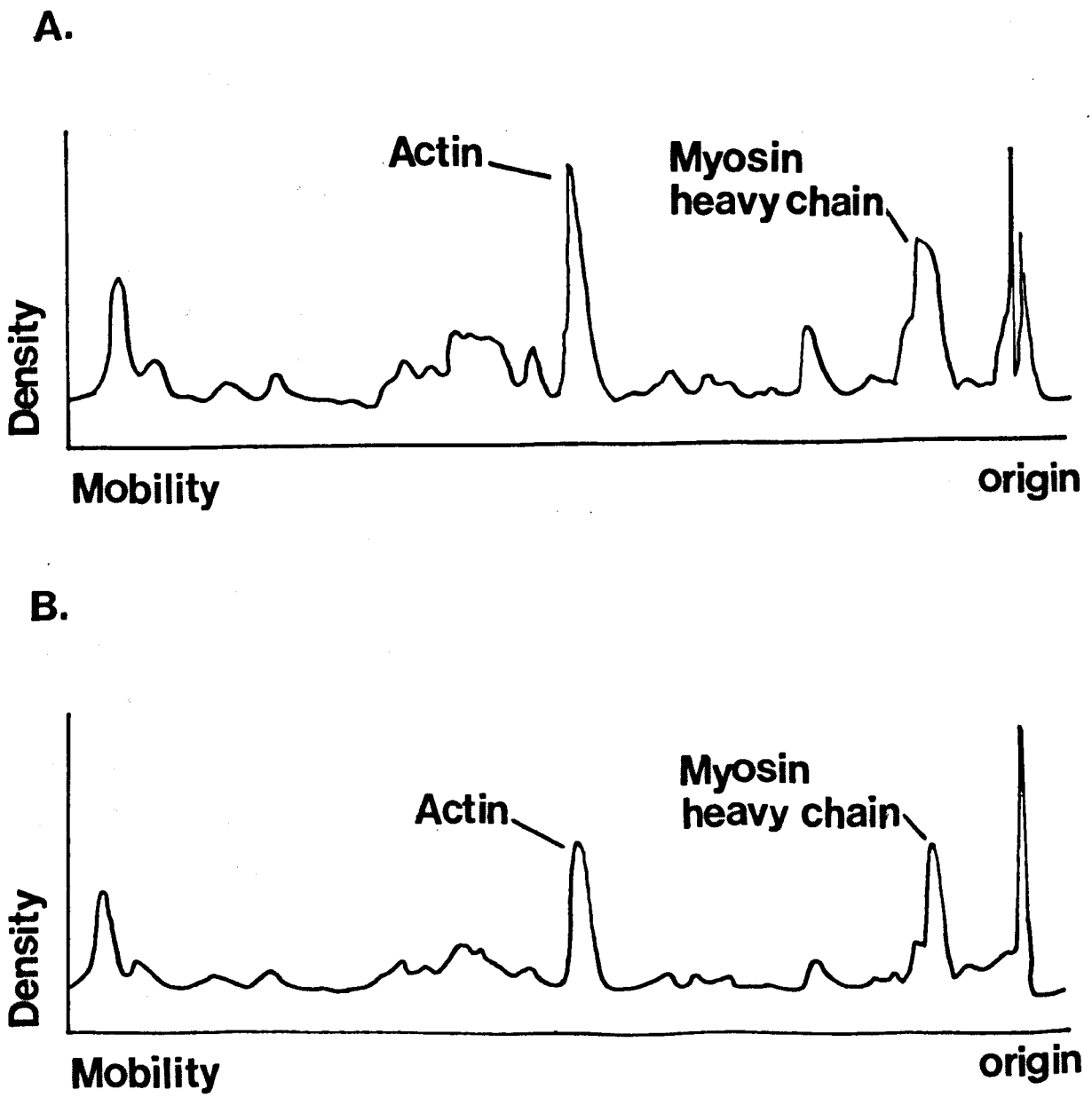


Figure 5.9. Densitometer traces of the electrophoretogram (figure 6.8) of bovine L.dorsi muscle.

A: Control (no heat-treatment).

B: 100°C for 10 minutes.



An approximately 30% reduction in the bands of interest had occurred when comparing the control (no heat-treatment) sample, with that heated at 100 C for 10min. The vast difference in the electrophoretic pattern of these samples and the sample sterilised at 121 C for 5min (figure 5.7) is surprising, though the static retort used may not mimic commercial procedures in which the required temperature may be reached more rapidly. The increased pressure developed during retorting canned products may have some bearing on the situation as high pressure has been reported to affect muscle proteins (McFarlane, 1985; McFarlane, McKensie and Turner, 1986).

Actin-bound 3-methylhistidine, when expressed as mg/g nc N, and determined by the method described, gives a titre for lean meat which is relatively constant, even for bovine Masseter and Malaris. A titre of approximately 3 mg actin-bound 3-methylhistidine / g nc N, equivalent to an actin content of 2% of the fresh sample, was found. A new index of "lean meat" could be defined as: "that which contains 3mg actin-bound 3-methylhistidine / g nc N". Assuming a reasonably constant 3-methylhistidine titre for actin, and a constant weight ratio of actin compared with the remaining myofibrillar proteins, whatever the muscle, this index is a direct measure of the actin and therefore myofibrillar protein content of a sample.

The use of an index based on actin-bound 3-methylhistidine would be particularly useful to determine the meat content of products known to contain significant levels of ruminant Masseter and Malaris or other muscles in which the "red"

fibre content is unusually high. This is important since Masseter and Malaris (cheek meat) is now classed as "part of the carcass to be regarded as meat" (Statutory Instruments, 1984, 1986), although previously classed only as offal (Food Standards Committee, 1972). By the method described, any offals not classed as "meat" by the current regulations, which contain muscular tissue (such as stomach), would make a contribution towards the "lean meat" content; on the other hand, non-muscular offals, classed as "meat" (such as liver) would not. These contradictions would need to be considered in any new regulations.

Determination of actin-bound 3-methylhistidine by the method described was found not to be applicable to heat-sterilised meat, due to thermal degradation/non-extractability of the muscle proteins, although the actin in meat heated to 100°C for up to 10min remained essentially intact, indicating that in less severely heat treated meat the method could have applications.

6. HYDROXYPROLINE AS AN INDEX OF CONNECTIVE TISSUE IN FOOD.

6.1. INTRODUCTION.

The widespread use of hydroxyproline as an index of the connective tissue content of foods (section 2.5) has a number of drawbacks. The conversion of hydroxyproline to dry connective tissue depends on the use of a factor of 7.25 (Goll, Bray and Hoekstra, 1963), which is an average figure for muscle connective tissue. Determination of hydroxyproline by the standard colorimetric method of Stegeman and Stalder (1967) (Appendix I), and by other similar methods (section 2.5.2), are prone to interference, modifications being required depending on the nature of the sample being analysed.

With the above points in mind two investigations were carried out, one to determine if there was any interspecies or intracarcass variation in the hydroxyproline content of purified collagens, which would make the use of a single conversion factor invalid. The second investigation was to develop a method of quantitatively determining hydroxyproline by gas chromatography-mass spectrometry (GC-MS), which could be applicable to all types of samples.

6.2 INTERSPECIES AND INTRACARCASS LEVELS OF HYDROXYPROLINE IN COLLAGEN.

6.2.1 MATERIALS AND METHODS.

Native intramuscular collagens from bovine, ovine and porcine L.dorsi and Masseter and Malaris were purified by the method based on that of Fujii and Murota (1982).

All experimental work was carried out at 4°C.

Muscle samples were obtained from a local slaughter-house within 12 h of slaughter, trimmed of external fat and connective tissue, and washed twice with distilled water. The muscle was homogenised using an Ultraturrax TP 18/10 (Janke & Kunkel GmbH) in 5 vols. (w/v) of 10mM tris maleate (Sigma) / 0.1M KCl pH 7.2. The homogenate was stirred rapidly for 12 h and the fibrous crude sample was collected in a fine sieve. This fibrous material was extracted in 20 vols. Hasselbach-Schneider solution (which consisted of 0.47M KCl / 0.01M pyrophosphate (Sigma) / 0.1M potassium phosphate pH 6.2), for 12 h with stirring, the fibrous material being collected in the sieve. This extraction was carried out four times in total.

The resulting material was extracted three times in 20 vol. of 0.6M KI / 0.06M sodium thiosulphate, for 12 h, with stirring. The resulting white fibrous collagen was freeze-dried then ground in a Retsch ZM-1 ultracentrifugal mill (distributed by Glen Creston Ltd), with a 0.5mm screen, and stored at room temperature in a desiccator until required.

10mg of collagen was oven hydrolysed for 16 h in 15ml 6M HCl, in 14ml McCartney bottles with polypropylene

caps (Gallenkamp), at 110°C. The hydrolysates were filtered through Whatman no.541 paper, and their hydroxyproline content was determined by the standard method of Stegemann and Stalder (1967) (Appendix I). The nitrogen content of the collagen was determined on 50mg samples by the standard Kjeldahl method (Egan, Kirk and Sawyer, 1981) (Appendix I).

The data obtained was expressed in terms of g hydroxyproline / g nitrogen. Conversion factors for hydroxyproline to dry connective tissue were calculated, assuming that nitrogen (g) x 6.25 = protein (g) (Lord and Swan, 1984). It should be noted that lower factors have been suggested (such as a factor of 5.8, which was suggested, if tentatively, by N.Harrison, the County Analyst for Leicestershire, UK).

The presence of myofibrillar protein impurities in the collagen preparations was determined using gradient SDS PAGE (Pollard, 1982) (section 4.3.1.3). Samples were prepared by dissolving 10mg collagen in 2.5ml electrophoresis sample buffer and 2.5ml distilled water, heating at 60°C for 15min with occasional vortexing. The sample was then spun at approximately 5000g (5000rpm, MSE Multex bench centrifuge) in 12ml pyrex centrifuge tubes (Fisons), to pellet the insoluble material, the clear supernatant being used for electrophoresis at a loading of 20µl / track.

6.2.2 RESULTS AND DISCUSSION.

The role of the various extraction buffers used in the collagen preparation was to extract all of the myofibrillar proteins, leaving the collagen intact and in its native state. Strong KI solution is a particularly good solvent for this purpose. The concomitant use of Hasselbach-Schneider solution (Hasselbach and Schneider, 1951) enhances the efficacy of the KI treament (Etherington and Sims, 1981).

The hydroxyproline contents of the purified collagens are given in table 6.1, along with the conversion factors for hydroxyproline (g) to dry connective tissue (g). It is clear that the hydroxyproline contents of the purified collagens are similar. The conversion factors calculated are higher than the 7.25 used routinely (Goll, Bray and Hoekstra, 1963), even if the lower nitrogen to connective tissue factor of 5.8 (Harrison, personal communication) is used. This can be partially accounted for by the myofibrillar protein impurities, devoid of hydroxyproline, which were shown to be present by electrophoresis; these consisted mainly of actin and myosin heavy chain, and the level of impurities coincided with the slight differences in the hydroxyproline titres of the collagens. An alternative approach to the isolation of collagen would be extraction with denaturants such as SDS and 2-mercaptoethanol, which should dissolve the myofibrillar proteins, but leave the collagen essentially intact (Jonker, van Roon and den Hartog, 1987), although by this approach, some solubility of collagen might be expected.

TABLE 6.1. HYDROXYPROLINE CONTENTS OF PURIFIED COLLAGENS.

SPECIES	MUSCLE	OHPR CONTENT OF COLLAGEN (g/g nitrogen) MEAN*	SD(±)	CONVERSION FACTOR OHPR(g) to DRY CONNECTIVE TISSUE(g) MEAN*	SD(±)
Bovine	L.dorsi	0.605	0.013	10.17	0.22
	Masseter and Malaris	0.600	0.012	10.45	0.21
Ovine	L.dorsi	0.638	0.028	9.93	0.44
	Masseter and Malaris	0.589	0.020	10.57	0.35
Porcine	L.dorsi	0.489	0.012	12.77	0.32
	Masseter and Malaris	0.596	0.006	10.44	0.12

* Means of 4 determinations.

It is reasonable from the results presented to use the same conversion factor for hydroxyproline to dry connective tissue for all the muscles investigated. The data indicates that errors in the calculation of connective tissue content from hydroxyproline by the use of a single conversion factor are unlikely to contribute to the low 3-methylhistidine titre of ruminant Masseter and Malaris, when expressed in connective tissue free units. It is still a possibility that false assumptions could be made by using a mean factor, when determining the connective tissue content of samples containing atypically high levels of elastin (which is low in hydroxyproline), or containing high levels of one of the genetically distinct forms of collagen lower in this amino acid.

6.3 DEVELOPMENT OF A METHOD OF DETERMINING HYDROXYPROLINE BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY.

6.3.1 MATERIALS AND METHODS.

6.3.1.1 PREPARATION OF STANDARDS AND SAMPLES FOR DERIVATISATION.

All amino acid standards were obtained from Sigma, and were dissolved to the appropriate concentration in 0.1M HCl. A "standard amino acid mixture" was made of equal weights of 3-methylhistidine, hydroxyproline, glycine, tyrosine, histidine, aspartic acid, lysine and proline, in 0.1M HCl. In hydroxyproline standards used for calibration curve preparation, the total amino acids (mg) / derivatisation was made up to that of the samples using glycine, to ensure that the ratio of amino acids to reagents was the same for calibration standards and samples.

Meat hydrolysates were prepared as in the standard method of 3-methylhistidine determination (section 3.2.2). They were used for derivatisation without any prior treatment.

6.3.1.2 DERIVATISATION METHODS.

The use of a number of derivatisation methods was investigated, based on either silylation or acylation/alkyl esterificaton.

(a) TRIMETHYLSILYLATION USING BIS(TRIMETHYLSILYL)-TRIFLUOROACETAMIDE (BSTFA)-

This procedure was based on the method of Gehrke and Leimer (1971).

0.5mg total amino acids was added to a 14 X 100mm Pyrex culture tube fitted with a PTFE lined rigid plastic cap ("silylation tube") (Fisons). The sample was first dried in a 70°C sand bath under a stream of nitrogen, then subjected to azeotropic drying, carried out by adding 0.5ml dichloromethane (dried distilled grade; Fisons), and evaporating to dryness with a stream of nitrogen. Azeotropic drying was carried out three times in total, the sample needing to be free of all traces of water, since the silylating reagents and derivatives are susceptible to hydrolysis. All evaporations were carried out under nitrogen, since the presence of oxygen gives a reduced GC-MS response. 0.25ml dry acetonitrile (dried by refluxing with calcium chloride then distilling) and 0.25ml of the silylating reagent BSTFA (Sigma) were added to the sample tube. After sealing tightly the tube was ultra-sonicated (Sonicleaner type 6441AE, Dawe Instruments Ltd, USA) for 1min, then heated in an oil bath consisting of an 8.5cm X 3cm aluminium pan filled with silicon oil (supplied by Harrow Phoenix Ltd, Sheffield, UK), at 150°C for 2.5h, temperature was controlled by a thermostatically

controlled hot-plate/stirrer (Baird & Tatlock (London) Ltd); and using a magnetic stir bar. The tubes were immersed in the oil only to the level of the reagents, so that refluxing could occur. After cooling to room temperature the sample was ready for injection into the GC-MS. All derivatised samples were stored at 4°C in screw capped glass vials, which were sealed with Nescofilm (Nippon Shoji Kaisha Ltd), to prevent the entry of moisture.

(b) TRIMETHYLSILYLATION USING BSTFA + 1% TRIMETHYLCHLOROSILANE (TMCS) (Sigma)-

This catalysed formulation is a stronger silylator than BSTFA alone and is useful for compounds which are difficult to silylate (Pierce Chemical Company, 1987).

Derivatisation was as for silylation using BSTFA exept that 0.5ml silylator and 0.5ml of acetonitrile were used to try and ensure a complete reaction.

(c) TRIMETHYLSIYLATION USING TRIMETHYLSILYL-IMIDAZOLE (TMSI) IN DRY PYRIDINE, TRI-SIL "Z" (Pierce)-

The use of TRI-SIL "Z" was investigated, as TMSI is one of the strongest hydroxyl silylators available (although it will not silylate amine groups), and in the form of TRI-SIL "Z" is not as sensitive to moisture as BSTFA (Pierce Chemical Company, 1987).

Derivatisation was as for BSTFA exept that acetonitrile was omitted and 0.5ml of TRI-SIL "Z" was used as the silylator, refluxing at 70°C for 20min in a sand bath.

(d) t-BUTYLDIMETHYLSILYLATION USING N-METHYL-N-(t-BUTYLDIMETHYLSILYL)TRIFLUOROACETAMIDE (MTBSTFA)-

This procedure was based on the method of Schwenk et al. (1984).

t-Butyldimethylsilyl derivatives are reported to be far more stable than the corresponding trimethylsilyl derivatives (Pierce Chemical Company, 1987). They are particularly suited to GC-MS as they yield a high abundance of the M-57 ion on electron impact ionisation (Schwenk et al., 1984).

Derivatisation was as for BSTFA, exept that 0.2ml acetonitrile and 0.2ml MTBSTFA (Sigma) was used, silylation being at room temperature, overnight. An added advantage of this method is that the azeotropic drying steps are not necessary for maximum GC-MS response.

(e) N(O,S)-ACYLATION / ALKYL ESTERIFICATION USING TRIFLUOROACETIC ANHYDRIDE (TFAA) AND n-PROPANOL-

This procedure was based on the method of Gamerith (1983a,b) for the preparation of N(O)-trifluoroacetyl n-propyl ester (N-TFA n-propyl ester) derivatives.

0.5mg total amino acids in a "silylation tube" was dried in a sand bath at 70°C, with a stream of nitrogen, the residue being dried once, azeotropically, with dichloromethane (dried distilled grade; Fisons). 0.3ml of esterification reagent consisting of 3.5M HCl in n-propanol, made by adding 21.3ml acetyl chloride to 78.7ml n-propanol (dried distilled grade; Fisons) whilst cooled on ice, was added to the residue.

The sealed tubes were refluxed in an oil bath at 110°C for 25min, then cooled in cold water. The esterification reagents were evaporated at 40°C in a sand bath with a stream of nitrogen, the tube caps being dried in an oven at 90°C, to remove any traces of reagents which would interfere with the subsequent acylation step. Care must be taken not to heat the derivatised sample higher than recommended, as losses of some of the more volatile derivatives may occur.

Trifluoroacetylation was carried out by adding 0.4ml dichloromethane (dried distilled grade; Fisons) and 0.2ml TFAA (Sigma), the sealed tubes being refluxed in an oil bath at 150°C for 5-6min. After cooling to room temperature, the reagents were evaporated at 30°C in a sand bath with a stream of nitrogen. The derivative residue was dissolved in 0.5ml ethyl acetate (spectrograde; Fisons) ready for injection into the GC-MS.

6.3.1.3 EQUIPMENT.

GC-MS was performed using a Hewlett Packard (HP) (Pennsylvania, USA) 5890A Gas Chromatograph, a HP 5970 Mass Selective Detector (70 eV, electron impact ionisation) and a HP Think Jet printer, all controlled by a HP 59970 MS Chem Station.

Throughout the investigations the carrier gas was helium at a column flow rate of 1ml/min and a split ratio of 30:1, the sample injection volume was 1μl.

Narrow bore (0.2mm internal diameter) vitreous silica capillary columns were employed: namely, the BP-1 (25m) and BP-10 (12m) (SGE, Victoria, Australia) and the HP-1 (12m) (Hewlett Packard). Although these columns have slighty different

properties, results obtained on one column were comparable to those on the others, as standard calibration solutions were always derivatised and injected with each batch of samples.

6.3.1.4 METHOD DEVELOPMENT.

Initial investigations were carried out to find an appropriate derivatisation method for the determination of both hydroxyproline and 3-methylhistidine.

0.5mg (total amino acids) of hydroxyproline, 3-methylhistidine and bovine L.dorsi hydrolysate were trimethylsilylated using BSTFA and chromatographed with the GC-MS in the SCAN mode (detecting all ions m/e 30-500; comparable to flame ionisation detection), using the BP-1 column with the following temperature programme;

2min solvent delay,
100°C for 1min,
15°C/min to 300°C,
300°C for 1min.
Injector temperature 280°C,
detector temperature 300°C.

Similar investigations were carried out on trimethylsilyl derivatives made using BSTFA + 1% TCMS and TRI-SIL "Z", and on t-butyldimethylsilyl derivatives made using MTBSTFA. For these investigations 0.5mg (total amino acids) of the "standard amino acid mixture" was also analysed. GC-MS peaks were unequivocally identified by their mass spectra in comparison with Leimer, Rice and Gehrke, (1977b), and by predicted fragmentation patterns.

The use of acetylated / alkyl esterified derivatives was then investigated. 0.5mg (total amino acids) of

hydroxyproline, 3-methylhistidine, "standard amino acid mixture" and bovine L.dorsi hydrolysate were derivatised to the N(O)-TFA / n-propyl esters. Initially these were analysed using the BP-10 column, with the GC-MS in the SCAN mode, with the following temperature programme;

1.5min solvent delay,
100°C for 2min,
15°C/min to 250°C,
250°C for 2min.
Injector temperature 250°C,
detector temperature 250°C.

The GC-MS peaks were unequivocally identified by their mass spectra in comparison with Leimer, Rice and Gehrke (1977a) and by predicted fragmentation patterns.

The investigations using N(O)-TFA n-propyl esters were repeated, but with the GC-MS in the selected ion monitoring (SIM) mode, only detecting ions found to be uniquely characteristic of the hydroxyproline derivative (m/e 164, 276 and 365) and of the 3-methylhistidine derivative (m/e 95, 220 and 307). The SIM mode, being more sensitive than the SCAN mode, should aid the detection of minor amino acid constituents in the meat hydrolysates.

The detection of the methylated amino acid N-methyl-lysine, which had been suggested as a possible index of meat protein (Rangely and Lawrie, 1976, 1977) was attempted on bovine L.dorsi hydrolysate, using the method described above, but detecting ions uniquely characteristic of its derivative (m/e 194, 307 and 395) (Leimer, Rice and Gehrke, 1977a).

6.3.1.5 QUANTIFICATION OF HYDROXYPROLINE.

Effort was then concentrated on the quantification of hydroxyproline, using the BP-10 and HP-1 columns, and the temperature programme previously described for N(O)-TFA n-propyl derivatives (section 6.3.1.4). 6-Amino-n-caproic acid (ACA) was chosen as the internal standard. This choice was made as ACA was not found in the meat hydrolysates, it chromatographed close to (but not overlapping with) hydroxyproline and it had a characteristic ion (m/e 210) seen in only one other amino acid derivative; viz, that of hydroxyproline.

0.5mg (total amino acids) of hydrolysates of bovine L.dorsi, Masseter and Malaris and Semimembranosus were derivatised to N(O)-TFA n-propyl esters as previously described. The hydroxyproline standards for the calibration curve, of 0.5-16.5 μg (made up to 0.5mg total amino acids with glycine), were derivatised simultaneously with the samples to minimise variation due to derivatisation conditions. 10μg of internal standard (ACA) was included in each sample and standard. Chromatography was carried out in the SIM mode, detecting ions unique to hydroxyproline (m/e 164, 278 and 356) and the ion characteristic of the internal standard and hydroxyproline (m/e 210).

6.3.1.6 METHOD VALIDATION.

Quantification of the hydroxyproline and internal standard (ACA) ion peaks was carried out by integration using the facilities of the Hewlett Packard MS Chem Station. Calibration curves of hydroxyproline (μg) vs. selected ion peak areas (units)

were constructed from which the hydroxyproline levels in the hydrolysates could be interpolated, using the BBC Statistics "Linear Regression" computer programme. Reagent blanks and "spiked" samples were also analysed to determine reagent interference and hydroxyproline recovery.

Hydroxyproline values determined by GC-MS were compared with values determined by the standard colorimetric method (Stegemann and Stalder, 1967) (appendix I).

6.3.2 RESULTS AND DISCUSSION.

The trimethylsilylation methods (except that using TRI-SIL "Z", which gave no peaks in any of the samples) only gave a sharp peak in the SCAN mode for the 0.5mg hydroxyproline standard. 3-methylhistidine gave no peak. The "standard amino acid mixture" and meat hydrolysate gave only small peaks, which were hardly distiguishable from the base-line "noise".

t-Butyldimethylsilyl derivatives gave a sharp peak for the 0.5mg hydroxyproline standard, but no peak for 3-methylhistidine. Reasonable chromatographs were obtained for the "standard amino acid mixture" and the meat hydrolysate. The use of this derivative for the quantification of hydroxyproline might have been possible, except that repeated injection of these derivatives dramatically reduced the life of the column and the sensitivity of the mass spectrometer detector. This could have been due to the reagents used, or as a consequence of the direct derivatisation and injection of uncleaned hydrolysates, possibly resulting in fouling of the injection port, absorbtion of substances onto the column and fouling of the mass spectrometer

detector. The use of traditional cation exchange cleaning steps (eg. Adams, 1974) on the hydrolysates might have reduced this problem, but would have increased the time of sample preparation, to an extent unacceptable for a routine analytical method.

N(O)-TFA n-propyl esterification gave sharp peaks for both the 0.5mg hyroxyproline (HPro) and 0.5mg 3-methylhistidine (3MeHis) standards in the SCAN mode. Figure 6.1a, shows: (A) the SCAN mode (total ion) chromatogram of the hydroxyproline N-TFA n-propyl ester, (B) the mass spectrum of the derivative and, (C) the molecular structure of the derivative, with the expected electron impact ionisation pattern. The most abundant ion in the mass spectrum of the hydroxyproline derivative was m/e 164, which corresponds to the loss of the propoxy carbonyl group along with trifluoroacetic acid. Figure 6.1b gives similar details, but of the derivative of 3-methylhistidine. In the mass spectrum of the 3-methylhistidine derivative the high molecular weight ion m/e 307 represents the molecular ion (M^+).

The "amino acid standard mixture" and meat hydrolysates both gave reasonable chromatographs in the scan mode.

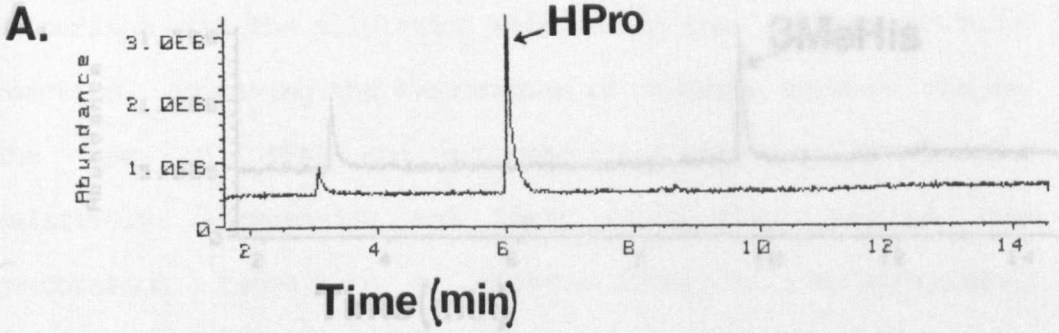
Analysis of bovine L.dorsi hydrolysate in the SIM mode, which detected ions specific to the hydroxyproline and 3-methylhistidine derivatives, unequivocally identified hydroxyproline by its retention time and selected ion spectra, although no peak corresponding to 3-methylhistidine could be identified. N-methyllysine could not be identified in bovine

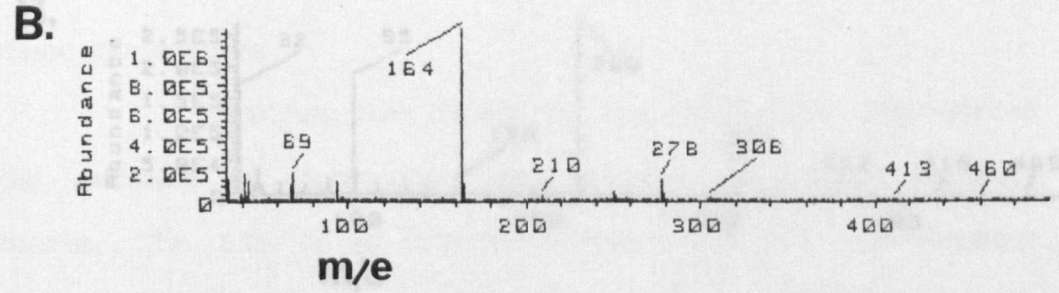
Figure 6.1a. A. Scan mode GC-MS chromatogram of hydroxyproline N-TFA n-propyl ester.

B. Mass spectrum of hydroxyproline N-TFA n-propyl ester on electron impact ionisation.

C. Fragmentation pattern of hydroxyproline N-TFA n-propyl ester on electron impact ionisation.

Experimental details as in text.





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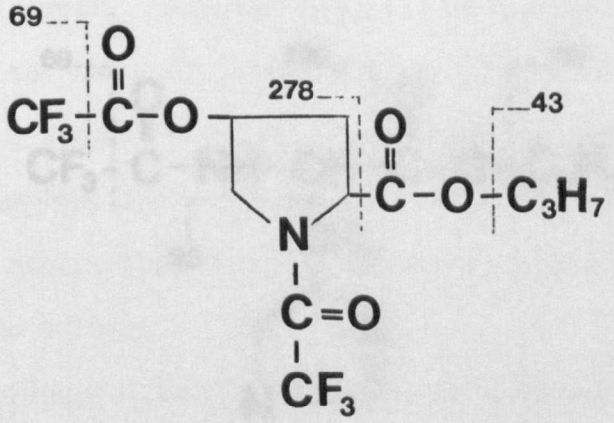
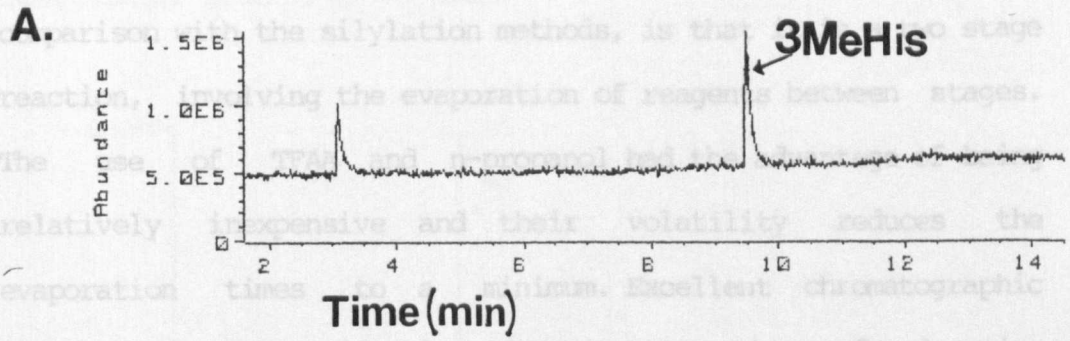


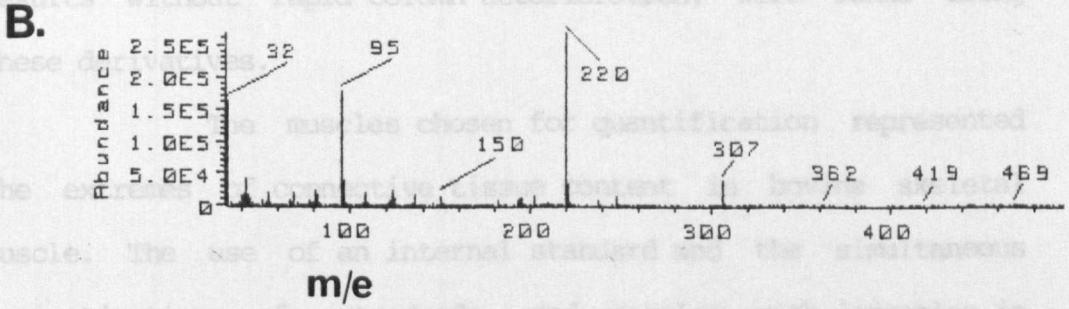
Figure 6.1b. A. Scan mode GC-MS chromatogram of 3-methylhistidine N-TFA n-propyl ester.

B. Mass spectrum of 3-methylhistidine N-TFA n-propyl ester on electron impact ionisation.

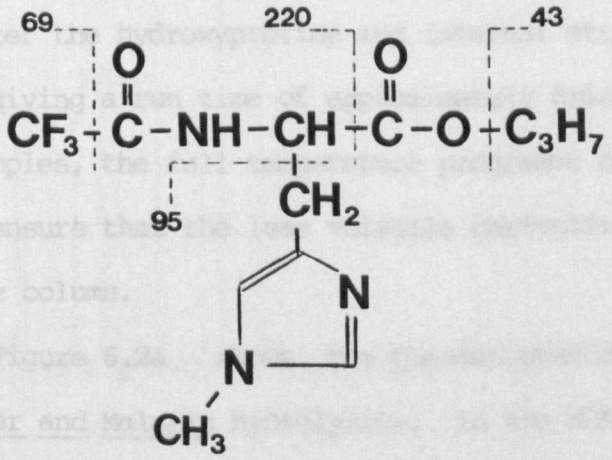
C. Fragmentation pattern of 3-methylhistidine N-TFA n-propyl ester on electron impact ionization.

Experimental details as in text.





C.



L.dorsi hydrolysate with the GC-MS in the SIM mode detecting ions specific to its derivative.

The results of the preliminary investigations indicated that the N(O)-TFA n-propyl ester derivatives would be most suitable for the quantitative determination of hydroxyproline. The only disadvantage of this method, in comparison with the silylation methods, is that it is a two stage reaction, involving the evaporation of reagents between stages. The use of TFAA and n-propanol had the advantage of being relatively inexpensive and their volatility reduces the evaporation times to a minimum. Excellent chromatographic results without rapid column deterioration, were found using these derivatives.

The muscles chosen for quantification represented the extremes of connective tissue content in bovine skeletal muscle. The use of an internal standard and the simultaneous derivatisation of standards and samples, with injection in duplicate, ensured maximum reproducibility of results. To reduce the time of analysis, standard chromatographic runs were stopped immediately after the hydroxyproline and internal standard peaks had eluted, giving a run time of approximately 6min. With the hydrolysate samples, the full temperature programme of 14min had to be run to ensure that the less volatile derivatives had been eluted from the column.

Figure 6.2a shows the chromatogram of uncleaned bovine Masseter and Malaris hydrolysate, in the SCAN mode, the peaks being unequivocally identified by their mass spectra (Leimer, Rice and Gehrke, 1977a). A comparison with figure 6.2b,

Figure 6.2a. SCAN mode GC-MS chromatogram of the N-TFA n-propyl esters of bovine Masseter and Malaris (cheek) hydrolysate.

Key: 1.Threonine; 2.Serine; 3.Valine; 4.Leucine; 5.Isoleucine; 6.Proline; 7.Hydroxyproline; 8.Aspartic acid; 9.Phenylalanine; 10.Glutamic acid; 11.Lysine; 12.Arginine.
(Glycine and alanine peaks are eluted during the solvent delay).

Experimental details as in text.

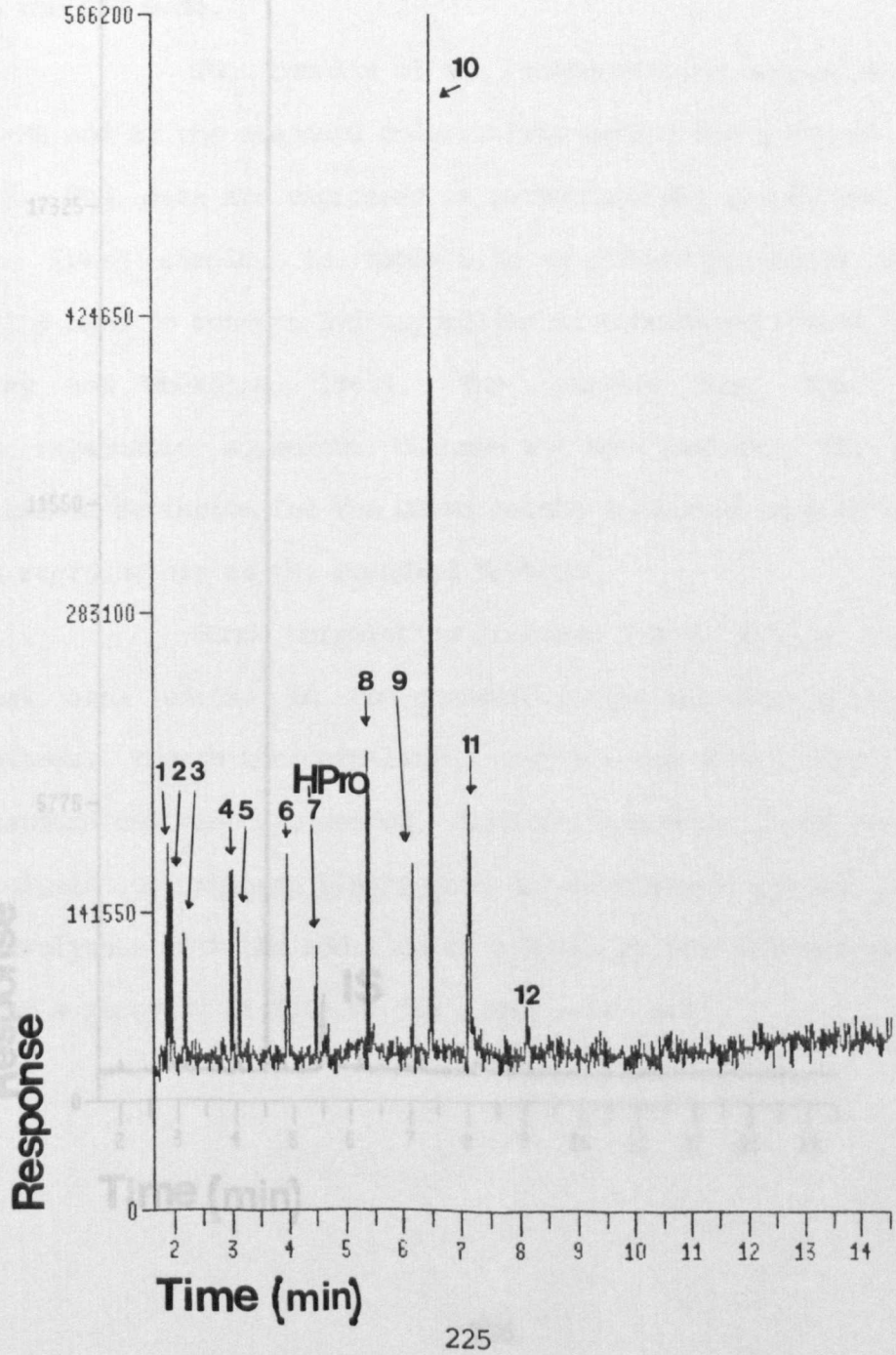
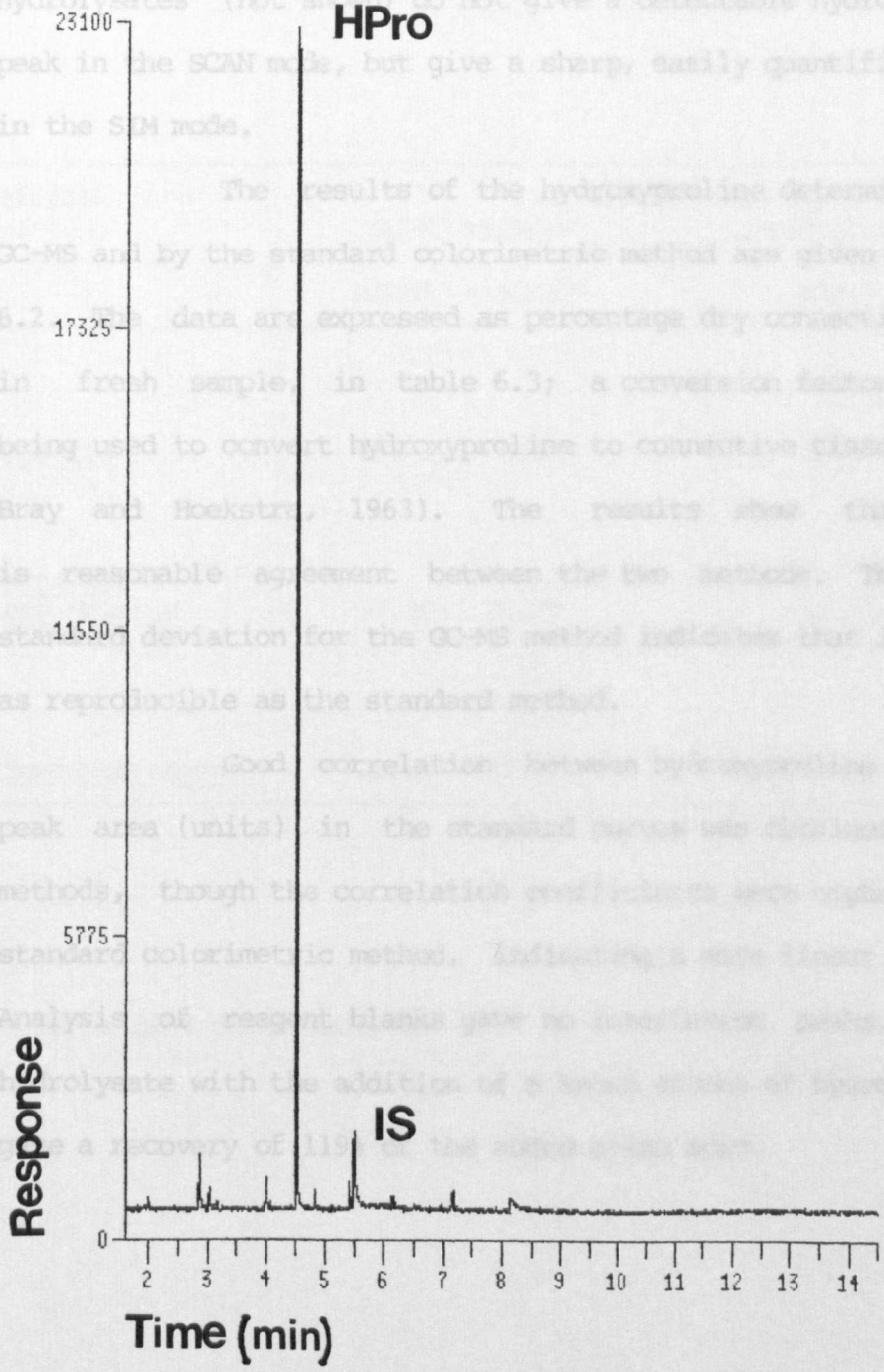


Figure 6.2b. SIM mode GC-MS chromatogram of the N-TFA n-propyl esters of bovine Masseter and Malaris (cheek) hydrolysate, detecting ions specific to the hydroxyproline (HPro) and internal standard (IS) derivatives.

Experimental details as in text.



carried out in the SIM mode, as used for quantification, shows the large sharp hydroxyproline peak, with a retention time of approximately 4.5min. This peak represents 158 picomoles of hydroxyproline, indicating the sensitivity and selectivity of the SIM mode response. L.dorsi and Semimembranosus hydrolysates (not shown) do not give a detectable hydroxyproline peak in the SCAN mode, but give a sharp, easily quantifiable peak in the SIM mode.

The results of the hydroxyproline determination by GC-MS and by the standard colorimetric method are given in table 6.2. The data are expressed as percentage dry connective tissue in fresh sample, in table 6.3; a conversion factor of 7.25 being used to convert hydroxyproline to connective tissue (Goll, Bray and Hoekstra, 1963). The results show that there is reasonable agreement between the two methods. The higher standard deviation for the GC-MS method indicates that it was not as reproducible as the standard method.

Good correlation between hydroxyproline (μg) and peak area (units) in the standard curves was obtained by both methods, though the correlation coefficients were higher for the standard colorimetric method, indicating a more linear response. Analysis of reagent blanks gave no interfering peaks. L.dorsi hydrolysate with the addition of a known amount of hydroxyproline gave a recovery of 119% of the added amino acid.

TABLE 6.2. HYDROXYPROLINE DETERMINATION OF BOVINE MUSCLE HYDROLYSATES BY GC-MS AND STANDARD COLORIMETRIC METHODS.

MUSCLE	[HYDROXYPROLINE] µg/ml			
	GC-MS METHOD		STANDARD COLORIMETRIC METHOD	
	MEAN*	SD(±)	MEAN*	SD(±)
L.dorsi	26.92	5.59	23.26	1.41
Masseter and Malaris	97.30	24.77	82.73	2.07
Semimembranosus	22.06	3.11	20.40	0.93

* Means of 4 determinations.

TABLE 6.3. DRY CONNECTIVE TISSUE CONTENTS OF BOVINE MUSCLES CALCULATED FROM GC-MS AND STANDARD COLORIMETRIC METHODS OF HYDROXYPROLINE DETERMINATION.

MUSCLE	DRY CONNECTIVE TISSUE CONTENT OF FRESH SAMPLE (g/100g)*			
	GC-MS METHOD		STANDARD COLORIMETRIC METHOD	
	MEAN**	SD(±)	MEAN**	SD(±)
L.dorsi	0.939	0.195	0.812	0.049
Masseter and Malaris	3.202	0.815	2.723	0.068
Semimembranosus	0.809	0.114	0.748	0.036

* Calculated as in appendix II, from compositional analyses (section 3.2.2.6 and appendix I).
** Means of 4 determinations.

The GC-MS method described has the attributes of sensitivity and specificity required for the determination of hydroxyproline in meat products. Although the method described has the advantage (over most GC methods for the determination of amino acids in biological samples), of not requiring laborious clean-up steps prior to analysis, further increase in the rapidity and reproducibility of the method is required if it is to be developed into a routine analytical procedure. Such developments might involve increasing the number of samples which could be simultaneously derivatised, and increased precision in the control of derivatisation conditions. The use of thermostatically controlled multi-sample hot-plates and evaporators enabling the simultaneous derivatisation of tens of samples could meet these requirements.

The precision requirement of the British Standards Institute (BSI) method for the determination of hydroxyproline in meat and meat products (BSI, 1979), is that "the difference between the two calculated values obtained simultaneously or in rapid succession from the duplicate test portions by the same analyst shall not exceed 5% of the arithmetic mean value". It was found that this precision requirement was generally fulfilled by the standard colorimetric method used. The precision of the GC-MS method was found to be lower than the requirements, though the data suggested that correction using the relative response of the internal standard, if included at appropriate concentration, would give duplicates of far greater precision.

Day to day variation was seen in the GC-MS response

to standards and samples, though this was corrected for by the simultaneous derivatisation of hydroxyproline standards with each batch of samples, better reproducibility could be obtained by correction using the relative peak area of the internal standard. This was not possible in this investigation due to the great difference in size of the internal standard and hydroxyproline peaks.

Quantitative determination of minor amino acid constituents of protein hydrolysates by SIM mode GC-MS, as illustrated here by hydroxyproline determination, was unsuccessful in the determination of 3-methylhistidine. The very low levels of this amino acid present, and the well documented difficulty in obtaining good derivatisation and gas chromatography of histidines (section 2.4.8), probably accounts for this. With future developments it may be possible to quantitatively determine both 3-methylhistidine and hydroxyproline in a single GC-MS analysis, representing in a single method, a possible alternative to the standard colorimetric assay of hydroxyproline and the HPLC / fluorescamine method for 3-methylhistidine determination.

7. GENERAL DISCUSSION AND CONCLUSION.

7. GENERAL DISCUSSION AND CONCLUSION.

The importance of establishing a robust, unequivocal method for the determination of meat in foods, has never been greater. Food processing technology has now reached a level of development which enables non-meat protein sources to be incorporated into meat and meat products to give analogues, which look and taste "traditional". The use of such non-meat proteins by a meat product manufacturer gives "added value", in economic terms, to the raw materials used, making relatively inexpensive protein sources, such as meat by-products, offals, and proteins of vegetable, microbiological and fungal origin (often unacceptable in their original form), into products commanding high prices. This makes fraudulent claims of meat content - important in determining the quality and therefore price of a product - an attractive proposition for the unscrupulous manufacturer.

Products in which part of the meat content is replaced by non-meat analogues, are not necessarily less nutritious than similar products containing no "foreign" proteins. Nevertheless, the consumer has a right, as enforced by regulation (Statutory Instruments, 1984, 1986), to be informed of the composition of the meat products purchased. This consumer awareness is particularly important since meat is commonly regarded as the main source of high quality protein, and therefore demands a high price. Certain population groups, such as the elderly, and families on low incomes, are particularly susceptible to false claims by fraudulent meat product manufacturers, since a large proportion of their total income is

spent on such products. Institutionalised groups are also particularly susceptible, since they have little control over their diet, and it is economically sensible to provide them with the best possible diet at the lowest cost.

Methods of determining meat in foods need to be based on parameters that are specific to meat proteins or the associated myofibrillar tissue. Protein-bound 3-methylhistidine is one such parameter and was proposed as an unequivocal index of meat in foods by Hibbert and Lawrie (1972). The more or less constant levels of this amino acid in meat, its absence from other food proteins, and its robustness to even the most severest processing conditions made this proposition attractive.

For 3-methylhistidine to be a truly unequivocal index of meat, the protein-bound levels of this amino acid in all tissues accepted as "meat" by the current regulations (Statutory Instruments, 1984, 1986) need to be more or less constant when corrected for the varying levels of fat and connective tissue found in different cuts (neither of which contain 3-methylhistidine). By regulation, "meat", is defined as the flesh of any mammal or bird, which is normally used for human consumption, including the offals: diaphragm, head meat, heart, kidney, liver, pancreas, tail meat, thymus and tongue of the mammalian carcass, and the gizzard, heart, liver and neck of the avian carcass. Discrepancies in the total protein-bound 3-methylhistidine titres of certain meats; viz, flank and Masseter and Malaris (cheek), as well as other "meat" offals, were reported by Jones et al. (1985) and White and Lawrie

(1985b). On further investigation (section 3), it has been found that only ruminant Masseter and Malaris (cheek) has a low total protein-bound 3-methylhistidine titre, along with the diaphragm of both ruminants (ox and sheep) and non-ruminants (pig). Non-ruminant (porcine) cheek and bovine flank appeared to have normal, prime-cut levels. These discrepancies required fundamental investigation, to determine the feasibility of utilising total protein-bound 3-methylhistidine levels as an index of meat content.

"Meat" offals, such as kidney and liver, have been shown to contain only low levels of total protein-bound 3-methylhistidine, whereas fish, not classed as "meat" by current regulations, appears to contain levels of this amino acid similar to that found in "prime-cuts" of meat. From these findings it is apparent that an index of "meat" content based on total protein-bound 3-methylhistidine would contradict the current definitions of "meat" as set out in the Statutory Instruments (1984, 1986). A possible answer to these contradictions would be to re-define "lean-meat content" as "myofibrillar protein content". This is a reasonable proposition, since myofibrillar protein is the essence of "meat". In most instances the high level of myofibrillar protein associated with muscular tissue would be expected to give a comparably, high level of total protein-bound 3-methylhistidine, whereas low levels of myofibrillar protein would give correspondingly low levels of this amino acid. Unfortunately a definition of "lean meat", based on myofibrillar protein content, would exclude the non-muscular offals, such as kidney and liver, which do

provide valuable, high quality protein, as well as essential vitamins and minerals.

The contribution of fat and connective tissue to meat needs to be considered, particularly the level of connective tissue which constitutes "amounts naturally associated with the flesh used", in the definition of "meat", and the level of fat in meat "free of visible fat", in the definition of "lean meat". Both fat and connective tissue can make valuable contributions to the flavour, texture and nutritional value of meat, when included at the appropriate levels. It was formerly believed that, because of its deficiency in essential amino acids, the presence of collagen in meat products must result in a lowering of the nutritional value. However, there is consistent evidence that a mixture of up to 30% collagen with muscle protein meets the nutritional requirements as the sole protein source for weaning children (Jobling, 1984).

The myofibrillar proteins, actin and myosin, are known to be the only proteins to contain 3-methylhistidine in significant amounts (section 2.1). Since the ratio of the different (connective tissue=free) myofibrillar proteins was expected to be similar in all striated muscle, regardless of the source, any differences in the total protein-bound titres of this amino acid in muscular tissue were expected to be solely due to corresponding differences in the degree of histidine methylation in actin and/or myosin.

As was expected from the conserved nature of actin (section 2.1.4), little intracarcass or interspecies variaton was

found in the level of histidine methylation in this protein. On the other hand the myosin purified from ruminant; viz, ovine and bovine; (White and Lawrie, 1985b), but not non-ruminant (porcine) Masseter and Malaris (cheek) muscle, had a dramatically lower 3-methylhistidine content than that usually associated with adult striated muscle (section 2.1.4). In fact 3-methylhistidine may be virtually absent from ruminant Masseter and Malaris myosin; the low levels detected could have arisen from actin contamination.

Localizing the variation reported in the histidine methylation of myofibrillar proteins in meat to the myosin, was not a particularly suprising finding, since the methylation of this protein has been shown to be under developmental control, depending on the age and tissue type composition of the muscle concerned. The myosin extracted from mammalian foetal, adult cardiac and adult skeletal muscle, represents three isoenzyme forms of the heavy chain of this protein, only the latter containing a methylated histidine residue (section 2.1.5). The reportedly low levels of 3-methylhistidine in the myosin of muscles of predominantly "red" fibre type; eg. cat Soleus (Kuehl and Adelstein, 1970) indicated that the level of histidine methylation in any striated muscle myosin preparation would depend upon the ratio of the fibre types in the tissue used. The results obtained for the fibre typing of meat samples (section 4.4) supported this proposition, in that ruminant Masseter (which has a low total protein-bound 3-methylhistidine titre), was unusual, being composed almost entirely of "red" (oxidative)

fibres. On the other hand non-ruminant Masseter, and the L.dorsi of all of the species investigated (all of which have high total protein-bound 3-methylhistidine titres typical of "prime" cuts), consisted of predominantly "white" (glycolytic fibres).

On the basis of the experimental work in sections 3 and 4, a relationship has been established between fibre type composition, myosin histidine methylation and total protein-bound 3-methylhistidine content of mammalian muscle meat. Skeletal muscle "red" fibre myosin appears to be extremely deficient or entirely lacking in 3-methylhistidine. This suggests the occurance of at least two myosin heavy chain isoforms in adult mammalian striated muscle, one methylated and one not.

Since it appeared that there was a correlation between the fibre type composition of a muscle meat, the 3-methylhistidine content of its myosin, and its total protein-bound 3-methylhistidine titre, it was reasonable to assume that the interspecies and intercarcass variation in the latter was due solely to the myosin. The measurement of the 3-methylhistidine of the actin present would therefore be expected to be a more unequivocal index of myofibrillar protein content of striated muscle (assuming that the ratio of actin to other myofibrilar proteins was constant), since the 3-methylhistidine titre of actin is relatively constant.

The development of a method for the determination of actin-bound 3-methylhistidine in meat required the

quantitative isolation of the actin from other 3-methylhistidine containing constituents; viz, the free amino acid, myosin heavy chain and balenine. Since there is a great difference between the molecular weight of actin and those of the free amino acid and balenine (much lower) and of myosin heavy chain (much higher), this was the parameter chosen for the basis of separation.

Using a method based on that of Stein et al. (1974), meat proteins were separated by SDS PAGE. The stained actin band was then excised and its 3-methylhistidine content determined by adaptations of the standard HPLC/fluorescamine method of Jones, Shorley and Hitchcock (1982a,b) as modified by White and Lawrie (1985a). This method for the determination of actin-bound 3-methylhistidine was found to be only semi-quantitative, even when an external standard calibration curve was generated by applying the method to known amounts of a standard actin preparation.

Gel filtration held promise of a means of quantitatively isolating actin. The relatively low solubility of the muscle proteins meant that sample buffers and eluents containing SDS were required, to solublise the meat proteins and prevent precipitation during chromatography, since precipitation would result in column blocking.

Separation of actin from the other 3-methylhistidine containing components was accomplished using conventional (low flow rate) gel filtration (section 5.2), although each separation took many hours (approximately 16h). This necessitated the collection of many dilute fractions,

requiring concentration by freeze-drying before detectable levels of 3-methylhistidine could be obtained. From electrophoretic evidence, it was shown that the actin and myosin heavy chain could be separated and isolated, although only low and variable yields of the expected total 3-methylhistidine of the sample prior to separation were recovered after chromatography. Uncertainty concerning the recovery from the column of the total protein loaded onto it, and the laborious work-up of the sample (required to concentrate it to a level at which the 3-methylhistidine was detectable), were probably the main causes of the low and variable yields of the amino acid. This made accurate quantification of actin-bound 3-methylhistidine by this method not possible.

The principle of applying SDS gel filtration to the isolation of the actin in meat appeared sound, since the actin appeared to be virtually completely solublised by the sample buffer used, and isolation of the actin from other 3-methylhistidine containing components could be accomplished. To improve the accuracy of quantification, Pharmacia fast protein liquid chromatography (FPLC) was used. This could more efficiently and quickly isolate the actin from relatively large samples of muscle protein, with the collection of a relatively concentrated actin fraction. A rapid and complete isolation of actin from the other 3-methylhistidine-containing components was obtained, yielding a single fraction containing the total actin of the sample. To further improve the accuracy of quantification, the protein in the fraction was rapidly and

easily concentrated using Centrisart "centrifugal filtration" tubes to a level at which the 3-methylhistidine content could be accurately assessed. Centrisart "centrifugal filtration" tubes, have the important characteristic of not concentrating the low molecular weight buffer components in the fraction, such as SDS, which at high concentrations would interfere with the 3-methylhistidine assay.

Levels of actin-bound 3-methylhistidine have been found to be similar, when expressed as mg/g non-connective tissue nitrogen, in bovine L.dorsi, Masseter and Malaris and Semimembranosus muscles. This indicates that actin-bound 3-methylhistidine, when expressed in these units, could be the basis of an index of "meat" (myofibrillar protein) content.

The use of actin-bound 3-methylhistidine as an index of meat in food, like the use of the total protein-bound titre of this amino acid, contradicts the definition of "meat" as defined by the current regulations. All muscular tissues, regardless of species or intracarcass origin, would be expected to have actin-bound 3-methylhistidine titres similar to those of "prime cuts", whereas non-muscle offals, even if presently classsed as "meat", would have low titres. Cardiac muscle (heart), Masseter and Malaris (cheek) and diaphragm, which are defined as "meat" offals, but have low total protein-bound 3-methylhistidine titres (due to the low level of histidine methylation in their myosins), should have "normal", "prime cut" levels of actin-bound 3-methylhistidine. On the other hand, smooth muscle offals, such as stomach and intestine, which are not classed as "meat", may exhibit higher than "prime-cut" levels

of actin-bound 3-methylhistidine, since the ratio of actin : myosin in smooth muscle is reported to be higher than in striated muscle (Lawrie, 1985).

The method developed for the determination of actin-bound 3-methylhistidine is applicable to raw samples, and from electrophoretic evidence (section 5.3.3), may be applicable to samples heated to at least as high as 100°C for 10 min, when the actin is still extractable and non-degraded. Unfortunately the method could not be applied successfully to canned, retorted (120°C internal temperature for 5 min) samples, under which conditions the integrity of the actin was almost completely lost, due to inextractability and/or degradation. In such cases of severe processing the total protein-bound 3-methylhistidine titre would appear to be the best index of meat content, since this titre is robust under these conditions (Hibbert and Lawrie, 1972).

The actin-bound 3-methylhistidine content of a food is a direct measure of its actin content, as theoretically all muscle actin has one residue of this amino acid per actin monomer. Alternative methods of measuring actin content, other than those based on its 3-methylhistidine content, have been proposed. Double radio-isotope labelling with peptide isolation (section 2.4.7) could be a very accurate method, since it depends on the ratio of radioactivity originating from a mixture of standard and sample actin. Losses in actin, due to the experimental procedure, should not affect this ratio. Unfortunately, double radio-isotope labelling with peptide

isolation depends on a series of rather complex radio-isotopic, chromatographic and electrophoretic methods, limiting its possibilities of development into a routine analytical procedure. Like the determination of actin-bound 3-methylhistidine, it also relies on the integrity of the actin in the sample for accurate quantification.

There is a wide range of published methods for the determination of meat in foods (section 2.4), all of which have their advantages and disadvantages compared with an index based on actin-bound 3-methylhistidine. By measuring actin-bound 3-methylhistidine, one is directly measuring a unique component of muscle which is present at a constant level, regardless of the source of the meat, and which is absent from all vegetable protein sources. Since actin is fundamental in the mechanism of action of striated muscle, constant levels of this protein, compared with the levels of other myofibrillar proteins, would be expected, regardless of the source of the striated muscle. Methods of meat determination based on nitrogen content (section 2.4.1) do not involve the direct determination of a unique component of muscle. These methods involve the accurate determination of total nitrogen usually by the Kjeldahl method, with the conversion of nitrogen to meat by mean nitrogen factors, determined from the statistical analysis of large numbers of samples. Correction for non-protein nitrogen is based on numerous assumptions including the calculation of carbohydrate associated nitrogen. No correction is made for the presence of non-meat protein sources, other than connective tissue when added at levels which exceed those "naturally

associated with the flesh used", which can be determined by its hydroxyproline content.

Electrophoretic methods (2.4.2) based on the densitometric quantification of stained protein bands corresponding to either "meat-specific" proteins (eg. actin) or non-meat, "foreign" proteins, are subject to the inherent variations in the staining of proteins on electrophoretic gels (Fishbein, 1972). Even with the use of internal standard proteins to counteract such variations, the inaccuracy of the method still means that it is only at an interim stage of development, requiring further modifications and refinements, before it can truly classed as a routine analytical method (Olsman, Dobbelaere, and Hitchcock, 1985). Electrophoretic methods, like those based on actin-bound 3-methylhistidine, require the integrity of the protein being quantified, and thus are also not applicable to severely heat-treated samples.

The use of immunochemical methods in the analysis of meat products (section 2.4.3) has created much interest in recent years, to the extent that, in the form of ELISA, it is becoming a well established method, particularly for the determination of soya in meat products and for meat species identification. Immunochemical methods can only be used to quantify a single protein or closeley related group of proteins, which means that separate assays are required to determine the presence of each of the wide range of possible "foreign" proteins which could be present in a product. One advantage of the immunochemical determination of soya, is that it can be carried out, with reasonable accuracy, on processed products,

if the sample and soya standard are similarly chemically denatured and then renatured to the "nearly native" state, giving similar immunoreactivity, regardless of previous processing. An alternative approach to the immunochemical determination of processed soya protein, is the use of antisera generated against "soya specific" peptides, produced on enzymatic degradation (Yasumoto, Hiroshi and Suzuki, 1985; Yasumoto et al., 1986). The levels of such peptides should not be greatly affected by even the severest processing conditions. A "meat specific" peptide "MP-1", produced by enzymatic degradation, has been characterised, and used as a means of determining the meat content of foods (Llewellyn et al., 1978). It may be possible to develop a robust unequivocal ELISA type immunoassy based on a "meat specific" peptide, such as "MP-1", or more specifically a peptide derived from actin, such as the chymotryptic, C-terminal carboxymethylated peptide. This peptide has been previously used by Anderson (1981) to quantify actin, by a complex method involving double radio-isotope labelling and peptide isolation. The immunoreactivity of such a peptide would be unlikely to vary greatly, regardless of the severity of the sample processing, or the intracarcass or interspecies origin of the meat. The sequence of such a peptide would be expected to be conserved throughout the muscles and species commonly used as meat, and it is unlikely that even the severest processing would degrade the primary structure of the actin to a degree that would cause a significant decrease in the level of this peptide on chymotryptic digestion.

Quantitative analysis of meat products using

histochemical methods (section 2.4.4) relies on the microscopically visible contrast between the components of interest and the sample matrix. Quantification is normally carried out by stereological point-counting methods, by which information about the 3-dimensional composition of a sample can be obtained from 2-dimensional sections. It has been proposed that "lean meat" content could be determined histologically, by the counting of muscle fibres (Flint and Meech, 1978), since muscle fibres are the essence of "lean meat". The accuracy of meat determination by stereological point-counting of muscle fibres relies heavily on the preparation of a representative sample of the material as a whole, and can only be regarded as giving an estimate of "lean meat" content. Histochemical methods were regarded by Griffiths, Billington and Griffith (1981) as being most suitable for the initial qualitative examination of products to determine the constituents present, prior to more critical analysis by other methods.

Of the wide array of other chemical and physical methods reported for the determination of the composition of meat products (section 2.4.5), none have the required characteristics of specificity and robustness possessed by 3-methylhistidine for the determination of meat content. The HPLC determination of the levels of "meat specific" peptides produced by enzymatic degradation (Griffiths, Billington and Griffiths, 1981) has prospects for the determination of meat in foods; especially, since recent developments in the automation of chromatographic apparatus, have made complex separations,

easier, more rapid, and less expensive once the investment in the apparatus has been made. Although near infra-red (NIR) reflectance spectroscopy, has found commercial applications in the rapid analysis of many components of foodstuffs (including protein), it does not have the capability to distinguish meat from non-meat protein which, like methods based on nitrogen content, limits its use in the determination of meat content of comminuted products.

The use of gas chromatography-mass spectrometry (GC-MS) (section 2.4.8) has been successfully applied to the determination of hydroxyproline in meat, without the laborious hydrolysate "clean-up" and amino acid isolation steps normally associated with the gas chromatographic analysis of amino acids in biological samples. The determination of 3-methylhistidine by GC-MS was not accomplished, due to the relatively low levels of this amino acid in the meat hydrolysates, and the uncertainty surrounding the derivatisation and chromatographic behaviour of histidine and its derivatives. Fortunately the basic nature of histidine and its derivatives allows the relatively simple isolation of them from complex hydrolysates, using micro-scale ion-exchange chromatography columns (Rogoskin, Krylov and Khlebnikova, 1987). The addition of a known amount of an internal standard, such as deuterated 3-methylhistidine (Matthews et al., 1981) to the hydrolysate, can be used to correct for any losses of 3-methylhistidine during the ion-exchange isolation, derivatisation and gas chromatographic stages, allowing accurate quantification, even when the efficiency of the process is variable and uncertain. Rapid

developments in the interfacing of high-efficiency separation techniques with the versatility of mass spectroscopic detection (Smith and Udseth, 1988) have led to the development of liquid chromatography-mass spectrometry apparatus, which may provide useful methods for the determination of very low levels of 3-methylhistidine, particularly the very low actin-bound levels of this amino acid which would be present in products with low meat contents.

The determination of total protein-bound 3-methylhistidine using methods described in this thesis has been applied commercially, for a number of manufacturers. A problem with maintaining the quality of reformed meat by-product / vegetable protein pieces used in meat analogue production was experienced by one manufacturer. The level of 3-methylhistidine in the incoming raw material of animal origin (chicken by-products), of which the composition varies considerably, was correlated to the ability of such materials to form meat analogues with the required rheological properties. This was presumably since 3-methylhistidine was a direct indicator of the myofibrillar protein content of the starting material, the myofibrillar proteins being important in maintaining the stability of the meat analogues, especially under processing conditions which degrade other meat associated proteins such as collagen, which is then lost on processing. Total protein-bound 3-methylhistidine levels have also been used to investigate advertised claims of "higher meat content" in leading brands of meat products, and for the comparison

of "meat" content of foreign brands, with those available on the home market; such comparisons will be particularly important with the lifting of the European trade barriers in 1992, when products will need to be of a consistent "quality" throughout the participating nations.

The method for the determination of 3-methylhistidine originally developed by Jones, Shorley and Hitchcock (1982a,b), as modified by White and Lawrie (1985a) and more recently by Jones, Homan and Favell (1987) has reached an interim stage of development into a routine analytical method. The results of a Ministry of Agriculture Fisheries and Food collaborative trial, into the repeatability and reproducibility of the method (the experimental details of which were reported by White, 1986), should indicate what if any, further modifications need to be made to the method.

Methods of meat determination based on total protein-bound 3-methylhistidine, although robust to the processing conditions found in the food industry, now appear to be unequivocal due to the variation in the histidine methylation of myosin. On the other hand the method based on the titre of actin-bound 3-methylhistidine appears to be an unequivocal index of myofibrillar protein (meat) content of food, and is robust to all but the severest processing conditions, when the integrity of the actin is destroyed. Of the alternative methods of meat determination, procedures based on the quantitative assessment of actin-specific peptides, produced by enzymatic degradation, appear promising; whether determined by chromatographic or immunochemical methods, or possibly by their 3-methylhistidine

content. The quantification of actin-specific peptides would be unequivocal, since it is based on actin content, and robust, as although severe processing may destroy the integrity of the actin molecule as a whole, it is unlikely to disrupt its primary structure to an extent that the levels of a short peptide sequence will be altered.

The experimental techniques used, and the assumptions made in the "novel" method developed for the determination of actin-bound 3-methylhistidine as an index of myofibrillar protein content of food, appear sound. Further developmental work and refinements to the method need to be made, and its applicability to the analysis of meat products, both raw and processed, needs to be critically investigated. The validity of the mean factors used for "classical" methods of meat content determination based on nitrogen content, show significant intercarcass variation. An index based on actin-bound 3-methylhistidine would not rely on such nitrogen factors and appears to be the most robust, unequivocal and specific method yet proposed for the determination of "meat" in food.

APPENDICES.

APPENDICES.

APPENDIX I. ROUTINE METHODS OF COMPOSITIONAL ANALYSIS.

(a). MOISTURE DETERMINATION.

10g of sample was placed in a pre-weighed aluminium pan 70mm diameter, 12.5mm deep, with an aluminium foil cover, pierced to allow the escape of water vapour. This was placed in a vacuum oven (Gallenkamp) at 70°C for 16 h (overnight). The sample was then cooled in a desiccator, and weighed. The sample was then dried for a further 2 h and re-weighed. The weighings were repeated until consistent readings were obtained indicating that the sample was completely dried.

$$\% \text{ moisture} = \left[1 - \frac{\text{Dry weight (g)}}{\text{Wet weight (g)}}\right] \times 100.$$

(b). LIPID DETERMINATION BY SOXHLET ETHER EXTRACTION.

Lipid determination by Soxhlet ether extraction was based on the standard method (Egan, Kirk and Sawyer 1981).

2g dried sample was weighed into a Soxhlet extraction thimble (Whatman). Extraction was carried out into a pre-weighed 250ml spherical flask using standard Soxhlet distillation apparatus. The solvent used was petroleum ether (boiling range 40-60°C). Due to the hazardous nature of this extraction it was performed in a purpose built "hazards laboratory", isolated from the main buildings. After 8h extraction at 60°C the solvent was carefully evaporated from the lipid in the spherical flask. Last traces of solvent were removed by heating the flasks at 60°C for 2h. The flasks were then cooled in a desiccator and weighed.

$$\text{\% lipid (in fresh sample)} = \frac{\text{Weight of lipid (g)}}{\text{Equivalent weight of fresh sample used (g)*}} \times 100$$

* Determined from weight of dried sample used for extraction and % moisture of fresh sample.

(c) CONNECTIVE TISSUE DETERMINATION.

Connective tissue determination was based on the method of Stegemann and Standler (1967), by which hydroxyproline which is unique to connective tissue is determined colorimetrically.

0.5 - 1.0 ml of hydrolysate, depending on the expected level of connective tissue, was pipetted into a 50ml beaker. 10μl of universal indicator (BHD) and 5ml distilled water were added. The solution was then neutralised to pH 6.5 by drop-wise addition of sodium hydroxide solutions of descending molarities. The total solution was then made up to 25ml by transferring to a 25ml volumetric flask.

A series of standards was prepared of from 0-5.0 μg/ml hydroxyproline (Sigma) and 0.4 μl/ml Universal indicator (BDH), in distilled water.

2ml of sample or standard was transferred to a test-tube. In the space of 1 minute, 1ml of "oxidation reagent" was added to each tube.

"Oxidation reagent" =	1.41g	chloromine T (Sigma).
	10 ml	water.
	10 ml	propan-1-ol.
	80 ml	citrate/acetate buffer pH 6*.

* Citrate/acetate buffer pH 6 =	50g	citric acid.
	12ml	glacial acetic acid.
	120g	sodium acetate trihydrate.
	34g	sodium hydroxide.

Made up to 1000 ml with distilled water, then add 200ml distilled water and 300ml propan-1-ol.

The tubes were mixed and left to stand at room temperature for exactly 20 min. 1ml of "colour reagent"** was then added to each tube , all within 1 minute.

** "Colour reagent" = 10g dimethylaminobenzaldeyde (Sigma).
35ml 60% perchloric acid.
65ml propan-2-ol.

The tubes were quickly mixed and transferred to a thermostatically controlled water bath at 60°C. Evaporation from the tubes was prevented by the use of glass marbles.

After exactly 15 min. at 60°C the tubes were cooled under running tap water for 3 min, to stop the reaction.

The optical density of the samples and standards at 558nm was determined in 1cm wide borosilicate glass cuvettes using a Unicam (Cambridge, UK) SP500 (Series 2) spectrophotometer.

The concentration of hydroxyproline in the samples could be interpolated from the standard curve of hydroxyproline standard concentration (µg/ml) against absorbance at 558nm. Using data obtained the hydroxyproline content of the original sample can be determined and thus the dry connective tissue content, as it is assumed that;

% dry connective tissue = % hydroxyproline X 7.25*

* (Goll, Bray and Hoekstra, 1963).

(d). NITROGEN DETERMINATION BY KJELDAHL DISTILLATION.

Nitrogen determination by Kjeldahl distillation was based on wet combustion with the reduction of organic nitrogen to ammonia as ammonium sulphate. In the presence of excess alkali the ammonia is released, distilled, trapped and titrated.

A known weight of sample equivalent to approximately 50mg protein was weighed onto Whatman no.541, ashless filter paper. The sample was placed in a Kjeldahl distillation tube. Blanks consisting of the filter paper alone were also made. To the tubes were added 2 Kjeldahl catalyst tablets (Kjeltabs, Thompson and Capper Ltd, Runcorn, UK), and 20ml concentrated sulphuric acid (SG 1.84). The tubes were then heated on a purpose-built heating block; the Tecator (Hoganas, Sweden) Digestion system 6, 1007 digestor, at 420°C for 1 h. Waste gases from the digestion were carried away by tube covers attached to a water powered vacuum line. After digestion the tubes were cooled and approximately 80ml distilled water was added to each.

The tube contents were distilled using a Tecator Kjeltec system, 1002 distilling unit, by neutralising with 40% sodium hydroxide and steam distilling, the distillate being trapped in 4% boric acid with 2% methyl red indicator (0.016% methyl red (Fisons) / 0.083% bromocresol green (Fisons) in ethanol). This was titrated with hydrochloric acid (0.1M or 0.05M).

The nitrogen content of the sample was calculated using the following formula:

$$\%\ \text{nitrogen (in fresh sample)} = \frac{14 \times [\text{sample titre(ml)} - \text{blank titre(ml)}] \times M^{*}}{\text{Weight of sample (g)} \times 10}$$

* Molarity of acid used for titrations.

APPENDIX II. FORMULAE FOR CALCULATION OF TOTAL PROTEIN-BOUND 3-METHYLHISTIDINE TITRES.

A : 3-Methylhistidine concentration of hydrolysate (μg/ml).

NB. It was necessary to correct for the fact that 0.1ml of the hydrolysate was used, whereas 0.2ml of the standard 3-methylhistidine solution was used for the fluorescamine derivatisation.

B : Weight of acetone powder taken for hydrolysis (g).

C : $\frac{\text{Weight of acetone powder (g)}}{\text{Weight fresh sample used for acetone powder preparation(g)}}$

= acetone powder ratio.

D : $\frac{A}{B} \times C \times 15$ (μg/g). (15 = total volume of hydrolysate in ml).

E : Lipid content of fresh sample (g/100g).

F : Hydroxyproline content of hydrolysate (μg/ml).

G : $\frac{F}{B} \times C \times 15 \times 10^{-4} \times 7.25$ (g/100g)

= % Dry connective tissue in fresh sample.

G': $\frac{F}{B} \times 15 \times 10^{-4} \times 7.25$ (g/100g)

= % Dry connective tissue in acetone powder.

H : $\frac{D \times 100}{100 - (E + G)}$ (μg/g)

= 3-Methylhistidine titre; μg/g ff, cf, fsam.

J : Nitrogen content of acetone powder (g/100g).

K : J X C X 6.25 (g/100g).

= % Protein content of fresh sample.

$$M : \frac{A}{(0.667 \times B) \times J - (G' \times 0.16)} \quad (mg/g)$$

= 3-Methylhistidine titre; mg/g nc N.*

* The calculation of connective tissue nitrogen was based on a nitrogen to connective tissue factor of 6.25 (Lord and Swan, 1984). Although lower factors have been suggested (eg. 5.8, Harrison, Personal Communication), their use would have very little effect on calculated values.

APPENDIX III. DATA FROM COMPOSITIONAL ANALYSIS.

SAMPLE	PERCENTAGE IN FRESH SAMPLE*			
	LIPID	DRY CONNECTIVE TISSUE	PROTEIN	MOISTURE
Bovine:				
L.dorsi	7.01	0.68	17.09	70.99
Semimembranosus	3.48	0.71	17.54	73.47
Sternomandibularis	4.00	1.57	15.60	74.14
Masseter and Malaris	2.12	2.64	15.70	76.26
Aponeurosis	6.11	0.51	15.99	72.10
Diaphragm	9.13	1.01	16.47	69.49
Ovine:				
Cheek	2.14	1.67	17.35	76.99
Diaphragm	3.82	0.69	15.54	77.30
Porcine:				
Neck	2.28	0.37	15.33	76.41
Shoulder	1.74	0.48	15.58	75.92
Cheek	4.80	1.82	16.80	74.89
Diaphragm	3.33	0.78	16.22	76.67
Cod	0.17	0.08	16.41	79.79
Redfish	3.63	0.24	15.57	76.91
Red Snapper	0.08	0.50	16.90	79.98
Bovine:				
Heart	3.58	0.75	15.00	76.46
"Tripe"	0.30	4.60	6.35	93.80
Kidney	5.79	1.70	13.69	75.54
Liver	1.36	0.10	15.99	69.84
Tongue	1.47	2.70	17.34	77.67
Porcine:				
Melts	13.41	1.20	12.93	68.27
Soya:				
TVP	1.17	(1.10)**	45.69	8.42

* All values are the means of at least 2 determinations.
** Apparent connective tissue content.

PUBLICATIONS.

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