OCCURRENCE, MEASUREMENT AND ORIGINS OF GELATINE COLOUR AS DETERMINED BY FLUORESCENCE AND ELECTROPHORESIS.

by

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Submitted to the Faculty of Biological and Agricultural Sciences.
(Department of Food Science)
University of Pretoria

In partial fulfilment of the requirements for the
Degree of Ph.D (Food Science).

PRETORIA

1995

ACKNOWLEDGEMENTS.

The author wishes to thank to the following:

° Ann my wife without whose tolerance and encouragement this would not have seen the light of day.

° Leiner Davis Gelatin (South Africa), Krugersdorp, for partial sponsorship of this study and for allowing the author time in which to complete the work.

° My coworkers at DGI, Krugersdorp, who helped with laboratory analyses and who accepted my absences without complaint.

° The Wheat Board, Pretoria, for the generous use of their laboratory facilities and equipment.

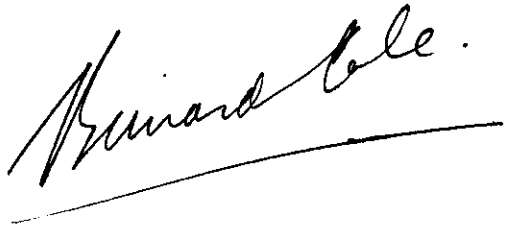
° The Department of Agriculture, Plant Protection Research Institute, Pretoria, for making their fluorospectrophotometer available for use.

° The Department of Microbiology and Plant Pathology for making equipment available to the author.

° The Animal and Dairy Sciences Research Institute for the provision of hides from animals of known age.

° Prof JJ Roberts for his advice and direction as supervisor during the course of this investigation.

I declare that the thesis herewith submitted for the degree of Ph.D (Food Science) at the University of Pretoria, has not been submitted previously by me for a degree at any other University.



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Summary.

It was known that gelatine produced from bovine hide was darker in colour than that produced from competitive raw materials like pigskin or ossein (demineralised bone). It was also known that the spectrophotometric measurement of the colour of gelatine solutions gave results that were in poor agreement with the subjective visual assessments of colour. The objectives of this study were to define or identify the parameters responsible for the production of unwanted dark colour. It was then necessary:

(i) to elucidate why there was a poor correlation between the spectrophotometric absorbance and visual colour
and
(ii) to develop a method that would allow the objective instrumental measurement of gelatine colour which would be in good agreement with the visual assessment of colour.

Variations in the lime-sulphide conditioning process and breed were found to have little or no effect on the colour of gelatines produced from bovine hide raw material. Colour was found to be mainly a function of animal age with good correlations between animal age and overall colour and animal age and first extract colour.

The problem of gelatine colour measurement was found to be one of variable light scatter due to molecular mass and imperfect filtration. The initial solution of the problem was sought in the enzymic proteolysis of the gelatine to a constant low molecular weight profile followed by filtration to standard clarity using membrane filtration. Good correlation between visual and instrumental colours was achieved when the origin of the absorbance curve was taken as the 700 nm absorbance instead of as the solvent blank. A

prerequisite of the method was that at least two enzymes were necessary to achieve hydrolysis of all normal gelatines with pHs' in the range of 4 to 8. Extension of the study to the BYK-Gardner Tristimulus Reflectance Spectrophotometer showed that gelatine solution colour could be measured by this instrument with even better reliability than with the single beam spectrophotometer. Intrinsic to the BYK-Gardner instrument's operation was a large amount of light scatter. It was found that as long as the scatter by the gelatine solution was small in comparison to the intrinsic scatter, the response of the instrument was proportional to colour. Hence, the colour of 6.67% gelatine solutions (from the Bloom gel strength determination) with a clarity of better than 80 NTU could be measured satisfactorily over a range of colours from almost colourless to dark amber. The correlation coefficient between visual and instrumental colour was 0.96.

Gelatine overall colour was found to be well correlated with animal age and it was proposed that the origin of the colour was probably the Maillard reaction *in vivo*. It was known from the literature that there was a senescence related (335/385 nm) fluorescent cross-link "pentosidine" that was formed in collagen. The hypothesis was that if this cross-link survived the gelatine manufacturing process then it could well be responsible for the colour of gelatine. A range of gelatines from the study of the origins of colour were subjected to analysis in a fluorospectrophotometer and it was found that gelatine did exhibit the pentosidine fluorescence. Furthermore, the fluorescence intensity was well correlated with gelatine colour and animal age for the paler top quality gelatines but not well correlated with the colour of low quality (darkest) gelatines. From this it was concluded that there were at least two causes of gelatine colour only one of which was related to the Maillard reaction. In addition, it was found that anion exchange resin absorbed a marked amount of the non-Maillard colour. The Maillard reaction with gelatine was further studied by reacting gelatine with glucose and ribose at pH 6 and pH 9 and measuring the development of colour and fluorescence and the change in pH with time. It was ascertained that the colour produced with glucose was identical to the natural colour of gelatine whereas the colour produced by ribose was markedly redder than the natural colour of gelatine. This indicated that *in vivo* the source of aldose for the Maillard reaction formation of pentosidine was in fact glucose and not ribose although this meant that one carbon atom from glucose had to be removed in the process. The fluorescent pyridinoline collagen cross-link was found only in gelatine derived from calf skin by the "acid conditioning process". This lead to the conclusion that this cross-link was labile in alkali thus explaining the extractability phenomena encountered during the investigation of the

occurrence of dark gelatine.

The gelatines from the hides of animals of various ages were subjected to SDS-PAGE electrophoresis from which it was shown that gelatines containing the most intact collagen α chain subunits were the palest, in line with the conclusion that the most easily converted (denatured) collagen gave gelatine of the best colour. This also confirmed that collagen cross-links were a source of colour. Furthermore, this study demonstrated that the role of sodium sulphide in lime-sulphide conditioning was to accelerate the hydrolysis of the alkali labile cross-links in collagen but it did not have any additional conditioning effect nor was there any evidence of sulphide having any effect on the colour of the gelatine produced.

UNIVERSITEIT VAN PRETORIA
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VOORKOMS, METING EN HERKOMS VAN DIE KLEUR VAN GELATIEN SOOS BEPAAL MET FLUORESSENSIE EN ELEKTROFORESE.

deur

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Opsomming.

Dit is bekend dat gelatien geproduseer vanaf beesvelle donkerder van kleur is as dié wat vanaf kompeterende rou materiale soos varkvel of osseïen (gedemineraliseerde been) geproduseer is. Dit is ook bekend dat die spektrofotometriese meting van die kleur van gelatienoplossings resultate lewer wat swak vergelyk met die subjektiewe visuele beoordeling van kleur. Die doelwitte van hierdie studie was om die parameters verantwoordelik vir die ongewenste donker kleur te definieer of te identifiseer. Dit was voorts nodig om:

(i) te verduidelik waarom daar 'n swak korrelasie was tussen die spektrofotometriese absorbansie en sigbare kleur, en

(ii) 'n metode te ontwikkel wat die objektiewe instrumentele meting van die kleur van gelatien moontlik sou maak en wat goed sou ooreenstem met die visuele beoordeling van kleur.

Variasies in die kalksulfiedkondisioneringsproses en beesras het baie min of geen effek gehad op die kleur van gelatien wat vanaf beesvel geproduseer is nie. Dis gevind dat kleur hoofsaaklik 'n funksie van die ouderdom van die dier was met goeie korrelasies tussen die ouderdom van die dier en globale kleur, en tussen ouderdom van die dier en kleur van die eerste ekstrak.

Die probleem van die meting van die kleur van gelatien was toe te skryf aan die variërende ligverstrooiing as gevolg van molekulêre massa en onvoldoende filtrasie. Die aanvanklike oplossing vir die probleem is gesoek by die ensimatiese proteolise van die gelatien na 'n konstante lae molmassaprofiel gevolg deur filtrasie tot 'n standaard helderheid deur middel van membraanfiltrasie. Goeie korrelasie is verkry tussen visuele en instrumentele kleure as die oorsprong van die absorbansiekromme as 700 nm geneem is pleks

van om die oplosmiddel as blanko te neem. 'n Voorvereiste van die metode was dat minstens twee ensieme nodig was vir die hidrolise van alle normale gelatine met pH-waardes in die strek van 4 tot 8. Uitbreiding van die studie na die BYK-Gardner Tristimulus Refleksiespektrofotometer het getoon dat die kleur van gelatienoplossings met hierdie instrument gemeet kon word met selfs beter betroubaarheid as met die enkelstraalspektrofotometer. 'n Intrinsieke kenmerk van die BYK-Gardner-instrument se werking is 'n groot mate van ligverstrooiing. Dis gevind dat, solank die verstrooiing deur die gelatienoplossing klein was as die intrinsieke verstrooiing, die responsie van die instrument proporsioneel was met die kleur. Gevolglik kon die kleur van 6.67% gelatienoplossings (van die gel sterkte bepaling) met 'n helderheid beter as 80 NTE bevredigend gemeet word oor 'n bestek van kleure van feitlik kleurloos tot donker amberkleurig. Die korrelasiekoëffisiënt tussen visuele en instrumentele kleur was 0.96.

Die globale kleur van gelatien het goed gekorreleer met die ouderdom van die dier en dis voorgestel dat die oorsprong van die kleur waarskynlik die Maillard-reaksie *in vivo* is. Uit die literatuur is dit bekend dat daar 'n verouderingsverwante fluoresserende (335/385 nm) kruisbinding-"pentosidien" in die kollageen gevorm word. Die hipotese was dat as hierdie kruisbinding die gelatienvervaardigingsproses sou oorleef, dit waarskynlik vir die kleur van die gelatien verantwoordelik kon wees. 'n Reeks gelatine van die studie oor die oorsprong van die kleur, is ontleed met 'n fluorospektrofotometer en dis gevind dat gelatien wel die pentosidien-fluoressensie toon. Daarbenewens het die intensiteit van die fluoressensie sterk gekorreleer met die kleur van die gelatien en ouderdom van die dier in die geval van die bleker topgehalte gelatine, maar met die kleur van die swak gehalte (donkerste) gelatine was die korrelasie maar swak. Hieruit is afgelei dat daar minstens twee oorsake vir die kleur van gelatien is en dat slegs een daarvan verbandhou met die Maillard-reaksie. Daarbenewens is gevind dat anioonuitruilhars 'n aansienlike hoeveelheid van die nie-Maillardkleur geabsorbeer het. Die Maillard-reaksie met gelatien is verder bestudeer deur gelatien te laat reageer met glukose en ribose by pH 6 en pH 9 en die ontwikkeling van kleur en fluoressensie, en ook pH-verandering met tydsverloop, te meet. Dis vasgestel dat die kleur wat deur glukose geproduseer is, identies was aan die natuurlike kleur van gelatien terwyl die kleur wat deur ribose geproduseer is, opvallend rooier was as die natuurlike kleur van gelatien. Dit dui daarop dat die *in vivo* bron van aldose vir die Maillard-reaksiegevormde pentosidien inderdaad glukose was en nie ribose nie, alhoewel dit beteken dat een koolstofatoom van glukose in die proses verwyder moes word. Die fluoresserende piridinolien-kollageenkruisbinding is slegs in gelatien gevind wat met die

"suurkondisioneringsproses" uit kalfsvel verkry is. Dit het gelei tot die gevolgtrekking dat hierdie kruisbinding labiel was in alkali en dat dit die ekstraheerbaarheidsverskynsel, wat tydens die ondersoek na die voorkoms van donkerkleurige gelatien teegekom is, verklaar.

Die gelatine van die velle van diere van verskillende ouderdomme is onderwerp aan SDS-PAGE-elektroforese waarmee aangetoon is dat gelatine wat meeste ongeskonde kollageen-α-ketting subeenhede bevat het, die bleekste was, dit wil sê in lyn met die gevolgtrekking dat die kollageen wat maklikste verander (gedenatureer) word, gelatien met die beste kleur gee. Dit bevestig ook dat kollageenkruisbindings 'n bron van kleur was. Hierdie studie het verder getoon dat natriumsulfied se rol in kalksulfiedkondisioneringsprosesse die versnelling van die hidrolise van die alkali-labiele kruisbindings in die kollageen was maar dat dit nie enige verdere kondisioneringseffek gehad het nie en dat daar geen getuienis was dat sulfied enige effek op die kleur van die gelatien gehad het nie.

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List of abbreviations.

3Y, 5Y, 6Y	Series of experiments on the hide of 3, 5 and 6 year old animals.
√	square root.
ADSRI	Animal and Dairy Sciences Research Institute. (Irene, RSA)
BGGRA	British Gelatine and Glue Research Association.
Bloom	Bloom gel strength.
BS	British Standards.
ca.	(circa) approximately.
CALF-A	Calf skin used for making Type A gelatine.
CT	Series of experiments to show the effect of conditioning time.
CTO	Series of experiments to show the effect of conditioning time on old animal hide.
DGI	Davis Gelatine Industries (Pty) Ltd. Later known as Leiner Davis Gelatin (South Africa).
Da.	Dalton - unit of molecular mass.
et al.	*et alia* - and others.
Exp.	Experiment.
GAG	Glucoseaminoglycan.
GGRA	Gelatine and Glue Research Association.
GF/GR	Green face pieces and green hide from the rest of the animal.
IAPI	Irene Animal Production Institute. (Irene, RSA) (Same as ADSRI)
INO	Experiments on the hide of an old Inguni animal.
JSLTC	Journal of the society of leather technologists and chemists.
KTO	Series of experiments on an old animal hide after dehairing at Krugersdorp Tannery.
LIRI	Leather Industries Research Institute. Rhodes University. Grahamstown.
loc. cit.	*loco citado* - Here in.
RSA	Republic of South Africa.
SDS	Sodium dodecylsulphate.
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis.
SEM	Scanning electron microscopy.
Sig.	Significance.
SF/SR	Salted face pieces and salted hide from the rest of the animal.
Soln.	Solution.

SPA	A process for the conversion of chrome tanned leather into gelatine.
ST	Series of experiments to show the effects of sodium sulphide concentration and time of conditioning.
Str.	Strength.
TEMED	N,N,N',N',Tetramethylethylenediamine.
Tris	Tris-(hydroxymethyl) methylamine.
VS	Volatile solids.
WT	Series of experiments conducted during winter to investigate the effect of temperature on the conditioning of hide.
YS	Series of experiments to show the effect of sulphide concentration and time of conditioning on young animal hide.

CHAPTER ONE. Introduction and literature review.

GELATINE AND ITS USES.

Gelatine is a protein derived from the natural mammalian protein, collagen, which is most abundant in connective tissues. According to Siebert (1992) the world consumption was estimated at 200 thousand tons per annum with a growth rate of about 2% per annum. Although gelatine was used in a very wide range of products, some two thirds of the production was used in the food sector of the economy where it was considered to be an ingredient rather than an additive, and of the food sector, some 70% of the usage was for confectionary (sweets).

Gelatine had a wide range of properties which could be exploited in foods. The most important property was that of reversible gelation at about blood temperature (37°C) which gave products "melt in the mouth" characteristics accompanied by excellent flavour release. Associated with gelation was the property of water binding. Gelatine gels were formed at about 2% concentration in water hence gelatine could bind up to 50 times its own weight of water. This property was used particularly in canning and in cake and pie fillings. Being a high molecular weight polymer gelatine could be used to impart body to soups. Gelatine, having positive and negative charges on the molecule, was amphoteric and had hydrophilic and hydrophobic areas on the polymer chain so it had emulsifying properties which complemented the gelation properties when it was used as a stabiliser in low fat spreads and the manufacture of toffees. The emulsifying properties of gelatine extend beyond oil and water and one of the main uses was as a foaming agent - air in water emulsion - where small quantities (2 - 3%) were used to generate and stabilise the foam of marshmallows and similar products. In yoghurt gelatine complexed with casein to overcome the problems of syneresis (Modler and Kalab, 1983). The oldest use of gelatine was as an animal glue. Its adhesive properties could be used for sticking together the layers of "liquorice all-sorts" as well as in pharmaceutical tableting. One property of gelatine that was partly surpassed by superior products was that of film former. However, that property could be coming back into use in the microencapsulation of flavours and vitamins. The edible film forming property of gelatine had long been utilized

in the manufacture of both hard and soft pharmaceutical capsules and the property was essential in forming the light sensitive film on an acetate or paper backing used in photography. The one property of gelatine that was not used in the food industry was that of flame retarder which was most important in the use of gelatine for match heads (Ward and Courts, 1977).

RAW MATERIAL FOR GELATINE MANUFACTURE.

As diverse as were the uses of gelatine, so were the sources from which it was obtained. Gelatine was traditionally manufactured from materials rich in Type I collagen which were "ossein" from degreased, demineralized bone and from the skin of pigs, bovines, and in China, donkeys. To a lesser extent sheep, goat and game skins were used in the manufacture of gelatine and recently, due to the Kosher implications, fish skin gelatine was gaining some prominence in the market place. In all cases the raw material for gelatine was waste material unwanted or unsuitable for more profitable uses. For example, the gelatine manufacturer received the skin from the head, legs and tails of bovines (2.5 to 5 kg per animal), while the rest of the hide which could vary between 20 to 36 kg per animal (Forrest, Aberle, Hendrik, Judge, and Merkel, 1975a) was sold at a much higher price to the leather manufacturer. Pigskin in excess of requirements for food manufacture (sausages) and leather was disposed of to the gelatine manufacturer. The implication was that in some instances the gelatine manufacturer, as a waste disposal industry, did not have much control over what was received as raw material.

The range of sources of raw material was further complicated by the fact that all skins could be fresh off the animal, or preserved by drying, salting, or other treatment. In the case of material of bovine origins, the gelatine manufacturer had to handle fresh hairy hide from the abattoir, salted hide from more distant sources, hide subjected to short term preservation using sodium metabisulphite, frozen sinews, dry hide and hide that had been pretreated in a tannery with lime and sodium sulphide, fleshed and then split to the required thickness. Unwanted corium (splits) and trimmings were used for gelatine manufacture and could be received either direct from the tannery or preserved by drying. Furthermore; a low grade gelatine could be recovered from chrome tanned leather waste using the SPA process (Drew, 1930). Under the circumstances it was not surprising that detailed analytical data on the precise nature of the raw material used in gelatine manufacture was not

considered of importance, hence its unavailability. However some proximate analyses would be forthcoming from this study.

This study, was primarily concerned with the colour of gelatine derived from wet salted bovine hide (cowhide) using a lime-sulphide conditioning process.

COLLAGEN.

According to Bailey and Light (1989a) and Forrest, Aberle, Hendrik, Judge, and Merkel (1975b) collagen, which was the protein from which gelatine was derived, was the most abundant protein in the animal body and constituted 20-25% of the total protein. It was the principal structural protein of connective tissue and was a major component of tendons, ligaments, bones and cartilage. It was classified as a glycoprotein by Forrest et al. (1975b) as it contained a small amounts of galactose and glucose. Collagen was characterized by the fact that one third of it was composed of glycine and another third of proline or hydroxyproline. It was also the only protein, besides elastin, to contain hydroxyproline (13-14%) and the amount of collagen or gelatine in a product or tissue could be reliably estimated from the hydroxyproline content. The structure and development of collagen was well covered in these as well as a number of other texts like Swatland (1985). Collagen was the subject of much current research mainly in the field of medicine, as collagen defects lead to many diseases (Bailey, 1992 or Igarashi, Uzuka and Nakajima, 1989). Knowledge of collagen chemistry had led to the mitigation of some of these effects, such as the treatment of the accelerated ageing in diabetes (Ulrich and Cerami, 1992). Furthermore, Bailey (1994) in a lecture to the Northampton group of the Society of Leather Technologists and Chemists was reported as saying that "if collagen was not hydroxylated, using vitamin C, scurvy resulted, (Peterkofsky, 1991), and that other problems could occur if collagen was not formed correctly, such as the hypermobile joints of the so called India rubber men in circus acts".

Collagen was a family of large extracellular proteins of closely related molecular structure. It was composed of three polypeptide chains (called α-chains) each of which contained long sequences of repeating tripeptides based on the general structure Gly-X-Y, where X was commonly proline and Y could represent any amino acid but was often the modified amino acid hydroxyproline. All collagens also contained varying quantities of the amino acid hydroxylysine which was formed by the enzyme-catalysed hydroxylation of lysine

residues at C5 in newly synthesised α-chains. Many of the hydroxylysine residues were subsequently glycosylated during intracellular processing and contained O-linked β-D-galactopyranoside or β-D-glucopyranosyl-β-D-galactopyranoside. The individual polypeptide α-chains were unstable but when three chains were wrapped round each other they formed a very stable right-handed triple helix characteristic of collagen (Bailey and Light, 1989b).

In order for the collagen triple helices to form fibres it was necessary that the helix be further stabilised by inter- and intramolecular cross-links. Most of the cross-links had been shown to follow the oxidative deamination of lysine or hydroxylysine by the enzyme lysyl oxidase. This enzyme replaced the ε-amino group of lysine with an aldehyde group to produce lysine aldehyde commonly called allysine. Adjacent aldehyde groups in the N-terminus telopeptide region of collagen α-chains had been shown to form aldol links between chains thus increasing the stability of the triple helix (Bailey and Light, 1989c).

In order to give stability to the collagen fibre, intermolecular cross-links were necessary. An example of this was the reaction of allysine in the telopeptide of one collagen helix with an ε-amino group of hydroxylysine (residue 103 or 946 of the α-chain) in the overlaps with an adjacent helix to form dehydro-hydroxylysinonorleucine (dehydro-HLNL). In the case of extensive hydroxylation of lysine the double bond formed between lysine and aldehyde could undergo the Amadori rearrangement. The C=N bond became a C-N bond while the adjacent C-OH became C=O giving the oxo-imine hydroxylysino-5-oxo-norleucine (HLONL). Both HLONL and dehydro-HLNL could be reduced by sodium borohydride to give acid stable products which had been isolated by chromatography. (Bailey and Light, 1989c)

With age, mature collagen matrices became progressively stronger and more rigid and the quantity of sodium borohydride reducible cross-links was reduced. It had been suggested that histidine combined with and stabilised dehydro-HLNL cross-links. Similarly in hydroxylysine rich collagens, hydroxylysine aldehyde reacted with the C-N bond of an oxo-imine cross-link to form the fluorescent pyridinoline trivalent cross-link between α-chains. As skin Type I collagen was not rich in hydroxylysine it was said that the fluorescent mature pyridinoline cross-link was not found in skin but was only found in Type I collagen from bone. (Bailey and Light, 1989c). Sell and Monnier, (1989) had shown the formation with age of a non-enzymic cross-link

in collagen producing the fluorescent product pentosidine. This linked the amino acids lysine and arginine by means of a 5-carbon chain derived from a sugar.

From the gelatine manufacturer's point of view the variation in the response of hide collagen to chemical pretreatment could be loosely attributed to variations in the cross-linking of the collagen. However, other than from the study of Reich, Walther, and Stather (1962b) there was no precise information on the effect of animal age and collagen cross-linking on the manufacturing process or the quality of gelatine produced. It was known that the divalent cross-links dehydro-HLNL and HLONL were acid labile (Bailey and Light, 1989c) but more important to the bovine hide gelatine manufacturer would be to know what links were alkali labile.

RAW MATERIAL COMPOSITION.

The constituents of cowhide and gelatine raw materials were variable as indicated below:

1. According to Bailey and Light (1989d) the collagen content of skin was approximately 50% (with 5% elastin) whereas tendon contained 95% collagen (based on dry masses).

2. According to Cooper, Russel, Shuttleworth and Boast (1984) cowhide consisted of 65% moisture and 35% dry substance. Salted hide consisted of 44% moisture, 14% salt and 42% hide substance. During salting there was a byproduct, brine, derived from the moisture in the hide which contained some salt soluble organic matter.

3. In the twentieth Proctor Memorial Lecture, Haines (1984) reviewed the subject of "The skin before tannage-Proctor's view and now." This review dealt with the micro-structure of skin as revealed in SEM micrographs and how this was affected by animal breed and age. Scott (according to Haines, 1984) had shown that the proteoglycan, dermatan sulphate was distributed over the surface of collagen fibrils in a regular and highly organized manner. Dermatan sulphate had a marked influence on collagen stability and its removal by liming was accompanied by a reduction in shrinkage temperature. A schematic diagram of the cross-section of the epidermal / dermal boundary of skin

showing 9 layers was given. This included layers of keratinous cells of the epidermis, layers of hyparin sulphate, laminin, and Types III and IV collagen in the "basement membrane". The latter, were all outside the dermis proper and it was apparent that the Type III collagen was responsible for the smooth grain layer of leather (Thompson, 1988 and Bailey, 1992).

4. Bowes, Elliot and Moss (1957) presented the constituents of fresh skin as:

Water 60-65%
Protein 30-35% - excluding hair.
Grease 1-10%
Carbohydrate (Sugars) 1%
Mucopolysaccharide 0.5-1%

Bowes, Elliot and Moss (1958) studied both acid and alkaline extracts of calf skin. Acid extraction yielded, besides acid soluble collagen which could be precipitated by salt, at least two other proteins. Furthermore, there were "plasma proteins" extractable using 0.1M phosphate buffer. Based on their study, calf skin also contained non-collagenous proteins soluble in alkali at 4° to 20°C. In summary, they showed that calf skin contained plasma proteins (8-10%), other non-collagenous proteins (10-15%) and collagen (75%). The amounts of hexosamine and hexose in the alkali extracts were relatively small and the greater part of the material extracted by alkali was non-collagenous protein rather than polysaccharide. "Of the 30-35% protein about 4-6% was made up of the interfibrillar proteins such as albumins and globulins of the tissue fluids, about 0.5-1% represented the epidermis and there were small amounts of muscle protein, elastin and reticulin.....but by far the greater part of the protein was collagen (90-95%)".

5. Shirai and Wada (1981) found that on liming pigskin, about 7.5 % of the protein as nitrogen was dissolved after 60 days. After 7 and 14 days, amino acid analysis of the protein in solution showed it to be different from collagen in that extraordinarily large amounts of tyrosine and low amounts of hydroxyproline were found. Thereafter the amino acid analysis was closer to that of collagen. The non-collagenous protein was estimated at 1% of total nitrogen and was shown to contain

glucosaminoglycan or acid mucopolysaccharide and to be of a molecular size far smaller than the α-chain released from collagen.

6. Komanowsky (1989) stated that "hide" contained at least 0.8% glucosaminoglycan (GAG) chondroitin sulphate in addition to about 0.5% hexose which was responsible for the cross-linking of hide during drying (particularly at high temperatures). Furthermore, there were indications that hyaluronic acid (a polymer of a repeating disaccharide unit of glucuronic acid and N-acetyl glucosamine) in salted hides was converted enzymically into smaller polysaccharides which exposed additional carbonyl groups for cross-linking reactions.

Ultrafiltration trials using a 2000 Dalton (unit of atomic mass) nominal cut-off membrane, were conducted at Davis Gelatine Industries (Cole, 1988) on spent conditioning liquor containing 1.5% ash and 1.5% organic matter. The liquor was concentrated to 9.5% solids and this concentrate contained 65% of the organic matter in the original solution. After drying, this organic matter was insoluble in water and it was concluded that spent conditioning liquor contained mainly non-collagenous protein which was denatured and rendered insoluble by drying at up to 60°C. It was felt that the findings in this study confirmed the findings of Bowes *et al*. (1958) that most of the low temperature alkali soluble proteins from skin were non-collagenous as long as the pH did not exceed 12.7 and the temperature did not go above 20-25°C. In this instance, the alkaline liquors contained sodium sulphide, hence the products of the hydrolysis of keratin (hair) must have also contaminated the alkali soluble proteins from the skin.

THE MANUFACTURE OF GELATINE.

This subject was covered in the literature (Bailey and Light, 1989d; Hinterwaldner, 1977; Glicksman, 1969; Veis, 1964a). There were essentially two main types of gelatine: Type A and Type B. Type A gelatine was produced by the acid process in which the conditioning function of the acid was simply one of pH adjustment to that required for extraction (Reich, Walther and Stather, 1962a). Type B gelatine was the product of an alkali conditioning process which largely hydrolysed the glutamine and asparagine residues to glutamic and aspartic acids. This resulted in a drop in iso-ionic pH from about 9 to about 5 (Veis, 1964b). In the traditional alkali process the conditioned material was extracted at a "neutral" pH of about 6. Variation of the extraction pH had

a dramatic effect on the relationship between gel strength and viscosity and this could be used to produce a gelatine most suited to its application. (Fysh and Goodwin, 1955).

The details of the laboratory manufacturing procedure follow later.

THE COLOUR OF GELATINE.

Commercial gelatin in solution had a colour varying from a very pale yellow to dark amber. The same variation was observed with the dry material but the particle size influenced the apparent dry colour.

It was accepted at Davis Gelatine Industries (DGI) that gelatines derived from pigskin or ossein (demineralised bone) had paler colours than those derived from bovine hide. Furthermore, tannery waste from bovine hide was recognised as yielding a paler gelatine than the whole hide. In gelatine derived from whole hide, there was found to be also a large variance in gelatine colour which was correlated with the other quality parameters of gel strength and viscosity. There was, in addition, a smaller random variance in the colour of gelatines with the same gel strength and viscosity. The availability of indigenous raw material dictated that in South Africa gelatine was made largely from the whole hide.

Although not a "functional property" of gelatine, the presence or absence of colour affected its use in many applications. Generally gelatine colour was an unwanted attribute in confectionary both because lack of colour was associated with "purity" and because it was impossible to make colourless articles with a yellow to amber coloured gelatine. Also, it stood to reason that variable colour militated against the production of a uniform product or the variation in results obtained with added colourants would be minimised if the gelatine was colourless. Colour was also important in some other uses of gelatine as in the production of art papers and string where gelatine was used as a size and in the production of decorative picture frame mouldings. For these reasons bovine hide gelatin could find itself disadvantaged in the market place. Hence, it was decided to try and approach the matter systematically.

Saunders and Ward (1953) studied the measurement of gelatine colour and made some suggestions for the instrumental measurement of colour but they had not

correlated their instrumental values with visual colour measurements. Blake and Plaster (1950) in a series of experiments involving heating gelatine solution with sugars at 134°C, found that colour was produced and therefore they suggested that the colour of gelatine could be as a result of protein/sugar reaction *in vivo*, however, they thought that lactose was the most likely sugar to be involved and they said that "if sugars were removed from raw material then the resulting gelatines would be better in colour ...."

From a comprehensive literature search it was found that most references covered the colouring of products containing gelatine (Hegasy and Winter, 1987) and the effects of gelatine on the colour of beverages due to the fining effects of gelatine (Weissenbach, 1985). One or two references mentioned colour as an attribute of gelatine. These were by the employees of a European manufacturer who made their readers aware of the perceived advantages of pigskin gelatine (Hoffmann, 1984). Otherwise there was no indication that gelatine colour had been a subject of study either from the point of view of its origins or its measurement.

At the start of this study the thinking with regard to gelatine colour was:

1. DGI production statistics showed that paler gelatines were produced at the start of a production cycle at the lowest extraction temperatures. These gelatines also had the best Bloom gel strength and viscosity. The darkest gelatines were produced at the end of a production cycle at the higher extraction temperatures. These gelatines also had the lowest Bloom strength and viscosity.

2. Gelatine produced from chrome tanned leather by the SPA process (Drew, 1930), at relatively high temperatures, had very low Bloom strength and viscosity and it was almost colourless. The results of Heidemann (1982) showed that chromium was attached exclusively to the aspartic and glutamic acid side chains of collagen. In the SPA process the chromium-collagen complex did not dissolve hence the aspartic and glutamic acid groups must have been in some way associated with gelatine colour.

3. Neutralisation with sulphurous acid after alkali conditioning produced gelatine of better colour than processes using sulphuric or hydrochloric acid, prior to extraction.

4. An enzymic conditioning process seemed to produce darker gelatine even with the sulphurous acid acidulation process.

5. Raw material soaked in hydrogen peroxide solution after alkaline conditioning and before acidulation gave a gelatine of better colour. The cost was high and the effect was variable.

6. Gelatine solution (liquor) passed through an anion exchange column exhibited variable colour reductions.

7. From the little data available in the author's M.Sc. thesis, (Cole, 1986), there was an indication that animal age could play a significant role in gelatine colour.

8. Investigations done at DGI, Cole (1983, 1983-1984, 1986, 1988) had shown that, in twinned experiments (duplicate experiments on raw material that had been carefully halved to eliminate raw material as a variable), wet salted hide stored for 1 month exhibited a small decrease in extractability and an increase in gelatine colour which was not considered significant. However, wet salted hide stored for 12 months in a sealed plastic bag exhibited decreased extractability from 24% to 16% at 50°C and increased gelatine colour from 6.4 to 11.2 for the first extraction. In a parallel experiment, wet limed splits showed a small improvement in gelatine colour accompanied by a drop in extractability but the material had been allowed to dry out because wet splits did not have an adequate shelf life.

9. Iron contamination of gelatine had been shown to have an adverse effect on colour (Williams, 1957), but as current iron contents of gelatine were less than 50 ppm this was not considered to be a factor in the colour of gelatine today. An experiment was conducted to re-establish the effect of iron contamination on colour. Chromium was also known to be a possible cause of colour but the colour was greenish whereas the colour of gelatine was yellowish to brown. Also from regular analyses, because of the use of chrome tanned leather as a raw material, it was known that chromium levels in gelatine were below 5 ppm at which level any colour produced by chromium was not visible. From experience it was known that the colour produced by 200 ppm Cr as $Cr_2(SO_4)_3$ was only just visible to the eye.

There were many subjective observations on which theories with regard to gelatine colour were based, but there was a lack of scientific investigation to verify these theories:

(a) One of the more plausible theories was a relationship between the observation of dark coloured fat during extraction and dark gelatine production. Dark fat could be due to breed or to the oxidation or decomposition of glycerides with the production of peroxides. If the latter was the cause of dark fat then the Maillard type of reactions with the protein could be envisaged. Dark fat due to breed, age or nutrition was believed to be due to the accumulation of a derivative of carotene (lutein - Xanthophyll). Because carotene was fat soluble, it was not considered a likely source of colour in gelatine.

(b) A second possible source of gelatine colour could be the melanocytes associated with the hair follicles which were part of the dermis, (Lyne and Short, 1965). These would not be lost during conditioning depilation. The melanin pigments causing skin pigmentation were found above the "basement membrane". If not completely removed by liming, they could also be a source of gelatin colour, particularly, in lower quality gelatines which were derived at higher temperatures. At these temperatures the complete disintegration of the skin occurs. On the other hand melanin pigments were water insoluble polymers (Brady, Duncan and Russell, 1989), hence their effect on the colour of a water soluble polymer like gelatine should be negligible.

OBJECTIVES.

The reason for undertaking this study was the fact that the colour of gelatine produced from bovine hide was dark in comparison to competitive gelatines produced from different raw materials and it was necessary to know the parameters that were responsible for the dark product. The primary objectives were to determine the parameters influencing the occurrence of gelatine colour and to overcome the problem of the instrumental measurement of colour. Thereafter, the gelatines produced from precisely known origins and by known methods were studied by fluorescence spectrophotometry and by electrophoresis in order to try to determine on the molecular origins of gelatine colour.

1. The origins of gelatine colour.

Gelatine colour had always been a concern to DGI and continuous efforts to try to improve colour, had resulted in few significant achievements. In most cases improvements had been small and not always reproducible. It was felt that in view of the little that was known about gelatine colour, the first requirement was to establish why gelatines from different sources exhibited different colours. With this knowledge it was possible that efforts at colour improvement could be made systematically or logically rather than in the random or *ad hoc* approaches of the past.

Firstly, it was necessary to determine how to produce dark coloured gelatine on a consistent basis. (It might appear trite to say it, but it was often forgotten that if one wanted to examine the effect of any process modification on colour one needed to know that the unmodified process would produce a coloured product, or in other words, taken to absurdity, one could not improve the colour of a colourless product). The essence of the problem was not only the production of dark gelatine *per se* but also the variation in the colour of the palest top quality gelatine produced by a standardised process.

Secondly, it was required to establish how far the alkali conditioning process affected colour. Production gelatine colours seemed to be cyclic as was the annual change in alkali conditioning with ambient temperature, hence it seemed probable that conditioning was a factor in gelatine colour.

1.1. Fluorescence and Electrophoresis.

As the study progressed the indications were that animal age (which was accompanied by reduced gelatine extractability) was at the root of the colour problem. In addition the increased degree of collagen cross-linking with senescence, (Sell and Monnier, 1989; Uchiyama, Ohishi, Takahashi, Kushida, Inoue, Jugie and Horiuchi, 1991), was appearing repeatedly in the literature as was collagen cross-linking and meat toughness (Bailey and Light, 1989e). The presentation by Monnier, Sell, Miyata, and Nagaraj (1990), showed that collagen cross-linking, senescence and the Maillard reaction were related. This was the basis on which a reasoned theory on the cause of colour in gelatine could be developed. There was no known literature on gelatine fluorescence although much had been published on collagen by Tanaka, Avigad, Eikenberry and Brodsky (1988), Sell and Monnier (1989) and others. Hence, it

was decided to study the fluorescence spectrum of gelatine with a view to establishing whether the pentosidine cross-link described by Sell and Monnier (1989) could be present in Types A and B gelatines. If so, was the fluorescence related to colour and/or animal age?

Electrophoresis had been used successfully by Tanaka *et al.* (1988), Chalepakis, Tanay and Heidemann (1985), Koepff (1984) to study collagen and gelatine at the molecular level, hence it was decided that this technique could yield additional insights into the effects of cross-linking and animal age on gelatine colour if applied to the range of gelatines produced above.

2. Gelatine colour measurement.

The measurement of colour was usually based on the absorption of light as measured by a spectrophotometer. With gelatine the variable scatter of light, due to molecular size and imperfections in clarity, interfered with the light absorption due to colour. This resulted in there being no generally accepted instrumental method for measuring the colour of gelatine. The measurement of gelatine colour by visual comparison to gelatine standards was the current subjective method used by internal quality control staff. As this comparison method was subjective it could not be used for specification purposes. Hence one of the objectives of this study was to investigate alternative instrumental methods for the measurement of gelatine colour, bearing in mind that a high degree of correlation with the visual methods was desirable.

REFERENCES.

Bailey, A.J., and Light, N.D. 1989a. Connective tissue in meat and meat products. p. 4. Elsevier Applied Science. London and New York.

Bailey, A.J. and Light, N.D. 1989. Molecular and fibre structure of collagen. Ch. 2, In *Connective tissue in meat and meat products*. Elsevier Applied Science. London & New York.

Bailey, A.J. and Light, N.D. 1989. Fibre formation and stabilisation of collagen. Ch. 4, In *Connective tissue in meat and meat products*. Elsevier Applied Science. London & New York.

Bailey, A.J., and Light, N.D. 1989d. Connective tissue in meat and meat products. p. 239-242. Elsevier Applied Science. London and New York.

Bailey, A.J., and Light, N.D. 1989e. Connective tissue in meat and meat products. p. 180-182. Elsevier Applied Science. London and New York.

Bailey, A.J. 1992. Proctor Memorial Lecture. Collagen - Nature's framework in the medical, food and leather industries. Journal of the Society of Leather Technologists and Chemists 76: 111-127.

Bailey, A.J. 1994. Reported in a news item. Journal of the Society of Leather Technologists and Chemists 78: 126-127.

Blake, J.N., and Plaster, F.H. 1950. Colour formation in gelatin manufacture. Journal of the Society of Leather Trades' Chemists 34: 177-186.

Bowes, J.H., Elliot, R.G., and Moss, J.A. 1957. Collagen and the more soluble constituents of skin. Journal of the Society of Leather Trades' and Chemists XLI(7): 249-266.

Bowes, J.H., Elliot, R.G., and Moss, J.A. 1958. The extraction of alkali-soluble protein from calf skin. British Leather Manufacturers' Research Association Laboratory Reports 37(1): 148-162.

Brady, D., Duncan, J.R., and Russell, A.E. 1989. Wool yellowing studies: The formation of melanin-like structures in solutions of tryptophan or tyrosine during exposure to sunlight. Leather Industries Research Institute Research Bulletin No 980.

Chalepakis.Von, G., Tanay, I., and Heidemann, E. 1985. Wie spezifisch ist der kollagenabbau bei der gelatineherstellung. Das Leder 36: 2-10.

Cole, B. 1983. Enzyme conditioning of wet salted hide - SA DAVAC 9 - The effect of ageing on drying and conditioning. Davis Gelatine Industries Internal Report dated 20 July 1993. Krugersdorp. RSA.

Cole, B. 1984. The effect of ageing raw material for 1 year. Davis Gelatine Industries Internal Reports SA DAVAC 13 dated 21 September 1983. and follow-up report dated 30 October 1984. Krugersdorp. RSA.

Cole, C.G.B. 1986. A study of the properties of gelatines derived by various chemical and enzymic methods. M.Sc Thesis. University of Pretoria. Pretoria. RSA.

Cole, B. 1988. The Application of Ultrafiltration to Pit Liquor. Davis Gelatine Industries Internal Report dated 7 January 1988. Krugersdorp. RSA.

Cooper, D.R., Russel, A.E., Shuttleworth, S.H., and Boast, D.A. 1984. Closed systems for salt and saline waste water in curing and tanning. Leather Industries Research Institute Research Bulletin No 877.

Drew, R.B. 1930. An improved process for extracting glue and gelatine from chrome leather. British Patent Specification 338,584. Nov.17, 1930. His Majesty's Stationery Office. Redhill. UK.

Forrest, J.C., Aberle, D.E., Hendrik, H.B., Judge, M.D., and Merkel, R.A. 1975a. In *Principles of meat science*. Schweigert, B.S. (Ed.). p. 356. W.H. Freeman and Company. New York.

Forrest, J.C., Aberle, D.E., Hendrik, H.B., Judge, M.D., and Merkel, R.A. 1975b. In *Principles of meat science*. Schweigert, B.S. (Ed.). p. 53. W.H. Freeman and Company. New York.

Fysh, D., and Goodwin, J.W. 1955. The influence of pH on the extraction of hides and the gelatine obtainable from them. Gelatine and Glue Research Association Research Report C6. August 1955.

Glicksman, M. 1969. Gum technology in the food industry. p. 364-366. Food Science and Technology, 8. Academic Press. New York.

Haines, B.M. 1984. The skin before tannage-Proctor's view and now. Journal of the Society of Leather Technologists and Chemists 68: 57-70.

Hegasy, A., and Winter, M. 1987. Beta-Carotin enthaltende gelatine. European Patent Application EP0221277A2. [In Food Science and Technology SilverPlatter 88-01-V0291]

Heidemann, E. 1982. Newer developments in the chemistry and structure of collagenous connective tissues and their impact on leather manufacture. The Journal of the Society of Leather Technologists and Chemists 66: 21-29.

Hinterwaldner, R. 1977. Technology of gelatine manufacture. In *The science and technology of gelatine*. Ward, A.G., and Courts, A. (Ed.). p. 315-361. Academic Press. London, New York, San Francisco.

Hoffmann, P. 1984. Der einsatz von gelatinprodukten in lebensmitteln. Deutsche Milchwirtschaft. 35: 269-273. [In Food science and Technology Abstracts SilverPlatter 85-12-T0026]

Igarashi, A., Uzuka, M., and Nakajima, K. 1989. The effects of vitamin E deficiency on rat skin. British Journal of Dermatology 121: 43-49.

Koepff, P. 1984. The use of electrophoresis in gelatine manufacture. In *International Working Group for Photographic Gelatin Reports 1970-1982*. Amman-Brass, H., and Pouradier, J. (Ed.). p. 198-208

Komanowsky, M 1989. The Maillard Reaction - Its possible influence on the physical properties of leather. Journal of the American Leather Chemists Association 84: 369-380.

Lyne, A.G., and Short, B.F. 1965. Biology of skin and hair growth. pp. 252-256. Angus and Robertson. Sydney.

Modler, H.W., and Kalab, M. 1983. Microstructure of yogurt stabilized with milk proteins. Dairy Science 66: 430-437.

Monnier, V.M., Sell, D.R., Miyata, S., and Nagaraj, R.H. 1990: The Maillard reaction as a basis for a theory of ageing. In *The Maillard reaction in food processing, human nutrition and physiology*. Finot, P.A., Aeschbacher, H.K., Hurrell, R.F., Liardon, R. (Ed.). p. 393-414. Advances in Life Sciences. Birkhauser Verlag Basel.

Peterkofsky, B. 1991. Ascorbate requirements for hydroxylation of procollagen: relationship to inhibition of collagen synthesis in scurvey. American Journal of Clinical Nutrition 54: 1135S-1140S.

Reich, G., Walther, S., and Stather, F. 1962a. (Translated) On some of the laws governing the production of gelatine from pigskin according to the acid conditioning process. Deutsche Lederinstitut, Freiberg/SA. 18: 15-23.

Reich, von G., Walther, S., und Stather, F. 1962b. Über den einÆuß des alters von rinds- und Schweinshäuten auf ausbeute und eigenschaften daraus nach dem sauren aufschlußverfahren gewonnener gelatine. Untersuchungen über kollagen und gelatine IV. [The influence of the age of cattle and pigskin on the yield and the quality of the gelatines obtained after the acid conditioning process. Investigation of collagen and gelatine IV.] Deutsche Lederinstitut, Freiberg/SA. 18: 24-30.

Saunders, P.R., and Ward, A.G. 1953. The colour and clarity of gelatin and glue solutions. Journal of the Science of Food and Agriculture 4: 523-527.

Sell, D.R., and Monnier, V.M. 1989. Structure elucidation of a senescence cross-link from human extracellular matrix. Journal of Biological Chemistry 264: 21597-21602.

Shirai, K., and Wada, K. 1981. Changes in the properties of pigskin collagen by liming. Japanese Journal of Zootech Science 52(10): 819-831.

Siebert, J. 1992. Ein stoff mit vielen eigenschaften. Chemische Industrie 115: 32-34.

Swatland, H.J. 1985. Structure and development of meat animals. p. 57-63. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

Tanaka, S., Avigad, G., Eikenberry, E.F., and Brodsky, B. 1988. Isolation and partial characterization of collagen chains dimerized by sugar-derived cross-links. Journal of Biological Chemistry 236: 17650-17657.

Thompson, G.A. 1988. The Bacteriology of hide and skin preservation: Past, Present and Future. Leather Industries Research Institute Research Bulletin No 961.

Uchiyama, A., Ohishi, T., Takahashi, M., Kushida, K., Inoue, T., Jugie, M., and Horiuchi, K. 1991. Fluorophores from ageing human articular cartilage. Journal of Biochemistry (Tokyo). 110: 714-718.

Ulrich, P.C., and Cerami, A. 1992. Amino acids useful as inhibitors of advanced glycosylation of protein. US Patent 825 598.

Veis, A. 1964a. The Macromolecular Chemistry of Gelatin. p. 121-227. Academic Press-New York and London.

Veis, A. 1964b. The Macromolecular Chemistry of Gelatin. p. 194-198. Academic Press-New York and London.

Ward, A.G. and Courts, A. (Ed.). 1977. The Science and Technology of Gelatin. Ch. 10 to 12. 315-413. Academic Press. London, New York, San Francisco.

Weissenbach. M.J. 1985. Gelatine-Bierschoenung in der praxis. Brauwelt. 125: 1030-1031, 1034, 1036, 1038, 1040, 1042-1044. [In Food Science and Technology Abstracts SilverPlatter 85-10-H0200].

Williams, H.V. 1957. The effect of iron on the colour of gelatine. DGI Research Report No 32. Dated 14 March 1957.

CHAPTER TWO. The Occurrence of Dark Coloured Gelatine.

INTRODUCTION.

The aim of this study was to determine the principal factors influencing the production of dark gelatine colour.

From Davis Gelatine Industries (DGI) production statistics it was known that paler gelatines were produced at the start of each day's production. This gelatine was extracted at the lowest temperature (45-50°C) and also had the best Bloom gel strength and viscosity as determined by British Standards Institution (1975) (BS) methods. By increasing extraction temperature more gelatine could be extracted from the raw material, however, the Bloom gel strength, viscosity and colour deteriorated as shown in Table 1.

Table 1. *The variation in gelatine colour with Bloom gel strength and viscosity.*

Bloom[2] (g)	Colour (DGI[1])	Clarity (DGI[1])	Viscosity[2] (ms @ 60°C)
269	7.5	11.0	35.0
247	9.0	9.2	32.4
220	11.0	10.0	31.1
182	12.6	9.9	27.0
145	13.3	10.0	22.2
66	14.8	10.8	18.9

Bloom gel strength / colour correlation coefficient r = -0.95

[1] Colour and clarity as determined by the DGI methods.

[2] As determined by BS 757 (1975) methods.

The best gelatine colour of 7.5 was variable between 6 and 9 and the reason for the variation was not well defined. In the laboratory "splits" (pg 2) from the tannery gave the best coloured gelatine often in the range 4 to 4.8 and the reason was thought to be associated with the tannery pretreatment of the material. However, although the tannery process was well known the colour

effect could not be reproduced using the whole hide. Also, from experience, even a colour of 4 was inferior in comparison with pigskin gelatine where colours of ≤ 3.2 were not uncommon.

Based on the observation that the best bovine hide gelatine colours appeared to come from tannery splits, there was a feeling that gelatine colour must be associated, at least in part, with the alkali conditioning process. Many attempts to prove this theory had failed or the experimental results had been misinterpreted because of deficiencies in the "twinned" experiment method. In this method face pieces or masks were divided into halves and the difference between the gelatines from the two halves could be attributed to differences in the process. A critical evaluation of the results from two different twinned experiments suggested that they could not be compared as the raw materials were not identical.

In leather manufacture, the leathers from different parts of the same hide were different - thin from the belly, thick from the butt, and of intermediate thickness from other parts. However, from the gelatine manufacturer's point of view this may not be as important. Hence, a series of experiments was conducted in which the same weight of hide from faces (the normal hide used for gelatine manufacture) and the rest of the hide were processed in parallel (by identical methods) to ascertain what differences may exist in yield, extractability and gelatine properties. This study showed that hide from the same animal was in fact a uniform (invariant) source of gelatine.(Experiments GF, GR, SF, SR)

Based on this finding the following studies were undertaken:

1. The conditioning process was investigated to determine whether liming time was a factor in gelatine colour and whether variances in the concentration of sodium sulphide had an effect on gelatine colour.

2. The hide structure was investigated by processing hide in a tannery. The flesh, corium split and grain layers were recovered and converted into gelatine.

3. To investigate the correlation between animal senescence and gelatine colour, animals of various ages and breeds were examined to determine the effects of these factors.

Heavy metals as a source of colour.

Gelatine, being a protein, was an amphoteric polyvalent polymer with the ability to complex polyvalent ions. Tanning made use of the ability to complex polyvalent cations as the complexes resisted microbiological degradation. It was possible therefore, that complexed cations could be responsible for the colour of gelatine. It was known that gelatine formed complexes with aluminium, zirconium and titanium and these complexes were used in the production of colourless leathers. Calcium was also thought to form a complex with gelatine which modified its gelling properties but there was no evidence for calcium forming a coloured complex (Cole, 1980).

Table 2. *The effect of iron contamination on gelatine colour.*

Iron added. ppm.	Davis Colour.
20	4.4
70	5.2
220	8.5
520	13.0
720	14.5

From Williams (1957).

Known coloured complexes formed by collagen or gelatine and cations were those formed with chromium and iron. The Cr(IV) complex used for the production of chrome tanned leather imparts a pale blue-green colour to collagen or gelatine which in gelatine was just visible at 200 ppm. As production gelatines had chromium contents well below 10 ppm and as the colour imparted by chromium was so different to the normal colour of gelatine it was decided that further investigation of this cation was not warranted. Iron on the other hand was known to impart red-brown stains to leather and colour to gelatine. From a study at DGI (Williams, 1957), it was known that low levels of iron contamination (50 ppm) had a negligible effect on gelatine colour. Higher levels of contamination could have a marked effect as shown in Table 2.

Linear regression analysis of the data in Table 2 gave the following result:

DGI Colour = 4.5 + 0.015 x Fe ppm. r = 0.988.

Because of the potential significance of iron contamination to this study it was decided to reinvestigate the effect of iron contamination on gelatine and to analyse different coloured gelatines from old and young animals for iron contamination.

The effect of colour on amino acid analysis.

It has been mentioned elsewhere that gelatine from chrome tanned leather was of an exceptionally pale colour. Heidemann (1982) had shown that in tanning, chromium was complexed by the glutamic and aspartic acid side chains of collagen. When gelatine was made from chrome tanned leather, the collagen was extracted at relatively high temperatures (80° to 100°C), and at approximately pH 9. The gelatine contained less than 10 ppm chromium, and the original (approximately 3%) chromium in the leather remained as an insoluble residue after extraction. From this it appeared that gelatine from chrome tanned leather could be low in glutamic and or aspartic acid residues. Furthermore, as this study progressed it became evident that there was an inverse correlation between animal age and hide extractability and a correlation between animal age and colour. Hence, it seemed probable that there could be a correlation between cross-links and gelatine colour. As lysine seemed to be involved with the formation of most if not all collagen cross-links (Dyer, Blackledge, Katz, Hull, Adkisson, Thorpe, Lyons and Baynes, 1991) it seemed reasonable to try and show a correlation between colour and amino acid analysis of gelatine. This prompted the submission of appropriate samples of gelatine to a consultant for amino acid analysis.

METHODS.

Lime slaking and conditioning liquor preparation.

The required quantities of quick lime (CaO), usually 640 g, and commercial sodium sulphide (60%) flakes (sulphide), were combined in a 5 l beaker. Two point six litres of water was added and the mixture was stirred with a rod until hot (approx. 60°C) and gelatinous. The mixture was then allowed to stand for about 1 h before being transferred to a 40 l plastic bin and diluted with water to 20 kg. After standing for at least 1 h, 10 ml aliquots were pipetted from the supernatant for sulphide determination using the Leather Industries Research Institute (LIRI) method for sulphide determination.

Preparation of hide for conditioning.

In this study, salted bovine hide was the starting material. In the few cases where green (fresh/untreated) hide was received it was salted by tumbling with 50% (w/w) coarse salt for at least 18 hr, after which it was drained for about 72 hr. Salted hide was stored in a sealed plastic bag to prevent drying and at ambient temperature, until required for conditioning. In only one instance, where the difference between green face pieces and the rest of the hide was being investigated, was refrigerated green hide put into process immediately upon receipt.

Two stainless steel tumblers were available for processing hide. These consisted of cylindrical vessels of about 200 l capacity which were rotated mechanically about their diameter at about 15 revolutions per minute. Each vessel had a door in its side which was used for adding hide and chemicals. The door could be closed with a perforated stainless steel plate which allowed drainage as the drum rotated. The perforated plate could be covered with a sheet of rubber if the drum was to be used without drainage. Furthermore, the axles of the drum were hollow allowing for the introduction of water or chemicals during rotation. In addition, the drum was fitted with internal "raisers" which ensured mixing during rotation.

Whole salted hides were cut into approximately 100 x 100 mm pieces which were then placed into a tumbler. The door was closed with a perforated plate and the hide was tumbled for 15 to 30 min to remove loose salt and to mix the pieces thoroughly. The hide was then weighed into aliquots, sealed in plastic bags and stored at ambient temperature until required. Before starting an experiment with salted hide the hide was washed as described later. The hide was then removed from the tumbler, allowed to drain for at least an hour, weighed and then placed into prepared conditioning liquor.

Hide washing.

Hide was placed in the tumbler. The door was closed. The tumbler rotation was then started as was the continuous flow of water. Washing (by decantation) was continued overnight (16 hr) unless otherwise stated.

Hide conditioning.

The quantities of hide and conditioning liquors used are stated in the experimental detail in the ADDENDA. The hide was placed in conditioning liquor in a constant temperature room (22°C) for the required conditioning period of 1 to 10 weeks. During this period the bins were kept covered with a polythene sheet to minimise losses due to evaporation. Three times a week (on Mondays, Wednesdays and Fridays) the bins were agitated by hand which was protected by an elbow-length PVC glove. The temperature of one or two bins was measured, using an electronic digital thermometer and recorded. The average liquor temperature for the conditioning period was used as the conditioning temperature. At the end of the conditioning period the bin was weighed for the determination of losses due to evaporation. A sample of the spent conditioning liquor was taken for analysis and then the contents of a bin were tipped into a stainless steel tumbler for washing. The washed hide was then transferred to a clean 40 l polyethylene bin for acidulation.

Acidulation.

After washing the conditioned hide was covered with 5 x 20 l lots of 0.1M sulphurous acid solution of at least 8 hr duration each. Acidulation usually lasted for 4 days which gave complete acid penetration of the hide. On the 5th day, the hide was washed with tap water for one hour using a hose placed in the bin. The hide was then soaked in the bin full of water for approximately 20 hr before the commencement of extraction. From experience, this process gave an extraction pH of about 3 and if the extractability of the hide was reasonable then the ash content of the final dry gelatine was approximately 1% W/W.

Extraction.

The soak water from the final acidulation step was sampled for pH determination before being discarded. The hide was packed into 5 l glass beakers which were filled to the 4.5 l mark with hot tap water and then placed in a thermostatically controlled waterbath set to the required extraction temperature (45°C etc.). During the extraction period the beakers were stirred gently by hand using a stirring rod. The temperature was noted using a digital thermometer with stainless steel probe. Towards the end of the extraction time fat was carefully skimmed (using a stainless steel ladle) and the volume was

recorded. At the end of the extraction time the liquor and skins were separated using a colander. The volume of liquor from each extraction was measured before commencing liquor processing. The residual skin was returned to the beakers for a second and third extraction, and finally placed in a stainless steel pot for boiling.

At the start of this series of experiments the boiled liquor was separated from the unextractable residue comprising epidermis, fat, hair, bone etc. The liquor volume was measured and a sample filtered through filter paper (Whatman 541) for the determination of gelatine concentration. This allowed the calculation of the amount of gelatine available in the raw material. Later it was found that on most occasions (if boiled for long enough) the boiled liquor could be filtered to a good clarity using Whatman GF/A paper in a Büchner funnel. If this liquor sample had a concentration of greater than 4 % gelatine then it could also used for colour determination by the normal DGI method, (after dilution). Thus, on many occasions, the colour of all the gelatine was available, making it possible to calculate the overall colours.

LIQUOR PROCESSING.

Resumé.

After the separation of the extraction liquor from the residual solids, the extraction liquor volume was determined. A sample (100 ml) was taken for pH determination. After filtration of the liquor, a further (100 ml) sample was used for the gravimetric determination of gelatine concentration.

After filtration, liquors were either concentrated immediately by vacuum evaporation or they were stored in a refrigerator (5°C) overnight and then processed. (After storage gelatine solutions were warmed in a 45°C bath before evaporation). After evaporation to approximately 10 % gelatine the solution was refiltered, the pH was adjusted and the gelatine solution was then set in a refrigerator. The set gel was cut into slices which were dried to about 10 % moisture content in a current of air. The dry gelatine was then ground to a powder for analysis.

Evaporation.

The evaporator was a purpose built single effect, steam heated, rising film

glass evaporator connected to a water vacuum pump and a condenser (cooled by mains water). The evaporator had a capacity of 2 to 3 l condensate per hour (depending on the temperature of the cooling water) and was run at a maximum liquor recirculation temperature of 42°C.

Heavy liquor filtration.

After evaporation the liquor was refiltered by the heavy liquor procedure described below. In the few instances where the amount of gelatine extracted was low and filtration through paper pulp would have led to unacceptable losses, the heavy liquor was vacuum filtered through Whatman GF/A paper in a Büchner funnel.

Laboratory filtration procedure:
EQUIPMENT.
40 l plastic open top container (bin).
Sheets of cotton linters paper pulp 680 x 800 mm, ex Carlson Ford U.K. (Rosenmeyer.W.H & Co. Joubert Park, Johannesburg).
Water Vacuum Pump.
Pressure hose to connect vacuum pump to Büchner flask.

For light (approx. 4%) liquor filtration:

Büchner Flask - 10 l size.
Porcelain Büchner Funnel - 270 mm ID.
Rubber bung to attach the flask to the funnel.

For heavy (approx. 12%) Liquor Filtration:

Büchner Flask - 2 l size.
Porcelain/plastic Büchner funnel - 130 to 160 mm ID.
Rubber bung to attach the funnel to the flask.

PROCEDURE.

1. Sheets of paper pulp were torn into approximately 100 mm pieces and disperse in approximately 20 l water in a plastic bin. After soaking for about 15 min the paper pulp was rubbed between the palms of the hands and stirred into the water until all resemblance to the original sheets was destroyed.

2. A Büchner funnel was attached to a flask. The funnel was filled with a maximum amount of dispersed pulp. The vacuum supply was attached and as the water was sucked out of the pulp, hand pressure was applied to the pulp such that it formed a pad of uniform thickness. Once most of the water had been removed from the pad, the pulp was compressed as far as was possible using the fist or a porcelain pestle while continuing with the suction. After compression a light liquor filter pad was at least 50 mm thick and a heavy liquor pad at least 30 mm thick. The pad was washed with cold water until there was no loose pulp in the filtrate.

3. Immediately before starting filtration, the filter was washed with sufficient hot water (60°C) such that the surface of the funnel was hot to the touch. The surface of the pad was sucked dry and then liquor filtration was commenced. The water displaced from the filter was wasted and the filtered liquor was collected.

4. If concentration was to be determined on the filtrate then the liquor was divided into 3 lots such that the first two lots were used for "washing" the filter pad and equipment and only the third lot was sampled for concentration determination.

Heavy liquor SO_2 & pH adjustment.

In order to ensure compliance with the requirements of the National Specification for edible gelatine heavy liquors were treated with 5% H_2O_2 to an approximate excess of 30ppm as indicated by Merck (Merck (Pty) Ltd. Midrand) test strips. The liquors were then treated with 5% NH_3 solution to give a pH of 5 to 5.5 as indicated by Merck test strips.

Heavy liquor drying.

The heavy liquor in a glass beaker was gelled in a refrigerator (5°C). The gelled liquor was cut into slices which were placed on trays. The trays were dried in a constant current of ambient air in a purpose built drying tunnel. The dry sheets were ground to a powder in a Waring Blender. The physical properties of the ground gelatines were determined by BS 757 (1975) methods as well as in house methods for colour and clarity.

ANALYSES.

Unless otherwise stated the analytical methods used on gelatine were those of the British Standards Association (1975).

Spent conditioning liquor analysis.

The sample of each spent conditioning liquor was vacuum filtered through Whatman GF/A paper. Duplicate aliquots of the filtered liquor were pipetted into tared silica crucibles and dried at 105°C overnight. After cooling in a desiccator and weighing the crucibles were placed in a 550°C muffle furnace for 24 hr before cooling and reweighing to give the total solids and ash contents of the conditioning liquor. Duplicate aliquots were also pipetted for the determination of the final sulphide concentration using the LIRI ferricyanide method.

LIRI method for sulphide analysis.

This method, obtained from the Leather Industries Research Institute (Rhodes University, Grahamstown, RSA) was intended for the determination of sulphide in alkaline solutions. The sulphide was oxidised to sulphur by titration with standard potassium ferricyanide solution in the presence of a ferrous dimethylglyoxime complex as indicator. Sulphite was known to interfere and was removed by precipitation with barium chloride. Thiosulphate was known not to interfere under the conditions of the determination.

REAGENTS.

Potassium ferricyanide 0.05N: 16.4625 g/l (Analitical Reagent grade, dried at 105°C for 2 hr). (Solution kept in the dark was stable for at least 30 days).

Buffer: 200 g NH_4Cl
200 ml ammonia (S.G. 0.88) per litre.

Barium chloride solution: 12.5 g/l. (10 ml precipitates 0.3 g sodium sulphite).

Indicator: 10 ml 0.6% $FeSO_4$.
50 ml 1% dimethylglyoxime in ethanol.
0.5 ml conc. H_2SO_4.

PROCEDURE.

1. The sample was filtered through glass wool if it contained suspended solids.
2. To a 250 ml stoppered flask was add 20 ml buffer and 20 ml barium chloride solution.
3. To the flask was added a suitable aliquot (equivalent to about 0.04 g Na_2S) of alkaline sulphide solution, by pipette. The flask was stoppered, swirled, and allowed to stand for one minute.
4. Indicator solution (1 ml) was added and the sulphide was titrated with standard ferricyanide solution until the pink colour was converted to a green colour which persisted for 15-30 seconds.

1 ml 0.05N ferricyanide = 0.00195 g Na_2S.

Note. If it was necessary to standardise the potassium ferricyanide solution this could be accomplished by treating an aliquot (20 ml) with 2 g KI, 10 ml 30% sulphuric acid and 10 ml 20% zinc sulphate and titrating the liberated iodine with standard sodium thiosulphate solution using starch indicator.

Light liquor concentration determination.

The liquor was warmed to 40°C. Duplicate 10 ml aliquots were pipetted into weighed stainless steel dishes. The dishes were dried at 105°C for 48 hr. After cooling in a desiccator the dishes were weighed and the gelatine concentration (% w/v) at 12.5% (m/m) moisture content was calculated by multiplying the weight of dry gelatine by a factor of 11.4286 in order to express the result as commercial gelatine rather than anhydrous gelatine.

Determination of gelatine colour (DGI Method).

EQUIPMENT:
Fluorescent table lamp.
Sheet of white filter pulp.
1 matched pair of 100 ml Nessler Tubes.
3 x 100 ml measuring cylinders.
Waterbath. Thermostatically controlled at ca. 45°C.
Beaker of distilled water at ca. 45°C.

METHOD.

Gelatine colour standard (7.5g) was weighed into a Bloom gel strength bottle and dissolved as for the Bloom gel strength determination. This standard solution was measured using a measuring cylinder (60 ml) and diluted to 100 ml with warm distilled water. The dilute standard solution was transferred to the first Nessler tube, taking care to avoid the formation of bubbles, and ensuring a homogeneous mixture.

Melted Bloom gel strength sample (60 ml) of an unknown gelatine in another measuring cylinder was diluted to 100 ml with warm water and transfer to the second Nessler tube.

The Nessler tubes were compared down their length against white paper illuminated by the fluorescent light. Solution was poured from the darker tube into the appropriate measuring cylinder until a colour match was achieved.

If the colour value of the Standard was C_1 and the colour value of the unknown was C_2 then:

$$C_2 = C_1 \times (\text{Volume of Standard}) / (\text{Volume of Unknown})$$

Determination of gelatine clarity (DGI Method).

EQUIPMENT.

Nephelometer and glass cuvette. (ICM Turbidimeter. ICM. 163 S.W. Freeman, Hillsboro. OR 97123. USA)
40 NTU (National Turbidity Units) standard for the Nephelometer.

METHOD.

The Nephelometer was set to the to 0 to 100 scale. The 40 NTU standard supplied with the instrument was inserted into the sample compartment and the instrument was set to read 40 using the sensitivity adjustment. The Nephelometer cuvette was filled with the clarity standard gelatine solution (6.67%). Cleanliness of the cuvette was ensured by wiping with a paper tissue. The cuvette was then inserted into the Nephelometer cuvette holder. If the reading given by the standard was correct then the determination of the

clarity of the melted 6.67% Bloom gel strength samples was undertaken. The results were reported in NTU units (0 to 100) which could then be scaled to Davis Gelatine units (14.0 to 2.5):

$$\text{DGI Clarity} = 13.9 - 0.133 \times \text{NTU} \quad \ldots\ldots\ldots\ldots\ldots\ldots 1$$

Processing of analytical results:

In calculations using a gel strength, the square root ($\sqrt{}$) of the gel strength was used. The reason for this was that gel strengths were known not follow the laws of simple proportions but the $\sqrt{}$ (gel strength) was known to be closely proportional to concentration over a wide range of gel strengths, Veis (1964a), Jones (1977).

1). Overall quality.

Results were expressed as required by the methods, however for most extractions "overalls" were calculated. This procedure calculated the value of the parameter as if all the gelatine from a particular raw material had been composited as a single product. In order to do this the contribution of each gelatine from the raw material was taken into consideration according to the amount extracted. For example:

$$\Sigma\ (\text{Colour} \times \text{Mass}) \div \Sigma\ \text{Mass} = \text{Overall Colour}.$$

2). Corrected Bloom gel strength value and viscosity.

The reason for this calculation was that gelatine concentration had a marked influence on the particular values. Hence to simplify comparisons, the value at 12.5 % non-protein components in the starting gelatine, was calculated to give the "corrected" value (e.g. gel strength). (Non-protein components of gelatine were moisture and ash.)

$$\text{Corrected Gel Strength} = ((\sqrt{\text{Gel Str.}}) \times 87.5 \div \text{concentration})^2.$$
$$\text{Corrected Viscosity} = \text{Viscosity} \times 87.5 \div \text{concentration}.$$

3). Yield corrections.

Firstly as concentrations were expressed as gelatine containing 12.5% moisture it was necessary to apply a factor of 0.875 to obtain the yield on an anhydrous basis. Thereafter a factor of 0.95 was applied because in various trials it had been found impossible to recover more than 95% of the gelatine

indicated by volume and concentration determinations.

Iron analysis.

The method used was developed by Eastoe and Eastoe (1951) of The British Gelatine and Glue Research Association (BGGRA).

Silica crucibles were prepared by boiling in 32% HCl and then standing in the solution overnight. After rinsing with distilled water several times and drying, the crucibles were heated in a muffle furnace at 550°C for 1 hr, cooled and weighed.

Test Solution.

Duplicate 5 g samples of gelatine were weighed into crucibles. The gelatine was ashed in a muffle furnace at 550°C, overnight. The ash was cooled in a desiccator, weighed and then moistened with a few drops of distilled water. The ash was then digested with 5 ml cHCl on a hot plate until dryness was just achieved. Hydrochloric acid (N - 10 ml) was then added and the residue in the crucible was dissolved with gentle warming. The solution was transferred into a 50 ml volumetric flask through a Whatman 541 filter paper which was washed 3 times with distilled water. The flasks were then made up to the mark with distilled water.

Colour development:

10 ml test solution.
2 ml 10% hydroxylamine hydrochloride solution.
2 ml 2M sodium acetate solution.
2 ml 0.25% O-phenanthroline solution.

These were mixed in a 25 ml volumetric flask, which was then made up to the mark with distilled water. After 10 min the absorbances were read using a Jenway (Jenway Ltd. Dunmow, Essex. UK.) Colorimeter with No 3 filter (490 nm) and water as the blank.

100 ppm Fe-III solution:

Standard 1000 ppm Fe solution (5 ml) (ex SaarChem (Pty) Ltd. Krugersdorp. RSA.) was pipetted into a 50 ml volumetric flask. Potassium permanganate solution (N/50 - 4 drops) was added to impart a permanent pink colour. The solution was then made up to the mark with distilled water to give a 100 ppm solution of Iron-III.

Standard curve.

Suitable aliquots of 100 ppm Fe solution were used to prepare 50 ml solutions containing 0, 50, 100, 200 and 300 μg Fe, equivalent to 0, 10, 20, 40 and 60 ppm Fe in 5 g of gelatine.

Aliquots (10 ml) of these solutions were used for colour development as above. A standard curve was generated from which the iron contents of the unknowns could be read. Average results for the duplicate samples were reported.

Effect of iron on gelatine colour.

Four gram samples of a pale gelatine 155/1 were weighed into Bloom gel strength bottles. Various amounts of Fe solution (0, 0.8, 1.6, 2.4, 3.2, and 4 ml of 100 ppm Fe) were added to different samples. The samples were then diluted to 100 g with distilled water. After soaking the samples were dissolved in a 45°C waterbath. The colour of the solutions was then determined by comparison to 100 g if a 4% w/w solution of the colour standard with an ascribed colour value of 8, in 100 ml Nessler tubes. Three ml aliquots of these solutions were scanned using the Jenway Colorimeter in 1 cm plastic cells. The remainder of the gelatine solutions were used for pH measurement.

Amino acid analysis.

Stevens and Stevens (1992) (Applied Science and Technology, 169 Havannah St, Bathurst, NSW 2795. Australia.) were experienced in the amino acid (AA) analysis of gelatine and had worked for Davis Gelatine Australia on a number of occasions. Hence it was agreed to make use of their services as long as there were no prescriptions as to what had to be divulged about the

samples prior to analysis. It was decided to submit 6 samples as a preliminary trial as follows:

One pale gelatine from chrome tanned leather. Labelled "A".

Two pale gelatines from a young animal. YSA/1 and YSA/3. Labelled "B" and "C".

One pale duplicate sample. YSA/3. Labelled "D".

Two dark gelatines from an old animal. WT3/1 & WT3/3. Labelled "E" & "F".

Prior to the commencement of the investigation they were advised that the variation in amino acid analysis with gelatine colour was of importance as was the possible detection of minor peaks that could be attributable to cross-link residues.

Details of the methods used were not made available, however they advised that the sample preparation involved hydrolysis to reduce the protein to amino acids and the amino acid separation involved "Picotag precolumn derivatisation". The hydrolysis procedure caused destruction of methionine and cystine. Furthermore as histidine was a minor constituent with its peak close to glycine its quantisation was problematical.

The amino acid data was received in two lots. The first lot (Report "Interim Report on South African Gelatin Samples. Amino acid and crosslink analysis." dated 11.6.92) was on samples B to F and is designated B1 to F1 in ADDENDUM 13. This data did not contain results for the ornithine content of the gelatines.

The second lot of data (Report c:\wp51\gfwi\aacole dated 1 July 1992) contained duplicate results from duplicate hydrolyses of the gelatines. This data is designated A2 and A3 to F2 and F3 in ADDENDUM 13. Sample D3 was lost and the aspartic acid result for sample C2 was obviously in error.

The results received (Addendum C13) were entered in a Quattro Pro (Version 5. Borland International, Inc. 1800 Green Hills Road, Scotts Valley, CA 95067-0001, USA) spreadsheet. The means and relative standard deviations (% RSD) for each amino acid were calculated. The % RSD was the standard deviation as a percentage of the mean.

1. "Experimental Error" (EE) used the values for samples C and D only to give a % RSD. due to experimental error only, as the samples were identical.

2. "All" included all data. Thus if ALL-RSD was substantially larger than the EE-RSD it would indicate an effect due to the samples A, B, E & F.

3. "All - A" (All minus A) included all data except that for the chrome gelatines of sample A. Thus if All - A RSD was less than the ALL-RSD it would indicate an effect due to Chrome gelatine.

The data in ADDENDUM 14 was calculated from the data in ADDENDUM 13 by:

1. Correcting for the variable protein content of the samples due to variable moisture and ash contents. Each AA value was divided by the % protein and multiplied by 100. This gave the % (AA) by mass.

2. The results from 1 above were converted to moles amino acid by dividing mass of each amino acid by its molecular weight and multiplying by 100. This gave the number of moles of amino acid per 100 g protein. This value was then converted to moles of each amino acid per 100 moles.

Due to the obvious error in the aspartic acid value of gelatine C2 in ADDENDUM 13 this value was replaced by the average molar numbers of the aspartic acid values of samples C1, C3, D1 and D2, i.e. the value of 4.07 was used.

ADDENDUM 15 is a compressed form of ADDENDUM 14 in which the molar percentages for each sample were averaged. The mean and % RSD data were copied from the values in ADDENDUM 14. Table 18 was copied from ADDENDUM 15.

EXPERIMENTAL CODES.

Each series of experiments was given a code which was a mnemonic of the main variable/s for the series. For example:

CT = conditioning time.
G or S = green or salted hide.
ST = sulphide usage and time variables.
WT = winter temperature experiments.
3Y = Three year-old animal.

EXPERIMENT GR. Face Pieces versus the rest of the hide.
(GF, GR, SF, SR).

This experiment was designed to determine whether there were any significant differences between the hide of the head and the hide of the rest of the animal. The Animal and Dairy Sciences Research Institute (ADSRI) abattoir was asked to provide the mask of an animal and a piece of the hide of an equal mass in a plastic bag. This was done with 6 animals. The hide was stored in the abattoir cold room overnight. The next day the samples were checked for equal weights of mask and the rest. Three of the masks were salted in a tumbler (SF) and the corresponding "rest" pieces were salted in a second tumbler (SR). The 3 green face pieces (GF) and rest pieces (GR) were placed in lime/sulphide conditioning liquor immediately. For the detailed data on this experiment see Addendum C1.

EXPERIMENT CT. - The effect of conditioning time.

This experiment was designed to determine the effect of conditioning time on hide extractability, yield, and gelatine properties. A large salted hide was required to conduct the number of experiments envisaged and it was obtained from a hide merchant. The supplier could only advise that the animal was a Brahman and it had been reared on a "feed lot". From this it was surmised that the animal was approximately 18 months old at slaughter. This size of hide allowed conditioning experiments to be carried out for 1 to 6 weeks while keeping all other variables constant. There was a small amount of hide left over for examining the effect of replacing the conditioning liquors weekly for three weeks. For detailed data on this experiment see Addendum C2.

EXPERIMENT CTO. - Old animal hide and conditioning time.

This experiment was designed as a replicate of experiment CT to examine the effect of conditioning time on the hide of an old animal. A salted hide from a 13 year-old animal was provided by ADSRI. This hide was divided into four equal parts (within 5 days of slaughter) which were conditioned for 2, 4, 7 and 10 weeks. For detailed data on this experiment see Addendum C3.

EXPERIMENT ST1. - The effect of time and sulphide concentration on the conditioning of the hide of an old animal.

Sodium sulphide was considered to be a "sharpener" or an accelerator of the conditioning process. In the past, work had been done using the "twinned experiment" technique which had often lead to confusing results due to the variation in the raw material used between the pairs of each trial. In experiments CT and CTO it had been shown that conditioning time did not appear to have had a significant effect on gelatine colour. Hence, a statistically designed factorial experiment Montgomery (1985) was executed to evaluate the effect of varying both time and sodium sulphide concentration using the F statistic. F values were based on (variance of the means) / (mean variance due to a treatment). It was required that the experiments be conducted in random order, so, for random permutations of 9 numbers reference was made to Cochran and Cox (1957) from which it was found that the experiments could be started in a convenient random order #3, #1, #8, #5, #9, #4, #2, #6, #7 as shown in Table 3.

A Quattro Pro (*loc. sit*) spread sheet was used to calculate the F ratios for the variables of interest (Bloom gel strength value, yield, extractability etc.) using the formulas provided by Montgomery (1985) and Freund and Williams (1964a).

Table 3. *Factorial design of experiment ST.*

Time in Weeks	2	4	6
Sulphide Conc. in g/l			
1.5	#1(1-2)	#2(1-4)	#3(1-6)
2.2	#4(2-2)	#5(2-4)	#6(2-6)
2.9	#7(3-6)	#8(2-4)	#9(3-6)

Code #7(3-6) denoted:
Experiment No. 7;
Sulphide Concentration No. 3 (or 2.9 g/l);
Time = 6 weeks.

Details of this experiment are given in ADDENDUM C4. The raw material for this experiment was from 2 salted hides provided by ADSRI. Both animals were 12

years old at slaughter. 9 x 3 kg lots were obtained from the first hide to which was added 9 x 2.7 kg from the second. Random punchings were taken from each of the 9 lots for duplicate moisture and ash determinations.

Due to the poor extractability resulting from 2 weeks conditioning the "Boil" of Experiment #1 was conducted in 2 parts. After 15 minutes of boiling the liquor was separated from the hide to give the fourth liquor. After an additional 7 hours of boiling the final boil liquor was separated from the scutch residue to give the fifth liquor ST1-2/5.

EXPERIMENT ST2 (or WT). The effect of temperature and sulphide concentration on conditioning.

In experiment ST1 it was found that variation in sulphide concentration did not vary the degree of extractability of hide. This finding was at variance with the accepted DGI practices of decades and it was necessary to determine whether sulphide had any effect on conditioning at all. The fact that it was a depilatory was important from the point of view of production but it could be that the cost of the benefit was not justified.

Sodium sulphide was a reducing agent and the initial Maillard reaction was oxidative hence the role of sulphide could simply be one of inhibiting the Maillard cross-linking and darkening during the alkaline conditioning process by reducing the availability of oxygen to the system. To investigate the reduction theory it was decided to conduct a conditioning in lime only under nitrogen to reduce the oxygen tension of the conditioning system and determine whether this would duplicate the effect of sulphide.

To verify the lack of conditioning effect due to sulphide concentration it was decided to determine the effect of a 6:1 variance in sulphide concentration at winter conditioning temperatures which was far greater than the 2.5:1 variance normally used in production to "compensate" for reduced conditioning temperatures of winter.

Details of this series of experiments are in ADDENDUM C5. They were conducted on the salted hide of a single 12 year-old animal provided by ADSRI and the hide was divided into 5 x 4.0 kg lots.

For Experiment WT1 the lime slurry was made using water that had been boiled

and then cooled in full, sealed plastic bottles to prevent oxygen absorption. The hide was placed in a glass carboy. The neck was sealed with a rubber bung which was wired to prevent loosening. The tap at the bottom of the carboy was also wired and after adding the lime to the hide. It was used to evacuate the air above the lime using a water vacuum pump for 1 hr. The vacuum was then replaced with nitrogen from a cylinder. On the first day the evacuation and nitrogen flushing was repeated once and then again on day 2, day 3, day 6 and day 13.

During conditioning it was noted that the carboy surface temperature was always 2° to 3°C higher than the conditioning liquors in the bins. This could have been due to the elevation of the carboy relative to the heaters used to control the temperature of the room and could have caused a slight enhancement in the degree of conditioning.

In summary:
WT1 was a pure lime anaerobic conditioning.
WT2 was a pure lime aerobic conditioning.
WT3 was a control conditioning with a normal 1.8 g/l Na_2S at 22°C.
WT4 was a conditioning with 1.8 g/l Na_2S at 12°C.
WT5 was a conditioning with 11.1 g/l Na_2S at 12°C.

EXPERIMENT YS. The effects of animal age - 10 month old animal salted hide from ADSRI.

As experiments CTO, ST1 and ST2 had been conducted on old animals' hide it was decided to do a comparative experiment on young animal's hide. Hence, a suitable large hide was obtained from ADSRI.

Details of this experiment are in ADDENDUM C6. As the animal was so young it was decided to condition with a nominal 2 and 6 g/l Na_2S, for 2 weeks, (YSA & YSB) and 3 weeks, (YSC & YSD). A four weeks conditioning with no sulphide, (YSE) was also conducted to confirm the effect of sodium sulphide on conditioning.

EXPERIMENT KTO. Gelatine quality from various layers of the salted hide from a 12 year-old animal.

It was often maintained that the best gelatine colours were obtained from "splits" raw material. To investigate this perception it was necessary to

split a hide as in tanning into the flesh layer, the corium or "split" layer and the grain layer. The help of a tannery with equipment for splitting a hide after pretreatment was obtained. In a previous trial (Cole, 1989) with similar aims, a hide provided by the tanner was used. The extractabilities indicated that the skin was from a young animal so the colours of the gelatines recovered were pale but the experiment yielded the following information:

Flesh split yielded gelatine of darkest colour. (8.5 to 12.0)
Middle split yielded gelatine of palest colour. (4.0 to 5.2)
Epidermis split (grain) yielded gelatine of intermediate colour. (5.2 to 5.0)
The whole hide yielded gelatine with colour 5.2 to 5.0.

Furthermore, from this experiment it was concluded that all portions of the hide should receive similar conditioning treatments and if an old animal was used perhaps the colour differences would be more pronounced. Hence a salted hide from a 12 year-old animal was obtained from ADSRI.

The tannery operations were conducted by Kwiktan, Delporton, Krugersdorp as follows:
Tumbler washed overnight to rehydrate the hide.
Treated with 3% w/w (60%) Na_2S + 3% $Ca(OH)_2$ in a 100% float:
Drummed 3 hr and then every 30 min. for approx. 69 hr.
Washed by decantation with continuous water flow for 10 min.
The hide was then cut into halves down the backbone and one half was split into flesh, middle and grain splits.

The details of this series of experiments are recorded in ADDENDUM C7. The 4 lots, whole hide, and flesh, middle and grain splits, could not be treated simultaneously, so parts KTO3 and KTO4 (middle and grain splits) were placed in lime only for 7 days and then the sulphide was added in order to obtain substantially the same conditioning on all four parts. Conditioning procedures were otherwise normal.

As there was no hair on the hide during conditioning this experiment afforded the possibility of determining how much colour the conditioning liquor removed from the hide during conditioning. When the spent conditioning liquor samples were filtered, a portion of the liquor was used for the determination of its absorbance (colour) using the Colorimeter with the No 2 (470 nm) filter and water as the blank. Furthermore, as there was no hair, the sulphide

determination, performed two hours after the addition of sulphide solution to the limed hide mixtures KT03 and KT04, gave good estimates of the initial sulphide concentration.

EXPERIMENT CALF-A. Type A gelatine from calf skin.

It was well known that pigskin gelatine is made from animals that were slaughtered at about 6 months of age. From this study it had become clear that young animals gave paler gelatines than old animals. Hence there was a need to determine the quality of gelatine that could be produced from calf skin by the acid process. Reich, Walther, and Stather (1962a,b) had covered the "acid process" in detail, however, they did not concern themselves with the colour of the gelatines produced. It was of interest to note that they concluded that the "acid conditioning process" was in fact no more than an acid treatment to equilibrate the skin to the required acid extraction pH and to quote "with the acid process there is no possibility to compensate for age-related differences in the stability of skin collagen as is the case with the alkaline conditioning process".

In order to show whether there was any difference in colour between calf skin Type A gelatine and pigskin gelatine, three salted calf skins were obtained from a merchant. The skins had Friesland black and white colouring and from their size it was evident that they were from calves of less than six months of age. One skin still had the umbilical cord attached indicating that it was from an animal of between one week and one month of age. The skins were stored in a sealed plastic bag for seven days to equilibrate the moisture content. The skins were very hairy and so to make liquor drainage efficient and to maximize yield and recovery it was decided that they should be subjected to a very quick tannery dehairing process the details of which are given in Addendum C8. This was followed by acidulation with 0.1N sulphuric acid overnight using the method of Reich *et al.* (1962a). The hide was then washed with tap water (pH 7.5 to 8) by upflow, in a static washer for 2.5 hours and soaked in 20 l water until the next day to give an extraction pH of close to 4.0. Extraction and liquor handling were normal, except that after filtration it was decided to raise the pH of the light liquors by passing a portion of the liquor through a mixed bed ion-exchange column and then mixing this with untreated liquor to obtain a liquor pH of approximately 5 before evaporation. From experience it was known that paper pulp filtration could have a marked

effect on gelatine liquor pH after mixed bed ion exchange which was presumably due to the ion exchange effects of cellulose. It was concluded that this was the reason for the relatively high pHs of the final gelatines. In the case of the third extraction too much liquor was deionised hence the need to add sulphuric acid to the heavy liquor after evaporation.

In order to determine the isoionic point of the gelatines produced the ion-exchange method described by Veis (1964b) was used. A 4.5 cm diameter glass column was charged with 400 ml of Rohm & Haas (ACIX, Germiston) mixed bed MB3 ion exchange resin. After warming with 2 bed volumes of warm distilled water the column was used to treat 600 ml of 1% (w/v) gelatine solution at a flow rate of about 5 bed volumes per hour. The last 50 ml of eluate was collected and the pH determined. This pH was taken as the isoionic pH of the gelatine.

EXPERIMENT 3Y & 6Y. The effect of 3 & 6 years old animal's hide on conditioning response and gelatine quality.

It was decided that it was necessary to include animals of ages between 18 and 144 months to complete the data on the effect of animal age on the response to conditioning and resultant variances in gelatine quality. Furthermore it would be important to know whether breed would affect the results significantly. Hence, salted hides from Friesland animals of 40 and 78 months of age were obtained from ADSRI for inclusion in the study.

This experiment was also used to confirm the previous findings with regard to the role of sodium sulphide in lime-sulphide conditioning. The 4 week conditionings 3Y4 and 6Y4 were split into two parts, part A being conditioned with 2 g/l sodium sulphide and part B with 4 g/l sodium sulphide. The six week conditionings (3Y6 and 6Y6) were similarly split into the A part with 2 g/l sodium sulphide and the B part with no sodium sulphide. Details of this series of experiments are in ADDENDUM C9.

EXPERIMENT 5Y. Effect of age and breed on conditioning response and gelatine quality.

The hide from a 58 month old Chianina cow was available from ADSRI and was included in the study. Details of this series of experiments are in ADDENDUM C10.

EXPERIMENT INO. A 12 year-old Inguni cow's hide was made available by ADSRI for inclusion in the study. Details of this experiment are in ADDENDUM C11.

RESULTS and DISCUSSION.

EXPERIMENT GR.
(Green face pieces v/s the rest of the hide.)

In the past the procedure of "twinning" hide had been found to give two halves which responded very similarly to processing. In the procedure each piece of hide was halved as equally as possible using the same approach as in tanning where a hide could be "sided" by halving down the backbone to give two halves which would be expected to behave similarly in processing. To the tanner however different parts of the hide were not identical. For example the belly area was thin and the haunch area was thick. The face or mask was of very variable thickness and was never used by the tanner, nor were the irregular pieces covering the legs and tails. It was considered that if hide from two closely similar areas from "twinning" behaved similarly towards processing then perhaps the whole hide might be considered a uniform piece of raw material as far as gelatine manufacture was concerned. This experiment was designed to test the theory that the hide of a single animal was a uniform raw material from the point of view of gelatine manufacture. As a further consideration, it was known that the salting of hide caused it to exude an amount of serum equivalent to some 15 % of the weight of the raw hide (see Introduction). This exudate would contain salt soluble proteins (Na, Phillips and Freire, 1989) and as a result salted hide could be different in response to green hide, hence both types of material were investigated.

From a comparison of the detailed data in ADDENDUM C1 on green hide - GR and GF - the difference in 45°C extractability of 6% might seem "significant" but in light of the virtually identical yields and identical gelatine properties of corrected Bloom, viscosity, and colour the difference in extractability was considered to be due to random variation.

In the salted hide comparison, SR and SF, the differences in extractabilities and yield were negligible but there was an apparently significant difference in the corrected Bloom gel strength values of 12 g on the first run gelatines. However, at 280 g Bloom gel strength the standard deviation of the

determination was known to be of the order of 4 g, hence, the significance of a Bloom gel strength difference of 12 g was minimal. From the detailed data in ADDENDUM C1 and from experience of the errors inherent in the estimation of extractability, yield and quality parameters, it was concluded that there were no significant differences in the response of either green or salted hide to processing or to the quality of the gelatine produced. Hence, it was accepted that the hide of a single animal was a uniform raw material from the point of view of gelatine manufacture. However, in using this finding it was decided that in all cases the hide would firstly be reduced to small pieces which would then be randomised by tumbling before the hide was divided between the parts of an experiment. Finally, it was considered that the experiments which followed, especially experiment ST, completely vindicated the assumption that the hide of a single animal was a uniform raw material.

EXPERIMENT CT.

In the absence of evidence to the contrary it was presumed that the conditioning process must play a role in determining the colour of gelatine produced. Conditioning for 1 to 6 weeks was considered to be adequate to show the expected effects on colour and detailed data on extractability and yield would be an added benefit.

From the detailed results in ADDENDUM C2 extracted into Table 4 below it was evident that the colour of gelatine was largely invariant with respect to the time of conditioning. Furthermore, the small change in gelatine colour with extraction temperature was totally at variance with experience and as a result it had to be concluded that gelatine colour was almost entirely a function of animal age. Finally, the observation that part CT7 gave the same gelatine colours as parts CT1 to CT6, even after weekly changes in conditioning liquor, was a very strong confirmation that "conditioning" played no direct role as far as gelatine colour was concerned.

Table 4. *The effect of hide conditioning time on the (DGI) colours of the extracted gelatines.*

Exp. No	Conditioning. Time in Weeks	GELATINE COLOUR				
		1st Extract	2nd Extract	3rd Extract	4th Extract	Overall
CT1	ONE	5.6	5.6	5.2	6.8	6.5
CT3	THREE	5.2	5.6	6.4	7.2	6.2
CT7	THREE	5.6	5.6	6.4	-	-
CT5	FIVE	6.0	6.4	6.4	8.0	6.4

- Not available.

This experiment was of great value because it illustrated the many effects of conditioning time:

The drop in the sulphide content of the conditioning liquor of 1.8 g/l down to 1.1 g/l after 2 weeks and 0.8 g/l after 6 weeks was thought to be significant. The exact role of sulphide during conditioning was the subject of speculation, however, the Maillard reaction was known to be associated with oxidation so it was considered that reducing conditions during conditioning should be significant and this was investigated in experiments ST and WT.

The change in conditioning liquor volatile solids (VS) (dissolved organic matter) with conditioning time from 0.8% after 2 weeks to 1.4% after 5 weeks was also of interest as it could indicate loss of "collagen contaminants" from the hide. However, as the increase in VS was accompanied by a small drop in yield from 27% to 26% the change in conditioning liquor volatile solids could be due to losses of collagen. However, the similarity in VS between CT5 and CT6 with a further loss of yield indicated that in fact loss of collagen into conditioning liquor was not the cause of VS but rather it was most probably (see experiments 3Y and 6Y) due to the dissolution of hair caused by the presence of sulphide. From this it followed that the hair content of the hide was about 288 g or 5% and also that these "hair burn" contaminants of conditioning liquor had no effect on the colour of the final gelatine.

From the point of view of the proximate analysis of hide it was

noteworthy that the gelatine yield on dry hide substance averaged 79% which could be taken as an estimate of the collagen content of the "hide substance".

EXPERIMENT CTO.

To confirm the indications in the author's thesis (Cole, 1986) that animal age was a cause of gelatine colour experiment CT was repeated using the hide of a 13 year-old animal. The detailed data is recorded in Addendum C3.

This data showed the normal darkening in gelatine colour as extraction temperature increased and confirmed the tentative conclusion that animal age was a most important contributor to gelatine colour. Furthermore, with this experiment it was observed that the first extraction gelatine colour increased with conditioning time as did extractability but most importantly the overall colour, that is the colour expected from combining all the gelatine extracted from the hide, was substantially constant and independent of conditioning time. This indicated that the most easily converted collagen, which was presumably the most recently produced collagen, yielded the best gelatine colour but in a relatively small quantity and as the extractability was increased by conditioning so the older collagen was caused to dissolve yielding darker gelatine.

The conditioning liquor volatile solids (VS) in this instance reached a maximum of 358 g indicating 8% hair plus alkali soluble organics on the sample weight. This represented some 22% of the anhydrous hide substance.

The average anhydrous gelatine yield was only 54% and in this instance the fat recovered during extraction was between 5 and 20 ml from approximately 1450g of anhydrous hide substance.

EXPERIMENT ST1.

Experiment ST1 was designed to investigate the effect of sodium sulphide on conditioning. There were no clear indications of the effect of sodium sulphide. It was thought to have had a "sharpening" effect on conditioning as it clearly improved extractability when compared to conditioning with lime only. In removing the hair from the hide it also facilitated processing. Finally if the Maillard reaction was in any way responsible for the colour of

gelatine then Na_2S could possibly play a role in preventing *post mortem* colour development. The detailed results are in Addendum C4.

The design of the experiment allowed for statistical assessment of the effect of time and sodium sulphide concentration on gelatine colour and the other attributes/parameters. A Quattro Pro spreadsheet was developed to perform the necessary calculations as shown in Tables 5 and 6.

Table 5. *Analysis of variance - Two factor factorial design. First extraction colour.*

Na_2S g/l	TIME IN WEEKS			SUM	MEAN	VAR.	MEAN VARIANCE	
	2	4	6					
1.5	6.4	10.0	10.7	27.1	9.0	5.3		
2.2	11.4	8.9	11.4	31.7	10.6	2.1	2.8	
2.9	10.0	9.4	11.4	30.8	10.3	1.1	$F_{SULPHIDE}$ = 0.7	
					10.0	0.7	(0.7 X 3 / 2.8)	
SUM	27.8	28.3	33.5	89.6				
MEAN	9.3	9.4	11.2		10.0	1.11		
VAR.	6.7	0.3	0.2					
MEAN VAR.		2.4					F_{TIME} = 0.4 (1.11 X 3 / 2.4)	

Table 6. *Analysis of variance - Two factor factorial design. Overall colour*

Na_2S g/l	TIME IN WEEKS			SUM	MEAN	VAR.	MEAN VARIANCE	
	2	4	6					
1.5	18.1	14.2	13.1	45.4	15.1	6.9		
2.2	14.6	15.0	15.8	45.4	15.1	0.4	2.9	
2.9	14.7	14.3	14.6	45.5	15.2	1.4	$F_{SULPHIDE}$ = 0.0	
					15.1	0.0	(0.0 X 3 / 2.9)	
SUM	47.4	43.5	45.4	136.3				
MEAN	15.8	14.5	15.1		15.1	0.42		
VAR.	4.0	0.2	3.2					
MEAN VAR.		2.5					F_{TIME} = 0.52 (0.42 X 3 / 2.5)	

From the low values of $F_{0.05\ (2.2)} < 19$ (Freund and Williams, 1964b) in both tables it was concluded that there was no statistically significant correlation between conditioning time, or sulphide concentration and first extraction colour or overall colour.

Comparison of the colours of ST1-2/4 and ST1-2/5 (17.8 and 22.8) boil gelatines in ADDENDUM C4, showed the deterioration in colour with the progress of extraction as was normally experienced in production. Hence, it appeared that the production of dark gelatines at the end of the production cycle was a consequence of the hide from old animals being part of the raw material mixture.

EXPERIMENT ST2 / WT.
(Old animal and the effects of sulphide and temperature).

It was accepted that the Maillard reaction was an oxidation reaction and that SO_2 could inhibit colour formation (Monnier, Sell, Miyata and Nagaraj, 1990). Hence, it was proposed that sulphide in conditioning might also have had an effect on colour due to its reducing properties. This experiment was designed to compare conditioning with lime only under nitrogen against aerobic lime only and lime plus sulphide. As there was material available it was decided to investigate the effect of temperature as well, by conditioning with two levels of sulphide under ambient winter conditions. Based on the previous findings that old animal hide yielded the darkest gelatine it was considered that a hide from an old animal would best exhibit any effect due to the treatments, so the hide of a 12 year-old animal from ADSRI was used. Details of the results are in ADDENDUM C5.

Comparing the overall colours of the WT1 (15.7), WT2 (12.7) and WT3 (14.3) gelatines it appeared that liming with lime under nitrogen (WT1), gave the worst overall colour. The same observation was made with respect to first extraction colours!

Comparing WT2 and WT3 the conditioning enhancing effect of sodium sulphide was evident from the increase in the first extraction proportion of WT2 (aerobic lime only) from 4.1% to 10.4% in WT3. The effect of sulphide on colour appeared to be one of darkening as shown by the increase in overall colour from 12.7 with lime only to 14.3 with lime-sulphide conditioning. However, as

this was only just larger than the 1.5 units accepted as the error of visual determinations of colour, it would need confirmatory data before it could be accepted as significant. Nevertheless, it could be concluded that sodium sulphide in conditioning had no beneficial effect on gelatine colour.

Comparing WT3 conditioned at 22°C and WT4 conditioned at 12°C, the effect of temperature on conditioning could be clearly seen from the drop in first run extractability from 10% to 4% and an increase in gelatine recovered in the boil from 49% to 71%. Comparison of the extractability data of WT3 and WT4 with ST1-2 and ST1-4 (Experiment ST - ADDENDUM C4) shows that they were very similar. As both experiments were conducted on "old" animal hide it could also be proposed that a 10°C drop in conditioning temperature was approximately equivalent to a 2 week drop in conditioning time at 22°C.

The effect of the 10°C drop in temperature on overall colour was apparently also one of darkening. This was the first indication that conditioning parameters could have had an effect on the colour of the gelatine but the indication was not unequivocal because the poor clarity of the boil liquor would probably have lead to an overestimation of the colour of 71% of the gelatine in the boil from the low temperature conditioning.

Comparing WT4 and WT5 it was apparent that the 6 fold increase in sulphide concentration in WT5 had only a marginal effect on extractability and no significant effect on colour which confirmed the findings in experiment ST1.

If the overall colour result of 15.7 in WT1 was taken with the overall colour results of 16.3 and 16.6 in WT4 and WT5 it appeared that possibly the low overall colour results in WT2 and WT3 could be considered due to random variations.

The WT series of experiments was particularly important in showing that high levels of dissolved organic matter (volatile solids) in conditioning liquor had not had a deleterious effect on gelatine colour. Furthermore, if it was accepted that there was no solubilization of hair in the absence of sulphide then this experiment indicated that liming with sulphide could solubilise some 13.5% of the hide substance but lime only, solubilised only 7% of the hide substance. Hence, by subtraction, some 6.5% of hide substance was hair, and 7% was alkali soluble non-collagen organic matter.

EXPERIMENT YS.

(Young animal - effect of sodium sulphide)

Most of the previous trials had been conducted on the hide of old animals in order to ensure that any treatment effects on colour would be evident. This experiment was designed to demonstrate the best possible colour obtainable from young bovine hide and from the one part in which no sodium sulphide was used it was hoped to obtain a measure of the conditioning enhancing effect of this additive. From experience it was known that the conditioning time required by the hide of such a young animal was very much less than that for older animals, hence conditioning was carried out for 2 and 3 weeks with lime and sulphide and for 4 weeks without sulphide. The detailed results are in ADDENDUM C6.

The observations made during this experiment were:

1. The effect on extractability of increasing the sulphide concentration, from 1.6 to 5.7 g/l (YSA & YSC v/s YSB & YSD), was 4.5 %. The size of this effect was such as to make it impossible to say whether the change in extractability was due to the treatment or to experimental error. It was noted that there was no effect on gelatine colour.

2. The young animal hide gave gelatines with colours almost as good those expected from Type A pigskin gelatines. In a later investigation gelatine YSB/1 was measured as having an absorbance area of 4.41, whereas the best result on American pigskin gelatines was an absorbance area of 4.57, hence, it would appear that the best colour from Type B calf skin would be equal to that of the best Type A pigskin.

3. Four weeks of lime only (part YSE) gave a 45°C extractability of 30%. Part YSA which received 2 weeks of lime sulphide conditioning had an extractability of 35%. Both parts had the same proportion recovered in the residue boil. Hence, it would seem that the presence of sulphide during conditioning had an effect equivalent to about 2 weeks of lime only at 22°C.

This experiment confirmed that the effect of sulphide was not proportional to concentration so it could be proposed that the effect was probably one of

inhibiting the primary Maillard reaction which would be promoted by the low pH of liming and would result in cross linking of the collagen by any available aldose. The lack of effect on gelatine colour would mean that the sulphide did not inhibit the formation of coloured byproducts of the Maillard cross-linking that had occurred prior to liming. From data to be reported under the fluorescence study it could be deduced that the amount of colour that would be generated by the Maillard reaction during 4 weeks of liming at 22°C would probably not be noticeable especially if the aldose available was mainly glucose as will be seen from the discussion of the gelatine/glucose interaction (*loc cit*).

EXPERIMENT KTO.

(Tannery treatment of an old animal hide).

Details of this experiment are shown in Addendum C7. The experiment was designed to investigate the colours of the gelatines that were recovered from different layers of the same hide. In order to maximise the effects on colour the experiment was conducted on the salted hide of a 12 year-old animal from ADSRI.

It had long been the experience at DGI that good quality dry splits gave gelatine of superior colour to that obtained from whole hide. Also those gelatine manufacturers that produced almost entirely from tannery wet limed splits, had a gelatine colour advantage over those who used the whole hide. However, whether this observation could be attributable to the use of corium only needed to be substantiated. The results obtained are shown in Tables 7 and 8.

Table 7. *Gelatine colours from various layers of the same hide.*

RAW Material	Overall Colour	First Extract Colour	Second Extract Colour	Third Extract Colour	Boil Extract Colour
Flesh	--	8.4	10.0	16.0	NM[a]
Middle	12.3	6.4	6.0	6.4	16.0
Grain	12.6	6.0	6.0	6.8	16.7
Whole Hide	11.6	8.0	7.2	9.4	13.3

-- Not available.

[a] Not measurable.

Table 8. *Gelatine Clarity from various layers of the same hide.*

Raw Material	Overall Clarity	First Extract Clarity	Second Extract Clarity	Third Extract Clarity	Boil Extract Clarity
Flesh	--	11.1	7.0	3.5	NM[a]
Middle	14.0	12.5	12.5	11.1	15.4
Grain	10.1	12.5	12.5	11.1	9.0
Whole Hide	12.4	11.8	11.8	9.0	14.3

-- Not available.

[a] Not measurable.

From Tables 7 and 8, it was concluded that the flesh associated with the hide made the biggest contribution to the colour of the extracted gelatine as well as being responsible for the worst clarity. The poor colour of gelatine from the flesh split was in agreement with a previous experiment (not reported) on hide of unknown origins.

The colour of the first extract gelatine from the whole hide may appear dark but the expected colour, calculated from the weighted contributions from the three layers, of 7.8, was in good agreement with the 8.0 measured colour of this gelatine.

It was of particular interest to note that the grain (epidermis) split and the middle split gave gelatines of very similar colour and clarity and that this colour was markedly better than that of the whole hide. The fact that the middle split gelatine colour was not as good as could be expected from pigskin was probably due to the age of the animal.

With lime-sulphide conditioning of hairy hide, the conditioning liquor normally becomes very dark in colour and it was a matter of conjecture whether this darkening was due to "hair burn" by the sulphide or whether it was due to the removal of colour from the collagen. In this experiment all the hair had been removed by the tannery pretreatment, hence, the very low variance in the absorbance data recorded on the filtered conditioning liquors indicated that lime-sulphide conditioning did not remove significant amounts of coloured substances from the hide.

Finally, the high extractability of the flesh split was interesting as it indicated that this layer contained the most recently formed collagen. However, the dark colour was at variance with the finding so far, namely, that the younger the animal the paler the gelatine.

EXPERIMENT CALF-A.

(Type A gelatine from calf skin).

The detailed results of this experiment are in ADDENDUM C8.
Due to the dehairing step used, the proximate composition of the salted hides was found to be moisture 44.8%, ash 17.0%, hair 17.6%, gelatine 16.8%, fat 0.7%, acid solubles (collagen) 0.8% and an unknown balance of 2.3%(Table 10).

It should be noted that the gelatine yield of 44% on an anhydrous, ash free basis was markedly low. More normal yields were in the range 56% to 78%. Furthermore, the residue after the boil was abnormally high in spite of the raw material being calf skin. This residue could easily account for the 2.3% (175g) of the original material that was otherwise unaccounted for.

Based on the isoionic points of the gelatines produced it was concluded that the short dehairing process had had a negligible effect on the acid amides of the collagen. Also the drop in pI with extraction temperature was in line with the results of Toda (1986).

The Type A calf skin gelatines had an undoubtedly good colour but the three run overall colour (4.1) was no better than that recorded for Type B gelatine from 10 month old animal skin of 3.4 for experiment YSD above.

EXPERIMENT 3Y & 6Y.

(3 and 6 year-old Friesland animal's hide).

This experiment was designed to show the effect of Friesland breed and animal age on gelatine colour as well as to act as a confirmatory experiment on the role of sulphide in lime-sulphide conditioning. Detailed data is in ADDENDUM C9.

Gelatine Colour.

From the point of view of gelatine colour the 3 year-old animal (3Y) gave very

similar gelatine colours irrespective of conditioning time, with overall colours close to 5 which was even better than the overall colour of about 6 obtained in experiment CT on 18 month old animal, above. This appeared to indicate that breed does in fact play a role in gelatine colour.

The six year-old animal hide gave rather erratic first extraction colours with the best colour being associated with the shortest conditioning time and lowest extractability. In this experiment the overall colours were about 7.5 which was markedly darker than the overall colour produced from the 3 year-old Friesland.

Role of Sodium Sulphide.

Comparison of the **4A and **4B parts of these experiments confirmed that doubling the sodium sulphide concentration during conditioning had virtually no effect on gelatine extractability and colour. The 3Y6 and 6Y6, B parts which were conditioned for six weeks with no sulphide, again gave gelatines of the same colour as was produced in the A parts with 2 g/l of sulphide in the conditioning liquor. This confirmed that sulphide had no effect on gelatine colour.

The effect of sulphide on conditioning was shown by both experiments (3Y & 6Y). The first extraction proportions after 6 weeks with lime only were intermediate between 2 and 4 weeks with sulphide. This, combined with the result given by experiment YS, indicated that the presence of sulphide during conditioning was equivalent to about 3 weeks of extra time at 22°C without sulphide. Hence, the previous conclusions with regard to the role of sulphide in conditioning, namely, that it inhibited the Maillard cross-linking promoted by the alkaline conditions, were unaffected.

EXPERIMENTS 5Y and INO.
(Five year-old Chianina hide and a 12 year-old Inguni hide)

The details of these experiments are recorded in ADDENDUM C10 and ADDENDUM C11. The main point of interest of these experiments was that they were conducted on a Chianina hide and an Inguni hide. The results obtained were in no way unusual, hence, it was concluded that breed had not had a significant effect on conditioning response or gelatine colour.

THE EFFECT OF ANIMAL AGE AND PROCESSING ON GELATINE COLOUR.

The detailed colour data extracted from ADDENDA C1 to C11 is presented in ADDENDUM C12. From this table it might appear that there was no systematic change in first extraction gelatine colour with conditioning time or with extractability, hence there was no justification for separating the data using conditioning time. For this reason the average colours produced from a single hide, as shown in Table 9, were used to evaluate whether there was a statistical correlation between animal age and gelatine colour.

The first extraction colours were subjected to polynomial regression analysis against animal age. The first and second order correlation coefficients (0.892 and 0.898) were very similar hence there was no reason to use the second order regression equation and the linear coefficients were:
B(0) = 4.117093
B(1) = 0.0366585.
The correlation coefficient (r) of 0.89 for 8 degrees of freedom was significant at the highest (0.0005) level of probability, however the fact that r was not closer to 1 indicated that there was variance in the data due to other factors.

Table 9. *Gelatine colour response to animal age.*

EXPERIMENT No	ANIMAL AGE MONTHS	1ST EXTRACT COLOUR	OVERALL COLOUR
CALF-A	3	4	
YS	10	3.3	4
CT	18	5.5	6.3
3Y	40	6.3	5.8
5Y	58	6.1	7.6
6Y	78	6.9	7.6
CTO	152	8.4	15.1
ST1	144	10	15.1
ST2	144	11.4	15.1
INOE	143	9.4	14.9
KTO	144	8	11.6

In the case of Experiment CTO, from inspection, it appeared that one of the "other" factors causing darker gelatine could be an increase in extractability due to the increase in conditioning time. In the case of the other experiments either the animal was too young to show the effect, or the changes in conditioning times were insufficiently varied to demonstrate the effect. There was additional evidence that the gelatine was darker as the degree of extraction increases in the ST1-2/4 and ST1-2/5 (ADDENDUM C4) boil data.

In the case of the correlation between average overall colour and animal age data, the second order polynomial regression correlation coefficient was 0.966 and the equation coefficients were:
B(0) = 4.68104
B(1) = 0.0221079
B(2) = 0.00030458
This correlation coefficient indicates that the other factors affecting overall colour were probably quite small and possibly limited to random or experimental error.

Also the data in Table 9 indicated that there was no marked effect on gelatine colour attributable to breed differences.

THE EFFECT OF ANIMAL AGE ON GELATINE EXTRACTABILITY.

The significance of the extractability data in Table 10 below, lies in the understanding it provides of the reasons behind the variable response of raw material to a conditioning process. The data was obtained using a single conditioning process controlled at 22°C, and a single extraction regime, hence the variables were minimal and the differences in extractability could be attributed to the differences in animal age and conditioning time.

When the data in Table 10 was submitted to 3 way linear regression analysis, however, the correlation coefficient between extractability and conditioning time was only 0.27 (26 degrees of freedom) which was only significant with a probability of 0.1, whereas, the correlation between extractability and age was 0.645 which was significant at the 0.0005 level of probability. Hence, statistically, animal age was the most important factor when it came to extractability as a result of lime/sulphide conditioning.

Table 10. *The changes in the 45°C extractability of hide due to conditioning time and animal age.*

		CONDITIONING TIME IN WEEKS.								
EXP. No.	ANIMAL AGE MONTHS	1	2	3	4	5	6	7	8	10
YS	10	-	35.3	45.9	-	-	-	-	-	-
CT	18	3.3	11.3	21.2	22.5	35.0	39.5	-	-	-
3Y	40	-	9.6	-	28.3		36.1	-	36.9	-
5Y	58	-	7.5	-	20.0	-	21.4	-	-	-
6Y	78	-	6.6	-	15.3	-	21.4	-	-	-
CTO	152	-	4.4	-	8.4	-	-	10.9	-	10
ST1	144	-	3.6	-	9.6	-	11.5	-	-	-
ST2	144	-	-	-	10.4	-	-	-	-	-
INOE	143	-	-	-	11.4	-	-	-	-	-
KTO	144	-	-	-	10.5	-	-	-	-	-

- Not applicable.
Lime-sulphide conditioning using 2 g/l Na_2S at 22°C.

From an inspection of the data in Table 10, however, it could be seen that conditioning time was a major factor in determining gelatine extractability for animals under the age of about 5 years but for old animals conditioning time beyond about 4 weeks at 22°C had a negligible effect on extractability.

THE COMPOSITION OF HIDE.

Due to the possible variation in the amount of hide substance in any sample, gelatine yields etc. were expressed in terms of anhydrous ash-free hide substance for comparative purposes.

Due to the single acidulation solution used in the production of Type A calf skin gelatine it was practical to estimate the losses due to acidulation as acid soluble collagen by determining the amount of organic matter in the solution as volatile solids (at 550°C). Furthermore, it was apparent that the 2.1 kg of residue after extraction could easily have been responsible for the 2.3% of the raw material not accounted for.

Table 11. *Composition of calf skin ex the acid process.*

ATTRIBUTE	% of Raw Material	% of Anhydrous Ash-free Hide
Moisture	44.8	-
Ash	17.0	-
Hair	17.6	44.5
Gelatine	16.8	44.0
Fat	0.7	1.8
Acid Soluble Collagen	0.8	2.1
Extraction Residue. (by difference)	2.3	6.0

Data from Addendum C7.
- Not applicable

In the case of experiment YS it was evident that unaccounted losses were considerably higher than with the calf skin used for acid processing, in spite of the extraction residues being only about 300 g. The reason for this was thought to be due to losses of collagen as eucollagen (Balian and Bowes, 1977) into the large amounts of sulphurous acid solution used in acidulation. (The

term eucollagen was used to distinguish acid soluble collagen from alkali treated hide, from acid soluble collagen from untreated (calf) hide).

The hair content of the alkali processed calf hide (YS) was determined by the difference between the organic content of spent conditioning liquor where no sulphide was used (YSE) and where a high level of sulphide was used (YSB). The large difference in this attribute between the two lots of calf skin was noteworthy and this was obviously a factor in the lower gelatine yield indicated in Table 11. However, in general the data in Tables 11 and 12 was in good agreement with the findings of Bowes, Elliot and Moss (1958).

Table 12. *Composition of calf skin ex the alkaline process.*

ATTRIBUTE	% of Raw Material	% of Hide Substance
Moisture.	61.0	-
Ash.	0.2	-
Hide Substance.	38.8	-
Gelatine.	21.3	54.9
Hair.	1.2	3.0
Lime solubles.	2.8	7.3
Fat.	2.0	5.2
Unaccounted / Losses.	11.2	29.6

- Not applicable.

The data in Table 13 on the composition of hide used in alkali conditioning was extracted from the data in the addenda. The gross composition of hide was limited to the major constituents, namely moisture and ash with the residue being "hide substance".

Table 13. *Average crude composition of adult bovine hide.*

TYPE	Green Hide	Washed Hide	Salted Hide
Number of Samples.	1	5	6
Moisture %	65.5	59 - 69	38 - 48
Ash %	0.6	0.2 - 0.6	14 - 17
Hide Substance % (Anhydrous Ash Free).	33.9	30 - 40	38 - 44

The average composition of bovine (anhydrous, ash-free) organic hide substance is given in Table 14. The variability of the amount of hair in hide substance has been mentioned above. The large variability of gelatine yield (or collagen content) from 40% to 80 % was also noteworthy. The highest yield (80%) was given by an 18 month old Brahman and the lowest (42%) by 6 year-old Friesland. It was also observed that with the high yield of the Brahman went a very low fat recovery from the extraction liquors.

A part of the 21% of the hide unaccounted for could be losses due to eucollagen dissolved during the sulphurous acid acidulation process. Another part would be the "scutch" or insoluble residue remaining at the end of extraction. A further part of the losses could be material passing through the tumbler screens during washing, however, unless the material was grossly over conditioned, this loss was very small.

The "lime soluble material" determined from those experiments where no sulphide had been used (WT1, WT2, YSE, 3Y6B and 6Y6B) was assumed to be a measure of the albumins, globulins and products of the destruction of glucoseaminoglycans (GAGs) and elastin components of skin (Bowes and Elliot, 1958; Haines, 1984). Where sulphide was used this value was much higher and therefore included much of the products of keratin destruction as well. The residue after spent conditioning liquor filtration was always black, hence, part of the hair including the melanin pigments of hair were not solubilised by sulphide.

Table 14. *The constituents of anhydrous ash-free bovine hide.*

ATTRIBUTE	MEAN	STANDARD DEVIATION	NUMBER OF SAMPLES	RANGE
Gelatine %	58.7	11.1	40	42.4 - 82.7
Fat %	3.8	2.0	6	Nil - 6.7
Lime/Sulphide Solubles % = A.	16.4	3.8	6	11.2 - 21.6
Lime Solubles % = B.	7.35	2.15	6	4.3 - 9.8
A - B = Hair %	9.0	-	-	-
Unaccounted / Losses %.	21.1	-	-	-

GELATINE IRON CONTENT.

Linear regression was performed on the calibration data giving:

Fe ppm = 2.82 + 113.9 x Absorbance. r = 0.997.

Slope error = ± 13 hence Fe error = ± 7 ppm.

The error of 7 ppm was considered satisfactory for quality control purposes of estimating gelatine iron contents in the range of 10 to 50 ppm.

Table 15. *Gelatine iron content and colour.*

Sample	Absorbance	Fe ppm.	Corrected ppm Fe	Colour Value.
40 ppm Instrument Control	0.309	40		
Reagent Blank.	0.014	4.4	0	
Gelatine 155/1	0.187	24	20	6.4
Gelatine YSA/1	0.159	21	17	3.6
Gelatine YSA/3	0.165	22	18	4.8
Gelatine WT3/1	0.301	37	34	10.7
Gelatine WT3/3	0.220	28	24	16.0

Blank = Distilled water.

Table 16. *The effect of added iron on the colour of gelatine 155/1.*

Fe added. ppm.	Fe content. ppm.	Colour Value.	pH
0	20*	6.4	5.63
20	40	6.8	5.46
40	60	7.6	5.33
60	80	8.0	5.21
80	100	8.0	5.13
100	120	8.9	5.03

* From Table 15.

Linear regression analysis of colour and iron content from Table 16. gave the following result:

Colour Value = 6.0 + 0.0236 x ppm Fe. r = 0.975.

From this one could conclude that each ppm Fe contributed 0.02 units to the colour value of the gelatine in the range 0 to 120 ppm Fe. However, as can be noted from Table 15, most gelatines had an iron content well below 50 ppm. Hence, the contribution of iron to the normal colour of gelatine would be less than one unit. However, due to the subjective nature of the colour determination the error of the method was generally accepted as 1.5 units, hence the contribution of iron to gelatine colour could be negligible when iron contents were less than 50 ppm.

Finally, it was possible that pH could have played a part in the observed changes in colour with iron content in Table 16. However, the pHs of 5.0 to 5.6 were in the normal range and the differences were considered too small to be significant.

The gelatines chosen above were:

155/1 a normal production gelatine with a colour value of 6.4.
YSA/- Pale gelatines from a 10 month old animal skin.
WT3/- Dark gelatines from a 144 month old animal skin.

From Table 15, it was evident that there was no correlation between gelatine colour and iron content and that iron content alone was certainly not the cause of gelatine colour.

GELATINE AMINO ACID ANALYSIS.

The results of the amino acid analyses in ADDENDUM 13 had quite large standard deviations. It was evident that by removing the variables due to moisture and ash content the standard deviations were markedly reduced as shown in Table 17.

The standard deviation due to analytical errors was calculated from the standard deviation on analysis of the same gelatine, namely samples C and D. Thus any increase in standard deviation, when all the data was taken into consideration could possibly be attributed to variation due to the different samples. The results in Table 17 where "%-RSD" changes exceeded 1% were marked with a *. Similarly differences in "%-RSD" between all samples and all samples without A (chrome tanned leather gelatine) could be interpreted as due to chrome gelatine.

From the Experimental Error %RSDs (EE-RSD) it was concluded that all the values for methionine and cystine should be ignored and (as stated by Stevens and Stevens, 1992) the data for histidine was very unreliable.

Comparing EE-RSD and ALL-RSD the largest discrepancy was in the ornithine data. This result was in agreement with the progressive alkaline conversion of arginine to ornithine, with the liberation of urea (Veis, 1964c). In sample A (gelatine from tanned leather waste), the collagen had received tannery treatment plus extraction at 70° to 90°C at pH 9. Samples B and C had been limed for 2 weeks. From the ornithine contents these treatments seem to have been similar in effect. In samples E and F the collagen had been limed for 4 weeks (*loc cit*) and the ornithine content of the gelatine was double that of samples B and C. As was expected this phenomenon also lead to a marked change in the arginine %-RSDs.

Table 17. *The molar % amino acid content of gelatines A to F.*

	SAMPLE						EXPERI-MENTAL ERROR % RSD OF SAMPLES C-D	MEAN OF ALL SAMPLES	% RSD OF ALL SAMPLES		MEAN WITHOUT SAMPLE A	% RSD WITHOUT SAMPLE A	
AMINO ACID	A	B	C	D	E	F							
ASP	3.9	4.5	4.5	4.4	4.1	4.3	2.6	4.3	5.2	*	4.4	4.3	*
GLU	6.4	6.7	6.7	6.6	6.6	6.5	3.6	6.6	3.5		6.6	3.4	
HOPRO	10.1	9.8	10.7	10.1	10.6	10.2	3.0	10.3	3.3		10.3	3.5	
SER	3.2	3.3	3.5	3.5	3.4	3.4	2.4	3.4	3.0		3.4	2.6	
GLY	32.6	32.8	33.2	33.0	33.4	32.9	2.6	33.0	2.5		33.1	2.7	
HIS	0.2	0.2	0.2	0.3	0.2	0.2	123.3	0.2	130.1		0.2	134.8	
ARG	6.4	5.9	5.8	5.6	5.8	5.6	5.9	5.8	7.1	*	5.7	6.6	
METSO1	0.0	0.0	0.0	0.0	0.1	0.1	er	0.0	267.3		0.0	247.5	
METSO2	0.0	0.0	0.0	0.0	0.1	0.0	er	0.0	387.3		0.0	360.6	
THR	1.3	1.6	1.6	1.6	1.6	1.6	5.5	1.5	6.7	*	1.6	4.4	*
ALA	11.4	10.9	10.6	10.6	10.5	10.6	0.8	10.7	2.9	*	10.6	1.7	*
PRO	13.5	13.3	12.9	13.1	12.9	13.4	3.3	13.1	3.8		13.1	3.9	
TYR	0.0	0.2	0.2	0.4	0.1	0.1	50.7	0.2	82.4		0.2	68.5	
VAL	2.1	2.1	1.9	2.0	2.0	2.0	2.2	2.0	3.7	*	2.0	3.5	
MET	0.0	0.0	0.0	0.0	0.0	0.1	er	0.0	276.3		0.0	256.0	
CYS	0.0	0.0	0.0	0.0	0.0	0.0	er	0.0	er		0.0	er	
ILE	1.2	1.2	1.2	1.3	1.2	1.3	6.1	1.2	4.4	*	1.2	4.7	
LEU	2.4	2.4	2.2	2.3	2.3	2.4	5.3	2.3	5.6		2.3	6.0	
HOLYS1	0.8	0.8	0.7	0.7	0.8	0.7	10.8	0.8	9.1		0.8	9.7	
HOLYS2	0.2	0.2	0.2	0.2	0.2	0.2	19.6	0.2	18.9		0.2	18.5	
PHE	1.2	1.3	1.2	1.3	1.1	1.2	5.9	1.2	5.2		1.2	5.6	
LYS	3.0	2.7	2.7	2.9	2.7	2.9	5.8	2.8	4.5		2.8	4.2	
ORN	0.1	0.2	0.1	0.2	0.4	0.4	9.9	0.2	51.4	*	0.3	44.0	*
TOTAL	100	100	100	100	100	100	-	-	-		-	-	

- Not applicable. er = Error result of calculation..

Comparison of the ALL-RSD and the RSD-A lead to the conclusion that aspartic acid, threonine with an hydroxyl group in the side chain and possibly alanine with no functional groups in the side chain could be involved in the chrome tanning process. It was important to note that glutamic acid did not appear to be involved with the binding of chromium.

Hence, when in addition the hide from an animal was cut into 100 x 100 mm pieces and these were randomised by tumbling, this allowed several experiments to be conducted without interference from the effects from variance in raw material.

2. Alkaline conditioning variables had no significant effect on the overall colour of the gelatine extracted. In particular, the inclusion of sulphide in the conditioning liquor could not be shown to have had any effect on gelatine colour.

3. Possibly the most significant conclusion to be drawn from this study was that the apparent inverse correlation between gelatine colour and the other quality parameters of Bloom gel strength and viscosity was only valid when the gelatine was extracted from old animals. When gelatine was extracted from young animal's hide the colour of the product was comparatively invariant.

4. When the extractability of old animal hide was increased by long liming time, the colour of the first extract (45°C) gelatine darkened with liming time. This effect was only demonstrated once but it could account for some of the random variation in first extraction gelatine colours.

5. The correlation between gelatine overall colour and animal age was 0.97 with 8 degrees of freedom which besides being significant at the 0.9995 level of probability was also high enough to indicate that other factors contributing to the colour were hardly significant.

6. From the data there was no indication that breed played a significant role in determining gelatine colour.

7. Of importance to the manufacturer was the extractability data showing that as animal age increased so the extractability decreased to the extent that there was little advantage to be gained from increasing the conditioning time with lime and sodium sulphide beyond four weeks at 22°C. The demonstration that the effect of sodium sulphide in conditioning was equivalent to an extra 3 weeks in lime only, was also of economic significance. Finally, from experiment WT it was concluded that a drop of 10°C in liming temperature caused a drop in

extractability which could be compensated for by approximately 2 weeks extra conditioning time.

8. The average proximate analysis of anhydrous hide substance was collagen/gelatine 58.7%, fat 3.8%, lime soluble substances 7.4% and hair 9%.

Although it was recognised that iron contamination could contribute to the colour of gelatine, this study showed that at normal levels of iron contamination (<50 ppm) this source of colour was small and possibly negligible.

It was hoped that there would be a correlation between amino acid analysis (particularly lysine) and gelatine colour. The only correlations found were the well known correlations between arginine, ornithine and liming time. Also, gelatine from chrome tanned leather showed a slight depletion of the aspartic acid content, but how this was related to its pale colour was not evident.

REFERENCES.

Balian, G., and Bowes, J.H. 1977. The Structure and Properties of Collagen. In *The science and technology of gelatin*. A.G. Ward and A. Courts. (Ed.). p. 21. Academic Press. London, New York, San Francisco.

Bowes, J.H., and Elliot, R.G. 1958. The extraction of soluble proteins from skin-III. The extraction of alkali soluble protein from calf skin. . British Leather Manufacturer's Research Association Laboratory Reports. 37: 148-162.

Bowes, J.H., Elliot, R.G., and Moss, J.A. 1958. The Extraction of Alkali-Soluble Protein from Calf Skin. British Leather Manufacturers' Research Association Laboratory Reports. 37(1): 148-162.

British Standards Institution, 1975. (BS 757:1975). Methods for sampling and testing gelatine. British Standards Institution. London.

Cochran, W.G., and Cox, G.M. 1957. Experimental designs. John Wiley & Sons Inc. London, Sydney.

Cole, B. 1980. The effects of sulphate on gelatine Rating value. Davis Gelatine Industries Departmental Memorandum dated 10/11/1980. Krugersdorp. RSA. (Not in the public domain).

Cole, C.G.B. 1986. A study of the properties of gelatines derived by various chemical and enzymic methods. M.Sc. Thesis. University of Pretoria. RSA.

Cole, B. 1989. Gelatine quality - derived from various layers of the same hide. Davis Gelatine Industries Departmental Memorandum dated 17 November 1989. (Not in the public domain).

Dyer, D.G., Blackledge, J.A., Katz, B.M., Hull, C.J., Adkisson, H.D., Thorpe, S.R., Lyons, T.J., and Baynes, J.W. 1991. The Maillard reaction in vivo. Zeitschrift für Ernährungswissenschraft 30(1): 29-45.

Eastoe, J.E., and Eastoe, B. 1951. The Determination of Trace Elements in Gelatine - I. Colorimetric Methods for Copper and Iron. British Gelatine and Glue Research Association Research Report B2. UK.

Freund, J.E., and Williams, F.J. 1964a. Elementary business statistics - The modern approach. p 384-389. Prentice-Hall Inc. New Jersey.

Freund, J.E., and Williams, F.J. 1964b. Elementary business statistics - The modern approach. p. 440-441. Prentice-Hall Inc. New Jersey.

Haines, B.M. 1984. The skin before tannage-Proctor's view and now. Journal of the Society of Leather Technologists and Chemists 68: 57-70.

Heidemann, E. 1982. Proctor Memorial Lecture. Newer developments in the chemistry and structure of collagenous connective tissues and their impact on leather manufacture. Journal of the Society of Leather Technologists and Chemists 66: 21-29.

Jones, N.R. 1977. Uses of gelatin in edible products. In *The Science and Technology of Gelatin*. A.G. Ward and A. Courts (Ed). p.393. Academic Press. London, New York, San Francisco.

Monnier, V.M., Sell, D.R., Miyata, S., and Nagaraj, R.H. 1990. The Maillard reaction as a basis for a theory of ageing. In *The Maillard reaction in food processing, human nutrition and physiology*. Finot, P.A., Aeschbacher, H.K., Hurrell, R.F., Liardon, R. (Ed.). p. 393-414. Advances in Life Sciences. Birkhauser Verlag Basel.

Montgomery, D.C. 1985. Design and analysis of experiments. John Wiley & Sons. New York, Singapore.

Na, C.G., Phillips, L.J., and Freire, E.I. 1989. In vitro collagen fibril assembly: Thermodynamic studies. Biochemistry 28: 7153-7161.

Reich, G., Walther., S., and Stather, F. 1962a. On some of the laws governing the production of gelatine from pigskin according to the acid conditioning process. Deutsche Lederinstitut, Frieberg/SA. 18: 15-23.

Reich, G., Walther, S., and Stather, F. 1962b. The influence of the age of cattle and pigskin on the yield and quality of gelatine obtained after the acid conditioning process. Deutsche Lederinstitut, Frieberg/SA. 18: 24-30.

Stevens, P.V., and Stevens, J.A. 1992. Interim report on South African gelatine samples. Amino acid and cross-link analysis and Further results on six Sth African gelatins. (Private communications dated 11.6.92 and 1.7.1992)

Toda, Y. 1983. The Relationship between the isoelectric point distribution and molecular weight distribution of gelatin. Proceedings of the 4th IAG Conference on Photographic Gelatin. Int. Arbeitsgem. Photogelatine, Fribourg. Switzerland.

Veis, A. 1964a. The macromelecular chemistry of gelatine. p. 393. Academic Press, New York & London.

Veis, A. 1964b. The macromelecular chemistry of gelatine. p. 107. Academic Press, New York & London.

Veis, A. 1964c. The macromolecular chemistry of gelatin. p. 200-202. Academic Press-New York & London.

Williams, H.V. 1957. The effect of iron on the colour of gelatine. DGI Research Report No 32. Dated 14 March 1957. Krugersdorp. RSA. (Not in the public domain).

ADDENDA.

ADDENDUM C1. The effect of position of the hide on the animal.

EXPERIMENT FR

Green Face pieces v/s Green Rest of the hide.

Raw Material and Conditioning.

Raw Material **Green Hide from IAPI.**

Moisture Content	65.45
Ash Content.	0.58
Sample GF	10.05 kg
Sample GR	10.00 kg

Conditioning.
Conditioning Liquor: CaO 640 g
Na_2S 60% 120 g
Water to 20 kg.

Sample No	GF	GR	
Cond: Time (Days)	27	27	
Cond: Temperature (°C)	23.0	23.0 ±1.2	
Init: Sulphide (g Na_2S/l)	2.29	2.35	
Final Sulphide (g Na_2S/l)	1.35	1.50	
Final Liquor Total Solids (%)	4.24	3.52	Error <0.02%
Final Liquor Volatile Solids (%)	3.19	2.52	Error <0.02%
Final Liquor Organic Solids (g)	210	200	
Ex-Lime Wash for 16hrs			
Limed Mass (kg)	17.55	16.95	
Swelling (%)	175	170	
Acidulation 5 coats of H_2SO_3 soln. (days)	3	3	
Wash 1hr.			
Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	22.0	22.35	
Soak Water (pH)	1.98	1.92	

ADDENDUM C1. Continued...

Raw Material **Salted Hide from IAPI.**

Moisture Content	48.09 %
Ash Content	14.10 %
Sample SF	4.75 kg
Sample SR	4.70 kg

Conditioning.

Conditioning Liquor: CaO 640 g
Na_2S 60% 120 g
Water to 20 kg.

Sample No	SF	SR	
Cond: Time (Days)	29	29	
Cond: Temperature (°C)	23.0	23.0 ±1.0	
Init. Sulphide (g Na_2S/l)	2.98	2.93	
Final Sulphide (g Na_2S/l)	1.43	1.34	
Final Liquor Total Solids (%)	5.63	6.27	Error <0.01%
Final Liquor Volatile Solids (%)	1.94	2.07	Error <0.01%
Final Liquor Organic Solids (g)	388	414	
Ex-Lime Wash for 16hrs			
Limed Mass (kg)	10.05	10.70	
Swelling (%)	12	228	
Acidulation 5 coats of H_2SO_3 soln.. (days)	3	3	
Wash 1hr.			
Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	12.3	12.65	
Soak Water (pH)	2.07	2.05	

ADDENDUM C1. Continued...

Extraction & Quality Data. Experiment GF.

Extraction.

Run No	1	2	3
Time hrs	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	21.68	7.39	6.27
Liquor pH	2.88	3.14	
Liquor Concentration (%w/v)	5.15	7.46	5.57
Scutch (g)			310
Gelatine (g)	1116.5	551.3	349.2
Gelatine % Proportion	55.4	27.3	17.3
Total Gelatine Recovered (%)			2017
Total Gelatine Yield (%)			20.07
Anhydrous Gelatine Recovered (g) (f 0.875)			1674.9
Anhydrous Gelatine Corrected (g) (f 0.95)			1676.6
Anhydrous Gelatine Yield on Raw Material (%)			49.1

Gelatine Quality.

Run No	1	2
Bloom	285	304
Colour (DGI)	4.8	6.4
Clarity (DGI)	8.5	9.0
pH	5.2	5.8
Moisture (%)	10.54	8.87
Ash (%)	1.14	0.72
SO_2 (ppm)	80	56
Viscosity (ms @ 60°C)	55.5	65.7
Corrected* Bloom	274	284
Corrected* Viscosity	55	64

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C1. Continued...

Extraction & Quality Data. Experiment GR.

Extraction.

Run No	1	2	3
Time hrs	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	21.43	8.42	8.01
Liquor pH	2.76	3.04	
Liquor Concentration (%w/v)	5.74	6.69	2.48
Scutch (g)			40
Gelatine (g)	1230.1	563.3	198.6
Gelatine % Proportion	61.7	28.3	10.0
Total Gelatine Recovered (g)			1992.0
Total Gelatine Yield (%)			19.9
Anhydrous Gelatine Recovered (g) (f 0.875)			1743.0
Anhydrous Gelatine Corrected (g) (f 0.95)			1655.9
Anhydrous Gelatine Yield on Raw Material (%)			48.7

Gelatine Quality.

Run No	1	2
Bloom	286	301
Colour (DGI)	4.8	5.2
Clarity (DGI)	10.0	12.0
pH	5.0	5.7
Moisture (%)	11.08	8.57
Ash (%)	1.00	0.62
SO_2 (ppm)	56	112
Viscosity (ms @ 60°C)	56.7	54.5
Corrected* Bloom	283	279
Corrected* Viscosity	56	53

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C1. Continued...

Extraction & Quality Data. Experiment SF.

Extraction.

Run No	1	2	3
Time hrs	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	13.78	7.18	7.07
Liquor pH	3.02	3.26	
Liquor Concentration (%w/v)	5.04	5.14	3.03
Scutch (g)			160
Gelatine (g)	694.5	369.1	214.2
Gelatine % Proportion	54.3	28.9	16.8

Total Gelatine Recovered (g)	1227.8
Total Gelatine Yield (%)	26.9
Anhydrous Gelatine Recovered (g) (f 0.875)	1118.1
Anhydrous Gelatine Corrected (g) (f 0.95)	1062.2
Anhydrous Gelatine Yield on Raw Material (%)	59.1

Gelatine Quality.

Run No	1	2
Bloom	292	278
Colour (DGI)	6.4	8.5
Clarity (DGI)	10.5	4.5
pH	5.3	4.5
Moisture (%)	11.30	11.54
Ash (%)	0.78	0.75
SO_2 (ppm)	152	112
Viscosity (ms @ 60°C)	52.1	48.1
Corrected* Bloom	289	277
Corrected* Viscosity	52	48

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C1. Continued...

Extraction & Quality Data. Experiment SR.

Extraction.

Run No	1	2	3
Time hrs	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	13.95	7.28	7.16
Liquor pH	2.99	3.24	
Liquor Concentration (%w/v)	4.78	5.46	2.76
Scutch (g)			160
Gelatine (g)	668.2	397.5	197.6
Gelatine % Proportion	52.9	31.5	15.6
Total Gelatine Recovered (g)			1263.3
Total Gelatine Yield (%)			26.9
Anhydrous Gelatine Recovered (g) (f 0.875)			1105.4
Anhydrous Gelatine Corrected (g) (f 0.95)			1050.1
Anhydrous Gelatine Yield on Raw Material (%)			59.1

Gelatine Quality.

Run No	1	2
Bloom	275	272
Colour (DGI)	6.4	7.2
Clarity (DGI)	10.5	8.0
pH	5.4	4.5
Moisture (%)	11.31	10.9
Ash (%)	0.62	0.83
SO_2 (ppm)	64	72
Viscosity (ms @ 60°C)	53.1	48.7
Corrected* Bloom	272	267
Corrected* Viscosity	53	48

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C2. The effect of Conditioning Time.

EXPERIMENT CT

Raw Material and Conditioning.

Raw Material Brahman Bull - Feed Lot animal approx 18 months old.

Washed Salted Hide:

Moisture Content	69.2 ± 0.5%
Ash Content	0.18 ± 0.08%
Sample 1 to 6 Mass (kg)	5.75 kg
Sample 7 Mass (kg)	1.85 kg
Anhyd: Hide Subs:	1760 ± 32 g

Conditioning.

Conditioning Liquor: CaO 640 g
Na_2S 60% 70 g
Water to 20 kg.

Sample No	CT1	CT2	CT3
Cond: Time (Weeks)	1	2	3
Cond: Temperature (°C)	21.0	21.5	21.9
Init. Sulphide (g Na_2S/l)	1.7	1.76	1.83
Final Sulphide (g Na_2S/l)		1.12	1.13
Final Liquor Total Solids (%)		1.373	1.54
Final Liquor Volatile Solids (%)		0.794	1.09
Final Liquor Organic Solids (g)		159	218
Ex-Lime Wash for 18hrs			
Limed Mass (kg)	9.1	9.7	11.1
Swelling (%)	160	168	193
Acidulation 5 coats of H_2SO_3 soln. (days)	4	4	4
Wash 1hr.			
Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	10.25	15.55	12.15
Soak Water pH	2.54	2.24	2.33

Sample No	CT4	CT5	CT6
Cond: Time Weeks	4	5	6
Cond: Temperature (°C)	21.9	22.0	22.0
Init: Sulphide (g Na_2S/l)	1.77	1.7	1.76
Final Sulphide (g Na_2S/l)	0.95	0.80.	0.81
Final Liquor Total Solids (%)	1.62	1.91	1.93
Final Liquor Volatile Solids (%)	1.15	1.44	1.43
Final Liquor Organic Solids (g)	230	288	286
Ex-Lime Wash for 18hrs			
Limed Mass (kg)	11.05	11.1	10.35
Swelling (%)	192	193	180
Acidulation 5 coats of H_2SO_3 soln. (days).	4	4	4
Wash 1hr.			
Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	12.0	12.55	11.55
Soak Water pH	2.11	2.14	2.02

ADDENDUM C2. Continued...

Sample No	CT7
Cond: Time (Weeks)	3
Cond: Temperature (°C)	21.9
Init: Sulphide (g Na_2S/l)	1.72

Conditioning liquor (20 l) changed at the end of week 1 and week 2.

Final Sulphide (g Na_2S/l)	1.61	Errors ±0.015 to 0.003%
Final Liquor Total Solids (%)	0.478	Errors ±0.02 to 0.002%
Final Liquor Volatile Solids (%)	0.019	Errors ±0.02 to 0.003%
Final Liquor Organic Solids (g)	4 g	

Ex-Lime Wash for 18hrs

Limed Mass (kg)	3.45
Swelling (%)	186

Acidulation 5 coats of H_2SO_3 soln. (days).	4
Wash 1hr.	
Soak in fresh water ± 22hr.	
Wt: for Extraction (kg)	3.85
Soak Water pH	2.73

ADDENDUM C2. Continued...

Extraction & Quality Data. Experiment CT1. (1 weeks liming)

Extraction.

Run No	1	2	3	4
Time hrs	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	9.44	9.69	7.04	11.32
Liquor pH	2.78	3.48	3.69	
Liquor Concentration (%w/v)	0.60	1.42	2.79	11.63
Scutch (g)				390
Gelatine (g)	56.5	137.6	196.4	1316.5
Gelatine (%) Proportion	3.3	8.1	11.5	77.1
Heavy Liquor Volume (ml)	700	1300	600	
Heavy Liquor Conc: (%)				
5% H_2O_2 (ml)	9	8	2	
5% NH_3 (ml)	12	10	8	

Total Gelatine Recovered (g)	1707
Total Gelatine Yield (%)	29.7
Anhydrous Gelatine Recovered (g) (f 0.875)	1493.6
Anhydrous Gelatine Corrected (g) (f 0.95)	1418.9
Anhydrous Gelatine Yield on Raw Material (%)	80.6

Gelatine Quality.

Run No	1	2	3	4
Bloom	285	332	331	
Colour (DGI)	5.6	5.6	5.2	6.8
Clarity (DGI)	5.0	9.5	11.5	7.5
pH	5.6	5.6	5.9	4.4
Moisture (%)	11.44	9.5	10.72	
Ash (%)	5.29	1.82	0.80	
SO_2 (ppm)	**	56	272	
Viscosity (ms @ 60°C)	30.5	33.6	33.2	
Corrected* Bloom	314	323	323	
Corrected* Viscosity	32	33	32	
Overall Colour.		6.5		

** Insufficient Sample

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C2. Continued...

Extraction & Quality Data. Experiment CT2. (2 weeks liming)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	11.22	6.76	7.65	12.03
Liquor pH	2.76	3.37	3.66	
Liquor Concentration (%w/v)	1.72	3.57	4.28	7.82
Scutch (g)				290
Gelatine (g)	193.0	241.3	327.4	940.7
Gelatine % Proportion	11.3	14.2	19.2	55.2
Heavy Liquor Volume (ml)	1200	1050	1050	
5% H_2O_2 (ml)	8	2	2	
5% NH_3 (ml)	10	12	22	

Total Gelatine Recovered (g)	1702.4
Total Gelatine Yield (%)	29.7
Anhydrous Gelatine Recovered (g) (f 0.875)	1489.6
Anhydrous Gelatine Corrected (g) (f 0.95)	1415.1
Anhydrous Gelatine Yield on Raw Material (%)	80.4

Gelatine Quality.

Run No	1	2	3	4
Bloom	332	330	302	
Colour (DGI)	5.2	5.2	6.0	6.4
Clarity (DGI)	10.0	12.0	11.5	13.5
pH	5.4	5.6	5.2	4.2
Moisture (%)	11.16	11.22	11.30	
Ash (%)	1.92	0.74	0.55	
SO_2 (ppm)	88	24	192	
Viscosity (ms @ 60°C)	36.7	37.3	41.2	
Corrected* Bloom	336	325	297	
Corrected* Viscosity	37	37	41	
Overall Colour.		6.0		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C2. Continued...

Extraction & Quality Data. Experiment CT3. (3 weeks liming)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	12.36	8.84	8.99	7.08
Liquor pH	2.78	3.21	3.51	
Liquor Concentration (%w/v)	3.00	4.81	5.28	6.79
Scutch (g)				180
Gelatine (g)	370.8	425.2	474.7	480.7
Gelatine % Proportion	21.2	24.3	27.1	27.4
Heavy Liquor Volume (ml)	1600	1900	1400	
Heavy Liquor Conc: (%)			11	
5% H_2O_2 (ml)	5	6	3	
5% NH_3 (ml)	11	35	30	
Total Gelatine Recovered (g)			1751.4	
Total Gelatine Yield (%)			30.5	
Anhydrous Gelatine Recovered (g) (f 0.875)			1532.5	
Anhydrous Gelatine Corrected (g) (f 0.95)			1455.9	
Anhydrous Gelatine Yield on Raw Material (%)			82.7	
Gelatine Quality.				
Run No	1	2	3	4
Bloom	317	309	291	
Colour (DGI)	5.2	5.6	6.4	7.2
Clarity (DGI)	12.0	11.0	10.0	13.0
pH	5.5	5.3	5.3	4.3
Moisture (%)	9.89	10.84	11.26	
Ash (%)	0.94	0.52	0.46	
SO_2 (ppm)	312	104	264	
Viscosity (ms @ 60°C)	42.8	43.5	39.5	
Corrected* Bloom	305	301	286	
Corrected* Viscosity	42	43	39	
Overall Colour.		6.2		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C2. Continued...

Extraction & Quality Data. Experiment CT4. (4 weeks liming)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	13.02	8.85	9.36	8.20
Liquor pH	2.89	3.22	3.45	
Liquor Concentration (%w/v)	3.47	5.36	4.95	4.35
Scutch (g)				40
Gelatine (g)	376.3	474.4	463.3	356.7
Gelatine % Proportion	22.5	28.4	27.7	21.4
Heavy Liquor Volume (ml)	1400	1300	1500	
Heavy Liquor Conc: (%)	9	11	9	
5% H_2O_2 (ml)	6	3	2	
5% NH_3 (ml)	27	25	30	
Total Gelatine Recovered (g)				1670.7
Total Gelatine Yield (%)				29.1
Anhydrous Gelatine Recovered (g) (f 0.875)				1461.8
Anhydrous Gelatine Corrected (g) (f 0.95)				1338.8
Anhydrous Gelatine Yield on Raw Material (%)				78.9

Gelatine Quality.				
Run No	1	2	3	4
Bloom	310	324	299	
Colour (DGI)	5.6	5.6	5.6	**
Clarity (DGI)	9.0	11.0	11.5	**
pH	5.4	5.4	5.3	4.4
Moisture (%)	8.68	8.93	11.15	
Ash (%)	1.21	0.58	0.56	
SO_2 (ppm)	24	168	128	
Viscosity (ms @ 60°C)	46.3	48.0	39.4	
Corrected* Bloom	292	302	294	
Corrected* Viscosity	45	46	39	

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C2. Continued...

Extraction Data. Experiment CT5. (5 weeks liming)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	14.37	9.10	9.14	1.24
Liquor pH	2.87	3.23	3.39	
Liquor Concentration (%w/v)	4.03	5.62	5.03	8.23
Scutch (g)				Nil
Gelatine (g)	579.1	511.4	459.7	102.1
Gelatine % Proportion	35.0	31.0	27.8	6.2
Heavy Liquor Volume (ml)	1500	1100	1500	
Heavy Liquor Conc: (%)	9	13	11	
5% H_2O_2 (ml)	4	2	2	
5% NH_3 (ml)	20	30	40	
Total Gelatine Recovered (g)				1652.3
Total Gelatine Yield (%)				28.7
Anhydrous Gelatine Recovered (g) (f 0.875)				1445.8
Anhydrous Gelatine Corrected (g) (f 0.95)				1373.5
Anhydrous Gelatine Yield on Raw Material (%)				78.0

Gelatine Quality.				
Run No	1	2	3	4
Bloom	324	298	275	
Colour (DGI)	6.0	6.4	6.4	8.0
Clarity (DGI)	11.5	11.5	12.5	13.5
pH	5.7	5.4	5.6	4.5
Moisture (%)	9.59	11.78	9.96	
Ash (%)	0.94	0.49	0.53	
SO_2 (ppm)	40	144	464	
Viscosity (ms @ 60°C)	51.5	49.5	50.1	
Corrected* Bloom	310	296	263	
Corrected* Viscosity	51	49	49	
Overall Colour.		6.4		

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C2. Continued...

Extraction Data. Experiment CT6. (6 weeks liming)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	13.70	9.58	8.14	2.90
Liquor pH	2.84	3.30	3.57	
Liquor Concentration (%w/v)	4.45	5.20	4.42	2.60
Scutch (g)				Nil
Gelatine (g)	609.7	498.2	359.8	75.4
Gelatine % Proportion	39.5	32.3	23.3	4.9
Heavy Liquor Volume (ml)	1400	1700	1300	
Heavy Liquor Conc: (%)	9	9	11	
5% H_2O_2 (ml)	4	3	2	
5% NH_3 (ml)	20	35	35	
Total Gelatine Recovered (g)				1543.1
Total Gelatine Yield (%)				26.8
Anhydrous Gelatine Recovered (g) (f 0.875)				1350.2
Anhydrous Gelatine Corrected (g) (f 0.95)				1282.7
Anhydrous Gelatine Yield on Raw Material (%)				72.9

Gelatine Quality.				
Run No	1	2	3	4
Bloom	327	308	277	
Colour (DGI)	5.2	5.6	6.0	**
Clarity (DGI)	11.5	11.5	12.5	**
pH	5.3	5.6	5.6	4.4
Moisture (%)	10.14	9.34	9.62	
Ash (%)	0.85	0.64	0.59	
SO_2 (ppm)	360	352	296	
Viscosity (ms @ 60°C)	48.0	55.6	49.7	
Corrected* Bloom	316	291	263	
Corrected* Viscosity	47	54	48	

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C2. Continued...

Extraction Data. Experiment CT7. (3 weeks liming)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	2.92	3.22	3.74	4.20
Liquor pH	2.89	3.17	3.45	
Liquor Concentration (%w/v)	3.70	3.90	3.73	3.35
Scutch (g)				50
Gelatine (g)	108.0	125.6	139.5	157.5
Gelatine % Proportion	20.4	23.7	26.3	29.6
Heavy Liquor Volume (ml)	1000	1200	900	
Heavy Liquor Conc: (%)		8	7	
5% H_2O_2 (ml)	2	2	2	
5% NH_3 (ml)	10	17	17	
Total Gelatine Recovered (g)				530.6
Total Gelatine Yield (%)				28.7
Anhydrous Gelatine Recovered (g) (f 0.875)				464.3
Anhydrous Gelatine Corrected (g) (f 0.95)				441.1
Anhydrous Gelatine Yield on Raw Material (%)				77.8

Gelatine Quality.				
Run No	1	2	3	4
Bloom	333	318	279	
Colour (DGI)	5.6	5.6	6.4	**
Clarity (DGI)	9.0	11.0	11.5	**
pH	5.6	5.5	5.4	
Moisture (%)	10.19	10.72	10.87	
Ash (%)	0.86	0.60	0.61	
SO_2 (ppm)	56	144	192	
Viscosity (ms @ 60°C)	43.8	44.6	39.7	
Corrected* Bloom	322	310	273	
Corrected* Viscosity	43	44	39	

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C3. Old animal hide & conditioning time.

EXPERIMENT CTO.

Raw Material and Conditioning Data.

Salted Hide ex ADSRI from a beef animal 13 years of age. The hide was cut into small pieces, washed in a tumbler overnight, drained and then separated into 4 lots.

Moisture Content	69.3 ± 0.3%
Ash Content	0.28 ± 0.07%

Sample No	CTO1	CTO2	CTO3	CTO4
Sample Mass (kg)	4.6	4.8	4.8	4.8
Anhyd: Hide Subs: (g)	1399	1460	1460	1460

Conditioning.

Conditioning Liquor: CaO	640 g
Na_2S 60%	70 g
Water	to 20 kg.

Sample No	CTO1	CTO2	CTO3	CTO4
Cond: Time Weeks	2	4	7	10
Cond: Temperature (°C)	21.8	22.0	21.8	21.8
Init: Sulphide (Na_2S g/l)	1.81	1.84	1.85	1.81
Final Sulphide (Na_2S g/l)	1.14	0.68	0.46	0.27
Sulphide consumed (g/l)	0.67	1.16	1.39	1.54
Spent Liquor Solids (%w/v)	1.69	2.25	2.41	2.56
" " Ash (%w/v)	0.53	0.66	0.85	0.76
" " Volatiles (%w/v)	1.16	1.59	1.56	1.79
" " Organic Matter (g)	232	318	312	358
" " Absorb: (470nm)		0.12	0.08	0.08
Ex-Lime Wash for 16hrs.				
Limed Mass (kg)	6.1	7.5	6.9	.15
Swelling (%)	132	156	144	149
Acidulation. 5 coats of H_2SO_3 soln. over 4 days.				
Wash 1hr.				
Soak in fresh water ± 22hr.				
Wt: for Extraction (kg)	7.05	7.65	7.35	7.3
Swelling (%)	153	159	153	152
Soak Water pH	2.33	2.41	2.43	2.45

ADDENDUM C3. Continued...

CTO1 Extraction and Quality Data.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	7.76	7.44	6.58	9.32
Liquor pH	2.76	3.31	3.39	
Liquor Concentration (%w/v)	0.55	0.96	1.58	8.03
Fat (ml)	10	4	6	
Scutch (g)				280
Gelatine (g)	42.7	71.4	103.9	748.4
Gelatine % Proportion	4.4	7.4	10.8	77.4
Heavy Liquor Volume (ml)	1100	700	800	
Heavy Liquor Conc:	1.5	6	7.5	
5% H_2O_2 (ml)	6.5	3	--	
5% NH_3 (ml)	5	5	--	

Total Gelatine Recovered (g)	966.4
Total Gelatine Yield (%)	21.0
Anhydrous Gelatine Recovered (g) (f 0.875)	845.6
Anhydrous Gelatine Corrected (g) (f 0.95)	803.3
Anhydrous Gelatine Yield on Raw Material (%)	57.4
Total Anhydrous Solids Recovered (g)	1035.3
Total Anhydrous solids Recovered (%)	74.0

Gelatine Quality.

Run No	1	2	3	4
Bloom	300	306	286	
Colour	6.4	7.2	10.0	16.0
Clarity	10.5	11.5	10.5	10.5
pH	5.5	6.1	5.9	4.3
Moisture (%)	10.26	10.90	9.66	
Ash (%)	4.63	1.76	1.43	
SO_2 (ppm) ϕ	---	48+	216	
Viscosity (ms @ 60°C)	30.8	30.6	28.4	
Corrected* Bloom	317	294	277	
Corrected* Viscosity	31.6	30.0	27.9	
Overall Colour.		12.4		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ϕ + Indicates peroxide positive on Starch/KI test.

ADDENDUM C3. Continued...

CTO2 Extraction and Quality Data.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	8.40	7.69	7.44	7.36
Liquor pH	2.88	3.27	3.53	
Liquor Concentration w/v	0.98	1.36	1.72	9.11
Fat (ml)	3.5	2.0	1.0	
Scutch (g)				40
Gelatine (g)	82.3	104.6	128.0	670.5
Gelatine % Proportion	8.4	10.6	13.0	68.0
Heavy Liquor Volume (ml)	1200	800	1000	
Heavy Liquor Conc:	3	6	-	
5% H_2O_2 (ml)	4	1.4	1	
5% NH_3 (ml)	6	8	10	
Total Gelatine Recovered (g)			985.4	
Total Gelatine Yield (%)			20.5	
Anhydrous Gelatine Recovered (g) (f 0.875)			862.2	
Anhydrous Gelatine Corrected (g) (f 0.95)			819.1	
Anhydrous Gelatine Yield on Raw Material (%)			56.1	
Total Anhydrous Solids Recovered (g)			1137.1	
Total Anhydrous solids Recovered (%)			77.9	

Gelatine Quality.				
Run No	1	2	3	4
Bloom	330	306	262	
Colour	8.0	8.9	10.0	20.0
Clarity	4.0	10.5	10.5	12.5
pH	5.8	5.9	5.8	4.3
Moisture (%)	8.66	8.90	8.85	
Ash (%)	2.51	1.32	1.10	
SO_2 (ppm) ϕ	80+	64	64	
Viscosity (ms @ 60°C)	38.3	32.9	31.1	
Corrected* Bloom	320	291	247	
Corrected* Viscosity	37.7	32.1	30.2	
Overall Colour.		16.5		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ϕ + Indicates peroxide positive on Starch/KI test.

ADDENDUM C3. Continued...

CT03 Extraction and Quality Data.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	8.78	6.85	6.3	5.67
Liquor pH	2.88	3.26	3.45	
Liquor Concentration (%w/v)	1.12	1.59	2.15	9.94
Fat (ml)	2.0	1.5	1.5	
Scutch (g)				Nil
Gelatine (g)	98.3	108.6	135.1	563.6
Gelatine % Proportion	10.9	12.0	14.9	62.2
Heavy Liquor Volume (ml)	1000	800	1200	
Heavy Liquor Conc: (%)	4	5	6	
5% H_2O_2 (ml)	3	1	1	
5% NH_3 (ml)	7	8	20	
Total Gelatine Recovered (g)			905.6	
Total Gelatine Yield (%)			18.9	
Anhydrous Gelatine Recovered (g) (f 0.875)			792.4	
Anhydrous Gelatine Corrected (g) (f 0.95)			752.8	
Anhydrous Gelatine Yield on Raw Material (%)			51.6	
Total Anhydrous Solids Recovered (g)			1064.8	
Total Anhydrous solids Recovered (%)			72.9	
Gelatine Quality.				
Run No	1	2	3	4
Bloom	311	273	236	
Colour	9.4	10.7	12.3	17.8
Clarity	8.5	10.5	9.0	12.0
pH	5.6	5.6	5.6	4.2
Moisture (%).	8.31	7.98	9.29	
Ash (%)	1.6	1.17	0.96	
SO_2 (ppm) ϕ	160	64+	80	
Viscosity (ms @ 60°C)	37.8	27.5	30.7	
Corrected* Bloom	293	253	224	
Corrected* Viscosity	36.7	26.5	29.9	
Overall Colour.		15.2		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ϕ + Indicates peroxide positive with Starch/KI test.

ADDENDUM C3. Continued...

CTO4 Extraction and Quality Data.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	8.5	7.11	6.16	4.89
Liquor pH	2.84	3.41	3.54	
Liquor Concentration (%w/v)	1.03	1.33	2.08	11.99
Fat (ml)	6	2	1	
Scutch (g)				Nil
Gelatine (g)	87.6	94.6	128.1	586.3
Gelatine (%) Proportion	9.8	10.5	14.3	65.4
Heavy Liquor Volume (ml)	900	800	1050	
Heavy Liquor Conc:	4	4.4	5	
5% H_2O_2 (ml)	1.5	1.0	0.5	
5% NH_3 (ml)	3.5	8.0	11.0	
Total Gelatine Recovered (g)			896.6	
Total Gelatine Yield (%)			18.7	
Anhydrous Gelatine Recovered (g) (f 0.875)			784.5	
Anhydrous Gelatine Corrected (g) (f 0.95)			745.3	
Anhydrous Gelatine Yield on Raw Material (%)			51.0	
Total Anhydrous Solids Recovered (g)			1103.3	
Total Anhydrous solids Recovered (%)			75.5	

Gelatine Quality.				
Run No	1	2	3	4
Bloom	302 Φ	246	213	
Colour	10.0	9.4	10.0	20.0
Clarity	8.5	10.0	9.0	6.0
pH	5.4	5.5	5.2	4.1
Moisture (%)	8.32	11.444	9.72	
Ash (%)	1.98	1.33	0.89	
SO_2 (ppm)	32	64	80	
Viscosity (ms @ 60°C)	39.1	32.0	28.9	
Corrected* Bloom	287	247	204	
Corrected* Viscosity	38.1	32.1	28.3	
Overall Colour.		16.4		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

Φ Evaporation temperature rose to 50°C for a short time due to a leaf in the water vacuum pump.

ADDENDUM C4. Time & sodium sulphide concentration.

EXPERIMENT ST.

Raw Material.

Two salted hides were supplied by ADSRI from 12 year-old Afrikaners. These were cut into 100 x 100 mm pieces and randomised by tumbling. Equal amounts were taken from each hide to make up the 9 x 5.7 kg lots required for the experiment.

Mass of hide for each experiment.	5700 g.
Moisture Content	38.6 ± 1.4%
Ash Content	17.1 ± 0.7%
Organic Content	44.3 %
Organic Content of samples.	2525 g

Prewashing 18hrs minimum.

Hide Conditioning with 50 g Sodium Sulphide

Conditioning Liquor: CaO 640 g
Na_2S 50 g
Water to 20.0 kg.

Experiment No	ST1-2	ST1-4	ST1-6
Washed Hide (kg)	7.1	7.2	7.3
Conditioning Time -Weeks.	2	4	6
Cond: Temperature (°C)	22.2	21.9	22.0
Init: Sulphide (g Na_2S/l)	1.13±0.00	1.12±0.01	1.14±0.01
Ex-Lime Wash for 18hrs			
Limed Mass (kg)	10.15	11.25	11.2
Swelling (%)	143	156	153
Acidulation 5 coats of H_2SO_3 soln. over 4 days.			
Wash 1hr.			
Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	10.9	11.65	11.6
Swelling (%)	153	162	159
Soak Water pH	2.28	2.24	2.19
Spent Conditioning Liquor:			
Mass (kg)	20	19.7	19.55
Na_2S Conc: (g/l)	0.72±0.01	0.54±0.01	0.38±0.01
Sulphide consumed (g/l)	0.41	0.58	0.76
Spent Liquor Solids (g/l)	13.73±0.01	16.37±0.03	19.55±0.1
" " Ash (g/l)	3.53±0.03	5.32±0.04	4.8 ±0.6
" " Volatiles (g/l)	10.2	11.05	14.8
" " Organic Matter (g)	204	221	296
Organic Matter (%)	8.1	8.8	11.7

ADDENDUM C4. Continued...

Hide Conditioning with 74 g Sodium Sulphide.

Conditioning Liquor:	CaO	640 g
	Na_2S	74 g
	Water to	20.0 kg.

Experiment No	ST2-2	ST2-4	ST2-6
Washed Hide (kg)	7.05	6.8	7.4
Conditioning Time (Weeks)	2	4	6
Cond: Temperature (°C)	21.6	21.8	21.9
Init: Sulphide (g Na_2S/l)	1.91±0.01	1.90±0.01	1.84±0.01
Ex-Lime Wash for 18 hr.			
Limed Mass (kg)	10.45	10.8	10.75
Swelling (%)	148	159	145
Acidulation 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	11.1	11.2	11.3
Swelling (%)	157	164	152
Soak Water pH	2.22	2.36	2.24
Spent Conditioning Liquor:			
Mass (kg)	20.0	19.75	19.8
Na_2S Conc: (g/l)	1.17±0.01	0.90±0.00	0.50±0.01
Sulphide consumed (g/l)	0.74	1.00	1.34
Spent Liquor Solids (g/l)	18.26±0.08	20.1±0.1	26.14±0.01
" " Ash (g/l)	5.80±0.1	6.3±0.1	9.00±0.01
" " Volatiles (g/l)	12.46	13.8	17.14
" " Organic Matter (g)	249	276	343
Organic Matter (%)	9.9	10.9	13.6

ADDENDUM C4. Continued...

Hide Conditioning with 98 g Sodium Sulphide.

Conditioning Liquor:	CaO	640 g		
	Na_2S	98 g		
	Water to	20.0 kg.		
Experiment No		ST3-2	ST3-4	ST3-6
Washed Hide (kg)		7.0	7.8	7.35
Conditioning Time (Weeks)		2	4	6
Cond: Temperature (°C)		21.6	21.9	21.9
Init: Sulphide (g Na_2S/l)		2.59±0.01	2.64±0.1	2.38±0.01
Ex-Lime Wash for 18hrs				
Limed Mass (kg)		9.9	10.6	10.55
Swelling (%)		141	135	144
Acidulation 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.				
Wt: for Extraction (kg)		10.9	11.3	10.9
Swelling (%)		155	145	148
Soak Water pH		2.24	2.05	2.2
Spent Conditioning Liquor:				
Mass (kg)		19.6	19.4	19.9
Na_2S Conc: (g/l)		1.72	0.95	0.59
Sulphide consumed (g/l)		0.87	1.69	1.79
Spent Liquor Solids (g/l)		19.33±0.1	29.28±0.09	30.21±0.03
" "	Ash (g/l)	6.11±0.05	11.59±0.2	11.53
" "	Volatiles (g/l)	13.22	17.69	18.68
" "	Organic Matter (g)	264	354	374
	Organic Matter (%)	10.4	14.0	14.8

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST1-2.

Extraction.

Run No	1	2	3	4	5
Time (hrs)	5	5	5	1	6
Temperature (°C)	45	50	60	93	93
Liquor Volume (l)	9.23	8.58	7.90	5.81	3.26
Liquor pH	2.57	3.00	3.48	3.83	4.2
Liquor Concentration (%w/v)	0.79	1.46	3.68	5.95	26.73
Fat (ml)	60	20	25		
Scutch (g)					325
Gelatine (g)	72.9	125.2	290.3	345.7	871.4
Gelatine % Proportion	4.3	7.3	17.0	20.3	51.1
Heavy Liquor Volume (ml)	600	900	1050	900	
Heavy Liquor Conc:	7	9	11	10	
5% H_2O_2 (ml)	40	20	8	4	
5% NH_3 (ml)	15	8	7	10	

Total Gelatine Recovered (g)	1705.5
Total Gelatine Yield (%)	29.9
Anhydrous Gelatine Recovered (g) (f 0.875)	1492.3
Anhydrous Gelatine Corrected (g) (f 0.95)	1417.7
Anhydrous Gelatine Yield on Raw Material (%)	56.1
Total Anhydrous Solids Recovered (g)	1726.7
Total Anhydrous solids Recovered (%)	68.3

Gelatine Quality.

Run No	1	2	3	4	5
Bloom	179	92	237	146	
Colour	6.4f	10.0f	10.7	17.8	22.8
Clarity	12.5	12.5	12.5	6.5	9.0
pH	5.1	5.0	5.3	5.6	4.2
Moisture (%)	14.7	12.1	11.8	11.0	
Ash (%)	7.84	4.45	2.23	1.96	
SO_2 (ppm)	+	224	96	152	
Viscosity (ms @ 60°C)	15.8	13.9	19.8	19.2	
Corrected* Bloom	228	101	246	147	
Corrected* Viscosity	17.8	14.6	20.1	19.3	
Overall Colour.	18.1				

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
f Bloom sample filtered; Whatman GF/A
\+ H_2O_2 Positive.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST1-4.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	10.95	8.80	7.49	8.64
Liquor pH	2.63	3.13	3.56	
Liquor Concentration (%w/v)	1.47	2.73	5.73	10.96
Fat (ml)	25	20	15	
Scutch (g)				75
Gelatine (g)	160.9	239.8	428.8	946.9
Gelatine % Proportion	9.1	13.5	24.1	53.3
Heavy Liquor Volume (ml)	600	1000	1200	
Heavy Liquor Conc:	8	10	13	
5% H_2O_2 (ml)	5	6	3	
5% NH_3 (ml)	3	7	18	

Total Gelatine Recovered (g)	1776.4
Total Gelatine Yield (%)	31.2
Anhydrous Gelatine Recovered (g) (f 0.875)	1554.4
Anhydrous Gelatine Corrected (g) (f 0.95)	1476.6
Anhydrous Gelatine Yield on Raw Material (%)	58.5
Total Anhydrous Solids Recovered (g)	1757.6
Total Anhydrous solids Recovered (%)	69.6

Gelatine Quality.

Run No	1	2	3	
Bloom	332	303	247	
Colour	10.0	11.4	13.3	16.0
Clarity	12.5	12.5	11.8	12.5
pH	5.4	5.4	5.5	4.4
Moisture (%)	9.6	9.4	11.3	
Ash (%)	2.81	2.39	1.39	
SO_2 (ppm)	nd	64	360	
Viscosity (ms @ 60°C)	33.2	29.8	27.5	
Corrected* Bloom	331	298	248	
Corrected* Viscosity	33.1	29.5	27.5	
Overall Colour.	14.2			

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST1-6.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	11.27	9.03	7.54	6.35
Liquor pH	2.69	3.20	3.61	
Liquor Concentration (%w/v)	1.81	3.23	6.00	12.50
Fat (ml)	15	15	10	
Scutch (g)				Nil
Gelatine (g)	204.4	291.2	452.4	793.8
Gelatine % Proportion	11.7	16.7	26.0	45.6
Heavy Liquor Volume (ml)	900	1000	1000	
Heavy Liquor Conc:	7	12	11	
5% H_2O_2 (ml)	4	8	2	
5% NH_3 (ml)	6	8	15	
Total Gelatine Recovered (g)				1741.8
Total Gelatine Yield (%)				30.6
Anhydrous Gelatine Recovered (g) (f 0.875)				1524.1
Anhydrous Gelatine Corrected (g) (f 0.95)				1447.9
Anhydrous Gelatine Yield on Raw Material (%)				57.3
Total Anhydrous Solids Recovered (g)				1783.9
Total Anhydrous solids Recovered (%)				70.6

Gelatine Quality.				
Run No	1	2	3	
Bloom	331	288	240	
Colour	10.7	12.3	14.5	13.3
Clarity	11.1	11.8	10.5	11.1
pH	5.5	5.4	5.5	4.1
Moisture (%) .	9.3	9.7	11.5	
Ash (%)	1.74	2.72	1.29	
SO_2 (ppm)	nd	200	456	
Viscosity (ms @ 60°C)	36.3	31.7	32.1	
Corrected* Bloom	320	288	241	
Corrected* Viscosity	35.7	31.7	32.2	
Overall Colour.	13.1			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
nd Not determined.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST2-2.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	10.66	10.30	8.07	8.83
Liquor pH	2.56	3.03	3.38	4.27
Liquor Concentration (%w/v)	0.61	1.37	3.91	14.70
Fat (ml)	35	15	20	
Scutch (g)				335
Gelatine (g)	65	141.1	315.5	1298.0
Gelatine (%) Proportion	3.6	7.8	17.3	71.3
Heavy Liquor Volume (ml)	600	1150	1150	
Heavy Liquor Conc:	6	8	10	
5% H_2O_2 (ml)	18.5	23	5.5	
5% NH_3 (ml)	12.5	15	8	
Total Gelatine Recovered (g)			1819.6	
Total Gelatine Yield (%)			31.9	
Anhydrous Gelatine Recovered (g) (f 0.875)			1592.2	
Anhydrous Gelatine Corrected (g) (f 0.95)			1512.5	
Anhydrous Gelatine Yield on Raw Material (%)			59.9	
Total Anhydrous Solids Recovered (g)			1831.7	
Total Anhydrous solids Recovered (%)			72.5	

Gelatine Quality.				
Run No	1	2	3	
Bloom	146	265	204	
Colour	11.4f	10.7f	11.4	16.0
Clarity	12.5	13.3	12.5	9.5
pH	5.1	5.3	5.2	4.1
Moisture (%)	10.2	10.1	10.9	
Ash (%)	8.39	4.29	1.92	
SO_2 (ppm)	+	40+	320	
Viscosity (ms @ 60°C)	15.0	20.7	17.8	
Corrected* Bloom	168	277	206	
Corrected* Viscosity	16	21.1	17.9	
Overall Colour.	14.6			

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.
f Bloom sample filtered; Whatman GF/A
+ H_2O_2 Positive.
ns Not set.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST2-4.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	11.36	9.21	8.59	7.08
Liquor pH	2.73	3.26	3.47	4.1
Liquor Concentration (%w/v)	1.49	2.50	5.42	12.67
Fat (ml)	15	10	20	
Scutch (g)				30
Gelatine (g)	169.3	230.3	465.6	897.0
Gelatine % Proportion	9.6	13.1	26.4	50.9
Heavy Liquor Volume (ml)	1200	1150	1050	
Heavy Liquor Conc:	9	9	15	
5% H_2O_2 (ml)		15	5	
5% NH_3 (ml)	10	5	12	
Total Gelatine Recovered (g)			1762.2	
Total Gelatine Yield (%)			30.9	
Anhydrous Gelatine Recovered (g) (f 0.875)			1541.9	
Anhydrous Gelatine Corrected (g) (f 0.95)			1464.8	
Anhydrous Gelatine Yield on Raw Material (%)			58.0	
Total Anhydrous Solids Recovered (g)			1785.8	
Total Anhydrous solids Recovered (%)			70.7	
Gelatine Quality.				
Run No	1	2	3	
Bloom	322	315	230	
Colour	8.9	12.3	13.3	17.8
Clarity	11.1	12.5	11.1	12.5
pH	5.9	5.3	5.2	4.1
Moisture (%)	8.4	8.1	10.9	
Ash (%)	3.64	3.16	1.56	
SO_2 (ppm)	+	8+	64	
Viscosity (ms @ 60°C)	32.5	31.7	23.2	
Corrected* Bloom	318	306	230	
Corrected* Viscosity	32.3	31.3	23.2	
Overall Colour.	15.0			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
\+ H_2O_2 Positive.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST2-6.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	11.63	9.87	8.42	5.04
Liquor pH	2.80	3.23	3.60	4.21
Liquor Concentration (%w/v)	1.70	2.88	5.49	15.35
Fat (ml)	20	10	15	
Scutch (g)				Nil
Gelatine (g)	197.7	284.3	462.6	772.9
Gelatine % Proportion	11.5	16.6	26.9	45.0
Heavy Liquor Volume (ml)	1450	900	1000	
Heavy Liquor Conc:	6	13	12	
5% H_2O_2 (ml)	15	5	3	
5% NH_3 (ml)	7	7	8	
Total Gelatine Recovered (g)				1717.5
Total Gelatine Yield (%)				30.1
Anhydrous Gelatine Recovered (g) (f 0.875)				1502.8
Anhydrous Gelatine Corrected (g) (f 0.95)				1427.7
Anhydrous Gelatine Yield on Raw Material (%)				56.5
Total Anhydrous Solids Recovered (g)				1815.7
Total Anhydrous solids Recovered (%)				71.9

Gelatine Quality.				
Run No	1	2	3	
Bloom	307	280	246	
Colour	11.4	11.4	16.0	20.0
Clarity	11.8	11.8	10.0	14.3
pH	5.2	5.1	5.2	4.15
Moisture (%)	12.5	12.4	9.6	
Ash (%)	3.44	1.82	2.14	
SO_2 (ppm)	40	104	144	
Viscosity (ms @ 60°C)	30.5	28.9	31.5	
Corrected* Bloom	332	291	242	
Corrected* Viscosity	31.8	29.5	31.2	
Overall Colour.	16.5			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST3-2.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	10.60	9.26	8.18	8.49
Liquor pH	2.60	3.02	3.49	4.14
Liquor Concentration (%w/v)	0.62	1.56	3.74	14.41
Fat (ml)	40	30	10	
Scutch (g)				430
Gelatine (g)	65.7	144.5	305.9	1223.4
Gelatine % Proportion	3.8	8.3	17.6	70.3
Heavy Liquor Volume (ml)	550	1000	1350	
Heavy Liquor Conc:	7	8	9	
5% H_2O_2 (ml)	20	10	11.5	
5% NH_3 (ml)	15	7.5	10	
Total Gelatine Recovered (g)			1739.5	
Total Gelatine Yield (%)			30.5	
Anhydrous Gelatine Recovered (g) (f 0.875)			1522.1	
Anhydrous Gelatine Corrected (g) (f 0.95)			1446.0	
Anhydrous Gelatine Yield on Raw Material (%)			57.3	
Total Anhydrous Solids Recovered (g)			1790.0	
Total Anhydrous solids Recovered (%)			70.9	
Gelatine Quality.				
Run No	1	2	3	
Bloom	267	287	265	
Colour	10.0	11.4	12.3	16.0
Clarity	8.0	12.5	12.5	5.5
pH	5.7	5.5	5.3	4.2
Moisture (%)	11.2	10.0	10.5	
Ash (%)	8.28f	2.93	2.66	
SO_2 (ppm)	nd	48+	88	
Viscosity (ms @ 60°C)	25.4	22.4	25.1	
Corrected* Bloom	315	290	269	
Corrected* Viscosity	27.6	22.5	35.3	
Overall Colour.	14.7			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
f Bloom sample filtered; Whatman GFA
+ H_2O_2 Positive.
nd Not determined.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST3-4.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	10.97	9.80	7.51	5.76
Liquor pH	2.72	3.07	3.48	4.16
Liquor Concentration (%w/v)	1.32	2.63	5.22	17.11
Fat (ml)	15	10	10	
Scutch (g)				55
Gelatine (g)	144.8	257.7	392.0	985.5
Gelatine % Proportion	8.1	14.5	22.0	55.4
Heavy Liquor Volume (ml)	1000	1100		
Heavy Liquor Conc:	8	8		
5% H_2O_2 (ml)	19	10		
5% NH_3 (ml)	10	8		

Total Gelatine Recovered (g)	1780.0
Total Gelatine Yield (%)	31.2
Anhydrous Gelatine Recovered (g) (f 0.875)	1557.5
Anhydrous Gelatine Corrected (g) (f 0.95)	1479.6
Anhydrous Gelatine Yield on Raw Material (%)	58.6
Total Anhydrous Solids Recovered (g)	1868.6
Total Anhydrous solids Recovered (%)	74.0

Gelatine Quality.

Run No	1	2	3	
Bloom	305	295	252	
Colour	9.4	12.3	13.3	16.0
Clarity	12.5	12.5	111.1	12.5
pH	5.1	5.3	5.2	4.1
Moisture (%)	10.13	9.7	10.5	
Ash (%)	4.81	2.94	1.40	
SO_2 (ppm)	144	224	32	
Viscosity (ms @ 60°C)	29.0	26.2	27.4	
Corrected* Bloom	322	296	248	
Corrected* Viscosity	29.8	26.2	27.2	
Overall Colour.	14.3			

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C4. Continued...

Extraction & Quality Data. Experiment ST3-6.

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	11.85	8.92	7.84	7.63
Liquor pH	2.60	3.35	3.64	4.20
Liquor Concentration (%w/v)	1.79	3.01	5.80	9.78
Fat (ml)	30	15	30	
Scutch (g)				Nil
Gelatine (g)	212.1	268.5	454.7	746.2
Gelatine % Proportion	12.6	16.0	27.0	44.4
Heavy Liquor Volume (ml)	1250	1000	1600	
Heavy Liquor Conc:(%)	8	10	12	
5% H_2O_2 (ml)	14	6	5	
5% NH_3 (ml)	10	8	25	

Total Gelatine Recovered (g)	1681.5
Total Gelatine Yield (%)	29.5
Anhydrous Gelatine Recovered (g) (f 0.875)	1471.3
Anhydrous Gelatine Corrected (g) (f 0.95)	1397.7
Anhydrous Gelatine Yield on Raw Material (%)	55.4
Total Anhydrous Solids Recovered (g)	1846.7
Total Anhydrous solids Recovered (%)	73.1

Gelatine Quality.

Run No	1	2	3	
Bloom	305	290	222	
Colour	11.4	13.3	16.0	17.8
Clarity	11.1	11.8	10.0	14.3
pH	5.3	5.4	4.8	4.14
Moisture (%)	9.0	9.0	11.6	
Ash (%)	3.42f	2.39	1.53	
SO_2 (ppm)	152	120	48	
Viscosity (ms @ 60°C)	30.4	31.3	29.0	
Corrected* Bloom	305	283	225	
Corrected* Viscosity	30.4	30.9	29.2	
Overall Colour.	15.8			

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C5. Conditioning temperature & sulphide concentration.

EXPERIMENT WT.

Raw Material.

The raw material for this experiment was a salted hide from a 12 year-old Afrikaner animal supplied by ADSRI. The hide had been stored for 72 days before being used.

Mass of hide for each experiment.	4000 g.
Moisture Content	41.1± 0.6%
Ash Content	17.2± 0.2%
Organic Content	41.7%
Organic Content of samples.	1668 g ± 32 g
Prewashing	18hrs minimum.

Sundry Data.

pH of Lime Slurry.

18 g CaO (commercial) + 90 ml Water to slake. Cooled overnight.
pH @ ± 20°C = 12.69
$Ca(OH)_2$ Dissociation Constant $Ka_2 = 3.1 \times 10^2$
Solubility @ 20°C = 1.64 g/l = 2.2×10^2M
$[Ca] \times [OH^-]^2 = 3.1 \times 10^{-2} \times [Ca(OH)_2]$
Hence Theoretical pH = 12.94

pH of Na_2S Solutions:

18 g comm/l. (0.14M) pH = 12.79
0.33 g comm / 100 ml = 2 g Na_2S/l. pH = 12.8
0.6 g AR $Na_2S.9H_2O$(99%) = 2 g Na_2S/l. pH = 12.15

pH of NaOH Solutions:

2.0 g/l = 0.05M Theoretical pH = 12.70
Measured pH = 12.84
20.0 g/l = 0.50M Theoretical pH = 13.70
Measured pH = 13.57
(Error of pH measurements was < ±0.2 pH units.)

Dissolved Oxygen Measurements.

33 g CaO/l as freshly made. DO = 7.8 mg/l
33 g CaO/l boiled & cooled. Do = 1.0 mg/l
WT1 Spent conditioning liquor. DO = 0.1 mg/l
WT2 Spent conditioning liquor. DO = 0.1 mg/l

ADDENDUM C5. Continued...

Hide Conditioning at 22 deg C.

Conditioning Liquor:	CaO 510 g Na_2S qs Water to 16.0 kg.		
Experiment No	WT1	WT2	WT3
Washed Hide (kg)	5.3	5.3	5.25
Conditioning Time (Weeks)	4	4	4
Cond: Temperature (°C)	24.1	22.0	22.0
Init: Sulphide (g Na_2S/l)			1.77±0.03
Init: pH		12.23	12.79
Ex-Lime Wash for 18hrs			
Limed Mass (kg)	6.95	6.8	7.85
Swelling (%)	131	128	150
Acidulation 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	7.8	7.2	8.15
Swelling (%)	147	136	155
Soak Water pH	2.33	2.31	2.16
Spent Conditioning Liquor:			
Mass (kg)	16	15.5	15.7
pH	12.39	12.46	12.37
Na_2S Conc: (g/l)	0.03	0.02	0.73
Sulphide consumed (g/l)			1.04
Spent Liquor Solids (g/l)	11.45±0.02	8.74±0.07	18.87±0.05
" " Ash (g/l)	3.87±0.07	2.85±0.5	6.07±0.3
" " Volatiles (g/l)	7.6	5.9	12.8
" " Organic Matter (g)	152	118	256
Organic Matter (%)	9.1	7.0	15.3

ADDENDUM C5. Continued...

Hide Conditioning at 12 deg: C.

Conditioning Liquor:	CaO 510 g Na_2S qs Water to 16.0 kg.	
Experiment No	WT4	WT5
Washed Hide (kg)	4.95	5.1
Conditioning Time -Weeks.	4	4
Cond: Temperature (°C)	12.4±2.7	11.9±3.4
Init: Sulphide (g Na_2S/l)	1.83±0.01	11.1 ±0.0
Init: pH	12.78	12.89
Ex-Lime Wash for 18hrs		
Limed Mass (kg)	6.95	7.6
Swelling (%)	140	149
Acidulation 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.		
Wt: for Extraction (kg)	7.5	7.75
Swelling (%)	151	152
Soak Water pH	2.18	2.28
Spent Conditioning Liquor:		
Mass (kg)	15.5	15.9
pH	12.64	12.99
Na_2S Conc: (g/l)	0.91±0.01	7.66±0.00
Sulphide consumed (g/l)	0.92	3.44
Spent Liquor Solids (g/l)	15.05±0.00	24.42±0.01
" " Ash (g/l)	4.67±0.03	13.84±0.03
" " Volatiles (g/l)	10.38	10.58
" " Organic Matter (g)	208	212
Organic Matter (%)	12.5	12.7

ADDENDUM C5. Continued...

Extraction & Quality Data Experiment WT1.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	93
Liquor Volume (l)	7.92	7.77	6.12	8.95
Liquor pH	2.83	3.16	3.55	4.23
Liquor Concentration (%w/v)	0.92	1.63	4.46	9.24
Fat (ml)	15	5	11	
Scutch (g)				155 (Mainly hair)
Gelatine (g)	72.9	126.7	273.0	827.0
Gelatine % Proportion	5.6	9.7	21.0	63.6
Heavy Liquor Volume (ml)	600	900	900	
Heavy Liquor Conc:	8	8	15	
5% H_2O_2 (ml)	15	12	5	
5% NH_3 (ml)	10	7.5	10	

Total Gelatine Recovered (g)	1299.6
Total Gelatine Yield (%)	32.5
Anhydrous Gelatine Recovered (g) (f 0.875)	1137.2
Anhydrous Gelatine Corrected (g) (f 0.95)	1080.3
Anhydrous Gelatine Yield on Raw Material (%)	64.7
Total Anhydrous Solids Recovered (g)	1262.9
Total Anhydrous solids Recovered (%)	75.6

Gelatine Quality.

Run No	1	2	3	4
Bloom	263	253	230	
Colour	12.3f	11.4	12.3	17.8 (Darker than)
Clarity	12.5	12.5	12.5	4.0 (WT2/4)
pH	5.8	5.4	5.3	4.2
Moisture (%)	12.5	12.8	13.8	
Ash (%)	5.60	3.19	1.44	
SO_2 (ppm)		40	32	
Viscosity (ms @ 60°C)	23.8	26.1	25.2	
Corrected* Bloom	300	274	245	
Corrected* Viscosity	25.4	27.1	26.0	

Overall Colour. 15.7

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
f Bloom sample filtered; Whatman GFA

ADDENDUM C5. Continued...

Extraction & Quality Data Experiment WT2.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	8.43	8.41	6.87	7.33
Liquor pH	2.74	3.11	3.48	4.15
Liquor Concentration (%w/v)	0.65	1.54	3.87	12.13
Fat (ml)	6	2	2	
Scutch (g)			120 (epidermis)	
Gelatine (g)	54.8	129.5	265.9	889.1
Gelatine % Proportion	4.1	9.7	19.8	66.4
Heavy Liquor Volume (ml)	600	800	1200	
Heavy Liquor Conc:	6	11	12	
5% H_2O_2 (ml)	16	12	5	
5% NH_3 (ml)	10	7.5	10	

Total Gelatine Recovered (g)	1339.3
Total Gelatine Yield (%)	33.5
Anhydrous Gelatine Recovered (g) (f 0.875)	1171.9
Anhydrous Gelatine Corrected (g) (f 0.95)	1113.3
Anhydrous Gelatine Yield on Raw Material (%)	66.6
Total Anhydrous Solids Recovered (g)	1241.1
Total Anhydrous solids Recovered (%)	74.3

Gelatine Quality.

Run No	1	2	3	
Bloom	240	257	227	
Colour	11.4f	10.0	12.3	13.0
Clarity	7.0	12.5	12.5	nm
pH	5.2	5.4	5.4	4.0
Moisture (%)	12.39	13.50	12.98	
Ash (%)	6.16	3.20	1.76	
SO_2 (ppm)	40	64	64	
Viscosity (ms @ 60°C)	21.9	24.8	25.3	
Corrected* Bloom	277	283	239	
Corrected* Viscosity	23.5	26.1	25.9	
Overall Colour.	12.7			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
f Bloom sample filtered; Whatman GFA
nm Not measurable.

ADDENDUM C5. Continued...

Extraction & Quality Data Experiment WT3.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	8.63	6.90	7.14	7.83
Liquor pH	2.69	3.17	3.57	4.25
Liquor Concentration (%w/v)	1.53	2.58	4.80	7.88
Fat (ml)	3	0	3	
Scutch (g)				30
Gelatine (g)	132.0	178.0	342.7	617.0
Gelatine (%) Proportion	10.4	14.0	27.0	48.6
Heavy Liquor Volume (ml)	750	1150	1500	
Heavy Liquor Conc:	10	9	12	
5% H_2O_2 (ml)	10	12	10	
5% NH_3 (ml)	8	10	20	

Total Gelatine Recovered (g)	1269.7
Total Gelatine Yield (%)	31.7
Anhydrous Gelatine Recovered (g) (f 0.875)	1111.0
Anhydrous Gelatine Corrected (g) (f 0.95)	1055.4
Anhydrous Gelatine Yield on Raw Material (%)	63.2
Total Anhydrous Solids Recovered (g)	1317.4
Total Anhydrous solids Recovered (%)	78.9

Gelatine Quality.

Run No	1	2	3	
Bloom	295	273	241	
Colour	10.7	13.3	16.0	14.5
Clarity	13.3	12.5	4.8	12.5
pH	5.5	5.4	5.5	4.1
Moisture (%)	12.67	10.13	10.60	
Ash (%)	2.99	3.18	1.74	
SO_2 (ppm)	nd	184	88	
Viscosity (ms @ 60°C)	27.9	30.1	32.1	
Corrected* Bloom	316	278	240	
Corrected* Viscosity	28.8	30.4	32.0	
Overall Colour.	14.3			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
nd Not determined.

ADDENDUM C5. Continued...

Extraction & Quality Data Experiment WT4.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	7.92	7.86	7.49	6.67
Liquor pH	2.61	3.04	3.55	4.35
Liquor Concentration (%w/v)	0.61	1.35	3.06	14.05
Fat (ml)	10	3	3	
Scutch (g)				230
Gelatine (g)	48.3	106.1	229.2	937.1
Gelatine % Proportion	3.6	8.0	17.4	71.0
Heavy Liquor Volume (ml)	600	900	1250	
Heavy Liquor Conc:	4	6	10	
5% H_2O_2 (ml)	14	16	8	
5% NH_3 (ml)	10	10	8	

Total Gelatine Recovered (g)	1320.7
Total Gelatine Yield (%)	33.0
Anhydrous Gelatine Recovered (g) (f 0.875)	1155.6
Anhydrous Gelatine Corrected (g) (f 0.95)	1097.8
Anhydrous Gelatine Yield on Raw Material (%)	65.7
Total Anhydrous Solids Recovered (g)	1321.4
Total Anhydrous solids Recovered (%)	79.1

Gelatine Quality.

Run No	1	2	3	
Bloom	262	278	267	
Colour	11.4f	12.3f	13.3	17.8
Clarity	12.5	13.3	12.5	nm
pH	5.6	5.5	5.4	4.2
Moisture (%)	10.00	9.84	9.97	
Ash (%)	7.99	4.88	2.08	
SO_2 (ppm)	---	24+	128	
Viscosity (ms @ 60°C)	26.9	26.2	28.2	
Corrected* Bloom	298	293	264	
Corrected* Viscosity	28.7	26.9	18.1	
Overall Colour.	16.3			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
f Bloom sample filtered; Whatman GFA
\+ H_2O_2 Positive.
nm Not measurable.

ADDENDUM C5. Continued...

Extraction & Quality Data Experiment WT5.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	8.42	8.88	7.04	7.13
Liquor pH	2.78	3.31	3.44	---
Liquor Concentration (%w/v)	0.80	1.47	3.47	11.06
Fat (ml)	10	5	3	
Scutch (g)				210
Gelatine (g)	67.4	130.5	244.2	788.6
Gelatine % Proportion	5.5	10.6	19.8	64.1
Heavy Liquor Volume (ml)	600	1100	1300	
Heavy Liquor Conc:	7	7	10	
5% H_2O_2 (ml)	15	14	6	
5% NH_3 (ml)	8	8	8	

Total Gelatine Recovered (g)	1230.7
Total Gelatine Yield (%)	30.8
Anhydrous Gelatine Recovered (g) (f 0.875)	1076.8
Anhydrous Gelatine Corrected (g) (f 0.95)	1023.0
Anhydrous Gelatine Yield on Raw Material (%)	61.2
Total Anhydrous Solids Recovered (g)	1252.6
Total Anhydrous solids Recovered (%)	75.0

Gelatine Quality.

Run No	1	2	3	
Bloom	271	294	261	
Colour	11.4f	13.3f	16.0	17.8
Clarity	12.5	12.5	11.1	nm
pH	5.5	5.4	5.2	4.2
Moisture (%)	11.63	9.05	9.44	
Ash (%)	5.48	3.94	1.86	
SO_2 (ppm)	0	24	176	
Viscosity (ms @ 60°C)	23.4	29.1	29.4	
Corrected* Bloom	301	297	254	
Corrected* Viscosity	24.7	29.3	29.0	
Overall Colour.	16.6			

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
nm Not measurable.
f Bloom sample filtered; Whatman GF/A

ADDENDUM C6. Young (10.5 month old) animal hide and sodium sulphide level.

EXPERIMENT YS.

Raw Material and Conditioning.

For 2 g/l Na_2S 67 g sodium sulphide flake was used.
For 6 g/l Na_2S 200 g sodium sulphide flake was used.

Conditioning Liquor data.

Bin	Na2S g/l Analyzed	Tare Wt	Gross Wt + liquor	Gross Wt + hide
A	1.63	3.00	23.0	29.05
B	5.70	3.25	23.25	29.15
C	1.60	3.20	23.20	29.10
D	5.73	3.70	23.70	29.50
E	Nil	3.70	23.70	29.65

Raw Material.

Young - 10.5 month old - animal salted hide ex ADSRI.

Prewashing	18hrs minimum.
Mass of hide for each experiment.	6000 g.
Moisture Content	61.01± 0.4%
Ash Content	0.23± 0.03%
Organic Content	38.76%
Organic Content of samples.	2326 g

ADDENDUM C6. Continued...

Hide Conditioning at 22 degree Centigrade.

Conditioning Liquor:	CaO	640 g	
	Na_2S	qs	
	Water to	20.0 kg.	
Experiment No	YSA	YSB	YSC
Washed Hide (kg)	6.0	6.0	6.0
Conditioning Time -Weeks.	2	2	3
Cond: Temperature (°C)	21.4	21.4	21.6
Init: Sulphide (g Na_2S/l)	1.63	5.70	1.60
Ex-Lime Wash for 17 hrs			
Limed Mass (kg)	9.0	9.9	9.3
Swelling (%)	150	165	155
Acidulation 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	10.35	11.0	11.85
Swelling (%)	173	183	198
Soak Water pH	2.44	2.35	2.23
Spent Conditioning Liquor:			
Mass (kg)	19.65	19.80	19.95
pH	12.28	12.48	12.30
Na_2S Conc: (g/l)	1.17	4.30	1.00
Sulphide consumed (g/l)	0.46	1.40	0.60
Spent Liquor Solids (g/l)	13.94±0.03	21.47±0.03	16.14±0.00
" " Ash (g/l)	5.01±0.02	9.38±0.03	5.10±0.05
" " Volatiles (g/l)	8.93	12.09	11.04
" " Organic Matter (g)	179	241	220
Organic Matter (%)	7.7	10.4	9.5

ADDENDUM C6. Continued...

Hide Conditioning at 22 degree Centigrade.

Conditioning Liquor:	CaO	640 g	
	Na_2S	qs.	
	Water to	20.0 kg.	
Experiment No		YSD	YSE
Washed Hide (kg)		6.0	6.0
Conditioning Time -Weeks.		3	4
Cond: Temperature (°C)		21.6	22.0
Init: Sulphide (g Na_2S/l)		5.73	Nil
Ex-Lime Wash for 18hrs			
Limed Mass (kg)		9.3	9.1
Swelling (%)		155	152
Acidulation 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)		12.25	10.2
Swelling (%)		204	170
Soak Water pH		2.31	2.38
Spent Conditioning Liquor:			
Mass (kg)		19.95	19.85
pH		12.53	12.46
Na_2S Conc: (g/l)		3.90	0.04
Sulphide consumed (g/l)		1.83	-0.04
Spent Liquor Solids (g/l)		22.82±0.07	9.4±0.6
" "	Ash (g/l)	9.81	3.07±0.04
" "	Volatiles (g/l)	13.01	6.36
" "	Organic Matter (g)	260	127
	Organic Matter (%)	11.2	5.5

ADDENDUM C6. Continued...

Extraction & Quality Data Experiment YSA.(2 weeks liming, 1.63 g/l Na_2S)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume l	12.45	6.04	5.36	4.45
Liquor pH	3.06	3.08	3.28	
Liquor Concentration (%w/v)	4.41	6.85	6.06	6.02
Fat (ml)	40	45	35	
Scutch (g)				360
Gelatine (g)	549.0	413.7	324.8	267.9
Gelatine % Proportion	35.3	26.6	20.9	17.2
Heavy Liquor Volume (ml)	1000	1400	1650	
Heavy Liquor Conc:(%)	10	13	9	
5% H_2O_2 (ml)	4	6	4	
5% NH_3 (ml)	10	20	20	

Total Gelatine Recovered (g)	1555.4
Total Gelatine Yield (%)	25.9
Anhydrous Gelatine Recovered (g) (f 0.875)	1360.9
Anhydrous Gelatine Corrected (g) (f 0.95)	1292.9
Anhydrous Gelatine Yield on Raw Material (%)	55.6
Total Anhydrous Solids Recovered (g)	1591.5
Total Anhydrous solids Recovered (%)	68.4

Gelatine Quality.

Run No	1	2	3	4
Bloom	326	313	286	
Colour	3.6	4.4	4.8	4.8
Clarity	8.0	8.0	7.5	12.5
pH	5.8	5.6	5.6	4.5
Moisture (%)	9.1	9.9	10.8	
Ash (%)	0.46	0.28	0.58	
SO_2 (ppm)	120	632	600	
Viscosity (ms @ 60°C)	55.1	50.8	47.5	
Corrected* Bloom	305	297	279	
Corrected* Viscosity	53	49	47	
Overall Colour.		4.3		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C6. Continued...

Extraction & Quality Data Experiment YSB. (2 weeks liming, 5.7 g/l Na_2S)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	13.04	6.12	5.90	4.04
Liquor pH	3.03	3.02	3.23	
Liquor Concentration (%w/v)	4.70	6.71	5.24	5.16
Fat (ml)	20	45	50	
Scutch (g)				255
Gelatine (g)	612.9	410.7	309.2	208.5
Gelatine % Proportion	39.8	26.6	20.1	13.5
Heavy Liquor Volume (ml)	1250	1600	1400	
Heavy Liquor Conc:(%)	11	13	10	
5% H_2O_2 (ml)	4	6	3	
5% NH_3 (ml)	12.5	25	17.5	

Total Gelatine Recovered (g)	1541.3
Total Gelatine Yield (%)	25.7
Anhydrous Gelatine Recovered (g) (f 0.875)	1348.6
Anhydrous Gelatine Corrected (g) (f 0.95)	1281.2
Anhydrous Gelatine Yield on Raw Material (%)	55.1
Total Anhydrous Solids Recovered (g)	1638.0
Total Anhydrous solids Recovered (%)	70.4

Gelatine Quality.

Run No	1	2	3	
Bloom	314	312	287	
Colour	<3.2	5.2	4.8	4.4
Clarity	10.0	7.5	9.0	11.8
pH	5.5	5.5	5.6	4.5
Moisture (%)	9.08	9.76	10.79	
Ash (%)	0.47	0.43	0.42	
SO_2 (ppm)	504	616	184	
Viscosity (ms @ 60°C)	53.7	52.6	38.7	
Corrected* Bloom	294	296	279	
Corrected* Viscosity	52	51	38	
Overall Colour.		4.2		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C6. Continued...

Extraction & Quality Data Experiment YSC. (3 weeks liming, 1.6 g/l Na_2S)

Extraction.				
Run No	1	2	3	
Time (hrs)	5	5	7	
Temperature (°C)	45	50	Boil	
Liquor Volume (l)	14.32	7.15	3.56	
Liquor pH	3.11	3.30		
Liquor Concentration (%w/v)	4.81	6.65	9.45	
Fat (ml)	6	27		
Scutch (g)			310	
Gelatine (g)	688.8	475.5	336.4	
Gelatine % Proportion	45.9	31.6	22.4	
Heavy Liquor Volume (ml)	1350	1400		
Heavy Liquor Conc:(%)	-	10.5		
5% H_2O_2 (ml)	4	4		
5% NH_3 (ml)	15	17.5		
Total Gelatine Recovered (g)				1500.7
Total Gelatine Yield (%)				25.0
Anhydrous Gelatine Recovered (g) (f 0.875)				1313.1
Anhydrous Gelatine Corrected (g) (f 0.95)				1247.5
Anhydrous Gelatine Yield on Raw Material (%)				53.6
Total Anhydrous Solids Recovered (g)				1495.5
Total Anhydrous solids Recovered (%)				64.3

Gelatine Quality.

Run No	1	2	3
Bloom	326	323	
Colour	3.2	4.0	4.4
Clarity	11.1	10.5	11.8
pH	5.5	5.6	4.4
Moisture (%)	9.14	8.60	
Ash (%)	0.34	0.31	
SO_2 (ppm)	160	680	
Viscosity (ms @ 60°C)	53.1	51.9	
Corrected* Bloom	305	298	
Corrected* Viscosity	51	50	
Overall Colour.		4.0	

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C6. Continued...

Extraction & Quality Data Experiment YSD. (3 weeks liming, 5.7 g/l Na_2S)

Extraction.			
Run No	1	2	3
Time (hrs)	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	15.34	8.00	4.20
Liquor pH	2.97	3.20	
Liquor Concentration (%w/v)	4.81	5.73	4.22
Fat (ml)	3	35	
Scutch (g)			110
Gelatine (g)	737.9	458.4	177.2
Gelatine % Proportion	53.7	33.4	12.9
Heavy Liquor Volume (ml)	1350	1300	
Heavy Liquor Conc:(%)	10	12	
5% H_2O_2 (ml)	4	2	
5% NH_3 (ml)	15	20	
Total Gelatine Recovered (g)			1373.5
Total Gelatine Yield (%)			22.9
Anhydrous Gelatine Recovered (g) (f 0.875)			1201.8
Anhydrous Gelatine Corrected (g) (f 0.95)			1141.7
Anhydrous Gelatine Yield on Raw Material (%)			49.1
Total Anhydrous Solids Recovered (g)			1441.9
Total Anhydrous solids Recovered (%)			62.0

Gelatine Quality.

Run No	1	2	3
Bloom	322	323	
Colour	3.2	3.2	4.8
Clarity	11.1	11.8	13.3
pH	5.5	5.4	4.4
Moisture (%)	9.21	8.55	
Ash (%)	0.37	0.26	
SO_2 (ppm)	96	1184	
Viscosity (ms @ 60°C)	55.5	48.7	
Corrected* Bloom	332	297	
Corrected* Viscosity	54	47	
Overall Colour.		3.4	

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C6. Continued...

Extraction & Quality Data Experiment YSE. (4 weeks liming, No Na_2S)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	13.23	8.24	6.50	2.48
Liquor pH	3.14	3.27	3.41	
Liquor Concentration (%w/v)	3.51	5.02	6.12	6.04
Fat (ml)	30	20	Not Measurable.	
Scutch (g)		161.7 Dry. **		
Gelatine (g)	464.4	413.6	397.8	(274.2)
Gelatine % Proportion	30.0	26.7	25.7	17.6
Heavy Liquor Volume (ml)	1000	1400	1300	
Heavy Liquor Conc:(%)	10	9	14	
5% H_2O_2 (ml)	2	2	2	
5% NH_3 (ml)	10	17.5	25	

Total Gelatine Recovered (g)	1550 estimated.
Total Gelatine Yield (%)	25.8
Anhydrous Gelatine Recovered (g) (f 0.875)	1356.3
Anhydrous Gelatine Corrected (g) (f 0.95)	1288.4
Anhydrous Gelatine Yield on Raw Material (%)	55.4
Total Anhydrous Solids Recovered (g)	1465.6
Total Anhydrous solids Recovered (%)	63.0

Gelatine Quality.

Run No	1	2	3
Bloom	340	319	292
Colour	<3.2	4.0	5.2
Clarity	10.5	7.5	7.5
pH	5.6	5.5	5.3
Moisture (%)	9.56	10.73	10.2
Ash (%)	0.62	0.40	0.36
SO_2 (ppm)	48	40	80
Viscosity (ms @ 60°C)	44.0	46.8	43.4
Corrected* Bloom	323	309	279
Corrected* Viscosity	43	46	42
Overall Colour.		±4.0	

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

**The residue boil nearly boiled dry, thus some of the gelatine was rendered insoluble. For this reason the insoluble residue was air dried and weighed. The total recovery was 1587.3 g which included all the insolubilized gelatine and scutch. For this reason a gelatine yield of 1550 g was taken for calculation purposes.

ADDENDUM C7. Gelatine from various layers of a hide.

EXPERIMENT KTO.
(Krugersdorp Tannery Old animal hide).

Raw Material and Conditioning Data.

Sample	KT01 Whole Hide	KT02 Flesh Split	KT03 Middle Split	KT04 Grain Split
Moisture Content (%)	69.84	71.6	68.26	71.77
Ash Content (%)	3.82	17.70	2.69	2.24
Sample Mass (kg)	8.55	2.40	2.75	3.15
Anhyd: Hide Subs: (g)	2315	257	799	819

Conditioning.

Conditioning Liquor: 640 g CaO in 20 kg water.

Sample No	KT01	KT02	KT03	KT04
Cond: Time (Weeks) *	6	6	6	6
Cond: Temperature (°C)	21.5	21.5	21.0	21.0
Init: Sulphide Na_2S (g/l)	2.19	2.07	1.94	1.99
Final Sulphide Na_2S (g/l)	1.18	1.33	1.29	1.19
Sulphide consumed (g/l)	1.01	0.74	0.65	0.80
Spent Liquor Solids (%w/v)	1.96	1.00	0.83	1.00
" " Ash (%w/v)	0.98	0.68	0.64	0.74
" " Volatiles (%w/v)	0.98	0.32	0.19	0.26
" " Organic Matter (g)	196	64	37	51
" " Absorb: (470nm)	0.050	0.000	0.001	0.006
Ex-Lime Wash for 16hrs.				
Limed Mass (kg)	14.2	5.85	4.10	4.6
Swelling (%)	167	244	149	146
Acidulation. 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.				
Wt: for Extraction (kg)	13.5	5.5	5.1	5.1
Swelling (%)	158	229	185	162
Soak Water pH	2.82	2.12	2.91	2.83

* See experimental procedures.

ADDENDUM C7. Continued...

KT01 Whole Hide Extraction & Quality Data.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	13.66	10.80	10.05	5.66
Liquor pH	2.73	3.36	3.66	
Liquor Concentration (%w/v)	1.49	2.24	4.63	7.94
Fat (ml)	Nil	Nil	Nil	
Scutch (g)				Nil
Gelatine (g)	203.5	241.9	465.3	1015.1
Gelatine % Proportion	10.5	12.6	24.2	52.7
Heavy Liquor Volume (ml)	1200	1200	1300	
Heavy Liquor Conc:	7	5.5	8	
5% H_2O_2 (ml)	2.5	1	0.5	
5% NH_3 (ml)	9	12	24	

Total Gelatine Recovered (g)	1925.8
Total Gelatine Yield (%)	22.5
Anhydrous Gelatine Recovered (g) (f 0.875)	1685.1
Anhydrous Gelatine Corrected (g) (f 0.95)	1600.8
Anhydrous Gelatine Yield on Raw Material (%)	69.1
Total Anhydrous Solids Recovered (g)	1796.8
Total Anhydrous solids Recovered (%)	77.6

Gelatine Quality.

Run No	1	2	3	4
Bloom	324	271	222	
Colour	8	7.2	9.4	13.3
Clarity	11.8	11.8	9.0	14.3
pH	5.5	5.5	5.5	4.08
Moisture (%)	8.06	11.81	12.12	
Ash (%)	1.23	0.71	0.40	
SO_2 (ppm) ϕ	16	376	272	
Viscosity (ms @ 60°C)	35.0	30.8	30.9	
Corrected* Bloom	301	271	222	
Corrected* Viscosity	33.7	30.8	30.9	

Overall Colour.	11.6

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ϕ + Indicates peroxide positive on Starch/KI test.

ADDENDUM C7. Continued...

KT02 Flesh Split Extraction & Quality Data.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	6.95	4.66	4.88	1.44
Liquor pH	3.35	3.69	3.9	
Liquor Concentration (%w/v)	0.72	1.035	1.33	6.795
Fat (ml)	Nil	Nil	Nil	
Scutch (g)				Nil
Gelatine (g)	50	48.2	64.9	97.8
Gelatine % Proportion	19.2	18.5	24.9	37.4
Heavy Liquor Volume (ml)	600	600	900	
Heavy Liquor Conc:	5	5	3	
5% H_2O_2 (ml)	0.5	0.5	0.5	
5% NH_3 (ml)	5	6	8	

Total Gelatine Recovered (g)	260.9
Total Gelatine Yield (%)	10.9
Anhydrous Gelatine Recovered (g) (f 0.875)	228.3
Anhydrous Gelatine Corrected (g) (f 0.95)	216.9
Anhydrous Gelatine Yield on Raw Material (%)	84.3
Total Anhydrous Solids Recovered (g)	280.9
Total Anhydrous solids Recovered (%)	109.3

Gelatine Quality.

Run No	1	2	3	4
Bloom	310	267	234	
Colour	8.4	10.0	16.0	NM
Clarity	11.1	7.0	3.5	NM
pH	5.3	5.2	5.3	4.3
Moisture (%)	8.58	12.12	11.05	
Ash (%)	2.14	1.62	1.30	
SO_2 (ppm) ϕ	368	272	464	
Viscosity (ms @ 60°C)	41.0	39.4	41.6	
Corrected* Bloom	298	274	233	
Corrected* Viscosity	40.2	39.9	41.5	
Overall Colour.		---		

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.
ϕ + Indicates peroxide positive on Starch/KI test.

ADDENDUM C7. Continued...

KT03 Middle Split Extraction & Quality Data.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	6.8	5.25	4.3	2.4
Liquor pH	3.27	3.5	3.74	
Liquor Concentration (%w/v)	0.86	1.44	3.73	20.15
Fat (ml)	Nil	Nil	Nil	
Scutch (g)				Nil
Gelatine (g)	58.5	75.6	160.4	483.6
Gelatine % Proportion	7.5	9.7	20.6	62.2
Heavy Liquor Volume (ml)	650	450	950	
Heavy Liquor Conc:	6	12	9	
5% H_2O_2 (ml)	0.5	0.25	0.1	
5% NH_3 (ml)	8	14	23	

Total Gelatine Recovered (g)	778.1
Total Gelatine Yield (%)	28.3
Anhydrous Gelatine Recovered (g) (f 0.875)	680.8
Anhydrous Gelatine Corrected (g) (f 0.95)	646.8
Anhydrous Gelatine Yield on Raw Material (%)	80.9
Total Anhydrous Solids Recovered (g)	683.8
Total Anhydrous solids Recovered (%)	85.6

Gelatine Quality.

Run No	1	2	3	4
Bloom	313	252	218	
Colour	6.4	6.0	6.4	16.0
Clarity	12.5	12.5	11.1	15.45
pH	5.3	5.3	5.3	4.15
Moisture (%)	9.98	10.21	9.46	
Ash (%)	2.02	1.08	0.36	
SO_2 (ppm) ϕ	160	192	240	
Viscosity (ms @ 60°C)	30.3	28.8	28.8	
Corrected* Bloom	309	245	205	
Corrected* Viscosity	30.1	28.4	27.9	

Overall Colour.	12.3

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.
ϕ + Indicates peroxide positive with Starch/KI test.

ADDENDUM C7. Continued...

KT04 Grain Split Extraction & Quality Data.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	6.65	4.92	4.01	3.36
Liquor pH	3.13	3.51	3.68	
Liquor Concentration (%w/v)	1.01	1.71	3.93	13.95
Fat (ml)	Nil	Nil	Nil	
Scutch (g)				Nil
Gelatine (g)	67.2	84.1	157.6	468.7
Gelatine % Proportion	8.6	10.8	20.3	60.3
Heavy Liquor Volume (ml)	675	600	800	
Heavy Liquor Conc:	7	11	10	
5% H_2O_2 (ml)	0.5	0.25	0.1	
5% NH_3 (ml)	10	16	20	

Total Gelatine Recovered (g)	777.6
Total Gelatine Yield (%)	24.77
Anhydrous Gelatine Recovered (g) (f 0.875)	680.4
Anhydrous Gelatine Corrected (g) (f 0.95)	646.4
Anhydrous Gelatine Yield on Raw Material (%)	78.9
Total Anhydrous Solids Recovered (g)	697.6
Total Anhydrous solids Recovered (%)	85.1

Gelatine Quality.

Run No	1	2	3	4
Bloom	316	259	216	
Colour	6.0	6.0	6.8	16.7
Clarity	12.5	12.5	11.1	9.0
pH	5.5	5.5	5.5	4.15
Moisture (%)	9.96	10.01	9.73	
Ash (%)	1.49	0.91	0.34	
SO_2 (ppm)	32	144	160	
Viscosity (ms @ 60°C)	30.0	29.8	29.7	
Corrected* Bloom	308	249	204	
Corrected* Viscosity	29.6	29.3	28.9	

Overall Colour.	12.6

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C8. Type A calf skin gelatine.

EXPERIMENT CALF-A

Conditioning.

Raw Material Weight. 7.6 kg.
Moisture content. 44.75%
Ash content. 17.03%
Organic Matter. 38.22% = 2905 g.

Tumbler washed for 17 (hrs).
Dehairing. Solution (3%) = 20 kg containing 1 kg 60% Na_2S.
Time. 30 minutes with occasional stirring.
Tumbler wash. 6 (hrs).

Dehaired & Washed Weight. 11.25 kg.
Moisture content. 85.93%
Ash Content. 0.13%
Organic Matter. 13.94% (= 1568 g).
Hair + epidermis 1337 g (= 17.6% of Raw Material).

Acidulation.

57 (ml) Conc: H_2SO_4 diluted to 20 kg. By titration = 0.103N.
= 2.06 Eq.
Acidulation time 17 hr.
Spent acid = 0.019N. (pH = 1.7).
" " Concentration = 0.33% acid soluble collagen.
or ±66 g.
Total water volume = 20 (l) acid solution
+ 9.67 (l) in washed skin.
Residual acid = 29.66 (l) @ 0.019N = 0.564 Eq.
Acid consumed = 2.06 - 0.56 = 1.50 Eq
or 0.96 Eq/kg collagen.

ADDENDUM C8. Continued...

CALF-A

Extraction.

Weight for extraction = 17.0 kg.
Soak water pH = 3.2.

Run No	1	2	3	4
Extraction Time. (Hours)	5	5	5	7
Extraction Temperature. (°C)	50	60	70	Boil
Extract Volume. (l).	16.24	6.84	5.42	4.41
Extract pH.	3.63	3.73	3.78	
Extract Concentration. (%)	3.40	4.58	3.97	4.46
Extract proportion (%).	43.2	24.5	16.8	15.4
Fat. (ml).	40	15		
Residue (kg).				2.1
Heavy Liquor Volume. (l).	2.4	1.8	1.05	
Heavy Liquor Conc: (%).	10.5	9	8.5	
5% NH_3 added to >pH 5	5 (ml)	0 (ml)	*45 (ml)	
* 0.1N H_2SO_4				
Total Gelatine Produced.		1277 g		
Yield		16.8 %		
Corrected Yield (f= 0.92)		15.5 %		
Yield on Anhyd: Organic matter.		43.9 % (Normally 60% min:)		

Gelatine Quality

Run No.	1	2	3
Bloom	351	333	284
Colour DGI	4.0	3.6	5.2
Clarity DGI	7.5	10.5	9.0
pH	6.2	6.3	6.5
Moisture (%).	10.08	11.07	13.79
Ash (%)	0.27	0.21	0.11
SO_2 ppm Titre.	S 22	S 31	S 17
SO_2 ppm Distillation.	72	128	56
Viscosity ms.	55.9	63.5	32.6
Isoelectric Point	9.4	9.2	8.9

ADDENDUM C9. Three & six year-old Friesland's hides ex ADSRI.

EXPERIMENTS 3Y and 6Y.

3 Year-old - Raw Material and Conditioning.

Raw Material: Hide No 8901 - Washed and Drained.
3 year-old Friesland hide.

Moisture Content	65.6 ± 3.8%
Ash Content	0.28 ± 0.1%
Sample Mass (kg)	5.0
Anhyd: Hide Subs:	1705 g

Conditioning.

Conditioning Liquor: CaO 640 g
Na_2S 60% 75 g (150 g)
Water to 20 kg.

Sample No	3Y2	3Y4A	3Y4B
Cond: Time Weeks	2	4	4
Cond: Temperature (°C)	21.7	21.8	21.8
Init: Sulphide (g Na_2S/l)	1.99	1.95	4.25
Final Sulphide (g Na_2S/l)	1.43	1.12	2.78
Sulphide consumed (g/l)	0.56	0.83	1.47
Spent Liquor Solids (%w/v)	1.62	1.90	2.10
" " Ash (%w/v)	0.60	0.44	0.66
" " (%w/v) Volatiles	1.02	1.46	1.44
" " Organic Matter (g)	204	292	288
Evaporative Loss (kg)	0.35	0.3	0.45
Ex-Lime Wash for 16hrs			
Limed Mass (kg)	7.1	7.75	7.55
Swelling (%)	142	155	151
Acidulation 5 coats of			
H_2SO_3 soln. over 4 days.			
Wash 1hr.			
Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	7.3	9.8	8.15
Soak Water pH	2.34	2.1	2.04

Sample No	3Y6A	3Y6B	3Y8
Cond: Time Weeks	6	6	8
Cond: Temperature (°C)	21.8	21.8	21.8
Init: Sulphide (g Na_2S/l)	1.99	0.01	2.03
Final Sulphide (g Na_2S/l)	0.96	0.05	0.69
Sulphide consumed (g/l)	1.03	-0.04	1.34
Spent Liquor Solids (%w/v)	2.24	1.12	2.44
" " Ash (%w/v)	0.56	0.30	0.75
" " pH	12.38	12.27	
" " (%w/v) Volatiles	1.68	0.83	1.69
" " Organic Matter (g)	336	166	338
Evaporative Loss (kg)			0.6
Ex-Lime Wash for 16hrs			
Limed Mass (kg)			7.05
Swelling (%)			141
Acidulation 5 coats of			
H_2SO_3 soln. over 4 days.			
Wash 1hr.			
Soak in fresh water ± 22hr			

ADDENDUM C9. Continued...

Six Year-old - Raw Material and Conditioning.

Raw Material: Hide No 8551 - Washed and Drained.
6 year-old Friesland hide.

Moisture Content	59.3 ± 3.8%
Ash Content	0.59 ± 0.01%

Sample No	6Y2	6Y4A	6Y4B
Sample Mass (kg)	6.0	6.0	6.0
Anhyd: Hide Subs: (g)	2406	2406	2406

Conditioning.
Conditioning Liquor: CaO 640 g
Na_2S 60% 75 g (150 g)
Water to 20 kg.

Sample No	6Y2	6Y4A	6Y4B
Cond: Time (Weeks)	2	4	4
Cond: Temperature (°C)	21.6	21.7	21.7
Init: Sulphide (g Na_2S/l)	2.05	2.02	4.41
Final Sulphide (g Na_2S/l)	1.37	1.03	2.44
Sulphide consumed (g/l)	0.69	0.99	1.96
Spent Liquor Solids (%w/v)	1.78	2.14	2.83
" " Ash (%w/v)	0.65	0.43	0.78
" " (%w/v) Volatiles	1.13	1.71	2.05
" " Organic Matter (g)	226	332	394
Evaporation Loss (kg)	0.15	0.6	0.8
Ex-Lime Wash for 16hrs			
Limed Mass (kg)	8.55	8.75	9.10
Swelling (%)	143	146	152
Acidulation 5 coats of			
H_2SO_3 soln. over 4 days.			
Wash 1hr.			
Soak in fresh water ± 22hr.			
Wt: for Extraction (kg)	8.85	9.05	8.9
Soak Water pH	2.07	2.25	2.14

ADDENDUM C9. Continued...

Sample No	6Y6A	6Y6B	6Y8
Sample Mass (kg)	6.0	2.4	6.0
Anhyd: Hide Substance (g)	2406	962	2406
Cond: Time Weeks	6	6	8
Cond: Temperature (°C)	21.8	21.8	21.7
Init: Sulphide (g Na_2S/l)	2.04	0.0	2.03
Final Sulphide (g Na_2S/l)	0.81	0.02	0.70
Sulphide consumed (g/l)	1.23	-0.02	1.33
Spent Liquor Solids (%w/v)	2.48	0.59	2.69
" " Ash (%w/v)	0.61	0.19	0.62
" " (%w/v) Volatiles	1.87	0.40	2.07
" " pH	13.09	13.31	
" " Organic Matter (g)	374	80	414
Evaporation Loss (kg)			0.1
Ex-Lime Wash for 16hrs			
Limed Mass (kg)	8.45	3.00	
Swelling (%)	141	125	
Acidulation 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.			
Weight for Extraction (kg)	9.0	3.25	8.6
Soak Water pH	2.07	2.22	2.26

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 3Y2. (2 weeks liming)

Extraction.				
Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	55	Boil
Liquor Volume (l)	8.98	7.35	7.05	5.24
Liquor pH	2.65	3.21	3.57	
Liquor Concentration (%w/v)	1.27	2.42	3.82	11.99
Fat (ml)	35	15	10	
Scutch (g)				210
Gelatine (g)	114.0	177.9	269.3	628.3
Gelatine % Proportion	9.6	15.0	22.6	52.8
Heavy Liquor Volume (ml)	600	1200	1000	
Heavy Liquor Conc: (%)	8	9	12	
5% H_2O_2 (ml)	14	14	10	
5% NH_3 (ml)	7	7	5	

Total Gelatine Recovered (g)	1189.5
Total Gelatine Yield (%)	23.8
Anhydrous Gelatine Recovered (g) (f 0.875)	1040.8
Anhydrous Gelatine Corrected (g) (f 0.95)	988.8
Anhydrous Gelatine Yield on Raw Material (%)	58.0
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	1252.8
Total Anhydrous solids Recovered (%)	73.5

Gelatine Quality.

Run No	1	2	3	4
Bloom	295	296	292	
Colour	6.4	6.0	6.4/7.2	4.8
Clarity	12.5	11.1	11.1	10.5
pH	5.5	5.4	5.4	4.5
Moisture (%)	13.25	12.58	12.3	
Ash (%)	4.58	2.69	2.51	
SO_2 (ppm)				
Viscosity (ms @ 60°C)	23.0	32.5	38.5	
Corrected* Bloom	335	316	308	
Corrected* Viscosity	25	34	40	
Overall Colour.		5.5		

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 3Y4A. (4 weeks liming, 2 g/l Na_2S)

Extraction.			
Run No	1	2	3
Time (hrs)	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	10.01	7.47	3.97
Liquor pH	2.89	3.31	
Liquor Concentration (%w/v)	3.33	4.72	12.39
Fat (ml)	15	15	
Scutch (g)			50
Gelatine (g)	333.3	352.6	491.9
Gelatine % Proportion	28.3	29.9	41.8
Heavy Liquor Volume (ml)	1250	1000	
Heavy Liquor Conc: (%)	9	11	
5% H_2O_2 (ml)	15	7	
5% NH_3 (ml)	10	8	
Total Gelatine Recovered (g)			1177.8
Total Gelatine Yield (%)			23.6
Anhydrous Gelatine Recovered (g) (f 0.875)			1030.6
Anhydrous Gelatine Corrected (g) (f 0.95)			979.0
Anhydrous Gelatine Yield on Raw Material (%)			57.4
Total Anhydrous Solids Recovered (g) (Fat + Cond:)			1301
Total Anhydrous solids Recovered (%)			76.3

Gelatine Quality.

Run No	1	2	3
Bloom	266	272	
Colour	6.4/6.8	6.0	3.6
Clarity	10.0	11.1	14.3
pH	5.4	5.7	4.3
Moisture (%)	15.5	14.8	
Ash (%)	3.01	2.74	
SO_2 (ppm)	448	624	
Viscosity (ms @ 60°C)	37.5	40.6	
Corrected* Bloom	307	306	
Corrected* Viscosity	40	43	
Overall Colour.		5.2	

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 3Y4B. (4 weeks liming, 4g/l Na_2S)

Extraction.			
Run No	1	2	3
Time (hrs)	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	9.82	7.96	3.47
Liquor pH	3.00	3.29	
Liquor Concentration (%w/v)	3.58	5.02	13.44
Fat (ml)	5	15	
Scutch (g)			50
Gelatine (g)	351.6	399.6	466.4
Gelatine % Proportion	28.9	32.8	38.3
Heavy Liquor Volume (ml)	1200	1200	
Heavy Liquor Conc: (%)	9	10	
5% H_2O_2 (ml)	12	12	
5% NH_3 (ml)	8	5	

Total Gelatine Recovered (g)	1217.6
Total Gelatine Yield (%)	24.3
Anhydrous Gelatine Recovered (g) (f 0.875)	1065.4
Anhydrous Gelatine Corrected (g) (f 0.95)	1023.1
Anhydrous Gelatine Yield on Raw Material (%)	59.4
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	1331.1
Total Anhydrous solids Recovered (%)	78.1

Gelatine Quality.

Run No	1	2	3
Bloom	277	277	
Colour	6.4	6.4	5.6
Clarity	9.5	10.5	14.3
pH	5.4	5.3	4.4
Moisture (%)	15.89	12.74	
Ash (%)	2.97	3.15	
SO_2 (ppm)	440	384	
Viscosity (ms @ 60°C)	34.3	41.2	
Corrected* Bloom	322	300	
Corrected* Viscosity	37	43	
Overall Colour.		6.1	

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 3Y6A. (6 weeks liming)

Extraction.

Run No	1	2	3
Time (hrs)	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	10.51	8.18	4.51
Liquor pH	2.90	3.24	
Liquor Concentration (%w/v)	3.98	Est*4.92	7.48
Fat (ml)	1	Nil	
Scutch (g)			Nil
Gelatine (g)	418.3	402.4	337.3
Gelatine % Proportion	36.1	34.8	29.1
Heavy Liquor Volume (ml)	1300	1250	
Heavy Liquor Conc: (%)	9	10	
5% H_2O_2 (ml)	13	10	
5% NH_3 (ml)	7.5	5	

Total Gelatine Recovered (g)	Est*1158.0
Total Gelatine Yield (%)	23.2
Anhydrous Gelatine Recovered (g) (f 0.875)	
Anhydrous Gelatine Corrected (g) (f 0.95)	
Anhydrous Gelatine Yield on Raw Material (%)	
Total Anhydrous Solids Recovered (g)	(1322.1)
Total Anhydrous solids Recovered (%)	(77.5)

* Due to loss of the sample, estimates were based on the average yields for the other 5 lots of hide from the same animal.

Gelatine Quality.

Run No	1	2	3
Bloom	305	270	
Colour	6.0	6.8	
Clarity	12.5	11.8	
pH	5.4	5.4	
Moisture (%)	11.3	12.55	
Ash (%)	3.58	2.67	
SO_2 (ppm)	936	96	
Viscosity (ms @ 60°C)	29.5	39.2	
Corrected* Bloom	322	288	
Corrected* Viscosity	34	41	
Overall Colour.		Not Available.	

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 3Y6B. (6 weeks liming, no Na_2S)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	8.76	8.31	6.81	1.92
Liquor pH	2.89	3.22	3.5	
Liquor Concentration (%w/v)	2.34	3.00	6.61	15.3
Fat (ml)	20	7	20	
Scutch (g)				Nil
Gelatine (g)	205.0	249.3	450.1	293.8
Gelatine % Proportion	17.1	20.8	37.6	24.5
Heavy Liquor Volume (ml)	1050	1050	1115	
Heavy Liquor Conc: (%)	6	11		
5% H_2O_2 (ml)	9	14	5	
5% NH_3 (ml)	5	7	7	

Total Gelatine Recovered (g)	1198.2
Total Gelatine Yield (%)	24.0
Anhydrous Gelatine Recovered (g) (f 0.875)	1048.4
Anhydrous Gelatine Corrected (g) (f 0.95)	996.0
Anhydrous Gelatine Yield on Raw Material (%)	58.4
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	1208.6
Total Anhydrous solids Recovered (%)	70.9

Gelatine Quality.

Run No	1	2	3
Bloom	296	270	223
Colour	6.0	6.0	6.8
Clarity	11.1	12.5	10.5
pH	5.3	5.3	5.3
Moisture (%)	12.16	12.74	13.66
Ash (%)	6.44	2.85	2.01
SO_2 (ppm)	80	56	240
Viscosity (ms @ 60°C)	25.0	28.4	39.4
Corrected* Bloom	342	290	240
Corrected* Viscosity	27	29	41

Overall Colour. Not Available.

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 3Y8. (8 weeks liming)

Extraction.

Run No	1	2	3
Time (hrs)	5	5	7
Temperature (°C)	45	50	Boil
Liquor Volume (l)	10.0	6.48	3.24
Liquor pH	2.86	3.29	
Liquor Concentration (%w/v)	3.69	5.16	9.12
Fat (ml)	Nil	Nil	
Scutch (g)			Nil
Gelatine (g)	369.0	334.4	295.5
Gelatine % Proportion	36.9	33.5	29.6
Heavy Liquor Volume (ml)	1050		
Heavy Liquor Conc: (%)	10.5		
5% H_2O_2 (ml)	10	8.5	
5% NH_3 (ml)	7	7.5	

Total Gelatine Recovered (g)	998.9
Total Gelatine Yield (%)	20.0
Anhydrous Gelatine Recovered (g) (f 0.875)	874.0
Anhydrous Gelatine Corrected (g) (f 0.95)	830.3
Anhydrous Gelatine Yield on Raw Material (%)	48.7
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	1167.3
Total Anhydrous solids Recovered (%)	68.4

Gelatine Quality.

Run No	1	2	3
Bloom	288	236	
Colour	6.4	6.8	
Clarity	11.8	12.5	
pH	5.4	5.3	
Moisture (%)	12.74	13.14	
Ash (%)	3.19	2.72	
SO_2 (ppm)	80	24	
Viscosity (ms @ 60°C)	35.9	31.6	
Corrected* Bloom	312	255	
Corrected* Viscosity	37	33	
Overall Colour.		Not Available.	

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 6Y2. (2 weeks liming)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	7.62	7.92	7.08	3.51
Liquor pH	2.72	2.99	3.42	
Liquor Concentration (%w/v)	1.11	2.26	4.85	19.31
Fat (ml)	100	35	25	
Scutch (g)				315
Gelatine (g)	84.6	179	343.4	677.8
Gelatine % Proportion	6.6	13.9	26.7	52.8
Heavy Liquor Volume (ml)	625	1100	1400	
Heavy Liquor Conc: (%)	7	8	13	
5% H_2O_2 (ml)	11	14	10	
5% NH_3 (ml)	10	7	7	

Total Gelatine Recovered (g)	1284.8
Total Gelatine Yield (%)	21.4
Anhydrous Gelatine Recovered (g) (f 0.875)	1121.5
Anhydrous Gelatine Corrected (g) (f 0.95)	1065.5
Anhydrous Gelatine Yield on Raw Material (%)	44.28
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	1451.5
Total Anhydrous solids Recovered (%)	60.3

Gelatine Quality.

Run No	1	2	3	4
Bloom	98	303	289	
Colour	5.2(F)	6.8	7.6/8.0	7.6
Clarity	10.0	11.1	10.5	10.0
pH	6.1	5.3	5.3	4.5
Moisture (%)	11.3	10.94	9.79	
Ash (%)	5.37	3.08	2.22	
SO_2 (ppm)				
Viscosity (ms @ 60°C)		27.0	31.9	
Corrected* Bloom	108	314	286	
Corrected* Viscosity		28	32	
Overall Colour.		7.4		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
(F) Filtered using GFA paper before colour/clarity determination.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 6Y4A. (4 weeks liming, 2 g/l Na_2S)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	9.00	7.16	7.41	5.47
Liquor pH	2.73	3.4	3.62	
Liquor Concentration (%w/v)	2.09	3.57	5.70	6.63
Fat (ml)	50	15	35	
Scutch (g)				40
Gelatine (g)	188.1	255.6	422.4	362.7
Gelatine % Proportion	15.3	20.8	34.4	29.5
Heavy Liquor Volume (ml)	900	1100	1200	
Heavy Liquor Conc: (%)	6	9	10	
5% H_2O_2 (ml)	5	8	9	
5% NH_3 (ml)	2.5	5	7	

Total Gelatine Recovered (g)	1228.8
Total Gelatine Yield (%)	20.5
Anhydrous Gelatine Recovered (g) (f 0.875)	1075.2
Anhydrous Gelatine Corrected (g) (f 0.95)	1021.4
Anhydrous Gelatine Yield on Raw Material (%)	42.4
Total Anhydrous Solids Recovered (g)	1453.4
Total Anhydrous solids Recovered (%)	60.4

Gelatine Quality.

Run No	1	2	3	4
Bloom	312	284	249	
Colour	8.4	7.6	8.9	6.8
Clarity	10.5	13.3	12.5	14.3
pH	5.4	5.5	5.5	4.4
Moisture (%)	9.8	11.42	11.26	
Ash (%)	2.82	2.45	2.90	
SO_2 (ppm)	120	8	240	
Viscosity (ms @ 60°C)	38.4	29.4	33.7	
Corrected* Bloom	313	293	259	
Corrected* Viscosity	39	30	34	
Overall Colour.		7.9		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 6Y4B. (4 weeks liming, 4 g/l Na_2S)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	8.91	7.50	7.02	4.83
Liquor pH	2.75	3.17	3.53	
Liquor Concentration (%w/v)	2.48	3.63	6.13	7.80
Fat (ml)	25	10	25	
Scutch (g)				30
Gelatine (g)	221	272.3	430.3	376.7
Gelatine % Proportion	17.0	20.9	33.1	29.0
Heavy Liquor Volume (ml)	1100	1600	1100	
Heavy Liquor Conc: (%)	8	7	13	
5% H_2O_2 (ml)	13	17	7	
5% NH_3 (ml)	5	7.5	4	

Total Gelatine Recovered (g)	1300.3
Total Gelatine Yield (%)	21.7
Anhydrous Gelatine Recovered (g) (f 0.875)	1137.8
Anhydrous Gelatine Corrected (g) (f 0.95)	1080.9
Anhydrous Gelatine Yield on Raw Material (%)	44.9
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	1550.9
Total Anhydrous solids Recovered (%)	64.5

Gelatine Quality.

Run No	1	2	3	4
Bloom	289	288	247	
Colour	6.4	8.0	9.0/10.0	4.8
Clarity	11.1	11.8	12.5	15.4
pH	5.3	5.2	5.2	4.4
Moisture (%)	13.18	12.36	11.44	
Ash (%)	3.21	2.96	2.52	
SO_2 (ppm)	216	56	184	
Viscosity (ms @ 60°C)	35.3	33.1	34.0	
Corrected* Bloom	317	308	255	
Corrected* Viscosity	37	34	35	

Overall Colour. 7.3

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 6Y6A. (6 weeks liming)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	9.19	9.03	6.45	4.05
Liquor pH	2.77	3.25	3.6	
Liquor Concentration (%w/v)	2.88	3.37	6.74	5.73
Fat (ml)	40	4	25	
Scutch (g)				Nil
Gelatine (g)	264.7	304.3	434.7	232.1
Gelatine % Proportion	21.4	24.6	35.2	18.8
Heavy Liquor Volume (ml)	1300	1150	1400	
Heavy Liquor Conc: (%)	8	9	11	
5% H_2O_2 (ml)	15	5	11	
5% NH_3 (ml)	7	5	7	

Total Gelatine Recovered (g)	1235.8
Total Gelatine Yield (%)	20.6
Anhydrous Gelatine Recovered (g) (f 0.875)	1081.3
Anhydrous Gelatine Corrected (g) (f 0.95)	1027.3
Anhydrous Gelatine Yield on Raw Material (%)	42.7
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	1470.5
Total Anhydrous solids Recovered (%)	61.1

Gelatine Quality.

Run No	1	2	3	4
Bloom	300	282	235	
Colour	8.0	7.2	8.4/9.0	6.4
Clarity	10.5	10.0	11.1	12.5
pH	5.2	5.3	5.1	4.4
Moisture (%)	11.33	11.77	8.43	
Ash (%)	3.26	1.83	2.59	
SO_2 (ppm)	72	256	296	
Viscosity (ms @ 60°C)	34.8	40.7	29.4	
Corrected* Bloom	315	289	227	
Corrected* Viscosity	36	41	29	

Overall Colour. 7.7

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 6Y6B. (6 weeks liming, no Na_2S)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	2.66	3.00	3.39	3.13
Liquor pH	2.70	3.13	3.50	
Liquor Concentration (%w/v)	1.80	2.49	4.66	7.39
Fat (ml)	45	6	6	
Scutch (g)				Nil
Gelatine (g)	47.9	74.7	158.0	231.3
Gelatine % Proportion	9.4	14.6	30.8	45.2
Heavy Liquor Volume (ml)	600	600	1000	
Heavy Liquor Conc: (%)	4	9	9	
5% H_2O_2 (ml)	9	6	3	
5% NH_3 (ml)	4	3	5	

Total Gelatine Recovered (g)	511.9
Total Gelatine Yield (%)	21.3
Anhydrous Gelatine Recovered (g) (f 0.875)	447.9
Anhydrous Gelatine Corrected (g) (f 0.95)	425.5
Anhydrous Gelatine Yield on Raw Material (%)	44.2
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	562.5
Total Anhydrous solids Recovered (%)	58.4

Gelatine Quality.

Run No	1	2	3	4
Bloom	No Material	279	248	
Colour		6.4	10.0	NM
Clarity		10.5	7.0	NM
pH		5.1	5.3	4.2
Moisture (%)	10.54	10.96	8.12	
Ash (%)	5.27	2.94	2.64	
SO_2 (ppm)	128	96	504	
Viscosity (ms @ 60°C)		28.5	37.1	
Corrected* Bloom		288	238	
Corrected* Viscosity		29	36	

Overall Colour. Not Available.

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C9. Continued...

Extraction & Quality Data. Experiment 6Y8. (8 weeks liming)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	9.26Est*	10.75	6.96	2.29
Liquor pH	2.75	3.24	3.55	
Liquor Concentration (%w/v)	2.68	3.16	6.40	10.10
Fat (ml)	3	Nil	9	
Scutch (g)				Nil
Gelatine (g)	248.2	(339.7)	445.4	231.3
Gelatine % Proportion	19.6	(26.9)	35.2	18.3
Heavy Liquor Volume (ml)	1000	1000	950	
Heavy Liquor Conc: (%)	10	8	11	
5% H_2O_2 (ml)	11	14	5	
5% NH_3 (ml)	5	7.5	5	

Total Gelatine Recovered (g)	Est *1264.8
Total Gelatine Yield (%)	21.1
Anhydrous Gelatine Recovered (g) (f 0.875)	
Anhydrous Gelatine Corrected (g) (f 0.95)	
Anhydrous Gelatine Yield on Raw Material (%)	(1051.4)
Total Anhydrous Solids Recovered (g) (Fat + Cond:)	(1477.4)
Total Anhydrous solids Recovered (%)	(61.4)

* Light Liquor spilled. Estimate based on the average Yield of the other five samples.

Gelatine Quality.

Run No	1	2	3	4
Bloom	293	238	221	
Colour	6.4	8.9	9.4	
Clarity	12.5	11.8	11.1	
pH	5.2	5.2	5.2	
Moisture (%)	8.8	10.82	10.0	
Ash (%)	3.44	4.70	2.36	
SO_2 (ppm)	408	-	120	
Viscosity (ms @ 60°C)	29.6	24.8	31.9	
Corrected* Bloom	291	255	220	
Corrected* Viscosity	30	26	32	

Overall Colour. Not Available.

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C10. Five year-old Chianina hide ex ADSRI.

EXPERIMENT 5YC.

Raw Material and Conditioning.

Raw Material:	Chianina Hide No 11.	
Moisture Content		46.25 ± 3.1%
Ash Content		14.84 ± 1.03%
Sample Mass (kg)		5.6
Anhyd: Hide Subs:		2179 ± 116 g
Washed Weight (kg).		7.15
Conditioning.		
Conditioning Liquor:	CaO 640 g	
	Na_2S 60% 75 g	
	Water to 20 kg.	

Sample No	5Y2	5Y4	5Y6	
Conditioning Time (Weeks)	2	4	6	
Cond: Temperature (°C)	21.5	22.2	22.3	
Init: Sulphide (g Na_2S/l)	2.01	1.97	2.00	
Evaporative Loss (kg)	0.2			
Ex-Lime Wash for 16hrs				
Limed Mass (kg)	10.1	10.3	9.6	
Swelling (%)	141	144	134	
Sample			5Y6A	5Y6B
Weight (kg)			4.8	4.7
Acidulation. 5 coats of H_2SO_3 soln. over 4 days. Wash 1hr. Soak in fresh water ± 22hr.				
Wt: for Extraction (kg)	10.35	10.9	5.4	5.3
Soak Water pH		2.55	2.76	2.52

ADDENDUM C10. Continued...

Extraction & Quality Data. Experiment 5Y2. (2 weeks liming)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	10.14	10.27	9.30	7.46
Liquor pH	2.74	3.32	3.58	
Liquor Concentration (%w/v)	1.27	2.42	3.82	11.99
Fat (ml)	21	9	3	
Scutch (g)				460
Gelatine (g)	143.0	230.0	423.2	1118.3
Gelatine % Proportion	7.5	12.0	22.1	58.4
Heavy Liquor Volume (ml)	750	750	1000	
Heavy Liquor Conc: (%)	7	10.5	12.2	
5% H_2O_2 (ml)	18	15	2	
5% NH_3 (ml)	10	7.5	6	
Isoionic point	5.2			

Total Gelatine Recovered (g)	1914.5
Total Gelatine Yield (%)	34.2
Anhydrous Gelatine Recovered (g) (f 0.875)	1675.2
Anhydrous Gelatine Corrected (g) (f 0.95)	1591.4
Anhydrous Gelatine Yield on Raw Material (%)	73.0

Gelatine Quality.

Run No	1DI$^{\Phi}$	1	2	3	4
Bloom	377	280	280	291	
Colour	4.8	6.4	5.6	8.0	6.0
Clarity (NTU)	36	15	22	23	11
Clarity (DGI)	9.0	12.0	11.0	11.0	12.5
pH	5.7	5.4	5.2	5.0	4.1
Moisture (%)	8.3	8.93	10.16	8.57	
Ash (%)	0.19	6.24	4.17	1.58	
SO_2 (ppm)	17	528	344	232	
Viscosity (ms @ 60°C)	38.4	32.6	25.9	33.7	
Corrected* Bloom	345	297	292	276	
Corrected* Viscosity	37	34	26	33	

Overall Colour. 6.4

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.
Φ Mixed bed ion exchanged.

ADDENDUM C10. Continued...

Extraction & Quality Data. Experiment 5Y4. (4 weeks liming)

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	13.02	9.14	6.05	5.57
Liquor pH	2.99	3.37	3.61	
Liquor Concentration (%w/v)	2.90	4.31	8.91	10.42
Fat (ml)	6	5	28	
Scutch (g)				20
Gelatine (g)	377.6	393.9	539.1	580.4
Gelatine % Proportion	20.0	20.8	28.5	30.7
Heavy Liquor Volume (ml)	800	1000		
Heavy Liquor Conc: (%)	11.5	10		
5% H_2O_2 (ml)	5	7.5		
5% NH_3 (ml)	5	12		

Total Gelatine Recovered (g)	1891.0
Total Gelatine Yield (%)	33.8
Anhydrous Gelatine Recovered (g) (f 0.875)	1654.6
Anhydrous Gelatine Corrected (g) (f 0.95)	1571.9
Anhydrous Gelatine Yield on Raw Material (%)	72.1

Gelatine Quality.

Run No	1	2	3	4
Bloom	311	269	226	
Colour	5.6	6.8	8.4	7.2
Clarity (NTU)	19	21	20	9
Clarity (DGI)	11.0	11.0	11.0	13.0
pH	5.9	5.8	5.6	4.12
Moisture (%)	10.0	9.9	11.5	
Ash (%)	2.32	3.94	2.41	
SO_2 (ppm)	8	48	80	
Viscosity (ms @ 60°C)	35.7	34.5	33.2	
Corrected* Bloom	310	277	233	
Corrected* Viscosity	35	35	34	

Overall Colour. 7.2

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C10. Continued...

Extraction & Quality Data. Experiment 5Y6A. (6 weeks liming)

NB Experiment 5Y6A - Acidulation over 4 days.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	6.53	2.76	2.94	1.78
Liquor pH	2.91	3.30	3.62	
Liquor Concentration (%w/v)	2.94	6.75	9.27	13.96
Fat (ml)				
Scutch (g)				Nil
Gelatine (g)	192.0	186.3	272.5	248.5
Gelatine % Proportion	21.4	20.7	30.3	27.6
Heavy Liquor Volume (ml)	900	800	1200	
Heavy Liquor Conc: (%)	10.5	13	12.5	
5% H_2O_2 (ml)	6	6	4	
5% NH_3 (ml)	5	10	15	

Total Gelatine Recovered (g) 899.3

Gelatine Quality.

Run No	1	2	3	4
Bloom	317	288	225	
Colour	6.4	8.4	9.4	11.4
Clarity (NTU)	12	23	32	43
Clarity (DGI)	12.5	10.5	9.5	8.0
pH	5.8	5.3	5.3	4.03
Moisture (%)	10.1	10.8	11.0	
Ash (%)	2.57	1.51	1.36	
SO_2 (ppm)	32	0	48	
Viscosity (ms @ 60°C)	41.5	37.5	24.6	
Corrected* Bloom	318	287	224	
Corrected* Viscosity	42	37	25	

Overall Colour. 9.1 (Boil clarity was poor)

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C10. Continued...

Extraction & Quality Data. Experiment 5Y6B. (6 weeks liming)

NB Experiment 5Y6B - Acidulation over 3 days.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Liquor Volume (l)	6.04	2.71	2.94	1.60
Liquor pH	2.96	3.26	?	
Liquor Concentration (%w/v)	3.0	6.89	9.06	14.26
Fat (ml)				
Scutch (g)				Nil
Gelatine (g)	181.2	186.7	267.2	228.2
Gelatine % Proportion	21.0	21.6	31.0	26.4
Heavy Liquor Volume (ml)	1200	700	1100	
Heavy Liquor Conc: (%)	10.4	14	13	
5% H_2O_2 (ml)	9	4	5	
5% NH_3 (ml)	5	10	15	
Total Gelatine Recovered (g)			863.9	

Gelatine Quality.

Run No	1	2	3	4
Bloom	321	279	226	
Colour	6.8	7.2	8.9	9.4
Clarity (NTU)	20	29	29	7
Clarity (DGI)	11.0	10.0	10.0	13.0
pH	5.3	5.3	5.3	4.20
Moisture (%) .	9.43	11.5	11.8	
Ash (%)	2.61	1.35	1.47	
SO_2 (ppm)	48	64	64	
Viscosity (ms @ 60°C)	32.1	37.4	30.4	
Corrected* Bloom	310	277	233	
Corrected* Viscosity	35	35	34	
Overall Colour.		8.2		

* Corrected to 12.5% non gelatine (moisture + ash) using √ for gel strengths.

ADDENDUM C10. Continued...

Extraction Data. Experiment 5Y6. (6 weeks liming)

NB Combined data for Experiment 5Y6.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	45	50	60	Boil
Scutch (g)				Nil
Gelatine (g)	373.2	373.0	539.7	476.7
Gelatine % Proportion	21.2	21.2	30.6	27.0

Total Gelatine Recovered (g)	1762.6
Total Gelatine Yield (%)	31.5
Anhydrous Gelatine Recovered (g) (f 0.875)	1542.3
Anhydrous Gelatine Corrected (g) (f 0.95)	1465.1
Anhydrous Gelatine Yield on Raw Material (%)	67.2

ADDENDUM C11. 12 year-old Inguni's hide ex ADSRI.

EXPERIMENT INO.

Raw Material.

Hide from Inguni Cow, 143 months of age.
Cut into 100 x 100 mm pieces.

Moisture Content	41.4 ± 0.2%
Ash Content	16.5 ± 0.13%
Hide Substance	42.1 ± 0.1%
Each Lot Weight	3700 g; Hide Substance 1558 ± 3 g.

Conditioning.

Lot	E
Na_2S (g)	75
CaO (g)	640
Water to (kg)	20
Time (days).	28
Ave. Temp. (°C)	22.2
Tumbler Wash (hours).	16
Cond. Weight (kg)	6.65
Swelling (%)	180

Acidulation.

Lot	**INOE**
5 Coats of H_2SO_3 soln. over 4 days.	
Wash 1 hr.	
Soak in 40 (l) for ± 20 hr.	
Wt. for Extn.	6.75 kg
Soak Water pH	2.48

ADDENDUM C11. Continued...

Lot INOE. Extraction and Quality.

Extraction.

Run No	1	2	3	4
Time (hrs)	5	5	5	7
Temperature (°C)	50	60	70	Boil
Liquor Volume (l)	8.90	6.00	4.98	3.18
Liquor pH	3.00	3.45	3.57	
Liquor Concentration (%w/v)	1.40	4.18	8.14	9.80
Fat (ml)	8	2	-	
Scutch (g)				95
Gelatine (g)	124.2	250.5	405.1	311.5
Gelatine % Proportion	11.4	23.0	37.1	28.5
Heavy Liquor Volume (ml)	1200	1200	1600	
Heavy Liquor Conc: (%)	6.8	13.5	15	
5% H_2O_2 (ml)	2	1	1	
Initial pH	<4	4.1	3.9	
5% NH_3 (ml)	6.5	20	34	
Final pH	5.4	5.2	5.2	

Total Gelatine Recovered (g)	1091.3
Total Gelatine Yield (%)	29.5
Anhydrous Gelatine Recovered (g) (f 0.875)	954.9
Anhydrous Gelatine Corrected (g) (f 0.95)	907.1
Anhydrous Gelatine Yield on Raw Material (%)	58.2

Gelatine Quality.

Run No	1	2	3	4
Bloom	302	233	154	
Colour	9.4	12.3	16.0	17.8
Clarity (NTU)	13	21	25	20
Clarity (DGI)	12	11	10.5	11
pH	5.5	5.3	5.1	4.1
Moisture (%)	10.38	10.89	10.72	
Ash (%)	1.69	0.62	0.38	
SO_2 (ppm)	96	48	64	
Viscosity (ms @ 60°C)	29.9	26.9	24.3	
Corrected* Bloom	299	228	149	
Corrected* Viscosity	29.8	26.6	23.9	
Overall Colour.		14.9		

* Corrected to 12.5% non gelatine (moisture + ash) using ✓ for gel strengths.

ADDENDUM C12. The effect of animal age and processing on gelatine colour.

EXP	AGE MONTHS	CONDIT. TIME WKS.	COLOURS 1ST	2ND	3RD	BOIL	OVERALL
YSA	10	2	3.6	4.4	4.8	4.8	4.3
YSB	10	2	<3.2	5.2	4.8	4.4	4.2
YSC	10	3	3.2	4.0		4.4	4.0
YSD	10	3	3.2	3.2		4.8	3.3
YSE	10	4	<3.2	4.0	5.2	?	?
		AVE	3.3			AVE	4.0
CT	18	1	5.6	5.6	5.2	6.8	6.5
		2	5.2	5.2	6.0	6.4	6.0
		3	5.2	5.6	6.4	7.2	6.2
		4	5.6	5.6	5.6	?	?
		5	6.0	6.4	6.4	8.0	6.4
		6	5.2	5.6	6.0	?	?
		AVE	5.5			AVE	6.3
3Y	40	2	6.4	6.0	6.8	4.8	5.5
		4	6.6	6.0		3.6	5.2
		4	6.4	6.4		5.6	6.1
		6A	6.0	6.8		LOST	?
		6B	6.0	6.0	6.8	?	?
		8	6.4	6.8		LOST	?
		AVE	6.3			AVE	5.8
5Y	58	2	6.4	5.6	8.0	6.0	6.4
		4	5.6	6.8	8.4	7.2	7.2
		6	6.4	8.4	9.4	11.4	9.1
		AVE	6.1			AVE	7.6
6Y	78	2	5.2	6.8	7.8	7.6	7.4
		4A	8.4	7.6	8.9	6.8	7.9
		4B	6.4	8.0	9.5	4.8	7.3
		6A	8.0	7.2	8.7	6.4	7.7
		6B	?	6.4	10.0	NM	?
		8	6.4	8.9	9.4	?	?
		AVE	6.9			AVE	7.6
INOE	143	4	9.4	12.3	16.0	17.8	14.9
CTO	152	2	6.4	7.2	10.0	16.0	12.4
		4	8.0	8.9	10.0	20.0	16.5
		7	9.4	10.7	12.3	17.8	15.2
		10	10.0	9.4	10.0	20.0	16.4
		AVE	8.5			AVE	15.1
KTO	144	6	8.0	7.2	9.4	13.3	11.6
ST1	144	2	6.4	10.0	10.7	22.8	18.1
		2	11.4	10.7	11.4	16.0	14.6
		2	10.0	11.4	12.3	16.0	14.7
		4	10.0	11.4	13.3	16.0	14.2
		4	8.9	12.3	13.3	17.8	15.0
		4	9.4	12.3	13.3	16.0	14.3
		6	10.7	12.3	14.5	13.3	13.1
		6	11.4	11.4	16.0	20.0	16.5
		6	11.4	13.3	16.0	17.8	15.8
		AVE	10.0			AVE	15.1

ADDENDUM C12. Continued...

EXP.	AGE MONTHS	CONDIT. TIME WKS.	COLOURS 1ST	2ND	3RD	BOIL	OVERALL
ST2/WT	144	4	12.3	11.4	12.3	17.8	15.7
		4	11.4	10.0	12.3	13.0	12.7
		4	10.7	13.3	16.0	14.5	14.3
		4	11.4	12.3	13.3	17.8	16.3
		4	11.4	13.3	16.0	17.8	16.6
		AVE	11.4			AVE	15.1
CALF-A	3 (ACID PROC)		4.0	3.6	5.2	?	?

ADDENDUM C13. Amino acid analysis of 6 samples of gelatine by Stevens and Stevens - Consultants.

AMINO ACID AND PROTEIN CONTENT BY WEIGHT. mg/g sample.

		SAMPLES														
Mw	AMINO ACID	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	E1	E2	E3	F1	F2	F3
133.1	ASP	42.7	42.1	51.2	46.3	47.6	48.6	37.5	51.1	48.5	45.7	42.9	41.9	41.4	46.6	42.6	46.7
165.2	GLU	84.5	85.6	96.5	87.3	87.3	94.0	87.3	90.2	90.7	84.6	89.2	82.5	80.5	87.2	81.9	86.0
131.1	HOPRO	104.6	108.8	107.2	102.8	105.3	111.8	117.9	113.0	107.1	105.5	109.7	104.9	105.5	105.1	103.4	109.0
105.1	SER	27.3	27.6	30.4	27.8	27.7	29.2	30.6	29.4	30.1	28.3	28.6	27.2	26.8	28.8	27.6	28.2
75.1	GLY	193.6	201.1	212.5	192.8	197.2	206.9	201.5	203.0	205.0	191.3	201.0	187.3	189.2	199.7	187.2	197.3
155.2	HIS	5.6	0.0	0.0	6.5	0.0	0.0	6.1	0.0	0.0	7.1	0.0	7.2	0.0	0.0	6.7	0.0
174.2	ARG	89.4	89.6	78.9	84.3	86.2	75.4	83.4	87.1	73.4	83.5	70.5	85.4	77.8	70.3	77.6	81.5
165.2	METSO1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.7	0.0	0.0	4.9	0.0	0.0
165.2	METSO2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0	0.0	0.0	0.0
119.1	THR	12.6	13.0	17.2	14.6	15.0	16.3	14.9	14.8	15.9	14.1	14.8	13.9	14.1	15.0	14.5	14.5
89.1	ALA	80.9	83.2	80.8	77.9	78.5	76.0	77.5	78.4	74.7	76.3	70.9	72.3	72.2	72.8	76.3	74.8
115.1	PRO	120.9	129.1	124.1	124.5	125.2	115.8	123.7	124.0	116.0	125.4	108.1	115.9	116.7	114.8	127.4	122.9
181.2	TYR	0.0	0.0	4.5	3.5	0.0	4.9	4.5	0.0	5.4	4.8	2.8	2.8	0.0	3.3	2.9	0.0
117.2	VAL	20.0	20.2	21.3	19.8	19.6	18.2	18.8	18.6	18.9	19.0	17.4	18.4	17.9	18.6	19.1	18.2
149.2	MET	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0
121.2	CYS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
131.2	ILE	12.9	13.0	13.6	12.4	12.3	12.8	11.9	12.6	13.1	13.9	12.1	12.7	12.1	13.0	13.5	12.4
131.2	LEU	24.9	25.6	25.4	26.5	26.3	22.2	23.7	25.2	23.1	25.6	21.6	24.5	23.7	22.6	26.0	24.4
162.2	HOLYS1	9.8	10.0	9.5	11.0	10.7	8.8	9.0	11.6	8.8	10.3	9.5	10.8	9.2	8.1	9.8	9.9
162.2	HOLYS2	3.0	2.7	2.1	2.8	2.6	1.8	2.0	2.8	1.8	2.7	1.7	2.6	1.9	1.6	2.5	2.4
165.2	PHE	15.8	16.0	18.1	16.4	16.5	16.1	15.0	16.3	17.2	17.2	14.5	14.7	14.3	16.1	16.6	14.8
146.2	LYS	34.9	35.0	34.6	31.9	31.7	32.1	29.9	34.7	33.2	34.6	29.9	31.5	30.7	32.0	33.9	33.4
132.2	ORN	1.0	1.2	0.0	1.6	1.8		1.4	1.8		1.6		3.9	3.9		3.8	3.8
	% PROTEIN	88.4	90.4	92.9	89.1	89.2	89.1	89.7	91.5	88.3	89.2	85.2	86.0	83.8	86.1	87.6	88.0

ADDENDUM C13. Continued.

Mean values and Relative Standard Deviation %-RSD of amino acids in gelatine.

AMINO ACID	MEAN ALL	%-RSD ALL	MEAN -A	%-RSD -A
ASP	45.2	8.1	45.6	8.3
GLU	87.2	4.7	87.5	4.9
HOPRO	107.6	3.6	107.7	3.7
SER	28.5	4.0	28.6	4.0
GLY	197.9	3.6	198.0	3.7
HIS	2.5	129.8	2.4	134.5
ARG	80.9	7.5	79.7	6.9
METSO1	0.5	267.5	0.6	247.7
METSO2	0.2	387.3	0.2	360.6
THR	14.7	7.5	15.0	5.9
ALA	76.5	4.4	75.7	3.7
PRO	120.9	4.6	120.3	4.5
TYR	2.5	83.0	2.8	69.2
VAL	19.0	5.0	18.8	4.9
MET	0.3	273.9	0.3	253.7
CYS	0.0	ERR	0.0	ERR
ILE	12.8	4.3	12.7	4.6
LEU	24.5	6.0	24.3	6.2
HOLYS1	9.8	9.1	9.8	9.8
HOLYS2	2.3	19.3	2.2	18.8
PHE	16.0	6.5	16.0	7.0
LYS	32.8	5.2	32.4	4.9
ORN	2.2	59.8	2.6	42.1
% PROTEIN	88.4		86.1	

ADDENDUM C14. Transformation of amino acid data from ADDENDUM 13.

MOLAR % OF AMINO ACIDS IN GELATINES.

AMINO ACID	SAMPLES A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	E1	E2	E3	F1	F2	F3
ASP	4.03	3.87	4.57	4.35	4.45	4.52	4.46	4.66	4.55	4.30	4.17	4.08	4.10	4.48	4.07	4.41
GLU	6.42	6.34	6.94	6.61	6.57	7.04	6.46	6.63	6.85	6.41	6.98	6.48	6.42	6.76	6.30	6.55
HOPRO	10.01	10.15	9.70	9.81	9.98	10.55	10.98	10.45	10.19	10.07	10.82	10.37	10.60	10.26	10.02	10.45
SER	3.26	3.21	3.43	3.31	3.28	3.44	3.56	3.39	3.57	3.37	3.52	3.36	3.36	3.51	3.34	3.37
GLY	32.38	32.78	33.60	32.13	32.66	34.11	32.79	32.81	34.08	31.89	34.63	32.35	33.22	34.05	31.69	33.04
HIS	0.45	0.00	0.00	0.52	0.00	0.00	0.48	0.00	0.00	0.57	0.00	0.60	0.00	0.00	0.55	0.00
ARG	6.44	6.29	5.38	6.05	6.15	5.36	5.85	6.07	5.26	6.00	5.23	6.36	5.89	5.17	5.66	5.88
METSO1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.38	0.00	0.00
METSO2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00
THR	1.33	1.34	1.71	1.53	1.57	1.69	1.53	1.51	1.67	1.48	1.61	1.51	1.56	1.61	1.55	1.53
ALA	11.40	11.43	10.77	10.94	10.96	10.56	10.63	10.68	10.47	10.72	10.29	10.52	10.68	10.46	10.88	10.55
PRO	13.18	13.72	12.80	13.53	13.52	12.45	13.13	13.07	12.58	13.63	12.14	13.05	13.36	12.76	14.06	13.42
TYR	0.00	0.00	0.29	0.24	0.00	0.33	0.30	0.00	0.37	0.33	0.20	0.20	0.00	0.23	0.20	0.00
VAL	2.14	2.11	2.16	2.11	2.08	1.92	1.96	1.93	2.01	2.03	1.92	2.04	2.01	2.03	2.07	1.95
MET	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.00
CYS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ILE	1.23	1.21	1.23	1.18	1.17	1.21	1.11	1.17	1.25	1.33	1.19	1.26	1.22	1.27	1.31	1.19
LEU	2.38	2.39	2.30	2.53	2.49	2.09	2.21	2.33	2.20	2.44	2.13	2.42	2.38	2.21	2.52	2.34
HOLYS1	0.76	0.75	0.70	0.85	0.82	0.67	0.68	0.87	0.68	0.79	0.76	0.86	0.75	0.64	0.77	0.77
HOLYS2	0.23	0.20	0.15	0.22	0.20	0.14	0.15	0.21	0.14	0.21	0.14	0.21	0.15	0.13	0.20	0.19
PHE	1.20	1.19	1.30	1.24	1.24	1.21	1.11	1.20	1.30	1.30	1.14	1.15	1.14	1.25	1.28	1.13
LYS	3.00	2.93	2.81	2.73	2.70	2.72	2.50	2.88	2.83	2.96	2.64	2.79	2.77	2.80	2.95	2.87
ORN	0.09	0.11		0.15	0.17		0.13	0.17		0.15		0.38	0.39		0.37	0.36
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	90.1	90.4	90.6	89.8	90.2	90.7	91.3	90.1	90.7	89.6	90.8	89.6	90.6	90.8	89.8	90.4

ADDENDUM C14. Continued...

AMINO ACID	EXPERIMENTAL ERROR %-RSD C-D	MEAN ALL	%-RSD ALL		MEAN -A	%-RSD -A	
ASP	2.6	4.3	5.2	*	4.4	4.3	*
GLU	3.6	6.6	3.5		6.6	3.4	
HOPRO	3.0	10.3	3.3		10.3	3.5	
SER	2.4	3.4	3.0		3.4	2.6	
GLY	2.6	33.0	2.5		33.1	2.7	
HIS	123.3	0.2	130.1		0.2	134.8	
ARG	5.9	5.8	7.1	*	5.7	6.6	
METSO1	ERR	0.0	267.3		0.0	247.5	
METSO2	ERR	0.0	387.3		0.0	360.6	
THR	5.5	1.5	6.7	*	1.6	4.4	*
ALA	0.8	10.7	2.9	*	10.6	1.7	*
PRO	3.3	13.1	3.8		13.1	3.9	
TYR	50.7	0.2	82.4		0.2	68.5	
VAL	2.2	2.0	3.7	*	2.0	3.5	
MET	ERR	0.0	276.3		0.0	256.0	
CYS	ERR	0.0	ERR		0.0	ERR	
ILE	6.1	1.2	4.4	*	1.2	4.7	
LEU	5.3	2.3	5.6		2.3	6.0	
HOLYS1	10.8	0.8	9.1		0.8	9.7	
HOLYS2	19.6	0.2	18.9		0.2	18.5	
PHE	5.9	1.2	5.2		1.2	5.6	
LYS	5.8	2.8	4.5		2.8	4.2	
ORN	9.9	0.2	51.4	*	0.3	44.0	*

ADDENDUM C15. Mean molar % amino acids in gelatine.

AMINO ACID	SAMPLES. A	B	C	D	E	F	EXPERIMENTAL ERROR %RSD C-D	MEAN ALL	% RSD ALL	MEAN -A	%RSD -A
ASP	3.9	4.5	4.5	4.4	4.1	4.3	2.6	4.3	5.2 *	4.4	4.3 *
GLU	6.4	6.7	6.7	6.6	6.6	6.5	3.6	6.6	3.5	6.6	3.4
HOPRO	10.1	9.8	10.7	10.1	10.6	10.2	3.0	10.3	3.3	10.3	3.5
SER	3.2	3.3	3.5	3.5	3.4	3.4	2.4	3.4	3.0	3.4	2.6
GLY	32.6	32.8	33.2	33.0	33.4	32.9	2.6	33.0	2.5	33.1	2.7
HIS	0.2	0.2	0.2	0.3	0.2	0.2	123.3	0.2	130.1	0.2	134.8
ARG	6.4	5.9	5.8	5.6	5.8	5.6	5.9	5.8	7.1 *	5.7	6.6
METSO1	0.0	0.0	0.0	0.0	0.1	0.1	ERR	0.0	267.3	0.0	247.5
METSO2	0.0	0.0	0.0	0.0	0.1	0.0	ERR	0.0	387.3	0.0	360.6
THR	1.3	1.6	1.6	1.6	1.6	1.6	5.5	1.5	6.7 *	1.6	4.4 *
ALA	11.4	10.9	10.6	10.6	10.5	10.6	0.8	10.7	2.9 *	10.6	1.7 *
PRO	13.5	13.3	12.9	13.1	12.9	13.4	3.3	13.1	3.8	13.1	3.9
TYR	0.0	0.2	0.2	0.4	0.1	0.1	50.7	0.2	82.4	0.2	68.5
VAL	2.1	2.1	1.9	2.0	2.0	2.0	2.2	2.0	3.7 *	2.0	3.5
MET	0.0	0.0	0.0	0.0	0.0	0.1	ERR	0.0	276.3	0.0	256.0
CYS	0.0	0.0	0.0	0.0	0.0	0.0	ERR	0.0	ERR	0.0	ERR
ILE	1.2	1.2	1.2	1.3	1.2	1.3	6.1	1.2	4.4 *	1.2	4.7
LEU	2.4	2.4	2.2	2.3	2.3	2.4	5.3	2.3	5.6	2.3	6.0
HOLYS1	0.8	0.8	0.7	0.7	0.8	0.7	10.8	0.8	9.1	0.8	9.7
HOLYS2	0.2	0.2	0.2	0.2	0.2	0.2	19.6	0.2	18.9	0.2	18.5
PHE	1.2	1.3	1.2	1.3	1.1	1.2	5.9	1.2	5.2	1.2	5.6
LYS	3.0	2.7	2.7	2.9	2.7	2.9	5.8	2.8	4.5	2.8	4.2
ORN	0.1	0.2	0.1	0.2	0.4	0.4	9.9	0.2	51.4 *	0.3	44.0 *

CHAPTER THREE. The Instrumental Measurement of Gelatine Colour.

INTRODUCTION.

The colour of gelatine was of commercial importance for two reasons:

1. It was possibly a cultural phenomenon but white or colourlessness had a connotation of purity and it was therefore desirable. Hence, all other things being equal a paler gelatine colour had the advantage in the market place.

2. There was a technical demand for pale or non-coloured gelatines for the production of non-coloured confectionery jellies of high clarity, furthermore, the paler the gelatine the less it modified the colours of dyes used in the manufacture of gelatine-based gels and films.

Currently, there was no generally accepted instrumental or absolute method for the measurement of the colour of gelatine and it was apparent that such a system would be of value to the industry as a whole.

Without detailed knowledge of gelatine there would appear to be little difficulty in the instrumental measurement of gelatine colour (Saunders and Ward, 1953). However, there was considerable difficulty in obtaining spectrophotometer values that were well correlated with the subjective or visual colour of dry gelatine or gelatine in solution.

Because of the commercial importance of gelatine colour, manufacturers had developed "in house" methods for comparing gelatines to colour standards by eye using methods which conformed to Beer's Law or which were completely arbitrary. Hence, the commercial "specification" of gelatine colour by the manufacturer or the consumer was extremely difficult.

In the dry state gelatine was produced in many forms from sheets or pieces of dried film to powders with a particle size of 100 microns or finer. As the physical form of the gelatine played a significant role in the visual

assessment of dry colour and because gelatine was mostly used as a solution in water for food applications, it was most common for gelatine colour to be assessed in aqueous solution. As the gel state could introduce additional problems to colour assessment due to a variable loss of clarity close to the isoelectric pH (pH 5 for Type B gelatine) (Veis, 1964) it was therefore common practice to assess gelatine colour in solution at a temperature above the gel melting temperature i.e. in the vicinity of 40°C.

The visual assessment of gelatine colour could be achieved but the repeatability was affected by day-to-day personal errors and bias. Also it was not practical, on a day-to-day basis, to have several experienced assessors determine the colours of all the production gelatines being tested and as a consequence it was accepted that there would be differences between two assessments of colour. In this study a 10% error was accepted or ± 0.8 colour units between colours of 0 and 12. Above 12 an error of approximately 1.6 units was regarded as acceptable, hence, the agreement between the "instrumental" colour and the visual colour should have been within 1.5 units up to colour 12 and within 3 units above colour 12.

The eye had a unique ability to compensate for small differences in solution turbidity which was caused by the scattering of light due to molecular size and by insoluble colloidal matter in suspension. To the spectrophotometer scattered light was "absorbed" and it was common knowledge that the amount of scatter was a variable depending on the wavelength of the light (Sears and Zemansky, 1964).

In this study an attempt was made to solve the problems associated with the instrumental determination of gelatine colour.

LITERATURE REVIEW.

A literature search showed that there was only one reference made to the instrumental measurement of gelatine colour. Saunders and Ward (1953) determined the absorption spectrum of gelatine between 350 and 900 nm. They also recognized the problem of light scatter due to suspended matter and recommended that gelatine solutions be filtered through a "Seitz" (sterilizing) filter before absorbance determination. They claimed that colour could be expressed as optical densities (OD) at approximately 425, 520 and 700 nm using a colorimeter and filters. No statistical correlations of absorbance

at the different wavelengths and visual colour were performed. The failure of the industry to adopt the above method was probably due to a lack of suitable data to correlate the instrumental determination of gelatine colour with conventional visual methods.

EXPERIMENTAL MATERIALS and METHODS.

Unless otherwise stated Reagent Grade chemicals were used in all the experiments. The analytical data on the gelatines used in this study are detailed in ADDENDUM 6. In order to ascertain that the methods were generally applicable, gelatines from a number of other manufacturers, as shown in ADDENDUM 6, were included in the study.

Solution of gelatine.

Due to the low absorbance of the paler gelatines it was an advantage to use a high concentration of gelatine in order to maximize the sensitivity of the method. Hence, it was decided to use a concentration of 6.67 %. This was the internationally accepted concentration used for the determination of Bloom gel strength (British Standards Institution, 1975).

Gelatine solutions were made by weighing 0.714 ± 0.0005 g aliquots of gelatine into 50 ml glass containers fitted with screw caps or into 30 ml test tubes. Water (10 ml) or an aqueous solvent (see below) was pipetted into the containers. These were swirled using a mechanical vortex mixer to wet the particles of gelatine. The gelatine was allowed to swell for 20 to 30 min. It was then placed in a 40°C to 50°C waterbath. The mixtures were swirled from time to time until completely dissolved (usually 10 min.). The presence of striations at the bottom of a sample indicated that solution was incomplete. When a proteolytic enzyme (Alcalase, Trypsin, Papain) solution was used an additional reaction time of 1 hour at 40°C was allowed for the enzymic hydrolysis of the gelatine samples (see below).

Visual measurement of gelatine colour.

The traditional method of estimating colour was conducted by comparing 100 ml of a 4% solution of an unknown sample to 100 ml of a 4% solution of a standard sample in Nessler tubes at a temperature of approximately 40°C. The standard sample had been arbitrarily assigned the colour value or colour concentration

of 8. The tubes were examined down their length against a white background and solution was poured out of the darker tube until a match was obtained. According to Beer's law:

$$V_1C_1 = V_2C_2$$

Where V = volume and C = concentration.

If 50 ml of unknown sample was equal to 100 ml of the standard sample then the colour of the unknown was 100 x 8 ÷ 50 = 16, or if 70 ml standard sample = 100 ml unknown sample, then the colour of the unknown was 70 x 8 ÷ 100 = 5.6 etc.

Instrumental measurement of gelatine colour.

Preliminary transmittance results were obtained using a Perkin-Elmar 500 UV-VIS double beam spectrophotometer with a 1 cm path length and 1mm beam width. Quartz cells were used. The 6.67% gelatine solutions were scanned manually at 10 nm intervals between 150 and 700 nm.

Appraisal of the problem of gelatine colour measurement.

The basis of the problem of the measurement of gelatine colour appeared to be linked to the following:

1. Gelatine did not have a convenient absorption maximum in the visible region of the spectrum, hence, its visible colour was the cumulative result of the absorption of light over the visible spectrum. To imitate this optical effect it would be necessary to scan the light absorbance over the visible spectrum and integrate the area under the absorption curve. A Beckman DU70 spectrophotometer (Beckman Instruments, Tonetti St, Halfway House, Johannesburg, R.S.A.) with these capabilities was selected.

2. Variable light scattering by solutions of gelatine would be mainly due to variances in gelatine clarity which was largely the result of suspended impurities. In addition scatter due to molecular size was particularly noticeable at the isoelectric point and was known as the "isoelectric effect". This effect was enhanced by high gelatine quality (high Bloom strength and viscosity), low concentration and low temperature (Veis, 1964).

3. High viscosity gelatine solutions were difficult to filter, hence filtration through sterilizing 0.45μm filters to obtain a uniform clarity was often quite impractical. A possible solution could be to hydrolyse the gelatine to a uniform low molecular weight prior to filtration. Filtration to uniform clarity could then be practical. Furthermore, such a procedure could reduce the scatter due to molecular size to a constant. Hence, if the procedure did not affect the colour of the gelatine it could well lead to a practical pretreatment which would permit the instrumental measurement of colour.

It was decided to use Novo Alcalase 0.6L (Novo Enzymes ex Enzymes S.A. (Pty) Ltd, PO Box 651216, Benmore 2010. R.S.A.) as a practical pretreatment to hydrolyse gelatine prior to colour measurement using the Beckman DU70 spectrophotometer. Alcalase was widely used commercially hence it was readily available. It was also a proteolytic enzyme of good stability when kept refrigerated. The cost of approximately R 15/kg was low when compared to proteolytic enzymes like Trypsin at R 1/g. The only objection to Alcalase was its intrinsic colour but this could be compensated for by using the enzyme solution as the blank. The quantity chosen for use was based on personal experience, as were the conditions required for gelatine hydrolysis. Also, it was known that from the point of view of viscosity reduction, the hydrolysis of gelatine was practically complete in less than 10 minutes, however, an hydrolysis time of 1 hour was allowed throughout this study to ensure complete hydrolysis.

Beckman DU70 Spectrophotometer.

The Beckman DU70 was a single beam spectrophotometer with 2 nm slit width, using a 1 cm path length cell. A glass cell was used for readings in the visible region of the spectrum (400 to 700 nm) and a quartz cell was used for scans with readings below 400 nm. The operation of the instrument was computer controlled and permitted spectral scans to be down-loaded to other computers for manipulation or analysis of data. The Beckman Data Leader software (Ver 2.3) provided facilities for base line modification which was used to move the base line to the 700 nm absorbance value. The software also allowed the calculation of the area under the curve between 400 and 700 nm. Also, use was made of the Zoom facility which enabled detailed curve analysis, and of the Trace function for the determination of the absorbance at any required wavelength. In this study, the instrument was set to scan between 400 and 700

nm at a scan speed of 600 nm/min giving two data points per nm. There were no other variable parameters.

Procedures.

The cuvette was washed three times with approximately 1 ml aliquots of the gelatine solution to be tested. It was then fillet with test solution and scanned by the spectrophotometer. The machine automatically recorded the scan. At the end of a series of scans or when the spectrophotometer's memory was full the scans were transferred from the spectrophotometer's memory to a computer, running the Data Leader software, for permanent storage and manipulation. During each scan the 700 nm absorbance value was noted from the instrument. As a precaution the 400 nm absorbance was also noted. If either of these values was missed then it could be obtained using the software Trace Home or Trace End facilities. It was occasionally noted during the course of this study that the Manual Baseline facility had not always contained the correct 700 nm absorbance value for area calculations. It was necessary, therefore, to check that the value displayed was the same as that recorded during the scanning of the solution. If it was not correct the Manual Baseline value for the 700 nm absorbance could be edited appropriately.

To avoid the formation of a gel in the cuvette the absorbance or transmittance of 40°C gelatine solutions in water were measured in duplicate as quickly as possible. Hydrolysed gelatine solutions were allowed to cool to room temperature (20-25°C) prior to taking readings. The optical densities of determinations on duplicate samples were recorded and averaged.

On each day that absorbance data was determined there was one sample which was used as a control to establish that the instrument and the procedure were giving substantially constant results.

The usefulness of the instrumental data for the measurement of gelatine colour was evaluated by entering the instrumental and visual colour data into a Quattro spreadsheet (Borland International Inc, 1800 Green Hills Rd, Scotts Valley CA 95067 USA) and calculating the linear correlation between the instrumental and visual data. Data on gelatines with colours of less than 3.2 and gelatines of very poor clarity were excluded from the correlation calculations. By definition a zero colour gelatine must have zero absorbance at all wavelengths, hence the linear regression constant must be zero. The

Quattro regression calculation allowed this to be specified.

Aqueous Solvents.

Enzyme solution No. 1. (ES1).

Enzyme Solution No.1 (ES1) was prepared by diluting 1.0 ml of Novo Alcalase 0.6L (*loc sit*). with distilled water to 1000 cm^3 in a volumetric flask. Alcalase 0.6L had a specified activity of 0.6 AU/ml. (AU = Anson Unit).

Trypsin solution.

The Trypsin enzyme preparation was made by dissolving 0.1g Crystalline Porcine Trypsin Novo (Novo Enzymes *loc sit*) in distilled water containing 0.2 ml of 0.3N hydrochloric acid according to the manufacturers instructions and diluting to 500 ml in a volumetric flask. Trypsin solutions were always slightly hazy. The PTN 3.0 S, Trypsin had a specified activity of 3.0 AU/g.

In the trials using Trypsin, duplicate samples of gelatine were treated with the two solvents, ES1 and Trypsin solution, and the areas under the absorbance curves were recorded as well as the 400 nm and 700 nm absorbances.

Enzyme solution No. 2 (ES2).

The development of ES2 was necessary because on treatment of pigskin gelatines with ES1 it was found that they could not be filtered easily and on refrigeration the samples gelled indicating inadequate hydrolysis. The reason was attributed to the low pH (4.5) of these gelatines which was on the edge of the activity range of Alcalase. ES2 was prepared with 0.05% ammonia to adjust gelatine solutions with a pH of about 4.5 to a pH of about 6. Ammonia was chosen as the preferred alkali because it was a weak base. Thus it would alter the pH of gelatine gently. Also Alcalase could be exposed to ammonia solutions (pH 11) without damage whereas dilute sodium hydroxide (pH 13) could inactivate Alcalase.

The solution was prepared as follows:

Alcalase 0.6L 0.5 ml,
1:4 Ammonia soln. (5%) 5.0 ml.
Distilled water to 500 ml in a volumetric flask.

Enzyme solution No. 3 (ES3).

Due to the precipitation caused with some gelatines by the use of solvent ES2 it was decided that this was not a viable solution to the problem of inadequate hydrolysis of gelatines with a low pH. ES3 was made up by dissolving 0.62 g Papain 6100P (supplied by Enzymes S.A. Pty Ltd. *loc cit*) in distilled water and diluting to 200 ml in a volumetric flask. Papain was a vegetable protease derived from *circa papaya* with a specified activity of 6100 National Formulary units (NFu/mg). This enzyme solution was also slightly hazy.

Acid hydrolysis.

Hydrochloric acid (20%) for 16 hours at 100°C, was normally used for the hydrolysis of gelatine for amino acid analysis (Heidemann, 1981). As only partial hydrolysis was required for the colour determination, duplicate 0.714 g samples of gelatine were treated with 10 ml x 15% hydrochloric acid for 16 hours at 100°C. Another pair of samples of the same gelatine were treated with ES1 in the usual way. As membrane filters cannot be used with strong acid, the acid hydrolysed gelatine samples were filtered using Whatman GF/A filter papers prior to absorbance determination.

Acid hydrolysis solution (1:1 HCl) was prepared by diluting concentrated hydrochloric acid (30%) with an equal volume of distilled water.

Gelatine Filtration.

In preliminary trials an attempt was made to simplify sample preparation by filtration of gelatine to standard clarity using 10 cm circles of Whatman GF/A filter paper (1.6 μm pore size). Subsequently it was found to be necessary to use 0.45μm membrane filters. The procedure used was to fit Millipore Swinex filter holders (Microsep. PO Box 391647 Bramley 2018.) with Millipore 25 mm

HVLP 02500 low protein binding membrane filters. The 10 ml samples of enzyme treated gelatine solution were poured into the barrel of a 10 ml plastic syringe while closing the outlet with a finger. The syringe plunger was fitted and the air expelled from the syringe. A filter holder was then pressed onto the syringe outlet and the contents of the syringe were filtered into the spectrophotometer cuvette using 3 x 1ml portions to wash out the cuvette and then approximately 4 ml to fill the cuvette for the absorbance scan. The remaining solution in the syringe was filtered into the original glass container which was then placed into a refrigerator (2°C). Failure to gel ensured that the gelatine solution had been hydrolysed sufficiently.

The BYK-Gardner Color-View Reflectance Spectrophotometer.

In a study on gelatine colour measurement it was considered that one of the "tristimulus" (L, a, b system) instruments should be used, especially when the conventional spectrophotometric approach was giving controversial results.

A BYK-Gardner Colour-View was obtained on loan (from Premier Technologies (Pty) Ltd. PO Box 173, Northriding, Randburg). This instrument is described in the attachments in Addendum 7. The main features were:

a. 45° circumferential illumination, with viewing normal to the sample surface. Illumination was achieved by the use of multiple fibre optics.

b. Analysis of the light reflected / transmitted by the sample was on the L, a, b, colour scale. The computer program was used to convert this data to the ASTM and DIN indices of "Yellowness".

c. The L, a, b, colour of a sample could be obtained as an absolute value or as a difference between the sample value and a standard value. For this investigation it was decided to maintain the normal spectrophotometric procedure and use the difference between the solvent (blank) and the sample.

d. The instrument had to be connected to a host computer running under Microsoft Windows (Ver 3.1) (Microsoft SA (Pty) Ltd. Katherine St, Sandown, Rivonia, R.S.A.). The software provided was Gardner-soft QC Manager (Ver 1.3) which both controlled the instrument and recorded the results. Each colour value was the average of three readings taken on each sample.

e. The sample cup was a quartz glass cylindrical vessel with an external diameter of 60 mm and a capacity of 90 ml. The base through which the light entered the sample was a high quality optically plane surface. In order that most of the light entering a clear sample was not lost from the upper surface, the top of the sample cup was covered with the white standard tile provided with the instrument.

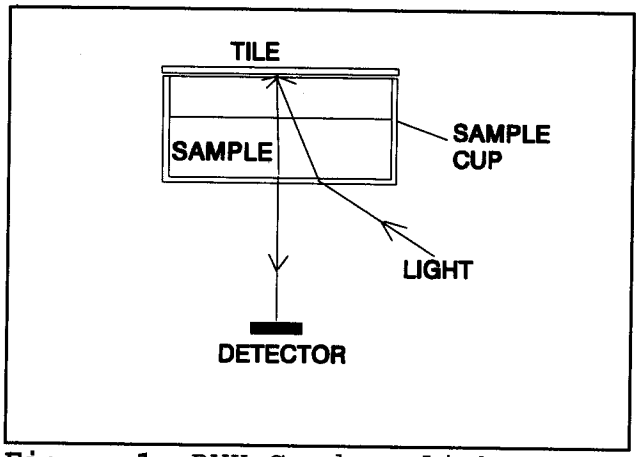


Figure 1. BYK-Gardner light path.

To establish the effect of changing the cell, trials were made using a "Grainer" cylindrical vessel of similar dimensions filled to the same depth as the quartz cell.

The effect of sample depth was established by comparing a 75 ml sample and a 50 ml sample in the Gardner sample cup.

As could be seen from Figure 1, the light entering the sample was scattered by the tile and a large portion was reflected back to the detector. This had two consequences:

1. Moisture condensed on the tile hence the amount of light scattered back to the detector was not constant resulting in reduced repeatability.

2. The amount of light scattered by the tile was considerably more than was scattered by a solution of normal clarity (<80 NTU) hence the Color-View readings appeared to be relatively insensitive to variances in gelatine molecular scatter and clarity.

Experimental procedure.

Each day the instrument was calibrated against the black and white tiles provided. The program was set to record the results of each reading. Using a 50 ml grade B measuring cylinder, 50 ml water was measured into the sample cup. After use the measuring cylinder was inverted so that it could drain between samples. The white tile was placed on top of the cup and then the water was read as the standard. The instrument was then set to read samples.

Each sample consisted of a 6.67% solution of molten gelatine at about 40°C. (This allowed the direct measurement of molten Bloom samples). Amounts of 50 ml were measured into the sample cup which was then placed on the instrument. The white tile was positioned on top of the cup. The sample was then read. The sample was returned to the original container and the cup was inverted so that it could drain on absorbent tissue until required for the next sample. The L, a, b, and "Yellowness" (differences from standard) were noted from the computer screen (CRT). The values were entered into a Quattro spread sheet for statistical analysis. Each day a control sample was included in order to be able to assess the instrument's performance and the repeatability of the method.

RESULTS and DISCUSSION.

Gelatine spectra.

Figure 1, depicts the transmission spectra of three gelatines. It was noted that:

1. A higher %Transmission was recorded for the pale gelatine N33/3 than the dark gelatine N33/1 over the spectral range 150 to 750 nm.
2. Chrome gelatine C542/2 showed a spectral anomaly in the region 300 to 330 nm which had been observed with most of the SPA process (Drew, 1930) gelatines derived from chrome tanned leather. No explanation for the anomaly could be provided.

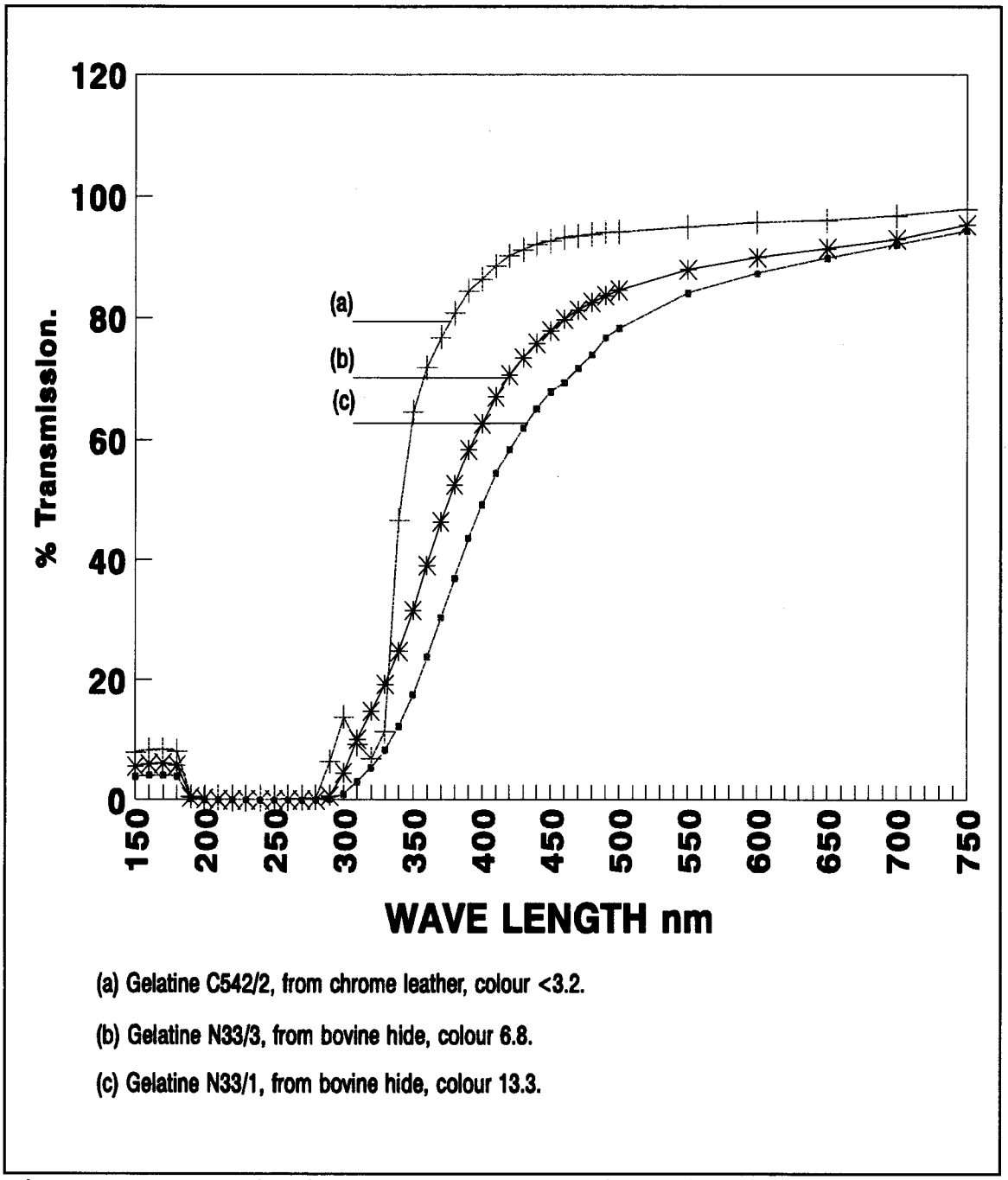


Figure 2 Transmission spectra of bovine hide gelatins.

Introduction to the problem of gelatine colour measurement.

From Figure 2 it was observed that gelatine transmits light relatively well in the visible region of the spectrum (400 to 750nm). Hence, the integrated transmission data should be related to visual colour intensity better than the transmission at a single wavelength. For this reason it was decided to evaluate the integration of the %T values over the visible spectrum. To this end the %T values for a number of gelatines at 400, 450, 500, 550, 600, 650, 700 and 750nm were summed. It was assumed that if gelatine had 100% transmission at all wavelengths (Σ%T = 800) this would equate to colour 0.0. The second order polynomial regression equation coefficients for the correlation of the integrated %T and visual colour were:

B(0) = 211.3185

B(1) = -0.4839819, B(2) = 2.741259E-04

R Square = 0.8256, R = 0.909

Using these coefficients and the values of Σ%T, the colour values for the various gelatines were calculated as shown in Table 1.

Table 1. *The correlation of visual colour and colour value calculated from Σ%T.*

Sample	Visual Colour	Σ%T	Calculated Colour Value
N33/4	14.5	627.6	15.5
N33/1	13.3	643.3	13.4
WT3/3	13.3	667.9	10.4
WT3/1	10.7	684.2	8.5*
N33/3	6.8	685.2	8.4
M8884	6.8	687.8	8.1
N33/2	9.4	702.0	6.7*
ES1	3.2	703.9	6.5*
ES1	3.2	729.0	4.1
	0.0	800.0	-0.4

The data in Table 1 showed that there were too many instances (*) where the disparity between the visual colour and the "calculated" colour was unacceptably large from the commercial point of view for the procedure to be acceptable.

Verification of the problems of colour determination.

The data in Table 2 shows that the correlation of area under the absorbance curve and visual colour was slightly better than the correlation of 400 nm absorbance and colour. Furthermore, the value of the "standard error of the estimate" of colour derived from the instrumental data, was very significant. This was the standard deviation of the difference between the estimated colour from the regression equation and the visually measured value of the colour.

Table 2. *The correlation of visual colour and absorbance data for gelatines dissolved in water.*

Absorbance Parameter X.	700 nm	400 nm	Area under the Curve
Regression Constant.	0	0	0
Std. Error of Estimated Colour.	8.54	4.48	4.37
Correlation Coefficient.	0.0	.555	0.586
Number of Observations.	83	83	83
Degrees of Freedom.	82	82	82
X Coefficient.	113.4	30.58	0.4922
Std. Error of Coefficient.	18.04	1.61	0.0251

For additional details see Addendum 1

The use of enzymic hydrolysis and filtration.

Tables 3 & 4 show the results of a series of preliminary comparisons of the area under absorbance curve for gelatines dissolved in water and in solvent ES1 (after filtration through a GF/A paper).

Table 3. *The area under the absorbance curves between 400 and 700 nm for three gelatines.*

Gelatine.	178(a)		183(b)		542(c)	
Solvent	Absorbance area	Mean	Absorbance area	Mean	Absorbance Area.	Mean.
Water	30.416	31.4	22.120		5.307	
Water	32.167	[0.89]	23.007	22.7	6.430	5.8
Water	31.541		23.095	[0.51]	5.711	[0.57]
ES1	28.026	29.0	22.972	21.9	3.543	4.2
ES1	29.951	[1.36]	20.758	[1.57]	4.774	[0.87]

(a) colour = 17.8; (b) colour = 9.0; (c) colour = 3
[ ] = Standard deviation.

Comments:

(i) The standard deviations of sample 542 were far too high for the area under the curve to be meaningful as an estimate of colour.

(ii) Based on water solvent, the area/colour regression equation was: Colour = -1.75 + 0.62 x Area. (r = 0.939 - not significant; probability = 0.1).

(iii) Based on ES1 solvent, the regression equation was: Colour = - 0.75 + 0.62 x Area. (r = 0.91 - not significant).

Table 3. shows that the regression equation correlation coefficient had not improved with the use of enzyme in the solvent. This was problematic, however, the absorbance curves shown in Figure 3. suggested that the area between the absorbance at 700 nm and the baseline could contribute to unwanted variation. The invisible absorbance at 700 nm and higher wavelengths could not contribute to the visible colour hence it became apparent that the baseline should be at the 700 nm absorbance value and not at 0.00 absorbance.

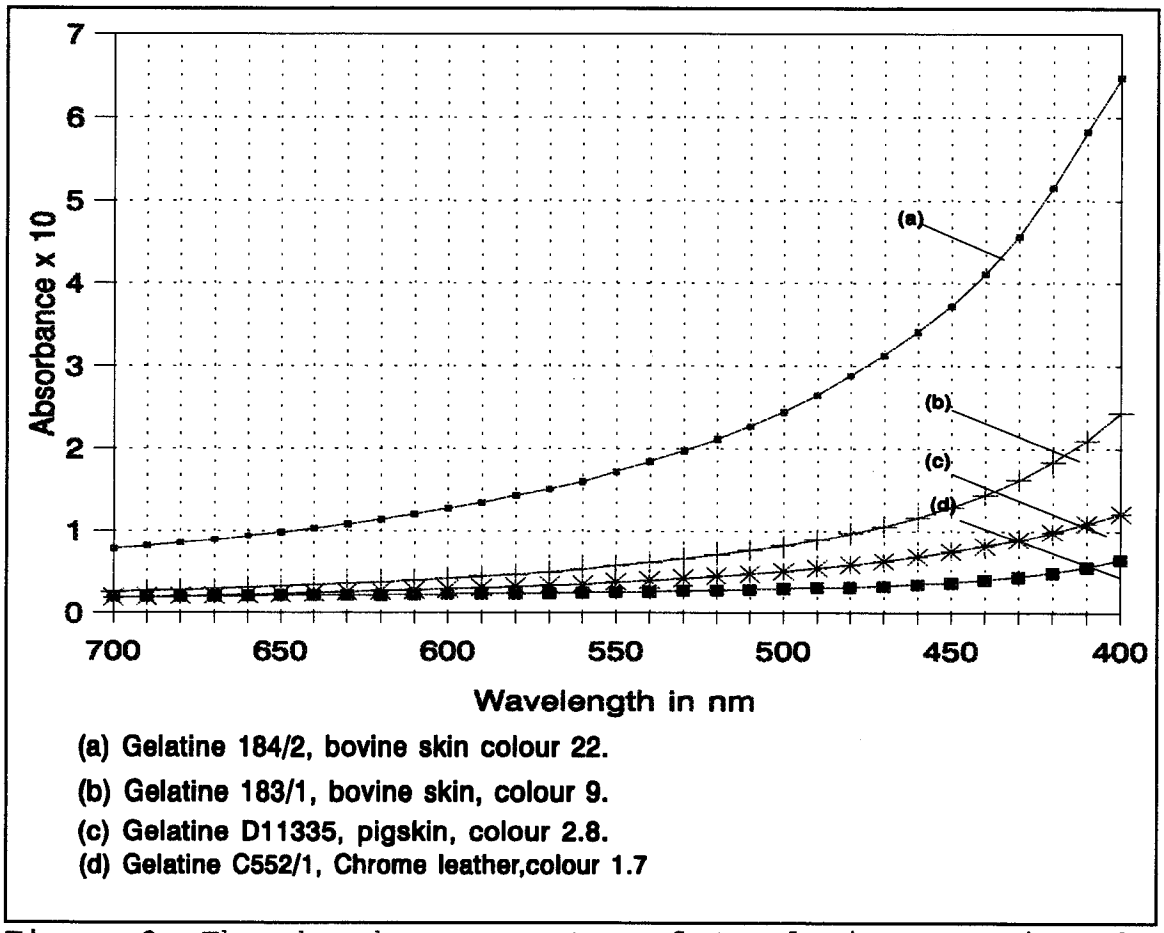


Figure 3. The absorbance spectra of 4 gelatins spanning the normal colour range.

Table 4. *The area under the absorbance curves between 400 and 700 nm for three gelatines, calculated with the baseline at the 700 nm absorbance value.*

Gelatine.	178(a)		183(b)		542(c)	
Solvent	Absorbance area	Mean	Absorbance area	Mean	Absorbance area.	Mean
Water	24.371	24.585	15.820		3.522	
Water	24.982	[0.34]	15.766	15.770	4.279	3.808
Water	24.401		15.626	[0.05]	3.624	[0.41]
ES1	22.347	22.379	(15.43)	13.54	2.942	2.951
ES1	22.411	[0.05]	13.543		2.959	[0.01]

(a) colour = 17.8; (b) colour = 9.0; (c) colour = 3
[ ] = Standard deviation.

The results shown in Table 4 were a distinct improvement on those shown in Table 3 and were attributable to the moving of the baseline to the 700 nm absorbance value:

(i) They show an improvement in repeatabilities. The standard deviations using solvent ES1 became acceptable because a weighing variance of ±0.002 g on 0.714 g would be expected to result in variances 0.3% in absorbance i.e. 0.01 on an area of 3.00 or 0.1 on an area of 30 which was the order of magnitude of the standard deviations of the ES1 samples.

(ii) It was noted that even with the pale colour and good clarity of sample 542, the enzyme-filtration combination treatment had the effect of markedly reducing the sample absorbance indicating reduced light scattering.

(iii) The colour / area regression line for water as solvent was:

Col. = -1.176 + 0.803 x Area. r = 0.968

The colour / area regression line for ES solvent was:

Col. = - 0.541 + 0.865 x Area. r = 0.9756.

These values of r were significant at the 0.1 level of probability (1 degree of freedom).

Based on these preliminary findings all subsequent determinations of the area under the absorbance curve were performed with the base line at the 700 nm absorbance.

Analysis of the use of ES1 solvent & filtration (GF/A)

Having shown that enzyme treatment and filtration of the gelatine solutions together with changing the base line for the absorbance area determination would probably give a useful correlation to visual colour, the next step was to apply the technique to a large number of gelatine samples. Using the data in Addendum 2 but excluding data from gelatines of unknown visual colour value, the linear regression results shown in Table 5. were obtained.

In comparison to the unfiltered data (Table 2) there was a small improvement in the standard error of the estimate of the colour but otherwise the improvement in correlation coefficients was disappointing due largely to the poor clarity achieved by filtration of the G35 and G36 samples using GF/A paper. The decision was then made to try 0.45μm membrane filters.

Table 5. *The correlation of visual colour and absorbance data for gelatines dissolved in solvent ES1 and then filtered using GF/A paper.*

Absorbance Parameter X.	700 nm	400 nm	Area under the Curve
Regression Constant.	0	0	0
Std. Error of Estimated Colour.	6.21	3.49	4.01
Correlation Coefficient.	0.0	0.637	0.466
Number of Observations.	31	31	31
Degrees of Freedom.	30	30	30
X Coefficient.	259	35.68	0.54
Std. Error of Coefficient.	39	2.51	0.04

Solvent = ES1.
Filtration = Whatman GF/A papers. (For detail see Addendum 2.)

A comparison of the results obtained using GF/A filter papers and 0.45μm membrane filters is shown in Table 6. from which it was observed that:
1. Fresh enzyme solution daily gave more reproducible results.
2. The repeatability of absorbance area determinations were better than 1 unit of absorbance using 0.45μm filters.
3. That the area under the absorbance curve increased from 13.6 using GF/A filters to 14.1 using membrane filters. This indicated that if the removal of suspended matter by the membrane was the same or better than with the GF/A filters then it was likely that the GF/A had removed more colour from solution than had the membrane.

Furthermore, by comparing Tables 5 and 7 it was concluded that the use of 0.45μ filters greatly improved the accuracy of the estimate of colour from the absorbance data and statistically the 400 nm absorbance could be expected to give better results than the area under the absorbance curve.

It should be mentioned that initially the results using the 0.45μ filters were hardly better than those using the GF/A papers.

Table 6. *A comparison of the daily repeatability of absorbance data on gelatine control sample 183/1.*

	Solvent = Water			Solvent = ES1			
Day Number	Absorbance			Filter	Absorbance.		
	400 nm	700 nm	Area		400 nm	700 nm	Area
0	-	-	15.77		-	-	14.49
1	- -	0.024 0.029	15.47 17.38	GF/A	0.230 0.232	0.024 0.024	15.03 14.92
2	0.236 0.239	0.021 0.022	15.42 15.55	GF/A *	0.200 0.193	0.021 0.020	12.02 11.56
3	0.247 -	0.024 0.025	15.79 16.19	GF/A	0.225 0.223	0.024 0.023	14.36 14.33
4	0.242	0.025	15.70	GF/A *	0.204 0.200	0.019 0.021	13.03 12.55
	Mean, [Standard Deviation]		15.91 [0.64]		Mean, [Standard Deviation]		13.59 [1.31]
5	0.241	0.023	15.91	0.45μm*	0.212	0.021	14.06
6	0.251 0.251	0.026 0.027	16.43 16.16	0.45μm*	0.222 0.221	0.020 0.021	14.35 14.13
7	0.254	0.025	16.23	0.45μm*	0.224 0.213	0.019 0.019	14.65 13.55
	Mean, [Standard Deviation]		16.18 [0.21]		Mean, [Standard Deviation]		14.15 [0.41]

* Fresh Enzyme Solution.
\- Data not recorded.

It was noted that the poor correlation was mainly due to 6 (dark) gelatines, hence it was decided to ask the Davis Gelatine Laboratory to reassess the visual colours of these gelatines with the proviso that two experienced lab assistants should agree on the results. Additionally it was noted that the G35 and G36 gelatines could not be adequately filtered (700 nm absorbance > 0.04) so they were excluded from the correlation as well.

Table 7. *The correlation of visual colour and absorbance data for gelatine dissolved in ES1 and filtered using 0.45 µ membrane filters.*

Absorbance Parameter X.	700 nm	400 nm	Area under the Curve
Regression Constant.	0	0	0
Std. Error of Estimated Colour.	4.61	1.308	1.685
Correlation Coefficient.	0.535	0.971	0.951
Number of Observations.	31	31	31
Degrees of Freedom.	30	30	30
X Coefficient.	647	44.2	0.6946
Std. Error of Coefficient.	49.28	0.880	0.0178

Solvent = ES1.
Filtration = Millipore 0.45µm membrane filters. (For detail see Addendum 3.)

The results of the reassessment of the visual colours mentioned above are shown in Table 8. These result were in line with the anticipated error of 5 to 10 ml when using 100 ml Nessler Tubes, and the correlation coefficients were improved to 0.95 and 0.97 as a result.

Table 8. *The result of the reassessments of the visual colours of some gelatines.*

Sample Number	Original Colour	Reassessed Colour	Change in Volume to equal Standard. - ml.
LF752/1	6.0	5.2	10
850/6	16.0	18.8	5
183/4	14.5	13.3	5
186/6	16.0	16.0	0
178/12	20.0	17.8	5
184/10	22.8	22.8	0

The inclusion of Type A (pigskin) gelatines.

Due to the apparent success of the enzyme filtration combination treatment for the instrumental determination of gelatine colour, it was decided to assess whether Type A gelatines would present any problems to this colour assessment

technique. To this end a manufacturer was asked to provide pigskin gelatines with a range of colours. The nine samples they provided were assessed using solvent ES1. The result of the inclusion of this data with the data used to generate the correlation shown in Table 7 was to reduce the 400 nm correlation coefficient to 0.95 and the Absorbance area correlation coefficient to 0.93.

Based on the observation that the solutions were very difficult to filter it was decided to modify solvent ES1 by including some ammonia to give solvent ES2. This change, however, required the reassessment of the Type B gelatines under the same circumstances. The results are summarised in Table 9. The complete data is presented in Addendum 4.

Table 9. *The correlation of visual colour and absorbance data for gelatines dissolved in solvent ES2.*

Absorbance Parameter X.	700 nm	400 nm	Area under the Curve
Regression Constant.	0	0	0
Std. Error of Estimated Colour.	5.718	1.808	2.173
Correlation Coefficient.	0.387	0.571	0.9366
Number of Observations.	34	34	34
Degrees of Freedom.	33	33	33

Solvent = ES2.
Filtration = Millipore 0.45μm membrane filters. (For detail see Addendum 4.)

A comparison of the data in Tables 9 and 7 indicated that the use of ES2 solvent system had not improved the correlation coefficients for the instrumental method. The main reason was attributed to the observation that the change in pH sometimes caused precipitation and consequent loss of colour with the Type B gelatines.

Summary of data using solvent system ES1.

Some gelatine absorbances were measured repeatedly in order to assess the reproducibility of the method and to act as a check on the instrument. It was considered that the inclusion of the data on a single gelatine several times could distort the calculated correlation coefficients. It was decided therefore, to average the results of multiple absorbance measurements made using solvent ES1 and to use this to calculate the correlations between

absorbance data and visual colour as shown in Table 10. The X coefficient was then used to calculate Table 11.

Table 10. *The correlation of visual colour and absorbance data for both Type A and Type B gelatines dissolved in solvent ES1 and filtered through 0.45µ filters.*

Absorbance Parameter X.	400 nm	Area under the Curve
Regression Constant.	0	0
Std. Error of Estimated Colour.	1.463	1.769
Correlation Coefficient.	0.959	0.939
Number of Observations.	35	35
Degrees of Freedom.	34	34
X Coefficient.	43.66	0.6851
Std. Error of Coefficient.	1.771	0.0223

Solvent = ES1. (Alcalase solution)
Filtration = Millipore 0.45µm membrane filters. (For detail see Addendum 3.)
Each gelatine was included only once.

Table 11. *The correlation of the visual and estimated colours of gelatines based on their absorbance data when dissolved in solvent ES1 and filtered through 0.45µ membrane filters.*

Gelatine Sample	Visual Colour	400 nm Absorbance Colour Estimate		Absorbance Area Colour Estimate	
178/12	17.8	16.2		15.7	
180/6	16.0	17.3		17.1	
183/1	9.0	9.5		9.7	
184/10	22.8	23.7		25.5	
184/5	10.0	9.8		9.7	
185/2	6.8	7.6		7.1	
CT6/1	5.2	5.4		4.8	
CT6/2	5.6	7.0		6.6	
CT6/3	6.0	6.9		6.2	
D11335	2.8	3.0		3.4	
D1287	2.0	3.4		3.7	
D147	2.0	3.6	*H	4.1	*H
D1512	2.4	3.5		3.9	
D2116	2.4	2.9		3.1	
D2227	2.0	3.1		3.4	
D2249	2.0	3.0	*H	3.3	
D31108	2.8	5.4	*H	6.0	*H
D32117	4.0	6.8	*H	7.6	*H
D3264	3.6	5.9		6.5	*H
N - TY/A	4.8	5.4		4.9	
FISH 824/1	11.4	6.9	*L	6.7	*L
HORSE/1	14.0	12.5		12.5	
L752/1	5.2	5.4		5.2	
LCS	7.6	9.9	*H	8.9	
LF752/1	6.0	5.2		4.2	*L
LF752/1	6.0	5.0		4.1	*L

Table 11 Contd.

Gelatine Sample	Visual Colour	400 nm Absorbance Colour Estimate		Absorbance Area Colour Estimate	
826/1	8.0	7.5		6.9	
OCS	8.0	8.7		8.3	
PIS PP1	11.4	9.0	*L	7.5	*L
SBG	4.8	4.1		3.9	
ST26/1	11.4	10.9		10.6	
ST26/2	11.4	11.7		11.4	
ST26/3	16.0	15.4		15.0	

*H unacceptable high estimate.
*L unacceptable low estimate.

Table 11 showed that:

1. In 21% of cases the colours estimated from 400 nm absorbance were not acceptable (*) and that there were both high and low estimates.

2. In 24% of cases the colours estimated from absorbance area were not acceptable (*) and that there were both high and low estimates.

These differences were in line with the differences in correlation coefficients and the conclusion reached was that in spite of the highly significant correlation coefficients of 0.95 and 0.93 the instrumental method of colour measurement was not commercially viable.

The next approach to the problem was to find a proteolytic enzyme that could hydrolyse acidic gelatines.

The use of solvent ES3 on Gelatines with a pH of 4 to 5.

The enzyme Papain which had good proteolytic activity in the pH range of 4 to 5 was evaluated using gelatine. Although the enzyme was said to have had adequate activity in the range pH 3 to 10 it was found that above pH 5 the hydrolysis of Type B gelatines was inadequate, that is, the gelation of 6.67%

solutions at room temperature was prevented but gelation at 2°C still occurred and the solution viscosity at room temperature was such as to make filtration very difficult. A mixed solution of Alcalase and Papain was made. It appeared to work well on both Type A and Type B gelatines from the point of view of hydrolysis. The mixture, however, was markedly cloudy and particularly with gelatine 178/12 with which it caused a filterable precipitate. The absorbance of the filtrate was low, indicating loss of colour with the precipitate. Hence, Enzyme Solution (ES3) which only contained Papain, was used on the acid process pigskin gelatines. The results are shown in Table 12.

Table 12. *A comparison of the areas under the absorbance curves between 400 and 700 nm for low pH gelatines using solvents ES1 and ES3.*

Solvent	DGI	ES1	ES3	Estimated Colour
Sample Number	Colour	Absorbance Area	Absorbance Area	= ES3 Abs. x 0.7
D2227	2.0	5.01	3.92	2.7
D3264	3.6	9.53	6.90	4.8
D1512	2.4	5.62	3.81	2.7
D32117	4.0	11.13	8.15	5.7*
D2116	2.4	4.57	3.54	2.5
D147	2.0	5.99	4.55	3.2
D31108	2.8	8.82	6.64	4.6*
D1287	2.0	5.39	4.29	3.0
D2249	2.0	4.75	3.78	2.6

* denotes an unacceptable instrumental estimate of colour.

It should be noted that there were differences in clarity between solvents ES1 and ES3 but as all absorbances were measured with the solvent as the blank the absorbance values at 400 nm could also be correlated with visual colour.

On average, the absorbance areas of low pH gelatines determined in solvent ES1 were 1.7 absorbance units higher than in solvent ES3 presumably due to inadequate hydrolysis in solvent ES1. This equates to an average of 1.2 colour units darker than the visual colour which was very significant when the visual colour was about 3.

The absorbance area data obtained by the use of ES3 with the low pH gelatines,

was substituted for the data obtained with ES1 on these gelatines the statistical results shown in Table 13 were obtained.

Table 13. *The correlation of visual colour and absorbance data for gelatines with pH <5 dissolved in solvent ES3 and gelatines with pH >5 dissolved in solvent ES1.*

Absorbance Parameter X.	400 nm	Area under the Curve
Regression Constant.	0	0
Std. Error of Estimated Colour.	1.290	1.509
Correlation Coefficient.	0.968	0.953
Number of Observations.	35	35
Degrees of Freedom.	34	34
X Coefficient.	44.299	0.6999
Std. Error of Coefficient.	1.044	0.01936

Each gelatine was included only once.
Solvent = ES3.(Papain solution).
Filtration = Millipore 0.45μm membrane filters. (For detail see Addendum 5.)

The small improvements in correlation coefficients due to the use of ES3 absorbance values in place of ES1 absorbance values resulted in the "unacceptable" estimates of colour being reduced from 24% to 18%.

The effect of ES1 and ES2 on solution pH.

The pHs of a number of pairs of gelatine samples in water and in solvent ES1 and ES2 are recorded in Table 14. As can be seen the change in pH due to enzymic hydrolysis (ES1) were negligible due to the strong buffering properties of gelatine and the limited ability of enzymes to hydrolyse gelatine.

Table 14. *A comparison of gelatine pHs in water and solvents ES1 and ES2.*

Sample	pH in Water	pH in ES1	pH in ES2
185/2	5.15	5.14	-
178/12	5.30	5.29	-
183/1	4.92	4.91	-
185/2	5.12	-	7.30
824/1	5.65	-	7.42
D1512	4.52	-	6.64
N - TyA	5.78	-	6.18
N - TyA	5.81	-	6.2
D2227	4.50	-	6.8

- Not applicable.

Repeatability of the area under the absorbance curve.

The daily control sample data presented in Table 15 shows the repeatability of colour values. From the data it could be concluded with a probability of 0.95, that determinations of absorbance area should not differ by more than 2 units of absorbance (which equates to 1.4 colour units on the Davis scale). In confirmation it was found that in ADDENDUM 2 there were a number of gelatines with two or more determinations of absorbance area all of which agreed to better than 1 unit of absorbance.

Table 15. *The repeatability of absorbance data from day-to-day.*

Sample	Date	Absorbance Area	Date	Absorbance Area
183/1	17 JAN 19 JAN 28 JAN 02 FEB	14.8, 15.0 14.3, 14.3 14.1 14.6, 13.6	18 JAN 20 JAN 01 FEB	12.0, 11.8 13.0, 12.8 14.4, 14.2
183/1		Mean = 13.75	Standard Deviation = 1.04	Number of Determinations = 13
178/12	25 MAR 12 APR 20 APR 27 APR	22.6 24.3, 24.4 24.6, 24.1 23.0, 23.0	11 APR 14 APR 22 APR	22.8, 22.6 24.2, 24.1 24.4, 24.4
178/12		Mean = 23.73	Standard Deviation = 0.79	Number of Determinations = 13

The use of an alternative enzyme.

It was desired to show whether the method was dependent on the use of Alcalase. Trials were undertaken using Pancreatic Trypsin Novo in place of Alcalase in solvent ES1. Using the computer program Epistat (T.L. Gustafson, 2011 Cap Rock Circle, Richardson, Texas, 75080, USA), the Student's t-test was applied to the Absorbance Areas in Table 16. The value of t(8) for the difference between the two means was 1.061445, with a probability exceeding 0.3. This indicated that there was no significant difference between the means.

In this experiment, due to the observed haze in the Trypsin solution both enzyme solution blanks were filtered (0.45μ) prior to use. However, as a result of residual haze in the trypsin solution there was a statistically significant difference between both the 400 nm and the 700 nm absorbances obtained with ES1 or Trypsin. This demonstrated the value of absorbance area determination.

Table 16. *A comparison of the absorbance data obtained with Alcalase and Trypsin hydrolysis of Type B gelatines.*

	A L C A L A S E Absorbances.			T R Y P S I N Absorbances.		
Sample.	400 nm.	700 nm.	Area 400 - 700 nm.	400 nm.	700 nm.	Area 400 - 700 nm.
C200/1	0.0392	0.001	2.09	0.041	0.001	2.35
WT3/3	0.3262	0.017	20.58	0.3292	0.018	20.65
YSA/1	0.0843	0.007	5.29	0.0805	0.007	5.04
INOE/2	0.2815	0.013	18.70	0.2918	0.025	18.26
877/1	0.1432	0.003	8.14	0.1562	0.014	8.33
155/1	0.1657	0.006	9.90	0.1771	0.018	9.80
5Y4/3	0.2022	0.012	12.61	0.2127	0.023	12.57
178/12	0.3690	0.011	22.65	0.3774	0.028	22.47
CT4/2	0.1524	0.011	9.16	0.1592	0.020	8.98

The Effect of acid hydrolysis of gelatine.

Again it was desirable to know whether a hydrolysis system other than an enzymic system could be used in preparation for the instrumental determination of colour. The results are shown in Table 17.

Table 17. *A comparison of the absorbance data for gelatines hydrolysed with 1:1 hydrochloric acid and Alcalase solution ES1.*

SOLVENT - 15% Hydrochloric Acid.				
	Colour	A B S O R B A N C E.		
Sample		400 nm.	700 nm.	Area 400 - 700 nm.
178/12	17.8	1.57, 1.53	0.10, 0.09	124.5, 120.7
185/2	6.0	1.12, 1.12	0.07, 0.06	89.7, 89.6
SOLVENT - ES1.				
178/12	17.8	0.37, 0.37	0.02, 0.02	23.01, 23.00
185/5	6.0	0.18, 0.18	0.01, 0.01	10.44, 10.46

From Table 17 it was clear that partial acid hydrolysis was accompanied by considerable darkening and for this reason alone it was decided that the

procedure was not worth pursuing. It was noted, however, that the paler gelatine hydrolysate had a markedly lower absorbance than the darker, hence there did seem to be a relationship between gelatine colour and hydrolysate colour.

The BYK-Gardner Color-View Reflectance Spectrophotometer.

The results of the measurements made on a range of gelatines are recorded in Addendum 8.

Preliminary trials were made comparing the results using 50 and 75 ml of sample. The L values of the samples was some 3 units or 6% paler using 75 ml sample size indicating a need to control the sample size used for the determination and the need for the smaller sample size to maximise the effect of colour. Furthermore, the differences caused by a change from the Gardner sample cell to a Grainer glass cell filled to the same depth were such as to indicate the need to use the same cell for all determinations. This aspect would preclude the possibility of the routine determination of colour on gelled solutions.

The sample quantity of 50 ml was chosen because it half filled the Gardner cell which was convenient from the point of view of handling and measurement and it appeared better than 75 ml from the point of view of the optics of the system. The maximum sample size would have been 90 ml which was the capacity of the cell but this would have been impractical from the handling point of view.

Initially trials were made using the white tile as the standard and the following linear correlation coefficients were obtained after excluding the G35, G36 and G37 gelatines with unmeasurable clarities:

L and Colour.	r = 0.98;	a and Colour.	r = 0.56
b and Colour.	r = 0.91;	Y1 and Colour.	r = 0.95
Y2 and Colour.	r = 0.92		

It was realised, however, that it would be best to stay with the conventional procedure of comparing the colour of the solution to a solvent blank thus eliminating any differences which might arise due to differences in standard tiles.

Using water as the standard, the following linear correlation coefficients were obtained after eliminating gelatines with clarities of ≥ 100 NTU:

L and Colour.	r = 0.96;	a and Colour.	r = 0.84
b and Colour.	r = 0.89;	Y1 and Colour.	r = 0.91
Y2 and Colour.	r = 0.88		

From the above it was concluded that for the 86 gelatines tested, the L or darkness component gave the best correlation to visual colour and the correlation coefficient was very slightly better than that obtained in Table 10. The Davis colours of all the gelatines were calculated from the linear coefficients (Colour = -1.345 -1.582 x L) and there were 15% where the instrumental colour deviated unacceptably (marked with a *) from the visual colour. From inspection it was clear that where the instrumental colour was low, in most cases the clarity was also unusually low. Furthermore, the gelatines with clarities > 100 NTU caused the instrumental colour to be high.

The gelatines used in this experiment were as diverse as those used previously and included the pigskin and fish skin gelatines as well as Type A cow hide gelatines, and gelatines from other manufacturers, hence, it appeared that the BYK-Gardner reflectance spectrophotometer gave better results than the Beckman DU70 spectrophotometer without the need to prepare samples specially for colour measurement. In addition, it was concluded that given the inherent error of the subjective measurement of colour, the BYK-Gardner instrument would give colour results as reliable or with a better reliability than visual assessment, as long as the results obtained with poor clarity gelatines (>80 NTU) were accepted as being unreliable, as were visual colours on poor clarity gelatines. Finally, examination of the data in ADDENDUM 8 showed that the maximum error between determinations of colour should not exceed 0.7 Davis colour units. This error could largely be attributed to differences in reflection from the white tile (with water in the cell, the difference in colour between a clean tile and one with condensed moisture on it, was 0.4 Davis colour units).

CONCLUSIONS.

1. Table 8 shows the uncertainty associated with the subjective visual determination of the colour of gelatine in solution. To this must be added the fact that such a system of colour determination was comparative and hence it was very difficult or impractical to use it for "specification" purposes. Hence, it would be an advantage to be able to measure gelatine solution colour instrumentally.

2. Table 2 showed that without the elimination of interferences there was a poor correlation between optical density (OD) and visual colour. Table 3 showed that even with filtration there was little improvement in the correlation coefficient. Table 4 showed that if in addition the base line was moved, then a significant correlation between:

a) the area under the absorbance curve between 400 and 700 nm and visual colour, and

b) the 400 nm absorbance and visual colour.

could be obtained.

3. The use of enzymic hydrolysis and filtration of the gelatine solution through a 0.45μ membrane filter refined the correlation between 400 nm absorbance or 'area under the curve and colour to the level where a commercially useful method was available for Type B gelatine with a pH of greater than 5.

4. The inclusion of acid pigskin gelatines upset the correlation between absorbance measurements and visual colour. It was realised that the low pH of these gelatines made the hydrolysis by Alcalase ineffective. It was found that the enzyme papain could be used on gelatines with a pH between 4 and 5 and this modification returned the correlation coefficients between absorbance area and visual colour to 0.95. The correlation coefficient between 400 nm OD and colour was increased to almost 0.97 (Table 13). In other words, absorbance data and visual colour agreed reasonably in 88% of the determinations even though every effort had been made to upset the correlation by including a variety of gelatines from all available sources.

The method was based on the principle of reducing the light scatter of

gelatine solutions to a constant. This was not possible with the bad clarity G35, G36 and G37 gelatines and these had to be eliminated from the correlation. Hence, it must be stated that the method could only be applied to gelatines with a natural clarity of better than 80 NTU measured on 6.67% solutions at about 40°C. This translates to a filtered OD at 700 nm not exceeding 0.030.

5. The BYK-Gardner Color-View reflectance spectrophotometer gave a better correlation of the L component of colour to the visual colour than was obtained with the Beckman DU70 spectrophotometer. Given the inherent error of the visual measurement of colour and that solution colour could be reliably measured on remelted 6.67% Bloom sample without any further manipulation. This instrument could be recommended for the measurement of gelatine colour with the probability that the difference between two determinations would not exceed 0.7 Davis colour units which was half the error currently accepted using the visual determination of colour.

6. A number of minor observations were made:

a) Whatman GF/A filter papers absorbed some of the colour from darker gelatine solutions.

b) The enzymes Alcalase and Trypsin could be used interchangeably (Table 6). If absorbance data from different solvent systems was to be used to measure gelatine colours then the best correlation was given by the absorbance area between 400 and 700 nm. The reason for this was that the effective subtraction of the 700 nm absorbance had not fully compensate for differences in solvent clarity.

c) If, during enzymic hydrolysis there was precipitation then this was likely to be accompanied by absorption of some of the colour by the precipitate, resulting in a low OD or colour value (Addendum 5 & Table 9).

d) The pH change in 6.67% gelatine solutions due to enzymic hydrolysis was small (Table 14) both because of the buffering power of gelatine and because the enzymes could only partially hydrolyse the protein.

e) The possibility of acid hydrolysis was investigated (Table 17) but it was found that this led to a marked darkening of the solutions so the process was

not pursued.

REFERENCES.

British Standards Institution, 1975. (BS 757:1975). Methods for sampling and testing gelatine. British Standards Institution. London.

Drew, B.R. 1930. An improved process for extracting glue and gelatine from chrome leather. British Patent 338,589. London. Application 17/8/1929. Accepted 17/11/1930.

Heidemann, E. 1981. The determination of hydroxyproline in materials containing collagen. The Journal of the Society of Leather Technologists and Chemists 64: 57-59.

Saunders, P.R., and Ward, A.G. 1953. The colour and clarity of gelatin and glue solutions. Journal of the Science of Food and Agriculture 4: 523-527.

Sears, F.W., and Zemansky, M.W. 1964. College Physics. Third Edition. p 946-947.Addison-Wesley Publishing Company Inc. Reading, Massachusetts, Palo Alto, London.

Veis, A. 1964. The Macromolecular Chemistry of Gelatin. p. 108-9. Academic Press. New York & London.

ADDENDA.

ADDENDUM 1. DU70 Absorbance data on gelatines.
Solvent = Water.
Filtration. Nil.

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
12/1	178/12	17.8	7.5	0.393		31.4
12/1	183/1	9	6.5	0.24		22.7
12/1	C542/2L	<3.2	13	0.064		5.8
	BASE LINE = ABS 700nm					
13/1	C542/2L	<3.2	13			3.808
19/1	G35	4.8	NM	1.1101	0.37	71.8
19/1	G36	8.9	NM	0.6687	0.153	45.71
18/1	YSB/1	<3.2	10	0.0953	0.016	5.92
19/1	C552/1	<3.2	12.5	0.0502	0.011	2.57
20/1	C542/1L	<3.2	13.3	0.0738	0.016	3.99
19/1	C542/3L			0.114	0.015	6.47
28/1	C542/3L			0.1244	0.012	5.42
28/1	YSB/1	<3.2	10	0.0845	0.012	5.22
02/2	C542/1L	<3.2	13	0.074	0.016	3.8
02/2	C542/2L	<3.2	13.3	0.0671	0.008	3.99
13/1	178/12	17.8	7.5			24.58
13/1	183/1	9	6.5			15.77
17/1	183/1	9	6.5		0.026	16.42
17/1	184/10	22.8	4	0.6412	0.077	44.25
17/1	LF752/1	6	11	0.178	0.03	8.63
17/1	185/2	6.8	10.5	0.1838	0.01	10.95
17/1	L752/1	4.8	10	0.0293	0.066	10.29
17/1	180/6	16	6.5	0.4239	0.034	26.69
17/1	183/4	14.5	10.5	0.3023	0.021	18.69
18/1	183/1	9	6.5	0.2374	0.022	15.48
18/1	CT6/1	5.2	11.5	0.1471	0.02	8.52
18/1	CT6/2	5.6	11.5	0.1865	0.032	9.81
18/1	CT6/3	6	12.5	0.1757	0.016	9.93
18/1	YSB/3	4.8	9	0.1394	0.027	8.37
18/1	ST26/1	11.4	11.8	0.2826	0.033	16.79
18/1	ST26/2	11.4	11.8	0.285	0.022	17.01
18/1	ST26/3	16	10	0.371	0.027	22.69
19/1	183/1	9	6.5	0.247	0.025	16.09
19/1	826/2	8	26	0.211	0.027	11.96
19/1	OCS	8	20	0.2204	0.016	13.33
19/1	LCS	7.6	7.5	0.2635	0.015	15.73
19/1	YSB/2	5.2	7.5	0.1621	0.047	9.73
19/1	184/5	10	11.1	0.242	0.021	15.33
20/1	183/1	9	6.5	0.2421	0.025	15.7

ADDENDUM 1. Continued...

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
20/1	D11335	2.8	10	0.1204	0.017	8.452
20/1	D94025	2.4	9	0.1903	0.042	13.61
20/1	HORSE/90	14	4.5	0.4034	0.017	25.47
20/1	CALF-A/3	5.2	9	0.2474	0.052	16.77
20/1	PIS-PP1	11.4	10.5	0.273	0.036	14.14
20/1	PIZ/1	3.6	9	0.1652	0.049	9.1
20/1	SBG	6.4	8	0.1544	0.019	9.72
20/1	N CALF-TyB	5.6	12	0.1581	0.015	9.27
28/1	183/1	9	6.5	0.243	0.022	16.08
28/1	L752/1	4.8	10	0.1868	0.054	9.13
28/1	N CALF TyB	5.6	12	0.1552	0.011	9.44
28/1	LF752/1	6	11	0.1725	0.023	9.41
28/1	178/12	17.8	7.5	0.3974	0.022	24.67
28/1	183/4	14.5	10.5	0.3001	0.018	19.03
28/1	185/2	6.8	10.5	0.1804	0.008	10.78
28/1	184/10	22.8	4	0.6442	0.076	44.52
28/1	YSB/3	4.8	9	0.1431	0.025	9.26
28/1	180/6	16	6.5	0.408	0.023	26.17
28/1	YSB/2	5.2	7.5	0.1436	0.033	9.03
28/1	826/2	8	9	0.1947	0.015	11.21
28/1	CT6/3	6	12.5	0.1654	0.012	8.93
28/1	CT6/1	5.2	11.5	0.1344	0.012	8
28/1	184/5	10	11.1	0.2198	0.008	13.93
28/1	OCS	8	10	0.2008	0.007	12.39
28/1	CT6/2	5.6	11.5	0.1625	0.015	9.39
01/2	183/1	9	6.5	0.2511	0.027	16.3
01/2	SBG	6.4	8	0.6136	0.019	10.57
01/2	HORSE/1	14	4.5	0.4139	0.075	25.66
01/2	G35	4.8	NM	1.1143	0.369	72.65
01/2	LF752/1	6	11	0.1802	0.03	8.91
01/2	184/10	22.8	4	0.6483	0.079	44.76
01/2	LCS	7.6	7.5	0.2682	0.016	15.87
01/2	180/6	16	6.5	0.4274	0.034	26.9
01/2	178/12	20	7.5	0.4098	0.025	25.37
01/2	183/4	14.5	10.5	0.3093	0.021	19.2
02/2	183/1	9	6.5	0.2537	0.025	16.23
02/2	D11335	2.8	10	0.114	0.014	8.14
02/2	D94025	2.4	9	0.1884	0.038	13.7
02/2	PIS PP1	11.4	10.5	0.2667	0.036	14.91
02/2	ST26/2	11.4	11.8	0.2864	0.022	17.73
02/2	ST26/3	16	10	0.3761	0.028	23.28
02/2	ST26/1	11.4	11.8	0.2987	0.04	17.43
02/2	850/6 + C	17.8	5	0.4876	0.063	28.63
02/2	850/6	17.8	5	0.4999	0.063	30.45
02/2	852/1 + C	6	11	0.1783	0.027	9.39
02/2	852/1	6	11	0.1719	0.031	9.56
11/4	178/12	17.8	7.5	0.3964	0.025	24.33
11/4	D31108	2.8	36	0.1914	0.035	13.65
11/4	D2249	2	11	0.0731	0.006	5.05
11/4	D1512	2.4	30	0.1284	0.022	9.55
11/4	D2116	2.4	17	0.0897	0.011	6.52
11/4	D2227	2	21	0.096	0.015	6.81

ADDENDUM 1. Continued...

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
11/4	N - TyA	4.8	17	0.1545	0.016	9.44
28/4	D32117	4	50	0.2334	0.046	16.21
28/4	D1287	2	31	0.1171	0.019	8.31
28/4	D3264	3.6	42	0.1946	0.035	13.66
28/4	D147	2	44	0.1571	0.031	11.36
28/4	FISH 824/1	11.4	8.5	0.2157	0.038	12.3
28/4	FISH COD2/1	6.0	11.0	0.1532	0.012	9.07
28/4	178/12	17.8	7.5	0.3957	0.022	24.73
28/4	185/2	6	11	0.1838	0.01	10.66

ADDENDUM 2. Solvent = ES1 (1 ml Alcalase 0.6L/l)
Filtration = Whatman papers (GF/A).
BASE LINE = ABS. 700nm for area calculation.

DU70 Gelatine Absorbance Data.

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
12/1	C542/2L	<3.2	13	0.051		4.2
13/1	C542/2L	<3.2	13			2.95
18/1	YSB/1	<3.2	10	0.085	0.018	4.73
19/1	C542/3L			0.0964	0.006	5.24
19/1	C552/1	<3.2	12.5	0.0435	0.006	2.34
20/1	C542/1L	<3.2	13.3	0.052	0.008	2.52
12/1	178/12	17.8	7.5	0.371		29
12/1	183/1	9	6.5	0.238		21.9
13/1	178/12	17.8	7.5			22.38
13/1	183/1	9	6.5			13.54
17/1	183/1	9	6.5	0.2309	0.024	14.92
17/1	184/10	22.8	4	0.4843	0.037	32.2
17/1	LF752/1	6	11	0.1272	0.01	6.34
17/1	185/2	6.8	10.5	0.1886	0.014	10.98
17/1	L752/1	4.8	10	0.1313	0.012	7.44
17/1	180/6	16	6.5	0.3504	0.024	21.03
17/1	183/4	14.5	10.5	0.2798	0.018	17
18/1	183/1	9	6.5	0.1962	0.021	11.79
18/1	CT6/1	5.2	11.5	0.1413	0.017	8.29
18/1	CT6/2	5.6	11.5	0.1671	0.017	9.74
18/1	CT6/3	6	12.5	0.174	0.018	9.82
18/1	YSB/3	4.8	9	0.1094	0.014	6.61
18/1	ST26/1	11.4	11.8	0.2591	0.018	16.12
18/1	ST26/2	11.4	11.8	0.2843	0.021	17.34
18/1	ST26/3	16	10	0.3703	0.028	22.65
19/1	183/1	9	6.5	0.2237	0.024	14.35
19/1	826/2	8	26	0.186	0.013	10.74
19/1	OCS	8	20	0.2155	0.018	13.01
19/1	LCS	7.6	7.5	0.2329	0.014	13.39
19/1	YSB/2	5.2	7.5	0.1151	0.021	7.2
19/1	184/5	10	11.1	0.2341	0.018	14.38
19/1	G35	4.8	NM	0.4721	0.077	32.73
19/1	G36	8.9	NM	0.5282	0.088	37.26
20/1	183/1	9	6.5	0.202	0.02	12.79
20/1	D11335	2.8	10	0.0662	0.007	4.37
20/1	D94025	2.4	9	0.0535	0.011	3.47
20/1	HORSE/90	14	4.5	0.322	0.039	20.64
20/1	CALF-A/3	5.2	9	0.1675	0.023	10.36
20/1	PIS-PP1	11.4	10.5	0.2072	0.02	10.52
20/1	PIZ/1	3.6	9	0.0801	0.007	4.09
20/1	SBG	6.4	8	0.1256	0.016	7.63
20/1	N CALF-TyB	5.6	12	0.1396	0.011	7.86

ADDENDUM 3. Solvent = ES1.
Filtration = membrane (0.45 μm).

DU70 Gelatine absorbance data.

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
28/1	C542/3L			0.0794	-0.004	4.41
28/1	YSB/1	<3.2	10	0.0624	0.005	3.76
02/2	C542/1L	<3.2	13	0.0454	0	2.25
02/2	C542/2L	<3.2	13.3	0.0457	0	2.71
01/2	G35	4.8	NM	0.3956	0.052	27.09
28/1	183/1	9	6.5	0.2115	0.012	14.07
28/1	L752/1	5.2	10	0.1237	0.001	7.59
28/1	LF752/1	6	11	0.1138	-0.004	6.05
28/1	178/12	17.8	7.5	0.3662	0.01	22.67
28/1	183/4	13.3	10.5	0.2808	0.009	17.78
28/1	185/2	6.8	10.5	0.1681	0	10.31
28/1	184/10	22.8	4	0.538	0.037	37.22
28/1	YSB/3	4.8	9	0.0995	0.006	6.41
28/1	180/6	16	6.5	0.3924	0.025	24.63
28/1	YSB/2	5.2	7.5	0.093	0.009	5.94
28/1	826/2	8	9	0.1724	0.006	10.02
28/1	CT6/3	6	12.5	0.1581	0.008	9.07
28/1	CT6/1	5.2	11.5	0.1228	0.009	7.06
28/1	184/5	10	11.1	0.2254	0.014	14.09
28/1	OLD C/S	8	10	0.2001	0.012	12.11
28/1	CT6/2	5.6	11.5	0.1601	0.012	9.57
01/2	183/1	9	6.5	0.2215	0.021	14.24
01/2	SBG	6.4	8	0.0946	0.003	5.74
01/2	HORSE/1	14	4.5	0.2871	0.027	18.22
01/2	LF752/1	6	11	0.1222	0.006	6.08
01/2	184/10	22.8	4	0.5453	0.045	37.2
01/2	LCS	7.6	7.5	0.2271	0.009	12.97
01/2	180/6	16	6.5	0.4012	0.027	25.16
01/2	178/12	20	7.5	0.3774	0.017	22.99
01/2	183/4	14.5	10.5	0.2925	0.017	18.12
02/2	183/1	9	6.5	0.2183	0.019	14.1
02/2	D11335	2.8	10	0.0687	0.005	5
02/2	PIK SHP	11.4	10.5	0.205	0.007	10.9
02/2	ST26/2	11.4	11.8	0.2679	0.013	16.6
02/2	ST26/3	16	10	0.3517	0.019	21.83
02/2	ST26/1	11.4	11.8	0.2495	0.013	15.49

SOLVENT ES1, FILTRATION 0.45μm.
ABSORBANCE AREA v/s Colour. Regression Output:

Constant		0
Std Err of Y Est		1.684898
R Squared		0.904779
No. of Observations		31
Degrees of Freedom		30
X Coefficient(s)	0.691605	
Std Err of Coef.	0.017813	

ADDENDUM 3. Continued...

ABSORBANCE-400nm v/s Colour. Regression Output:

Constant	0
Std Err of Y Est	1.308228
R Squared	0.942595
No. of Observations	31
Degrees of Freedom	30
X Coefficient(s)	44.20141
Std Err of Coef.	0.880448

ABSORBANCE-700nm v/s Colour. Regression Output:

Constant	0
Std Err of Y Est	4.613375
R Squared	0.286124
No. of Observations	31
Degrees of Freedom	30
X Coefficient(s)	647.7928
Std Err of Coef.	48.95671

Additional data including pigskin gelatines.
SOLVENT =.ES1. FILTER = 0.45μm.

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
11/4	178/12	17.8	7.5	0.3679	0.016	22.74
11/4	D31108	3.8	36	0.124	0.011	8.82
11/4	D2249	2.4	11	0.0676	0.005	4.75
11/4	D1512	2.2	30	0.0791	0.009	5.62
11/4	D2116	2	17	0.0662	0.005	4.57
11/4	D2227	2.2	21	0.0711	0.006	5.01
11/4	N - TyA	4.8	17	0.1247	0.006	7.17
28/4	D32117	4.7	50	0.1566	0.017	11.13
28/4	D1287	2.2	31	0.077	0.007	5.39
28/4	D3264	4	42	0.1351	0.014	9.53
28/4	D147	2.4	44	0.0826	0.01	5.99
28/4	FISH 824/1	11.4	8.5	0.1579	0.004	9.85
28/4	FISH COD2/1	6	11	0.1718	0.016	10.7
28/4	178/12	17.8	7.5	0.3748	0.018	23.01
28/4	185/2	6.8	11	0.1795	0.011	10.45

ABSORBANCE AREA V/S COLOUR. Regression Output:

Constant	0
Std Err of Y Est	1.820664
R Squared	0.901143
No. of Observations	46
Degrees of Freedom	45
X Coefficient(s)	0.69094
Std Err of Coef.	0.0175

ADDENDUM 3. Continued...

ABSORBANCE - 400 nm V/S COLOUR.	Regression Output:
Constant	0
Std Err of Y Est	1.472233
R Squared	0.93536
No. of Observations	46
Degrees of Freedom	45
X Coefficient(s)	44.15308
Std Err of Coef.	0.899812

ABSORBANCE - 700nm v/s COLOUR.	**Regression Output:**
Constant	0
Std Err of Y Est	4.106796
R Squared	0.579047
No. of Observations	57
Degrees of Freedom	56
X Coefficient(s)	635.9171
Std Err of Coef.	39.49756

ADDENDUM 4. Solvent = ES2. (Contained ammonia).
Filtration. = membrane (0.45 μm).

DU70 Gelatine Absorbance data.

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
14/4	C200/1	<3.2	11	0.048	0	2.74
14/4	YSB/1	<3.2		0.072	0.005	4.72
12/4	D2116	2.4		0.055	0.004	3.84
12/4	D2249	2		0.055	0.003	3.87
14/4	D1287	2	10	0.058	0.005	4.05
12/4	D2227	2		0.061	0.006	4.27
12/4	D1512	2.4		0.062	0.006	4.5
14/4	D147	2	8	0.065	0.007	4.7
12/4	D31108	2.8	36	0.103	0.01	7.19
14/4	5Y4/3	8.4	5.6	0.126	0.005	7.24
14/4	D3264	3.6	8	0.102	0.009	7.31
14/4	D32117	4	7	0.125	0.013	9.09
14/4	N -TyA	4.8	11.5	0.167	0.003	10.02
14/4	WT3/3	16	4.8	0.308	0.008	19.17
14/4	IOE/2	12.3	11	0.319	0.015	21.29
12/4	178/12	17.8	7.5	0.386	0.014	24.02
14/4	178/12	17.8		0.384	0.014	24.4
20/4	ST24/1	8.9		0.1163	-0.001	6.6
20/4	5Y4/2	6.8		0.1287	0	7.49
20/4	5Y4/3	8.4		0.1694	0.003	10.4
20/4	ST24/3	13.3		0.3015	0.007	18.99
20/4	ST24/2	12.3		0.2122	0.003	12.82
20/4	5Y4/1	5.6		0.0942	0	5.71
20/4	YSB/3	4.8		0.1157	0.01	7.91
20/4	YSB/2	5.2		0.1053	0.012	7.283
20/4	178/12	17.8	7.5	0.3853	0.014	24.32
22/4	SBG	6.4	8	0.1026	0.002	6.7
22/4	178/12	17.8	7.5	0.3837	0.014	24.38
22/4	850/6+C	17.8	5	0.409	0.019	25.87
22/4	850/6	17.8	5	0.4282	0.025	27.66
22/4	FISH824/1	11.4	8.5	0.1423	0.004	8.22
22/4	183/4	13.3	10.5	0.3056	0.013	19.94
22/4	180/6	16	6.5	0.3899	0.021	24.95
22/4	184/10	22.8	4	0.5721	0.045	40
22/4	184/5	10	11	0.2451	0.015	16.21
22/4	185/2	6	11	0.1957	0.012	12

ADDENDUM 5. Pigskin gelatines.
Solvent = ES3. (Contained papain).
Filtration = membrane (0.45μm).

DU70 absorbance data on Pigskin gelatines with pH < 5.

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
6/6	D2227	2.2		0.0559	0.001	3.92
6/6	D3264	4		0.1023	0.006	6.9
6/6	D1512	2.2		0.0563	0.003	3.81
6/6	D32117	4.7		0.1191	0.008	8.15
6/6	D2116	2		0.0524	0.001	3.54
9/6	D147	2.4		0.0656	0.006	4.55
9/6	D31108	3.8		0.1	0.005	6.64
9/6	D1287	2.2		0.0623	0.005	4.29
9/6	D2249	2.4		0.0537	0.001	3.78
9/6	OCS	8		0.1751	0.007	10.11
9/6	UNFILTERED ES3			0.025	0.0131	1.34

MIXED ENZYME Solution.
0.62g PAPAIN + 0.2 ml ALCALASE TO 200 ml.
10 ml per sample.

DATE	SAMPLE	COLOUR	CLARITY	ABS 400 nm	ABS 700 nm	AREA 400-700
6/6	178/12	17.8		0.2979	0.012	17.57
6/6	877/1	5.6		0.139	0.003	7.77
6/6	155/1	6.8		0.1612	0.006	9.24
6/6	D2227	2.2		0.0527	0.002	3.39
6/6	D3264	4		0.0938	0.005	6.11
6/6	D1512	2.2		0.049	0.001	3.27
6/6	UNFILTERED MIXED ENZYME.			0.0984	0.046	6.57

ADDENDUM 6. Gelatine analytical data.

NOTES - ORIGINS	SAMPLE	COLOUR	CLAR	BLOOM	pH	% Moist	% Ash	Visc.ms	Type
Bovine hide Prodn. Gelatine	155/1	6.8	12.5	269	5.7	10.36	0.4	36.4	B
Bovine hide Prodn. Gelatine	178/12	17.8	7.5	125	5.7		1.3		B
Bovine hide Prodn. Gelatine	180/6	16	6.5	204	5.2		0.98		B
Bovine hide Prodn. Gelatine	183/1	9	6.5	256	5.3	8.68	0.78	36.5	B
Bovine hide Prodn. Gelatine	183/4	14.5	10.5	160	5	7.9	0.89		B
Bovine hide Prodn. Gelatine	184/10	22.8	4	98	5.9	9.3	1.56		B
Bovine hide Prodn. Gelatine	184/5	10	11.1	219	5.2		1.25		B
Bovine hide Prodn. Gelatine	185/2	6.8	10.5	266	5.2		0.4	39.5	B
5 year old bovine gelatine	5Y4/3	8.4	11	226	5.6	11.5	2.41	33.2	B
Bovine hide Prodn. Gelatine	826/2	8	26	263	5.1	9.55	0.82	39.8	B
Bovine hide Prodn. Gelatine	850/6	17.8	5	182	5.3	10.4	0.76	29.8	B
Bovine hide Prodn. Gelatine	850/6 + C	17.8	5	178	5.3	9.25	0.78	29.9	B
Bovine hide Prodn. Gelatine	852/1	6	11	240	5.3	13.23	0.35	42.4	B
Bovine hide Prodn. Gelatine	852/1 + C	6	11	231	5.1	15.46	0.34	40.1	B
Bovine hide Prodn. Gelatine	877/1	5.6	11.5	267	5.1	11.59	0.42	42.1	B
Calf Skin - Type A	CALF-A/3	5.2	9	284	6.5	13.8	0.1	32.6	A
18 month old Brahman	CT4/2	5.6	11	324	5.4	8.93	0.58	48	B
18 month old Brahman skin	CT6/1	5.2	11.5	327	5.3	10.04	0.85	48	B
18 month old Brahman skin	CT6/2	5.6	11.5	308	5.6	9.34	0.64	55.6	B
18 month old Brahman skin	CT6/3	6	12.5	277	5.6	9.62	0.59	49.7	B
SPA process gelatine	ES1	3.2	12.5	0	Not Analysed				
SPA process gelatines	C200/1	<3.2	11.1	15	6.3	11.4	1.75	15.6	B
SPA process gelatines	C542/1L	<3.2	13.3	60	5.7	12.02	1.28	12.5	B
SPA process gelatines	C542/2L	<3.2	13.3	6	4.3	10.75	1.36	12.5	B
SPA process gelatines	C542/3L			0	NOT	ANALYS-	ED		B
SPA process gelatines	C552/1	<3.2	12.5	4	5.6	14.2	0.92		B
Pigskin Gelatine.	D11335	2.8	10	233	4.6	9.5	0.21	35	A
Pigskin Gelatine.	D1287	2	31	268	4.6	8.68	0.27	44	A
Pigskin Gelatine.	D147	2	44	289	4.5	8.44	0.2	58.4	A
Pigskin Gelatine.	D1512	2.4	30	281	4.4	7.88	0.16	47.4	A
Pigskin Gelatine.	D2116	2.4	17	246	4.5	8.48	0.14	36.1	A
Pigskin Gelatine.	D2227	2	21	206	4.5	8.86	0.22	30.6	A
Pigskin Gelatine.	D2249	2	11	227	4.5	8.26	0.17	32.1	A
Pigskin Gelatine.	D31108	2.8	36	109	4.2	8.45	0.33	25.3	A
Pigskin Gelatine.	D32117	4	50	86	4.3	9.78	0.3	21.5	A
Pigskin Gelatine.	D3264	3.6	42	121	4.2	8.38	0.29	25	A
Pigskin Gelatine.	D94025	2.4	9	282	4.6	8.6	0.14	69	A
Type A bovine hide gelatine	N - TyA	4.8	17	228	4.4	10.3	0.77	34.2	A
Fish Skin Gelatine	FISH 824/1	11.4	8.5	210	5.1	7.71	1.46	61.5	A
Fish Skin Gelatine	FISH COD2/1	6	11	243	5	10.95	0.49	23.5	A
Bovine hide Prodn. Gelatine	G35	4.8	NM	205	5.3	9.1	1.52	36.3	B
Bovine hide Prodn. Gelatine	G36	8.9	NM	245	5.2	8.8	1.02	34.3	B
Horse Skin Gelatine	HORSE/90	14	4.5	296	5.4	10.4	3	28.8	B
Bovine hide Prodn. Gelatine	LCS	7.6	7.5	86	5.3	12.5	0.74	22.4	B
Bovine hide Prodn. Gelatine	M8884	6.8	6.5	236	5.2	10.7	0.56	41.0	B
Lab gelatine.	LF752/1	6	11	267	5.5	12.35	4.12	22.2	B
Production Calf - Type B	N CALF TyB	5.6	12	232	4.7	9.97	1.66	32	B
Bovine hide Prodn. Gelatine	N33/3	6.8	11	245	4.5	11.5	0.89	34.8	B
Bovine hide Prodn. Gelatine	N33/1	13.3	11	109	4.3	10.8	0.86	20.7	B
Bovine hide Prodn. Gelatine	N33/4	14.5	11	135	4.6	10.8	0.92	27.3	B
Bovine hide Prodn. Gelatine	OCS	8	10		4.8				B

ADDENDUM 6. Continued....

NOTES - ORIGINS	SAMPLE	COLOUR	CLAR	BLOOM	pH	% Moist	% Ash	Visc.ms	Type
Pickled Sheep skin	PIS-PP1	11.4	10.5	307	5.3	8.6	4.3	39.4	B
Ex Pizzles.	PIZ/1	3.6	9	328	5	10.8	2.2	36	B
Bone Glue	SBG	4.8	8	77	6.1	12.5	0.34	25.7	
12 year old bovine hide	ST26/1	11.4	11.8	307	5.2	12.5	3.44	30.5	B
12 year old bovine hide	ST26/2	11.4	11.8	280	5.1	12.4	1.82	28.9	B
12 year old bovine hide	ST26/3	16	10	246	5.2	9.6	2.14	31.5	B
12 year old bovine hide	WT3/3	16	4.8	241	5.5	10.6	1.74	32.1	B
10 month old bovine hide	YSB/1	<3.2	10	314	5.5	9.08	0.47	53.7	B
10 month old bovine hide	YSB/2	5.2	7.5	312	5.5	9.76	0.43	52.6	B
10 month old bovine hide	YSB/3	4.8	9	287	5.6	10.79	0.42	38.7	B
12 year old Inguni	IOE/2	12.3	11	233	5.3	10.89	0.62	26.9	B
Lab extracted	L752/1	4.8	10	289	5.4	11.64	3.54	127.1	B

Included in the above samples were production or experimental gelatines from the following manufacturers:

DynaGel Incorporated, Calumet City, USA.
Gelatinas Ecuatorianas S.A. (Gelec S.A.). Quito, Ecuador.
Leiner Davis Gelatin (Brasil). Sao Paulo, Brasil.
Leiner Davis Gelatin (South Africa). Krugersdorp, South Africa.
Leiner Davis Gelatin (New Zealand). Christchurch, New Zealand.
Productoria de Gelatina SA (Progel). Manizales, Colombia.
Systemes Bio-Industries (SANOFI). Boulogne Cedex, France.
Smits Vuren B.V. Gorinchem, Nederland.

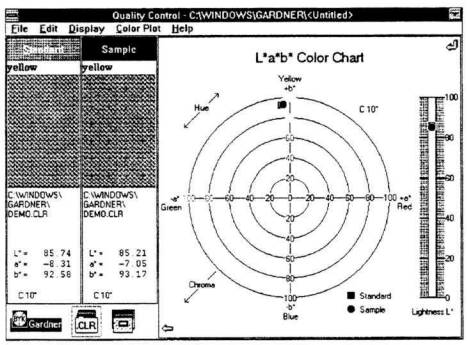
ADDENDUM 7. BYK-Gardner Color-View Reflectance Spectrophotometer.

BYK Gardner

15
15.1.1

Color
color-view™
Spectrophotometer

Catalog 92

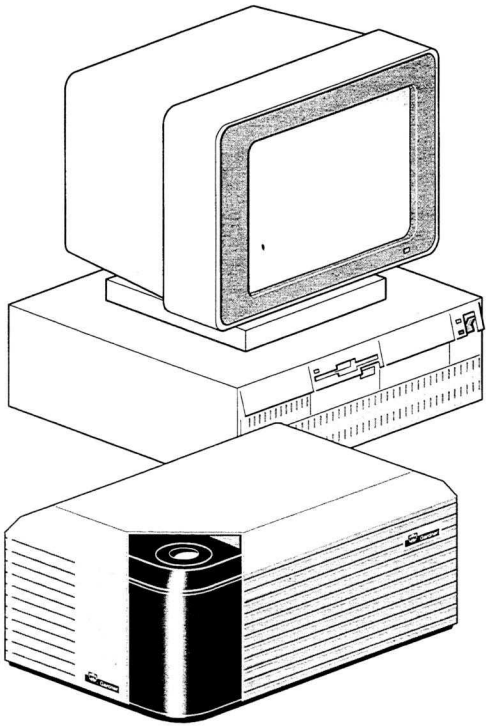


Color Measurement
in accordance with DIN 5033, DIN 5036, ISO 7724, NF X 08-012, ASTM D 2244, E 97, E 308, E 313,

color-view is a compact and versatile array spectrophotometer which integrates gardner-soft color-insights, a new Microsoft® Windows™* based quality control software to provide the user a cost-effective solution for quality control needs in virtually any application.

color-view is designed using 45/0 geometry (45° circumferential illumination / 0° viewing), which provides the best agreement with visual assessment.

Experiences gained from the reliable **TCM** spectrophotometer led to the consistent development of the **color-view**. Circumferential illumination minimizes the need for multiple measurements of directional or textured products.



color-view allows the rapid color measurement of a wide variety of materials. Such materials include:

- Paint and Coatings
- Automotive
- Plastics
- Raw Materials
- Adhesives
- Inks & Graphic Arts
- Agricultural
- Ceramics
- Paper
- Food
- Detergents
- Textiles

An internal paddle mechanism blocks the light path except for the rapid measurement cycle and minimizes the risk of material falling into the sensing head.
In addition, glass fiber optics prevent transmission of IR radiation, thus minimizing the effect of photochromism and thermochromism respectively, which can adversely effect sample measurement.

color-view provides colorimetric measurements under commonly used standardized illuminants, color scales, observers, and indices.

Software
- gardner-soft color-insights software
- Communications library to facilitate programming for custom link to third party color quality control packages (Option)

*Windows™ is a registered trademark of Microsoft® Corporation

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ADDENDUM 7 Continued....

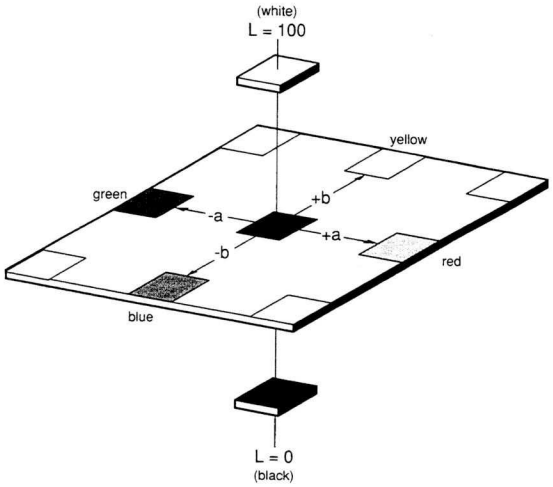
BYK Gardner

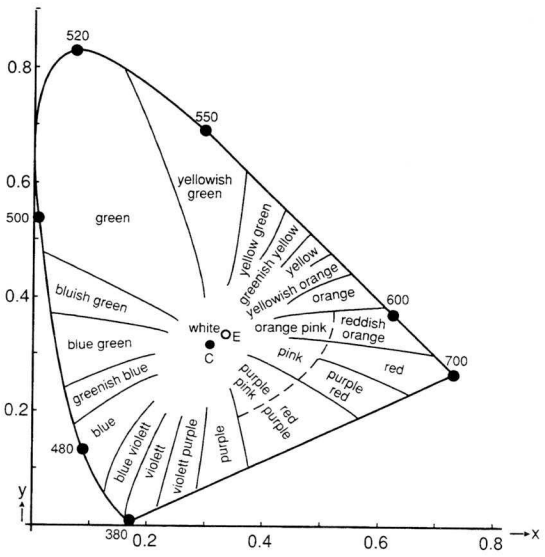
15
15.1.2

Color
color-view™ Spectrophotometer

Catalog 92

L, a, b - System





Chromaticity Diagram in accordance with CIE publication 15.2, ASTM E 308 and DIN 5033

Features

• Compact Size
color-view is one of the smallest 45/0 bench-top spectrophotometers produced today. It takes up less bench space while allowing convenient positioning of the sensor.

• Patented Auto-standardization
An internal paddle mechanism performs a reference standardization prior to every measurement, reducing operator error while increasing certainty of correct calibration.

• Rugged Construction
With its enclosed optics and its temperature and humidity operating range, **color-view** can go in more environments than other bench top instruments of the same class.

• Sample Viewer (Standard Accessory)
The sample viewer enables the operator to positively place and view small specimen areas prior to measurement.

• Quality Control Software
color-view incorporates gardner-soft color-insights software for applications ranging from pass/fail to in depth color analysis. color-insights, based on the Microsoft® Windows™ operating environment, is both extensive in performance and easy to use.

• Patented 60° Gloss Measurement Option
With this option, the user simultaneously measures 60° gloss values and color values according to international standards. Use of this option will save the operator's time.

• Foot Switch for Remote Control (Option)
The Unsolicited Data Option gives the operator the ability to measure samples directly at the sensor by use of a foot switch or switch closure. This option improves at-line measurement and allows the sensor to be placed into more rugged environments than a computer can withstand.

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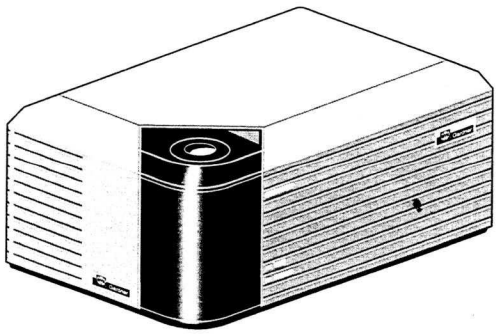
ADDENDUM 7 Continued....

BYK Gardner

15
15.1.3

Color
color-view™ Spectrophotometer

Catalog 92



BYK-Gardner color-view
Color Measuring System
- Spectrophotometer, 45° circumferential/0° geometry
- in accordance with DIN, ISO, ASTM
- **PC or Printer not Included**

Principle: Reflectance measurement of visible spectrum from 380 nm to 720 nm in 10 nm intervals. 60° gloss measurement.

Catalog No.	Voltage	Gloss Measurement 60°
6501	230 V/50 Hz	no
6502	230 V/50 Hz	yes

Standard System Configuration
color-view 45/0 grating/diode array spectrophotometer,
color-insights QC Manager software,
Integrated sample viewer,
Standard 1" (32 mm) area of view,
Holder with scratch-resistant calibration standards,
Inspection standard, Spare lamp and fuses,
Mains cable, Serial cable,
Instrument User's guide, Software operator's manual

Options:
60° gloss measurement per ASTM D 523,
Port down configuration with stand and sample presentation elevator (on request),
Small area of view (11 mm),
Microsoft® Windows™,
Foot switch for remote control,
Foreign language options: English, German, French, Italian, Spanish

The complete system requires:
IBM-PC, PS/2 or true compatible
- 386 processor, 2 MB RAM,
10 MB available on hard drive,
Enhanced Color Graphics (VGA) or any Microsoft Windows supported monitor,
DOS version 3.3 is required, 5.0 is recommended,
Microsoft Windows 3.X,
Microsoft Windows supported printer,
Microsoft Mouse,
Math coprocessor recommended

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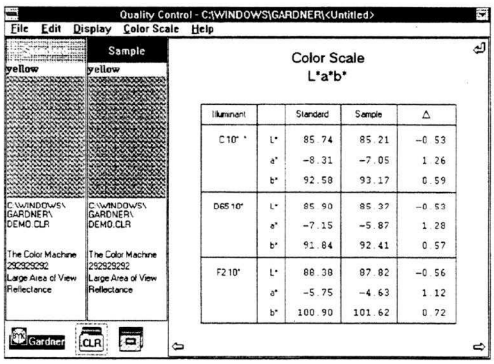
ADDENDUM 7 Continued....

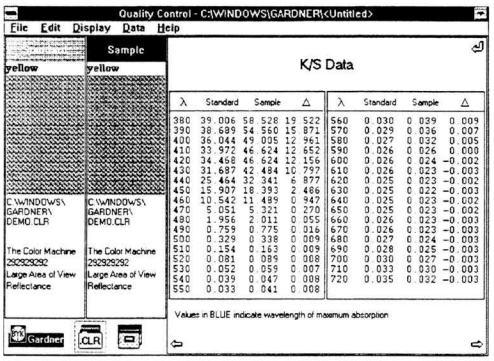
BYK Gardner

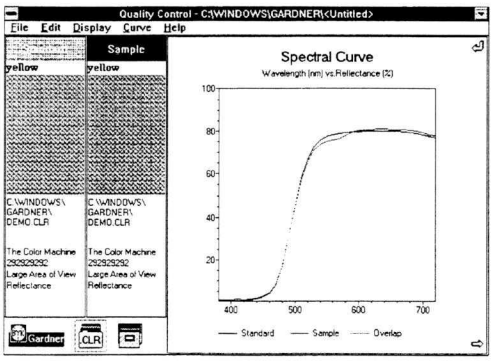
15
15.1.4

Color
color-view™ Spectrophotometer

Catalog 92







Technical Data

Power Supply:	230 volts 50/60 Hz or 115 volts 50/60 Hz
Repeatability:	Less than 0.01 ΔE* 1 σ * Standard Deviation Based on 300 consecutive readings of white tile
Reproducibility:	0.1 ΔE*, 1σ * Standard Deviation Based on average of 12 BCRA II tiles (complete set)
Standardization:	Uses internal white and black standards as continuous reference to minimize the use of external standards. External calibration also achievable
Measurement Geometry:	45° circumferential illumination 0° receiver optics
Sample Port Aperture: Options:	32 mm ø (1.26 in.) 11 mm ø (0.43 in.)
Spectral Response:	10 nm data collection with 10 nm bandpass measures visible spectrum from 380 to 720 nm
Illumination:	Tungsten-Halogen Lamp
Illuminants*:	A, C, D65 & F
Color Scales*:	X, Y, Z L*, a*, b* (CIELAB) L, a, b (Hunter) L*, C*, h Y, x, y L*, u*, v*
Indices*:	Metamerism Yellowness per DIN & ASTM Whiteness per CIE & ASTM
Color Differences*:	Component Color Scale Differences FMC II, CMC
CIE Observers:	2° & 10°
Dimensions: H x W x D Instrument Weight:	162 x 381 x 273 mm 6.4 x 15.0 x 10.8 in. 11.1 kgs / 24.6 lbs

Operating Environment

Operating Temp.:	+15 °C to +35 °C (65 °F to 95 °F)
Relative Humidity:	5 % to 85 % (non-condensing)

* A wide variety of illuminants, scales, indices and differences for special applications are standard. Please contact BYK-Gardner for information.

ADDENDUM 8. Gardner Tri-stimulus data.

GARDNER SPECTROPHOTOMETER. STANDARD = WHITE TILE
6.67% GELATINE SOLUTIONS COVERED WITH THE WHITE TILE.
(CONDENSATION WAS NOTED)

VOLUME ml	SAMPLE	COLOUR	CALC. COLOUR	CLARITY	L	a	b	Y1	Y2
75 ml	155/1	6.8		10	-51.8	-2.19	16.23	42.42	38.01
75 ml	970/3	8.7		15	-52.94	-0.2	26.07	66.89	56.52
50 ml	WATER	0.0		0	-50.47	-1.44	-2.43	-10.71	-7.28
50 ml	C79/1-2	4.8		14	-52.95	-2.46	7.65	19.84	19.45
50 ml	C79/1A	4.8		14	-52.71	-2.45	7.20	18.51	18.28
50 ml	C6/1-3	5.2		25	-53.58	-2.45	8.67	23.06	22.16
50 ml	C6/1-2	5.2		25	-54.42	-2.35	8.57	23.20	22.18
50 ml	C6/1	5.2		25	-54.09	-2.33	8.72	23.57	22.45
50 ml	764/1	5.2		13	-53.19	-2.56	8.03	20.88	20.43
50 ml	764/1-2	5.2		13	-53.13	-2.54	8.11	21.14	20.62
50 ml	877/1-2	5.6		17	-54.05	-2.53	10.5	28.22	26.6
50 ml	877/1	5.6		17	-53.83	-2.66	10.22	27.16	25.84
50 ml	155/1	6.8		10	-55.18	-2.35	10.90	30.13	28.01
50 ml	764/6	12.3		25	-58.10	-0.86	18.65	54.32	46.72
50 ml	764/6-2	12.3		25	-58.12	-0.87	18.66	54.36	46.76
50 ml	183/4-2	14.5		25	-58.07	-1.19	17.49	50.99	44.30
50 ml	183/4	14.5		25	-58.04	-1.16	17.65	51.39	44.60
50 ml	180/6	16.0		54	-59.68	-0.22	18.54	56.46	47.69
50 ml	111/3A-2	20.0		21	-62.51	1.84	23.68	74.17	59.92
50 ml	111/3A	20.0		21	-62.29	1.79	23.64	73.76	59.66
50 ml	184/10	22.8		73	-64.77	-2.48	19.7	68.76	54.24
50 ml	184/10	22.8		73	-64.47	-2.23	18.96	66.26	52.49
50 ml	D2249	2.4		11	-52.08	-2.31	3.85	8.51	9.81
50 ml	OCS	8		29	-56.01	-2.04	12.71	35.97	32.61
50 ml	155/1	6.8		10	-55.02	-2.43	11.71	32.1	29.79
50 ml	D1287	2.2		31	-53.04	-1.96	3.91	9.38	10.19
50 ml	L752/1	4.8		10	-55.19	-2.17	8.61	23.82	22.54
50 ml	D147	2.4		44	-53.9	-1.71	4.23	10.95	11.26
50 ml	970/3	8.7		15	-56.36	-1.8	16.53	46.38	41.14
50 ml	970/3-2	8.7		15	-56.36	-1.77	16.67	46.77	41.44
50 ml	G36	8.9		NM	-63.25	0.64	13.58	47.76	39.23
50 ml	G37	10		NM	-60.73	-0.43	10.69	35.51	30.43
50 ml	G35	4.8		NM	-66.8	0.8	11.09	43.31	35.19

Linear Regression - visual Colour v/s Gardner values. Blank = White Tile.

COL/L Regression Output:	
Constant	-85.98
Std Err of Y Est	1.2444
R Squared	0.9643
No. of Observations	28
Degrees of Freedom	26

X Coefficient(s) -1.693
Std Err of Coef. 0.0639

COL/a Regression Output:	
Constant	14.36923
Std Err of Y Est	5.41556
R Squared	0.323752
No. of Observations	28
Degrees of Freedom	26

X Coefficient 3.135633
Std Err of Coef. 0.8887

ADDENDUM 8. Continued.

SAMPLE SIZE = 50 ml. COLOUR STANDARD = 50 ml WATER
SAMPLES COVERED WITH THE WHITE TILE.

SAMPLE	COLOUR	CALC: COLOUR	CLARITY	L	a	b	Y1	Y2
111/3A	20.0	18.4	21	-12.5	3.33	26.16	85.2	67.41
111/3A	20.0	18.6	21	-12.6	3.4	26.18	85.46	67.52
143/1	7	6.5	60	-4.97	-0.65	15.1	46.09	39.44
143/1	7	6.3	60	-4.86	-0.59	15.36	46.93	40.08
155/1	6.8	6.5	10	-4.96	-0.99	14.37	43.51	37.66
155/1	6.8	6.5	10	-4.99	-0.97	14.46	43.79	37.87
155/1	6.8	6.7	25	-5.1	-0.93	14.33	43.56	37.63
155/1	6.8	6.1	10	-4.68	-1.01	14.19	43.07	37.3
155/1	6.8	6.8	10	-5.18	-0.98	14.08	42.95	37.18
157/1	7.2	6.7	21	-5.09	-0.65	17.87	53.45	45.65
160/7	11.7	11.9	10	-8.36	0.84	24.28	73.19	60.61
161/1	5.2	5.3	9	-4.22	-0.97	13.22	40.16	34.79
161/2	6.1	6.1	10	-4.68	-0.93	14.74	44.6	38.48
161/3	6.1	6.4	13	-4.88	-0.91	14.77	44.8	38.63
161/4	7.9	9.0	10	-6.52	0.38	18.98	57.49	48.78
161/5	9.7	10.8	9	-7.68	0.17	21.84	66.06	55.39
161/6	10.9	12.0	8	-8.44	0.62	23.25	70.61	58.68
161/7	10.9	11.8	7	-8.28	0.55	23.9	71.82	59.79
162/1	5.2	5.0	7	-3.99	-1.07	13.3	40.14	34.89
162/2	5.7	5.8	9	-4.53	-0.98	14.19	42.95	37.17
162/3	6.6	7.2	12	-5.4	-0.77	15.98	48.52	41.64
162/4	9.2	10.3	12	-7.34	-0.03	20.38	62.02	52.22
162/5	11.7	12.3	12	-8.63	0.77	23.83	72.32	59.96
162/6	12.5	12.7	12	-8.9	0.98	24.64	74.66	61.67
163/7	10.9	10.1	10	-7.21	0.32	22.86	68.39	57.16
164/1	6.1	5.3	10	-4.22	-1.25	15.21	45.16	39.3
164/1	6.1	5.3	10	-4.23	-1.24	15.19	45.14	39.27
164/2	7.4	6.9	16	-5.21	-0.98	16.89	50.54	43.56
164/3	10.3	10.9	16	-7.71	0.36	22.09	67.1	56.02
164/4	12.5	13.0	21	-9.09	1.21	24.68	75.44	62.04
164/5	14.6	15.4	24	-10.59	2.1	27.03	83.25	67.47
177/2	10	8.4 *L	40	-6.18	-0.29	16.19	50.25	42.52
179/6	12.3	10.7 *L	36	-7.62	0.03	19.2	59.43	49.94
180/6	16.0	14.2	54	-9.84	1.37	21.87	69.74	57.02
183/1	8.9	8.5	54	-6.24	-0.27	14.48	45.72	38.74
183/1	8.9	8.7	54	-6.35	-0.24	14.43	45.7	38.69
183/4	14.5	12.6 *L	25	-8.82	0.39	20.72	64.51	53.82
184/10	22.8	21.9	73	-14.7	3.96	22.42	80.3	62.19
752/1	4.8	6.8 *H	10	-5.16	-0.74	11.09	34.68	29.96
764/1	5.2	3.4 *L	13	-3.02	-1.13	11.45	34.37	30.05
764/6	12.3	11.4	25	-8.05	0.61	21.63	66.45	55.18
824/1-FISH	11.8	9.7 *L	39	-6.98	-0.65	20.54	42.32	35.41
877/1	5.6	5.0	17	-3.98	-1.08	12.93	38.95	33.9
970/3	8.7	8.6	15	-6.29	-0.35	19.31	58.05	49.23
970/3	8.7	8.8	15	-6.41	-0.31	19.32	58.22	49.32
BL 539	7	6.8	28	-5.12	-0.58	15.9	48.59	41.48
BL 539	7	6.9 1	28	-5.2	-0.62	15.93	48.63	41.57
BL 540	6.1	5.2	19	-4.11	-1.03	14.81	44.42	38.45
BL-534	7.9	8.6	24	-6.27	-0.32	18.29	55.7	47.21

ADDENDUM 8 Continued..

SAMPLE	COLOUR	CALC: COLOUR		CLARITY	L	a	b	Y1	Y2
BL-536	6.1	6.1		33	-4.72	-0.89	13.75	41.98	36.25
BL-537	6.1	6.4		16	-4.92	-0.83	15.62	47.21	40.6
C6/1	5.2	4.4		25	-3.65	-0.99	11.22	34.14	29.73
C6/1	5.2	4.4		25	-3.64	-1.01	11.19	34.03	29.66
C79/1	4.8	3.2	*L	12	-2.88	-1.01	10.24	31.05	27.13
C82/1L	2.8	2.8		21	-2.59	-0.48	6.37	20.19	17.5
C82/1L	2.8	2.8		21	-2.59	-0.46	6.45	20.47	17.71
C92/2	5.7	4.5		12	-3.68	-1.15	14.04	41.97	36.52
C93/3	2.6	2.3		6	-2.31	-1.12	8.7	26.24	23.21
C94/2	2.8	2.3		8	-2.28	-1.15	8.94	26.93	23.82
C95/3	5.2	4.5		16	-3.69	-1.25	13.54	40.45	35.33
D1287	2.2	3.4		31	-3.02	-0.51	6.44	20.42	17.73
D1287	2.2	3.3		31	-2.92	-0.51	6.51	20.62	17.9
D147	2.4	4.7	*H	44	-3.83	-0.26	6.69	21.73	18.59
D1512	2.2	3.4		30	-3.02	-0.49	6.29	20.03	17.37
D2116	2	2.5		17	-2.4	-0.71	6.1	18.97	16.72
D2227	2.2	2.7		21	-2.57	-0.72	6.56	20.45	17.98
D2227	2.2	2.8		21	-2.65	-0.7	6.55	20.47	17.97
D2249	2.4	1.9		11	-2.03	-0.83	6.3	19.32	17.14
D2249	2.4	2.2		11	-2.27	-0.83	6.22	19.1	16.96
D31108	3.8	5.4	*H	36	-4.26	-0.77	10.26	32.05	27.82
D32117	4.7	7.3	*H	50	-5.47	0.75	11.64	36.63	31.7
D3264	4	5.9	*H	42	-4.56	-0.74	10.45	32.77	28.4
LCS	7.6	7.5		48	-5.62	-0.88	18.31	54.48	46.81
LDP	7.2	6.5		44	-4.98	-1.04	14.18	43	37.26
LPC	7.2	7.1		48	-5.32	-0.86	13.84	42.53	36.7
LPG	7.4	9.1	*H	50	-6.63	-0.6	15.91	49.27	42.1
LPJ	6.1	6.4		36	-4.88	-0.87	12.62	38.91	33.64
OCS	8	8.1		29	-5.99	-0.62	15.04	46.45	39.73
OCS	8	8.0		29	-5.91	-0.61	15.22	46.9	40
PG94	5.2	12.1	*H	89	-8.47	-0.18	12.62	61.27	52.41
SFI	3.5	2.8		26	-2.6	-0.7	8	24.98	21.75
SFI	3.5	3.5		26	-3.06	-0.62	8.44	26.54	22.98
SBG	4.8	4.3		44	-3.58	-0.81	10.91	33.75	29.24
SBG	4.8	3.9		44	-3.33	-0.85	10.9	33.57	29.13
X084	6.1	5.5		11	-4.3	-0.95	14.67	44.27	38.23
X091	7	6.4		13	-4.92	-0.82	15.75	47.67	40.98
GELATINES WITH UNMEASURABLE CLARITY									
STD-185	15.9	18.8		NM	-12.75	1.7	11.22	58.34	46.89
STD-185	15.9	19.0		NM	-12.88	1.71	16.19	58.18	46.76
G37	10	15.9	*H	NM	-10.88	0.99	13.19	46.62	38.01
G36	8.9	19.9	*H	NM	-13.46	2.07	16.02	58.61	46.65
G35	4.8	25.5	*H	NM	-16.97	2.24	13.48	54.04	42.5

ADDENDUM 8 Continued..

WATER STANDARD MEASURED AS A SAMPLE

	Calc: Colour	L	a	b	Y1	Y2
WATER	-1.9	0.34	-0.05	-0.10	-0.35	-0.24
WATER	-1.9	0.35	-0.05	-0.09	-0.33	-0.23
CLEAN TILE + WATER	-1.5	0.10	-0.03	-0.09	-0.33	-0.25
TILE ONLY		50.39	1.42	2.52	11.03	7.57

STANDARD = 50 ml WATER FOR THE GARDNER CELL, 57 ml WATER FOR THE GRAINER CELL.

	SAMPLE	COLOUR	CALC. COLOUR	CLARITY	L	a	b	Y1	Y2
GRAINER	752/1	4.8		10	-3.7	-0.68	7.21	29.84	26.09
GARDNER		4.8		10	-5.16	-0.74	11.09	34.68	29.96
GRAINER	970/3	8.7		15	-4.15	-0.64	12.81	51.33	44.15
GARDNER		8.7		15	-6.41	-0.31	19.32	58.22	49.32

Linear Regression - Gardner L value and visual Colour. Blank = 50 ml water.

COL/L Regression Output:		COL/a Regression Output:	
Constant	-1.345	Constant	8.483555
Std Err of Y Est	1.2225	Std Err of Y Est	2.2578
R Squared	0.9124	R Squared	0.701165
No. of Observations	86	No. of Observations	86
Degrees of Freedom	84	Degrees of Freedom	84
X Coefficient(s)	-1.582	X Coefficient	3.4322
Std Err of Coef.	0.0535	Std Err of Coef	0.2445

Included in the above samples were production or experimental gelatines from the following manufacturers:
DynaGel Incorporated, Calumet City, USA.
Gelatinas Ecuatorianas S.A. (Gelec S.A.). Quito, Ecuador.
Leiner Davis Gelatin (Brasil). Sao Paulo, Brasil.
Leiner Davis Gelatin (South Africa). Krugersdorp, South Africa.
Leiner Davis Gelatin (New Zealand). Christchurch, New Zealand.
Productoria de Gelatina SA (Progel). Manizales, Colombia.
Systemes Bio-Industries (SANOFI). Boulogne Cedex, France.
Smits Vuren B.V. Gorinchem, Nederland.

CHAPTER FOUR. Fluorescence in gelatine.

INTRODUCTION.

The only mention of gelatine and fluorescence in the literature appeared to be the Gelatine and Glue Research Association Research Report A33 (Thornton, 1966) in which home-made equipment using filters was used to demonstrate gelatine sol and gel fluorescence depolarisation.

More recently there had been many reports on collagen fluorescence. These included:

Tanaka, Avigad, Eikenberry and Brodsky (1988) reported an increase in fluorescence with time, for rat tail collagen incubated in the presence of ribose at 35°C for up to two weeks. They showed that a similar increase in collagen fluorescence occurred as a result of animal age. In addition, when soluble collagen was incubated with ribose, dimers and trimers of the collagen α-chain were formed which confirmed that the fluorescence was associated with cross-linking.

Sell and Monnier (1989) isolated the "pentosidine" collagen cross-link from human collagen (dura mater), correlated the increase in quantity with age and elucidated the structure of the cross-link by synthesis which involved heating equimolar quantities of lysine, arginine and ribose at pH 7.2 and 80°C for one hour. They claimed that similar treatment of a mixture of lysine, arginine and glucose had not resulted in the formation of a fluorescent product hence there had been a "problem" in accounting for the source of the ribose used in cross-link formation.

Monnier, Sell, Miyata and Nagaraj (1990) used the 335/385 nm fluorescent collagen "pentosidine" cross-link as the basis of a theory that senescence was the result of the Maillard reaction between functional proteins and ribose, the resulting impaired functionality of the protein being seen as the well known manifestations of age.

Dyer, Blackledge, Katz, Hull, Adkisson, Thorpe, Lyons and Baynes (1991), in

a review "The Maillard reaction in vivo", stated that "since pentosidine contains only 5 carbons from the sugar component, its formation from glucose must involve the loss of a carbon atom either from glucose itself or from a later intermediate in the reaction. ...while its (pentosidine's) origins from ribose v/s glucose and other sugars may be uncertain....." indicated that there had been evidence for the formation of pentosidine from glucose attributed to Baynes *et al.* (1990).

In the study of Uchiyama, Ohishi, Takahashi, Kushida, Inoue, Jugie and Horiuchi (1991) human articular cartilage was treated with collagenase. The fluorescence of the digests was shown to correlate with age. Furthermore, the authors demonstrated that the 295/395 nm fluorescence was due to the pyridinoline collagen cross-link and that the amount was largely invariant with age. However, the 335/385 nm fluorophore was shown to be identical with the "pentosidine" of Sell and Monnier (1989) and the amount increased linearly with age.

Eyre (1987) gave the details of the methodology for the isolation of hydroxylysylpyridinoline (HP) and lysylpyridinolin (LP) cross-links of collagen which were fluorescent. It was stated that these cross-links could be detected in PAGE gels using an excitation wavelength of 330 nm and an emission wavelength of 395 nm. However, these cross-links were not found in skin collagen (Yamauchi Woodley and Mechanic, 1988), hence, they should not interfere with the detection of the pentosidine cross-link in hide gelatine using fluorescence.

Based on the observation that gelatine colour also increased with animal age (*loc sit*) it appeared reasonable to hypothesize that gelatine colour could be the result of the Maillard reaction (*in vivo*). One method of verifying this would be to observe the fluorescence of gelatine. If gelatine exhibited fluorescence at 335/385 nm and the fluorescence increased with colour and animal age, it would follow from the work of Uchiyama *et al.* (1991) that the colour could be attributable to the formation of pentosidine collagen cross-links. If this was indeed the case then, as with collagen, gelatine should darken and fluorescence should increase on incubation with glucose or ribose sugars.

Although a number of texts (Munck and de Francisco, 1989; Lacowits, 1983) on fluorimetry were studied, "Fluorescence Analysis in Foods" (Munck and de

Francisco, 1989) appeared to be the most relevant with respect to the advantages and disadvantages of the method:

1. The main advantages were the extreme sensitivity and excellent specificity of the method. Only a few of the compounds absorbing light emit fluorescent light, and in contrast to spectrophotometry, two wavelengths were used in fluorimetry (Munck and de Francisco, 1989a).
2. The main disadvantage (Munck and de Francisco, 1989b) of fluorimetry was the phenomenon of "quenching" or reduction of the emission intensity. Quenching could be caused by high concentration of a fluorophore. This causes absorption of the emitted fluorescence and attenuation of the excitation beam in the areas of the solution in front of the detection system. Quenching could also be caused by oxygen, impurities and temperature and pH. In the current study, temperature in particular could have been a problem as there were no facilities to control sample temperature (temperature increase causes fluorescence decrease). Sell and Monnier (1989) also noted that the fluorescence of pentosidine was pH dependent and that the fluorescence was completely quenched at pH 9.
3. Another disadvantage (Munck and de Francisco, 1989c) of fluorimetry as an analytical tool was its serious dependence on environmental factors such as temperature, pH, ionic strength, and previous exposure to photochemical decomposition (sun light, fluorescent laboratory lights etc.). The latter required that fluorescence be measured immediately after excitation and accounted for the fact that the intensity of gelatine fluorescence was observed to decrease with time of exposure (i.e. a steady reading of fluorescence intensity was not obtained and the readings that were recorded were instantaneous readings taken immediately after inserting samples into the measuring compartment of the instrument).

Hence, whereas fluorimetry was advantageous from the point of view of product identification it was evident from the above that the probable error of quantitative results was large unless all variables were rigorously controlled.

From the report by Odetti, Pronzato, Noberasco, Cosso, Traverso, Cottalasso and Marinari (1994), it appeared that fluorimetry was an accepted technique for studying pentosidine's relationship to ageing phenomena.

METHODS AND MATERIALS.

Unless otherwise stated reagent grade chemicals were used throughout this part of the study.

Choice of solution concentration.

Dyer *et al.* (1991) pointed out that for every mole of pentosidine there must be an additional 1000 cross-links in the dimerised protein (ß chains). Hence, one must expect the concentration of pentosidine to be low, and a high concentration of gelatine would be required. High concentrations would however be expected to give practical problems associated with gelation hence a compromise, using a nominal concentration of 1% w/v, was decided upon (i.e. 0.20 g dissolved in 20 ml distilled water). This concentration was found to be satisfactory as it gave fluorescence intensities of about 50 for calf skin gelatine to about 200 for old animal skin gelatine. The high fluorescence intensities encountered with gelatines after incubation with ribose or glucose required further dilution to 0.01% concentration (0.1 ml 1% w/v solution added to 10 ml distilled water).

Instrumentation.

The instrument used in this study was a SCHIMADZU RF-5000 Spectrofluorophotometer (ILSA (Pty) Ltd. PO Box 8166, Johannesburg.) capable of scanning both emission and excitation spectra. In "Multispectra" mode the instrument was preprogrammed to run 5 scans of the emission spectrum at 5 preset intervals of excitation wave length. The scans were then presented together on one printout. This facilitated the rough determination of the existence of excitation and emission peaks. These peaks were refined by running scans of the excitation and emission spectra at very slightly different wave lengths. For readings at fixed wavelengths the excitation and emission wavelengths could be set and the fluorescence intensity read on the computer screen (CRT). No facility to download data for storage or reanalysis was available. The only method of data storage was a printout of a scan using the attached thermal printer or the manual recording of numerical data. All the determinations were carried out using 5 nm excitation and emission slit widths.

Fluorescence determination.

Aliquots (0.20 g) of gelatine powder were weighed into 50 ml clear glass bottles. Distilled water (20 ml) was added by pipette. Screw caps were applied to the bottles and the gelatine was allowed to swell for 20 min. The bottles were then placed in a 40°C waterbath and swirled several times over 30 min. to achieve complete solution of the gelatine. For the measurement of the sample fluorescence intensity on the spectrofluorophotometer, a 1 cm quartz cuvette was rinsed three times with the sample and then filled and placed in the instrument's cuvette holder. The sample compartment was closed and then the sample was scanned, or the fixed wavelength intensities were read from the CRT.

The effect of incubating gelatine with glucose.

1. Outline.

Two pale, unblended, first extraction, Type B commercial gelatines were incubated with glucose at 50°C and at pHs 6 and 9 for up to 12 weeks. The changes in colour and fluorescence were monitored with time. The collagen/glucose Maillard reaction was known to be very slow presumably due to the low concentration of glucose in the aldohexose configuration (0.002%). (Tanaka *et al.* 1988). The temperature of 50°C was chosen both to accelerate the reaction and to reduce markedly the probability of interference by microbiological proliferation. Tanaka *et al.* (1988) chose to add thymol as a bacteriastat. The pH of 6 was chosen as this was close to the physiological pH (Lawrie, 1985) and the pH of 9 was chosen as this was the pH used for the "Formol" titration determination of free amino groups in protein (Cobbet Gibbs and Leach, 1963 and Leach, 1965). At this pH the amine/aldehyde reaction was virtually instantaneous due to the absence of a charge on the ϵ amino side chain of lysine.

In the planning of this experiment the development of very high intensity fluorescence was not anticipated hence determination of the fluorescence on 0.01% gelatine solutions was an added procedure as was the determination of the pH of the reaction mixture at the same time that the fluorescence was determined.

2. Controls.

The following controls were also planned:

a) Glucose at pH 6 and 8.75 was incubated at 50°C to ensure that "glucose caramelization" could not be a factor in any colour change.

b) Each time an incubated sample was taken for assessment a fresh sample of the gelatine was dissolved to act as a control on instrument performance.

c) Gelatine at pH 6 and 8.75 were included as the effect of 50°C incubation on the colour of gelatine solutions had not been documented.

3. Experimental detail.

Two similar commercial Type B bovine hide gelatines (ex Davis Gelatine Industries (Pty) Ltd, P.O. Box 5019, West Krugersdorp, 1742.) which had been extracted in 1992 and 1994, were used. Gelatines 155/1 and 877/1, with similar Bloom gel strengths and colours, were chosen. Aliquots of gelatine (25 g) were mixed with 9 g glucose (BDH Laboratory Reagent Grade) and 150 ml distilled water in a 500 ml beaker. The gelatine was allowed to swell for 30 min. The mixture was then placed in a 50°C water-bath and stirred occasionally over 30 min. until the gelatine had dissolved and the solution was homogenous. Using a pH meter, 0.28N NaOH was added from a burette with stirring until the required pH was reached - 6.0 or 8.75. The pH of 8.75 was used because the quantity of NaOH consumed was greater than anticipated. The contents of the beaker were then adjusted with distilled water to a net weight of 250 g. After mixing well at 50°C, 15 ml aliquots were dispensed into sterile Mc Cartney bottles which were then placed in an oven thermostatically controlled at 50°C ± 2°C.

25 g	gelatine.	10.0%
9 g	glucose.	3.6% or 0.2 Molar
216 g	NaOH soln. + distilled water.	86.4%
250 g		

On commencement of the experiment and at weekly intervals, one sample of each

of the 8 lots (gelatins 155 and 877 at pHs 6.0 and 8.75 both with and without glucose) was taken. From each sample aliquots of 7.15 ml and 2.0 ml were pipetted into sample tubes for colour and fluorescence determinations. Enzyme solution (ES1) was made up by taking 0.52 ml Alcalase 0.6L (Novo Enzymes. Enzymes S.A. (Pty) Ltd. PO Box 651216. Benmore 2010) and diluting it to 200 ml with distilled water in a volumetric flask.

The first tube containing 7.15 ml gelatine solution was diluted by the addition of 3.85 ml freshly prepared ES1 solution containing 0.01 ml Alcalase. The mixture was held at 40°C for 1 hr to complete the hydrolysis reaction. After filtration of the sample through a 0.45 micron membrane filter and using ES1 as the blank, the absorbance was scanned between 400 nm and 700 nm. The area under the absorbance curve after moving the base line to the 700 nm absorbance, was taken as the measure of the gelatine colour.

Note: Gelatine reaction mixture (7.15 ml) contained 0.715 g gelatine and 6.15 ml water. The addition of 3.85 ml ES1 soln. gave a water volume of 10.0 ml. .715/10.715x100 = 6.67% gelatine solution, the chosen concentration for colour determinations.

A control consisting of 0.714 g gelatine, 3.85 ml ES1, and 6.15 ml water, was measured each day to insure that the instrument and procedure were behaving normally. This sample was allowed to swell for 30 min. and dissolved at 40°C. A 1 hr reaction time at 40°C was allowed before the sample absorbance was determined.

The second tube containing 2.0 ml of reaction mixture was diluted with 18.3 ml distilled water for fluorescence determination as detailed above. When the fluorescence intensities increased to >1000 units the instrument ceased to respond and it was decided to dilute the sample to 0.01% by taking 0.1 ml of the above solution and adding this to 10 ml of distilled water. Later the apparent fluorescence of the 1% samples decreased due to concentration quenching as shown by the continued increase in 0.01% solution fluorescence.

Note: Incubated gelatine reaction mixture (2 ml) contained 0.2 g gelatine and 1.7 ml of water which had to be increased to 20 ml for the fluorescence determination at 1% concentration.

Tanaka *et al.* (1988) noted 370/445 nm fluorescence in ribose treated collagen.

hence this pair of wave lengths was also monitored with the glucose treated gelatine.

THE EFFECT OF INCUBATING GELATINE WITH RIBOSE.

1. Preliminary study No 1.

Based on the researches of Tanaka *et al.* (1988) the effects on the absorbance spectrum of gelatine, caused by incubating gelatine solution at pH 5 and 9 with glucose and ribose, were determined.

Two 6.67% gelatine solutions were made up using "Colour Standard" gelatine (Colour 8, Clarity 10) at 45°C. One of these was adjusted from pH 5.0 to 9.0 using approx. 10 ml 0.2N NaOH solution. Three test tubes were filled with 10 ml aliquots of the pH 5 solution and three tubes were filled with 10 ml aliquots of the pH 9 solution. One of the pH 5 and one of the pH 9 tubes was treated with a small amount of dry glucose after which the tubes were inverted several times to achieve solution and mixing. Similarly two tubes were treated with ribose and the two tubes that received no addition acted as controls. The tubes were labelled, capped with aluminium caps and then placed in a 44°C oven. After 7 days and after 14 days 2 ml aliquots were withdrawn from each tube for measurement of absorbance using the Jenway Colorimeter (Jenway Ltd. Dunmow, Essex, UK) and Filter No 2 with water as the blank. These samples were also used to determine the absorption spectrum when required.

2. Preliminary study No 2.

The object of this series of experiments was to observe the effects of the gelatine-ribose interaction at 37°C on colour and other gelatine quality parameters at pH ±5 and pH ±8. The following solutions were made up in quadruplicate in Bloom bottles:

Solution 1 (pH ± 5)

Gelatine 23/2L	7.50g	
Ribose	3.15g	(Tanaka *et al.*, 1988, 0.2M)
Thymol	0.10g*	(arbitrary quantity of preservative)
Distilled water	105.0g	

Solution 2 (pH ± 8)

Gelatine 23/2	7.50g
Ribose	3.15g
Thymol	0.10g*
0.2N NaOH	15 ml (by pipette = 15.09 ± 0.01g)
Distilled water	90.0g (NaOH + water = 105.0g)

* was not immediately soluble at 40°C, but globules disappeared with time at 37°C.

The samples were dissolved at 40°C and then incubated at 37°C. After the required period of incubation, samples were taken and placed in the 10°C Bloom bath for 16 to 18 hr and then the Bloom strengths were measured. The samples were then melted and the other normal quality measurements were made on the solution as well as the absorbance of the 6.67% solution using the Jenway Colorimeter (*loc cit*) with distilled water as the blank.

3. Detailed study.

In this study ribose and gelatine were incubated at 37°C and at pHs 6 and 9 for up to 12 days. Due to the higher reactivity of ribose as found in the preliminary study No 1, incubation was carried out at 37°C and samples were removed from the oven daily for measurement of colour and fluorescence. Due to the lower molecular weight of ribose the following mixtures were made up using gelatines 155/1 (RA @ pH 6, RB @ pH 9) and 877/1 (RC @ pH 6, RD @ pH 9):

Gelatine	25g	
Ribose	7.5g	
Thymol	0.25g	(0.1% as preservative)
Water	150g	
0.28N NaOH	qs	(± 17 ml to pH 6 or ± 45 ml to pH 9)
Water	qs	to 250g total weight.

Controls samples (gelatine 155/1, RE and RF and gelatine 877/1, RG and RH) in which ribose was replaced by water, were made up similarly in order to be able to assess the effects of the experimental conditions on colour and fluorescence.

Pentosidine synthesis.

Solutions of L-lysine hydrochloride and L-arginine hydrochloride (ex Sigma Chemical Corp.) were made up to 0.02 molar concentration in 0.02 molar ammonia solution. (Ammonia was used to neutralise the HCl associated with the amino acid). Similarly 0.02M D-ribose was made up in 0.02M ammonia. Equal aliquots (8 ml) of each solution were mixed in a 50 ml sample bottle and the fluorescence at zero time was determined. The cuvette was closed with a teflon cap and left in the spectrofluorophotometer. The bottle containing the balance of the mixture was sealed with a screw cap and placed in a 37°C oven. A second mixture was made for pH determination. After 3 and 10 days the fluorescence of the samples incubated at room temperature and at 37°C were measured. Furthermore after 10 days, the 37°C sample was diluted 1 to 100 and the fluorescence redetermined.

Anion exchange.

Aliquots of commercial dry gelatine, 120 g, were suspended in 4 l of distilled water and soaked for 30 min. The mixtures were then heated to 40°C in a waterbath with occasional stirring until dissolved.

A 45 mm diameter column was partially filled with 437 ml (Bayer S.A., Isando) Lewatit MP62 macroreticular weak base anion exchange resin and washed with warm ± 60°C distilled water and then fed with the gelatine solution at a rate of 8 to 10 bed volumes per hour using a peristaltic pump. Initially the eluate had a pH of about 8 and this dropped steadily as the amount of liquor treated increased. The treated liquor pH was adjusted to pH 5 to 5.5 with 5N sulphuric acid and then it was concentrated in 4 l lots to about 10% concentration in a vacuum evaporator. The concentrated liquor was filtered through paper pulp, set, cut and dried before grinding to a powder for analysis. The column was used to treat many hundreds of bed volumes of gelatine solution and as the column became exhausted so the pH of the eluate approached the pH of the feed liquor.

Three samples of treated dry gelatine K939, Q939 and F972 were submitted to spectrophotometric and fluorescence analysis for which gelatine 178/12 acted as a control.

After treating some 1200 bed volumes of 3% w/v gelatine solution or 15.7 kg

of gelatine the column was regenerated with 1 l of 5% NaOH solution. The 1 l of regenerant solution collected from the column was very dark. The pH was measured. A 200 ml aliquot required 140 ml N/10 HCl to reduce the pH to 6.0. A 1:50 dilution of the original and the acidified regenerant solutions were examined for fluorescence.

After finding that anion exchange had not affected the fluorescence of gelatine (samples 939 and 972) it was decided to determine whether anion exchange affected the colour of gelatines darkened by reaction with ribose:

A 25 mm diameter column was partly filled with 100 ml Lewatit MP 62 resin. The column was used to treat various (225 g) lots of gelatine solution at a flow rate of 10 to 16 ml/min and a temperature of about 40°C. The absorbance of the gelatine feed to the column and the eluate was determined using the Jenway Colorimeter with filter No 2, and a plastic 1 cm path length cell. In preliminary trials it was found that differences due to different cells made it necessary to use only one cell which was washed three times with aliquots of solution to be tested before a reading was taken. It was noted that absorbance values given by the colorimeter were very different to those given by the Beckman DU 70 spectrophotometer, on the same gelatine.

Gelatine solutions were made using the following formula:

Gelatine	15.0 g
Ribose	6.3 g
Thymol	0.2 g
0.2N NaOH	± 10 ml to pH 6
Water	qs to 225.0 g.

Gelatine solutions with and without ribose were made up in duplicate, in glass jars fitted with screw caps. After soaking for 30 min. and then dissolving at 40°C the sealed glass jars were placed in a 37°C oven. After incubation, the solutions without ribose were cloudy (due to precipitation of salts) hence all the solutions were filtered through Whatman GF/A papers prior to the measurement of their absorbance before anion resin treatment. The remainder of the filtered solution was passed through the anion column and the last 50 ml of eluate was used for measurements of the absorbance after the pHs had been adjusted to 5.0 to 5.5 by adding 3 drops of 5N sulphuric acid. This ensured that absorbance measurements before and after anion treatment were made on solutions of similar pH. The column was washed with warm water between

samples of gelatine.

RESULTS and DISCUSSION.

The detailed results are presented in the Addenda as follows:
Addendum 1. The origins of the gelatines used.
Addendum 2. Detailed analytical data on some of the gelatines used.
Addendum 3. Colour and fluorescence data on all the gelatines used.
Addendum 4. Data on the interaction of gelatine with GLUCOSE.
Addendum 5. Data on the interaction of gelatine and RIBOSE.

A phenomenon encountered working with colloidal gelatine solutions was that the second-order Rayleigh scatter was very pronounced i.e. if the excitation wavelength was 300 nm then the emission spectrum showed a peak at 300 nm due to first order Rayleigh scattering and another at 600 nm due to second-order Rayleigh scatter (Munck and de Francisco, 1989d). Similarly when the excitation wavelength was set to 600 nm a Rayleigh scatter peak was seen at 300 nm.

1. Type B Gelatine fluorescence.

The fluorescence excitation and emission spectra of a pale and a dark Type B gelatine from bovine hide were examined. From the "Multi-spectra" it was clear that between the emission wavelengths of 200 to 700 nm there was significant fluorescence only at excitation wavelengths of about 300 nm and emission wavelengths of about 400 nm. Detailed analysis showed that the excitation maximum was at about 335 nm which gave an emission maximum at 385 nm - see Table 1 below. (Due to quenching a steady, repeatable emission output was not obtained). As could be seen from Figure 4 a pale gelatine (YSA/2). showed a weak emission peak (50 units) at 385 nm and continued lesser emission (49 units) to 410 nm and a dark gelatine INOB/1 (Figure 5) showed a distinct emission peak (169 units) at 385 nm and a second peak (168 units) at 410 nm. (Both emission spectra showed narrow peaks of the same intensity at 360 nm. These Raleigh scatter peaks were caused by the emission scan starting so close to the excitation wavelength of 335nm hence they were ignored).

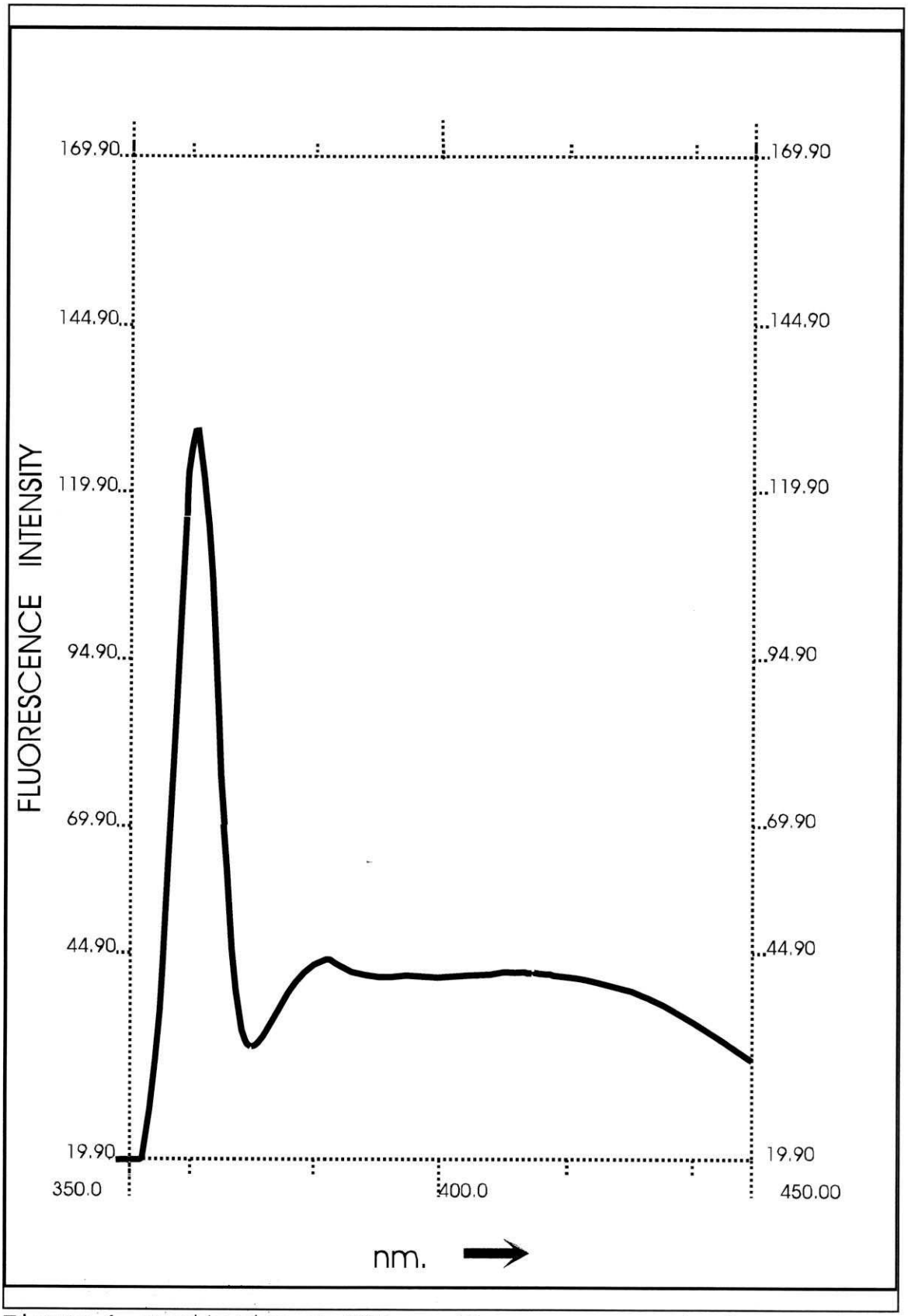


Figure 4. Excitation at 335 nm. Fluorescence emission spectrum of a 1% aqueous solution of a pale (Colour 4.4) Type B gelatine from 10 month old calf skin (YSA/2). Excitation at 335 nm.

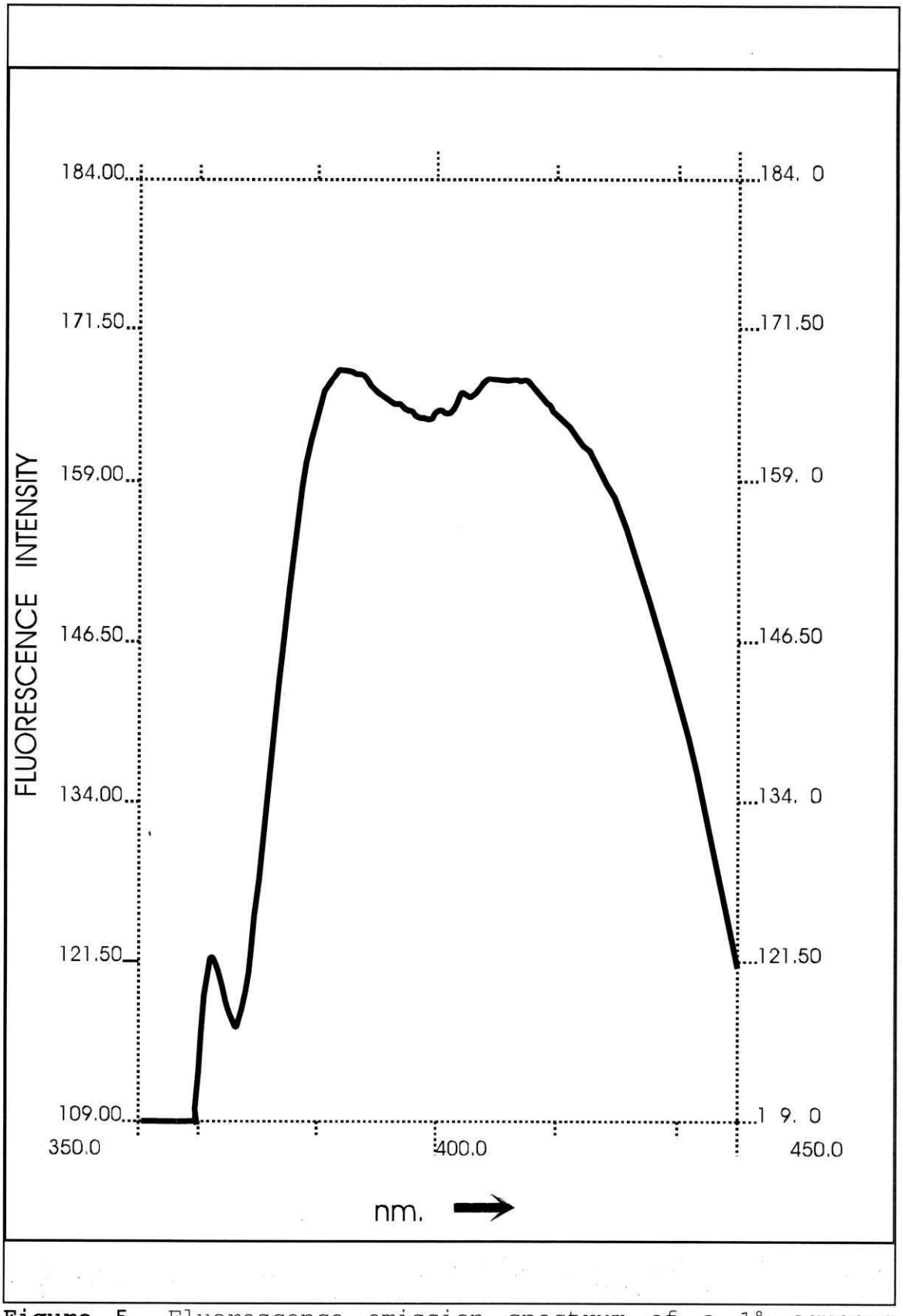


Figure 5. Fluorescence emission spectrum of a 1% aqueous solution of dark (Colour 16.0) Type B gelatine from 12 year old Inguni cow skin (INOB/1). Excitation at 335 nm.

An attempt was made to find (Table 1) the 295/395 pyridinoline cross-link peak reported by Uchiyama *et al.* (1991) for collagen, but this peak was not observed even with the palest gelatine (YSA/1 and YSA/2). Excitation at 295 nm gave emission peaks which bore a striking resemblance to the peaks due to 335 nm excitation only at a slightly lower wavelength, hence they could not be attributed to a different fluorophore. It was concluded that if the pyridinoline cross-link survived the Davis extraction process, its presence would only be detectable after separation from the pentosidine cross-link.

From Addendum 3 it could be seen that linear correlation of all the 335/385 (Maillard) fluorescence data with gelatine colour gave a highly significant correlation coefficient r = 0.78 with 73 degrees of freedom and a correlation equation:

Colour = 0.0066 + 0.0579 x Fluorescence Intensity.

That the value of r was not higher was interpreted as indicating that there was some other factor as well as fluorescence contributing to gelatine colour.

In summary:

Type B gelatine made by the lime-sulphide alkali conditioning process followed by sulphurous acid acidulation had an excitation maximum at about 335 nm and emission maxima at 385 nm and about 410 nm. Both these maxima became stronger and more pronounced with increasing animal age hence it would appear that both were associated with Maillard cross-linking. From the investigations of Sell and Monnier (1989) it was evident that the reaction of lysine, arginine and ribose had not given only one product and from this study it would appear that there could be at least two forms of the Maillard cross-link with similar but not identical structure.

Table 1. *Gelatine fluorescence emission peak data for excitation at 335 and 295nm.*

Sample	Visual Colour	Excitation at 335 nm				Excitation at 295 nm			
		Peak 1		Peak 2		Peak 1		Peak 2	
		nm	Height	nm	Height	nm	Height	nm	Heigh
6Y4B/3	9.5	385	174	410	172	383	106	412	99
6Y4B/2	8.0	387	164	407	160	385	114	-	-
6Y4B/1	6.4	385	183	415	185	380	104	418	98
CT6/2	5.6	388	144	412	147	381	89	410	81
CT6/3	6.0	385	148	415	154	381	88	410	87
3Y4A/1	6.6	385	102	405	99	381	73	-	-
5Y6A/1	6.4	385	118	410	118	378	73	-	-
5Y6A/3	9.4	385	158	415	166	378	90	423	87
5Y6A/2	8.4	385	134	410	139	378	86	410	77
CT6/1	5.2	385	115	-	116	379	83	-	-
3Y4/2A	6.0	385	138	-	-	389	70	405	62
YSA/2	4.4	385	50	410	49	361	34	410	30
YSA/1	3.6	382	41	412	40	361	32	410	25
YSA/3	4.8	385	75	-	-	378	43	410	39
5Y4/3	8.4	385	151	410	156	380	86	420	83
5Y4/2	6.8	385	135	410	136	382	77	415	70
IOB/1	10.0	385	169	410	168	382	112	408	104
IOB/2	16.0	385	207	410	201	385	112	415	110
IOB/3	20.0	385	254	410	249	385	127	428	132
AVERAGE		385.1		410.7		378.9		413.6	

The analytical data on each sample is recorded in Addendum 3.
- indicates that the data could not be read from the graphs.

2. Type A calf skin gelatine.

Tanaka et al. (1988) showed a ribose induced 370/440 nm fluorescence in collagen. In this study there was one instance of this fluorescence occurring naturally and that was with Calf skin Type A gelatines where the 370/440 fluorescence intensity of 121 was many times stronger than the pentosidine

335/385 nm fluorescence of 21. It was observed that the excitation spectrum of gelatine Calf-A/1 monitored at 385 nm (Figure 7) had peaks of 24 units and 21 units at about 330 nm and 290 nm. When monitored at 440 nm there was very much stronger excitation peak (125 units) at 370 nm (Figure 6). For excitation at 335 nm the emission spectrum shows two clear peaks of 22 units at ± 380 nm and 65 units at 440 nm (Figure 8).

The 290 nm excitation maximum in Figure 7 was significant because:

1. This was the only instance of an excitation peak at this wavelength. According to Uchiyama *et al.* (1991) this fluorescence was characteristic of the pyridinoline cross-link and according to Eyre (1987) this cross-link was not found in skin.

2. From the work of Uchiyama *et al.* (1991) it was clear that this fluorescence was caused by the presence of the pyridinoline cross-link which was found here in Type A bovine gelatine but not in Type B gelatine.

It was concluded that both the 295/395 and the 370/440 fluorophores only found in Type A calf skin gelatine were destroyed by lime-sulphide conditioning followed by sulphurous acid acidulation of the collagen but they were not affected by sulphuric acid treatment for the production of Type A gelatine.

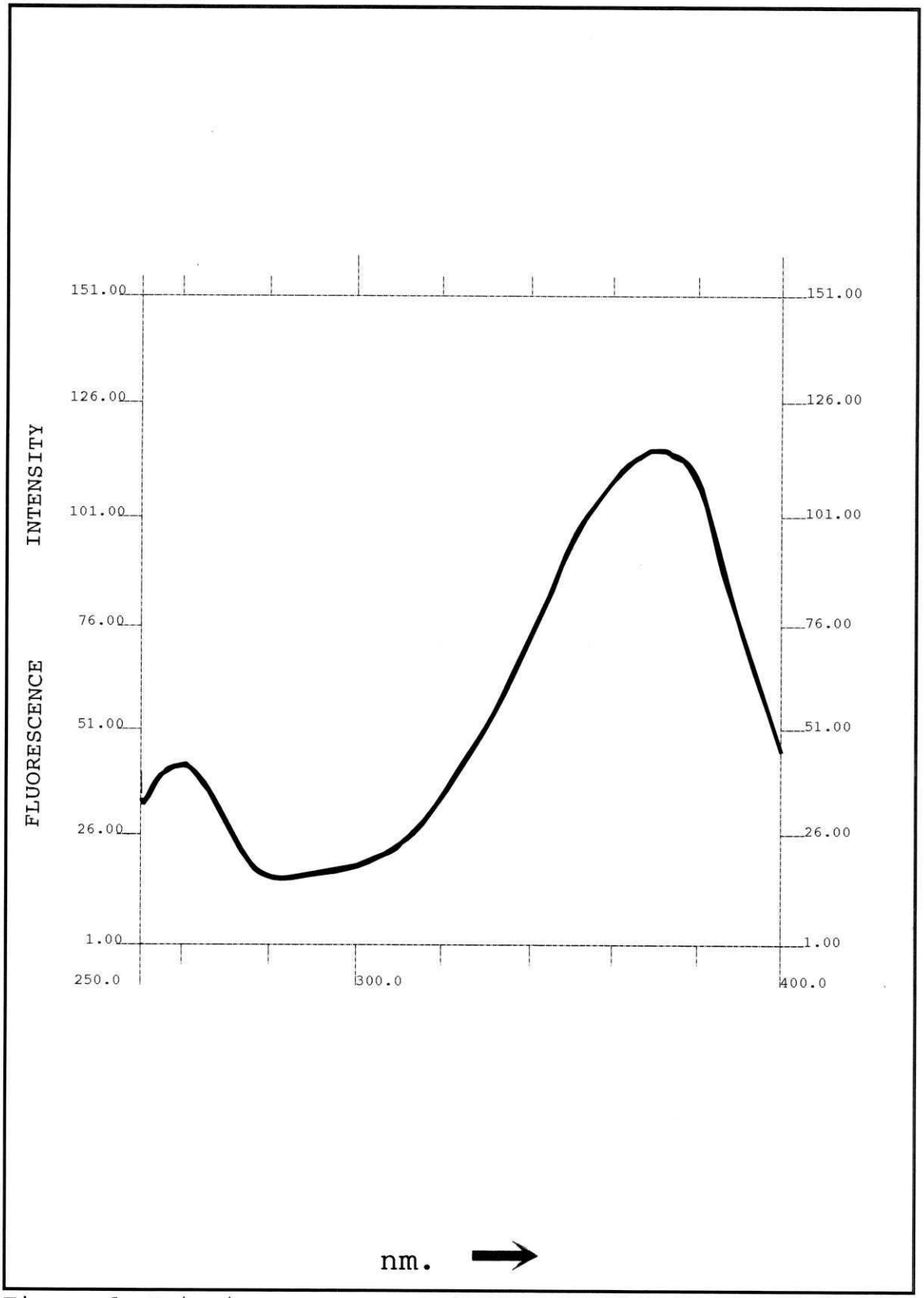


Figure 6. Emission at 440 nm. Fluorescence excitation spectrum of a 1% aqueous solution of Type A calf skin gelatine (Calf Type A/1). Note the excitation maximum at 370 nm.

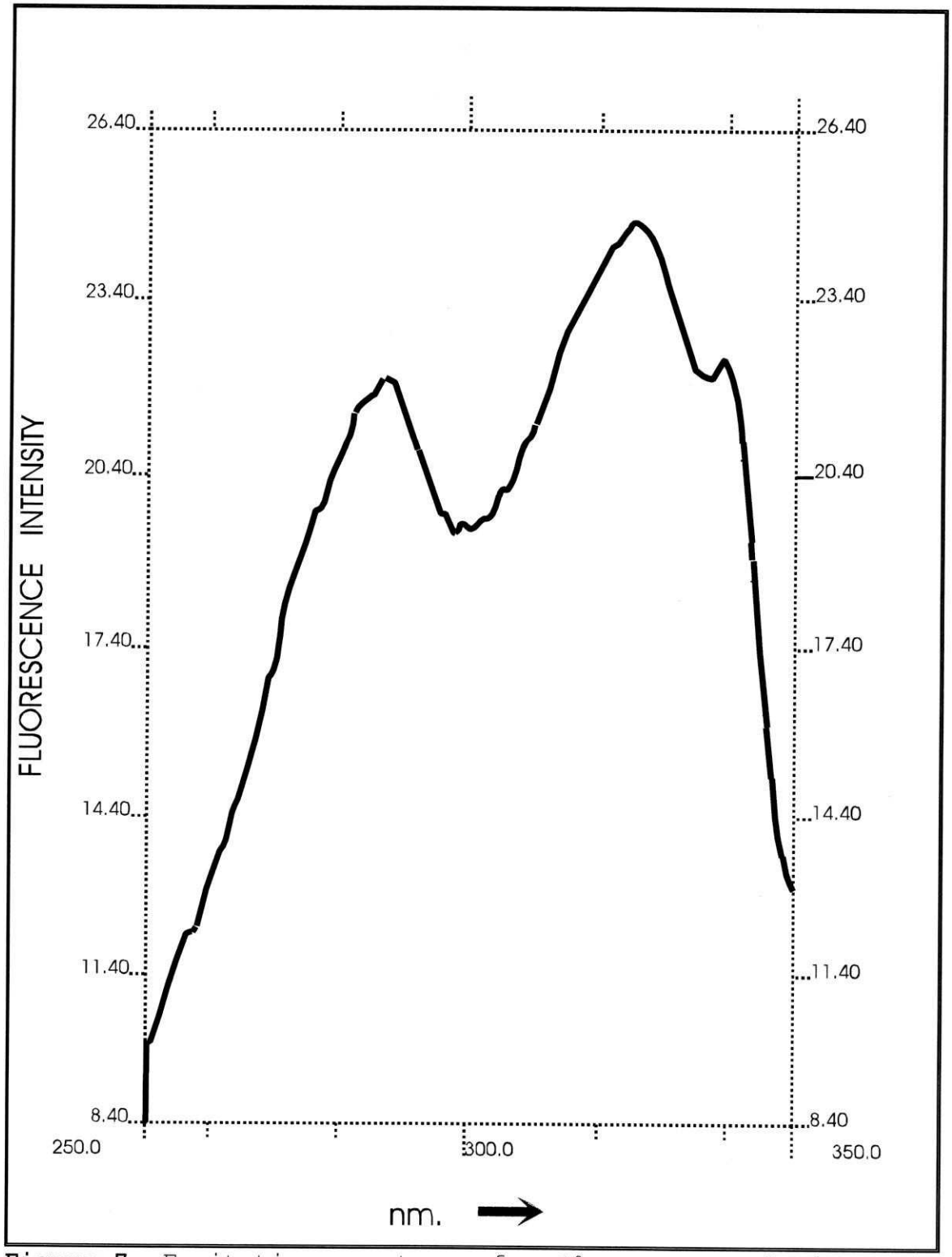


Figure 7. Excitation spectrum of a 1% aqueous solution of a Type A calf skin gelatine. Note - excitation maxima at 290-295 nm and at 335 nm. Emission at 385 nm.

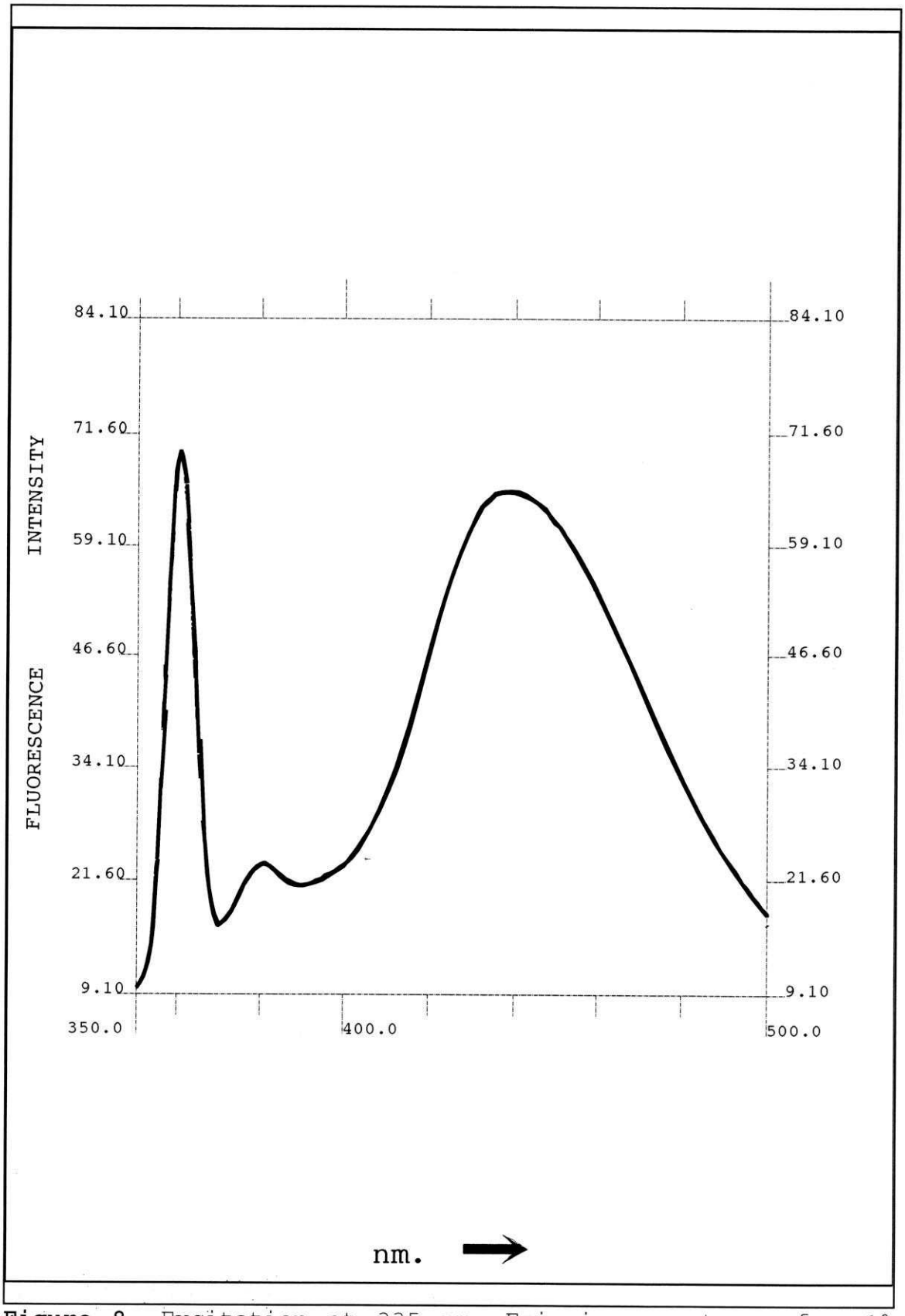


Figure 8. Excitation at 335 nm. Emission spectrum of a 1% aqueous solution of Type A calf skin gelatine. Note - emission maxima at 385 and 440 nm.

3. Pigskin gelatine.

Type A pigskin gelatine was expected to be similar to calf skin Type A gelatine, however, when pigskin gelatines were scanned only one excitation maximum was found at 335 nm (Figure 9) but these gelatines had a stronger emission peak at 420 nm than at 385 nm with only a shoulder to indicate that there was a peak at 385 nm as shown in Figure 10. i.e. the Maillard 335/385 fluorescence seems to be more marked in bovines than in pigs, however the pigskin gelatines (designated D...) had shown a significant (0.005) correlation between colour and pentosidine fluorescence ($r = 0.895$).

4. Fish skin gelatine.

The two fish skin gelatines made by the acid pigskin process also had a stronger fluorescence at 420 nm than at 385 nm but there was no indication of a relationship between colour and pentosidine fluorescence (see Addendum 3.)

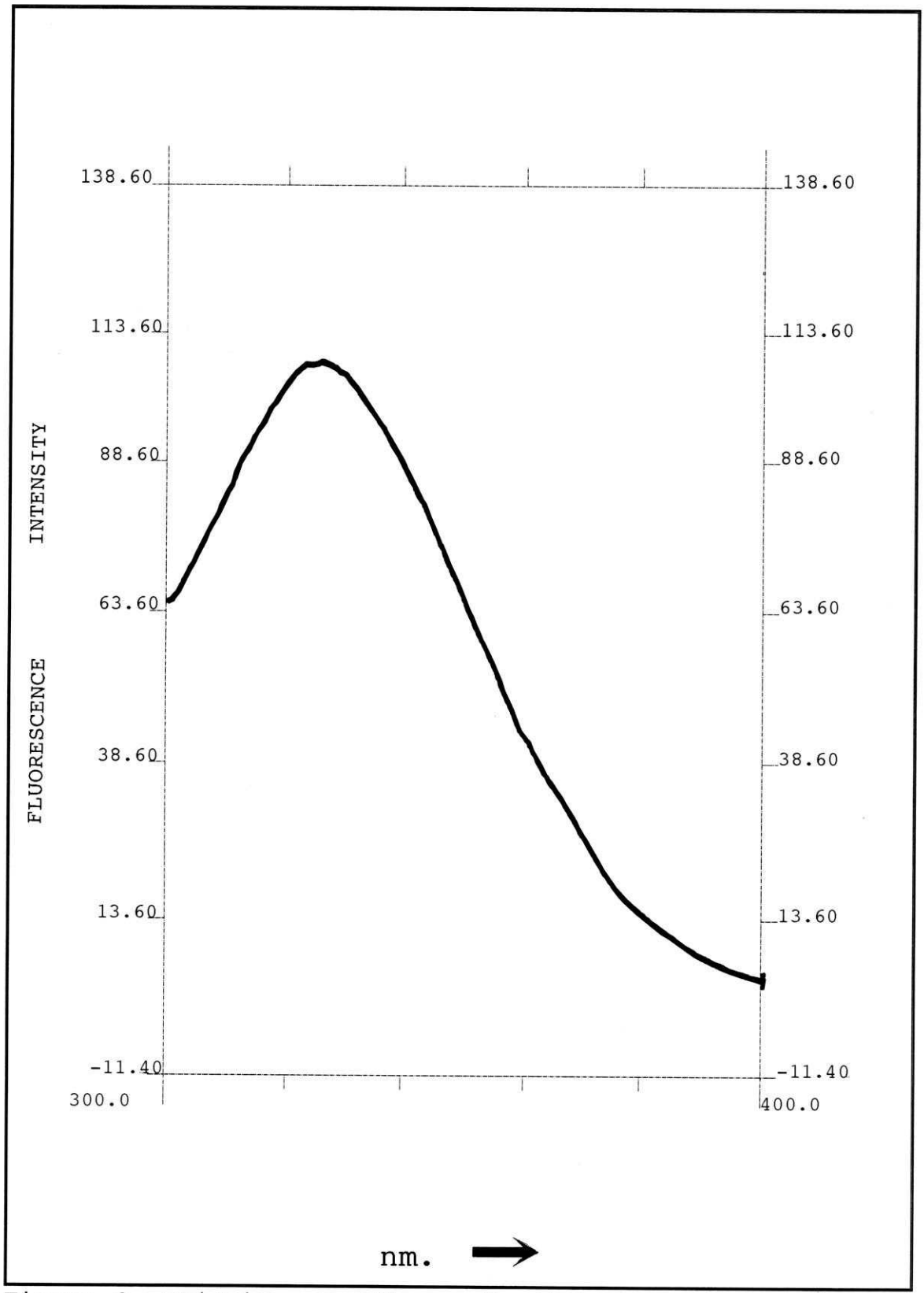


Figure 9. Emission at 420 nm. Excitation spectrum of a 1% aqueous solution of pigskin gelatine (D1512). Note excitation maximum at ± 330 nm.

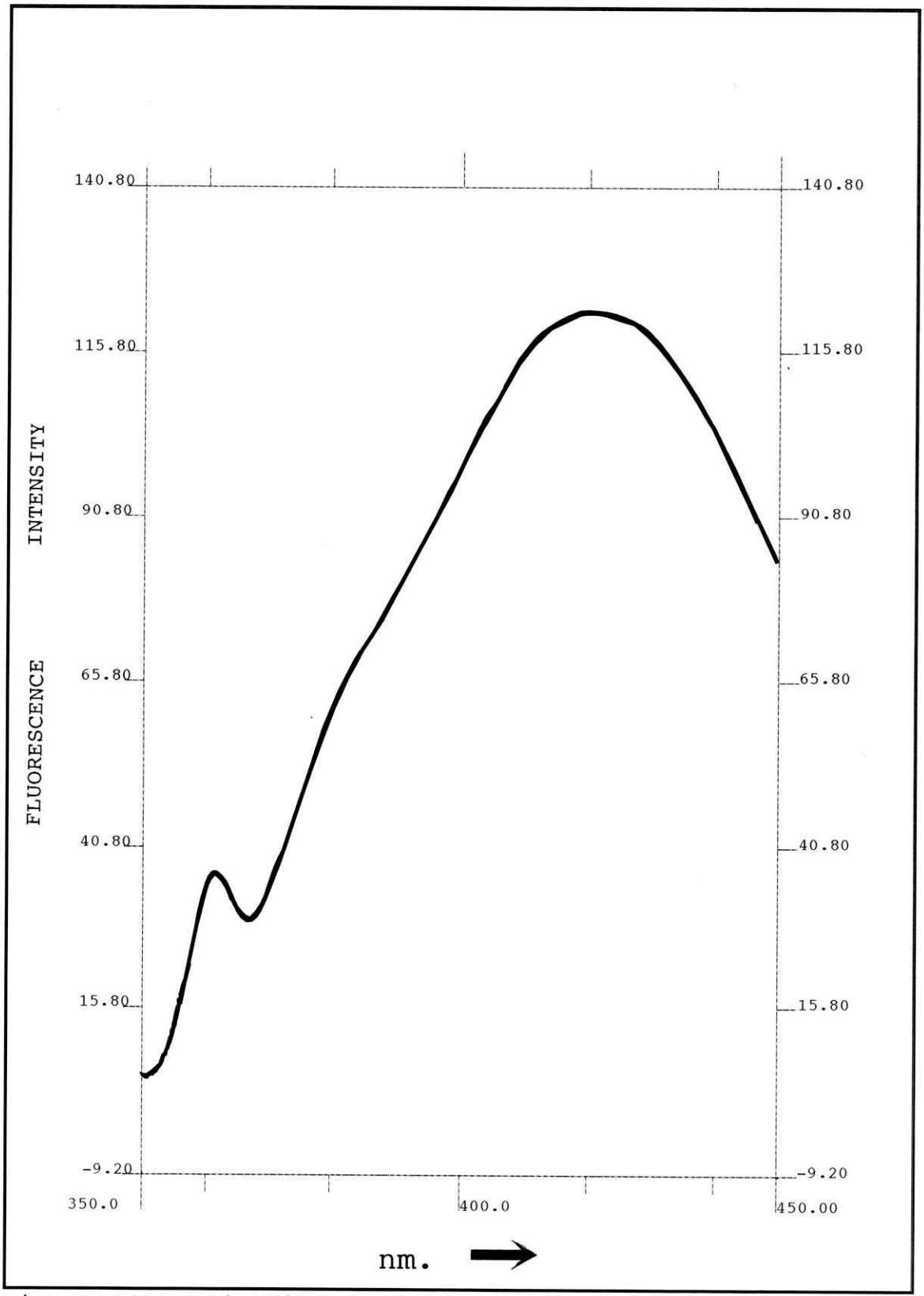


Figure 10. Excitation at 335 nm. Emission spectrum of a 1% aqueous solution of pigskin gelatine. Note shoulder at ± 385 nm and maximum at 410 nm.

Table 2. *Colour and fluorescence data for gelatines derived from animals of known age.*

SAMPLEϕ	ANIMAL AGE MONTHS	OVERALL COLOUR	% EXTRACTABILITY						COLOUR in Davis Units.			Fluorescence Emission Intensity - Excitation @ 335 nm.		
			45°C	50°C	55°C	60°C	70°C	3 RUNS	1ST	2ND	3RD	1ST	2ND	3RD
YSA	10	4.3	35	26	20	-	-	83	3.6	4.4	4.8	41	50	75
CT6	18	6.4	40	32	23	-	-	95	5.2	5.6	6.0	115	144	148
5Y4	58	7.2	20	21	-	-	-	70	5.6	6.8	8.4	104	126	140
5Y4	58	7.2	20	21	-	-	-	70	5.6	6.8	8.4	94	129	142
5Y6A	58	8.2	21	21	-	-	-	72	6.4	8.4	9.4	118	134	158
5Y6A	58	8.2	21	21	-	-	-	72	6.4	8.4	9.4	115	128	148
ST12	144	18.1	4	7	-	-	-	29	6.4	10.0	10.7	105	124	167
ST16	144	13.1	12	17	-	-	-	55	10.7	12.3	14.5	174	192	228
INOB	143	(21.7)		9	-	20	27	57	16.0	16.0	20.0	169	207	254
6Y4B	78	7.3	17		21	33	-	71	6.4	8.0	9.5	183	164	174
YSA*	10	4.3	35	26	20	-	-	83	3.6	4.4	4.8	38	48	68
CT4*	18	6.4	23	28	28	-	-	76	5.6	5.6	5.6	104	133	142
5Y4*	58	7.2	20	21	-	29	-	70	5.6	6.8	8.4	104	126	149
ST24*	144	15.0	10	13	-	26	-	49	8.9	12.3	13.3	133	168	214
WT4*	144	16.3	4	8	-	17	-	29	11.4	12.3	13.3	114	130	172
INOE*	143	14.9		11	-	23	37	72	9.4	12.3	16.0	175	207	NA
CTO4*	156	16.4	10	11	14	-	-	35	10.0	9.4	10.0	187	223	239
3Y4A*	40	5.2	28	30	-	-	-	-	6.6	6.0	-	102	138	-
6Y4A*	78	7.9	15	21	-	34	-	70	8.4	7.6	8.9	141	165	174

ϕ See Addendum 2 for details of origins etc.
* Samples had equal conditioning - 4 weeks, except for YSA.
() Not included in the correlation due to process differences.
- Not applicable.

Table 2 contains the colour and fluorescence data for the gelatines extracted from the skin of animals of known age enabling statistical analysis for linear correlations between animal age, colour and fluorescence.

Correlations:

1. Animal Age & Overall Colour.

   Samples *. $r = 0.976$, $df = 6$, Sig. > 0.01

   Samples All. $r = 0.953$, $df = 15$, Sig. > 0.01

2. 45°C Extractability & 1st Colour.

   Samples *. $r = -0.933$, $df = 5$, Sig. > 0.01

   Samples All $r = -0.779$, $df = 14$, Sig. > 0.01

3. 50°C Extractability & 2nd Colour.
   Samples *. r = -0.915, df = 5, Sig. > 0.01
   Samples All r = -0.816, df = 9, Sig. > 0.01

4. 1st Colour & 1st Fluorescence.
   Samples All. r = 0.6934, df = 15, Sig. > 0.01

5. 2nd colour & 2nd Fluorescence.
   Samples All. r = 0.6979, df = 16, Sig. > 0.01

6. 3rd Colour & 3rd Fluorescence.
   Samples All. r = 0.3852, df = 14, Not Significant.

The above shows that there were significant correlations between animal age and overall colour, between gelatine 1st and 2nd run extractability and colour, and between 1st and 2nd extraction gelatine colour and pentosidine fluorescence intensity. In other words, the Maillard cross-linking as determined by fluorescence intensity affected hide extractability and gelatine colour similarly - a not unexpected observation, and it did confirm that, to an extent, colour was a part of the protein and not a removable contaminant.

EFFECT OF INCUBATING Gelatines WITH GLUCOSE.

This was the first experiment of this type that was conducted, hence it showed some of the characteristics of a preliminary experiment in that experimental determinations were added after the start of the experiment to analyse or explain the emerging data.

The detailed results of the monitoring of the colour, fluorescence and pH of the gelatine-glucose solutions with starting pHs of 6 and 8.75 are given in ADDENDUM 4. These results are summarised in Figure 11.

Glucose gelatine interaction - Effect on Colour.

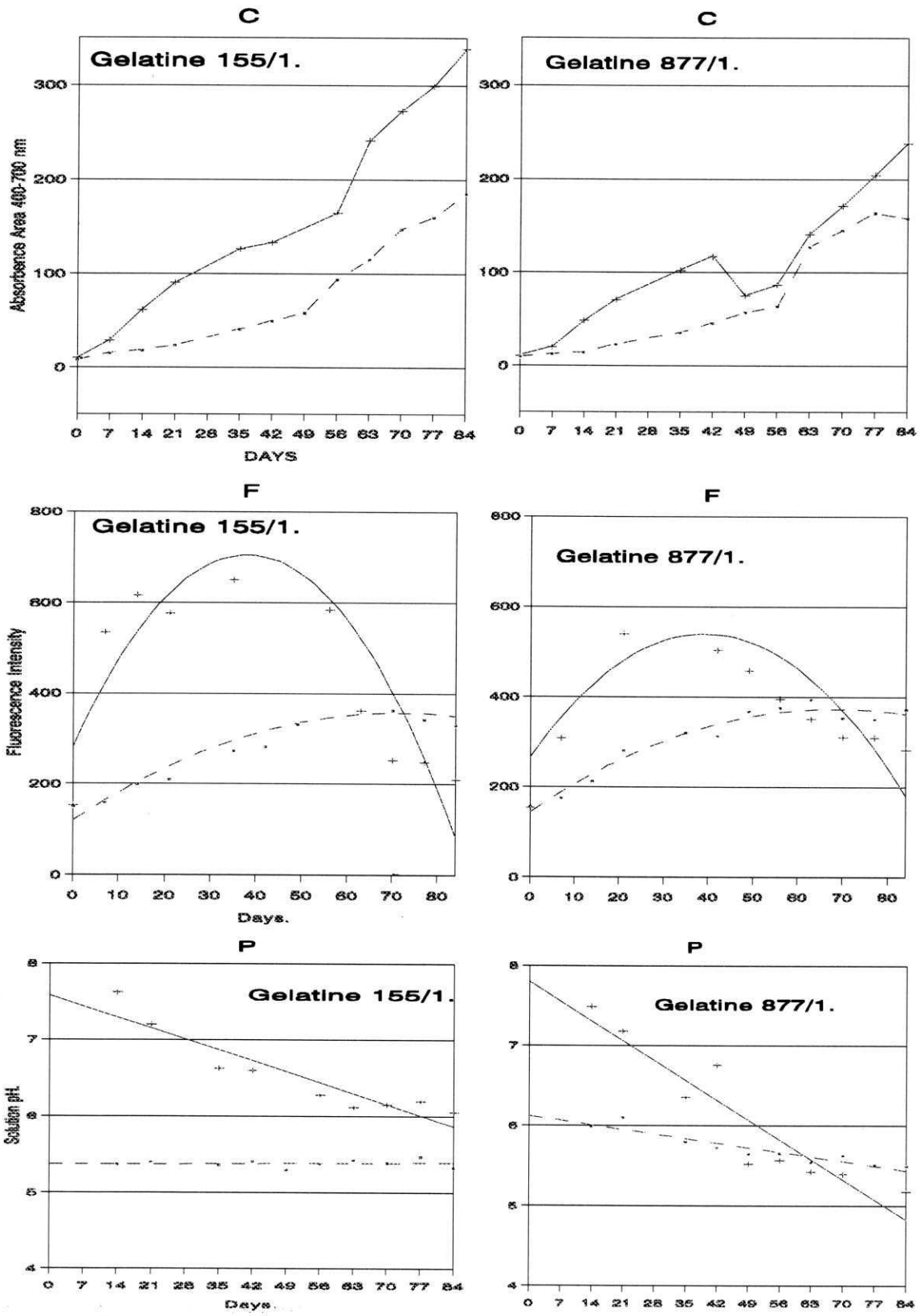
In the previous section it was shown that the Gardner instrument gave the best correlation between instrumental and visual colour measurements. The Gardner instrument was however not available for this series of experiments, hence, the alternative colour measurement using a spectrophotometer and measuring the area under the curve between 400 nm and 700 nm had to be used.

The apparent "lag phase" to the development of colour in the gelatine-glucose solutions, particularly with the samples starting at pH6 was noted. It was also apparent that the starting pH of 6 resulted in an overall lesser development of colour than with a starting pH of 8.75.

A comparison of the absorbance spectra of a naturally pale gelatine 155/1 and a naturally dark gelatine 178/12 and glucose darkened gelatines 155/1 and 877/1 are shown in Figure 12. These spectra show a smooth increase in absorbance from the light 155/1 to the darkest 178/12 over the whole spectral range which would tend to confirm the contention that pentosidine and gelatine colour originate from glucose *in vivo* (Dyer *et al.*, 1991).

Finally, the two gelatines 155/1 and 877/1 behaved similarly but not identically. After 84 days gelatine 877/2 and 155/1 had produced similar amounts of colour at pH 6 as evidenced by the 400 nm absorbances of 2.11 and 2.36, but at pH 9 the colour produced by gelatine 155/1 was an order of magnitude greater (with a 400 nm absorbance of 4.68) than was the colour produced by gelatine 877/1 (with a 400 nm absorbance of 3.01).

It was also noteworthy that the 400 nm absorbance was, as with colour, closely related to the area under the absorbance curve between 400 and 700 nm (r=0.999) as shown in Table 3.



The effects of gelatine glucose interaction on (C) colour, (F) fluorescence intensity and (P) pH at starting — pH 8.75 and - - pH 6. Gelatines 155/1 & 877/1 had similar gel strength and colour.

Figure 11. Gelatine glucose interaction. (10% w/w gelatine in 0.2M aqueous glucose solution incubated at 50°C).

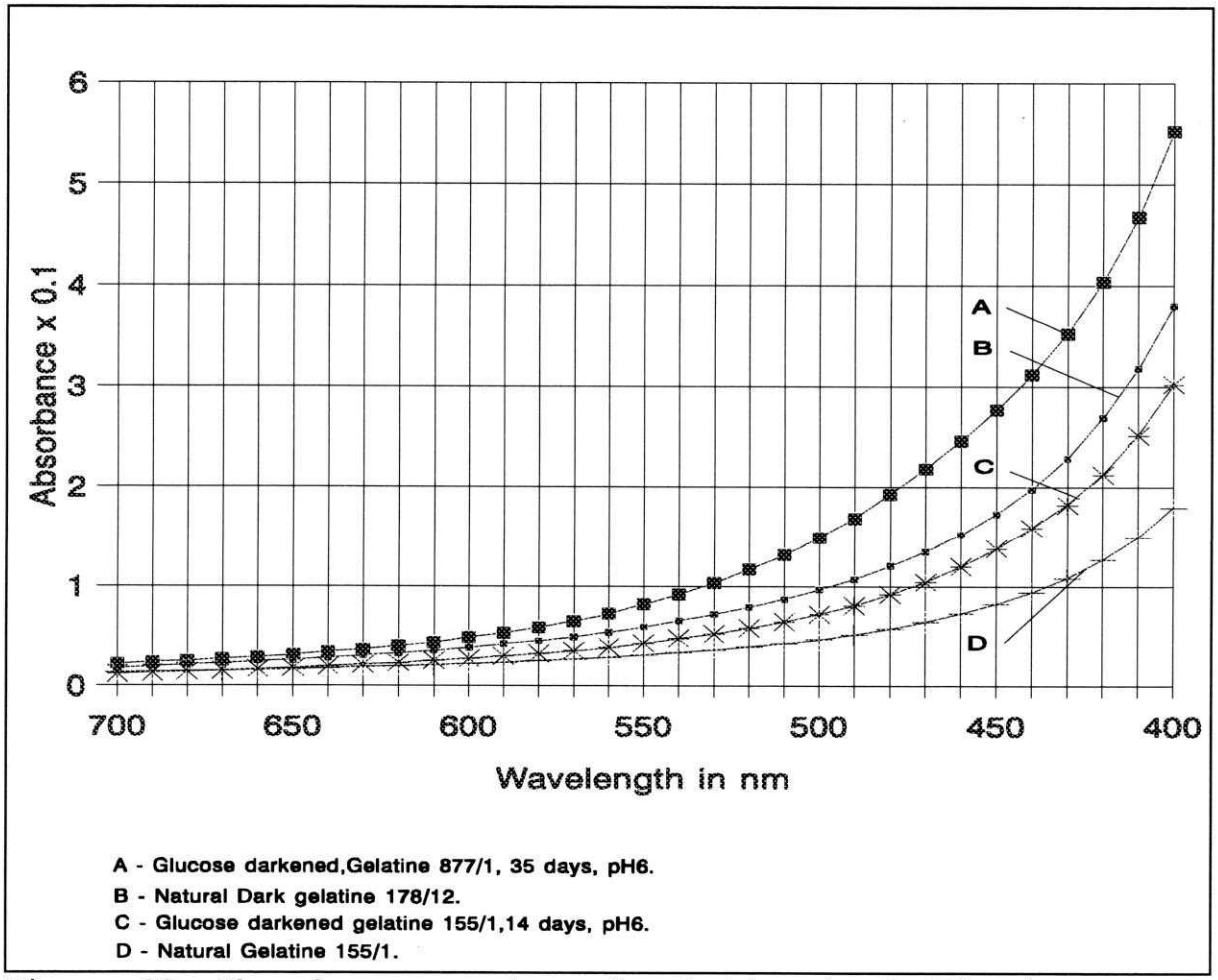


Figure 12. Absorbance spectra of gelatine in 6.67% w/v aqueous solution. Glucose darkened by incubating 10% w/w gelatine in aqueous glucose solution (0.2M) at 50°C.

Table 3. *A comparison of the area under the absorbance curve between 400 and 700 nm and the absorbance at 400 nm for glucose darkened gelatine 155/1 starting at pH 9.*

Absorbance at 400 nm	Curve Area 400 to 700 nm.	Absorbance at 400 nm	Curve Area 400 to 700 nm.
0.18	10	0.5	28
0.94	61	1.35	90
1.89	126	2.31	164
3.74	272	4.68	339

Absorbances of > 4.7 were outside the range of the instrument.
see ADDENDUM 4, Sample B.

Glucose gelatine interaction - Effect on Fluorescence.

The increase in 335/385 nm fluorescence intensity of the gelatine-glucose solutions with time was evident from Figure 11. As with colour, there was a markedly greater production of fluorophore in solutions with a starting pH of 8.75 than in solutions with a starting pH of 6.

The eventual decrease in fluorescence intensity with time, which was particularly noticeable with the solutions starting at pH 8.75, was typical of concentration quenching (Munck and de Francisco, 1989e). From the continuously increasing fluorescence intensities of the 0.01% gelatine solutions it was concluded that concentration quenching was the reason for these observations.

In Figure 11 the fluorescence v/s time data is shown as curves derived using second order polynomial regression with the following correlation coefficients (n = 12):

Gelatine 155/1 at pH 6. r = 0.94
Gelatine 155/1 at pH 8.75. r = 0.81
Gelatine 877/1 at pH 6. r = 0.97
Gelatine 877/1 at pH 8.75. r = 0.77

Tanaka et al. (1988) noted 370/445 nm fluorescence in ribose treated collagen. Hence, these wavelengths were also monitored. After 8 weeks, no excitation maximum other than that at about 350 nm could be observed, as shown in Figure 13. Hence, the data for these wavelengths has not been recorded. The apparent movement of the excitation maximum from 335 nm was due to observation of the emission at 420 nm instead of 385 nm.

Glucose gelatine interaction - Effect on pH.

It is evident from Figure 11. that there were marked changes in the pH of the solutions with time. A change in solution pH would be expected to result from the reaction of glucose with the amine side chains of lysine and arginine but such a pH change would be expected to be smooth and consistent even if not linear. From the graphs it is evident that the changes in solution pH were somewhat erratic. For example gelatine 155/1 starting at pH 8.75 had, by day 49, a pH of 5.40, whereas at day 56 the pH was 6.27. It was concluded that

this variance could only be attributed to experimental error. As the pHs of the daily standards were constant it appeared that this error had to be due to inadequate mixing after pH adjustment at the start of the experiment (possibly due to the onset of gelation). Similarly the variances in the gelatine 877/1 pHs (starting at pH 8.75) indicate that there was also considerable "experimental error" associated with the results. Measurement of solution pH was not part of the original experimental plan, hence it was not easy to show inconsistencies in pH resulting in inconsistencies in fluorescence or colour, however, the fluorescence maximum for gelatine 877/1 at 35 days coincided with an anomalously low solution pH. This high fluorescence value could be the result of a lower concentration of fluorophore resulting in less quenching.

Glucose gelatine interaction - General observations.

As a general observation it was noted that after about 28 days at 50°C the solutions in the Mc Cartney bottles removed from the incubator had not geled at room temperature. In addition, there was no sign of gelation of the incubated samples as was observed with the ribose interaction. From experience, this was probably due to the effects of thermal degradation at 50°C being greater than those of cross-linking. There was a possibility that the loss of gelling properties could have been due also to microbiological activity. Three of the four controls, after 61 days, (samples F, G, H) exhibited cloudiness. However, these samples were not malodorous, so the cloudiness could have been due also to thermal degradation of the gelatine resulting in precipitation of $CaSO_4$ ash.

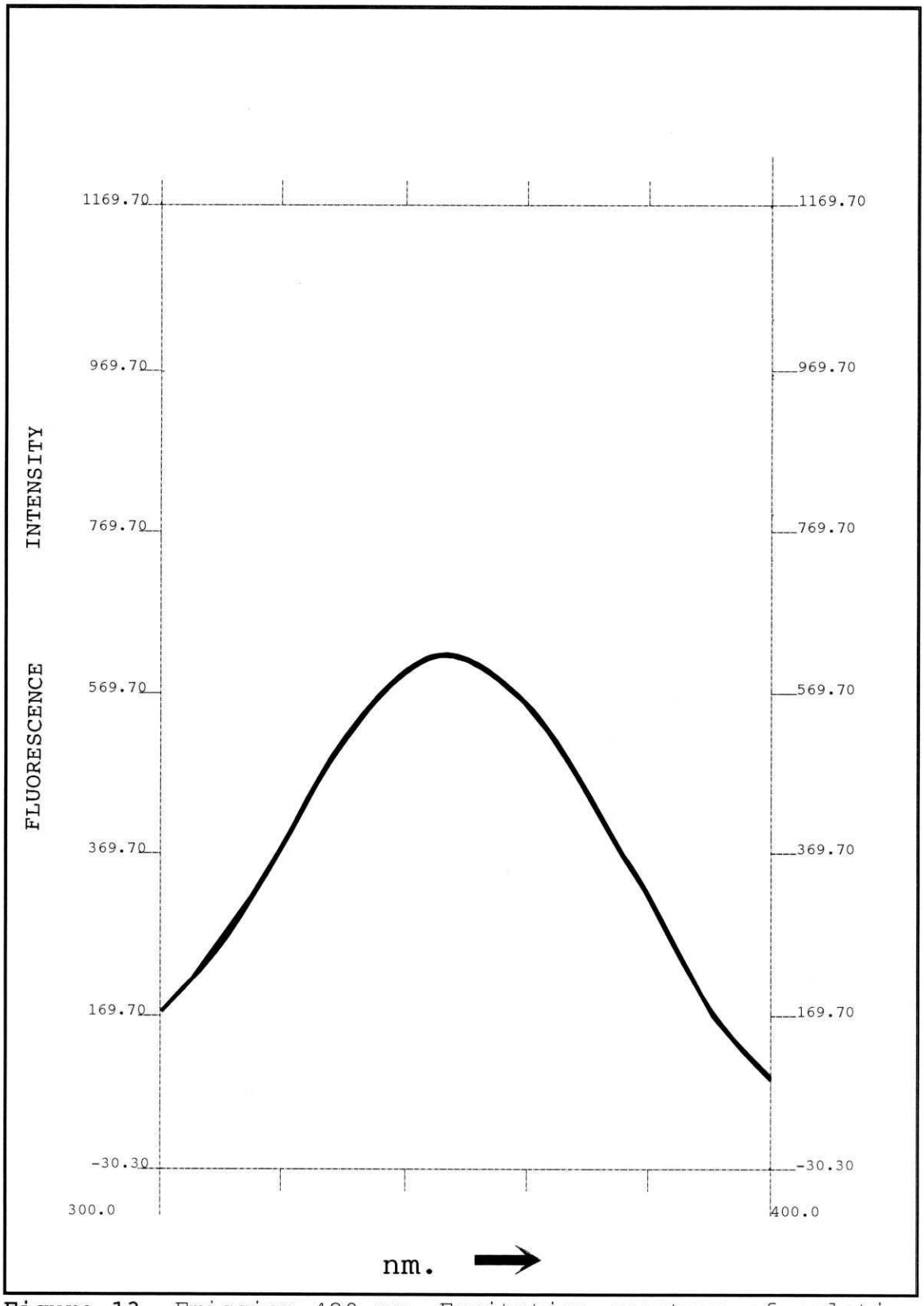


Figure 13. Emission 420 nm. Excitation spectrum of gelatine (155/1) after darkening with glucose for 56 days at 50°C (initial pH = 6.0)

Glucose gelatine interaction - Controls.

As can be seen from the data on the Controls in ADDENDUM 4, Gelatine solutions at pH 6 and 8.75 showed a small increase in colour or area under the absorbance curve of the order of 2 absorbance units at the start of incubation at 50°C whereafter the colour increased slowly by an additional 4 units of absorbance over some 60 days of incubation. In contrast the glucose treated gelatine increased in area under the absorbance curve by some 150 to 300 units, hence, this large increase could not be due to "natural" darkening under the experimental conditions. In other words some 1% of the darkening observed with the glucose - gelatine interaction could have been due to the "natural" darkening of gelatine when it was incubated under the same conditions. It was also noteworthy that whereas glucose treated gelatine exhibited more darkening in the solutions starting at pH 8.75 than those starting at pH 6, the reverse was observed in the absence of glucose.

The fluorescence intensity of the controls increased very slightly with time of incubation and solution pH decreased very slowly with time. These results could be interpreted as indicating that the gelatine contained a small amount of Amadori reaction products or sugar which could continue with the Maillard reaction in solution yielding a small amount of colour and fluorescence. However, gelatine hydrolysis at 50°C would be expected to also contribute to variance in pH.

Glucose caramelization.

For the sake of completion it was decided to establish whether glucose alone underwent any significant changes caused by the experimental conditions. From Table 4 it is clear that there was no caramelization of glucose. The apparent change in fluorescence intensity was well within the limits of experimental error and therefore negligible. The glucose solution pHs were 6.47 and 7.87, respectively, at the end of the test period. The differences from the starting pHs were noted but this did not affect the conclusions that glucose alone had not changed colour or degree of fluorescence under the conditions of the experiment.

Table 4. *The effect of 50°C incubation on a 3.6% glucose solution, at pH 6 and pH 8.75, on the colour and fluorescence intensity of the solutions.*

	FLUORESCENCE INTENSITY			
WEEK No.	0	6	9	14
pH 6	2.0	1.4	4.2	3.0
pH 8.75	2.0	1.4	2.4	3.0
	C O L O U R			
WEEK No.	0	6	9	14
pH 6	1.0	0.8	0.9	0.5
pH 8.75	0.9	0.7	0.8	0.4

THE EFFECT OF INCUBATING GELATINE WITH RIBOSE.

Ribose is a pentose sugar, hence it forms a ring structure that is less stable than the gluco-pyranose ring structure and for this reason, the pentoses had a greater proportion of molecules in the reactive aldehyde form which would presumably account for the greater Maillard reactivity of ribose (Tanaka *et al.*, 1988).

The reasons for choosing pH 9 for use in the preliminary study No1 was because it was known that the reaction of dilute gelatine solution with formaldehyde, in the Formol titration, was conducted at pH 9 because the reaction with formaldehyde was instantaneous at this pH (Cobbet *et al.*, 1963). Also, from experience in the cross-linking of gelatine with formaldehyde (to form an ether cross-link) it was known that the reaction was very fast at pH 9 while at pH 5 the mixture takes many hours before forming an insoluble gel. Hence, the Maillard reaction with sugar aldehyde would probably be facilitated or accelerated at pH 9.

Preliminary study No 1.

Table 5. *The changes in absorbance of gelatine treated with reducing sugars monitored using a Colorimeter and filter No. 2 (470 nm).*

Treatment	Weeks at 44°C	Absorbance.	
		pH5	pH9
Blank	1	0.097	0.089
Glucose	1	0.103	0.101
Ribose	1	0.146	0.176
Blank	2	0.137	0.145 *
Glucose	2	0.131	0.122 ϕ
Ribose	2	0.417	0.289

* Solution became cloudy with a precipitate probably due to bacterial growth.
ϕ Solution only just gelled at 17°C. All other solutions were strongly gelled at 17°C after two weeks at 44°C.

From Table 5 it was concluded that:

(i) Ribose caused marked darkening of gelatine while glucose did not appear to react. This was in line with common practice of using glucose in gelatine confectionery.

(ii) Although pH 9 seemed to accelerate the rate of colour production with ribose after one week's incubation. this trend seemed to be reversed after incubation for two weeks.

(iii) It was noted that the use of aluminium caps on test tubes did not prevent evaporation which could account for the darkening of the blank after two weeks incubation.

Table 6. *The absorbance characteristics of a pale and a dark gelatine for comparison with the colour of ribose treated gelatine.*

Filter nm.	GELATINE			
	Colour = 8	Colour = 20	Ribose treated @ pH 5 *	Ribose treated @ pH 9 *
400	0.080	0.145	0.347	0.266
470	0.086	0.153	0.417	0.289
490	0.074	0.130	0.328	0.236
520	0.058	0.103	0.220	0.174
540	0.048	0.081	0.149	0.129
580	0.039	o.064	0.106	0.097
600	0.031	0.049	0.070	0.065
710	0.017	0.034	0.041	0.037

*Colour 8 gelatine treated with ribose for 2 weeks at 44°C.

From Table 6 it was concluded that the colour production due to the Maillard reaction had absorbance characteristics similar to that of ordinary dark gelatine. It was evident also that colours darker than Davis Colour 20 were quite possible.

Preliminary study No 2.

Table 7. *The effect of ribose treatment on the physical properties of gelatine at pH 5 and 8.*

Property	Solution 1. pH 5.			Solution 2. pH 8.		
	Days at 37°C.			Days at 37°C		
	0	3	6	0	3	6
Bloom	233	195	188	229	170	168
Colour	7.6	8.9	26.7	11.4	22.8	>32
Clarity	11.1	11.8	12.5	11.8	12.5	13.3
pH	5.5	5.3	5.3	8.4	7.9	7.5
Viscosity	34	33	31	39	44	48
Absorbance with Filter No.						
1	0.058	0.076	0.263	0.068	0.256	0.433
2	0.061	0.080	0.277	0.073	0.295	0.539
3	0.050	0.067	0.231	0.060	0.255	0.492
4	0.041	0.052	0.179	0.041	0.180	0.358
5	0.032	0.039	0.140	0.029	0.108	0.219
6	0.022	0.031	0.117	0.020	0.065	0.133
7	0.016	0.026	0.097	0.010	0.039	0.071
8	0.010	0.015	0.080	0.008	0.019	0.035

From Table 7 it was observed that there was:

1. Markedly faster darkening at pH 8 than at pH 5.
2. Increasing viscosity with time indicating cross-linking at pH 8.
3. A drop in pH with incubation time for the pH 8 sample. The source of acid was not evident, however, the removal of amine side chains due to the Maillard reaction would be expected to have had the same effect.
4. Such a fast reaction at pH 8 that there was some darkening during the time required for the Bloom determination, as evidenced by Solution 2 with a colour of 11.4 at time 0. Solution 1 had a colour of 7.6 at the same time and the original colour of the gelatine was 8.6.

5. A large change in the short wavelength absorbance, using the No 2 filter, due to (the Maillard) reaction but there was relatively little change in the No 8 filter long wavelength absorbance.

Detailed study - Overview.

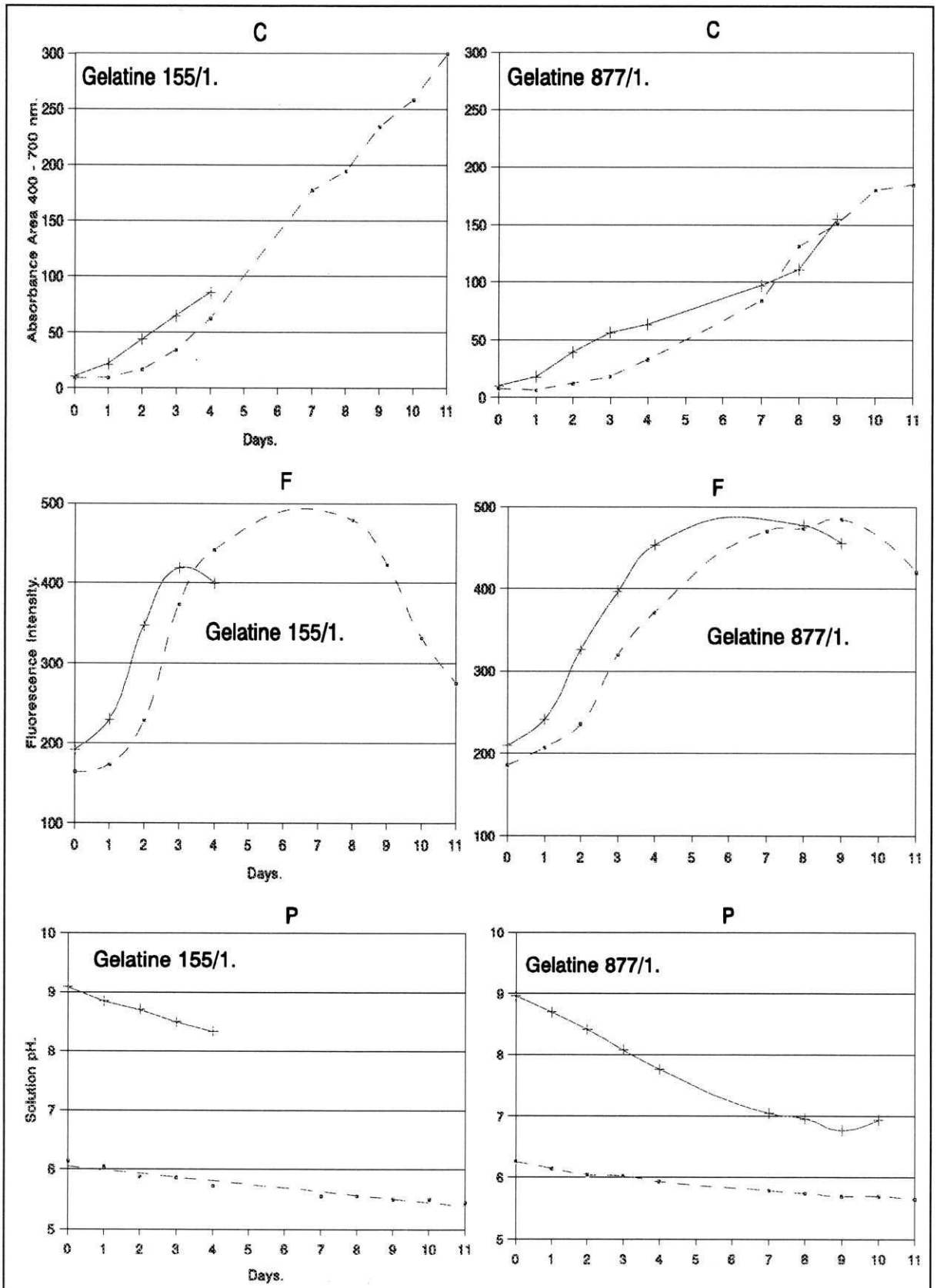
In this study gelatine and ribose were incubated at 37°C and at pH 6 and 9 for up to 12 days with controls. The solution colour, pH, and fluorescence were monitored. The detailed data is given in ADDENDUM 5, and Figure 14 shows the most pertinent results.

Ribose gelatine interaction - Gelation.

All four of the ribose treated gelatines gelled during the course of the trial. Gelatine 155/1 at pH 9 gelled between the 4th and 7th days. Gelatine 877/1 at pH 9 gelled on the 10th day and both gelatines at pH 6 gelled between the 11th and 14th days. The gels were not "insoluble" like formalin induced gels, and they could be diluted with warm water to produce apparently normal solutions. They could not, however, be pipetted so the results obtained on gelatine 877/1 on day 10 that were obtained by taking 7.2 g and 2.0 g of sample and diluting them appropriately for colour and fluorescence determinations, must be considered suspect.

Ribose gelatine interaction - Induced colour.

The development of colour due to the Maillard reaction was not the same for the two different gelatines. The gelatines chosen for this study were normal gelatines of similar colour and from the start of extraction. The difference in Maillard reaction colour development must be primarily a function of differences in the availability of amine groups for the reaction. As amine groups were involved in all known cross-linking reactions, for example, Kuypers, Tyler, Kurth and Horgan (1994), it was of interest to note that not all reactions at the lysine side chains led to colour development. Conversely it was concluded that differences in the cross-linking of collagen could limit the development of colour due to the Maillard reaction.



The effects of gelatine ribose interaction on (C) colour, (F) Fluorescence intensity and (P) solution pH, at starting — pH 9 & - - pH 6. Gelatines 155/1 & 877/1 had similar gel strength and colour.

Figure 14. Gelatine ribose interaction. (10% gelatine w/w in 0.2M aqueous ribose solution, incubated at 37°C).

It was also noteworthy that although colour development during days 1 to 4 was faster at pH 9 than at pH 6, eventually the pH 6 solution colour caught up with and outstripped the pH 9 colour which solved the apparent anomaly observed in the "Preliminary Study No 1" above. Furthermore, the development of colour in Figure 14 showed a distinct "lag phase" at pH 6 which was hardly visible at pH 9, particularily with gelatine 155/1.

It was noted that the absorbance spectra of the ribose treated gelatines exhibited an anomaly at about 470 nm when compared to normal gelatine absorbance spectra. This resulted in the ribose containing reaction mixtures having a distinctly reddish tinge when compared to normal or glucose treated gelatine as shown in Figure 15 below. Towards the end of the reaction time however, the solutions became too dark for the reddishness to be evident.

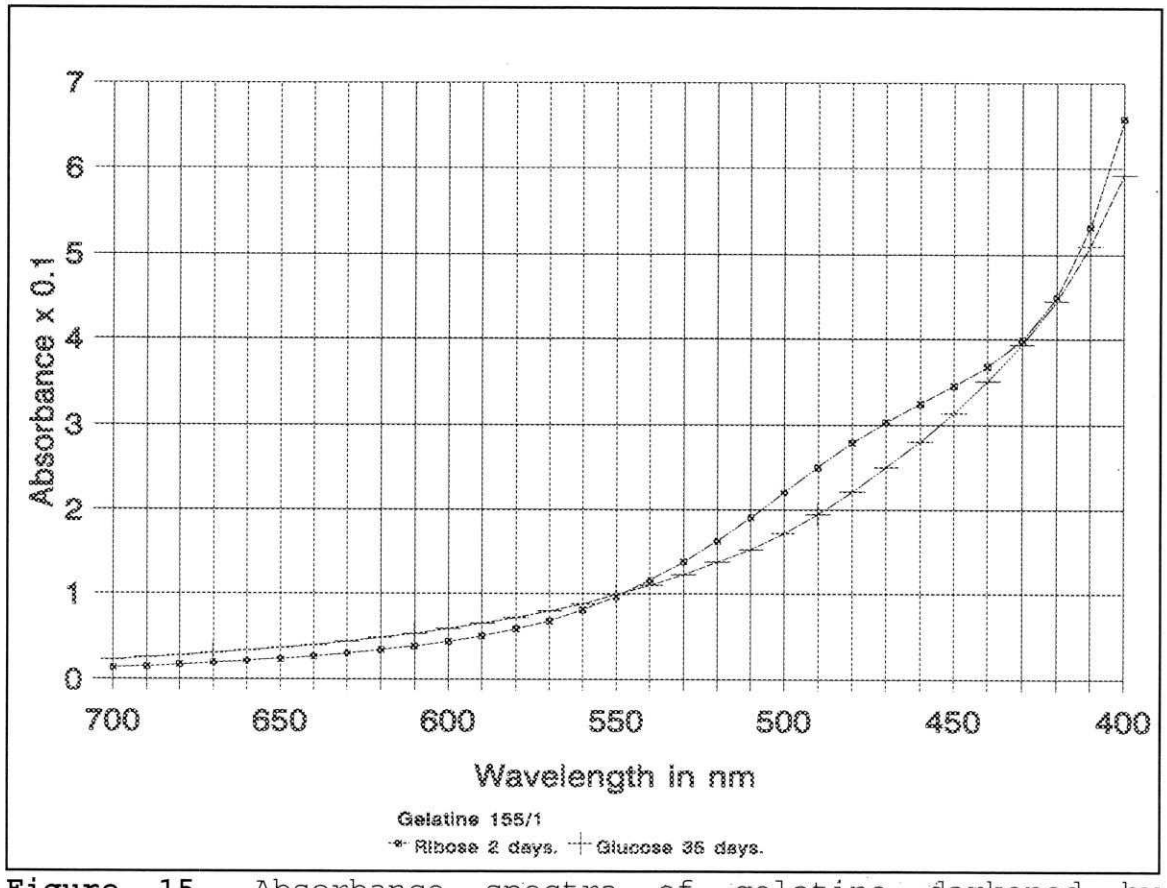


Figure 15. Absorbance spectra of gelatine darkened by incubating 10% w/w gelatine in 0.2 M glucose or ribose aqueous solution at 50°C or 37°C respectively.

Finally, from a comparison of the slopes of the glucose and ribose induced absorbance areas against time for gelatines 155/1 and 877/1 at pH 6:

Glucose averaged 1.94 absorbance units per day for 84 days at 50°C.
Ribose average 21.3 absorbance units per day for 11 days at 37°C.

Hence, it was concluded that the reaction rate of gelatine with ribose at 37°C was some 10.9 times faster than the reaction between gelatine and glucose at 50°C. If one then accepts that the reaction rate doubles for every 10°C (Findlay, 1947) then the ribose reaction rate was some 28 times faster than the glucose reaction rate at the same temperature which was in reasonable agreement with the factor of 25 given by Tanaka *et al*. (1989).

Ribose gelatine interaction - Induced fluorescence.

As with colour, it was evident (Figure 14) that both gelatines had not responded identically in their reaction with ribose. Again gelatine 155/1 reacted faster than 877/1 reaching the maximum fluorescence at pH 6 in about 7 days as against 9 days for 877/1.

The downturn in the fluorescence intensity plots was again an example of the phenomenon of concentration quenching because the fluorescence intensities of the pH 9 solutions diluted to 0.01% (0.1 ml to 10 ml water) showed a continuous increase to a maximum intensity and then a levelling off as gelation occurred. (See detailed results in APPENDIX 5. RB-FI/100 and RD-FI/100).

The fluorescence spectra were studied in detail from time to time. Although efforts were made to detect the 370 nm excitation maximum with emission at 445 nm which Tanaka *et al*. (1988) found with ribose reaction on collagen, no sign of these fluorescence peaks could be found. The excitation spectrum shown in Figure 16 shows a single maximum at ±345 nm (due to monitoring the emission at 420 nm) but no sign of a further maximum near 370 nm. On all occasions the excitation maximum was close to 335 nm and the emission spectrum showed an inflection at 385 nm. This was consistent with the generation of the pentosidine cross-link, and a continuously increasing emission to about 410 nm as shown in Figure 17.

Ribose gelatine interaction - Induced pH changes.

It was evident from Figure 14 that there was an approximately linear drop in the pH of the reaction mixtures with time. This would be consistent with the removal of amino groups due to the Maillard reaction. The only anomaly was the pH of the gelatine 877/1, pH9 reaction mixture after 10 days. Perhaps as gelation occurred the Maillard reaction slowed markedly or ceaseed. This also seemed to be the case with colour development because the increase in colour production of this mixture also appeared to cease after gelation, although no measurements could be made to substantiate this observation.

Ribose gelatine interaction - Controls.

From ADDENDUM 5. it could be seen that for the reaction mixtures without ribose RE, RF, RG, RH, there was initially a small increase in colour and fluorescence and a decrease in pH over the first few days of incubation, whereafter the values remained substantially constant. This would be consistent with the completion of any incomplete Maillard reaction inherent in the gelatine. Against this, however, it must be stated that the gelatines were the product of long liming, that is, the raw material had been immersed in lime at greater than pH 11 for at least 30 days, hence, it was hard to envisage how there could be any sugars present that had not completely reacted with the collagen prior to its conversion to gelatine, unless, as indicated above, the Maillard reaction becomes particularly slow in the solid or gel state due to steric hindrance and the inability of the reactants to come together in the required conformation. It was also possible that sulphide in the lime liquor could have been responsible for inhibiting the Maillard reaction during conditioning, but the failure to observe an effect on colour due to sulphide would probably discount this possibility.

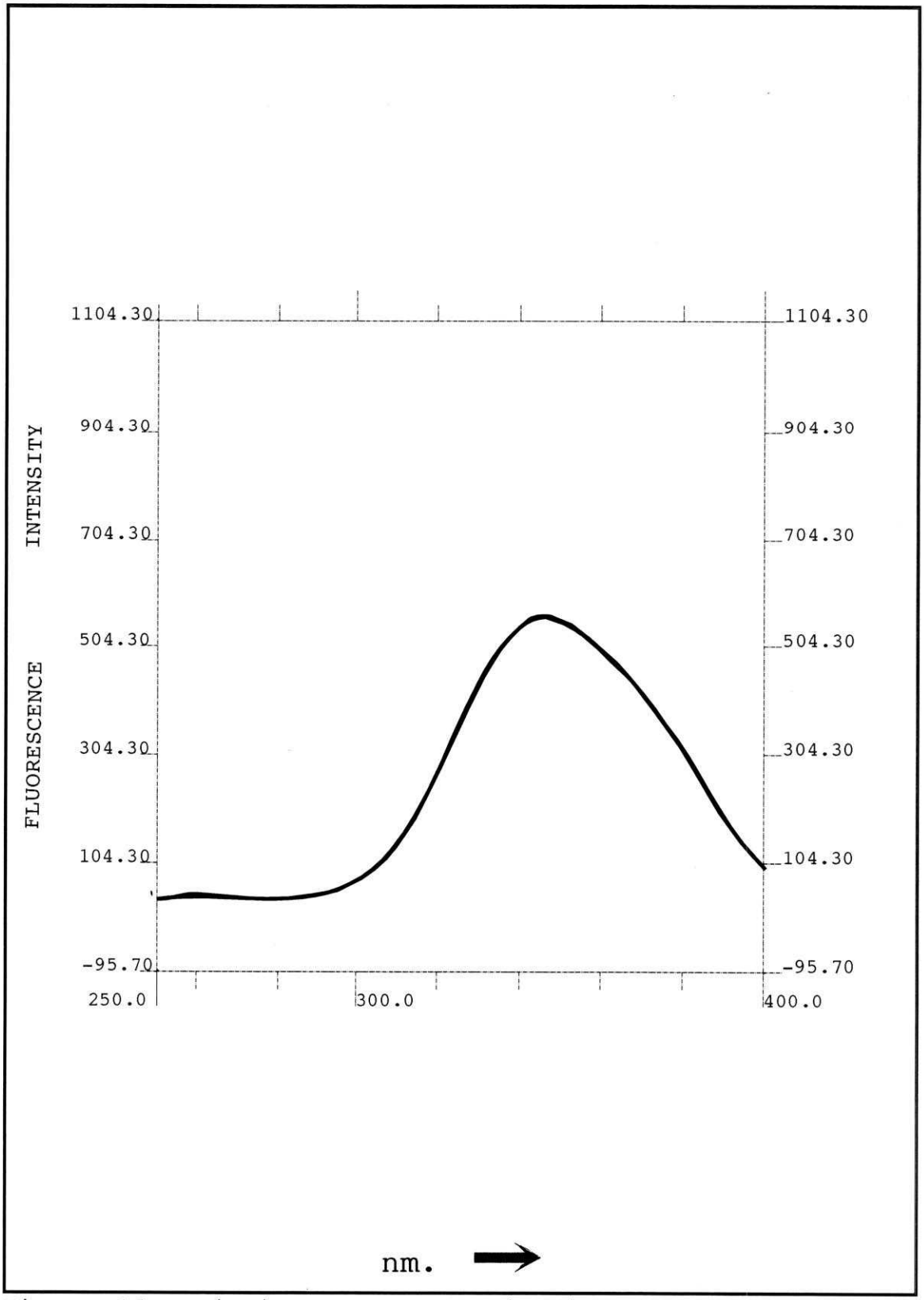


Figure 16. Emission 420 nm. Excitation spectrum of ribose darkened gelatine (155/1). A 10% w/w solution of gelatine in 0.2 M ribose at an initial pH of 6.0 incubated at 37°C for 7 days.

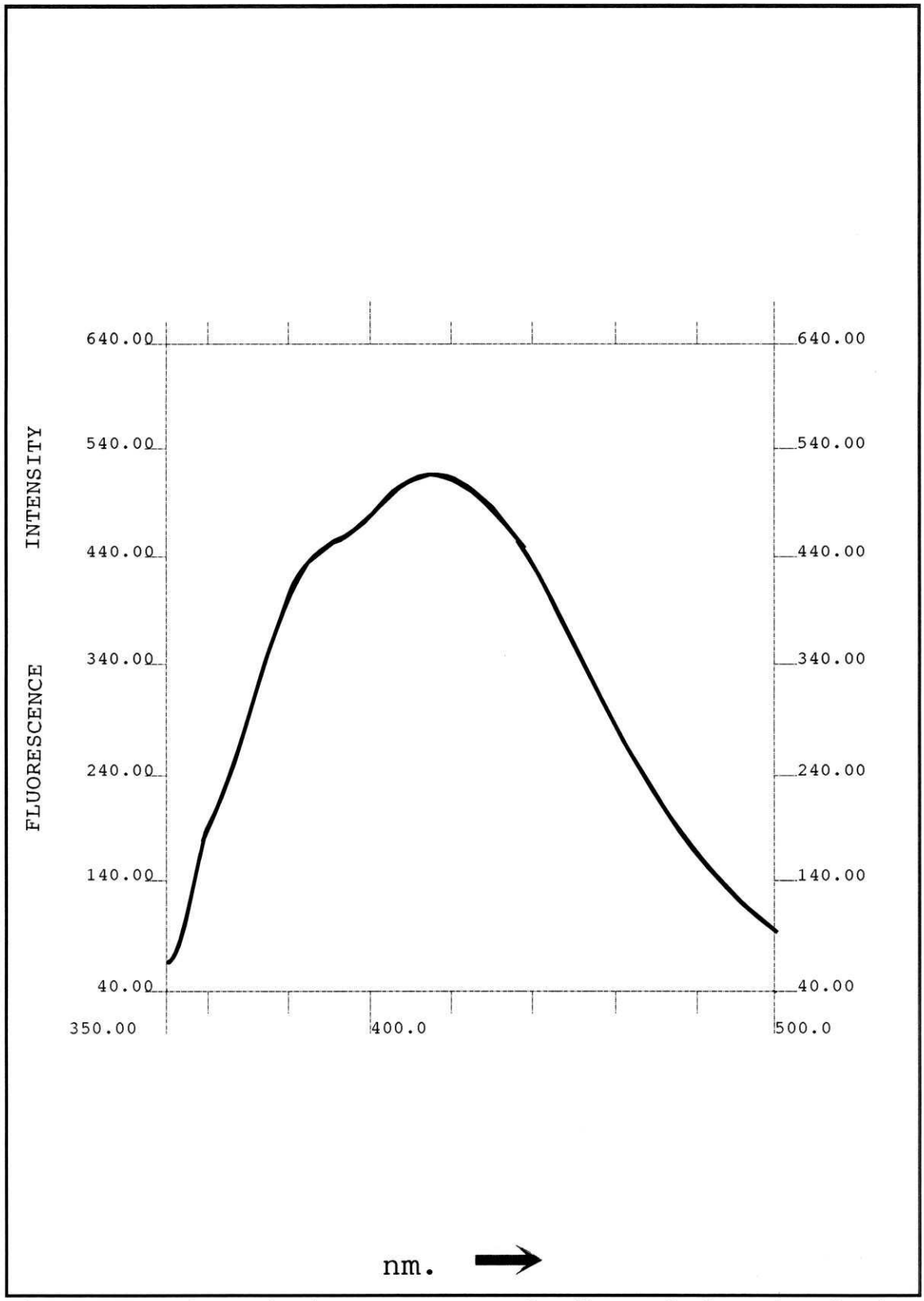


Figure 17. The emission spectrum of ribose darkened gelatine (155/1) at 1% concentration. Gelatine 10% w/w in 0.2 M ribose solution, after 7 days at 37°C with a starting pH of 6.0. Excitation at 335 nm. Note the shoulder at ca. 385 nm and the peak at 410 nm.

PENTOSIDINE SYNTHESIS.

In the study of Sell and Monnier (1989) "pentosidine" was synthesised by heating 100 mM quantities of L-arginine, L-lysine and D-ribose in 3 litres of water for 1 hr at 80°C (pH 7.3) and then purifying the product by ion exchange and chromatography. They showed that the purified product was identical to the natural "pentosidine" from human dura mater, however, it was clear from the results that pentosidine was not the only product of reaction. Hence, it was decided to repeat the Sell and Monnier synthesis under conditions more similar to those employed in the gelatine aldose reactions above. The reaction mixture consisted of equal amounts of 0.02M lysine.HCl, arginine.HCl, and ribose in 0.02M ammonia. The solution had a pH of 9.5.

Table 8. *Fluorescence development in an equimolar mixture of lysine, arginine and ribose at room temperature (± 20°C) and at 37°C.*

Fluorescence Intensity due to Excitation at 335 nm.				
Emission at	385 nm.		405 nm.	
Time.	Reaction Temperature.		Reaction Temperature.	
	Ambient.	37°C	Ambient.	37°C
Zero	5.0			
10 min.	5.0			
3 days.	15	100		
10 days.	135	982	156	797
10 days. Diluted 1/100		103		88

The conclusions drawn from Table 8. were:

1. The formation of fluorescent products was greatly accelerated by the increase in temperature from ambient (20°C) to 37°C.

2. The 10 day fluorescence exhibited marked concentration quenching as shown by a 100 times dilution exhibiting only a 10 times reduction in fluorescence intensity.

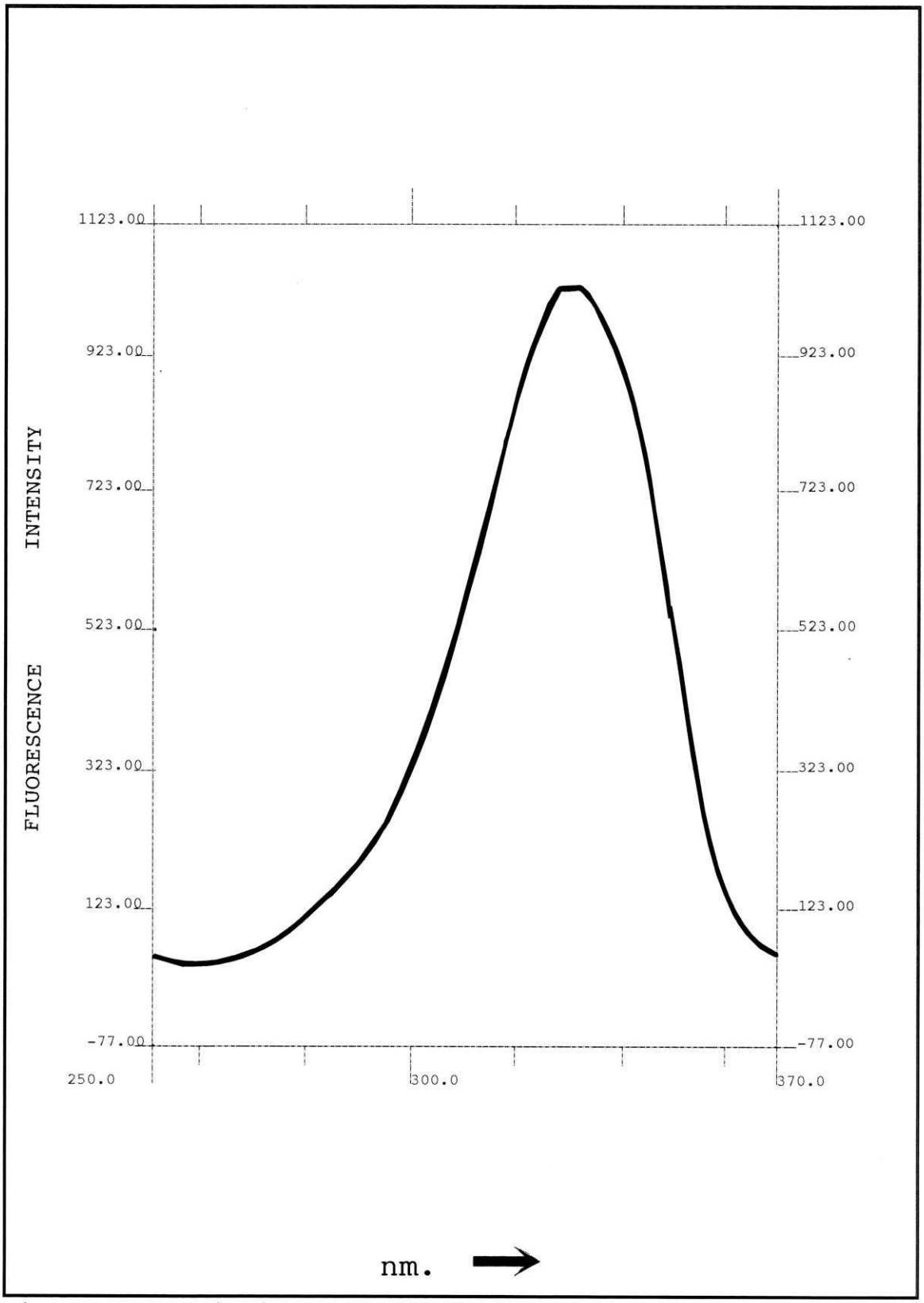


Figure 18. Emission at 385 nm. Excitation spectrum of the "pentosidine" reaction mixture after 10 days at 37°C. Note the 335 nm excitation peak. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9).

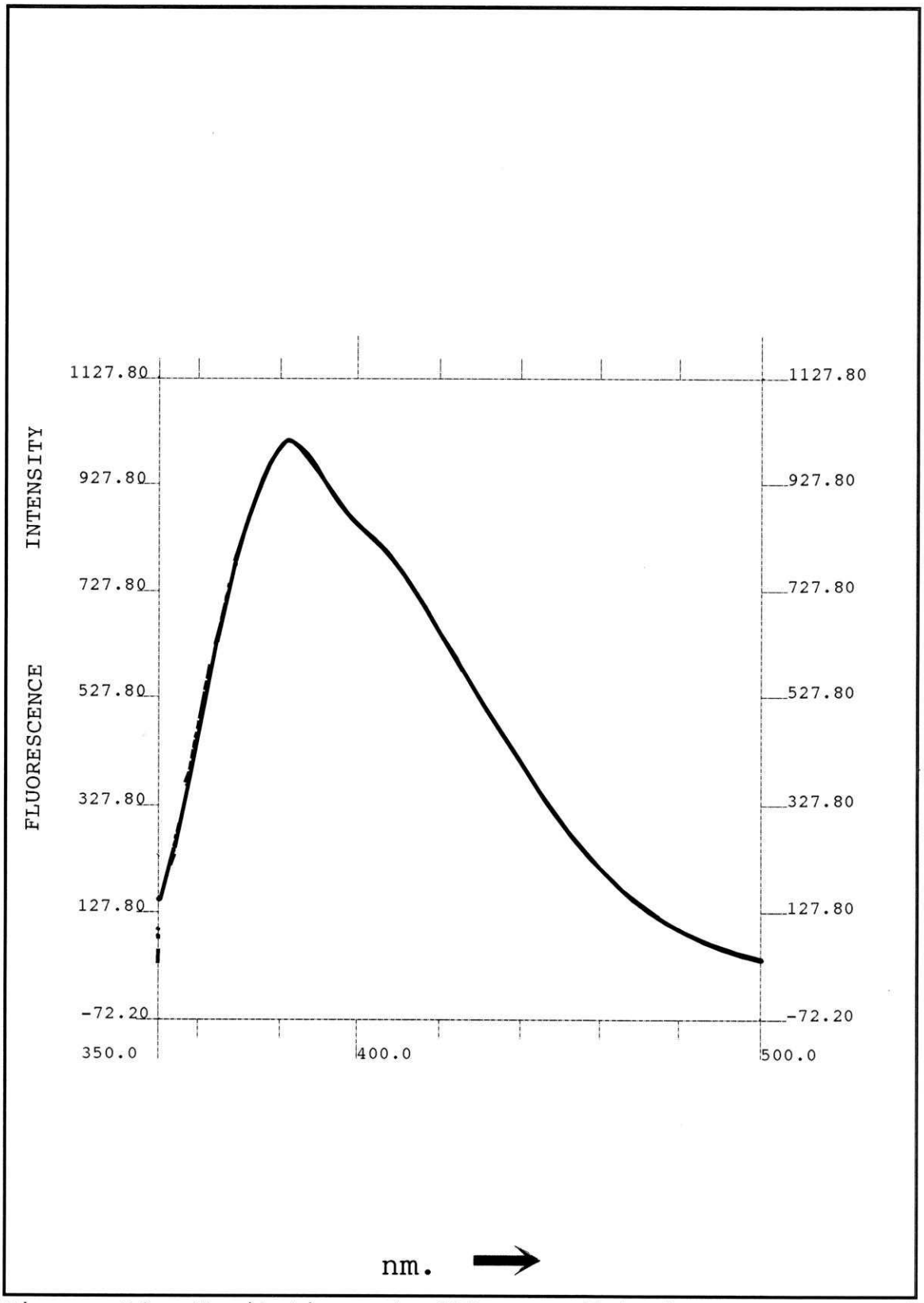


Figure 19. Excitation at 335 nm. Emission spectrum of "pentosidine" reaction mixture after 10 days at 37°C. Note the 385 nm emission maximum and the shoulder at ± 410 nm. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9)

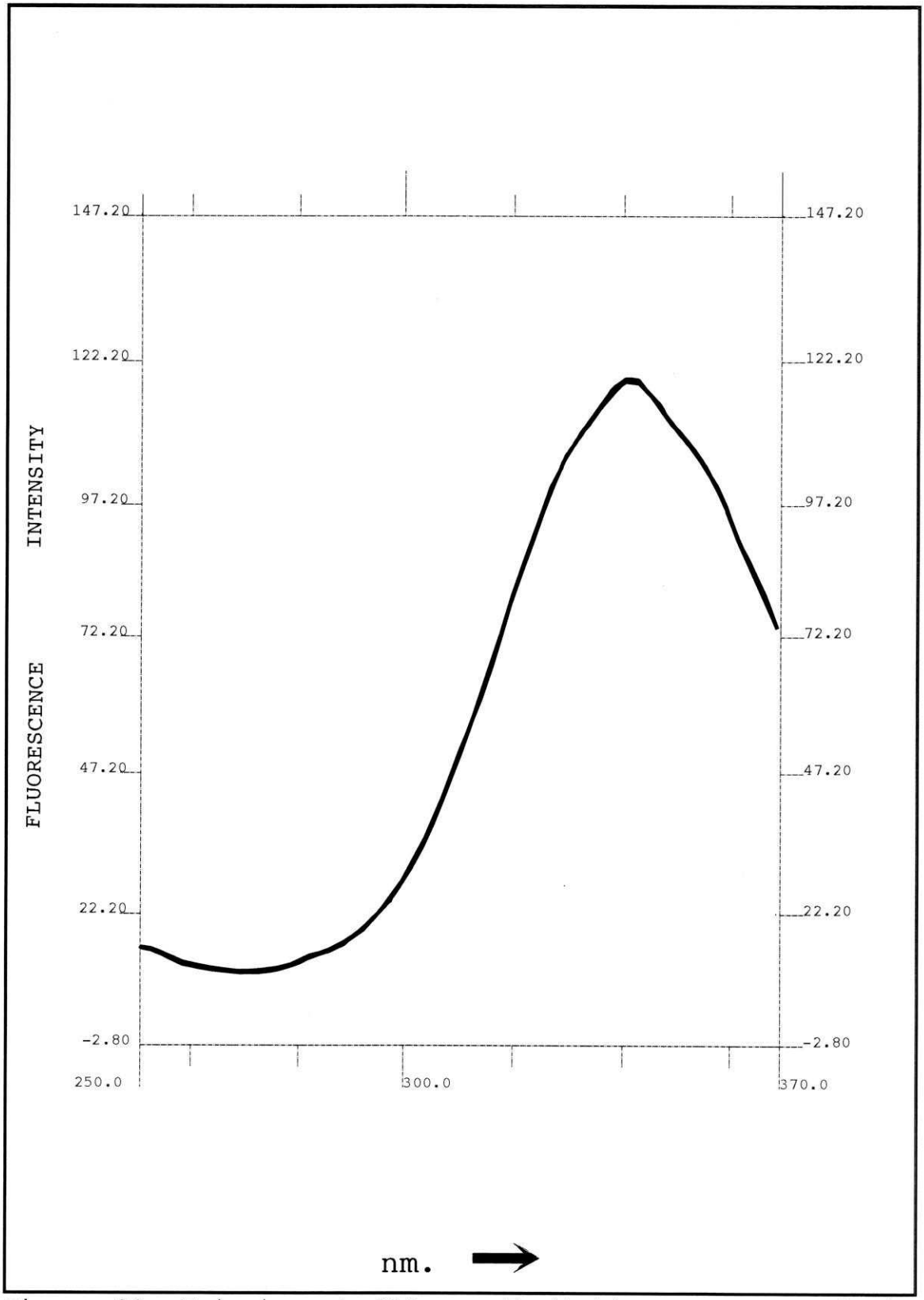


Figure 20. Emission at 385 nm. Excitation spectrum of the "pentosidine" reaction mixture after 10 days at ambient temperature (20°C). Note the 340 nm excitation maximum. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9).

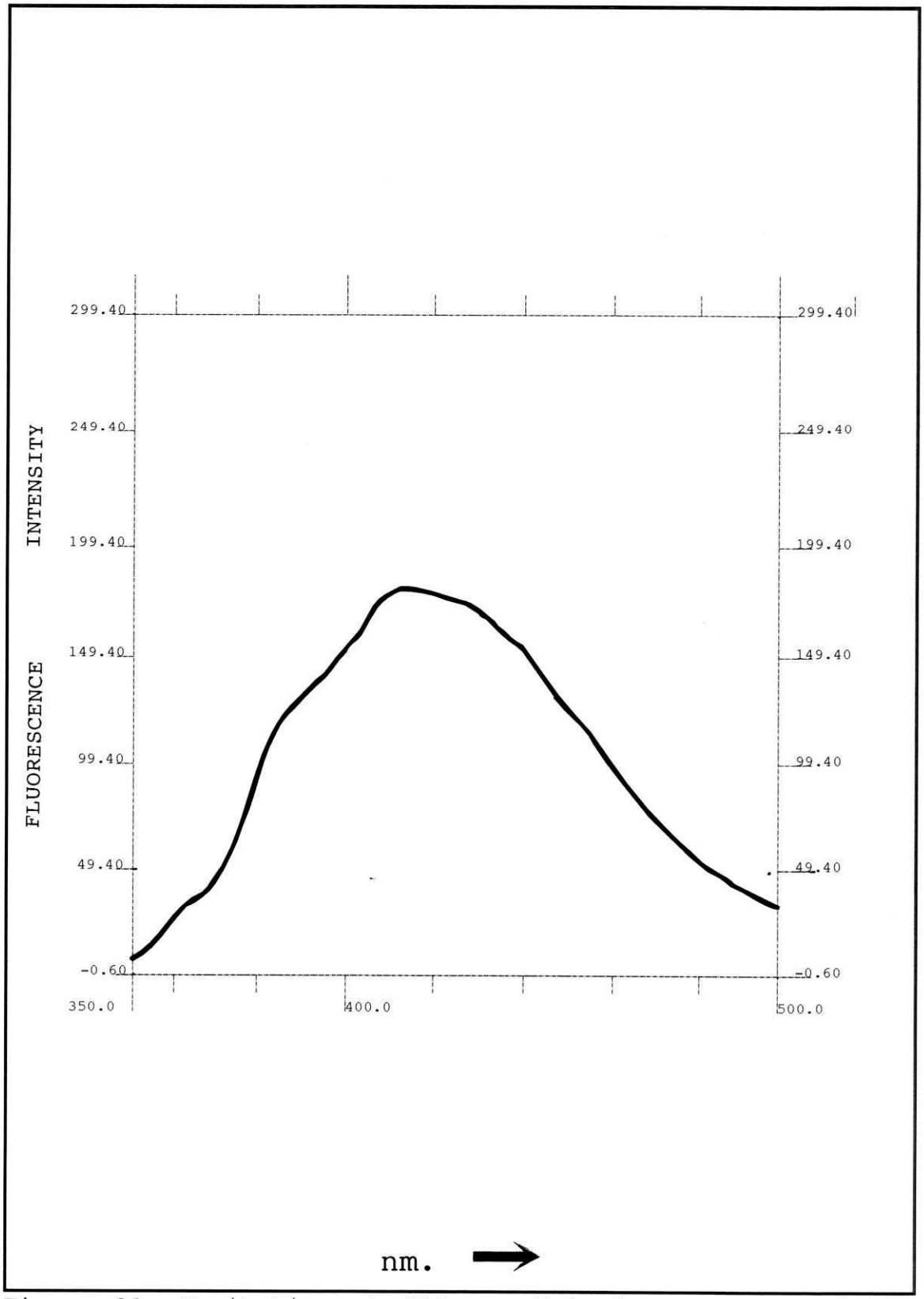


Figure 21. Excitation at 335 nm. Emission spectrum of the "pentosidine" reaction mixture after 10 days at ambient temperature (20°C). Note the 410 nm emission maximum. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9)

3. As with colour formation the pentosidine reaction appeared to have had an initiation phase followed by a much faster reaction phase which was typical of free radical catalysed chain reactions such as the decomposition of acetaldehyde (Glasstone, 1952).

From Figures 18 to 21 it was observed that fluorescence spectra similar to those of gelatine (Figure 5) were produced from the slow reaction of lysine, arginine and ribose at ambient temperature. That is, fluorescence with excitation at 335 nm and emission from 385 to 410 nm was produced at lower temperature while at 37°C the emission intensity peaked at 385 nm and fell away rapidly at higher wavelengths. This latter was characteristic of the production of pentosidine.

THE EFFECT OF ANION EXCHANGE.

As can be seen from Addendum 2 and Table 9 the effect of anion exchange on the colour of gelatines 939/4 and 972/6 was marked. This phenomenon had been found to be most obvious in the case of dark, low quality (Bloom strength and viscosity) gelatines.

Table 9. *The spectrophotometric data on two gelatines before and after anion exchange.*

	Visual Colour	Estimated Colour*	Absorbance# 400 nm.	Absorbance# 700 nm.	Absorbance# Area 400-700 nm.
Sample	Before Anion Exchange				
939/4	14.5	15.6	0.366	0.017	22.37
972/6	17.5	16.4	0.369	0.017	23.46
178/12 Control	17.8	15.7	0.366	0.017	22.37
Sample	After Anion Exchange				
K939	9.4	10.3	0.232	0.009	14.71
Q939	9.2	10.1	0.229	0.010	14.4
F972	11.7	11.9	0.266	0.013	16.79

Solvent = ES1. Concentration = 6.67%. Filter = 0.45μ membrane.
* Absorbance Area x 0.7

Table 9 showed that the bleaching effect due to anion exchange was variable

and in terms of reduction in absorbance was about 37% for gelatine 939/4 and only 27% for gelatine 972/6. Table 9 also showed that anion exchange resulted in colour reductions of 5 to 6 Davis units and from Table 10 it was clear that there was virtually no change in the 335/385 nm fluorescence intensities of the gelatines as a result of anion exchange. From the fluorescence/colour correlation determined earlier, the fluorescence data of K939 and Q939 indicated that these gelatines should have had colours of about 14.6 and the F972 gelatine a colour of 15.9 which was correct for the original gelatines but not for the anion exchanged ones. This result clearly showed that there was a component of the colour of gelatine that was not related to fluorescence. This finding helped to account for the relatively poor correlation coefficient between fluorescence and colour. The fact that this component had been found in dark, low quality, gelatines also helped to explain why the correlation between colour and fluorescence was not significant for third extraction gelatines as noted in the discussion on Table 2.

Table 10. *Fluorescence Intensity data for 1% w/v solutions of two gelatines before and after anion exchange.*

FLUORESCENCE INTENSITY		
Excitation Wavelength. Emission Wavelength.	335 385	370 440
Sample.	Before Anion Exchange.	
939/4	253	141
972/6	274	149
178/12 Control.	288	177
Sample.	After Anion Exchange.	
K939	269	128
Q939	254	129
F972	272	137

The very dark regenerant solution from regeneration of the column after it had treated about 1200 bed volumes of 3% w/v gelatine liquor or 15.7 kg of gelatine, had a pH of 12.6, and was only about 0.07 N NaOH after it had regenerated the resin. Normally fluorescence determinations were done on 1% w/v solutions of gelatine. The fluorescence determination on the alkaline

regenerant solution was equivalent to determining the fluorescence of a 31% solution of gelatine. The determination was also done on the acidified regenerant because Sell and Monnier (1989) had found that the fluorescence of pentosidine was completely quenched at pH 9. Hence due to dilution the fluorescence of the acidified regenerant should have been about 59% that of the alkaline regenerant solution if no "quenching" was involved. The 335/385 fluorescence intensities of 87 for the alkaline regenerant and 102 for the acid regenerant indicated that pH quenching was a factor, however the low values were taken to indicate that little of the colour removed by anion exchange was associated with pentosidine or Maillard fluorescence.

Anion exchange of ribose darkened gelatine.

In order to ascertain that the 100 ml column was bleaching a dark gelatine normally the column was used to treat solutions of gelatine 178/12, which had acted as a "control" in the colour determination study, with the results shown in Table 11.

Table 11. *The effect of anion exchange on gelatine 178/12.*

Absorbance using Filter No. 2.		
Before Anion Exchange Treatment	After Anion Exchange Treatment	Decrease
0.134	0.102	0.032 (24%)
0.136	0.104	0.032 (24%)

The pale gelatine 877/1 that had been used extensively in the glucose and ribose interaction experiments was chosen for the following investigation involving ribose darkening because it was known that the gelatine exhibited minimal bleaching due to anion exchange. Hence, any observed bleaching after ribose darkening could not be attributed to similar bleaching prior to ribose treatment. Furthermore, controls without ribose were included in the experiment.

Table 12. *The effect of anion exchange on ribose darkened gelatine 877/1.*

Incubation Days.	Absorbance using Filter No. 2.			
	Sample with Ribose	Before Anion Exchange.	After Anion Exchange.	Decrease due to Anion Exchange.
6	Excluded	0.048	0.037	0.011 (23%)
	Included	0.255	0.229	0.026 (10%)
7	Excluded	0.054	0.040	0.014 (26%)
	Included	0.310	0.264	0.046 (15%)

Table 11 showed that gelatine 178/12 lost 24% of its absorbance due to anion exchange which was of the same order of magnitude of the reduction obtained with gelatines 939/4 and 972/6 in Table 9. From Table 12 it was concluded that ribose darkened gelatine after 6 and 7 days incubation lost only 10% to 15% of its colour after anion exchange which was considerably less than the anion exchange bleaching of normally dark gelatines. Hence it seems probable that gelatine colour produced by the Maillard reaction was only slightly susceptible, if at all, to bleaching or absorption using anion exchange resin.

CONCLUSIONS.

1. The observation that gelatine exhibited 335/385 "pentosidine" fluorescence was evident from Table 1, however, most gelatines also had a second peak in the emission spectrum at about 410 nm. This pair of peaks was also produced by the Sell and Monnier (1989) pentosidine synthesis mixture when it was held at ambient temperature for 10 days. Hence, it could be proposed that both the fluorescence peaks found in gelatine were the products of the Maillard reaction with slightly different molecular structures.

In the case of pigskin gelatines, the second emission peak due to 335 nm excitation was closer to 420 nm, as was the case with fishskin gelatines. The small change from 410 to 420 nm could possibly be a function of the difference in processing (the presence or absence of alkaline conditioning).

2. Tanaka *et al*. (1988) produced fluorescence and cross-linking in collagen by reacting it with ribose and glucose. This experiment was repeated with gelatine in place of collagen and it was found that the darkening of the solutions with time was accompanied by an increase in 335/385 fluorescence intensity. In the case of ribose, the reaction mixtures showed a marked lag phase at pH6. Also these mixtures gelled at 37°C which could not be a clearer demonstration that the Maillard reaction caused cross-linking. The increase in 335/385 fluorescence was accepted as showing that the cross-linking was due to formation of pentosidine.

3. The absorption spectrum of ribose cross-linked gelatine was quite different from the spectrum of the glucose cross-linked gelatine. The absorption spectrum of glucose cross-linked gelatine and the spectrum of naturally dark gelatine were virtually identical and this was interpreted as indicating that the natural pentosidine cross-links in collagen and gelatine were derived from glucose rather than ribose *in vivo*. This confirmed the contention of Dyer *et al*. (1991) that pentosidine was formed from glucose with the loss of one carbon atom, rather than from ribose. Hence, the scarcity of ribose to take part in cross-linking, as mentioned by Sell and Monnier (1989), was not relevant.

4. In the case of glucose, the rate of reaction with gelatine was about 28 times slower than with ribose. This had implications in production where even with raised temperatures, as in evaporation or sterilization, the amount of the Maillard darkening should be expected to be small to negligible due to the short times involved. The low reaction rate also explains how confectionery manufacturers could process glucose and gelatine at high temperatures without any apparent darkening.

5. There was a significant correlation between overall gelatine colour and animal age and between gelatine colour and 335/385 nm fluorescence. Based on the findings of Uchiyama *et al*. (1991), that 335/385 nm fluorescence was due only to pentosidine, it was concluded that the colour of gelatine was a function of the formation of the Maillard pentosidine cross-links. Furthermore, there was a significant inverse correlation between gelatine colour and hide extractability. This was additional evidence of the formation of stable cross-links (including pentosidine) in collagen, with senescence.

It could be argued that only the isolation of pentosidine from gelatine would

unequivocally prove that the colour of gelatine was caused by the pentosidine cross-link however Odetti *et al.* (1994) also based their conclusions on fluorescence data only, hence, fluorescence data alone was accepted as sufficient for meaningful conclusions to be drawn.

6. The significant correlation between first and second extraction high quality gelatine colour and fluorescence but the failure of the correlation with low quality gelatine was explained by the finding that the bleaching action of anion exchange resin had virtually no effect on the fluorescence of the gelatine. This was interpreted as showing that there were at least two sources of colour in gelatine; the first was the Maillard reaction which was not susceptible to anion exchange bleaching and the second was an as yet unknown source of colour which was susceptible to anion exchange bleaching.

7. Tanaka *et al.* (1988) noted the development of 370/440 nm fluorescence with the ribose treatment of collagen. This fluorescence was sought throughout this study of gelatine, and it was only found with Type A gelatine from calf skin, that is, from animals of less than 6 months of age. This fluorescence was not found with Type A gelatines from pigskin nor was it found in Type B gelatine from 10 month old animal skin. There was insufficient information on which to base theories as to the origins of the fluorescence other than to suggest it could be associated with immature collagen or that it was caused by a very unstable or short-lived cross-link.

8. The absence of 295/395 nm pyridinoline fluorescence in Type B gelatines was expected (Eyre, 1988) hence finding it in Type A calf skin even if only at a low intensity indicated that the pyridinium cross-link was present in bovine hide collagen and this possibly explained the conditioning phenomena encountered in this study:

(i) Eyre, Paz and Gallop (1984) stated that the pyridinium cross-link was labile in alkali.

(ii) The amount of pyridinium cross-link in collagen was invariant. (Uchiyama *et al.* 1991).

(iii) This study showed that young animal hide extractability increased steadily with conditioning time, but old animal hide exhibited a limit to the extractability after which conditioning time seemed to have had no effect.

Hence, if bovine hide contained a small amount of the alkali labile pyridinium

cross-link which predominated in younger animals and which was later swamped by pentosidine and other mature cross-links as the animal aged, then it would be expected that the time related response to alkali conditioning in younger animals would decrease as the animal became older and as the alkali labile cross-links became relatively fewer.

9. The ribose interaction with gelatine at pH 8 was of particular interest because it indicated that after gelation the pH and colour formation slowed dramatically indicating that steric hindrance may play an important role in the rate of the Maillard reaction *in vivo*. Current treatments to control the effects of high glucose levels in diabetes were based on making amine groups available for reaction with excess glucose, hence the patient could be medicated with amino acids (Ulrich and Cerami, 1992). Possibly some means of using steric hindrance to limit the effects of glucose in the body could be considered. Likewise this study highlighted the effect of pH on the rate of the Maillard reaction and perhaps this effect could also be applied medicinally.

The sugar/gelatine interaction experiments also indicated that two similar gelatines reacted with the glucose or ribose at very different rates. It could only be surmised that this effect indicated different availabilities of amine groups for reaction but the as yet unknown reason for this difference in availability could be significant when it came to differences in rates of senescence.

10. The controls for the glucose/gelatine interaction experiments indicated only a small amount of darkening of gelatine solutions due to storage at pHs of 6 and 8.75 at 50°C. for many weeks. The observation that the darkening was greater at pH 6 than at pH 9 for both gelatines could indicate that the chromophore was not related to the Maillard reaction. This was confirmed by the pH 6 fluorescence (335/385 nm) being less than or equal to the pH 9 fluorescence for both gelatines. (Addendum 4). The ribose/gelatine interaction controls were held at 37°C for only 14 days which was not long enough for material discolouration etc. to occur.

REFERENCES.

Baynes, J.W., Dunn, J.A., Dyer, D.G., Knecht, K.J., Ahmed, M.U., and Thorpe, S.R. 1990. In: Glycated Proteins in Diabetes Mellitus, Ryall, R.G. (Ed) 221-236. Adelaide University Press, Australia.

Cobbet, W.T., Gibbs, J.A., and Leach, A.A. 1963. Chemical derivatives of gelatin and glue Part III. The determination of the degree of substitution of sulphonyl gelatines and glues. Gelatine and Glue Research Association Report C33.

Dyer, D.G., Blackledge, J.A., Katz, B.M., Hull, C.J., Adkisson, H.D., Thorpe, S.R., Lyons, T.J., and Baynes, J.W. 1991. The Maillard reaction in vivo. Zeitschrift für Ernährungswissenschaft 30(1): 29-45.

Eyre, R.E., Paz, M.A., and Gallup, P.M. 1984. Cross-linking in collagen and elastin. Annual Review of Biochemistry 53: 717-748.

Eyre, D. 1987. Collagen cross-linking Amino Acids. Methods in Enzymology 144: 115-139.

Findlay, A. 1947. Practical physical chemistry. 7th Ed. p.257. Longmans Green & Co. London. New York. Toronto.

Glasstone, S. 1952. Textbook of Physical Chemistry. Second Edition. p 1084. Macmillan and Co. Ltd. St Martins St, London.

Kuypers, R., Tyler, M., Kurth, B.L., and Horgan, D.J. 1994. The Molecular location of Ehrlich Chromogen and pyridinoline cross-links in bovine perimysial collagen. Meat Science 37: 67-89.

Lacowitz, J.R. 1983. Principles of fluorescence spectroscopy. Plenum Press. New York & London.

Leach, A.A. 1965. Further aspects of the determination of the degree of substitution at gelatin amino groups. Gelatin and Glue Research Association. 1st supplement to Research Report C33.

Lawrie, R.A. 1985. Meat Science. 4th Ed. p. 90-92. Pergamon Press. Oxford. New York. Toronto. Sydney. Paris. Frankfurt.

Monnier, V.M., Sell, D.R., Miyata, S., and Nagaraj, R.H. 1990. The Maillard reaction as a basis for a theory of ageing. In: The Maillard reaction in food processing, human nutrition and physiology. Finot, P.A., Aeschbacher, H.K., Hurrell, R.F., Liardon, R. (Ed.). p. 393-414. Advances in Life Sciences. Birkhauser Verlag Basel.

Munck, L., and de Francisco, A. 1989. Fluorescence analysis in foods. Longman Scientific & Technical and John Wiley & Sons Inc. New York.

Munck, L., and de Francisco, A. 1989a. Fluorescence analysis in foods. p. 44. Longman Scientific & Technical and John Wiley & Sons Inc. New York.

Munck, L., and de Francisco, A. 1989b. Fluorescence analysis in foods. p. 41. Longman Scientific & Technical and John Wiley & Sons Inc. New York.

Munck, L., and de Francisco, A. 1989c. Fluorescence analysis in foods. p. 45-46. Longman Scientific & Technical and John Wiley & Sons Inc. New York.

Munck, L., and de Francisco, A. 1989d. Fluorescence analysis in foods. p. 37. Longman Scientific & Technical and John Wiley & Sons Inc. New York.

Munck, L., and de Francisco, A. 1989e. Fluorescence analysis in foods. p. 42. Longman Scientific & Technical and John Wiley & Sons Inc. New York.

Odetti, P., Pronzato, M.A., Noberasco, G., Cosso, L., Traverso, N., Cottalasso, D., and Marinari, U.M. 1994. Relationships between glycation and oxidation related fluorescence in rat collagen during ageing. An *in vivo* and *in vitro* Study. Laboratory Investigation 70(1): 61-67.

Sell, D.R., and Monnier, V.M. 1989. Structure elucidation of a senescence cross-link from human extracellular matrix. Journal of Biological Chemistry 264: 21597-21602.

Tanaka, S., Avigad, G., Eikenberry, E.F., and Brodsky, B. 1988. Isolation and partial characterization of collagen chains dimerized by sugar-derived cross-links. Journal of Biological Chemistry 263: 17650-17657.

Thornton, A.C.R. 1966. Depolarisation of fluorescence studies on gelatin sols and gels. Gelatine and Glue Research Association Research Report A.33. April 1966.

Uchiyama, A., Ohishi, T., Takahashi, M., Kushida, K., Inoue, T., Jugie, M., and Horiuchi, K. 1991. Fluorophores from ageing human articular cartilage. Journal of Biochemistry (Tokyo) 110: 714-718.

Ulrich, P.C., and Cerami, A. 1992. Amino acids useful as inhibitors of advanced glycosylation of protein. US Patent 825 598.

Yamauchi, M., Woodley, D.T., and Mechanic, G.L. 1988. Ageing and cross-linking of skin collagen. Biochemical and Biophysical Research Communications. 152: 898-903.

A D D E N D A

ADDENDUM 1. Origins of the gelatines used in this study.

Laboratory Gelatines:

YS	10 month old Afrikaner - conditioned with lime sulphide.
CT	18 month old Brahman - conditioned with lime & sulphide
CTO	12 yr. old Afrikaner - " " "
5Y	5 yr. old Afrikaner - " " "
ST	12 yr. old Afrikaner - " " "
IOB	12 yr. old Inguni - " " "
6Y	6 yr. old Friesland - " " "
WT	12 yr. old Afrikaner - " " "
3Y	3 yr. old Friesland - " " "
C	Gelatine from chrome tanned leather.
FISH	Acid process gelatine from fish skin.
D	Acid process pigskin gelatines.

For complete processing and quality details see the Chapter 2 on the origins of gelatine colour.

155/1 First extraction gelatine.
877/1 First extraction gelatine.
(Both gelatines were chosen for their normal but pale colour.)

143/1 First extraction gelatine.
23/2L Gelatine of medium colour.
939/4 Dark gelatine.
K939 & Q939 Gelatine recovered from an anion exchange column after treatment of solutions of gelatine 939/4.
972/6 Dark gelatine.
F972. Gelatine recovered from an anion exchange column after treatment of a solution of gelatine 972/6.

ADDENDUM 2. Analysis Data of Gelatines.

Gelatine	155/1	877/1	23/2L	143/1
Bloom	269	267	241	216
Colour	6.8	5.6	8.6	12.3
Clarity	12.5	11.5	12.5	10.5
pH 1%	5.7	5.1	5.3	5.2
Moisture %	10.4	10.6	10.8	9.5
Ash %	0.4	0.4	0.23	0.65
Viscosity ms.	36	42	33.9	32.5

Gelatine	939/4	K939	Q939	972/6	F972
Bloom	170	140	159	75	74
Colour	14.5	9.4	9.2	17.5	11.7
Clarity	11.5	11.0	12.0	11.0	10.5
pH 1%	5.6	6.0	5.8	5.5	5.5
Moisture %	7.9	9.8	10.9	10.7	9.4
Ash %	1.6	2.2	1.8	1.7	2.3
Viscosity ms.	27.8	19.2	24.1	19.7	17.7

ADDENDUM 3. Fluorescence data.

EXCITATION EMISSION		335 nm 385 nm	295 nm 380 nm	370 nm 440 nm	335 nm 420 nm
SAMPLE CODE	COLOUR	Peak Ht.	Peak Ht.	Peak Ht.	Peak Ht.
YSA/1	3.6	41	32		
YSA/2	4.4	50	34		
YSA/3	4.8	75	43		
3Y4A/1	6.6	102	73		
CT6/1	5.2	115	83		
5Y6A/1	6.4	118	73		
5Y6A/2	4.4	134	86		
5Y4/2	6.8	135	77		
3Y4A/2	6	138	70		
CT6/2	5.6	144	89		
CT6/3	6	148	88		
5Y4/3	8.4	151	86		
5Y6A/3	9.4	158	90		
6Y4B/2	8	164	114		
IOB/1	16	169	112		
6Y4B/3	9.5	174	106		
6Y4B/1	6.4	183	104		
IOB/2	16	207	112		
IOB/3	20	254	127		
5Y4/1	5.6	104			
5Y4/2	6.8	126			
5Y4/3	8.4	149			
5Y6/1	6.4	115			
5Y6/2	8.4	128			
5Y6/3	9.4	148			
ST16/1	10.7	174			
ST16/2	12.3	192			
ST16/3	14.5	228			
178/12	17.8	261			
ST12/1	6.4	105			
ST12/2	10	124			
ST12/3	10.7	167			
INOE/1	9.4	175			
INOE/2	12.3	207			
850/6	17.8	249			
850/6C	17.8	250			
C542/1L	1.6	63			
C542/2L	1.9	64			
5Y4/1	5.6	94			
5Y4/2	6.8	129			
5Y4/3	8.4	142			
6Y4A/1	8.4	141			
6Y4A/2	7.6	165			
6Y4A/3	8.6	174			
YSA/1	3.6	38			
YSA/2	4.4	48			

ADDENDUM 3. Continued.

EXCITATION EMISSION		335 nm 385 nm	295 nm 380 nm	370 nm 440 nm	335 nm 420 nm
SAMPLE CODE	COLOUR	Peak Ht.	Peak Ht.	Peak Ht.	Peak Ht.
YSA/3	4.8	68			
ST24/1	8.9	133			
ST24/2	12.3	168			
ST24/3	13.3	214			
WT4/1	11.4	117			
WT4/2	12.3	130			
WT4/3	13.3	172			
CT4/1	5.6	104			
CT4/2	5.6	133			
CT4/3	5.6	142			
CT04/1	10	187			
CT04/2	9.4	223			
CT04/3	10	239			
CALF TyA/1	4	21		121	
CALF TyA/2	3.6	27		183	
CALF TyA/3	5.2	49		211	
155/1	6.8	147		86	
877/1	5.6	162		90	
D31108	3.8	127			245
D2227	2.2	112			182
D2116	2	87			140
D1512	2.2	78			142
D2249	2.4	104			153
D147	2.4	81			135
D32117	4.7	156			267
D1278	2.2	85			136
D3264	4	122			212
FISH-COD2/1	6	50			67
FISH-824/1	11.4	51			72
C200/1	<3.2	61			

385 nm Fluorescence/ Colour - Regression Output:

Constant	0.006579	
Std Err of Y Est	2.677614	
R Squared	0.60754	R = 0.78
No. of Observations	75	
Degrees of Freedom	73	
X Coefficient(s)	0.057929	
Std Err of Coef.	0.005449	

ADDENDUM 4. Glucose gelatine interaction.

GELATINE + GLUCOSE
AREA UNDER THE ABSORBANCE CURVE 400 TO 700 nm. (Colour)

SAMPLE	A	B	C	D
Gelatine	155	155	877	877
	pH-6	pH-9	pH-6	pH-9
Day No.				
0	7.8	10.3	9.3	10.7
7	15.5	28.8	12.5	19.9
14	18.2	61.3	14.2	48.3
21	23.5	90.7	22.9	71.2
28				
35	40.6	126.2	35.3	102.3
42	49.8	132.8	45.6	116.9
49	58.1	97.1	56.8	75.4
56	94	165	63.5	86.7
63	114.9	241.5	126.8	140.6
70	147.4	272.6	144.4	171.3
77	160.2	299.6	163.7	204.6
84	185.2	338.7	157.8	238.3

GELATINE + GLUCOSE
FLUORESCENCE INTENSITY.

SAMPLE	A	B	C	D	*B/100	D/100
Gelatine	155	155	877	877		
	pH-6	pH-9	pH-6	pH-9		
Day No.						
0	151	154	157	155		
7	160	535	176	308		
14	200	617	212	562		
21	210	577	280	540	23	16
28						
35	272	650	320	701	31	34?
42	282	602	313	503		
49	331	>1000	368	458		
56	402	585	376	396	11?	
63	402	362	394	351	41	14
70	363	252	353	310		
77	343	248	350	309	33?	21
84	330	211	373	282	43	21

*Samples B and D diluted 1/100.

ADDENDUM 4. Continued...

GELATINE + GLUCOSE
1% SOLUTION pH

SAMPLE Day No	A	B	C	D
0	-	-	-	-
7	-	-	-	-
14	5.36	7.62	5.99	7.49
21	5.39	7.2	6.1	7.18
28	-	-	-	-
35	5.35	6.62	5.8	6.35
42	5.4	6.59	5.72	6.75
49	5.29	5.4	5.64	5.52
56	5.37	6.27	5.65	5.56
63	5.42	6.11	5.54	5.42
70	5.38	6.15	5.62	5.39
77	5.46	6.19	5.5	-
84	5.32	6.05	5.49	5.18

- Values not determined

C O N T R O L S

DAILY INSTRUMENT CONTROLS.*

Gelatine. Day No.	COLOUR AREA 155	COLOUR AREA 877	FLUORESCENCE 155	FLUORESCENCE 877	pH 155	pH 877
0	9.72	7.79	152	156	-	-
7	10.57	8.7	140	147	-	-
14	10.46	8.09	147	162	5.69	5.28
21	9.48	7.82	136	148	5.83	5.33
28	-	-	-	-	-	-
35	9.76	8.27	151	168	5.51	5.28
42	10.94	9.35	142	140	5.49	5.25
49	9.82	8.21	158	167	5.41	5.26
56	8.8	6.18	152	181	5.46	5.23
63	7.68	6.15	161	190	5.42	5.22
70	-	6.94	152	183	5.64	5.33
77	7.6	6.0	166	185	5.56	5.25
84	7.8	6.34	162	182	5.51	5.22
Mean	9.33	7.49	152	167	5.55	5.26
Std Dev.	1.14	1.08	8.8	16.2	0.13	0.04

* Gelatine samples were dissolved and measured as daily instrument/method controls.
- Values not determined.

ADDENDUM 4. Continued.

CONTROL - SOLUTIONS OF GELATINE WITHOUT GLUCOSE INCUBATED AT 50°C.

GELATINE COLOUR = ABSORBANCE CURVE AREA 400 - 700 nm.

Sample Gelatine.	E 155 pH-6	F 155 pH-9	G 877 pH-6	H 877 pH-9
Day No.				
0	7.9	9.3	6.2	8.3
5	10.5	10.1	10.8	10.7
12	11.1	9.7	10.1	10
19	12.6	10.2	12	10.5
26	12.6	9.4	12.4	10.7
40	15.3	12.2	12.6	13.1
61	16.4	13.2	15.9	14.8

335/385 nm FLUORESCENCE INTENSITY

Sample. Gelatine.	E 155 pH-6	F 155 pH-9	G 877 pH-6	H 877 pH-9
Day No.				
0	164	144	140	187
5	181	220	206	220
12	191	230		226
19	200	266	219	232
26	217	285	240	236
40	223	309	221	262
61	233	318	252	252

1% SOLUTION pH

Sample. Gelatine.	E 155 pH-6	F 155 pH-9	G 877 pH-6	H 877 pH-9
Day No.				
0	6.31	8.81	6.33	8.8
5	6.02	8.68	6.14	8.07
12	6.1	8.59	-	7.64
19	6.14	8.66	6.14	7.78
26	6.12	8.51	6.48	7.77
40	6.07	8.50	6.18	8.08
61	6.03	*8.36	*9.09	*7.76

* Cloudy
- Value not determined.

ADDENDUM 5. Ribose gelatine interaction.

GELATINE + RIBOSE INCUBATED AT 37°C

GELATINE 155

Day	pH 6 (RA)			:	pH9 (RB)			
No	Abs-Areaϕ	Flu-Int	pH	:	Abs-Areaϕ	Flu-Int	RB-FI/100*	pH
0	9	164	6.13	:	10.9	192		9.08
1	9.6	173	6.04	:	21.8	230	4.3	8.85
2	16.7	229	5.87	:	44.2	348	8.7	8.71
3	34.5	374	5.86	:	65.1	419	13.5	8.5
4	62.6	442	5.72	:	85.4	400	16.5	8.33
5				:				
6				:				
7	177	413	5.55	:				
8	194	479	5.56	:	(122)	(425)	(23)	(7.72)
9	234.4	423	5.5	:				
10	258.3	332	5.5	:				
11	300.6	275	5.44	:				

* Fluorescence Intensity sample RB diluted 0.1 ml into 10 ml water.
ϕ Area under the absorbance curve 400 - 700 nm.
Flu-Int = Fluorescence Intensity.

GELATINE 877

Day	pH6 (RC)			:	pH9 (RD)			
No	Abs-Areaϕ	Flu-Int	pH	:	Abs-Areaϕ	Flu-Int	RD-FI/100*	pH
0	7.6	185	6.25	:	9.8	210		8.96
1	6.25	207	6.14	:	17.8	242	4.6	8.7
2	12.1	236	6.04	:	39.4	327	7.3	8.42
3	18.1	320	6.02	:	56.6	397	11.0	8.08
4	33.1	372	5.93	:	63.5	454	15.5	7.76
5				:				
6				:				
7	83.7	471	5.79	:	96.9	412	25	7.05
8	131	474	5.74	:	111	478	26	6.96
9	151.2	485	5.7	:	155.1	456	(21.4)	6.77
10	179.8	400	5.7	:	(124.6)	(417)	27.1	6.94
11	184.1	420	5.65	:				

* Fluorescence sample RD diluted 0.1 ml into 10 ml water.
ϕ Area under the absorbance curve 400 - 700 nm.
Flu-Int = Fluorescence Intensity.

ADDENDUM 5. Continued.

C O N T R O L S.

GELATINE SOLUTIONS WITHOUT RIBOSE INCUBATED AT 37°C.

GELATINE 155

Day No	pH 6 (RE) Abs-Areaϕ	Flu-Int	pH	pH9 (RF) Abs-Areaϕ	Flu-Int	pH
0	8.37	164	6.17	9.91	170	9.00
1	8.55	170	6.13	10.09	179	8.94
2	9.89	166	6.08	11.51	183	8.90
3	8.85	178	6.06	10.35	209	8.91
4	9.38	178	6.07	10.48	197	8.90
5						
6						
7	9.34	173	6.06	10.57	196	8.98
8						
9	9.43	181	6.05			
10						
11	9.76	183	6.09	11.21	204	8.97

GELATINE 877

Day No	pH6 (RG) Abs-Areaϕ	Flu-Int	pH	pH9 (RH) Abs-Areaϕ	Flu-Int	pH
0	6.90	167	6.26	9.43	197	8.94
1	7.21	199	6.15	10.14	227	8.93
2	8.45	198	6.12	10.83	230	8.90
3	8.14	206	6.13	10.48	243	8.92
4	8.08	194	6.12	10.04	230	8.88
5						
6						
7	8.53	206	6.13	10.19	244	8.93
8						
9	8.16	196	6.11	10.11	253	8.94
10						
11	8.21	192	6.13	10.19	245	8.95

ϕ Area under the absorbance curve 400 - 700 nm.
Flu-Int = Fluorescence Intensity.

CHAPTER FIVE.
Electrophoresis.

INTRODUCTION.

The study reported in Chapter Two has shown that darker gelatines were derived from older animals. Electrophoresis was recognized as a primary method for investigating proteins at the molecular level (Hames and Rickwood, 1981; Koepff, 1984; Chalepakis Tanay and Heidemann, 1985; Tanaka, Avigad, Eikenberry, and Brodsky, 1988). Hence, it was hoped that electrophoresis could yield information about differences between gelatines of different colour at the molecular level.

It was accepted that gelatine was a protein derived from collagen by the hydrolysis of an infinite polymer. Chalepakis *et al*. (1985) showed that for acid conditioned pigskin gelatine, there was little specificity in the molecular weight profile. In contrast, alkaline conditioning gave gelatine containing far larger quantities of the specific collagen sub-units, alpha, beta and gamma chains, of 100k, 200k and 300k Dalton, respectively. Furthermore, Chalepakis *et al*. (1985) showed by means of SDS-PAGE, that the α-chain material from acid conditioned pigskin had a faster migration rate than α-chains from alkaline conditioned material. From the above observations it could be proposed that in the gelatine manufacturing process, the action of alkaline conditioning resulted in the hydrolysis of "reducible"/labile collagen cross-links. This freed some of the α-chain subunits allowing them to dissolve as soon as the stabilizing hydrogen bonds were sufficiently weakened by temperature and/or the presence of hydrogen bond breakers in the environment. Additional increase in temperature caused protein hydrolysis and conversion of collagen into gelatine which then had a polydisperse molecular weight profile.

In Type A gelatine manufacture, the acid conditioning of pigskin was simply a process of equilibration of the collagen to an acid pH prior to heating for extraction (Reich, Walther and Stather, 1962). This was not a conditioning process in the terms of the classic alkaline conditioning process. Heating caused thermal hydrolysis and denaturation of the protein resulting in

solubilization and production of gelatine which consisted of peptides with a largely random distribution of molecular weights (or polydisperse protein). The finding of some α, ß and gamma chain material could be due to the rupture of unstable collagen cross-links which would be present due to the low age of pigs at slaughter. More recently Muller and Heidemann (1993) showed that the hydrolysis of collagen (that occurs during acid extraction) was not random but that there were preferential points for thermal hydrolysis (which were possibly pH dependant).

Based on the above it was argued that darker gelatine from old animals could be distinguishable from paler gelatines from young animals. Old animal gelatine should exhibit greater amounts of "random" proteolysis. In this regard, note was taken of the study by Hodney, Struzinsky and Deyl (1992) in which they distinguished age related changes in collagen due to glycation by the degree of silver staining after SDS-PAGE. However, due to the generally accepted difficulty of silver staining gels, no attempt was made to repeat their findings.

An earlier study involving the electrophoresis of gelatine, (Cole, 1985), was conducted using PAGE in acid buffers. Hames and Rickwood (1981a), stated that SDS complexes with most proteins in the ratio of 1.4 g SDS to 1 g protein. The result was that the intrinsic charge of the protein was then insignificant compared to the negative charges provided by the detergent. Thus, due to uniform charge density, the rate of migration of the protein in polyacrylamide gel became strictly dependent on molecular size. Furthermore, Hames and Rickwood (1981b) explained the theoretical advantages of the Laemmli discontinuous buffer system used by Chalepakis *et al.* (1985). Based on the above it was decided to adopt their procedure. It was only realised after the initial trials were complete that Hames and Rickwood (1981c) advised against the storage of gels using this technique due to diffusion of the different buffers between the stacking and running gels. (Running gels could be stored if the facility existed for addition of the stacking gel immediately prior to use.) Finally, in this study only polyacrylamide gels of 5% w/v concentration were used because unfractionated gelatines were to be examined. Gels of 5% concentration were recommended by Hames and Rickwood (1981d) for proteins with molecular weights in the range 200k to 25k Dalton and were also used by Chalepakis *et al.* (1985) for gelatine.

The statemant by Hames and Rickwood (1981a) that proteins complex with SDS in

the ratio of 1 to 1.4 needs to be qualified in the light of the study of Kao, Prockop and Berg (1979) who showed that collagen α-chain mobility was modified by the degree of hydroxylation of the proline residues and the study of Bonadio and Byers (1984) who showed that small variances in collagen α-chains due to mutations caused small changes in the migration rate under the conditions of SDS-PAGE. Hence it was apparent that small changes in α-chain mobility were significant and that these changes could be related to changes in the secondary and tertiary structure of the molecule without significant change in molecular weight.

MATERIALS AND METHODS.

INITIAL STUDY WITH PHARMACIA APPARATUS.

The gelatines used in this study were:

ST../. Gelatines produced from 144 month old animal hides (*Loc cit* Chapter 2.) in a series of experiments in which sulphide levels and time were varied.

WT../. Gelatines produced from 144 month old animal in a series of experiments (*Loc cit* Chapter 2.) in which sulphide levels, time of conditioning and temperature of conditioning were varied.

YS../. Gelatines produced in a series of experiments (*Loc cit* Chapter 2.) using the hide of a 10 month old animal in which sulphide concentration and time were varied.

CT../. Gelatines produced in a series of experiments (*Loc cit* Chapter 2.) on an 18 month old animal hide in which conditioning time was the only variable.

CTO../. Gelatines produced in a series of experiments (*Loc cit* Chapter 2.) on the hide of a 156 month old animal in which conditioning time was the only variable.

AC../. Gelatines produced by an accelerated conditioning process using sodium sulphide and caustic soda for 5 days at 27°C.

185/2 = 266 Bloom gelatine.
184/3 = 160 Bloom gelatine.

184/5 = 219 Bloom gelatine.
184/10 = 98 Bloom gelatine.
155/1 = 269 Bloom gelatine.
C200/1 = 15 Bloom gelatine from chrome tanned leather.
R/Z* = Gelatine Blend. 230 Bloom, 39 ms Viscosity.
R/K* DGI Gelatine Blend. 230 Bloom, 31 ms Viscosity.

The samples marked * were of interest because although they had the same Bloom gel strength they gave very different results in a 4 hr jelly desert performance tests. R/K was markedly superior.

PHARMACIA APPARATUS.

Pharmacia GE 2/4 electrophoresis apparatus for 2 gels 180 x 140 mm. (Separations Scientific cc, PO Box 4328, Honeydew, 2040) was fitted with a coil for cooling/heating the buffer and a pump for buffer circulation between the anode and cathode compartments. In normal mode the lower reservoir contained the anode. The total buffer requirement of the apparatus was 3500 ml.

Pharmacia GSC-2 slab gel casting apparatus:
This apparatus allowed the casting of two cassettes (gels) at a time and required the casting of the stacking gel first and then the running gel. Stacking gel solution (70 ml) was required and 180 ml of Running gel solution.

A DC power supply capable of being run in constant voltage or constant amperage modes. The maximum current available was 80 ma. The supply was used in constant voltage mode.

A waterbath with a "cold finger" to provide continuous cooling, and a circulating pump with integral thermostat and heater unit, was used. The bath contained a submersible pump to circulate water to the electrophoresis apparatus' coil. The initial temperature of the bath was set to 14°C. The cold finger did not have sufficient cooling capacity to maintain this temperature during electrophoresis.

Gel cassettes were assembled using glass plates 180 x 140 mm, separated by 1.5 mm spacers. The 18 mm wide PVC tape used to assemble the cassettes according to the method given in the Pharmacia handbook, was purchased locally. A

scalpel was used for cutting the tape as needed. After assembly the cassettes were warmed at 75°C for 30 minutes to ensure adhesion of the tape to the glass in the presence of SDS solution. (80°C caused excessive shrinkage of the tape; Pharmacia recommend 60°C for 1 to 16 hr). The cassettes were cooled, fitted with well-formers (which had to be tight enough not to fall out when the cassette was inverted) and then assembled into the gel casting apparatus.

Great care was taken with cassette assembly in that the glass plates were scrupulously clean, (only handled with gloves after washing with detergent, rinsing with distilled water and air drying) assembled tightly with the spacers and the PVC tape was stuck both to the glass and the spacers, without bubbles or creases.

Preparation of gels.

Electrophoresis grade acrylamide, bis-acrylamide, TEMED and Coomassie Blue 250-R were used. All other materials were analytical reagent grade chemicals. Tris-base was used in all formulations.

Reagents and buffer solutions.

The method of Chalepakis *et al.* (1985) was followed. The items marked ** were made using formulae from Miflin and Shewry (1977). Solutions A, and B were stored at 4°C. Solution C was made up fresh each day.

Resolving gel:

Solution A: 30 g Acrylamide
0.6 g Bis-Acrylamide
diluted to 100 ml with water. (%C = 2%)

Solution B: 4.64 g Boric acid
12.114 g Tris
0.3 ml TEMED
diluted to 100 ml with water. (1 M Tris; pH=8.8).

Solution C:** 1.5 g Ammonium persulphate diluted to 50 ml with water.

Gel strength in %:

5% (T 5%) 30 ml A: 18 ml B: 9 ml 2% SDS: 9 ml C.
diluted to 180 ml with water.

Stacking gel:

Solution A: 10 g Acrylamide
0.26 g Bis-Acrylamide
diluted to 100 ml with water. (%C = 2.5)

Solution B: 12.114 g Tris
0.3 ml TEMED
adjusted to pH 6.8 with HCl and
diluted to 100 ml with water. (1 M Tris)

Solution C: 1.5 g Ammonium persulphate diluted to 50 ml with water.

Gel solution T 3%.

21 ml A; 7 ml B; 3.5 ml 2% SDS; 3.5 ml C. diluted to 70 ml with water.

Sample buffer.

2.42 g Tris
1.24 g Boric acid
20 g SDS
120.12 g Urea
diluted to 1 l with water. (0.02 M Tris; 2 M Urea;) (pH 9.2) - stored under refrigeration. (Hames and Rickwood, 1981).

Stock buffer.

5.41 g Boric acid
141.3 g Tris
11.7 g SDS
diluted to 1 l with distilled water.
(1.166 M Tris; pH 9.3)

Electrophoresis buffer.

300 ml Stock buffer
diluted to 3.5 l with distilled water. (0.1 M Tris)

Fixing Solution.**

250 g Trichloroacetic acid
1000 ml Methanol
diluted to 2500 ml with distilled water.

Staining Solution. ** (Stegmann stain).

0.53 g Coomassie Blue R250 (0.025%)
dissolved in 400 ml methanol. (20%)
50 ml diluted to 250 ml with distilled water for staining of each gel.

Destaining Solution.

175 ml Methanol (8.75%)
200 ml Glacial acetic acid (10%)
diluted to 2 l with water.

Gel storage solution. (7% HOAc)**

140 ml Glacial Acetic Acid
1860 ml distilled water.

Sucrose Solution. **(40%)

40 g Sucrose
0.2 g SDS
Diluted to 100 ml with distilled water.

Marker dye.

0.05 g Bromophenol blue
dissolved in 10 ml sample buffer.

Preparation of Gelatine Samples.

Gelatine (0.10 g)was dispersed in 10 ml sample buffer (1% w/v). After 30 minutes soaking at ambient temperature (20° - 28°C) the samples were heated in a 40°C waterbath with intermittent swirling to complete the solution of the gelatine. Samples were stored at 0°to 4°C and warmed to ambient temperature before electrophoresis. Due to the urea the samples did not gel when refrigerated.

For electrophoresis, 1 ml of gelatine sample solution was mixed with 1 ml sucrose solution. Aliquots (20 to 50 μl) were deposited in the required sample well of the electrophoresis gel, using a Hamilton syringe. (0.5% = 5 μg/μl; 20 to 50 μl = 100 to 250 μg gelatine per well.)

Electrophoresis Methods.

Stacking gel solutions A, B, and SDS were measured into a 100 ml measuring cylinder and made up to 66.5 ml with distilled water. The mix was transferred into a 250 ml Büchner flask. Vacuum was applied for 5 minutes with occasional swirling. Solution C was added by pipette below the surface of the degassed

solution and mixed in with gentle swirling. The solution was then carefully poured, minimizing turbulence, into the assembled gel casting apparatus such that the well-former was covered to a depth of approximately 10 mm. The apparatus was tapped to release any trapped bubbles and then allowed to stand undisturbed for 60 minutes (Oxygen inhibits gelation of the acrylamide) after which the unpolymerized liquid on the surface was poured to waste.

Resolving gel solutions A, B, and 2% SDS were measured into a 250 ml measuring cylinder and made up to 171 ml with distilled water. The mixture was degassed in a 500 ml Büchner flask for 5 min. after which solution C was added, mixed in and poured onto the gelled stacking gel in the gel casting apparatus. The apparatus was allowed to stand for 60 minutes by which time gelation was complete. The apparatus was then stored in the refrigerator overnight. The cassettes were removed from the casting apparatus, by carefully cutting away excess gel at the top and bottom of the cassettes using a scalpel. The cassettes were then wrapped in moist paper before placing in a polyethylene bag for storage under refrigeration or they were used immediately after removal from the casting apparatus.

Starting electrophoresis.

The electrophoresis apparatus was filled to the anode wire with dilute electrophoresis buffer cooled to 14°C. The apparatus' cooling coil was connected to the pump circulating water at 14°C. A cast gel was taken and the well-former was carefully removed under a stream of tap water. The locations of the cassette(/s) wells were marked on the glass plate using a waterproof marker pen and then the cassette was inserted into the gasket of the upper buffer reservoir. The cassette was then levelled and pushed down such that it was below the level of the cathode wire. The electrolyte was then pumped into the upper cathode reservoir and electrolyte was added such that the anode wire in the lower reservoir was well covered. For "pre-electrophoresis" the power pack was connected and the system was equilibrated for 30 min at 70 V (constant voltage), after which the power pack was switched off and disconnected.

Loading samples.

The electrolyte pump was switched to circulate buffer in the lower reservoir. A Hamilton syringe was used to add 5 μl of marker dye/sucrose (50:50) to

locate each well clearly, then the required quantity of gelatine/sucrose solution was added to the wells. On completion of the loading of the samples the power leads were reconnected and the power was switched on in the "constant voltage" mode. The voltage was set such that the initial amperage was 80 mA. This was about 125 V for one gel or 75 V for two gels. After 15 minutes the dye had moved into the stacking gel and the circulation pump valve was switched to circulate buffer between the anode and cathode chambers.

Electrophoresis.

Electrophoresis was continued until the marker dye reached the bottom of the gel(s). If low molecular weight material was considered to be important, electrophoresis was stopped at this point, otherwise electrophoresis was continued arbitarily for an additional hour. Details of anode reservoir temperature, voltage and amperage were recorded from time to time.

The electrophoresis buffer was used twice and then discarded.

Post-electrophoresis treatment.

At the completion of electrophoresis the cassettes were removed from the upper cathode reservoir gaskets. The tape was stripped from one side of the cassette and with care the glass plates were opened by inserting the tip of a spatula and twisting. The plate to which the gel adhered was freed of spacers and tape, marked by removing the "bottom right hand corner" as the samples were loaded and then it was placed in a basin containing 250 ml fixing solution. Using the spatula the gel was then carefully separated from the supporting plate. After 60 min the fixing solution was poured off for reuse and replaced with 250 ml dye solution which remained in contact with the gel overnight. The dye solution was then sucked out of the tray using a pipette and a water vacuum pump. The gel was then treated with destaining solution for 1 to 2 hours after which the destaining solution was discarded and replaced with fresh solution. When destaining was considered to be complete (±6 h diffusion destaining) the destaining solution was replaced with 7% acetic acid. The gels were then photographed using a light box, Illford Pan F (50 ASA) film and a medium-red filter on the lens. It was found that the camera's automatic light meter tended to over expose the film and reducing exposures by 1 and 2 stops was found to be advisable. After photographing, the gels were placed in plastic bags with a minimum amount of acetic acid solution. The bags were then

heat sealed. Thus the gels were preserved and could be re-photographed if necessary. There was no densitometer available for quantification of the gels.

The α, and β bands on the photographs were marked based on the studies of Tanaka *et al.* (1988), and Hodney *et al.* (1992).

Electrophoresis Details.

Gel No 1:

Pre-electrophoresis.	70V	40-36 mA 30 minutes.		
Electrophoresis.	Start	150 V	78 mA	16°C
	60 min	150 V	55 mA	
	120 min	150 V	53 mA	19°C

Fix 1 hr. Stain 1 hr. Destain 1 hr. Gel was insufficiently stained.
Stain 16 hr. Destain 24 hr. No bands visible.
Stain 8 hr. Destain 16 hr. Feint tracks photographed.

Gels No 2 and 3:

Pre-electrophoresis.	30 min.	70 V.	56 mA.	17.5°C
Electrophoresis.	Start	100 V.	80 mA.	18°C
	90 min	100 V.	76 mA.	21°C
	180 min	112 V.	79 mA.	21°C
	225 min.	Marker dye exits.		
	275 min.	112 V.	69 mA.	25°C

Fix 1 hr. Stain 16 hr. Destain ± 6 hr. Photographed.

Gel No 4:

Pre-electrophoresis.	30 min.	70 V.	48 mA.	
Electrophoresis.	Start	130 V.	80 mA.	17°C
	85 min	130 V.	59 mA.	20°C
	150 min	130 V.	57 mA.	22°C Marker dye exits.
	210 min	130 V.	59 mA.	23°C Stop.

Fix 1 hr. Amido Black stain 1 hr. 7% Acetic acid destain 30 min. Change destain solution and destain 16 hr. No bands were visible. Gel was re-stained with Coomassie Blue.

Gels No 6 and 7:

Pre-electrophoresis.	30 min	70 V.	72 - 68 ma.	
Electrophoresis.	Start	80 V.	80 mA	18°C
	90 min	80 V.	78 mA	21°C
	285 min	95 V.	80 mA	25.5°C. Marker Dye exits.
	330 min	Stop.		

Fix 1 hr. Stain 16 hr. Destain 4 hr.
The bands were again inexplicably faint and the anomaly at the top of the resolving gel was very evident.

Gels No 8 and 9:

Pre-electrophoresis	30 min.	60 V.	80 mA	
Electrophoresis	Start.	70 V.	80 mA.	24°C
	15 min.		start circulation.	
	160 min.	80 V.	80 mA	26°C
	270 min.	80 V.	80 mA.	
	305 min		Marker dye exited.	

Fix 30 min. Stain 16 hr. Destain 4 to 6 hr. Photograph gels.

It was noted that in spite of using 50 μl samples the tracks were very pale. Later it was concluded that this could have been due to the inadvertently reduced fixing time.

The low starting voltage was possibly a consequence of the high starting temperature for the electrophoresis.

FINAL RUN WITH BIO-RAD APPARATUS.

Soluble collagen standard.

In the first study Type B calf skin gelatine was used as a standard with each gel. Based on the findings of Tanaka *et al.* (1988), Chalepakis *et al.* (1985), the main bands were presumed to be the collagen α- and β- and gamma chains. It was decided that it would be preferable to produce a soluble collagen standard for this study using the method described by Na, Phillips and Freire (1989) as a guide. As they worked at 4°C throughout the process, it was not possible to follow their method precisely. Furthermore Johns (1977) warned

that if steps were not taken to remove neutral salt soluble collagen prior to the extraction of acid soluble collagen, then both types were extracted by acid solution.

Reagents:

0.5 M Acetic Acid - 29 ml glacial acetic acid per l.
Saturated Salt Solution - 300 g NaCl/l
0.05 M NaP buffer pH7:
0.05 M NaH_2PO_4 in 1.0 M NaCl (pH 3.4) - 160 ml
0.05 M Na_2HPO_4 in 1.0 M NaCl (pH 8.4) - 800 ml

Method

The method of Na et al. (1989) was modified as follows. A fresh calf skin was obtained from the abattoir and it was immediately tumbled overnight with 50% of its weight of coarse salt. The hide was then allowed to drain for 24 hrs before being cut into approx. 20 mm x 20 mm pieces which were stored in a sealed plastic bag in a ca. 5°C refrigerator until required.

For extraction of collagen, 125 g hide was taken and washed overnight in a continuous flow of water. The hide was then drained of excess water and placed in a 2 l flask together with 1 l x 0.5 M acetic acid and a magnetic stirrer bar. The hide was gently agitated for 8 h at ambient temperature using the magnetic stirrer. It was then allowed to stand for a further 16 h. The insoluble residue was separated from the very viscous liquid using 300μ nylon mesh. The liquid was then centrifuged at 63 hz (approx 2500 g) for 30 min. The 475 ml of supernatant was recovered and the collagen was precipitated by adding 1/5th volume (95 ml) of saturated salt solution very slowly with stirring. The precipitated collagen was separated by centrifugation to yield single precipitated acid soluble collagen (SPASC).

An attempt was made to follow the purification process of Na *et al.* (1989) by dissolving the SPASC in NaP buffer with stirring overnight, centrifuging and then adding NaCl to 3M (174 g/l). It was found that there was no sediment to remove by centrifugation nor was there any precipitation of collagen from the solution by this method. The reason for the failure of the process was possibly because the procedure was carried out at ambient temperature. (It was later observed that where acid soluble collagen was completely soluble in electrophoresis buffer at ambient (15°C) temperatures, it precipitated under

refrigerated storage conditions).

The SPASC was purified by redissolving in 250 ml 0.5M acetic acid with stirring for 30 min. The solution was vacuum filtered through Whatman GF/A filter paper in a Büchner funnel. The collagen was precipitated by slow addition of 1/5th volume of 30% NaCl and was recovered by centrifugation for 30 min. The supernatant was discarded and the solid was transferred to a glass bottle for storage at 2°C until required as acid soluble collagen (ASC).

Gelatines used in this series of experiments:

ASC - Acid soluble collagen control.
Calf A/1 to A/3 - Type A gelatine from calf skin. For the details see Chapter 2.
YSA/1 to YSA/3 - Type B gelatine from 10 month old calf skin. For the details see Chapter 2.
CT4/1 to CT4/3 - Type B gelatine from an 18 month old Brahman after 4 weeks of lime-sulphide conditioning. For the details see Chapter 2.
5Y4/1 to 5Y4/3 - Type B gelatine from a 5 year-old Chianina after 4 weeks of lime-sulphide conditioning. For the details see Chapter 2.
ST24/1 to ST24/3 - Type B gelatine from a 12 year-old bovines after 4 weeks of lime-sulphide conditioning. For the details see Chapter 2.
D147 - High quality Type A pigskin gelatine.

These gelatines were chosen to span the animal age range for Type B gelatine manufacture as well as the three extraction temperatures of 45°C, 50°C, and 55°C for each raw material. Furthermore it enabled a comparison of Type A gelatines from bovine and pig skin. Finally, it enabled a comparison of Type A and Type B gelatine from calf skin. This range of gelatines amounted to 17 samples hence they could all be run at the same time on one gel. As the apparatus allowed the electrophoresis of two gels at the same time each gelatine could be run in duplicate.

Reagents and solutions - II.

Electrophoresis grade acrylamide, bis-acrylamide, TEMED and Coomassie Blue 250-R were used. All other materials were analytical reagent grade chemicals. Tris-base was used in all formulations.

The reagents of Chalepakis *et al.* (1985) were used except for the staining of the gels. Solutions A, B and sample buffer were stored at 4°C. Solution C was made up fresh each day.

1. Resolving gel:

Solution A: 30 g Acrylamide
0.6 g Bis-Acrylamide
diluted to 100 ml with water. (%C = 2%)
Solution B: 4.64 g Boric acid
12.114 g Tris
0.3 ml TEMED
diluted to 100 ml with water. (1 M Tris; pH=8.8).
Solution C: 0.5 g Ammonium persulphate diluted to 50 ml with water.

Gel strength in %:
5% (T 5%) 16.7 ml A; 10 ml B; 5 ml 2% SDS; 5 ml C.
diluted to 100 ml with water.

2. Stacking gel:

Solution A: 10 g Acrylamide
0.26 g Bis-Acrylamide
diluted to 100 ml with water. (%C = 2.5)
Solution B: 12.114 g Tris
0.3 ml TEMED
adjusted to pH 6.8 with HCl and
diluted to 100 ml with water. (1 M Tris)
Solution C: (APS solution)
0.5 g Ammonium persulphate diluted to 50 ml with water.

Gel solution T 3%:
15 ml A; 5 ml B; 2.5 ml 2% SDS; 5.0 ml C.
diluted to 50 ml with water.

3. Sample buffer.

2.42 g Tris
1.24 g Boric acid
20 g SDS
120.12 g Urea
diluted to 1 l with water. (0.02 M Tris; 2 M Urea;)
(pH 9.2)

4. Stock buffer.

5.41 g Boric acid
141.3 g Tris
11.7 g SDS
diluted to 1 l with distilled water.
(1.166 M Tris; pH 9.3)

5. Electrophoresis buffer.

214 ml Stock buffer
diluted to 2.5 l with distilled water. (0.1 M Tris).
Used once and discarded.

6. Destaining Solution.

175 ml Methanol (8.75%)
200 ml Glacial Acetic acid (10%)
diluted to 2 l with water.

7. Marker dye.

0.05 g Bromophenol blue dissolved in 10 ml sample buffer.

8. Layering Solution.

50 ml t-Butanol
50 ml distilled water
Mixed well in a sealed glass container and then allowed to separate into a butanol upper layer saturated with water and a water rich lower layer. The upper layer was used for "layering".

9. Stain Solution.- Made up fresh daily, one lot for each gel.

100 g Trichloroacetic acid dissolved in 200 ml distilled water.
1.25 g Coomassie Blue dissolved in 200 ml methanol.
Immediately prior to use the above were mixed in a 500 ml volumetric flask and then diluted to the mark with distilled water.

10. Sugar solution.

40 g sucrose.
0.2 g SDS
5 ml Marker Dye
Diluted to 100 ml with distilled water.

11. Destain Solution.
200 ml Glacial acetic acid.
175 ml Methanol
Diluted to 2000 ml with distilled water.

Procedures. II

The BIO-RAD apparatus was used as instructed by the manual. The glass plates with spacers were assembled in the gel-casting apparatus. Resolving Gel A (16.5 ml) was mixed with 10 ml Resolving Buffer B and 5 ml 2% SDS in a measuring cylinder and diluted to 95 ml with distilled water. After degassing for 15 min. at 13" vacuum in a Büchner flask, 5 ml Solution C was added below the surface of the liquid and mixed in by swirling gently. The plates were filled to 1.5 cm below the bottom of the combs before carefully overlaying with 3 ml water saturated t-butanol added down the spacer between the plates, using a pipette. This gave a very smooth upper surface to the running gel about 20 mm below the bottom of the comb. After 60 min. the butanol was poured from the gels and completely removed by repeated washing with distilled water.

Stacking gel was prepared by mixing 15 ml Stacking Gel A, 5 ml Stacking Buffer B, 2.5 ml 2% SDS in a measuring cylinder and diluting to 45 ml with distilled water. After deaeration in a Büchner flask for 15 min at 13" vacuum, 5 ml Solution C was added below the surface of the solution which was then swirled gently to mix. This stacking gel solution was then used to nearly fill the space above the polymerised running gel. The comb was positioned and the stacking gel was allowed to polymerise. After 60 min, shrinkage was filled with distilled water and the plates were covered with a moist cloth and allowed to stand at ambient temperature until used within 24 hrs.

The casting apparatus produced 2 gels 200 mm wide by 160 mm long and 1.5 mm thick. The comb produced 20 wells in the stacking gel each 5 mm wide by 27 mm deep which was very different to the 8 mm x 8 mm x 2.8 mm wells of the Pharmacia apparatus.

Sample preparation.

Gelatine samples. Gelatine (0.1 g) was dispersed in 10 ml Sample Buffer. After soaking for 30 min the samples were placed in a 40°C waterbath. The solutions were homogenised by mixing using a vortex mixer.

ASC Standard. ASC (2 g) was weighed into a test tube and dispersed in sufficient (15 ml) boric acid/Tris buffer (pH 8.8) to give a pH of 6 on a Merck pH strip. Saturated NaCl solution (approx. 10 ml) was added slowly with agitation until a precipitate was formed. The collagen was recovered by centrifugation at 50 hz for 10 min. The recovered solid was dissolved in 15 ml sample buffer (pH 9.2).

For electrophoresis, 0.5 ml of sample and 0.5 ml 40% sugar solution in a test tube were mixed well using a vortex mixer. 15 μl was loaded into each well.

Electrophoresis.

A waterbath fitted with a cooling coil, thermostatically controlled heater and a circulating pump was set to maintain 14°C. The outlet of the circulating pump was connected to the central cooling tank of the electrophoresis apparatus and the outlet from the tank was returned to the waterbath using rubber tubing. Electrophoresis buffer (2.5 l) was made up in an Erlenmeyer flask and cooled overnight in the waterbath. At the start of each run 2.15 l of cold buffer was poured into the lower buffer chamber (outer shell) of the apparatus.

The cast gels were released from the casting apparatus and installed onto the central cooling tank of the electrophoresis apparatus to form the upper buffer (cathode) chamber. Care was taken to moisten the gaskets with buffer before clipping the gels into place. The position of the wells in the stacking gel were marked on the outer plate of the gel using a waterproof marking pen. The combs were carefully removed from the gels and the central tank was placed in the outer tank. The remainder of the cold buffer (350 ml) was used to substantially fill the upper buffer compartment formed by the gels and the top of the inner tank. Each well was loaded with 15μl of sample using a Hamilton syringe. The cover was then fitted to the apparatus and the leads connected to the power pack.
Electrophoresis was run with a current of 50 ma until the marker dye had moved into the resolving gel. The current was then increased to 70 ma (for 2 gels). The details of current, voltage and bath temperature were noted from time to time. As the marker dye reached the bottom of the gels the power pack was switched off and disconnected.

Post electrophoresis treatment.

The gels were removed from the electrophoresis apparatus. The glass plates were removed from the casting holders and the plates were separated. Each gel was then marked by removing the bottom right corner so that the order of the lanes could be identified. The gel attached to one plate was placed in a polythene container and covered with 500 ml fresh dye solution. The container was rocked from time to time over 30 min; during this rocking the gel and support plate were separated. Dying of the gel was allowed to continue overnight. The dye solution was sucked from the container using a water vacuum pump and was replaced with 250 ml destaining solution. The glass plate was also removed at this stage. After 15 min. the gel was manipulated onto a clean glass plate and transferred to a clean container in which destaining was completed over 7 to 8 h using a total of 1 l of destaining solution per gel in 250 ml lots.

The gels were photographed on a light box as described under Pharmacea Apparatus, Post Electrophoresis Treatment. They were also scanned using a Hoefer Scientific Instruments (San Francisco) GS 300 Transmittance/Reflectance Scanning Densitometer with gain set to minimum. The output was plotted using a Lloyd Scientific Graphic 1000 (Lloyd Scientific, Johannesburg) plotter set to the 0 to 100 mv range and with gain set to a low level in order to minimise noise and to keep the plot within the width of the recording paper. As this setting was arbitrary it was not possible to re-scan a gel and obtain the identical plot after any setting on the recorder had been modified.

Following the guidelines provided by Ewing (1969) the densitometer traces were photocopied and where peaks were overlapping the Gaussian curves were completed manually. The peaks were then cut out with a pair of scissors and weighed on an analytical balance (accurate to 0.1 mg), as an estimate of the curve area which represented the quantity of material belonging to the peak. Finally the traces were digitised manually. The data was entered into Harvard Graphics (Ver 3.0) (SPC Software Publishing, 3165 Kifer Ave., Santa Clara, California, 95052 USA) and are shown in Addendum 1.

Gels 12 & 13.

Start. 8:30. 50 ma. 14.9°C
9:30. Dye front moves into resolving gel.
9:30. 70 ma. 210 V. 14.8°C.
10:15 68 ma. 230 V. 14.9°C
11:25 54 ma. 290 V. 14.8°C
11:30 Dye front at bottom of the gels. Stopped.

Gels stained overnight.
Destain 1. 250 ml. 30 min.
Destain 2. 250 ml. 90 min.
Destain 3. 250 ml. 4 hr.
Destain 4. 250 ml. overnight.
Gels were stored in 100 ml destain solution at 4°C until scanned by the densitometer. The gels were then photographed using Illford Pan F film (50 ASA) and a Kenko W12 Red filter on the camera.

RESULTS AND DISCUSSION.

PRELIMINARY STUDY WITH PHARMACIA APPARATUS.

Table 1. *Loading details of Gel No.1*

GEL No 1		
SAMPLE WELL LOADING		
WELL No	SAMPLE	Volume µl
3	YSA/1	20
4	WT3/1	20
5	155/1	20
7	YSA/1	12
8	WT3/1	12
9	155/1	12
10	YSA/1	6
12	WT3/1	6
13	155/1	6

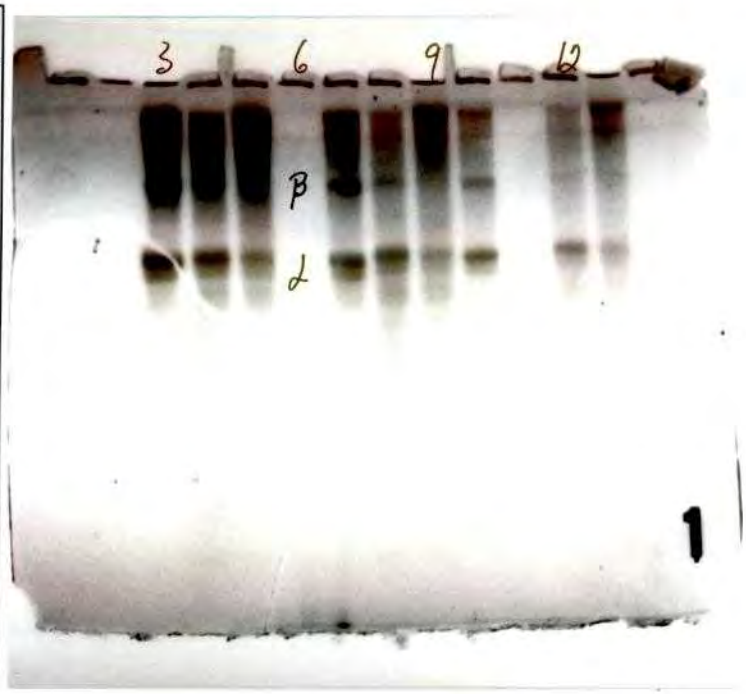


Figure P1. Photograph of Gel No 1.

Observation on Gel No 1.

The object of this experiment was to establish the correct gel loading and to see if there were any obvious differences between young animal YSA/1, old animal WT3/1 and production 155/1, gelatines:

1. The gelatines migrated through the stacking gel completely.

2. All the gelatines contained material of very low mobility in the resolving gel.

3. The YSA gelatine contained a lot more of the α and ß chain components than the WT3 gelatine which in it's turn contained more of these components than the production gelatine.

4. It was evident that considerably higher gel loading could be used and it was decided to use 30 to 50 μl in future trials.

5. Based on the studies of Tanaka *et al.* (1988) and Hodney *et al.* (1992), YSA/1 contained clearly recognizable α and ß chain bands it was decided to use this gelatine as a reference or control in the following experiments.

Observations on Gels 2 and 3:

The object of this experiment was to investigate the effect of conditioning time at constant sodium sulphide usage level on young animal hide (CT) and old animal hide (CTO):

1. When compared to Gel 1, there was considerably greater movement of the α-chain doublet and the ß chain band due to the extra hour of electrophoresis after the marker dye had run out of the gels. This resulted in the anticipated definition of a gamma chain band at the top of the resolving gel.

2. All the gelatines exhibited α-, β-, and gamma chain bands, particularily in gel 3. Furthermore the first extraction samples (*/1) exhibited strong α- and ß-chain bands. However, with second extractions (*/2) there was a marked reduction in α-chain band density, and third extractions (*/3) exhibited only very weak α-chain bands, indicating an increase in the polydispersity of the gelatine with extraction temperature. No differences could be observed between

young animal (CT) gelatines in Gel 2 and old animal (CTO) gelatines in Gel 3.

Table 2. *Loading details.*
Gel No. 2.

GEL No 2 LOADING	
30µl per WELL	
WELL No	SAMPLE
1	YSA/1
3	CT2/1
4	CT2/2
5	CT2/3
7	CT4/3
8	CT4/2
9	CT4/1
11	CT6/1
12	CT6/3
13	CT6/2

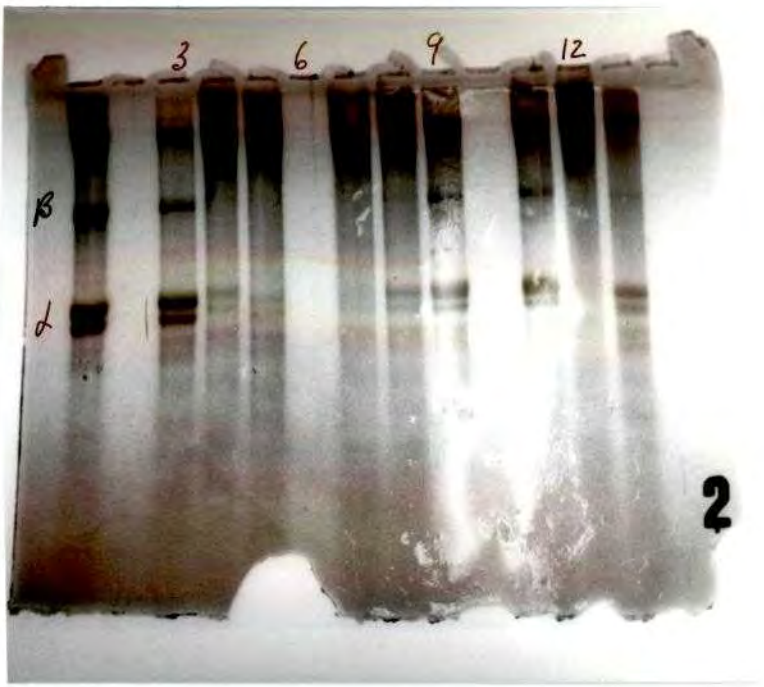


Figure P2. Photograph of Gel No. 2.

Table 3. *Loading details.*
Gel No.3.

GEL No 3 LOADING	
30µl per WELL	
WELL No	SAMPLE
2	YSA/1
3	CTO2/1
4	CTO2/2
5	CTO2/3
7	CTO4/1
8	CTO4/2
9	CTO4/3
11	CTO3/1
12	CTO3/2
13	CTO3/3

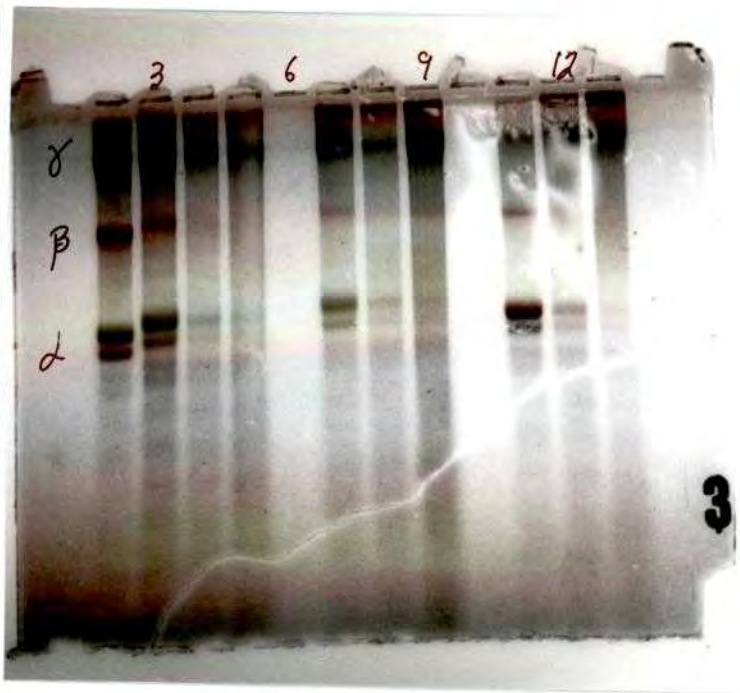


Figure P3. Photograph of Gel No 3.

3. Comparing the first run α-chain bands and the control it appeared that the CT gelatine α-chain bands moved at the same rate as the YSA α-chain bands but the CTO α-chain bands moved more slowly. This could parallel the finding of Tanaka *et al*. (1988) in that after the reaction with ribose, the α-chain collagen moved more slowly than did the natural α-chain collagen. This observation was confirmed in Gel 4.

4. All samples contained protein less mobile than ß chain protein which stayed at the top of the resolving gel.

5. No differences, attributable to conditioning time were observed. This could be very significant. With the CT gelatines the amount of first extract gelatine was a function of conditioning time (*loc cit* Chapter 2, experiment CT). Hence, an apparently constant amount of α-chain material indicated that the main function of conditioning was to release α-chains from the collagen polymer. This would be achieved by hydrolysing the primary telopeptide cross-links. The fact that this hydrolysis was limited in old animals was clear from the limited extractability of old animal hide (see Chapter 2. CTO for details), yet apparently the amount of α-chain protein was roughly the same as in the first extract gelatine. Hence, it followed that with animal age, the mature cross-links, as observed by many workers (e.g. Bailey and Light, 1989), were also stable to alkaline hydrolysis, or else, new cross-links that were stable to alkali were formed with animal age.

Observations on Gel 4:

The object of the experiment was to compare the rate of migration, using a single gel, of gelatine from a young animal (CT) with that from an old animal (CTO) both materials having had the same conditioning treatment.

As in gel No 3. the migration rate of the CTO/1 α-chains from an old animal was less than the control YSA/1 α-chains and it was also less than the migration rate of the CT/1 α-chains from a young animal. Hence, there appeared to be a progressive change in the collagen α-chain with animal age at the same level of conditioning (4 weeks). Although the observed differences were small, based on the studies of Kao *et al* (1979) and Bonadio and Byers (1985), they were evidently significant and in conformity with the observation in the fluorescence study (*loc sit*) that collagen undergoes cross-linking on ageing that bring about changes in the secondary and tertiary structure of the α-

chains.

Table 4. *Loading details.*
Gel No. 4.

GEL No 4 LOADING	
30µl per WELL	
WELL No	SAMPLE
2	YSA/1
4	CT4/1
5	CT4/2
6	CT4/3
8	CTO2/1
9	CTO2/2
10	CTO2/3
12	YSA/1
13	155/1

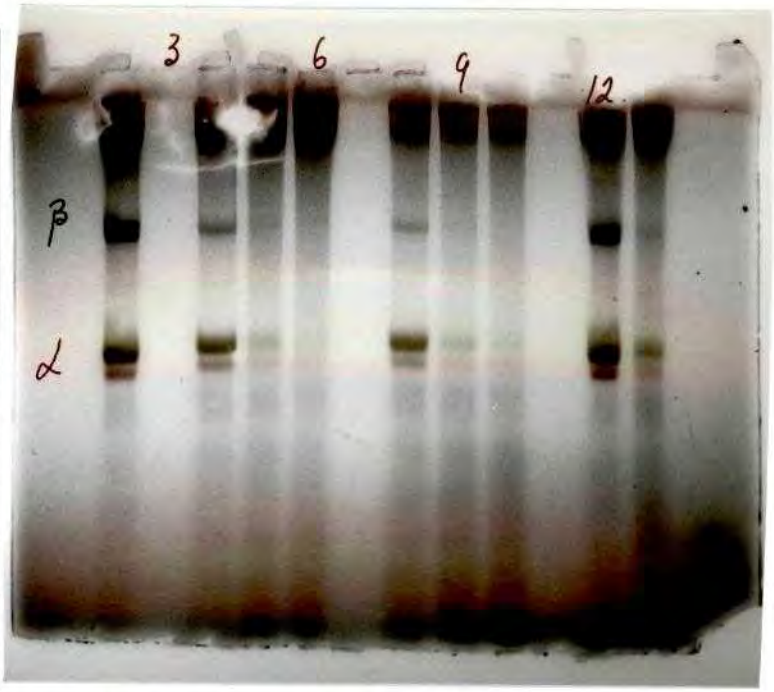


Figure P4. Photograph of Gel No. 4.

Due to the poor visualization of the protein no conclusions could be drawn from Gel No 5.

Observations on Gel Nos 6 and 7:

The object of the experiment was to scan gelatines derived from old animals with both conditioning time and sulphide concentration as variables. Furthermore the YSA/YSE comparison was repeated.

1. It was noteworthy that only the ST12/* gelatines from old animal hide conditioned for 2 weeks with the lowest level of sulphide exhibited no α-chain bands at all. These gelatines, extracted at 45°, 50°and 60°C had Bloom strengths of 179, 92 and 237 which indicated marked under-conditioning. This observation was in line with the work of Chalepakis *et al.* (1985) who demonstrated that alkaline conditioning was necessary to release α-chains into solution. Gelatines ST14/1 and ST32/1 both exhibited normal α-chain content so both an increase in sulphide level from 1.1 to 2.5 g/l or an increase in time from 2 to 4 weeks at 1.1 g/l sulphide achieved sufficient additional

"conditioning" to release α-chains into solution.

Table 5. *Loading details.*
Gel No. 6.

GEL No 6 LOADING	
30μl per WELL	
WELL No	SAMPLE
1	YSA/1
2	ST12/2
3	ST12/3
4	ST12/1
5	ST34/1
6	ST34/2
7	ST34/3
8	ST16/1
9	ST16/2
10	ST16/3
11	ST32/3
12	ST32/1
13	ST32/2

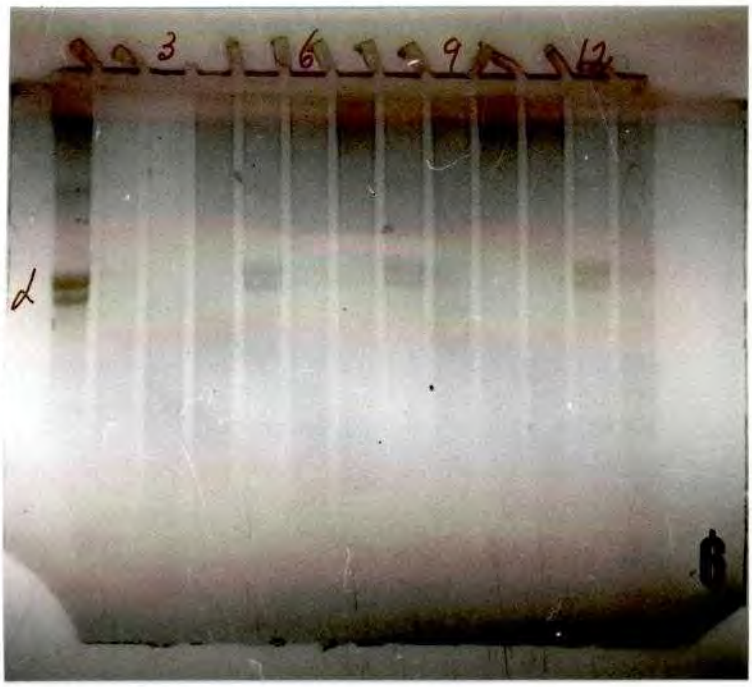


Figure P5. Photograph of Gel No. 6.

Another aspect was demonstrated by the observation that ST12/1, ST14/1 and ST16/1 all exhibited similar extractability but only ST14/1 and ST16/1 exhibited a normal amount of α-chain material in the gelatine. From this it was concluded that alkaline conditioning hydrolysed the cross links that stabilize the collagen triple helix α-chains. (Once destabilized, the α-chain could then dissolve at 45°C). The lack of a parallel change in extractability indicated that there was another mechanism which was not susceptible to alkaline hydrolysis which controled extractability.

2. From Gel 7 it was apparent that there was slightly more β-chain material in YSA/1 than in YSE/1. Hence again, at equal extractability, it appears that the presence of sulphide during conditioning contributed to the rupture of some cross-links that normally prevent the dissolution of collagen as α- and β-chains.

Table 6. *Loading details.*
Gel No. 7.

GEL No 7 LOADING	
30µl per WELL	
WELL No	SAMPLE
1	YSA/1
2	ST14/3
3	ST14/2
4	ST14/1
5	ST36/1
6	ST36/2
7	ST36/3
8	YSA/1
9	YSA/2
10	YSA/3
11	YSE/1
12	YSE/2
13	YSE/3

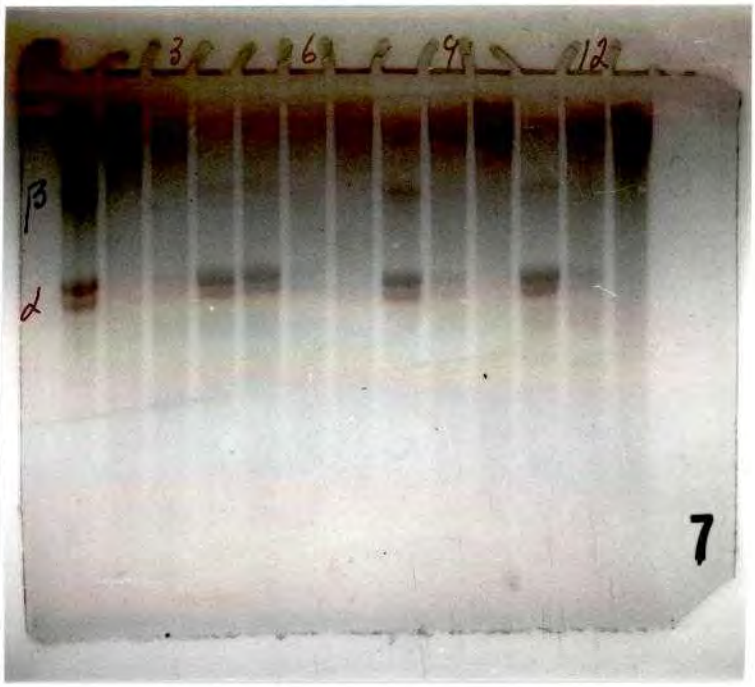


Figure P6. Photograph of Gel No. 7.

Observations on Gel Nos 6 and 7 continued.

3. It was noted that the young animal first extraction gelatines (YS*/1) exhibited ß-chain bands whereas the old animal gelatines (ST**/1) did not. Gels 2 and 3 did not exhibit this phenomenon hence the observation was attributed to the poor visualization of gels 6 and 7, however, the observation did imply less ß-chain protein in gelatine from old animals.

Table 7. *Loading details.*
Gel No. 8.

GEL No 8 LOADING	
50µl per WELL	
WELL No	SAMPLE
2	YSA/1
3	WT1/1
4	WT1/2
5	WT1/3
6	WT3/1
7	WT3/2
8	WT3/3
9	AC3A/1
10	AC3A/2
11	AC3A/3
12	R/Z
13	R/K

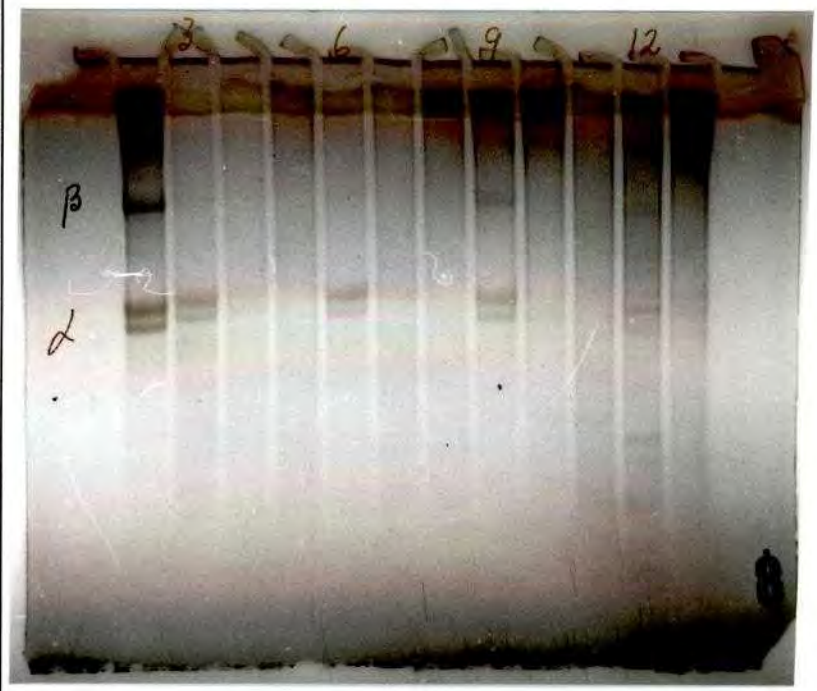


Figure P7. Photograph of Gel No. 8.

Observations - Gels No 8 and 9.

The object of the experiment was firstly to investigate the WT series of gelatines made from an old animal hide but with variances in conditioning temperatures and sulphide levels. (WT1/* gelatines were conditioned without sulphide). Secondly gelatines from hide conditioned by the accelerated conditioning process (AC**)were included for interest.

The R gelatine blends were also of interest due to the difference in their performance characteristics in spite of similar Bloom values:

1. The WT gelatines appeared quite normal in that the first extraction gelatines (WT*/1) exhibited the α-chain double band, the second extraction gelatines (WT*/2) less α-chain material and the third extraction gelatines were almost completely polydisperse.

2. The AC**/1 first extraction gelatines both exhibited ß chain bands. This coupled with the high extractabilities (42% & 53% at 50°C) indicated that the

Table 8. *Loading details.*
Gel No. 9.

GEL No 9 LOADING	
50µl per WELL	
WELL No	SAMPLE
2	YSA/1
3	WT4/1
4	WT4/2
5	WT4/3
6	WT5/1
7	WT5/2
8	WT5/3
9	AC3B/1
10	AC3B/2
11	AC3B/3
12	C200/1

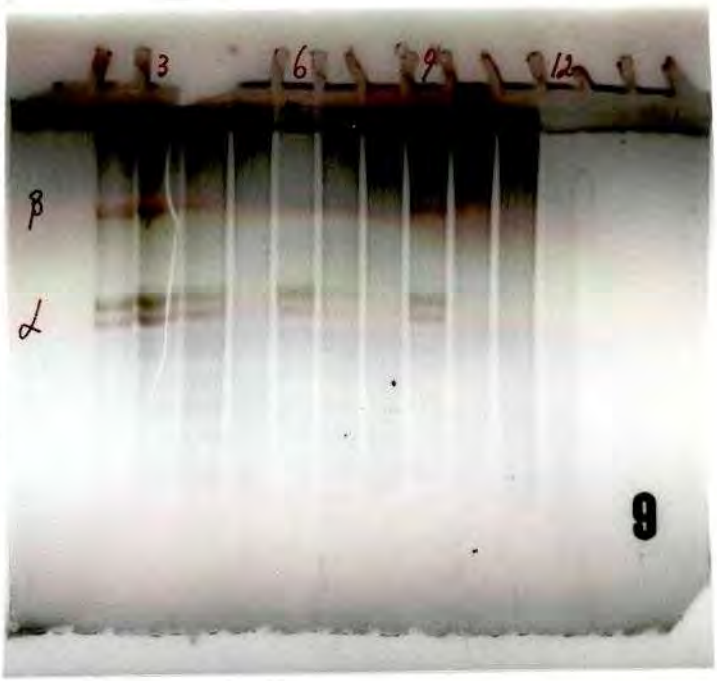


Figure P8. Photograph of Gel No. 9.

Observations - Gels No 8 and 9 continued:

hide was from young animals. This observation was significant in that it showed that the first extraction contained the α- and ß-chain collagen sub-units liberated by conditioning. Thereafter, the gelatine consisted of protein liberated by thermal hydrolysis of the collagen which was apparently polydisperse. Furthermore, α- and ß-chains appeared to have an intrinsic stability which enableed them to resist thermal hydrolysis at a temperature which was sufficient to release polydisperse protein from the "mature" collagen. This raised the question of what temperature was sufficient to dissolve α-chains from collagen without any thermal hydrolysis? From the procedure for the production of acid soluble collagen, Tanaka *et al.* (1988) and Na *et al.* (1987), it was evident that for calf skin some whole collagen molecules were soluble at ambient temperature if the pH was low enough (0.5 M HOAc).

3. The R gelatines had similar Bloom and viscosity values but the R/K material was markedly superior on a 4 hr performance test. The electrophoretograms were very similar but if anything the R/Z material contained a visible amount of

α-chain material whereas the R/K local gelatine was completely polydisperse. The work of Graesser (1985) and Huang, Miao, and Zhang (1989) indicated that the gelation rate of gelation was related to amount of protein with molecular weight greater than α-chain, however, this aspect could not be assessed without a densitometer to measure the amounts of the different components in the gelatine. (Later from Part II of this investigation, it was concluded that the number of bands with greater mobility than α-chain would suggest that R/Z was probably a Type A gelatine).

4. WT4/1 exhibited some ß chain material which was absent in WT5/1. WT4 was conditioned at 12°C with 1.8 g/l sodium sulphide while WT5 received 11.1 g/l sodium sulphide. Extractabilities were hardly different (3.6% and 5.5%). Hence, it may be that very high levels of sulphide usage could militate against the production of ß chain peptide by promoting the hydrolysis of the telopeptide region of the α-chain.

5. Gel No 9, track 12. Chrome gelatine C200/1. This very low Bloom strength gelatine evidently contained protein of too low a molecular weight to be separated on a 5% acrylamide gel. The low visibility of the track even at the bottom of the gel was indicative of the ability of gelatine to diffuse out of the gel during staining and destaining.

Summary of Observations on Gels 1 to 9.

1. The difficulty and variability of gel staining was recognized from the start. It was eventually decided that the stain formula used by Chalepakis *et al*. (1985) should be tried. It appeared that time in TCA Fixer was important and hence TCA as part of the stain solution could be of advantage.

2. The absence of a collagen standard in this work was a source of criticism and this would be corrected in any future work.

3. The discontinuous buffer system gave good separation of the α-chain doublet but apparently due to storage there were anomalies in the resolving gel at various distances below the start. There did not seem to be any evidence of high molecular weight species being held in the stacking gel.

4. The previously used Amido Black staining system, (Cole, 1986) did not stain the gelatine at all using the SDS/Urea system for denaturing the protein.

5. Due to poor and variable staining only the most obvious effects may have been observed, which were:

a. First extraction gelatines contain marked quantities of protein very similar to the double band of α-chain protein demonstrated by others. (Bartley and Marrs, 1974; Murray, Wait and Freire, 1982; Koepff, 1984).

b. First extraction gelatine from well conditioned material (>30% in the first extraction) also contained amounts of protein recognizable as ß chain material.

c. Second extraction and top quality commercial gelatines contained small amounts of α-chain protein, however most of these gelatines were polydisperse protein.

d. Poor conditioning resulting in poor extractability could consequentially result in the absence of α-chain protein even from first extraction gelatines.

e. Where the first extraction temperature was 50°C, the gelatine still contained marked amounts of α-chain protein, (i.e. 50°C did not destroy the molecular species). Hence, it appeared that alkali conditioning hydrolyses the primary (telopeptide) collagen cross-links such that α-chains could be released into solution provided that the temperature was high enough to disrupt the hydrogen bonding (which stabilizes the triple helix of α-chains of procollagen, Heidemann, 1982). After dissolution of α-/β-chains, continued dissolution of collagen was apparently a consequence of thermal hydrolysis resulting in proteins of largely polydisperse molecular weight going in solution. Müller and Heideman (1993) have produced evidence that thermal hydrolysis was selective rather than random for acid extraction of collagen.

f. From the conditioning data in Chapter 2 it was concluded that liming over 1 to 10 weeks had a conditioning effect that was very dependent on the age of the animal. In the case of older animals the first (45°C) extractability was low and independent of liming times of greater than 4 weeks. The electrophoretic data showed a substantially constant amount of α-chain material in first extractions irrespective of animal age. From this it could be hypothesised that above a certain age of about 6 yrs the skin collagen replacement rate slowed to a constant such that only about 10% of the collagen was extractable at 45°C. The alternative deduced from observation on the

ST**/1 gelatines (Gels 6 & 7) was that there were two types of cross-links:

(a) The alkali labile cross-links which release α-chains when hydrolysed.

(b) The stable cross-links which, when formed with age, limit the conversion of collagen to gelatine to thermal hydrolysis of the peptide.

SECOND STUDY WITH BIO-RAD APPARATUS.

The objectives of this study were to:

1. Solve the staining problems encountered in the preliminary series of experiments.

2. To compare Type B gelatines derived from young, middle-aged and old bovine's hide after similar alkaline conditioning treatments.

3. Compare Type A and Type B gelatines from calf skin.

4. Compare Type A gelatines from calf skin and pigskin.

5. Use acid soluble collagen as the reference standard for collagen component electrophoretic mobility.

6. Gels 12 and 13 were to be loaded identically and run together, thus demonstrating the repeatability of the results.

Observations on Gels 12 & 13.

Gels 12 and 13 were marked C and D before being photographed.

Possibly the most significant observation made was that the combination of TCA and Coomassie Blue in the stain resulted in very good visualisation of the separation achieved by electrophoresis. The combination had the disadvantage that methanol and TCA reacted to form compounds of reduced solubility which had an initially "messy" appearance. However, this did not detract from the efficiency of destaining. The visualisation of the low molecular weight bands of Type A gelatine clearly indicated the value of the procedure when highly soluble proteins were involved.

Lane 1 contained the Acid Soluble Collagen (ASC) control. This material showed the gamma chain 300k Dalton material at the top of the gel then the β-chain doublet at 200k Dalton and the well separated α-chain doublet of 100k Dalton at about 1/3 of the length of the track from the top of the gel.

Lanes 3, 4, 5 and 18 contained the acid process gelatines and were notable for the number of distinct bands with greater mobility and lower molecular weights than the α-chain material. This confirmed the findings of Müller and Heideman (1993) that with acid conditioning, extraction caused the formation of discrete subunits of the α-chain rather than random proteolysis.

Table 9. *Loading details. Gel Nos. 12 & 13.*

GELS No 12 & 13 LOADING	
15μl per WELL	
WELL No	SAMPLE
1	ASC
3	CalfA/1
4	CalfA/2
5	CalfA/3
6	YSA/1
7	YSA/2
8	YSA/3
9	CT4/1
10	CT4/2
11	CT4/3
12	5Y4/1
13	5Y4/2
14	5Y4/3
15	ST24/1
16	ST24/2
17	ST24/3
18	D147

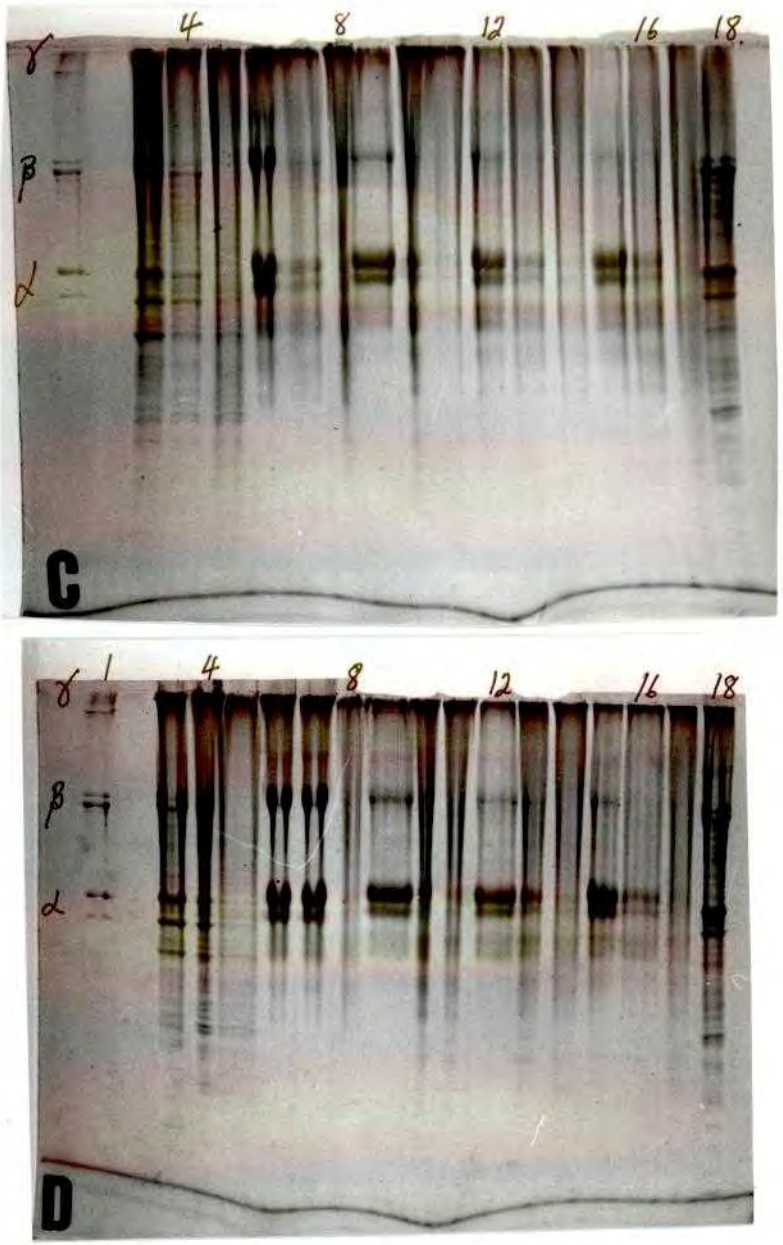


Figure P9. Photographs of Gels 12 & 13.

Observations on Gels 12 & 13 continued.

These Type A gelatines were also notable in that the α-chain material had the same mobility as ASC whereas the α-chain material from mature Type B gelatine had a slightly lower mobility than ASC. This was in agreement with the work of Chalepakis *et al*. and indicated that the α-chain material in Type A gelatin was largely due to the presence of acid soluble collagen from the acid extraction conditions. In Type B gelatines however the alkaline conditioning process hydrolysed the unstable crosslinks giving α-chains that were modified or of higher molecular weight, to go into solution. This was in overall agreement with the theory of Ward (1978) that gelatine manufacture was largely a process of reversing the collagen formation process.

Lanes 6, 9, 12 and 15 showed that by comparison, first extraction Type B gelatines contain far more of the α- and β-chain gelatine than the subsequent extractions. The 12 year-old animal (lanes 15, 16, 17) exhibited a marked amount of α-chain material which indicated that the production of fresh collagen was an on-going process and/or that α-chain material was produced by the hydrolysis of alkali labile cross-links.

Lanes 11, 14 and 17 were of interest in that they showed that with mature animals the third extraction material contained only "polydisperse" protein which must have been derived from the "random" hydrolysis of the peptide bonds of collagen.

Another observation made with every gelatine was that there was a marked amount of material with a discrete molecular weight less than that of α-chain material. In the case of Type B gelatine, from the densitometer data this gelatine had a molecular weight of between 100k and 80k Dalton which would point to a weakness in a particular peptide bond after alkaline conditioning.

Densitometry.

It must be accepted that the data in Table 10. is purely relative. Also only the tracks with a "normal" appearance were subjected to densitometry. Bearing in mind these limitations, the following observations were made:

1. The greatest amount of α-chain gelatine was seen in Type B calf skin gelatine extracted at 50°C (Sample YSA/2). This was also a very pale gelatine

with a colour of 4.4. Other than sample YSA/1 all the other gelatines exhibited markedly lesser α-chain contents and were considerably darker in colour. Similarly CT4/1 from 18 month old animal contained more α-chain material than the other gelatines. Furthermore, with a colour of 5.6 YSA/1 was markedly paler than ST2-4/1 with a colour of 8.9. Hence, again it was evident that increased α-chain availability was accompanied by reduced colour or in other words cross-linking reduced α-chain availability and was associated with darker gelatine colour .

2. The high Bloom Type A gelatines exhibit both β1 and β2 peaks while Type B gelatines only exhibit one β-chain peak in a position approx. equal to β2.

3. The main peaks were often not distinguishable with Type B second and third extraction gelatines.

4. All the gelatines exhibited a peak below α2 but its position was relatively erratic, especially with Type A gelatines. However, with Type B gelatines it was positioned at about 56 mm from the origin equivalent to about 82k Dalton.

Table 10. *Data for molecular weight calculation from ASC mobility.*

Chain	Molecular Weight Da.	Log Mw	Migration Distance mm.	Linear correlation.
Gamma	300 000	5.477	6.5	A = 5.565
β	200 000	5.301	24	B = -0.0118
α	100 000	5.000	47	r = 0.997

From the linear correlation coefficients in Table 10, the peaks at 56 mm from the origin had a molecular weight of about 82k Da.

Log Mw = 5.565 - 0.0118 x distance. ($p > 0.975$)

Table 11. *Densitometer data measured on gel 13 (D).*

T Y P E A gelatine peaks.												
PEAK	GAMMA		β1		β2		α1		α2		<α	
SAMPLE	D-O mm	Area mg	D-O mm	Area mg	D-O mm	Area mg	D-O mm	Area mg	D-O mm	Area mg	D-O mm	Area mg
ASC	6.5	15	23	10	25	19	45	19	49	3	-	-
Pigskin D147	-	-	-	-	25	36	44	104	49	53	72	29
Calf A/1	-	-	22	28	24	15	45	72	49	50	56	35
T Y P E B gelatin peaks.												
PEAK	Origin	Gamma		β		α1		α2		<α		
SAMPLE	Ht mm	D-O mm	Area mg	D-O mm	Area mg	D-O mm	Area mg	D-O mm	Area mg	D-O mm	Area mg	
YSA/1	52	-	-	25	49	46	188	49	118	56	28	
CT4/1	39	-	-	22	32	43	188	46	98	56	10	
5Y4/1	32	-	-	22	16	43	68	46	142	56	17	
ST24/1	43	-	-	22	13	43	144	46	83	55	9	
YSA/2	66	4	39	24	230	43	423	47	281	56	57	
5Y4/2	57	-	-	-	-	44	64	46	43	53	34	
ST24/2	46	-	-	-	-	44	33	45	17	55	10	
YSA/3	71	-	-	-	-	-	-	-	-	58	10	
5Y4/3	40	-	-	-	-	-	-	-	-	61	20	
ST24/3	54	-	-	24	4	42	7	-	-	51	6	

D-O mm = Distance from the origin (Top of the gel) in mm.

Ht mm = Peak height in mm. **Area mg** = Peak weight in mg x 10

\- = Peak not identifiable.

CONCLUSIONS.

The correlation of the electrophoresis data and gelatine colour was through the observation that darker first extraction gelatines showed lower levels of α-chain units in the gelatine due to the increased levels of (pentosidine) cross-linking. However, the electrophoretic results have provided additional insights into the nature of the alkaline conditioning process:

(a) in agreement with the results of Müller and Heidemann (1993), Type A gelatines exhibited a large number of bands with mobilities greater than α-chain.

(b) Type B gelatines show a considerable amount of polydisperse gelatine with molecular weight between 100k and 80k Dalton and little material with a lesser molecular weight, except for gelatines extracted at high temperatures.

2. First extraction gelatines from 5 year-old and 12 year-old animals exhibit similar amounts of α-chain material which implied a continuous slow production of new collagen in aged animals or the presence of an alkali labile mature cross-link.

3. From gels 3 and 4 differences attributable to animal age were observed in that the α-chain mobility was observed to decrease progressively with animal age. This observation was confirmed in Gels 12 & 13. Based on the finding of Tanaka *et al*. (1988) (i.e. that α-chain moved more slowly after incubation with ribose) it could be proposed that the slower movement of mature animal Type B gelatine could be due to the additional size of α-chains (i.e. due to the attachment of sugars to the protein chain). From the fluorescence data in Chapter 4, it was evident that glycosylation was a very slow process, hence, with Type A gelatines and Type B gelatine from young animals (YSA/1), it was observed that the α-chains migrated at the same rate as the ASC α-chain. However, with older animals, glycosylation resulted in modification of the α-chains and a progressive reduction in their SDS-PAGE migration rate.

4. As animals aged, it was accepted that collagen cross-links matured and became more stable. The decrease in α-chain material in gelatine as animals matured together with the increase in 335/385 nm fluorescence and colour intensity indicated that at least some of the stable cross-links were due to the formation of Maillard pentosidine.

5. The ST gelatines in Gels 6 & 7 showed that both conditioning time and sulphide concentration caused the release of α-chain material from collagen, hence, they must both hydrolyse some cross-link/s that stabilise the collagen triple-helix. The failure to observe any sulphide related effect on gelatine colour must mean that the cross-links hydrolysed by sulphide were not those associated with colour. The theory that sulphide, as a reducing agent, could inhibit the Maillard reaction like SO_2 (Ames, 1987) was not substantiated

possibly due to the very low rate of the glucose/protein reaction.

It was also noted that gels 6 and 7 indicated the presence of two separate effects of alkali conditioning. One effect was the release of α-chains from the helix and a separate effect was the enabeling of protein denaturation and solution as gelatine. It was of particular interest to note that the one effect could occur without the other.

6. The role of sodium sulphide in conditioning had not been understood. A comparison of ST12 and ST32 (Gel 6), showed that for equal time the increase in α-chain material could only be attributed to increased sulphide level giving an increased amount of cross-link hydrolysis. However, from extraction data (Chapter 2) it was evident that the effect of sulphide was limited so it had to be concluded that only a few cross-links were susceptible to sulphide hydrolysis. That sulphide caused the release of α-chain material indicated that the hydrolysis was in the telopeptide part of the molecule. This small conditioning effect of sulphide was confirmed in Gel 7 by a comparison of the separations on YSA and YSE gelatines.

7. This study indicated that at extraction temperatures of 45°C or 50°C the Type B gelatine extracted from mature collagen was largely due to the reversal of cross-links by alkaline conditioning. This was followed at higher temperatures by thermal proteolysis resulting in gelatine with a polydisperse molecular weight profile.

REFERENCES.

Ames, J. 1987. The Maillard reaction. Food. September 1987: 67-72.

Bailey, A.J., and Light, N.D. 1989. Connective tissue in meat and meat products. p. 86-95. Elsevier Applied Science. London & New York.

Bartley, J.P., and Marrs, W.M. 1974. The electrophoretic separation of gelatin. Part 2. British Food Manufacturing Industry Research Association Technical Circular No 582.

Bonadio, J. and Byers, P.H. 1985. Subtle structural alterations in the chains of Type I procollagen produce osteogenesis imperfecta type II. Nature. 316: 363-366.

Chalepakis, von G., Tanay, I., and Heidemann, E. 1985. How specific is the decomposition of collagen in the preparation of gelatine. Das Leder 36(1): 2-10.

Cole, C.G.B. 1985. A study of the properties of gelatines derived by various chemical and enzymic methods. M.Sc. Thesis. University of Pretoria. Pretoria. RSA.

Ewing, G.W. 1969. Instrumental Methods of Chemical Analysis, 3rd ed. McGraw-Hill Book Company. New York, St. Louis, San Francisco, London, Mexico, Panama, Sydney, Totonto, Kogakusha Co. Ltd. Tokyo.

Graesser, W. 1985. Connections between physio-chemical properties of photographic gelatines and their molecular weight distribution. Presented at the symposium "Photographic Gelatin" of The Royal Photographic Society. September 1985. Oxford.

Hames, B.D., and Rickwood, D. 1981. Gel electrophoresis of proteins: a practical approach. IRL Press Limited, Oxford and Washington DC.

Hames, B.D., and Rickwood, D. 1981a. Gel electrophoresis of proteins: a practical approach. p. 6. IRL Press Limited, Oxford and Washington DC.

Hames, B.D., and Rickwood, D. 1981b. Gel electrophoresis of proteins: a practical approach. p. 8-11. IRL Press Limited, Oxford and Washington DC.

Hames, B.D., and Rickwood, D. 1981c. Gel electrophoresis of proteins: a practical approach. p. 33. IRL Press Limited, Oxford and Washington DC.

Hames, B.D., and Rickwood, D. 1981d. Gel electrophoresis of proteins: a practical approach. p. 15. IRL Press Limited, Oxford and Washington DC.

Hames, B.D., and Rickwood, D. 1981e. Gel electrophoresis of proteins: a practical approach. p. 44. IRL Press Limited, Oxford and Washington DC.

Heidemann, E. 1982. Newer developments in the chemistry and structure of collagenous connective tissues and their impact on leather manufacture. The Journal of the Society of Leather Technologists and Chemists 66:

21-29.

Hodney, Z., Struzinsky, R., and Deyl, Z. 1992. Silver staining of collagen type I after sodium dodecylsulphate polyacrylamide gel electrophoresis: effect of Maillard reaction. Journal of Chromatography 578: 53-62.

Huang, M., Miao, J., and Zhang, C. 1989. Study of gelatin manufacture technique - extracting process - II. Gelation properties of extracted gelatines. Proceedings 5th IAG conference. Ammann-Brass, H., Pouradier, J. (Ed.). Vol 1 pp 201-16.

Johns, P. 1977. Structure and composition of collagen containing tissues. In: *The Science and Technology of Gelatin*. Ward, A.G., Courts, A. (Ed.). p. 41-42. Academic Press. London, New York, San Francisco.

Kao, W.W-Y., Prockop, D.J., Berg, R.A. 1979. Kinetics for the secretion of nonhelical procollagen by freshly isolated tendon cells. The Journal of Biological Chemistry. 254: 2234-2243.

Koepff, P. 1984. The use of electrophoresis in the gelatin manufacture. International working group for photographic gelatin reports 1970 - 1982. Ammann-Brass, H. and Pouradier, J. (Ed.) p. 197-208.

Miflin, B.J., and Shewry, P.R. 1977. Techniques for the separation of barley and maize seed protein. p. 42-47. Workshop in the EEC Program of coordination of Research on Plant Proteins. Commission of European Communities. Luxenbourg.

Müller, H.-T., and Heidemann, E. 1993. (Translated from German) 2 An investigation of the laws governing the acid decomposition of skin collagen and the identification of collagen splitting points in the acid gelatin process. Das Leder 44: 69-79

Murray, L.W., Wait, J.H., and Freire, E.I. 1982. Preparation and characterization of invertebrate collagens. Methods in Enzymology 82: 65-96. Academic Press. Inc.

Na, C.G., Phillips, L.J., and Freire, E.I. 1989. In vitro collagen fibril assembly: Thermodynamic studies. Biochemistry 28: 7153-7161.

Reich, G., Walther, S., and Stather, F. 1962. On the laws governing the production of gelatine from pigskin according to the acid conditioning process. Investigations of collagen and gelatine III. Deutsche Lederinstitut Freiberg/SA. 18: 15-23.

Tanaka, S., Avigad, G., Eikenberry, E.F., and Brodsky, B. 1988. Isolation and partial characterization of collagen chains dimerized by sugar-derived cross-links. The Journal of Biological Chemistry 263: 17650- 17657.

Ward, A.G., 1978. XVII Proctor Memorial Lecture: Collagen 1891-1977. Retropect and Prospect. The Journal of the Society of Leather Technologists and Chemists. 62: 1-13.

ADDENDA.

ADDENDUM 1. Densitograms of the electrophoretic separations on Gel 13.

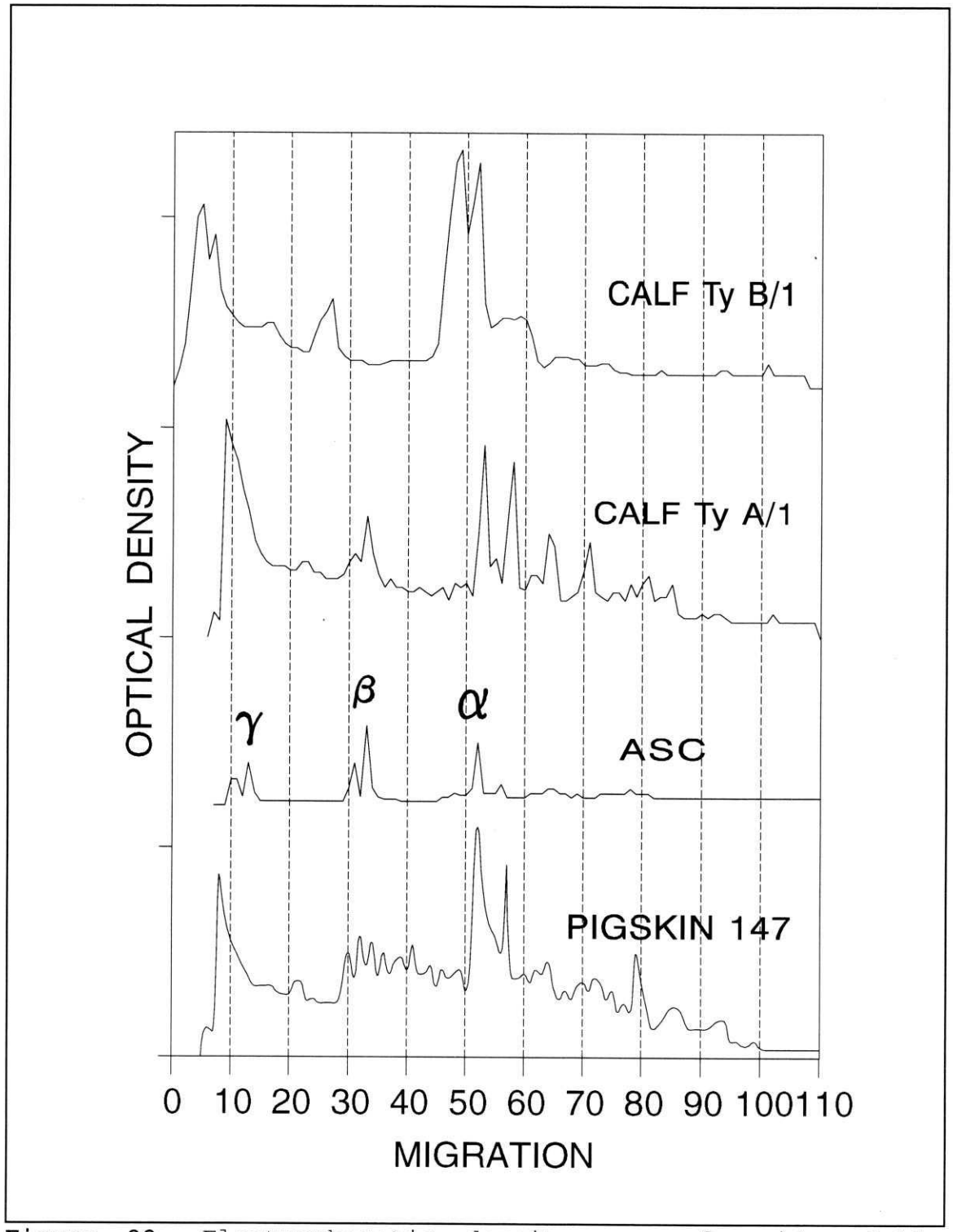


Figure 22. Electrophoretic densitograms of acid soluble collagen (ASC), Pigskin gelatine 147 (PIGSKIN 147), and first extraction gelatines from calf skin, Type A = CA/1 (CALF Ty A/1) and Type B = YSA/1 (CALF Ty B/1). Collagen gamma, β- and α-chains are designated.

Addendum 1 Continued....

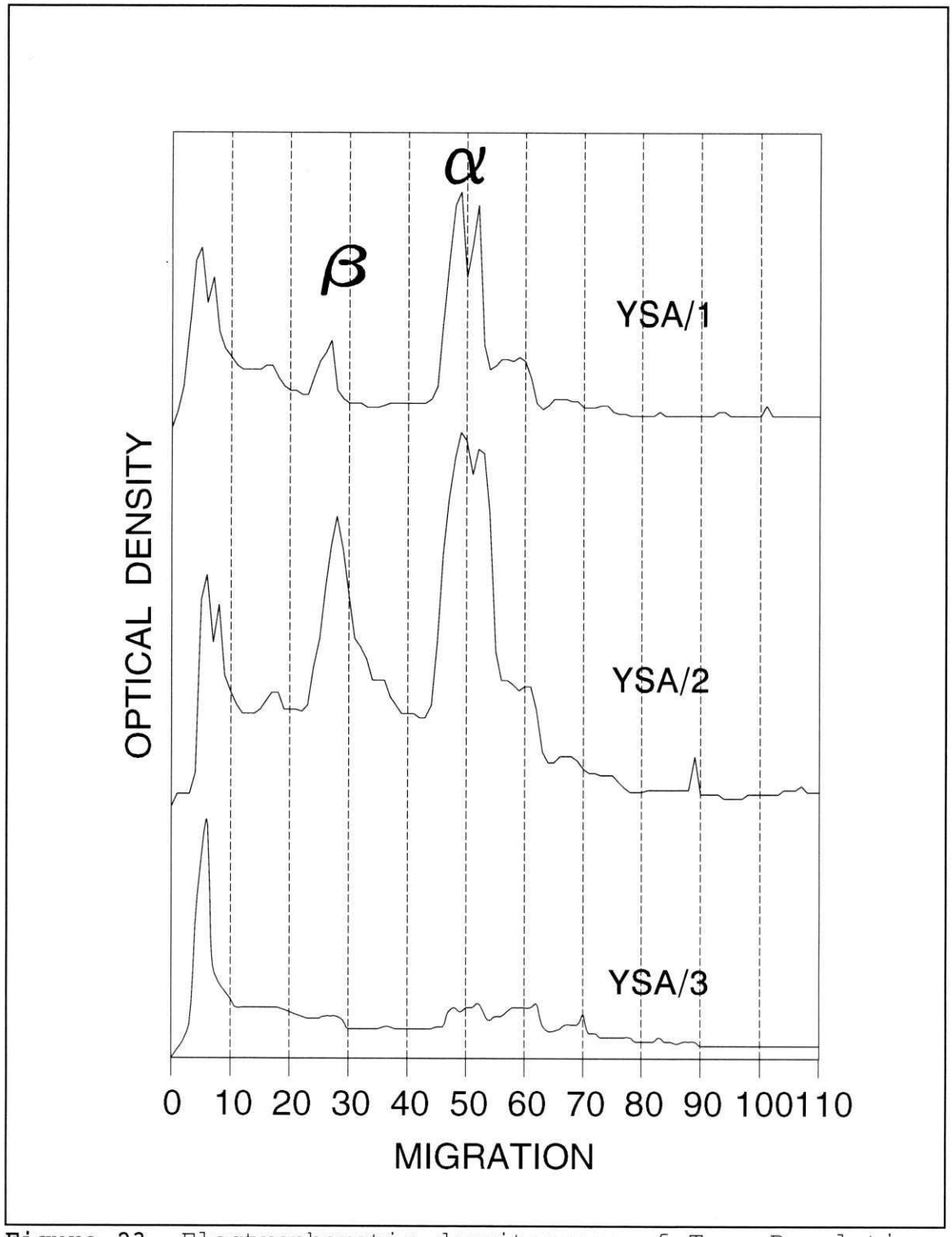


Figure 23. Electrophoretic densitograms of Type B gelatines from 10 month-old bovine hide. Collagen α- and β-chains designated. YSA/1 first extraction, YSA/2 second extraction and YSA/3 third extraction.

Addendum 1 Continued ....

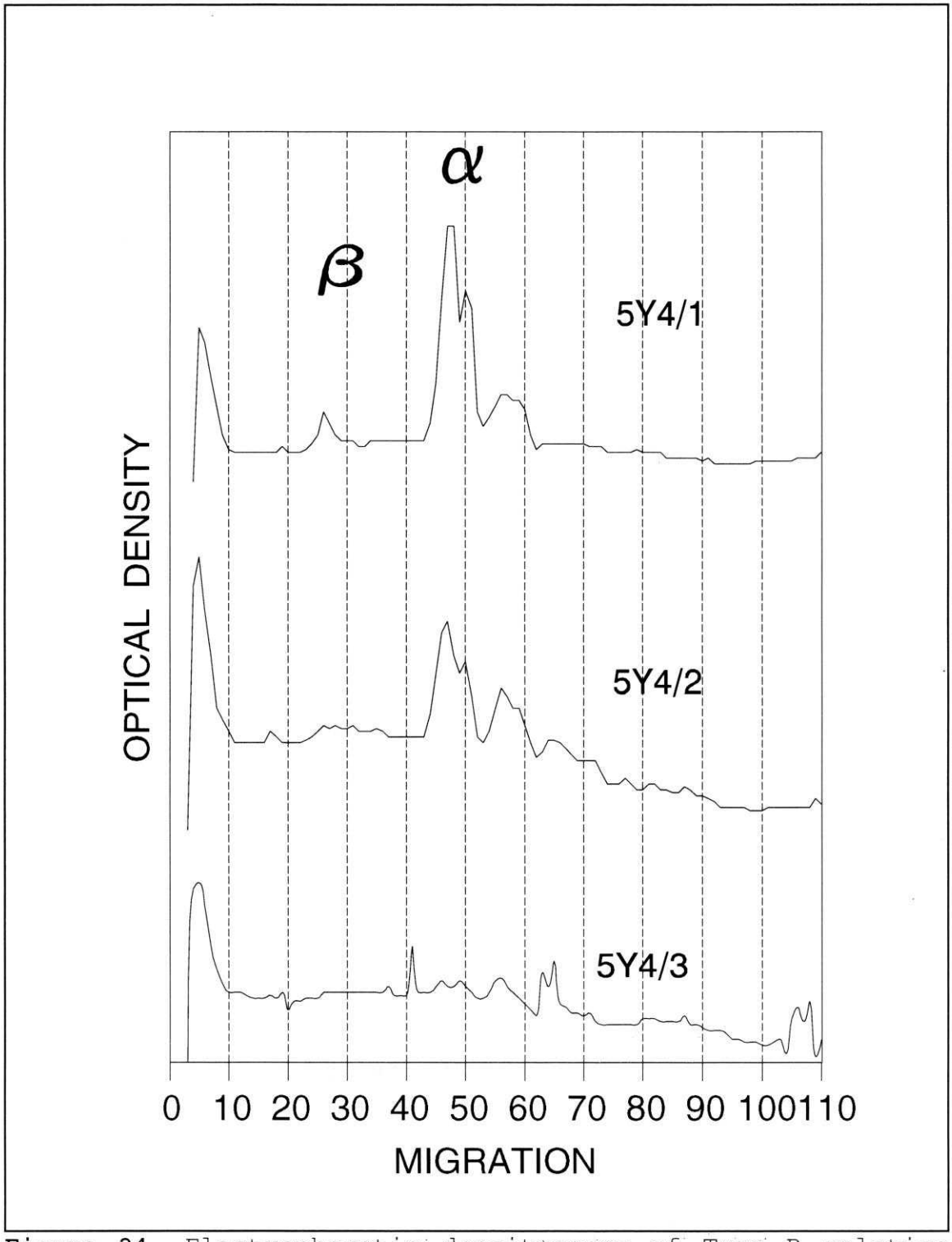


Figure 24. Electrophoretic densitograms of Type B gelatine from 58 month-old bovine's hide. Collagen β- and α-chains designated. 5Y4/1 first extraction, 5Y4/2 second extraction and 5Y4/3 third extraction.

Addendum 1 Continued ....

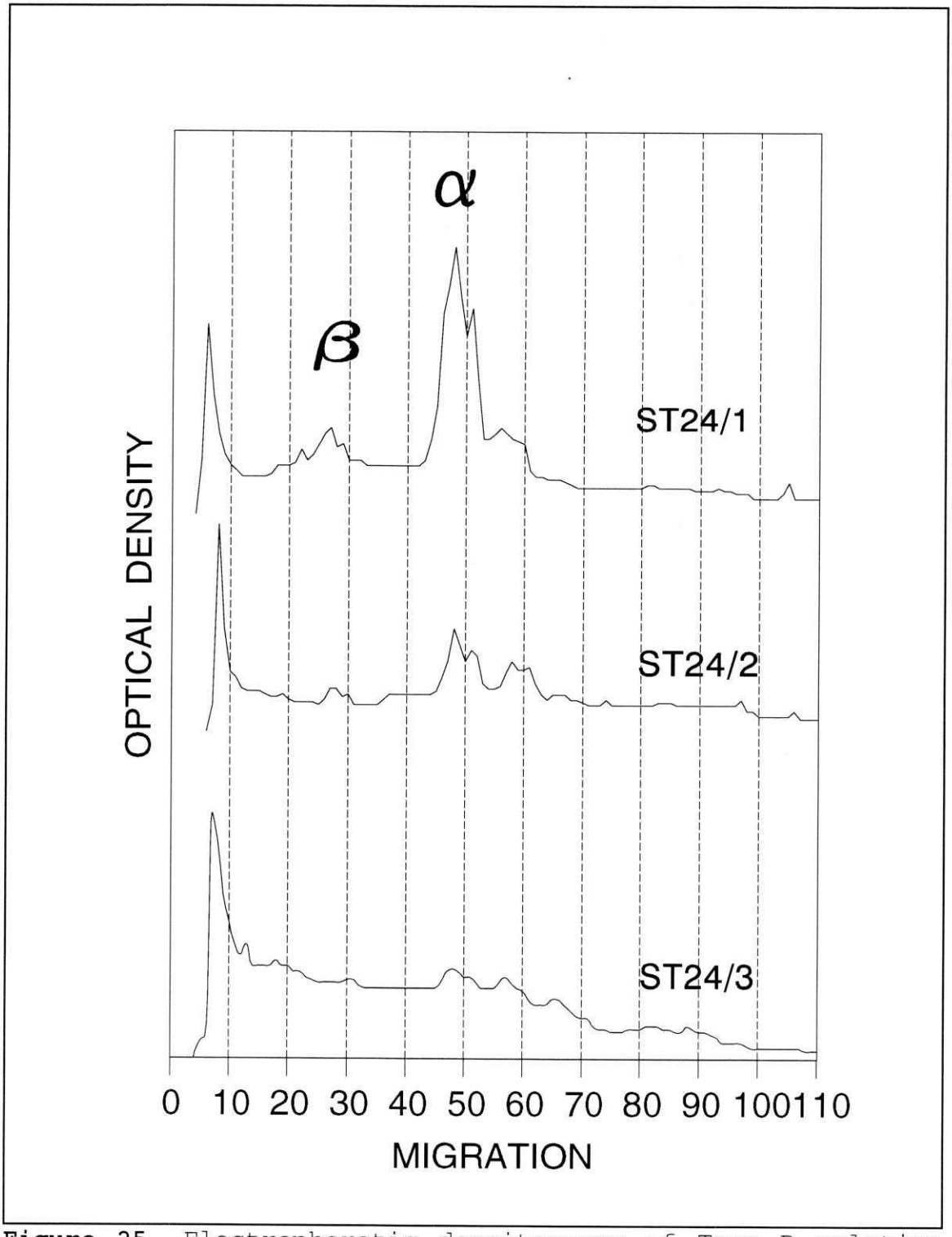


Figure 25. Electrophoretic densitograms of Type B gelatine from 144 month-old bovine's hide. Collagen α- and β-chains designated. ST24/1 first extraction, ST24/2 second extraction and ST24/3 third extraction.

Addendum 1 Continued ....

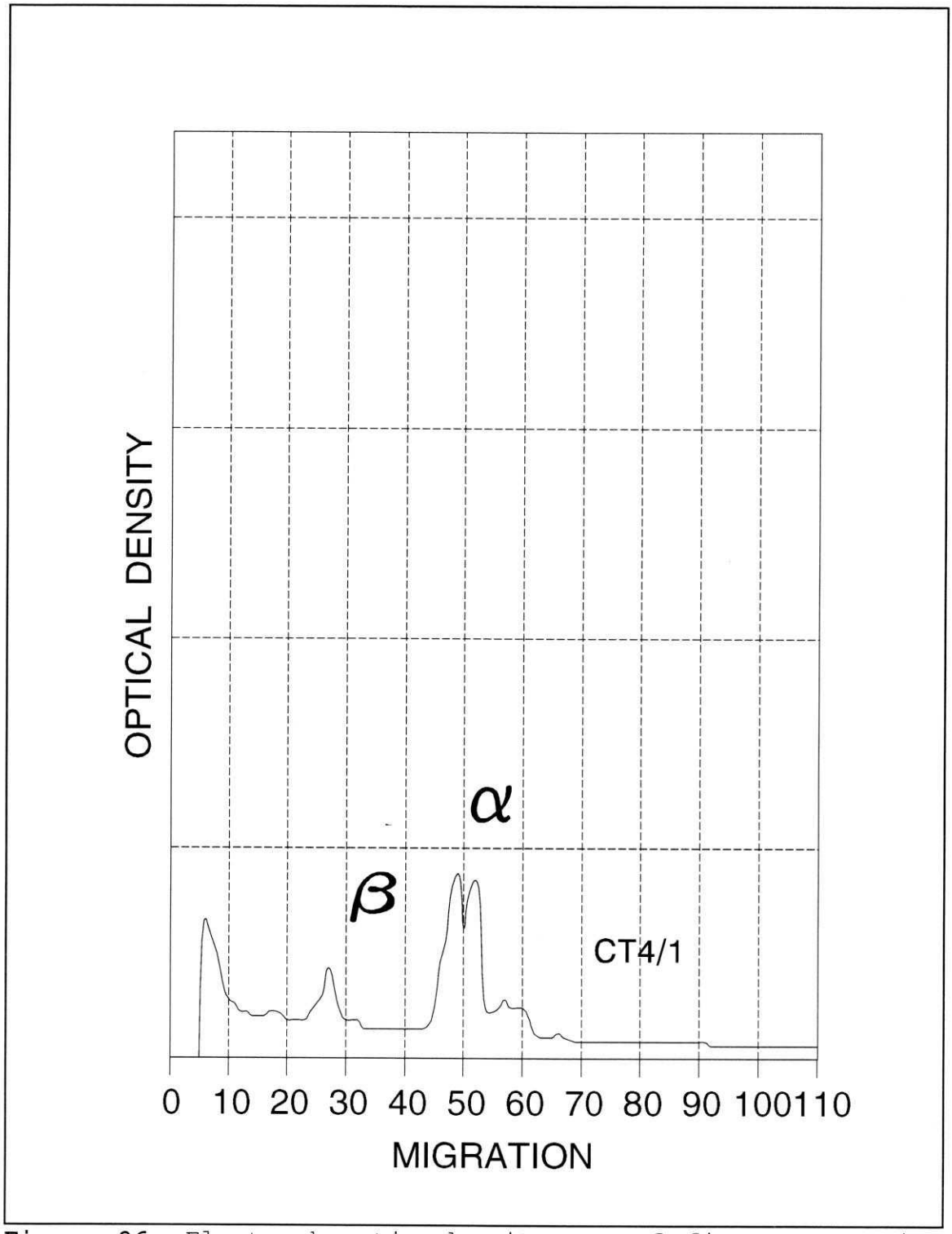


Figure 26. Electrophoretic densitogram of first extraction Type B gelatine from an 18 month-old animal. Collagen α- and β-chains are designated.

CHAPTER SIX. Discussion.

This study of gelatine colour was undertaken primarily because DGI was desirous of producing from its traditional beefskin raw material a gelatin that would be more competitive with the paler pigskin and ossein gelatines with which the European and American markets were more familiar.

Gelatine manufacturers tend to be secretive as evidenced by the scarcity of publications in the field, hence there was little background on which the study could be based, other than that based on personal experience.

The problem was approached mainly from the point of view of gelatine manufacture because this was the area of expertise. The other factor was that gelatine manufacturers seem to think that their processes were unique and that their process was responsible for the uniqueness of their product. Of course, to an extent, this was true as any manufacturer trying to duplicate the gelatine from another source will know. The differences were due mainly to Bloom - viscosity relationships, ash contents and pI which were all known factors of the production processes. Hence, it was natural to suspect that differences in colour were also a function of the processing of the raw material. From experience it was also known that, to a small extent, gelatine colour was a function of the raw material used (tannery splits usually gave pale gelatine).

The occurrence of dark gelatines.

Having possibly the unique ability and experience to produce gelatine on a small scale, the first approach to the investigation of the process was to develop a technique that would allow the comparison of a number of processing variables without interference from the raw material. Gelatine manufacturers utilise waste collagenous materials, hence, they usually do not use whole hides. For this study however, most of the investigation was conducted on whole hides as it had the advantage of eliminating the raw material as a variable in a series of experiments. The result of this was that for the first time, a detailed study of the alkaline conditioning process was possible. The next factor which was of great assistance was the ability to obtain the hide of animals of known origins and age from the Irene Animal Production Institute

(IAPI) previously known as Animal and Dairy Sciences Research Institute.

The process used for the manufacture of gelatine was a traditional one, namely, lime-sulphide conditioning followed by sulphurous acid acidulation and then batchwise extraction. It was known that sulphurous acid acidulation had a marked effect on gelatine colour and if, for example, hydrochloric acid were used in its place the colour of the gelatine was very much darker. This effect was recently quantified in experiment IN0 (Cole, 1993) in which sulphuric acid acidulation was used. Sulphuric acid gave first extraction colours of 16 and overall colours of 21 compared to the sulphurous acid first extraction colour of 9.4 and overall colour of 15. Hence, in using the results of this study it must be borne in mind that the actual colours produced depended to an extent on the gelatine manufacturing process used.

Besides the sulphurous acid acidulation process it was known that alkaline peroxide bleaching of the raw material could give some additional colour improvement, however, variable decomposition of the peroxide meant that the results were variable. Alkaline peroxide bleaching of gelatine solution was also effective but the process was time dependent, hence there was a consequential quality deterioration. Activated carbon treatment of gelatine solution was also of limited effect but it added considerably to filtration difficulties. Anion exchange also, was known to have had a limited bleaching effect on dark low quality gelatine solutions but hardly any effect on the top quality paler gelatine solutions. Combination of all these processes could provide quite marked colour improvements but the cost was prohibitive and the greatest effect was on the low quality gelatines whereas the colour improvement was most desired on the naturally paler top quality gelatines.

The major finding of the investigation of the conditioning process was that variations within the process had a small to negligible effect on the colour of the resultant gelatine and animal age was shown to have been the major contributor to the colour. Furthermore, the acid conditioning process gave Type A gelatines from calf skin with colours equivalent to those from pigskin. Also there was virtually no difference in gelatine colour from calf skin whether produced by the acid or the alkaline conditioning processes, provided that the extraction pH and temperatures were the same.

The small effect on gelatine colour contributed by variations in the process was associated with darker gelatine being produced at higher levels of

extractability, in line with the most easily converted collagen yielding gelatine of the best colour. The overall colour was found to be substantially unaffected by variations within the process and no evidence was found for any effect on gelatine colour attributable to the use of sodium sulphide in conditioning.

Finally, it was found that the large variation in gelatine colour apparently related to the quality parameters of Bloom strength and viscosity was only observed with gelatines from old animal hide. Young animal's hide produced gelatines of pale and comparatively invariant colour.

The study was extended to different breeds of bovines and it was found that within the breeds available there were no statistically significant differences in gelatine colour with breed. This did not preclude the possibility that other breeds, not investigated, could give gelatines of a different colour.

Other results from the investigation.

Possibly by far the greatest importance of the study on the alkaline conditioning process was the knowledge gained of the effect of animal age. It was never suspected that lime-sulphide conditioning could have had such a small conditioning effect on old animal hide nor that the degree of conditioning was largely independent of conditioning time above 4 weeks, for older animals. Furthermore, the demonstration that the effect of sodium sulphide in conditioning was not concentration dependent above a concentration of about 1.5 g/l made considerable savings in sulphide usage possible.

Another result of the investigation was quantification of the variance in collagen (gelatine) content of hide and the resultant proximate analysis of anhydrous, ash-free, hide:

Gelatine / Collagen	59 %
Fat	4 %
Hair	9 %
Non Collagen Protein	8 %
Unknown Losses.	20 %

Gelatine colour measurement.

The second colour problem applicable to the gelatine industry as a whole was the measurement of gelatine colour. The problem, as previously explained, was due to the interference with spectrophotometric absorbance by variances in light scatter caused by variation in gelatine clarity and molecular weight.

In approaching the problem of the measurement of gelatine colour, cognisance was taken of the need for a method that was applicable to the widest possible range of gelatines. For this reason, in developing a method, production Type A and Type B gelatines were included in the assessment as well as experimental gelatines derived from horse hide, sheep skin, fish skin and chrome tanned leather as well as gelatines from other manufacturers. Regrettably only one ossein gelatine (derived from bone) was available, however, this one sample did not behave abnormally.

Firstly the problem was tackled by enzymic hydrolysis of gelatine solutions to a uniform low molecular weight and then membrane filtration to uniform clarity. The process gave an acceptable correlation ($r=0.95$, $n=35$) between instrumental and visual colour assessment provided that the initial gelatine clarity was not worse than 100 NTU on a 6.67% solution. Also it was found that there were problems when the gelatine pH was out of the effective pH range of the enzyme being used, hence for the full range of gelatines at least two enzymes were required.

In continued investigations it was found that with the same clarity limitation as above, the BYK-Gardner Tristimulus Reflectance Spectrophotometer could give as good a statistical correlation ($r = 0.96$, $n=86$) between the visual colour and the instrumental colour, without any special sample preparation. The reason for the success with the Gardner instrument was that there was an inherently large amount of light loss due to scatter, with the result that small changes in scatter due to variances in gelatine clarity and molecular weight were negligible.

The main advantage of the instrumental measurement of gelatine solution colour was its reproducibility due to the elimination of the personal errors of the visual method. As the aim was to come as close as possible to the visual colour there was neither the need nor the opportunity to remeasure all the colours of the gelatines produced in this study over the span of several

years, using the Gardner instrument.

Ancillary to the measurement of the colour of gelatine was a study of the absorbance spectra of a large number of gelatines between 150 and 700 nm. It was of interest to note that the absorbance of gelatine in the ultraviolet region between 280 and 400 nm was markedly variable, with darker gelatines exhibiting more absorbance than pale gelatines. The result of this was that after enzymic hydrolysis and filtration the 400 nm absorbance was almost as good a measure of colour as was the total area under the absorbance curve between 400 and 700 nm. Furthermore, poor clarity gelatines showed greater absorbance at 700 nm than clear gelatines. These effects of clarity variance could be reduced by moving the origin of the absorbance curve up to the 700 nm absorbance before measuring the area under the curve. This study also exhibited a spectral anomaly at 320 nm only exhibited by gelatines derived from chrome tanned leather which would point to some molecular peculiarity associated with these SPA process gelatines.

Cause of gelatine colour.

Having produced a large number of gelatines with a large variance in colour the investigation of the cause of the colour was based on the techniques available.

Past investigations using chromatography of gelatine did not lead us to have faith in that approach. The investigations of Sell & Monnier (1989), Uchiyama, Ohishi, Takahashi, Kushida, Inoue, Jugie and Horiuchi, (1991) and Kuypers, Tyler, Kurth and Horgan, (1993) lead us to realise that degradation and isolation of the chromophore was very much a dedicated team project and not within the means at our disposal.

Based on the investigations of Tanaka *et al*. (1988), Sell & Monnier (1989), Uchiyama *et al*. (1991) and later Odetti *el al* (1994), it appeared that the development of colour in gelatine could well be the result of increased degree of the Maillard cross-linking with age in which case this could be verified using spectrofluorophotometry as the Maillard "pentosidine" cross-link of Sell & Monnier (1989) was fluorescent at 335/385 nm. Furthermore, Tanaka *et al*. (1988) had shown that the Maillard cross-linking of collagen could be detected by a change in the electrophoretic mobility of the α-chain, hence, the application of SDS-PAGE could possibly yield interesting results.

Another approach that might have been applicable was the use of ELISA to detect the presence of pentosidine but it was only after the study was completed that the production of a suitable antibody by Taneda and Monnier (1994) was reported in chemical abstracts.

Fluorescence in gelatine.

No reference to gelatine fluorescence was available from the literature, hence there was nothing but collagen studies on which the investigation could be based. Collagen was not soluble, hence, it was presumed that those working in the field worked in reflectance mode rather than the transmittance mode which was possible with solutions. The reflectance mode would not, however, be as prone to concentration quenching but this mode would not be as sensitive from the quantitative point of view.

From the outset it was clear that gelatine, like collagen, did exhibit fluorescence, however the spectrum was not simply 335/385 nm "pentosidine" fluorescence but there was a second emission peak at about 410 nm. The excitation peak at 295 nm that Uchiyama *et al*. (1991), found to be due to the pyridinoline cross-link, was only observed in Type A calf skin gelatine, hence it was concluded that alkaline conditioning destroyed this cross-link. This was a most important finding and enabled a clear picture of the effect of alkaline conditioning in the gelatine manufacturing process as follows:

It was known from the research studies of Sell & Monnier (1989) and Uchiyama *et al*. (1991) that the pyridinoline cross-link remained constant with animal age but that the concentration of pentosidine cross-link increased with age. Hence, the finding that alkaline conditioning destroyed the pyridinoline but not the pentosidine cross-link explained the observation in the conditioning experiments that:

(i) liming time had a marked effect on the extractability of young animal hide low in pentosidine cross-links,
and
(ii) liming time had a comparatively limited effect on the extractability of old animal hide with a high concentration of pentosidine cross-links.

The observation that the 370/440 nm collagen fluorescence of Tanaka *et al*. (1988) was found also only in Type A calf skin gelatine indicated that the

molecule causing this fluorescence was also subject to alkaline degradation.

The second finding that was considered to be of importance was the good statistical correlation found between the 335/385 fluorescence intensity and first extraction gelatine colour and the absence of a correlation between the third extraction gelation colour and fluorescence intensity. This was interpreted as top quality, first extraction, gelatine colour being due to the Maillard-pentosidine cross-linking and lower quality gelatine colour being caused by a second unknown factor. This was confirmed experimentally by the anion exchange results presented in Chapter 4, Table 9. The multi-factor cause of gelatine colour explained the often variable response of gelatine to bleaching processes as it was probable that each process would only affect one of the two (or more) chromophores in gelatine.

The one disappointment of the investigation was the failure to throw any light on the reason for the abnormally pale colours of gelatines derived from chrome tanned leather.

Gelatine sugar interaction.

In order to verify that the Maillard reaction, as exhibited by pentosidine formation, was in fact a cause of gelatine colour, the reducing sugar experiments of Tanaka *et al*. (1988) were repeated at a physiological pH of 6 and at an alkaline pH of 9. (This latter pH was known to accelerate the aldehyde/lysine interaction). These experiments confirmed their findings by showing that the gelatine-ribose reaction rate at pH 6 was about 28 times faster than the gelatine-glucose reaction rate.

There were two important observations made from these experiments:

a. The 335/385 nm fluorescence intensity of the gelatines increased with time of reaction as did the colour of the gelatines and in the case of ribose, the reaction mixtures gelled.

b. The colour spectrum of glucose darkened gelatine was the same as the spectrum of dark gelatine, whereas the colour spectrum of ribose darkened gelatine was markedly redder than that of dark gelatine.

The first observation was expected and confirmed that gelatine darkening could certainly be caused by the Maillard cross-linking and pentosidine formation.

The second observation was interpreted as showing that *in vivo* glucose was probably the source of the five carbon atoms linking lysine and arginine to form pentosidine, even though this possibly requires the loss of 1 carbon atom. However, the fluorescence excitation and emission maxima were the same for both glucose and ribose darkened gelatine so the difference in colour could be associated possibly with a second chromophore and not the Maillard chromophore. This was not confirmed by the anion exchange bleaching experiments that followed. Furthermore, in contrasts to the finding of Sell & Monnier (1989) that a mixture of lysine, arginine and glucose did not form the fluorescent product, pentosidine, this experiment showed that with glucose and a complex protein like gelatine the Maillard reaction, with pentosidine formation, did proceed even if rather slowly.

The slowness of the Maillard reaction in solution, particularly between gelatine and glucose, was an explanation possibly of the failure to observe any effect on gelatine colour due to sulphide in conditioning. The slowness of the reaction rate would be multiplied if the steric hinderance to the Maillard reaction, (indicated by the ribose-gelatine reaction experiment to have resulted from gelation), was verified. The other explanation for the lack of a sulphide effect could be the possibility that SO_2 inhibition of the Maillard reaction was a function of the formation of a sulphite addition compound (with the aldehyde) rather than a function of the reducing properties of sulphite.

The control experiments conducted without sugars showed unequivocally that the darkening and fluorescence development were attributable to the presence of the reducing sugar in the reaction mixtures.

Pentosidine synthesis.

The pentosidine formation procedure of Sell & Monnier (1989) was modified by reducing the reaction temperature. Ambient temperature reaction of lysine, arginine and ribose favoured the formation of fluorescent products with the excitation maximum at 335 nm and an emission spectrum similar to that of gelatine (that is, with a prominent second peak at about 410 nm). At 37°C reaction favoured the 385 nm emission peak with a lesser shoulder at 410 nm.

Hence, this eliminated any suspicion that the gelatine fluorescence was not due to pentosidine because of the second peak at 410 nm, but rather it shows that there were possibly more than one isomer of pentosidine with slightly different emission spectra.

The effect of anion exchange.

It was accepted that anion exchange was useful as part of the de-ashing of gelatine solutions and the additional benefit of colour removal was demonstrated. An experiment was in progress to determine the capacity of a particular resin for colour removal hence a number of samples were available that had been anion exchanged, together with the original gelatines. The difference in the fluorescence intensities of treated and untreated gelatines were within the normal limits of experimental error, and it was concluded that the observed bleaching effect was not associated with removal of the Maillard induced chromophore.

When the anion exchange resin was regenerated with caustic soda, the very dark spent regenerant solution was examined using the spectrofluorophotometer and although the solution was mildly fluorescent, the amount of fluorescence was such as to confirm that the resin had accumulated very little of the intrinsic gelatine fluorescence. Furthermore, it was noted that the 335/385 nm fluorescence of the regenerant solution was greater at pH 6 than at pH 12 which agreed with the observation of Sell & Monnier (1989) that pentosidine fluorescence was quenched at pH 9.

An additional confirmatory experiment was undertaken to determine whether the colour produced by the Maillard reaction could be removed by anion exchange. If, as had been contended, gelatine colour was associated with a cross-link formation then the colour should not be removable by anion exchange. The experiment showed that anion exchange had a very small effect on the colour of ribose darkened gelatine solution thus confirming that the chromophore was tightly bound to the protein chain. This experiment also confirmed that because anion exchange did bleach darker low quality gelatines, the colour removed by the resin had to be due to a different chromophore from that formed by the Maillard cross-linking.

In summary:

1. Gelatine exhibited 335/385 nm pentosidine fluorescence as well as a

second emission peak at about 410 nm. The fluorescence intensity was correlated significantly to first extraction gelatine colour but not to third extraction gelatine colour.

2. Type A calfskin gelatine also exhibited weak 295/395 pyridinoline fluorescence which appeared to be destroyed by alkaline conditioning explaining the very limited time related response of old animal hide to conditioning.

3. Glucose-gelatine interaction gave colour production with a spectrum identical to that of naturally dark gelatines. Ribose-gelatine interaction produced a chromophore with a spectrum different to that of naturally dark gelatine. Hence natural pentosidine was probably the product of glucose and collagen interaction. The very slow rate of the gelatine-glucose Maillard reaction was demonstrated and it was concluded that this could be the reason for the failure to observe the Maillard reaction when it was expected.

4. The ribose-gelatine interaction at 37°C, besides exhibiting colour and pentosidine fluorescence development also exhibited gelation which clearly demonstrated the cross-linking effect of the Maillard reaction.

5. The low temperature reaction of lysine, arginine and ribose produced products with a fluorescence spectrum very similar to that of gelatine.

6. Anion exchange removed colour from low quality dark gelatine without effecting its fluorescence. It was unable to remove the colour from ribose darkened gelatine. Hence, there were at least two chromophores responsible for the colour of gelatine.

Electrophoresis.

The work of Tanaka *et al*. (1988) showed that the ribose collagen interaction caused an increase in the amount of α-chain dimer. The α-chains also appeared to migrate more slowly under the conditions of electrophoresis. Hence, it was anticipated that α-chains from dark gelatine might migrate more slowly than those from pale gelatines due to the additional size contributed by the Maillard addition products. The differences in molecular mobility observed were the previously reported differences between α-chains from Type A and Type B gelatines (Chalepakis, Tanay, and Heidemann, 1985) and also a progressive decrease in mobility of the α-chains with animal age.

The initial experiments were conducted using the methodology of Chalepakis *et al*. (1985) except for the staining where it was decided that the conventional

Stegman staining technique should be better (Miflin and Shewry, 1977). After some time it was found that staining was not good and it was realised that probably the reason for the inclusion of trichloroacetic acid (TCA) in the stain solution used by Chalepakis *et al*. (1985) was to prevent highly soluble gelatine from diffusing out of the gel during overnight staining. In order to obtain clean gels it was essential that the Coomassie blue be completely dissolved in the stain solution. Hence, as Coomassie blue was not readily soluble in TCA it was decided to try dissolving the Coomassie blue in methanol, the TCA in water and combining the two immediately prior to use. In spite of the reaction between TCA and methanol which formed an unsightly odorous oil on the surface of the polyethylene staining basins, the staining of the gels was good and the oil did not seem to adhere in any way to the polyacrylamide gel. After removing the stain solution the gel was washed in destaining solution, transferred to a clean glass plate and then transferred to a clean basin for destaining. The dirty basin was easily cleaned using methanol.

From gels 12 and 13 (Chapter 5) it was evident that the electrophoresis techniques had not been perfected however the gels were good enough to show that:

1. There was a marked difference between type A and Type B gelatines.
2. That all first extraction gelatines contained significant amounts of α-chain collagen, (hence, the most easily hydrolysed collagen was also the least coloured).
3. That all third extraction gelatines contained little or no α-chain collagen (and they were also the darkest).
4. What others seem to have failed to observe, a significant amount of a protein with a molecular weight of about 82 kD in all the gelatines.

Difference between Types A and B gelatine.

The difference between Type A and Type B gelatine was the higher mobility of the Type A α-chain material, as found by Chalepakis et al. (1985) and also the large number of discrete bands with a higher mobility (lower molecular weight) than α-chain material in Type A gelatine. Müller and Heidemann (1993) searched for and found discrete or preferred points of thermal hydrolysis of collagen.

These preferred points of hydrolysis were far less evident in Type B alkaline process gelatines than in Type A gelatines. Furthermore they found that the α, β, and gamma chain bands of Type B gelatine were far more prominent than those in Type A gelatine. This could now be attributed to the destruction of pyridinoline cross-links by liming freeing the basic collagen subunits to go into solution as Type B gelatine. In the Type A process, however, the presence of α-chains appeared to be a limited to the solution of acid-soluble collagen from young animal hide.

The amount of α-chain material in first extraction gelatines.

From the densitometer measurements it was evident that the amount of α-chain material in Type B gelatines decreased with increasing temperature of extraction or run number. However, the amount of α-chain material was surprisingly unaffected by animal age except for calf skin Type B gelatine (YSA/2) where extraction at 50°C seemed to produce almost pure α, β and gamma chain collagen. From the consistency of the amount of α-chain material in Type B gelatines extracted at 45°C it was concluded that at this temperature thermal proteolysis was negligible and protein going into solution was from new, minimally cross-linked collagen. The amount of first extraction gelatine available (after 4 to 6 weeks lime sulphide conditioning) from old animal hide was about 10 % of the total collagen which would be a measure of the collagen replacement rate in bovine hide.

Third extraction gelatines.

These gelatines evidently contained very low levels of the collagen subunits, hence it was concluded that these gelatines were truly protein with a random molecular weight profile. From the quality parameters it was evident that these gelatines could also have excellent properties like YSA/3 with a Bloom gel strength of 286 g and viscosity of 47 ms. This was a clear demonstration of the difficulty of relating electrophoretic profiles to physical quality parameters.

The 82 kD component of gelatine.

Gels 12 and 13 showed that all gelatines seemed to have had a significant amount of this component which could only be interpreted as indicating that there was a thermally unstable peptide bond some 18% down the length of the

α-chain or in the vicinity of amino acids in positions 180 or 820. It was significant that of the preferred splitting points found by Müller & Heidemann (1993), three were at amino acid number 779 of the α1 chain, one each was at No 208 and at 791 of the α1 chain and one was at No 838 (from the N terminal end) of the α2 chain.

Other known causes of colour in gelatine.

Other than the use of strong acids in the acidulation of limed hide, the only other known cause of dark colour was the presence of iron as a contaminant. The effect of iron was shown to be measurable only at about 50 ppm. Currently the iron content of edible gelatines did not exceed 50 ppm.

Based on the knowledge gained over the years it could be said that chromium was the only other inorganic contaminant which could contribute to colour and only at levels which were well above the 0 to 5 ppm currently found in edible gelatines.

The amino acid analyses on pale and dark gelatines provided by Stevens and Stevens (1992) did not show any significant change in lysine content with colour hence it was confirmed that a very small number of cross-links had a very marked effect on the properties of collagen as indicated by Dyer *et al.* (1991). The well known conversion of arginine to ornithine by alkaline treatment was also confirmed. The bonding of chromium to glutamic and aspartic acids in chrome tanning could only be seen as a small reduction in the aspartic acid content of gelatine from this source.

Finally it was worth mentioning that samples of dark and pale gelatines were given to an Australian laboratory that used Fourier Transform Infra-red analyses on a regular basis. They did observe differences in the spectra but were unable to interpret the differences in a meaningful way.

Conclusions and Recommendations.

1. Gelatine Colour.

a. On the main subject of this study it has been shown that at least in part the colour of gelatine was the result of the Maillard reaction *in vivo*. Hence, much of the colour of gelatine was a function of the age of the animal at

slaughter. As a result the prevention of this colour formation or its elimination from the gelatine did not seem to be practical.

b. Dark gelatine was a comparitavely rich source of the pentosidine cross-link and could be used for further study of this phenomenon. For example, the confirmation and location of the natural pentosidine cross-link in the helix or the telopeptide, as was done by Kuypers, Tyler, Kurth and Horgan (1994) for the Ehrlich Chromogen, would be of interest . Furthermore, the elucidation of the difference in the glucose and ribose mediated cross-links could have far-reaching significance.

c. It has been shown that there was at least a second source of colour in gelatine which was largely associated with lower quality product. This colour was not associated with fluorescence and it was removable by anion-exchange. It was felt that this would be a profitable subject for further study both from the point of view of the structure of the chromophore and its formation and also its inhibition.

d. This study was limited to gelatine produced by a liming process followed by sulphurous acid acidulation. It was known that the use of a strong acid in place of sulphurous acid had a deleterious effect on colour. Hence, the characterisation of the sulphurous acid bleached chromophore would be of interest.

e. One of the objectives of this study was to elucidate the reason for the pale colour of gelatine from chrome tanned leather produced by the SPA process (Drew, 1930). No light was shed on this phenomenon and it was felt that research in this direction would be most likely to lead to the desired gelatines of low colour.

f. There was no evidence from this study that the alkaline stage of the gelatine manufacturing process was in any way responsible for the dark colour of alkali process gelatine. In fact this study showed that alkali process Type B gelatine from calf skin (YS experiments) produced as good if not better gelatine colour than did the traditional acid process for Type A gelatine (CALF-A experiment). In fact all the evidence indicated that alkali treatment had no effect on gelatine colour and it was felt that the indications were that further studies in this area will be futile. There was however evidence from Hains (1984) that the treatment that collagen received between abattoir

and gelatine manufacture could be significant especially if processes were allowed to occur that caused the liberation of reducing sugars.

2. Gelatine colour measurement.

This study produced a commercially viable method for the instrumental and objective measurement of edible gelatine colour. It will be interesting to see whether the industry adopts the technique.

3. Hide conditioning.

The studies did much to characterise the variances in response to the alkali conditioning process, of collagen from animals of various ages . Further study of the variances in the alkali process would be valuable from the point of view of the manufacturer's ability to control the process. Currently it would appear that the traditional liming process was giving way to faster conditioning processes involving stronger alkalis.

This study showed that the apparent inverse correlation between gelatine colour and Bloom gel strength or viscosity was only valid for gelatine extracted from old animals. From young animals the colour of the gelatine was remarkably invariant.

4. Gelatine Fluorescence.

The fluorescence study established the correlation between the cross-linking of hide due to the Maillard reaction and animal age. The study also provided evidence that the source of the sugar for the Maillard reaction was in fact glucose rather than ribose, as suggested by Sell and Monnier (1989). The implications of the Maillard reaction cross-linking for gelatine extractability and quality was discussed. Further research on the correlation of extractability and fluorescence could be of great value to the manufacturer for the control of the process, in that the age of the animal at slaughter could be determined.

5. Gelatine Electrophoresis.

The electrophoresis of gelatine had been used for controlling quality in sensitive applications like photographic emulsions (Koepff, 1984). In this

study, the effects of animal age and extraction temperature were clearly shown by use of this technique. From the point of view of quality control it appeared that the technique could also be used to distinguish between Type A and Type B gelatine, however pI determination would be a simpler approach to that problem.

References.

Chalepakis, von G., Tanay, I., and Heidemann, E. 1985. How specific is the decomposition of collagen in the preparation of gelatine. Das Leder 36(1): 2-10.

Cole, B. 1993. The effect of various acid treatments on the colour of gelatine. Davis Gelatine Industries Research Report dated 12 December 1993.

Dyer, D.G., Blackledge, J.A., Katz, B.M., Hull, C.J., Adkisson, H.D., Thorpe, S.R., Lyons, T.J., and Baynes, J.W. 1991. The Maillard reaction in vivo. Zeitschrift für Ernährungswissenschaft 30(1): 29-45.

Drew, R.B. 1930. An improved process for extracting glue and gelatine from chrome leather. British Patent Specification 338,584. Nov.17, 1930. His Majesty's Stationery Office, Redhill, UK.

Haines, B.M. 1984. The skin before tannage-Proctor's view and now. Journal of the Society of Leather Technologists and Chemists 68: 57-70.

Kuypers, R., Tyler, M., Kurth, B.L., and Horgan, D.J. 1994. The Molecular location of Ehrlich Chromogen and pyridinoline cross-links in bovine perimysial collagen. Meat Science 37: 67-89.

Miflin, B.J., and Shewry, P.R. 1977. Techniques for the separation of barley and maize seed protein. p. 42-47. Workshop in the EEC Program of coordination of Research on Plant Proteins. Comission of European Communities, Luxenbourg.

Müller, H.T., and Heidemann, E. 1993. (Translated from the German) An investigation of the laws governing the acid decomposition of skin collagen and the identification of collagen splitting points in the

acid gelatine process. Das Leder 44: 69-79.

Odetti, P., Pronzato, M.A., Noberasco, G., Cosso, L., Traverso, N., Cottalasso, D., and Marinari, U.M. 1994. Relationships between glycation and oxidation related fluorescence in rat collagen during ageing. An *In vivo* and *In vitro* Study. Laboratory Investigation 70(1): 61-67.

Sell, D.R., and Monnier, V.M. 1989. Structure elucidation of a senescence cross-link from human extracellular matrix. Journal of Biological Chemistry 264: 21597-21602.

Stevens, P.V. & Stevens, J.A. 1992. Interim report on South African gelatine samples. Amino acid and crosslink analysis and Further results on six Sth African gelatines. (Private communications dated 11.6.92 and 1.7.1992)

Tanaka, S., Avigad, G., Eikenberry, E.F., and Brodsky, B. 1988. Isolation and partial characterization of collagen chains dimerized by sugar-derived cross-links. The Journal of Biological Chemistry 263: 17650-17657.

Taneda, S. and Monnier, V.M. 1994. ELISA of pentosidine, an advanced glycation end product, in biological specimens. Clinical Chemistry 40: 1766-1773.

Uchiyama, A., Ohishi, T., Takahashi, M., Kushida, K., Inoue, T., Jugie, M., and Horiuchi, K. 1991. Fluorophores from ageing human articular cartilage. Journal of Biochemistry (Tokyo) 110: 714-718.