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

by

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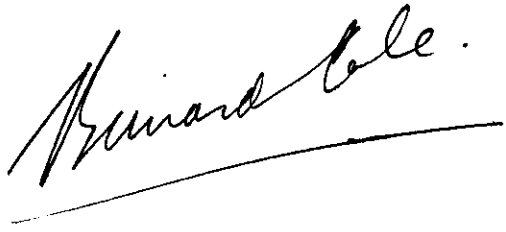
I declare that the thesis herewith submitted for the degree of Ph.D (Food Science) at the University of Pretoria, has not been submitted previously by me for a degree at any other University.



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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.

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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.

TABLE OF CONTENTS.

INDEX OF TABLES.

CHAPTER TWO.

CHAPTER THREE.

CHAPTER FOUR.

CHAPTER FIVE.

INDEX OF FIGURES.

CHAPTER THREE.

CHAPTER FOUR

CHAPTER FIVE.

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.