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STUDIES ON EQUINE MILK AND COMPARATIVE STUDIES ON EQUINE AND BOVINE MILK SYSTEMS

Thesis presented by

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for the degree of

Doctor of Philosophy

in

Food Chemistry

June, 2011

Table of Contents

Abstract

Acknowledgements

Abbreviations

		Page
Chapter 1	Equid Milk	**1**
Chapter 2	Equine Milk Proteins	**106**
	Thesis Objectives	**181**
Chapter 3	Compositional, Chemical and Physico-Chemical Properties of Equine Milk	**183**
Chapter 4	Stability of Equine Casein Micelles. I. Acid-induced Coagulation of Equine Milk	**244**
Chapter 5	Stability of Equine Casein Micelles. II. Rennet-induced Coagulation of Equine Milk	**278**
Chapter 6	Stability of Equine Casein Micelles. III. Dissociation of Equine Casein Micelles by Various Treatments	**339**
Chapter 7	Effect of Heat or High Pressure on the Whey Proteins and Casein Micelles of Equine Milk	**382**
Chapter 8	Proteomic Study of Equine Milk Proteins and Comparison of Changes in Equine and Bovine Milk Proteins on Renneting	**409**
Chapter 9	Electron Microscopy Studies on Equine and Bovine Casein Micelles	**458**
Chapter 10	Digestion of Equine β-casein and Examination of Peptides for the Presence of Casomorphins	**493**
Chapter 11	General Discussion	**517**
	Proposed Future Work	**531**
Appendix		**535**
	Publications	i
	Co-authored publications	ii

Abstract

The composition of equine milk differs considerably from that of the milk of the principal dairying species, i.e., the cow, buffalo, goat and sheep. Because equine milk resembles human milk in many respects and is claimed to have special therapeutic properties, it is becoming increasingly popular in Western Europe, where it is produced on large farms in several countries. Equine milk is considered to be highly digestible, rich in essential nutrients and to possess an optimum whey protein:casein ratio, making it very suitable as a substitute for bovine milk in paediatric dietetics. There is some scientific basis for the special nutritional and health-giving properties of equine milk but this study provides a comprehensive analysis of the composition and physico-chemical properties of equine milk which is required to fully exploit its potential in human nutrition. Quantification and distribution of the nitrogenous components and principal salts of equine milk are reported. The effects of the high concentration of ionic calcium, large casein micelles (~ 260 nm), low protein, lack of a sulphydryl group in equine β-lactoglobulin and a very low level of κ-casein on the physico-chemical properties of equine milk are reported. This thesis provides an insight into the stability of equine casein micelles to heat, ethanol, high pressure, rennet or acid. Differences in rennet- and acid-induced coagulation between equine and bovine milk are attributed not only to the low casein content of equine milk but also to differences in the mechanism by which the respective micelles are stabilized. It has been reported that β-casein plays a role in the stabilization of equine casein micelles and proteomic techniques support this view. In this study, equine κ-casein appeared to be resistant to hydrolysis by calf chymosin but equine β-casein was readily hydrolysed. Resolution of equine milk proteins by urea-PAGE showed the multi-phosphorylated isoforms of equine α_s- and β-caseins and capillary zone electrophoresis showed 3 to 7 phosphorylated residues in equine β-casein. *In vitro* digestion of equine β-casein by pepsin and Corolase PP™ did not produce casomorphins BCM-5 or BCM-7, believed to be harmful to human health. Electron microscopy provided very clear, detailed images of equine casein micelles in their native state and when renneted or acidified. Equine milk formed flocs rather then a gel when renneted or acidified which is supported by dynamic oscillatory analysis. The results presented in this thesis will assist in the development of new products from equine milk for human consumption which will retain some of its unique compositional and health-giving properties.

Abbreviations

ANOVA	analysis of variance
α-la	α-lactalbumin
BCM	β-casomorphin
β-lg	β-lactoglobulin
CCP	colloidal calcium phosphate
CZE	capillary zone electrophoresis
CMP	caseinomacropeptide
CN	casein
CP	cold-precipitated
cryo-SEM	cryo scanning electron microscopy
cryo-STEM	cryo scanning transmission electron microscopy
CSLM	confocal scanning laser microscopy
CTRL	control
Da	dalton
1-DE	one dimensional electrophoresis
2-DE	two dimensional electrophoresis
DLAOR	dynamic low amplitude oscillatory rheometry
DLS	dynamic light scattering
DWS	diffusing wave spectroscopy
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
G′	storage modulus
G′′	loss modulus
GDL	glucono-δ-lactone
HCT	heat coagulation time
HP	high pressure
IDF	International Dairy Federation
IMCU	international milk clotting unit
J	creep compliance
MALDI	matrix-assisted laser desorption/ionization

MCP	micellar calcium phosphate (or CCP)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
Nano-LC	nano liquid chromatography
NPN	non-protein nitrogen
η*	complex dynamic viscosity
Pa	pascal
Pa s	pascal second
PAGE	polyacrylamide gel electrophoresis
Pi	inorganic phosphate
PCA	principal component analysis
PMF	peptide mass fingerprinting
PTA	phosphotungstic acid
RCT	rennet coagulation time
RP-HPLC	reverse phase high performance liquid chromatography
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SGID	simulated gastrointestinal digestion
SMUF	synthetic milk ultrafiltrate
tan δ	tangent of phase angle
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TEM	transmission electron microscopy
TOF	time of flight
TS	temperature sensitive
ω	frequency
ζ-potential	zeta potential

Amino acid abbreviations

One-letter symbol	Three-letter symbol	Amino acid
A	Ala	Alanine
B	Asx	Aspartic acid or asparagine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	Xaa	Unknown
Y	Tyr	Tyrosine

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CHAPTER 1

Equid Milk:

Chemistry, Biochemistry and Processing

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Chapter 1 contents:

1.1. Introduction
- 1.1.1. Equid evolution
- 1.1.2. Equid domestication
- 1.1.3. Ruminants and non-ruminants
- 1.1.4. Why equid milk is used in human nutrition
- 1.1.5. Production of equid milk

1.2. Composition of Equid Milk
- 1.2.1. Factors that affect the composition of equid milk
  - 1.2.1.1. Effect of stage of lactation
  - 1.2.1.2. Effect of equid breed on milk composition

1.3. Proteins of Equid Milk
- 1.3.1. Caseins
  - 1.3.1.1. α_{S1}-Casein
  - 1.3.1.2. α_{S2}-Casein
  - 1.3.1.3. β-Casein
  - 1.3.1.4. κ-Casein
    - 1.3.1.4.1. Glycosylation of κ-casein
    - 1.3.1.4.2. Hydrolysis of κ-casein
- 1.3.2. Casein Micelles
  - 1.3.2.1. Stability of equid casein micelles
  - 1.3.2.2. Enzymatic coagulation of equid milk
  - 1.3.2.3. Acid-induced coagulation of equid milk
  - 1.3.2.4. Heat-induced coagulation of equine milk
  - 1.3.2.5. Stability of equine milk to ethanol
- 1.3.3. Whey Proteins
  - 1.3.3.1. β-Lactoglobulin
  - 1.3.3.2. α-Lactalbumin
  - 1.3.3.3. Immunoglobulins
  - 1.3.3.4. Lactoferrin
  - 1.3.3.5. Whey protein denaturation

1.4. Digestibility of Equid Milk

1.5. Total amino acids

1.6. Non-protein Nitrogen
- 1.6.1. Free amino acids
- 1.6.2. Bioactive peptides
- 1.6.3. Leptin and related products
- 1.6.4. Amyloid A

1.7. Indigenous Enzymes
- 1.7.1. Lysozyme
- 1.7.2. Other indigenous enzymes in equine milk

1.8. Carbohydrates
    1.8.1. Lactose and glucose
    1.8.2. Oligosaccharides
1.9. Lipids
    1.9.1. Free fatty acids
    1.9.2. Structure of triglycerides
    1.9.3. Equid milk fat globules
    1.9.4. Rheology of equid milk fat
1.10. Vitamins
1.11. Minerals
    1.11.1. Macro elements
    1.11.2. Trace elements
1.12. Physical properties of equid milk
    1.12.1. Density
    1.12.2. Refractive index
    1.12.3. pH
    1.12.4. Freezing point
    1.12.5. Viscosity
    1.12.6. Colour
1.13. Processing of equid milk
    1.13.1. Koumiss
    1.13.2. Other products from equid milk
1.14. Nutritional and biomedical properties of equid milk
    1.14.1. Cow milk protein allergy
    1.14.2. Cross-reactivity of milk proteins
1.15. Summary
1.16. References

1.1. Introduction

Approximately one third of all mammalian genera are herbivores, more than half of which belong to 2 orders, the Perissodactyla [odd-toed ungulates (hoofed animals)] and the Artiodactyla (even-toed ungulates) (Savage and Long, 1986). The horse and donkey belong to the Order Perissodactyla which has three families: Equidae (nine species of horses, donkey and zebras), Tapiridae (four species of tapirs) and Rhinocerotidae (five species of rhinoceros). Uniquely among species, all Equidae can interbreed, but the hybrid offspring are almost always infertile because horses have 64 chromosomes, donkeys have 62 (Trujillo *et al.*, 1962). Zebras have between 32 and 46 chromosomes, depending on breed [Burchelli's zebra, *Equus burchelli*, has 44 chromosomes, (Carbone *et al.*, 2006)] and viable hybrids with donkeys have been produced where gene combinations have allowed for embryonic development to birth. Differences in chromosome numbers between horses and zebras are most likely due to horses having 2 longer chromosomes that contain single gene content compared to 4 zebra chromosomes (Benirschke *et al.*, 1964).

This article presents a brief historical overview of some aspects of the domestication of equid species, a review of the chemistry and biochemistry of the principal constituents of equine milk and, to a lesser extent, of asinine milk, with comparative data for bovine and human milk. The technological properties of equid milk, with reference to processing of the milk are examined and, finally, a synopsis of the nutritional and biological significance of equid milks in the human diet is presented.

1.1.1. Equid evolution

Perissodactyla species evolved during the early Eocene (~ 55 M years ago) and were the dominant ungulate order until ~ 15 M years ago when numbers declined, probably due to climatic factors rather than competition with emerging Artiodactyla species (including the Ruminantia family) (MacDonald, 2001). Nevertheless, wild equids ranged in vast herds across the grasslands of the northern hemisphere and in South America until the end of the ice age, ~ 10,000 years ago. Wild horses were the chief prey of increasing human populations and numbers decreased until they became extinct in North America ~ 7,000 years ago and in Europe, herds of horses were pushed eastwards into central Asia where the last few Przewalski horses (*Equus ferus przewalski*) survived until the 20th Century (Clutton-Brock, 1992). It is generally assumed that equids were domesticated 5,000 years ago but

archaeologists believe man did not ride horses until 1,000 BC, although horses replaced the ox as draft animals 2,000 BC (Clutton-Brock, 1992). One of the early records of the domestic horse in Western Europe comes from horse remains found at the late Neolithic site at Newgrange, County Meath, Ireland (Clutton-Brock, 1992). Outram *et al.* (2009) demonstrated domestication of the horse in the Eneolithic Botai culture of Kazakhstan ~3,500 years ago and analysis of organic residues based on characterization of stable isotopes of carbon, $\delta^{13}C$ (which allows differentiation of non-ruminant and ruminant carcass and dairy fats) and deuterium δD, values of fatty acid analysis revealed processing of mares' milk and meat in ceramics at that time. The donkey (*Equus asinus*) is believed to have evolved from the Nubian and Somalian sub-species of African wild asses ~4-5000 years ago and has since been an integral part of human life as a pack and riding animal. Today, perissodactyls are a poor second to artiodactyls in terms of numbers of species, geographical distribution, variety of form and ecological diversity (MacDonald, 2001). For further details on equid evolution and genetic lineages of domestic horse species see Vilà *et al.* (2001) and references therein.

1.1.2. Equid domestication

Only 5 major species of large, plant-eating mammals have been widely domesticated: sheep, goat, cattle, pig and horse. Nine additional minor species have been domesticated but are restricted to certain geographical areas: Arabian and Bactrian camels, llama, alpaca, donkey, reindeer, water buffalo, yak, Bali cattle of Southeast Asia and the gaur (mithan) of India and Burma (Bruns, 1999). Despite taxonomic congruity and behavioural similarities between equid species, only two equids have been domesticated: the horse (*Equus ferus*) and the donkey (*Equus asinus africanus*) (Clutton-Brock, 1992). Because milk and products derived from it provide up to 30% of dietary protein in developed countries, to meet this demand man has genetically improved some species for milk production (Mercier, 1986). Domesticated animals have been modified from their wild ancestors through being kept and selectively bred for use by humans who control the animals' breeding and feeding. Domestication of dairy species has meant that different breeds of farm animals, e.g., dairy cows and goats, can be developed and maintained to optimize certain hereditary factors, e.g., length of lactation, period of gestation and milk yield, as well as protein and fat levels in the milk produced. Genetic selection of breeds of horse and donkey for milk production has not occurred, as yet, and consequently there is high variability for milk yield and length of lactation, as well as high individual variability.

In some regions of the world, e.g., Mongolia and southern Russia, Hungary, France and Belgium, the horse and, less frequently, the donkey, is an important source of meat and milk. Steppe Mongols, forest-steppe Kazakhs, the Hadza hunter-gatherers of Tanzania and the urban French all regard horses as a valuable food source and believe that horse flesh and milk have special nutritional and medicinal attributes (Levine, 1998). The use of donkeys as a dairy species can be traced back to Roman times when the nutritional value of its milk and beneficial properties in skin care were first recognized (Salimei, 2011).

The importance of the horse in leisure activities, especially racing, has led to the scientific breeding and nutrition of horses, including foals, and has created the need to characterise the composition and properties of equine milk, which are now relatively well known. The fact that the horse is spread throughout the world, has been domesticated, is relatively easily handled, and produces large amounts of milk, which can be obtained relatively easily, makes equine milk a relatively easy subject for research. Although the donkey is now less widely distributed than the horse, its milk can be obtained readily. The zebra has not been domesticated and although it is widespread in the wild in Africa and in captivity elsewhere, apparently it is very difficult to obtain zebra milk, even from captive animals. Studies of the social behaviour of equids have shown that there are no intrinsic reasons why the zebra has never been domesticated but it is believed that the peoples of Africa may have had cultural rather than biological reasons not to use zebras as pack animals (Clutton-Brock, 1992).

1.1.3. Ruminants and non-ruminants

The horse and donkey are non-ruminant herbivores and digest portions of their feed first enzymatically in a foregut and then ferment it in a very large sacculated hindgut. Limited digestion occurs in the equid stomach which liquefies incoming feed and secretes gastric acid and pepsin to initiate breakdown of feed components. The equid digestive system is designed to process small amounts of food frequently (Sneddon and Argenzio, 1998). Equids rarely fast for more than 2 to 4 hours at a time and naturally forage for 16 to 18 hours per day. Horses in the wild roam widely, grazing both day and at night on immature, easily digested food and exhibit few digestive problems in comparison to domesticated horses (Sellnow, 2006). Unlike ruminants, and in keeping with their status as prey animals, horses do not require periods of rest to stop and ruminate. Ruminants have very efficient digestive systems with microbial breakdown of fibrous food at the start of the gastro-intestinal tract and nutrient absorption

along the entire intestine. Ruminants can digest fiber and carbohydrate more completely than any other species.

Donkeys, like horses, generally survive on a diet high in fiber and low in soluble carbohydrate and protein but while donkeys are not as efficient as ruminants at digesting cell-wall components, they are far more efficient than horses (Izraely *et al.*, 1989). Donkeys are capable of consuming large amounts of forage and gain more digestible energy from it than even goats fed a similar diet (Smith and Sherman, 2009). The donkey achieves this by substantially increasing its forage intake rate to compensate for a low quality diet. A donkey uses its narrower muzzle and prehensile lips for greater selectivity of its food, thereby maximizing feed quality rather than quantity (Aganga *et al.*, 2000). Donkeys have a much lower water requirement per unit weight than any other mammal, except the camel, and can rehydrate quickly with large volumes of water without complications. Donkeys can work while suffering from severe dehydration by reducing water and energy turnover rates while maintaining feed intake and its plasma volume can be maintained by drawing on the substantial fluid reservoir in the hind-gut (Sneddon *et al.*, 2006). The zebra, on the other hand needs a constant source of water for survival (Aganga *et al.*, 2000).

1.1.4. Why Equid Milk in Human Nutrition?

The benefits of equine milk for human health is an ancient idea and there is much literature from the former Soviet Union on this subject, although it is now accepted that the results of experimental work are dubious (Doreau and Martin-Rosset, 2002). Because equine milk resembles human milk in many respects and is claimed to have special therapeutic properties, it is becoming increasingly important in Western Europe, especially in France, Italy, Hungary and the Netherlands. Equine milk (and koumiss, fermented equine milk) is often used for the treatment of a myriad of ailments including anaemia, nephritis, diarrhoea, gastritis disorders, cardiovascular disease and in post-operative care, as well as for stimulation of the immune system (Lozovich, 1995). In Mongolia, where koumiss is the national drink, people have a saying that ‘kumys cures 40 diseases’ (Levine, 1998). In Italy, equine milk has been recommended as a possible substitute for bovine milk for allergic children (Curadi *et al.*, 2001). Equine milk is considered to be highly digestible, rich in essential nutrients and possesses an optimum whey protein:casein ratio, making it very suitable as a substitute for bovine milk in paediatric dietetics. Estimates suggest that more than 30 million people drink

equine milk regularly, with this figure increasing significantly annually (Doreau and Martin-Rosset, 2002).

The use of asinine milk by humans for alimentary and cosmetic purposes has been popular since Egyptian antiquity. Cleopatra is reputed to have bathed daily in asinine milk and kept a herd of 700 to fill her bath. Hippocrates (460-370 BC) was a strong advocate of the use of asinine milk as a medicine and used it to cure many ailments including, liver disease, oedema, nose bleeds, poisoning and wounds. Today, asinine milk is consumed mainly in countries where donkeys were traditionally bred, Asia, Africa and eastern Europe but more recently it has been used successfully as a substitute for human milk in western Europe (Vincenzetti *et al.*, 2008) and is the milk of choice in Italy for children with severe IgE-mediated cows' milk allergy. (Iacono *et al.*, 1992; Businco *et al.*, 2000).

1.1.5. Production of equid milk

The production of equine milk and the factors that affect it have been the subject of several reviews, including Doreau and Boulot, (1989); Doreau *et al.*(1990); Doreau, (1994); Doreau and Martin-Rosset, (2002) and Park et al. (2006) and, therefore, is considered only briefly here.

World milk production was ~ 695 million tonnes in 2009, of which 84% was bovine, 13% buffalo and 3% sheep, goat and other species (IDF, 2009). Statistics for milk production from species other than cows and buffalo are not very reliable and are available only from countries where these milks are processed industrially although it is accepted that, while bovine milk production figures have changed little in recent years, the production of milk from buffalo, camels, horses and donkeys is increasing. In Europe, it is estimated that about 1-1.3 million litres of equine milk are currently produced per annum but in countries such as Mongolia the figure is considerably higher, probably ~ 9 million litres.

A decade ago, equine milk was produced only in isolated small holdings in parts of Eastern Europe and Mongolia but now there are large-scale operations in France, Belgium, Germany, Austria and the Netherlands. Asinine milk is produced in large donkey farms in Italy, France, Spain, Belgium, Xinjiang and Shanxi provinces of China, Ethiopia and Pakistan (Salimei, 2011).

For equine milk production, milking begins when the foal reaches ~ 8 weeks and is eating some hay and grass. The mare is separated from the foal by day and milked ~5 times at intervals of about 2.5 h and produces 1-1.5 L of milk at each milking. At night, the foal feeds

freely (van Laar, Orchid's Paardenmelkerij, the Netherlands, personal communication). Milk production is thus very labour intensive and expensive, with the result that equine milk is priced as a delicacy typically, €11 per litre. Milking schedules are similar for asinine milk, but the yield is lower than that of the horse, ~350 to 850 ml of milk per milking, depending on several factors including: foal and mare management, milking procedure, stage of lactation, body size and condition and feeding regime (Salimei, 2011). Mastitis is rarely a problem with equids and occurs only if teat injury occurs during milking. Furthermore, equid species appear to be relatively resistant to brucellosis and tuberculosis, which is advantageous from a dairy farming point of view (Stoyanova *et al.*, 1988).

1.2. Composition of Equid Milk

With the exception of the major domesticated dairy species and humans, information on milk composition is poor and of >4,500 mammalian species in existence, milk compositional data are available for ~ 200 species, of which, data for only ~55 species appears to be reliable. The milk of all mammals contains the same principal components: water, salts, vitamins, fats, carbohydrate and proteins, but these constituents differ significantly both quantitatively and qualitatively between species (Table 1.1) although species from the same taxonomic order e.g., equids, produce milk of similar composition (Table 1.1). Equid milk is similar in composition to human milk but considerably different from that of other dairy mammals, e.g., cow, buffalo, sheep, goat, camel, llama and yak (Table 1.1). Why equid milk is so similar in macro composition to that of human milk is unclear, especially as equids and humans are phylogenetically distantly related. Inter-species differences in milk composition reflect very divergent patterns of nutrient transfer to the young and presumably reflect adaptations in maternal rearing of off-spring to physiological constraints and environmental conditions (Oftedal and Iverson, 1995). In all situations, lactation must be effective in providing nourishment to the off-spring without over-taxing maternal resources.

The protein content of milk varies considerably between species and reflects the growth rate of the young. Bernhart (1961) found a linear correlation between the % calories derived from protein and the logarithm of the days to double birth weight for 12 mammalian species. For humans, one of the slowest growing and slowest maturing species, it takes 120-180 days to double birth weigh and only 7% of calories come from protein. In contrast, carnivores can double their birth weigh in as little as 7 days and acquire > 30% of their energy from protein. Equid species take between 30 and 60 days to double their birth weight and, like humans,

have an exceptionally low level of protein in their milk (Table 1.1). The high metabolic needs of the foal are met through frequent feeding. Equid milks have a significantly lower energy value than human milk, owing to their low fat content (Table 1.1).

The fat content of milk across species shows large variation and ranges from ~ 0.6% for some breeds of donkey and less than 1% for rhinoceros to ~ 50% in the milk of some seals. High-fat milks are important for some species, e.g., dessert mammals, when maternal water economy is important and energy needs to be transferred to the young in an efficient manner. Equid milk has one of the highest contents of carbohydrate, which is similar to that of human milk. In equid species lactation lasts naturally for ~ 7 months.

1.2.1. Factors that affect the composition of equid milk

1.2.1.1. Stage of Lactation

Within species, the stage of lactation is the most important determinant of milk composition and the difference between colostrum and mid-lactation milk shows the most significant difference but absolute values and the direction of change vary among species (Casey, 1989). Shorty after parturition, the mammary gland produces colostrum which is richer in dry matter, proteins, fat, vitamins and minerals (except calcium and phosphorus) but poorer in lactose than mature milk. One of the major biological benefits of colostrum is the presence of immunoglobulins, IgA, IgM and IgG, and high levels of some enzymes, including catalase, lipase and proteinase. Figure 1.1 shows the affect of lactation on the main constituents of equine milk and indicates a very rapid transition from equine colostrum to mature equine milk, i.e., within the first 24 h of lactation.

Table 1.1. Gross composition of the milk of equid species and some dairy species, with human and other selected species included for comparison. Values are expressed as *g.kg^{-1} or **g.L^{-1} milk**.**

Species	***Total Solids***	***Protein***	***Casein:Whey Ratio***	***Fat***	***Lactose***	***Ash***	***Gross Energy (kJ.kg-1 or kJ.L-1)***	***Days to double birth rate***
*Horse (*Equus caballus*)	102	21.4	1.1:1	12.1	63.7	4.2	1883	40-60
*Donkey (*Equus africanus asinus*)	88.4	17.2	1.28:1	14	68.8	3.9	1966	30-50
*Mountain zebra (*Equus zebra hartmannae*)	100	15.6	-	10.2	69	3	1800	-
*Plains zebra (*Equus burchelli*)	113	16.3	-	22	70	4	2273	-
*Przewalski horse (*Equus caballus przewalski*)	105	15.5	1.1:1	15	67	3	1946	-
**Cow (*Bos taurus*)	127	34	4.7:1	37	48	7	2763	30-47
*Buffalo (*Bubalus bubalis)*	172	46.5	4.6:1	81.4	48.5	8	4644	48-50
**Sheep (*Ovis aries)*	181	55.9	3.1:1	68.2	48.8	10	4309	10-15
*Goat (*Capra hircus)*[h]	122	35	3.5:1	38	41	8	2719	12-19
**Camel (*Camelus dromedarius*)	124.7	33.5	1.68:1	38.2	44.6	7.9	2745	250
** Llama (*Llama glama*)	131	34	3.1:1	27	65	5	2673	120
*Yak (*Bos grunniens*)	160	42.3	4.5:1	56	52.9	9.1	3702	60
*Man(*Homo sapiens*)	124	9	0.4:1	38	70	2	2763	120-180
**Pig (*Sus scrofa*)	188	36.5	1.4:1	65.8	49.6	10	3917	9
**Rabbit *(Oryctolagus cuniculus*)	328	139	2.0:1	183	21	18	9581	4-6
**Blue whale (*Balaenoptera musculus*)	550	119	2.0:1	409	13	14	17614	10
**Northern Fur Seal (*Callorhinus ursinus*)	633	103	1.1:1	507	1	5	20836	5
*Rat (*Rattus norvegicus)*	210	84	3.2:1	103	26	13	5732	2-5
*Elephant (*Loxodonta africana africana*)	176.9	47.3	0.61:1	60.7	38.8	7	3975	100-260
*Rhinoceros (*Ceratotherium simum*)	77.5	16.2	0.22:1	7.4	61	3	1589	25-35

(modified from Uniacke *et al.*, 2010)

The concentration of protein in equine milk is very high, >15 g per 100 g milk, immediately *post-partum*, but decreases rapidly to <4 g per 100 g milk after 24 h of lactation and to less than 2 g per 100 g milk after 4 weeks of lactation (Fig. 1.1A). The casein to whey protein ratio in equine colostrum is 0.2:1 immediately *postpartum* and this changes to ~1.1:1 within one week. The protein content of bovine milk decreases during the first 3 months of lactation, but increases subsequently (Walstra *et al.*, 2006a). The concentration of lactose in equine more than doubles during the first 24 h (Fig. 1.1C), as also observed for bovine milk (Walstra *et al.*, 2006a). The concentration of lactose in equine milk subsequently increases steadily throughout further lactation (Fig. 1.1C), a trend which is different from that for bovine milk in which lactose content decreases progressively (Walstra *et al.*, 2006a). Close agreement is observed between the data of various studies for the level of protein and lactose in equine milk but considerable differences are reported for the lipid content of equine milk between different studies (Csapó *et al.*, 1995; Doreau *et al.*, 1986). This may be due to an increase in the fat content of equine milk which occurs during a milking session and, in some cases, the use of the hormone, oxytocin, which promotes complete evacuation of the udder (Doreau *et al.*, 1986). Hence, the volume of milk drawn and the degree of evacuation of the udder will significantly influence the lipid content of the milk and thus explain differences in lipid content observed between different studies. However, all studies indicated in Fig. 1.1B show the same trend, i.e., a decrease in the lipid content of equine milk with advancing lactation, whereas that the lipid content in bovine milk shows a distinct minimum after ~3 months of lactation (Walstra et al., 2006a). The fat content of asinine milk increases from ~ 0.5 to 1.5% from days 15 to 105 but decreases sharply thereafter (Guo et al., 2007; Salimei *et al.*, 2004). Piccione *et al.* (2008) reported a decrease in fat in the milk of Ragusana donkeys throughout lactation and observed a daily rhythmicity, similar to that found in bovine and human milk, for the levels of fat, lactose and protein in asinine milk, with fat and lactose peaking at night and protein reaching a maximum level during the daytime.

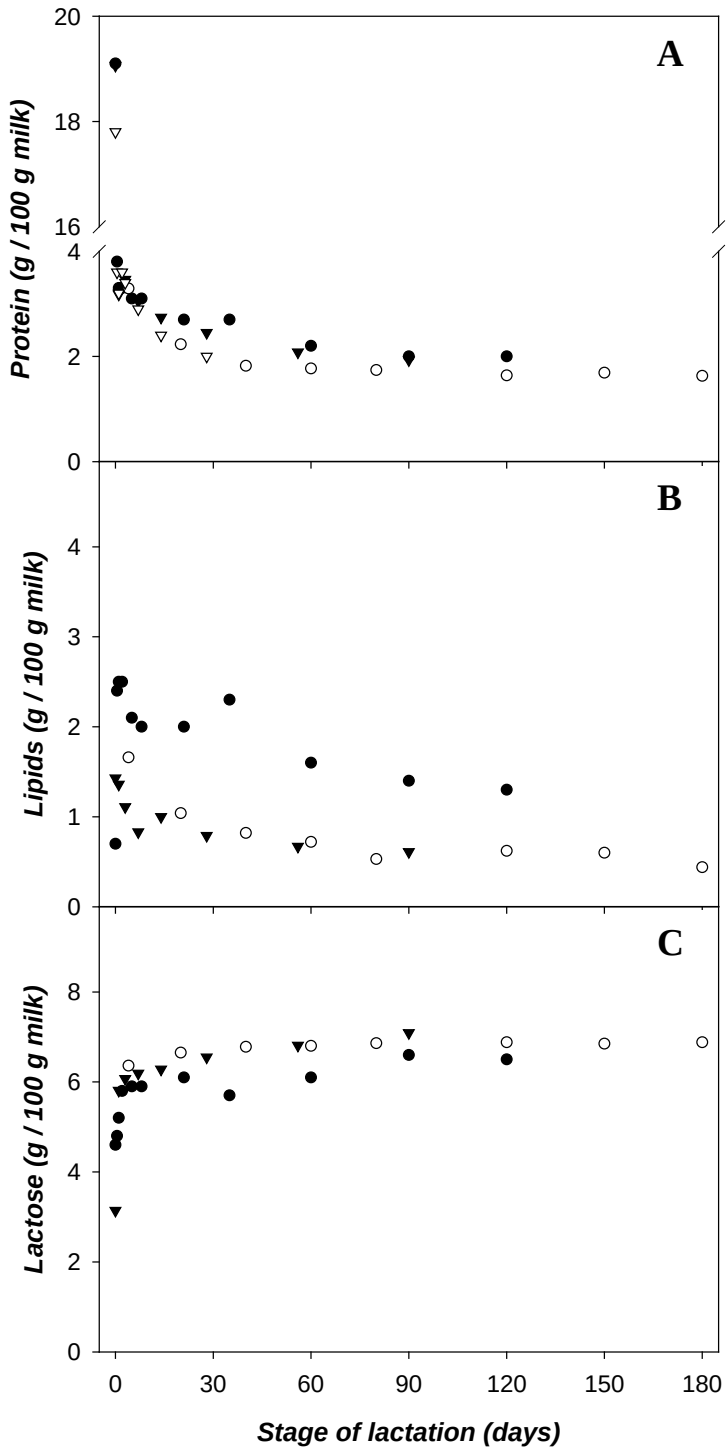

Figure 1.1 Influence of lactation stage on the concentration of (A) protein, (B) lipids, or (C) lactose in equine milk. Data from Ullrey *et al.* (1966, ●), Mariani *et al.* (2001, ○) Smolders *et al.* (1990, ▼) and Zicker & Lönnerdal (1994, ▽)

1.2.1.2. Effect of equid breed on milk composition

Data in the literature are not conclusive as to whether or not the breed of mare has an effect on the concentration of protein in milk. Kulisa (1977), Doreau *et al.* (1990), Csapó-Kiss *et al.* (1995) and Csapó *et al.* (1995) reported no effect of breed on the concentration of proteins or lipids in equine milk throughout lactation. On the other hand, Boulot (1987) and Formaggioni *et al.* (2003) have reported significant differences in protein content between breeds. Civardi *et al.* (2002), who compared Arabian, Haflinger, Trotter and Norico breeds, found that Norico milk had significantly lower α-lactalbumin, highest lysozyme and β-lactoglobulin and highest thermal resistance of the breeds studied. Pelizzola *et al.* (2006), who compared the milk of Haflinger, Quarter horse, Sella/Salto and Rapid Heavy Draft, found that Quarter horse milk had significantly higher concentrations of the main constituents and higher concentrations of linoleic and α-linolenic fatty acids than in the milk of the other species.

Asinine milk shows variability in fat content among breeds and is reported to be as low as 0.4% for Martina Franca mares, 0.6% in Ragusana mares and as high as 1.7% in Jiangyue donkeys (for these donkeys an increase from 0.5 to 1.7% was recorded in the fat content of the milk over 180 days of lactation (Guo *et al.*, 2007). Milk yield is significantly lower for Jiangyue donkeys than for Martina Franca and Ragusana breeds and the protein pattern of Jangyue milk is significantly different from the other breeds (Guo *et al.*, 2007)1.3. Proteins

While the protein content of mature equid milk is lower than that of bovine milk, there is a strong qualitative resemblance, the principal classes of proteins, i.e., caseins and whey proteins are similar in both milks. However, while the caseins are the predominant class of proteins in bovine milk (~80% of total milk protein), equid milk contains less casein and more whey proteins. The distribution of casein and whey proteins in equid milk is shown in Table 1.2, with comparative data for bovine and human milk.

1.3.1. Caseins

About 80% of the proteins in bovine milk are caseins which are primarily a source of amino acids, calcium, phosphate and bioactive peptides for neonates (Shekar *et al.*, 2006). The low casein concentration in mature equine milk (~55% of total protein) has many implications which will be discussed below. The traditional method for separating caseins from whey proteins is isoelectric precipitation of the caseins at pH ~4.6. The casein fraction of most milks consists of four gene products: α_{s1}-, α_{s2}-, β- and κ-caseins, of which the first three are calcium sensitive. All caseins lack secondary structure, which led Holt and Sawyer (1993) to

consider them as rheomorphic proteins. The lack of secondary structure may be attributed, at least partially, to the relatively high level of proline residues in casein. As a result, caseins do not denature or associate on heating (Paulson and Dejmek, 1990). The biological function of the caseins lies in their ability to form macromolecular structures, casein micelles, which transfer large amounts of calcium to the neonate with a minimal risk of pathological calcification of the mammary gland. The individual caseins will be discussed separately in the following sections with focus on their interactions to form casein micelles and the colloidal stability thereof.

Fractionation and characterization of individual equine caseins has been poorly researched to date in comparison to those of bovine milk and it had been reported that equine, and presumably asinine, caseins exhibit greater heterogeneity and a higher level of post-translational modifications than those of bovine milk (Miranda, 2004). Table 1.3 shows the biochemical properties of individual casein proteins which are discussed further below.

Table 1.2. Concentration of caseins and whey proteins (g. kg^{-1}) in equine, asinine, human and bovine milk.

	Equine	**Asinine**	**Human**	**Bovine**
Total casein	**13.56**	**7.8**	**2.4**	**26**
α_{s1}-casein	2.4	identified	0.77	10.7
α_{s2}-casein	0.20	unknown	-	2.8
β-casein	10.66	identified	3.87	8.6
κ-casein	0.24	unknown	0.14	3.1
γ-casein	identified	unknown	-	0.8
Total whey protein	**8.3**	**5.8**	**6.2**	**6.3**
β-lactoglobulin	2.55	3.3	-	3.2
α-lactalbumin	2.37	1.9	2.5	1.2
Serum albumin	0.37	0.4	0.48	0.4
Proteose peptone	-	-	-	0.8
Immunoglobulins	**1.63**	**1.30**	**0.96**	**0.80**
$IgG_{1,2}$	0.38		0.03	0.65
IgA	0.47		0.96	0.14
IgM	0.03		0.02	0.05
Lactoferrin	0.58	0.37	1.65	0.10
Lysozyme	0.87	1.00	0.34	126×10^{-6}
NPN ($mg.L^{-1}$)	**375**	**455**	**454**	**266**
Casein Micelle Size (nm)	**255**	**~100-200**	**64**	**182**

(modified from Uniacke *et al.*, 2010, with asinine data from Guo et al., 2007 and Salimei *et al.*, 2004)

1.3.1.1. α_{S1}-Casein

The amino acid sequence of equine α_{s1}-casein has been deduced from its cDNA sequence (Lenasi *et al.*, 2003). The protein contains 205 amino acids and has a molecular mass of 24,614.4 Da prior to post-translational modification, i.e., it is considerably larger than its bovine or human counterpart (Table 1.3). Two smaller isoforms of α_{s1}-casein have been identified in equine milk, which probably result from the skipping of exons during transcription (Miranda *et al.*, 2004). Equine α_{s1}-casein contains six potential phosphorylation sites (Lenasi *et al.*, 2003), five of which are in very close proximity (Ser_{75}, Ser_{77}, Ser_{79}, Ser_{80}, Ser_{81}) and can thus form a phosphorylation centre, which is important in the structure of casein micelles. Matéos *et al.* (2009a) determined the different phosphorylation levels of the native isoforms of equine α_{s1}-casein and identified 36 different variants with several phosphate groups ranging from two to six or eight which, like equine β-casein, present a complex pattern on 1D and 2D electrophoresis. Bovine α_{s1}-casein contains eight or nine phosphorylation sites (Swaisgood, 2003), which form two phosphorylation centres (De Kruif and Holt, 2003). Bovine α_{s1}-casein contains three distinct hydrophobic regions, roughly including residues 1-44, 90-113 and 132-199 (Swaisgood, 2003). These regions are characterized by positive values for hydropathy. Likewise, equine α_{s1}-casein has three domains with a high hydropathy value, i.e., around residues 25-30, 95-105 and 150-205 and therefore it probably has association properties similar to those of bovine α_{s1}-casein. Furthermore, equine α_{s1}-casein contains two regions with very low hydropathy, i.e., around residues 45-55 and 125-135, which are expected to behave hydrophilically. Human α_{s1}-casein does not appear to have distinct hydrophobic regions. Overall, equine and human α_{s1}-casein have comparable grand average hydropathy (GRAVY) score, which are lower than that of bovine α_{s1}-casein (Table 1.3), indicating an overall higher hydrophobicity for the latter. GRAVY scores reflect the relative ratio of hydrophobic and hydrophilic amino acid residues in a protein, with a positive value reflecting an overall hydrophobic and a negative value an overall hydrophilic nature of the protein. Prior to the mid-1990s, it was generally assumed that human milk contains mainly β- and κ-caseins with little or no α_s-casein (Kunz and Lönnerdal, 1990). A minor casein component has since been identified and is considered to be the human equivalent of α_{s1}-casein, although this identification highlights several inconsistencies in comparison with the equivalent casein in other species. Uniquely, human α_{s1}-casein appears to contain at least two cysteine residues and exists as a multimer in complex with κ-casein (Cavaletto *et al.*, 1994; Rasmussen *et al.*, 1995).

Table 1.3. Properties of equine, bovine and human α_{s1}-,β-and κ-caseins

Protein	Species	Primary accession number*	Amino acid residues	MW (Da)	pI	GRAVY**	Cysteine residues
α_{s1}-casein	Equine	-	205	24,614.4	5.47	-1.127	0
	Bovine	P02662	199	22,974.8	4.99	-0.704	0
	Human	P47710	170	20,089.4	5.17	-1.013	3
β-casein	Equine	Q9GKK3	226	25,511.4	5.78	-0.415	0
	Bovine	P02666	209	23,583.2	5.13	-0.355	0
	Human	P05814	211	23,857.8	5.33	-0.289	0
κ-casein	Equine	P82187	165	18,844.7	8.03	-0.313	2
	Bovine	P02668	169	18,974.4	5.93	-0.557	2
	Human	P07498	162	18,162.6	8.68	-0.528	1

*Primary accession number for the protein in SWISS-PROT database
**Grand average hydropathy (GRAVY) score using the scale of Kyte and Doolittle (1982)

(modified from Uniacke *et al.*, 2010)

Johnsen *et al.* (1995) identified three cysteine residues in human α_{s1}-casein and provided a molecular explanation for α_{s1}-κ-casein complex formation. Martin *et al.* (1996) provided definitive evidence for the presence of a functional α_{s1}-casein locus in the human genome which is expressed in the mammary gland during lactation, while Sørensen *et al.* (2003) determined the phosphorylation pattern of human α_{s1}-casein. In bovine milk, α_{s1}- casein is a major structural component of the casein micelle and plays a functional role in curd formation (Walstra and Jenness, 1984). The relatively low level of α_{s1}-casein in equine milk (Table 1.2), and similarly in human milk, may be significant and, coupled with the low protein content, could be responsible for the soft curd produced in the stomach of the infant or foal (Dr. Ursula Fogarty, National Equine Centre, Ireland – personal communication). Goat milk lacking α_{s1}-casein has poor coagulation properties compared to milk containing α_{s1}-casein (Clark and Sherbon, 2000). Bevilaçqua *et al.* (2001), who assessed the capacity of goats milk with a low or high α_{s1}-casein content to induce milk protein sensitization in guinea pigs, found significantly less sensitization in milk with low α_{s1}-casein. This may represent another important attribute of the low α_{s1}-casein content of equine milk for use in human allergology. An α_{s1}-like protein of ~ 31-33 kDa has been identified in asinine milk although Criscione *et al.* (2009) reported its absence in one Ragusana donkey under investigation.

1.3.1.2. α_{S2}-Casein

The complete amino acid sequence of equine α_{s2}-casein is unknown, but Ochirkhuyag *et al.* (2000) published the sequence of the N-terminal 15 amino acid residues (Lys-His-Lys-Met-Glu-His-Phe-Ala-Pro-???-Tyr-???-Gln-Val-Leu). Only five of these amino acids were confirmed by Miranda *et al.* (2004). Isoelectric focusing showed two major bands for equine α_{s2}-casein, with isoelectric points in the pH range 4.3-5.1 (Ochirkhuyag *et al.*, 2000). Bovine α_{s2}-casein is the most highly phosphorylated casein, usually containing 11 phosphorylated serine residues, with lesser amounts containing 10, 12 or 13 phosphate groups (Swaisgood, 2003). There are no reports on the presence of α_{s2}-casein in human milk. Using three different methods for protein identification, Criscione *et al.* (2009) could not detect α_{s2}- or κ-casein in asinine milk.

1.3.1.3. β-Casein

The amino acid sequence of equine β-casein, derived from the cDNA, has been reported by Lenasi *et al.* (2003), and revised by Girardet *et al.* (2006) with the insertion of eight amino acids (Glu_{27} to Lys_{34}). The theoretical molecular mass of this 226 amino acid polypeptide is 25,511.4 Da (Table 1.3). Bovine and human β-casein contain 209 and 211 amino acid residues, respectively (Table 1.3). Two smaller variants of equine β-casein, which probably result from casual exon-skipping during transcription, were reported by Miranda *et al.* (2004). The 28 C-terminal amino acids contain seven potential phosphorylation sites (Ser_9, Ser_{15}, Ser_{18}, Ser_{23}, Ser_{24}, Ser_{25}, Ser_{28}) and multiple-phosphorylated isoforms of equine β-casein containing three to seven phosphoserine residues have been reported, with the isoelectric point varying from pH 4.74 to 5.30 (Girardet *et al.*, 2006; Matéos *et al.*, 2009b). Bovine β-casein, which contains four or five phosphorylated serine residues, has an isoelectric point of 5.0-5.5 (Swaisgood, 2003). Human β-casein has up to six levels of phosphorylation, i.e., 0, 1, 2, 3, 4 or 5 phosphorylated serine residues (Sood and Slattery, 2000). Equine, bovine and human β-casein have a very hydrophilic N-terminus, followed by a relatively random hydropathy distribution in the rest of the protein, leading to an amphiphilic protein with a hydrophilic N-terminus and a hydrophobic C-terminus. In equine sodium caseinate, the Lys_{47}-Ile_{48}, bond of β-casein is hydrolysed readily by bovine plasmin whereas no cleavage of the corresponding bond, Lys_{48}-Ile_{49} in bovine β-casein has been shown (Egito *et al.*, 2003). In bovine β -casein, Lys_{28}-Lys_{29} is readily cleaved by plasmin but the equivalent, Lys_{28}-Leu_{29}, in equine β-casein, is insensitive (Egito *et al.*, 2002). Other plasmin cleavage sites in equine β-casein are Lys_{103}-Arg_{104}, Arg_{104}-Lys_{105} and Lys_{105}-Val_{196} (Egito *et al.*, 2002). Equine β-casein is readily hydrolysed by chymosin at Leu_{190}-Tyr_{191} (Egito *et al.*, 2001).

Equine β-casein and equine α-La undergo spontaneous deamidation under physiological conditions at Asn_{135}-Gly_{136} and Asn_{45}-Gly_{46}, respectively (Girardet *et al.*, 2004), which has been reported also for canine milk lysozyme (Nonaka et al., 2008) and human lactoferrin (Belizy *et al.*, 2001) but not, to our knowledge, for bovine or human β-casein or α-la. Recent research has shown that temperature may be an important factor controlling the spontaneous deamidation process and at 10^{o}C, the phenomenon is strongly reduced (Matéos *et al.*, 2009b). Spontaneous deamidation represents an important modification of equine milk proteins under certain conditions where bovine milk proteins, which do not contain a potential site for deamidation, remain unaffected. Equine Lf also contains the Asn-Gly sequence and may be susceptible to spontaneous deamidation (Girardet *et al.*, 2006).

Unique to equine milk and apparently absent from the milk of other species, including ruminants, is a low-MW multi-phosphorylated β-casein variant which accounts for 4% of the total casein (Miclo *et al.*, 2007). This short protein (94 amino acid residues) is the result of a large deletion (residues 50-181) from full-length equine β-casein. No spontaneous deamidation of this low-MW form of β-casein has been found. Multiphosphorylated isoforms of β-casein, ~ 34-35.4 kDa, have been identified in asinine milk but no further characterization has been reported to date (Criscione *et al.*, 2009).

1.3.1.4. κ-Casein

The presence of κ-casein in equine milk was an issue of debate for several years, with several authors (Visser *et al.*, 1982; Ono *et al.*, 1989; Ochirkhuyag *et al.*, 2000) reporting its absence. However, other studies (Kotts and Jenness, 1976; Malacarne *et al.*, 2000; Iametti *et al.*, 2001; Egito *et al.*, 2001) showed its presence, albeit at a low concentration. The primary structure of equine κ-casein has been derived (Iametti *et al.*, 2001; Lenasi et al., 2003; Miranda *et al.*, 2004); it contains 165 amino acids residues, i.e., four less than bovine κ-casein but three more than human κ-casein (Table 1.3). The MW of equine κ-casein, prior to post-translational modification, is 18,844.7 Da. Equine and human κ-casein have a considerably higher isoelectric pH than bovine κ-casein (Table 1.3), and they have a net positive charge at physiological pH, whereas bovine κ-casein has a net negative charge. The GRAVY score of bovine κ-casein is considerably lower than that of equine κ-casein (Table 1.3), indicating that the latter is more hydrophilic. Bovine κ-casein is characterized by a hydrophilic C-terminus, which is very important for the manner in which bovine casein micelles are stabilized, but a comparison of the hydropathy distribution of bovine and equine κ-caseins indicates that the C-terminus of equine κ-casein is far less hydrophilic, particularly as a result of the absence of a strong hydrophilic region at residues 110-120. Human κ-casein appears to be more like equine than bovine κ -casein in terms of the distribution of hydropathy along the polypeptide chain. Studies on asinine milk have not identified κ-casein (Vincenzetti *et al.*, 2008; Chianese *et al.*, 2010).

1.3.1.4.1. Glycosylation of κ-Casein

κ-Casein, the only glycosylated member of the casein family, exhibits microheterogeneity due to the level of glycosylation (Saito and Itoh, 1992). Tri- or tetra-saccharides consisting of N-acetylneuraminic acid (NANA), galactose and N-acetylgalactosamine are attached to κ -casein *via 0*-glycosidic linkages to threonine residues in the C-terminal portion of the molecule (the glycomacropeptide, GMP, region). About two-thirds of bovine κ-casein molecules are glycosylated at one of six threonyl residues, i.e., Thr_{121}, Thr_{131}, Thr_{133}, Thr_{135}, Thr_{136} [only in bovine κ-casein variant A] or Thr_{142} (Pisano *et al.*, 1994); Ser_{141} is also a potential glycosylation site (Kanamori *et al.*, 1981). Human κ-casein has seven glycosylation sites, Thr_{113}, Thr_{123}, Thr_{128}, Thr_{131}, Thr_{137}, Thr_{147} and Thr_{149} (Fiat *et al.*, 1980). Although no direct information is available, lectin-binding studies indicate that equine κ-casein is glycosylated (Iametti *et al.*, 2001), possibly at residues Thr_{123}, Thr_{127}, Thr_{131}, Thr_{149} and Thr_{153} (Lenasi *et al.*, 2003) [these glycosylation sites are not fully in agreement with those proposed by Egito *et al.* (2001)]. To date, no non-glycosylated κ -casein has been identified in equine milk (Martuzzi and Doreau, 2006).

κ-Casein is located mainly on the surface of the casein micelles and is responsible for their stability (Walstra, 1990). The presence of a glycan moiety in the C-terminal region of κ-casein enhances its ability to stabilize the micelle, by electrostatic repulsion, and may increase the resistance of the protein to proteolytic enzymes and high temperatures (Minkiewicz *et al.*, 1993; Dziuba and Minkiewicz, 1996). Biologically, NANA residues have antibacterial properties and act as a bifidogenic factor (Dziuba and Minkiewicz, 1996). κ-Casein is thought to play a major role in preventing the adhesion of *Helicobacter pylori* to human gastric mucosa (Strömqvist *et al.*, 1995). It is likely that heavily glycosylated κ-casein provides protection due to its carbohydrate content and in breast-feeding infants is thought to be important, especially as *H. pylori* infection is occurring at an increasingly younger age (Lönnerdal, 2003).

1.3.1.4.2. Hydrolysis of κ-casein

The hydrolysis of bovine κ-casein by chymosin at Phe_{105}-Met_{106} leads to the production of the hydrophobic N-terminal para-κ-casein and the hydrophilic C-terminal caseinomacropeptide (CMP) (Walstra and Jenness, 1984). Chymosin hydrolyses the Phe_{97}-Ile_{98} bond of equine

κ-casein (Egito *et al.*, 2001) and slowly hydrolyses the Phe_{105}-Ile_{106} bond of human κ-casein (Plowman *et al.*, 1999). However, as summarized in Table 1.4, the CMPs released from equine and human κ-caseins are considerably less hydrophilic than bovine CMP. The sequence 97-116 of κ-casein is highly conserved across species, suggesting that the limited proteolysis of κ-casein and subsequent coagulation of milk are of major biological significance (Mercier *et al.*, 1976; Martin *et al.*, 2011).

A grouping system for mammals based on κ-casein structure and the site of cleavage by chymosin has been suggested (Mercier *et al.*, 1976; Nakhasi *et al.*, 1984). Group I species (cow, goat, sheep and buffalo) have a higher content of dicarboxylic amino acids and low hydrophobicity and carbohydrate content and κ-casein is cleaved at Phe_{105}-Met_{106}, while Group II species (horse, human, mouse, pig, rat) have a high proline content, less dicarboxylic amino acids and a much higher hydrophobicity and carbohydrate content and are cleaved at Phe_{97}-Ile_{98} or Phe_{105}-Leu_{106}. Marsupial κ -casein appears to form a separate group with a cleavage site different from that in eutherian mammals (Stasiuk *et al.*, 2000). Cleavage of equine milk at Phe_{97}-$Ile_{98,}$ as well as other characteristics of its κ -casein, place the horse in Group II. The divergence between species into groups I and II could account for differences in the clotting mechanisms of ruminant and non-ruminant milks (Herskovits, 1966). In addition to the differences in cleavage site, the grouping system also divides species based on the number of *0*-glycosylation sites in κ-caseins. As equine and human κ-casein are considerably more highly glycosylated than bovine κ-casein and non-glycosylated κ-casein has not been found in equine milk (Egito *et al.*, 2001), equine and human κ -caseins belong to the same group. The level of glycosylation does not affect micelle structure but it does affect the susceptibility of κ-casein to hydrolysis by chymosin, with susceptibility decreasing as the level of glycosylation increases (Doi *et al.*, 1979; Addeo *et al.*, 1984; Van Hooydonk *et al.*, 1984; Vreeman *et al.*, 1986; Zbikowska *et al.*, 1992). Therefore, equine milk probably has a different clotting mechanism by chymosin than bovine milk.

Table 1.4. Properties of equine, bovine and human para-κ-casein and caseinomacropeptide.

Protein	Species	Residues	Amino acid residues	Mw (Da)	pI	GRAVY*
Para-κ-casein	Equine	1-97	97	11,693.3	8.96	-0.675
	Bovine	1-105	105	12,285.0	9.33	-0.617
	Human	1-105	97	11,456.9	9.63	-1.004
CMP	Equine	98-165	68	7,169.3	4.72	0.203
	Bovine	106-169	63	6,707.4	4.04	-0.370
	Human	106-162	65	6,723.7	4.24	0.182

*Grand average hydropathy (GRAVY) score using the scale of Kyte and Doolittle (1982).

(modified from Uniacke *et al.*, 2010)

1.3.2. Equid casein micelles

In the milk of all species studied in sufficient detail, the caseins exist predominantly as micelles, which are hydrated spherical structures with dimensions in the sub-micron region. The dry matter of casein micelles consists predominantly (>90%) of proteins, with small amounts of inorganic matter, collectively referred to as micellar calcium phosphate (MCP). The structure and sub-structure of bovine casein micelles has been studied in detail and reviews include: Holt and Horne (1996); Horne (1998, 2006); De Kruif and Holt (2003); Phadungath (2005); Farrell *et al.* (2006); Qi (2007); Fox and Brodkorb (2008).

Equine casein micelles are larger than bovine or human micelles (Table 1.2) (Welsch *et al.*, 1988; Buchheim *et al.*, 1989) while those of asinine milk are similar in size to bovine micelles (Salimei, 2011). Electron microscopy shows that bovine and equine micelles have a similar 'spongy' appearance, while human micelles seem to have a much 'looser', more open structure (Jasińska and Jaworska, 1991). Such a loose open structure may affect the susceptibility to hydrolysis by pepsin. Jasińska and Jaworska (1991) reported that human micelles are much more susceptible to pepsin hydrolysis than either equine or bovine micelles. There are no specific reports on the sub-structure of equine casein micelles although equine milk does contain ~10.1 mmol L^{-1} micellar calcium and ~2.6 mmol L^{-1} micellar inorganic phosphorus, suggesting a micellar calcium:casein ratio of >20:1 which, on a molar

basis, far exceeds the calcium-binding capacity of equine casein molecules. Hence, it may be assumed that equine micelles, like bovine casein micelles, contain nanoclusters of calcium phosphate. Since equine milk contains little or no κ-casein, unphosphorylated β-casein may play a role in micellar stability (Ochirkhuyag *et al.*, 2000; Doreau and Martin-Rosset, 2002). Both equine α_{s1}-casein (residues 75-81) and β-casein (residues 23-28) contain a phosphorylation centre, which is required for the formation of nanoclusters; furthermore, both proteins also contain distinct hydrophobic regions through which solvent-mediated protein-protein interactions may occur. Equine α_{s2}-casein may have similar properties to equine α_{s1}-casein, pending further characterization. The ratio of micellar calcium: micellar inorganic phosphorus is 2.0 in equine milk, but ~3.9 in bovine milk (Holt and Jenness, 1984) and might indicate that either a smaller proportion of micellar calcium is incorporated into nanoclusters in equine milk, or that equine nanoclusters contain a higher proportion of casein-bound phosphate which would imply smaller nanoclusters. However, unlike bovine κ-casein, equine κ-casein does not have a distinctly hydrophilic C-terminal domain; thus, it is unclear if this part of the protein is capable of protruding from the micellar surface to sterically stabilize the micelles. Furthermore, given that the size of casein micelles and the content of κ-casein are inversely related (Yoshikawa *et al.*, 1982; Dalgleish, 1998.), a low level of κ -casein would be expected in equine milk compared to bovine milk. The situation for asinine micelles is less clear as the micelles are reported as being similar in size to bovine micelles but no κ-casein has been reported (Salimei, 2011). Further research is required to elucidate the structure of equine and asinine casein micelles as destabilization of the micelles is the basis for the successful conversion of milk into a range of dairy products, e.g., cheese or yoghurt.

1.3.2.1. Stability of equid casein micelles

Coagulation of milk occurs when the colloidal stability of the casein micelles is destroyed and may be desirable or undesirable. Coagulation is desirable in the manufacture of yoghurt and cheese and is also important from a nutritional point of view, as clotting of the caseins in the stomach, and the type and structure of the resultant coagulum strongly affect digestibility. In contrast, heat-induced coagulation of casein micelles, which can occur at a temperature >120ºC, is undesirable. In this section, common types of micellar instability will be described. Bovine casein micelles are sterically stabilized by a brush of predominantly κ-casein

(De Kruif and Zhulina, 1996), which protrudes from the micelle surface. Coagulation of casein micelles can occur only following collapse of the brush, which occurs on acidification of milk, i.e., in the manufacture of yoghurt or on removal of the brush which occurs on rennet-induced coagulation of milk. The combined process of enzyme- and acid-induced coagulation is likely to contribute to coagulation of casein micelles in the stomach.

1.3.2.2. Enzymatic coagulation of equid milk

Enzymatic coagulation of milk is the first step in the manufacture of most cheese varieties and also plays an important role in the flocculation of casein micelles in the stomach. For cheese manufacture, the process involves the addition of a milk-clotting enzyme, e.g., chymosin, to the milk, followed by incubation at a temperature $\geq$30ºC. During the incubation of bovine milk with rennet, chymosin hydrolyses the Phe_{105}-Met_{106} bond of κ-casein, leading to the formation of two fragments, the hydrophobic N-terminal fragment, f1-105, which remains attached to the casein micelles and is referred to as para- κ -casein, and the hydrophilic C-terminal fragment, f106-169, which is released into the milk serum and is referred to as the caseinomacropeptide (CMP). As a result, the micelles lose steric stabilization and become susceptible to aggregation, particularly in the presence of Ca^{2+} (for reviews see Walstra and Jenness, 1984; Wong *et al.*, 1988; Walstra, 1990; Fox and McSweeney, 1998; Walstra *et al.*, 2006b). Equine κ-casein is hydrolysed slowly by chymosin (Kotts and Jenness, 1976) at the Phe_{97}-Ile_{98} bond (Egito *et al.*, 2001), without gel formation, and it appears that either the chymosin-sensitive bond of equine κ-casein is located in the micelle in a manner which renders it inaccessible to chymosin, or that the equine casein micelle derives colloidal stability from constituents other than κ-casein. The high degree of glycosylation may also affect the ability of chymosin to hydrolyze equine κ -casein. Figure 1.2 illustrates the coagulation of equine, asinine and bovine milk by calf chymosin at 30ºC. While it is clear that no gel is formed from equine milk, as judged by lack of an increase in storage modulus, G', asinine milk seems to form a gel, although it is very weak compared to the gel formed from bovine milk. Further investigation is warranted to determine if there are differences in the coagulation properties of asinine and equine milk.

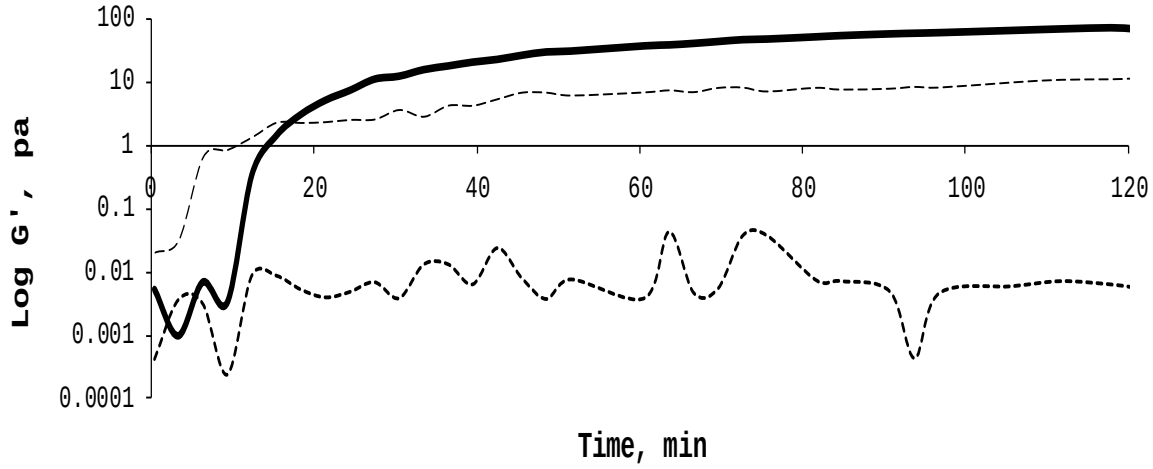

Figure 1.2 Rennet-induced coagulation of equine milk (----), asinine milk (- - -) and bovine milk (——) at 30°C.

1.3.2.3. Acid-induced coagulation of equid milk

When bovine milk is acidified to a pH below 5.0, flocculation of casein micelles occurs, leading ultimately to gel formation. This process is the basis of the manufacture of yoghurt, in which acidification is induced by the production of lactic acid by lactic acid bacteria, and also occurs at the low pH of the stomach (for review see Lucey and Singh, 2003). Acid-induced flocculation of bovine casein micelles is believed to result from a reduction in the solvency of the κ-casein brush on the micellar surface due to protonation of the negatively charged carboxylic acid groups of Glu and Asp. Equine casein micelles are considerably less susceptible to acid-induced flocculation. Di Cagno *et al.* (2004) reported that equine milk acidified at pH 4.2, the point of minimum solubility of equine caseins (Egito *et al.*, 2001), had an apparent viscosity only ~ 7 times higher than that of equine milk at its natural pH (Waelchli *et al.*, 1990) and is probably indicative of micellar flocculation rather than gelation. By comparison, the viscosity of acidified bovine milk is ~ 100 times higher than that of bovine milk at natural pH. Differences in acid-induced flocculation between equine and bovine casein micelles may be related to differences in the mechanism by which they are sterically stabilized. Figure 1.3 shows the effect of acidification of bovine, equine and asinine milk at 30^{o}C and 3% glucono-δ-lactone (GDL). Asinine milk appears to form a weak gel when treated with GDL, unlike equine milk which shows little or no gel formation. Elucidation of the mechanism of steric stabilization of equine casein micelles is likely to shed further light on this subject.

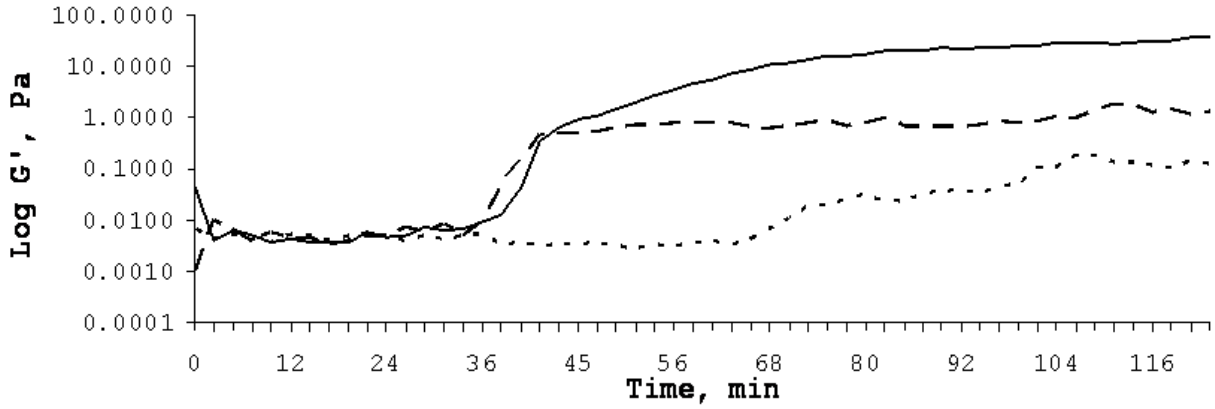

Figure 1.3 Coagulation of equine milk (----), asinine milk (- - -) and bovine milk (——) acidified with 3% GDL at 30°C.

1.3.2.4. Heat-induced coagulation of equine milk

Although milk, compared to most other foods, is extremely heat-stable, coagulation does occur when heated for a sufficiently long time at >120ºC. Unconcentrated bovine milk, usually assayed at 140ºC, displays a typical profile, with a heat coagulation time (HCT) maximum (~20 min) at pH ~6.7 and a minimum at pH ~6.9 (O'Connell & Fox, 2003). In contrast, the HCT of unconcentrated equine milk at 140°C increases with pH, i.e., it has an almost sigmoidal pH-HCT profile (Figure 1.4), from < 2 min at pH 6.3-6.9 to > 20 min at pH 6.9-7.1; a slight maximum is observed at pH 7.2. Pre-heating equine milk shifts the pH-HCT profile and reduces the HCT in the pH region around the maximum, similar to the effect reported for bovine milk (O'Connell and Fox, 2003). The HCT of concentrated equine milk at 120°C increases up to pH 7.1 but decreases progressively at higher pH values. While the profile for concentrated bovine milk is somewhat similar, the maximum HCT occurs at a considerably lower pH, i.e. ~ 6.6.

Differences in heat stability between equine and bovine milk may be related to differences in steric stabilization of the micelles and, while heat-induced complexation of β-Lg with κ-casein greatly affects the heat stability of bovine milk (O'Connell & Fox, 2003), it is unlikely to do so in equine milk due to lack of a sulphydryl group in equine β-Lg. The lower protein, particularly casein, concentration in equine milk is also likely to contribute to its higher heat stability.

The colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles, which may have significant implications for the conversion of equine milk into dairy products. Based on the evidence outlined, manufacture of cheese and yoghurt from equine milk is unlikely to be successful using conventional manufacturing protocols.

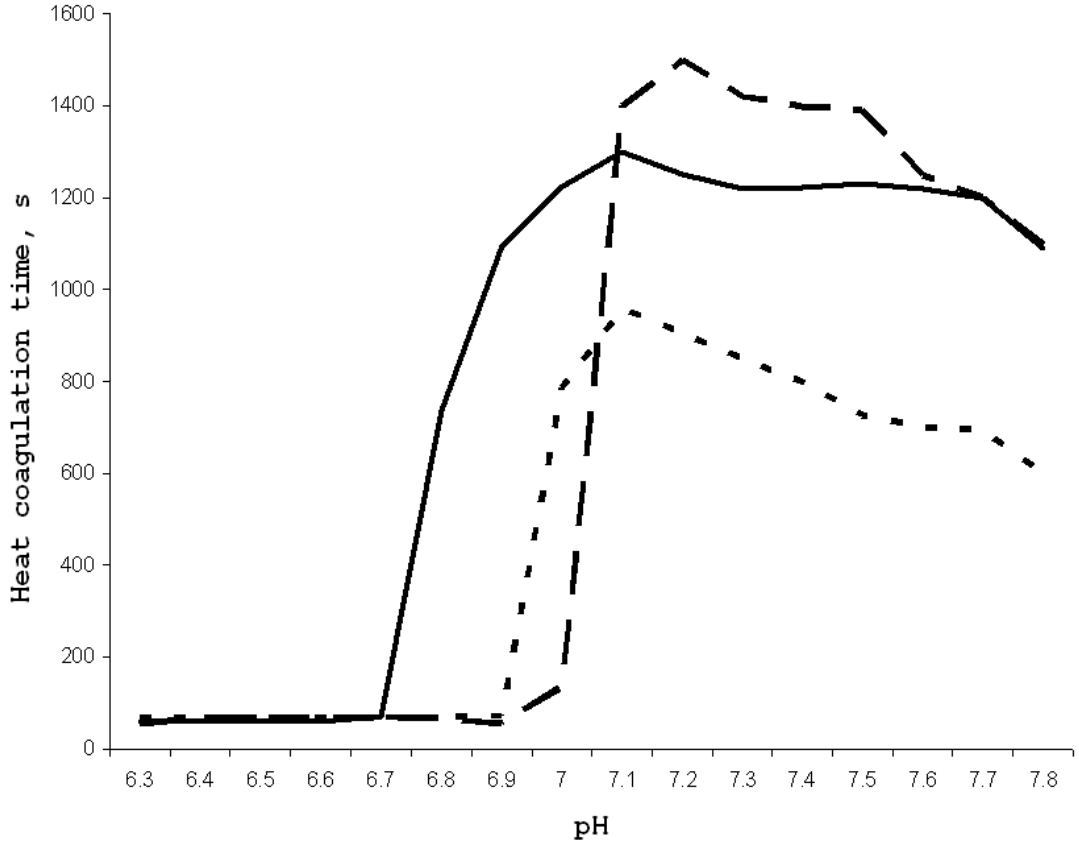

Figure 1.4 Heat coagulation time (HCT)-pH profile of raw unconcentrated skimmed equine milk at 140°C (----), preheated and unconcentrated milk (——) and concentrated milk at 120°C (- - -).

1.3.2.5. Stability of equine milk to ethanol

The ethanol stability of bovine milk (for review see Horne, 2003), defined as the minimum concentration of added aqueous ethanol which causes it to coagulate at its natural pH (~6.7), is 70-75% (added 1:1 to milk), whereas the ethanol stability of equine milk (pH ~7.2) is 40-45% (Chapter 3). The high concentration of ionic calcium and low level of κ-casein in equine milk probably contribute to its low ethanol stability.

1.3.3. Whey Proteins

Similar to bovine milk, the major whey proteins in equine and asinine milk are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (Igs), blood serum albumin (BSA), lactoferrin (Lf) and lysozyme (Lyz) (Bell *et al.*, 1981a; Salimei *et al.*, 2004; Guo *et al.*, 2007). Except for β-Lg, all these proteins are also present in human milk. However, the relative amounts of the whey proteins differ considerably between these milks (Table 1.2). Compared to bovine milk, equine milk contains less β-Lg but more α-La and Igs. The principal anti-microbial agent in equine milk is Lyz and to a lesser extent Lf (which predominates in human milk (Table 1.2). Both Lf and Lyz are present at low levels in bovine milk, in which Igs form the main defense against microbes (Malacarne *et al.*, 2002). Together, Ig A, G, M, Lf and Lyz provide the neonate with immune and non-immune protection against infection (Baldi *et al.*, 2005).

1.3.3.1. β-Lactoglobulin

β-Lg is the major whey protein in the milk of most ruminants and is also present in the milk of some monogastrics and marsupials, but is absent from the milk of humans, camels, lagomorphs and rodents. β-Lg is synthesized in the secretory epithelial cells of the mammary gland under the control of prolactin. Although several biological roles for β-Lg have been proposed, e.g., facilitator of vitamin A (retinol) uptake and an inhibitor, modifier or promoter of enzyme activity, conclusive evidence for a specific biological function of β-Lg is not available (Sawyer, 2003; Creamer and Sawyer, 2011). β-Lg of all species studied binds retinol; β-Lg of many species, but not equine or porcine, binds fatty acids also (Pérez *et al.*, 1993). During digestion, milk lipids are hydrolysed by pre-gastric and pancreatic lipases, greatly increasing the amount of free fatty acids which could potentially bind to β-Lg, displacing any bound retinol, and implying that fatty acid metabolism, rather than retinol transport, is the more important function of β-Lg (Pérez and Calvo, 1995). Bovine β-Lg is

very resistant to peptic digestion and can cause allergenic reactions on consumption. Resistance to digestion is not consistant among species, with ovine β-Lg being far more digestible than bovine β-Lg (El-Zahar *et al.*, 2005). The digestibility of equine β-Lg, which has, to our knowledge, not been studied, warrants research, particularly considering the potential applications of equine milk as a hypo-allergenic dairy product.

Two isoforms of equine β-Lg have been isolated, β-Lg I and II, which contain 162 and 163 amino acids, respectively. The extra amino acid in equine β-Lg II is a glycine residue inserted after position 116 of β-Lg I (Halliday *et al.*, 1991). Asinine milk also has two forms of β-Lg,I and II (MW. 18.5 and 18.2 kDa, respectively); two variants of β-Lg I, i.e., A and B, are known and 4 variants of β-Lg II, A, B, C and D (Cunsolo *et al.*, 2007). Godovac-Zimmerman *et al.* (1985, 1988a, 1988b) reported that β-Lg I from asinine milk has 162 amino acids, similar to equine β-Lg I (from which it differs by 5 amino acids). β-Lg II in both asinine and equine milks has 163 amino acids and shows substantial differences between both milks, with only 6 clusters of amino acid residues conserved (Godovac-Zimmerman *et al.*, 1990). Criscione *et al.* (2009) reported the absence of β-Lg II from more than 23% of Ragusana donkeys in one study.

Bovine β-Lg occurs mainly as two genetic variants, A and B, both of which contain 162 amino acids and differ only at positions 63 (Asp in variant A, Gly in variant B) and 117 (Val in variant A, Ala in variant B); a further 11, less common, genetic variants of bovine β-Lg have also been reported (Sawyer, 2003). Based on its amino acid sequence, unmodified equine β-Lg I has a molecular mass of 18,500 Da and an isoelectric pH of 4.85, whereas equine β-Lg II, despite having one more amino acid, has a molecular mass of 18,262 Da (ExPASy ProtParam Tool, 2009), and an isoelectric pH of 4.71 (Table 1.5). Bovine β-Lg A and B have a molecular mass of 18,367 and 18,281 Da, respectively, and an isoelectric pH of 4.76 and 4.83, respectively (Table 1.5). Using the hydropathy scale proposed by Kyte and Doolittle (1982), equine β-Lg I and II have a GRAVY score of -0.386 and -0.300, respectively (Table 1.5). Bovine β-Lg A and B have a GRAVY score of -0.167 and -0.162, respectively (Table 1.5), and are, therefore, considered to be less hydrophilic than equine β-Lg I and II. Both equine and bovine β-Lg contain two intramolecular disulphide bridges, linking Cys_{66} to Cys_{160} and Cys_{106} to Cys_{119} in equine β-Lg I, Cys_{66} to Cys_{161} and Cys_{106} to Cys_{120} in equine β-Lg II and Cys_{66} to Cys_{160} and Cys_{106} to Cys_{119} or Cys_{121} in bovine β-Lg A and B. Bovine β-Lg contains one sulphydryl group at Cys_{119} or Cys_{121}. Equine β-Lg contains only

four cysteine residues and lacks a sulphydryl group which has major implications for denaturation and aggregation of the protein (see below).

At physiological conditions (neutral pH and β-Lg concentration > 50μM), bovine β-Lg occurs predominantly in dimeric form and at its isoelectric point (pH 3.7 to 5.2) the dimers associate into octamers but below pH 3.4 and above pH 8.0 the protein dissociates into its monomeric form (Gottschalk *et al.*, 2003). Equine and asinine β-Lg I exist in the monomeric form only (Godovac-Zimmermann *et al.*, 1990).

Table 1.5. Properties of equine and bovine β-Lg and equine bovine and human α-La and lactoferrin. Values were calculated from the amino acid sequences of the mature proteins provided on http://au.expasy.org/tools.

Protein	Species	Variant	Primary accession number[a]	Amino acid residues	Molecular mass (Da)	pI	GRAVY score[b]	Disulphide bridges
β-Lg	Equine	I	P02758	162	18500.2	4.85	-0.386	2
		II	P07380	163	18261.6	4.71	-0.300	2
	Bovine	A	P02754	162	18367.3	4.76	-0.167	2
		B	P02754	162	18281.2	4.83	-0.162	2
α-La	Equine	A	P08334	123	14223.2	4.95	-0.416	4[c]
		B	P08896	123	14251.2	4.95	-0.503	4[c]
		C	P08896	123	14249.3	5.11	-0.438	4[c]
	Bovine		P00711	123	14186.0	4.80	-0.453	4
	Human		P00709	123	14078.1	4.70	-0.255	4
Lactoferrin	Equine		O77811	689	75420.4	8.32	-0.376	17
	Bovine		P24627	689	76143.9	8.67	-0.350	16[d]
	Human		P02788	691	76165.2	8.47	-0.415	16

[a] Primary accession number for the protein in SWISS-PROT database.
[b] Grand average hydropathy (GRAVY) score using the scale of Kyte and Doolittle (1982).
[c] Estimated from structural similarity with bovine and human α-La.
[d] Estimated from structural similarity with human lactoferrin.

(modified from Uniacke *et al.*, 2010)

1.3.3.2. α-Lactalbumin

α-La, a unique milk protein, is homologous with the well-characterized c-type lysozymes. It is a calcium metalloprotein, in which the Ca^{2+} plays a crucial role in folding and structure and has a regulatory function in the synthesis of lactose (Larson, 1979; Brew, 2003; Neville, 2009).

Similar to the α-La of asinine, bovine, caprine, ovine, camelid and human milk, equine α-La contains 123 amino acids (Brew, 2003). Equine α-La occurs as three genetic variants, A, B and C, which differ by only a few single amino acid replacements (Godovac-Zimmerman *et al.*, 1987). Bovine α-La occurs as two, or possibly three, genetic variants (Bell *et al.*, 1981b) and human α-La has two genetic variants, one of which has been identified only recently (Chowanadisai *et al.*, 2005). The primary structure of equine, bovine and human α-La differ only by a few single amino acid replacements, and the proteins have similar properties (Table 1.5). Equine α-La A, B and C have an isoelectric point at pH 4.95, 4.95 and 5.11, respectively, whereas bovine and human α-La have pIs at pH 4.80 and 4.70, respectively (Table 1.5). The GRAVY scores of equine and bovine α-La are comparable, whereas that of human α-La is distinctly higher (Table 1.5), indicating a lower hydrophobicity. The eight cysteine residues of bovine and human α-La form four intramolecular disulphide bonds, linking Cys_6 to Cys_{120}, Cys_{28} to Cys_{111}, Cys_{61} to Cys_{77} and Cys_{73} to Cys_{93}. Based on the very high similarity between equine, bovine and human α-Las, as well as the α-La of other species, it is very likely that equine α-La also contains four intramolecular disulphide bridges, in the aforementioned positions. Equine, bovine or human α-La does not contain a sulphydryl group. Three genetic variants of equine α-La have been reported but asinine milk has only 1 (123 amino acid residues, MW ~14.2 kDa and 4 disulphide bonds), although some heterogeneity has been shown. Two isoforms, A and B of asinine α-La (whose isoelectric points differ by 0.23 units) have been reported but subsequent analysis showed that the protein has only one form and misidentification in earlier work was probably due to differences in calcium binding by asinine α-La (Giuffrida *et al.*, 1992). The primary structure of asinine α-La has been determined and differs from those of equine and bovine proteins with 39 and 40 amino acid substitutions, respectively (Godovac-Zimmerman *et al.* 1987).

1.3.3.3. Immunoglobulins

The concentration of whey proteins is significantly elevated in the colostrum of all ruminants and equids as maternal Igs are passed from mother to neonate after birth when the small intestine is capable of absorbing intact proteins. After a few days, the gut 'closes' and further significant passage of proteins is prevented and within 2 to 3 days, the serum level of IgG in the neonate is similar to adult levels (Widdowson, 1984). In contrast, *in utero* transfer of Igs occurs in humans and in some carnivores Igs are passed to the newborn both before and after birth. The milk of species that provide prenatal passive immunization tends to have relatively small differences in protein content between colostrum and mature milk compared to species that depend on *post-natal* passage of maternal Igs. In the latter cases, of which all ungulates are typical, colostrum is rich in Igs and there are large quantitative differences in protein content between colostrum and mature milk (Langer, 2009).

Three classes of immunoglobulins, which form part of a mammal's natural defense against infection, are commonly found in milk, immunoglobulin G (IgG), A (IgA) and M (IgM); IgG is often sub-divided into two subclasses, IgG_1 and IgG_2 (Hurley, 2003; Madureira *et al.*, 2007). All monomeric Igs consist of a similar basic structure of four polypeptides, two heavy chains and two light chains, linked by disulphide bridges, yielding a subunit with a molecular mass of ~160 kDa. IgG consists of one subunit, while IgA and IgM consist of two or five subunits, with a molecular mass of ~400 or ~1000 kDa, respectively. The relative proportions of the Igs in milk differ considerably between species (Table 1.2). IgG is the principal Ig in equine colostrum, but IgA is the principal form in equine milk. In bovine milk and colostrum, IgG is the principal immunoglobulin, while IgA is the predominant Ig in human colostrum and milk.

1.3.3.4. Lactoferrin

Lactoferrin (Lf) is an iron-binding glycoprotein, comprised of a single polypeptide chain of MW ~ 78 kDa (Conneely, 2001). Lf is structurally very similar to transferrin (Tf), a plasma iron transport protein, but has a much higher (~300 fold) affinity for iron (Brock, 1997). Lf is not unique to milk although it is especially abundant in colostrum, with small amounts in tears, saliva and mucus secretions and in the secondary granules of neutrophils. The expression of Lf in the bovine mammary gland is dependent on prolactin (Green and Pastewka, 1978); its concentration is very high during early pregnancy and involution and is expressed predominantly in the ductal epithelium close to the teat (Molenaar *et al.*, 1996).

Human, equine, asinine and bovine milk contain 1.65 g, 0.58 g, 0.37 and 0.1 g Lf per kg, respectively (Table 1.2). The concentration of lactoferrin (Lf) in asinine milk, which comprises ~ 4% of total whey protein, is significantly lower than in equine milk (Table 1.2).

Shimazaki *et al*. (1994) purified Lf from equine milk and compared its iron-binding ability with that of human and bovine Lfs and with bovine Tf. The iron-binding capacity of equine Lf is similar to that of human Lf but higher than that of bovine Lf and Tf. Various biological functions have been attributed to Lf but the exact role of Lf in iron-binding in milk is unknown and there is no relationship between the concentrations of Lf and Tf and the concentration of iron in milk (human milk is very rich in Lf but low in iron) (Masson and Heremans, 1971).

Lf is a bioactive protein with nutritional and health-promoting properties (Baldi *et al.*, 2005). Bacterial growth is inhibited by its ability to sequester iron and also to permeabilize bacterial cell walls by binding to lipopolysaccharides through its N-terminus. Lf can inhibit viral infection by binding tightly to the envelope proteins of viruses and is also thought to stimulate the establishment of a beneficial microflora in the gastrointestinal tract (Baldi *et al.*, 2005). Ellison and Giehl (1991) suggested that Lf and Lyz work synergistically to effectively eliminate Gram-negative bacteria; Lf binds oligosaccharides in the outer bacterial membrane, thereby opening 'pores' for Lyz to hydrolyse glycosidic linkages in the interior of the peptidoglycan matrix. This synergistic process leads to inactivation of both Gram-negative bacteria, e.g., *E. coli* (Rainhard, 1986) and Gram-positive bacteria, e.g., *Staph. epidermis* (Leithch and Willcox, 1999). Furthermore, a proteolytic digestion product of bovine and human Lf, i.e., lactoferricin, has bactericidal activity (Bellamy *et al.*, 1992). Bovine and human Lf are reported to have antiviral activity and a role as a growth factor (Lönnerdal, 2003). The specific biological function of equine Lf has not been studied, but is likely to be similar to that of bovine and human Lf.

Equine Lf contains 689 amino acid residues, which is similar to bovine Lf and two more than human Lf (Table 1.5). Compared to most other milk proteins, Lf has a high isoelectric point, i.e., at pH 8.32, 8.67 or 8.47 for equine, bovine or human Lf (Table 1.5). As a result, the protein is positively charged at the pH of milk and may associate with negatively-charged proteins *via* electrostatic interactions. GRAVY scores are comparable for equine, bovine and human Lf (Table 1.5). Equine and human Lf contain 17 and 16 intra-molecular disulphide bonds, respectively (Table 1.5). Based on structural similarities with human Lf, it has been assumed that bovine Lf contains 16 intra-molecular disulphide bonds (Table 1.5). The iron-

binding capacity of equine, bovine and human Lfs are equivalent, although the pH-dependence of the iron-binding capacity of bovine Lf differs from that of equine and human Lf (Shimazaki *et al.*, 1994).

All Lfs studied to date are glycosylated, but the location and number of potential glycosylation sites, as well as the number of sites actually glycosylated, vary. In bovine Lf, four out of five potential glycosylation sites, i.e., Asn_{223}, Asn_{368}, Asn_{476} and Asn_{545}, are glycosylated (Moore *et al.*, 1997), whereas in human Lf, two of three potential glycosylation sites, i.e., Asn_{137} and Asn_{478}, are glycosylated (Haridas *et al.*, 1995). Glycosylation of equine Lf has not been studied, but using the consensus sequence, Asn-Xaa-Ser/Thr (where Xaa is not Pro), for glycosylation, three potential glycosylation sites are likely in equine Lf, i.e., Asn_{137}, Asn_{281} and Asn_{476}.

1.3.3.5. Whey Protein Denaturation

Whey proteins are susceptible to heat-induced denaturation. The thermal stability of equine Lf and BSA is comparable to that of the bovine proteins but equine β-Lg and α-La are more heat stable than the bovine proteins (Bonomi *et al.*, 1994). Equine β-Lg is more thermally stable than equine α-La, which is different from bovine milk, where α-La is more thermally stable than β-Lg (Civardi *et al.*, 2007). The high thermal stability of equine β-Lg may be related to its lack of a sulphydryl group. Thermal denaturation of bovine β-Lg is a two-stage process, unfolding of the polypeptide chain and exposure of the sulphydryl group, followed by self-association or interaction with other proteins *via* sulphydryl-disulphide interchange (Sawyer, 2003). Owing to the lack of a sulphydryl group, equine β-Lg can not undergo the second denaturation step and therefore its structure may refold on cooling. Denaturation of α-La is commonly a result of complex formation with β-Lg *via* sulphydryl-disulphide interchange and its higher thermal stability may therefore be a result of differences in its environment, rather than its molecular structure. Recent research suggests that equine α-La and β-Lg are also less susceptible to denaturation than their bovine counterparts under high pressure (Uniacke-Lowe and Huppertz, unpublished data).

1.4. Digestibility of Equid Milk

Milk is highly digestible and, because it is liquid, the gastrointestinal tract of mammals has mechanisms for delaying its passage; coagulation of milk in the stomach delays the degradation of proteins and improves their assimilation by the body. Caseins are precipitated by gastric acid and enzymes, forming a clot in the stomach which entraps fat. The hardness of this clot depends on the casein content of the milk; high casein-containing milks will produce firm clots. Generally, species that nurse their young at frequent intervals, e.g., equids and humans, tend to produce dilute milk in which < 60% of total protein is casein and which form a soft clot, whereas those that nurse infrequently, e.g., cattle and sheep, produce milk which is high in fat and casein and has much longer gastric retention (Jenness, 1986). Degradation of casein in the gastrointestinal tract is slow but extensive and while β-Lg is relatively resistant to gastric proteolysis, α-lactalbumin is readily hydrolysed when the gastric pH is ~ 3.5 (Savalle *et al.*, 1988).

The physico-chemical differences between human and bovine caseins result in the formation of different types of curd in the stomach (Hambræus, 1982) and because the protein profile of equine milk is quite similar to that of human milk, equine milk may be more suitable in human nutrition than bovine milk. Turner (1945) compared the digestibility of equine, human and bovine milk based on the average percentage conversion of acid-insoluble protein to acid-soluble protein during digestion. Equine and human milk have a much lower buffering capacity than bovine milk and, while equine milk is very digestible, it was slightly less so than human milk but significantly better than bovine milk. Turner (1945) concluded that both equine and human milk form soft curds in the stomach which pass through the digestive tract more quickly than bovine milk curd. Kalliala et al.(1951) also reported that the overall digestibility of equine and human milk (by *in-vitro* experiments) appeared to be quite similar and both were easier to digest than bovine milk. Human milk forms fine, soft flocs in the stomach with an evacuation time of 2 to 2.5 h, whereas bovine milk forms compact hard curds with a digestion time of 3 to 5 h.

1.5. Total amino acids

Guo *et al.* (2007) investigated the total amino acid composition of asinine milk and expressed the results both in grams of individual amino acids per 100 grams of milk and per 100 grams of protein and compared values to those for equine, bovine and human milk (Table 1.6). Results expressed per 100 g of milk demonstrated differences related, most likely, to

differences in the total protein content between the milks, but when expressed as g per 100 g protein, the differences were not so apparent. It has been reported that glycine is exceptionally high in equine casein (Lauer and Baker, 1977) and other studies have reported that the mean values of peptide-bound amino acids in equid milks are generally higher than those in camel and buffalo milks and may indicate that equid milks are more suitable for human consumption than other milks studied to date. Asinine milk has noticeably higher levels of serine, glutamate, arginine and valine and much less cystine and the percentage of 7 of the 8 essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, valine) is also higher than those of equine and bovine milk (Guo *et al.*, 2007). In equine, bovine and human milk cystine, glycine, serine, threonine and alanine decrease as lactation progresses while glutamate, methionine, isoleucine, lysine tend to increase (Davis *et al.*, 1994).

1.6. Non-protein Nitrogen

The non protein-nitrogen (NPN) of milk consists primarily of urea, peptides, amino acids and ammonia. NPN constitutes 10 to 15% of the total nitrogen in mature equine milk which is intermediate between the values for human milk and ruminant milk, 25 and 5%, respectively (Hambræus 1984; Oftedal *et al.*, 1983; Atkinson *et al.*, 1989; Walstra *et al.*, 2006b). In equine milk, NPN increases from <2% of total nitrogen at parturition to >10% after two weeks (Zicker and Lönnerdal, 1984). The components of the NPN in human and bovine milk have been characterized (see Atkinson *et al.*, 1989; Atkinson and Lönnerdal, 1995; Rudloff, S. and Kunz, C., 1997; Carratù, *et al.*, 2003), but the NPN of equine milk has not been studied in detail.

Up to 50% of the NPN of human milk is urea and free amino acids, the exact function of which is, as yet, unknown. Asinine milk has a significantly higher level of NPN than equine milk and is close to that of human milk (Table 1.2). Equine and asinine milk have similar urea levels.

Table 1.6. Amino acid composition of asinine and equine milk expressed as g AA per 100 g protein, with comparative data for bovine and human milk.

Amino acid	Asinine	Equine	Bovine	Human
Aspartic acid	8.9	10.4	7.8	8.3
Serine	6.2	6.2	4.8	5.1
Glutamic acid	22.8	20.1	23.2	17.8
Glycine	1.2	1.9	1.8	2.6
Histidine	2.3	2.4	3.0	2.3
Arginine	4.6	5.2	3.3	4.0
Threonine	3.6	4.3	4.5	4.6
Alanine	3.5	3.2	3.0	4.0
Proline	8.8	8.4	9.6	8.6
Cystine	0.4	0.6	0.6	1.7
Tyrosine	3.7	4.3	4.5	4.7
Valine	6.5	4.1	4.8	6.0
Methionine	1.8	1.5	1.8	1.8
Lysine	7.3	8.0	8.1	6.2
Isoleucine	5.5	3.8	4.2	5.8
Leucine	8.6	9.7	8.7	10.1
Phenylalanine	4.3	4.7	4.8	4.4
Tryptophan	-	1.2	1.5	1.8
Essential AA	38.2	36.7	37.5	40.7

(modified from Guo *et al*., 2007)

1.6.1. Free Amino Acids

The free amino acid content of equine, bovine and human milk are 1960, 578 and 3019 μmol.L^{-1}, respectively (Rassin *et al.*, 1978; Agostini *et al.*, 2000) (Table 1.7). Glutamine, glutamate, glycine, alanine and serine are the most abundant free amino acids in equine, bovine and human milk, and taurine also is exceptionally high in human milk (Rassin *et al.*, 1978; Sarwar *et al.*, 1998; Carratù *et al.*, 2003). Taurine is an essential metabolite for the human infant and may be involved in the structure and function of retinal photoreceptors (Agostini *et al.*, 2000). Compared to bovine milk, equine milk has an appreciable amount of taurine although it is 10 times less than that of human milk (Table 1.7). In contrast to total amino acid composition, which is essentially similar in equine, bovine and human milks, free amino acids show a pattern characteristic of each species (Table 1.7) which may be important for early post-natal development in different animals. Free amino acids are more easily absorbed than protein-derived amino acids and glutamic acid and glutamine, which comprise > 50% of the total free amino acids of human milk, are a source of α-ketoglutaric acid for the citric acid cycle and also act as neurotransmitters in the brain (Levy, 1998; Agostini *et al.*, 2000).

Table 1.7. Free amino acids ($\mu M.L^{-1}$) of equine, bovine and human milk

Amino acid	Equine[a]	Bovine[a]	Human[b]
Alanine	105	30.0	227.5
Arginine	14.0	10.0	35.4
Aspartic acid	40.0	15.0	183.2
Cystine	2.0	21.0	56.0
Glutamic acid	568.0	117.0	1184.1
Glutamine	485.0	12.0	284.8
Glycine	100.0	88.0	124.6
Histidine	46.0	9.0	7.7
Isoleucine	8.0	3.0	33.4
Leucine	16.0	3.0	55.6
Lysine	26.0	15.0	39.0
Methionine	~0	~0	8.8
Phenylalanine	5.0	3.0	23.6
Proline	1.61	-	64.3
Serine	175	23.0	273.7
Taurine	32.0	13.0	301.1
Threonine	137.0	16.0	97.6
Tyrosine	3.0	0.3	2.5
Valine	45.0	5.0	72.7
Total	~1960.0	578.0	3019.7

[a] Rassin *et al.*, 1978
[b] Agostini *et al.*, 2000

1.6.2. Bioactive Peptides

Both caseins and whey proteins are believed to contribute to human health through latent biological activity produced enzymatically during digestion, fermentation with specific starter cultures or enzymatic hydrolysis by microorganisms, resulting in the formation of bioactive peptides. These peptides are important for their physiological roles, their opioid-like features as well as their immunostimulating and anti-hypertensive activities and their ability to enhance Ca^{2+} absorption and are released or activated during gastrointestinal digestion. Several peptides generated by the hydrolysis of milk proteins are known to regulate the overall immune function of the neonate (Baldi *et al.*, 2005). A detailed discussion on bioactive peptides in milk is outside the scope of this article, for reviews see: Donnet-Hughes *et al.*, 2000; Shah, 2000; Malkoski *et al.*, 2001; Fitzgerald and Meisel, 2003; Silva and Malcata, 2005; Fitzgerald and Murray, 2006; Lopéz *et al.*, 2006; Michaelidou and Steijns, 2006; Thöma-Worringer *et al.*, 2006; Phelan *et al.*, 2009. Research on the bioactive peptides derived from equid milk is very limited. Peptides from the hydrolysis of equine β-casein may have a positive action on human health (Doreau and Martin-Rosset, 2002). Chen *et al.* (2010) reported the presence of 4 novel ACE-inhibitory peptides in koumiss which may enhance the beneficial effects of koumiss on cardiovascular health. Peptides with trophic or protective activity have been identified in asinine milk (Salimei, 2011).

1.6.3. Hormones and Growth Factors

Leptin is a protein hormone of ~ 16kDa which has been discovered recently in human milk and plays a key role in the regulation of energy intake and energy expenditure, as well as functioning in mammary cell proliferation, differentiation and apoptosis. Human-like leptin has been isolated from equine milk at a level of 3.2-5.4 $ng.mL^{-1}$ which is similar to levels reported for other mammals and showed little variation throughout lactation (Salimei *et al.*, 2002).

Levels of the bioactive peptides, ghrelin and insulin growth factor I, which play a direct role in metabolism, body composition and food intake, have also been reported for asinine milk at 4.5 $pg.\ mL^{-1}$ and 11.5 $ng.\ mL^{-1}$, respectively, similar to levels in human milk (Salimei, 2011).

1.6.4. Amyloid A

Amyloid A3 (AA3) is a protein produced in the mammary gland and is encoded by a separate gene from that for serum amyloid A (serum AA) (Duggan *et al.,* 2008). AA3 is believed to prevent attachment of pathogenic bacteria to the intestinal cell wall (Mack *et al.*, 2003) and may prevent necrotizing enterocolitis in human infants (Larson *et al.*, 2003). McDonald *et al.* (2001) demonstrated the presence of AA3 in the colostrum of cows, ewes, sows and horses. Bovine colostrum has a high concentration of AA3 but by ~ 3 days post-partum the level declines. In bovine milk the presence of serum AA in milk is an indicator of mastitic infection (Kaneko *et al.*, 2004; Winter *et al.*, 2006). In equine colostrum, the concentration of AA3 is considerably lower than in milk and consequently it may play a crucial role in intestinal cell protection in the foal especially after gut closure (Duggan *et al.*, 2008).

1.7. Indigenous Enzymes

Milk contains many indigenous enzymes which originate from the mammal's blood plasma, leucocytes (somatic cells), or cytoplasm of the secretory cells and the milk fat globule membrane (MFGM)(Fox and Kelly, 2006). The indigenous enzymes in bovine and human milks have been studied extensively but the enzymes in the milk of other species have been studied only sporadically. Equine milk probably contains all the enzymes that have been identified in bovine milk but relatively few studies have been reported.

1.7.1. Lysozyme

Lysozyme (Lyz, EC 3.1.2.17) occurs at high levels in equine, asinine and human milk (Table 1.2). Human, equine and asinine milk contain 3,000, 6,000 and >6,000 times more lysozyme, respectively, than bovine milk (Salimei *et al*., 2004; Guo *et al.*, 2007) with levels as high as 0.4 g.100g^{-1} reported for Martina Franca donkeys (Coppola *et al.*, 2002) although 0.1 g. 100 g^{-1} is reported more commonly found in asinine milk (Vincenzetti *et al.*, 2008). The concentration of Lyz in human milk increases strongly after the second month of lactation, suggesting that Lyz and Lf play major roles in fighting infection in breast-fed infants during late lactation, and in protecting the mammary gland (Montagne *et al.*, 1998).

Equine milk Lyz is more stable to denaturation than human Lyz during pasteurization at 62^{o}C for 30 min but at 71^{o}C for 2 min or 82^{o}C for 15 s, the inactivation of both were similar (Jauregui-Adell, 1975). It has been suggested, but research is scarce, that while the

composition of breast milk varies widely between well-nourished and poorly-nourished mothers, the amount of Lyz is conserved.

Lyz found in egg-white, tears and saliva do not generally bind calcium but equine and canine milk Lyz do and this is believed to enhance the stability and activity of the enzyme (Nitta *et al.*, 1987). The binding of a Ca^{2+} by Lyz is considered to be an evolutionary linkage between non-Ca^{2+}-binding lysozymes and α-La (Tada *et al.*, 2002; Chowdbury *et al.*, 2004). The conformation of the calcium-binding loop of equine Lyz is similar to that of α-La (Tsuge et al., 1992; Tada *et al.*, 2002) and both equine Lyz and α-La form stable, partially folded, "molten globules" under various denaturing conditions (Koshiba *et al.*, 2001) althoughequine Lyz is considerably more stable than α -La (Lyster, 1992; Morozova-Roche, 2007). The molten state of canine Lyz is significantly more stable than that of equine Lyz (Koshiba *et al.*, 2000; Spencer *et al.*,1999). Equine milk Lyz is very resistant to acid (Jauregui-Adell 1975) and proteolysis (Kuroki *et al.*, 1989), and may reach the gut relatively intact.

Asinine Lyz contains 129 amino acids, is a C-type lysozyme, binds calcium strongly and has 51% homology to human Lyz (Godovac-Zimmerman *et al.*, 1988b). Two genetic variants of Lyz, A and B, have been reported in asinine milk (Herrouin *et al.*, 2000) but only one is found in equine milk. Asinine Lyz is remarkably heat stable and requires 121^0C for 10 min for inactivation. The Lyz content of equid milks is one of the main attractions for use of these milks in cosmetology as it is reputed to have a smoothing effect on the skin and may reduce scalp inflammation when incorporated into shampoo. Equid milk has very good antibacterial activity, presumably due to its high level of lysozyme.

1.7.2. Other indigenous enzymes in equine milk

Lactoperoxidase (LPO), catalase, amylase, proteinase (plasmin), lipase, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) have been reported in equine milk. Bovine milk is a rich source of xanthine oxidoreductase (XOR) but the milk of other species for which data are available have much lower XOR activity, because in non-bovine species, most (up to 98% in human milk) of the enzyme molecules lack Mo and are inactive. XOR has not been reported in equine milk which is unusual considering the role of XOR in the excretion of fat globules from the secretory cells and also considering that equine milk contains quite a high level of Mo, which presumably is present exclusively in XOR. Chilliard and Doreau, (1985) characterized the lipoprotein lipase activity of equine milk and reported that the milk has high lipolytic activity, comparable to that in bovine milk and higher that in caprine milk. There are

no reports on hydrolytic rancidity in equine milk which is potentially a serious problem in equine milk products and warrants investigation.

Plasmin, a serine proteinases, is one of a number of proteolytic enzymes in milk. Visser *et al.* (1982) and Egito *et al.* (2002) reported γ-caseins in equine milk and, it is therefore assumed, that equine milk contains plasmin. Humbert *et al.* (2005) reported that equine milk contains five times more plasmin activity than bovine milk and 90% of total potential plasmin activity was plasmin, with 10% as plasminogen; the plasmin:plasminogen ratio in bovine and human milk is 18:82 and 28:72, respectively.

Alkaline phosphatase (ALP) is regarded as the most important indigenous enzyme of bovine milk because ALP activity is used as the index of the efficiency of HTST pasteurization. About 40% of ALP activity in bovine milk is associated with the MFGM. Equine milk has 35-350 times less ALP activity than bovine milk and there are no reports on ALP in the equine MFGM. Due to the low level of ALP in equine milk it has been suggested that it is not suitable as an indicator of pasteurisation efficiency of equine milk (Marchand *et al.*, 2009) although one would expect that once the exact initial concentration of ALP is known, the use of a larger sample size or a longer incubation period would overcome the low levels of enzyme.

1.8. Carbohydrates

1.8.1. Lactose and Glucose

The chemistry, properties and applications of lactose have been reviewed extensively elsewhere e.g., Fox (1985, 1997) and McSweeney and Fox (2009) and will not be considered here. The concentration of lactose in asinine milk is high (51-72.5 $g.kg^{-1}$), probably marginally higher than equine milk (~ 64 $g. kg^{-1}$) (Table 1.2) which is similar to the level in human milk and significantly higher than that in bovine milk. As an energy source, lactose is far less metabolically complicated than lipids but the latter provides significantly more energy per unit mass. As well as being a major energy source for the neonate, lactose affects bone mineralization during the first few months *post-partum* as it stimulates the intestinal absorption of calcium (Schaafsma, 2003). Equine milk contains a significant concentration of glucose, ~ 50 mg per L in colostrum which increases to ~ 150 mg per L 10 days *post-partum* and then decreases gradually to ~ 120 mg per L (Enbergs *et al.*, 1999). Although the lactose content of equid milks is high, the physico-chemical properties of lactose which cause problems in the processing of bovine milk are of no consequence for equid milks, which are

consumed either fresh or fermented. In Mongolia, where ~88% of the population is lactose intolerant (Yongfa *et al.*, 1984), lactose intolerance is not a problem with fermented equine milk, koumiss, as ~30% of lactose is converted to lactic acid, ethanol and carbon dioxide during fermentation.

1.8.2. Oligosaccharides

The milk of all species examined contains oligosaccharides (OSs) but the concentration varies markedly (Urashima *et al.*, 2009). The OSs in milk contain 3 to 10 monosaccharides and may be linear or branched; they contain lactose at the reducing end and also contain fucose, galactosamine and N-acetylneuraminic acid. The highest levels are in the milk of monotremes, marsupials, marine mammals, humans, elephants and bears. Oligosaccharides (OSs) are the third most abundant constituent of human milk which has an exceptionally high content (~ 20 $g.L^{-1}$ in colostrum, which decreases to 5-10 g. L^{-1} in milk) and structural diversity of OSs (> 200 molecular species) which have a range of functions, including as important components of our immune system and as prebiotics to promote a healthy gut microflora (Donovan, 2009). Bovine, ovine, caprine and equine milk contain relatively low levels of OSs, which have been characterized (see Urashima *et al.*, 2001). The OSs identified in equine colostrum are summarized in Table 1.8. The OSs in mature equine milk have not been reported but it can be assumed that the level is considerably lower than in colostrum which has ~ 18.6 g per L (Nakamura *et al.*, 2001). The neutral OSs, lacto-*N*-neotetraose and lacto-*N*-neohexaose, in equine colostrum are also abundant in human milk, while iso-lacto-*N*-neotetraose and lacto-*N*-novopentanose 1 are not, but have been identified in bovine colostrum; the latter has been identified also in the milk of the Tammar wallaby and brown capuchin monkey (Urashima *et al.*, 2009).

1.9. Lipids

Milk fat is important for the provision of energy to the newborn as well as being the vehicle for fat-soluble vitamins and essential fatty acids. From a practical point of view, milk lipids are important as they confer distinctive nutritional, textural and organoleptic properties on dairy products. Dietary composition is considered one of the major determinants of the fatty acid composition of equid milk and non-dietary factors such as stage of lactation, age and parity of the mare play minor roles.

Triglycerides (TGs) represent ~ 80-85% of the lipids in equine and asinine milk, while ~ 9.5% are free fatty acids (FFAs) and ~ 5-10% are phospholipids (Jahreis *et al.*, 1999). In contrast, ~97-98% of the lipids in bovine and human milk are triglycerides, with low levels of phospholipids and free fatty acids, 1.3 and 1.5 $g.100g^{-1}$, respectively. The high level of free fatty acids in equid milks implies that rancidity is a problem with these milks and is dealt with in section 1.9.4.1. below.

Table 1.8. Principal oligosaccharides of equine colostrum.

Oligosaccharide	**mg/L**
Acidic	
Neu5Ac(α2-3) Gal(β1-4)Glc	n/a
Gal(β1-4)GlcNAcα1-diphosphate (N-acetyllactosamine-α1-phospahte)	n/a
Neutral	
Gal(β1-3)Gal(β1-4)Glc (β3'-galactosyllactose)	7.8
Gal(β1-6)Gal(β 1-4)Glc (β6'-galactosyllactose)	4.8
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc (lacto-N-neotetraose)	n/a
Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)Glc (iso-lacto-N-neotetraose)	0.5
Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]Gal(β1-4)Glc (lacto-N-novopentanose 1)	1.1
Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)GlcNAc(β1-3)]Gal(β1-4)Glc (lacto-N-neohe	1.1
Gal(β1-4)GlcNAc-1-phosphate (N-acetyllactosamine-1-0-phosphate)	n/a
Neu5Ac(α2-3)Gal(β1-4)Glc (3'-N-acetylneuraminyllactose)	n/a

Abbreviations: 'Gal'=D-galactose, 'Glc'=D-glucose, 'GlcNAc'=N-acetylglucosamine, 'Neu5A'=N-acetylneuraminic acid, n/a = not available

(from Urashima *et al*., 1989, 2001; Nakamura *et al*., 2001)

The relatively high content of phospholipids in equid milk is thought to contribute to its buffering properties. TGs, the primary transport and storage form of lipids, are synthesized in the mammary gland from fatty acids that originate from three sources: *de novo* synthesis (C8:0, C10:0 and C12:0), direct uptake from the blood (> 14 carbons) and modification of fatty acids in the mammary gland by desaturation and/or elongation. Circulating fatty acids in the blood may originate from dietary fat or from lipids mobilized from body fat stores. The principal phospholipids of equid milk are phosphatidylcholine (19%), phosphatidylethanolamine (31%), phosphatidylserine (16%) and sphingomylin (34%); the corresponding values for bovine milk are 35%, 32%, 3% and 25% and for human milk are 28, 20, 8 and 39%, respectively. The high level of FFAs in equid milk implies considerable lipolysis but this has not been suggested; if lipolysis was responsible for the high level of FFAs, they should be accompanied by high levels of mono- and di-glycerides but these are reported to be quite low at ~1.8% of total lipids.

Table 1.9 shows the monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the milk fat of some ruminant and non-ruminant species. The milk fat of non-ruminants contains substantially higher levels of PUFAs than ruminant milks due to the lack of biohydrogenation of fatty acids in the former, and for the two equid species shown, the horse has considerably more PUFAs in its milk than the donkey. Saturated fatty acids are the dominant class in asinine milk and levels are significantly higher than those in equine or human milk (Table 1.9).

1.9.1. Fatty acids in equid milks

The fatty acid profile of equid milk (Table 1.10) differs from that of bovine and human milk fat in a number of respects. Like human, and unlike bovine milk, equid milk is characterized by low proportions of saturated fatty acids with low or higher numbers of carbons, i.e., $C_{4:0}$, $C_{6:0}$, $C_{16:0}$ and $C_{18:0}$ (Pikul and Wójtowski, 2008). Butyric acid ($C_{4:0}$) is present at high levels in bovine and other ruminant milk fats, produced from 3-hydroxybutanoic acid, which is synthesized by bacteria in the rumen (Pikul and Wójtowski, 2008). Caprylic acid, $C_{8:0}$, is very high in equid milk compared to the level in human and bovine milk (Table 1.10). Levels of middle chain-length FAs, especially $C_{10:0}$ and $C_{12:0}$, are high in equid milk (20-35% of all FAs contain < 16 C) and in all non-ruminant herbivores, suggesting that they arise from the use of glucose as the principal precursor for fatty acid synthesis (Palmquist, 2006). Mammalian

de novo fatty acid synthesis requires a carbon source (acetyl-CoA) and reducing equivalents in the form of NADPH + H^+. In ruminants, acetate and β-hydroxybutyrate are the primary sources of carbon while glucose and acetate are the primary sources of reducing equivalents; in non-ruminants, e.g., equid species, glucose is the primary source of both carbon and reducing equivalents and also supplies some of the glycerol for milk triglycerides (Dils, 1986). When horses were infused with either glucose or acetate and palmitate, $C_{12:0}$ and $C_{14:0}$ were formed exclusively from acetate, as in ruminants, and $C_{16:0}$ was formed, partly from acetate and partly from palmitate; unlike ruminants, 44% of $C_{18:0}$ and 7% of $C_{18:1}$ are formed from acetate in the horse (Palmquist, 2006). If this is so, acetate and 3-hydroxybutyrate are presumably produced by bacterial fermentation in the lower intestine of the horse and why 3-hydroxybutyrate is not converted to butanoic acid, as in ruminants, is unclear. Fatty acids from $C_{6:0}$ to $C_{16:0}$ are released from the Fatty-Acid-Synthesis complex by acyl-specific thioesterases; presumably, the middle chain-length-specific thioesterases are particularly active in equids; investigation of this possibility is warranted.

Equine milk-fat contains a relatively high level of $C_{16:1}$ (2-10%, w/w) and $C_{18:1}$, reflecting high Δ-9 desaturase activity. Equid milk fats contain a very high level of *n*-3-octadecatrienoic acid (linolenic acid), which reflects the high level of PUFAs in the diet and the lack of biohydrogenation, as occurs with ruminants. In the rumen, extensive hydrogenation of double bonds occurs and most fatty acids taken up from the intestinal tract are saturated. The large intestine of equids shows significant differences in the relative rate of transport of volatile fatty acids compared to ruminants and *de novo* synthesis of $C_{18:0}$ fatty acids occurs with a further high proportion of $C_{6:0}$ to $C_{14:0}$ carbon fatty acids and some $C_{16:0}$ arising from products of their large bowel fermentation.

Table 1.9. Monounsaturated and polyunsaturated fatty acids (% of total fatty acids ± standard deviations) in the milk fat of some ruminants and non-ruminants.

	MUFAs	PUFAs	CLA*
Non-ruminants			
Equine	20.70	36.80	0.09
Asinine	15.30	16.00	-
Porcine	51.80	12.40	0.23
Human	33.20	12.50	0.39
Ruminants			
Caprine	26.90	2.58	0.65
Bovine	23.20	2.42	1.01
Ovine	23.00	3.85	1.08

* CLA = conjugated linoleic acid

(from Jahreis *et al*., 1999 and Salimei *et al*., 2004)

Table 1.10. Typical fatty acid (% of total fatty acids) composition in the milk of equid species; bovine and human milk are included for comparison.

Fatty Acid	Common Name	Equine	Asinine	Zebra	Bovine	Human
Saturates						
$C_{4:0}$	Butyric	0.09	0.6		3.9	0.19
$C_{6:0}$	Caproic	0.24	1.22		2.5	0.15
$C_{8:0}$	Caprylic	3.15	12.8	8.2	1.5	0.46
$C_{10:0}$	Capric	6.48	18.65	15.3	3.2	1.03
$C_{12:0}$	Lauric	6.65	10.67	9.2	3.6	4.4
$C_{13i:0}$			0.22			
$C_{13:0}$		0.17	3.92		0.19	0.06
$C_{14i:0}$			0.12			0.04
$C_{14:0}$	Myristic	7.04	5.77	6.5	11.1	6.27
$C_{15a:0}$						0.21
$C_{15\ i:0}$		0.16	0.07			
$C_{15:0}$	Pentadecanoic	0.39	0.32	0.5	1.2	0.43
$C_{16i:0}$			0.12			0.17
$C_{16:0}$	Palmitic	20.43	11.47	13.3	27.9	22
$C_{17i:0}$		0.31	0.2			0.23
$C_{17:0}$	Margaric	0.38	0.22		0.6	0.58
$C_{18i:0}$						0.11
$C_{18:0}$	Stearic	1.18	1.12	2	12.2	8.06
$C_{20:0}$			0.12		0.35	0.44
$C_{21:0}$					0.04	0.13
$C_{22:0}$			0.05		0.2	0.12
$C_{24:0}$					0.14	0.25
Total		**46.67**	**67.66**	**55**	**68.62**	**45.33**
Monounsaurates						
$C_{10:1}$		1.46	2.2		0.15	
$C_{12:1}$		0.2	0.25		0.06	
$C_{14:1c\ n\text{-}5}$	Myristoleic	0.52	0.22		0.8	0.41
$C_{14:1t\ n\text{-}5}$						0.07
$C_{15:1}$	Pentadecanoic	0.22			0.3	0.11
$C_{16:1c\ n\text{-}7}$	Palmitoleic	5.68	2.37	4.2	1.5	3.29
$C_{16:1c\ n\text{-}9}$		0.56				
$C_{16:1t\ n\text{-}7}$						0.36
$C_{17:1}$	Heptadecanoic	0.62	0.27		0.36	0.37
$C_{18:1c\ n\text{-}9}$	Oleic	20.26	9.65	20.4	17.2	31.3
$C_{18:1c\ n\text{-}11}$		1.31				
$C_{18:1t\ n\text{-}9}$						2.67
$C_{18:1t\ n\text{-}11}$	Vaccenic				3.9	
$C_{20:1\ n\text{-}9}$		0.4			0.32	0.67
$C_{20:1\ n\text{-}11}$			0.35			
$C_{22:1\ n\text{-}9}$					0.06	0.08
$C_{24:1\ n\text{-}9}$						0.12
Total		**31.23**	**15.31**	**24.65**	**24.65**	**39.45**

Cont.

(Table 1.10 continued)

Polyunsaturates						
n-6 Series						
$C_{18:2cc}$	Linoleic (LA)	10.1	8.15	16.04	1.4	10.85
C18:2 conj.	CLA			0.07	1.1	
$C_{18:2tt}$						0.46
$C_{18:2ct}$						0.69
$C_{18:3}$	γ-linolenic (GLA)		0.15	0.61	1	0.25
$C_{20:2}$			0.35	0.37	0.07	0.27
$C_{20:3}$	dihomo-γ-linolenic			0.1	0.1	0.32
$C_{20:4}$	Arachidonic			0.11	0.14	0.46
$C_{22:2}$					0.04	0.11
$C_{22:4}$					0.03	0.09
$C_{22:5}$					0.04	0.09
n-3 Series						
$C_{18:3}$	α-linolenic(ALA)	8	6.32	5.31	1.8	1.03
$C_{18:4}$			0.22			
$C_{20:3}$			0.12			
$C_{20:4}$			0.07			0.09
$C_{20:5}$	Eicosapentaenoic(EPA)		0.27	0.02	0.09	0.12
$C_{22:5}$			0.07	0.1		0.19
$C_{22:6}$	Docosahexaenoic(DHA)		0.3	0.04	0.01	0.25
Total PUFA		**18**	**16.02**	**22.77**	**5.82**	**15.27**
Ratio *n*-6:*n*-3		**1.26**	**1.17**	**3.14**	**2.06**	**8.09**
Ratio $C_{18:2}$ to $C_{18:3}$		**1.26**	**1.28**	**2.72**	**1.55**	**9.37**

Abbrevations: *c*, cis; *t* ,trans; *i*, iso; *a*, anteiso

(modified from Uniacke and Fox, 2011)

Equine and asinine milks have similar fatty acid profiles although the former has a higher content of monosaturated fatty acids (Tables 1.9 and 1.10). Both equine and asinine milk have characteristic low levels of stearic acid, and oleic acid is exceptionally low in asinine milk. Asinine and zebra milk fat contain a high level of PUFAs, although considerably lower than in equine milk. The well-balanced ratio of *n*-6: *n*-3 of 1.17:1 in asinine milk compared to 3.14:1 in equine milk makes it an interesting product for human nutrition. *n*-6 and *n*-3 fatty acids are essential in human metabolism as components of membrane phospholipids, precursors of eicosanoids, ligands for membrane receptors and transcription factors that regulate gene expression. The importance of *n*-6 $C_{18:2}$, linoleic acid (LA), has been known for many years but the significance of *n*-3 $C_{18:3}$, α-linolenic acid (ALA) was not recognized until the late 1980s and has since been identified as a key component in the diet for the prevention of atopic dermatitis (Horrobin, 2000). LA and ALA are not interconvertible but are the parent acids of the *n*-6 and *n*-3 series of long chain (LC) polyunsaturated fatty acids, respectively [e.g., *n*-6 $C_{20:4}$, arachidonic acid (AA); *n*-3 $C_{20:5}$, eicosapentaenoic acid (EPA) and *n*-3 $C_{22:6}$, docosahexaenoic acid (DHA)] which are components of cellular membranes and precursors of other essential metabolites such as prostaglandins and prostacyclins (Cuthbertson, 1999; Innis, 2007). DHA and AA are now recognised as being crucial for normal neurological development (Carlson, 2001). Humans have evolved on a diet with a ratio of *n*-6 to *n*-3 fatty acids of ~ 1:1 but Western diets nowadays have a ratio of 15:1 to 16.7:1. As a species, humans are generally deficient in *n*-3 fatty acids and have excessive levels of *n*-6 which is associated with the pathogenesis of cardiovascular, cancerous, inflammatory and autoimmune diseases (Simopoulos, 2002).

Equine milk-fat contains a very low level of conjugated linoleic acid (CLA; rumenic acid, Tables 1.9 and 1.10) which is virtually absent from asinine milk but is high in ruminant milk-fats, being produced in the rumen by abortive biohydrogenation of *n*-6 octadecadienoic acid (LA) (see Whigham *et al.*, 2000; Bauman and Lock, 2006; Collomb *et al.*, 2006). CLA has several desirable effects in the diet; some of the positive health effects attributed to it include: suppression of carcinogenesis, anti-obesity agent, modulator of the immune system and control of artherogenesis and diabetes. Small amounts of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) are present in asinine milk whereas equine milk has only trace amounts. EPA and DHA are especially important in infant nutrition but their absence from

equine milk is not considered to be a problem as an infant's liver can desaturate linoleic and α-linolenic acids to form EPA and DHA.

1.9.2. Structure of Triglycerides

The distribution of fatty acids in animal triglycerides is non-random, apparently so that the TGs will be liquid at body temperature. Inter-species comparison of the positional distribution of fatty acids has been determined, using fatty acid and stereospecific analysis, for 11 species: echidna, koala, Tammar wallaby, guinea pig, dog, cat, Weddell seal, horse, pig, cow and human (Parodi, 1982). Generally, the positional distribution of fatty acids is similar, except for the echidna, with short chain fatty acids preferentially esterified at *sn*-3, saturated fatty acids at *sn*-1 and unsaturated fatty acids generally at *sn*-2. In equine milk fat, $C_{10:0}$ occurs at the *sn*-3 position whereas in bovine milk fat, more $C_{10:0}$ is found at the *sn*-2 than at the *sn*-3 position. In human and equine milk, $C_{16:0}$ is located predominantly at the *sn*-2 position which is regarded as favourable for assimilation by infants and children whereas in bovine milk, $C_{16:0}$ is equally distributed between the *sn*-1 and *sn*-2 positions. In the milk fat of the Weddell seal and horse, $C_{18:1}$ is esterified preferentially at *sn*-1 but for all other species studied it occurs mainly at *sn*-3 (Parodi, 1982). No information is available on the stereospecific distribution of fatty acids in the triglycerides of asinine milk

1.9.3. Equid Milk Fat Globules and MFGM

The fat in milk is emulsified as globules which are surrounded and stabilized by a very complex emulsifying layer, consisting of phospholipids and proteins, called the milk fat globule membrane (MFGM). Many of the indigenous enzymes in milk are concentrated in the MFGM. The glycoproteins in the MFGM of human, rhesus monkey, chimpanzee, dog, sheep, goat, cow, grey seal, camel, horse and alpaca have been studied; large intra- and inter-species differences have been found (see Keenan and Mather, 2006). Very highly glycosylated proteins occur in the MFGM of primates, horse, donkey, camel and dog. Long (0.5-1μm) filamentous structures, comprised of mucins (highly glycosylated proteins), extend from the surface of the fat globules in equine and human milk (Welsch *et al.*, 1988). These filaments dissociate from the surface into the milk serum on cooling and are lost on heating. For unknown reasons, the filaments on bovine milk fat globules are lost much more easily than those in equine or human milk. The filaments facilitate the adherence of fat globules to the intestinal epithelium and probably improve the digestion of fat (Welsch *et al.*, 1988). The

mucins prevent bacterial adhesion and may protect mammary tissue against tumors (Patton, 1999). The milk fat globules (MFGs) of asinine milk can be up to ~ 10μm in diameter, whereas those of equine milk are generally smaller at 2-3 μm on average; the MFGs of bovine and human milk are 3-3.5 μm and ~ 4 μm, respectively. Little is known about the proteins of the MFGM of equids but they are known to play a major role in neonatal defense mechanisms in humans (see Mather, 2000).

Butyrophilin, acidophilin and xanthine oxidoreductase have been identified in the equine MFGM and appear to be similar to the corresponding proteins of the human MFGM, as does lactadherin which shares 74% identity with that of the human lactadherin (Barello *et al.*, 2008). Both xanthine oxidoreductase and acidophilin are involved in fat globule secretion with butyrophylin while lactadherin is thought to have a protective function against rotovirus in the intestinal tract (Barello *et al.*, 2008). Like ovine and buffalo milk, equine milk does not cream due to the lack of cryoglobulin.

1.9.4. Rheology Equid Milk Fat

The temperature-dependent melting characteristics of bovine milk-fat have been studied thoroughly but since equid milk is not used for the production of butter, the spreadability, rheology and melting characteristics of these fats have not been studied in detail (see Chandan *et al.*, 1971). Considering the rather unusual fatty acid profile of equid milk fats, they should have interesting melting and rheological properties.

1.9.4.1. Stability of Equine Milk Fat

Lipids generally are susceptible to two forms of chemical spoilage, lipid oxidation (oxidative rancidity) and lipolysis (hydrolytic rancidity) which are of great commercial significance to the dairy industry and have been studied in detail (see Fox and McSweeney, 2006). No studies on the chemical spoilage of equid milk-fat have been reported. Considering the high content of PUFAs in these fats, they are probably quite susceptible to oxidation. Since equine milk contains a lipase, hydrolytic rancidity would be expected under certain conditions.

1.10. Vitamins

The overall vitamin content of any milk depends on maternal vitamin status but water-soluble vitamins are more responsive to the maternal diet than fat-soluble vitamins. Vitamin levels in the milk of some species are shown in Table 1.11.

Table 1.11. Vitamin levels (mg L^{-1}) in the milk of some species.

Vitamin	Buffalo	Goat	Sheep	Donkey	Cow	Horse	Human
Water-soluble							
Thiamine,B_1	0.5	0.49	0.48	0.41	0.37	0.3	0.15
Riboflavin,B_2	1.0	1.5	2.3	0.64	1.8	0.3	0.38
Niacin,B_3	0.8	3.2	4.5	0.74	0.9	1.4	1.7
Pantothenic acid,B_5	3.7	3.1	3.5	-	3.5	3	2.7
Pyridoxine,B_6	0.25	0.27	0.27	-	0.64	0.3	0.14
Biotin,B_7	0.11	0.039	0.09	-	0.035	-	0.006
Folic acid,B_9					0.18		0.16
Cobalamin,B_{12}	3.0	0.7	.007[a]	1.1	0.004	0.003	0.5
Ascorbic acid,C	-	9.0	4.25[b]	-	21	17.2[c]	43
Fat-soluble							
Vitamin A & β-carotene	-	0.5	0.5	-	0.32- 0.50	0.12	2.0
Cholecalciferol, D_3	-	-	-	-	0.003	0.003	0.001
α-Tocopherol, E	-	-	-	-	0.98-1.28	1.128	6.6
Phylloquinone, K	-	-	-	-	0.011	0.020	0.002

(modified from Walstra and Jenness 1984; Souci *et al*., 2000;[a]Ramos *et al*., 1994; [b]Recio *et al*., 2009; [c]Csapo *et al*., 1995)

Equine milk contains a significantly higher level of vitamin C than the other milks shown, except for ovine milk. The level of vitamin E is low in asinine milk (~0.05 $mg.L^{-1}$) and is reduced further if the milk is heated. Concentrations of fat-soluble vitamins are generally similar in equine and bovine milks (Table 1.12). The levels of vitamins A, D_3, K and C are significantly higher in equine colostrum than in equine milk, whereas the concentration of vitamin E remains unchanged throughout lactation (Table 1.12)(Csapó *et al.*, 1995).

Table 1.12 Vitamins ($mg.\ L^{-1}$) in equine milk

Vitamin	Equine Colostrum	Equine Milk	Bovine Milk
A	0.88	0.34	0.352
D3	0.0054	0.0032	0.0029
E	1.342	1.128	1.135
K3	0.043	0.029	0.032
C	23.8	17.2	15.32

(modified from Csapó *et al.*, 1995)

1.11. Minerals

1.11.1. Macro-elements

The levels of ash in equine and asinine milk are similar (Table 1.2) and the levels of inorganic elements are close to those in human milk except for higher concentrations of Ca and P (Table 1.13) (Holt and Jenness, 1984). The principal salts in equine milk are phosphates, chlorides, carbonates and citrates of potassium, sodium, calcium and magnesium. However, there are considerable quantitative inter-species differences in milk salts, (Table 1.13). The concentrations of all macro-elements, except potassium, are higher in equine and asinine milk than in human milk but all are considerably lower than in bovine, caprine, ovine or porcine milk. The low level of salts in equid milk reduces renal load, making it suitable in infant nutrition. Pieszka and Kulisa (2005) reported on the low tolerance of equine species to imbalances in mineral concentrations in milk *post-partum*; slight increases in some minerals can cause severe deformation of teeth and bones in horses and affect metabolism and protein synthesis.

Table 1.13. Total concentrations of inorganic elements ($mmol.L^{-1}$) and citrate in the milk of 8 different species

Species	Calcium	Magnesium	Sodium	Potassium	Phosphorus (inorganic)	Citrate	Chloride
Horse	16.5	1.6	5.7	11.9	6.7	3.1	6.6
Cow	29.4	5.1	24.2	34.7	20.9	9.2	30.2
Man	7.8	1.1	5.0	16.5	2.5	2.8	6.2
Goat	23.1	5.0	20.5	46.6	15.6	5.4	34.2
Sheep	56.8	9.0	20.5	31.7	39.7	4.9	17.0
Pig	104.1	9.6	14.4	31.4	51.2	8.9	28.7
Rat	80.4	8.8	38.3	43.6	93.3	0.06	36.1
Rabbit	214.4	19.5	83.7	89.5	54.2	17.4	80.0

(modified from Holt and Jenness 1984)

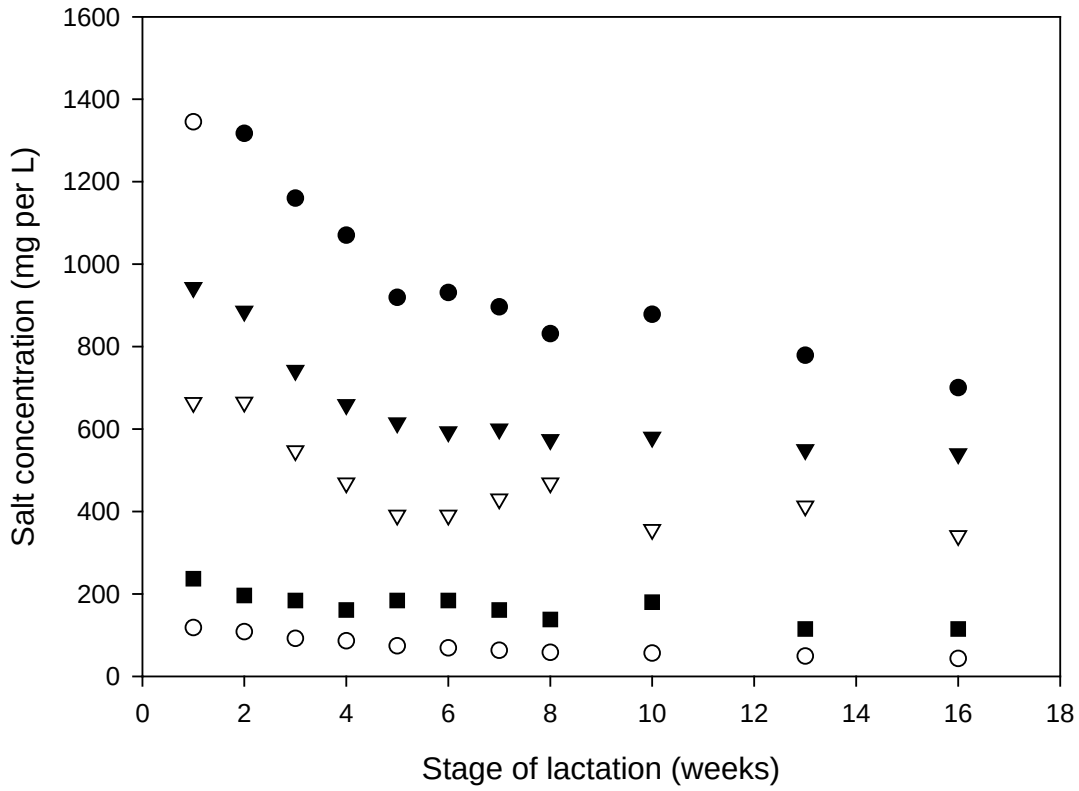

Figure 1.5 Influence of the stage of lactation on the concentration of calcium (●), magnesium (○), phosphorus (▼), potassium (▽) or sodium (■) in equine milk (data from Schryver *et al.* 1986).

The concentration of macro-elements in equine milk is comparable to that in zebra milk (*Equus zebra*), whereas in early lactation, the concentrations of calcium and phosphorus are considerably higher in domestic horse milk (*Equus caballus*)(Table 1.14) than in zebra milk (Schryver *et al.*, 1986). The concentrations of macro-elements in equine milk is strongly influenced by the stage of lactation (Figure 1.5) and there is a progressive decrease in the concentrations of calcium, magnesium, phosphorus, sodium and potassium from the end of the first week of lactation.

Table 1.14. Concentration of inorganic elements ($\mu g.g^{-1}$ whole milk) in early and late lactation milk of various equid species.

Species	Total solids	Ash	Ca	P	Mg	Na	K	Cu	Zn	**Fe**
Early Lactation										
Przewalski horse	11.6	0.58	1380	790	104	220	590	0.42	4.1	1.3
Hartmann's zebra	11.3	0.50	1100	800	120	290	590	1.13	2.4	1.1
Domestic horse	11.6	0.58	1327	884	102	198	655	0.64	2.7	0.37
Domestic pony	9.6	0.48	1036	600	70	189	483	0.26	1.8	-
Late Lactation										
Przewalski horse	10.3	0.33	804	419	62	137	344	0.23	1.9	1.1
Hartmann's zebra	10.0	0.32	840	550	80	200	422	0.26	1.9	3.6
Grant's zebra	10.7	0.35	690	490	93	277	187	1.0	2.9	2.2
Domestic horse	10.2	0.36	811	566	53	140	410	0.25	1.9	0.27
Domestic pony	9.4	0.36	857	418	77	127	250	0.37	1.7	-

(modified from Schryver *et al*., 1986)

The concentration of calcium in equine milk increases during the first week of lactation, before decreasing steadily thereafter (Ullrey *et al*., 1966). As in the milk from all mammals which have been studied in sufficient detail, the concentrations of calcium and phosphate in equine milk far exceeds the solubility of calcium phosphate in milk. As a result, part of the calcium and phosphate exist in a non-ultrafiltrable, micellar, form, i.e., micellar calcium phosphate. Holt and Jenness, (1984) reported that ~60, 20 or 40% of total calcium, magnesium or inorganic phosphorus, respectively, in equine milk are non-ultrafiltrable, compared to ~70, 35 or 45% of total calcium, magnesium and inorganic phosphorus in bovine milk. Based on measurements of the distribution of salts between the ultrafiltrable and non-ultrafiltrable phase of milk, Holt and Jenness (1984) estimated the concentrations of ionic calcium and magnesium in equine milk at pH 7.0 are 2.5 and 0.6 mmol L^{-1}, respectively, compared to 2.0 or 0.8 mmol L^{-1}, respectively, in bovine milk at pH 6.7. For human infant nutrition, a Ca:P ratio of ~ 2:1 is considered optimal; for bovine milk, the ratio is ~ 1:1, but in equine milk it is about 2:1, and is very close to that in human milk.

Table 1.15. Concentrations of trace elements ($\mu g.L^{-1}$) in equine, bovine and human milk

Element	Species		
	Equine	**Bovine**	**Human**
Aluminum	123	98	125
Boron	97	333	273
Barium	76	188	149
Copper	155	52	314
Iron	224	194	260
Lithium	15	24	7
Molybdenum	16	22	17
Manganese	14	21	7
Silicon	161	434	472
Strontium	442	417	60
Titanium	145	111	25
Zinc	1835	3960	2150

(modified from Anderson, 1992)

1.11.2. Trace elements

Data on concentrations of trace elements, i.e., those elements present at concentrations less than 30 mg L^{-1}, in equine milk are sporadic. The concentrations of trace elements in equine, bovine and human milk are compared in Table 1.15. Compared to bovine milk, equine milk contains markedly higher levels of aluminum, copper, iron and titanium but lower levels of boron, barium, lithium, molybdenum, manganese, silicon and zinc. Human milk contains more boron, barium, copper, iron, silicon and zinc, but less lithium, manganese, strontium and titanium than equine milk (Table 1.15). Concentrations of zinc, iron and copper in equine milk decrease progressively with advancing lactation, whereas the concentration of manganese increases during the first 5 days of lactation, after which it decreases progressively (Csapó-Kiss *et al.*, 1995; Ullrey *et al.*, 1974).

1.12. Physical Properties of Equid Milk

The physical properties of the milk of some species are compared in Table 1.16.

1.12.1. Density

The density (kg m^{-3}) of equine colostrum is higher than that of equine milk (Waelchli *et al.*, 1990; Ullrey *et al.*, 1966; Mariani *et al.*, 2001), due primarily to its considerably higher protein content. Values for colostrum can reach up to ~1080 kg m^{-3} (Ullrey *et al.*, 1966) and show a significant linear correlation with the IgG content of colostrum (Waelchi *et al.*, 1990; Le Blanc *et al.*, 1986). Density is highest immediately *post-partum* and decreases rapidly during the first 12 h (Ullrey *et al.*, 1966); considerably smaller decreases in density are observed during the rest of lactation (Ullrey *et al.*, 1966; Mariani *et al.*, 2001). Density values reported for mature equine milk range from ~1028 to 1035 kg m^{-3}. The density of whole mature bovine milk normally ranges from 1027 to 1033 kg m^{-3} (Singh *et al.*, 1997).

1.12.2. Refractive index

The refractive index for equine colostrum ranges from 1.340 to 1.354, whereas that of mature equine milk is ~1.339 (Waelchi *et al.* 1990). The higher refractive index of colostrum than of mature milk is probably related to its higher total solids content, since the refractive index increases with increasing mass fraction of each solute. The refractive index of bovine milk is in the range 1.344-1.349 (Singh *et al.*, 1997).

1.12.3. pH

There is considerable variation among reported values for the pH of equine milk. Mariani *et al.* (2001) reported that its pH 4 days *post-partum* is ~6.6 and increases to ~6.9 after 20 days and to ~7.1 at 180 days *post-partum*. A value of ~ 7.0 for the pH for mature equine milk was reported by Kücükcetin *et al.* (2003), but Pagliarini *et al.* (1993) reported an average value of ~7.2. The pH of bovine milk is generally between 6.5 and 6.7 (Singh *et al.*, 1997) and increases during lactation (Tsioulpas *et al.*, 2007). These differences are presumably related to differences in protein and salt composition of the milks.

1.12.4. Freezing point

The freezing point of milk is directly related to the concentrations of water-soluble compounds therein. Fat globules and proteins have a negligible influence on freezing point, with the main effect arising from lactose and minerals. A freezing point of -0.554°C (Pagliarini *et al.*, 1993) or -0.548°C (Nesini *et al.*, 1958) has been reported for equine milk, whereas the vast majority of bovine milk samples have a freezing point in the range -0.512 to -0.550°C (Singh *et al.*, 1997). The lower freezing point of equine milk is probably related to its higher lactose content.

Table 1.16 Physical properties of the milk of equid species, with comparative data for bovine and human milk.

Property	Equine Milk	Equine Colostrum	Asinine Milk	Bovine Milk	Human milk
Freezing Point (^{o}C)	-0.525 to -0.554	-	-0.55 to -0.49	-0.512 to -0.55	-
pH (25^{o}C)	7.1 - 7.3	-	7.01-7.35	6.5-6.7	6.8
Density (kg m^{-3}), (20^{o}C)	1032	1080	1029-1037	1027-1033	1031
Refractive Index, n_D^{20}	1.3394	1.340-1.354	-	1.344-1.349	-
Viscosity (mPa s)	1.5031	-	-	1.6314	-
Zeta potential, mV	-10.3	-	-	-20.0	-
Colour L*,a*,b*	86.52, -2.34, -0.15	-	80.88, -2.27, -3.53	79.12, -7.46, -2.31	-

(modified from Uniacke and Fox, 2011)

1.13. Processing of equid milk

Due to its unique physico-chemical properties, outlined earlier, the processing of equine and asinine milk into traditional dairy products is not possible; cheese is not produced from these milks as a firm curd is not formed on renneting. Equid milk will form a weak coagulum under acidic conditions and this is exploited in the production of yoghurt-type products with reputed probiotic and therapeutic properties. Traditionally, and to date, the only significant product from equine milk is the fermented product, koumiss, the production and properties of which are described below (1.13.1). Interest in koumiss production has grown recently which may be attributed to the fact that, worldwide, the overall consumption of fermented milk products has grown faster than the consumption of fresh milk (IDF, 2009).

Fermentation is one of the oldest methods for preserving milk and probably dates back ~ 10,000 to the Middle East where the first organized agriculture occurred. Traditional fermented milk products have been developed independently worldwide and were, and continue to be, especially important in areas where transportation, pasteurization and refrigeration facilities are inadequate. Worldwide, milk from eight species of domesticated mammals (cow, buffalo, sheep, goat, camel, horse, reindeer and yak) have been used to produce traditional fermented milk products. There are three categories of fermented milks, those resulting from lactic fermentations, yeast-lactic fermentations and mould (*Geotrichum candidum*)-lactic fermentations. Koumiss and kefir belong to the yeast-lactic fermentation group where alcoholic fermentation by yeasts is used in combination with a lactic acid fermentation (Tamine and Marshall, 1984). The conversion of milk into a fermented product has several important advantages; as well as being a means of preservation, it also improves taste and digestibility and increases the variety of food. Current interest in the health benefits of fermented milks started with the theory of longevity proposed by the Russian microbiologist Elie Metchnikoff (1845-1918); he claimed that people who consumed fermented milks lived longer as lactic acid bacteria from the fermented product colonized the intestine and inhibited 'putrefaction' caused by harmful bacteria (a probiotic effect), thereby slowing down the aging process. Nowadays, the principal effects of probiotics are thought to be: improved gastrointestinal transit time of digesta, bowel function and glycemic index; some reports claim that they have an anticarcinogenic effect (see McIntosh, 1996).

Further details on the fermentation of milk can be found in many publications including: Tamine and Marshall (1984); Koroleva (1991); Kurmann *et al.* (1992); Nakazawa and Hosono (1992); Surono and Hosono (2002).

1.13.1. Koumiss

Koumiss (Kumys), fermented equine milk, is widely consumed in Russia, Mongolia and Kazakhstan, primarily for its therapeutic value. Russians, in particular, have long advocated the use of koumiss for a wide variety of illnesses but the variable microbiology of these products has made it difficult to confirm any theoretical basis for the claims (Tamine and Robinson, 1999). In Mongolia, koumiss is the national drink (Airag) and a high-alcoholic drink made by distilling koumiss, called Arkhi is also produced (Kanbe, 1992). *Per caput* consumption of koumiss in Mongolia is estimated to be about 50 L per year.

Koumiss is still manufactured in remote areas of Mongolia by traditional methods but with increased demand elsewhere it is now produced under more controlled and regulated conditions. Traditional koumiss (from fresh raw milk) was prepared by seeding milk with a mixture of bacteria and yeasts using part of the previous day's product as an inoculum. The milk was held in a leather sack called a 'turdusk' (also called a 'saba' or 'burduk'), made from smoked horsehide taken from the thigh area i.e., it has a broad bottom and a narrow, long, sleeve with a capacity of 25-30 L, fermentation takes from 3 to 8 hours. In the 1960s the microbial population was analysed and found to consist mainly of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei*, *Lactococcus lactis* subsp. *lactis*, *Kluveromyces fragilis* and *Saccharomyces unisporus* (Tamine and Marshall, 1984). The lactic acid bacteria are responsible for acid production and the yeasts for the production of ethanol and carbon dioxide. During the mixing and maturation stages of production, more equine milk is usually added to control the levels of acidity and ethanol. The whole process is poorly controlled and often results in a product with an unpleasant taste due to the presence of too much yeast or excessive acidification. Turdusks, often containing fermented caprine milk from the previous season, were stored in a cool place over winter and the culture was reactivated when required by gradually filling the turdusk with equine milk over about 5 days (Tamine and Marshall, 1984).

Koumiss contains about 90% moisture, 2-2.5% protein (1.2 % casein and 0.9% whey proteins), 4.5-5.5% lactose, 1-1.3% fat and 0.4-0.7% ash, as well as the end-products of microbial fermentation, i.e., lactic acid (1.8%), ethanol (0.6-2.5%) and CO_2 (0.5-0.9%) and provides 37 to 40 kCal per 100 mL). After production, koumiss contains between 0.6 and 3 % ethanol and is effervescent. Koumiss is thought to be more effective than raw equine milk in disease treatment due to the additional peptides and bactericidal substances produced during microbial metabolism (Doreau and Marti-Rosset, 2002).

In the last decade, technological advances have been made in the manufacture of koumiss, such as the development of blends of microorganisms in starter cultures that enhance flavour development and extend the shelf-life up to 14 days. The production of koumiss and other fermented milk products is carried out using a more standardized protocol for manufacture and is of considerable interest for increasing the market and consumption of equine milk products in countries where it has not normally been consumed (Di Cagno *et al.*, 2004). As well as pasteurizing the raw equine milk, pure cultures of lactobacilli such as *Lb.delbrueckii* subsp. *bulgaricus* and yeasts are used for koumiss manufacture. The use of *Saccharomyces lactis* is considered best for ethanol production (2-5%) and *S. cartilaginosus* is sometimes used for its antibiotic activity against *Mycobacterium tuberculosis* (Park *et al.*, 2006). Other microorganisms such as *Candida* spp., *Torula* spp., *Lb. acidophilus* and *Lb. lactis* may also be used in koumiss production (Surono and Hosono, 2002). A protocol for the manufacture of commercial koumiss is presented in Figure 1.6. The characteristics of a good koumiss are optimal when lactic and alcoholic fermentations proceed simultaneously so that the products of fermentation occur in definite proportions. As well as lactic acid, ethanol and CO_2, volatile acids and other compounds are formed which are important for aroma and taste and ~10% of equine milk proteins are hydrolysed. Products with varying amounts of lactic acid and ethanol are produced and generally 3 categories of koumiss are recognised: mild (0.6-0.8% acidity, 0.7-1.0 % alcohol; medium (0.8-1.0% acidity, 1.1-1.8% alcohol) and strong (1.0-1.2% acidity, 1.8-2.5% alcohol (Tamine and Marshall, 1984).

The presence of a high level of thermo-stable lysozyme in equine milk may interfere with the activity of some starter microorganisms in the production of fermented products and thus cause problems in the processing of equine milk. Di Cagno *et al.* (2004) who heated equine milk to 90^{o}C for 3 min to inactivate lysozyme, produced an acceptable fermented product. In sensory tests, fermented equine milk generally scores low and, in an attempt to enhance the rheological and sensory properties of fermented products made from equine milk, DiCagno *et al.* (2004) fortified equine milk with bovine Na caseinate (1.5 g.100^{-1}g), pectin (0.25 g.100^{-1}g) and threonine (0.08 g.100^{-1}g). The resultant product had good microbiological, rheological and sensory characteristics after 45 days at 4^{o}C. Fermented unmodified equine milk had an unacceptable viscosity and scored very low in comparison to fortified products for appearance, consistency and taste.

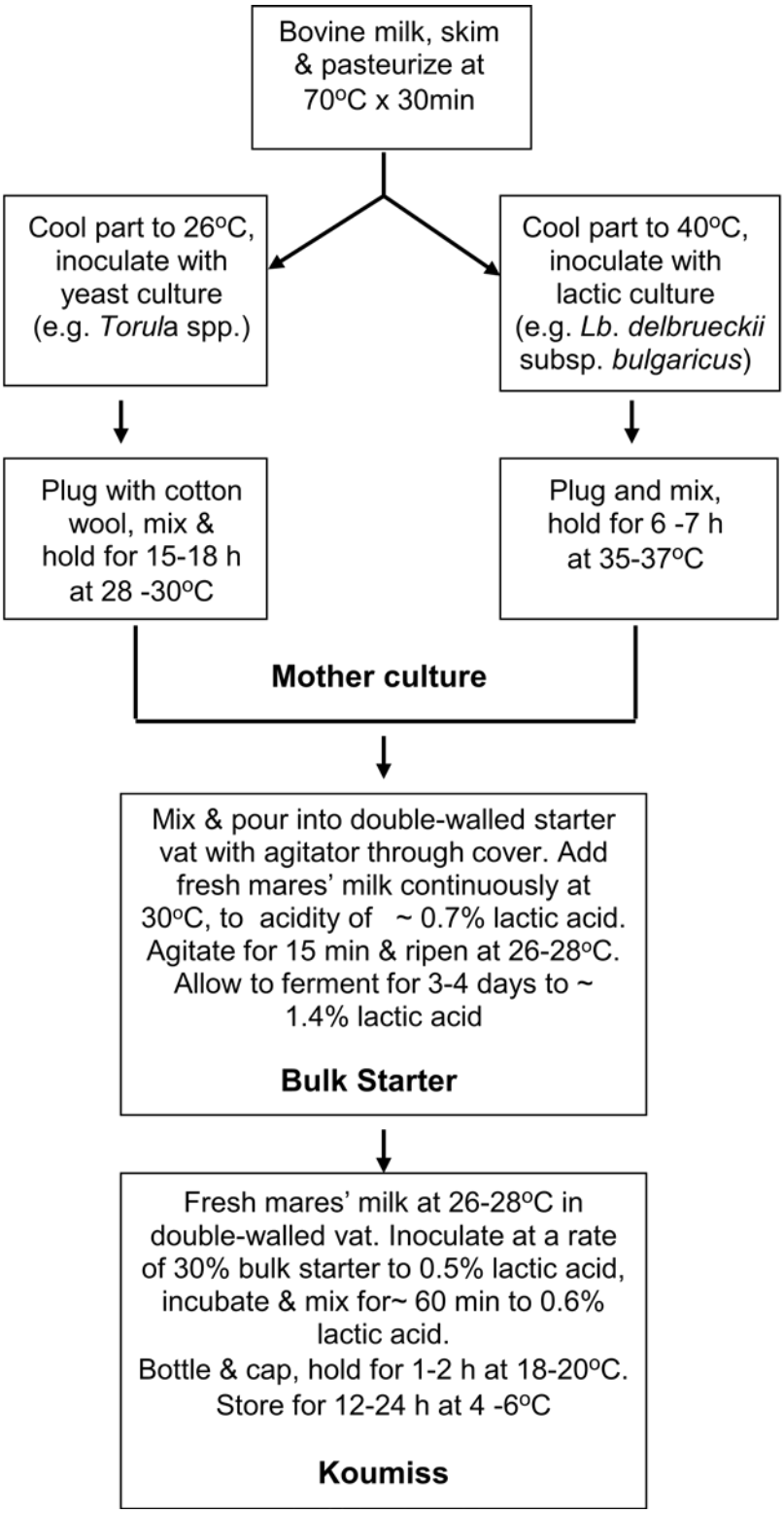

Figure 1.6 Schematic for the production of koumiss (based on Berlin, 1962)

Research has turned also to producing koumiss-like products from bovine milk, which must be modified to make it suitable for koumiss production. Methods have been developed, with varying degrees of success, where a single constituent of bovine milk has been altered to resemble that of equine milk, e.g., the carbohydrate content has been increased or the protein content reduced but, until recently both had not been altered simultaneously. Koumiss of reasonable quality has been produced successfully from whole or skimmed bovine milk containing added sucrose using a mixture of *Lb. acidophilus*, *Lb*. *delbrueckii* ssp. *bulgaricus* and *Kluyveromyces marxianus* var. *marxianus* or var. *lactis* as starter culture (Kücükcetin *et al.*, 2003). Koumiss has also been made from diluted bovine milk supplemented with lactose and, more successfully, from bovine milk mixed with concentrated whey using a starter culture of *Kluyveromyces lactis* (AT CC 56498), *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. acidophilus*. Starter cultures for koumiss manufacture from bovine milk may also include *Saccharomyces lactis* (high antimicrobial activity against *Mycobacterium tuberculosis*) in order to retain the 'anti-tuberculosis image' of equine milk (Kücükcetin *et al.*, 2003). More recently, bovine milk has been modified to approximate the composition of mares' milk using a series of membrane filtration steps and a starter culture (*Kluyveromyces lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. acidophilus*) that ensures consistent fermentation; the resulting product was found to be very similar to koumiss with respect to pH, titratable acidity, ethanol content, proteolytic activity, apparent viscosity and microbial composition, both when fresh or stored (15 days at 4^{o}C) (Kücükcetin *et al.*, 2003).

The physico-chemical and microbiological properties of asinine milk, similar to equine milk, such as low microbiological load and high lysozyme make it a good substrate for the production of fermented products with probiotic *Lactobacillus* strains. Coppola *et al.* (2002) incubated asinine milk with the probiotic *Lb. rhamnosus* (AT 194, GTI/1, GT 1/3) and found that the strains are unaffected by the high lysozyme activity in the milk and remained viable after 15 days at 4^{o}C and pH 3.7-3.8. *Lb. rhamnosus* inhibits the growth of most harmful bacteria in the intestine and acts as a natural preservative in yoghurt-type products, considerably extending shelf-life. Chiavari *et al.* (2005) produced fermented beverages from asinine milk using a mixed culture of *Lb. rhamnosus* (AT 194, CLT 2.2) and *Lb. casei* (LC 88) and in all cases found a high level of viable bacteria after 30 days storage. Some sensory differences were recorded for the fermented drinks and those made with the *Lb. casei* strain developed a more acceptable and balanced aroma than the boiled vegetable/acidic taste and aroma of the products made with *Lb*. *rhamnosus*.

1.13.2. Other Products from Equid Milk

As sales of equine milk have increased considerably in recent years, research is now focused on the development of new fermented products or new methods for extending the shelf-life of existing products, while maintaining some of the unique components of equine milk. The ability of milk to withstand relatively high processing temperatures is very important from a technological point of view. The whey proteins in equine milk are much more thermo-stable than those of bovine milk. Heat treatment at 80^{o}C x 80 s causes only a 10-15% decrease in non-casein nitrogen, with a marked decrease evident only when the temperature is increased above 100^{o}C (Bonomi *et al.*, 1994). Lactoferrin and equine BSA are the most heat-sensitive but are not completely denatured until the temperature reaches 130^{o}C x 10 min. β-Lg and α–La are almost completely denatured at temperatures over 130^{o}C and lysozyme at temperatures greater than 110^{o}C (68% residual lysozyme activity after heating at 82^{o}C for 15 min (Jauregui-Adell, 1975).

1.14. Nutritional and biomedical properties of equid milk

It is claimed that fresh and fermented equid milk relieve metabolic and intestinal problems while having a gut-cleansing effect coupled with 'repair' of the intestinal micro-flora. It is claimed to give relief from stomach ulcers, high blood pressure, high cholesterol and liver problems and is also recommended as an aid in the treatment of cancer patients. The recommended amount of equine milk is 250 mL per day. The most significant use of equid milk is as a substitute for bovine milk for patients with cows' milk protein allergy (CMPA); limited research has shown that both equine and asinine milks are generally tolerated well by CMPA patients (see below, 1.14.1.) The use of equine milk in the production of cosmetics is relatively new and includes soaps, creams and moisturisers (Doreau & Martin-Rosset, 2002). At present, equine milk is available in several forms: frozen milk, frozen yoghurt-type drink, lyopholized powder, shampoos and various cosmetic and medicinal creams. Lyopholized equine colostrum is available and used in the high-value horse industry to feed orphaned foals. Marconi and Panfili (1998) suggested that research is required to identify optimum drying and storage conditions for powder equine milk for retention of some of the unique characteristics of raw milk, including high levels of whey proteins, PUFAs, lysine, tocopherols and vitamin C which are partially destroyed in the preparation of commercial powdered equine milk. Asinine milk is used to make ice-cream and other desserts and also a fermented milk product. To improve the nutritive value of asinine milk and increase its

overall energy content for human nutrition, it is frequently supplemented with ~ 4% medium-chain triglycerides (Salimei, 2011).

1.14.1. Cow Milk Protein Allergy

Equine and asinine milk, with a composition close to that of human milk, may be good nutritional sources for the neonate when breast milk is not available. Bovine milk or bovine milk products are used traditionally as substitutes for human milk in infant nutrition but bovine milk is considerably different from human milk in terms of its macro- and micro-nutrients and the absorption rates of vitamins and minerals from the two milks are different, which can be problematic for infants. CMPA is an IgE-mediated type I allergy, which may be life-threatening, and is defined as a set of immunologically-mediated adverse reactions which occur following the ingestion of milk, affecting from 2 to 6% of children in their first year of life. About 50% of affected children recover after the age of one and 80-90% of those affected recover by 5 years of age (Caffarelli *et al.*, 2010). The high frequency of CMPA in infants and children is thought to be due to an incomplete gut mucosal barrier, increased gut permeability to large molecules and immature local and systemic responses which are aided by breast-milk which facilitates gut maturation and provides passive protection against bacteria and antigens (Hill, 1994).

The difference between bovine milk protein allergy and lactose intolerance is of particular interest and it is an area which causes much confusion. CMPA is a food allergy, i.e., an adverse immune reaction to a food protein that is normally harmless to the non-allergic individual. Lactose intolerance is a non-allergic food hypersensitivity due to a deficiency of the enzyme β-galactosidase (lactase), required to hydrolyze lactose. Lactase deficiency manifests as abdominal symptoms and chronic diarrhoea after ingestion of milk (see Bindslev-Jensen, 1998; Vesa *et al.*, 2000). Lactose intolerance is not a disease or malady; 70% of the world's population is lactose-intolerant. Adverse effects of lactose intolerance occur at a much higher level of milk consumption than that which causes milk allergy. CMPA is important because bovine milk is the first foreign antigen ingested in large quantities in early infancy. Reviews on CMPA include: Hill (1994); Hill & Hosking (1996); Taylor 1986; Høst (1988), (1991); Bindslev-Jensen (1998); Wal (2002, 2004); El-Agamy (2007); Apps and Beattie (2009). Because β-Lg is absent from human milk, it has commonly been considered to be the most important cows' milk allergen (Goldman *et al.*, 1963; Ghosh *et al.*, 1989) although other whey proteins (Jarvinen *et al.*, 2001) and caseins (Savilahti and Kuitunen,

1992; Restani *et al.*, 1995) have also been implicated in allergic reactions. In children, β-Lg is the major allergen whereas casein appears to be the most allergenic for adults. The resistance of β-Lg to proteolysis allows the protein to remain intact through the gastrointestinal tract with the possibility of being absorbed across the gut mucosa. Ingested β-Lg has been detected in human milk and could be responsible for colic in breast-fed infants and the sensitization of infants, predisposing them to allergies (Jakobsson and Lindberg, 1978; Kilshaw and Cant, 1984; Stuart *et al.*, 1984; Jakobsson *et al.*, 1985; Axelsson *et al.*, 1986).

The choice of substitute for cows' milk in cases of CMPA depends on two major factors, i.e., nutritional adequacy and allergenicity; cost and taste must also be taken into account. Many soy or hydrolysate (casein-based and more recently whey protein-based) formulae are available for treatment of CMPA but they can themselves induce allergic reactions. Heat treatment of milk may destroy heat-labile proteins, especially BSA and Igs, and change the antigenic properties of other whey proteins, such as β-Lg and α-La, although caseins need severe heat treatment (121^{o}C x 20 min) to reduce sensitizing capacity (Hill, 1994). Enzymatic treatment of milk proteins may result in products with an unacceptable taste due to bitterness arising from the production of peptides and amino acids and such peptides may, in fact, be allergenic (Schmidt *et al*., 1995; Sélo *et al*., 1999; El-Agamy, 2007).

Many clinical studies have been carried out on the use of the milk of different species in infant nutrition, e.g., goat, sheep (Restani *et al.*, 2002), camel (El-Agamy, 2007), buffalo (El-Agamy, 2007), horse and donkey (Iacono *et al.*, 1992; Carroccio, *et al.*, 2000; El-Agamy *et al.*, 1997; Businco *et al.*, 2000; Monti *et al.*, 2007). Results on the benefits of such milks are conflicting and infants with CMPA may suffer allergic reactions to buffalo, goat, sheep, donkey or mare milk proteins due to positive immunological cross-reaction with their counterparts in cows' milk (El-Agamy, 2007). Lara-Villoslada *et al.*(2005) found that the balance between casein and whey proteins may be important in determining the allergenicity of bovine milk proteins in humans and that modification of this balance may reduce the allergenicity of bovine milk; a readjustment of the casein:whey proteins ratio to 40:60 was found to make the bovine milk significantly less allergenic. Presumably, equine and asinine milk, with a ratio of casein:whey proteins reasonably close to that in human milk, are potentially good substitutes for human milk. It is noteworthy that the study by Lara-Villoslada *et al.* (2005) was carried out using mice as subjects and the increased level of β-lactoglobulin (a major bovine milk allergen) in bovine milk adjusted to 40:60, casein:whey was not considered.

1.14.2. Cross reactivity of milk proteins

Cross-reaction occurs when two food proteins have similar amino acid sequences or when the three-dimensional conformation makes two molecules similar in their capacity to bind specific antibodies (Restani *et al.*, 2002). Cross reactivity of proteins from different species generally reflects the phylogenetic relations between animal species, e.g., homologous proteins from vertebrates often cross-react. A comprehensive study on the subject by Jenkins *et al.* (2007) highlights some interesting points, especially concerning the potential allergenicity of caseins from different species. The authors set out to determine how closely a foreign protein has to resemble a human homologue before it actually looses its allergenic affect. A high degree of similarity to human homologues would, presumably, imply that a foreign animal food protein would be much less likely than a protein with little or no similarity to its human homologue to be allergenic in human subjects. In addition, the study of potential animal allergens must take into account the ability of the human immune system to discriminate between its own proteins, i.e., an autoimmune response, and those from another species which have a high similarity, i.e., how closely a foreign protein has to resemble a human homologue before it loses its ability to act as an allergen? (Spitzauer, 1999). Table 1.17 gives the percentage identity of α_{S1}-, α-$_{S2}$ and β-caseins from different species to bovine and human homologues. Known allergens are less than 53% identical to human sequences. Natale *et al.* (2004) found that 90% of a group of infants with CMPA had serum IgE against bovine α_{S2}-casein, 55% against bovine α_{S1}-casein and only 15% against bovine β-casein, which is closest in amino acid composition to human β-casein. Caprine and ovine milk proteins are more closely related to each other than either is to bovine milk proteins, thus explaining why an individual allergic to goats' milk cheese may exhibit high IgE cross-reactivity with sheep's milk proteins but could tolerate cow's milk and its products.

Allergy to equine milk appears to be rare and, to date,only two documented cases have been reported. Fanta and Ebner (1998) reported the case of an individual who experienced sensitization to horse dander allergen and subsequently produced IgE antibodies on ingestion of equine milk which was prescribed to 'strengthen' her immune system. Gall *et al.* (1996) demonstrated the existence of an IgE-mediated equine milk allergy in one patient, caused by low MW heat-labile proteins, most likely α-la and β-Lg, without cross-reaction to the corresponding whey proteins from bovine milk. Presumably, the above cases are not isolated incidents and as the consumption of equine milk and its products increases, it is likely that further cases will be reported.

Bevilaçqua *et al.* (2001) tested the capacity of goats' milk with low or high α_{s1}-casein content to induce milk protein sensitization in guinea pigs and found significantly less sensitization by milk with low α_{s1}-casein. This may represent an important attribute of the low α_{s1}-casein content of equine milk for use in human allergology. The absence of α_{s2}-casein (and lack of α_{s1} casein in one donkey) and β-lg II in donkey milk reported by Criscione *et al.* (2009) could be potentially interesting for future research on the allergenicity of asinine milk; α_{s1}-casein and β-lg are scarce or absent in human milk and are considered to be the most significant proteins causing allergic reactions in children and adults.

Table 1.17. Homology (%) between milk proteins from different species and their human homologues.

		% Identity to Closest	
Casein	**Accession Code**	**Bovine Homolog**	**Human Homolog**
α_{S1}-Casein			
Cow	P02662	100	29
Goat	Q8M1H4	88	29
Sheep	P04653	88	28
Horse	Q8SPR1	39	44
Human	P47710	29	100
Rat	PO2661	22	27
Camel	O97943	41	36
Rabbit	PO9115	37	37
α_{S2}-Casein			
Cow	P02663	100	16
Goat	P33049	88	17
Sheep	P04654	89	17
Camel	097944	56	11
Rabbit	P50418	36	16
β-Casein			
Cow	P02666	100	53
Goat	Q712N8	91	54
Sheep	P11839	91	54
Horse	Q9GKK3	56	58
Human	P05814	53	100
Camel	Q9TVD0	66	58
Rabbit	PO9116	52	55

(modified from Jenkins *et al*., 2007)

1.15. Summary

The characteristics of equine and asinine milk of interest in human nutrition include an exceptionally high concentration of polyunsaturated fatty acids, low cholesterol content, high lactose and low protein levels (Solaroli *et al.*, 1993; Salimei *et al.*, 2004), as well as high levels of vitamins A, B and C. The low fat and unique fatty acid profile of both equine and asinine milk results in low atherogenic and thrombogenic indices. Research has shown that human health is considerably improved when dietary fat intake is reduced and, more importantly, when the ratio of saturated to unsaturated fatty acids is reduced. The high lactose content of equid milk gives good palatability and improves intestinal absorption of calcium which is important for bone mineralization in children. The renal load of equine milk, based on levels of protein and inorganic substances, is equal to that of human milk, a further indication of its suitability as an infant food. Equine and asinine milk can be used for their prebiotic and probiotic activity and as alternatives for infants and children with CMPA and multiple food intolerances (Iacono *et al.*, 1992; Carroccio et al., 2000).

The invigorating effect of equine milk may be, at least partially, due to its immuno-stimulating ability. Lysozyme, lactoferrin and *n*-3 fatty acids have long been associated with the regulation of phagocytosis of human neutrophils *in vitro* (Ellinger *et al.*, 2002). The concentration of these compounds is exceptionally high in equine milk and the consumption of frozen equine milk significantly inhibits chemotaxis and respiratory burst, two important phases of the phagocytic process (Ellinger *et al.*, 2002). This result suggests a potential anti-inflammatory effect by equine milk.

To be successful as a substitute for human milk in infant nutrition, equine milk must be capable of performing many biological functions associated with human milk. The presence of high concentrations of lactoferrin, lysozyme, *n*-3 and *n*-6 fatty acids in equine milk are good indicators of its potential role. However, the lack of research must be addressed to develop the potential of equine milk in the health and nutritional markets. Studies are required to bring the health claims for equine milk out of the realms of regional folklore. It seems reasonable to suggest that equine milk could be marketed as a dietary aid where the immune system is already depleted, i.e., as a type of ‘immuno-boost’. More than 30% of customers who purchase equine milk in the Netherlands are patients undergoing chemotherapy, who find equine milk helpful in counteracting the effects of the treatment. The composition of equine milk suggests a product with interesting nutritional characteristics with potential use in dietetics and therapeutics, especially in diets for the elderly, convalescent and newborns.

Future research should also include comprehensive characterization of the proteins of different breeds of horse and donkey, with the possibility of selection of animals for specific proteins which could, in turn, optimize the nutritional and technological properties of the milks; the genetic control of α_s-, β- and κ-caseins as well as β-Lg in bovine and caprine milk has been researched, albeit in a somewhat limited manner, as has the effects of these proteins on the technological properties of both types of milk, e.g., colloidal stability, coagulation and curd strength. These characteristics can determine the physical and chemical behaviour of milk in the infant gastrointestinal tract with the result that digestion and availability of nutrients to the young may be affected (Cuthbertson, 1999). Furthermore, genetic selection of certain breeds of horse and donkey may improve milk yield and lactation pattern and make the production of milk more cost effective.

1.15. References

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CHAPTER 2

Equine milk proteins:

Chemistry, structure and nutritional significance

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Abstract

Equine milk has important nutritional and therapeutic properties which can benefit the diet of the elderly, convalescent or newborn. The protein content of equine milk is lower than that of bovine milk but similar to that of human milk. In this review qualitative and quantitative differences between the casein and whey proteins of equine, bovine and human milk are discussed. Important biological and functional properties of specific proteins are reviewed and their significance in human nutrition considered. As well as characterizing equine milk proteins in the context of human nutrition and allergology, the potential industrial exploitation of equine milk is explored. Cross-reactivity of proteins from different species is discussed in relation to the treatment of cow's milk protein allergy. While there is some scientific basis for the special nutritional and health-beneficial properties of equine milk based on its protein composition and similarity to human milk, further research is required to fully exploit its potential in human nutrition.

Contents

1. Introduction
2. Gross composition of equine milk
3. Factors that affect the composition of equine milk
4. Equine milk proteins
   - 4.1. Whey proteins
     - 4.1.1. β-Lactoglobulin
     - 4.1.2. α-Lactalbumin
     - 4.1.3. Lactoferrin
     - 4.1.4. Lysozyme
     - 4.1.5. Immunoglobulins
   - 4.2. Denaturation of whey proteins
   - 4.3. Caseins
     - 4.3.1. α_{S1}-Casein
     - 4.3.2. α_{S2}-Casein
     - 4.3.3. β-Casein
     - 4.3.4. κ-Casein
     - 4.3.5. Glycosylation of κ-casein
     - 4.3.6. Hydrolysis of κ-casein
5. Equine casein micelles
   - 5.1. Colloidal stability of casein micelles
   - 5.2. Enzymatic coagulation of equine milk
   - 5.3. Acid-induced flocculation of equine milk
   - 5.4. Heat-induced coagulation of equine milk
6. Non-protein nitrogen of equine milk
   - 6.1. Bioactive peptides
   - 6.2. Free amino acids in equine milk
7. Digestibility of equine protein
8. Equine milk in human nutrition
   - 8.1. Cow milk protein allergy
   - 8.2. Cross-reactivity of proteins
9. Products from equine milk
   - 9.1. Koumis
   - 9.2. Processing of equine milk
10. Conclusions

References

1. *Introduction*

Although horses are of minor importance for milk production in comparison with cows, buffalo, sheep and goats, they have been traditionally important dairy animals in Mongolia and in the southern states of the former Soviet Union, e.g., Kazakhstan, Kyrgyzstan and Tajikistan. Because equine milk resembles human milk in many respects and is claimed to have special therapeutic properties (Freudenberg, 1948a, b; Kalliala, Seleste, & Hallman, 1951; Stoyonova, Abramova, & Ladoto, 1988; Lozovich, 1995), it is becoming increasingly important in Europe, especially in France, Italy, Hungary and the Netherlands. Koumiss (fermented equine milk) is used in Russia and Mongolia for the management of digestive and cardiovascular diseases (Lozovich, 1995; Levine, 1998); in Italy, equine milk is recommended as a substitute for bovine milk for allergic children (Curadi, Giampietro, Lucenti, & Orlandi, 2001). Overall, equine milk is considered to be highly digestible, rich in essential nutrients and possesses an optimum whey protein/casein ratio, making it suitable in paediatric dietetics. Estimates suggest that more than 30 million people worldwide drink equine milk regularly, with that figure increasing significantly annually (Doreau & Martin-Rosset, 2002). However, in comparison to bovine milk, equine milk is very expensive to produce. One of the main constraints of the horse as a dairy animal is the high frequency of milkings required; generally about 5 times per day or every 2 hours, with the foal feeding through the night. The capacity of the mare's udder is low, about 2 litres, and the foal must be in close proximity during milking (Solaroli, Pagliarini, & Peri, 1993). Unlike dairy cows, mastitis is not a major factor in equine milk production due to the small udder size which limits exposure of the teats to infection (Doreau & Martin-Rosset, 2002) and despite its high level of unsaturated fatty acids, equine milk has better keeping quality than human or bovine milk (Kallila *et al*., 1951). For details on the production of equine milk and the factors that affect it, the reader is referred to the reviews by Doreau and Boulot (1989), Doreau, Boulet, Bartlet, and Patureau-Mirand (1990), Doreau (1994), Doreau and Martin-Rosset (2002) and Park, Zhang, Zhang, and Zhang, (2006).

2. Gross composition of equine milk

Young mammals are born at very different stages of maturity and their maternal milk differs greatly in composition (Table 1), although milk from species in the same taxonomic order, e.g., the equids, tend to be fairly similar (Table 2) (Jenness, 1974a). The composition of equine milk differs considerably from that of the milk of the principal dairying species, i.e., the cow, buffalo, goat and sheep (Table 1). In comparison with bovine milk, equine milk contains less fat, protein, inorganic salts but more lactose, with a concentration close to that in human milk. A mare produces 2 - 3.5 kg milk per 100 kg live-weight per day to sustain the rapid growth of the foal (Oftedal, Hintz, & Schryver, 1983; Doreau, 1994). A healthy foal will consume 10 to 25% of its body weight of milk per day (Gibbs, Potter, Blake, & McMullan, 1982; Oftedal *et al*., 1983; Zicker & Lönnerdal, 1994) which, for an average 45 kg foal, is 9 - 13 L of milk daily (Paradis, 2003). The composition and constituents of equine milk have been studied thoroughly; reviews include Linton (1931), Neseni, Flade, Heidler, & Steger (1958), Neuhaus (1959), Doreau & Boulot (1989), Solaroli, Pagliarini, & Peri (1993), Malacarne, Martuzzi, Summer, & Mariani (2002) and Park, *et al*. (2006). This article presents a detailed review of the proteins in equine milk, with comparative data for bovine and human milk, and the nutritional significance thereof.

Table 1. Gross composition of the milk of selected species. Values are expressed as *g. kg^{-1} or **g. L^{-1} milk**.**

Species	*Total Solids*	*Protein*	*Casein/Whey Ratio*	*Fat*	*Lactose*	*Ash*	*Gross Energy* (kJ. kg^{-1} or kJ. L^{-1})
*Man (*Homo sapiens*)[a]	124.0	9.0	0.4:1	38.0	70.0	2.0	2763
**Cow (*Bos taurus*) [b]	127.0	34.0	4.7:1	37.0	48.0	7.0	2763
*Horse (*Equus caballus*) [c]	102.0	21.4	1.1:1	12.1	63.7	4.2	1883
*Donkey (*Equus africanus asinus*) [d]	88.4	17.2	1.3:1	3.8	68.8	3.9	
*Buffalo (*Bubalus bubalis)* [e,f]	172.0	46.5	4.6:1	81.4	48.5	8.0	4644
**Sheep (*Ovis aries)* [g]	181.0	55.9	3.1:1	68.2	48.8	10.0	4309
*Goat (*Capra hircus)* [h]	122.0	35.0	3.5:1	38.0	41.0	8.0	2719
**Camel (*Camelus dromedarius*)[i,j]	124.7	33.5	1.68:1	38.2	44.6	7.9	2745
** Llama (*Llama glama*)[k]	131.0	34.0	3.1:1	27.0	65.0	5.0	2673
*Yak (*Bos grunniens*)[l,m]	160.0	42.3	4.5:1	56.0	52.9	9.1	3702

[a] Hambræus, 1982, 1984; Picciano, 2001; [b] Jenness, 1974b; [c] Malacarne *et al*., 2002; Schryver *et al*., 1986; [d] Salimei et al., 2004; [e]Jenness & Sloan, 1970; [f] Tufarelli *et al*., 2008; [g] Raynal-Ljutovac *et al*., 2008. [h] Park, 2006; [i] Konuspayeva *et al*., 2009; [j] Vaisman *et al*., 2006; [k]Rosenberg, 2006. [l] Silk *et al*., 2006; [m] Sheng *et al*., 2008.

Table 2. Gross composition (g. kg^{-1}) of the milk of some equid species.

	Total Solids	Fat	Protein	Casein/Whey Ratio	Lactose	Ash	Gross energy (kJ. kg^{-1})
Donkey [a]	88.9	5.6	16.6	~1.28:1	65.2	4.2	2710
Mountain zebra [b]	100.0	10.0	16.0	-	69.0	3.0	2000
Plains zebra [b]	113.0	22.0	16.0	-	70.0	4.0	2400
Przewalski horse [b]	105.0	15.0	16.0	1.1:1	67.0	3.0	2100
Pony [b]	104.0	15.0	18.0	1.1:1	67.0	5.0	2200

[a] values averaged from Guo *et al*., 2007, Salimei *et al*., 2004 and Piccione *et al*., 2008, using a value for density of 1029 kg m^{-3} (Chiavari *et al*., 2005), [b] Oftedal & Jenness, 1988.

3. Factors that affect the composition of equine milk

Both genetic and environmental factors affect the gross composition of milk; including the breed of mammal, individuality of animals, stage of lactation, frequency and completeness of milking, maternal age, health and type of feed. Data in the literature are not conclusive on whether or not the breed of mare has an effect on the concentration of protein in milk. Kulisa (1977), Doreau *et al*. (1990), Csapó-Kiss, Stefler, Martin, Makray, and Csapó (1995) and Csapó, Stefler, Martin, Makray, and Csapó-Kiss (1995) reported no effect of breed on the concentration of proteins or lipids in equine milk throughout lactation. On the other hand, Boulot (1987), Formaggioni, Malacarne, Martuzzi, Summer, and Mariani (2003), Civardi, Curadi, Orlandi, Cattaneo, and Giangiacomo (2002) and Pelizzola, Contarini, Povolo, and Giangiacomo (2006) reported significant differences in protein content between breeds. Table 3 shows the gross composition of the milk of a number of horse breeds; differences are evident between breeds in the concentration of some milk constituents, especially proteins. Assessment of the effect of breed on milk composition is difficult owing to differences between individuals, as well as between feeding regimens and herd conditions (Martuzzi & Doreau, 2006). The stage of lactation has a marked effect on the composition of equine milk. In the first few hours after parturition, the mammary gland produces colostrum which is richer in dry matter, protein, fat, vitamins and minerals (except calcium and phosphorus) but poorer in lactose than mature milk. One of the major biological benefits of colostrum is the presence of immunoglobulins, IgA, IgM and IgG, and high levels of several enzymes, including catalase, lipase and proteinase. The transition of colostrum into mature equine milk occurs within 2 days of parturition. The concentration of protein in equine colostrum is very high, >150 g. kg^{-1}, immediately *post-partum* (due primarily to the high concentration of immunoglobulins), but decreases rapidly to < 40 g. kg^{-1} within 24 h and to < 20 g. kg^{-1} after 4 weeks of lactation (Linton, 1937; Mariani, Martuzzi, & Catalano, 1993). The casein to whey protein ratio in equine milk is 0.2:1 immediately *post-partum* and changes to 1.2:1 during the first week of lactation (Zicker & Lönnerdal, 1984). In bovine milk, the protein content decreases during the first 3 months of lactation, but increases subsequently (Jenness, 1974b; Fox & McSweeney, 1998; Tsioulpas, Grandison, & Lewis, 2007).

Table 3. Gross composition of the milk of various breeds of mid-lactation, multiparous mares. Values expressed as *g. kg^{-1} or **g. L^{-1} milk.

Species	Sample Size (n)	Total Solids	Protein	Ratio of Casein/Whey	Fat	Lactose	Ash	Reference
**Andalusian	18	122.1	19.3	ND	24.0	66.3	ND	Fuentes-Garcia *et al*., 1991
**Arabian Mare	-	110.9	20.3	ND	17.0	63.6	ND	Pieszka & Kulisa, 2003; Pieszka *et al*., 2004
**Dutch Saddle	39	ND	21.0	ND	6.3	68.4	ND	Smolders *et al*.,1990
**Finnish Native	15	ND	20.0	ND	10.0	70.0	5.0	Antila *et al*., 1971
**Indigenous Italian	-	103.0	22.0	1.1:1	15.0	62.0	3.6	Marconi & Panfili, 1998
**Murgese	8	ND	18.5	0.86:1.0	10.6	70.4	ND	Caroprese *et al*., 2007
**Orlov Trotter	-	ND	15.8	0.84:1	ND	ND	ND	Kudryashov & Krylova, 1965
**Quarter Horse	7	104.0	21.0	ND	15.0	ND	ND	Burns *et al*., 1992
**Quarter Horse	14	105.0	21.0	ND	13.0	ND	ND	Gibbs *et al*., 1982
**Russian Heavy	-	109.5	20.9	1.35:1	18.0	64.0	ND	Stoyanova *et al*., 1987
**Thoroughbred	10	105.0	19.3	ND	12.9	69.1	ND	Oftedal *et a*l., 1983
*Bretonnes & Comtoises	10	ND	33.8	ND	11.3	62.7	ND	Doreau *et al*., 1986
*Bretonnes & Comtoises	11	ND	20.6	ND	12.5	67.3	ND	Doreau et al., 1990
*Clydesdale	-	114.1	26.4	ND	19.0	68.5	3.58	Linton, 1937
*Haflinger	-	96.8	18.3	1.33:1	9.0	64.7	3.9	Pelizzola *et al*., 2006
*Haflinger	5	ND	18.2	ND	8.2	67.8	3.9	Mariani *et al*., 2001
*Lusitano	48	ND	18.4	ND	5.9	60.8	ND	Santos & Silvestre, 2008
*Murgese	-	ND	20.5	ND	19.3	63.1	4.2	Di Cagno *et al.*, 2004
*Quarter Horse	-	104.9	23.1	1.42:1	14.0	70.2	5.5	Pelizzola *et al*., 2006
*Rapid Heavy Draft	-	ND	18.4	1.12:1	11.0	67.8	3.6	Pelizzola *et al*., 2006
*Sella & Salto	-	ND	26.6	1.63:1	7.0	63.3	5.3	Pelizzola *et al*., 2006
*Sella &Trotter	11	111.6	23.9	1.13:1	13.6	69.5	4.6	Mariani *et al*., 1993
*Shetland Pony	-	100.6	17.8	ND	8.0	72.3	2.5	Linton, 1937

ND, not determined

4. Equine milk proteins

While the protein content of mature equine milk is lower than that of bovine milk, there is a strong qualitative resemblance, the principal classes of proteins, i.e., caseins and whey proteins are similar in both types of milk. However, while the caseins are the predominant class of proteins in bovine milk (~80% of total milk protein), equine milk contains less casein and more whey proteins. In the past, the proportion of casein in equine milk has been underestimated as determinations were made by acid precipitation at pH 4.6, as in bovine milk, but maximum precipitation of equine casein occurs at pH 4.2 (Egito *et al*., 2002). The concentrations and distribution of the casein and whey proteins of equine milk are shown in Table 4, with comparative data for human and bovine milk.

4.1 Whey proteins

The major whey proteins in equine milk are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (Igs), blood serum albumin (BSA), lactoferrin (Lf) and lysozyme (Lyz) (Bell, McKenzie, Muller, Rogers, & Shaw, 1981a), which is similar to bovine milk. Except for β-Lg, all these proteins are also present in human milk. However, the relative amounts of the whey proteins differ considerably between these species (Table 4). Compared to bovine milk, equine milk contains less β-Lg and more α-La and Igs. The principal anti-microbial agent in equine milk is Lyz and to a lesser extent Lf, which predominates in human milk (Table 4). Both Lf and Lyz are very low in bovine milk, in which Igs form the main defence against microbes (Malacarne *et al.*, 2002). Together, IgA, IgG, IgM, Lf and Lyz provide the neonate with immune and non-immune protection against infection (Baldi *et al*., 2005).

Table 4. Concentrations of caseins and whey proteins (g. kg $^{-1}$) in equine, human and bovine milk.

	Equine[a]	**Human**[b]	**Bovine**[c]
Total casein	**13.56**	**2.4**	**26.0**
α_{s1}-casein	2.4	0.77[a]	10.7
α_{s2}-casein	0.20	-	2.8
β-casein	10.66	3.87[a] (> 85%)[h]	8.6
κ-casein	0.24	(< 15%)[h]	3.1
γ-casein	-	-	0.8
Total whey protein	**8.3**[d]	**6.2**[d]	**6.3**
β-lactoglobulin	2.55	-	3.2
α-lactalbumin	2.37	2.5	1.2
Serum albumin	0.37	0.48	0.4
Proteose peptone	-	-	0.8
Immunoglobulins	**1.63**[d]	**0.96**[d]	**0.80**
$IgG_{1,2}$	0.38[e]	0.03	0.65
IgA	0.47[e]	0.96	0.14
IgM	0.03[e]	0.02	0.05
Lactoferrin	0.58[f]	1.65	0.10
Lysozyme	0.87[f]	0.34[k]	126 x 10^{-6}[i]
NPN	**0.381**	**0.485**	**0.296**[j]
Casein Micelle Size (nm)[g]	**255**	**64**	**182**

note: where necessary values have been adjusted using density values ,1032, 1031 and 1033 kg m^{-3} for equine (Uniacke & Fox, unpublished data), human and bovine milks (Neville & Jensen, 1995), respectively.

[a] adapted from Miranda *et al*., 2004; [b] adapted from Hambræus, 1984 ; [c] adapted from Walstra *et al*., 2006; [d] from Park *et al*., 2006; [e] Hurley, 2003; [f] Pagliarini *et al*., 1993; [g] Malacarne *et al*., 2002; [h] Hambræus & Lönnderdal, 2003; [i] ElAgamy *et al*., 1996; [j] DePeters & Ferguson, 1992. [k] Montagne *et al*., 1998, 2000.

4.1.1. β-Lactoglobulin

β-Lg is the major whey protein in the milk of most ruminants and is also present in milk of monogastrics and marsupials, but is absent from the milk of humans, camels, lagomorphs and rodents. β-Lg is synthesized in the secretory epithelial cells of the mammary gland under the control of prolactin. Although several biological roles for β-Lg have been proposed, e.g., facilitator of vitamin uptake and an inhibitor, modifier or promoter of enzyme activity, conclusive evidence for a specific biological function of β-Lg is not available (Sawyer, 2003). β-Lg from all species studied binds retinol; β-Lg of many species, but not equine or porcine, binds fatty acids also (Pérez, Puyol, Ena, & Calvo, 1993). During digestion, milk lipids are hydrolysed by pre-duodenal lipases, greatly increasing the amount of free fatty acids which could potentially bind to β-Lg, displacing any bound retinol, and implying that fatty acid metabolism, rather than retinol transport, is the more important function of β-Lg (Pérez & Calvo, 1995). Bovine β-Lg is very resistant to peptic digestion and can cause allergenic reactions on consumption. Resistance to digestion is not uniform among species, with ovine β-Lg being far more digestible than bovine β-Lg (El-Zahar *et al.,* 2005). The digestibility of equine β-Lg, which has to our knowledge not been studied, warrants research, particularly considering the potential applications of equine milk as a hypo-allergenic dairy product.

Two isoforms of equine β-Lg have been isolated, β-Lg I and II, which contain 162 and 163 amino acids, respectively. The extra amino acid in equine β-Lg II is a glycine residue inserted after position 116 (Halliday, Bell, & Shaw, 1991). Bovine β-Lg occurs mainly as two genetic variants, A and B, both of which contain 162 amino acids and differ only at positions 63 (Asp in variant A, Gly in variant B) and 117 (Val in variant A, Ala in variant B); a further 11, less common, genetic variants of bovine β-Lg have also been reported (Sawyer, 2003). The amino acid sequences of equine β-Lg I and II are shown in Fig. 1. Based on its amino acid sequence, unmodified equine β-Lg I has a molecular mass of 18,500 Da and an isoelectric pH of 4.85, whereas equine β-Lg II, despite having one more amino acid, has a molecular mass of 18,262 Da (ExPASy ProtParam Tool, 2009), and an isoelectric pH of 4.71 (Table 5). Bovine β-Lg A and B have a molecular mass of 18,367 and 18,281 Da, respectively, and an isoelectric pH of 4.76 and 4.83, respectively (Table 5).

Using the hydropathy scale proposed by Kyte and Doolittle (1982), equine β-Lg I and II have a grand average hydropathy (GRAVY) score of -0.386 and -0.300, respectively (Table 5). This scale reflects the relative ratio of hydrophobic and hydrophilic amino acid residues in a protein, with a positive value reflecting an overall hydrophobic and a negative value an overall hydrophilic nature of the protein. Bovine β-Lg A and B have a GRAVY score of -0.167 and -0.162, respectively (Table 5), and are, therefore, considered to be less hydrophilic than equine β-Lg I and II. Both equine and bovine β-Lg contain two intramolecular disulphide bridges, linking Cys_{66} to Cys_{160} and Cys_{106} to Cys_{119} in equine β-Lg I, Cys_{66} to Cys_{161} and Cys_{106} to Cys_{120} in equine β-Lg II and Cys_{66} to Cys_{160} and Cys_{106} to Cys_{119} or Cys_{121} in bovine β-Lg A and B.. Unlike bovine β-Lg equine β-Lg lacks a sulphydryl group, which has large implications for denaturation and aggregation of the protein, as discussed in Section 4.2. Also, unlike bovine β-Lg, equine β-Lg does not dimerize (Sawyer, 2003).

	1									**10**									**20**
I	Thr-	Asn-	Ile-	Pro-	Gln-	Thr-	Met-	Gln-	Asp-	Leu-	Asp-	Leu-	Gln-	Glu-	Val-	Ala-	Gly-	Lys-	Trp-	His-
II	Thr-	Asp-	Ile-	Pro-	Gln-	Thr-	Met-	Gln-	Asp-	Leu-	Asp-	Leu-	Gln-	Glu-	Val-	Ala-	Gly-	Arg-	Trp-	His-
	21									**30**									**40**
I	Ser-	Val-	Ala-	Met-	Ala-	Ala-	Ser-	Asp-	Ile-	Ser-	Leu-	Leu-	Asp-	Ser-	Glu-	Glu-	Ala-	Pro-	Leu-	Arg-
II	Ser-	Val-	Ala-	Met-	Val-	Ala-	Ser-	Asp-	Ile-	Ser-	Leu-	Leu-	Asp-	Ser-	Glu-	Glu-	Ala-	Pro-	Leu-	Arg-
	41									**50**									**60**
I	Val-	Tyr-	Ile-	Glu-	Lys-	Leu	Arg-	Pro-	Thr-	Pro-	Glu-	Asp-	Asn-	Leu-	Glu-	Ile-	Ile-	Leu-	Arg-	Glu-
II	Val-	Tyr-	Val-	Glu-	Glu-	Leu	Arg-	Pro-	Thr-	Pro-	Glu-	Gly-	Asn-	Leu-	Glu-	Ile-	Ile-	Leu-	Arg-	Glu-
	61									**70**									**80**
I	Gly-	Glu-	Asn-	Lys-	Gly-	Cys-	Ala-	Glu-	Lys-	Lys-	Ile-	Phe-	Ala-	Glu-	Lys-	Thr-	Gln-	Ser-	Pro-	Ala-
II	Gly-	Ala-	Asn-	Lys-	Gly-	Cys-	Ala-	Glu-	Arg-	Asn-	Ile-	Val-	Ala-	Gln-	Lys-	Thr-	Glu-	Ser-	Pro-	Ala-
	81									**90**									**100**
I	Gln-	Phe-	Lys-	Ile-	Asn-	Ala-	Leu-	Asp-	Glu-	Asp-	Thr-	Val-	Phe-	Tyr-	Leu-	Asp-	Thr-	Asp-	Tyr-	Lys-
II	Val-	Phe-	Thr-	Val-	Asn-	Tyr-	Gln-	Gly-	Glu-	Arg-	Lys-	Ile-	Ser-	Val-	Leu-	Asp-	Thr-	Asp-	Tyr-	Ala-
	101									**110**									**120**
I	Asn-	Tyr-	Leu-	Phe-	Leu-	Cys-	Met-	Lys-	Asn-	Ala-	Ala-	Thr-	Pro-	Gly-	Gln-	Ser-	-	Leu-	Val-	Cys-
II	His-	Tyr-	Met-	Phe-	Phe-	Cys-	Val-	Gly-	Pro-	Pro-	Leu-	Pro-	Ser-	Ala-	Lys-	His-	Gly-	Met-	Val-	Cys-
	121									**130**									**140**
I	Gln-	Tyr-	Leu-	Ala-	Arg-	Thr-	Gln-	Met-	Val-	Asp-	Glu-	Glu-	Ile-	Met-	Glu-	Lys-	Phe-	Arg-	Arg-	Ala-
	Gln-	Tyr-	Leu-	Ala-	Arg-	Thr-	Gln-	Lys-	Val-	Asp-	Glu-	Glu-	Met-	Glu-	Lys-	Phe-	Ser-	Arg-	Ala-	Arg-
	141									**150**									**160**
I	Leu-	Gln-	Pro-	Leu-	Pro-	Gly-	Arg-	Val-	Gln-	Ile-	Val-	Pro-	Asp-	Leu-	Thr-	Arg-	Met-	Ala-	Glu-	Arg-
II	Leu-	Gln-	Pro-	Leu-	Pro-	Gly-	Arg-	Val-	Gln-	Ile-	Val-	Glu-	Asp-	Pro-	Ser-	Gly-	Gly-	Gln-	Glu-	Arg-
	161		**163**																	
I	Cys-	Arg-	Ile																	
II	Cys-	Gly-	Phe																	

Figure 1. Primary structure of equine β-lactoglobulin I (from Conti, Godovac-Zimmerman, Liberatori, & Braunitzer, 1984) and II (from Godovac-Zimmerman, Conti, Liberatori, & Braunitzer, 1985 and Halliday, Bell, & Shaw,1991)

Table 5. Properties of equine and bovine β-Lg. Values were calculated from the amino acid sequences of the mature proteins provided on http://au.expasy.org/tools.

Species	Variant	Primary accession number[a]	Amino acid residues	Molecular mass (Da)	pI	GRAVY score[b]	Disulphide bridges	Free sulphydryl groups
Equine	I	P02758	162	18,500.2	4.85	-0.386	2	0
	II	P07380	163	18,261.6	4.71	-0.300	2	0
Bovine	A	P02754	162	18,367.3	4.76	-0.167	2	1
	B	P02754	162	18,281.2	4.83	-0.162	2	1

[a] Primary accession number for the protein in SWISS-PROT database
[b] Grand average hydropathy (GRAVY) score calculated using the scale of Kyte & Doolittle (1982)

4.1.2. α-Lactalbumin

α-La, a unique protein in the milk of mammals, is homologous with the well-characterized c-type lysozymes. It is a calcium metalloprotein, in which Ca^{2+} plays a crucial role in folding and structure. α-La is synthesised in the rough endoplasmic reticulum, from where it is transported to the Golgi apparatus, where it has a regulatory function in the synthesis of lactose. Together with β-1, 4 galactosyltranferase, which is the catalytic component of lactose synthetase, α-La enhances enzymatic affinity for glucose 1000 fold in the final step of lactose synthesis, when glucose is linked to galactose (Larson, 1979; Brew, 2003; Neville, 2009).

Similar to the α-La of bovine, caprine, ovine, asinine, camelid and human milk, equine α-La contains 123 amino acids (Brew, 2003). Equine α-La occurs as three genetic variants, A, B and C, which differ by only a few single amino acids (Fig. 2) (Godovac-Zimmerman, Shaw, Conti, & McKenzie, 1987). Bovine α-La occurs as two, or possibly three, genetic variants (Bell, Hopper & McKenzie, 1981b) and human α-La has two genetic variants, one of which has been identified only recently (Chowanadisai *et al*., 2005). The primary structure of equine, bovine and human α-La differ only by a few single amino acid replacements, and the proteins have similar properties (Table 6). Equine α-La A, B and C have an isoelectric point at pH 4.95, 4.95 and 5.11, respectively, whereas bovine and human α-La have pIs at pH 4.80 and 4.70, respectively (Table 6). The GRAVY scores of equine and bovine α-La are comparable, whereas that of human α-La is distinctly higher (Table 6), indicating a lower hydrophobicity. The eight cysteine residues of bovine and human α-La form four intramolecular disulphide bonds, linking Cys_6 to Cys_{120}, Cys_{28} to Cys_{111}, Cys_{61} to Cys_{77} and Cys_{73} to Cys_{93}. Based on the very high similarity between equine, bovine and human α-Las, as well as the α-La from other species, it is highly likely that equine α-La also contains four intramolecular disulphide bridges, at the aforementioned positions.

	1									**10**									**20**
A	Lys-	Gln-	Phe-	Thr-	Lys-	Cys-	Glu-	Leu-	Ser-	Glu-	Val-	Leu-	Lys-	Ser-	Met-	Asp-	Gly-	Tyr-	Lys-	Gly-
B	Lys-	Gln-	Phe-	Thr-	Lys-	Cys-	Gln-	Leu-	Ser-	Glu-	Val-	Leu-	Lys-	Ser-	Met-	Asp-	Gly-	Tyr-	Lys-	Gly-
C	Lys-	Gln-	Phe-	Thr-	Lys-	Cys-	Gln-	Leu-	Ser-	Glu-	Val-	Leu-	Lys-	Ser-	Met-	Asp-	Gly-	Tyr-	Lys-	Gly-
	21									**30**									**40**
A	Val-	Thr-	Leu-	Pro-	Glu-	Trp-	Ile-	Cys-	Thr-	Ile-	Phe-	His-	Ser-	Ser-	Gly-	Tyr-	Asp-	Thr-	Gln-	Thr-
B	Val-	Thr-	Leu-	Pro-	Glu-	Trp-	Ile-	Cys-	Thr-	Ile-	Phe-	His-	Asn-	Ser-	Gly-	Tyr-	Asp-	Thr-	Gln-	Thr-
C	Val-	Thr-	Leu-	Pro-	Glu-	Trp-	Ile-	Cys-	Thr-	Ile-	Phe-	His-	Asn-	Ser-	Gly-	Tyr-	Asp-	Thr-	Gln-	Thr-
	41									**50**									**60**
A	Ile-	Val-	Lys-	Asn-	Asn-	Gly-	Lys-	Thr-	Glu-	Tyr-	Gly-	Leu-	Phe-	Gln-	Ile-	Asn-	Asn-	Lys-	Met-	Trp-
B	Ile-	Val-	Lys-	Asn-	Asn-	Gly-	Lys-	Thr-	Glu-	Tyr-	Gly-	Leu-	Phe-	Gln-	Ile-	Asn-	Asn-	Lys-	Met-	Trp-
C	Ile-	Val-	Lys-	Asn-	Asn-	Gly-	Lys-	Thr-	Glu-	Tyr-	Gly-	Leu-	Phe-	Gln-	Ile-	Asn-	Asn-	Lys-	Met-	Trp-
	61									**70**									**80**
A	Cys-	Arg-	Asp-	Asn-	Gln-	Ile-	Leu-	Pro-	Ser-	Arg-	Asn-	Ile-	Cys-	Gly-	Ile-	Ser-	Cys-	Asp-	Lys-	Phe-
B	Cys-	Arg-	Asp-	Asn-	Gln-	Ile-	Leu-	Pro-	Ser-	Arg-	Asn-	Ile-	Cys-	Gly-	Ile-	Ser-	Cys-	Asn-	Lys-	Phe-
C	Cys-	Arg-	Asp-	Asn-	Gln-	Ile-	Leu-	Pro-	Ser-	Arg-	Asn-	Ile-	Cys-	Gly-	Ile-	Ser-	Cys-	Asn-	Lys-	Phe-
	81									**90**									**100**
A	Leu-	Asp-	Asp-	Asp-	Leu-	Thr-	Asp-	Asp-	Val-	Met-	Cys-	Ala-	Lys-	Lys-	Ile-	Leu-	Asp-	Ser-	Glu-	Gly-
B	Leu-	Asp-	Asp-	Asp-	Leu-	Thr-	Asp-	Asp-	Val-	Met-	Cys-	Ala-	Lys-	Lys-	Asp-	Leu-	Asp-	Ser-	Glu-	Gly-
C	Leu-	Asp-	Asp-	Asp-	Leu-	Thr-	Asp-	Asp-	Val-	Met-	Cys-	Ala-	Lys-	Lys-	Ile-	Leu-	Asp-	Ser-	Glu-	Gly-
	101									**110**									**120**
A	Ile-	Asp-	Tyr-	Trp-	Leu-	Ala-	His-	Lys-	Pro-	Leu-	Cys-	Ser-	Glu-	Lys-	Leu-	Glu-	Gln-	Trp-	Leu-	Cys-
B	Ile-	Asp-	Tyr-	Trp-	Leu-	Ala-	His-	Lys-	Pro-	Leu-	Cys-	Ser-	Glu-	Lys-	Leu-	Glu-	Gln-	Trp-	Leu-	Cys-
C	Ile-	Asp-	Tyr-	Trp-	Leu-	Ala-	His-	Lys-	Pro-	Leu-	Cys-	Ser-	Glu-	Lys-	Leu-	Glu-	Gln-	Trp-	Leu-	Cys-
	121		**123**																	
A	Glu-	Glu-	Leu-																	
B	Glu-	Glu-	Leu-																	
C	Glu-	Glu-	Leu-																	

Figure 2. Amino acid sequence of equine α-lactalbumin A (from Kaminogawa, McKenzie, & Shaw,1984), B and C (from Godovac-Zimmerman, Shaw, Conti, & McKenzie, 1987). Amino acid positions at which the genetic variants differ are shaded.

Table 6. Properties of equine, bovine and human α-La. Values were calculated from the amino acid sequences of the mature proteins provided on http://au.expasy.org/tools.

Species	Variant	Primary accession number[a]	Amino acid residues	Molecular mass (Da)	pI	GRAVY score[b]	Disulphide bridges	Sulphydryl groups
Equine	A	P08334	123	14223.2	4.95	-0.416	4[c]	0
	B	P08896	123	14251.2	4.95	-0.503	4[c]	0
	C	P08896	123	14249.3	5.11	-0.438	4[c]	0
Bovine		P00711	123	14186.0	4.80	-0.453	4	0
Human		P00709	123	14078.1	4.70	-0.255	4	0

[a] Primary accession number for the protein in SWISS-PROT database
[b] Grand average hydropathy (GRAVY) score calculated using the scale of Kyte & Doolittle (1982)
[c] Estimated from structural similarity with bovine and human α-La.

4.1.3. Lactoferrin

Lactoferrin (Lf) is an iron-binding glycoprotein, comprised of a single polypeptide chain of MW ~ 78 kDa (Conneely, 2001). Lf is structurally very similar to transferrin (Tf), a plasma iron transport protein, but has a much higher (~300 fold) affinity for iron (Brock, 1997). Lf is not unique to milk, although it is especially abundant in colostrum; small amounts are also found in tears, saliva and mucus secretions and in the secondary granules of neutrophils. The expression of Lf in the bovine mammary gland is dependent on prolactin (Green & Pastewka, 1978); its concentration is very high during early pregnancy and involution and is expressed predominantly in the ductal epithelium close to the teat (Molenaar *et al*., 1996). Equine, human and bovine milk contain approximately 0.6 g, 1.6 g and 0.1 g Lf per kg, respectively (Table 4). Shimazaki, Oota, Nitta, and Ke (1994) purified Lf from equine milk and compared its iron-binding ability with that of human and bovine Lf and with bovine Tf. The iron-binding capacity of equine Lf is similar to that of human Lf but higher than that of bovine Lf and Tf. Various biological functions have been attributed to Lf but the exact role of Lf in iron-binding in milk is unknown and there is no relationship between the concentrations of Lf and Tf and the concentration of iron in milk (human milk is very rich in Lf but low in iron) (Masson & Heremans, 1971).

Bacterial growth is inhibited by the ability of Lf to sequester iron and also to permeabilize bacterial cell walls by binding to lipopolysaccharides through its N-terminus. Lf can inhibit viral infection by binding tightly to the envelope proteins of viruses and is also thought to stimulate the establishment of a beneficial microflora in the gastrointestinal tract (Baldi *et al*., 2005). Ellison and Giehl (1991) suggested that Lf and Lyz work synergistically to effectively eliminate Gram-negative bacteria; Lf binds oligosaccharides in the outer bacterial membrane, thereby opening ‘pores’ for Lyz to disrupt glycosidic linkages in the interior of the peptidoglycan matrix. This synergistic process leads to inactivation of both Gram-negative bacteria, e.g., *E. coli* (Rainhard, 1986) and Gram-positive bacteria, e.g., *Staph. epidermis* (Leithch & Willcox, 1999). A proteolytic digestion product of bovine and human Lf, i.e., lactoferricin, has bactericidal activity (Bellamy *et al*., 1992). Bovine and human Lf are reported to have antiviral activity and a role as a growth factor (Lönnerdal, 2003).

The specific biological function of equine Lf has not been studied, but is likely to be similar to that of bovine and human Lf.

The amino acid sequence of equine Lf, as reported by Sharma, Paramasivan, Srinivasan, Yadav, and Singh (1998), is shown in Fig. 3. Equine Lf contains 689 amino acid residues, which is similar to bovine Lf and two more than human Lf (Table 7). Compared to most other milk proteins, Lf has a high isoelectric point, i.e., at pH 8.32, 8.67 or 8.47 for equine, bovine or human Lf (Table 7). As a result, the protein is positively charged at the pH of milk and may associate with negatively-charged proteins *via* electrostatic interactions. GRAVY scores are comparable for equine, bovine and human Lf (Table 7). Equine and human Lf contain 17 and 16 intra-molecular disulphide bonds, respectively (Table 7). The iron-binding capacity of equine, bovine and human Lf is equivalent, although the pH-dependence of the iron-binding capacity of bovine Lf differs from that of equine and human Lf (Shimazaki *et al*, 1994).

All Lf types studied to date are glycosylated, but the location and number of potential glycosylation sites, as well as the number of sites actually glycosylated, vary. In bovine Lf, four out of five potential glycosylation sites, i.e., Asn_{223}, Asn_{368}, Asn_{476} and Asn_{545}, are glycosylated (Moore, Anderson, Groom, Haridas, & Baker, 1997), whereas in human Lf, two of three potential glycosylation sites, i.e., Asn_{137} and Asn_{478}, are glycosylated (Haridas, Anderson, & Baker, 1995). Glycosylation of equine Lf has not been studied, but using the consensus sequence, Asn-Xaa-Ser/Thr (where Xaa is not Pro), for glycosylation, three potential glycosylation sites are likely in equine Lf, i.e., Asn_{137}, Asn_{281} and Asn_{476}.

1									**10**									**20**
Ala-	Pro-	Arg-	Lys-	Ser-	Val-	Arg-	Trp-	Cys-	Thr-	Ile-	Ser-	Pro-	Ala-	Glu-	Ala-	Ala-	Lys-	Cys-	Ala-
21									**30**									**40**
Lys-	Phe-	Gln-	Arg-	Asn-	Met-	Lys-	Lys-	Val-	Arg-	Gly-	Pro-	Ser-	Val-	Ser-	Cys-	Ile-	Arg-	Lys-	Thr-
41									**50**									**60**	
Ser-	Ser-	Phe-	Glu-	Cys-	Ile-	Gln-	Ala-	Ile-	Ala-	Ala-	Asn-	Lys	Ala-	Asp-	Ala-	Val-	Thr-	Leu-	Asp-
61									**70**									**80**	
Gly-	Gly-	Leu-	Val-	Tyr-	Glu-	Ala-	Gly-	Leu-	His-	Pro-	Tyr-	Lys-	Leu-	Arg-	Pro-	Val-	Ala-	Ala-	Glu-
81									**90**									**100**	
Val-	Tyr-	Gln-	Thr-	Arg-	Gly-	Lys-	Pro-	Gln-	Thr-	Arg-	Tyr-	Tyr-	Ala-	Val-	Ala-	Val-	Val-	Lys-	Lys-
101									**110**									**120**	
Gly-	Ser-	Gly-	Phe-	Gln-	Leu-	Asn-	Gln-	Leu-	Gln-	Gly-	Val-	Lys-	Ser-	Cys-	His-	Thr-	Gly-	Leu-	Gly-
121									**130**									**140**	
Arg-	Ser-	Ala-	Gly-	Trp-	Asn-	Ile-	Pro-	Ile-	Gly-	Thr-	Leu-	Arg-	Pro-	Tyr-	Leu-	Asn-	Trp-	Thr-	Gly-
141									**150**									**160**	
Pro-	Pro-	Glu-	Pro-	Leu-	Gln-	Lys-	Ala-	Val-	Ala-	Asn-	Phe-	Phe-	Ser-	Ala-	Ser-	Cys-	Val-	Pro-	Cys-
161									**170**									**180**	
Ala-	Asp-	Gly-	Lys-	Gln-	Tyr-	Pro-	Asn-	Leu-	Cys-	Arg-	Leu-	Cys-	Ala-	Gly-	Thr-	Glu-	Ala-	Asp-	Lys-
181									**190**									**200**	
Cys-	Ala-	Cys-	Ser-	Ser-	Gln-	Glu-	Pro-	Tyr-	Phe-	Gly-	Tyr-	Ser-	Gly-	Ala-	Phe-	Lys-	Cys-	Leu-	Glu-
201									**210**									**220**	
Asn-	Gly-	Ala-	Gly-	Asp-	Val-	Ala-	Phe-	Val-	Lys-	Asp-	Ser-	Thr-	Val-	Phe-	Glu-	Asn-	Leu-	Pro-	Asp-
221									**230**									**240**	
Glu-	Ala-	Asp-	Arg-	Asp-	Lys-	Tyr-	Glu-	Leu-	Leu-	Cys-	Pro-	Asp-	Asn-	Thr-	Arg-	Lys-	Pro-	Val-	Asp-
241									**250**									**260**	
Ala-	Phe-	Lys-	Glu-	Cys-	His-	Leu-	Ala-	Arg-	Val-	Pro-	Ser-	His-	Ala-	Val-	Val-	Ala-	Arg-	Ser-	Val-
261									**270**									**280**	
Asp-	Gly-	Arg-	Glu-	Asp-	Leu-	Ile-	Trp-	Arg-	Leu-	Leu-	His-	Arg-	Ala-	Gln-	Glu-	Glu-	Phe-	Gly-	Arg-
281									**290**									**300**	
Asn-	Lys-	Ser-	Ser-	Ala-	Phe-	Gln-	Leu-	Phe-	Lys-	Ser-	Thr-	Pro-	Glu-	Asn-	Lys-	Asp-	Leu-	Leu-	Phe-
301									**310**									**320**	
Lys-	Asp-	Ser-	Ala-	Leu-	Gly-	Phe-	Val-	Arg-	Ile-	Pro-	Ser-	Gln-	Ile-	Asp-	Ser-	Gly-	Leu-	Tyr-	Leu-
321									**330**									**340**	
Gly-	Ala-	Asn-	Tyr-	Leu-	Thr-	Ala-	Thr-	Gln-	Asn-	Leu-	Arg-	Glu-	Thr-	Ala-	Ala-	Glu-	Val-	Ala-	Ala-
341									**350**									**360**	
Arg-	Arg-	Glu-	Arg-	Val-	Val-	Trp-	Cys-	Ala-	Val-	Gly-	Pro-	Glu-	Glu-	Glu-	Arg-	Lys-	Cys-	Lys-	Gln-
361									**370**									**380**	
Trp-	Ser-	Asp-	Val-	Ser-	Asn-	Arg-	Lys-	Val-	Ala-	Cys-	Ala-	Ser-	Ala-	Ser-	Thr-	Thr-	Glu-	Glu-	Cys-
381									**390**									**400**	
Ile-	Ala-	Leu-	Val-	Leu-	Lys-	Gly-	Glu-	Ala-	Asp-	Ala-	Leu-	Asn-	Leu-	Asp-	Gly-	Gly-	Phe-	Ile-	Tyr-
401									**410**									**420**	
Val-	Ala-	Gly-	Lys-	Cys-	Gly-	Leu-	Val-	Pro-	Val-	Leu-	Ala-	Glu-	Asn-	Gln-	Lys-	Ser-	Gln-	Asn-	Ser-
421									**430**									**440**	
Asn-	Ala-	Pro-	Asp-	Cys-	Val-	His-	Arg-	Pro-	Pro-	Glu-	Gly-	Tyr-	Leu-	Ala-	Val-	Ala-	Val-	Val-	Arg-
441									**450**									**460**	
Lys-	Ser-	Asp-	Ala-	Asp-	Leu-	Thr-	Trp-	Asn-	Ser-	Leu-	Ser-	Gly-	Lys-	Lys-	Ser-	Cys-	His-	Thr-	Gly-
461									**470**									**480**	
Val-	Gly-	Arg-	Thr-	Ala-	Ala-	Trp-	Asn-	Ile-	Pro-	Met-	Gly-	Leu-	Leu-	Phe-	Asn-	Gln-	Thr-	Gly-	Ser-
481									**490**									**500**	
Cys-	Lys-	Phe-	Asp-	Lys-	Phe-	Phe-	Ser-	Gln-	Ser-	Cys-	Ala-	Pro-	Gly-	Ala-	Asp-	Pro-	Gln-	Ser-	Ser-
501									**510**									**520**	
Leu-	Cys-	Ala-	Leu-	Cys-	Val-	Gly-	Asn-	Asn-	Glu-	Asn-	Glu-	Asn-	Lys-	Cys-	Met-	Pro-	Asn-	Ser-	Glu-
521									**530**									**540**	
Glu-	Arg-	Tyr-	Tyr-	Gly-	Tyr-	Thr-	Gly-	Ala-	Phe-	Arg-	Cys-	Leu-	Ala-	Glu-	Lys-	Ala-	Gly-	Asp-	Val-
541									**550**									**560**	
Ala-	Phe-	Val-	Lys-	Asp-	Val-	Thr-	Val-	Leu-	Gln-	Asn-	Thr-	Asp-	Gly-	Lys-	Asn-	Ser-	Glu-	Pro-	Trp-
561									**570**									**580**	
Ala-	Lys-	Asp-	Leu-	Lys-	Gln-	Glu-	Asp-	Phe-	Glu-	Leu-	Leu-	Cys-	Leu-	Asp-	Gly-	Thr-	Arg-	Lys-	Pro-
581									**590**									**600**	
Val-	Ala-	Glu-	Ala-	Glu-	Ser-	Cys-	His-	Leu-	Ala-	Arg-	Ala-	Pro-	Asn-	His-	Ala-	Val-	Val-	Ser-	Gln-
601									**610**									**620**	
Ser-	Asp-	Arg-	Ala-	Gln-	His-	Leu-	Lys-	Lys-	Val-	Leu-	Phe-	Leu-	Gln-	Gln-	Asp-	Gln-	Phe-	Gly-	Gly-
621									**630**									**640**	
Asn-	Gly-	Pro-	Asp-	Cys-	Pro-	Gly-	Lys-	Phe-	Cys-	Leu-	Phe-	Lys-	Ser-	Glu-	Thr-	Lys-	Asn-	Leu-	Leu-
641									**650**									**660**	
Phe-	Asn-	Asp-	Asn-	Thr-	Glu-	Cys-	Leu-	Ala-	Glu-	Leu-	Gln-	Gly-	Lys-	Thr-	Thr-	Tyr-	Glu-	Gln-	Tyr-
661									**670**									**680**	
Leu-	Gly-	Ser-	Glu-	Tyr-	Val-	Thr-	Ser-	Ile-	Thr-	Asn-	Leu-	Arg-	Arg-	Cys	Ser-	Ser-	Ser-	Pro-	Leu-
681								**689**											
Leu-	Glu-	Ala-	Cys-	Ala-	Phe-	Leu-	Arg-	Ala-											

Figure 3. Amino acid sequence of equine lactoferrin (from Sharma, Paramasivan, Srinivasan, Yadav, & Singh, 1998).

Table 7. Properties of equine, bovine and human lactoferrin. Values were calculated from the amino acid sequences of the mature proteins provided on http://au.expasy.org/tools.

Species	Primary accession number[a]	Amino acid residues	Molecular mass (Da)	pI	GRAVY score[b]	Disulphide bridges	Sulphydryl groups
Equine	O77811	689	75420.4	8.32	-0.376	17	0
Bovine	P24627	689	76143.9	8.67	-0.350	16[c]	2
Human	P02788	691	76165.2	8.47	-0.415	16	0

[a] Primary accession number for the protein in SWISS-PROT database
[b] Grand average hydropathy (GRAVY) score calculated using the scale of Kyte & Doolittle (1982)
[c] Estimated from structural similarity with human lactoferrin.

4.1.4 Lysozyme

Equine milk contains approximately 0.8 - 1.1 g. kg^{-1} lysozyme (Lyz) (Jauregui-Adell, 1975; Miranda, Mahé, Leroux, & Martin, 2004) (Table 4). Human milk contains ~ 0.3 g. kg^{-1} (Lyz), slightly lower than the level in colostrum (Montagne, Cuillière, Molé, Béné, & Faure, 1998) but bovine milk contains only ~70 - 130 μg. kg^{-1} (Chandan, Shahani & Holly, 1964; El-Agamy, Ruppanner, Ismail, Champagne, & Assaf, 1996). The concentration of Lyz in human milk increases strongly after the second month of lactation, suggesting that Lyz and Lf play major roles in fighting infection in breast-fed infants during late lactation, and protect the mammary gland (Montagne *et al*., 1998). Equine milk Lyz is more stable to denaturation than human Lyz during pasteurization at 62^{o}C for 30 min, but at 71^{o}C for 2 min or 82^{o}C for 15 s, the inactivation of both were similar (Jauregui-Adell, 1975). It has been suggested, but research is scarce, that while the composition of breast milk varies widely between well-nourished and poorly-nourished mothers, the amount of Lyz is conserved.

Equine milk Lyz binds calcium, which increases the stability and activity of the enzyme (Nitta, Tsuge, Sugai, & Shimazaki, 1987). The binding of a Ca^{2+} by Lyz is considered to be an evolutionary linkage between non-Ca^{2+}-binding lysozymes and α-La (Tada *et al*., 2002; Chowdbury, Fairman, Bi, Rigotti & Raleigh, 2004). The conformation of the calcium-binding loop of equine Lyz is similar to that of α-la (Tsuge *et al*., 1992; Tada *et al*., 2002) and both equine Lyz and α-la form stable, partially folded, "molten globule" states under various denaturing conditions (Koshiba, Kobashigawa, Demura, & Nitta, 2001) with that of equine Lyz being considerably more stable than α-la (Lyster, 1992; Morozova-Roche, 2007). However, the molten state of canine Lyz is significantly more stable than that of equine Lyz (Koshiba *et al*., 2000; Spencer *et al*., 1999). Equine milk Lyz is very resistant to acid (Jauregui-Adell, 1975) and protease digestion (Kuroki, Taniyama, Seko, Nakamura, & Kikuchi, 1989), and may reach the gut relatively intact.

4.1.5. Immunoglobulins

Three classes of immunoglobulins, which form part of the body's natural defense against infection, are commonly found in milk, immunoglobulin G (IgG), A (IgA) and M (IgM); IgG is often sub-divided into two subclasses, IgG_1 and IgG_2 (Hurley, 2003; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007). All monomeric Igs consist of a similar basic structure of four polypeptides, two heavy chains and two light chains, linked by disulphide bridges, yielding a subunit with a molecular mass of ~160 kDa. IgG consists of one subunit, while IgA and IgM consist of two or five subunits, with a molecular mass of ~400 or ~1000 kDa, respectively. The relative proportions of the Igs in milk differ considerably between species (Table 4). IgG is the principal immunoglobulin in equine colostrum, but IgA is the principal form in equine milk. In bovine milk and colostrum, IgG is the principal immunoglobulin, while IgA is the predominant immunoglobulin in human colostrum and milk. In some species, e.g., humans, maternal IgG is transferred to the foetus *in utero* and the intestines are relatively impermeable to proteins in a full-term newborn. In contrast, in ruminants and equids, IgG is not transferred *in utero* and the newborn depends on colostrum to supply IgG, so that within 2 to 3 days, the serum level of IgG is similar to adult levels, after which the intestinal tract 'closes' and further significant passage of proteins is prevented (Widdowson, 1984).

4.2. Denaturation of whey proteins

Whey proteins are susceptible to heat-induced denaturation. The thermal stability of equine Lf and BSA is comparable to that of their bovine counterparts but equine β-Lg and α-La are more heat stable than corresponding bovine proteins (Bonomi, Iametti, Pagliarini, & Solaroli, 1994). Equine β-Lg is reported to be more thermally stable than equine α-La (Civardi, Curadi, Orlandi, Cattaneo, & Giangiacomo, 2007). For bovine milk, the definition of thermal stability based on the assay method may give contradictory results; the α-La of bovine milk is the first protein to unfold at high temperature but the last to aggregate; therefore if thermal denaturation is assessed by differential scanning calorimetry, bovine α-La will appear less stable to heat compared to bovine β-Llg (Rüegg, Moor & Blanc, 1977; De Wit & Klarenbeek,

1984). If the thermal denaturation of bovine whey proteins is assessed by HPLC or SDS-PAGE of the pH 4.5 insoluble whey proteins the reverse is found (Donovan & Mulvihill, 1987). The high thermal stability of equine β-Lg may be related to its lack of a sulphydryl group. Thermal denaturation of bovine β-Lg is a two-stage process, unfolding of the polypeptide chain and exposure of the sulphydryl group, followed by self-association or with other proteins *via* sulphydryl-disulphide interchange (Sawyer, 2003). Owing to the lack of a sulphydryl group, equine β-Lg cannot undergo sulphydryl-disulphide interchange reactions. Denaturation of α-La is a result of complex formation with β-Lg *via* sulphydryl-disulphide interchange and its higher thermal stability may therefore be a result of differences in its environment, rather than its molecular structure. Equine α-La and β-Lg are also less susceptible to high pressure-induced denaturation than their bovine counterparts (Uniacke-Lowe & Huppertz, unpublished data).

4.3. Caseins

Caseins are primarily a source of amino acids, calcium, phosphate and bioactive peptides for neonates (Shekar *et al*., 2006). The lower casein concentration in mature equine milk (~55% of total protein) compared to bovine milk (~ 80% of total protein) has many implications which will be discussed below. The traditional method for separating caseins from whey proteins is isoelectric precipitation of the caseins at pH ~4.6. The casein fraction of most milk types consists of four gene products: α_{s1}-, α_{s2}-, β- and κ-caseins, of which the first three are calcium-sensitive. All caseins display a distinct lack of secondary structure, which led Holt and Sawyer (1993) to classify them as rheomorphic proteins. The lack of secondary structure may be attributed, at least partially, to the relatively high level of proline residues in casein. As a result, caseins do not denature or associate on heating (Paulson & Dejmek, 1990). The biological function of the caseins lies in their ability to form macromolecular structures, casein micelles, which transfer large amounts of calcium to the neonate with a minimal risk of pathological calcification of the mammary gland. The following sections will discuss the individual caseins separately and focus on their interactions to form casein micelles and the colloidal stability thereof.

4.3.1. α_{s1}-Casein

The amino acid sequence of equine α_{s1}-casein, derived from its cDNA sequence by Lenasi, Rogelj, and Dovc (2003), is presented in Fig. 4. The protein contains 205 amino acids and has a molecular mass 24,614.4 Da prior to post-translational modification, as such, it is considerably larger than its bovine or human counterpart (Table 8). Two smaller isoforms of α_{s1}-casein have been identified in equine milk, which probably result from the skipping of exons during transcription (Miranda *et al*., 2004). Equine α_{s1}-casein contains six potential phosphorylation sites (Lenasi *et al*., 2003; Fig. 4), five of which are in very close proximity (Ser_{75}, Ser_{77}, Ser_{79}, Ser_{80}, Ser_{81}) and can thus form a phosphorylation centre, which is important in the structure of casein micelles. Matéos *et al*. (2009a) determined the different phosphorylation levels of the native isoforms of equine α_{s1}-casein and identified 36 different isoforms with several phosphate groups ranging from two to six or eight which, like for equine β-casein, present a complex pattern on 1D and 2D electrophoresis. Bovine α_{s1}-casein contains eight or nine phosphorylation sites (Swaisgood, 2003), which form two phosphorylation centres (De Kruif & Holt, 2003). Equine α_{s1}-casein appears to have three domains characterized by a high hydropathy value, i.e., around residues 25-30, 95-105 and 150-205 and therefore probably displays association properties similar to those of bovine α_{s1}-casein which also contains three distinct hydrophobic regions. Furthermore, equine α_{s1}-casein contains two regions with very low hydropathy, i.e., around residues 45-55 and 125-135, and therefore are expected to behave hydrophilically. Human α_{s1}-casein does not appear to have distinct hydrophobic regions on its N-terminus. Overall, equine and human α_{s1}-casein have comparable GRAVY scores, which are lower than that of bovine α_{s1}-casein (Table 8), indicating an overall higher hydrophobicity for the latter.

1 **10** **20**
Arg- Pro- Lys- Leu- Pro- His- Arg- Gln- Pro- Glu- Ile- Ile- Gln- Asn- Glu- Gln- Asp- Ser- Arg- Glu-
21 **30** **40**
Lys- Val- Leu- Lys- Glu- Arg- Lys- Phe- Pro- Ser- Phe- Ala- Leu- Glu- Tyr- Ile- Asn- Glu- Leu- Asn-
41 **50** **60**
Arg- Gln- Arg- Glu- Leu- Leu- Lys- Glu- Lys- Gln- Lys- Asp- Glu- His- Lys- Asp- Thr- Ser- Asn- Glu-
61 **70** **80**
Ser- Thr- Glu- Glu- Tyr- Leu- Ile- Glu- Asp- Pro- Glu- Gln- Gln- Glu- Ser- Ser- Ser- Thr- Ser- Ser-
81 **90** **100**
Ser- Glu- Glu- Val- Val- Pro- Ile- Asn- Thr- Glu- Gln- Lys- Arg- Ile- Pro- Arg- Glu- Asp- Met- Leu-
101 **110** **120**
Tyr- Gln- His- Thr- Leu- Glu- Gln- Leu- Arg- Arg- Leu- Ser- Lys- Tyr- Asn- Gln- Leu- Gln- Leu- Gln-
121 **130** **140**
Ala- Ile- His- Ala- Gln- Glu- Gln- Leu- Ile- Arg- Met- Lys- Glu- Asn- Ser- Gln- Arg- Lys- Pro- Met-
141 **150** **160**
Arg- Val- Val- Asn- Gln- Glu- Gln- Ala- Tyr- Phe- Tyr- Leu- Glu- Pro- Phe- Gln- Pro- Ser- Tyr- Gln-
161 **170** **180**
Leu- Asp- Val- Tyr- Pro- Tyr- Ala- Ala- Trp- Phe- His- Pro- Ala- Gln- Ile- Met- Gln- His- Val- Ala-
181 **190** **200**
Tyr- Ser- Pro- Phe- His- Asp- Thr- Ala- Lys- Leu- Ile- Ala- Ser- Glu- Asn- Ser- Glu- Lys- Tyr- Asp-
201 **210**
Ile- Ile- Pro- Glu- Trp-

Figure 4. Amino acid sequence of equine α_{s1}-casein (from Lenasi, Rogelj, & Dovc, 2003). Potential phosphorylation sites are shaded.

Table 8. Properties of equine, bovine and human α_{s1}-, β- and κ-caseins

Protein	Species	Primary accession number [a]	Amino acid residues	MW (Da)	pI	GRAVY [b]	Cysteine residues
α_{s1}-casein	Equine	-	205	24,614.4	5.47	-1.127	0
	Bovine	P02662	199	22,974.8	4.99	-0.704	0
	Human	P47710	170	20,089.4	5.17	-1.013	3
β-casein	Equine	Q9GKK3	226	25,511.4	5.78	-0.415	0
	Bovine	P02666	209	23,583.2	5.13	-0.355	0
	Human	P05814	211	23,857.8	5.33	-0.289	0
κ-casein	Equine	P82187	165	18,844.7	8.03	-0.313	2
	Bovine	P02668	169	18,974.4	5.93	-0.557	2
	Human	P07498	162	18,162.6	8.68	-0.528	1

[a] Primary accession number for the protein in SWISS-PROT database
[b] Grand average hydropathy (GRAVY) score calculated using the scale of Kyte &Doolittle (1982)

Prior to the mid-1990's it was generally accepted that human milk contained mainly β- and κ-caseins with little or no α_s-casein (Kunz & Lönnerdal, 1990). A minor casein component has since been identified and is considered to be the human equivalent of α_{s1}-casein although this identification highlights several inconsistencies when compared with the equivalent casein in other species. Uniquely, this human α_{s1}-casein appears to contain at least two cysteine residues and exists as a multimer in complex with κ-casein (Cavaletto, Cantisani, Gluffrida, Napolitano & Conti, 1994; Rasmussen, Due & Petersen, 1995). Johnsen, Rasmussen, Petersen, and Berglund (1995) identified three cysteine residues in human α_{s1}-casein which explains α_{s1}-κ-casein complex formation. Martin, Brignon, Furet, and Leroux (1996) provided definitive evidence for the presence of a functional α_{s1}-casein locus in the human genome which is expressed in the mammary gland during lactation while Sørensen, Møller, Vinther, Petersen, and Rasmussen (2003) reported the phosphorylation pattern of human α_{s1}-casein. In bovine milk, α_{s1}- casein is a major structural component of the casein micelle and plays a functional role in curd formation (Walstra & Jenness, 1984). The relatively low level of α_{s1}-casein in equine compared to bovine milk (Table 4) may be significant and, coupled with the low protein content, may be responsible for the soft curd produced in the infant stomach and the foal (Dr. Ursula Fogarty, National Equine Centre, Ireland – personal communication). Goat milk lacking α_{s1}-casein has poor coagulation properties compared to milk containing α_{s1}-casein (Clark & Sherbon, 2000).

4.3.2. α_{s2}-Casein

The complete amino acid sequence of equine α_{s2}-casein is unknown, but Ochirkhuyag, Chobert, Dalgalarrondo, and Haertlé (2000) published the sequence of the N-terminal 15 amino acid residues (Lys-His-Lys-Met-Glu-His-Phe-Ala-Pro-???-Tyr-???-Gln-Val-Leu). Only five of these amino acids (Lys-His-Asn-Met-Glu-His-Arg-Ser-Ser-Ser-Glu-Glu) were confirmed by Miranda *et al*. (2004). Isoelectric focusing showed two major bands for equine α_{s2}-casein, with isoelectric points in the pH-range 4.3-5.1 (Ochirkhuyag *et al*., 2000). Bovine α_{s2}-casein is the most highly phosphorylated casein, usually containing eleven phosphorylated serine residues,

with lesser amounts containing ten, twelve or thirteen phosphate groups (Swaisgood, 2003). There are no reports on the presence of α_{s2}-casein in human milk.

4.3.3. β-Casein

The amino acid sequence of equine β-casein, derived from the cDNA, reported by Lenasi *et al*. (2003), and revised by Girardet, Miclo, Florent, Mollé, and Gaillard (2006) by the insertion of eight amino acids (Glu_{27} to Lys_{34}), is shown in Fig. 5. The theoretical molecular mass of this 226 amino acid polypeptide is 25,511.4 Da (Table 8). Bovine and human β-casein contain 209 and 211 amino acid residues, respectively (Table 8). Two smaller variants of equine β-casein, which probably result from casual exon-skipping during transcription, were reported by Miranda *et al*. (2004). The 28 C-terminal amino acids contain seven potential phosphorylation sites (Ser_9, Ser_{15}, Ser_{18}, Ser_{23}, Ser_{24}, Ser_{25}, Ser_{28}) and multiple-phosphorylated isoforms of equine β-casein, containing three to seven phosphoserine residues, have been reported, with an isoelectric point varying from pH 4.74 to 5.30 (Girardet *et al*., 2006; Matéos *et al*., 2009b). Bovine β-casein, which contains four or five phosphorylated serine residues, has an isoelectric point of 5.0-5.5 (Swaisgood, 2003). Human β-casein has up to six phosphorylation levels, i.e., 0, 1, 2, 3, 4 or 5 phosphorylated serine residues (Sood & Slattery, 2000). Equine, bovine and human β-casein have a very hydrophilic N-terminus, followed by a relatively random hydropathy distribution in the rest of the protein, leading to an amphiphilic protein with a hydrophilic N-terminus and a hydrophobic C-terminus. In equine sodium caseinate, the Lys_{47}-Ile_{48} bond of equine β-casein is readily hydrolysed by bovine plasmin whereas no cleavage of a corresponding bond, Lys_{48}-Ile_{49}, in bovine β-casein, occurs (Egito *et al*., 2003). In bovine β-casein, Lys_{28}-Lys_{29} is readily cleaved by plasmin but the equivalent, Lys_{28}-Leu_{29}, in equine β-casein, is insensitive (Egito *et al*., 2002). Other plasmin cleavage sites in equine β-casein are Lys_{103}-Arg_{104}, Arg_{104}-Lys_{105} and Lys_{105}-Val_{196} (Egito *et al*., 2002). Equine β-casein is readily hydrolysed by chymosin at Leu_{190}-Tyr_{191} (Egito *et al*., 2001).

1 **10** **20**
Arg- Glu- Lys- Glu- Glu- Leu- Asn- Val- Ser- Ser- Glu- Thr- Val- Glu- Ser- Leu- Ser- Ser- Asn- Glu-
21 **30** **40**
Pro- Asp- Ser- Ser- Ser- Glu- Glu- Ser- Ile- Thr- His- Ile- Asn- Lys- Glu- Lys- Leu- Gln- Lys- Phe-
41 **50** **60**
Lys- His- Gln- Gly- Gln- Gln- Gln- Arg- Gln- Val- Gln- Arg- Gln- Asp- Lys- Ile- Ser- Arg- Phe- Val-
61 **70** **80**
Gln- Pro- Gln- Pro- Val- Val- Tyr- Pro- Tyr- Ala- Glu- Pro- Val- Pro- Tyr- Ala- Val- Val- Pro- Gln-
81 **90** **100**
Ser- Ile- Leu- Pro- Leu- Ala Gln- Pro- Pro- Ile- Leu- Pro- Phe- Leu- Gln- Pro- Glu- Ile- Met- Glu-
101 **110** **120**
Val- Ser- Gln- Ala- Lys- Glu- Thr- Ile- Leu- Pro- Lys- Arg- Lys- Val- Met- Pro- Phe- Leu- Lys- Ser-
121 **130** **140**
Pro- Ile- Val- Pro- Phe- Ser- Glu- Arg- Gln- Ile- Leu- Asn- Pro- Thr- **Asn-** Gly- Glu- Asn- Leu- Arg-
141 **150** **160**
Leu- Pro- Val- His- Leu- Ile- Gln- Pro- Phe- Met- His- Gln- Val- Pro- Gln- Ser- Leu- Leu- Gln- Thr-
161 **170** **180**
Leu- Met- Leu- Pro- Ser- Gln- Pro- Val Leu- Ser- Pro- Pro- Gln- Ser- Lys- Val- Ala- Pro- Phe- Pro-
181 **190** **200**
Gln- Pro- Val- Val- Pro- Tyr- Pro- Gln- Arg- Asp- Thr- Pro- Val- Gln- Ala- Phe- Leu- Leu- Tyr- Gln-
201 **210** **220**
Asp- Pro- Arg- Leu- Gly- Pro- Thr- Gly- Glu- Leu- Asp- Pro- Ala- Thr- Gln- Pro- Ile- Val- Ala- Val-
221 **226**
His- Asn- Pro- Val- Ile- Val

Figure 5. Amino acid sequence of equine β-casein (from Lenasi *et al*., 2003; Girardet, Miclo, Florent, Mollé, & Gaillard , 2006). Potential phosphorylation sites are shown grey; the plasmin-sensitive bond is underlined and the Asn residue susceptible to deamidation is in bold.

Equine β-casein and equine α-la undergo spontaneous deamidation under physiological conditions at Asn_{135}-Gly_{136} and Asn_{45}-Gly_{46}, respectively (Girardet *et al*., 2004; Girardet *et al*, 2006) which has been reported also for canine milk Lyz (Nonaka *et al*., 2008) and human Lf (Belizy *et al*., 2001) but not, to our knowledge, for bovine or human β-casein. Recent research has demonstrated that temperature may be an important factor controlling the spontaneous deamidation process and at 10^{o}C the phenomenon is strongly reduced (Matéos *et al*., 2009). Spontaneous deamidation represents an important modification of equine milk proteins under certain conditions where bovine milk proteins, which do not contain a potential site for deamidation, remain unaffected. Equine Lf also contains the Asn-Gly sequence and may be susceptible to spontaneous deamidation (Girardet *et al*., 2006).

Unique to equine milk and apparently absent from the milk of other species, including ruminants, is a low-MW multi-phosphorylated β-casein variant which accounts for 4% of the total casein (Miclo *et al*., 2007). This short protein (94 amino acid residues) is the result of a large deletion (residues 50-181) from full-length equine β-casein. No spontaneous deamidation of this low MW form of β-casein has been found (Miclo *et al*., 2007).

4.3.4. κ-Casein

The presence of κ-casein in equine milk was an issue of debate for several years, with several authors (Visser, Jenness & Mullin, 1982; Ono, Kohno, Odagiri, & Takagi, 1989; Ochirkhuyag *et al*., 2000) reporting its absence. However, other studies (Kotts & Jenness, 1976; Malacarne, Summer, Formaggioni, & Mariani, 2000; Iametti, Tedeschi, Oungre, & Bonomi, 2001; Egito *et al*., 2001) showed its presence, albeit at a low concentration. The primary structure of equine κ-casein has been derived (Fig. 8) (Iametti *et al*., 2001; Lenasi *et al*., 2003; Miranda *et al*., 2004); it contains 165 amino acids residues, i.e., four less than bovine κ-casein but three more than human κ-casein (Table 8). The MW of equine κ-casein, prior to post-translational modification, is 18,844.7 Da. Equine and human κ-casein have a considerably higher isoelectric pH than bovine κ-casein (Table 8), and they have a net positive charge at physiological pH, whereas bovine κ-casein has a net negative

charge. The GRAVY score of bovine κ-casein is considerably lower than that of equine κ-casein (Table 8), indicating equine κ-casein is less hydrophilic than its bovine counterpart. Bovine κ-casein is characterized by a hydrophilic C-terminus, which is very important for the stabilization of bovine casein micelles, but a comparison of the hydropathy distribution of bovine and equine κ-caseins indicates that the C-terminus of equine κ-casein is far less hydrophilic, particularly as a result of the absence of a strong hydrophilic region at residues 110-120. Equine κ-casein appears to be more like human than bovine κ-casein in terms of the distribution of hydropathy along the polypeptide chain.

4.3.5. Glycosylation of κ-Casein

κ-Casein is the only glycosylated member of the casein family and exhibits micro-heterogeneity due to the level of glycosylation (Saito & Itoh, 1992). Oligosaccharides consisting of N-acetylneuraminic acid (NANA), galactose and N-acetylgalactosamine are attached to *κ*-casein *via 0*-glycosidic linkages to threonine residues in the C-terminal portion of the molecule (glycomacropeptide, GMP, region). Although no direct information is available, lectin-binding studies indicate that equine κ-casein is glycosylated (Iametti *et al*., 2001), possibly at residues Thr_{123}, Thr_{127}, Thr_{131}, Thr_{149} and Thr_{153} (Fig. 6; Lenasi *et al*., 2003) [these glycosylation sites are not fully in agreement with those proposed by Egito *et al*., 2001]. About two-thirds of bovine κ-casein molecules are glycosylated at one of six threonyl residues, i.e., Thr_{121}, Thr_{131}, Thr_{133}, Thr_{135}, Thr_{136} [only in bovine κ-casein variant A] or Thr_{142} (Pisano, Packer, Redmond, Williams, & Gooley, 1994); Ser_{141} is also a potential glycosylation site (Kanamori, Doi, Ideno, & Ibuki, 1981). Human κ-casein has seven glycosylation sites, Thr_{113}, Thr_{123}, Thr_{128}, Thr_{131}, Thr_{137}, Thr_{147} and Thr_{149} (Fiat, Jollès, Aubert, Loucheux-Lefebvre, & Jollès, 1980). To date, no non-glycosylated

κ-casein has been identified in equine milk (Martuzzi & Doreau, 2006).

κ-Casein is located mainly on the surface of the casein micelles and is responsible for their stability (Walstra, 1990). The presence of a glycan moiety in the C-terminal region of κ-casein enhances its ability to stabilize the micelle, by electrostatic

repulsion, and may increase the resistance by the protein to proteolytic enzymes and high temperatures (Minkiewicz, Dziuba, & Muzińska, 1993; Dziuba & Minkiewicz, 1996). Biologically, NANA residues have antibacterial properties and act as a bifidogenic factor (Dziuba & Minkievicz, 1996). κ-Casein is thought to play a major role in preventing adhesion of *Helicobacter pylori* to human gastric mucosa (Strömqvist *et al*., 1995). It is likely that heavily glycosylated κ-casein provides protection due to its carbohydrate content and breast-feeding infants is thought to provide some protection, especially as *H. pylori* infection is occurring at an increasingly younger age (Lönnerdal, 2003).

4.3.6. Hydrolysis of κ-casein

The hydrolysis of bovine κ-casein by chymosin at Phe_{105}-Met_{106} leads to the production of the hydrophobic C-terminal para-κ-casein and the hydrophilic N-terminal caseinomacropeptide (CMP) (Walstra & Jenness, 1984). Calf chymosin hydrolyses the Phe_{97}-Ile_{98} bond of equine κ-casein (Egito *et al*., 2001) and slowly hydrolyses the Phe_{105}-Ile_{106} bond of human κ-casein (Plowman, Creamer, Liddell, & Cross, 1999). However, as summarized in Table 9, the CMPs released from equine or human κ-casein are considerably less hydrophilic than bovine CMP. The sequence 97-116 of κ-casein is highly conserved across species, suggesting that the limited proteolysis of κ-casein and subsequent coagulation of milk are of major biological significance (Mercier, Chobert, & Addeo, 1976)

Table 9. Properties of equine, bovine and human para-κ-casein and caseinomacropeptide.

Protein	Species	Residues	Amino acid residues	Mw (Da)	pI	GRAVY [a]
Para-κ-casein	Equine	1-97	97	11,693.3	8.96	-0.675
	Bovine	1-105	105	12,285.0	9.33	-0.617
	Human	1-105	97	11,456.9	9.63	-1.004
CMP	Equine	98-165	68	7,169.3	4.72	0.203
	Bovine	106-169	63	6,707.4	4.04	-0.370
	Human	106-162	65	6,723.7	4.24	0.182

[a] Grand average hydropathy (GRAVY) score calculated using the scale of Kyte &Doolittle (1982)

A grouping system for mammals based on κ-casein structure and the site of cleavage by chymosin has been suggested (Mercier *et al*., 1976; Nakhasi, Grantham, & Gullino, 1984). Group I species (cow, goat, sheep, buffalo) have a higher content of dicarboxylic amino acids and low hydrophobicity and carbohydrate content and κ-casein is cleaved at Phe_{105}-Met_{106}, while Group II species (human, mouse, pig, rat) have a high proline content, less dicarboxylic amino acids and a much higher hydrophobicity and carbohydrate content and are cleaved at Phe_{97}-Ile_{98} or Phe_{105}-Leu_{106}. Marsupial κ-casein appears to form a separate group, with a putative chymosin cleavage site of Phe-Ala, which is different from that in eutherian mammals (Stasiuk, Summers, & Demmer, 2000). Cleavage of equine milk at Phe_{97}-$Ile_{98,}$ as well as other characteristics of its κ-casein, place the horse in Group II. The divergence between species into groups I and II could account for differences in the clotting mechanisms of ruminant and non-ruminant milk (Herskovitis, 1966). In addition to the differences in cleavage site, the grouping system also divides species based on the number of *0*-glycosylation sites. As equine and human κ-caseins are considerably more highly glycosylated than bovine κ-casein and non-glycosylated κ-casein has not been found in equine milk (Egito *et al*., 2001), equine and human κ-casein belong to the same group. The level of glycosylation does not affect micelle structure but it does affect the susceptibility of κ-casein to hydrolysis by chymosin, with susceptibility decreasing as the level of glycosylation increases (Doi, Ibuki, & Kanamori, 1979; Addeo, Martin, & Ribadeau-Dumas, 1984; Van Hooydonk, Olieman, & Hagedoorn, 1984; Vreeman, Visser, Slangen, & van Riel, 1984; Zbikowska, Dziuba, Jaworska, & Zaborniak, 1992). Therefore, equine milk probably has a different chymosin-induced clotting mechanism than bovine milk.

5. Equine casein micelles

In the milk of all species studied in sufficient detail, the caseins exist predominantly as micelles, which are hydrated spherical structures with dimensions in the sub-micron region. The dry matter of casein micelles consists predominantly (>90%) of proteins, with small amounts of inorganic matter, collectively referred to as micellar

calcium phosphate (MCP). The structure and sub-structure of bovine casein micelles has been studied in detail and reviews include Holt and Horne, 1996; Horne, 1998, 2006; De Kruif and Holt, 2003; Phadungath, 2005; Farrell, Malin, Brown, and Qi, 2006; Qi, 2007; Fox and Brodkorb, 2008. The casein micelles are best described as sterically stabilized association colloids (De Kruif & Holt, 2003). MCP exists as nanometer-sized clusters of amorphous calcium phosphate which are stabilized by a shell of caseins (α_{s1}-, α_{s2}- and β-caseins). This core-and-shell structure is commonly referred to as a nanocluster. Such nanoclusters may associate to form particles of colloidal dimensions, either *via* cross-linking through caseins which contain more than one phosphorylation centre, or through solvent-mediated association of the proteins of the shell of the nanoclusters. Growth of casein micelles is terminated by the solvent-mediated adsorption of κ-casein onto the micellar surface. The hydrophilic C-terminal region of κ-casein or 'brush' protrudes from the surface of the micelles and sterically stabilizes them against aggregation (De Kruif & Zhulina, 1996).

Equine casein micelles have an average diameter of ~ 255 nm (Welsch, Buchheim, Schumacher, Schinko, & Patton 1988; Buchheim, Lund & Scholtissek 1989) while bovine casein micelles have an average diameter of ~180 nm (Table 4). Human casein micelles are considerably smaller, with an average diameter of 60-80 nm (Table 4).

Electron microscopy shows a 'spongy' appearance for equine and bovine micelles, although bovine micelles appear more ordered and equine micelles 'looser'; human micelles are considerably looser than equine micelles (Jasińska & Jaworska, 1991). Such a loose open structure may affect the susceptibility to hydrolysis by pepsin. Jasińska and Jaworska (1991) reported that human micelles were much more susceptible to peptic hydrolysis than either equine or bovine micelles.

The sub-structure of equine casein micelles has not been studied in detail but some information may be derived from comparison with bovine milk. Equine milk contains ~10.1 mmol L^{-1} micellar calcium and ~2.6 mmol L^{-1} micellar inorganic phosphorus, compared to ~20.2 and 9.7 mmol L^{-1} in bovine milk (Holt & Jenness, 1984). Considering that equine milk contains <0.5 mmol casein L^{-1}, this suggests a micellar calcium: casein molar ratio of >20:1, which far exceeds the calcium-binding

capacity of equine casein. Hence, it may be assumed that like bovine micelles, equine micelles contain nanoclusters of calcium phosphate. Both equine α_{s1}-casein (residues 75-81; Fig. 4) and β-casein (residues 23-28; Fig. 6) contain a phosphorylation centre, which is required for the formation of nanoclusters (De Kruif & Holt, 2003); furthermore, both proteins also contain distinct hydrophobic regions through which solvent-mediated protein-protein interactions may occur. Equine α_{s2}-casein may have similar properties, pending further characterization. The ratio of micellar calcium: micellar inorganic phosphorus is 2.0 in equine milk, but ~3.9 in bovine milk (Holt & Jenness, 1984) and might indicate that either a smaller proportion of micellar calcium is incorporated into nanoclusters in equine milk, or that equine nanoclusters contain a higher proportion of casein-bound phosphate. The latter would imply smaller nanoclusters, since the casein-bound phosphate can participate only at the surface of the core. However, unlike bovine κ-casein, equine κ-casein does not have a distinctly hydrophilic C-terminal domain; thus, it is unclear if this part of the protein is capable of protruding from the micellar surface to sterically stabilize the micelle. Ochirkhuyag *et al*. (2000) and Doreau and Martin-Rosset (2002) concluded that the steric stabilization of equine casein micelles by κ-casein may be aided by non-phosphorlated β-casein on the surface of the micelle, thus compensating for the low κ-casein content. A similar conclusion was reported by Dev, Satish, Sood, DeWind, and Slattery, (1994) for the stabilization of human casein micelles which also have a very low content of κ-casein. Further research is required to elucidate the structure of the equine casein micelle as destabilization of the micelles is the basis for the successful conversion of milk into a range of dairy products, e.g., cheese or yoghurt.

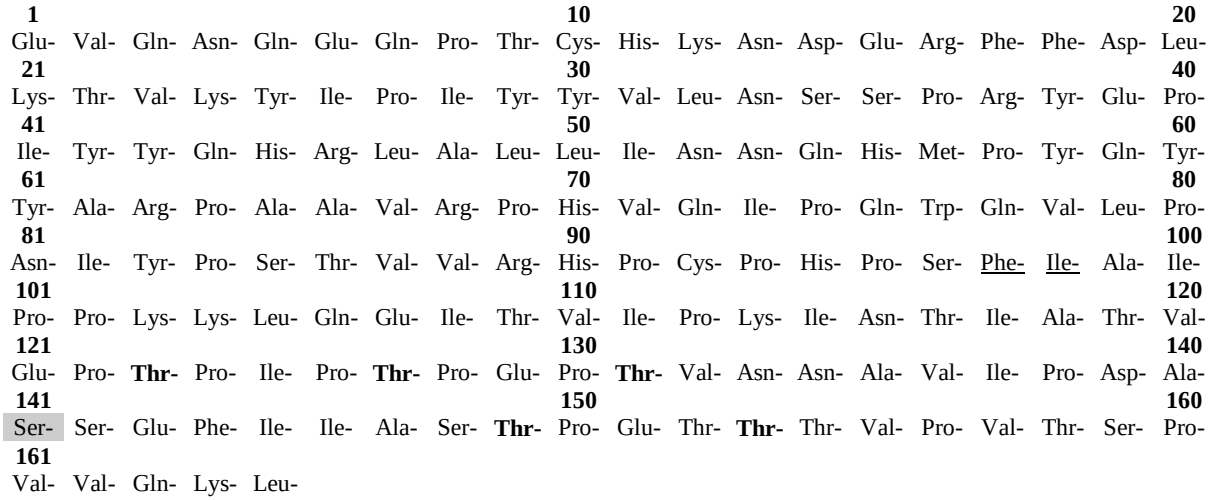

Figure 6. Amino acid sequence of equine κ-casein. Potential phosphorylation sites are shaded grey. Potential glycosylation sites are printed in bold font (from Lenasi *et al*., 2003). The chymosin-sensitive bond is underlined.

5.1. Colloidal stability of casein micelles

Coagulation of milk occurs when colloidal stability is destroyed and may be desirable or undesirable. Coagulation is desirable in the manufacture of yoghurt and cheese. Coagulation of milk is also important from a nutritional point of view, as clotting of the caseins in the stomach, and the type and structure of the resultant coagulum, strongly affects digestibility. In contrast, heat-induced coagulation of casein micelles, which can occur at a temperature >120ºC, e.g., during the retort sterilization of liquid products for infant or clinical nutrition, is undesirable. In this section, common types of micellar instability will be described.

As outlined above, bovine casein micelles are sterically stabilized by a brush of predominantly κ-casein, which protrudes from the micelle surface. Coagulation of casein micelles can occur only following collapse of the brush, which occurs on acidification of milk, i.e., in the manufacture of yoghurt or on removal of the brush which occurs on rennet-induced coagulation of milk. The combined process of enzyme and acid-induced coagulation is likely to contribute to coagulation of casein micelles in the stomach.

5.2. Enzymatic coagulation of equine milk

Enzymatic coagulation of milk is the first step in the manufacture of most cheese varieties and also plays a role in the flocculation of casein micelles in the stomach. For cheese manufacture, the process involves the addition of a milk-clotting enzyme, e.g., chymosin, to the milk, followed by incubation at a temperature $\geq$30ºC. During incubation of bovine milk with rennet, chymosin hydrolyses the Phe_{105}-Met_{106} bond in κ-casein, leading to the formation of two fragments, the hydrophobic N-terminal fragment, f1-105, which remains attached to the casein micelles and is referred to as para-κ-casein, and the hydrophilic C-terminal fragment, f106-169, which is released into the milk serum and is referred to as the caseinomacropeptide (CMP). As a result, the micelles lose steric stabilization and become susceptible to aggregation, particularly in the presence of Ca^{2+}. Since equine κ-casein is hydrolysable by chymosin at the Phe_{97}-Ile_{98} bond (Egito *et al*., 2001), albeit slowly (Kotts & Jenness, 1976) and without gel formation (Uniacke-Lowe & Fox, unpublished data), it appears that either the chymosin-sensitive bond of equine κ-casein is located in the

micelle in a manner which renders it inaccessible to chymosin, or the equine casein micelle derives colloidal stability from constituents other than κ-casein. The high degree of glycosylation may also affect the ability of chymosin to hydrolyse equine κ-casein.

5.3. Acid-induced flocculation of equine milk

When bovine milk is acidified to a pH below 5.0, flocculation of casein micelles occurs, leading ultimately to gel formation. This process is the basis of the manufacture of yoghurt, in which acidification is induced by the production of lactic acid by lactic acid bacteria and also occurs at the low pH of the stomach. Equine casein micelles are considerably less susceptible to acid-induced flocculation than bovine casein micelles and equine casein has a lower isoelectric pH (4.2) than bovine casein (pH 4.6) due to the slightly more acidic character of equine β- and κ-caseins (Egito *et al*., 2001; Egito *et al*., 2002). Acidification of equine milk causes a relatively small increase in viscosity (Di Cagno *et al*., 2004) compared to that of acidified bovine milk and, in the case of the former, is probably indicative of micellar flocculation rather than gelation. Differences in acid-induced flocculation between equine and bovine casein micelles may be related to differences in the mechanism by which they are sterically stabilized. Acid-induced flocculation of bovine casein micelles is believed to result from a reduction in the solvency of the κ-casein brush on the micellar surface due to protonation of the negatively charged carboxylic acid groups of Glu and Asp. Elucidation of the mechanism of steric stabilization of equine casein micelles is likely to shed further light on this subject.

5.4. Heat-induced coagulation of equine milk

Heat-induced coagulation of milk occurs when milk is heated sufficiently long at >120ºC. Unconcentrated bovine milk, usually assayed at 140ºC, displays a typical profile, with a heat coagulation time (HCT) maximum (~20 min) at pH ~6.7 and a minimum at pH ~6.9 (O'Connell & Fox, 2003). In contrast, the HCT of unconcentrated equine milk at 140^{o}C, increases with pH, i.e., it has a sigmoidal pH-HCT profile, with a mid-point around pH 6.7. HCT at pH 7 is ~60 min (Uniacke-

Lowe, Huppertz & Fox, unpublished data). Differences in heat stability between equine and bovine milk may be related to differences in steric stabilization of the micelles; in addition heat-induced complexation of β-Lg with κ-casein greatly affects the heat stability of bovine milk (O'Connell & Fox, 2003), but is unlikely to occur in equine milk due to lack of a sulphydryl group in equine β-Lg. Finally, the lower casein concentration in equine milk is also likely to contribute to its higher heat stability.

6. *Non-protein nitrogen of equine milk*

The non protein-nitrogen (NPN) of milk consists primarily of urea, peptides, amino acids and ammonia. NPN constitutes 10 to 15% of the total nitrogen in mature equine milk which is intermediate between the values for human milk and ruminant milk, 25 and 5%, respectively (Hambræus, 1984; Oftedal *et al*., 1983; Atkinson, Schnurr, Donovan, & Lönnerdal, 1989; Walstra, Wouters, & Geurts, 2006). In equine milk, NPN increases from <2% of total nitrogen at parturition to >10% after two weeks (Zicker & Lönnerdal, 1984). The components of the NPN in human milk have been characterized (see Atkinson *et al*., 1989; Atkinson & Lönnerdal, 1995; Carratù, Bongilia, Scalise, Ambruzzi, & Sanzini, 2003), but the NPN of equine milk has not been studied in detail.

6.1 Bioactive peptides

Both caseins and whey proteins are believed to contribute to human health through latent biological activity after enzymatic hydrolysis by digestive enzymes, fermentation with specific starter cultures or enzymatic hydrolysis by enzymes derived from microorganisms or plants (Phelan, Aherne, Fitzgerald, & O'Brien, 2009). Some of the peptides released, termed bioactive peptides, are capable of modulating specific physiological functions: anti-hypertensive, opioid, mineral-binding, anti-bacterial and immunomodulatory activities have been reported for casein-derived bioactive peptides (Abd El-Salam, El-Shibinyand, & Buchheim, 1996; Dziuba & Minkiewicz, 1996; Brody, 2000; Malkoski *et al*., 2001; Baldi *et al*., 2005; Silva & Malcata, 2005; Thomä-Worringer, Sørensen, & López-Fandiño, 2006;

Michaelidou, 2008) and whey-protein derived peptides (Nagaoka, Kanamaru, & Kuzuya, 1991; Mullally, Meisel, & Fitzgerald, 1996; Pellegrini, Dettling, Thomas, & Hunziker, 2001; Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Chatterton, Smithers, Roupas, & Brodkorb, 2006; Yamauchi, Wada, Yamada, Yoshikawa, & Wada, 2006; Hernández-Ledesma, Recio, & Amigo 2008). Detail on bioactive peptides is outside the scope of this article but general reviews include: Shah, 2000; Dziuba and Darewich, 2007; Shahidi and Zhong, 2008; Haque, Chand, and Kapila, 2009 and Phelan *et al*., 2009. Research on the bioactive peptides from equine milk is very limited but it has been reported that peptides derived from equine β-casein may have a positive affect on human health (Doreau & Martin-Rosset, 2002).

6.2. Free amino acids in equine milk

The free amino acid content of equine, bovine and human milk are 1960, 578 and 3020 μML^{-1}, respectively (Rassin, Sturman, & Gaull, 1978; Agostini, Carratù, Boniglia, Riva, & Sanzini, 2000) (Table 10). Glutamine, glutamate, glycine, alanine and serine are the most abundant free amino acids in equine, bovine and human milk while taurine is also exceptionally high in human milk (Rassin *et al.*, 1978; Sarwar, Botting, Davis, Darling, & Pencharz, 1998; Carratù *et al*., 2003). Taurine is as an essential metabolite for the human infant and may be involved in the structure and function of retinal photoreceptors (Agostini *et al*., 2000). Compared to bovine milk, equine milk has an appreciable amount of taurine although it is 10 times less than that of human milk (Table 10). By contrast to total amino acid composition, which is essentially similar between equine, bovine and human milk, free amino acids show a pattern characteristic for each species (Table 10) which may be important for early post-natal development in different animals. Free amino acids are more easily absorbed than protein-derived amino acids and glutamic acid and glutamine, which comprise > 50% of the total free amino acids of human milk, are a source of ketoglutaric acid for the citric acid cycle and also act as neurotransmitters in the brain (Levy, 1998; Agostini *et al*., 2000).

7. Digestibility of equine protein

In vivo digestion of milk proteins is initiated in the stomach by pepsin and, in young animals, by chymosin. Coagulation of milk in the stomach delays the degradation of proteins and allows for their better assimilation by the body. Degradation of casein is slow but extensive and while β-Lg is relatively resistant to gastric proteolysis, α-lactalbumin is readily hydrolysed when the gastric pH is ~ 3.5 (Savalle, Miranda & Pélissier, 1988). The structure of the coagulum formed in the stomach depends on the casein content of ingested milk; high-casein milk yields firm, tough clots. Species that nurse their young at frequent intervals, e.g., horses and humans, produce dilute milk with a casein content <60% of the total protein and the coagulum formed in the stomach is soft and disintegrates quickly (Oftedal, 1980).

The physico-chemical differences between human and bovine caseins result in the formation of different types of curd in the stomach (Hambræus, 1984) and because the protein profile of equine milk is quite similar to that of human milk, equine milk may be more appropriate in human nutrition than bovine milk. Turner (1945) compared the digestibility of equine, human and bovine milk based on the average percentage conversion of acid-insoluble protein to acid-soluble protein during digestion. Equine and human milk have a much lower buffering capacity than bovine milk and, while equine milk is very digestible, it was slightly less so than human milk but significantly better than bovine milk. Turner (1945) concluded that both equine and human milk form soft curds in the stomach which pass through the digestive tract more quickly than bovine milk curd. Kallila *et al*. (1951) also reported that the overall digestibility of equine and human milk (by *in-vitro* experiments) appeared quite similar and both were easier to digest compared to bovine milk. Human milk forms fine, soft flocs in the stomach with an evacuation time of 2 to 2.5 h, whereas bovine milk forms compact hard curds with a digestion time of 3 to 5 h.

Table 10. The free amino acids (μM L^{-1}) of equine, bovine and human milk

Amino acid	Equine[a]	Bovine[a]	Human[b]
Alanine	105	30.0	227.5
Arginine	14.0	10.0	35.4
Aspartic acid	40.0	15.0	183.2
Cystine	2.0	21.0	(56) [a]
Glutamic acid	568.0	117.0	1184.1
Glutamine	485.0	12.0	284.8
Glycine	100.0	88.0	124.6
Histidine	46.0	9.0	7.7
Isoleucine	8.0	3.0	33.4
Leucine	16.0	3.0	55.6
Lysine	26.0	15.0	39.0
Methionine	~0	~0	8.8
Phenylalanine	5.0	3.0	23.6
Proline	1.61	-	64.3
Serine	175	23.0	273.7
Taurine	32.0	13.0	301.1
Threonine	137.0	16.0	97.6
Tyrosine	3.0	0.3	2.5
Valine	45.0	5.0	72.7
Total	~1960	578	3019.7

[a] Rassin *et al*., 1978
[b] Agostini *et al*., 2000

8. Equine milk in human nutrition

The human infant has a much slower growth rate than other animals, except other primates (Emmett & Rogers, 1997), and the concentration of nutrients in human milk reflects this (Table 1). Equine milk, with a composition close to that of human milk, may be a good nutritional source for the neonate when breast milk is unavailable. Bovine milk or milk products are used traditionally as substitutes for human milk in infant nutrition. However, bovine milk is considerably different from human milk in terms of its macronutrients and micronutrients and the absorption rates of vitamins and minerals from the two milks are different, which can be problematic for infants. Bovine milk has lower concentrations of lactose, polyunsaturated fatty acids, free amino acids and vitamins A, D and K than human milk. Lf and Tf, while present in both types of milk, occur in different ratios, which may alter their effectiveness (Emmet & Rogers, 1997).

8.1. Cow milk protein allergy

Cows' milk allergy (CMA) is an IgE-mediated type I allergy, which may be life-threatening, and is defined as a set of immunologically-mediated adverse reactions which occur following the ingestion of milk. CMA is important because bovine milk is the first foreign antigen ingested in large quantities in early infancy (Hill & Hosking, 1996). CMA is the subject of many articles and reviews including: Hill, 1994; Taylor, 1986; Høst, 1988, 1991; Bindslev-Jensen, 1998; Wal, 2002, 2004; El-Agamy, 2007; Apps and Beattie, 2009.

Because β-Lg is absent from human milk, it has commonly been considered to be the most important cow's milk allergen (Goldman *et al*., 1963, Ghosh, Malhotra, & Mathur, 1989) although other whey proteins (Jarvinen, Chatchatee, Bardina, Beyer & Sampson, 2001) and caseins (Savilahti & Kuitunen, 1992; Restani *et al*., 1995) have also been implicated in allergic reactions. The resistance of β-Lg to proteolysis allows the protein to remain intact after digestion with the possibility of it being absorbed across the gut mucosa (Wal, 2002). Ingested β-Lg has been detected in human milk and could be responsible for colic in breast-fed infants and the sensitization of infants, predisposing them to allergies (Jakobsson & Lindberg, 1978;

Kilshaw & Cant, 1984; Stuart, Twiselton, Nicholas, & Hide, 1984; Jakobsson, Lindberg, Benediktsson, & Hansson, 1985; Axelsson, Jakobsson, Lindberg, & Benediksson, 1986).

The choice of substitute for cows' milk in cases of CMA depends on two major factors, i.e., nutritional adequacy and allergenicity; cost and taste must also be taken into account (Businco, 1993). Many soy or hydrolysate (casein-based and more recently whey protein-based) formulae are available for treatment of CMA but they can themselves induce allergic reactions (Eastman, 1989; Hill, Heine, Cameron, Francis, & Bines, 1999; Restani *et al*., 1999). Heat treatment of milk may destroy heat-labile proteins, especially BSA and Igs, and change the antigenic properties of other whey proteins, such as β-Lg and α-la although caseins need severe heat treatment (121^{o}C x 20 min) to reduce sensitizing capacity (Hill, 1994). Enzymatic treatment of milk proteins has resulted in products with unacceptable taste due to bitterness arising from the production of peptides and amino acids and such peptides may, in fact, be allergenic (Schmidt, Meijer, Slangen, & van Beresteijn, 1995; Sélo *et al*. 1999; El-Agamy, 2007).

Many clinical studies have been carried out on the use of the milk of different species in infant nutrition, e.g., goat, sheep (Restani, Beretta, Fiocchi, Ballabio, & Gali, 2002), camel (El-Agamy, 2007), buffalo (El-Agamy, 2007), horse and donkey (Iacono *et al*. 1992; Carroccio, Cavataio, & Iacono, 2000; El-Agamy, Abou-Shloue, & Abdel-Kader, 1997; Businco *et al*., 2000; Monti *et al*., 2007). Results on the benefits of such milk types are conflicting and infants with CMA may suffer allergic reactions to buffalo, goat, sheep, donkey and horse milk proteins due to the presence of positive immunological cross-reaction with their counterparts in cow's milk (El-Agamy, 2007; Businco, 1993; Bellioni-Businco *et al*., 1999). Lara-Villoslada, Olivares, and Xaus (2005) found that the balance between casein and whey proteins may be an important factor in determining the allergenicity of bovine milk proteins in humans and that modification of this balance may reduce the allergenicity of bovine milk. Presumably, equine milk, with a ratio of whey proteins to casein close to that in human milk, would potentially be a good substitute for human milk.

The difference between CMA and lactose intolerance is of particular interest to many people and is an area which causes much confusion. CMA is a food allergy, an

adverse immune reaction to a food protein that is normally harmless to the non-allergic individual. Lactose intolerance is a non-immunological adverse reaction, due to a lack of the enzyme, β-galactosidase, required to digest the predominant sugar, lactose, in milk. Lactose intolerance manifests as abdominal symptoms and chronic diarrhoea after ingestion of milk (Bindslev-Jensen, 1998). Lactose intolerance is not a disease; 70% of the world's population is lactose-intolerant (for reviews: Ingram & Swallow, 2003; Ingram, Mulcare, Ital, Thomas, & Swallow, 2009). Adverse effects of lactose intolerance occur at much higher levels of milk consumption than milk protein allergy. From a positive viewpoint, lactose is thought to have a major affect on bone mineralization during the first few months after birth as it stimulates the intestinal absorption of calcium (Wasserman & Lengemann, 1960). With high lactose content similar to human milk, equine milk would seem to be suitable for infant nutrition, especially as lactose intolerance is uncommon in infants and children under two years of age.

8.2. Cross reactivity of proteins

Cross-reaction occurs when two food proteins have similar amino acid sequences or when the three-dimensional conformation makes two molecules similar in their capacity to bind specific antibodies (Restani *et al*., 2002). Cross reactivity of proteins from different species generally reflects the phylogenetic relations between animal species, e.g., homologous proteins from vertebrates often cross-react. A comprehensive study on the subject by Jenkins, Breiteneder, and Mills (2007) highlights some interesting points, especially concerning the potential allergenicity of caseins from different species. The authors set out to determine how closely a foreign protein had to resemble a human homologue before it actually looses its allergenic affect. A high degree of similarity to human homologues would, presumably, imply that a foreign animal food protein would be much less likely than a protein with little or no similarity to its human homologue to be immunogenic in human subjects. In addition, the study of potential animal allergens must take into account the capability of the human immune system to discriminate between its own proteins and those from another species (i.e., an autoimmune response) which have a high similarity, i.e., how closely does a foreign protein have to resemble a human

homologue before it loses its ability to act as an allergen? (Spitzauer, 1999; Jenkins *et al*., 2007). Table 11 gives the percentage identity of α_{S1}-, α-$_{S2}$ and β-caseins from different species to bovine and human homologues. Known allergens are less than 53% identical to human sequences (Jenkins *et al*., 2007). Natale *et al*. (2004) found that 90% of a group of infants with CMA had serum IgE against bovine α_{S2}-casein, 55% against bovine α_{S1}-casein and only15% against bovine β-casein which is closest to human β-casein. Caprine and ovine milk proteins are more closely related to each other than either is to bovine milk proteins, thus explaining why an individual allergic to goat's cheese may exhibit high IgE cross-reactivity with sheep's milk proteins but could, in fact, tolerate cow's milk and its products (Jenkins *et al*., 2007).

Allergy to equine milk appears to be rare and, to date, only two documented cases have been reported. Fanta and Ebner (1998) reported on the case of an individual who experienced sensitization to horse dander allergen and subsequently produced IgE antibodies on ingestion of equine milk which was prescribed to 'strengthen' her immune system. Horse serum albumin has been identified as an allergen in horse serum and dander, hair and skin have been found to be allergenic (Spitzauer, 1999). IgE antibodies to animal-derived proteins are known to occur in about 2% of the population and in about 40% of atopic individuals making any animal an important source of inhalant allergens (Spitzauer, 1999). Gall, Kalveram, Sick, and Sterry (1996) demonstrated the existence of an IgE-mediated equine milk allergy in one patient, caused by low MW heat-labile proteins, most likely α-la and β-Lg, without cross-reaction to the corresponding whey proteins from bovine milk. Presumably, the above cases are not isolated incidents and as the consumption of equine milk and its products increases, it is likely that further cases will be reported.

Bevilacqua *et al*. (2001) tested the capacity of goat's milk with low and high α_{s1}-casein content to induce milk protein sensitization in guinea pigs and found significantly less sensitization in milk with low α_{s1}-casein. This may represent an important attribute of the low α_{s1}-casein content of equine milk for use in human allergology.

Table 11. Relationships (%) between proteins from different species milks and their human homologues

Casein	Primary Accession Number [a]	% Identity to Closest	
		Bovine Homolog	Human Homolog
α_{S1}-Casein			
Cow	P02662	100	29
Goat	Q8M1H4	88	29
Sheep	P04653	88	28
Horse	Q8SPR1	39	44
Human	P47710	29	100
Camel	O97943	41	36
α_{S2}-Casein			
Cow	P02663	100	16
Goat	P33049	88	17
Sheep	P04654	89	17
Camel	097944	56	11
β-Casein			
Cow	P02666	100	53
Goat	Q712N8	91	54
Sheep	P11839	91	54
Horse	Q9GKK3	56	58
Human	P05814	53	100
Camel	Q9TVD0	66	58

(from Jenkins *et al*., 2007). [a] Primary accession number for the protein in SWISS-PROT database

9. *Products from equine milk*

9.1 Koumiss

Unlike other milk, cheese is not produced from equine milk as no curd is formed on addition of rennet. It forms a weak coagulum under acidic conditions and this is exploited in the production of yoghurt-type products, especially in the Netherlands, where it is generally flavoured with concentrated fruit extract. Koumiss (Kumys), a fermented equine milk product, is widely consumed in Russia, Western Asia (e.g., Kazakhstan), primarily for its therapeutic value. In Mongolia, koumiss, called Airag, is the national drink and distilled koumiss, Arkhi, is also produced (Kanbe, 1992; Ørskov, 1995). Koumiss and kefir belong to the yeast-lactic fermentation group where alcoholic fermentation using yeasts is used in combination with lactic acid fermentation (Tamine & Marshall, 1984). In traditional Koumiss manufacture, part of the previous days batch is used to inoculate fresh mare's milk and fermentation takes place over three to eight hours with the indigenous microbial population which include: *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei*, *Lactococcus lactis* subsp. *lactis*, *Kluyveromyces fragilis* and *Saccharomyces unisporusis* (Litopoulou-Tzanetaki & Tzanetakis, 2000). Koumiss is still manufactured in remote areas of Mongolia by this traditional method but with increased demand elsewhere, it is now produced under carefully controlled and regulated conditions. A starter for the production of Koumiss was patented in 1990 in the former USSR; it consists of *Lactococcus lactis* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and the yeast *Torula* spp. (Surono & Hosono, 2003)

Koumiss contains about 900 g. kg^{-1} moisture, 21 g. kg^{-1} protein (12 g. kg^{-1} casein and 9 g. kg^{-1} whey proteins) , 55 g. kg^{-1} lactose, 12 g. kg^{-1} fat and 3 g. kg^{-1} ash, as well as the end-products of microbial fermentation, i.e., lactic acid, 18 g. kg^{-1}, ethanol, 6 to 25 g. kg^{-1} and CO_2, 5 to 9 g. kg^{-1} (Doreau & Boulot, 1989; Pagliarini, Solaroli, & Peri, 1993; Lozovich, 1995; Litopoulou-Tzanetaki & Tzanetakis, 2000).

Good quality koumiss is produced when lactic and alcoholic fermentations proceed simultaneously so that the products of fermentation occur in definite proportions (Berlin, 1962). During fermentation, 0.7-1.8% lactic acid, 0.6-2.5% ethanol and 0.5-0.9% CO_2, volatile acids and other compounds are formed which are important for aroma and taste. Up to 10% of the equine milk proteins are hydrolysed after 96 h but

the fat remains unchanged (Berlin, 1962; Tamine & Marshall, 1984). Koumiss is a milky, grey, fizzy liquid with a sharp alcohol and acidic taste (Berlin, 1962; Tamine & Marshall, 1984). Products with varying amounts of lactic acid and ethanol are produced and generally 3 categories of koumiss, mild, medium and strong, are known. Koumiss is thought to be more effective than raw equine milk in the treatment of various illnesses due to the additional peptides and bactericidal substances from microbial metabolism (Doreau & Martin-Rosset, 2002). Nowadays, the main interest in fermented foods such as koumiss is their apparent ability to promote functions of the human digestive system in a positive way, i.e., to have a probiotic effect (Sahlin, 1999).

9.2. Processing of equine milk products

As sales of equine milk have increased considerably during recent years, research is now focused on the development of new products or new methods for extending the shelf-life, while maintaining some of the unique components of equine milk. The ability of milk to withstand relatively high processing temperatures is very important from a technological point of view. Whey proteins in equine milk are much more thermo-stable than those of bovine milk, making equine milk less sensitive to thermal processing. Heat treatment at 80^{o}C x 80 s caused only a 10-15% decrease in non-casein nitrogen, with a marked decrease evident only when the temperature was increased above 100^{o}C (Bonomi *et al*., 1994). Lactoferrin and equine BSA appeared to be the most sensitive but were not completely denatured until the temperature reached 130^{o}C. β-Lg and α-La are almost completely denatured at temperatures over 130^{o}C and lysozyme at temperatures greater than 110^{o}C. The latter is in agreement with work by Jauregui-Adell (1975) who found 68% residual lysozyme activity after heating at 82^{o}C for 15 min. The presence of a high level of thermo-stable lysozyme in equine milk may interfere with the microbial activity of starter cultures in the production of fermented products (Jaurequi-Adell, 1975) and thus cause technological problems in the processing of equine milk. Di Cagno *et al*. (2004) heated equine milk to 90^{o}C for 3 min to inactivate lysozyme and, using yoghurt cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptomyces thermophilus*, produced an acceptable fermented product. They found that

fortification of equine milk with Na caseinate (1.5 $g.100^{-1}g$), pectin (0.25 $g.100^{-1}g$) and threonine (0.08 $g.100^{-1}g$) enhanced the rheological and sensory properties of fermented products made from equine milk. The resultant products had good microbiological, rheological and sensory characteristics after 45 days at $4^{o}C$. Fermented unmodified equine milk alone had an unacceptable viscosity and scored very low in comparison to fortified products for appearance, consistency and taste. Addition of sucrose and Na caseinate had a positive effect on the rheological properties of a fermented equine milk product due to strengthening of the protein network.

In health food shops and some pharmacies in Western Europe, equine milk is sold frozen or as capsules of lyophilised milk. Other products from equine milk include frozen or lyophilised colostrum which is used mostly in the high-value horse industry to feed orphaned foals. It is claimed that many of the products relieve metabolic and intestinal problems while having a gut-cleansing effect coupled with 'repair' of intestinal flora. Relief from stomach ulcers, high blood pressure, high cholesterol and liver problems are also reported and equine milk is recommended as an aid in the treatment of cancer patients. The recommended amount of equine milk is 250 mL per day. The use of equine milk in the production of cosmetics is relatively new and includes soaps, creams and moisturisers (Doreau & Martin-Rosset, 2002).

10. Conclusions

The characteristics of equine milk of interest in human nutrition include an exceptionally high concentration of polyunsaturated fatty acids, low cholesterol content, high lactose and low protein contents (Solaroli *et al.,* 1993), as well as high levels of vitamins A, B and C. The renal load of equine milk, based on levels of protein and inorganic substances, is equal to human milk, providing further indication of its suitability as an infant food (Iacono *et al*., 1992).

The claimed invigorating effect of equine milk is thought to be, at least in part, due to its immuno-stimulating ability. Lyz, Lf and ω-3 fatty acids have long been associated with the regulation of phagocytosis of human neutrophils *in vitro* (Ellinger, Linscheid, Jahnecke, Goerlich, & Endbergs, 2002). The concentration of these compounds is exceptionally high in equine milk and in a comparative study of

of the effects of deep-frozen or lyophilised equine milk on phagocytosis, the consumption of frozen equine milk significantly inhibited chemotaxis and respiratory burst, two important phases of the phagocytic process whereas the same effect was not found for lyophilised equine milk. (Ellinger *et al*., 2002). This result suggests a potential anti-inflammatory effect by equine milk.

To be successful as a substitute for human milk in infant nutrition, equine milk must be capable of performing many biological functions associated with human milk. The presence of high concentrations of lactoferrin, lysozyme, ω-3 and ω -6 fatty acids in equine milk are good indicators of its potential role. However, the lack of research must be addressed to develop the potential of equine milk in the health and nutritional markets. Scientific studies are required to bring the health claims for equine milk out of the realms of regional folklore. It seems reasonable that equine milk could be marketed as a dietary aid where the immune system is already depleted, i.e., as a type of 'immuno-boost'. Data suggest that more than 30% of customers who purchase equine milk in the Netherlands are patients undergoing chemotherapy, and find equine milk helpful in counteracting the effects of cancer treatment. The composition of equine milk suggests a product with interesting nutritional characteristics with potential use in dietetics and therapeutics, especially in diets for the elderly, convalescent and newborns.

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Thesis Objectives

Based on the review of literature on equine milk, it was clear that there were many gaps in the overall knowledge and fundamental properties of equine milk. In particular, there is little or no information on the physico-chemical properties of equine milk. The gross composition of equine milk has been reasonably well established and the caseins have been fractionated and well characterized, although the presence of κ-casein remains a contentious issue. It has also been established, although the analytical work is limited, that equine casein micelles are considerably larger than those in bovine milk. Furthermore, it has been reported that equine β-lactoglobulin lacks a free thiol (sulphydryl) group which would have major implications for the denaturation of equine whey proteins by treatments such as heating or the application of high pressures. The objectives of this thesis were to add to the body of knowledge on equine milk, in particular to provide a detailed study of the compositional, chemical and physico-chemical properties of equine milk; the principal objectives were:

- To study the composition of equine milk, with quantification and distribution of the nitrogeneous compounds and principal salts
- A detailed study of the physico-chemical properties of equine milk, including heat and ethanol stabilities, rennetability and effect of acidification
- To identify the principal proteins in equine milk using two-dimensional electrophoresis (2-DE) and to study the susceptibility of equine κ- and β-caseins to hydrolysis by chymosin using 2-DE, with comparative analysis of bovine caseins
- To study the rheological properties of ‘curd’ formed from equine milk by acidification or renneting
- To study the colloidal stability of equine casein micelles, including their dissociation by urea, trisodium citrate or low temperature and the effect of high pressure treatment, ethanol or heat
- To study the effect of heat or high pressure treatment on the whey proteins in equine milk

- To characterize equine casein micelles, including micelle size and the definitive identification of κ-casein in equine milk
- To study the effect of pH on the rennetability of equine milk by calf chymosin
- To prepare a crude extract of equine chymosin from foal vells and to determine if chymosin from some species improves the coagulability of milk from those species
- To separate the multi-phosphorylated forms of equine β-casein and to study the formation of certain casomorphins by *in vitro* digestion

CHAPTER 3

Composition, Chemical and Physico-Chemical Properties of Equine Milk

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Abstract

The consumption of equine milk by humans is increasing strongly, due to its considerably lower allergenicity than bovine milk and similarities in composition to human milk, but at present there is little knowledge of the physico-chemical properties of equine milk. In this study, compositional analysis of equine milk, including the quantification and distribution of its nitrogenous components and principal salts is reported, with comparative analysis of bovine milk. Some physical properties were studied, including fat globule and casein micelle size distribution. The heat-, ethanol-, rennet- and acid-induced coagulation of skimmed equine milk were studied, as well as the stability of equine proteins to pH and sedimentation. Heat-induced coagulation of equine milk at 140°C occurred rapidly at pH 6.3-6.9, but the coagulation time at pH 7.0-7.3 was > 45 min. The heat stability of 2-fold concentrated milk increased gradually from pH 6.7, up to a maximum at pH 7.1. Skimmed equine milk displayed a sigmoidal ethanol stability-pH profile, but the stability of equine milk was lower than that of bovine milk. Equine casein micelles were not coagulable by rennet at pH 6.6. Some micellar flocculation, but no gelation, was observed on acidification of equine milk.

Keywords: Equine milk; rennet coagulation; heat stability; ethanol stability; acid coagulation.

3.1 Introduction

Although horses are spread throughout the world, equine dairy herds are, traditionally, located primarily in Mongolia, Kazakhstan, Kyrgyzstan and Tajikistan and to a lesser extent in eastern and central Europe. There are few equine dairy herds in Western Europe or other countries (Doreau & Martin-Rosset, 2003), but in recent years, there has been an increasing interest in the use of equine milk in human nutrition, as a low-allergenic substitute for bovine milk (Businco *et al.*, 2000; Doreau & Martin-Rosset, 2003) or in the treatment of patients with certain illnesses (Nassal & Rembalski, 1980; Solaroli *et al.*1993). Equine milk resembles human milk in many respects and is claimed to have special therapeutic properties, hence increasing its popularity in Western Europe, especially in France, Italy, Belgium and the Netherlands. Lactic-alcoholic fermentation of equine milk produces koumiss, a basic foodstuff and therapeutic product for the nomadic people of Mongolia and Kazakhstan. Overall, equine milk is considered to be highly digestible, rich in essential nutrients and to possess an optimum whey protein:casein ratio, making it very suitable as a substitute for bovine milk in paediatric dietetics.

Equine and bovine milk show large quantitative differences; equine milk contains much less fat, protein and minerals, but more lactose, than bovine milk (for review see Malacarne *et al.*, 2002; Park, 2006). Compared to bovine milk, the protein fraction of equine milk contains proportionally less casein and more whey proteins. The relative proportions of whey proteins in equine milk also differ considerably from bovine milk, with more β-lactoglobulin (β-lg) and immunoglobulins and less α-lactalbumin (α-la) in equine milk (Malacarne *et al.*, 2002). Equine casein contains almost equal amounts of α_s- and β-caseins, but little κ-casein (Ochirkhuyag *et al.*, 2000; Egito *et al.*, 2001). The caseins in equine milk exist as micelles, which are considerably larger than those in bovine milk (Buchheim *et al.*, 1989).

There is little information on the physico-chemical properties of equine milk, which is required for the manufacture of various products from equine milk and thus for it to achieve its full potential in human nutrition. This study aims to provide definitive compositional analysis for equine milk, including the

distribution of the nitrogenous components and its principal salts. Furthermore, physico-chemical studies should provide a first insight into the stability of equine casein micelles to heat, ethanol, rennet or acid and form the basis for follow-up studies. Bovine milk analyses have been included where appropriate for comparative purposes.

3.2. Materials and Methods

3.2.1 Milk supply

Fresh equine milk was obtained from the Irish National Stud (Tully, Kildare, Ireland). Samples of milk were obtained from five multiparous mares in mid-lactation. The mares were hand-milked, without the use of oxytocin, after being separated from their foals for ~ 2 h; the milk was cooled to 4^{o}C, and was received at our laboratory within ~24 h of milking. Before use, the milk was filtered through glass wool to remove any extraneous material. Raw whole equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from five milkings over 24 h, from a herd of ~ 45 multiparous New Forest and New Forest/Arabian mares in mid-lactation, physically separated by day from their foals. Raw whole bovine milk was obtained from a local dairy farm.

Asinine milk (17 g L^{-1} protein, 3.8 g L^{-1} fat) used in acidification and rennetability experiments was a gift from the Dipartimento di Scienze Animali, Universita degli Studi del Molise, Campobasso, Italy. The milk was frozen prior to delivery and upon receipt was thawed gently overnight at 4^{o}C, followed by the addition of 0.5 g L^{-1} sodium azide and equilibration at room temperature (~ 20^{o}C) for several hours before analysis.

Due to the large size of equine casein micelles shown in preliminary analysis, and in agreement with values reported by Welsch *et al.* (1988), fresh equine milk was defatted at 1,000 g for 20 min at 20^{o}C using a Sorvall® RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) and filtered through glass wool to remove fat particles. Whole bovine milk was defatted by centrifugation at 2,000 g for 20 min at 20^{o}C followed by filtration through glass wool. It was

assumed that the micelles in asinine milk were probably larger than those of bovine milk and therefore the milk was defatted as described for equine milk.

Sodium azide (0.5 g L^{-1}) was added to skimmed milks to prevent microbial growth prior to storage at 4^{o}C until required, but for no longer than 3 days. In the case of equine milk, protein and fat were determined both before and after defatting to assess the efficiency of the skimming process and ensure that significant loss of protein did not occur; bovine milk was similarly analysed for comparative purposes.

Throughout this study, pH was measured using a Radiometer pHM 210 pH meter equipped with a Radiometer Meterlab® combined general purpose electrode (pHC 2001) with built-in temperature sensor (Radiometer Analytical SAS, Lyon, France). The pH meter was calibrated daily using pH 4.0 and pH 7.0 standards from Radiometer Analytical.

All chemicals used were of reagent grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

3.2.2. Sample preparation

Preparation of bovine and equine caseins for electrophoretic analysis

Raw skim bovine milk, prepared as described above, was warmed to 40^{o}C and then acidified to pH 4.6 with 1 M HCl, held for 45 min and then centrifuged at 1,000 x g for 20 min to separate the precipitated casein as a pellet. The pellet was washed with deionized water and recentrifuged; this step was repeated several times. The casein pellet was resuspended to its original volume in deionized water, the pH was adjusted to 7.0 with 1 M NaOH and held at 4^{o}C for 4 h for equilibration, after which the sample was frozen and lyophilized. Equine casein was prepared in a similar manner from skimmed bulk milk except that the milk was acidified to pH 4.2, as preliminary characterization indicated that the pH of minimum solubility of equine casein is ~ pH 4.2, in agreement with Egito *et al.* (2001).

Preparation of suspensions of equine and bovine casein micelles

Suspensions of bovine or equine casein micelles were prepared by sedimenting the micelles at 100,000 *g* for 90 min at 20°C using an Optima LE-80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a Beckman 50.2 Ti rotor (12 place) and resuspending the pellets at ~3-3.5% in the ultracentrifugal supernatant [from which residual micelles and fat globules were removed by filtration (0.22 μm syringe filter, Minisart®), Sartorius Stedim Biotech S.A., Aubagne, France)] using a tissue homogeniser, followed by stirring for ~20 h at 4°C. The suspensions were subsequently centrifuged at 500 g for 30 min at 20°C to remove any undissolved material and the casein content determined by the Kjeldahl method (IDF, 2001) and adjusted to 25 g L^{-1} by the addition of ultracentrifugal supernatant and subsequently adjusted to pH 6.6.

Preparation of micellar equine and bovine casein with interchanged serum phases

Casein pellets recovered by ultracentrifugation of equine or bovine milk were resuspended in the original weight of serum as described above and the serum phases were exchanged, i.e., equine micelles were resuspended in bovine serum and *vice versa.* Protein levels were adjusted to 25 g L^{-1} by the addition of either bovine or equine ultracentrifugal supernatant and adjusted to pH 6.6.

Preparation of equine and bovine milk for mineral analysis

For determination of soluble calcium, inorganic phosphate, magnesium and citrate the casein micellar and serum phases of equine and bovine milk were separated by ultracentrifugation at 100,000 g for 60 min at 20°C using an Optima LE-80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a Beckman 50.2 Ti rotor. Equine and bovine milk and their ultracentrifugal supernatants were deproteinated by the addition of an equal volume of 24% (w/v) trichloroacetic acid (TCA), left stand at room temperature for 30 min and subsequently centrifuged at 24,000 g for 30 min at 20°C; the supernatant was filtred through Whatman 542 paper.

3.2.3. Compositional analysis

The total nitrogen and fat content of the equine and bovine milk samples were determined by the Kjeldahl (IDF, 2001) and Gerber (IDF, 1981) methods, respectively. Total solids non-fat (TSNF) were determined using the AOAC (1995a) method after defatting the bovine and equine milk samples as described above. The ash content was measured using the AOAC (1995b) method. Lactose was determined by the Chloramine-T method (James, 1999).

3.2.4. Nitrogenous components of equine milk

The distribution of nitrogenous components in equine and bovine milk was determined as follows: non-casein nitrogen (NCN) was determined after precipitation of the casein fraction of milk with 10% acetic acid, followed by 1M sodium acetate to a final mixture pH of ~ 4.6 for bovine milk and pH 4.2 for equine milk (IDF, 2004). The nitrogen content of the filtrate (non-casein nitrogen components) was determined by the Kjeldahl method (IDF, 2001). The casein nitrogen content was calculated as the difference between the total nitrogen content and the non-casein nitrogen content of the milks. A multiplication factor of 6.38 was used in all cases to convert total nitrogen values to total protein content (IDF, 1993). Non-casein nitrogen (NCN) was calculated by difference between true total protein and casein protein values. Non-protein nitrogen (NPN) in equine and bovine milk was determined in a 12% tricholoacetic acid (TCA) extract of the milk according to the AOAC (1995c). The NPN, expressed as protein equivalents, was calculated from the % nitrogen multiplied by 6.38.

3.2.5. Concentration and distribution of the principal salts in equine milk

3.2.5.1. Determination of the concentration of total, soluble and colloidal calcium

The concentration of total and soluble (non-micellar serum) calcium in equine and bovine milk was determined by atomic absorption spectroscopy [(AAS) SpectraAA-100 atomic absorption spectrometer, Varian Inc., Walnut Creek, CA, USA], essentially as described by Huppertz *et al.* (2005), with some modifications.

The calcium content of the deproteinated filtrates, diluted 1:200 with lanthanum chloride solution [26.6% w/v, $LaCl_3.7H_2O$ BDH Spectrosol®, VWR International Ltd., Arlington Heights, IL, USA] to a final concentration of 0.5% La, was subsequently determined from a standard curve of 0 to 10 mg kg^{-1} calcium in deionized water containing 0.5% $LaCl_3$ prepared from a 1,000 mg kg^{-1} $CaCl_2$ standard solution for AAS (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). A sample blank, was prepared by adding an equal volume of 24% TCA to distilled water, followed by dilution as described above. The calcium content of each sample was determined in triplicate. Colloidal (micellar) calcium was calculated by subtracting the value for soluble calcium obtained for the ultracentrifugal supernatant from the value for total calcium.

3.2.5.2. Determination of the concentration of ionic calcium

The ionic calcium (Ca^{2+}) content of equine and bovine milk and their milk sera was measured using a calcium ion-selective electrode (ISE25Ca; Radiometer Analytical, Copenhagen, Denmark). A standard curve of mV plotted against the log concentration of $CaCl_2$ (1 to 10 mM) in 0.1 M KCl (used to maintain consistent ionic strength) was used to calculate the ionic calcium concentration in equine and bovine milk and serum. For each standard, the electrode was allowed to stabilize for 5 min. Prior to analysis, samples were gently mixed and, to minimize the effect of electrode drift, the probe was rinsed with deionized water between measurements and single-point calibrations made immediately before the next measurement. It took no longer than 40 s to obtain a stable reading for each sample.

Due to inherent difficulties in the measurement of the ionic calcium concentration in milk (Silanikove *et al.*, 2003; Lin *et al.*, 2006), for comparative purposes, two additional methods of analysis were used which were kindly carried out at the laboratory of Dr. Mike Lewis, Food & Nutritional Sciences, University of Reading. The second method for [Ca^{2+}] analysis used the Rapidlab 348 pH/Blood Gas analyser (Bayer Plc., Newbury, UK) with calcium standards in the range 0.05 to 5.0 mmol L^{-1}. The third method used was as per Lin *et al.* (2006) using a CIBA Corning 634 ISE CA^{2+}/pH analyser (Bayer Plc., Diagnostic

Division, Newbury, UK) using calcium standard solutions in imidazole buffer in the range 1.0 to 10.0 mM calcium. Due to experimental constraints and lack of fresh equine milk at the time of analysis, measurements with the 634 ISE analyser were carried out using equine milk that had been stored at -80^{o}C within 24 h after milking. The milk was thawed overnight at 4^{o}C and tempered at 20^{o}C for several hours prior to analysis to ensure adequate equilibration of minerals and proteins.

3.2.5.3. Determination of magnesium

The magnesium content of equine and bovine milk and milk sera was determined by atomic absorption spectroscopy (SpectraAA-100 atomic absorption spectrometer), Samples were prepared as described above for total and soluble calcium. A standard curve of 0 to 1 mg kg^{-1} Mg (at 0.2 mg kg^{-1} intervals) was prepared from a $MgCl_2$ (1000 mg kg^{-1} Mg) standard solution (BDH Aristar$^{®A}$, VWR International Ltd., Arlington Heights, IL,) for AAS.

3.2.5.4. Determination of citrate

The citrate content of equine and bovine milk and their respective milk sera was measured spectrophotometrically using an enzyme kit (UV-method for citric acid determination in foodstuffs and other material, Boehringer-Mannheim/R-Biopharm, R-Biopharm Rhône Ltd., Glasgow, Scotland; catalogue no. 139,076). Milk samples and sera (from ultracentrifugal supernatant, prepared as described in Section 3.2.2) were prepared as described by White & Davies (1963), filtered through Whatman No 42 paper, and diluted 1 to 9 with distilled water prior to analysis. Samples were prepared and analysed in triplicate

3.2.5.5. Determination of total and soluble inorganic phosphate

The total inorganic phosphate (Pi) content of equine and bovine milk and their ultracentrifugal supernatants (milk sera, prepared as described above) was determined by a modification of the method of Fiske & Subbarow (1925). Milk and ultracentrifugal supernatants were deproteinazed by addition of an equal volume of 24% (w/v) TCA followed by centrifugation at 24,000 g for 30 min at

20°C. A standard curve containing 0 to 0.12 mg P mL^{-1} prepared from anhydrous KH_2PO_4 was used and standards and samples were read at 830 nm using a Cary UV-Vis IE spectrophotometer. To ensure that sample values were within the range of the standard curve, a diluent of 1mL of molybdate reagent and 4 mL of sulfonic acid reagent brought to 100 mL was prepared and used, as necessary. Colloidal inorganic phosphate was calculated by difference.

3.3. Measurement of selected physical properties

3.3.1 Refractive index

The refractive index of equine and bovine milk was measured using a hand-held refractometer with a sodium lamp as the light source (Atago R5000, Atago Co., Ltd., Tokyo, Japan).

3.3.2 Measurement of colour

The colour of whole and skimmed equine or bovine milk samples was measured on 5 replicates of each sample at 20°C using a Minolta CR-300 colorimeter (Minolta Corp., Osaka, Japan). The CIELAB (CIE, 1976) standard measurement system, which measures colour using 3 coordinates, L*, a*, and b* was used. The instrument was calibrated with a white tile (standard tristimulus values: Y = 92.4; x = 0.3161; y = 0.3325) supplied by Minolta prior to measurement and at regular intervals throughout. Experimental error was ± 0.1 units and each sample was assessed five times.

3.3.3. Measurement of viscosity

The viscosity of whole equine and bovine milk was measured using a low-shear viscometer (Contraves Low Shear-30, Contraves AG, Zurich, Switzerland). For each sample, torque measurements were taken at 12 shear rates, spaced at equal logarithmic intervals, within the range 1.78-69.53 s^{-1}. The sample cup was maintained at 20°C using a circulating waterbath (Haake K15 with C10 temperature controller; Haake Mess-Technik GmbH, Karlsruhe, Germany). The instrument was calibrated prior to analysis using Milli-Q® water.

3.3.4. Determination of zeta potential

The zeta potential of skimmed equine and bovine milk at their natural pH and diluted 1:250 and 1:500, respectively, with lactose-free synthetic milk ultrafiltrate (SMUF; Jenness & Koops, 1962), was measured by laser-Doppler electrophoresis using a Malvern Zetamaster 3000 (Malvern Instruments, Malvern, Worcestershire, UK) at an applied voltage of 120 V and a modulation frequency of 250 Hz. The instrument was tested with a Malvern zeta potential transfer standard (carboxyl-modified polystyrene particles), provided by Malvern Instruments, with a surface potential of -50 mV ± 5 mV. To eliminate any effects of dilution, all samples were diluted immediately prior to analysis. To minimize the effect of any temperature increase, 3 readings per sample were taken for each sequence of measurements, the duration of which was no longer than 30 s with a delay between samples of ~ 20 s.

3.3.5. Determination of fat globule size and size distribution

The fat globule size distribution in equine milk was determined using a Malvern Mastersizer model S (Malvern Instruments Ltd., Malvern, Worchestershire, UK) equipped with a 300 RF (reverse Fourier; range 0.05 to 880 μm) lens and a He-Ne laser light source at a wavelength of 633 nm using a polydisperse optical analysis model. Distilled water (~ 25^{o}C) was used as the dispersant in a small volume sample presentation unit (MSXL 5). To ensure that the correct optical properties for equine milk were used, the refractive index of equine milk, measured as described in Section 3.3.1 was used to set up an instrument sample presentation model (i.e., a combination of the ratio of the relative refractive indices of the dispersed phase and water and the absorbance of the dispersed phase). The fat globule size of bovine milk was measured for comparative purposes using the manufacturer's preset 3NAD presentation; the refractive index i.e., the ratio of refractive index of fat globules (1.456) and that of the dispersion medium (1.333) was 1.095. Prior to analysis, milk samples were mixed 3:1 with 0.4 M trisodium citrate containing 4% (w/v) sodium dodecyl sulphate (SDS). The former dissociates the casein micelles (Walstra, 1990) and the SDS removes absorbed materials from the oil-water interfaces, thus

dissociating fat aggregates (DeFeijter *et al.*, 1987). The samples were tempered at 25 ± 0.5^{o}C for 15 min and introduced drop-wise to the presentation unit until an obscuration of 10 to 30% was attained. Results were recorded as the volume-weighted mean fat globule diameter, D [4,3], the surface to volume weighted mean diameter or Sauter-average mean diameter, D [3,2], and the arithmetic mean diameter values below which 10, 50% and 90% of fat globules have lower diameters (D [v, 0.1], D [v, 0.5] and D [v, 0.9], respectively). Results were deemed valid once a residual value (i.e., the overall fit of presentation data to sample measurement data) of < 1% was observed.

3.3.6. Determination of average casein micelle size and distribution

The size distribution and average size of casein micelles in equine and bovine milk diluted 1:250 and 1:500, respectively, with lactose-free synthetic milk ultrafiltrate (SMUF; Jenness & Koops, 1962), was determined at 20^{o}C by fixed-angle dynamic light scattering (DLS), using a Malvern Zetamaster 3000 instrument, equipped with a 632.8 nm He-Ne laser using a scattering angle of 90^{o}. Milk samples were diluted with SMUF and filtered through Whatman No. 40 paper immediately prior to analysis to eliminate any effects of dilution.

As a complementary technique, the size of equine casein micelles was also determined by multi-angle DLS using an ALV Compact Goniometer System (ALV-Laser; Vertriebsgesellschaft GmbH, Langen, Germany), as described by Huppertz & De Kruif (2008). DLS measures the hydrodynamic radius of casein micelles. Prior to analysis, equine milk dialysate was prepared as diluent by exhaustive dialysis of a 60 g L^{-1} lactose solution against 2 x 20 volumes of equine milk for 48 h at 20^{o}C. Sodium azide (0.5 g L^{-1}) was added as preservative.

3.4. Analytical techniques

3.4.1. Effect of pH on the solubility of equine milk proteins

Skimmed equine and bovine milk were adjusted to pH values in the range of 3.7 to 6.6 with 1M HCl at 4^{o}C and held overnight. Samples were tempered at 20^{o}C for 30 min and the pH rechecked and readjusted, if necessary, after which they

were centrifuged at 5,000 g x 30 min. The protein content of the supernatant was determined by the Kjeldahl method.

3.4.2. Influence of gravitational force on protein sedimentation

Skimmed equine and bovine milk samples were centrifuged at 3,000, 5,000, 10,000, 20,000 and 50,000 x g for 40 min at 20^{o}C using a Sorvall® RC 5B or an Optima LE-80K ultracentrifuge. Supernatants were removed carefully and the protein content of 100µL was determined by the Bio-Rad Protein Assay No. 500-0002 (Bio-Rad Laboratories, Hercules, CA., USA.) which is based on the Bradford method (Bradford, 1976) and uses bovine serum albumin (BSA) as standard. The bovine and equine milk filtrates were diluted 1:20 and 1:40, respectively, with distilled water prior to analysis.

3.4.3. Heat-induced coagulation of equine milk

The heat stability of unconcentrated or pre-heated (at 140°C) or concentrated (at 120°C) skimmed equine milk was determined as a function of milk pH (6.3-7.3; adjusted using 1.0 M NaOH or 1.0 M HCl) by the subjective method of Davies & White (1966). The time taken to cause coagulation of a milk sample as indicated by flocculation of the milk proteins was recorded as the heat coagulation time (HCT).

Pre-heated equine milk was prepared by heating unconcentrated skimmed equine milk at 90°C for 10 min, followed by rapid cooling to room temperature in ice-water. Statistical analysis of experimental data was not possible as small differences in pH can markedly affect the heat stability and assessment of the end-point is subjective, therefore the heat stability *vs* pH profile shown is typical and reproducible to the extent that similar patterns were found on all cases for unconcentrated, concentrated or unconcentrated and pre-heated equine milk.

3.4.4. Determination of the ethanol stability of milk

Four mL of aqueous ethanol (0 to 100%, v/v, in increments of 2.5%) were added to 4 mL portions of equine or bovine milk samples in Petri dishes. The minimum concentration of ethanol necessary to destabilise the milk proteins (visible

flocculation) was recorded as the ethanol stability (Davies & White, 1958; Horne & Parker, 1981a). Control samples were prepared as described above but with addition of distilled water instead of ethanol.

A second method was developed to determine the ethanol stability of equine milk. Two mL of milk sample at a particular pH and 2 mL of an ethanol solution were placed in thin-walled glass tubes with screw caps and PTFE seals. The tubes were tempered in a thermostatically controlled waterbath at 20^{o}C for 10 min. Immediately afterwards, the tubes were centrifuged at 500 g x 6 min in a Universal 32 benchtop centrifuge ((Hettich Zentrifugen, Tuttlingen, Germany). Samples were removed and visually assessed in an inverted position; destabilised protein remained at the base of the tubes when an ethanol concentration caused precipitation. A control sample consisted of equal volumes of milk and water. For all ethanol stability experiments, each analysis was performed on at least 3 occasions with at least 3 different milk samples.

3.4.5. Determination of the ethanol stability of milk as a function of pH

The ethanol stability of unconcentrated skimmed equine milk was determined as a function of pH adjusted in the range 5.0 to 7.5 with 1.0 M NaOH or 1.0 M HCl, as described by Huppertz *et al.* (2004). The minimum concentration of ethanol required to destabilize the milk proteins was recorded as the ethanol stability (Davies & White, 1958; Horne & Parker, 1981a).

3.4.6. Determination of the rennetability of equine milk

The rennetability of equine milk was studied in triplicate using a modification of the Berridge method (IDF, 1987). Two mL of either equine or bovine skim milk (adjusted to pH 6.6) were placed in thin-walled glass tubes and tempered for 15 min at 32^{o}C in a thermostatically controlled waterbath. Ten μL mL^{-1} of a 1:10 (v/v) aqueous dilution of fermentation-produced chymosin (Maxiren 180, 180 international milk-clotting units (IMCU) per ml, DSM Food Specialities, Delft, the Netherlands) were added and the tubes were oscillated gently on a rocking platform in the waterbath. Rennet coagulation time was recorded as the first sign of flocculation, i.e., visible curd flocs on the wall of the tube.

The rennet-induced flocculation of casein micelles was also monitored by diffusing wave spectroscopy (DWS) in transmission mode, as described by Huppertz & De Kruif (2007) with the modification that Maxiren 180 was added at a level of 6 μL mL^{-1}. Chymosin was added at lower levels than previously used due to the sensitivity of the instrument and the time scale of the experiment was expanded accordingly. The time at which the autocorrelation curve had decayed to 50% of its maximum plateau level is defined as $\tau_{½}$, the relaxation time, and reflects the restriction of particles in solution during the initial stages of flocculation, the relaxation time is therefore a direct measure of particle growth. All data were normalized relative to a control, which is the $\tau_{½}$ value of the sample at 32°C prior to addition of rennet.

3.4.7. Dynamic Measurement of Rennet Coagulation Properties

Dynamic oscillatory analysis (low amplitude oscillatory measurement) of renneted equine, bovine and asinine milk was performed as described by O'Connell *et al.* (2006), with some modifications. Eleven mL of each milk were pre-warmed in a waterbath at 32°C for 15 min and 120 μL of a 1:10 (v/v) dilution of Maxiren 180 were added immediately prior to analysis. Analysis was carried out using a controlled shear stress Carri-Med CSL^2 100 rheometer equipped with concentric cylinders and a recessed acrylic rotor (TA Instruments, Leatherhead, Surrey, U.K.). Frequency of oscillation was set at 6.283 rad s^{-1}. The storage modulus, G', of the sample was recorded continuously at a low amplitude shear strain (0.01) over 90 min.

3.4.8. Acid-induced coagulation of casein micelles in equine and bovine milk

The acid-induced coagulation of equine, asinine and bovine milk was monitored by dynamic oscillatory rheology using the same conditions described for measurement of the rennetability of equine milk except that the storage modulus, G', was recorded for 120 min after the addition of 25 g L^{-1} GDL. The pH of bovine and equine milk decreased to 4.17 and 3.5, respectively, after 120 min.

As an additional analytical technique, the acid-induced coagulation of equine casein micelles was followed by DWS, as described by Vasbinder *et al.* (2001)

with the modification that the acidulant, glucono-δ-lactone (GDL), was added at a level of 30 g L^{-1}. The rate of decrease in pH, subsequent to addition of GDL, was monitored. The hydrodynamic radius of casein micelles was determined by multi-angle dynamic light scattering (DLS), as described by Huppertz & De Kruif (2007).

3.4.9. Preliminary characterization of equine casein by gel electrophoresis

Urea polyacrylamide gel electrophoresis (Urea-PAGE) [12.5% total monomer concentration (T); 4% cross-linking monomer (C), pH 8.9] was performed using a Protean II xi vertical slab gel unit (Bio-Rad Laboratories Ltd.,, Hercules, CA., USA) according to the method of Andrews (1983), with the modifications of Shalabi & Fox (1987). One mm thick gels were poured and sufficient separating gel solution to allow ~ 1 cm of stacking gel below the level of the bottom of the wells. A layer of Milli-Q® water was poured over the separating gel and the gel allowed stand until polymerization was complete. The layer of water was removed carefully using Whatman No 113 filter paper and the stacking gel solution was poured and the slot former inserted, taking care to avoid air bubbles. Gels were pre-run at 280 V and after sample application, the gels were run at this voltage through the stacking gel, after which the voltage was increased to 300 V until the tracking dye front was ~1 cm from the bottom of the gel. Samples of bovine sodium caseinate, equine casein and equine milk were prepared in sample buffer at 10 mg mL^{-1} for the lyophilized samples and a 1:1 (v/v) dilution of equine milk. Three μL of bovine caseinate, 6 μl of equine casein and 10 μL equine milk were applied to the gel. Gels were stained with Coomassie Brilliant Blue G250 as described by Blakesely & Boezi (1977) with gentle agitation at 20 rpm on an orbital platform shaker (Heidolph Rotamix 120, Heidolph Canada Biotech Edge, Toronto, Canada) overnight. Gels were destained in several changes of distilled water, again with gentle agitation and scanned on a flat-bed scanner (Scanjet 6300C, HP, Singapore).

3.4.10. Data analysis

All experiments were repeated at least in triplicate on individual equine milk samples (N=5) or pooled bulk samples of equine and bovine milk. and average results with standard deviations reported. In addition, compositional analysis data reported for bulk equine and bovine milk was from milk collected on 3 separate occasions.

Descriptive statistical analysis of data (mean, standard deviation) was carried out using Minitab Statistical Software (Release 13.31; Minitab Inc., State College, PA., USA.). All results are reported in tabular form for the individual equine milk samples (N=5) with comparative results for bulk bovine milk. Fat, protein, lactose, total calcium ionic calcium and pH were measured for bulk equine milk from 3 separate batches and one-way analysis of variance (ANOVA) was performed for each parameter measured using Minitab Statistical Software (Release 13.31; Minitab Inc., State College, PA, USA)., at a significance level, p, of 0.05, to determine if there was a significant difference between individual equine milk samples and bulk equine milk. Prior to analysis, all data were tested for normality using the Anderson-Darling test.

Measurements of heat stability, rennet coagulation and ethanol stability of milk are subjective and for this reason and, due to the effect of very small differences in pH on heat and ethanol stabilities, detailed statistical analysis of replicate results is not reproducible; all assays were carried out at least 5 times and a similar result was recorded on all occasions. For clarity, data were presented from one analysis and the coefficient of variation was always < 5% of the reported value for each data point. For consistency of graphical presentation, error bars were not included as some graphs had data on a logarithmic scale where inclusion of error bars can be both incorrect and misleading.

3.5. Results and Discussion

3.5.1. Compositional analysis

Values for the gross composition of equine milk are presented in Table 3.1, with comparative values for bovine milk. The total solids (TS) content of equine milk

was 101 g L^{-1} and total milk solids non-fat (TSNF) were 87.7 g L^{-1}, while the corresponding values for bovine milk were 132.5 and 99.50 g L^{-1}. The values for equine and bovine milk are in agreement with those of Solaroli *et al.* (1993). Schryver *et al.* (1986) reported ~ 102 g L^{-1} total solids in mid-lactation equine milk, marginally lower than a value of 110 g L^{-1} reported by Pieszka & Kulisa (2003).

The protein content of equine milk, 22.98 g L^{-1}, was similar to that reported by others for milk from mares in mid-lactation (Ullrey *et al.*, 1966; Mariani *et al.*, 2001; Smolders *et al.*, 1990; Zicker & Lönnerdal, 1994). The distribution of the nitrogenous constituents of equine and bovine milk is included in Table 3.1.

The protein content of fresh bulk equine milk was 21.88 ± 1.08 g L^{-1} and no statistically significant difference ($p > 0.05$) was found for protein content between individual equine milk samples and bulk equine milk. Similarily, no significant difference was found for the protein content of bulk equine or bulk bovine milk from 3 separate collections ($P > 0.05$ for both cases).

Values for total casein protein and total whey protein were within the range of values reported by Solaroli *et al.* (1993) and Csapó-Kiss *et al.* (1995), while the value for non-casein nitrogen was similar to that reported by Mariani *et al.* (2001) for Haflinger mares. The NPN found here for bovine milk, representing ~ 5% of total N, was within the range found by de Peters & Ferguson (1992). On the other hand, Zicker & Lönnerdal (1994) reported values > 10% of total N for the NPN of mid-lactation human milk, while Shamsia (2009) estimated human milk NPN to be ~ 16% of its nitrogen fraction, one of the highest proportions found in mammalian milks.

The fat content of equine milk was 13.8 ± 0.25 g L^{-1}, almost one third that of bovine milk (36.7 ± 0.22 g L^{-1}); the fat content of the skimmed equine milk was 2.0 ± 0.05 g L^{-1}. Fresh bulk equine milk had a fat content of 13.6 ± 0.19 g L^{-1} and no statistically significant difference ($p > 0.05$) was found between fat values of individual equine milk samples and bulk equine milk. Similarily, no significant difference was found for the fat content of bulk equine or bulk bovine milk from 3 separate collections ($P > 0.05$ for both cases).

The ash content of equine milk, 4.1 g L^{-1}, was almost half that of bovine milk and the values for both milks were within the range of those reported by Solaroli *et al.* (1993) while the value for equine milk was very close to those of Schryver *et al.* (1986) and Csapó-Kiss *et al.* (1995) for mid-lactation milk. Lactose in equine milk, at 66 g L^{-1}, is considerably higher than that of bovine milk (49.1 g L^{-1}) and is in agreement with values reported by Solaroli *et al.* (1993), Malacarne *et al.* (2002) and Park *et al.* (2006). The lactose content of bulk equine milk was 66.1 ± 0.04 g L^{-1} and no statistically significant difference ($p > 0.05$) was found for the lactose content of individual or bulk equine milk samples.

Table 3.1: Average composition (g L^{-1}) for equine milk, with comparative data for bovine milk.

Constituent	Equine milk	Bovine milk†
Total solids	101.7 ± 0.90	132.50 ± 0.42
Total solids non-fat (TSNF)	87.7 ± 0.15	95..5 ± 0.22
Total nitrogen (x 6.38)	25.58 ± 1.22	35.9 ± 1.16
True protein	22.98 ± 1.60	34.1 ± 1.88
Non-casein nitrogen (NCN)	12.2 ± 0.31	8.2 ± 0.45
Non-protein nitrogen (NPN) *	2.6 ± 0.04	1.8 ± 0.03
Total casein	12.08 ± 0.60	28.1 ± 1.33
Total whey proteins	8.3 ± 0.54	6.1 ± 0.32
Casein :whey protein ratio	1.45:1	4.6:1
Fat content whole milk	13.8 ± 0.25	36.7 ± 0.22
Fat content skimmed milk	2.0 ± 0.05	5.5 ± 0.04
Ash	4.1 ± 2.00	7.5 ± 3.30
Lactose	66 ± 0.05	49.1 ± 0.05

Values are means of data for five individual equine milk samples, ± standard deviations. * Value for NPN expressed as protein equivalents (N x 6.38). † Bovine milk was from a bulk milk supply.

In comparison to human milk (Shamsia, 2009), equine milk had a similar content of total solids, including lactose, less fat, more ash, more protein, more casein and less whey proteins. The casein:whey protein ratio in this study was 1.45:1 while that of human milk, to which equine milk is frequently compared, has been reported as ~ 0.5-0.66:1 (Shamsia, 2009). The casein:whey protein ratio for bovine milk of 4.6:1, is close to the 80:20 ratio reported by Jenness (1974).

3.5.2. Concentration and distribution of the principal salts of equine milk

The minerals and caseins in milk are in dynamic equilibrium between the micellar and serum phases and the partitioning of these constituents is influenced by temperature, pH and various calcium-chelating agents (see Udabage *et al.*, 2000 and references therein). The principal buffering factors in milk are soluble phosphate, colloidal calcium phosphate (CCP), citrate and proteins (Lucey *et al.*, 1993; Singh *et al.*, 1997; Salaün *et al.*, 2005). The salts in milk and their distribution determine many of the functional properties, such as gel strength, rennet coagulation time, heat stability and the rate of pH change in milk products during processing. Table 3.2 shows the concentration and partitioning of the principal milk salts in equine and bovine milks. All values for bovine milk salts were within the range of those cited by White & Davis (1958), Holt (1997), Gaucheron (2005) and Tsioulpas *et al.* (2007b). Values for equine milk were very similar to those reported by Holt & Jenness (1984), except for the levels of total and serum citrate, which were lower in this study. Lower concentrations of calcium, magnesium, phosphate, potassium and sodium in equine milk compared to bovine milk found in this study were also reported by Holmes *et al.* (1947), Csapó-Kiss *et al.* (1995) and Marconi & Panfili (1998).

Table 3.2: Concentrations (mmol L^{-1}) and distribution of the principal salts in equine and bovine milk

Mineral	Equine milk	Bovine milk
Total calcium	19.35 ± 3.00	28.67 ± 2.60
Serum calcium	6.3 ± 0.52	7.19 ± 0.34
Colloidal calcium	13.05 ± 1.21	21.48 ± 1.17
Ionic calcium (Ca^{2+}) in milk†	2.66 ± 0.10	1.76 ± 0.20
Ionic calcium (Ca^{2+}) in milk serum†	2.89 ± 0.12	2.01 ± 0.20
Total magnesium	1.13 ± 0.04	5.04 ± 0.06
Serum magnesium	0.89 ± 0.03	3.42 ± 0.33
Colloidal magnesium	0.24 ± 0.01	1.62 ± 0.31
Total citrate	2.3 ± 0.08	9.10 ± 0.12
Serum citrate	2.15 ± 0.08	8.00 ± 0.12
Total inorganic phosphate (Pi)	6.81 ± 0.13	20.40 ± 1.12
Serum inorganic phosphate (Pi)	3.34 ± 0.12	10.06 ± 0.17
Colloidal inorganic phosphate (Pi)	3.47 ± 0.14	9.73 ± 0.12
Ca:Pi ratio	2.84:1	1.45:1

Values are means of data for five individual equine milk samples, ± standard deviations. † measured using a calcium ion-selective electrode.

3.5.3. Concentration of total and soluble calcium

Calcium is partitioned between the colloidal and serum phases and is in electrochemical equilibrium with several major milk components (Silanikove *et al.*, 2003). The distribution of Ca between the colloidal and serum phases in milk is governed by the level of casein in the milk (Holt & Jenness, 1984; Neville *et al.*, 1994). In human milk, the casein content is low and only ~ 25% of Ca is associated with it, while in bovine and caprine milk the figure is close to 65% (Neville *et al.*, 1995). Skimmed equine milk contained 19.35 mmol L^{-1} calcium, whereas its ultracentrifugal supernatant contained 6.3 mmol L^{-1}, suggesting that in equine milk, ~65% of total calcium is in the micellar phase, which agrees well with the data of Holt & Jenness (1984). The concentration of total calcium was considerably higher than that reported for Italian Saddle mare's milk (Martuzzi *et al.*, 1997) and mares of the Bardigiano breed (Martuzzi *et al.*, 1998), but is within the range reported by Anderson (1991) for the Quarter Horse breed. Bulk equine milk had 19.0 ± 1.3 mmol L^{1} total calcium which was not statistically different to the values from individual equine milk samples ($p > 0.05$). Bovine milk had 28.67 mmol L^{1} total Ca, which was within the range reported by Tsioulpas *et al.* (2007b). No statistically significant difference was found for the total calcium content of bulk equine or bovine milk ($P > 0.05$ in both cases) from 3 separate collections. Both equine and bovine milk had significantly higher levels of total calcium than the values reported for human milk (~ 7.5-8.0 mmol L^{-1}, Neville *et al.*, 1995; Silanikove *et al.*, 2003).

3.5.4. Concentration of ionic calcium

The concentration of ionic calcium, at the natural pH of equine milk, was 2.66 mmol L^{-1} when measured using a calcium ion-selective electrode (Table 3.2), which agrees well with the value predicted by Holt & Jenness (1984) and considerably higher than that obtained for bovine milk, 1.76 mmol L^{-1}, which agrees with other studies (Holt *et al.*, 1981; Holt & Jenness, 1984; Silanikove *et al.*, 2003; Tsioulpas *et al.*, 2007b; Faka *et al.*, 2009). No statistically significant difference ($p > 0.05$) was found for the ionic calcium concentration between individual equine milk samples and bulk equine milk. Similarily, no significant

difference was found for the ionic calcium concentration content of bulk equine or bulk bovine milk from 3 separate collections ($P > 0.05$ for both cases).

The concentration of ionic Ca in equine milk was very similar to that in human milk, i.e., 2.5-3.0 mmol L^{-1} (Allen & Neville, 1983; Neville *et al*, 1995; Silanikove *et al.*, 2003). Using the Rapidlab 348 Analyser, equine milk was found to have 2.10 ± 0.05 mmol L^{-1} $Ca^{2+}$and bovine milk 1.20 ± 0.05 mmol L^{-1} Ca^{2+}, both values were lower than those obtained using an ion-selective electrode. The [Ca^{2+}] in equine and bovine milk sera were higher than those of the respective milks (Table 3.2) which was observed throughout this study. With the RapidLab 348 analyser, the [Ca^{2+}] in equine serum at 2.22 mmol L^{-1} was also higher than that found in equine milk, while the pH was actually higher (7.18) compared to the equine milk (7.04). The increase in serum pH was also observed while measuring the [Ca^{2+}] using the ion-selective electrode (pH 7.27 for equine milk *vs* 7.34 for equine milk serum). It is possible that the increased [Ca^{2+}] observed in milk sera may be due to the absence of Ca-binding casein, however, one would expect this to be accompanied by a lower pH value which has been reported in the case of bovine milk (see Lewis, 2010). The [Ca^{2+}] in frozen equine milk and serum was found to be 1.89 ± 0.05 mmol L^{-1} and 2.0 ±0.05 mmol L^{-1}, respectively, using the 634 analyser. These values represent a decrease of ~10% compared to those obtained for fresh milk using the RapidLab 348 analyser; the decrease may be due to the effects of freezing rather than the different instrumentation. It has been reported that freezing and subsequent thawing reduces the [Ca^{2+}] in bovine milk by between 10 and 15% (Lewis, 2010). The concentration of [Ca^{2+}] in bulk equine milk was 2.70 ± 0.31 mmol L^{-1} which was not statistically different ($p > 0.05$) from the values reported for individual equine milk samples.

It is generally accepted that Ca^{2+} play an important role in the physico-chemical and functional properties of milk (Demott, 1968; Geerts *et al.*, 1983; Augustin, 2000; Jeurnink & de Kruif, 1995; Faka *et al.*, 2009) and there is a strong relationship between the [Ca^{2+}] and milk pH (Geerts *et al.*, 1983; Tsioulpas *et al.*, 2007b), whereby a reduction in the [Ca^{2+}] causes a loss of H^+ and an increase in pH (Augustin, 2000). While theoretically, the concentration of ionic Ca depends

on pH and increasing the pH should reduce the [Ca^{2+}] (which is contrary to that found for equine milk), its concentration is also highly dependent on serum levels of PO_4, citrate, Mg and the ionic strength.

The need to provide calcium to the neonate is imperative for mammals during lactation (Oftedal, 1980). The concentration of calcium is directly related to the growth rate of the neonate and is relatively high in horses while the concentration in humans and primates is low at 7-8 mmol L^{-1} (Lewis, 2010). Calcium in milk is found complexed with its constituents which must be broken down to release the calcium in a soluble and, probably, ionized form before it is absorbed; lactose in milk enhances calcium bioavailability (Allen, 1982) and therefore, the high lactose content of equine milk (Table 3.1), favours the absorption of calcium. The horse is born at an advanced stage of development, but with only 17% of their mature bone mineral content. At six months of age, a foal will reach 84% of its mature height (or ~ 68% of its maximum bone mineral content) and at 12 months of age it will attain 94% of its full height and 76% of maximum bone mineral content (Lawrence, 2005). With rapid skeletal development, calcification of bones will be retarded if the milk supply is deficient in calcium and phosphate. The results of this study suggest that a large proportion of equine calcium could be bound directly to casein, increasing its bioavailability (Guégan & Pointillart, 2000), and not in the form of micellar nanoclusters. Coupled with a low colloidal Pi concentration, the high calcium level in equine milk implies that the foal relies on casein-bound calcium for a substantial amount of its bioavailable calcium. Following ingestion of milk, casein-bound calcium could be made available relatively quickly in the foal's stomach through proteolysis of casein rather than (or in conjunction with) acid-induced solubilisation of calcium phosphate. Human milk contains a low level of Ca-binding casein and Ca^{2+} comprise a larger fraction of total Ca, hence while the total Ca in human milk may be significant nutritionally, the Ca^{2+} has important physiological implications (Neville & Peaker, 1981). It is therefore concluded that the exceptionally high content of ionic calcium in equine milk coupled with a large amount of Ca bound directly to casein is physiologically important for the foal.

The ratio of total calcium and total phosphate in any milk is critical for correct absorption and assimilation and is optimized for building of tissue in the neonate (Heaney, 2000). The ratio of total calcium to phosphate found in this study, 2.8:1, is higher than that reported by Anderson (1991) and Martuzzi *et al.* (1997, 1998). The calcium: phosphate ratio should be between 3:1 and 1:1 for proper development in the foal; higher or lower ratios are reported to be detrimental for bone development (Lawrence, 2005). For human infant nutrition, a Ca:P ratio of ~ 2:1 is considered optimal; for bovine milk, the ratio is ~ 1:1, but in equine milk it is about 2:1, i.e., very close to that in human milk.

3.5.5. Concentration of magnesium

Equine milk contained 1.13 mmol L^{-1} Mg, of which 0.89 mmol L^{-1} was in the serum phase and 0.24 mmol L^{-1} was colloidal (Table 3.2). These levels were considerably lower than those in bovine milk which had 5.04 mmol L^{-1} total Mg, with 3.42 and 1.62 mmol L^{-1} in the serum and colloidal phases, respectively; bovine milk has almost double the amount of ash compared to equine milk (Table 3.1). The values found for bovine milk were close to those reported by Tsioulpas *et al.* (2007b). The total Mg content of equine milk was significantly lower than the value of ~ 3.9 mmol L^{-1} reported by Pieszka & Kulisa (2005) but fell within the range of a comprehensive follow-up study by Pieszka & Kulisa (2006). The total concentration of Mg in equine milk is close to that reported for human milk, ~ 1.25 mmol L^{1} (Dórea, 2000). As a percentage of total ash, the Mg in equine milk represents significantly less (~ 0.1%) than that for human milk, in which it is reported to constitute 1.43-2.45% of total ash (Feeley *et al.*, 1983; Nagra, 1989).

3.5.6. Concentration of citrate

The levels of citrate in equine milk and serum were 2.3 and 2.15 mmol L^{-1}, respectively, compared to 9.1 and 8.0 mmol L^{-1} for bovine milk and serum, respectively; the latter values were close to those reported by Konar *et al.* (1971), Walstra *et al.* (1999), Izco *et al.* (2003) and Tsioulpas *et al.* (2007b). In bovine milk, 90% of citrate is in the serum phase, complexed with Ca and Mg, with the

remaining 10% being in the casein micelles (Walstra *et al.*, 1999). Citrate chelates Ca^{2+}, reducing their activity and improves micelle stability. Addition of 5 mmol L^{-1} citrate to bovine milk prevents its coagulation by rennet (Udabage *et al.*, 2001). Serum citrate chelates Ca^{2+} and Mg^{2+} and reduces Ca^{2+} activity, which improves micellar stability (Horne & Parker, 1981b).

3.5.7. Concentration of inorganic phosphate

Equine milk contained 6.81 mmol L^{-1} inorganic phosphate, significantly lower than that in bovine milk, 20.40 mmol L^{-1}. Reported values in the literature for total phosphate in milk do not always indicate whether the values were for total phosphate or total inorganic phosphate and some studies suggest that equine milk has ~ 24 mmol L^{-1} total phosphate. If this is the case, we believe that the organic phosphate content of equine milk is very high compared to ~ 10 mmol L^{-1} in bovine milk and is ~17 mmol L^{-1}, which agrees with Holt & Jenness (1984). Human milk has a low total phosphate content compared to bovine milk (Guo & Hendricks, 2008) but a relatively high proportion of organic phosphate (Lenstrup, 1926) suggests greater bioavailability of phosphorus in equine milk compared to other species.

3.6. ***Measurement of selected physical properties***

3.6.1. Refractive index

The physical properties of equine and bovine milk determined in this study are compared in Table 3.3. The refractive index of equine milk was 1.3420, i.e., similar to the refractive index reported by Waelchli *et al.* (1990) and marginally lower than that found for bovine milk, which agrees with the value reported by Walstra & Jenness (1984). The lower refractive index of equine milk compared to bovine milk is probably due to the lower level of total solids in the former (Table 3.1).

3.6.2. pH

The pH of the equine milk was 7.27 (Table 3.3), which was similar to the value reported by Mariani *et al.* (2001) and significantly higher than that found for

bovine milk, pH 6.63. The pH of bovine milk at 25°C is in the range 6.5-6.7, with a mean value of 6.63 (Fox & McSweeney, 1998; Tsioulpas *et al.*, 2007a). The pH of equine milk is very close to that of human milk, which is reported to be 7.0-7.1 at 3 months *post-partum* and up to 7.4 at 10 months *post-partum* (Morriss *et al.*, 1986). The pH of bulk equine milk, measured on 3 separate occasions, was 7.31 ± 0.04, which was statistically similar to the average value reported for individual equine milk samples.

3.6.3. Viscosity

The viscosity of milk is affected by the state and concentrations of fat and protein, temperature, pH and age of the milk (Jenness & Patton, 1976). Under most conditions, milk behaves as a Newtonian liquid with shear stress proportional to the shear rate (Walstra *et al.*, 1999). The viscosity of equine milk, 1.5031 mPa s, (Table 3.3) was lowered than that of bovine milk at 1.6314 mPa s, probably due to the lower content of total solids (especially protein) in equine milk. The value for bovine milk was similar to that reported by Neville & Jensen (1995) but lower than ~1.3 mPa s reported by Spreer (1998).

3.6.4. Zeta Potential

The zeta potential (ζ-potential) of skimmed equine and bovine milks were -10.3 and -20.5 mV, respectively, Table 3.3. The value for bovine milk was within the range of several studies (-19 to -20 mV; Dalgleish, 1984; Anema & Klostermayer, 1996; Famelart *et al.*, 2003; Gastaldi *et al.*, 2003; Philippe *et al.*, 2005; Glantz *et al.*, 2010). ζ-potential is regarded as an indicator of charge interactions and can be related to the stability of colloidal dispersions. It has been reported that colloids with a high ζ-potential are stable whereas those with a low value tend to aggregate (Walstra & Jenness, 1984). However, casein micelles are stabilized primarily sterically rather than electrostatically and, in the case of equine micelles, are relatively stable although the ζ-potential is low. The stability of bovine casein micelles is partially due to a net-negative charge on the micelle surface. If temperature or pH is increased, the electrophoretic mobility and the ζ-potential of the micelles increase (Darling & Dickson, 1979).

3.6.5. Colour of equine milk

Equine milk is significantly whiter than bovine milk (Table 3.3). The positive b* value for bovine milk is indicative of the presence of carotene in the sample. After the removal of fat, both milks became whiter/brighter. The large negative a* value for bovine milk is probably due to a combination of carotene and riboflavin in the milk and because of the increased contribution of the latter to milk colour after skimming, the value increases.

The opaque, white colour of milk is due to the scattering of light by the dispersed phase of fat globules and casein micelles. L* is primarily a measurement of turbidity at a scattering angle of 180^{o}. Turbidity scales to the first power of particle number but to the 6^{th} power of particle size; therefore, the influence of particle size, e.g., the considerably larger micelles of equine milk, will influence L*. The whiteness of milk is due to the size and number of these particles; smaller particles scatter light of shorter wavelengths as in the case of skimmed milk where casein micelles, in the absence of fat globules, scatter more blue light (Chandan, 2006).

Table 3.3: Physical properties of bovine and equine milk

Property	Equine milk	Bovine milk
pH	7.27 ± 0.02	6.63 ± 0.01
Refractive Index	1.3420	1.3440
L* a* b* whole milk		
L*	85.99 ± 0.03	75.54 ± 0.06
a*	-1.86 ± 0.01	-6.52 ± 0.02
b*	-0.61 ± 0.01	+ 2.79 ± 0.02
L*a* b* skimmed milk		
L*	87.12 ± 0.04	79.84 ± 0.02
a*	-2.32 ± 0.005	-7.46 ± 0.02
b*	-0.11 ± 0.006	-2.31 ± 0.02
Viscosity (mPa s)	1.5031 ± 0.03	1.6314 ± 0.024
Zeta (ζ)- potential (mV)*	-10.3 ± 0.9	-20.5 ± 1.6
Fat globule size (μm)		
D [4,3]	3.66 ± 0.23	3.43 ± 0.01
D [3,2]	1.05 ± 0.06	1.52 ± 0.71
D[v, 0.1];	0.33 ± 0.01	0.34 ± 0.01
D[v, 0.5]	3.27 ± 0.1	3.13 ± 0.25
D[v, 0.9]	6.43 ± 0.15	6.13 ± 0.35
Average micelle diameter (nm)	276.75 ± 1.90	179.00 ± 4.20

Values are means of data for five individual equine milk samples, ± standard deviations. * mean calculated from 5 replicates

3. 6.6. Average fat globule size and size distribution

The average volume surface mean diameters, D[4,3], of equine milk fat globules was 3.66 μm (Table 3.3), which is larger than the value , 2-3 μm, reported by Welsch *et al.*, (1988) and close to that (~ 4 μm) reported for human milk fat globules by Michalski *et al.* (2005). In this study, the D[4,3] value for bovine milk fat globules was 3.43 μm, which is within the 3-4 μm range reported by Mulder & Walstra (1974) and Couvreur *et al.* (2007). Figure 3.1 shows the fat globule size distribution of two representative samples of equine milk and a bovine milk sample for comparison. While the distributions were similar, bovine milk had a lower percentage of fat globules in the lowest range than equine milk and more fat globules at the higher end of the scale as indicated by a higher value of D [v, 0.9].

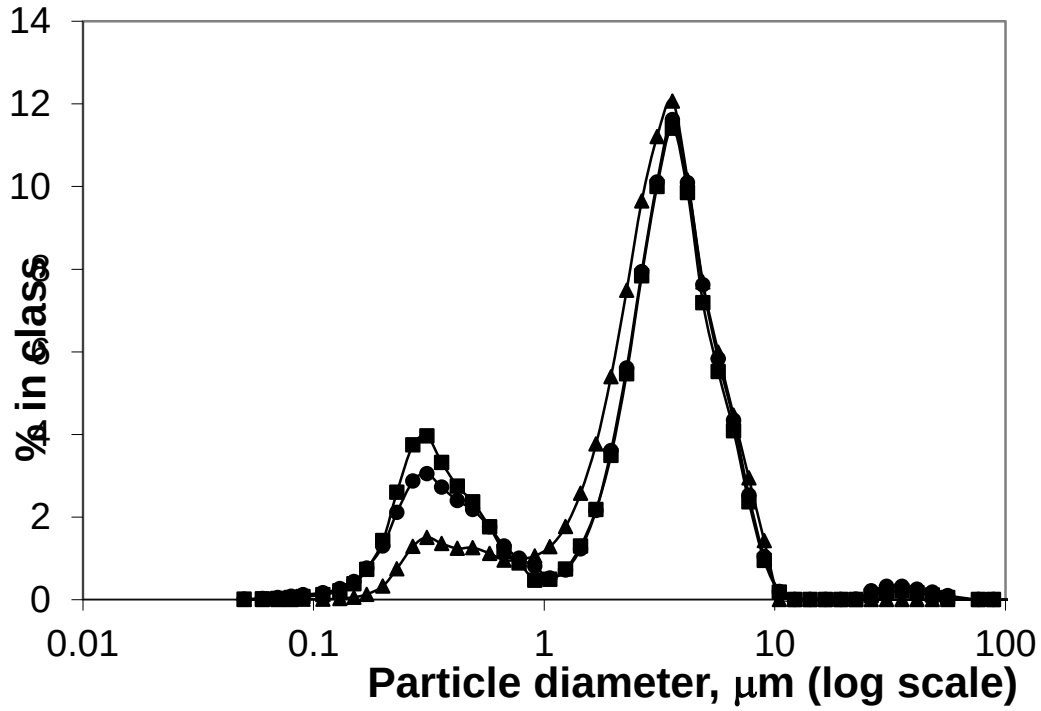

Figure 3.1. Fat globule size distribution for two samples of equine milk, (-●-), (-■-) and bovine milk, (-▲-).

3.6.6. Average casein micelle size and size distribution

The hydrodynamic radius of bovine and equine casein micelles, determined by multi-angle DLS, is expressed as a function of the scattering wave vector in Figure 3.2. In agreement with the results of Buchheim *et al.* (1989), equine casein micelles were considerably larger than bovine casein micelles. At a scattering angle of 90º, which is commonly used in single-angle DLS, the hydrodynamic radius of bovine and equine micelles was 88 or 127 nm, respectively.

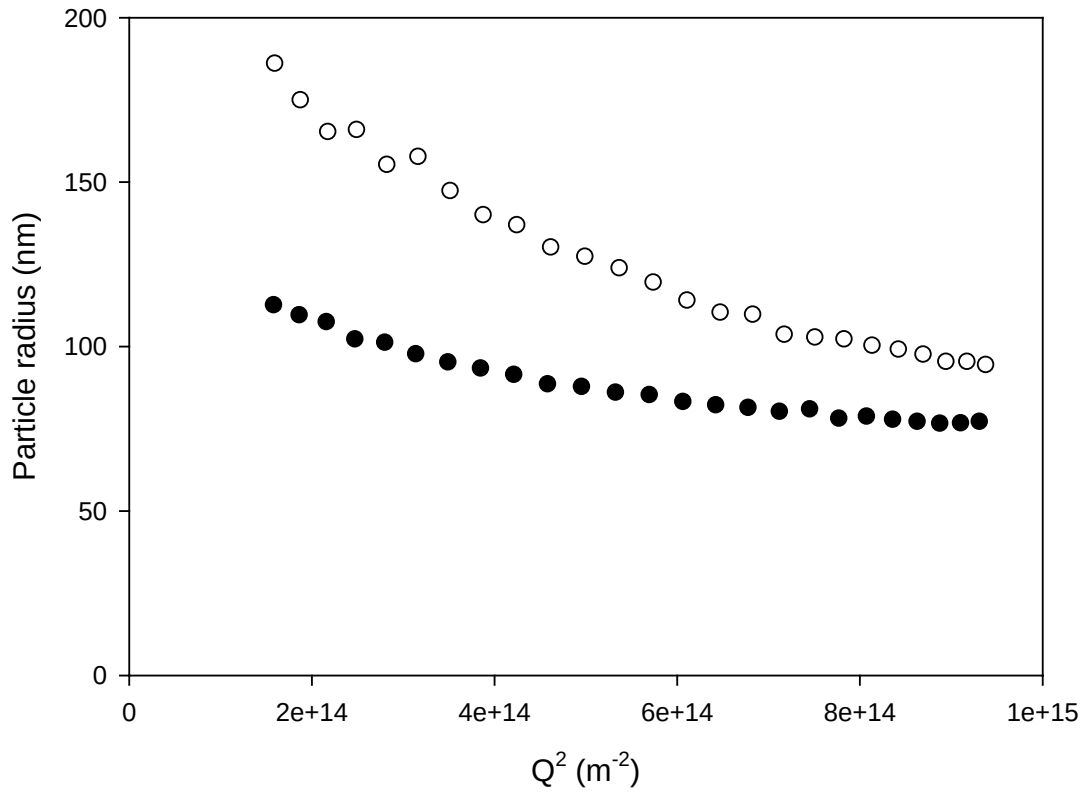

Figure 3.2. Average particle radius as a function of the squared scattering wave vector (Q^2) for skimmed bovine (●) or equine (○) milk. Values of means of data from experiments on five individual milk samples. The coefficient of variation was <5% of the reported value for each data point.

The higher dependence of the radius of equine micelles than that of bovine casein micelles on the scattering wave vector indicates a more polydisperse size distribution of casein micelles in equine milk (Finsy, 1994). Micelle size data determined by fixed angle DLS gave diameters of 179 and 277 nm for bovine and equine milks, respectively, which compared well with the multi-angle DLS results. The *Z*-average hydrodynamic diameter of bovine casein micelles found in this study was well within the range of values reported by Lin *et al.* (1971), Schmidt *et al.* (1973), Holt *et al.* (1978), Walstra & Jenness, 1984), de Kruif (1998) and Glantz *et al.* (2010), while the value for equine micelles was similar to that reported by Welsch *et al.* (1988). The use of lactose-free SMUF as diluent for fixed angle DLS measurements or equine milk dialysate in the case of multi-angle DLS measurements gave similar results. When particle size distribution is polydisperse, multi-angle DLS is generally a preferable method for measuring size distributions. Furthermore, Z-average diameter calculations from fixed-angle DLS are calculated from a correlation function and the fitting of data only takes into account the initial stage of the correlation function and hence may overestimate particle size. In the present study the multi-angle calculation was determined by fitting the second cumulant to the autocorrelation function to determine a diffusion coefficient and hence particle size which is generally considered to be more accurate, however, micelle size calculations using both methods in this study were very close. The casein micelle size distribution in samples of equine and bovine milk is shown in Figure 3.3, the equine casein micelles were larger and considerably more polydisperse

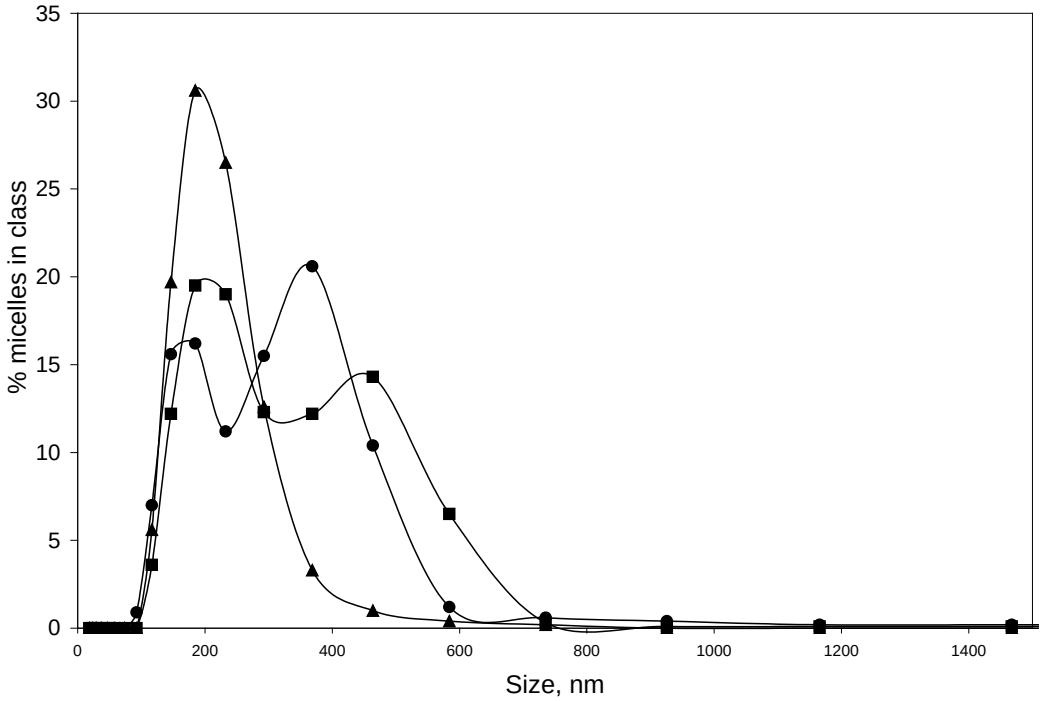

Figure 3.3. Casein micelle size distribution in two samples of equine milk, (-●-), (-■-) and a sample of bovine milk, (-▲-) determined by laser light scattering.

3.6.7. Effect of pH on the solubility of equine milk proteins

The concentration of soluble (non-sedimentable) protein in equine milk decreased with decreasing pH (Figure 3.4), reaching a pH minimum at ~ pH 4.2, which is in agreement with the value of the isoelectric point of equine casein reported by Egito *et al.* (2001). A considerable proportion of the protein in equine milk remained soluble at very low pH, due to the high whey protein content. From pH 3.6 to 3.4 the non-sedimentable protein content of equine milk increased. The non-sedimentable protein content of bovine milk decreased slowly to pH 5.5 and thereafter abruptly. Bovine non-sedimentable protein was at a minimum at pH ~ 4.5 and again at ~ pH 4.1, thereafter it increased from pH ~ 4.0 to 3.4. It is reported that as the pH of bovine milk decreases to the point of minimum solubility, the κ-casein brush on the micelle surface collapses as the glutamic residues become protonated close to their pKa value, with the result that the brush looses its solubility. As the pH decreases further, some of the previously uncharged residues become positively charged (pH < pKa) and this may increase the brush solvency again which will keep some protein in suspension. The mass of human κ-casein is reported to almost double when fully glycosylated and the same may be true for equine κ-casein. Therefore, when the isoelectric point of the polypeptide chain is reached as the pH is reduced, the solubility of κ-casein may be maintained to a certain extent due to the presence of sugar groups.

3.6.8. Effect of gravitational force on protein sedimentation in equine and bovine milk

Due to the large size of the casein micelles in equine milk, centrifugation at a gravitational force > 1,000 x g caused significant sedimentation of the protein, unlike bovine milk, where sedimentation occurred at forces > 10,000 x g (Figure 3.5). Throughout this study, it was found that equine milk was prone to significant sedimentation even on standing. Care was always taken to ensure redispersion of the protein and occasionally it was necessary to redisperse a small protein pellet formed on the removal of fat.

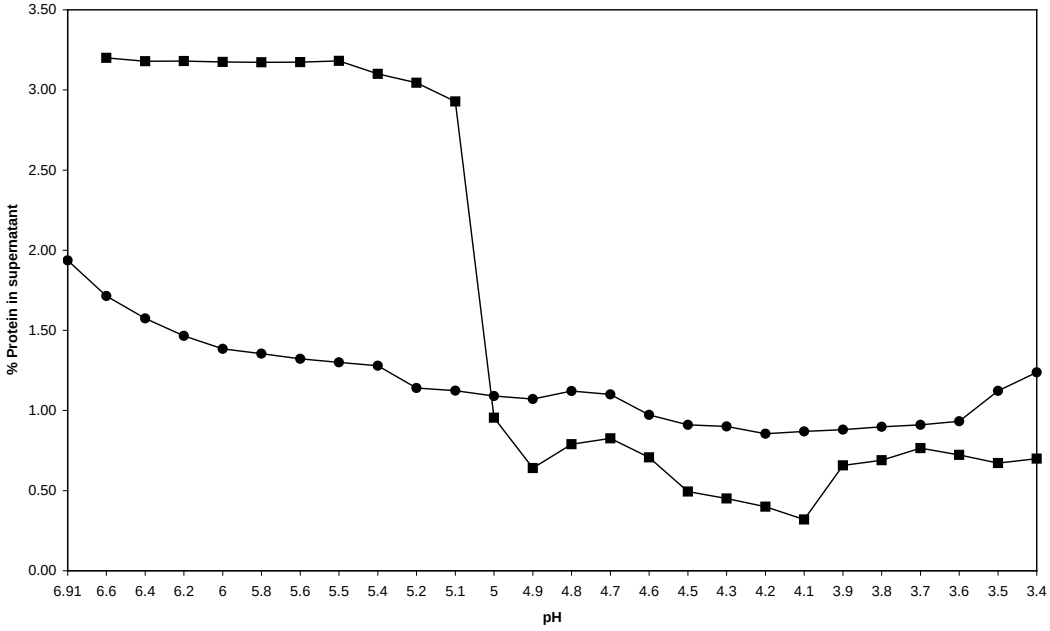

Figure 3.4. Influence of pH on the non-sedimentable protein in equine (-●-) and bovine (-■-) milk. The coefficient of variation was <5% for all data points

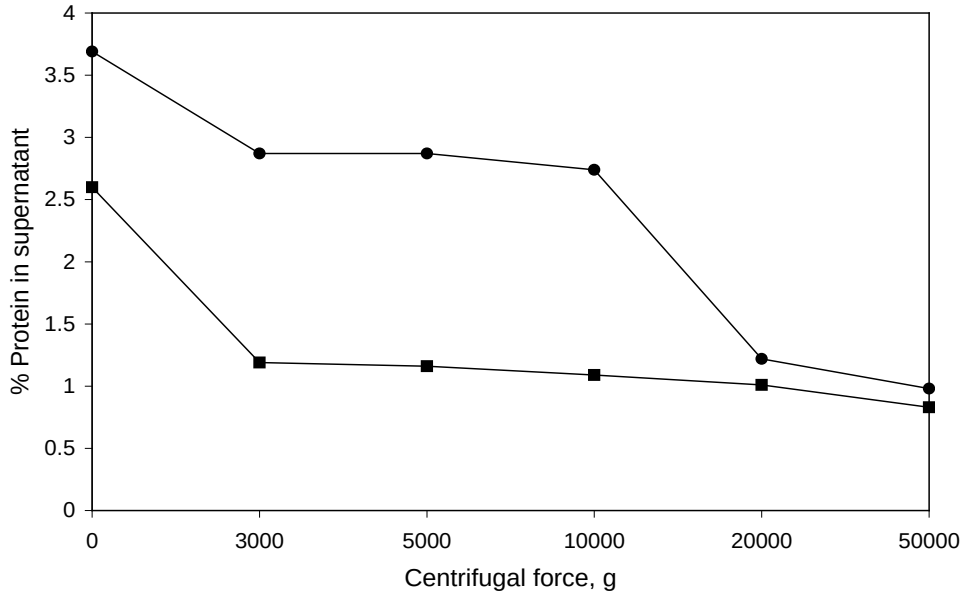

Figure 3.5. Influence of centrifugal force at 30^{o}C for 30 min on the % protein in the supernatants of bovine (●) or equine (■) milk. Data points are the average values from triplicate analysis. The coefficient of variation was <5% of the reported value for each data point.

3.6.9. Rennetability of equine milk

Figure 3.6 shows the rennetability of bovine, equine and asinine milk as indicated by the storage modulus, G', over 120 min at pH 6.6. Equine milk was not susceptible to rennet-induced coagulation at pH 6.6, but the asinine sample did coagulate, although the resulting gel was very weak. It has been reported that asinine casein micelles are considerably smaller than those of equine milk (Salimei, 2011) which would mean a higher content of κ-casein and potentially improved rennetability compared to equine milk.

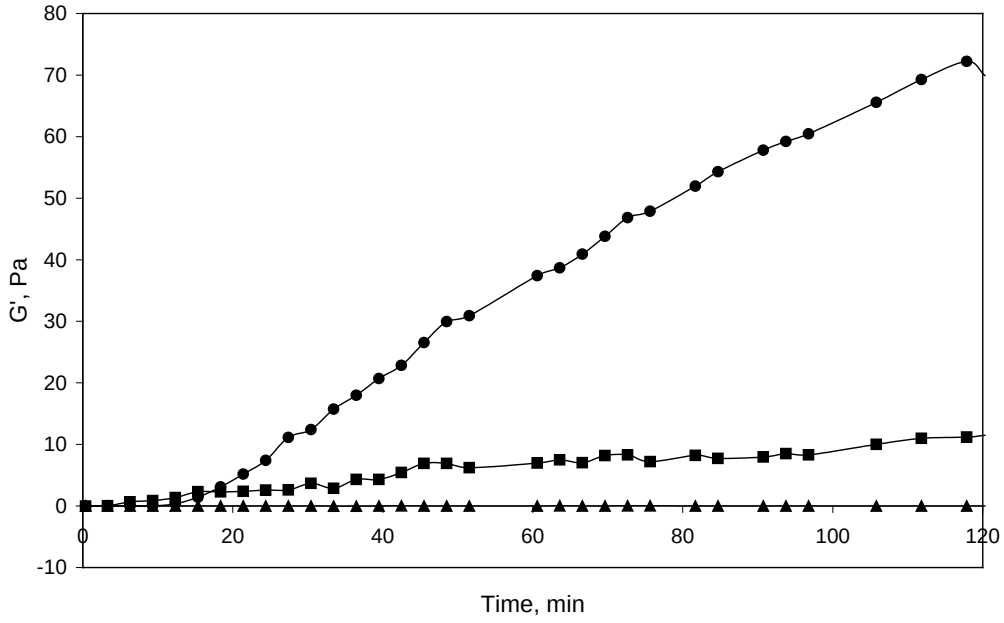

Figure 3.6. Effect of renneting with 6 μl mL^{-1} Maxiren 180 on the storage modulus, G´, of bovine (●), asinine (■) or equine (▲) milk at pH 6.6. The coefficient of variation was <5% of the reported value for each data point.

When suspended at 25 g L^{-1} casein (which approximates the casein content of bovine milk) in the ultracentrifugal supernatant of bovine or equine milk, no increase in $\tau_{½}$ from DWS for equine casein micelles was observed on renneting, whereas bovine casein micelles showed a steep increase in $\tau_{½}$ after ~8 min, which is indicative of micellar flocculation (Vasbinder *et al.*, 2001; Hemar *et al.*, 2004). Continued incubation of equine casein micelles dispersed in equine serum with chymosin for up to 36 h at 32°C did not affect $\tau_{½}$ (data not shown). DWS was a particularily useful technique as it has long been known that DWS is a very sensitive method for monitoring micelle flocculation and, in particular, the onset of milk coagulation (Dalgleish & Horne, 1991; Hemar *et al.*, 2004; Vasbinder *et al.*, 2001, 2003; Alexander &Dalgleish, 2004).

The failure of rennet to coagulate suspensions containing 25 g L^{-1} equine casein micelles at pH 6.6 (Figures 3.7A and B) is related to the equine micelles, rather than to the composition of the equine milk serum, since equine casein micelles suspended in bovine milk serum also failed to coagulate (Figure 3.7A), whereas bovine casein micelles suspended in equine milk serum (Figure 3.7B) coagulated in a manner similar to those suspended in bovine serum (Figure 3.7A). Equine κ-casein is susceptible to rennet-induced hydrolysis at the Phe_{97}-Ile_{98} bond (Egito *et al.*, 2001), but its hydrolysis is considerably slower than that of bovine κ-casein (Kotts & Jenness, 1976); the amount of calf chymosin required for comparable rates of κ-casein hydrolysis was ~5,000 times greater for equine than for bovine κ-casein (Kotts & Jenness, 1976).

The inability of calf chymosin to hydrolyse significant amounts of equine κ-casein within a relatively short time is probably, at least partially, responsible for the inability of chymosin to induce noticeable gelation of equine milk (Figure 3.6), even after renneting for 36 h (data not shown). Figure 3.8 shows the results from small deformation rheological measurements of the rennetability of bovine and equine micelles with reversed serum phases. The results were similar to those found by DWS but demonstrate the later stages of gel formation more clearly. One feature of interest is that bovine micelles in equine serum formed a firmer gel, as indicated by a higher G´ value than the bovine micelles suspended

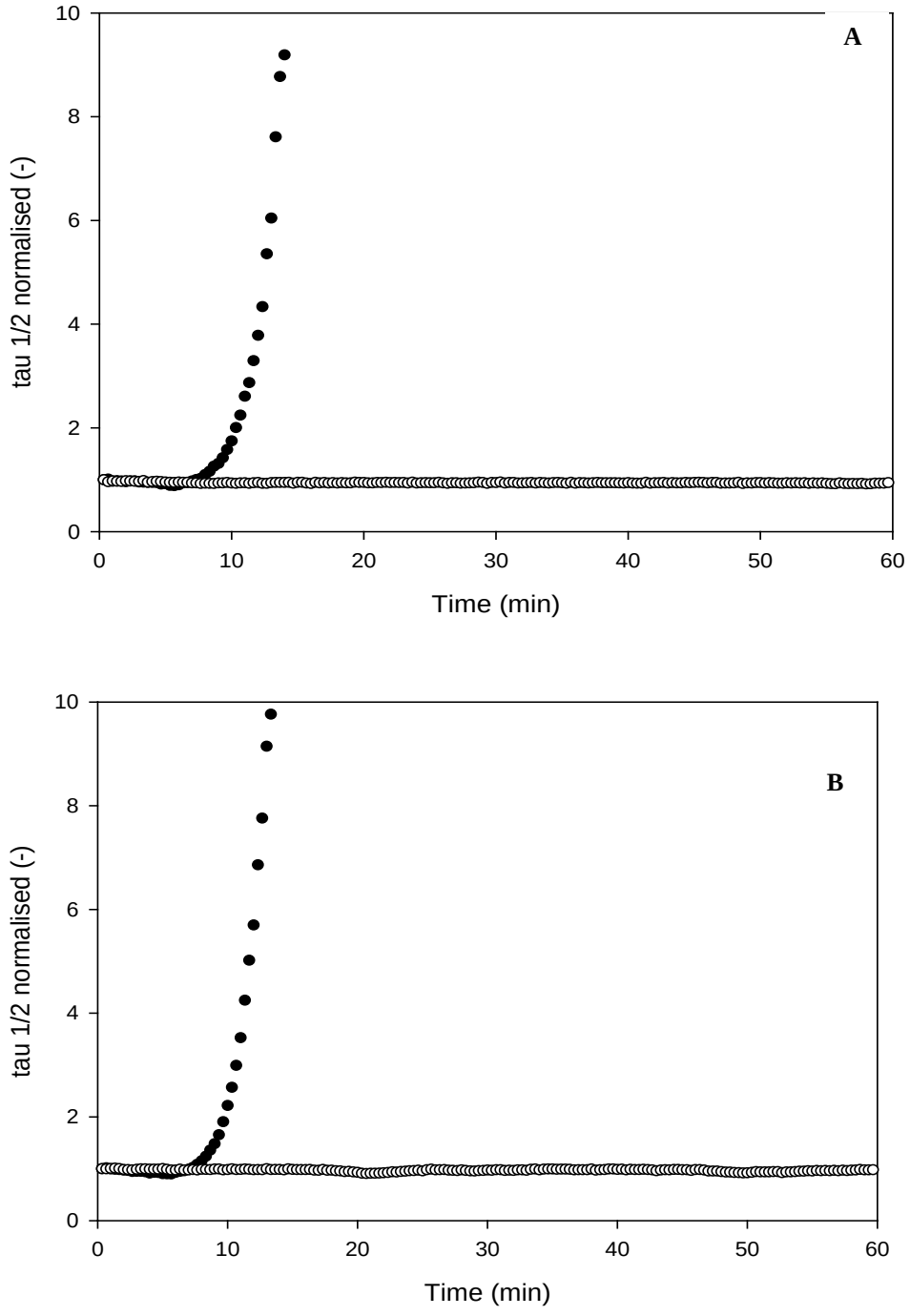

Figure 3.7. Effect of renneting with 6 μL mL^{-1} Maxiren 180 on the tau ½ ($\tau_{½}$) from DWS of 25 g L^{-1} suspensions of bovine (●) or equine (○) casein micelles suspended in the ultracentrifugal supernatant of bovine (A) or equine (B) milk. The coefficient of variation was <5% of the reported value for each data point.

in bovine serum. The higher concentration of ionic calcium in the equine serum would account for the difference in gel strength.

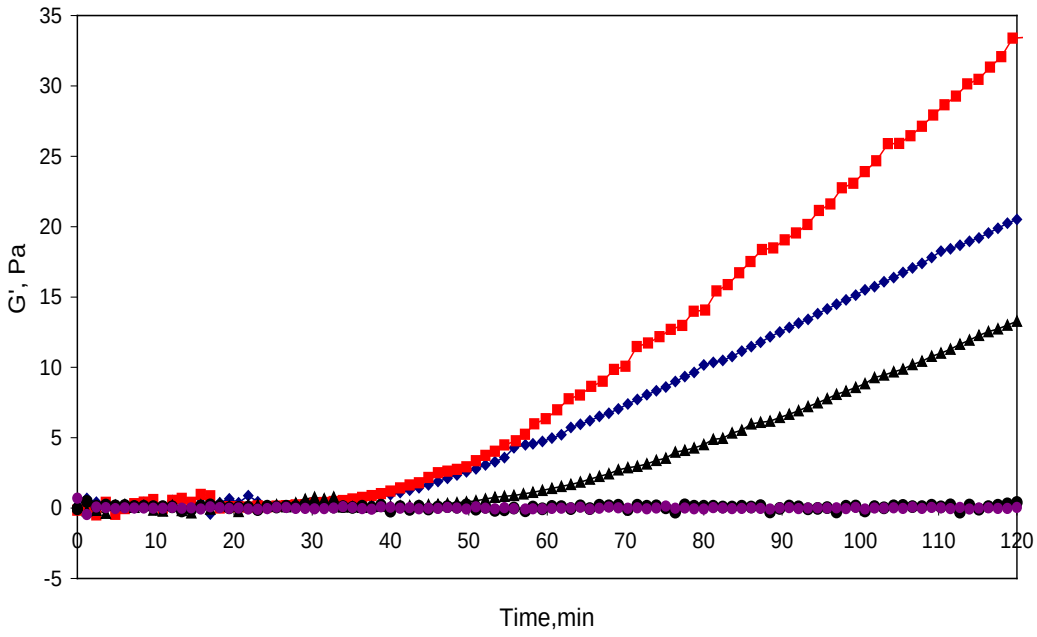

Figure 3.8. Effect of renneting with 6 μL mL^{-1} Maxiren 180 at pH 6.6 on the storage modulus, G´, of bovine skim control (-■-), 25 g L^{-1} bovine casein micelles in equine serum (-♦-) or bovine serum (-▲-), equine micelles in equine (-●-) or in bovine serum (-●-).The coefficient of variation was <5% of the reported value for each data point.

3.6.10. Acid-induced flocculation of equine casein micelles

DWS showed that equine casein micelles were susceptible to acid-induced flocculation, but the extent was considerably lower than that of bovine casein micelles (Figure 3.9). For bovine casein micelles, $\tau_{½}$ increased rapidly with decreasing pH when the point of flocculation was reached, which is indicative of acid-induced flocculation of casein micelles (Vasbinder *et al.*, 2001). However, for equine micelles, $\tau_{½}$ increased only gradually with decreasing pH; even at pH 4.6, below the gelation point of bovine micelles, $\tau_{½}$ had increased only 4-fold and the equine micelle suspension showed no signs of gelation.

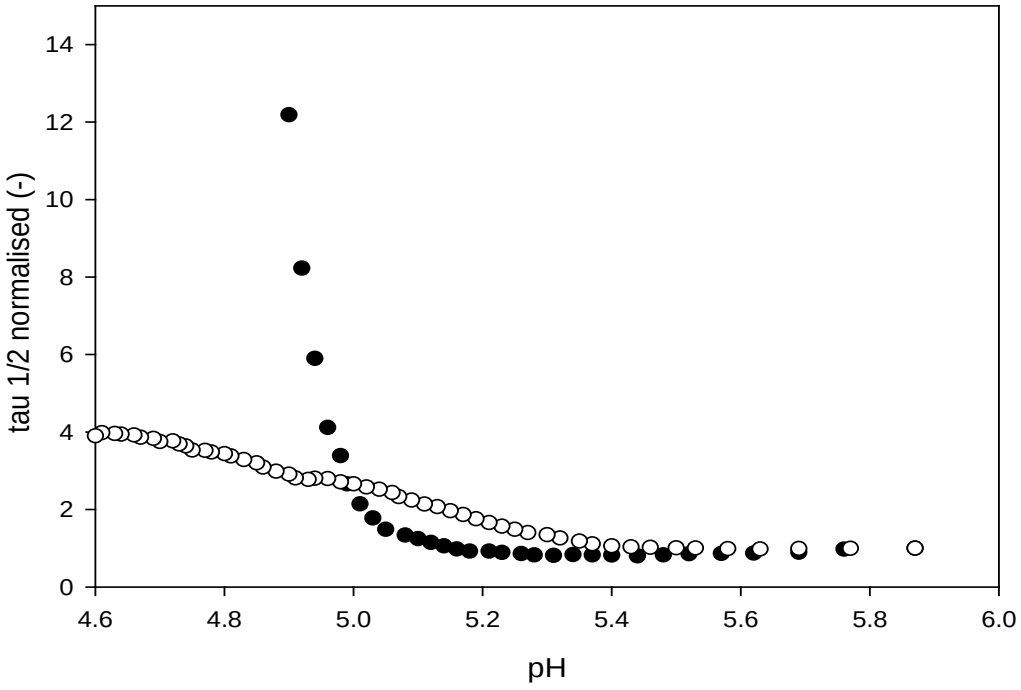

Figure 3.9. Influence of acidification by 30 g L^{-1} glucono-δ-lactone at 32°C on the tau ½ ($\tau_{½}$) of 25 g L^{-1} suspensions of bovine (●) or equine (○) casein micelles in their ultracentrifugal supernatants. The coefficient of variation was <5% of the reported value for each data point.

Di Cagno *et al.* (2004) reported that equine milk acidified at pH 4.2, which was determined to be the isoelectric point of equine casein in agreement with Egito *et al.* (2001), had an apparent viscosity of only ~10 mPa s which was only 7 times higher than the value found in this study (1.5031 mPa s) for equine milk at its natural pH and is probably indicative of micellar flocculation rather than gelation; this is further highlighted by the fact that the equine milk fermented to 4.2 by Di Cagno *et al.* (2004) showed near-Newtonian behaviour at a shear rate > 1 s^{-1} (i.e., apparent viscosity was independent of shear rate), which is atypical behaviour for a gelled system.

Rheological measurements (Figure 3.10) for equine, asinine and bovine milks following acidification confirmed the result obtained by DWS. Asinine milk formed a weak gel with considerably more structure than that from equine milk, which warrants further investigation. Reasons for the inability of equine casein micelles to form an acid-induced gel are unclear. The most important factors determining the structure of acid gels are casein content, pH and calcium content of the milk. At low pH, calcium is progressively dissociated from the casein micelles and, in addition, neutralization of negative charges on the casein favours extensive aggregation and fusion of micelles which tend to form a gel. In the case of bovine milk, a gel is formed at pH ~ 5.1 (Parry, 1974). The low casein content of equine milk is probably the most important factor preventing gelation. The high level of glycosylation of equine κ-casein could also be a factor; the molecular mass of equine κ-casein prior to post-translational modification is ~18.8 kDa (Lenasi *et al.*, 2003), whereas after post-translational modification it is ~25.3 kDa (Miranda *et al.*, 2004), suggesting that carbohydrate moieties represent ~35% of total mass of equine κ-casein. For bovine κ-casein, glycosylation increases the mass of κ-casein by <5% (Vreeman *et al.*, 1977). The degree of glycosylation of κ-casein can have considerable effects on the colloidal stability of casein micelles (Dziuba & Minkiewicz, 1996), including acid-induced coagulation (Cases *et al.*, 2003).

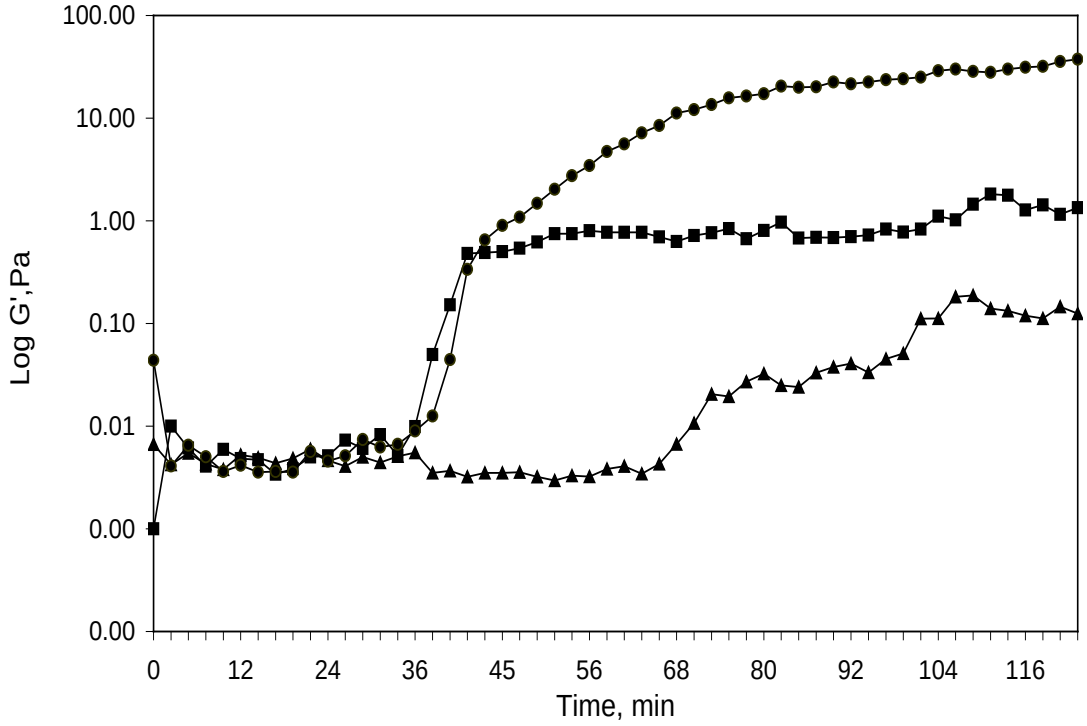

Figure 3.10. Influence of acidification by 30 g L^{-1} GDL at 32°C on the storage modulus, G′, of bovine (●), asinine (■) or equine (▲) milk. The coefficient of variation was <5% of the reported value for each data point.

3.6.11. Ethanol stability of equine milk

At its natural pH (~7.2), the ethanol stability of equine milk was ~45%, whereas that of bovine milk analysed under identical conditions at its natural pH (~6.7) is 70-75% (Huppertz *et al.*, 2004; Huppertz & De Kruif, 2007). Figure 3.11 shows the results for the ethanol stability test on equine milk. The ethanol stability of unconcentrated equine milk increased in a near-sigmoidal fashion with milk pH, from ~12% at pH 5.5 to ~55% at pH 7.5 (Figure 3.12). This sigmoidal profile was similar to that reported for bovine milk (see Horne, 2003), although the actual ethanol stability of equine milk (Figure 3.12) was lower than reported for bovine milk analysed under identical conditions (Huppertz *et al.* 2004; Huppertz & De Kruif, 2007).

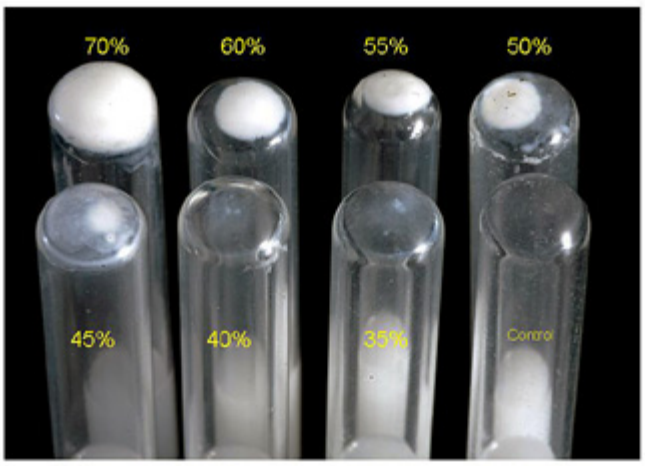

Figure 3.11. Assessment of the ethanol stability of equine milk. Percentages indicate that an ethanol content of 45% is the minimum concentration that destabilizes the milk proteins in equine milk. The control sample was a mixture of equine milk and an equal volume of water.

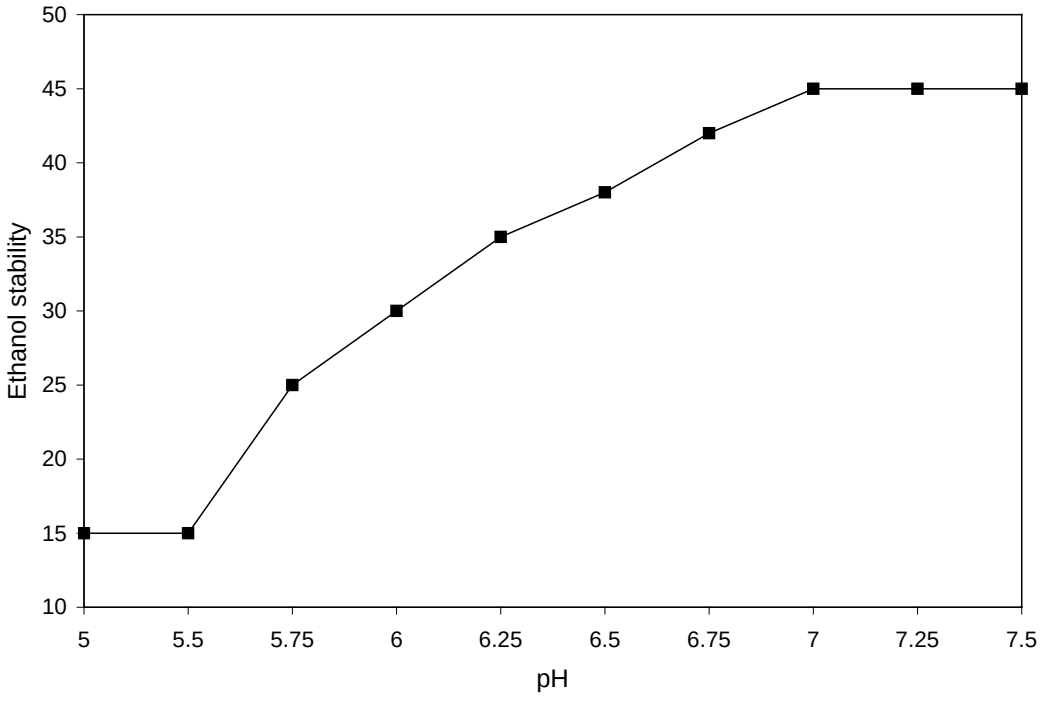

Figure 3.12. Ethanol stability-pH profile of skimmed equine milk. Values are means of data from triplicate experiments on 5 individual milk samples. The coefficient of variation was <5% of the reported value for each data point.

Horne & Parker (1981a) found that serum phase components govern the sigmoidal shape and position of the ethanol stability-pH profile in bovine milk and that the ionic calcium concentration had a significant affect on the profile. Calcium, magnesium, phosphate and citrate concentrations also influence the ethanol-pH stability curve (Donnelly & Horne, 1986; Horne, 1987) along with the ionic strength and pH of the milk (Horne, 1987; Horne, 1992; Horne & Parker, 1979). The high concentration of ionic calcium in equine milk (2.66 mmol L^{-1}) contributes significantly to the low ethanol stability (Horne & Parker, 1981a; Horne, 1987; Horne, 2003). Since micelle size *per se* does not influence the ethanol stability of bovine casein micelles (O'Connell & Fox, 2000), the lower ethanol stability of equine milk compared to bovine milk is unlikely to be related to the differences in micelle size (Table 3.3). . A lower surface coverage (brush density) of equine micelles is also likely to contribute to the low ethanol stability of equine milk as the ethanol stability of bovine casein micelles decreases progressively with decreasing density of κ-casein surface coverage of the micelles (De Kruif, 1998). Miranda *et al.* (2004) estimated that κ-casein represents <2% of total equine casein. In contrast, κ-casein represents ~12% of total casein in bovine milk (Walstra *et al.* 2006).

At present it is unclear whether the low content of κ-casein in equine milk has implications for micellar colloidal stability or whether the surface layer of equine casein micelles contains higher proportions of other caseins The high concentration of ionic calcium and the low level of κ-casein in equine milk probably contribute to its low ethanol stability.

3.6.12. Heat-induced coagulation of equine milk

The heat coagulation time (HCT) of unconcentrated skimmed equine milk at 140°C increased in an almost sigmoidal fashion as a function of pH, from < 2 min at pH 6.3-6.9 to > 45 min at pH 7.0-7.2; a slight maximum was observed at pH 7.2 (Figure 3.13). Fox & Hoynes (1976) reported that the heat stability of equine milk is very variable; all (n=10) individual equine milk samples analysed by them were very unstable at the natural pH, which ranged from 6.8 to 7.2, but

HCT increased with increasing pH. The HCT-pH profile of most samples showed a maximum, usually at pH 7.4 to 7.8, above which the HCT decreased and remained low at higher pH; however, some samples remained stable >pH 8.0 when assayed at 100ºC. The HCT-pH profile of unconcentrated equine milk (Figure 3.13) differs considerably from that generally observed for bulked unconcentrated bovine milk, which has a maximum at pH ~6.6 and a minimum at pH ~6.9 (O'Connell & Fox, 2003; Singh, 2004). Pre-heating unconcentrated milk shifted the pH-HCT profile of equine milk to higher pH values and reduced the HCT in the pH range around the maximum (Figure 3.13), similar to the effect in bovine milk (O'Connell & Fox, 2003; Singh, 2004).

The low heat stability of equine milk at pH < 6.9 (Figure 3.13) is probably related to the high concentration of ionic calcium in milk (~2.66 mmol L^{-1}; Table 3.2), as the heat stability of bovine milk, particularly at low pH, is reduced considerably by increasing ionic calcium content (O'Connell & Fox, 2003). The low casein content of equine milk found in this study and by Zicker & Lönnerdal (1994) and Park (2006) probably contributes to the high heat stability of unconcentrated equine milk at pH >6.9 (Figure 3.13). The distinct maximum and minimum which are observed in the pH-HCT profile of all bulked, and most individual, unconcentrated bovine milks (O'Connell & Fox, 2003; Singh, 2004) were not observed for equine milk (Figure 3.13).

The HCT maximum and minimum are caused primarily by the ability of bovine β-lg to reduce heat stability in the pH-range ~6.7-7.0 by promoting heat-induced dissociation of κ-casein, although changes in the mineral balance also contribute (O'Connell & Fox, 2003; Singh, 2004). The pH of maximum heat stability of bovine milk is most likely due to κ-casein and β-lg complexing at the micelle surface, stabilizing the micelle by reducing the dissociation of κ-casein, increasing steric stabilization, ζ-potential and hydration. The absence of a distinct maximum and minimum in the pH-HCT profile of unconcentrated equine milk (Figure 3.13) may indicate a much lower extent, or even absence, of heat-induced dissociation of equine κ-casein from the micellar surface. This may be related to the higher thermal stability of equine β-lg (Bonomi *et al.*, 1994), and

the absence of a free sulphydryl group therein, although further study is required to clarify this.

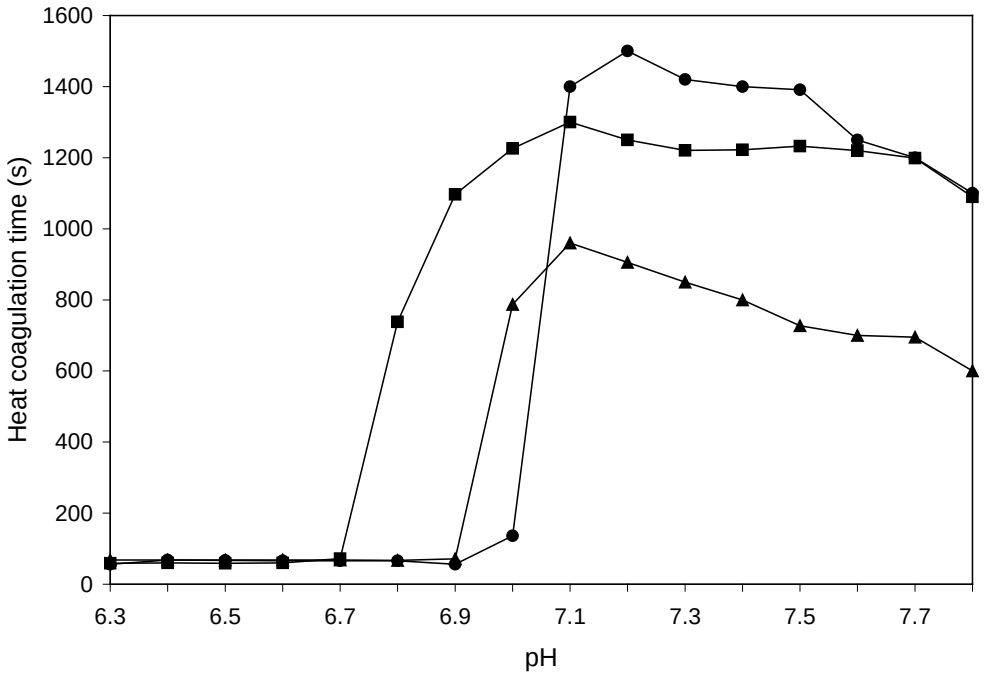

Figure 3.13. Heat coagulation time-pH profile of skimmed equine milk: unconcentrated unheated (-●-), unconcentrated pre-heated for 10 min at 90°C (-■-) or concentrated (2×) unheated (-▲-). Measurements were made at 140°C for unconcentrated milks or 120°C for concentrated milk. The coefficient of variation was <5% of the reported value for each data point.

It has been demonstrated for bovine milk that the ethanol stability test is not a good indicator of heat stability (Horne & Parker, 1979; Horne & Muir, 1990; Chavez *et al.*, 2004); ionic calcium concentration is widely accepted as contributing to both heat and ethanol stabilities and any increase in Ca^{2+} causes a decrease in both; however, the mechanisms and sensitivities involved may be

different (Horne & Parker, 1981a; Horne, 1987; Horne & Muir, 1990; Chavez *et al*., 2004).

Porcine milk has poor heat stability (< 2 min at 95^{o}C) and poor ethanol stability (66-68%, v/v) (Hoynes & Fox, 1975). Caprine milk is also reported to be much less stable to heat and ethanol (44%, v/v; Guo *et al.*, 1998) than bovine milk (Fox & Hoynes, 1976; Horne & Parker, 1982; Guo, 1985), probably due to differences in its chemical composition and casein profile (Horne & Parker, 1982; Guo *et al*., 1993) and its low ratio of sodium to potassium (Guo & Luo, 1992) or for some caprine breeds, the lack of α_{s1}-casein (Horne & Parker, 1982).

3.6.13. Preliminary characterization of equine casein by gel electrophoresis

Urea-PAGE (Figure 3.14) of equine caseins showed the greater heterogeneity of the α_s- and β-caseins of equine casein compared to those of bovine casein, indicative of a higher level of post-translational modifications of equine caseins than bovine caseins (Miranda, 2004). Multiply-phosphorylated isoforms of equine β-casein containing three to seven phosphoserine residues have been reported, with the isoelectric point varying from pH 4.74 to 5.30 (Girardet *et al.*, 2006; Matéos *et al.*, 2009b). Bovine β-casein, which contains 4 or 5 phosphorylated serine residues, has an isoelectric point of 5.0 to 5.5 (Swaisgood, 2003). Matéos *et al.* (2009a) determined the different levels of phosphorylation in the isoforms of equine α_{s1}-casein and identified 36 different variants with 2 to 8 phosphate groups including variants formed from post-transcriptional modifications, i.e., skipping involving exon 7 and/or exon 14 and variants involving a cryptic splice site located at the start of exon 11, corresponding to Gln_{91} (Lenasi *et al*., 2003).

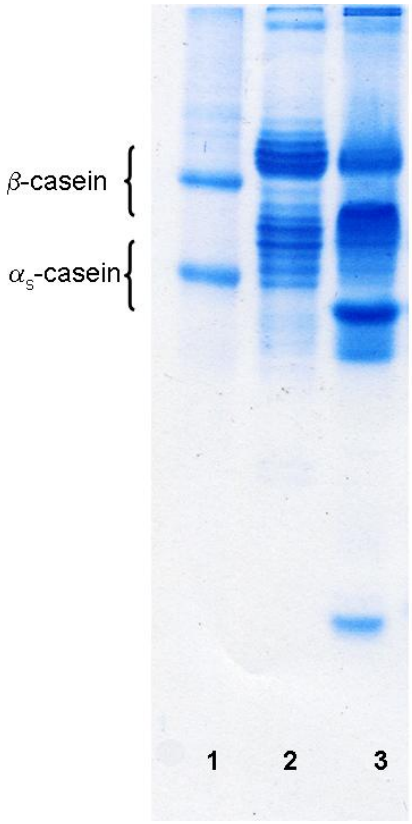

Figure 3.14. Urea-PAGE of bovine sodium caseinate (Lane 1), equine casein (Lane 2) and equine milk (Lane 3).

3.7. Conclusions

The composition of equine milk, including the quantity and distribution of its nitrogenous components and principal salts was considerably different to bovine milk. The compartmentalization and molecular forms of the minerals in equine milk appear to be associated with high bioavailability for the foal in the early stages of development. The colloidal stability of equine casein micelles differed considerably from that of bovine casein micelles. Equine casein micelles were more stable to coagulation by acid, rennet and, at certain pH values, to heat, but were less stable to ethanol-induced coagulation compared to bovine casein micelles. The results presented are a first step towards gaining fundamental understanding of the physico-chemical properties of equine milk, which is required for the successful exploitation of the full potential of equine milk, or products derived there from, as a hypo-allergenic food product.

Differences in protein composition and preliminary differences in micellar structure found in this study between equine and bovine milk will markedly influence the properties of coagula from acidified or renneted equine milk and hence influence the digestibility and bioavailability of milk nutrients. Equine milk, like human milk, forms very soft, fine curd which may be more suitable in infant nutrition due to ease of digestion, as indicated by Kalliala *et al.* (1951) and Solaroli *et al.* (1993).

A more detailed study of the properties of equine casein micelles, particularly the protein composition of the micellar surface, is warranted to provide better understanding of the colloidal stability of equine milk.

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CHAPTER 4

Stability of Equine Casein Micelles. I. Acid-induced Coagulation of Equine Milk

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Abstract

Equine milk has a chemical composition quite similar to human milk, especially its low fat, low protein, high whey protein:casein ratio compared to bovine milk and high lactose content. The low casein content contributes to the digestibility of equine and human milk with both milks forming a fine, flaky precipitate with a short transit time through the gastro-intestinal tract. Many studies have been undertaken on protein interactions, rheological and microstructural properties during acidification and gelation of bovine milk but very few have been carried out on the acidification of equine milk. Equine casein micelles are considerably less susceptible than bovine casein micelles to acid-induced flocculation. This study examined the rheological properties of acidified unheated equine milk, with comparative analysis of unheated bovine milk using small deformation rheological measurements and creep recovery tests. The influence of acidification by GDL on the storage modulus, G', of equine and bovine milk and an equine sample with an increased casein concentration of 25 g L^{-1} showed that even when the casein concentration in equine milk was increased, little structure was present in the sample following acidification. Creep recovery curves demonstrated that acidified equine milk flowed easily when small stresses were applied to the sample. L*a*b* values recorded during the acidification of equine and bovine milk showed that the L* value for equine milk decreased as the isoelectric point of equine casein was reached.

Keywords: Equine milk; acidification; GDL; dynamic oscillatory rheology; creep recovery; L* a*b*.

4.1. Introduction

Due to its unique physico-chemical properties, the processing of equine milk into traditional dairy products is difficult; cheese cannot be produced from equine milk as a firm curd is not formed on renneting (Section 3.6.9). Equine milk forms a weak coagulum under acidic conditions (Section 3.6.10) and this is exploited in the production of yoghurt-type products with reputed probiotic and therapeutic properties. Traditionally, and to date, the only significant product from equine milk is the fermented product, koumiss, the production and properties of which have been described in Section 1.13.1.

In the milk of all species studied in sufficient detail, the caseins exist predominantly as micelles, which are hydrated spherical structures with dimensions in the sub-micron region. The dry matter of casein micelles consists predominantly (>90%) of proteins, with small amounts of inorganic matter, collectively referred to as colloidal calcium phosphate (CCP). Coagulation of milk occurs when colloidal stability is destroyed and may be desirable or undesirable. Coagulation is desirable in the manufacture of yoghurt and cheese. Coagulation of milk is also important from a nutritional point of view, as clotting of the caseins in the stomach, and the type and structure of the resultant coagulum strongly affect protein digestibility. The structure and sub-structure of bovine casein micelles has been studied in detail and reviews include, Holt & Horne (1996), Horne, (1998, 2006), De Kruif & Holt (2003), Phadungath (2005), Farrell *et al*. (2006), Qi (2007) and Fox & Brodkorb (2008). The casein micelles are best described as sterically stabilized association colloids (De Kruif & Holt, 2003). CCP exists as nanometer-sized clusters of amorphous calcium phosphate which are stabilized by a shell of caseins (α_{s1}-, α_{s2}- and β-caseins). This core-and-shell structure is commonly referred to as a nanocluster. Such nanoclusters may associate to form particles of colloidal dimensions, either *via* cross-linking through caseins which contain more than one phosphorylation centre, or through solvent-mediated association of the proteins of the shell of the nanoclusters. Growth of casein micelles is terminated by the solvent-mediated adsorption of κ-casein onto the micellar surface (De Kruif & Holt, 2003). The hydrophilic C-terminal region of κ-casein or 'brush' protrudes from the surface of the micelles and sterically stabilizes them against aggregation (De Kruif & Zhulina, 1996). Casein micelles play a crucial role in the physico-chemical stability of milk, e.g., enzyme-, acid- , heat- or ethanol-induced coagulation, thereby affecting the manufacture and stability of products such as cheese, yoghurt, evaporated milk or cream liqueurs, respectively. Coagulation of casein micelles can occur only following collapse of the brush, which occurs on acidification of milk, i.e., in the manufacture of yoghurt or on removal of the brush which occurs on rennet-

induced coagulation of milk. The combined process of enzyme- and acid-induced coagulation contributes to coagulation of casein micelles in the stomach.

At the normal pH of bovine milk, the casein micelles carry a net negative charge which causes electrostatic and steric repulsion between protein molecules. As the pH is reduced, several changes occur. At pH 6.7 to 6.0, the net negative charge on the proteins decreases and electrostatic repulsion is reduced. From pH 6.0 to 5.0, the charged κ-casein hairs shrink due to a decrease in electrostatic repulsion and steric stabilization and the micelles flocculate as their isoelectric point (pH 4.6) is approached (van Vliet *et al.*, 1991; De Kruif, 1999). CCP is also solubilised within the pH range 6.0 to ~ 5.0, although the average hydrodynamic radius of the micelles is unchanged (Phadungath, 2005). Flocculation of micelles at the isoelectric point of the caseins leads to the formation of chains and clusters which link together to form a three-dimensional network (Mulvihill & Grufferty, 1995). Acidification can be carried out by using bacterial cultures which ferment lactose to lactic acid (yoghurt manufacture; see Lucey & Singh, 2003) or by direct addition of acids such as HCl or glucono-δ-lactone (GDL) (Lucey *et al*., 1997a; Phadungath, 2005).

Casein micelles undergo many changes to their physico-chemical properties when acidified (Phadungath, 2005); however, the central process in the conversion of milk to yoghurt is the agglomeration of casein micelles into a three-dimensional network. In traditional yoghurt manufacture the milk is preheated to 90^{o}C for 10-20 min which results in the association of denatured β-lg with κ-casein on the micelle surface. The firmness and viscosity of acid milk gels are related to the extent of denaturation of whey proteins. In unheated milk, gelation occurs at pH ~ 4.9 (Horne, 1998) whereas pre-heated milk gels at pH ~ 5.2-5.4 (Lucey, 2004) due to the higher isoelectric pH of heat-denatured whey proteins (e.g., β-lg has an isoelectric pH of ~ 5.3) associated with κ-casein at the micelle surface (Phadungath, 2005). During the acidification of preheated milk the solubilisation of CCP in casein particles, that are already part of an emerging gel network, can loosen the gel network as it forms leading to some syneresis; however, as the pH decreases further, electrostatic repulsion between micelles weakens which facilitates greater hydrophobic interactions and as a result the gel becomes firmer again with less syneresis, and at pH ~ 4.6 syneresis is virtually absent (Lucey, 2004). Acid-induced gels formed from preheated milk therefore develop a firm texture through disulphide bridging which leads to increased cross-linking through the gel network (Lucey, 2004) and have high whey retention capacity (Phadungath, 2005). Electron microscopy examination of acid gels prepared from unheated and preheated milks show that the latter

differ in the size of protein aggregates, the thickness of the protein strands and the diameter of the pores between the strands (van Vliet & Keetels, 1995).

Equine casein micelles are considerably less susceptible than bovine casein micelles to acid-induced flocculation (Section 3.6.10). Di Cagno *et al*. (2004) reported that equine milk acidified at pH 4.2, the point of minimum solubility of equine caseins (Egito *et al*., 2001; Section 3.6.7), had an apparent viscosity only ~ 7 times higher than that of equine milk at its natural pH (Waelchli *et al*., 1990) and is probably indicative of micellar flocculation rather than gelation. By comparison, the viscosity of acidified bovine milk is ~ 100 times higher than that of bovine milk at its natural pH. Differences in acid-induced flocculation between equine and bovine casein micelles may be related to differences in the mechanism by which they are sterically stabilized. Equine micelles are reported to contain very little κ-casein (Malacarne *et al*., 2000; Iametti *et al*., 2001; Egito *et al*., 2001) and a values of ~ 0.25 g L^{-1} has been reported by Miranda et al. (2004) which is < 2% of the total casein; equine casein micelles are also large (Section 3.6.6). Unlike bovine κ-casein, equine κ-casein does not have a distinctly hydrophilic C-terminal domain but the apparent high level of glycosylation (Section 2.3.5) would enhance the ability of κ-casein to stabilize the micelles (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996). It is therefore unclear if the C-terminal of equine κ-casein is capable of protruding from the micellar surface to sterically stabilize it. Ochirkhuyag *et al*. (2000) and Doreau & Martin-Rosset (2002) concluded that the steric stabilization of equine casein micelles by κ-casein may be aided by unphosphorlated β-casein on the surface of the micelle, thus compensating for the low κ-casein content. In human milk, β-casein exists as a single protein phosphorylated at various levels from zero to five (β-casein-OP to β-casein-5P) but in model systems, the OP– and 1P- forms of human β-casein have been reported not to show any stabilizing ability for 2P or 4P forms in the presence of Ca^{2+} ions (Sood & Slattery, 2002).

Gels formed by acid-induced coagulation of milk are weak particle gels (Flory, 1974; Horne, 1999), in which cross-links between chains formed during the aggregation process originate from physical interactions such as hydrogen bonds but electrostatic, van der Waals and hydrophobic interactive forces and calcium-bridging (de Gennes, 1979; Horne, 1998; Lucey, 2002) are also important. As aggregation proceeds, larger and larger aggregates of associated chains are formed until a critical point is reached where the aggregate spans the whole sample volume (de Gennes, 1979; Horne, 1999). After a sufficient length of time, a stable or quasi-stable rheological state is reached in the absence of any disturbing phenomena.

Acid milk gels are viscoelastic and exhibit shear thinning when sheared, with a slow recovery after shearing has stopped (Lucey, 2004). This viscoelastic behaviour of macromolecular gels is related to the nature and rate of conformational rearrangement of macromolecules and the type and number of intermolecular bonds formed (Ferry, 1980). In acid-induced gels, the dominant structural component is casein which is heterogeneously arranged as three-dimensional aggregates of strands and linkage nodes (Harwalker & Kalab, 1980; Roefs *et al*., 1985). The principal factors governing acid milk gel formation are casein concentration, pH, temperature and ionic strength (Roefs & van Vliet, 1990).

Many studies have been undertaken on protein interactions, rheological and microstructural properties during acidification and gelation of bovine milk (see Phadungath, 2005); however, very few have been carried out on the acidification of equine milk. One of the best techniques for monitoring structure formation as milk is acidified is dynamic, non-destructive, small amplitude oscillatory rheology (Lucey & Singh, 2003; Lucey, 2004) which allows measurement of the storage (elastic; G') and viscous (G") components as the gel is forming as long as the analysis is performed using very small strains ($\leq$ 1%) and oscillatory strain rate (< 0.1 Hz) to avoid destruction of the gel (van Marle & Zoon, 1995; Lucey *et al*., 1998). The purpose of this study was to examine the rheological properties of acidified unheated equine milk, with comparative analysis of unheated bovine milk.

4.2. Materials and Methods

4.2.1. Milk supply

Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from 5 milkings over 24 h, from a herd (> 45) of multiparous, New Forest and New Forest/Arabian mares in mid-lactation, physically separated by day from their foals. The milk was filtered through glass wool to remove any extraneous material and cooled to 4^{o}C. It was received at our laboratory within ~24 h of milking.

The milk was defatted by centrifugation at 1,000 g for 20 min at 20^{o}C using a Sorvall® RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) and filtered through glass wool to remove fat particles. Raw whole bovine milk, obtained from a local dairy farm, was defatted by centrifugation at 2,000 g for 20 min at 20^{o}C, followed by filtration through glass wool. Sodium azide, 0.5 g L^{-1} was added as preservative to skimmed milk samples which were stored for no longer than 3 days.

Throughout the study, pH was measured using a Radiometer pHM 210 standard pH meter equipped with a Radiometer Meterlab® combined general purpose electrode with a built-in temperature sensor (Radiometer Analytical SAS, Lyon, France).

All chemicals used were of reagent grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

All analyses, unless otherwise stated, were carried out in at least triplicate using bulk equine or bovine milk. For clarity, data presented is generally the result of a single analysis and the coefficient of variation was always <5% of the reported value for each data point.

The temperature was controlled within ± 0.1^{o}C.

4.2.2. Preparation of suspensions of equine casein micelles at 25 g L^{-1} casein

Suspensions of equine casein micelles were prepared by pelleting the micelles ultracentrifugally (100,000 *g* for 90 min at 20°C) using an Optima LE-80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a Beckman 50.2 Ti rotor (12 place) and resuspending them in the ultracentrifugal supernatant [from which residual micelles and fat globules were removed by ultrafiltration (0.22 μm syringe filter, Minisart®), Sartorius Stedim Biotech S.A., Aubagne, France)] at ~ 30-35 g L^{-1} casein using a tissue homogeniser, followed by stirring for ~20 h at 4^{o}C. The suspensions were subsequently centrifuged at 500 g for 30 min at 20°C to remove any undissolved material; the casein content was determined using the Kjeldahl method (IDF, 2001) and adjusted to 25 g L^{-1} (which approximates the casein concentration in bovine milk) with equine ultracentrifugal supernatant. The equine micellar suspensions were adjusted to pH 6.6 and sodium azide, 0.5 g L^{-1}, was added as preservative; the samples were stored for no longer than 3 days.

4.2.3. Glucono-δ-lactone- induced acidification of equine and bovine milk

Skimmed equine and bovine milk or equine casein suspended at 2.5 g L^{-1} in ultracentrifugal supernatant were acidified using 30 g L^{-1} glucono-δ-lactone (GDL). GDL converts to gluconic acid in water and slowly releases H^{+} over time, lowering the pH of the milk and providing a simplified and controlled model of milk fermentation (Lucey, 2002; Girard & Schaffer-Lequart, 2006). Addition of 30 g L^{-1} GDL to the milk samples ensures that all the colloidal inorganic phosphate in the milk is solubilised in ~ 90 min and calcium is completely removed from the casein micelles (Heertje *et al*., 1985). Acidification to the isoelectric point of the respective caseins took ~ 90 min for bovine and ~ 40 min for equine milk, although in the latter case the pH remained relatively unchanged from 40 to ~ 80 min.. The protein of

equine milk was previously found (Section 3.6.7) to have a minimum solubility at ~ 4.2 in agreement with the value of the isoelectric point of equine casein reported by Egito *et al*. (2001). Prior to addition of GDL, the pH of fresh skimmed equine milk was adjusted to pH 6.6, the natural pH of the skimmed bovine milk. GDL was added to the milk under study, previously warmed to 30^{o}C, and maintained at this temperature for 20 min and stirred vigorously for 1 min. In tandem with rheological measurements, the pH of a sub-sample of milk was monitored and recorded continuously.

4.3. Analytical methods

4.3.1. Dynamic oscillatory analysis of equine and bovine milk on acidification

Milk gels are viscoelastic and their rheological properties can be characterized by measuring both the viscous (G") and elastic (G') moduli (Lucey, 2002). Dynamic oscillatory analysis (small amplitude oscillatory measurement) of acidified equine or bovine milk was performed as described by O'Connell *et al.* (2006), with some modifications; 11 mL of each milk sample were pre-warmed in a waterbath at 30^{o}C for 15 min, GDL was added and the sample was placed immediately in the pre-heated cup (30^{o}C) of the rheometer (controlled shear stress Carri-Med CSL^{2} 100 rheometer equipped with concentric cylinders and a recessed acrylic rotor; TA Instruments, Leatherhead, Surrey, UK). The frequency of oscillation was set at 0.6283 rad s^{-1}. The storage modulus, G', of the sample was recorded continuously at a low amplitude shear strain (0.01) for ~ 120 min. The strain used ensured that the sample behaviour was linear throughout the study. Generally, the gel point observed during small amplitude oscillatory rheology is regarded as the point where the value of the storage modulus (G') crosses over the loss modulus (G") value or when G' increases to ~ 1 Pa. Alternatively, as milk has a low initial viscosity and G" values may not measurable due to the low sensitivity of the rheometer, the gel point can be regarded as the point at which dynamic moduli become greater than the background noise of the instrument (Horne, 1999); this method of gel point observation was used throughout the present study.

4.3.2. Effect of casein concentration on structure formation in acidified equine milk

Equine casein micelles, suspended at 25 g L^{-1} in ultracentrifugal equine supernatant were acidified with 30 g L^{-1} GDL and the elastic modulus, G', was monitored for ~ 60 min (to pH ~ 4.0). The pH of a sub-sample was monitored and recorded throughout this time.

4.3.3. Effect of acidification on tan δ of equine and bovine milk

The loss tangent, tan δ, which is the ratio of G" (loss modulus) to G' (storage modulus), i.e., the ratio of the viscous to the elastic properties of a sample (van Vliet *et al*., 1989; Roefs *et al*., 1990; Lucey *et al*., 1998; Lopez da Silva & Rao, 1999) was recorded as a function of time after GDL addition. Tan δ values > 1.0 are indicative of liquid-like behaviour (Tunick, 2011) and changes in tan δ, which is very sensitive to any shift in the slopes of the dynamic moduli (G" and G') over time, may be used to quantify the presence and extent of elasticity in a fluid. As a sample gels, tan δ is a useful indicator of the number and type of bonds forming between protein particles.

4.3.4. Effect of acidification on L, a* and b* values of equine and bovine milk*

The extent of dissociation of casein micelles in equine and bovine milk during acidification with 30 g L^{-1} GDL was measured by monitoring the changes in appearance or turbidity over time. Changes in the light scattering by casein micelles during acidification were observed by measuring the L*a*b* values of acidified equine and bovine milks. Measurements were made using a Minolta CR-300 colorimeter (Minolta Corp., Osaka, Japan) which was calibrated using a standardized white tile (chromaticity coordinates; Y = 88.2, x = 0.309 and y = 0.316). The CIELAB (Commission Internationale de l'Éclairage, 1976) standard measurement system was used; this measures colour using 3 coordinates, L*, a*, and b*. L* indicates sample brightness (where 0 is black and 100 is white), a* represents red/greenness of a sample (negative a* values are green while positive a* values are red) and b* represents yellow/blueness (negative b* values are blue while positive b* values are yellow). Zero values of a* and b* indicate neutral colour.

4.3.5. Mechanical spectra of acidified equine and bovine milk

Small-deformation oscillatory measurements (0.1% strain) of storage modulus (G') and loss modulus (G") were made using a highly truncated cone-and-plate geometry (diameter 50 mm; cone angle 0.05 rad; minimum gap, 1mm) on a sensitive prototype rheometer. Samples, loaded onto the plate, were coated with light silicon oil at their periphery to minimize evaporation. Measurements of G' and G" were made at a fixed frequency of 1 rad s^{-1} and, after 90 min, a mechanical spectrum was recorded to show the variation of G' and G" with frequency in the range 0.1-100 rad s^{-1} at a fixed strain of 0.5%.

Mechanical spectra demonstrate the frequency-dependence of storage modulus, G', loss modulus, G" and complex dynamic viscosity, η*, where

$$\eta^* = \frac{(G'^2 + G''^2)^{1/2}}{\omega}$$

and ω is the frequency of oscillation (in rad s^{-1})

4.3.6. Creep-recovery measurements of acidified equine and bovine milk

To assess the response of acidified equine and bovine milk to the application and removal of a constant stress, creep tests were carried out using a controlled shear stress Carri-Med CSL^2 100 rheometer. In creep recovery tests, a stress is applied virtually instantaneously to the sample and held constant for a fixed period during which strain values are recorded. The stress is removed and the strain again monitored to determine the extent to which the sample recovered its original structure. The recoverable strain, which corresponds to the initial sharp increase when stress is applied, gives a measure of the solid-like (elastic) character of the gel and the irrecoverable strain, which corresponds to the gradual increase in strain (flow) during the initial creep period, gives a measure of the liquid-like (viscous) character of the gel.

Milk samples were prepared by heating 11 mL of fresh skimmed equine or bovine milk (pH 6.6) to 30°C in a waterbath for 15 min. GDL (30 g L^{-1}) was added and the sample stirred vigorously for 1 min before pouring the samples into the preheated concentric cylinder of the rheometer. The pH of sub-samples was monitored under quiescent conditions. For analysis of acidified equine and bovine milk gels, creep-recovery experiments were carried out 90 min after the addition of GDL, a shear stress (τ) was applied for 10 min, with measurement of the resulting strain (γ); recovery after removal of the stress was monitored for a further 10 min. The applied stresses used for bovine samples were increased by 2-fold increments, from 0.2 to 102.4 Pa. In the case of acidified equine milk samples, the gels were significantly weaker than those of acidified bovine milk and the values of applied stress used were from 0.03 to 0.4 Pa. For both bovine and equine samples, creep-recovery curves are reported as compliance, *J*, where, $J = \gamma / \tau$.

The difference in the resistance of acidified equine and bovine gels to fracture and in their extent of deformation in response to lower stresses was analysed by plotting (double-logarithmically) maximum strain values reached at the end of the initial 10 min period against the applied stress.

4.4.Results and Discussion

4.4.1. Dynamic oscillatory analysis of the acidification of equine and bovine milk

The influence of acidification by GDL on the storage modulus, G', of equine and bovine milk and an equine sample with a casein concentration of 25 g L^{-1} is shown in Figure 4.1. The G' of bovine milk increased sharply from pH ~ 5.0 (the onset of gelation) to give a final G' of ~ 35 Pa. Equine milk did not have a significant increase in G' at the isoelectric point of the caseins (pH 4.2) which occurred at ~ 40 min. The final G' for equine milk was ~ 0.13 Pa, indicating that no gelation of the milk occurred. Increasing the casein concentration of equine milk to 25 g L^{-1} casein, which approximates the casein concentration in bovine milk, increased the final G' value approximately 10 fold compared to that of equine milk at its natural casein concentration (12 g L^{-1}; Section 3.5.1) and the G' *vs* pH profile showed a sharp increase in G' at the onset of gelation. Both equine milk with its natural casein concentration and with the casein concentration approximately doubled, had G' *vs* pH profiles which were not smooth lines once aggregation had occurred, which indicated that the structure was weak and, in the former case, was indicative of an aggregate rather than a true gelled structure.

Acidification of unheated bovine milk with GDL results in a longer gelation time, a lower pH values at gelation (pH < 5.0) (Lucey *et al*., 2000) and a low G' value, < 20 Pa (Lucey *et al*., 1997a, 1998, 2000), compared to preheated bovine milks. The final G' value for acidified bovine milk was higher in this study than that reported by Lucey *et al*. (1997a) which could be due to a slightly higher casein content in the bovine milk used in this study. It has been reported that acid milk gels made from heated bovine milk and acidified using bacterial cultures, have a higher pH at the onset of gelation which could mean that the casein particles are more mineralised, highly charged and less dissociated than those made with GDL and the latter form considerably softer gels (Renan *et al.*, 2008).

GDL-induced acidification of milk, which is a simplified and controlled model of fermentation (Lucey *et al*., 1997a, 1998; Lucey, 2002; Aichinger, 2005) is reported to differ from acidification by *in situ* bacterial cultures used in the production of fermented milk products; the method of acidification is believed to affect the rate of dissolution of colloidal calcium phosphate (CCP), dissociation of casein micelles, aggregation of micelles as well as the time available for rearrangement of aggregating protein particles (Lucey *et al*., 1998; Laligent *et al*., 2003) with the result that the gels formed by both types of acidification have different rheological and structural properties (Lucey, 2004). Gels made from unheated bovine milk have low G' values because the dense clusters of aggregated casein particles formed by the extensive rearrangement of particles during gel formation do not entirely cross-

link throughout the gel network. In heated milk samples, denatured whey proteins associated with casein micelles interact with each other and act as bridging material, increasing the strength and number of bonds between protein particles (Lucey, 2002). Preheating equine milk prior to acidification is unlikely to have a significant effect on the strength of the gel that forms as equine β-lactoglobulin lacks a sulphydryl group and is unable to associate with κ-casein on the micelle surface. Di Cagno *et al*. (2004) reported no significant increase in the viscosity of equine milk preheated at 90°C for 3 min and acidified with cultures of *Lactobacillus delbruechii* subsp. *bulgaricus* and *Streptococcus thermophilus* at 42°C whereas viscosities increased when equine milk was fortified with bovine milk, ovine milk, Na caseinate, pectin, Na caseinate and pectin, Na caseinate, pectin and sucrose or Na caseinate, pectin and threonine.

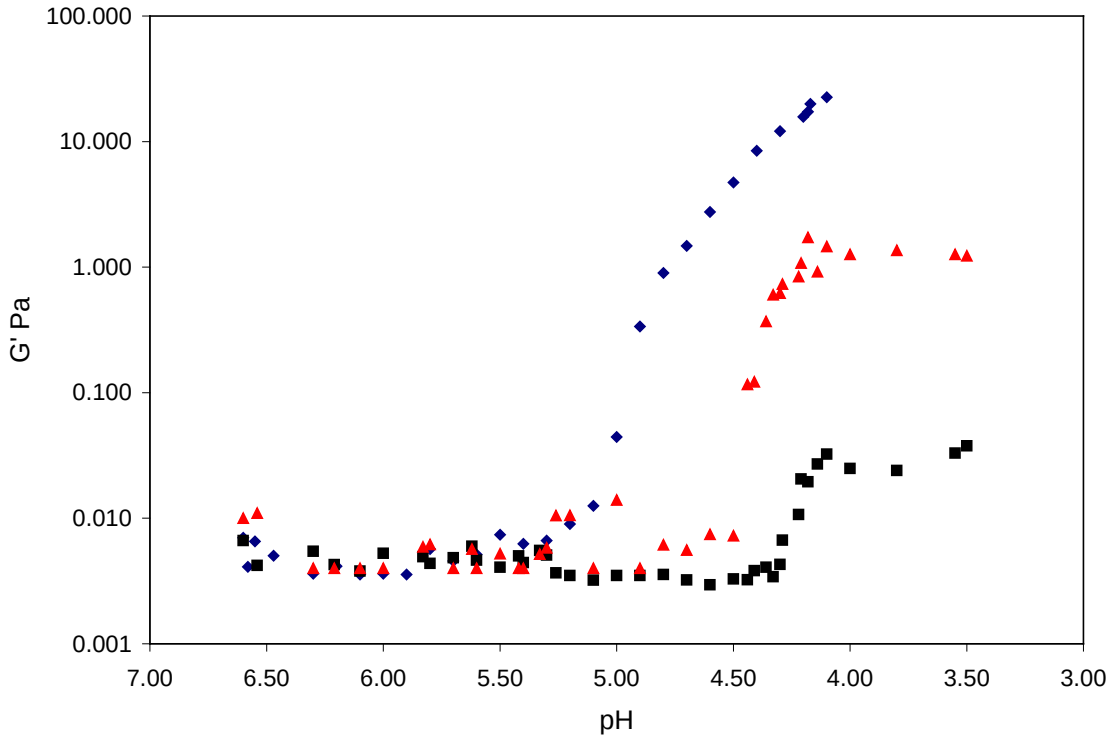

Figure 4.1. Influence of acidification by 30 g L^{-1} glucono-δ-lactone (GDL) at 30°C on the storage modulus, G′, of bovine (◆) and equine (■) milk or equine casein at 25 g L^{-1}(▲) in ultracentrifugal supernatant. The coefficient of variation was <5% of the reported value for each data point.

The rheological properties of casein gels depend on the number and strength of bonds formed between casein particles and on the structure and the spatial distribution of the strands making up these particles (Roefs *et al*., 1990; Lucey *et al*., 1997b; van Vliet et al., 1989; van Vliet, 1999). When more covalent bonds are present it is expected that the shear modulus will be higher (Lakemond & van Vliet, 2005). The concentration of protein affects gel structure and aggregation and gelation occurs only above a certain protein concentration, below this level there is a limited number of protein contacts (Ozer *et al*., 1999). As protein concentration increases, G' increases (Bremer *et al*., 1990; Roefs *et al*., 1990; Ross-Murphy, 1990; Walstra, 1990; Ozer *et al*., 1999). The protein content of equine milk is low compared to bovine milk (22.98 g L^{-1} *vs* 34.1 g L^{-1}; Section 3.5.1) and the casein content is low in equine milk compared to bovine milk (12.08 g L^{-1} *vs* 28.1 g L^{-1}) as a result little or no structure is formed on acidification of equine milk. It has been reported that the total solids and protein content of milk from different species have a major effect on the rheological properties of yoghurt, the viscosity of which is in the order, ovine > caprine>bovine >camel milk, which is in order of decreasing total solids and protein content, furthermore; camel milk (~ 1.8 g 100 g^{-1} protein) showed no significant increase in viscosity on acidification (Jumah *et al*., 2001).

The large size of equine casein micelles (~ 277 nm; Section 3.6.6) is unlikely to affect the strength of a gel formed and it has been reported that the number of contact points between casein aggregates is independent of their size but dependent on the protein concentration (Walstra *et al*., 1990); if the protein concentration is sufficiently high, a network structure is formed throughout the entire system.

The result of this study is in agreement with DWS analysis reported previously for the acidification of equine and bovine milk (Section 3.6.10). The $\tau_{½}$ *vs* time profile obtained showed that equine casein micelles are susceptible to acid-induced flocculation, but the extent was considerably lower than that for bovine casein micelles.

The most important factors in an acid curd structure are casein content, pH and calcium content of the milk. At low pH, calcium dissolves progressively from the casein micelle and, in addition, neutralization of negative charges on the casein favours extensive aggregation and fusion between micelles which tend to form a gel. In the case of bovine milk, a gel is formed at pH ~ 4.6 (Parry, 1974). It is likely that the charge distribution on the equine casein micelle is different to that of bovine micelles. Equine and human κ-casein have a considerably higher isoelectric pH than bovine κ-casein (Section 2.3.4.), and they have a net positive charge at physiological pH, whereas bovine κ-casein has a net negative charge. Bovine κ-casein is characterized by a hydrophilic C-terminus, which is very important for the manner in which bovine casein micelles are stabilized but the C-terminus of equine κ-casein is far less

hydrophilic although the exact level of glycosylation of equine κ-casein has not been determined. Lectin-binding studies indicate that equine κ-casein is glycosylated (Egito *et a*l., 2001; Iametti *et al*., 2001), possibly at residues Thr_{123}, Thr_{127}, Thr_{131}, Thr_{149} and Thr_{153}. The presence of these glycan moieties in the C-terminal region of κ-casein would enhance its ability to stabilize the micelle, by electrostatic repulsion, and may increase the resistance by the protein to proteolytic enzymes, acidification and high temperatures (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996). If equine κ-casein is highly glycosylated it would not form a firm gel on acidification or addition of rennet (Section 3.7.9). It has been reported (Roefs *et al*., 1990) that removal of the hydrophilic part of bovine κ-casein by renneting, followed by acidification, increases the pH at which gelation occurs; however the gel strength is low compared to that of unmodified milk. Another study (Cases *et al*., 2003) on the effect of κ-casein deglycosylation on the acid coagulabiliy of bovine milk found shorter gelation times, a higher rate of gel strengthening and higher final gel firmness for casein micelles modified by the release of part of the *N*-acetylneuraminic acid residues.

4.4.2. Effect of acidification on tan δ values of equine and bovine milk

The effect of acidification on the tan δ values of equine and bovine milk are shown in Figures 4.2 and 4.3, respectively. Prior to the onset of aggregation (~ 35 min), the tan δ values recorded for equine milk were high, indicating that the sample was unstructured and viscous. In contrast, the tan δ values for bovine milk were lower and < 1 when a structured gel began to form. For both equine and bovine milks, tan δ decreased continuously after the onset of gelation as the pH decreased. The result for bovine milk is in agreement with the data reported by Lucey *et al*. (1998, 2000) where tan δ deceased at gelation and continued to decrease during the aging of milk gels acidified with GDL.

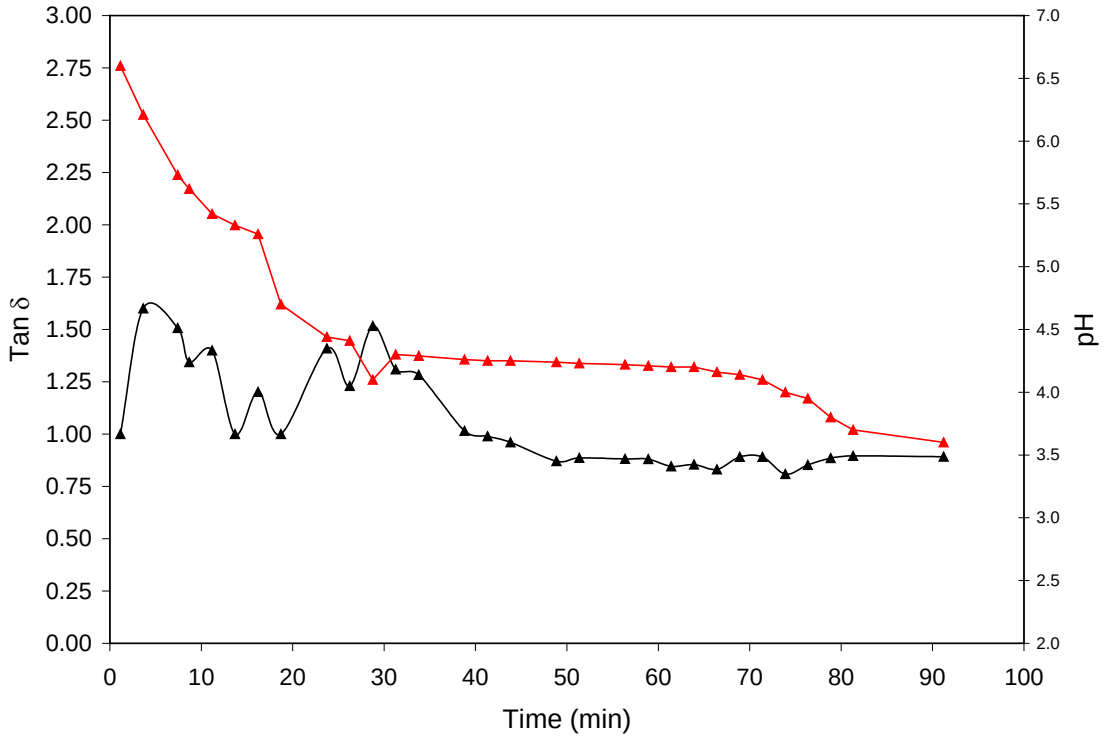

Figure 4.2. Loss tangent, tan δ (-▲-), as a function of time for equine milk acidified with 30 g L^{-1} glucono-δ-lactone (GDL) for 90 min at 30°C (ω of 0.6283 rad s^{-1} and strain of 1%). pH profile (-▲-). Analysis was carried out in triplicate. The coefficient of variation was <5% of the reported value for each data point.

In this study, tan δ values levelled out 80 min after acidification for both milks and the tan δ value was lower for bovine milk. The loss tangent, tan *δ,* is related to the spatial distribution and the number of protein-protein bonds formed during the gelation of milk and is therefore a good measure of the nature and strength of protein bonds between casein particles (Roefs, 1986). Milk gels with a high tan δ value at low frequencies of oscillation favour rearrangement of their protein network (van Vliet *et al*., 1991). Tan δ values > 1 (i.e. G" > G') are indicative of liquid-like behaviour (Rao, 1999). No maximum in the value of tan δ just after the onset of gelation of acidified bovine or equine milks was observed in this study which is in agreement with the results of Lucey *et al*. (1997c) on the acidification of bovine milk which had not been preheated. An increase in tan δ at the onset of gelation has been reported in gels formed by microbially acidified high heat-treated milk (Rönnegard & Dejmek, 1993; van Marle & Zoon, 1995) and in gels formed from heated bovine milk acidified with GDL (Lucey *et al*., 2000).

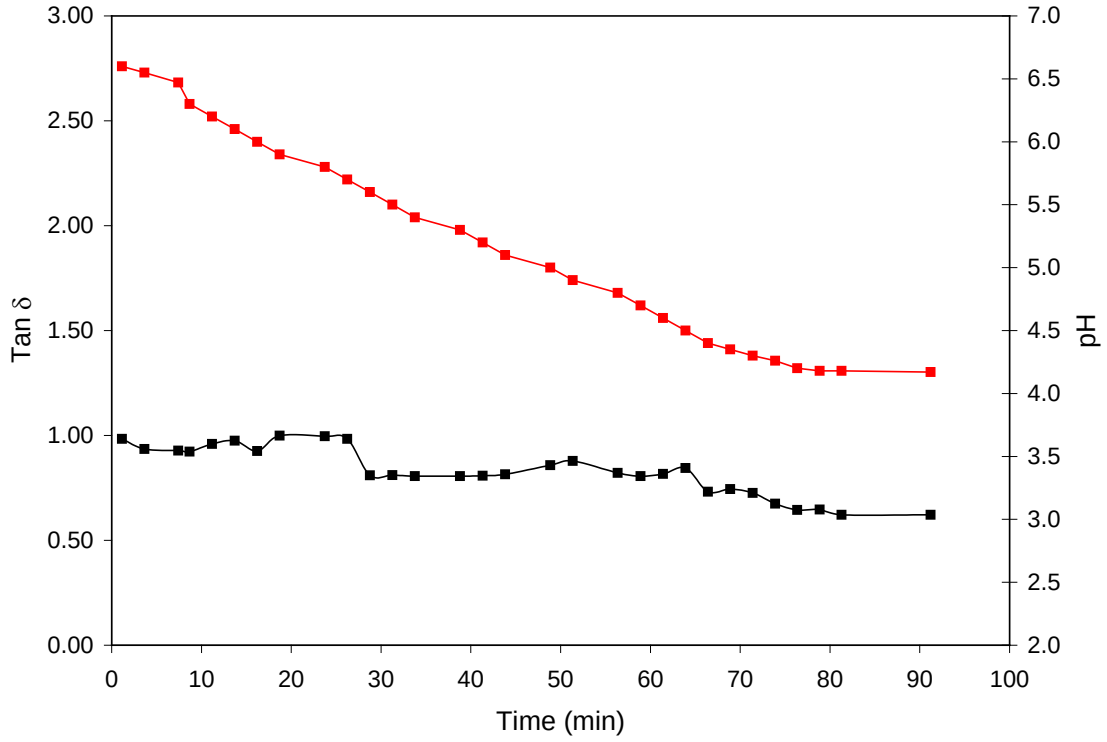

Figure 4.3. Loss tangent, tan δ (-■-), as a function of time for bovine milk acidified with 30 g L^{-1} glucono-δ-lactone (GDL)) for 90 min at 30°C (ω of 0.6283 rad s^{-1} and strain of 1%). pH profile (-■-). Analysis was carried out in triplicate. The coefficient of variation was <5% of the reported value for each data point.

In another study, using a combination of rennet and GDL, Lucey *et al*. (2000) observed a maximum in tan δ initially as the pH decreased and attributed it to a loosening of bonds between casein particles in the gel network due to solubilisation of colloidal calcium phosphate and the release of Ca^{2+} at pH < 6.0. Above the isoelectric pH, the ionic strength increases and Ca^{2+} may bind to negatively-charged casein micelles which could reduce protein-protein interactions by screening charged groups on the caseins (Lucey *et al*, 2000). At low pH (< 5.0), there is a decrease in the net negative charge on casein which results in increased electrostatic attraction between casein particles. Ca^{2+}-binding to casein decreases at pH < 6.6 and is low at pH $\leq$5.5 (Pyne, 1962).

4.4.4. Effect of acidification on L, a* and b* values of equine and bovine milk*

The influence of acidification on the L* of equine and bovine milk is shown in Figure 4.4. The L* of bovine milk remained unchanged throughout the acidification process; however, the L* of equine milk decreased at the onset of micellar flocculation and aggregation, indicating dissociation of the casein micelles. Due to its colloidal dimensions, milk scatters light and appears white. The whiteness is lost if any disruption of the casein micelles occurs (Fox & McSweeney, 1998). L* values relate to the extent of dissociation of casein micelles (Zadow, 1993; O'Connell *et al*., 2001; O'Sullivan *et al*., 2002); it has also been reported that if the number of micelles in a milk sample is reduced (e.g., by dilution), the L* value will decrease (Dunkerley *et al*., 1993).

As the pH decreased, in the range 5.9 to 5.2, electrostatic and hydrophobic bonds are weakened and caseins, especially β-casein, solubilise and all inorganic phosphate is in the serum phase, the micelles disintegrate and the reflectance of light is reduced. It appears from this study that the casein micelles in equine milk undergo significant dissociation as the isoelectric point of the caseins is approached. While the dissociation led to the formation of aggregates, the caseins did not form a structured gel. Differences have been reported in the dissociation of casein from the milk of different species. In a study of the acid coagulation of bovine, caprine and ovine milk with GDL (Ould Eleya *et al*., 1995), the β-casein of caprine milk dissociated more easily from the micelles as the pH decreased compared to the dissociation of β-casein from bovine and ovine micelles. The decrease in turbidity was more pronounced in caprine milk and the resultant gel appeared as a dispersion of coarse particles rather than a continuous network (Ould Eleya *et al*., 1995). The higher casein content of bovine, and especially ovine, milk increased the tendency for the casein particles to form firm gels (Ould Eleya *et al*., 1995). The casein composition, total calcium concentration and overall mineral balance of a milk are probably the most significant factors governing the aggregation and gel formation in acidified milks (Walstra & Jenness, 1984).

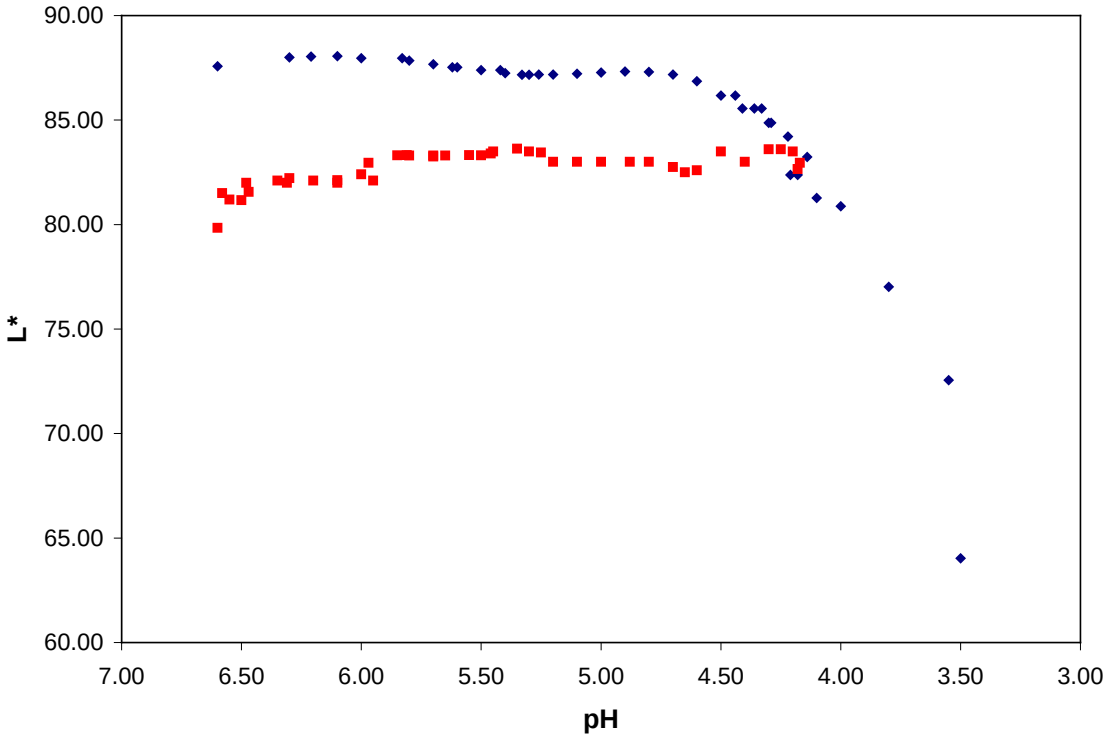

Figure 4.4. Influence of acidification with 30 g L^{-1} glucono-δ-lactone (GDL) on the L* value of equine (♦) and bovine (■) milks at 30°C measured using a Minolta CR-300 colorimeter. Analysis was carried out in triplicate. The coefficient of variation was <5% of the reported value for each data point. For clarity, data presented are the result from a single analysis.

The effect of acidification on the a* and b* values of equine and bovine milk are shown in Figures 4.5 and 4.6, respectively. For both milks, the a* value remained unchanged as the pH decreased. The b* value of equine milk increased slightly as the pH approached ~ 4.5 and at the same point where a decrease in L* value was recorded. The b* value of bovine milk increased sharply as the pH approached ~ 5.2, the onset of micellar aggregation (Figure 4.6). Delétang (2004) reported a sigmoidal pattern in the L* and b* values of bovine milk following rennet addition with a sharp increase in both parameters at the point of aggregation of casein micelles and when the aggregation was complete and a stable continuous network was formed, both the L* and b* values reached maximum values and levelled out. Dunkerley *et al*., 1993, recorded the L* a* b* values on dilution of bovine milk; the L* and a* decreased and increased, respectively, as the milk was diluted but the b* values showed a more complex pattern, decreasing as milk was diluted by ~ 20%, but increasing with further dilution.

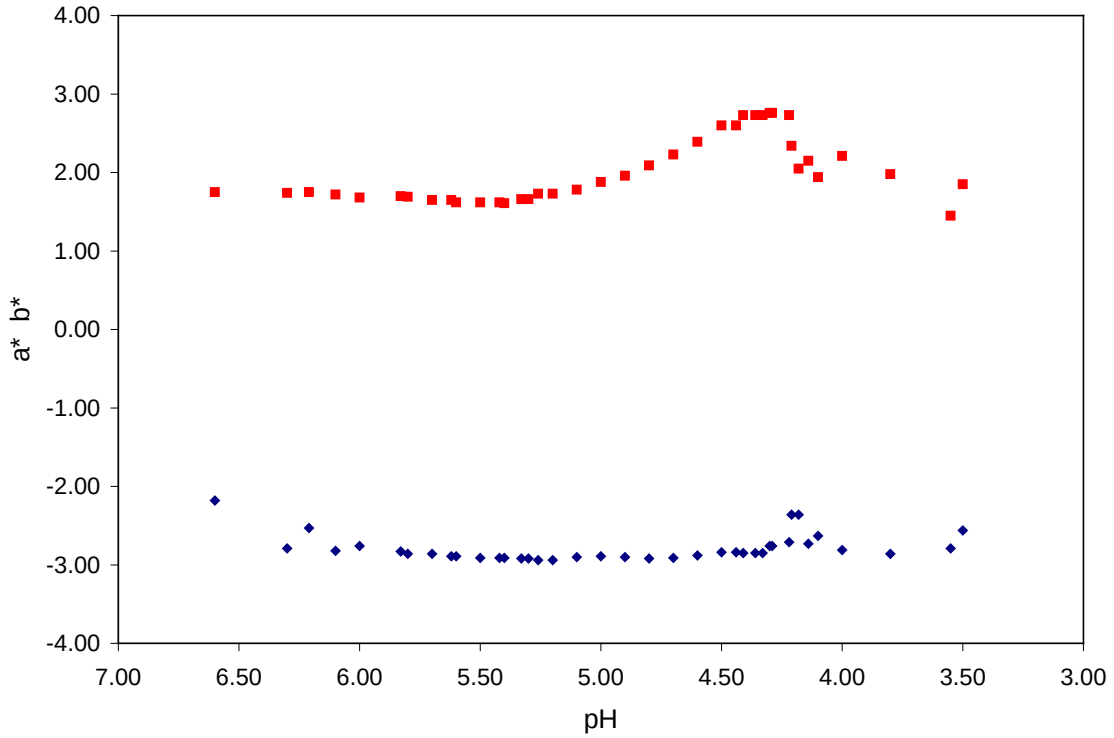

Figure 4.5. Effect of acidification at 30°C with 30 g L^{-1} glucono-δ-lactone (GDL) on the a* (-◆-) and b* (-■-) values of equine milk measured using a Minolta CR-300 colorimeter. Analysis was carried out in triplicate. The coefficient of variation was <5% of the reported value for each data point. For clarity, data presented are the result from a single analysis.

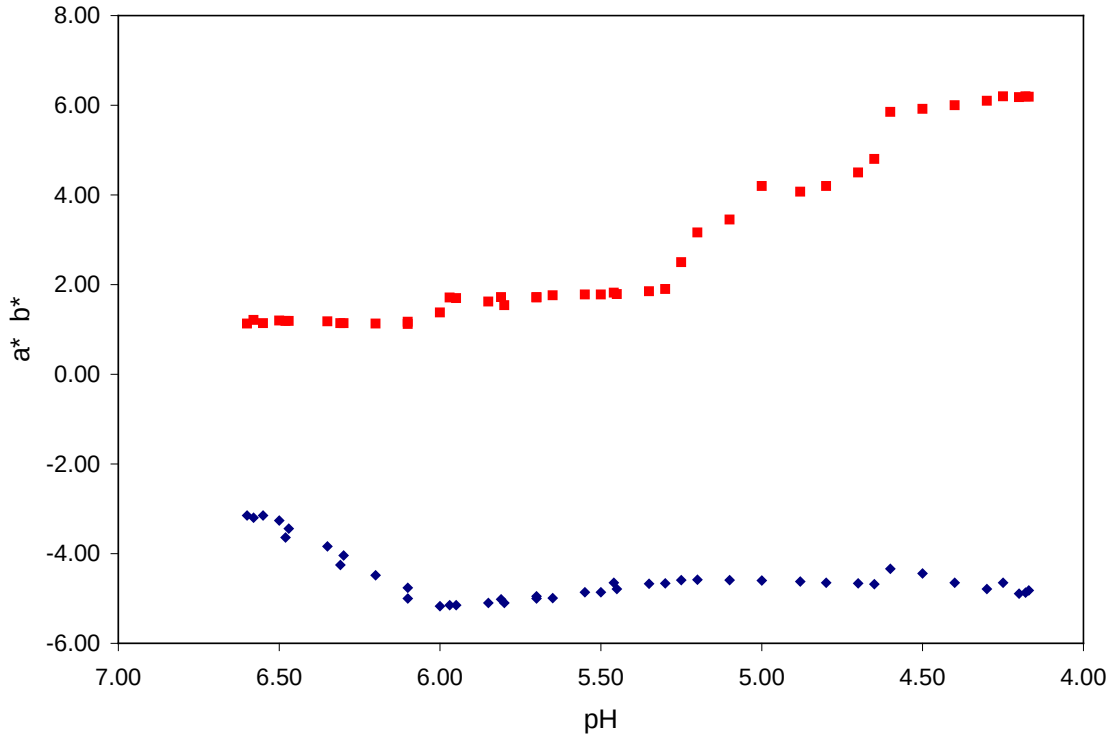

Figure 4.6. Effect of acidification at 30°C with 30 g L^{-1} glucono-δ-lactone (GDL) on the a* (-◆-) and b* (-■-) values of bovine milk measured using a Minolta CR-300 colorimeter. Analysis was carried out in triplicate. The coefficient of variation was <5% of the reported value for each data point. For clarity, data presented are the result from a single analysis.

4.4.5. Mechanical spectra of acidified equine and bovine milk

The frequency dependence of G', G" and η* of acidified equine and bovine milk are shown in Figures 4.7 and 4.8, respectively. For bovine milk, the maximum G' after 90 min acidification was ~ 37 Pa (Figure 4.1) and gel-like structure was observed as the frequency of oscillation was increased (Figure 4.8). The separation between G' and G" is smaller than in conventional, self-supporting biopolymer gels and the frequency dependence of both moduli increased as the frequency of oscillation increased indicating that the gel formed was weak (Ross-Murphy *et al.*, 1983). It was not possible to construct a similar graph from the data for acidified equine milk and it can be seen (Figure 4.7) that at all times during the frequency sweep, the G" was always greater than G' indicating viscous behaviour throughout and no true structure/gel formation. An insert graph (Figure 4.7) shows comparative data for bovine milk plotted without double-logarithmic axes.

A frequency sweep will distinguish between a weak and strong gel (Clark & Ross-Murphy, 1987). Weak gels are characterized by a high dependence of the dynamic moduli on frequency. In a true viscoelastic gel, the dynamic moduli (G' and G") are independent of frequency (ω) and in a typical biopolymer gel, the solid-like response exceeds the liquid-like response by an order of magnitude and a linear relationship exists between log η* and log ω with a slope of ~ -1 (Ross-Murphy, 1983). To form an ideal gel, permanent covalent bonding is required and the moduli are completely independent of frequency but in a protein gel a frequency dependence is observed (Ross-Murphy, 1983). Log G' *vs* log ω has a slope which is greater than zero but typically is < 0.1. More elastic systems will have lower slope values and more viscous systems will have higher slope values.

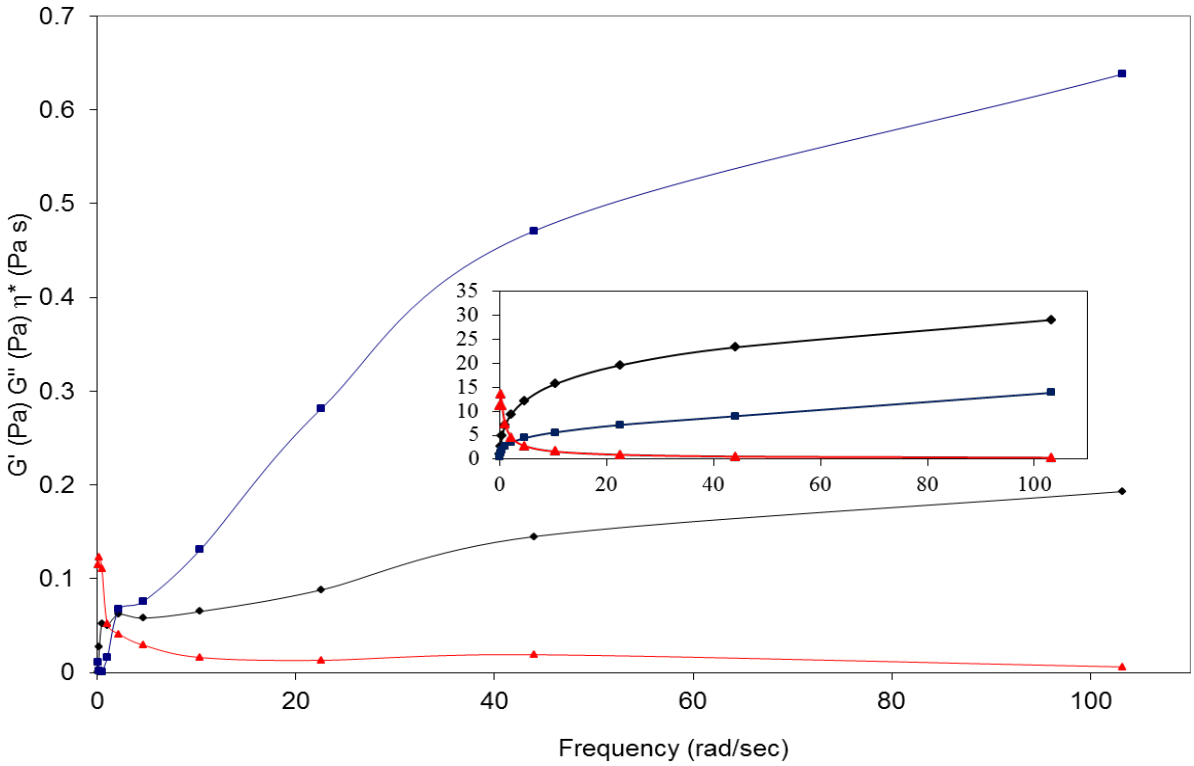

Figure 4.7. Mechanical spectra (0.1% strain) showing the frequency dependence of G' (-◆-), G" (-■-) and η* (-▲-) of equine milk acidified with 30 g L^{-1} glucono-δ-lactone (GDL) at 30°C for 90 min. Inset graph is the equivalent mechanical spectra of bovine milk. Analysis was carried out in triplicate and the coefficient of variation was <5% of the reported value for each data point. For clarity, data presented is the result from a single analysis.

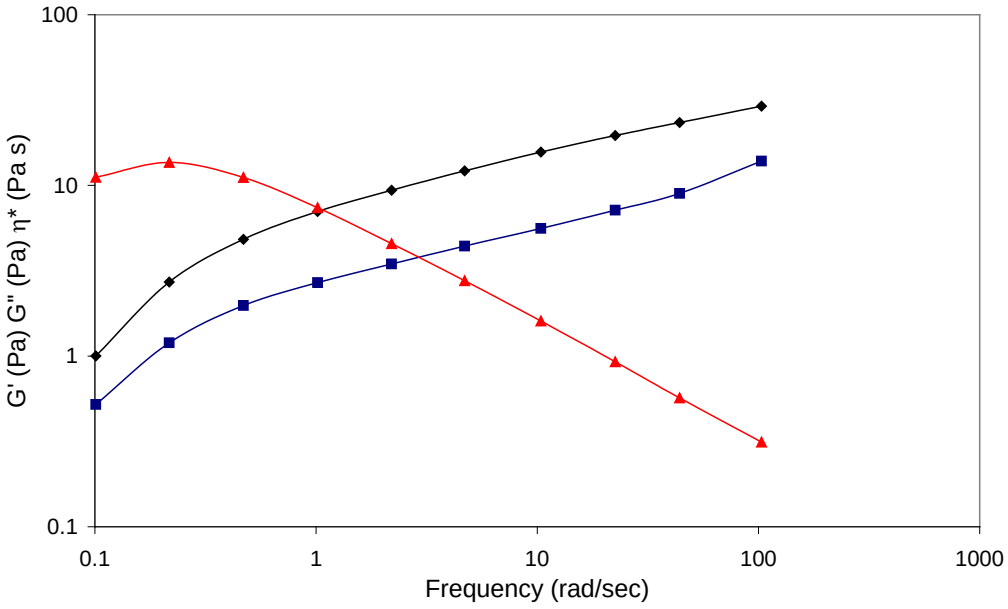

Figure 4.8. Mechanical spectra (0.1% strain) showing the frequency dependence (0.1 to 100 rad s^{-1}) of G' (-♦-), G" (-■-) and η* (-▲-) of bovine milk acidified with 30 g L^{-1} glucono-δ-lactone (GDL) at 30°C. Analysis was carried out in triplicate and the coefficient of variation was <5% of the reported value for each data point. For clarity, data presented are the result from a single analysis.

4.4.6. Creep-recovery measurements on acidified equine and bovine milk

The creep curves recorded for acidified equine and bovine milk are shown in Figures 4.9 and 4.10, respectively. In the case of bovine milk, the strains generated in response to an applied stress of 0.2 to 0.8 Pa were too small to be measured accurately but for the other stresses, the compliance (*J*) curves showed the form characteristics of a gel network with some elasticity. In a true viscoelastic gel, the compliance curves would superimpose closely and the irrecoverable strain would be similar for each applied stress. For the weak gel formed from bovine milk in this study, some irrecoverable deformation of the sample occurred for all stress levels and increased as the stress increased. At an applied stress of 102.4 Pa the compliance increased dramatically at the start of the creep period and the gel network fractured and flowed when a finite 'yield stress' value was exceeded.

In the case of acidified equine milk, the final G' value was only ~ 0.2 Pa (Figure 4.1) which is probably indicative of a very loose network of large aggregates rather than a gel. However, creep curves (Figure 4.9) were recorded at low applied stresses to determine if there was any

measurable structure present. Even at very low applied stress, the compliance value was extremely high; however some 'gel-like' structure was present and recovery from applied stresses occurred up to an applied stress value of 0.2 Pa, after which the structure fractured and flowed. These low applied stress values do not necessarily indicate viscoelastic or 'gel-like' behaviour but some casein aggregation was present in the acidified gels. Dynamic measurements on casein gels yield information about the short-range interactions, especially the conformation and structure of casein particles (Roefs *et al*., 1990). Creep measurements, on the other hand, provide information on the long-range properties of the casein network.

In creep tests, the protein network should rearrange to accommodate the applied deformation by dissociating pre-existing inter-chain junctions and replacing them with new interactions. For loose entangled coils typical of the network arrangement in weak gels, the response to an applied stress is dominated by liquid-like character with little, if any, recovery (solid-like response) when the stress is removed. Creep tests are the most reliable way for determining the yield stress and flow of a sample.

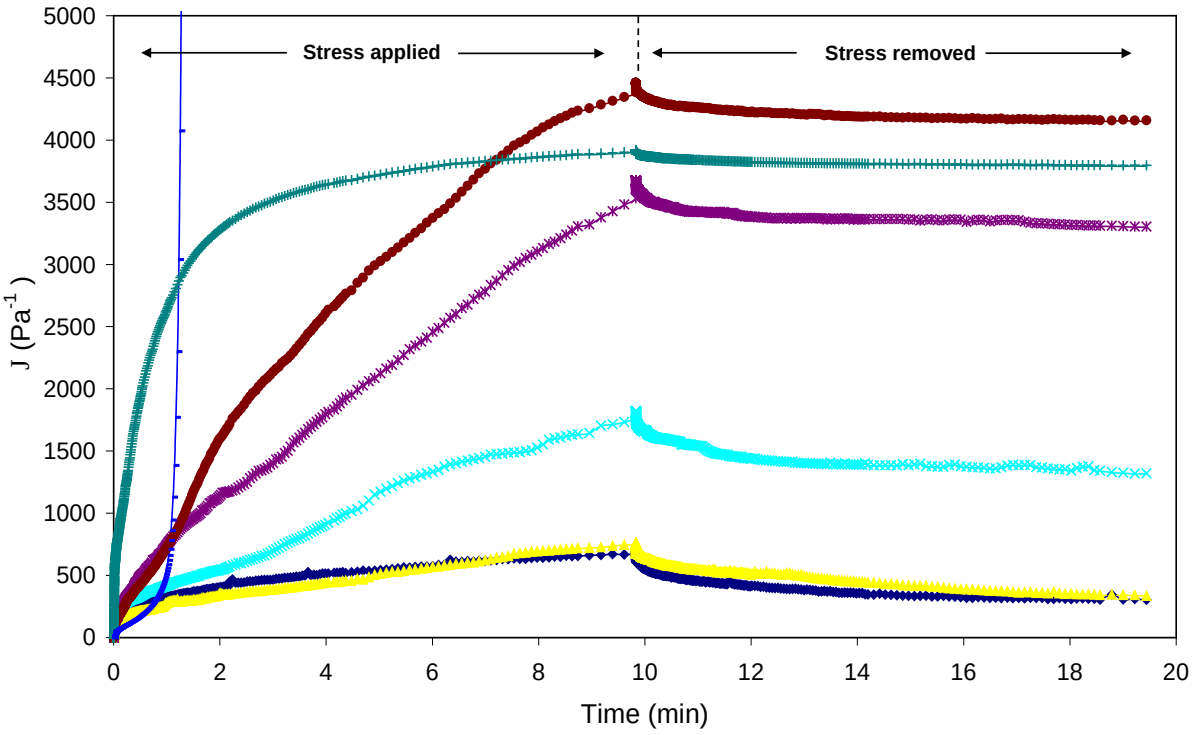

Figure 4.9. Creep-recovery curves (30°C) recorded for equine milk acidified with 30 g L^{-1} glucono-δ-lactone (GDL) The curves show the variation of compliance (*J*) in response to applied stress values (Pa) of 0.03 (-♦-), 0.05 (-▲-), 0.07 (-x-), 0.09 (-x-), 0.1 (-●-),0.2 (-|-) and 0.4 (-‒-). Stress was held constant for 10 min and recovery was monitored over a period of 10 min after the stress was removed. Analysis was carried out in triplicate and the coefficient of variation was <5% of the reported value for each data point. For clarity, data presented are the result from a single analysis.

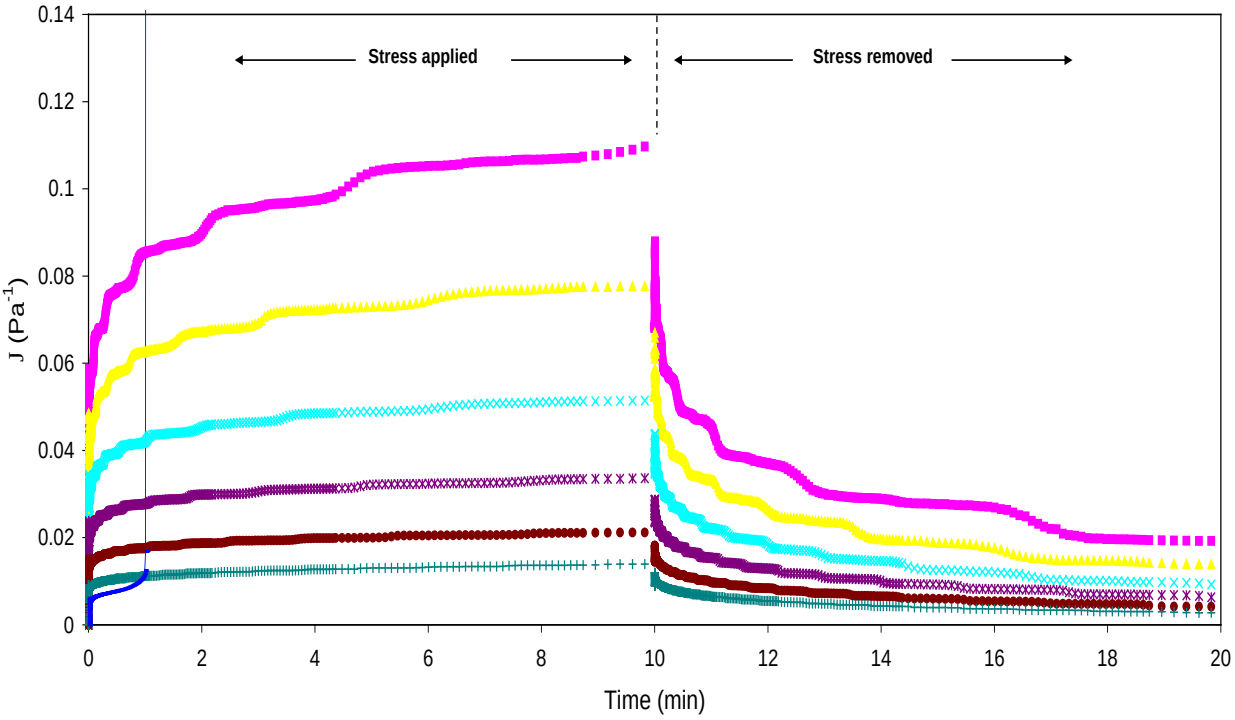

Figure 4.10. Creep-recovery curves (30°C) recorded for bovine milk acidified with 30 g L^{-1} glucono-δ-lactone (GDL) The curves show the variation of compliance (*J*) in response to applied stress values (Pa) of 1.6 (-+-), 3.2 (-●-), 6.4 (-⁎-), 12.8 (-x-), 25.6 (-▲-), 51.2 (-■-) and 102.4 (-▪-). Stress was held constant for 10 min and recovery was monitored over a period of 10 min after the stress was removed. Analysis was carried out in triplicate and the coefficient of variation was <5% of the reported value for each data point. For clarity, data presented are the result from a single analysis.

When no recovery is detected on removal of an applied stress, the elastic network, indicated by the low stress applied, fractured as a finite 'yield stress' value was exceeded. No superimposition of compliance curves occurred for acidified equine milk, indicating that the gel formed was extremely weak and not a true gel. No superimposition of compliance curves occurred for acidified bovine milk although the stresses applied were considerably greater than those for acidified equine milk and the compliance curves obtained at low applied stresses were close together indicating that the gel formed, while very weak, has some characteristics of a true gel.

The differences in resistance to fracture and in the extent of deformation in response to applied stresses of acidified equine and bovine milk gels was illustrated by plotting, double-logarithmically (Figure 4.11), the maximum strain reached at the end of the 10 min period of applied stress *vs* the applied stress. The gel formed from bovine milk was substantially stronger than that formed from equine milk and fractured at a higher stress value. The equine gel lacked significant cohesion and flowed easily. The strength of protein-protein bonds, the number of bonds per cross-sectional area of a protein strand, the relaxation times for the network bonds and the orientation of the strands in the protein matrix all contribute to the yield properties of gels (van Vliet *et al*., 1991). As the casein content of a milk increases for acid milk gelation, the yield stress of the gel increases and the firmness of a gel can be related to the number and strength of contact points (Anema, 2008). Before a gel ruptures, all the protein strands (bonds) in the network must be straightened and gel rupture results when the strands break (Mellema *et al*., 2002). Straightening of the protein strands depends on the extent to which they are curved which increases as the milk protein concentration is increased; such bonds have much greater breaking stresses than those formed when the protein content is lower. If the strands are formed from covalent bonds, greater force is required to break the strands as the bond energy is considerably higher than that of non-covalent bonds (Mellema *et al*., 2002).

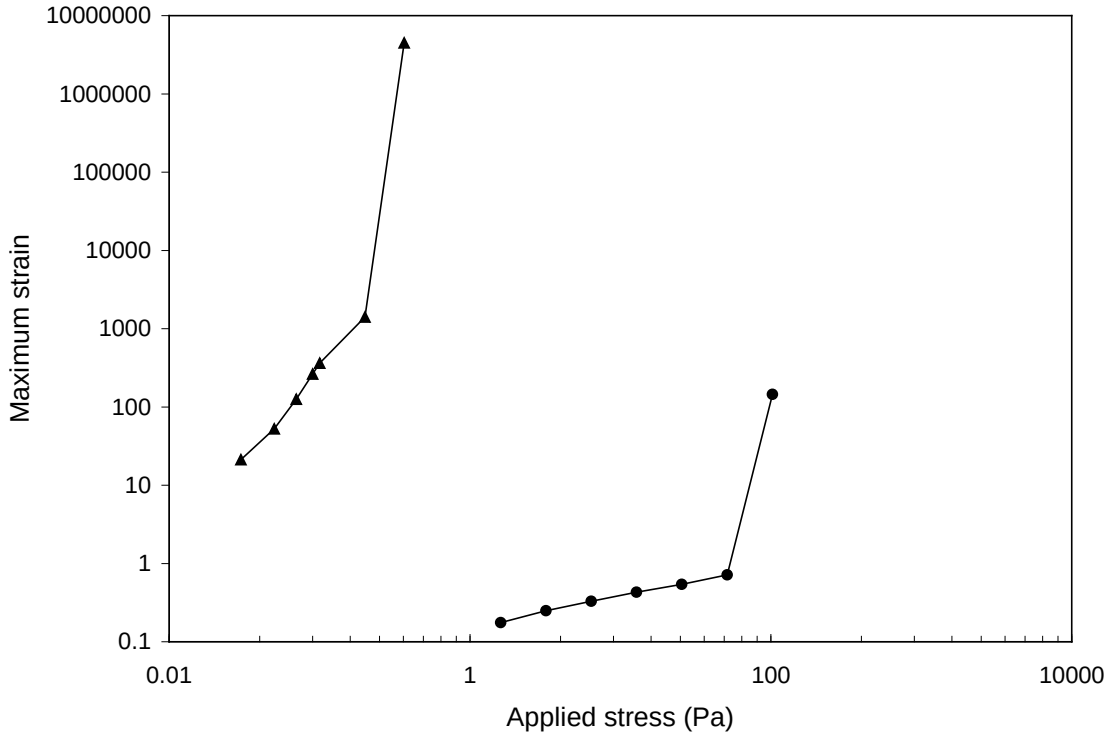

Figure 4.11. Variation of maximum strain (after 10 min of creep) with applied stress for equine (-▲-) and bovine (-●-) milk acidified with 30 g L^{-1} glucono-δ-lactone (GDL) for 90 min . Data is from triplicate analysis of 3 independent samples and the coefficient of variation was <5% of the reported value for each data point. Axes scales are $\log_{10}$.

4.5. Conclusions

Equine milk has a chemical composition quite similar to human milk, especially its low fat, low protein, high whey protein:casein ratio compared to bovine milk and high lactose content. The low casein content contributes to the digestibility of equine and human milk, with both milks forming a fine, flaky precipitate with a short transit time through the gastro-intestinal tract. In contrast, bovine milk forms a firm curd in the stomach which may be difficult to digest for some non-bovine species (Hambræus *et al*., 1977). Acidification or chymosin destabilizes bovine casein micelles which is essential in the stomach of the calf for digestion and assimilation and is exploited by man in the manufacture of cheese and fermented milk products (Horne, 2011). The poor gelation of equine milk on acidification and the formation of a fine coagulum is required for the physiological well-being of the foal. It is unlikely that pre-treatment of equine milk by heating (> 70^{o}C) would increase the gel strength of acidified equine milk. Equine β-lactoglobulin lacks a free thiol group (Section 2.1.1.) which in the case of heated bovine milk can form disulfide links with other proteins having a reactive thiol groups or through thiol group-disulfide bridge exchange reactions. In pre-heated bovine milk, denatured β-lactoglobulin covalently interacts with κ-casein at the micelle surface (Corredig & Dalgleish, 1996) and can interact with denatured β-lactoglobulin on other micelles and act as bridging agents by increasing the strength and number of bonds between protein particles with a resultant increase in the gel strength of acid-milk products (Vasbinder *et al*., 2003; Phadungath, 2005) and prevention of syneresis.

For the production of fermented products from equine milk, the high content of very thermo-stable lysozyme in equine milk must be taken into consideration as it may interfere with the microbial activity of starter cultures used in acidification (Jauregui-Adell, 1975). Fortification of equine milk with bovine or ovine milk, pectin, Na-caseinate and sucrose prior to acidification improved the gelation characteristics of acidified equine milk (Di Cagno *et al*., 2004).

The addition of pectin to equine milk for the production of a fermented product should favour gelation due to the high ionic calcium content (Section 3.5.4) of equine milk coupled with an additional increase of ionic calcium into the serum phase on acidification.

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CHAPTER 5

Stability of Equine Casein Micelles. II. Rennet-induced Coagulation of Equine Milk

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Abstract

The colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles, which may have significant implications for the conversion of equine milk into dairy products. This study examined the rennetability of equine milk under varying conditions such as pH, casein concentration, mixing with bovine milk and type of coagulant. Visual examination, particle size measurements, light and confocal microscopy and rheological characterization indicated that equine milk was coagulable slowly by calf chymosin at pH 6.6 but reducing the pH to 6.2 increased the rate of aggregation of equine protein although at both pH values no gel-like structure formed. Concentrating the casein in equine milk three and five fold had little effect on the coagulation process and the final G' remained low. A crude extract of chymosin from foal's stomach coagulated equine milk but without the formation of a strong coagulum. The equine chymosin extract had good activity on bovine milk although a weaker gel was formed than that formed with calf chymosin. Mixing bovine and equine milks at a 3:1 ratio is suggested as a means of producing some novel products from equine milk.

Keywords: equine milk; rennet-induced coagulation; casein; particle size distribution; light microscopy; confocal microscopy; zeta potential; chymosin; equine gastric extract.

5.1. Introduction

Milk is highly digestible and, because it is liquid, the gastrointestinal tract of mammals has mechanisms for delaying its passage; coagulation of milk in the stomach delays the degradation of proteins and improves their assimilation by the body. Caseins are precipitated by gastric acid and enzymes, forming a clot in the stomach which entraps fat. The strength of the curd, largely dependent on the casein concentration in the milk (high casein-containing milks will produce firm clots), which varies between species and will determine the kinetics of evacuation of peptides from the stomach. In the milk of all species studied to date, the casein fraction is present primarily in the form of colloidal particles known as micelles comprising ~ 94% protein and ~ 6% colloidal calcium phosphate (CCP) which contains calcium and phosphate with small amounts of magnesium and citrate (Fox, 2003). Casein micelles determine many of the physico-chemical properties and stability of milk, e.g., enzyme-, acid-, heat- or ethanol-induced coagulation, all of which in turn affect the manufacture and stability of products from milk. The structure and sub-structure of bovine casein micelles have been studied in detail and reviews include: Holt & Horne (1996); Horne (1998, 2006); De Kruif & Holt (2003); Phadungath (2005); Farrell *et al*. (2006); Qi (2007); Fox & Brodkorb (2008).

Bovine casein micelles are sterically stabilized by a brush of predominantly κ-casein (De Kruif & Zhulina, 1996), which protrudes from the micelle surface. Coagulation of casein micelles can occur only following collapse of the brush, which occurs on acidification of milk, i.e., in the manufacture of yoghurt, or on removal of the brush which occurs on rennet-induced coagulation of milk. The combined process of enzyme- and acid-induced coagulation is likely to contribute to the coagulation of casein micelles in the stomach.Chymosin (rennin; EC 3.4.23.4) is a neonatal gastric aspartic proteinase which is commercially important for cheese-making and its use as such probably dates back to ~ 6000BC and is one of the earliest application of enzymes in food processing. Chymosin is produced *in utero* in the abomasal mucosa of newborn mammals (Foltmann, 1970), whereas pepsin (EC 3.4.23.1) is predominant in adult mammalian gastric secretions (Rampili *et al*., 2005). Coagulation of casein micelles in milk can be achieved by various proteinases obtained from animal, plant or microbial sources. However, there are some reports in the literature that a clotting enzyme from a particular species is more effective at coagulating milk from that species than chymosin from an unrelated species. Rennet extracts from lambs or bovine calves where found to be most effective at coagulating the milk of the respective species (Herian & Krcal, 1971). Pig chymosin and pig pepsin showed higher clotting activity on porcine milk than on

bovine milk (Foltmann *et al*., 1981a). Chymosin exhibits a range of pH optima depending on the species but typically the pH optimum of bovine chymosin variants A and B is 4.2 and 3.7, respectively (Foltmann, 1992).The subject of rennets and their general and molecular characteristics have been reviewed widely (Sardinas, 1972; Ernstrom & Wong, 1974; Sternberg, 1976; Green, 1977; Phelan, 1985; Fox & McSweeney, 1997; Chitpinityol & Crabbe, 1998; Crabbe, 2004; Claverie-Martín & Vega-Hernández, 2007). During the incubation of bovine milk with rennet, chymosin hydrolyses the Phe_{105}-Met_{106} bond of κ-casein, leading to the formation of two fragments, the hydrophobic N-terminal fragment which remains attached to the casein micelles and is referred to as para-κ-casein, and the hydrophilic C-terminal fragment which is released into the milk serum and is referred to as the caseinomacropeptide (CMP) or glycomacropeptide (GMP) (Jollès & Alais, 1959; van Hooydonk & Walstra, 1987; Paynes, 1989; Walstra, 1990; Dalgleish, 1992). As a result, the micelles lose steric stabilization and become susceptible to aggregation (Dalgleish, 1992). Hydrophobic bonds are the main contributors to aggregation and gelation; Ca^{2+} and colloidal calcium phosphate (CCP) play essential roles (Dalgleish, 1983; Fox & McSweeney, 1997; Lucey, 2002a,b). The strength of a rennet-induced gel is dependent on the physical and chemical properties of the casein micelles, i.e., micelle size, protein content (i.e., casein), calcium concentration, pH and the ratio of casein to whey proteins (Lucey *et al*., 2003; Auldist *et al*., 2004; Amenu & Deeth, 2007). For detailed reviews on the renneting of bovine milk see Walstra & Jenness, (1984), Wong, (1988), Walstra, (1990) Fox & McSweeney, (1998) and Walstra *et al*. (2006). Physiologically, the release of CMP in the mammalian stomach is important. CMP inhibits acid gastric secretions and gastrin activity and has been found in blood plasma (Yvon *et al*., 1994; Chabance *et al*., 1995, 1998; Fosset *et al*., 2002) where it modifies blood concentrations of regulatory digestive peptides (Yvon *et al*., 1994). Research has shown that CMP is the only peptide released during the first hour after ingestion of milk by the calf, with fragments 165-199 of α_{s1}- and 193-209 of β-casein being released within ~ 90 min (Yvon & Pellissier, 1987). The release of CMP in the stomach is believed to increase the efficiency of the digestive process and control acid secretion (Stan *et al*., 1982) while preventing neonatal hypersensitivity to ingested proteins and inhibiting gastric pathogens (Rhoades *et al*., 2005). The presence of κ-casein in equine milk was a subject of debate for several years, with several authors (Visser *et al*., 1982; Ono *et al*., 1989; Ochirkhuyag *et al*., 2000) reporting its absence. However, other studies (Kotts & Jenness 1976; Malacarne *et al*., 2000; Iametti *et al*., 2001; Egito *et al*., 2001) reported its presence, albeit at a low concentration. The primary structure of equine κ-casein has been described

(Iametti *et al*., 2001; Lenasi *et al*., 2003; Miranda *et al*., 2004). Equine κ-casein is hydrolysed slowly by calf chymosin at the Phe_{97}-Ile_{98} bond (Egito *et al*., 2001), without gel formation (Section 3.7.9) while Phe_{105}-Ile_{106} bond of human κ-casein is reported to be slowly hydrolysed by calf chymosin (Plowman *et al*., 1999). The CMPs released from equine and human

κ-caseins are considerably less hydrophilic than bovine CMP. The sequence 97-116 of

κ-casein is highly conserved across species, suggesting that the limited proteolysis of κ-casein and subsequent coagulation of milk are of major biological significance (Mercier *et al*., 1976; Martin *et al*., 2011). The poor coagulabiltiy of equine milk by calf chymosin (Section 3.6.9) may be due to several factors, for example the chymosin-sensitive bond of equine κ-casein may be located in a manner which renders it inaccessible to chymosin, or the equine casein micelles derive colloidal stability from constituents other than κ-casein and it has been proposed that unphosphorylated β-casein plays a role in micellar stability (Ochirkhuyag *et al*., 2000; Doreau & Martin-Rosset, 2002). The high degree of glycosylation may also affect the ability of chymosin to hydrolyze equine κ-casein (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996).

In order to improve the gelation characteristics of equine milk and increase its economical importance it is necessary to understand which parameters and mechanisms are responsible for its poor coagulability by rennet. The purpose of this study was to examine the rennetability of equine milk under varying conditions such as pH, casein concentration, mixed with bovine milk and type of coagulant.

For clarity, the terminology used in this study is that adopted by Huppertz & de Kruif (2007) and is summarized as follows: *coagulation* refers to the process through which milk changes into a firm coagulum; *flocculation* refers to the process by which fine particulates clump together into floccules which may float or settle in the liquid. Throughout this study, flocculation may lead to aggregation and gelation or aggregation without gel formation depending on the system under study. The Berridge test, used in this study to monitor rennet activity, determines the flocculation time of rennet-treated milk, i.e., the time taken to observe, visually, speckles or ‘flocs’ on the wall of a test tube.

Generally, the gel point observed during small amplitude oscillatory rheology is regarded as the point where the storage modulus (G') and the loss modulus (G") cross-over or when G' increases to ~ 1 Pas. Alternatively, since milk has a low initial viscosity and G" values are not measurable due to low sensitivity of the rheometer , the gel point can be regarded as the point

when dynamic moduli responses become greater than the background noise of the instrument (Horne, 1999); this method of gel-point observation was adopted throughout the present study.

5.2. Materials and Methods

5. 2.1. Milk supply

Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from 5 milkings over 24 h, from a herd (N > 45) of multiparous, New Forest and New Forest/Arabian mares in mid-lactation, physically separated by day from their foals. The milk was filtered through glass wool to remove any extraneous material and cooled to 4^{o}C. It was received at our laboratory within ~24 h of milking.

The milk was defatted by centrifugation at 1,000 g for 20 min at 20^{o}C using a Sorvall® RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) and filtered through glass wool to remove fat particles. Raw whole bovine milk, obtained from a local dairy farm, was defatted by centrifugation at 2,000 g for 20 min at 20^{o}C followed by filtration through glass wool. Camel milk was obtained from Kamelenmelkerij Smits, Cromvoirt, the Netherlands and was defatted by the procedure used for equine milk. Sodium azide, 0.5 g L^{-1}, was added as preservative to skimmed milk samples which were stored for no longer than 3 days.

pH was measured using a Radiometer pHM 210 standard pH meter equipped with a Radiometer Meterlab® combined general purpose electrode with a built-in temperature sensor (Radiometer Analytical SAS, Lyon, France). All chemicals used were of reagent grade and obtained from Sigma-Aldrich. (St. Louis, MO, USA) unless otherwise stated. All analyses, unless otherwise stated, were carried out in at least triplicate and average results with standard deviations are reported where appropriate. Descriptive statistical analysis of data (mean, standard deviation) was carried out using Minitab Statistical Software (Release 13.31; Minitab Inc., State College, PA, USA). Graphical and photographic representations of results are presented from single analyses for clarity. Throughout this study the temperature was controlled within ± 0.1^{o}C.

5.2.2. Milk sample preparation

For some experimental work, equine milk powder (Lyempf BV, Kampen, the Netherlands) and bovine low-heat skim milk powder (Nilac) (NIZO, Ede, the Netherlands) were used. The Nilac powder was reconstituted in distilled water at
100 g L^{-1} as described by de Kruif (1997) which ensured that the renneting properties of the milk were equal to those of the fresh milk before drying. The equine milk powder was reconstituted in demineralised water at 110 g L^{-1}. Reconstituted milk was stirred at 20°C for 24 h to ensure complete equilibration of the mineral balance and hydration of the casein micelles, and subsequently skimmed by centrifugation at 1,000 × g for 20 min at 20°C, followed by filtration through glass wool. Sodium azide was added at a level of 0.5 g L^{-1} to both bovine and equine milk preparations to prevent microbial growth.

5.2.3. Assessment of whey protein denaturation in equine milk powder

Prior to use, the levels of denatured α-lactabumin (α-la) and β-lactoglobulin (β-lg) in equine milk powder were determined to ascertain if the milk was of low-heat grade and suitable for some experimental work in this study. The levels of denatured α-la and β-lg in reconstituted equine milk were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by McSwiney *et al*. (1994) with some modifications (Oldfield *et al.*, 1998). Samples were analysed by both reducing and non-reducing SDS-PAGE using a Bio-Rad Criterion™ mini-gel unit (Bio-Rad Laboratories, Hercules, CA, USA) and Criterion™ precast gels (18% Tris-HCl). The gels were run at a constant voltage of 100 V. Gels were stained with colloidal Coomassie Blue (Chevalier *et al*., 2004); the electrophoretograms were imaged with a GS-800 imaging densitometer (Bio-Rad) and the integrated intensities of α-la and β-lg from reducing and non-reducing SDS-PAGE were analysed with PDQuest software, V.7.3.1 (Bio-Rad).

5.2.4. Compositional analysis of equine milk

The total protein and fat content of the reconstituted equine milk was determined by the Kjeldahl (IDF, 2001) and Gerber (IDF, 1981) methods, respectively. The concentration of total calcium in milk and its ultracentrifugal supernatant (100,000 × g for 60 min at 20°C) was determined by atomic absorption spectroscopy as described in Section 3.2.5.1.The concentration of ionic calcium was determined using a calcium-selective electrode, as described in Section 3.2.5.2.

5.2.5. Preparation of suspensions of equine casein micelles

Suspensions of equine casein micelles were prepared in either equine or bovine ultracentrifugal supernatant by pelleting equine micelles ultracentrifugally (100,000 *g* for 90 min at 20°C) using an Optima LE-80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a Beckman 50.2 Ti rotor (12 place) and resuspending the pellets in the ultracentrifugal supernatant [from which residual micelles and fat globules were removed by ultrafiltration (0.22 μm syringe filter, Minisart®), Sartorius Stedim Biotech S.A., Aubagne, France)] of either equine or bovine milk, at a level of ~ 55 g L^{-1} casein, using a tissue homogeniser, followed by stirring for ~20 h at 4°C. The suspensions were subsequently centrifuged at 500 g for 30 min at 20°C to remove any undissolved material and the casein content determined using the Kjeldahl method (IDF, 2001) and adjusted to 35 or 50 g L^{-1}, which are approximately three and five times, respectively, the casein content of equine milk, by the addition of ultracentrifugal supernatant and subsequently adjusted to pH 6.6. Sodium azide, 0.5 g L^{-1} was added as preservative to the samples which were stored for no longer than 3 days.

5.2.6. Influence of renneting on the particle sizes of equine and bovine milk

The influence of renneting at 30°C with 5 μl mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 (DSM Food Specialities, Delft, the Netherlands) on the average particle size (Z-average) in fresh skimmed bovine or equine milk at pH 6.6 was examined using a particle size analyser (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcs, UK) on undiluted samples at a scattering angle of 173°. The instrument was tested with certified particle size standards (20 to 900 nm) (Nanosphere™ standards, Duke Scientific Corp., Palo Alto, CA, USA). The rennet was added to the bovine milk sample 30 min after its addition to equine milk due to the sensitivity of the instrument to small changes in particle size.

5.2.7. Microscopy studies of the structural changes in equine milk on renneting

Stereo light microscopy

The influence of renneting fresh skimmed equine milk at pH 6.6 and 30°C by

5 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 on the structure of fresh skimmed equine milk was examined using a Reichert-Jung Polyvar Met optical microscope (Leica Microsystems GmbH, Wetzlar, Germany) operated in bright-field mode. Images were obtained 120 min after rennet addition

Confocal microscopy

Fresh skimmed equine milk was renneted at pH 6.6 and 30°C using 5 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 and images of the renneted sample were recorded, using a confocal laser light microscope, 120 min after rennet addition. The instrument used was an Olympus Fluoview 1000 inverted confocal laser imaging system (Olympus America, Center Valley, PA, USA) mounted on an Olympus 1 x 81 microscope. Samples were placed in 35 mm glass bottom culture dishes with 14 mm microwells (MatTek Corp., Ashland, MA, USA). Some samples were imaged stained with a 50:50 mixture of Nile red and fluorescein isothiocyanate (FITC) stain and some samples were not. Excitation wavelengths of 488 (FITC) and 543 nm (Nile red) were used for imaging. Fluoview software (Olympus Microscopy, Southend-on-sea, Essex, UK) was used to capture images and record images at 100 K magnification and to create a Z-scale stacked image of the renneted milk.

5.2.8. Measurement of zeta potential of equine and bovine milk as a function of renneting

The zeta potential of fresh skimmed equine and bovine milk diluted 1:250 and 1:500, respectively, with lactose-free synthetic milk ultra-filtrate (SMUF; Jenness & Koops, 1962) was measured as a function of time after rennet addition by laser-Doppler electrophoresis using a Malvern Zetamaster 3000 instrument (Malvern Instruments, Malvern, Worcestershire, UK) as described in Section 3.3.4. Ten μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 were added to each milk (pH 6.6) which had been tempered at 30°C, and the zeta potential was measured at 5 min intervals on aliquots held at 30°C.

5.2.9. Rennetability of equine casein micelles at 35 or 50 g L^{-1}

Equine casein micelles, suspended at 35 or 50 g L^{-1} casein in either equine or bovine ultracentrifugal supernatant, were renneted at 30°C, pH 6.6 using 1:10 (v/v) diluted Maxiren 180. Dynamic oscillatory analysis was performed as described in Section 3.4.7 using a controlled shear stress Carri-Med CSL^2 100 rheometer equipped with concentric cylinders and a recessed acrylic rotor (TA Instruments, Leatherhead, Surrey, UK). Eleven mL of sample

were pre-warmed in a waterbath at 30°C for 15 min and 120 μL of a 1:10 (v/v) dilution of Maxiren 180 were added. The storage modulus, G', of the sample was recorded continuously at a low amplitude shear strain (0.01) over 90 min at a frequency of oscillation of 0.6283 rad s^{-1}; analysis was carried out in triplicate.

5.2.10. Rennetability of equine milk as a function of pH

Samples of fresh skimmed equine milk were adjusted to pH values in the range 6.0 to 7.2 at 0.1 pH unit intervals. The samples were held overnight at 4°C and tempered to 20°C prior to readjustment of pH, as necessary. After heating to 30°C, 1:10 (v/v) diluted Maxiren 180 was added at a level of 10 μl mL^{-1} to 2 mL sample at each pH value. The samples were rocked gently in a waterbath until the first signs of flocculation, after which the samples were placed in a rack and held under quiescent conditions for 15 min before visual assessment.

5.2.11. Effect of renneting on the particle size distribution of equine milk

The effect of rennet at pH 6.6 or 6.2 on the size distribution of equine casein micelles in fresh skimmed equine milk was monitored at 30°C by laser light scattering using a Malvern Mastersizer model S (Malvern Instruments Ltd., Malvern, Worchestershire, UK), as described in Section 3.3.5. Synthetic milk ultrafiltrate (Jenness & Koops, 1962) at 30°C was used as dispersant and particle size was measured at 10 min intervals, after addition of 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180, for the pH 6.6 samples and thereafter at two hourly intervals up to 12 h. For the milk sample renneted at pH 6.2, particle size measurement started 10 min after rennet addition and was measured up to 4 h, after which the large aggregates formed were outside the measuring range of the instrument.

5.2.12. Preparation of chymosin extract from foal stomach

Five foal stomachs obtained from the Irish Equine Centre (Johnstown, Co. Kildare, Ireland) were thawed and gastric extracts were prepared individually as follows: each stomach (~80 g) was cut into small pieces and homogenized in a Waring blender with an equal weight of 10% NaCL until thoroughly homogenized (~ 6 min). The homogenate was held at room temperature for 2 h and then centrifuged for 20 min at 3,000 g at 4°C. The pellet was recovered and the homogenization and centrifugation steps repeated. About 20 mL of crude extract (pH of 5.45 -5.50) were recovered at each step and pooled. The pH was reduced to 2.0 with 1M HCL to activate the proenzyme. A slight precipitate formed on acidification which was removed by centrifugation at 5,000 g for 30 min at 4°C. After holding for 30 min, the pH

was adjusted to 5.5 with 1M NaOH and the extract was stored at -80^{o}C. Without further purification, the clotting activity of the gastric extracts was measured and expressed as international milk clotting units per mL (IMCU; IDF,1997A). A liquid calf chymosin reference standard (> 99% chymosin; Chr. Hansen A/S, Hoersholm, Denmark) of 100 IMCUs mL^{-1} was used

5.2.13. Effect of pH on the activity of foal gastric extract.

Samples of 100 g L^{-1} reconstituted low-heat skimmed bovine milk were adjusted to pH values in the range of pH 6.0 to 6.8 and held overnight at 4^{o}C. Following equilibration at 20^{o}C, the pH was readjusted, if necessary. Two mL milk samples at each pH were renneted by the Berridge method (IDF, 1987) with either 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 or 50 μL of equine gastric extract and the time taken to the formation of visible floccs was recorded as the rennet coagulation time at a particular pH.

5.2.14. Coagulability of equine and bovine milk with foal gastric extract

Fresh skimmed equine or bovine milk (2 mL) was renneted at 30^{o}C with 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 or 50 μL mL^{-1} of equine chymosin extract (pH 5.5). The milk was renneted at its natural pH (7.2) and at pH 6.2. In a follow-up study, equine milk was stained with methylene blue (0.01%, w/v) and renneted with either Maxiren 180 or equine chymosin extract at pH 6.2.

5.2.15. Effect of equine casein concentration on the rennetability of equine milk

The effect of the concentration of equine casein on the rennetability of equine milk by either Maxiren 180 or foal gastric extract was studied on 2 mL samples of either fresh skimmed equine milk or equine micelles suspended in equine ultracentrifugal supernatant at 25 g L^{-1} casein at 30^{o}C and pH 6.2, prepared as described in Section 5.2.5.

5.2.16. Dynamic oscillatory analysis of equine and bovine milk renneted by foal gastric extract

Dynamic oscillatory measurements of the storage modulus, G', as a function of time after the addition of either Maxiren 180 or equine gastric extract to fresh skimmed bovine or equine milk were made as described in Section 3.4.7. The frequency of oscillation was set at 0.6283 rad s^{-1}. The storage modulus, G', of the sample was recorded continuously at a low amplitude

shear strain (0.01) over 90 min. Measurements were made at 30°C and pH 6.2 for 90 min. Maxiren 180 and equine gastric extract were added at levels of 120 μL mL^{-1} of a 1:10 dilution of Maxiren 180 and 1100 μL of foal gastric extract, per 11 mL substrate.

5.2.17. Rheological characterization of bovine milk renneted with foal gastric extract

The structure of a bovine milk gel formed after the addition of Maxiren 180 or equine gastric extract to fresh skimmed bovine milk was characterized by performing mechanical spectra on the samples renneted at pH 6.5, 60 min after the addition of rennet. The frequency-dependence of the storage modulus, G', loss modulus, G" and complex dynamic viscosity, η*, where

$$\eta^* = \frac{(G'^2 + G''^2)^{½}}{\omega}$$

and ω is frequency of oscillation (rad s^{-1}), were recorded to show the variation of G' and G" with frequency in the range 0.1-100 rad s^{-1} at a fixed strain of 0.5%.

Analysis was carried out using a controlled shear stress Carri-Med CSL^2 100 rheometer equipped with concentric cylinders and a recessed acrylic rotor (TA Instruments, Leatherhead, Surrey, UK) as described in Section 3.4.7. Data were plotted on a double logarithmic scale.

5.2.18. Coagulability of reconstituted equine and bovine milk by various rennets

Various milk-clotting enzymes were assessed for their ability to coagulate reconstituted equine milk or reconstituted bovine milk. The amount of each coagulant added was adjusted to give a rennet coagulation time (RCT) of ~ 7 min with the bovine substrate. The coagulants used, with the activity in international milk clotting units (IMCU) indicated in brackets, were: calf chymosin (> 90% chymosin, 113 IMCU mL^{-1}; Chr. Hansen); fermentation-produced calf chymosin, Maxiren-180, (180 IMCU mL^{-1}, DSM Food Specialties, Delft, the Netherlands); *Cryphonectria parasitica* proteinase (650 IMCU mL^{-1}, Suparen® DSM Food Specialties); Fromase 750 XL (750 IMCUs mL^{-1}), a fungal coagulant derived from *Rhizomucor miehei* (DSM Food Specialties); bovine pepsin (100 IMCUs mL^{-1}, Chr. Hansen); porcine pepsin (Sigma-Aldrich Chemical Co.) prepared at a concentration of 0.02% in Universal buffer at pH 5.0 (0.02 M each of citric acid, KH_2PO_4, boric acid and diethylbarbituric acid; Medham *et al*., 2000).

Determination of the rennetability of different milks by chymosin from either the same or another species was assessed by monitoring the storage modulus (G') over time at 30°C for 90

min after the addition of enzyme. Fresh skimmed camel, bovine and equine milks were renneted with either Maxiren 180 (10 μL mL $^{-1}$ of a 1: 10 dilution), equine chymosin (50 μL mL^{-1}) or camel chymosin (10 μL mL $^{-1}$ of a 1:100 dilution; CHY-MAX™ M, 1261 IMCU mL^{-1}; Chr. Hansen). Analysis was carried out at pH 6.6 except in the case of equine milk renneted with equine chymosin where the pH was 6.2. In one experiment, 0.02 g L^{-1} $CaCl_2$ was added to camel milk and the amount of Maxiren 180 was increased to 30 μL mL^{-1}.

5.2.19. Effect of mixing bovine milk and equine milks on curd formation by calf chymosin

Fresh skimmed equine and bovine milks were mixed at weight ratios of 1:1, 3:1, 5:1 and 7:1 equine:bovine milk and renneted at pH 6.2 with 10 μl mL^{-1} of 1:10 diluted Maxiren 180. Gel formation was monitored by dynamic oscillatory analysis of G' as a function of time after rennet addition.

5.2.20. Urea polyacrylamide gel electrophoresis of renneted mixtures of equine and bovine milk

To determine if equine proteins had been incorporated into the rennet gels formed from mixtures of equine and bovine milks, urea polyacrylamide gel electrophoresis (Urea-PAGE) was carried out on the pellets and supernatants. Samples of milk (2 mL) were renneted at pH 6.2 with 10 μL mL^{-1} of 1:10 diluted Maxiren 180 in Eppendorf tubes under quiescent conditions for 20 min after which they were heated quickly to 60^{o}C for 5 min to inactivate chymosin and centrifuged at 5,000 g for 30 min at 20^{o}C. The supernatants were removed carefully and diluted directly 1:1 with double-strength urea-PAGE sample buffer. The pellets were frozen, lyophilised and dissolved at 10 mg mL^{-1} in single-strength sample buffer. Bovine and equine milks were renneted under the test conditions as controls. Bovine sodium caseinate, prepared as described in Section 3.2.2 and fresh skimmed bovine and equine milks (diluted 1:1 with sample buffer) were included in the analysis. Pellets and supernatants were recovered from renneted equine and bovine milk and served as controls for image analysis.

Urea-PAGE [12.5% total monomer concentration (T); 4% cross-linking monomer concentration (C), pH 8.9] was performed using a Protean IIxi vertical slab gel unit (Bio-Rad Laboratories Ltd., Hercules, CA, USA) according to the method of Andrews (1983), as modified by Shalabi & Fox (1987) and some additional modifications described in Section 3.4.9. The gels were stained directly with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and destained in several changes of distilled water. Quantitative densitometric image

analysis was performed on the urea-PAGE gels using gel analysis software Total Lab Quant 1D version 11.4 (Total Lab Ltd.,, Newcastle-upon-Tyne, UK).

5.3. Results and Discussion

5.3.1. Compositional analysis of reconstituted equine milk

The protein content of the reconstituted skimmed equine milk was 20.3 ± 1.42 g L^{-1} which was within the range of that reported for fresh skimmed equine milk in Section 3.5.1. The fat content of the reconstituted skimmed milk was 0.8 g L^{-1}, indicating that the skimming procedure applied removed most of the fat from equine milk. The pH of the reconstituted skimmed equine milk was 6.87. The skimmed reconstituted equine milk contained 774 mg calcium L^{-1}, whereas its ultracentrifugal supernatant contained 252 mg calcium L^{-1}, suggesting that in equine milk, 65% of total calcium is in the micellar phase; which agrees well with results reported by Holt & Jenness (1984).

The concentration of ionic calcium, at the natural pH was 2.6 mmol L^{-1}, which agrees well with the value reported in Section 3.5.2. for fresh equine milk.

To determine the extent of denaturation of equine whey proteins during equine milk powder preparation, a densitometric comparison of equine milk after electrophoresis (figure not shown) by reducing and non-reducing SDS-PAGE indicated that 18 ± 4 % of β-lg and 14 ± 3 % of α-la were present as disulphide-linked multimeric proteins aggregates which probably resulted from thermal denaturation of these protein during the manufacture of the milk powder. Classification of equine milk powder as low-, medium- or high-heat milk powder according to the traditional whey protein nitrogen index (WPNI) used for bovine milk powders (see Kelly *et al*., 2003) is not possible due to the difference in initial whey protein content between bovine and equine milk and different heat denaturation patterns of the whey proteins in both milks. However, an approximation shows that for powdered bovine milk of average composition, denaturation of 15-20% of whey protein during processing yields a product with a WPNI sufficiently high to be classified as low-heat skim milk powder.

5.3.2. Influence of renneting on size of particles in equine and bovine milk

Figure 5.1 shows the effect of renneting on the particle sizes of equine and bovine milk. After 60 min renneting, the size of particles in equine milk began to increase and reached a value of 900 ± 17 nm for triplicate analysis at 140 min, which indicated that the casein micelles had begun to aggregate as a result of rennet action and the particle size continued to increase thereafter. For bovine milk, the use of laser light scattering indicated a change in casein micelle size almost immediately after chymosin addition which is not registered on a rheometer (Glantz *et al*., 2010). Therefore, rennet was not added to bovine milk until 30 min after the renneting of equine milk. The particles in bovine milk began to increase in size rapidly and the sizes recorded increased and reached values of ~ 1 μm (± 20 nm for triplicate analysis), 10 min after aggregation was first recorded. The result demonstrated that equine milk was susceptible to chymosin-induced aggregation although aggregation occurred at a much slower rate than bovine milk at pH 6.6.

5.3.3. Microscopy studies of the structural changes in equine milk on renneting

Stereo light microscopy

An image of renneted equine milk, recorded 120 min after the addition of rennet, is shown in Figure 5.2. The image showed that considerable aggregation of equine casein micelles occurred when the milk was renneted by chymosin at pH 6.6, in agreement with the increase in particle size which was recorded by laser light scattering. In bright-field mode, the dark background was visible between areas of aggregated protein.

Confocal microscopy

An unstained confocal micrograph of equine milk renneted with 5 μL mL^{-1} of 1:10 diluted Maxiren 180 at 30^{o}C is shown in Figure 5.3. The image, recorded 120 min after rennet addition, showed aggregated protein present throughout the renneted sample with several distinct dark areas of background without aggregated protein which indicated that a significant proportion of equine protein was incorporated into the aggregates. The image was essentailly the same as that of renneted equine milk obtained by stereo light microscopy (Fig. 5.2). By staining the renneted sample with FITC and Nile red, residual fat particles in the milk were stained orange/red and the protein stained green. The control equine milk micrograph (Figure 5.4) showed residual fat particles in the skimmed equine milk partially obscured by the background protein. When the sample was renneted (Figure 5.5), the aggregated protein

network was clearly visible with several fat particles incorporated into the aggregates. A set of Z-stack images (Figure 5.6) were recorded from consecutive optical sections taken at different depths in the renneted sample and provided a three-dimensional image of the sample structure. On renneting, aggregates of equine casein are dispersed evenly throughout the milk with residual fat globules of various sizes incorporated within the aggregates.

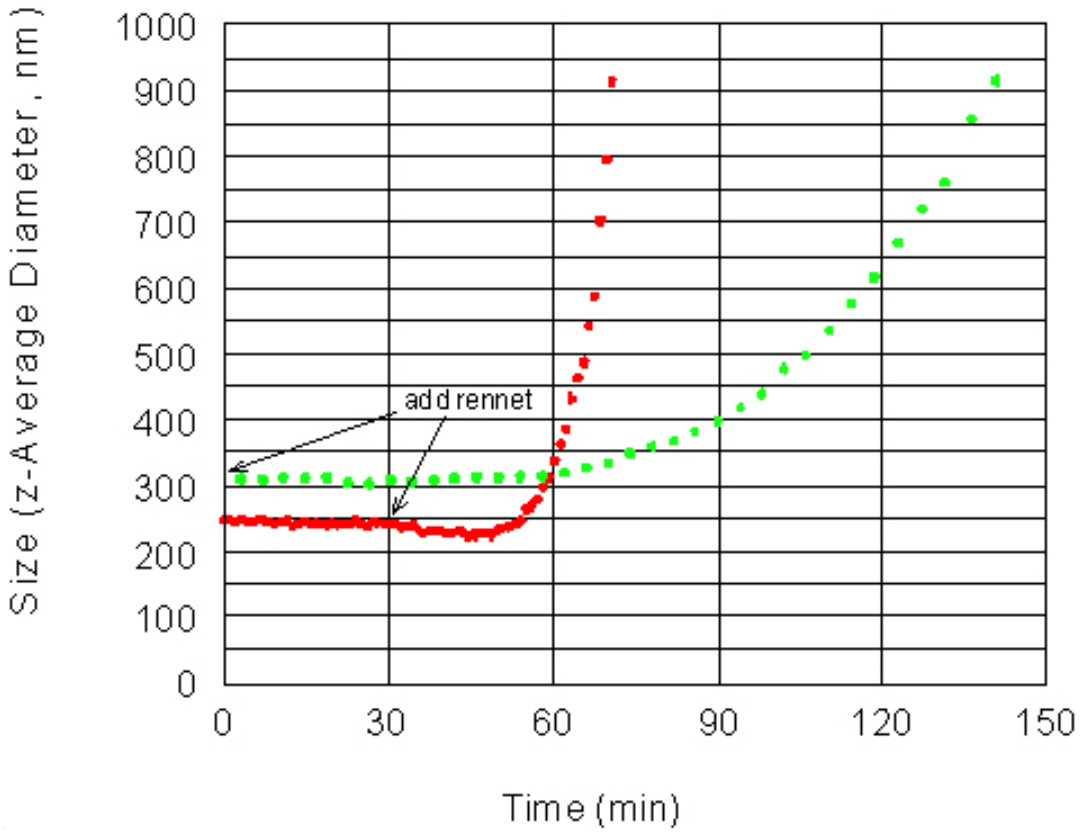

Figure 5.1. Influence of renneting with 5 μL mL^{-1} of 1:10 diluted Maxiren 180 at 30°C on the particle size in bovine (●) and equine (●) milk at pH 6.6 measured by laser light scattering. Rennet was added to the bovine milk sample 30 min after its addition to equine milk due to the sensitivity of the instrument. For clarity, the result of one set of analyses is shown.

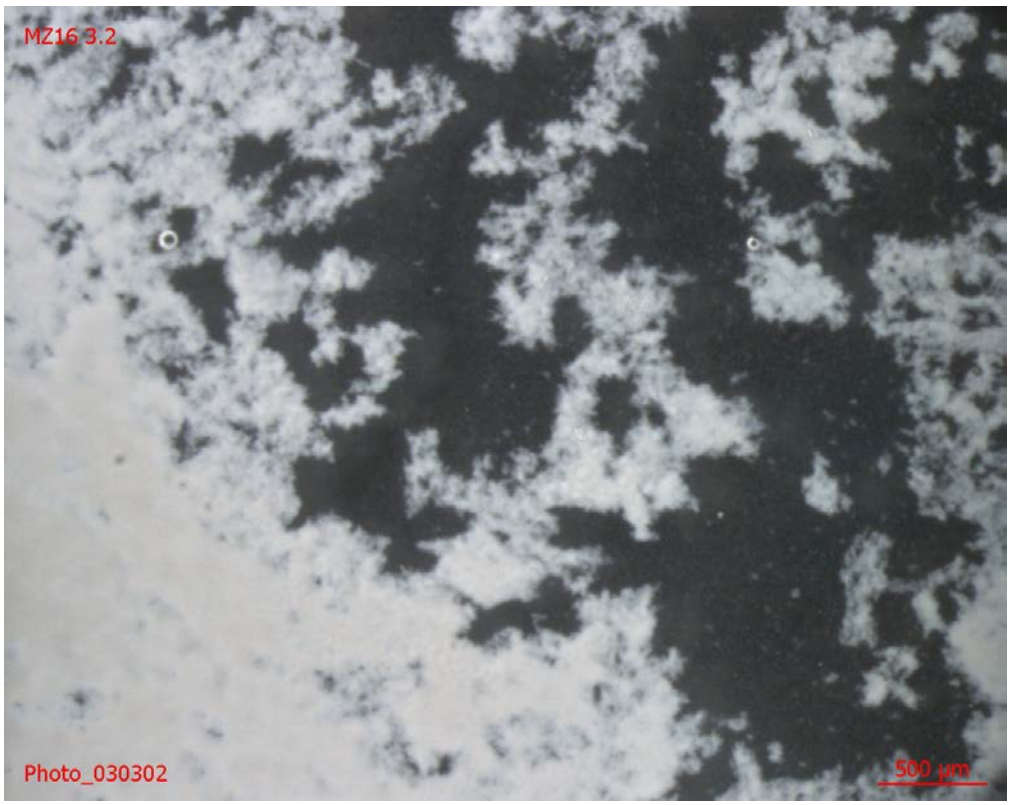

Figure 5.2. Stereo light micrograph of equine milk renneted with 5 μL mL^{-1} of 1:10 diluted Maxiren 180 at 30°C, pH 6.6 for 120 min. Dark areas are the aqueous phase and white areas are aggregated protein.

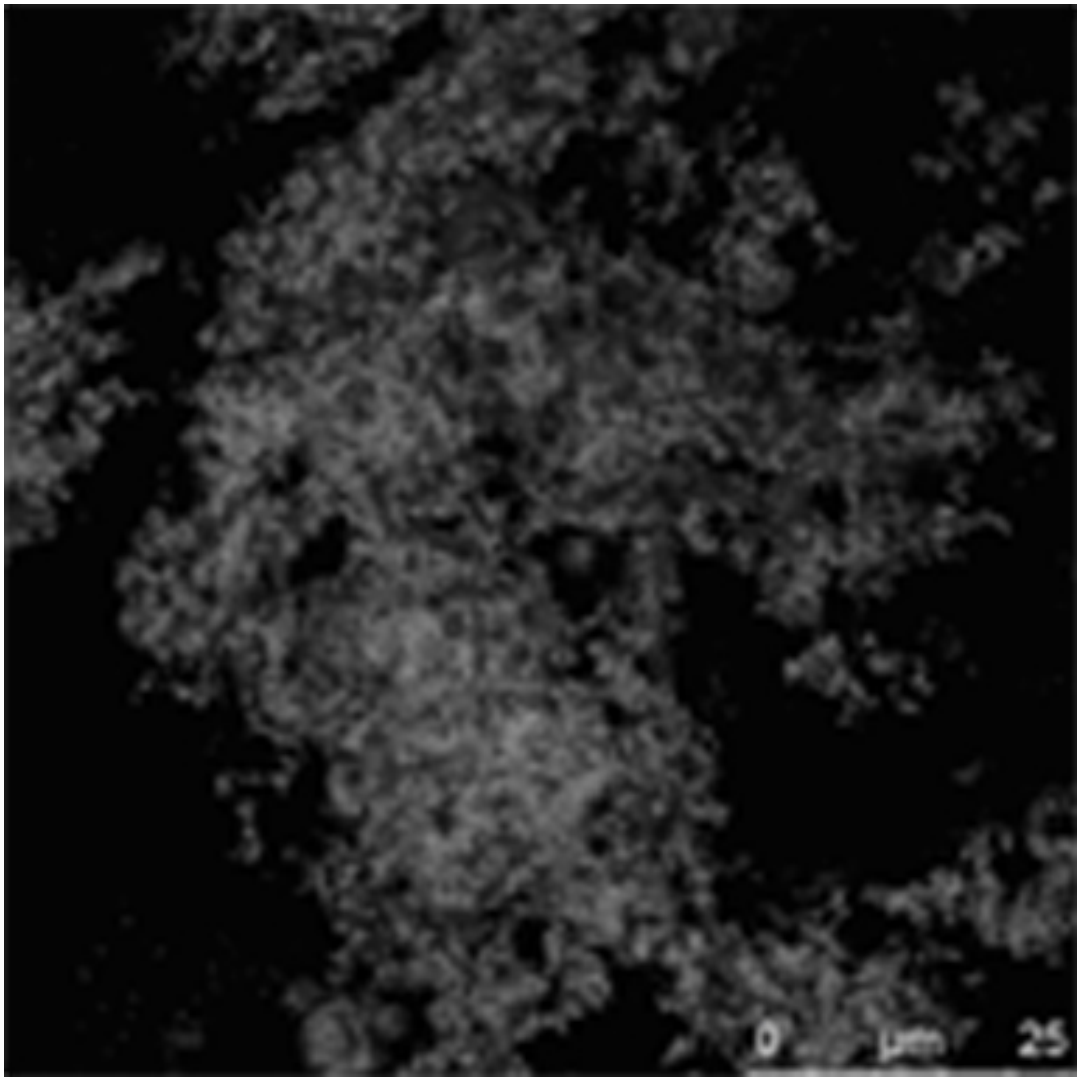

Figure 5.3. Confocal micrograph of equine milk renneted with 5 μL mL^{-1} of 1:10 diluted Maxiren 180 at 30^{o}C, pH 6.6 for 120 min.

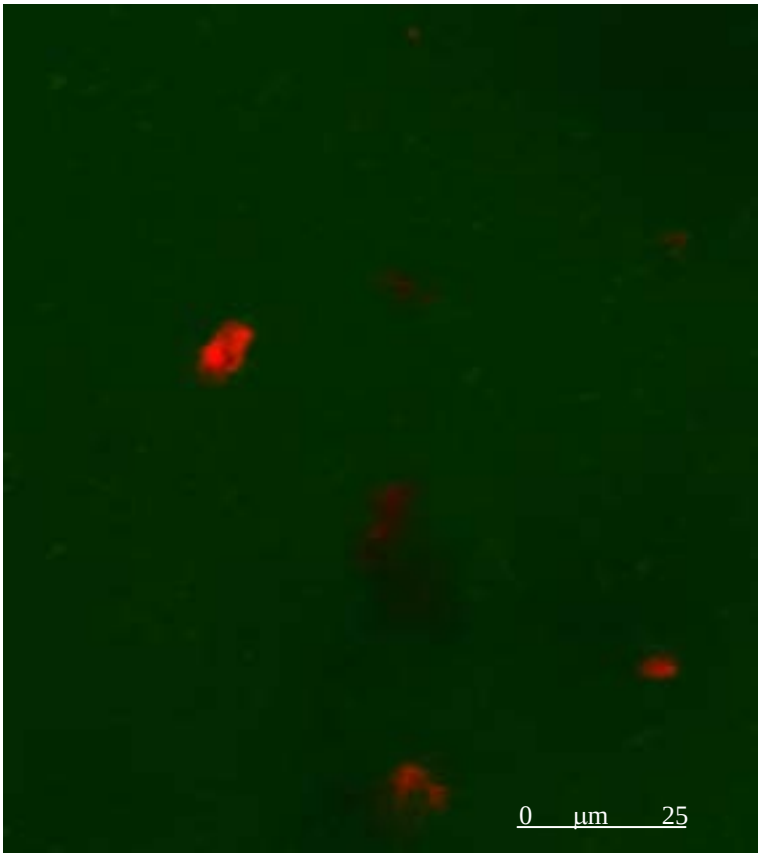

Figure 5.4. Confocal micrograph of untreated equine milk stained with FITC and Nile red.

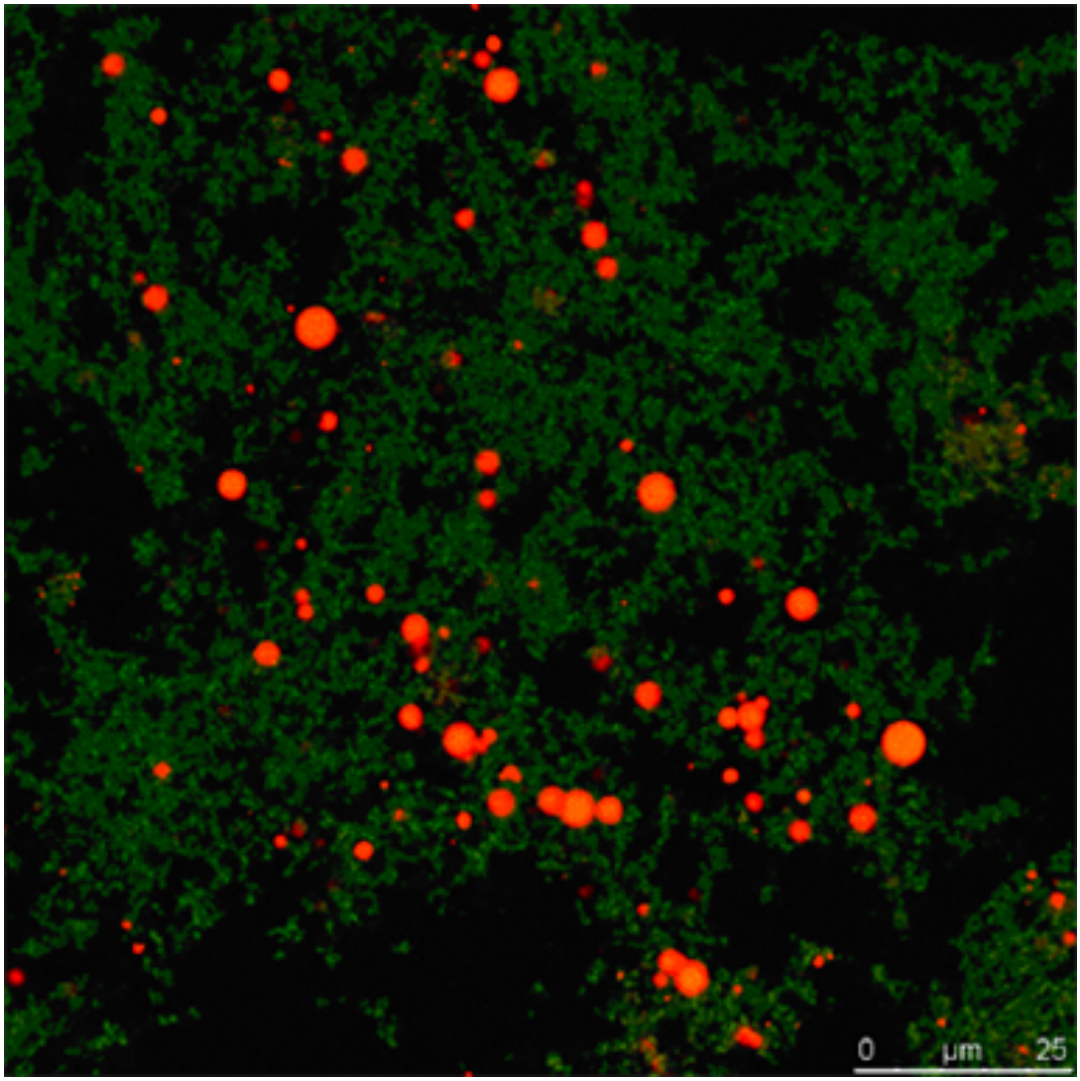

Figure 5.5. Confocal micrograph of equine milk renneted with 5 µL mL^{-1} of 1:10 diluted Maxiren 180 at 30°C for 120 min at pH 6.6 and stained with FITC/Nile red.

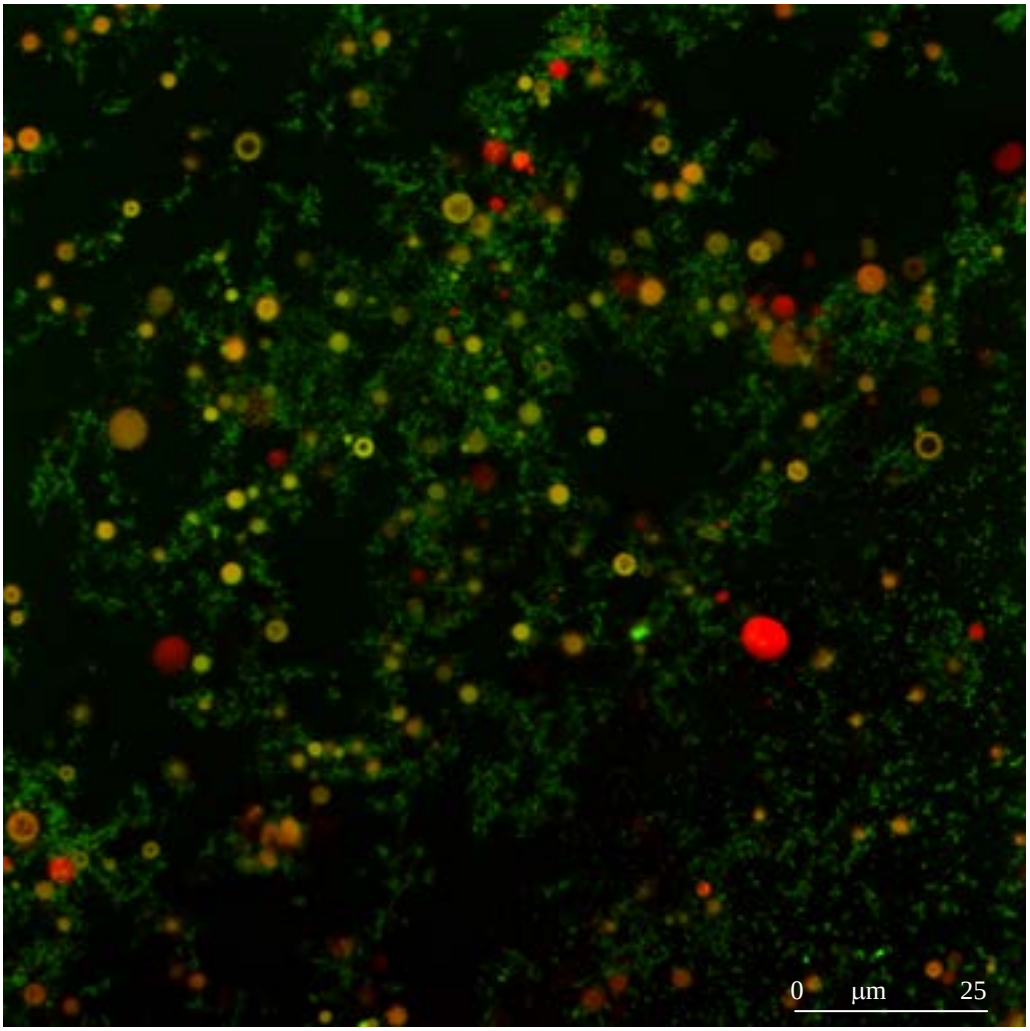

Figure 5.6. A Z-stack of confocal images from a set of consecutive optical sections recorded at different depths through a sample of equine milk renneted with 5 μL mL^{-1} of 1:10 diluted Maxiren 180 at 30^{o}C for 120 min at pH 6.6 and stained with FITC and Nile red.

5.3.4. Measurement of zeta potential as a function of renneting of equine and bovine milk

Figure 5.7 shows the ζ-potential of equine and bovine milk as a function of renneting time at 30°C; there was a significant decrease in the ζ-potential of bovine milk, from an initial value of -22 ± 1 mV, over time after rennet addition. At the point of visual flocculation of bovine milk, ~ 7 min after chymosin addition, the ζ-potential had decreased to -11.8 ± 0.2 mV and reached a minimum ζ-potential value of -9.9 ± 0.17 mV at the point of gelation and thereafter the ζ-potential increased slightly. In contrast, the ζ-potential of equine milk remained unchanged after chymosin addition from an initial value of -10.3 ± 0.15 to -9.6 ± 0.13 mV when some flocculation of the milk was observed ~ 180 min after chymosin addition.

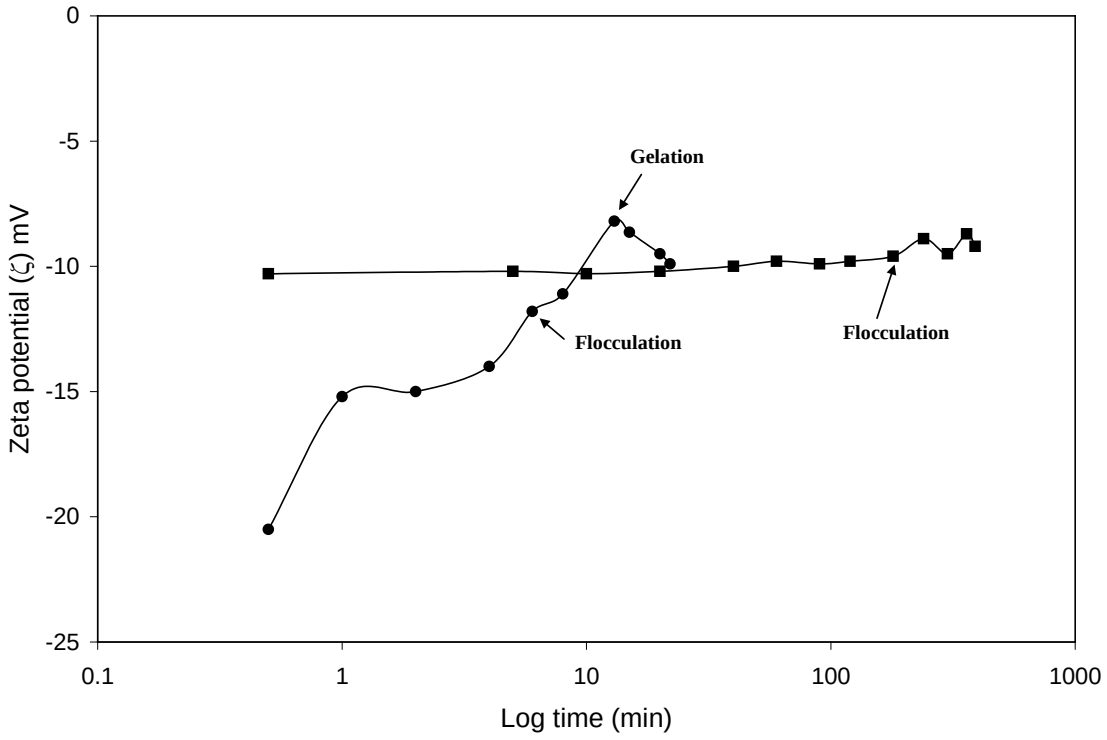

Figure 5.7. Zeta potential (ζ) as a function of time (log scale) for equine (-■-) and bovine (-●-) milk (pH 6.6) renneted with 10 μl mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 at 30°C. For clarity, the result from a single analyses is shown.

At 45°C and pH 6.8, the ζ-potential of bovine milk is ~ 22 mV and at 20°C and pH 5.7 it is ~ 8 mV; when bovine milk is renneted, the ζ-potential decreases progressively until all of the κ-casein has been cleaved by chymosin (Darling & Dickson, 1979; Gastaldi *et al*., 2003; Guillaume *et al*., 2004) but measurable aggregation of the casein micelles is observed only after ~ 85-90% of the κ-casein 'hairs' are removed. In a study by Gastaldi *et al*. (2003), partial hydrolysis of the κ-casein in bovine milk by chymosin reduced the net negative charge of the casein micelles from -20.20 mV in untreated milk to -13.75 mV in milk with ~ 50% of the κ-casein hydrolysed; the gelation pH on acidification of chymosin-treated milks was higher than that of a control sample and the storage (G') and loss (G") moduli were significantly higher in milks pre-treated with chymosin. Dalgleish (1984) also reported a reduction in ζ-potential for renneted bovine micelles.

ζ-Potential is regarded as an indicator of charge interactions and can be related to the stability of colloidal dispersions and colloids with a high ζ-potential value are electrically stabilized whereas those with low values tend to flocculate (Walstra & Jenness, 1984). The stability of bovine casein micelles is partially due to a net negative charge on the micelle surface. If temperature or pH is increased, the electrophoretic mobility of the micelles increase and ζ-potential decreases (Darling & Dickson, 1979). However, casein micelles are stabilized primarily sterically rather than electrostatically and, in the case of equine micelles, are relatively stable even with a low ζ-potential. It has been reported (Section 3.5.4) that equine milk contains a higher amount of casein-bound calcium than bovine milk which increase the ratio of Ca to P at the micelles surface and reduces the net negative charge (Darling & Dickson, 1979).

Equine micelles are reported to contain very little (~ 0.24 g L^{-1}) κ-casein (Malacarne *et al*., 2000; Iametti *et al*., 2001; Egito *et al*., 2001) and as a result, the casein micelles are large (Section 3.3.6). Unlike bovine κ-casein, equine κ-casein does not have a distinctly hydrophilic C-terminal domain and the level of glycosylation, which, if high, would enhance the ability of κ-casein to stabilize the micelle (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996) has not been established (Section 2.3.5). Ochirkhuyag *et al*. (2000) and Doreau & Martin-Rosset (2002) concluded that the steric stabilization of equine casein micelles by κ-casein may be aided by non-phosphorlated β-casein on the surface of the micelle, thus compensating for the low κ-casein content. Human milk β-casein exists as a single protein phosphorylated at various levels from zero to five (β-casein-OP to β-casein-5P). Azuma *et al*. (1985) reported that highly phosphorylated human β-casein can form casein micelles with κ-casein and the

highly phosphorylated β-casein is, in turn, stabilized by the co-operative function of lesser phosphorylated β-caseins and κ-casein.

5.3.5. Rennetability of equine milk containing 35 or 50 g L^{-1} casein

The effect of increasing the content of equine casein micelles suspended in equine or bovine ultracentrifugal supernatant to 35 or 50 g L^{-1} casein on rennet-induced gelation are shown in Figure 5.8. When suspended in equine supernatant, no increase in storage modulus, G', was found for suspensions containing 35 or 50 g L^{-1} casein which represent ~ 3 and 5 times the original casein content of equine milk, respectively. For equine casein micelles suspended in bovine ultracentrifugal supernatant, G' increased slightly for the 35 g L^{-1} casein suspension but was < 1 Pa for triplicate analyses after 90 min, which meant little or no structure had formed. At 50 g L^{-1} equine casein in bovine supernatant, the equine micellar suspension had a G' of ~ 4.9 ± 0.8 Pa after 90 min renneting which was indicative of a marginally more structured system compared to that of the 30 g L^{-1} equine casein suspension in bovine supernatant. It was been reported in Section 3.6.9 that equine milk and equine casein micelles suspended in the ultracentrifugal supernatant of bovine or equine milk at a casein concentration of 25 g $L^{-1}$are not coagulable by chymosin at pH 6.6 and 30^{o}C which was attributed to the equine micelles, rather than to the composition of the equine milk serum, since equine casein micelles suspended in bovine milk serum also failed to coagulate. It has been reported that the gelation rate and gel strength of bovine casein micelles increases as the micelle size decreases (Niki *et al*., 1994; Park *et al.*, 1999). Equine κ-casein is susceptible to rennet-induced hydrolysis at the Phe_{97}-Ile_{98} bond when in equine caseinate (Egito *et al.*, 2001), but its hydrolysis is considerably slower than that of bovine κ-casein. The amount of calf chymosin required for comparable rates of κ-casein hydrolysis was ~5,000 greater for equine than for bovine κ-casein (Kotts & Jenness, 1976). Equine and human κ-casein have a considerably higher isoelectric pH than bovine κ-casein (Section 2.3.4.), and they have a net positive charge at physiological pH, whereas bovine κ-casein has a net negative charge.

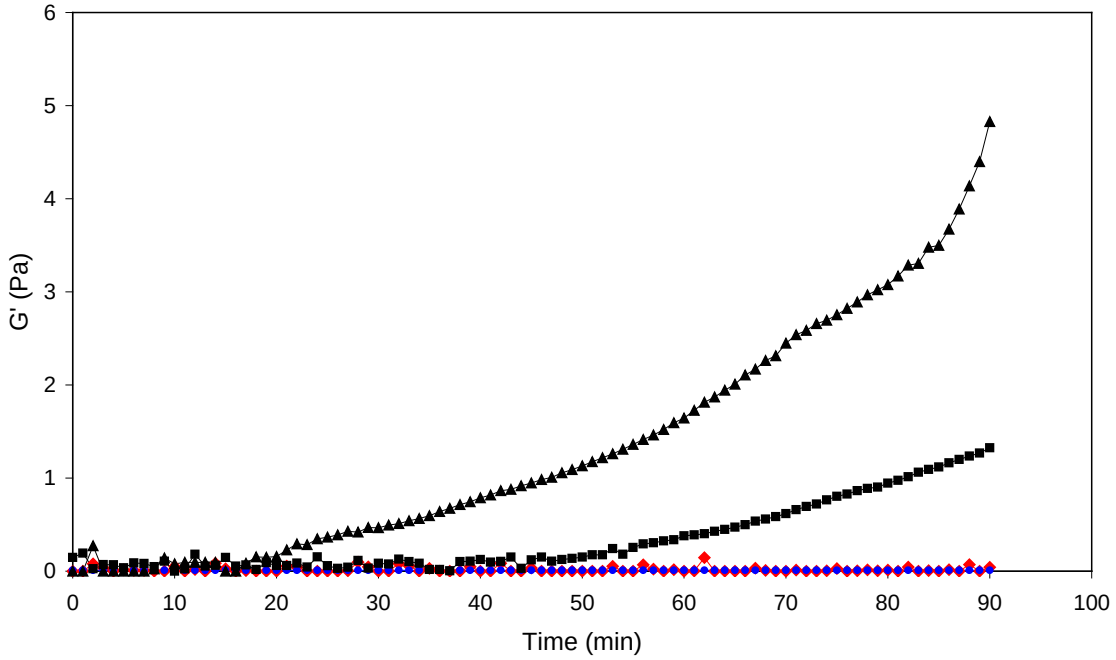

Figure 5.8. Rennetability of suspensions of equine casein micelles (35 or 50 g L^{-1} casein) suspended in bovine ultracentrifugal serum or equine serum at 30°C with 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180, pH 6.6. -▲-, 50 g L^{-1} in bovine serum; -■-, 35 g L^{-1} in bovine serum; -♦-, 50 g L^{-1} in equine serum; -●-, 35 g L^{-1} in equine serum.

Bovine κ-casein is characterized by a hydrophilic C-terminus, which is very important for the manner in which bovine casein micelles are stabilized but the C-terminus of equine κ-casein is far less hydrophilic, although the exact level of glycosylation is unknown. Equine κ-casein could be highly glycosylated, as a result of which it does not form a firm gel on acidification (Section 3.6.10) or on renneting. Lectin-binding studies indicate that equine κ-casein is glycosylated (Egito *et al*., 2001; Iametti *et al*., 2001), possibly at residues Thr_{123}, Thr_{127}, Thr_{131}, Thr_{149} and Thr_{153}. The presence of glycan moieties in the C-terminal region of κ-casein could significantly enhance its ability to stabilize the micelle, by electrostatic repulsion, and may increase the resistance by the protein to proteolytic enzymes, acidification or high temperatures (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996). A study on the effect of κ-casein deglycosylation on the acid coagulability of bovine milk found shorter gelation times, a higher rate of gel strengthening and a more firm gel for casein micelles modified by the release of part of the *N*-acetylneuraminic acid residues (Cases *et al*., 2003).

5.3.6. Rennetability of equine milk as a function of pH

The effect of pH on the rennetability of equine milk is shown in Figure 5.9. The pH of equine milk was reduced from its natural pH (7.2) to pH 6.0. At pH values > 6.4, no aggregation of equine milk was evident > 2 h after rennet addition. At pH 6.4, equine milk flocculated within ~ 20 min after chymosin addition and flocculation occurred more quickly as the pH decreased, reaching ~ 12 min at pH 6.0. From pH 6.4 to 6.0 large aggregates formed which sedimented quickly. From pH 6.2 to 6.0 the supernatant became clear very quickly. It was concluded that equine milk is coagulable by calf chymosin if the pH is reduced to ~ 6.4 but the coagulum formed was weak. The soft curd formed in the foal stomach is presumably designed to specifically meet the physiological needs of the foal. The foal's digestive system is designed to process small amounts of milk frequently (Sneddon & Argenzio, 1998).

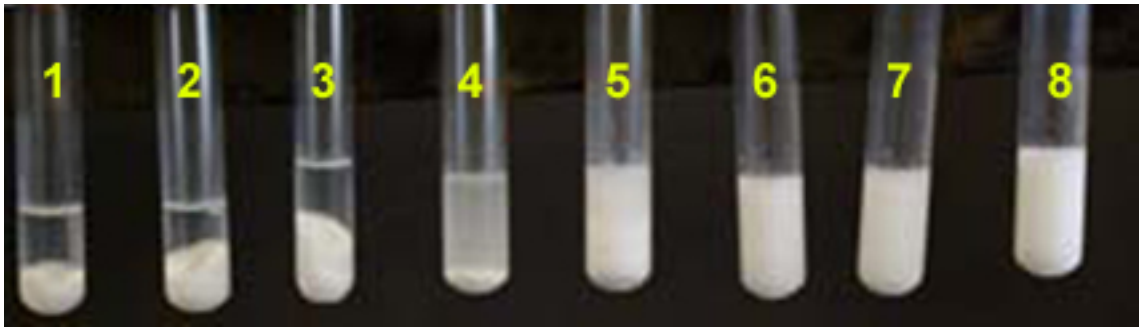

Figure 5.9. Effect of pH on the rennetability of equine milk with 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180. *Sample identification by pH*: **1**, 6.0. **2**, 6.1. **3**, 6.2. **4**, 6.3. **5**, 6.4. **6**, 6.5. **7**, 6.6. **8**, 7.2 (natural pH).

Equids rarely fast for more than 2 to 4 h and naturally forage for 16 to 18 hours per day. The strength of the coagulum formed in the foal's stomach is related to the casein content of equine milk; higher casein-containing milks produces firm clots.

It is believed that species that nurse their young at frequent intervals, e.g., equids and humans, tend to produce dilute milk in which < 60% of total protein is casein and which form a soft clot, whereas those that nurse infrequently produce milk which is high in fat and casein and has much longer gastric retention (Jenness, 1986; Oftedal, 2005). The different protein composition and micellar structure of equine milk compared to bovine milk has a marked effect on the rheological properties of curds formed from equine milk which in turn influences the digestibility of the milk and bioavailability of milk nutrients. Equine milk, like human milk, forms a soft fine curd in the stomach with an evacuation time of 2 to 2.5 h, whereas bovine milk forms compact hard curds with a digestion time of 3 to 5 h. In the case of equine milk, its ease of digestion and high bioavailability of nutrients makes it suitable in the diet of infants, convalescents and the elderly (Kallila *et al*., 1951; Solaroli *et al*., 1993). The relatively low level of α_{s1}-casein in equine compared to bovine milk (Section 2.4) may be significant and, coupled with the low protein content, may be responsible for the soft curd produced in the infant stomach and the foal (Dr. Ursula Fogarty, National Equine Centre, Ireland – personal communication). Goat milk lacking α_{s1}-casein has poor coagulation properties compared to goat milk containing α_{s1}-casein (Clark & Sherbon, 2000).

5.3.7. Changes in the particle size distribution of equine milk on renneting

The effect of renneting on the casein micelle size distribution in equine milk as a function of time after rennet addition was investigated at pH 6.6 or 6.2 using laser light scattering; the results are shown in Figures 5.10 and 5.11, respectively. When renneted at pH 6.6 (Figure 5.10) a redistribution of casein micelle sizes was evident after ~ 2 h and some floccs were recorded. Between 4 and 6h the floccs progressively increased in size and by 8 h a significant proportion of the original casein micelles were present as large aggregated particles. After 12 h, the size distribution profile showed the presence of large particles which indicated that most of the equine casein micelles were incorporated into large aggregates. When renneted at pH 6.2 (Figure 5.9) with calf chymosin, equine milk formed large aggregates of casein micelles within ~ 90 min and by 2h no smaller particles remained. As time after rennet addition increased, the aggregates formed got progressively bigger and by 4 h, the aggregates were outside the range of the instrument for measurement.

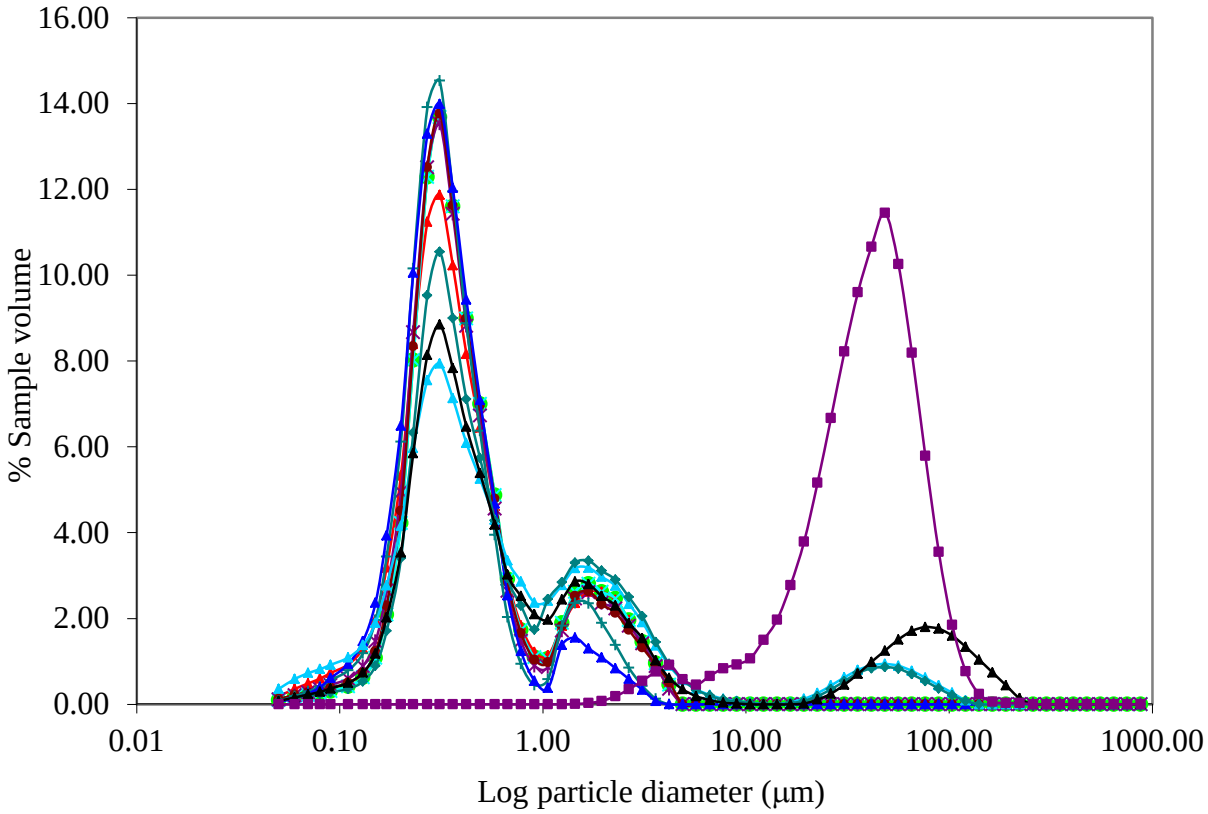

Figure 5.10. Changes in particle size distribution of equine milk renneted at pH 6.6 with 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 assayed by laser light scattering. Control (-●-), 20 min (-▲-), 30 min (-x-), 40 min (-∗-), 50 min (-●-), 60 min (-ı-), 2 h (-▲-), 4 h (-▲-), 6 h (-♦-), 8 h (-▲-) and 12 h (-■-).

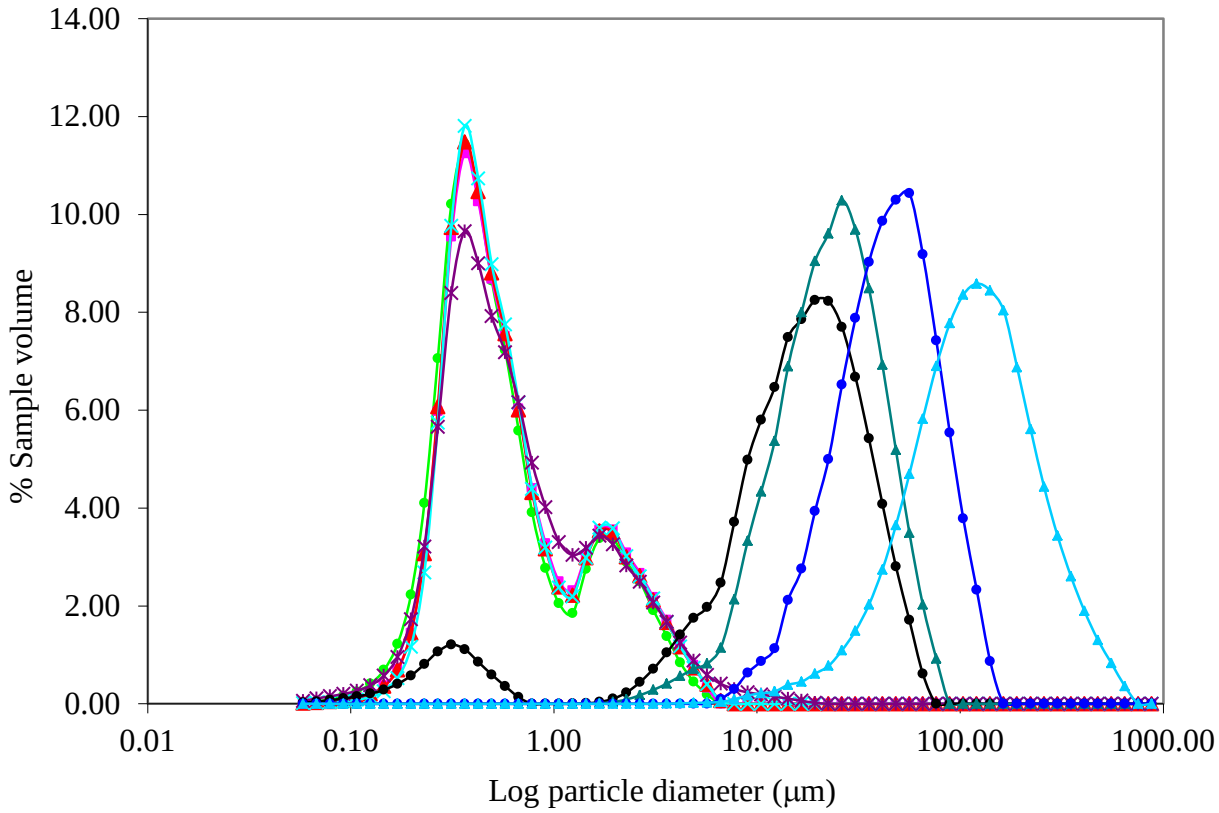

Figure 5.11. Changes in particle size distribution in equine milk renneted at pH 6.2 with 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 assayed by laser light scattering. Control (-●-), 10 min (-■-), 20 min (-▲-), 30 min (-x-), 60 min (-∗-), 90 min (-●-), 2 h (-▲-), 3 h (-●-), 4 h (-▲-).

5.3.8. The clotting activity of foal gastric extract

The clotting activity of equine gastric extract which was probably composed primarily of chymosin was examined by renneting 2 mL of reconstituted low-heat skimmed milk (Nilac) with gastric extract or Maxiren 180 at pH values of 6.8 to 6.0. The result obtained (Figure 5.12) showed that the gastric extract was inactive at pH > 6.6 but as the pH decreased from 6.6 to 6.0 the rennet coagulation time decreased and reached a minimum of ~ 50 s at pH 6.0. In comparison calf chymosin was active at pH > 6.6, although the rennet coagulation time increased significantly from pH 6.6 to 6.8. Lower pH values (< pH 6.5) favoured the coagulation of milk by calf chymosin and the equine extract coagulated the milk more quickly at pH 6.4-6.3 than calf chymosin. As the pH decreased further, the coagulation times were similar for both calf chymosin and equine gastric extract. The pH optimum for the hydrolysis of bovine κ-casein by calf chymosin is ~ 5.5 (van Hooydonk *et al*., 1984). The pH optimum of equine chymosin may be significantly lower; piglet chymosin has been reported to have optimal activity at ~ pH 3.5 (Foltmann *et al*., 1981; Houen *et al*., 1996) and is denatured at pH ~ 6.5 (O'Leary & Fox, 1975).

An investigation of the optimum storage pH for the equine gastric extract following activation of the zymogen was studied by renneting 2 mL of skimmed reconstituted low- heat milk powder (Nilac). Enzyme activity decreased on storage at pH values > 6.0 and it was completely inactive at pH 6.5 which is similar to the effect of pH reported for porcine chymosin (O'Leary & Fox, 1975). The optimum storage pH to maintain maximum activity was 5.0. However, to avoid auto-degradation, the enzyme preparation was stored frozen at -80^{o}C. Calf chymosin is stable at pH 5.3-6.3 and remains active at pH 2.0 although it may lose activity due to acidic auto-degradation and at pH > 9.8 it undergoes irreversible conformational change leading to loss of activity (Foltmann, 1959a, b; Danley & Geoghegan, 1988).

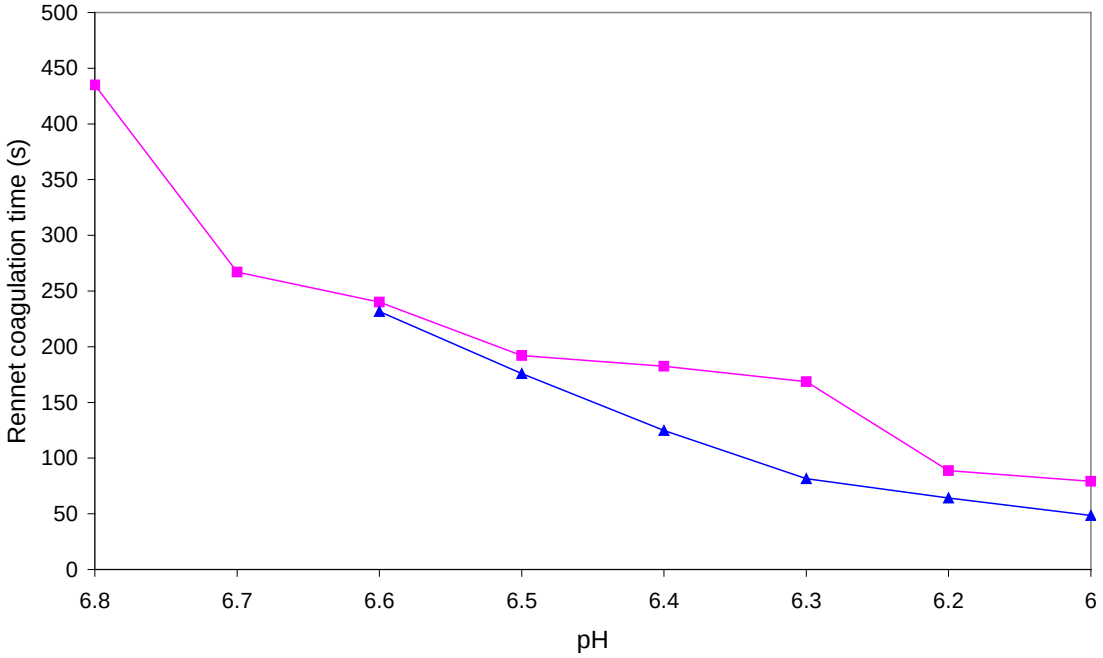

Figure 5.12. Effect of pH on the rennetability of 10% low-heat skimmed bovine milk (Nilac) with 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 (-■-) or 50 μL mL^{-1} of equine chymosin extract (-▲-) at 30°C.

The equine gastric extract probably contains little or no pepsin as the foals from which the stomachs were obtained died within 2 to 5 days *post-partum*. A previous study has shown that the stomach of newborn piglets contains chymosin and no pepsin during the first week of life (Foltmann *et al*., 1978). The chymosin content began to decline at ~ 2 weeks, and the pepsin content, which was very low initially, increased markedly from 2 to 4 weeks *post-partum* (Foltmann *et al*., 1978, 1981b). Piglet stomach has been reported to have very low chymosin activity (O'Leary & Fox, 1975) but porcine milk is coagulated rapidly by calf chymosin compared to the milk of other species (Hoynes & Fox, 1975), suggesting a relationship between the level of chymosin secreted by the piglet and the ease with which its mothers milk is coagulated by calf chymosin (O'Leary & Fox, 1975). It has been reported (Garnot *et al*., 1977) that the secretion of chymosin in the calf abomasa is activated by the presence of bovine milk in the calves stomach and that the casein fraction is responsible for this. The level of pepsin, which increases in the stomach as calves' age, is not affected by protein sources other than bovine milk (Garnot *et al*., 1977).

The foal gastric extract, without purification or concentration, had chymosin activity, about 50 times less than Maxiren 180; its activity in IMCU's mL^{-1} was 5 IMCU's mL^{-1}.

The coagulability of fresh equine and bovine milk with 50 μL mL^{-1} equine chymosin or 10 μL mL^{-1} of 1:10 (v/v) diluted Maxiren 180 is shown in Figure 5.13. Equine milk coagulated in ~ 4 min with added equine chymosin and in ~ 7 min with added Maxiren 180 at pH 6.2. In both samples, the flocculated micelles sedimented quickly and a clear supernatant was formed. As expected, equine milk was not coagulable by Maxiren or equine chymosin at pH 7.2, the milks natural pH. Equine chymosin coagulated bovine milk at pH 6.2 in ~ 5 min which was ~ 1 min faster than that of Maxiren 180 although in the former case the curd formed appeared to be softer. The result obtained when equine milk was stained with methylene blue (which stains aqueous phases blue) prior to renneting with equine chymosin or Maxiren 180 is shown in Figure 5.14. Renneting equine milk with equine chymosin at pH 6.2 appeared to form more aggregates in the milk than renneting with Maxiren 180 as indicated by the amount of flocculation visible in the test tube and the blue colour of the background liquid present in the sample renneted with Maxiren 180; the lighter blue colour of the aqueous phase of the sample renneted with equine chymosin was indicative of a greater proportion of aggregates in the serum and less aqueous phase.

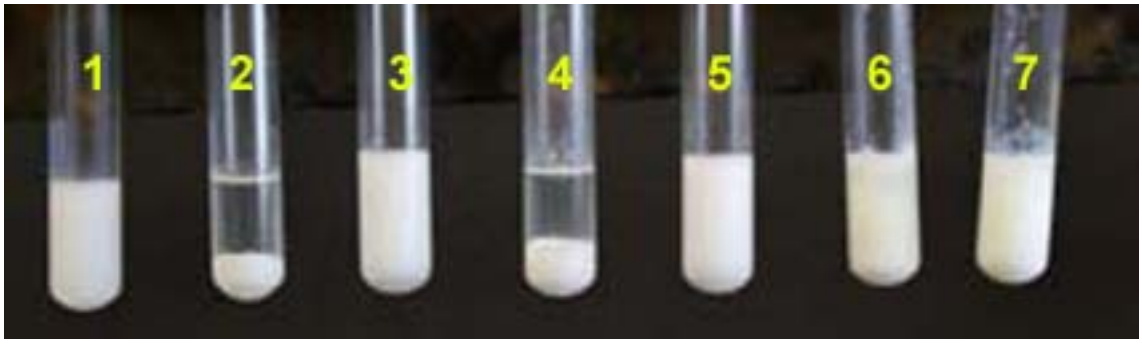

Figure 5.13. Coagulation of equine and bovine milk at 30°C by equine chymosin or Maxiren 180 (10μL mL^{-1} of a 1:10 (v/v) dilution). *Sample identification:* **1**, equine milk, pH 6.2. **2**, equine milk pH 6.2 with equine chymosin. **3**, equine milk pH 7.2 with equine chymosin. **4**, equine milk, pH 6.2 with Maxiren. **5**, equine milk, pH 7.2 with Maxiren. **6**, bovine milk, pH 6.2 with equine chymosin. **7**, bovine milk, pH 6.2 with Maxiren 180.

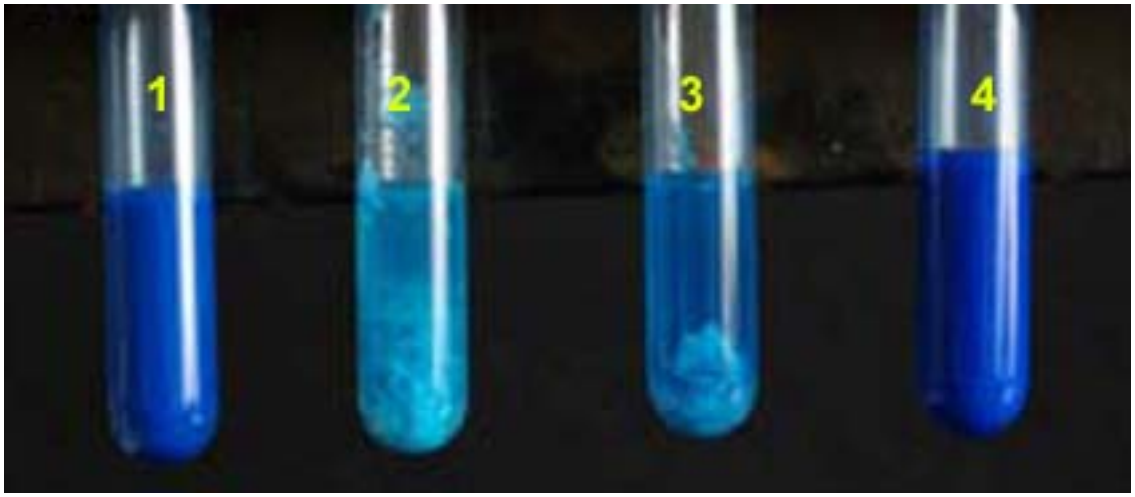

Figure 5.14. Coagulation of methylene blue-stained equine milk at 30°C with equine chymosin or Maxiren 180 (10μL mL^{-1} of a 1:10 (v/v) dilution). *Sample identification:* **1**, equine milk, pH 6.2. **2**, pH 6.2 with equine chymosin. **3**, pH 6.2 with Maxiren 180. **4**, equine milk, pH 7.2.

5.3.9. Effect of equine casein concentration on the rennetability of equine milk

The coagulation of equine milk or an equine micellar suspension of 25 g L^{-1} casein by Maxiren 180 or equine chymosin at pH 6.2 is shown in Figure 5.15. Compared to an untreated equine milk control, renneting equine milk with Maxiren 180 or equine chymosin created large aggregated particles which sedimented quickly. When renneted with equine chymosin, significantly more casein aggregation occurred in equine milk compared to samples renneted with Maxiren 180., which is in agreement with the result observed using methylene blue stain. When the casein concentration in equine milk was increased to 25 g L^{-1}, approximating that of bovine milk, the instability of equine milk at this casein concentration was evident even before the addition of rennet (Figure 5.15 B, control) and after ~ 30 min under quiescent conditions the casein began to sediment. This result is in agreement with a previous result (Section 3.6.8) where is was observed that equine protein sedimented from equine milk at very low gravitational force and even on storage of fresh equine milk for > 2 days, considerable sedimentation of equine protein was observed.

5.3.10. Dynamic oscillatory analysis of equine and bovine milk renneted with equine chymosin

The strength of gels formed by equine chymosin or Maxiren 180 on renneting fresh equine or bovine milk at pH 6.2 were measured using dynamic oscillatory analysis and the results are shown in Figure 5.16. The amount of equine chymosin added was chosen to achieve a clotting time for equine milk, similar to that of bovine milk renneted by Maxiren 180. The same amount of equine chymosin added to bovine milk coagulated the milk too quickly to obtain meaningful data at the initial stages of renneting and as a result the quantity of equine chymosin added to bovine milk was reduced. When renneted with equine chymosin, equine milk formed a very weak gel compared to that formed when bovine milk was renneted with equine chymosin. Bovine milk renneted by equine chymosin had a storage modulus (G') less than when bovine milk was renneted by Maxiren 180; in the former case, the G' *vs* time profile was not smooth after 50 min renneting which indicated sample syneresis.

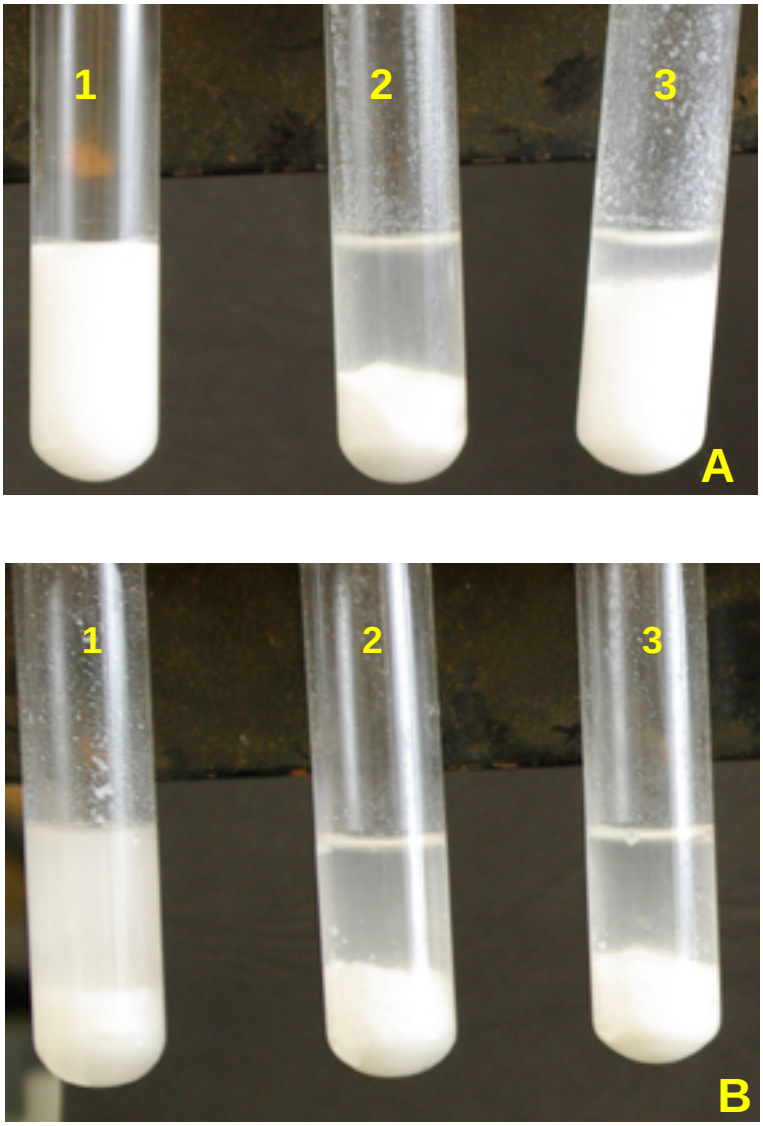

Figure 5.15. Effect of the concentration of equine casein on the coagulation of equine milk by equine chymosin or Maxiren 180 at pH 6.2 and 30°C. **A**: **1**, equine milk control. **2**, equine milk coagulated by Maxiren 180. **3**, equine milk coagulated by equine chymosin. **B**: equine casein, 25 g L^{1}; **1**, control. **2**, renneted by Maxiren 180. **3**, renneted by equine chymosin.

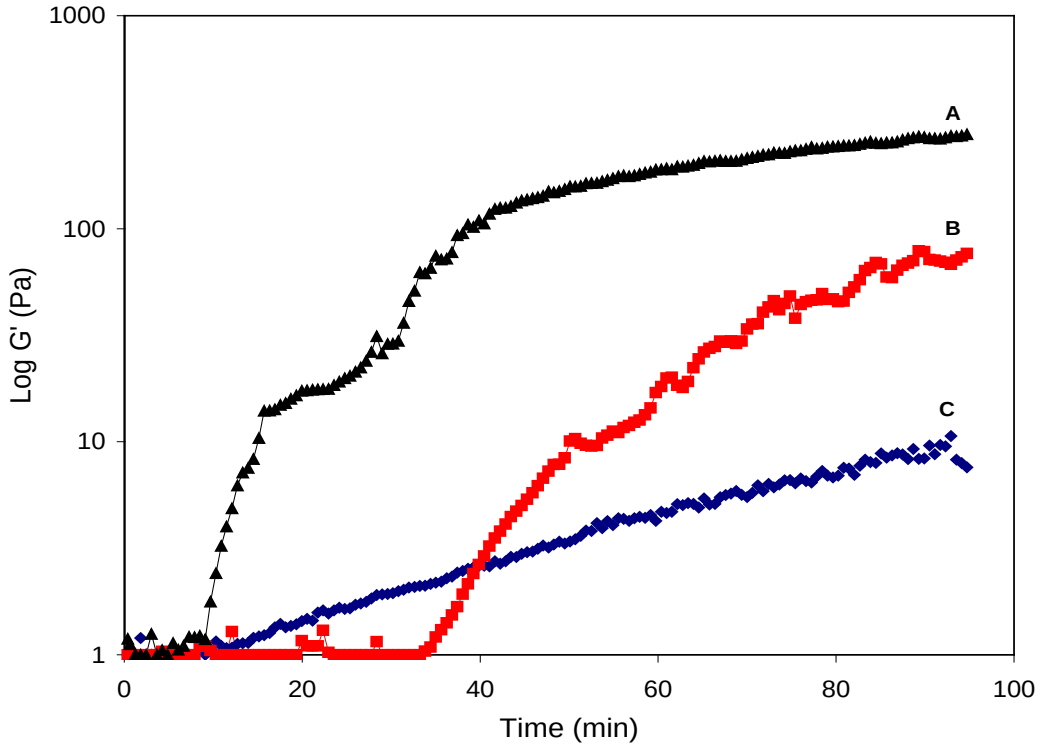

Figure 5.16. Coagulation of equine and bovine milk by equine chymosin at 30°C, pH 6.2. **A**: bovine control with 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180. **B**: bovine milk coagulated by equine chymosin and **C**, equine milk coagulated by equine chymosin. *Note*: the amount of equine chymosin used was chosen to give a clotting time for equine milk equal to the clotting time of bovine milk by Maxiren 180 when measured by by the Berridge method (IDF, 1987).

5.3.11. Rheological characterization of bovine milk renneted by equine chymosin

The frequency dependence of G', G" and η* of fresh bovine milk renneted by Maxiren 180 or equine chymosin at pH 6.5 are shown in Figure 5.17. The mechanical spectra for bovine milk renneted with equine or calf chymosin had the form characteristic of particle gels although, in both cases, the moduli show frequency dependence at higher frequencies and, in the case of bovine milk renneted with equine chymosin, G' almost crossed G" at high frequency which is characteristic of a weak gel network. The individual moduli were higher for bovine milk renneted with Maxiren 180 than with equine chymosin and the gelation rate for the latter was slower. The separation of moduli on the dynamic spectrum of the sample renneted with equine chymosin was smaller than that for the calf chymosin sample and their frequency dependence was greater, indicating a higher 'sol fraction' of material that does not form part of the continuous network. Food gels are self supporting if a three-dimensional network develops on renneting or acidification, such gels are termed 'true gels', or they may be characterized by a tenuous gel-like network which is easily broken when a stress is applied (Doublier *et al*., 1992). A frequency sweep will distinguish between a weak and a strong gel (Clark & Ross-Murphy, 1987). True physical gels have networks crosslinked by non-covalent bonds and G' is 10 times higher than G" (Ross-Murphy, 1983). When the separation between G' and G" is small on a mechanical spectrum and the frequency dependence of both moduli is high, the gel formed is weak (Ross-Murphy, 1983). In a true viscoelastic gel, the dynamic moduli (G' and G") are independent of frequency (ω) and in a typical biopolymer gel, the solid-like response exceeds the liquid-like response by an order of magnitude and a linear relationship exists between log η* and log ω with a slope of ~ -1 (Ross-Murphy, 1983).

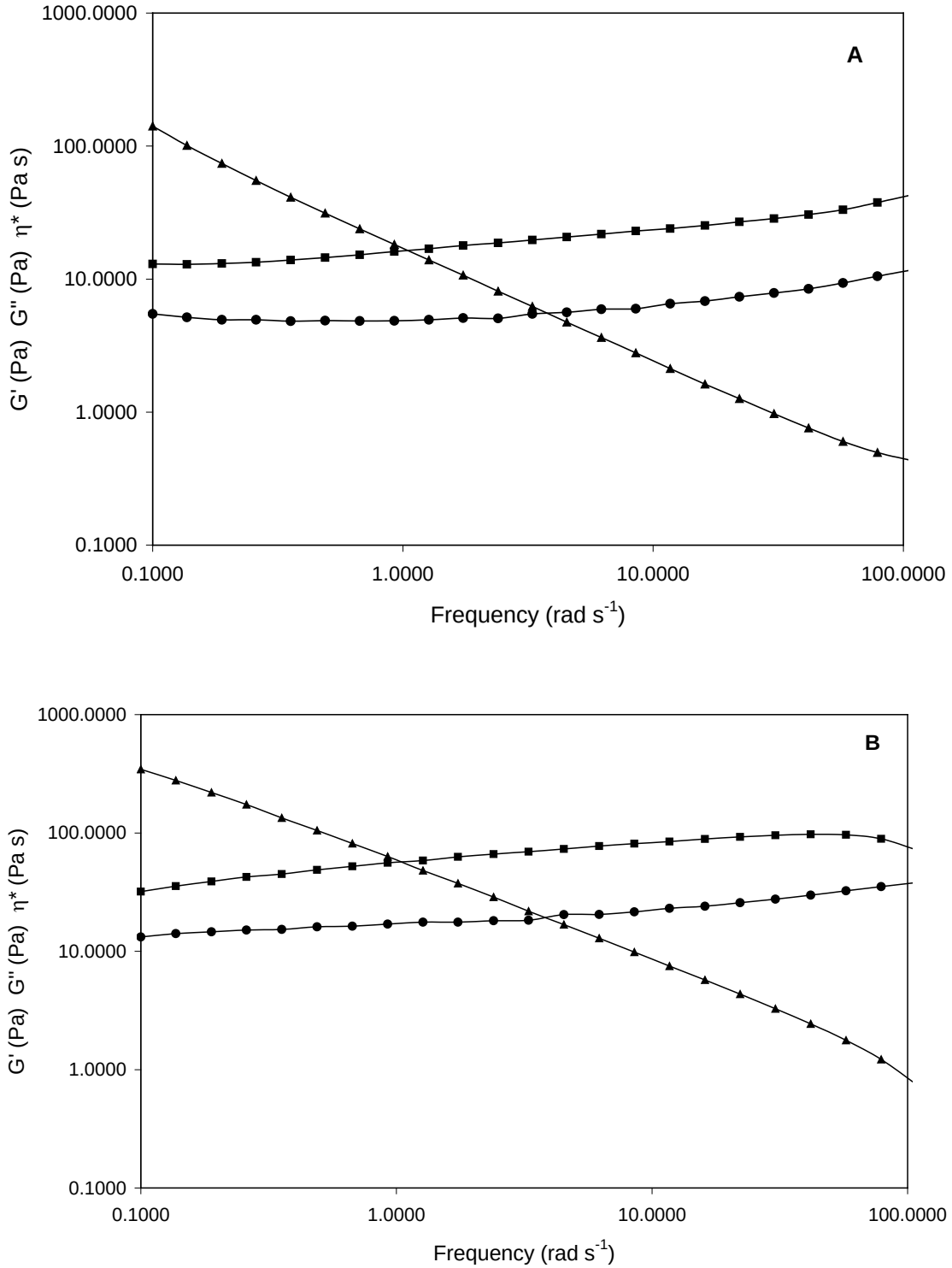

Figure 5.17. Mechanical spectra (30°C, 0.5 % strain) showing the frequency dependence of G' (-■-), G" (-●-) and η* (-▲-) for bovine milk renneted with Maxiren 180 (A) or equine chymosin (B) at pH 6.5.

5.3.12. Coagulability of reconstituted equine and bovine milk by various rennets

The rennetability of reconstituted equine milk by various coagulants was compared to that of bovine milk and the results are shown in Table 5.1. In the case of equine milk at pH 6.6, equine chymosin was the only coagulant that produced significant flocculation within a short period of time and the time at which flocculation occurred compared well with bovine milk renneted by various coagulants. *Cryphonectria parasitica* (Suparen) caused significant flocculation of equine milk at ~ 20 min after addition and within 60 min, the sample viscosity has increased considerably. *Cryphonectria parasitica*, a microbial coagulant, cleaves Ser_{104}-Phe_{105} of bovine κ-casein (Drøhse & Foltmann, 1970; Larson & Whitaker, 1970; Garg & Johri, 1994) and exhibits broad and different specificity compared to calf chymosin. In the manufacture of Mozzarella cheese, for example, *Cryphonectria parasitica* protease has been shown to be more proteolytic than either *Mucor miehei* protease or calf chymosin. Proteolysis of both α_{s1}- and β-caseins occurs in Mozzarella cheese made with *Cryphonectria parasitica* proteinases but α_{s1}-casein is hydrolyzed preferentially in cheeses made with chymosin and *Mucor miehei* protease (Yun *et al*., 1993). The change in the properties of cheese during storage is related to the combined effects of the hydrolysis of α_{s1}- and β-caseins (Tam & Whitaker, 1972; Vanderpoorten & Weckyx, 1972). The proteolytic activity of calf rennet and microbial-derived coagulants (*Rhizomucor miehei* and *Cryphonectria parasitica*) on bovine whole casein and casein fractions has been studied and it has been reported that on whole casein and on α_{s1}- and β-caseins, proteolytic activity decreased in the following order: *Cryphonectria parasitica* > *Rhizormucor miehei* > calf rennet (Tam & Whitaker, 1972; Vanderpoorten & Weckx; Ustunol & Zeckzer, 1996). In studies on the hydrolysis of ovine casein, rennet extracted from lamb stomach and *Cryphonectria parasitica* showed the lowest and the highest degree of proteolysis, respectively, on ovine casein (de Jong, 1977; Trujillo *et al*., 2000).

Equine β-casein has been reported to be readily hydrolysed by calf chymosin at Leu_{190}-Tyr_{191} (Egito *et al*., 2001) and is likely to be susceptible to hydrolysis by *Cryphonectria parasitica proteinase* which may have caused the flocculation seen in this study.

Table 5.1. Rennet coagulation time (min) of 2 mL skimmed reconstituted equine or bovine milk samples at pH 6.6 and 30°C with various coagulants. The amount of each coagulant was adjusted to give a rennet coagulation time of ~ 7 min for bovine milk.

	Rennet coagulation time (min)	
Enzyme	**Equine milk** (110 g L^{-1})	**Bovine milk** (100 g L^{-1})
Calf chymosin	flocculation in ~ 60 min	7.2 ± 0.13
Maxiren 180	flocculation in ~ 60 min	7.8 ± 0.2
Fromase	no flocculation in > 60 min	7.42 ± 0.15
Suparen	flocculation in ~ 20 min; significant viscosity increase in ~ 60 min	6.4 ± 0.22
Bovine pepsin	no flocculation in > 60 min	7.54 ± 0.14
Porcine pepsin	no flocculation in > 60 min	6.4 ± 0.23
Equine chymosin	9.14 ± 0.54 (flocculation)	6.8 ± 0.4

5.3.13. Coagulation of equine, bovine and camel milks by different chymosins

Bovine and camel milk were renneted with bovine and camel chymosins to investigate if chymosin from a particular species was better at coagulating the milk of that species. Equine milk renneted by equine chymosin at pH 6.2 was included in the analysis and in the case of equine milk, results obtained from renneting by camel chymosin (CHY-MAX™ M) and calf chymosin (Maxiren 180) have been omitted as, meaningful G' values were not recorded within the time period. The results obtained by dynamic oscillatory rheological measurements of G' *vs* time for the three milks renneted with various coagulants are shown in Figure 5.18. When bovine milk was coagulated by calf chymosin or camel chymosin the G' *vs* time profile was similar up to ~ 80 min after rennet addition; however, the gel formed by camel chymosin continued to strengthen linearly thereafter with a maximum G' value of ~ 75 Pa after 90 min compared to 55 Pa for bovine milk renneted with calf chymosin. In contrast, camel milk did not develop an appreciable G' value on renneting for 90 min with calf chymosin but a gel was formed when renneted with camel chymosin which had a maximum G' value of ~ 60 Pa after 80 min but thereafter appeared to whey-off and the G' began to decrease. These results were reproducible over at least triplicate analysis but the data presented are from a single analysis for clarity. As expected, equine milk was coagulable by the equine chymosin preparation but the strength of the gel was low and a maximum G' value of ~ 8.5 Pa was recorded 90 min after rennet addition. Camel chymosin coagulated camel milk and bovine milk and in the latter case the gel formed was stronger than that formed by calf chymosin. While camel milk was coagulable by camel chymosin only, bovine milk was coagulable by chymosin from another species (camel) as well as by calf chymosin. Rennets from ovine, caprine and porcine stomachs have been reported to be the most efficient at clotting the milk of their own species (Foltmann, 1981b).

Cheese is produced from bovine milk through specific cleavage of the Phe_{105}-Met_{106} bond of bovine κ-casein by rennet. Fermentation-produced calf chymosin is generally used due to its high specificity for this bond. Fermentation-produced camel chymosin has been demonstrated to provide a 7-fold higher ratio of milk clotting to general proteolytic activity in bovine milk (Kappler *et al*., 2006; Bansal *et al*., 2009) and hydrolyses bovine κ-casein at a significantly higher rate than bovine chymosin (Møller *et al.*, 2010).

The result for camel milk in this study is in agreement with those of previous studies which have reported that camel milk is relatively easily coagulated by camel chymosin but cannot be coagulated by calf chymosin (Mehaia, 1993). Camel milk can be coagulated by calf chymosin

if it is mixed with the milks of other species such as goats, ewes or buffaloes (Rao *et al*., 1970; Yagil, 1982) or if a high level of calf chymosin is used for coagulation (Chapman, 1985; Farah & Bachmann, 1987). It has been suggested (Ramet, 1987) that the addition of 1.5 mM $CaCl_2$ to camel milk prior to renneting with calf chymosin will reduce the rennet coagulation time of camel milk but more enzyme is required for coagulation compared to that used to coagulate bovine milk; however, the structure of the final coagulum does not change when the amount of chymosin is increased (Mohammed & Larsson-Raźnikiewicz, 1989). In this study, the addition of $CaCl_2$ to camel milk did not improve its coagulability by calf chymosin even when the amount of chymosin added was tripled. Camel milk has been reported to be coagulable by bovine rennet which contains pepsin primarily with a small amount of chymosin and the coagulability has been attributed to the pepsin fraction (Wangoh *et al*., 1993).

Camel milk has several compositional and physico-chemical properties that are similar to those of equine milk and a soft coagulum forms on renneting under optimal conditions for both milks.

Camel milk has a low casein content of which ~ 3.5 % is κ-casein compared to ~ 13% in bovine milk and camel κ-casein is cleaved by chymosin at Phe_{97}-Ile_{98} (Kappeler, 1998). The casein micelles in camel milk are large, ~ 280 nm, on average (Farah & Ruëgg, 1989; El-Agamy, 2006) as a result of which the κ-casein coverage is low (Ekstrand *et al*., 1980). Furthermore, the β-casein content is low (Kappeler, 1998). Equine milk forms a very soft curd when renneted by equine chymosin whereas the curd formed by camel chymosin from camel milk is firm, suggesting that despite several similarities between the milks, the type of curd formed is species-specific.

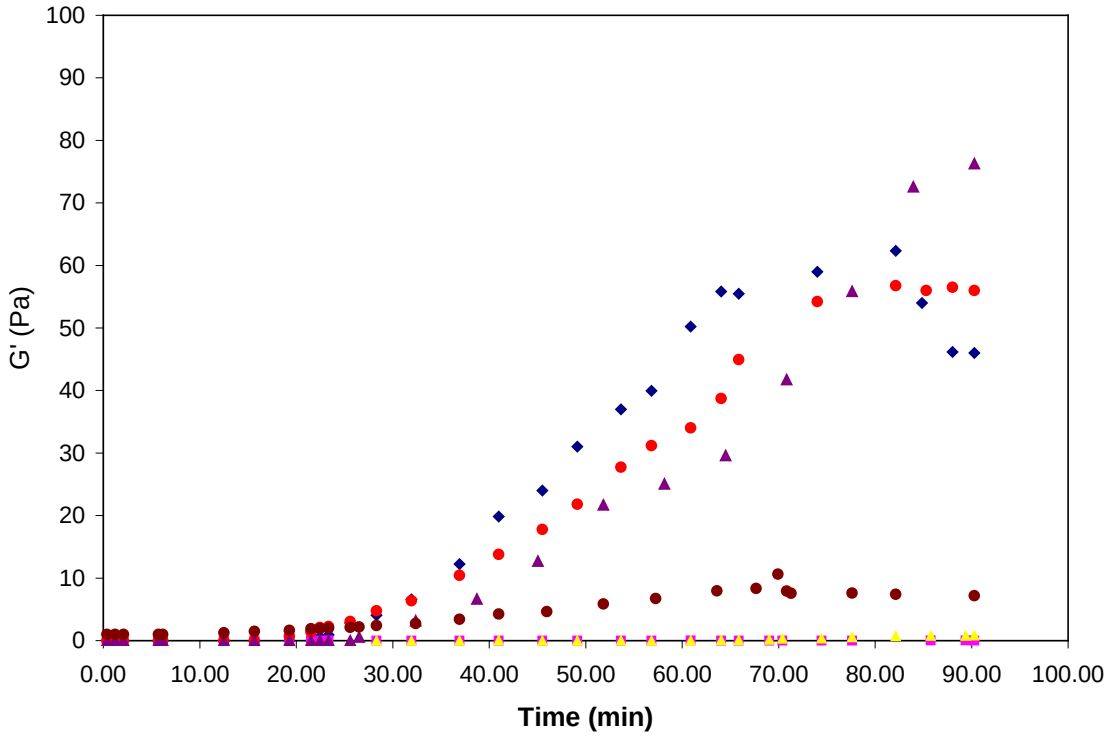

Figure 5.18. Rennetability at 30°C and pH 6.6 of bovine and camel milk with Maxiren 180, CHY-MAX™ M or equine chymosin. Equine milk renneted at pH 6.2 by equine chymosin is included in the analysis. Bovine milk coagulated by Maxiren 180 (●) or camel chymosin (▲), camel milk coagulated by CHY-MAX™ M (♦), Maxiren 180 (■) or Maxiren 180 (3X) with 0.02 g L^{-1} $CaCl_2$ (▲) and equine milk renneted with equine chymosin at pH 6.2 (●). Data are from triplicate analysis and the coefficient of variation was <5% of the reported value for each data point.

The low level of κ-casein in equine and camel milks is probably the most significant feature of the milks as regards coagulability by chymosin. It has been reported (Dalgleish, 1979; Green & Morant, 1981) that casein micelles will not aggregate if less than 80 % of κ-casein is hydrolysed. Above 80 %, the aggregating potential of the micelles increases and reaches a maximum when all the κ-casein has been hydrolysed which implies that there is a critical value for κ-casein hydrolysis below which casein micelles can not aggregate. The amount of κ-casein cleaved in equine milk by calf or camel chymosin or in camel milk by calf chymosin is not enough for effective aggregation of casein micelles. The failure to coagulate could also be due to non-specific interaction of the proteases with κ-casein. The results in this study suggest an adaptation of the specificity of the gastric proteinases and the structure of the

caseins of the different milks. The casein micelles in equine milk and camel milk are large as a result of low levels of κ-casein. In bovine milk, coagulation time has been reported to vary with micelle size, and is faster for small and medium size micelles which have higher κ-casein contents than the larger micelles (Ribadeau-Dumas & Garnier, 1970; Ekstrand *et al*. 1980). Smaller micelles form a firmer curd than larger micelles at the same casein concentration (Grandison, 1986).

5.3.14. Effect of mixing bovine milk with equine milk on curd formation by calf chymosin

The effect of mixing various weight ratios of equine and bovine milks, and renneting the mixtures with Maxiren 180 at pH 6.2, on gel strength are shown in Figure 5.19. A 1:1 mixture of equine and bovine milk had a curd strength of ~ 26 Pa, 60 min after addition of Maxiren 180, which was significantly lower than that of the bovine control (132 Pa) but represented a reasonably structured but weak gel. When the amount of equine milk was increased the gel strength decreased and at 5:1 equine:bovine milk, a weak gel with a final G' value of < 5 Pa was formed. It is concluded that it is possible to coagulate equine milk if it is mixed with bovine milk and the best ratio to use in order to retain some of the unique compositional properties of equine milk is a weight ratio of 3:1 equine:bovine milk. Urea-PAGE was used to investigate if equine proteins had been incorporated into the curd formed from blends of equine and bovine milk and the result is presented in Figure 5.20.

The electrophoretograms show that as the amount of equine milk was increased in a mixture of equine and bovine milks, the amount of equine proteins retained in the curd increased, especially some of the isoforms of equine α_s-and β-casein (Lanes 9, 11 and 13). The level of protein bands a and b, which have not been identified, also increased in renneted gels as the amount of equine milk was increased. Quantitative image analysis of the two unidentified protein bands in equine milk (a and b, Figure 5.20) and equine β- and α_s-caseins (c and d, respectively, Figure 5.20) as well as bovine α_{s1}- and β-caseins incorporated into the gels formed on renneting mixtures of equine and bovine milks is presented in Table 5.2. Approximately 32 and 21% of the original amounts of β- and α_s-caseins, respectively, found in equine milk renneted with Maxiren 180 at pH 6.2 (lane 7), were incorporated into the curd formed from a 3:1 mixture of equine and bovine milk and the amounts of both proteins increased, but not substantially, as the amount of equine milk in the mixture increased. At a 7:1 ratio of equine:bovine milk, ~ 44 and 46% of equine β- and α_s-casein were incorporated into an unstructured aggregate formed on renneting. This result confirmed that if a 3:1 weight

ratio of equine and bovine milk was renneted with chymosin at pH 6.2, a gel was formed which incorporates a significant amount of equine protein which may be of significance in the production of new products from equine milk.

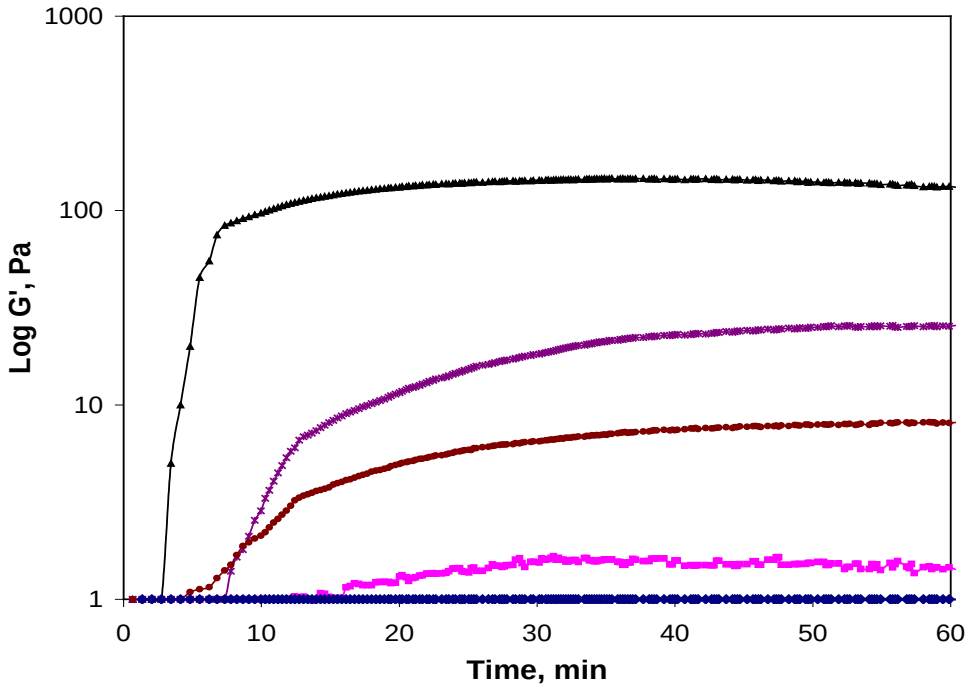

Figure 5.19. Coagulation of mixtures of equine and bovine milk at 30°C and pH 6.2 by 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180. Bovine control (-▲-); equine milk and bovine milk at weight ratios of 1:1 (-∗-), 3:1 (-●-), 5:1 (-■-) and equine milk (-♦-).

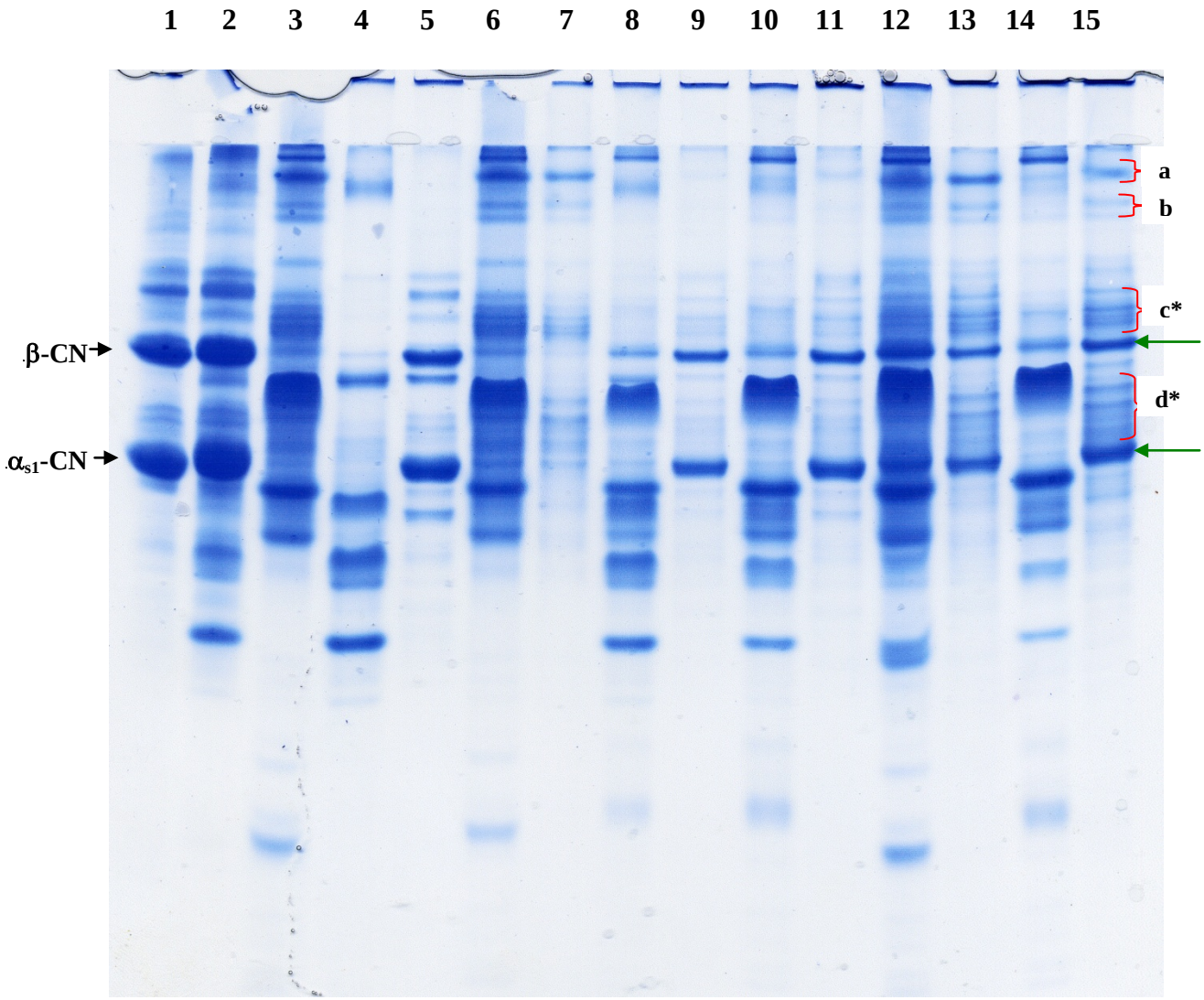

Figure 5.20. Urea polyacrylamide gel electrophoretograms of pellets and supernatants from equine and bovine milk and equine and bovine milk mixtures renneted at pH 6.2 and 30°C with 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180.
Lanes: Bovine caseinate (**1**), bovine milk (**2**) and equine milk (**3).** Bovine supernatant (**4**), pellet (**5**), equine supernatant (**6**) and equine pellet (**7**) from renneted controls after centrifugation at 5,000 g x 30 min at 20°C. 1:1 ratio of equine: bovine milk supernatant (**8**) and pellet (**9**). 3: 1 equine: bovine (**10**) and pellet (**11**). 5: 1 equine: bovine supernatant (**12**) and pellet (**13**). 7: 1 equine: bovine supernatant (**14**) and pellet (**15**).
Bracketed bands a and b have not been identified and c* and d* represent some of the isoforms of equine β- and α_s-caseins, respectively. Green arrows are the principal bovine caseins (β-casein and α-casein) which were quantified.

Table 5.2. Quantitative image analysis of the % equine casein (a-d of electrophoretogram) and bovine β- and α_{s1}-caseins in the curds of milks renneted with 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 at 30°C and pH 6.2. Analysis was carried out on 3 separate occasions with fresh skimmed milk samples.

	Ratio (w/w) of equine:bovine milk				
% in curd	**1:0**	**1:1**	**3:1**	**5:1**	**7:1**
a	51	0.0	17.71 ± 2.01	86.04 ± 4.22	77.53 ± 3.38
b	37.32 ± 4.42	0.0	0.0	5.68 ± 0.99	31.58 ± 2.27
c*	37.12 ± 3.33	20.53 ± 2.21	32.07 ± 3.52	31.07 ± 3.06	44.38 ± 2.49
d*	52.34 ± 4.47	14.12 ± 3.77	21.19 ± 2.29	38.47 ± 2.82	46.43 ± 3.21
Bovine β-CN	-	58.77 ± 1.46	60.58 ± 2.11	44.57 ± 1.22	49.76 ± 1.36
Bovine α_{s1}-CN	-	60.31 ± 2.55	78.43 ± 3.14	58.66 ± 2.28	59.17 ± 2.44

5.4.Conclusions

The colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles, which may have significant implications for the conversion of equine milk into dairy products. Based on the evidence outlined, manufacture of cheese and yoghurt from equine milk is unlikely to be successful using conventional manufacturing protocols but may be possible if, for example, bovine milk was mixed with equine milk at a ratio that allowed the formation of a coagulum while retaining an appreciable amount of equine protein. The results in this study demonstrated that equine milk is coagulable by rennet and while the κ-casein is low and has resulted in large micelles, it is likely that some isoforms of equine β-casein provides some stability at the micelle surface. It is also unlikely that equine milk is devoid of κ-casein; the absence of κ-casein in any mammalian milk would have serious implications for the well-being of the off-spring. κ-Casein deficient mice produced by genetic modification are unable to lactate due to destabilization of casein micelles in the lumina of the mammary gland (Shekar *et al*., 2006). By comparison, in another study, β-casein-deficient mice could lactate and successfully rear pups and, while the average micelle size was smaller than that of control mice, the content of other proteins increased to compensate for the lack of β-casein (Kumar *et al*., 1994).

The casein micelles in milk determine the colloidal stability of the polydisperse system in milk and rennet coagulation time varies with micelle size whereby small and medium-sized micelles, with higher levels of κ-casein, coagulate more easily with chymosin compared to larger micelles with less κ-casein (Ekstrand *et al*., 1980; Grandison, 1986). Increasing the casein micelle size in bovine milk has been shown to have a negative effect on gel strength with smaller micelles, with a much larger surface coverage of κ-casein, forming significantly stronger gels (Amenu & Deeth, 2007; Glantz *et a*l., 2010).

Research on the composition of poor- and non-coagulating bovine milks has shown that a low κ-casein content was one of the principal risk factors for non-coagulation (Wedholm *et al*., 2006; Hallén *et al*., 2010) although the enzymatic phase of the coagulation reaction proceeds in a similar manner for both non-coagulating and coagulating bovine milks (Tervala & Antila, 1985; van Hooydonk *et al*., 1986a,b; Hallén *et al*., 2010). A low concentration of individual caseins (Wedholm *et al*., 2006; Jõudu *et al*., 2008), casein concentration (Losi *et al*., 1982; Nsofor, 2000) or total protein concentration (Tyrisevä *et al*., 2003) have been reported as contributing to non-coagulation of bovine milk; however, Hallén *et al*. (2010) found no significant association between casein or protein content and non-coagulation of milk. Milk

will form a firmer curd when the relative content of α_{s1}- and β-casein is reduced, or the relative content of κ-casein in total casein is increased (Jõudu *et al*., 2008). No difference in total calcium has been reported for non-coagulating and coagulating bovine milks; however, addition of calcium (up to 0.05%, to significantly increase the concentration of free calcium ions) has been shown to improve coagulability of previously non-coagulating milk (van Hooydonk *et al*., 1986b; Hallén *et al*., 2010). It has been reported that the ratio of free calcium ions and colloidal calcium phosphate is different between non-coagulating and coagulating bovine milks (van Hooydonk *et al*., 1986b). Studies on the effect of protein content on gel strength and gelation time seem to be contradictory: some studies have shown that a high protein content improves gel strength (Pagnacco & Caroli, 1987; Auldist *et al*., 2004; Hallén *et al*., 2007) and decreases gelation time (Lindstrom *et al*., 1984), but others have found that a low protein content positively correlates with decreased gelation times (Pagnacco & Caroli, 1987; Ikonen *et al*., 2004; Glantz *et al*., 2010) but correlates negatively with gel strength (Glantz *et al*., 2010). Several inferences can be drawn from these studies on the poor rennetability of equine milk compared to bovine milk and it is likely that the most significant factors are the large equine casein micelle size, the poor coverage by κ-casein and the low protein (casein) content of the milk contribute to the failure to form a gel when renneted.

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CHAPTER 6

Stability of Equine Casein Micelles. III. Dissociation of equine casein micelles by various treatments

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Abstract

The colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles, which may have significant implications for the conversion of equine milk into dairy products. This study is part of a series studying the stability of equine casein micelles and studied the dissociation of the micelles by various treatments, including the addition of trisodium citrate and urea. The dissociation by trisodium citrate or urea of equine casein micelles, at their natural casein concentration or with the casein increased to 25 g L^{-1}, approximating that of bovine milk, followed a similar pattern to the dissociation of micelles in skimmed bovine milk. The effect of temperature on the dissociation of equine caseins was also examined and showed little dissociation of equine β-casein at 4 or 20°C compared to bovine β-casein while a considerable proportion of equine α_s-casein dissociated at both temperatures. Temperature-sensitive (TS) and cold-precipitated (CP) fractions of equine casein were prepared at pH 4.2 or 4.6, both of which stained positively for glycoproteins. Equine casein micelles were separated by differential centrifugation which also allowed positive identification of glycoprotein (κ-casein) in equine milk. Equine casein micelles were less stable to ethanol than bovine micelles and high ethanol concentrations coupled with high temperature caused irreversible changes to the micelles. When heated to 80°C with 100% ethanol, followed by rapid cooling on ice, bovine milk formed a firm gel but equine milk did not.

Keywords: Equine casein micelles; urea; trisodium citrate; temperature-dependent dissociation; temperature-sensitive casein; cold-precipitated casein; micelle size; differential centrifugation; ethanol stability.

6.1. Introduction

The biological function of caseins is to provide the mammalian neonate with a source of phosphate and calcium for the mineralization of bone and teeth, as well as amino acids and biologically active peptides. To this end, caseins occur in milk in the form of micelles which keep calcium phosphate in a soluble and bioavailable state, facilitating the transfer of large amounts of calcium and phosphate to the neonate with minimal risk of pathological calcification of the mammary gland. Bovine casein micelles, which have been studied extensively, are 50 -500 nm (mean 120 nm) in diameter and are composed of α_{s1}-, α_{s2}-, β- and κ-caseins (molar ratios ~ 4:1:4:1, respectively) (De Kruif & Holt, 2003; Fox, 2003). The caseins are held together by hydrophobic interactions and by calcium phosphate nanoclusters bound to serine phosphate residues of the caseins (De Kruif & Holt, 2003). The α_{s1}-, α_{s2}- and β-caseins are insoluble at the Ca^{2+} concentration in milk while κ-casein, which is located almost exclusively on the micelle surface, is insensitive to calcium and plays a role in maintaining the integrity and stability of the micelles. The hydrophobic *N*-terminal region of κ-casein is associated with the micelle core, while the hydrophilic, negatively-charged C-terminal protrudes from the surface as a highly charged flexible 'hair' or 'brush' and sterically stabilizes the micelles against aggregation (Schmidt, 1982; Walstra, 1990; Holt & Horne, 1996; De Kruif & Zhulina, 1996; Horne, 1998). This 'hairy layer' acts as a barrier against aggregation unless it is removed or neutralized (Walstra, 1979; Holt, 1992; Holt & Horne, 1996). Consequently, any process or environmental factor which destroys the stabilizing effect of κ-casein will significantly reduce the colloidal stability of casein micelles. The overall association of the caseins is governed by a balance between attractive hydrophobic forces and electrostatic repulsion (Horne, 1998). Although the casein micelle is extremely stable, its protein and inorganic salts exhibit a soluble-colloidal equilibrium which is affected by physico-chemical parameters such as temperature, pH and ionic strength (Pouliot *et al.*, 1994). Removal of calcium, which also solubilizes colloidal phosphate, results in the loss of micellar integrity and dissociation of the casein micelles (Pyne, 1962; Morr, 1967; Lin *et al*., 1972) which occurs in the order of β-casein, followed by κ-casein (Ono *et al*., 1978) and, ultimately, of α_{s1}- and α_{s2}-caseins (Lin *et al*., 1972). The addition of urea (McGann & Fox, 1974; Aoki *et al*., 1986), dialysis against phosphate-free buffer (Holt *et al*., 1986), acidification (Rose, 1968), lowering the temperature to 4^{o}C (Dalgleish & Law, 1988) and the addition of ethanol (Walstra, 1990, Holt & Horne, 1996; Horne, 1998.) will also dissociate casein micelles. The effect of Ca-chelating agents such as EDTA or citrate on the

properties of bovine milk has been investigated (Munyua & Larsson-Raznikiewicz, 1980; Ward *et al*., 1997; Udabage *et al.,* 2000; Ozcan-Yilsay *et al*., 2007). Chelating agents disrupt casein micelles by reducing the levels of ionic calcium and colloidal Ca phosphate (Munyua & Larsson-Raznikiewicz, 1980; Fox & Mulvihill, 1982; Visser *et al*., 1986; Goddard & Augustin, 1995; Udabage *et al*., 2000) which reduces micelle stability (Morr *et al*., 1967; Mohammad & Fox, 1983; Gaucheron, 2005)

Cold storage of milk leads to the solubilization of caseins, especially β-casein, from casein micelles (Rose, 1968; Creamer *et al*., 1977; Ali *et al*., 1980a; Pierre & Brulé, 1981; Ichilczyk-Leone *et al*., 1981; Davies & Law, 1983; Roefs *et al*., 1985; Dalgleish & Law, 1988) with a concomitant increase in the level of β-casein in ultracentrifugal supernatant (Dalgleish & Law, 1989a) and alteration of the mineral balance in milk (de la Fuente, 1998). Solubilization is generally considered to occur as a result of a decrease in hydrophobic interactions (Lenoir *et al*., 1974; Ali *et al*., 1980b; Davies & Law, 1983) and the solubilisation of calcium phosphate at low temperatures (Pyne, 1962; de la Fuente, 1998) with a concomitant reduction in micelle size reported in some studies (Lenoir *et al*., 1974; Davies & Law, 1983; Walstra & van Vliet, 1986) but not in others (Downey & Murphy, 1970; Puhan, 1989; Raynal & Remeuf, 2000). About 45% of the increase in serum casein at 4^{o}C is due to the dissociation of β-casein from the micelles and 30 and 23% to the dissociation of α_s- and κ-casein, respectively (Downey & Murphy, 1970). Cold-induced changes in micelles are partly reversible at room temperature within ~ 2h and β-casein can re-associate at the surface of the micelle and diffuse into the interior while solubilisation of calcium is partially reversible (Rose, 1968; Downey & Murphy, 1970; Creamer *et al*., 1977; Davies & Law, 1983).

The caseins in the milk of different species do not exhibit the same dissociation behaviour at low temperatures which has been attributed to the heterogeneity of β-casein (O'Connor & Fox, 1973). More casein dissociates on cooling bovine milk than in caprine milk, while little dissociation occurs in ovine milk (Raynal & Remeuf, 2000). The β–casein in equine milk has very limited dissociation at any temperature (O'Connor & Fox, 1973).

In Chapter 3 it was shown that the colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles. Equine micelles are more stable to coagulation by acid, rennet and, at certain pH values, heat, but are less stable to ethanol. It was concluded that these characteristics could be attributed to the exceptionally high level of ionic calcium (Section 3.5.2) and the low level of protein (Section 3.5.1) in equine milk

compared to bovine milk, coupled with the large size of equine casein micelles and the exceptionally low level of κ-casein in equine milk. The presence of κ-casein in equine milk was an issue of debate for several years, with several authors (Visser *et al*., 1982; Ono *et al*., 1989; Ochirkhuyag *et a*l., 2000) reporting its absence. However, other studies (Kotts & Jenness, 1976; Malacarne *et al*., 2000; Iametti *et al*., 2001; Egito *et al.*, 2001) demonstrated its presence, albeit at a low concentration. κ-Casein, the only glycosylated member of the casein family, exhibits microheterogeneity due to the level of glycosylation (Saito & Itoh, 1992). Although no direct information is available, lectin-binding studies indicate that equine κ-casein is glycosylated (Egito *et al*. 2001; Iametti *et al*., 2001), possibly at residues Thr_{123}, Thr_{127}, Thr_{131}, Thr_{149} and Thr_{153} (Lenasi *et al*., 2003).

This study is part of a series of studies on the stability of equine casein micelles. Here, the effects of added urea or trisodium citrate and low temperature on the stability of equine casein micelles were investigated. A fractionation method based on the dissociation of casein by pH and temperature was applied and the fractions were analyzed by urea-PAGE and the gels were stained by a modified staining procedure for glycoproteins to determine if any glycosylated casein (κ-casein) was present in equine milk. Equine casein micelles were also fractionated by differential centrifugation followed by staining of electrophoretograms for glycoproteins to determine if the level of glycosylated protein (κ-casein) increased with decreasing micelle size. Finally, the stability of equine casein micelles to ethanol-mediated temperature-dependent dissociation was studied.

6.2. Materials and Methods

6.2.1 Milk supply

Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from 5 milkings over 24 h, from a herd of multiparous, New Forest and New Forest/Arabian mares in mid-lactation, physically separated by day from their foals. The milk was cooled to 4^{o}C and was received at our laboratory within ~24 h of milking. Prior to use, the milk was filtered through glass wool to remove any extraneous material. Equine milk was defatted by centrifugation at 1,000 g for 20 min at 20^{o}C using a Sorvall® RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) and filtered through glass wool to remove fat particles. Raw whole bovine milk, obtained from a local dairy farm, was defatted by centrifugation at 2,000 g for 20 min at 20^{o}C followed by filtration through glass wool. Sodium azide, 0.5 g L^{-1}, was added as preservative to both equine and bovine milk and skimmed milk samples were stored for no longer than 3 days.

6.2.2. Milk sample preparation

6.2.2.1. Equine casein micelles for dissociation by urea and citrate

Raw skimmed equine milk was centrifuged at 100,000 g for 60 min at 20^{o}C using an Optima LE-80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA), equipped with a Beckman 50.2 Ti rotor (12 place); the pelleted micelles were resuspended in equine milk dialysate prepared by exhaustive dialysis of a 66 g L^{-1} lactose solution against 2 x 20 volumes of equine milk for 48 h at 20^{o}C. Sodium azide (0.5 g L^{-1}) was added as preservative. The protein content was determined by the Kjeldahl method (IDF, 2001).

For some experiments, suspensions of equine casein micelles were prepared as described above except that they were suspended in dialysate at ~ 30-35 g L^{-1} protein. After determination of the protein content by the Kjeldahl method (IDF, 2001), protein was adjusted to 25 g L^{-1}, which approximates the casein content of bovine milk, by the addition of dialysate and subsequently adjusted to pH 6.6.

6.3. Compositional analysis

The total protein content of the raw skimmed equine milk was determined by the Kjeldahl method. The ionic calcium (Ca^{2+}) content was measured using a calcium ion-selective electrode (ISE25Ca; Radiometer Analytical, Copenhagen, Denmark) as described in Section 3.2.5.2. A standard curve of mV plotted against the log concentration of $CaCl_2$ (1 to 10 mM) in 0.1 M KCl (to maintain consistent ionic strength) was used to calculate the concentration of ionic calcium. Throughout this study, pH was measured using a Radiometer pHM 210 standard pH meter equipped with a Radiometer Meterlab® combined general purpose electrode with built-in temperature sensor (Radiometer Analytical SAS, Lyon, France). All chemicals used were of reagent grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Descriptive statistical analysis of data (mean, standard deviation) was performed using Minitab Statistical Software (Release 13.31; Minitab Inc., State College, PA, USA). All analyses, unless otherwise stated, were carried out at least in triplicate and average results with standard deviations reported. The coefficient of variation was always < 5% for reported data. Measurement of the ethanol-mediated temperature-dependent dissociation of milk is subjective and for this reason and, because of the effect of very small differences in pH on ethanol stability, statistical analysis of replicate results was not possible; all assays were carried out at least 5 times and a similar result was recorded on all occasions.

6.4.1. Dissociation of equine casein micelles by urea

Equine casein micelles, suspended in dialysate, were made to concentrations of 0.0 to 8.0 mol L^{-1} urea, vortexed and left to stand for 10 min before centrifugation at 1,000 x g for 10 min at 20°C. The supernatant was filtered (0.45 μm) and diluted 1:5 with Milli-Q™ water. The absorbance of the suspensions in a 1 cm path-length glass micro-cuvette at 600 nm and 20°C was measured using a Cary IE double-beam UV-Vis spectrophotometer (Varian Scientific Instruments Inc., CA, USA.)

6.4.2. Dissociation of equine casein micelles by trisodium citrate

Dissociation of equine casein micelles reusupended in equine dialysate by trisodium citrate to final concentrations in the range 0.00-0.10 mol L^{-1} was determined. Samples were diluted

1:4 with Milli-Q™ water (200 μL sample + 800 μL water) and the absorbance at 600 nm and 20°C was measured in a 1 cm path length glass micro-cuvette.

6.4.3. Dissociation of equine caseins at 4 or 20°C.

Skimmed equine and bovine milks were ultracentrifuged at 20 or 4°C at 100,000 g for 1 h or 2 h, respectively. The centrifugation time at 4°C was increased to compensate for any increase in viscosity of the skimmed milks which occurs at low temperature (Davies & Law, 1983). The milks were centrifuged at their natural pH (7.23 and 6.63, respectively) in an Optima LE-80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA). The supernatant was removed carefully and the pellets were redispersed in SMUF. The ultracentrifugal supernatants and pellets, suspended in SMUF, were analysed by urea-PAGE. Samples were diluted 1:1, with urea sample buffer. Control samples were prepared by diluting skimmed equine and bovine milk 1:1 with sample buffer. The level of β-casein in the supernatants and pellets was quantified densitometrically using gel analysis software Total Lab Quant 1D version 11.4 (Total Lab Ltd., Newcastle-upon-Tyne, UK).

6.4.4. Fractionation of equine casein

The casein fraction of equine milk was prepared and separated according to the protocol in Figure 6.1. The fractionation is based on that of Visser *et al*. (1982) with several modifications. After the initial pH adjustment, samples were dialysed using Visking tubing with a molecular weight cut-off of 7,000 Da and a diameter of 16 mm (Medicell International Ltd., Liverpool Road, London, UK). The cold-precipitated casein (CP-casein), temperature-sensitive casein (TS-casein) and the whole casein fractions were characterized by urea-PAGE. Freeze-dried casein pellets were prepared at 10 mg mL^{-1} of urea-PAGE sample buffer.

Visser *et al*. (1982) were unable to identify κ-casein in equine milk; therefore, SDS-PAGE was carried out on the CP- and TS-fractions after separation at pH 4.6 and 4.2 and the gels were stained for glycoproteins. Two gels were run back-to-back to facilitate staining with protein stain and glycoprotein stain. Bovine Na-caseinate, equine ultracentrifugal casein and supernatant and equine casein and supernatant separated at pH 4.6 were included in the analysis.

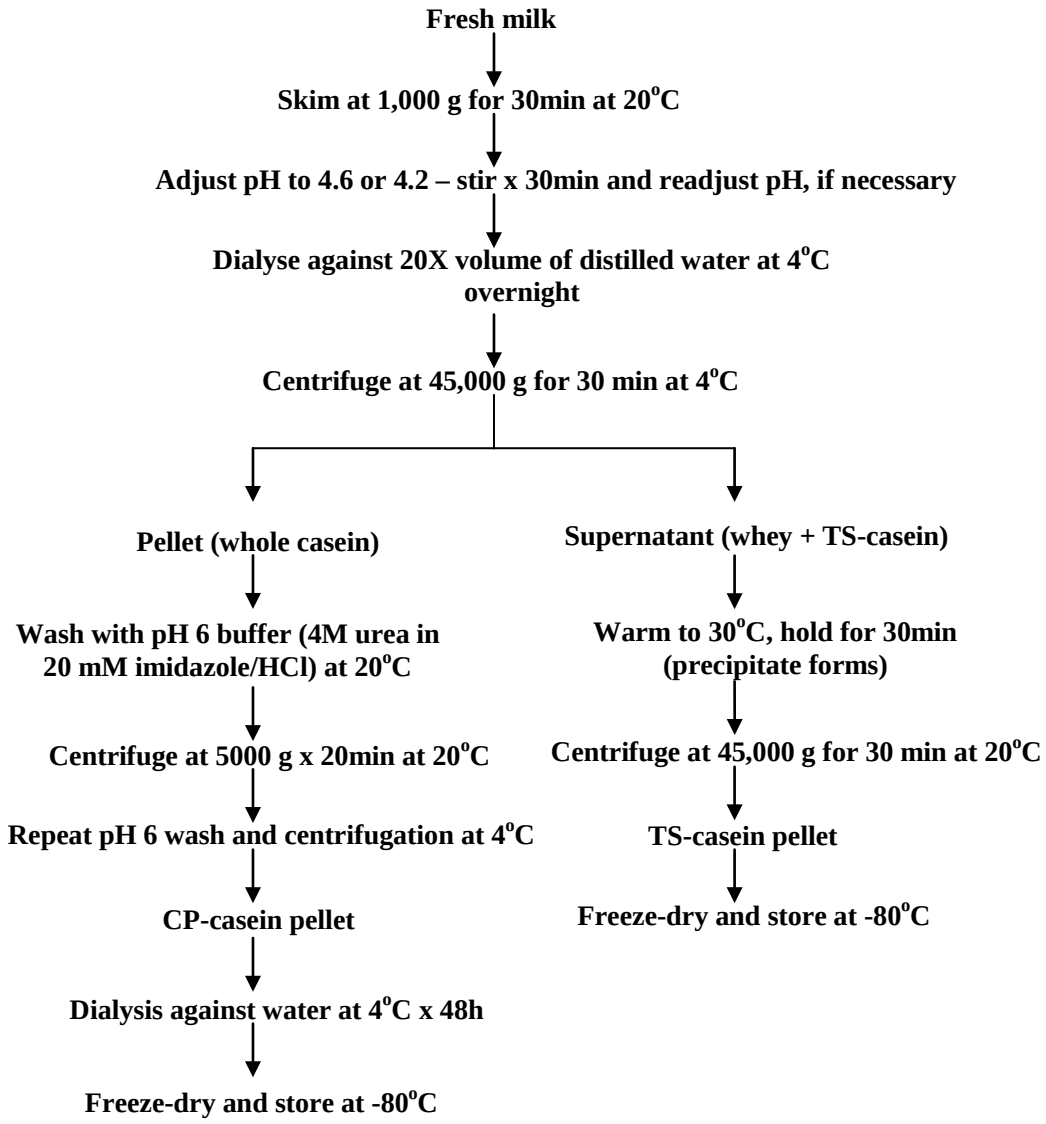

Figure 6.1. Fractionation of equine casein at pH 4.2 or 4.6. CP-casein, cold-precipitated casein; TS-casein, temperature-sensitive casein.

6.4.5. Fractionation of equine casein micelle by size

Equine milk was fractionated by differential centrifugation of raw skimmed milk at 5,000 g, 10,000 g, 20,000 g, 50,000 g for 30 min or 100,000 g for 60 min at 20^{o}C; the pellets obtained at each stage were washed several times with distilled water and redispersed in lactose-free SMUF (Jenness & Koops, 1962). Samples were diluted 1:1 with sample buffer for urea or SDS-PAGE, respectively. The samples for SDS-PAGE were overloaded on the gel as the level of equine κ-casein was expected to be low. The equine CP-pellet, fractionated according to the method of Visser *et al*. (1982), was included in the electrophoretic analysis.

6.4.6. Urea polyacrylamide gel electrophoresis (urea-PAGE)

Urea polyacrylamide gel electrophoresis (urea-PAGE) [12.5% total monomer (T); 4% cross-linking monomer (C), pH 8.9] was performed using a Protean II xi vertical slab gel unit (Bio-Rad Laboratories Ltd., Hercules, CA, USA) according to the method of Andrews (1983), with the modifications of Shalabi & Fox (1987). The gels were stained directly with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and destained in several changes of distilled water. Two, identically loaded gels, were run back-to-back in the electrophoresis unit to facilitate glycoprotein and protein staining.

6.4.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using separating and stacking gels containing 12% and 4% acrylamide, respectively in a Mini-Protean II cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the method of Laemmli (1970) with the modifications of Singh & Creamer (1991). Milk samples were mixed in the ratio 1:1 (v/v) with SDS sample buffer and heated at 100^{o}C for 10 min. Gels were run at a constant voltage of 200 V and were destained in several changes of a solution of methanol and glacial acetic acid (50% and 10% (v/v), respectively) until the background of the gel was clear. The gels were scanned using a flat-bed scanner and, in some cases, subjected to quantitative image analysis. An SDS molecular weight marker with molecular weights ranging from 6,500 to 205,000 Da (S8445, Sigma-Aldrich Inc.) was used. Two identical gels were run back-to-back to facilitate protein or glycoprotein staining.

6.4.7.1. Staining for glycoproteins

The glycoprotein staining protocol of Thornton *et al*. (1994) with several modifications was used to identify glycosylated protein (κ-casein) in equine milk. Following SDS-PAGE, the gels were fixed in acetic acid:methanol:Milli-Q® water (10:35:35, v/v) for 2 h and then rinsed once with Milli-Q® water. Subsequently, the gels were placed in a freshly prepared solution of 2.0% (v/v) periodic acid in 4% (v/v) acetic acid for 2 h. Gels were rinsed thoroughly with deionized water over a 40 min period with gentle agitation on an orbital platform shaker (Heidolph Rotamix 120, Heidolph Canada Biotech Edge, Toronto, Canada) at 20 rpm. Gels were rinsed twice with a freshly prepared solution of 0.1% (w/v) sodium metabisulfide in 10 mM HCl and allowed stand in the solution for 30 min, followed by incubation in Schiff's reagent for 2 h in the dark. Gels were again rinsed twice with 0.1% sodium metabisulfide in 10 mM HCl and left stand in this solution for 1 h in the dark at 20°C. The gels were rinsed once with deionised water and placed in a solution of 1.0 % sodium metabisulfide in 10 mM HCl for 2 h in the dark with several changes to the solution. The gels were rinsed with of 7.5 % (v/v) acetic acid, 5% (v/v) methanol solution in distilled water. Gels were scanned with a flat-bed scanner.

6.4.8. Ethanol-mediated temperature-induced dissociation of casein micelles.

As a preliminary experiment, equal volumes of ethanol (0-100%, at 10% intervals) and equine milk were mixed rapidly in sealed test tubes and left stand at 20°C for 10 min and then poured into polystyrene weighing boats (35 x 35 x 5 mm). L*, a* and b* values were measured in triplicate, as described in Section 4.3.4.

To study the ethanol-mediated temperature-induced dissociation of casein micelles, equal volumes of equine milk and ethanol (0-100% at 5% intervals) were mixed and heated in sealed tubes to a range of temperatures (30-80°C, at 10°C intervals) for 10 min. Milk samples, diluted with equal volumes of water, were used as controls. After heating, the samples were poured quickly into polystyrene weighing boats and L*, a* and b* values measured.

To study the reversibility of the effect of ethanol and heat treatment on equine milk, samples were prepared as described above and cooled to 20°C. Because ethanol caused significant precipitation of equine casein even at 40°C, results were assessed visually.

Finally, equal volumes of bovine or equine milk were mixed with 70 or 100% ethanol (v/v), heated to 70°C, held for 10 min and cooled rapidly on ice. The same procedure was used for

equine casein micelles resuspended in equine ultracentrifugal supernatant at a casein concentration adjusted to 25 g L^{-1}. Results were assessed visually.

6.5. Results and Discussion

6.5.1. Compositional analysis

The total protein content of skimmed equine milk was 18.50 ± 1.4 g L^{-1}, which was within the range of reported in Section 3.5.1. The pH was 7.23 ± 0.11 and was in the range of values reported in Section 3.6.2. The concentration of ionic calcium at the natural pH of equine milk was 2.89 ± 0.12 mmol L^{-1} which was higher than the value of 2.66 mmol L^{-1} reported in Section 3.5.4, but was within the range of values for equine milk predicted by Holt & Jenness (1984) and was considerably higher than that in bovine milk (1.76 mmol L^{-1}).

6.5.2. Dissociation of equine casein micelles by urea

The effect of added urea on the absorbance at 600 nm of skimmed bovine milk, equine casein micelles in equine dialysate (12.2 g L^{-1} protein) and equine micelles suspended in equine dialysate at a protein concentration of 25 g L^{-1} is shown in Figure 6.2. The extent of dissociation of the casein micelles in the bovine and equine samples was similar and as the concentration of urea was increased the dissociation increased, as indicated by a decrease in the absorbance at 600 nm. The dissociation of casein micelles reached a plateau at ~ 4 mol L^{-1} urea for both equine and bovine milk. Increasing the concentration of equine casein to 25 g L^{-1}, similar to that found in bovine milk, had little effect on the dissociation of equine casein micelles compared to that of equine micelles suspended at the natural casein concentration of equine milk. Addition of urea to milk dissociates the casein micelles by disrupting hydrogen and hydrophobic bonds in the micelles, without rupturing the linkages between casein and calcium phosphate (McGann & Fox, 1974; Holt, 1992). The decrease in the absorbance at 600 nm as the concentration of urea was increased was due to significant disruption of both equine and bovine casein micelles indicating that the strength of the hydrophobic and hydrogen of both milks were similar. Preliminary experimental work carried out using skimmed equine milk rather than resuspended equine casein micelles as substrate was unsuccessful as the residual fat in the sample led to high turbidity readings even after addition of 8 M urea.

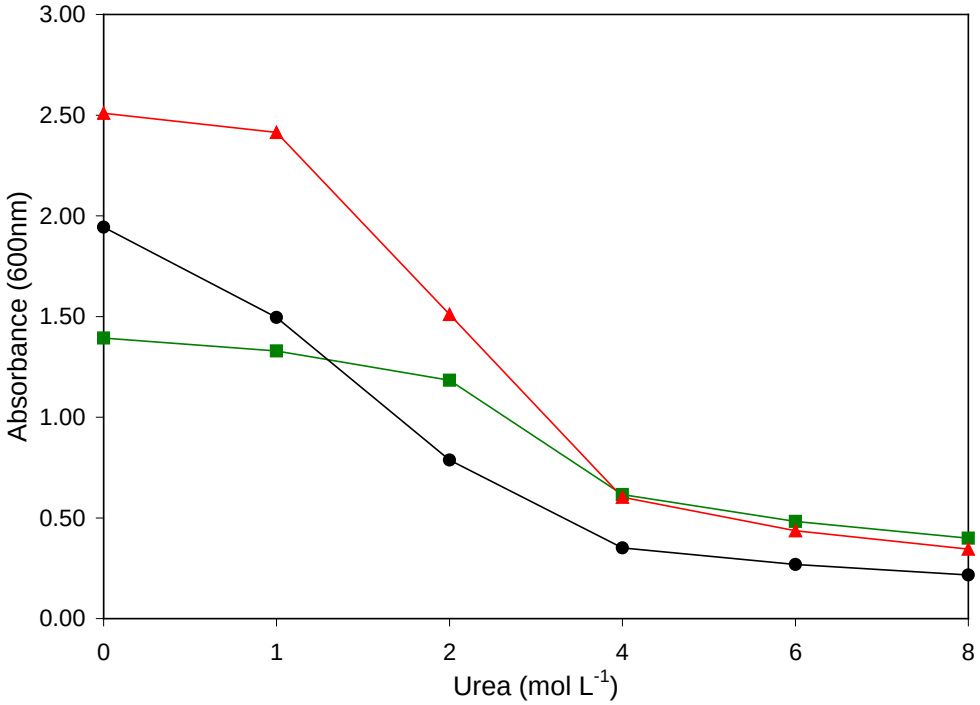

Figure 6.2. Influence of urea at a final concentration of 0.0-8.0 mol L^{-1} on the absorbance at 600 nm and 20°C, in a 1 cm path length glass micro-cuvette, of raw skimmed bovine milk (-■-), equine casein micelles in equine dialysate (-●-) or equine micelles at 25 g L^{-1} protein in equine dialysate (-▲-). Analysis was carried out in triplicate and the coefficient of variation was <5% of the reported value for each data point.

6.5.3. Dissociation of equine casein micelles by trisodium citrate

The dissociation of equine casein micelles suspended in dialysate at a casein concentration of 12.2 g L^{-1} or 25 g L^{-1} and of skimmed bovine milk by trisodium citrate is shown in Figure 6.3. Equine micelles suspended in dialysate at their natural casein concentration or at a casein concentration of 25 g L^{-1} showed a similar dissociation pattern as bovine milk with added trisodium citrate and the dissociation of both equine and bovine casein samples plateaued at ~ 0.04 mol L^{-1} citrate. Increasing the casein concentration of the equine sample to approximate that in bovine milk had no effect on the dissociation profile (Figure 6.3). Trisodium citrate chelates calcium which reduces free calcium and colloidal phosphate, with the loss of micelle integrity (Pyne, 1962; Morr, 1967; Walstra, 1990; Pouliot *et al*., 1994). The extent of dissociation is determined by the ratio of chelant to casein and, while the amount of calcium chelated is independent of temperature, the rate of dissociation of casein micelles has been reported to increase as temperature increases (Pitkowski *et al*., 2008).

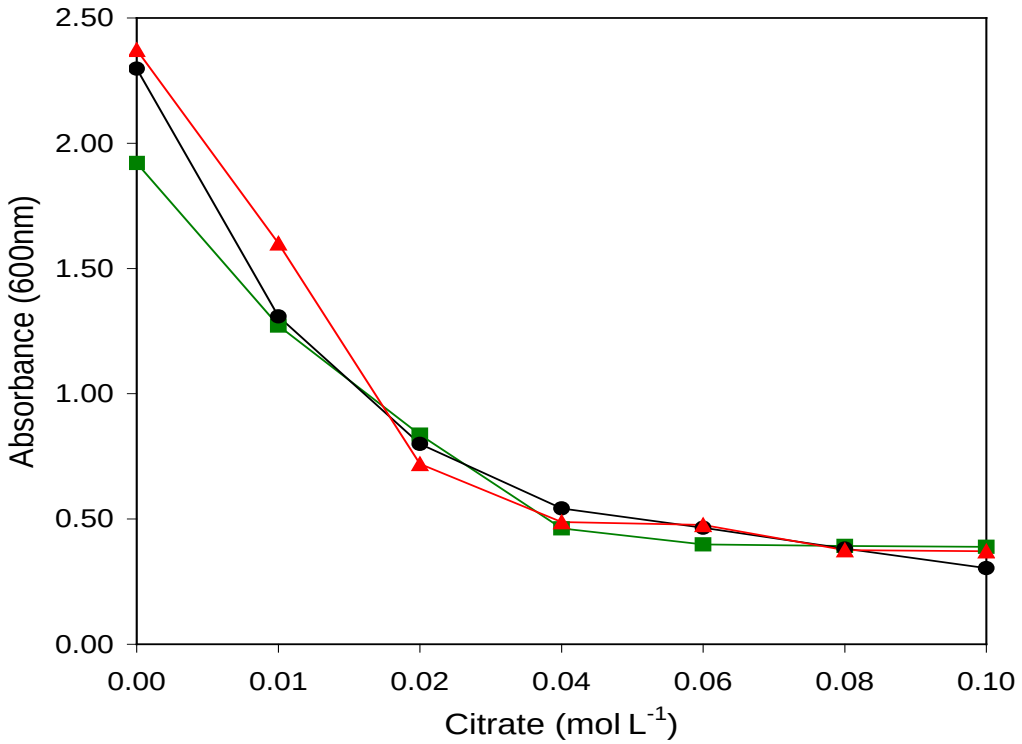

Figure 6.3. Influence of citrate added to a final concentration of 0.0-0.1 mol L^{-1} on the absorbance at 600 nm and 20°C, in a 1 cm path length glass micro-cuvette, of raw skimmed bovine milk (-■-), equine casein micelles in equine permeate (-●-) or equine micelles at 25 g L^{-1} protein in equine permeate (-▲-). Analysis was carried out in triplicate and the coefficient of variation was <5% of the reported value for each data point.

6.5.4. Dissociation of equine casein at 4 or 20°C

The dissociation of equine and bovine caseins at 4 and 20°C (at their natural pH) is shown in the urea-PAGE electrophoretogram in Figure 6.4. In comparison to an equine milk control sample (lane 1), very little β-casein dissociated from equine casein micelles at 4 or 20°C. Using quantitative image analysis (Table 6.1) it was estimated that 7.2 and 19.8 % of the original β-casein in equine milk was non-sedimentable at 20 and 4°C, respectively. By contrast, a considerable proportion of equine α_s-casein, 61.5 and 69.2% at 4 or 20°C respectively, (Table 6.1), was non-sedimentable under the conditions used in this study. The electrophoretograms showed that the less phosphorylated isoforms of equine α_s-casein, i.e., the bands with the slowest electrophoretic mobility, were more susceptible to dissociation at 4 or 20°C compared to isoforms with a higher degree of phosphorylation.

In comparison to equine β-casein, a considerable proportion of bovine β-casein dissociated at 4 and 20°C (Figure 6.4). Quantitatively, 34.1 and 66.6% of bovine β-casein was non-sedimentable at 20 and 4°C, respectively. The proportion of bovine β-casein which was non-sedimentable at 4°C in this study is in agreement with Downey (1973) who showed by repeated column chromatography of casein micelles on Sepharose 2B that up to ~ 60% of bovine β-casein could be removed from milk cooled at 4°C. Furthermore, bovine milk serum has been shown to contain increasing quantities of β-casein with increasing holding time up to 120 min at ~ 0°C (Creamer *et al*., 1977). Approximately 24% of the α_{s1}-casein in bovine milk was non-sedimentable at 20°C which was less than half the proportion of equine α_s-casein at the same temperature. At 4°C considerably more equine α_s-casein was non-sedimentable compared to bovine α_{s1}-casein (Table 6.1). The amount of bovine α_{s1}-casein which was non-sedimentable at 4°C is considerably higher than that previously reported (Downey, 1973) which may be due to the different experimental conditions of the present study or that the control sample here was close to saturation level for densiometric analysis.

Cold storage of bovine milk causes extensive dissociation of β-casein from the micelles with a concomitant increase in the level of β-casein in ultracentrifugal supernatant (Rose, 1968; Downey & Murphy, 1970; Creamer *et al*., 1977; Davies & Law, 1983; Dalgleish & Law, 1989a) although the extent of dissociation is dependent on the length of time for which the milk is cooled (Sood *et al*., 1997) and the milk pH (Dalgleish *et al*., 1989a). Storage of milk at 0 to 4°C weakens hydrophobic bonds (Davies & Law, 1983) and causes solubilisation of micellar calcium phosphate (Pyne, 1962) which contributes to the dissociation of the β-casein

while a considerable proportion of α_{s1}-, α_{s2}- and κ-casein remain intact. Increasing the ionic strength of the milk by addition of NaCl reduces the amount of Ca bound to β- and α_{s1}-caseins and increases the solubility of β-casein at low temperature (Pierre & Brulé, 1981). The effect of cooling milk can be partly likened to that of chelation of free Ca^{2+} in milk which leads to solubilisation of colloidal phosphate and loss of micelle integrity (Pyne, 1962; Morr, 1967), however, solubilisation of casein is primarily driven by reduced hydrophobic interactions at low temperature. In terms of time at low temperature, β-casein is solubilised first, followed by κ-casein (Ono *et al*., 1978) and ultimately α_s-caseins (Lin *et al*., 1972).

β-Casein released from casein micelles at a low temperature is probably not cross-linked by colloidal calcium phosphate which is the case for α_s-caseins and one of the key factors maintaining micellar integrity, at least in bovine milk (Aoki *et al*., 1990).

In contrast to the result reported in this study, O'Connor and Fox (1973) reported little tendency of the α_s-casein of equine, asinine, bovine, caprine, ovine, canine or porcine to dissociate at 30, 20, 10 or 5^{o}C, although proteolysis by rennin was the method used in their study to determine the extent of casein dissociation; differences in methodology could therefore account for the different dissociation patterns of α_s-casein found between this study and that of O'Connor & Fox (1973).

It has been reported (Ono *et al*., 1990) that the dissociation of β-casein on cooling bovine milk is closely related to micelle size and that β-casein dissociates easily from large micelles at 4^{o}C compared to 37^{o}C; however, equine casein micelles are significantly larger than those in bovine milk (Section 3.6.6) but β-casein does not dissociate to any great extent which probably indicates considerable differences in protein composition and micelle stabilizing factors between equine and bovine micelles.

Multiple-phosphorylated isoforms of equine β-casein, containing 3 to 7 phosphoserine residues, have been reported, with the isoelectric point varying from pH 4.74 to 5.30 (Girardet *et al*., 2006; Matéos *et al*., 2009). This multi-phosphorylation may account for some of the differences found in this study between the dissociation of equine and bovine β-casein at low temperature. In human milk the framework of casein micelles is made up primarily of β-casein, phosphorylated from 0 (β-CN-OP) to 5 (β-CN-5P), with < 5% α_s-casein (Greenberg & Groves, 1979; Sood *et al*., 1998).

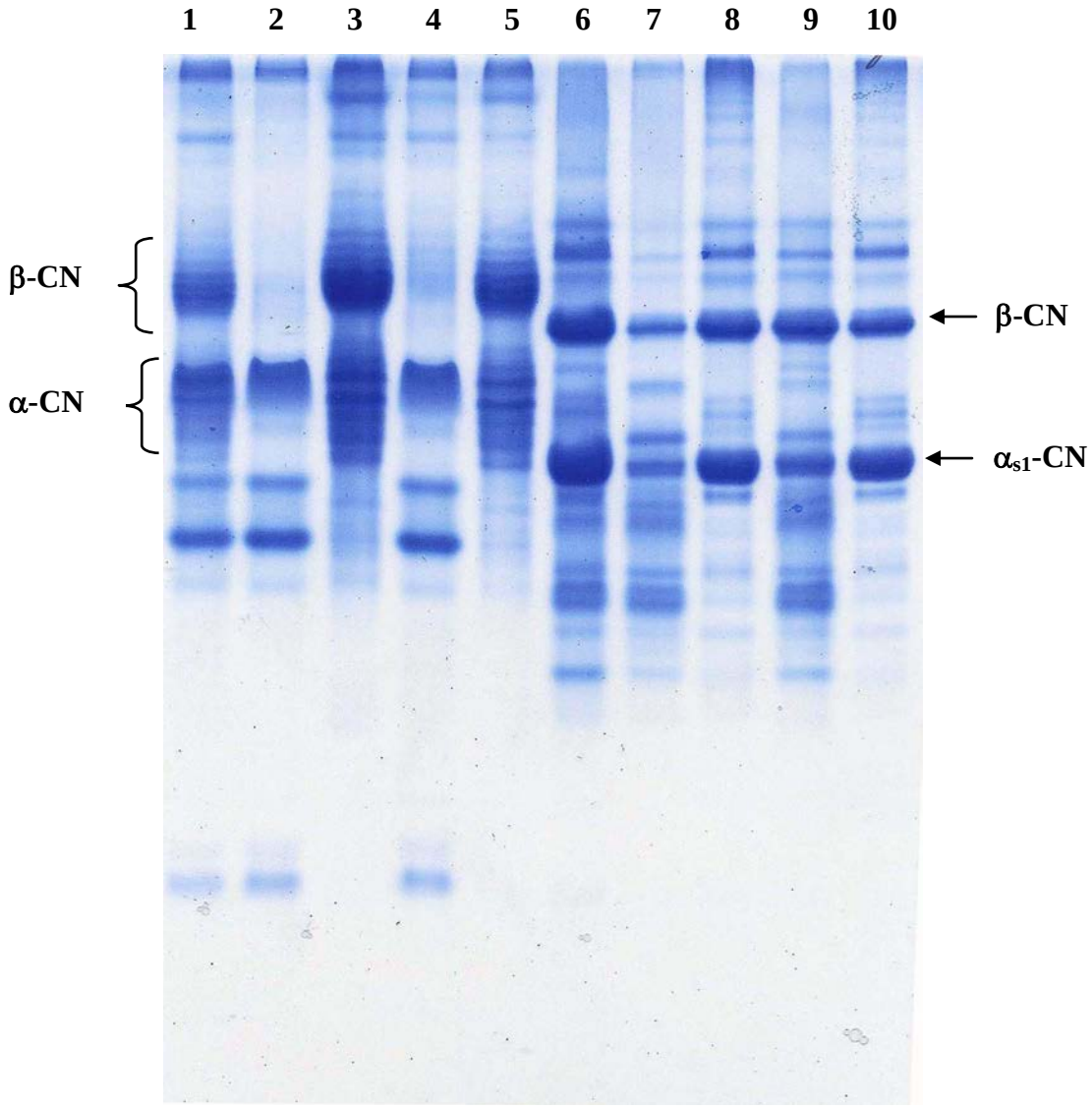

Figure 6.4. Urea-polyacrylamide gel electrophoretograms (12.5% T, 4% C, pH 8.9) of the sedimentable and non-sedimentable caseins of equine and bovine milk at 4 and 20°C at natural pH. *Lanes*: **1**, equine milk. **2**, equine supernatant 20°C, **3**, equine pellet 20°C. **4**, equine supernatant 4°C. **5**, equine pellet 4°C. **6**, bovine milk. **7**, bovine supernatant 20°C. **8**, bovine pellet 20°C. **9**, bovine supernatant 4°C. **10**, bovine pellet 4°C.

Table 6.1. Quantitative image analysis for the dissociation of β-casein and α_s-casein of bovine and equine milk following ultracentrifugation (100,000 x g) at 20 and 4°C as measured by imaging the quantity of each protein in the ultracentrifugal supernatant.

	Equine casein		**Bovine casein**	
	Non-sedimentable casein as a % of control*			
	20°C	**4°C**	**20°C**	**4°C**
β-casein	7.2 ± 1.2	19.8 ± 2.4	34.1 ± 1.2	66.6 ± 2.3
α_s-casein	61.53 ± 3.6	69.23 ± 2.7	23.8 ± 2.0	33.8 ± 21

Analysis was carried out on 3 separate occasions with fresh skimmed milk samples. *Control samples are equine and bovine milk (lanes 1 & 6 of the electrophoretogram)

At low temperature, β-CN-OP and β-CN-1P remain in human micelles when hydrophobic interactions are minimized (Sood et al., 1997) suggesting that these forms of β-casein may form the framework of human micelles.

Addition of EDTA to human milk causes a significant solubilisation of β-CN-3P, β-CN-4P and β-CN-5P indicating that electrostatic interactions between highly phosphorylated molecules also contribute to micellar stability to a greater extent than in bovine milk (Sood *et al*., 1997). Micellar integrity in equine milk is also likely to depend on electrostatic interactions between multiphosphorylated β-caseins for stability in a similar manner to human milk. By contrast, the micelle framework of bovine casein micelles consists of a high proportion of αs_1-casein with less phosphorylated forms of β-casein (Walstra & Jenness, 1984). The dissociation of bovine β-casein at low temperature is due, in part, to weakened hydrophobic interactions and the dissociation is reversible if the temperature is increased and, at 37°C for 3 h, most of the β-casein reassociates with the micelle (Sood *et al*., 1997).

6.5.5. Fractionation of equine casein

Figure 6.5 shows the urea-PAGE electrophoretograms of equine casein fractionated at pH 4.2 or 4.6 and 4 or 20°C using a modification of the method of Visser *et al*, (1982). The fractionation method separated the caseins of equine milk into a cold-precipitated fraction

(CP-casein) and a temperature-sensitive fraction (TS-fraction). The TS-casein fraction is much more soluble at 4°C than at 20°C whereas the CP-casein is insoluble unless the pH is raised or urea is added (Visser *et al*., 1982). For comparative purposes, whole equine casein prepared at pH 4.6 and 20°C was included in the electrophoretograms (lane 1). Under these conditions it was possible to isolate relatively pure β-casein from equine milk (lane 2, Figure 6.5) in the TS-casein fraction. pH 4.6 favoured the dissociation of β-casein with virtually no α_s-casein compared to the TS-casein fraction prepared at pH 4.2 (lane 3). At both pH values, the CP-casein fraction contained both β- and α_s-casein and the levels of each were more pronounced at pH 4.6.

Dalgleish & Law (1988) studied the effect of both temperature and pH on the dissociation of bovine casein micelles at 4, 20 or 30°C in the pH range 4.9-6.7 and reported that the amount and proportions of caseins that dissociated from micelles were both temperature and pH dependent. More caseins were non-sedimentable as the temperature was decreased from 30 to 4°C and a pH of maximum dissociation of caseins occurred between pH 5.6 and 5.1, which may be attributable to a partial loosening of bonds within and between caseins due to loss of CCP (Lucey, 2004). The pH of maximum dissociation of β-casein was higher at 20 than at 4°C (pH 5.4 *vs* 5.1, respectively), which is in agreement with earlier studies which showed that the solubility of β-casein, in the region of its ioselectric point, increased significantly as temperature is reduced (Bingham, 1971; Roefs *et al*., 1985; Law & Leaver, 1998;) . At 4, 20 and 30°C, only a small amount of α_{s1}-casein dissociated at pH > 6.2 (Dalgelish & Law, 1988). α_{s1}-, and α_{s2}-Casein associate with colloidal calcium phosphate *via* electrostatic interactions whereas β-casein associations are primarily *via* hydrophobic bonds which are weaken at low temperature.

Visser *et al*. (1982) were unable to identify κ-casein in equine milk. One fraction collected by ion-exchange chromatography of the TS-casein pellet was sensitive to chymosin although it was concluded based on other criteria that this fraction was γ-casein-like and not κ-casein-like.

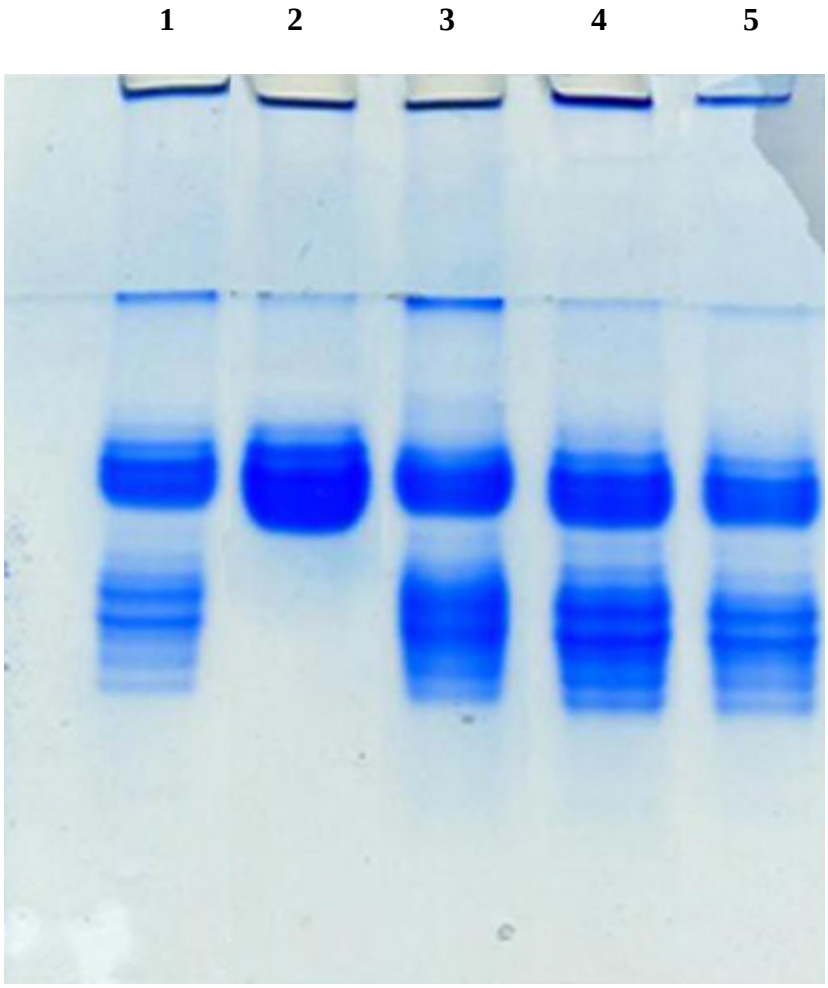

Figure 6.5. Urea polyacrylamide gel electrophoretograms (12.5% T, 4% C, pH 8.9) of equine caseins fractionated at pH 4.2 or 4.6 and 4 or 20°C. *Lanes*: **1**, whole casein, pH 4.6 (20°C). **2**, temperature-sensitive (TS) casein, pH 4.6. **3**, TS-casein, pH 4.2. **4**, cold-precipitated (CP) casein, pH 4.6. **5**, CP-casein, pH 4.2.

SDS-PAGE electrophoretogram of selected fractions of equine casein are shown in Figure 6.6. Included on the electrophoretogram, for comparative purposes, are an equine casein pellet and supernatant from ultracentrifugation at natural pH (pH 7.3) and whole equine casein prepared at pH 4.6 as well as bovine sodium caseinate, κ-casein, α-lactalbumin and β-lactoglobulin. The electrophoretograms showed several bands that could correspond to equine κ-casein; the TS-casein at both pH 4.6 and 4.2 had bands in the region of 25,000 Da. Equine κ-casein has a molecular weight of ~ 19,000 Da (Iametti *et al*., 2001; Lenasi *et al*., 2003; Miranda *et al*., 2004) prior to post-translational glycosylation and has been reported to

have a molecular weight of 25,300 Da when glycosylated (Miranda *et al*., 2004). Neither equine acid casein (lane 7) nor equine ultracentrifugal pellet (lane 5) appeared to have this band. However, staining with a highly sensitive glycoprotein detection technique showed the presence of glycosylated protein in several equine casein fractions (Figure 6.7). Equine ultracentrifugal pellet (lane 5), equine casein (lane 7), the TS-casein fraction, pH 4.6 (lanes 11 and 12) and the CP-casein fraction, pH 4.2 (lane 15) contained glycosylated casein. This result agrees with other reports that equine milk contains κ-casein (Malacarne, *et al*., 2000; Iametti *et al*., 2001; Egito *et al*., 2001; Lenasi *et al*., 2003; Miranda *et al*., 2004) and also agrees with Visser *et al*., (1982), i.e., that equine κ-casein is present in the CP-casein fraction but is also in the TS-casein. The CP-casein from pH 4.6 fractionation did not resolve well on SDS-PAGE.

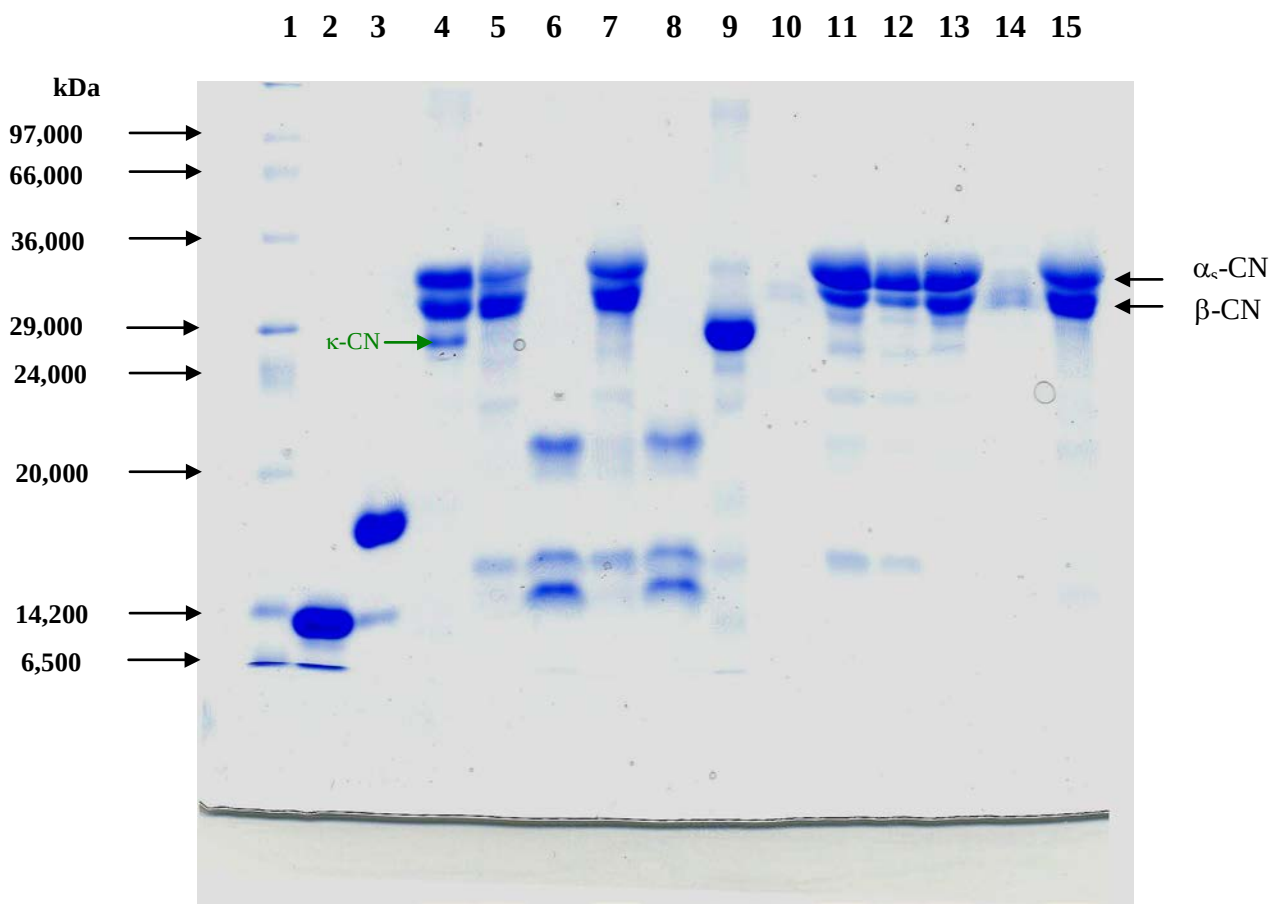

Figure 6.6. SDS-PAGE of selected fractions of equine casein. *Lanes:* **1**, molecular weight markers. **2**, bovine α-lactalbumin. **3**, bovine β-lactoglobulin. **4**, bovine sodium caseinate. Equine ultracentrifugal pellet, pH 7.3 (**5**) and supernatant (**6**). **7**, whole equine casein, pH 4.6 and supernatant (**8**). **9**, bovine κ-casein. **10**, CP-casein, pH 4.6. **11** & **12,** TS-casein, pH 4.6. **13**, TS-casein, pH 4.2. **15**, CP-casein, pH 4.2.

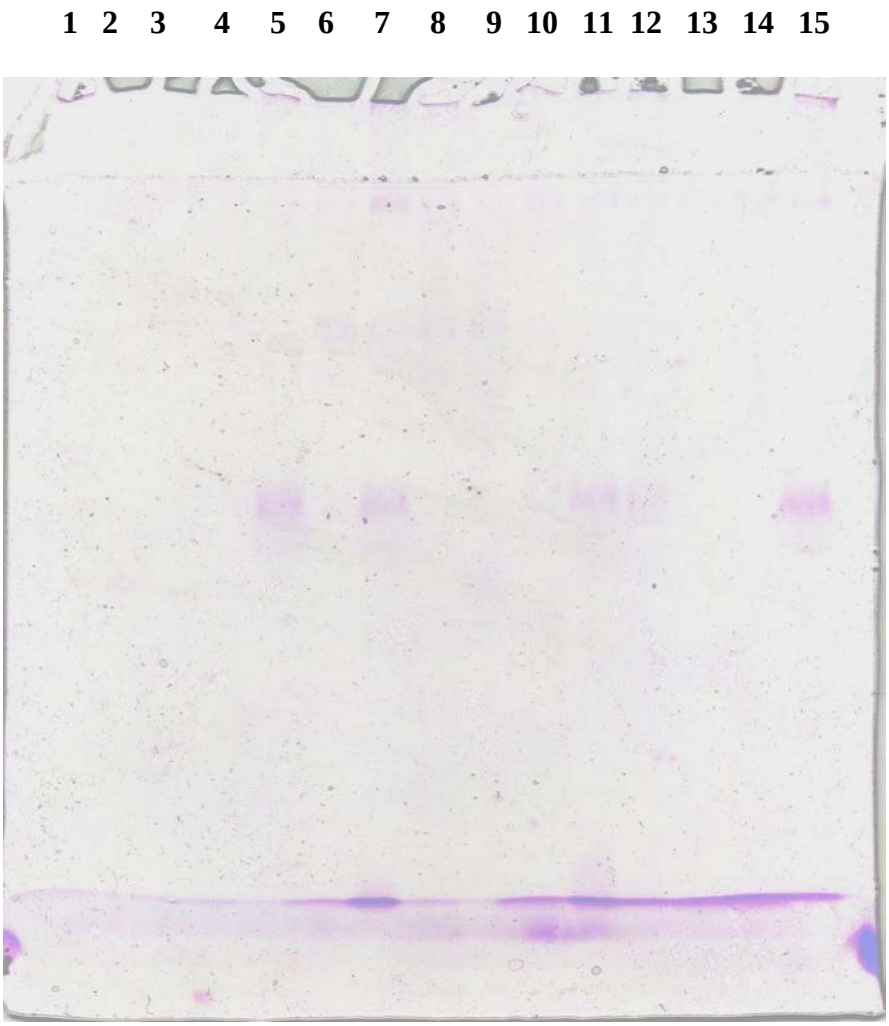

Figure 6.7. Glycoprotein detection using Sigma GLYCOPRO kit for SDS polyacrylamide gel electrophoresis of selected equine casein fractions. *Lanes*: **1**, molecular weight marker. **2**, bovine α-lactalbumin. **3**, bovine β-lactoglobulin. **4,** bovine sodium caseinate. Equine ultracentrifugal pellet (**5**) and supernatant (**6**), pH 7.3. **7**, whole equine casein, pH 4.6. **8**, supernatant pH 4.6. **9**, bovine κ-casein. **10**, CP-casein, pH 4.6. **11** & **12** TS-casein, pH 4.6. **13**, TS-casein, pH 4.2. **15**, CP-casein, pH 4.2. Bands with a pink colour indicate glycosylated components of equine casein (Lanes 5, 7, 11, 12 and 15).

6.5.6. Fractionation of equine casein micelles by size

To provide further confirmation of the presence of glycosylated protein in equine milk, equine casein micelles were fractionated according to size by differential centrifugation at progressively increasing gravitational force. The SDS-PAGE results are presented in Figure 6.8 stained with protein stain and in Figure 6.9, stained for glycoproteins. Casein micelles are heterogeneous in size and composition and can be fractionated by differential centrifugation. For bovine milk, as micelle size decreases, the content of κ-casein increases (Rose *et al*., 1969; Ekstrand & Larsson- Raźnikiewicz, 1978; McGann *et al*., 1980; Dalgleish *et al*., 1989) with a concomitant decrease in β-casein, while the level of α_{s1}-casein remains constant, at least in large or medium size micelles (Davies & Law, 1983; Donnelly *et al*., 1984; Dalgleish *et al*., 1989b). As micelle size decreases, further α_{s1}-casein decreases while the level of β-casein increases again (Ekstrand & Larsson-Raźnikiewicz, 1978). In this experiment, the presence of κ-casein in equine casein micelles was the main focus of attention, as a result of which the concentration of protein was overloaded for electrophoresis to compensate for the low level of κ-casein reported for equine milk, therefore it is difficult to determine, with certainty, whether β-casein concentration increased or decreased with changing micelle size (Figure 6.8). However, glycoprotein staining of the gel (Figure 6.9) showed an increase in the glycosylated band as the gravitational force was increased (lanes 7 to 12). It is concluded that as the size of equine micelles decreased as the centrifugal force increased, the κ-casein concentration increased. Equine casein (lane 4) and equine CP-casein (lane 5) also contained glycosylated protein (κ-casein). Bovine sodium caseinate (lanes 3 and 14) stained positively for glycoprotein in the κ-casein region.

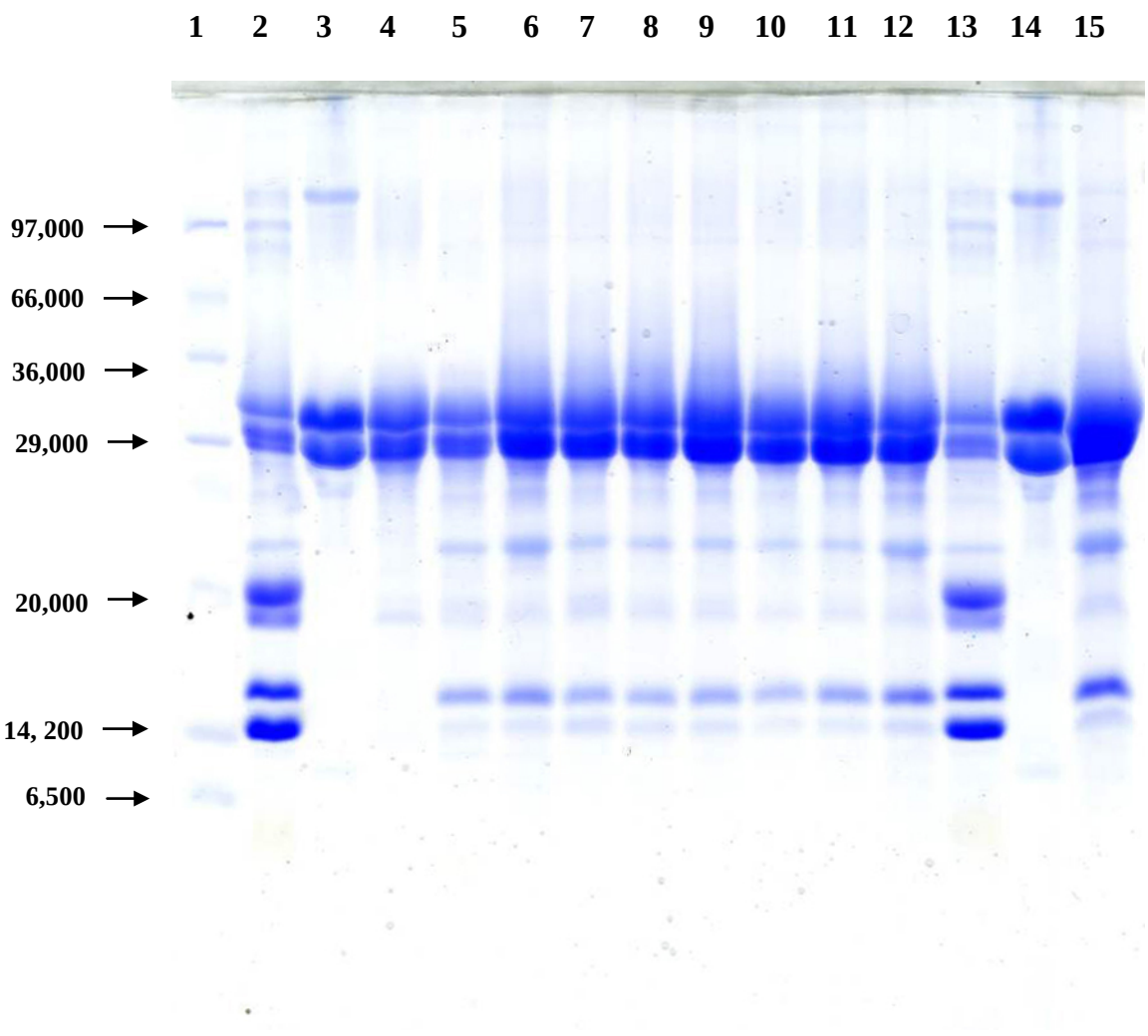

Figure 6.8. SDS-electrophoretograms of equine caseins separated using differential centrifugation at progressively increasing gravitational force. *Lanes*: **1**, molecular weight marker. **2 & 13**, skimmed equine milk. **3 & 14**, bovine sodium caseinate. **4,** equine whole casein fraction, pH 4.6. **5**, equine CP-casein , pH 4.6. **6, 12** & **15**, 100,000 x g pellet. **7**, 3,000 x g pellet. **8**, 5,000 x g. **9**, 10,000 x g. **10**, 20,000 x g. **11**, 50,000 x g. *Note*: samples have been intentionally overloaded for detection of glycosylated casein and the 100,000 x g pellet was loaded at various levels.

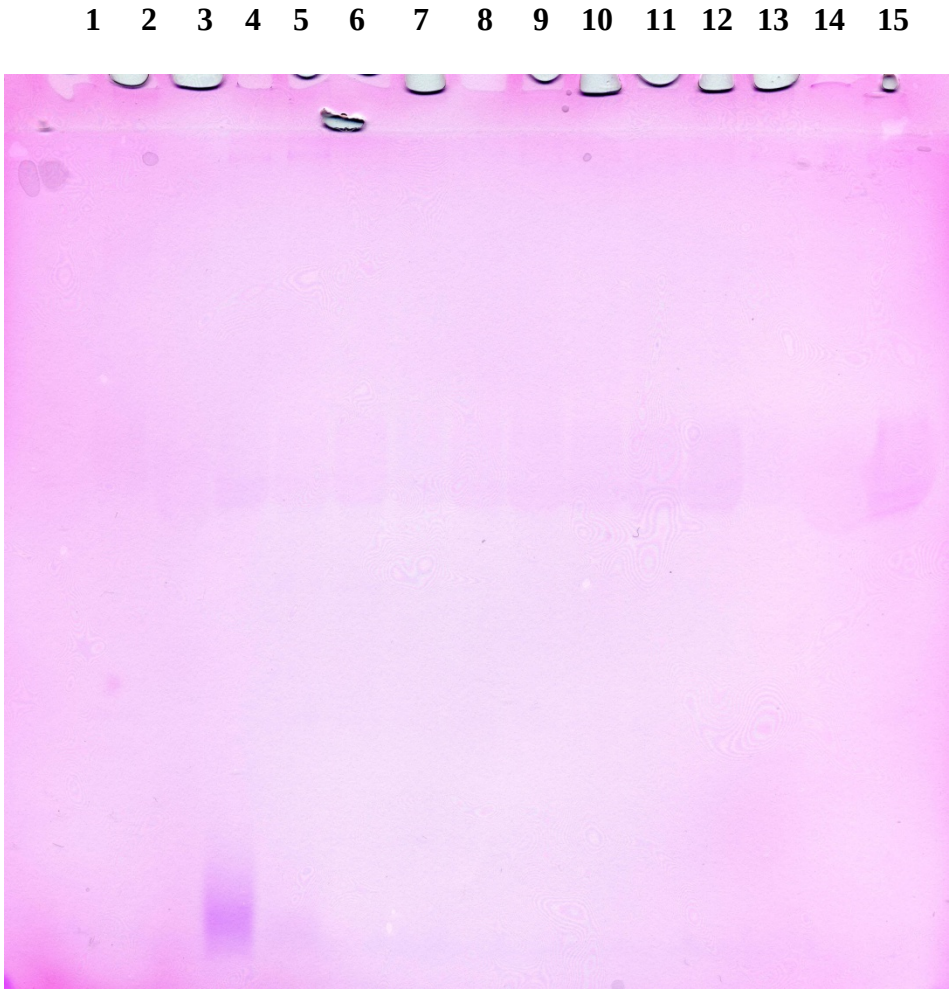

Figure 6.9. Glycoprotein detection using a modification of the method of Thornton *et al*. (1994) of equine caseins separated using differential centrifugation at progressively increasing gravitational force. *Lanes*: **1**, molecular weight marker. **2 & 13**, skimmed equine milk. **3 & 14**, bovine sodium caseinate. **4,** equine whole casein fraction, pH 4.6. **5**, equine CP-casein , pH 4.6. **6, 12** & **15**, 100,000 x g pellet. **7**, 3,000 x g pellet. **8**, 5,000 x g. **9**, 10,000 x g. **10**, 20,000 x g. **11**, 50,000 x g.

6.5.7. Ethanol-mediated temperature-induced dissociation of casein micelles in equine and bovine milk

The ethanol stability of equine milk at its natural pH (~7.2) was ~45% (Section 3.6.11), whereas that of bovine milk analysed under identical conditions at its natural pH (~6.7) is 70-75% (Huppertz *et al.*, 2004a; Huppertz & De Kruif, 2007). The low ethanol stability of equine milk was attributed to the high concentration of ionic calcium in equine milk (2.66 mmol L^{-1}) since ethanol stability decreases significantly with even small increases in ionic calcium content (Horne & Parker, 1981a; Horne, 1987, 2003). The ethanol stability of unconcentrated equine milk increased in a sigmoidal fashion with milk pH (Section 3.6.11), from ~12% at pH 5.5 to ~55% at pH 7.5 (Figure 3.11).

In the present experiment, the effect of the addition of ethanol to equine milk increasing temperature was investigated by recording changes in L*, a* or b* values. Figure 6.10 shows the L*, a* and b* values as a function of ethanol concentration at 20^{o}C. Changes in L*or lightness are related to the extent of dissociation of the casein micelles (Zadow, 1993; O'Connell *et al*., 2001a; O'Sullivan *et al*., 2002). If the number of micelles in a milk sample is reduced (e.g., by dilution), the L* value will decrease (Dunkerley *et al*., 1993). The a* (greenness to redness, i.e., -a* values are green) and b* (blueness to yellowness, i.e., - b* values are blue) are useful parameters for assessing colour changes in milk (Zadow, 1993)

The L* value of equine milk began to decrease at ~ 40% ethanol (Figure 6.10), which is approximately the ethanol stability value of equine milk, and continued to decrease as the concentration of ethanol increased. The addition of ethanol > 40% to equine milk caused significant precipitation of equine casein micelles even at 20^{o}C. The a* value for equine milk with added ethanol, remained unchanged as the ethanol concentration increased (Figure 6.10) but the b* value had a maximum at ~ 40% ethanol, which corresponded to the ethanol stability point of equine milk. A similar increase in b* value was reported previously at the point of flocculation of bovine and equine casein micelles after addition of glucono-δ-lactone (Section 4.4.4). The effect of ethanol on bovine milk is different and it has been reported that L*, a* and b* values for bovine milk at 20^{o}C do not change as the concentration of ethanol is increased even when the ethanol stability point is reached (Zadow, 1993).

The effect of ethanol at progressively increasing temperature on the L* value of equine milk is shown in Figure 6.11. At all temperatures, the L* value decreased significantly with 15-20% added ethanol and, at all temperatures, a further decrease occurred at ~ 40% added ethanol, which was more marked as the temperature was increased. It has been reported for

bovine milk that the L* value decreases at ~ 40%, with the decrease being greater at higher temperatures (Zadow, 1993). Addition of ethanol to equine milk caused considerable precipitation of equine casein micelles even at relatively low temperatures and low ethanol concentrations. The decrease in L* value of bovine milk on addition of ethanol with heating is due to the dissociation of casein micelles which has been attributed to an increase in the solubility of caseins as a consequence of reduced phosphoseryl-mediated cross-linking and increased protein hydrophobicity (O'Connell *et al*., 2001b). The a* value of equine milk with added ethanol showed little variation with increasing temperature (Figure 6.12). The b* value for equine milk with added ethanol increased for all temperatures at an ethanol concentration > 40% and at all temperatures was higher as the concentration of ethanol increased (Figure 6.13).

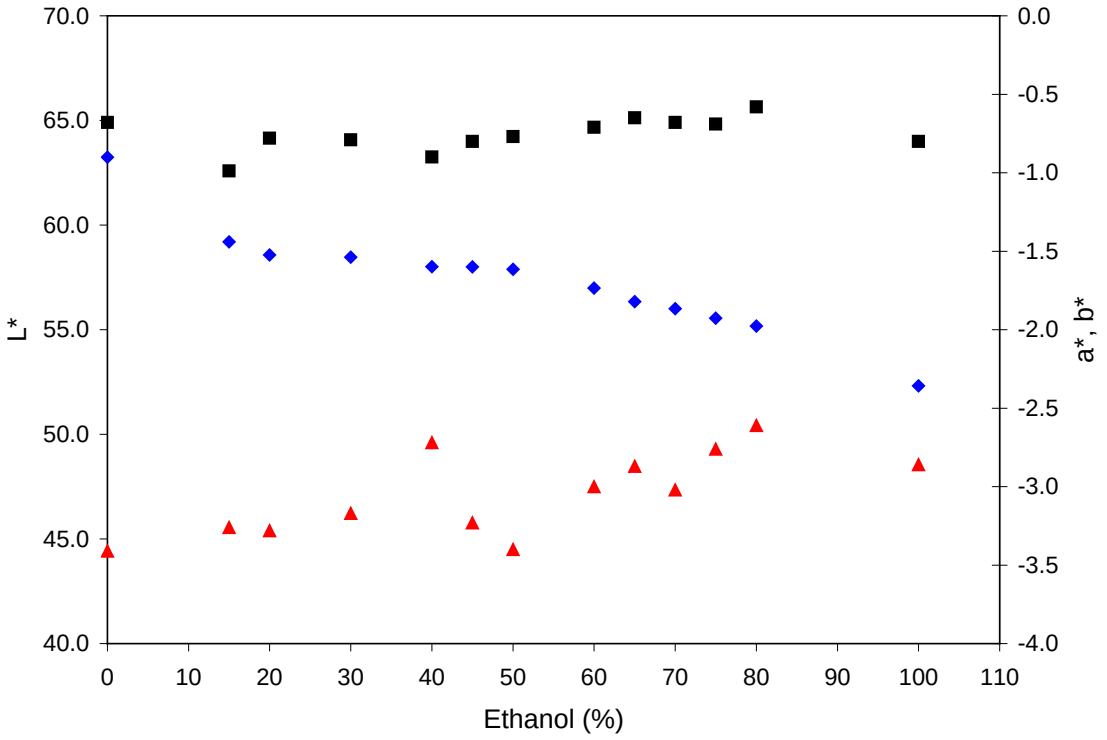

Figure 6.10. Effect of ethanol concentration on the L* (♦), a* (■) and b* (▲) values of a 1:1 mixture of skimmed equine milk and ethanol at 20°C. Analysis was performed in triplicate and the coefficient of variation was <5% of the reported value for each data point. For clarity, the results presented are from one sample.

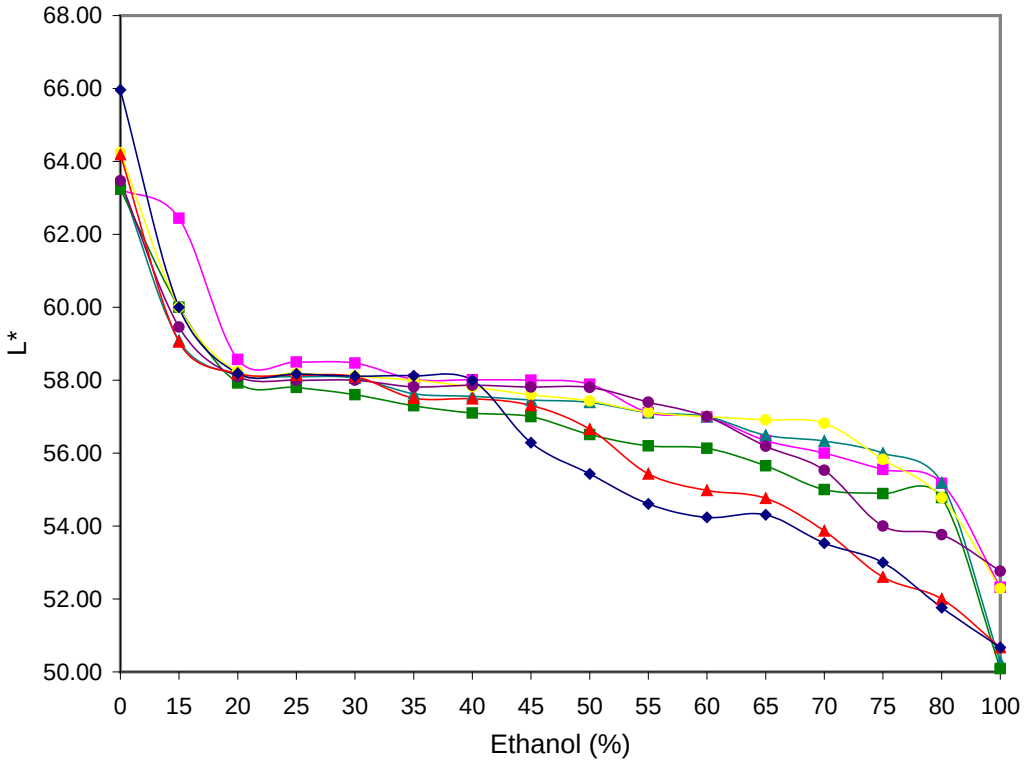

Figure 6.11. Effect of ethanol on the L* value of a 1:1 (v/v) mixture of equine milk and ethanol at 20 (-●-), 30, (-▲-), 40 (-■-), 50 (-●-), 60 (-■-), 70 (-▲-) or 80 (-♦-) ^{o}C. Analysis was performed in triplicate and the coefficient of variation was <5% of the reported value for each data point. The results presented are from one sample for clarity.

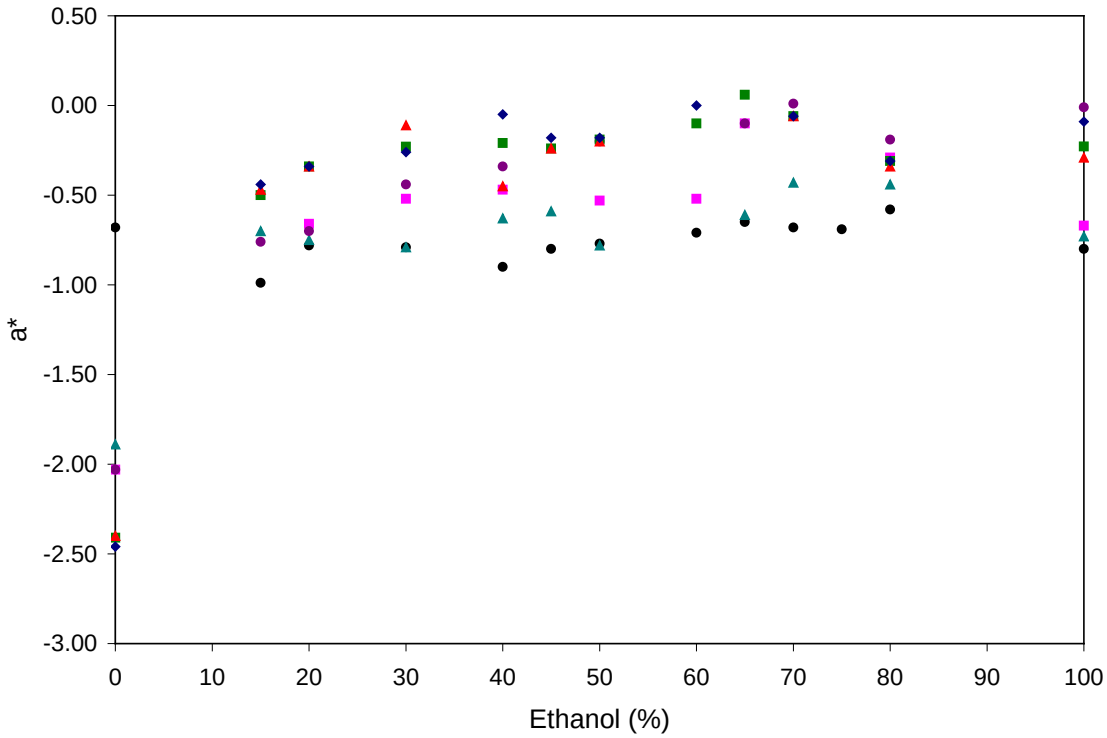

Figure 6.12. Effect of ethanol on the a* value of a 1:1 (v/v) mixture of equine milk and ethanol at 20 (-●-), 30, (-▲-), 40 (-■-), 50 (-●-), 60 (-■-), 70 (-▲-) or 80 (-♦-) ^{o}C. Analysis was performed in triplicate and the coefficient of variation was <5% of the reported value for each data point. The results presented are from one sample for clarity. *Note*: an increase in a* value is a tendency towards positive values which mean the sample is becoming less 'green' and more 'red'.

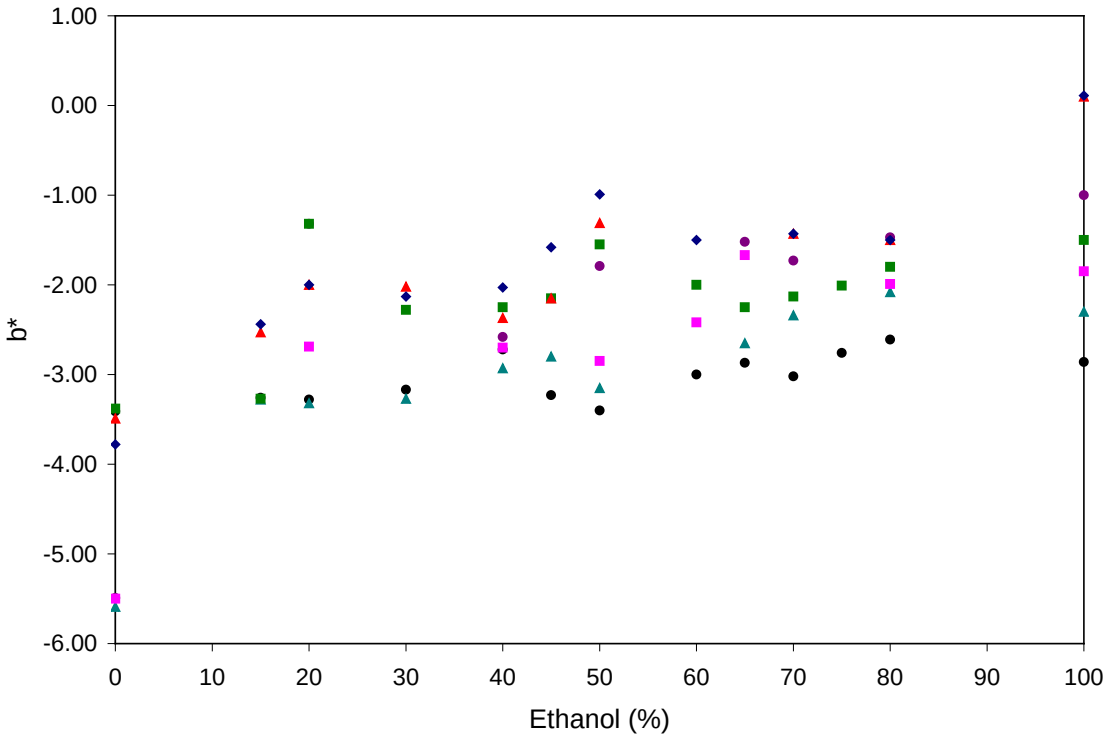

Figure 6.13. Effect of ethanol on the b* value of a 1:1 (v/v) mixture of equine milk and ethanol at 20 (-●-), 30, (-▲-), 40 (-■-), 50 (-●-), 60 (-■-), 70 (-▲-) or 80 (-♦-) ^{o}C. Analysis was performed in triplicate and the coefficient of variation was <5% of the reported value for each data point. The results presented are from one sample for clarity. *Note*: an increase in b* value is a tendency towards positive values which means the sample is becoming less 'blue' and more 'yellow'.

Addition of a 1:1 (v/v) 70% ethanol solution to equine milk and heating to 80°C caused irreversible precipitation of equine casein micelles when examined visually. In contrast, heating a 1:1 mixtures of bovine milk with 70% ethanol to 80°C caused a significant decrease in L* value which, on subsequent cooling, reversed within 20 min in agreement with observations by Zadow *et al*. (1993), O'Connell *et al*. (2001a, 2001b) and Huppertz *et al*. (2004b).

Cooling mixtures of equine milk and ethanol to 20°C after heating to temperatures in the range of 30-80°C showed that once the equine milk sample had precipitated, the effect was irreversible on cooling. As the temperature increased, the sample precipitated at lower concentrations of ethanol. Precipitation was extensive at high ethanol and high temperatures (> 65°C and 60% ethanol) and aggregates of casein sedimented quickly. Samples of equine and bovine milk mixed with 100% ethanol (v/v) at 20°C precipitated immediately. When the mixtures were heated to 70°C, the bovine sample dissolved and the solution became transparent, indicating extensive micellar dissociation, whereas the equine milk sample remained precipitated. Repeating the experiment and cooling both samples rapidly on ice resulted in the formation of an opaque stiff gel in the case of bovine milk but no gelation in the equine milk sample, where the casein remained precipitated. The effect of 100% ethanol on heating and cooling bovine milk is in agreement the results of O'Connell *et al*. (2001a). Addition of ethanol $\geq$ 35% (v/v) to equine milk resulted in irreversible precipitation of protein on heating.

The decrease in dielectric constant of the medium surrounding bovine casein micelles on addition of ethanol has been reported to reduce the energy barrier preventing coagulation (Horne & Parker, 1981b; Geerts *et al*., 1983; Zadow, 1993; O'Connell *et al*., 2001a) which, coupled with the high ionic Ca^{2+} concentration (Section 3.5.4) of equine milk, is sufficient to cause irreversible precipitation of equine casein micelles at relatively low temperature and ethanol concentrations. A low casein concentration has also been reported to be a factor characterizing bovine milk samples unstable to ethanol addition (Chavez *et al*., 2004); however, increasing the concentration of equine casein to 25 g L^{-1} followed by addition of 100% ethanol (v/v) with heating to 70°C, caused similar precipitation of equine casein micelles to samples at the natural casein concentration of equine milk.

Alcohol induces α-helical features in bovine α_{s}- and κ-caseins (Herskovits & Mescanti, 1965) as a result of which the tendency for the association of casein molecules into aggregates is eliminated (Horne & Davidson, 1987). More recent work has shown that the ethanol-

dependent temperature-induced effect is due to increasing casein solubility leading to dissociation of casein micelles essentially brought about by conformational transformations, i.e., an increase in α-helical structural features, but a temperature of ~70^{o}C are required for this effect (O'Connell *et al*., 2001b).

A reduction of pH or addition of NaCl markedly increases the dissociation temperature when ethanol is added to bovine milk, indicating that electrostatic interactions play a major role in the ethanol-dependent temperature-induced dissociation of casein micelles (O'Connell *et al*., 2001a). The phenomenon may also be explained in terms of the inhibition of hydrophobic bonding on addition of ethanol which leads to dissociation of casein micelles (Zadow, 1993) and may be of greater importance in the case of equine casein micelles than bovine micelles.

Another possible explanation for the irreversibility of the effect of ethanol with heating on equine casein micelles is that on heating, in the presence of ethanol, the micelles are dissociated into nanoclusters and probably some individual casein molecules. At room temperature, the addition of ethanol to milk would be expected to reduce overall solvent quality as the hydrophilic parts of caseins become less soluble; in addition, hydrophobic interactions may weaken because the solvency of the hydrophobic residues is improved in an ethanolic environment. Heating milk strengthens hydrophobic interactions up to ~ 80^{o}C in aqueous media, although increasing the temperature further diminishes hydrophobic interactions. Heating milk in the presence of ethanol shifts this transition temperature to a lower value and, combined with the fact that the ionic calcium concentration in the serum phase decreases as a result of both heating and ethanol addition, the serum may become a better solvent and thus result in dissociation of casein micelles. Furthermore, κ-casein may dissociate from the micelle surface as a result of increased solvent quality. On cooling, constituents of the original micelles undergo a reassembly, although the κ-casein of equine milk cannot reach the micelle surface quickly enough and larger particles are formed. For equine micelles, κ-casein appears to be far less important in micellar stability than in bovine milk and micellar constituents do not reassociate and undergo any form of reversal following addition of ethanol > 50% and temperatures > 55^{o}C.

6.5. Conclusions

The dissociation of β-casein and α_s-caseins from equine milk and bovine milks at 4 or 20^{o}C was different and, under the experimental conditions of this study, relatively little β-casein dissociated from equine casein micelles at either temperature, whereas considerable dissociation of bovine β-casein occurred, especially at 4^{o}C. Fractionation of equine casein micelles at pH 4.2 or 4.6 at 4 or 20^{o}C produced TS- and CP-casein fractions both of which stained positively for glycoproteins. Differential centrifugation followed by SDS-PAGE enabled the detection of glycoproteins, especially in the micelles fractionated at the higher centrifugal forces. It was concluded that equine milk contains κ-casein. The colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles, which may have significant implications for the conversion of equine milk into dairy products. Equine casein micelles were less stable to ethanol than bovine micelles and high ethanol concentrations and high temperature caused irreversible changes to equine casein micelles. The low casein content of equine milk and differences in micellar casein composition and stabilization compared to bovine micelles could account for the differences in ethanol stability and in the ethanol-induced temperature-dependent dissociation of the micelles of both milks. The high Ca^{2+} concentration of equine milk may also be a factor contributing to the difference in the stability of both milks as it is generally accepted that Ca^{2+} play an important role in many of the physico-chemical and functional properties of milk, especially stability to ethanol and heat (Demott, 1968; Geerts *et al*., 1983; Augustin, 2000; Jeurnink & de Kruif, 1995; Faka *et al.*, 2009).

6.6. References

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CHAPTER 7

Effect of Heat and High Pressure on the Whey Proteins and Casein Micelles in Equine Milk

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Abstract

The susceptibility of equine whey proteins to high pressure and heat treatment and the effects of high pressure treatment on equine micelle size were examined. A very significant increase in equine casein micelle size was evident at 400 MPa but size decreased at higher pressures. Equine α-lactalbumin (α-la) and β-lactoglobulin (β-lg) were less susceptible to high pressure-induced denaturation than their bovine counterparts. Equine α-la and β-lg showed less denaturation under high pressure than their bovine counterparts in the presence of 25 g L^{-1} equine or bovine micellar casein, respectively, or when the micellar casein phases were reversed. The resistance of equine β-lg to denaturation at high pressure is attributed to its lack of a free thiol group and its consequential inability to participate in sulphydryl-disulphide interchange reactions. Equine α-la had a similar denaturation pattern to its bovine counterpart when heated at 60 to 95^{o}C whereas equine β-lg was significantly more stable to heat than equine α-la or bovine β-lg.

Keywords: Equine milk; casein micelles; high pressure; alpha-lactalbumin; beta-lactoglobulin; heat treatment;

7.*1. Introduction*

High pressure (HP) processing of milk has been of interest for over a decade as a potential treatment for both the preservation and alteration of the properties of milk; its opportunities and challenges have been reviewed by Rastogi *et al.* (2007) and its effects on the constituents and properties of bovine milk and milk-based products have been reviewed by Balny & Masson (1993), Johnston (1995), Mozhaev *et al*. (1996), López-Fandiño *et al*. (1996, 1997), López-Fandiño & Olano (1998a,b), Balci & Wilbey (1999), Datta & Deeth (1999), Kruk *et al*. (2000), Needs *et al*., (2000a), Huppertz *et al*. (2002, 2004d, 2006a) and Trujillo *et al*. (2002). HP changes some of the functional properties of milk, destroys spoilage microorganisms (Patterson, 2005) and inactivates certain enzymes, significantly extending the shelf-life of milk (Indrawati *et al*., 2001; Anema *et al*., 2005) without impairing its nutritional quality (López-Fandiño, 2006). HP denatures whey proteins in milk, especially β-lactoglobulin (β-lg) (López-Fandiño *et al*., 1996; López-Fandiño & Olano, 1998a; Scollard *et al*., 2000; López-Fandiño, 2006; Considine *et al*., 2007; Anema, 2008) to an extent that depends on the protein structure, pressure, pH, ionic strength, solvent composition and temperature (Masson, 1992; Gross & Jaenicke, 1994; Hendrickx *et al*., 1998).

α-Lactalbumin (α-la) is significantly more baroresistant than β-lg (López-Fandiño *et al*., 1996; Felipe *et al*., 1997, Gaucheron *et al*., 1997; López-Fandiño & Olano, 1998a; García-Risco *et al*., 2000; Scollard *et al*., 2000; Huppertz *et al*., 2004a) and is resistant to denaturation at pressures up to 500 MPa (Huppertz *et al*., 2002; Anema, 2008). In bovine milk, the higher barostability of α-la has been related to its more rigid molecular structure, with four intra-molecular disulphide bonds compared to two in β-lg (Zeece *et al*., 2008). Bovine β-lg contains a free sulphydryl group at Cys_{119} or Cys_{121}, which can participate in sulphydryl oxidation or sulphydryl-disulphide interchange reactions (Hinrichs *et al*., 1996; López-Fandiño *et al*., 1996). HP promotes folding and aggregation of β-lg through sulphydryl-disulphide interchange reactions (Dumay *et al*., 1994; Funtenberger *et al*., 1997; van Camp *et al*., 1997; Belloque *et al*., 2000; Patel *et al*., 2005) but the effects are partially reversible on storage (Dumay *et al*., 1994; van Camp *et al*., 1997). α-Lactalbumin does not have a sulphydryl group and can undergo sulphydryl-disulphide interchange only with a protein that has a free sulphydryl group (Huppertz *et al*., 2004b).

The integrity of casein micelles is maintained by micro clusters of calcium phosphate and hydrophobic interactions (Horne, 2002; de Kruif & Holt, 2003), both of which are affected by HP treatment (Gaucheron *et al*., 1997; Law *et al*., 1998; Needs *et al*., 2000b; Huppertz *et al*., 2004a, 2004b, 2004c; 2004d; Regnault *et al*., 2004; Anema, 2005). HP causes solubilisation of colloidal calcium phosphate (CCP) (Schrader *et al*., 1997) and disrupts and reforms hydrogen bonds and hydrophobic interactions (Johnson *et al*., 1992; Gaucheron *et al*., 1997; Huppertz *et al*., 2004d; Orlien *et al*. 2006; Considine *et al*., 2007) with a concomitant redistribution of minerals and proteins between the soluble and colloidal phases of milk (Lee *et al*, 1996; López-Fandiño *et al*., 1998; Huppertz *et al*., 2002; Needs, 2002). It has been reported that HP has no effect on the mineral balance in HP-treated raw skim milk or phosphocaseinate dispersions when serum separation is carried out by ultrafiltration rather than ultracentrifugation suggesting that CCP is bound to caseins during its solubilisation or binds to soluble proteins in the soluble phase of raw skim milk or phosphocaseinate dispersions (Regnault *et al*., 2006). Light scattering by milk is reduced by HP due to the disruption of casein micelles, resulting in changes to their size and number (Johnston *et al*., 1992; Needs *et al*., 2000b; Anema *et al*., 2005; Huppertz *et al*., 2004c; Regnault *et al*., 2004; López-Fandiño, 2006; Huppertz *et al*., 2006b). Some dissociation of casein micelles by HP, especially of κ- and β-caseins, has been reported for bovine milk (López-Fandiño *et al*., 1998) although the increased transfer of individual caseins from the colloidal to the soluble phase of milk varies among species; in bovine milk, dissociation was in the order $\beta > \kappa > \alpha_{s1} > \alpha_{s2}$, whereas in caprine and ovine milk, the order was $\kappa > \beta > \alpha_{s1} > \alpha_{s2}$ (López-Fandiño *et al*., 1998).

Heat treatment of milk and dairy products is widely used to improve their microbiological quality, to extend shelf-life or improve some technological aspects related to functionality or quality (Jelen & Rattray, 1995). Mild heat treatment (65^{o}C x 30 s or 72^{o}C x 15 s) has little effect on milk proteins (Walstra & Jenness, 1984) but heating milk > 70^{o}C denatures whey proteins, especially β-lg, forming thiol-exposed monomers which react with each other or complex with casein micelles, primarily with κ-casein (Singh, 1995; Jelen & Rattray, 1995; Cho *et al*., 2003); this is exploited to improve the rheological properties of yoghurt (Lucey *et al*., 1998). Acid-induced gels formed from preheated milk develop a firm texture through disulphide bridging which leads to increased cross-linking through the gel network (Lucey, 2004), preventing syneresis (Phadungath, 2005). The sensitivity of the principal whey proteins to denaturation is different for HP and heat treatment (Felipe *et al*., 1997; Law *et al*.,

1998). Heat treatment causes a progressive loss in the solubility of all whey proteins at pH 4.6, whereas HP denatures β-lg significantly, but not the other whey proteins.

Casein micelles undergo little change at temperatures < 110^{o}C and denatured β-lg binds to the surface of the intact micelle (Law *et al*., 1994). HP has a similar effect on whey proteins as heat treatment although β-lg is denatured in the presence of disrupted micelles as a result of HP (Johnston *et al.*, 1992). α-La is very heat sensitive and easily denatured at 65^{o}C, pH 6.7, but its denaturation, even at temperatures up to 110^{o}C, is 80-90% reversible (Jelen & Rattray, 1995). α-La can not initiate heat-induced polymerization due to the absence of a thiol group but it is irreversibly denatured in the presence of β-lg due to thiol-disulfide bond exchange reactions. β-Lg also interacts with the casein micelles through thiol-disulfide interchange reactions with κ- and α_{s2}-casein.

The aim of this study was to investigate the effect of HP and heat treatment on the whey proteins of equine milk. Equine β-lg contains four cysteine residues but lacks a sulphydryl group which has major implications for denaturation and aggregation of the protein. The results should provide valuable information on the stability of equine milk to heat and HP and allow assessment of the benefits of either treatment for processing equine milk to improve its shelf-life, nutritional quality and to extend the variety of products that may be produced from equine milk, while maintaining its unique properties.

7.2: Materials and Methods

7.2.1. Milk supply

Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from 5 milkings over 24 h, from a herd of multiparous New Forest and New Forest/Arabian mares in mid-lactation, physically separated by day from their foals. Raw whole bovine milk was obtained from a local dairy farm. Equine and bovine milk were defatted at 1,000 and 2,000 x g, respectively, at 20^{o}C for 20 min followed by filtration through glass wool to remove fat particles. Sodium azide (0.5 g L^{-1}) was added to the skimmed milk to prevent microbial growth.

7.2.2. Compositional analysis

The protein and fat content of the equine and bovine milk samples were determined by the Kjeldahl (IDF, 2001) and Gerber (IDF, 1981) methods, respectively. pH was measured using a Radiometer pHM 210 pH meter fitted with a Radiometer Meterlab® combined general purpose electrode (pHC 2001) and a built-in temperature sensor (Radiometer Analytical SAS, Lyon, France).

7.2.3. Preparation of equine and bovine casein micelles with reversed sera

Suspensions of equine and bovine casein micelles were prepared by sedimenting the micelles at 100,000 x g for 90 min at 20^{o}C, as described in Section 3.2.2. Following ultracentrifugation, equine and bovine casein pellets were resuspended to their original volumes in their own ultracentrifugal supernatant or the supernatants were exchanged. In some instances, protein levels were adjusted to 25 g L^{-1} by the addition of sufficient bovine or equine supernatant and the pH was adjusted to 6.6.

7.2.4. High-pressure treatment

High pressure treatment of equine milk and suspensions of equine and bovine micelles in original and exchanged sera was carried out as described by Huppertz *et al*. (2004a), with some modifications, using a Stansted Fluid Power Iso-Lab 900 High Pressure Food Processor (2 L vessel capacity, internal diameter 100 mm, Stansted Fluid Power, Stansted, Essex, UK). Standard micro test tubes (Eppendorf 3810; Eppendorf UK Limited, Histon, Cambridge, UK) were filled with sample so that < 5% air remained in the tube. The tubes were wrapped in

parafilm and vacuum-sealed in a small vacuum bag containing ~ 150 ml water. Pressures of 50, 100, 250, 400, 600 or 800 MPa were applied and maintained for 30 min. Triplicates of each system were subjected to high pressure except for micellar suspensions which were treated in duplicate. To standardize the reversibility of β-lg denaturation, samples were fractionated exactly 10 min after high pressure treatment. Samples (1 mL) were placed in micro test tubes to which 30 μL of 33% acetic acid were added, followed by 30 μL 1M sodium acetate to a final mixture pH of ~ 4.6 (IDF, 2004) and the level of residual native α-la and β-lg determined as described below (7.3.2).

7.2.5. Heat-induced denaturation of equine and bovine whey proteins

The heat-induced denaturation of equine and bovine whey proteins was determined at temperatures from 60 to 95^{o}C at 5^{o}C intervals. Five mL of each sample were heated to the required temperature in capped test tubes and held for 10 min before cooling in ice-water to room temperature. Immediately after cooling, caseins and whey proteins in 1 mL of sample were fractionated at pH 4.6 and the levels of residual α-la and β-lg determined as described below (7.3.2).

7.2.6. Determination of the levels of residual α-la and β-lg

Following acidification to pH 4.6, heat- or high pressure-treated samples were vortexed for 1 min and held for 10 min. The sample was centrifuged in a micro-centrifuge (Sigma 1-15, Sigma Laborzentrifuge, Osterode am Harz, Germany) at 27,000 x g. The supernatant was removed carefully using a micro-pipette and diluted 1:10 with solvent A [0.1%, v/v, trifluoroacetic acid (TFA) in Milli-Q® water (Millipore Corp., Billerica, MA., USA)] and filtered through a Millex® syringe filter (0.45 μm; Millipore Corp.). A control milk sample was fractionated by the same method (7.3.2).

7.3. Analytical methods

7.3.1. Effect of high-pressure treatment on the size of equine casein micelles

The average size of equine casein micelles (Z-average) was determined by dynamic light scattering using a Malvern Zetamaster (Malvern Instruments Ltd., Malvern, Worcestershire, UK) as described in Section 3.3.4. Immediately following each pressure treatment, samples were diluted with lactose-free synthetic milk ultrafiltrate (SMUF; Jenness & Koops, 1962) at

levels that ensured that the Z-average readings were accurate and within the range of the instrument.

7.3.2. Determination of residual native α-lactalbumin and β-lactoglobulin in heat- or high pressure-treated samples

The denaturation of equine α-la and β-lg by heat or high pressure treatment was determined by measuring the levels of residual native α-la and β-lg in the pH 4.6-soluble fraction by reverse-phase high performance liquid chromatography (RP-HPLC), as described by Huppertz *et al*. (2004a), but with several modifications. RP-HPLC was performed using a Waters 626 non-metallic HPLC system (Waters Corp., Milford, MA, USA), consisting of a Waters 486 UV-Vis detector, a Waters 717^{+} autosampler, a Waters 626 pump with a 600S controller and an on-line Degasys DG-2410 degassing unit (Sanwa Tsusho Co., Tokyo, Japan). The HPLC system was controlled by Millenium^{32} Version 3.05.01 chromatography management software (Waters Corp.). A C_8 Nucleosil™ RP-HPLC column [250 x 4.6 mm, 5 μm particles size, 300 Å pore size (Macherey-Nagel GmbH & Co. KG, Düren, Germany)] with a guard column (10 x 4.6 mm) was used for protein separation and 100 μL of sample were applied to the column. Elution was at a flow rate of 0.75 mL per min, initially with 100% Solvent A for 5 min, followed by a linear gradient to 45% solvent B [0.1% TFA, v/v, in Far-UV HPLC-grade acetonitrile (Labscan Ltd., Gliwice, Poland)] over 5 min, increasing to 60% B over 15 min and holding at 60% B for 10 min with a further increase to 95% B over 5 min, holding for 3 min and returning to 100% solvent A over 2 min and holding for 15 min to ensure that the column was adequately re-equilibrated before injection of the next sample. The eluate was monitored at 214 nm. Peaks corresponding to the major bovine whey proteins were verified by analysing pure bovine α-la and β-lg (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Levels of residual native α-la and β-lg. were calculated from the decrease in total area of the respective peaks relative to untreated milk as indicated by the loss of solubility at pH 4.6 which occurs on denaturation (Jelen & Rattray, 1995).

7.3.3. Statistical analysis of data

Data were statistically analysed using Minitab Statistical Software (Release 13.31; Minitab Inc., State College, PA, USA). Prior to analysis, all data were tested for normality using the Anderson-Darling test. Having established that data were normally distributed, the influence of high pressure on casein micelle size and whey protein denaturation in equine milk, as well

as the influence of high pressure on equine and bovine whey proteins in their own or reversed sera and the influence of heat treatment on equine whey proteins, was examined using one-way analysis of variance (ANOVA) at a significance level of 0.05. Where statistically significant differences between sample means were found, i.e., there were differences between treatment means ($p < 0.05$), ANOVA was followed with Tukey's post-hoc pairwise multiple comparison test to determine which sample means were statistically different from others.

The results analysed statistically were from 3 independent experiments. Analysis of the effect of high pressure on bovine and equine whey proteins in the presence of their own or exchanged casein micelles was carried out in duplicate and the result presented graphically was reproducible and representative.

7.4.Results and Discussion

7.4.1. Compositional analysis

The protein content of equine and bovine milk was 21.78 ± 1.58 and 34.60 ± 1.79 g L^{-1}, respectively. The protein content of equine milk was within the range of an average value for five mares reported in Table 3.1 and close to values reported by Ullrey *et al.* (1966), Mariani *et al.* (2001), Smolders *et al.* (1990), Solaroli *et al.* (1993), Zicker & Lönnerdal (1994) and Csapó-Kiss *et al.* (1995). The protein content of bovine milk was within the range reported by Jenness (1974). The level of fat in equine and bovine milk was 13.00 ± 0.20 and 35.80 ± 0.23 g L^{1}, respectively, and were within the range reported in Table 3.1. The pH of bulk equine milk was 7.32, while that of bovine milk was 6.63.

7.4.2. Effect of high pressure on the size of equine casein micelles

The effect of high pressure on the average size of equine casein micelles together with the comparative result from one-way ANOVA are shown in Table 7.1. At 50-100 MPa, the size of equine casein micelles was similar to those in an untreated sample and increased significantly at 250 MPa which has been reported for bovine milk also (Needs *et al*., 2000b; Huppertz *et al*., 2004a). At 400 MPa equine micelles formed relatively large aggregates; this effect has not been reported for bovine milk for which micelle size was found to decrease at 300-800 MPa (Gaucheron *et al*., 1997; Needs *et al*., 2000b; Huppertz *et al*., 2004a). In this study, the size of equine casein micelle decreased at 600 MPa and decreased further at 800

MPa. Extensive disruption of caprine casein micelles has also been reported at 500 MPa (Law, *et al*., 1998) but buffalo casein micelles were not disrupted at 400 or 500 MPa (Huppertz *et al*., 2005).

Pressures of 250 to 300 MPa have been reported to cause broadening of the size distribution of casein micelles in bovine milk (Anema *et al*., 2005; Huppertz *et al*., 2004c; Regnault *et al*., 2004; Orlien *et al*., 2006) and a similar effect was evident in this study for equine micelles at 400 and 600 MPa (Figure 7.1). Multiple comparison testing of means using Tukey's post-hoc test is highly conservative and not tolerant of wide standard deviations such as those found for the treatment at 400 MPa; however, analysis of the data by other multiple comparison tests can present other statistical problems and for this reason Tukey's test was deemed the most suitable post-hoc test.

Table 7.1: Effect of treatment at 50-800 MPa for 30 min at 20^{o}C on the size of casein micelles in equine milk.

Pressure (MPa)	Casein micelle size (nm)
0	$248.90 \pm 7.2^{a,}$
50	244.10 ± 5.5^{a}
100	$237.33 \pm 4.27^{a,c}$
250	306.40 ± 5.8^{b}
400	648.80 ± 49.57^{d}
600	366.20 ± 9.2^{e}
800	290.92 ± 10.09^{b}

Values are means of data from experiments on 3 separate milk samples, ± standard deviation

^{a,b,c,d,e} Values in the casein micelle size column with a common superscript are not statistically different ($p > 0.05$).

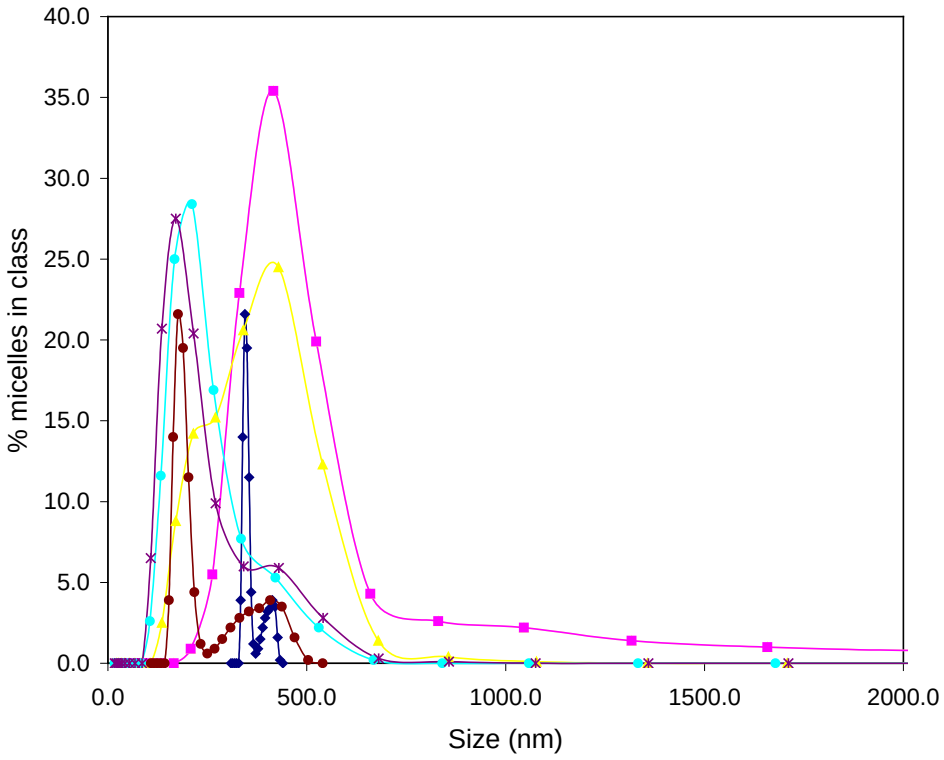

Figure 7.1. Effect of high pressure on the size distribution of equine casein micelles measured by laser light scattering. Control (-●-), 100 MPa (-●-), 250 MPa (-▲-), 400 MPa (-■-), 600 MPa (-⋆-) and 800 MPa (-♦-).

7.4.3. High pressure- induced denaturation of equine whey proteins

Results from ANOVA on the percentage residual native α-la and β-lg in equine milk following HP treatment are summarized in Table 7.2. The percentage of residual native α-la in equine milk following HP treatment was statistically different over the range of pressures applied. Significant differences in the level of residual α-la were found between the control and samples treated at 100 or 800 MPa. The high stability of equine α-la to pressure up to 600 MPa was due to the presence of 4 intra-molecular disulphide bridges. Denaturation of bovine β-lg and α-la occurs at pressures > 100 MPa or > 400 MPa, respectively (López-Fandiño *et al*., 1996; Gaucheron *et al*., 1997; Huppertz *et al*., 2004a).

While not confirmed in any study, based on the very high similarity between equine, bovine and human α-la, as well as the α-la from other species, it is highly likely that equine α-la contains 4 intra-molecular disulphide bonds. Some statistical differences were found for the level of residual native β-lg over the range of HP treatments (Table 7.2) but the differences were small in comparison to those reported for bovine milk (Huppertz *et al*., 2004a,b) and equine β-lg was quite stable to pressures in the range 100-800 MPa. Equine β-lg lacks a sulphydryl group and cannot undergo sulphydryl-disulphide interchange reactions, even at high pressures.

Table 7.2: Effect of treatment at 100-800 MPa for 30 min at 20°C on the level of residual native α-lactalbumin (α-la) and β-lactoglobulin (β-lg) in equine milk

Pressure (MPa)	% residual native α-la	% residual native β-lg
0	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
50	$93.50 \pm 4.20^{a,c}$	$94.22 \pm 3.20^{a,c}$
100	$89.80 \pm 3.10^{b,c,d}$	$86.63 \pm 3.60^{b,c,d}$
250	$93.30 \pm 2.40^{a,d}$	$87.76 \pm 4.20^{a,d}$
400	$97.20 \pm 1.40^{a,e}$	$95.08 \pm 2.70^{a,d}$
600	$93.20 \pm 3.01^{a,d}$	$89.61 \pm 8.72^{a,d}$
800	$90.20 \pm 1.20^{b,c,d,e}$	$85.17 \pm 5.80^{b,c,d,}$

Values are expressed as percentages of the total α-La or β-Lg in untreated milk and are the means of data from experiments on 3 separate milk samples, ± standard deviation.
[a,b,c,d,e] Values in columns with a common superscript are not statistically different ($p > 0.05$).

7.4.4. Effect of high pressure treatment of equine and bovine whey proteins in micellar suspensions with exchanged sera

In agreement with previous studies (Nakamura *et al*., 1993; Huppertz *et al*., 2004a,b; López-Fandiño *et al*., 1996; López-Fandiño & Olano, 1998; Scollard *et al*., 2000; López-Fandiño, 2006), bovine β-lg was significantly more susceptible to the effects of HP than bovine α-la (Figure 7.2 A and B). The effect of HP on β-lg is believed to occur in three stages: at pressures of up to 150 MPa, minor alterations of the protein occur, whereas at 200-450 MPa disulphide-linked dimers and some aggregates are formed (Zeece *et al*., 2008) but $\geq$ 500 MPa complete unfolding of β-lg occurs (Considine *et al*., 2005) with the formation of soluble disulphide-linked intermolecular aggregates (Funtenberger *et al*., 1997). Bovine β-lg was completely denatured at $\geq$ 600 MPa in bovine serum in the presence of bovine casein micelles (Figure 7.2, A); however, equine β-lg in equine serum was resistant to HP in the presence of bovine casein micelles, in agreement with the results in Table 7.2. The denaturation of bovine whey proteins in the presence of equine casein micelles followed a similar pattern to that of bovine whey proteins in the presence of bovine casein micelles, although the pressure of maximum denaturation was higher (800 MPa *vs.* 600 MPa). The β-lg in equine serum in the presence of equine casein micelles was resistant to HP treatment. Bovine α-la in bovine serum was resistant to HP at pressures < 500 MPa in the presence of bovine or equine casein micelles (Figure 7.2 B) but at higher pressures it denatured easily in both cases, which is in agreement with previous studies (López-Fandiño *et al*., 1996; Felipe *et al*., 1997, López-Fandiño & Olano, 1998; García-Risco *et al*., 2000; Scollard *et al*., 2000; Huppertz *et al*., 2004a). Equine α-la appeared to be more resistant to HP than bovine α-la in agreement with results in Table 7.2.

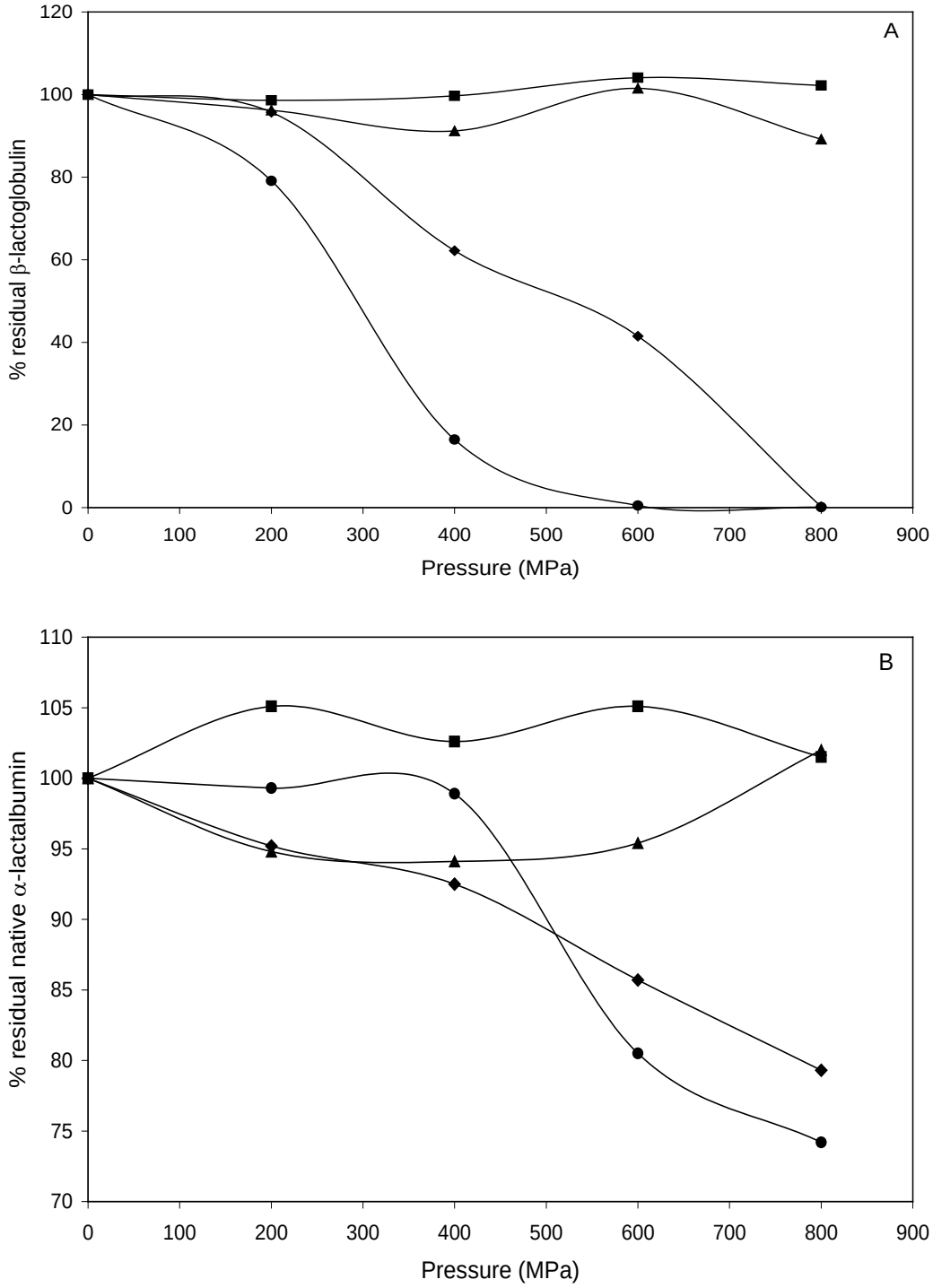

Figure 7.2: Effect of high pressure treatment at 100-800 MPa for 30 min at 20°C on the level of residual native β-lactoglobulin (A) and α-lactalbumin (B) when equine casein micelles were resuspended in equine serum (-■-) or bovine serum (-♦-) and when bovine casein micelles were suspended in bovine (-●-) or equine (-▲-) serum.

7.4.5. Heat-induced denaturation of equine and bovine whey proteins

The effect of heat treatment on equine and bovine α-la and β-lg is shown in Table 7.3. The effects of heat treatment from 60 to 95^{o}C were statistically different for the α-la and β-lg in both equine and bovine milk. . The α-la in equine and bovine milk showed similar thermal sensitivities at temperatures of 60 to 95^{o}C for 10 min, although equine α-la denatured significantly more than α-la in bovine milk at 75^{o}C, the reason for which is unclear. Equine α-la and β-lg have been reported to be more heat stable than their bovine counterparts (Bonomi *et al*., 1994); however, while that was the case for equine β-lg in this study, it was not for equine α-la. It has been reported (Civardi *et al*., 2007) that equine β-lg is more thermo-stable than equine α-la. For bovine milk, the definition of thermal stability based on the assay method may give contradictory results; the α-la of bovine milk is the first protein to unfold at high temperature but the last to aggregate; therefore if thermal denaturation is assessed by differential scanning calorimetry, bovine α-la appears less stable to heat than bovine β-lg (Rüegg *et al*., 1977; De Wit & Klarenbeek, 1984). However, if the thermal denaturation of bovine whey proteins is assessed by HPLC or SDS-PAGE of the pH 4.5 insoluble whey proteins the reverse is found (Donovan & Mulvihill, 1987). α-Lactalbumin is the only whey protein capable of renaturation following heating and renaturation levels of ~ 90% have been observed in model systems (Rüegg *et al*., 1977; De Wit & Klarenbeek, 1984) and this phenomenon has been used to explain the lower levels of denaturation of α-la compared to β-lg as measured by solubility at pH 4.5 (Mulvihill & Donovan, 1987). The thermostability of bovine α-la is thus interpreted in terms of a high degree of renaturation rather than a high temperature for denaturation (Donovan & Mulvihill, 1987). The higher thermo-stability of equine β-lg is probably related to its lack of a sulphydryl group. Thermal denaturation of bovine β-lg is a two-stage process, unfolding of the polypeptide and exposure of the sulphydryl group, followed by self-association or association with other proteins *via* sulphydryl-disulphide interchange (Sawyer, 2003). Heating $\geq$ 40^{o}C causes small reversible conformational changes to bovine β-lg, whereas heating at 50-85^{o}C causes extensive irreversible denaturation with exposure of a reactive thiol group due to conformational changes to the molecule (Jelen & Rattray, 1995). This reactive thiol group can form disulfide bonds with other proteins having a reactive thiol group or through thiol-disulfide interchange

reactions. The reaction makes the denaturation process irreversible, in contrast to the reversible denaturation of porcine β-lg which lacks a free thiol group (Burova *et al*., 2002; Ugolini *et al.,* 2001), a pattern also shown in this study for equine β-lg. The lack of a sulphydryl group in equine and porcine β-lg means the protein can not undergo the second denaturation step and therefore the structure may refold on cooling. Figure 7.2 shows the RP-HPLC chromatograms of the heat-induced denaturation of equine and bovine whey proteins from which the data for Table 7.3 were compiled. It has been possible to identify the bovine whey proteins with certainty by analysing standard α-la and β-lg but, due to lack of availability of equine whey protein standards, those of equine milk are tentatively called α-la and β-lg.

Table 7.3*:* Effect of heat treatment at 60-95^{o}C for 10 min on the level of residual native α-lactalbumin (α-la) and β-lactoglobulin (β-lg**)** in equine and bovine milk

	% residual native α-la		**% residual native β-lg**	
Temperature (^{o}C)	**Equine**	**Bovine**	**Equine**	**Bovine**
Control	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
60	99.91 ± 2.0^{a}	91.96 ± 0.8^{a}	99.69 ± 0.4^{a,c}	99.39 ± 0.9^{a}
65	98.23 ± 1.97^{a}	94.49 ± 1.6^{b}	101.64 ± 2.1^{a}	92.51 ± 1.2^{b}
70	92.54 ± 1.6^{b}	99.56 ± 1.1^{a}	99.38 ± 1.57^{c}	82.69 ± 2.0^{c}
75	79.29 ± 2.04^{c}	98.99 ± 2.04^{a}	93.02 ± 2.22^{b}	66.30 ± 1.8^{d}
80	60.76 ± 1.34^{d}	55.29 ± 1.87^{c}	82.69 ± 1.30^{d}	41.57 ± 3.1^{e}
85	44.91 ± 1.56^{e}	58.51 ± 1.43^{d}	74.47 ± 1.70^{e}	18.43 ± 1.7^{f}
90	37.02 ± 3.4^{d}	36.92 ± 2.7^{e}	52.78 ± 3.9^{f}	11.02 ± 1.1^{g}
95	9.63 ± 1.82^{e}	8.49 ± 1.43^{f}	29.23 ± 2.12^{g}	3.76 ± 1.0^{h}

Values are expressed as percentages of the total α-la and β-lg in untreated milk and are the means of data from three separate experiments, ± standard deviation

^{a,b,c,d,e,f,g,h} Values in columns with a common superscript are not statistically different (p >0.05).

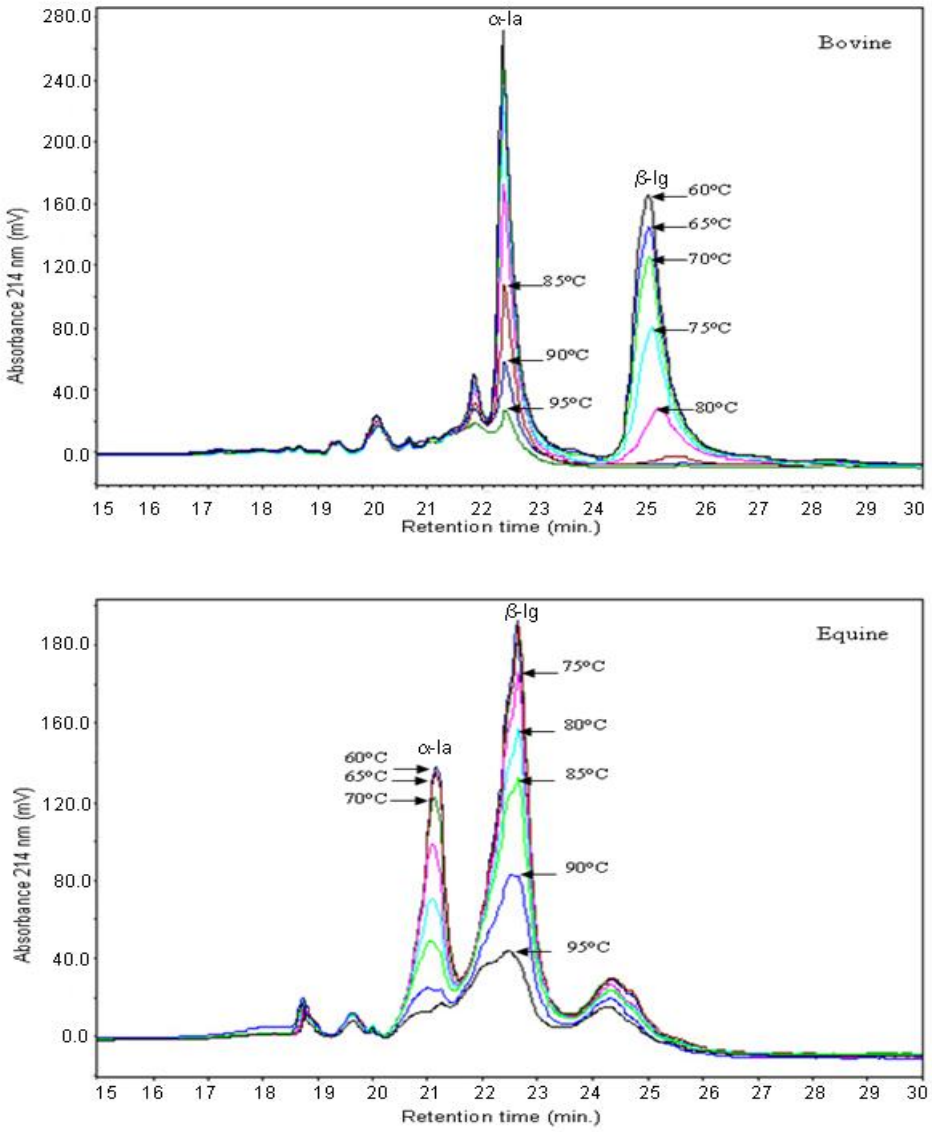

Figure 7.2: RP-HPLC chromatograms of the effect of heating at 60 to 95°C on the whey proteins in bovine and equine milk.

7.5 . Conclusions

Equine α-la had different susceptibility to denaturation by heat than to denaturation by high pressure and heating equine α-la caused considerable denaturation of the protein whereas it appeared relatively resistant to high pressures up to ~ 600 Mpa. β-Lg in equine milk was more resistant to heat and high pressure than its bovine counterparts and heat treatment of α-la in both milks caused a similar level of denaturation. The lack of a sulphydryl group in equine β-lg is responsible for the different responses of equine and bovine milk to heat or high pressure. High pressure treatment of equine milk could be used as a method of preservation while keeping the whey proteins and casein micelles relatively intact although, for infants with milk protein allergy, the presence of undenatured β-lg may be problematic. β-Lg, which is absent in human milk, is considered to be the principal allergen in bovine milk (Goldman *et al*., 1963, Ghosh *et al*., 1989). The resistance of β-lg to denaturation and proteolysis (Breiteneder & Mills, 2005) allows the protein to remain intact after digestion and it can be absorbed across the gut mucosa (Wal, 2002, 2004).

Heat treatment has been used to modify the functional properties of bovine whey proteins (e.g., gelation and emulsification) and to reduce the allergenicity of β-lg (Hill, 1994; Ehn *et al*., 2004) although, if a temperature of 90^{o}C is used, allergenicity may be increased due to exposure of internal antigenic sites in the protein (Kleber *et al*., 2007). In the last few years the sale of raw equine milk has commenced in health food shops and some pharmacies in western Europe but wider acceptance of the product will require heat treatment as a means of preservation. This study has shown that heat treatment denatures β-lg in equine milk and would therefore be a suitable method for preserving the milk while reducing its allergenicity and maintaining high levels of active lysozyme (Jauregui-Adell, 1975; Bonomi *et al*., 1994)

The effect of HP on equine milk lysozyme warrants investigation as the high lysozyme content of equine milk and its thermal stability is one of the unique characteristics of equine milk. Equine milk lysozyme is significantly more stable to heat denaturation than human lysozyme during pasteurization at 62^{o}C for 30 min but at 71^{o}C for 2 min or 82^{o}C for 15 s, the inactivation of both is similar (Jauregui-Adell, 1975). The stability of bovine lysozyme to HP has not been reported although the stability of several enzymes in bovine milk to HP have been reported, e.g., alkaline phosphatase (Rademacher & Hinrichs, 2006), lactoperoxidase (López-Fandiño *et al*., 1996), plasmin (García-Risco *et al*., 2000; Scollard *et al*., 2000), lipase (Pandey & Ramaswamy, 2004) and xanthine oxidase (Olsen *et al*., 2004).

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CHAPTER 8

Proteomic Study of Equine Milk Proteins and Comparison of Changes in Equine and Bovine Milk Proteins on Renneting

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Abstract

This study used a proteomic approach to examine the rennetability of equine milk by calf chymosin using two-dimensional electrophoresis (2-DE) followed by mass spectrometry with comparative analysis of renneted bovine milk. Mass spectrometry positively identified κ-casein in a dephosphorylated fraction collected from C4-RP-HPLC of skimmed equine milk treated with 0.1M bis-Tris buffer, pH 8.0 containing 8M urea, 1.3% trisodium citrate and 0.3% DTT. 2-DE separated and discriminated between the different individual proteins of equine milk and provided information on the range of isoforms of each protein as a result of post-translational modifications, as well as positively identifying several isoforms of κ-casein in equine milk. 2-DE gels coupled to MS demonstrated that equine milk was susceptible to hydrolysis by calf chymosin over 24 h at 30^{o}C and pH 6.5 but the peptides produced were from equine β-casein, which was also confirmed by MS analysis of C18-RP-HPLC peptides.

Keywords: Proteomics; equine milk; κ-casein; chymosin; MALDI-TOF, mass spectrometry; nano-LC MS/MS; posttranslational modifications.

8.1. Introduction

The milk from all species studied to date contains a heterogeneous mixture of proteins within which a few primary proteins dominate. In bovine milk these proteins are α_{s1}-, α_{s2}-, β- and κ-caseins, β-lactoglobulin and α-lactalbumin, with relative proportions of ~30:30:10:12:10:4, respectively (Fox & McSweeney, 1998). Proportions of these proteins vary greatly among species but, apart from whey acidic protein (WAP), no protein other than one of these families, has yet been found in the milk of any species; WAP has been identified in several species, including the mouse, rat, rabbit, camel, pig, tammar wallaby, bushtail possum, echidna and platypus (Hajjoubi *et al*., 2006) but in humans and ruminants the WAP gene has mutated into a pseudogene and the protein is not expressed (Rival-Gervier *et al*., 2003). The protein content of mature equine milk is lower than that of bovine milk (Section 3.5.1) but the principal classes of proteins, i.e., caseins and whey proteins, are similar in both milks.

The milk proteome is extremely complex due to post-translational modifications of proteins and the presence of many genetic variants (Eigel *et al*., 1984; Ng-Kwai-Hang & Grosclaude, 2003; Farrell *et al*., 2004). All milk proteins exhibit genetic polymorphism, usually due to the substitution of one or two amino acids, which do not have significant effects on protein functionality. Microheterogeneity of milk proteins also results from post-translational phosphorylation, glycosylation and proteolysis.

Phosphorylation of bovine caseins has been well characterized (Mercier *et al*., 1972; West, 1986; Mercier & Vilotte, 1993) and all caseins are phosphorylated at some level, with the level ranging from one phosphorylated residue (κ-casein) to as many as 13 (α_{s2}-casein).

κ-Casein is the only glycosylated member of the casein family and exhibits microheterogeneity due to the level of glycosylation (Saito & Itoh, 1992). About two-thirds of bovine κ-casein molecules are glycosylated at one or more of six threonyl residues while human κ-casein has 7 glycosylation sites (Fiat *et al*., 1980).

The principal proteins of equine milk have been fairly well characterized. The amino acid sequence of equine α_{s1}-casein has been derived from its cDNA sequence (Lenasi *et al*., 2003); it has 205 amino acids and a molecular mass 24,614.4 Da prior to post-translational modification, i.e., it is considerably larger than its bovine or human counterpart. Two smaller isoforms of α_{s1}-casein have been identified in equine milk, which probably result from the skipping of exons during transcription (Miranda *et al*., 2004). Equine α_{s1}-casein has 6 potential phosphorylation sites (Lenasi *et al*., 2003). Matéos *et al*. (2009a) identified 36

variants of equine α_{s1}-casein with 2 to 8 phosphate groups due to post-transcriptional modifications, i.e., skipping involving exon 7 and/or exon 14 and variants involving a cryptic splice site located at the start of exon 11, corresponding to Gln_{91} (Lenasi *et al*., 2003). Exon skipping is observed in the α_{s1}-casein of many species (Martin *et al*., 2002). Like equine β-casein, equine α_{s1}-casein presents a complex pattern on one dimensional (1-DE) and two dimensional electrophoresis (2-DE). Bovine α_{s1}-casein contains 8 or 9 phosphorylation sites (Swaisgood, 2003), which form 2 phosphorylation centres (De Kruif & Holt, 2003). The relatively low level of α_{s1}-casein in equine compared to bovine milk, may be significant and, coupled with the low protein content, could be responsible for the soft curd produced in the human infant and foal stomach. The complete amino acid sequence of equine α_{s2}-casein is unknown, but Ochirkhuyag *et al.* (2000) published the sequence of the N-terminal 15 amino acid residues of which only 5 residues were confirmed by Miranda *et al*. (2004).

The amino acid sequence of equine β-casein, derived from the cDNA sequence, was reported by Lenasi *et al*. (2003) and revised by Girardet *et al*. (2006) by the insertion of 8 amino acids. It has 226 amino acids and a theoretical mass of 25, 5114 Da. Two smaller variants of equine β-casein, which probably result from exon-skipping during transcription, were reported by Miranda *et al*. (2004). Multiple isoforms of equine β-casein containing 3 to 7 phosphoserine residues have been reported, with an isoelectric point ranging from pH 4.74 to 5.30 (Girardet *et al*., 2006; Matéos *et al*., 2009b). In equine sodium caseinate, the Lys_{47}-Ile_{48} bond of equine β-casein is readily hydrolysed by bovine plasmin whereas no cleavage of the corresponding bond, Lys_{48}-Ile_{49} in bovine β-casein, has been been reported (Egito *et al*., 2002). In bovine β-casein, Lys_{28}-Lys_{29} is readily cleaved by plasmin but the equivalent, Lys_{28}-Leu_{29}, in equine β-casein is insensitive (Egito *et al*., 2002). Other plasmin cleavage sites, Lys_{103}-Arg_{104}, Arg_{104}-Lys_{105} and Lys_{105}-Val_{106}, in equine β-casein have been reported by Egito *et al*. (2002). Equine β-casein is readily hydrolysed by chymosin at Leu_{190}-Tyr_{191} (Egito *et al*., 2001). Unique to equine milk and apparently absent from the milk of other species, including ruminants, is a low-MW multi-phosphorylated β-casein variant which accounts for 4% of the total casein (Miclo *et al*., 2007).

The presence of κ-casein in equine milk was an issue of debate for several years, with several authors (Visser *et al*., 1982; Ono *et al*., 1989; Ochirkhuyag *et al*., 2000) reporting its absence. However, other studies (Kotts & Jenness, 1976; Malacarne *et al*., 2000; Iametti *et al*., 2001; Egito *et al*., 2001, 2002) reported its presence, albeit at a low concentration. Equine κ-casein

was purified and fully sequenced by Iametti *et al*. (2001) and its entire cDNA sequence was reported by Lenasi *et al*. (2003); it contains 165 amino acid residues with a molecular weight of 18,844.7 Da. Equine and human κ-casein have a considerably higher isoelectric pH than bovine κ-casein and they have a net-positive charge at physiological pH, whereas bovine κ-casein has a net negative charge. Although no direct information is available, lectin-binding studies indicate that equine κ-casein is highly glycosylated (Egito *et al*., 2001; Iametti *et al*., 2001; Lenasi *et al*., 2003). To date, no non-glycosylated κ-casein has been identified in equine milk (Martuzzi & Doreau, 2006). The level of glycosylation may affect the susceptibility of κ-casein to hydrolysis by chymosin, with susceptibility decreasing as the level of glycosylation increases (Doi *et al*., 1979; Addeo *et al*., 1984; Van Hooydonk *et al*., 1984; Vreeman *et al*., 1986; Zbikowska *et al*., 1992) which could, at least in part, account for differences in the clotting mechanism of equine milk by chymosin compared to bovine milk (Section 3.6.9 and Chapter 5).

κ-Casein, which is located on the surface of casein micelles, plays an important role in the formation, stabilization and aggregation of the micelles, and alters the manufacturing properties and digestibility of milk. The presence of a glycan moiety in the C-terminal region of κ-casein enhances its ability to stabilize the micelle, by electrostatic repulsion, and may increase the resistance by the protein to proteolytic enzymes and high temperatures (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996). Unlike bovine κ-casein, equine κ-casein does not have a distinctly hydrophilic C-terminal domain, particularity due to the absence of a strong hydrophilic region at residues 110-120, and it is unclear if this part of the protein is capable of protruding from the micellar surface to sterically stabilize the micelle. It has been suggested (Ochirkhuyag *et al*., 2000; Doreau & Martin-Rosset, 2002) that the steric stabilization of equine casein micelles by κ-casein may be aided by non-phosphorlated β-casein on the surface of the micelles,

Chymosin (EC 3.4.23.4) is a neonatal gastric aspartyl proteinase which hydrolyses the κ-casein of bovine milk into a soluble glycopeptide (caseinomacropeptide (CMP): amino acid residues 106-171) and an insoluble part (para-κ-casein: amino acid residues 1-105) which is crucial for the production of cheese and for the nutrition of newborns (Mercier *et al*., 1973; Crabbe, 2004). The non-enzymatic secondary stage of the coagulation of milk by chymosin involves the aggregation and gelation of para-κ-casein under the influence of Ca^{2+} (Bringe &

Kinsella, 1986; Merin *et al*., 1989; Dalgelish, 1993). Apart from cleavage of the Phe_{105}-Met_{106} bond of κ-casein, no further hydrolysis of κ-casein has been reported (Crabbe, 2004). Calf chymosin hydrolyses the Phe_{97}-Ile_{98} bond of equine κ-casein (Egito *et al*., 2001) and slowly hydrolyses the Phe_{105}-Ile_{106} bond of human κ-casein (Plowman *et al*., 1999). While the CMPs released from equine and human κ-casein are less hydrophilic than bovine CMP, in all species CMP is believed to inhibit gastric acid secretion following milk intake (Mercier *et al*., 1976; Yvon *et al*., 1994; Guilloteau *et al*., 2010) as well as having antibacterial activity (Malkoski *et al*., 2001; Thomä-Worringer *et al*., 2006). The sequence 97-116 of κ-casein is highly conserved across species, suggesting that the limited proteolysis of κ-casein and subsequent coagulation of milk are of major biological significance (Mercier *et al*., 1976). The hydrolysis of other proteins in bovine milk by chymosin, α_{s1}-, α_{s2}-, β-caseins and α-lactalbumin, have been reported but at a much slower rate than the hydrolysis of κ-casein (Carles & Dumas, 1985; Miranda *et al*., 1989).

Over the last decade, milk from non-bovine mammals (goat, donkey, horse, camel) have been studied, generally, as a means of identifying the best substitute for human milk in infant nutrition (Businco *et al*., 2000; El-Agamy *et al*., 1997; Muraro *et al*., 2002; Restani *et al*., 2002). In recent years, 2-DE, combined with mass spectrometry (MS), has been used for the separation and characterization of the proteins in bovine milk or colostrum (Galvani *et al*., 2001; Lindmark-Månsson *et al*., 2005) or for identification of specific proteins and isoforms thereof (Holland *et al*., 2004; 2006; Chevalier & Kelly, 2010). Caseins in human milk have been resolved by 2-DE (Goldfarb, 1999; Poth *et al*., 2008) and a proteomic comparison between pre-term and term human milk has been reported (Armaforte *et al*., 2010). The proteins of equine colostrum and milk have been analysed by electrophoretic and immunological methods (Curadi *et al*., 2000) while Egito *et al.* (2001) characterized the casein fraction of equine milk using 1- DE and 2-DE of samples purified by anion exchange chromatography and reverse phase high performance liquid chromatography (RP-HPLC). Various mass spectrometric approaches have been used to characterize the proteins of asinine (Cunsolo *et al*., 2007, 2009; Marletta *et al*., 2007; Chianese *et al*., 2010; Criscione *et al*., 2010) and equine (Iametti *et al*., 2001; Girardet *et al*., 2006; Miclo *et al*., 2007) milks.

Proteomics and associated technologies are very powerful for the detection and characterization of different components in complex protein mixtures. Recently, extensive sequencing information in databases and the increased resolution and sensitivity of separation techniques has meant that it is possible to separate and identify proteins in the milks of different species.

Identification of individual proteins can be achieved *via* a four-step process consisting of protein separation (liquid phase separation or 2-DE), protein digestion (either before or after separation), MS analysis of resulting peptides and comparison of observed peptides to those in a database (O'Donnell *et al*., 2004). In a 2-DE map, it is possible to separate and discriminate individual proteins, but also to get information on the abundance of isoforms and post-translational modifications (Chevalier & Kelly, 2010). Holland *et al*. (2004) resolved and identified 10 glycoforms of κ-casein using 2-DE and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Phosphovariants of milk proteins are generally discernible following 2-DE with noticeable shifts along the horizontal axis of the gel corresponding to changes in the pI of the proteins (Holland *et al*., 2004).

The aim of this study was to separate and characterize the proteins of equine milk, especially to determine if equine milk contains κ-casein. Previous studies (Chapters 3 and 5) demonstrated the poor coagulability of equine milk by calf chymosin at pH 6.5 and the weak coagulum formed when equine milk was renneted with equine chymosin extract, therefore this study aimed to take a proteomic approach and examine the rennetability of equine milk by calf chymosin using 2-DE followed by mass spectrometry with comparative analysis of renneted bovine milk. A combined set of analytical procedures was used, including different electrophoretic methods, RP-HPLC, MALDI-TOF MS with peptide mass fingerprinting (PMF) and MALDI-TOF/TOF MS with PMF and nano-liquid chromatography tandem mass spectrometry (nano-LC-MS/MS analysis). MALDI-TOF PMF is the fastest and cheapest method of protein identification but for non-sequenced proteins the only possibility of identification is by MALDI-TOF/TOF or by nano-LC chromatography MS/MS (Sommerer *et al*., 2007).

8.2. Materials and Methods

8.2.1 Milk supply

Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from 5 milkings over 24 h, from a herd of multiparous New Forest and New Forest/Arabian mares in mid-lactation, physically separated by day from their foals. The milk was defatted by centrifugation at 1,000 x g using a Sorvall® RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 20°C for 20 min, followed by filtration through glass wool to remove fat particles. Raw whole bovine milk, obtained from a local dairy farm, was defatted by centrifugation at 2,000 g for 20 min at 20°C, followed by filtration through glass wool. Sodium azide (0.5 g L^{-1}) was added to the skimmed milks to prevent microbial growth. All chemicals used were of reagent grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

8.2.2 Renneting equine and bovine milks

8.2.2.1 Preparation of renneted equine and bovine milk for reverse phase high performance liquid chromatography (RP-HPLC)

The pH of skimmed equine and bovine milks was adjusted to pH 6.5 and the milks were tempered at 30°C for 20 min in thin-walled glass tubes in a thermostatically controlled waterbath. Ten μL mL^{-1} of a 1:10 (v/v) aqueous dilution of fermentation-produced chymosin (Maxiren 180; 180 international milk-clotting units (IMCU) per ml, DSM Food Specialities, Delft, the Netherlands) were added. Aliquots (1 mL) of renneted bovine milk were removed at 4, 10, 15, 20, 40, 60, 90 and 240 min and from renneted equine milk at 20, 30, 60, 90, 120, 240 min and at 24 h. Renneted milk samples were diluted (1:1) immediately with 4% trichloroacetic acid (TCA) in Eppendorf tubes and vortexed for 1 min, and then held at 20°C for 30 min. The samples were centrifuged at 5000 x g for 10 min in a micro-centrifuge (Sigma 1-15, Sigma Laborzentrifuge, Osterode am Harz, Germany). Supernatants were removed carefully and diluted 1:1 with buffer (0.1 M bis-Tris pH 7.0, 8M urea, 45 mM citrate) to a final pH of 3.0 (Vasbinder *et al*., 2003). Samples were filtered through 0.45 μm filters

(Millex®-HV, PVDF, 13 mm; Millipore Corp., Billerica, MA, USA) prior to injection onto the RP-HPLC column.

8.2.2.2 Preparation of renneted equine and bovine milk for two dimensional electrophoresis

Skimmed equine and bovine milks at pH 6.5 were tempered at 30°C for 20 min in thin-walled glass tubes. Ten μl mL^{-1} of a 1:10 (v/v) aqueous dilution of Maxiren 180 were added. Aliquots of renneted bovine milk were removed at 2 min intervals up to 16 min and at 20, 24 and 60 min. Samples of renneted equine milk were taken at 1, 2, 4, 8 and 24 h. Renneted milk samples were diluted (1:10) immediately with solubilisation buffer (9 M urea, 40 g L^{-1} 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 g L^{-1} Triton X100 and 65 mM dithiothreitol (DTT) according to the method of Chevalier *et al*. (2009). The protein content of 100 μL of each sample was determined by the Bio-Rad Protein Assay (No. 500-0002, Bio-Rad Laboratories, Hercules, CA, USA) which is based on the method of Bradford, (1976) and uses bovine serum albumin (BSA) as standard. Analysis was carried out in at least duplicate at each time point for both equine and bovine milk.

8.2.3. RP-HPLC analysis of renneted equine and bovine milk

RP-HPLC was preformed according to the method of Vasbinder *et al*. (2003) for RP-HPLC analysis of CMP, with some modificaions. Analysis was carried out using a Waters 626 non-metallic HPLC system (Waters Corp., Milford, MA, USA) consisting of a Waters 486 UV-Vis detector, a Waters 717^{+} autosampler, a Waters 626 pump with a 600S controller and an on-line Degasys DG-2410 degassing unit (Sanwa Tsusho Co., Tokyo, Japan). The HPLC system was controlled by Millenium^{32} Version 3.05.01 chromatography management software. A Varian Pursuit XRs C18 RP-HPLC column [250 x 4.6 mm, 5 μm particles size, 300 Å pore size (Varian Inc., Lake Forest, CA, USA)] was used for protein separation and 100 μl of sample were applied to the column. Elution was at a flow rate of 0.8 mL per min using the gradient described by Vasbinder *et al*. (2003). A wash step of 15 min with 70% solvent B, followed by 15 min equilibration with 85% solvent A, was included in the analysis between samples. The eluate was monitored at 220 nm.

8.2.4. Two-dimensional electrophoresis of renneted equine and bovine milk

Analytical 2-DE was carried out on renneted equine and bovine milk with 100 μg of protein using 7 cm immobilized pH gradient (IPG) strips (ReadyStrip, Bio-Rad, Hercules, CA, USA) with a linear pH gradient from 3 to 10 (Bio-Rad). Preparative 2-DE was carried out on equine and bovine milk with 300 μg of protein using 17 cm IPG strips and a linear pH gradient from 3 to 10 (Bio-Rad). The 7 or 17 cm IPG strips were rehydrated in the protein/solubilisation buffer. Isoelectric focussing was carried out according to the method of Armaforte *et al*. (2010) using a Protean IEF Cell isoelectric focussing system (Bio-Rad). The IPG strips were then embedded using 6 g L^{-1} low melting point agarose on top of a 12.5% acrylamide gel and sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) was carried out using a Criterion® Dodeca Cell electrophoresis unit (Bio-Rad) for the 7 cm strips or a Protean® II xi Cell electrophoresis unit (Bio-Rad) for the 17 cm strips. Gels were stained using colloidal Coomassie blue as described by Chevalier *et al*. (2006) and stained gels digitized at 300 dpi using a GS-800 densitometer (Bio-Rad).

8.2.5. In-gel digestion

Protein spots were selected for further analysis from preparative 2-DE and excised using a 2 mm diameter punch and gel pieces transferred to 1.5 mL Eppendorf tubes. The gel spots were washed sequentially with Milli-Q® water (Millipore Corporation, Billerica, MA, USA), 25 mM ammonium bicarbonate, acetonitrile/25 mM ammonium bicarbonate (1:1, v/v) and acetonitrile. Gel fragments were dried using a Speed-Vac (GMI Inc., MN, USA) and the proteins were digested by the addition of sequencing-grade trypsin (Promega, Charbonnieres, France) at 12.5 μg μL^{-1} in 25 mM ammonium carbonate which was added on ice. After 15 min of gel rehydration, the proteins were digested at 37°C for 2 h. Digested protein fragments were identified either by MALDI-TOF MS, MALDI-TOF MS/MS or nano-LC MS/MS.

8.2.6. Protein identification by MALDI-TOF mass spectrometry

Digested protein fragments were extracted by the addition of 20 μL of 0.1% TFA and sonicated for 15 min. The supernatants were transferred to 500 μL polypropylene microcentrifuge tubes. Final peptide extraction was carried out by the addition of 20 μL of a 3:2 (v/v) acetonitrile/TFA solution and sonicated for 15 min. Supernatants were concentrated using a Speed-Vac to a final volume of 10-20 μL. Peptides were simultaneously desalted and concentrated with C18 Zip-Tip microcolumns and supernatants were spotted directly on an

AnchorChip™ MALDI target with α-cyano-4-hydroxycinnamic acid matrix solution and allowed to co-crystallize. Crystallized samples were washed with 0.1% TFA in water and re-crystallized with 6/3/1 (v/v/v) ethanol/acetone/0.1% TFA in water. Peptide mass fingerprinting (PMF) was made using an UltraFlex MALDI-TOF/TOF mass spectrophotometer (Bruker, Bremen, Germany). Acquisition was performed with at least 200 laser shots which were summed to obtain a very specific and accurate mean spectra for each peptide. Peaks were annotated using Bruker's SNAP™ procedure. MASCOT search engine software (Matrix Science, London, UK) was used to search the NCBInr database. The following parameters were used for searching the database: a mass tolerance of 30 ppm which corresponds to ~ 0.1-0.2 Da, a minimum of five peptides matching the protein, one matched cleavage allowed, carbamidomethylation of cysteines as fixed modifications which occur during the experimental process and the taxonomy restricted to Mammalia.

To confirm PMF identification with low statistical scores, matching peptides from PMF were selected for TOF/TOF sequencing and database search. For non-identified proteins after PMF, a minimum of two peptides were selected for TOF/TOF sequencing. Parent ions were selected with a mass window of +/- 4 Da and a parent ion spectrum was surveyed, i.e., according to the first mass spectrum from MALDI-TOF, the most important peaks (parent ion) were selected and new spectra were created in TOF-TOF mode to obtain accurate fragmentation information (peptide sequences). Laser induced dissociation (LID) metastable fragments were generated using Bruker's LIFT™ mode. 1000 to 2000 fragment ion spectra were summed for each parent ion. Identification was made using MASCOT search engine software using the following parameters for the parent ion: a mass tolerance of 30 ppm for the parent ion, a mass tolerance of 0.6 Da for fragment ions, one missed cleavage allowed, carbamidomethylation of cysteines as fixed modification and the taxonomy search was restricted to Mammalia.

8.2.7. Protein identification by nano-LC mass spectrometry

When low abundant spots could not be identified by MALDI-TOF mass spectrometry, nano-LC MS was conducted. Protein digests were extracted using formic acid and peptides were analysed using an ion trap mass spectrometer (Esquire HCT plus; Bruker, Billerica, MA, USA) coupled to a nano-chromatography system (HPLC 1200, Agilent Technologies, Santa Clara, CA, USA) interfaced with an HPLC-Chip system (Chip Cube, Agilent Technologies). The samples were loaded at 4 μL/min in the C18 trapping column using 0.1% formic acid and

then flushed onto the nano-scale RP column (C18 Zorbax 300SB, 75 μm diameter, 43 mm length; Agilent Technologies) where separation was carried out at a flow rate of 0.3 μL/min with a mobile phase gradient of 97% solvent A (99.9% Milli-Q® water with 0.1% TFA) with

3% solvent B (89.95% acetonitrile with 9.95% water and 0.1% TFA), increasing to 45% solvent B in 7 min and an increase over 1 min to a washing step of 80% solvent B which was maintained for 3 min. The tandem mass spectrometer was an Esquire HCT+ (Bruker Daltonik GmBH, Bremen, Germany) operating in positive ion mode. The automated data dependent acquisition parameters were chosen such that only doubly and triply charged precursor ions were selected for C.I.D., excluding singly charged ions. Identification was made by querying the NCBI non-redundat database (NCBInr, release 20101018) using MASCOT search engine software. The following parameters were used for the database search: a mass tolerance of 0.6 Da for parent and fragment ions, one missed cleavage allowed, carbamidomethylation of cysteines as fixed modification, peptide charge fixed at 2+ and 3+ and the taxonomy was restricted to Mammalia.

8.2.8. RP-HPLC of skimmed equine milk

To determine if equine milk contained κ-casein, skimmed equine milk was analysed by RP-HPLC according to the method of Miranda *et al*. (2004). Prior to analysis the milk was treated 1:2 (v/v) with 0.1 M bis-Tris buffer, pH 8.0 containing 8 M urea, 1.3% trisodium citrate and 0.3% DTT. The sample was filtered through 0.45 μm filters and 30 μL were injected onto the column. Analysis was carried out using a Waters 600 HPLC pump with 600S controller, a Varian 9050 variable wavelength UV-Vis detector and a Waters 717^{+} autosampler. The HPLC system was controlled by Millenium^{32} Version 3.05.01 chromatography management software. A Microsorb™ C4 RP-HPLC column (250 x 4.6 mm, 300 Å pore size, 5 μm particle size) from Varian was used for protein separation. The column was held in a Shimadzu CTO-10AC column oven (Shimadzu Corp., Kyoto, Japan) at 40^{o}C.

Elution was achieved using the two step gradient outlined by Miranda *et al*. (2004) with the addition that a 20 min wash step with 60% solvent B was included after protein separation followed by 10 min at 100% solvent A before injection of subsequent samples. The eluate was monitored at 220 nm. A fraction was collected manually in the κ-casein region identified by Miranda *et a*l. (2004). The fraction was precipitated using TCA/acetone,

dephosphorylated, deglycosylated, digested with trypsin and subjected to MALDI-TOF/TOF and nanoLC-MS/MS analysis as described below.

8.2.9. Enzymatic treatment and SDS-PAGE of RP-HPLC fractions for mass spectrometry

A 10 mL fraction, collected from C4 RP-HPLC analysis, was cooled on ice, 750 μL of 100% TCA added and the mixture held on ice for 2 h. The sample was centrifuged at 10,000 x g for 10 min at 4^{o}C. The supernatant was removed carefully and 2 mL of acetone (pre-cooled on ice) were added to the pellet. The sample was vortexed for 2 min, followed by centrifugation at 10,000 x g (10 min at 4^{o}C). The supernatant was removed and the pellet was washed again with 2 mL ice-cold acetone and this step was repeated once. Finally, the pellet was dissolved in 45 μL distilled water and 2 μL of 20% SDS were added. Prior to electrophoresis, 3 samples (15 μL each) were prepared as follows: Sample 1, 15 μL were added to 15 μL of a 2X electrophoresis loading buffer. Sample 2 was dephosphorylated using the lambda protein phosphatase (lambda PP) and buffer system from New England Biolabs (Hitchin, Herts., UK); 2 μL of $MnCl_2$, 2 μL PPase buffer and 2 μL phosphatase were added to 15 μL of sample and the mixture was incubated overnight at 30^{o}C; 20 μL of single-strength electrophoresis buffer were added to 20 μL of the dephosphorylated sample solution. Sample 3 was deglycosylated as follows: 4 μL of 0.1M phosphate buffer, pH 7.5, containing 0.02% SDS and 10 mM 2-mercaptoethanol were added to 15 μL sample and the mixture was incubated at 100 ^{o}C for 10 min, after which 1 μL of PNGase F (Sigma-Aldrich) was added, followed by incubation at 37^{o}C for 2 h. Twenty μL of the solution were mixed with 20 μL of single-strength electrophoresis loading buffer. The samples were denatured by heating at 70^{o}C for 10 min. Electrophoresis was carried out using the NuPAGE® electrophoresis system from Invitrogen (Invitrogen Corp., Carlsbad, CA, USA) which is a ready-to-use system with denaturing loading buffer, 3-(N-morpholino) propanesulfonic acid- sodium dodecyl sulphate (MOBS-SDS) running buffer, precast polyacrylamide mini-gels (4-12% acrylamide). A molecular weight marker, SeeBlue® Plus2, from Invitrogen, was used in the analysis. Following electrophoresis, gels were stained using a colloidal Coomassie Blue staining procedure as described by Chevalier *et al*. (2006). A protein band from the phosphatase-treated sample with a molecular weight of ~ 24,000 Da (corresponding to the molecular weight of equine

κ-casein, Miranda *et al*., 2004) and a second band from the untreated sample were excised for further characterization by MS.

8.2.10. Image analysis of gels from analytical 2-DE

Preliminary investigation of the effect of renneting on the proteins of equine milk was carried out by analysing the 2-DE gels of renneted equine milk using Progenesis SameSpots V. 4.0 image analysis software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). The same gel area was selected from duplicate gels of each time point after rennet addition. Gel areas were compared and the protein spot volume was quantified as a mean of each replicate. To normalize the raw protein spot volume (evaluated in terms of optical density) and avoid experimental variation among 2-DE gels, each spot on the gel image was expressed relative to the total volume of all spots on that image that also matched and occurred in all of the gels of the experiment. Statistical analysis separated proteins that increased or decreased significantly after the treatments. Data analysis returned p-values from one-way analysis of variance (ANOVA), false discovery rate (FDR) q-values, principal component analysis (PCA) and power analysis values. FDR q-values are adjusted p-values calculated using an optimised FDR calculation, where characteristics of the p-value distribution are used to produce a list of q-values. Power analysis reflects confidence in the experimental data's ability to find differences that actually exist and is expressed as a percentage, where 80% power is an accepted level of confidence. Protein spots were ordered by p value from one-way ANOVA and the top-ranked proteins spots (p value < 0.05) were used for principal component analysis (PCA). PCA uses protein spot expression levels across gels to determine the principal axes of expression variation, allowing separation of gel samples according to the variation in protein expression.

In a second study image analysis was applied to renneted equine and bovine milk samples over time. Gel areas were selected and compared across duplicate gels for equine and bovine milk for each time point after rennet addition. Milk proteins were quantified using a 2-DE proteomic map of a control milk sample as a reference sample. Individual protein spots were quantified and values presented correspond to a percentage of the same protein on the proteomic map.

8.3 Results and Discussion

8.3.1. C18 RP-HPLC of renneted equine and bovine milk

Chromatograms of 2% TCA-soluble fractions of equine and bovine milk renneted using Maxiren 180 and analysed by RP-HPLC are shown in Figure 8.1. Compared to a control bovine milk sample, peaks at ~ 15 and ~16-17.5 min increased significantly in area from about 4 min after rennet addition (Figure 8.1A) and continued to increase in area up to 240 min. Renneted equine milk (Figure 8.1B) had a different RP-HPLC profile from that of bovine milk and it was ~ 20 min before peaks from protein hydrolysis appeared on the chromatogram. The time after rennet addition was therefore adjusted for equine milk and samples for RP-HPLC were taken up to 20 h after renneting. The retention times of peaks were close to those for bovine milk. Figure 8.2 is an enlarged chromatogram of the 30 min time-point for renneted equine and bovine milk and showed that one peak from the hydrolysis of equine milk by chymosin was evident relatively quickly after rennet addition, i.e., within a similar time period to the appearance of peaks on the renneted bovine milk chromatogram. Egito *et al*. (2001) reported that calf chymosin readily cleaved equine β-casein at Leu_{190}-Tyr_{191} and that the fragments produced were resistant to further hydrolysis, even after 24 h incubation with chymosin. Furthermore, Egito *et al* (2001) demonstrated the relatively slow hydrolysis of equine κ-casein using C18 RP-HPLC but only after whole equine casein was separated by affinity chromatography using agarose wheat germ agglutinin (WGA); hydrolysis of the WGA- bound fraction by chymosin produced fragments of equine κ-casein after cleavage of Phe_{97}-Ile_{98}. The fractions labelled a and b (Figure 8.1B) in this study were collected and analysed by MALDI-TOF MS/MS; the faster-eluting peak (a) was identified as having originated from equine β-casein (Table 8.1) which was produced quickly by calf chymosin, in agreement with Egito *et al*. (2001). Peptides from equine κ-casein were not identified in either fraction a or b analysed by MS.

8.3.2. C4 RP-HPLC of skimmed equine milk

To investigate if equine milk contains κ-casein, the C4-RPHPLC method of Miranda *et al* (2004) was used to fractionate the proteins and the fraction containing κ-casein, identified in their study, was collected (Figure 8.3) and was subsequently dephosphorylated. The electrophoretograms of the enzymatically-treated samples are shown in Figure 8.4. The phosphatase-treated sample had one band of ~ 24,000 Da which was selected for MS analysis along with a band of ~ 19,000 Da from the control sample. Using MALDI-TOF and MALDI TOF/TOF analysis, no result was obtained by PMF and multiple TOF-TOF analysis identified equine lysozyme; however, nano-LC MS/MS positively identified a peptide (YIPIYYVLNSSPR) from equine κ-casein in band b (Figure 8.5). Band a (Figure 8.4) was an equine milk sample which was not dephosphorylated and κ-casein was not identified therein. The κ-casein peptide, YIPIYYVLNSSPR, identified in band b contained several amino residues that could be phosphorylated; the low ion efficiency of phosphorylated peptides in complex samples makes it difficult to identify them by MS. Using NetPhos 2.0 (Blom *et al*., 1999) 11 phosphorylation sites in equine κ-casein (2 serine, 6 threonine, 3 tyrosine) were predicted which scored > 0.5 in the NetPhos scoring system. In the sequence, YIPIYYVLNS**S**PR, identified from equine κ-casein, the serine residue highlighted in bold scored 0.947 on the NetPhos scale which indicated a high probability of phosphorylation.

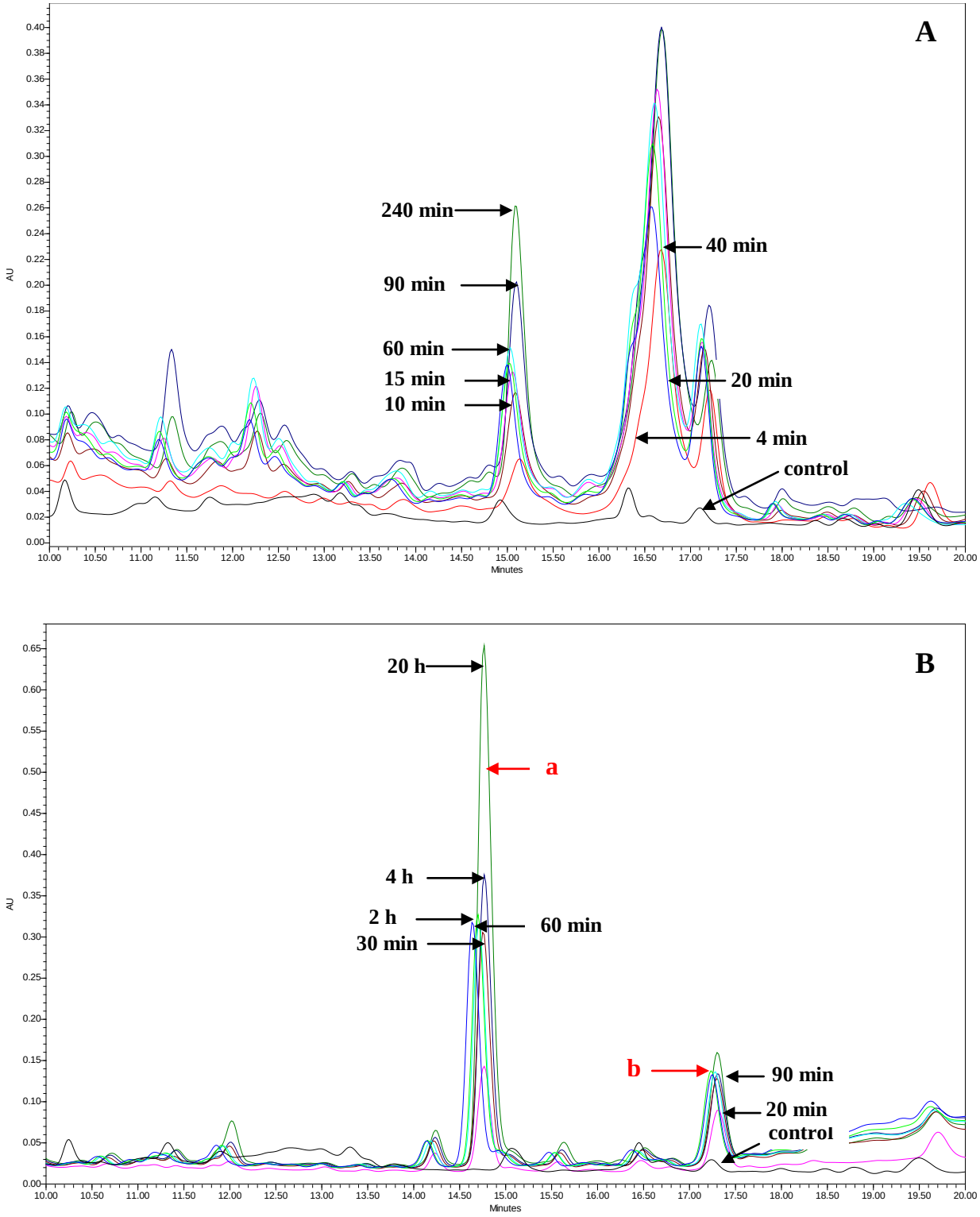

Figure 8.1. C18 RP-HPLC chromatograms of bovine (A) and equine (B) milk renneted with 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 at pH 6.5 and 30^{o}C. Red arrows indicate the fractions collected for MALDI-TOF MS/MS analysis.

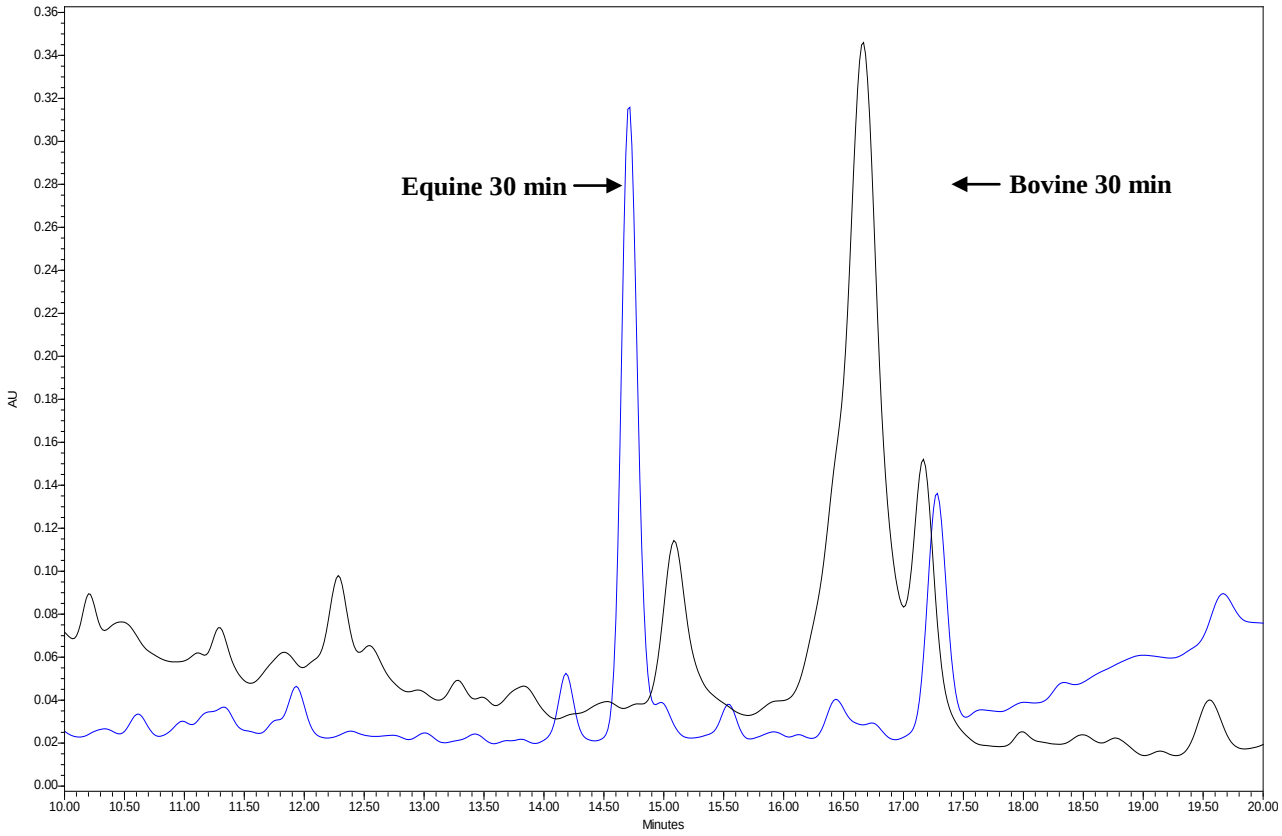

Figure 8.2. C18 RP-HPLC chromatograms of equine and bovine milk renneted with 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 at 30^{o}C and pH 6.5. Peaks formed at 30 min for each milk sample.

Table 8.1. Mass spectrometry results for fractions a and b from C18 RP-HPLC analysis of equine milk renneted with Maxiren 180 at pH 6.5 and 30°C; analysis carried out using MALDI-TOF MS/MS.

Fraction	Protein	Obs MW (Da)	Theor MW (Da)	Score	Sequence*	Amino acids†
a	β-casein *Equus caballus*	2336.289	2336.274	141	L-GPTGELDPATQPIVAVHNPVI-V	203-225
	β-casein *Equus caballus*	1593.759	1593.867	48	V-APFPQPVVPYPQ-R	175-188
b	β-lg I *Equus caballus*	1677.872	1677.941	61	L-RPTPEDNLEIIL-R	45-58

† peptides identified by mass spectrometry include the signal peptide (amino acids 1-15 for β-casein and 1-18 for β-lg
*cleavage sites indicated by red line

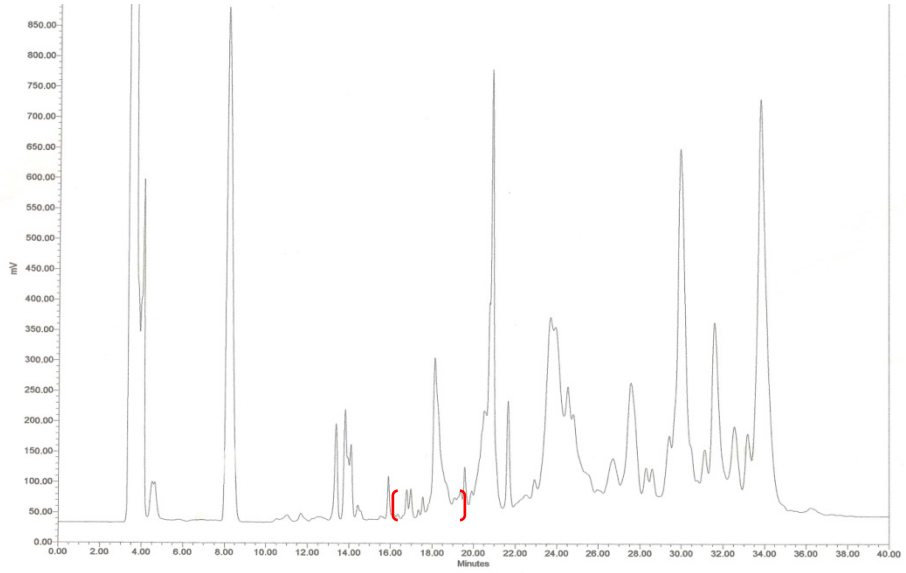

Figure 8.3. RP-HPLC chromatogram of a mixture (1:2, v/v) of skimmed equine milk and 0.1 M bis-Tris buffer, pH 8.0, containing 8 M urea, 1.3% trisodium citrate and 0.3% DTT separated on a C4 column. Red brackets indicate the fraction collected and submitted to mass spectrometry for the study of the presence of equine κ-casein.

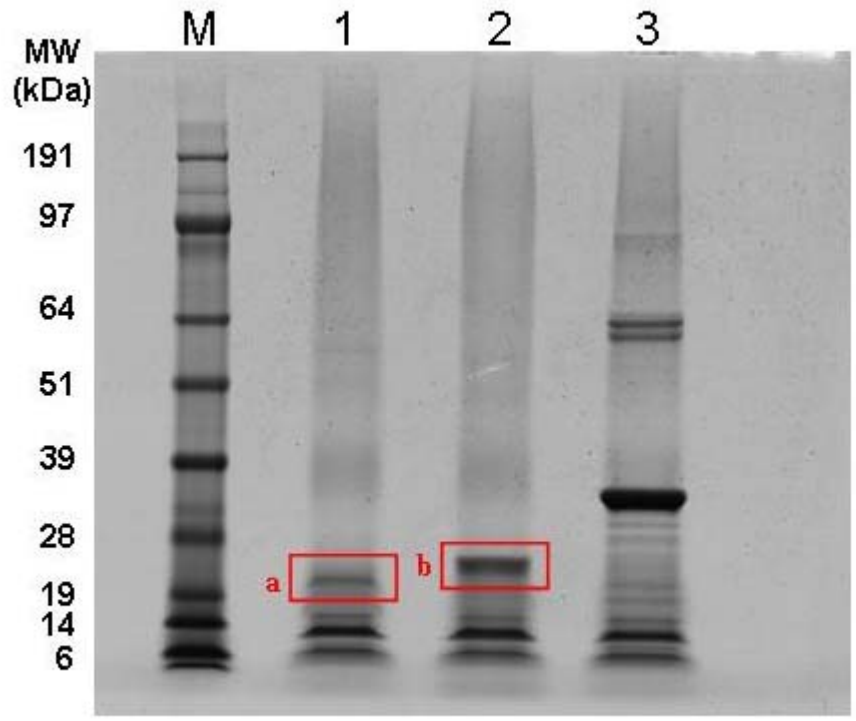

Figure 8.4. Electrophoretogram of fraction collected from C4 RPHPLC analysis of equine milk. Lanes: **M**. molecular weight marker, **1**. equine milk sample, **2**. sample treated with phosphatase, **3**. sample treated with glycosidase. Bands a and b were excised for identification by nano-LC-MS/MS.

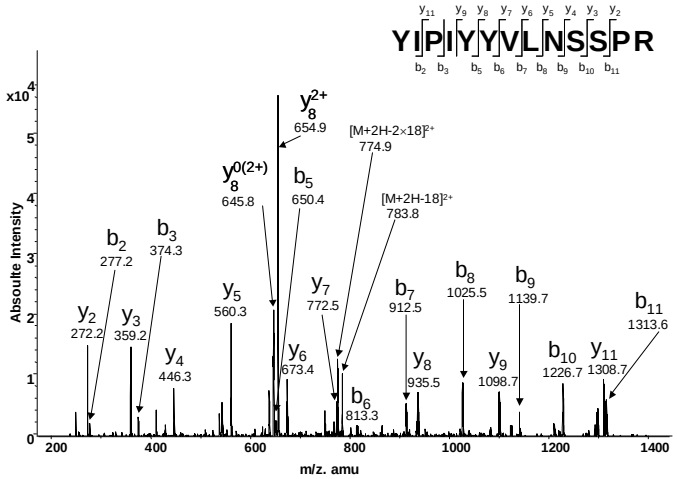

Figure 8.5. Nano-LC MS/MS spectra of the fragmentation process of the YIPIYYVLNSSPR peptide (with m/z 792.9) isolated from equine κ-casein; b^0, b-ion with loss of water (-18 Da); $[M+2H-18]^{2+}$, precursor ion with neutral loss of one molecule of water; $[M+2H-2x18]^{2+}$, precursor ion with neutral loss of 2 molecules of water. The sequence of the fragment if displayed with the fragment ions observed in the spectra

8.3.3. Image analysis of renneted equine milk

Preliminary image analysis was carried out on 2-DE analytical gels of equine milk renneted with Maxiren 180. Image analysis of the gels by Samepots V4.0 software is shown in Figures 8.6, 8.7 and 8.8. Figure 8.6A shows 140 protein spots identified by the software from duplicate analysis of each time point. Over the course of the ~ 20 h incubation with chymosin, 11 protein spots decreased in intensity (Figure 8.6B) and 7 increased in intensity (Figure 8.6C) and of these 18 spots, 10 varied significantly over time, i.e., ANOVA was < 0.05 for these spots, the q value was < 0.05 and the power was > 0.8. Figure 8.7 is the result for one of the spots which decreased significantly in intensity after renneting by chymosin; most of the decrease was recorded > 8 h after chymosin addition. Using data for all varying spots, principal component analysis (PCA) was carried out to produce a simplified graphical representation of the multidimensional proteomic data. For protein spots with significant differential amounts, the PCA bi-plot compared an experimental design grouping to observed relationships between groups (Figure 8.8). In the PCA plot, the control and 24 h samples were very different and were grouped at either end of the PCA plot. Samples renneted for 1 and 2 h were grouped closely together, as were samples renneted for 4 and 8 h.

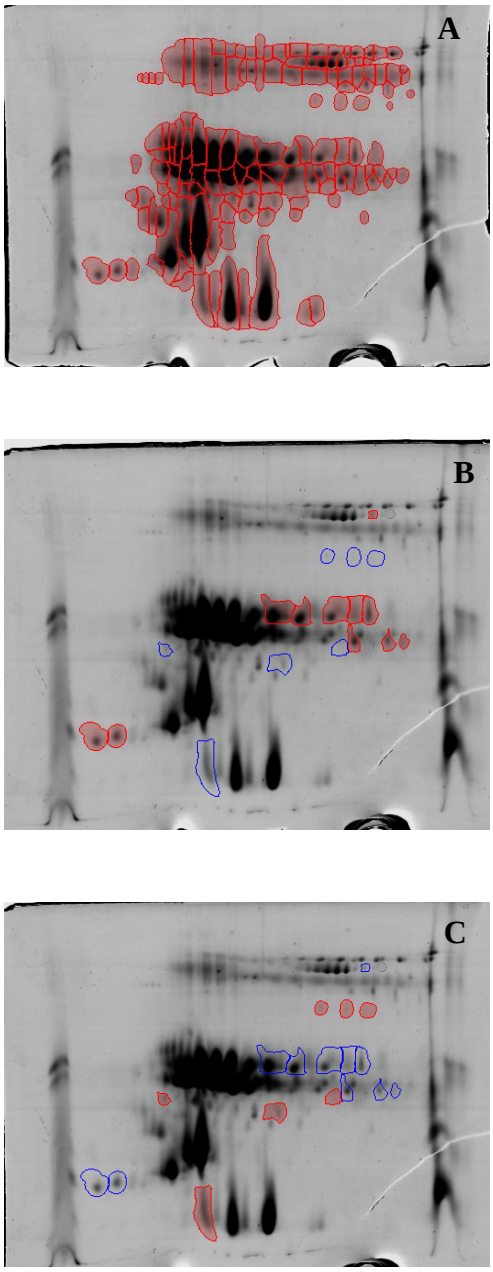

Figure 8.6. SameSpots V4.0 image analysis of equine milk renneted with 10 μL mL^{-1} of 1:10 (v/v) Maxiren 180 at 30°C and pH 6.5. 2-DE on analytical gels was carried out in duplicate at 1, 2, 4, 8 and 24 h after chymosin addition. **A**, 140 protein spots detected. **B**, 11 spots decreased in intensity over 24 h (highlighted in red). **C**, 7 protein spots increased in intensity over 24 h (in red).

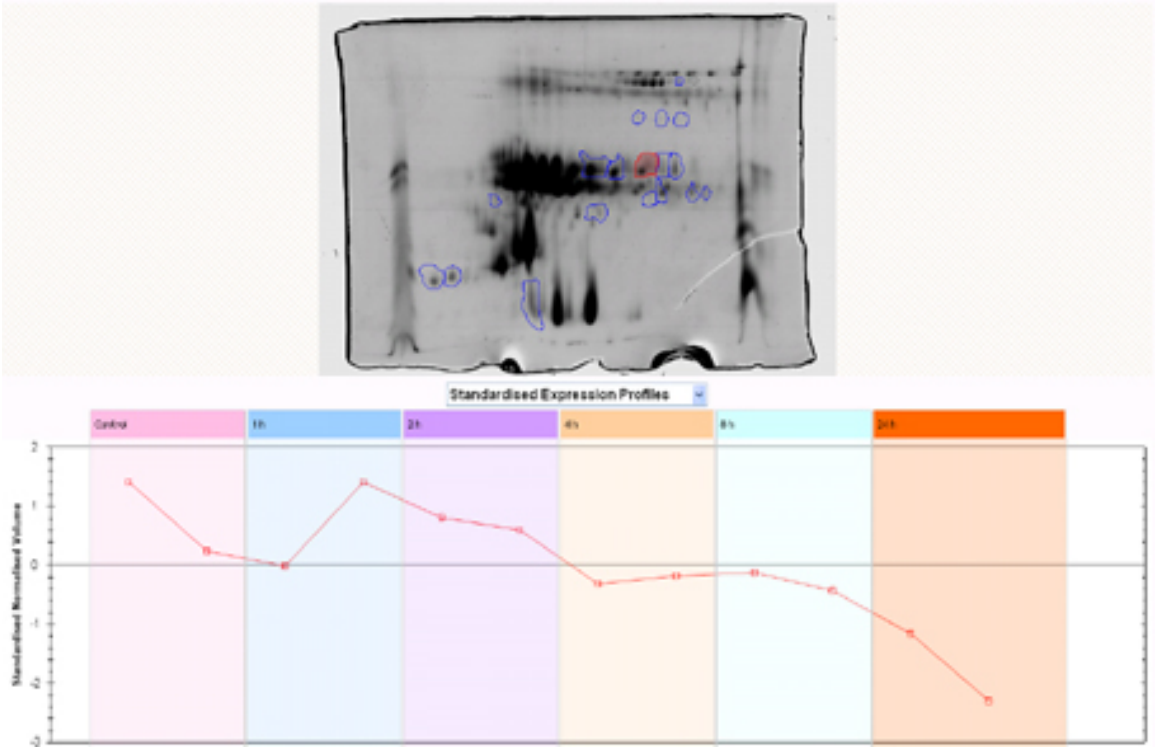

Figure 8.7. SameSpots V4.0 image analysis of a protein spot (highlighted in red) in equine milk which decreased significantly in intensity over time (0 to ~ 24 h) after addition of a 1:10 (v/v) dilution of Maxiren 180 at 30^{o}C and pH 6.5.

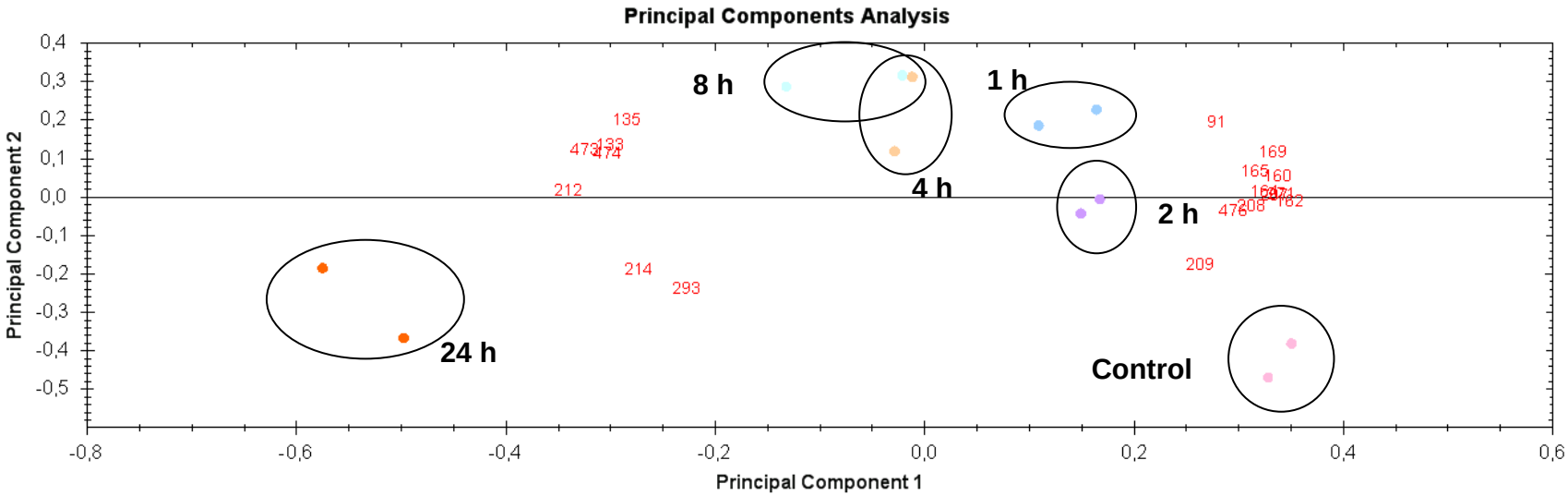

Figure 8.8. A bi-plot from principal component analysis (PCA) of transformed protein spot data from 2-DE gels of equine milk renneted with a 1:10 (v/v) dilution of Maxiren 180 at 30^{o}C and pH 6.5 for 1, 2, 4, 8 and 24 h. The points represent data from two gel replicates. Clustered protein spots had similar expression profiles. Duplicate 2-DE images are displayed as solid coloured circles and protein spots as numbers.

8.3.4. Preparative 2-DE analysis of bovine and equine milk

Preparative 2-DE gels of skimmed bovine and equine milk are shown in Figures 8.9 and 8.10, respectively. Protein spots in the bovine sample were labelled according to Chevalier & Kelly (2010); the spots in the κ-caseins region were identified by MALDI-TOF with PMF and the results are shown in Table 8.2 A. In this region, 5 κ-caseins, i.e., protein spots 1, 2, 3, 4 and 6 were identified by MALDI-TOF/TOF with PMF (Table 8.2A). It has been reported that up to 10 isoforms of κ-casein with pI's from 4.47 to 5.81 can be identified on 2-DE gels of bovine milk (Holland *et al*., 2004) and, if co-migrating α_s- and β-caseins are removed, up to 16 gel spots can be identified including κ-casein isoforms arising from post-translational modifications such as phosphorylation and glycosylation (Holland *et al*., 2006). In this study, within the region of the κ-casein isoforms, several β-caseins were identified (Table 8.2A,) which have been reported to migrate to similar regions on a 2-DE gel as the more acidic κ-casein isoforms (Holland *et al*., 2006). The purpose of this study was not to identify all the κ-casein isoforms in bovine milk, although the spot pattern was the same as that reported by Holland *et al* 2006 and Chevalier & Kelly (2010), but to identify the principal κ-casein spots and use this identification to study the effect of added chymosin over time.

Figure 8.10 is a 2-DE preparative gel of equine milk from which the most abundant protein spots were identified using peptide mass fingerprinting (PMF) with MALDI-TOF or nano LC-MS/MS. Equine κ-casein was identified in spots 25, 26 and 27 and some κ-casein co-migrated with β-casein in spots 40 and 41 (Table 8.2B). By comparison to bovine κ-casein, the isoelectric points of equine κ-caseins were significantly lower than their bovine counterparts. Protein spots 20, 21, 22, 23, 28 and 29 form a cluster of β-casein isoforms whereas protein spots 30, 31, 33, 34 and 35 represent isoforms of equine α_{s1}-casein (Figure 8.10 and Table 8.2B). Approximately 35% of protein spots were β-casein isoforms and a further 35% were α_{s1}-casein isoforms. As well as these two main milk proteins, other well-known milk proteins identified included α-lactalbumin (4 spots), β-lactoglobulin (5 spots) and lactoferrin (3 spots). This is the first study in which the most abundant proteins in equine milk have been identified.

Previously, 2-DE was used to demonstrate the micro-heterogeneity of equine α_{s1}- (Mateos *et al*., 2009a) and β-caseins (Girardet *et al*., 2006; Mateos *et al*., 2009b). The 2-DE gel of equine milk highlighted the complexity of the milk proteome, and while very different from that of bovine milk in general appearance, it contained a multitude of protein spots. The proteome of bovine milk, and the milk of most species, is dominated by 6 gene products that constitute ~ 95% of milk protein but > 150 protein spots can be detected using 2-DE of whole bovine milk. Many of the protein spots represent isoforms of the major gene products which have been produced by extensive post-translational modifications, including phosphorylation, glycosylation and proteolysis (Holland *et al*., 2004). D'Auria *et al*. (2005) conducted a proteomic evaluation of milk from different species, including equine milk, as a means of potentially identifying and evaluating the suitability of the milk of various species for infant nutrition. The study highlighted the complex pattern of proteins in 2-DE electrophoretograms of human, equine, asinine, caprine, ovine and bovine milks and reported that phylogenetically related species such as sheep/goats and horses/donkeys had quite similar protein expression. The 2-DE electrophoretogram of human milk was reported to be quite similar to those of the horse and donkey (D'Auria *et al*., 2005).

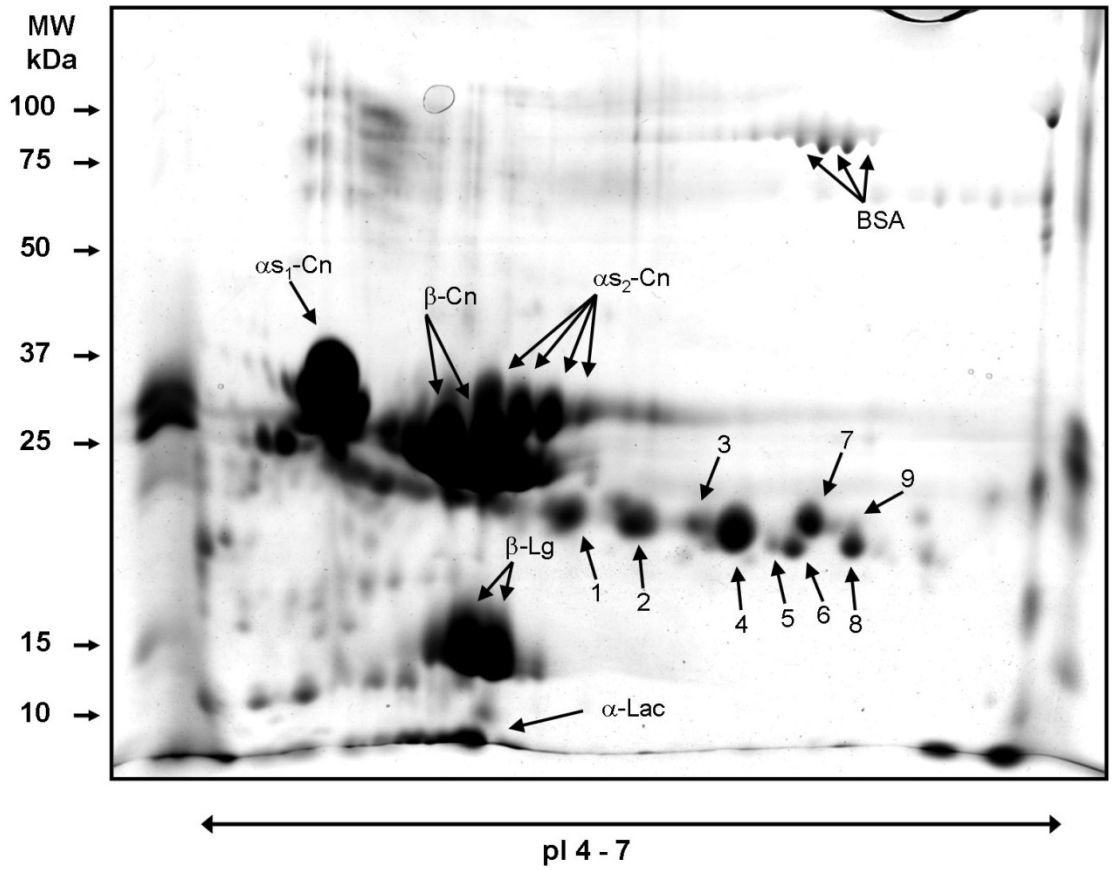

Figure 8.9. 2-DE preparative gel of a bovine milk sample (~ 300 μg protein) under reducing conditions using pH 4 to 7 pI range for the first dimension and a 12% acrylamide gel for the second dimension. The numbered spots were submitted to mass spectrometry by MALDI-TOF peptide mass fingerprint and/or nano-LC MS/MS (results in Table 8.2A). The proteins previously identified are from Chevalier & Kelly, (2010).

Table 8.2.A. Identification of the protein spots in the κ-casein region of bovine milk by 2-DE with peptide mass fingerprinting (PMF) using MALDI-TOF or TOF/TOF.

Spot	Protein	Accession number	Obs. pI	Obs. MW (kDa)	Theor. pI	Theor. MW (kDa)	Score	No. matching peptides	Sequence*	Amino acids†
1	κ-casein bovine	P02668	5.23	20	5.93	18.9	100	2	K-YIPIQYVLSR-Y	45-56
									R-SPAQILQWQVLSNTVPAK-S	89-108
2	κ-casein bovine	P02668	5.45	20	5.93	18.9	76	1	R-SPAQILQWQVLSNTVPAK-S	89-108
3	κ-casein bovine	P02668	5.66	20	5.93	18.9	46	1	K-YIPIQYVLSR-Y	45-56
4	κ-casein bovine	P02668	5.78	19	5.93	18.9	108	1	R-SPAQILQWQVLSNTVPAK-S	89-108
5	κ-casein bovine	P02668	5.94	19	5.93	18.9	55	1	K-YIPIQYVLSR-Y	45-56
6	β-casein fragment bovine	P02666	5.98	18	5.13	23.6	151	4	K-VLPVPQK-A	184-192
									K-AVPYPQR-D	191-199
									K-FQSEEQQQTEDELQDK-I	48-64
									R-DMPIQAFLLYQEPVLGPVR-G	198-218
7	κ-casein bovine	P02668	6.03	20	5.93	18.9	159	2	K-YIPIQYVLSR-Y	45-56
									R-SPAQILQWQVLSNTVPAK-S	89-108
8	β -casein fragment bovine	P02666	6.17	18	5.13	23.6	111	4	R-GPFPIIV.	217-224
									K-VLPVPQK-A	184-192
									K-AVPYPQR-D	191-199
									R-DMPIQAFLLYQEPVLGPVR-G	198-218
9	β -casein fragment bovine	P02666	6.17	19	5.13	23.6	50	2	K-VLPVPQK-A	184-192
									K-AVPYPQR-D	191-199

[a]Accession number corresponds to the Swiss-Prot/NCBI accession number.
[†] peptides identified by mass spectrometry include the signal peptide (amino acids 1-15 for β-casein and 1-18 for β-lg
*cleavage sites indicated by red line

Theoretical molecular mass and isoelectric point (pI) of proteins are based on the amino acid primary sequence without taking into account any post-translational and/or degradation modifications. Observed molecular mass and isoelectric points (pI) are those observed from the position of the corresponding spot on the two-dimensional electrophoresis gel.

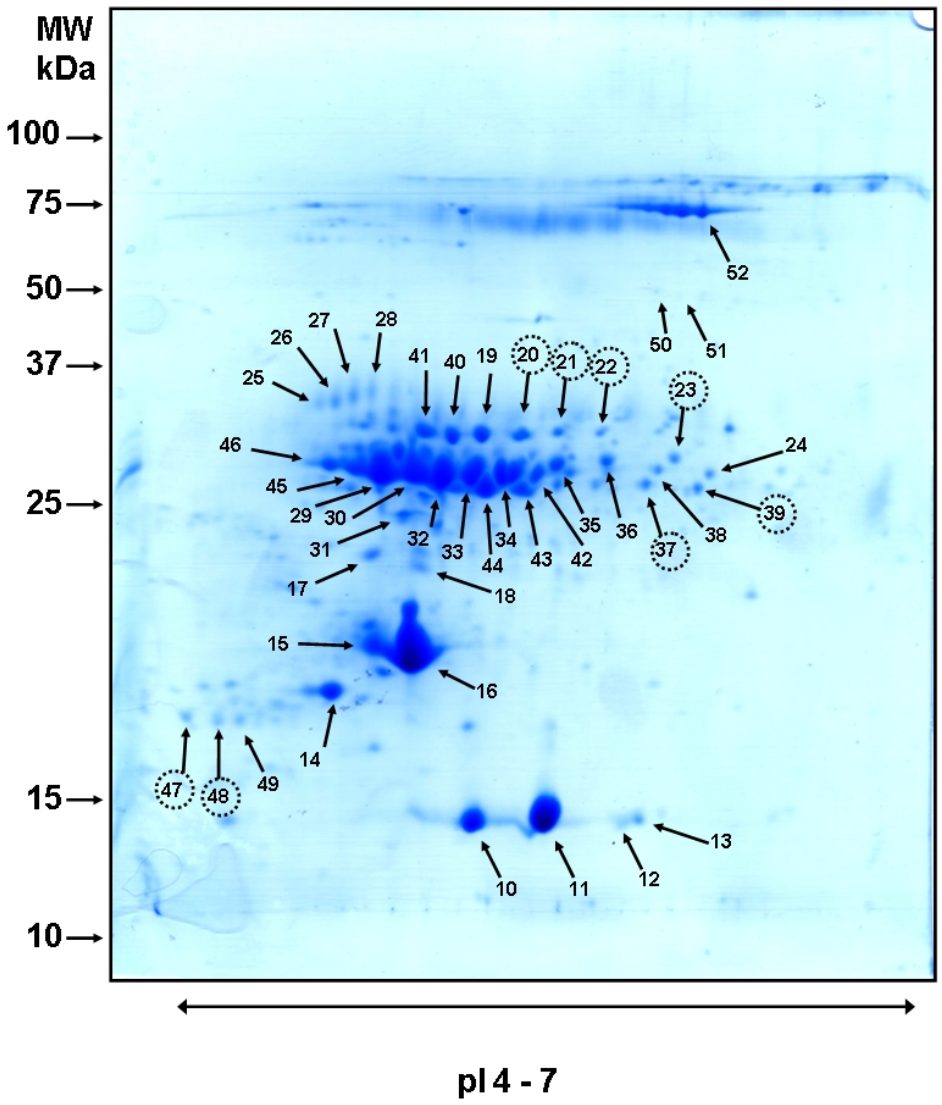

Figure 8.10. 2-DE preparative gel of equine milk (~ 300 μg proteins) under reducing conditions using a 17 cm pH 4-7 pI range strip in the first dimension and a 12% acrylamide gel for the second dimension. The most abundant spots, indicated with arrows were submitted to mass spectrometry identification by nano-LC/MS-MS (results in Table 8.2B). Gel spots with dashed circles were those identified in subsequent analysis as having decreased following renneting with Maxiren 180 at pH 6.5.

Table 8.2B. Identification of the most abundant spots from two-dimensional gel of equine milk by nano LC-MS/MS.

Spot	Protein	Accession number[a]	Obs. pI	Obs. MW(kDa)	Theor. pI	Theor. MW(kDa)	Score	Number matching peptides
10	alpha-lactalbumin - horse	P08334	5.25	14	4.95	14.2	424	8
11	alpha-lactalbumin - horse	P08334	5.55	14	4.95	14.2	561	6
12	alpha-lactalbumin - horse	P08334	5.89	14	4.95	14.2	401	9
	Heart-type fatty acid-binding protein	Q9XSI5	5.89	14	5.92	13.9	315	6
13	alpha-lactalbumin - horse	P08334	5.94	14	4.95	14.2	327	7
	Heart-type fatty acid-binding protein	Q9XSI5	5.94	14	5.92	13.9	115	3
14	beta-lactoglobulin II - horse	LGHO2	4.78	18	4.71	18.3	245	5
15	beta-lactoglobulin I - horse	P08334	4.91	19	4.85	18.5	415	9
16	beta-lactoglobulin I - horse	P08334	5.02	20	4.85	18.5	522	11
17	beta-lactoglobulin I - horse	P08334	4.87	25	4.85	18.5	330	7
	Beta-casein - horse	Q9GKK3	4.87	25	5.78	25.5	180	5
	AlphaS1-casein - horse	Q95KZ7	4.87	25	5.57	24.7	114	3
18	beta-lactoglobulin I - horse	P08334	4.97	24	4.85	18.5	266	6
	alpha-lactalbumin - horse	P08334	4.97	24	4.95	14.2	165	3
19	Beta-casein - horse	Q9GKK3	5.3	28	5.78	25.5	264	7
20	**Beta-casein - horse**	Q9GKK3	5.53	28	5.78	25.5	235	6
21	**Beta-casein - horse**	Q9GKK3	5.73	28	5.78	25.5	223	6
22	**Beta-casein - horse**	Q9GKK3	5.97	28	5.78	25.5	237	6
23	**Beta-casein - horse**	Q9GKK3	6.31	29	5.78	25.5	252	7
24	AlphaS1-casein - horse	Q95KZ7	6.53	26	5.57	24.7	329	7
25	kappa-casein - horse	P82187	4.72	33	8.03	18.8	138	3
26	kappa-casein - horse	P82187	4.75	33	8.03	18.8	181	4
27	kappa-casein - horse	P82187	4.79	33	8.03	18.8	61	1
28	Beta-casein - horse	Q9GKK3	4.85	32	5.78	25.5	82	2
29	Beta-casein - horse	Q9GKK3	4.9	26	5.78	25.5	212	6
	AlphaS1-casein - horse	Q95KZ7	4.9	26	5.57	24.7	167	2
30	Beta-casein - horse	Q9GKK3	5.02	27	5.78	25.5	252	6
	AlphaS1-casein - horse	Q95KZ7	5.02	27	5.57	24.7	95	2
31	AlphaS1-casein - horse	Q95KZ7	4.97	24	5.57	24.7	239	5
	Beta-casein - horse	Q9GKK3	4.97	24	5.78	25.5	177	4
32	Beta-casein - horse	Q9GKK3	5.1	26	5.78	25.5	168	5
	AlphaS1-casein - horse	Q95KZ7	5.1	26	5.57	24.7	107	2
33	Beta-casein - horse	Q9GKK3	5.31	26	5.78	25.5	259	7

Cont.

	AlphaS1-casein - horse	Q95KZ7	5.31	26	5.57	24.7	104	2
34	Beta-casein - horse	Q9GKK3	5.44	26	5.78	25.5	232	5
	AlphaS1-casein - horse	Q95KZ7	5.44	26	5.57	24.7	59	2
35	Beta-casein - horse	Q9GKK3	5.75	26	5.78	25.5	194	4
	AlphaS1-casein - horse	Q95KZ7	5.75	26	5.57	24.7	77	3
36	Beta-casein - horse	Q9GKK3	5.98	26	5.78	25.5	267	7
37	**Beta-casein - horse**	Q9GKK3	6.18	25	5.78	25.5	354	9
	AlphaS1-casein - horse	Q95KZ7	6.18	25	5.57	24.7	207	5
38	AlphaS1-casein - horse	Q95KZ7	6.23	26	5.57	24.7	245	5
	Beta-casein - horse	Q9GKK3	6.23	26	5.78	25.5	225	6
39	**AlphaS1-casein - horse**	Q95KZ7	6.48	25	5.57	24.7	454	8
	Beta-casein - horse	Q9GKK3	6.48	25	5.78	25.5	208	6
40	Beta-casein - horse	Q9GKK3	5.13	27	5.78	25.5	248	6
	kappa-casein - horse	P82187	5.13	27	8.03	18.8	180	3
41	Beta-casein - horse	Q9GKK3	5.01	28	5.78	25.5	254	6
	kappa-casein - horse	P82187	5.01	28	8.03	18.8	115	2
42	Beta-casein - horse	Q9GKK3	5.58	26	5.78	25.5	131	3
	AlphaS1-casein - horse	Q95KZ7	5.58	26	5.57	24.7	118	2
43	AlphaS1-casein - horse	Q95KZ7	5.53	26	5.57	24.7	96	1
	Beta-casein - horse	Q9GKK3	5.53	26	5.78	25.5	61	1
44	AlphaS1-casein - horse	Q95KZ7	5.38	25	5.57	24.7	282	3
45	Beta-casein - horse	Q9GKK3	4.85	26	5.78	25.5	220	5
	AlphaS1-casein - horse	Q95KZ7	4.85	26	5.57	24.7	145	3
46	Beta-casein - horse	Q9GKK3	4.74	27	5.78	25.5	239	5
	AlphaS1-casein - horse	Q95KZ7	4.74	27	5.57	24.7	93	2
47	Beta-casein - horse	Q9GKK3	4.25	17	5.78	25.5	87	3
48	Beta-casein - horse	Q9GKK3	4.36	17	5.78	25.5	64	1
49	Beta-casein - horse	Q9GKK3	4.46	17	5.78	25.5	75	2
50	Lactoferrin - horse	O97668	5.93	51	8.32	75.4	793	15
51	Lactoferrin - horse	O97668	6.15	50	8.32	75.4	863	16
52	Lactoferrin - horse	O97668	6.17	75	8.32	75.4	49	2

[a]Accession number corresponds to the Swiss-Prot/NCBI accession number.

Theoretical molecular mass and isoelectric point (pI) of proteins are based on the amino acid primary sequence without taking into account any post-translational and/or degradation modifications. Observed molecular mass and isoelectric points (pI) are those observed from the position of the corresponding spot on the two-dimensional electrophoresis gel.

8.3.5. *Image analysis of the effect of chymosin on the intensity of protein spots of equine and bovine milk*

Two-dimensional gels of renneted bovine and equine milk samples were scanned using SameSpots V4.0 software and protein spots that changed in intensity over time were identified. The protein spots susceptible to chymosin hydrolysis were selected for further analysis. In the case of renneted bovine milk, 6 κ-casein spots decreased over time after chymosin addition (Figure 8.11), and a bar chart was constructed from the combined means intensities of spots 1, 2, 3, 4, 5 and 7, previously identified as bovine κ-caseins (Table 8.2A), for each time point. The result showed the decrease in intensity of bovine κ-caseins with time after chymosin addition (Figure 8.12). Bovine β-caseins were not hydrolysed by chymosin over the time course of this experiment. After 60 min, ~ 75% of bovine κ-casein spots were hydrolysed (25% unhydrolysed by chymosin), except for spots 3 and 5 which were 55.88 and 68.32% unhydrolysed, respectively. The mean therefore of unhydrolysed bovine κ-casein was 36.53 ± 11 %, 60 min after chymosin addition. Spot intensity calculations were based on normalized volumes of κ-casein spots, independently for each spot. Therefore, calculated percentages do not take in to account the actual amount of each spot but are instead a mean value of remaining unhydrolysed κ-casein (%) of 6 independent spots. If calculations were based on remaining κ-casein after 60 min using normalized volumes of all 6 spots, a values of ~ 27% unhydrolysed κ-casein is found. Spots 3 and 5 are less abundant forms of κ-casein found in bovine milk and appear to be less susceptible to chymosin hydrolysis compared to other κ-casein isoforms.

The protein spots in equine milk that were hydrolysed by chymosin over 24 h were identified as β-caseins, i.e., protein spots 20, 21, 22, 23, 47 and 48 as well as 37 and 39 which were identified as mixtures of β and α_{s1}-caseins (Figure 8.10 and Table 8.2B). Image analysis was performed on the 2-DE gel area where the most significant change in the intensity of protein spots were detected (Figure 8.13) and a bar chart was constructed (Figure 8.14) which showed the decrease in intensity of spots of equine

β-casein over 24 h after chymosin addition with the most significant decrease having taken place ~ 4 h after chymosin addition. No change was observed over time with spots identified as κ-casein and there was no evidence that equine κ-casein was hydrolysed by calf chymosin under the conditions of this experiment. This result was in agreement with the C18-RPHPLC of renneted equine milk where the peak which appeared following chymosin hydrolysis was identified as β-casein. Egito *et al*. (2001) reported the slow hydrolysis of equine κ-casein by chymosin with peptides generated ~ 10-20 min after chymosin addition when analysed by C18 RP-HPLC. In Section 5.3.7 it was demonstrated that the particle size distribution of equine milk renneted with calf chymosin did not increase significantly at pH 6.6 and 30^{o}C until ~ 4 h after chymosin addition which is in agreement with the result in this study where most significant change in protein (β-casein) spot intensity occurred ~ 4 h after chymosin addition.

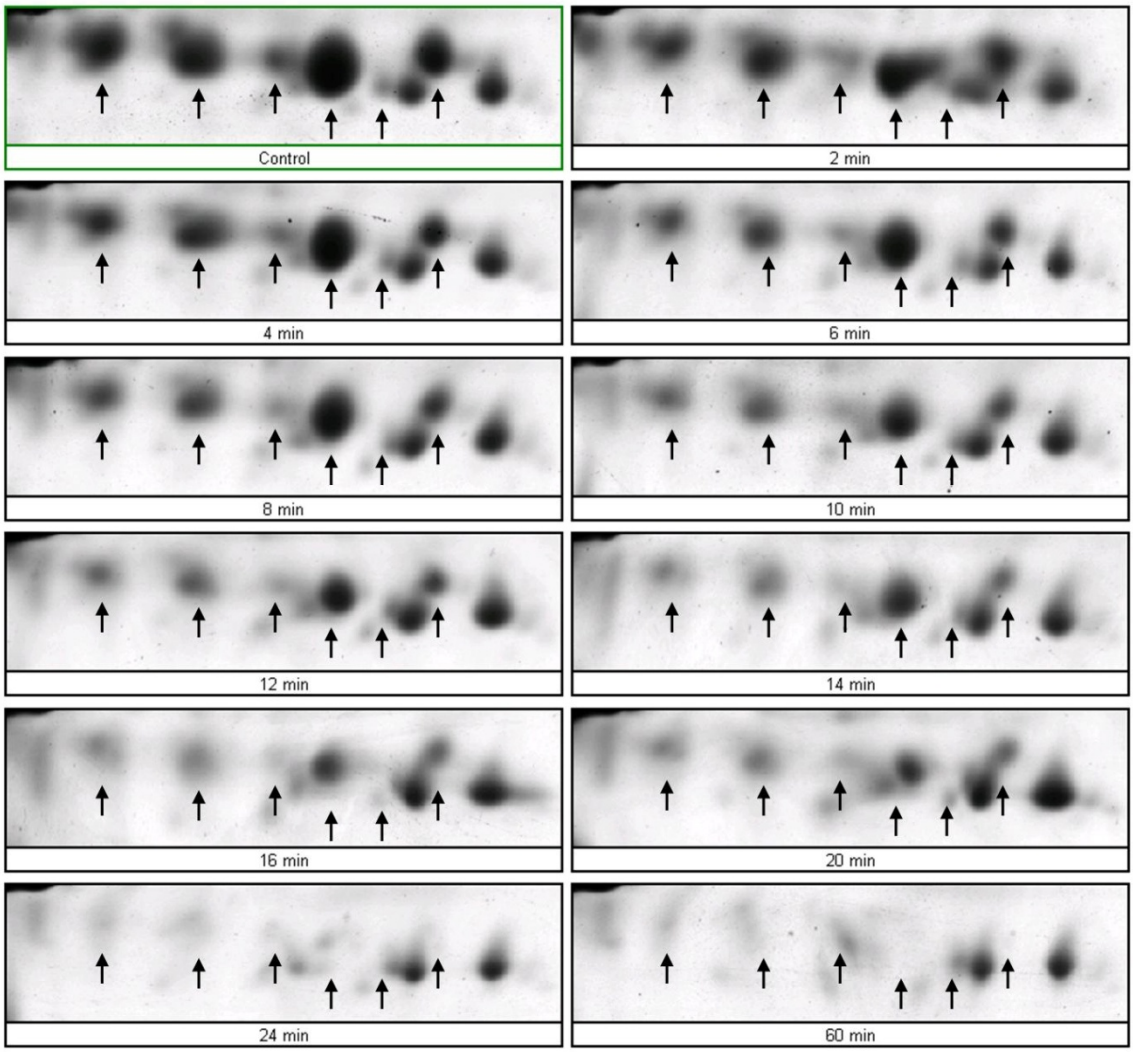

Figure 8.11. Image analysis of κ-casein spots from 2-DE gels of bovine milk renneted from 0 to 60 min. Arrows indicate the κ-caseins that changed in intensity (amount).

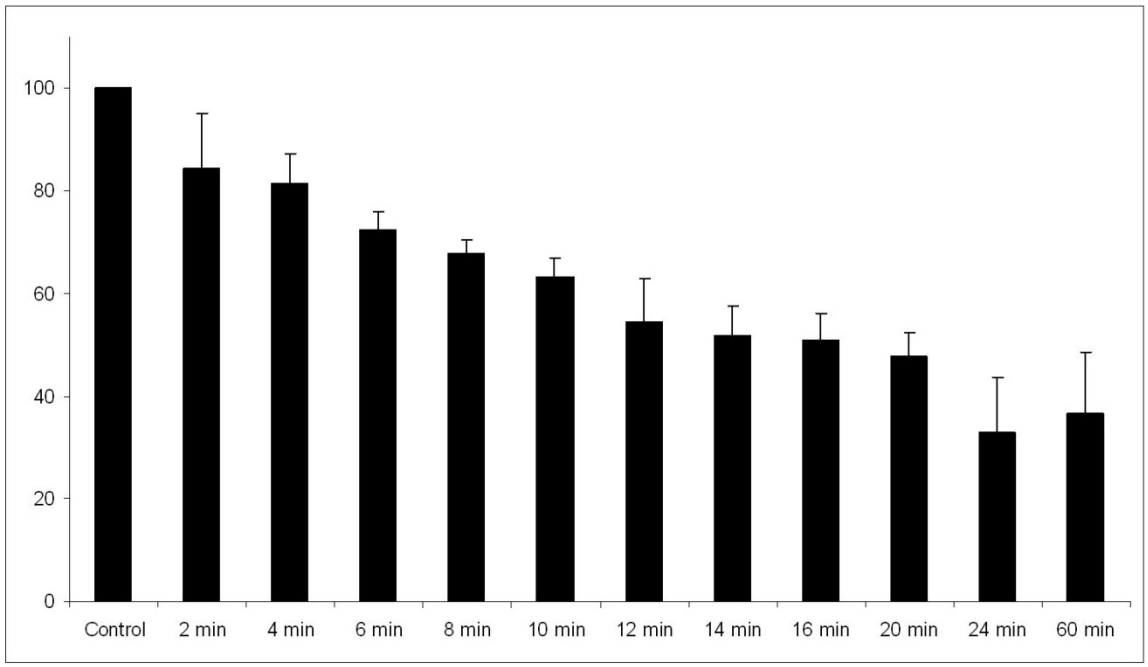

Figure 8.12. Quantitation of the intensities of bovine κ-casein spots over time after chymosin addition. Bars represent the combined means of intensities of spots 1, 2, 3, 4, 5 and 7 (Table 8.2A) at each time point with data variability represented by error bars. Selected protein spots were identified by MALDI-TOF analysis of a preparative 2-DE gel of bovine milk (Figure 8.9). Y-axis is the intensity of each protein spot calculated as a % of a control. Analysis was carried out on at least duplicate 2-DE gels for each time point.

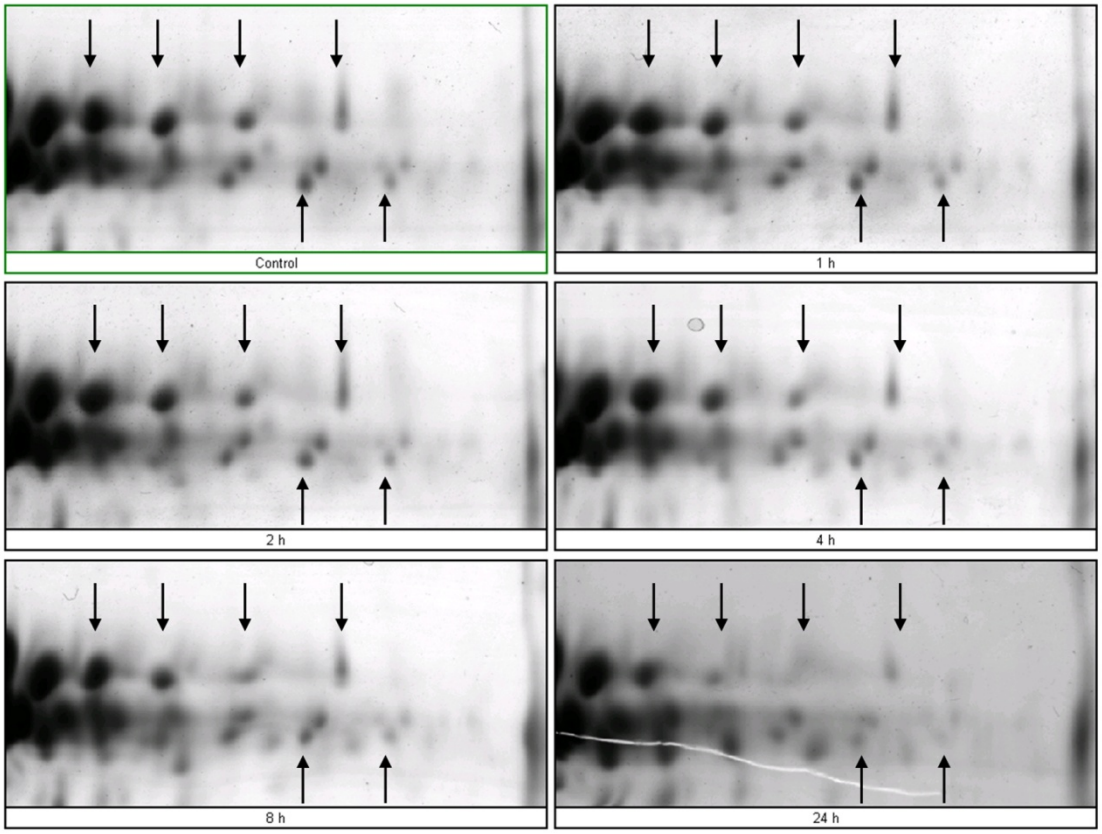

Figure 8.13. Image analysis of β-casein spots from 2-DE gels of equine milk renneted from 0 to 24 h. Arrows indicate the β-caseins spots that changed in intensity (amount).

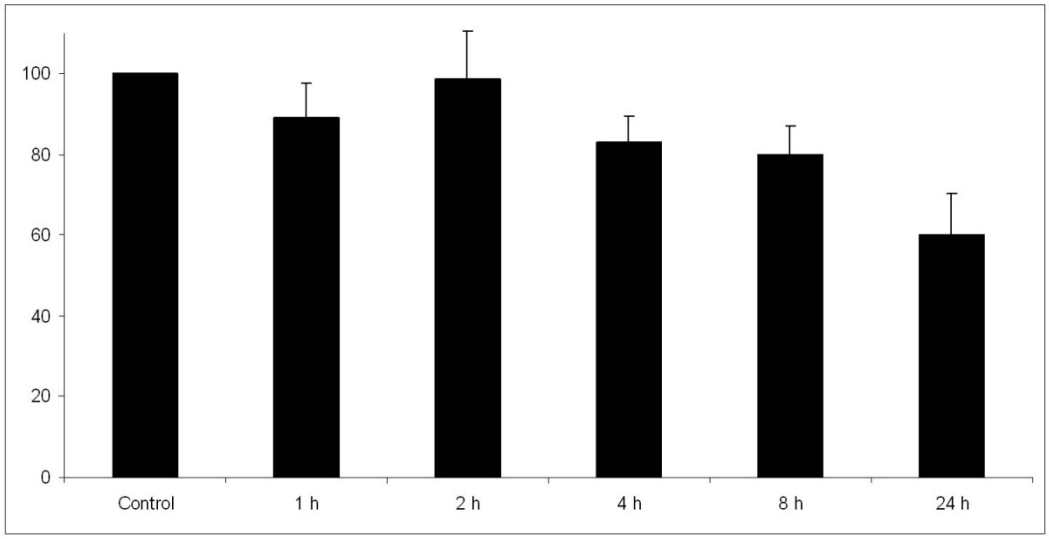

Figure 8.14. Quantification of the intensities of equine β-casein spots over time after rennet addition. Bars represent the combined means of intensities of spots 20, 21, 22, 23, 37, 39, 47 and 48 (Table 8.2B) at each time point with data variability represented by error bars. Selected protein spots were identified by nano-LC MS/MS analysis of a preparative 2-DE gel of equine milk (Figure 8.10). Y-axis is the intensity of each protein spot calculated as a percentage of a control.

The level of κ-casein in equine milk is low (Malacarne *et al*., 2000, 2002; Egito *et al*., 2001, 2002; Miranda *et al*., 2004) and it has been reported that the steric stabilization of equine casein micelles by κ-casein may be aided by non-phosphorlated β-casein (Ochirkhuyag *et al*., 2000). This study showed that at least some equine β-casein may occupy a surface location in the equine casein micelles and is readily hydrolysed by chymosin, compared to equine κ-casein which was not hydrolysed under the condition of this study. Whether it is phosphorylated or non-phosphorylated forms of equine β-casein that is hydrolysed by chymosin is unknown and it is speculative to assume that equine casein micelles are stabilized by β-casein but phosphorylated forms of β-casein are hydrophilic and, in theory, could contribute to micellar stability although they could be precipitated by calcium.

Conclusions

2-DE allows simultaneous detection and quantification of several thousand protein spots in the same gel (Galvani *et al*., 2000; Gorg *et al*., 2004; Chevalier, 2010) when combined with mass spectrometry. To date, analysis of the effects of various processing treatments on bovine milk proteins using proteomics has been confined to exploring the changes in disulfide bonds in milk proteins after heating or pressure treatment (Patel *et al*., 2006; Chevalier *et al*., 2009; Chevalier & Kelly, 2010). This study demonstrated the ability of 2-DE coupled with mass spectrometry and image analysis to follow the effects of renneting on equine and bovine milk proteins over time. 2-DE and MS allowed the simultaneous evaluation of the relative abundance and modification of the proteins in equine and bovine milk following hydrolysis by calf chymosin. Equine milk was susceptible to hydrolysis by calf chymosin over 24 h at 30^{o}C and pH 6.5 but the peptides produced were from equine β-casein, which was confirmed by MS analysis of C18-RPHPLC peptides. 2-DE separated and discriminated between the different proteins of equine milk and provided information on the range of isoforms of each protein as a result of post-translational modifications as well as positively identifying several isoforms of κ-casein in equine milk. Post-translational modifications of proteins can change the molecular weight, isoelectric points, solubility and sensitivity to staining methods. Equine proteins are highly phosphorylated and equine κ-casein has been reported to be highly glycosylated, making it difficult to identify by mass spectrometry. Glycosylation also limits protein staining on acrylamide using Coomassie Blue. Dephosphorylation of peptides produced by chymosin from equine proteins eliminated the problem of low ionization efficiency of phosphorylated peptides in complex mixtures (Poth *et al*., 2008) and enabled the positive identification of equine κ-casein.

8.5 References

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CHAPTER 9

Electron Microscopy Studies on Equine and Bovine Casein Micelles

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Abstract

In this study the casein micelles in equine and bovine milk were studied and compared using a variety of microscopy techniques and the effects of added trisodium citrate, rennet and, in the case of equine milk, glucono-δ-lactone on milk systems was studied. By combining several microscopy methods in one study it was hoped that the limitations of any one method would be minimized and that a clear idea of equine casein micelle structure, in particular, would emerge. One of the techniques used, cryo-scanning transmission electron microscopy (cryo-STEM), involves the preparation and imaging of casein micelles with the least disruption of micellar structure reported to date. However, simpler techniques, if undertaken carefully, such as negative staining and TEM imaging can yield detailed images of casein micelles. The high resolution obtained by cryo-SEM allowed very clear images to be obtained of equine and bovine casein micelles in their native state and when treated with acid or chymosin. The size of equine casein micelles found in this study using TEM with negative staining or TEM of ultra-thin sections were similar and compared well with the average equine casein micelle size measured by dynamic light scattering (~ 275 nm). Equine casein micelles appeared to be more uniform and symmetrical than bovine casein micelles. Electron micrographs showed the dense packing of bovine micelles in milk samples compared to far fewer and larger micelles in equine milk. Acidification or renneting of equine milk caused the formation of small clusters of micelles throughout the sample but no fusion or contact between clusters.

Keywords: Equine milk; casein micelles; cryo-fixation, STEM, SEM, TEM,

9.1. *Introduction*

In the milk of all species studied in sufficient detail, the caseins exist predominantly as micelles, which are hydrated spherical structures with dimensions in the sub-micron region (de Kruif & Holt, 2003; Farrell *et al*., 2006; Horne, 2006). The dry matter of casein micelles consists predominantly (>90%) of proteins (α_{s1}-, α_{s2}-, β- and κ-caseins), with small amounts of inorganic matter, collectively referred to as colloidal calcium phosphate (CCP). In some species, not all of these types of casein are present and the relative and absolute concentrations of the different caseins differ (Dalgleish, 2011). The protein sequences are not well-conserved between species (Ginger & Grigor, 1999). α_s- and β-Caseins are extensively phosphorylated and have many Ser-Xaa-Glu/PSer (Xaa denotes any amino acid; PSer represents phospho-serine) sequences in which the first serine residue is usually phosphorylated (Holland, 2009). A contiguous sequence of 3 phosphoseryl residues followed by two glutamic acid residues: -SerP-SerP-SerP-Glu-Glu- forms a phosphoseryl cluster (Meisel *et al*., 1989; Meisel, 1997). The level of phosphorylation enables the caseins to bind large amounts of calcium ions which can lead to their precipitation (Dalgleish, 2011). Phosphoseryl residues also enable the proteins to bind to calcium phosphate. κ-Casein is only singly phosphorylated and does not precipitate in the presence of Ca^{2+}; the molecule is also glycosylated (Dalgleish, 2011). The integrity of casein micelles is maintained by micro-clusters of calcium phosphate stabilized by a shell of highly phosphorylated caseins and hydrophobic interactions (Horne, 2002; de Kruif & Holt, 2003). Growth of casein micelles is terminated by the solvent-mediated adsorption of κ-casein onto the micellar surface (De Kruif & Holt, 2003). The average size of the casein micelles in milk is determined by the proportion of κ-casein in the caseins (Dalgleish, 2011). The hydrophilic C-terminal region of κ-casein or 'brush' protrudes from the surface of the micelles and sterically stabilizes them against aggregation (De Kruif & Zhulina, 1996). Casein micelles play a crucial role in the physico-chemical stability of milk, e.g., enzyme-, acid- , heat- or ethanol-induced coagulation, thereby affecting the manufacture and stability of products such as cheese, yoghurt, evaporated milk or cream liqueurs, respectively. Coagulation of casein micelles can occur only following collapse of the brush, which occurs on acidification of milk, e.g., in the manufacture of yoghurt or on removal of the brush which occurs on rennet-induced coagulation of milk. The combined process of enzyme- and acid-induced coagulation contributes to the coagulation of casein micelles in the stomach.

Bovine casein micelles are polydisperse, ranging in diameter from 80 to 500 nm with a mean diameter of ~ 150 nm and a molecular mass of 10^6 to 10^9 Da (Fox & McSweeney, 1998;

Walstra *et al*., 1999). Casein micelles in bovine milk are quite tightly packed, with 10^{14} to 10^{16} micelles per mL of milk with a distance apart of approximately two micelle diameters (Fox, 2003). The structure and sub-structure of bovine casein micelles has been studied in detail and reviews include; Holt (1992), Rollema (1992); Holt & Horne (1996), Horne (1998, 2002, 2006, 2011), McMahon & McManus, (1998), Walstra (1999), De Kruif & Holt (2003), Dalgleish *et al*., 2004, Phadungath (2005), Farrell *et al*. (2006), Qi (2007), Fox & Brodkorb (2008), McMahon & Oommen, 2008; Bouchoux *et al*. (2010), Dalgleish (2011) and Horne (2011).

Models of casein micelle structure fall into three general categories; the coat-core model (Waugh & Noble, 1965; Waugh, 1971; Waugh & Talbot, 1971), the internal structure model (Rose, 1969; Garnier & Ribadeau-Dumas, 1970) and the sub-micelle model (Morr, 1967; Slattery & Evard, 1973; Schmidt & Payens, 1976; Schmidt, 1980, 1982a; Walstra & Jenness, 1984; Walstra, 1990). While the three models differ considerably regarding the structure of the casein micelle, they agree that the surface of the casein micelle is composed primarily of κ-casein which protrudes into the serum phase and stabilizes the structure against aggregation by steric stabilization. The proposal of Holt (1992, 1994) that the casein micelle is almost spherical, highly hydrated and relatively open is one of the most widely accepted models and that the N-terminal of κ-casein is linked to the micelle *via* strong hydrophobic interaction. Horne (1998; 2006) proposed a dual-binding model for the casein micelle which differs from that of Holt in the size of the nanocluster and the number of phosphate clusters that the surface of the nanocluster can accommodate. The Horne model describes the association of caseins as a balance of attractive hydrophobic interaction and electrostatic repulsion.

Equine casein micelles have an average diameter of ~ 255-275 nm (Welsch *et al*., 1988; Buchheim *et al*., 1989; Section 3.6.6). Electron microscopy shows a 'spongy' appearance for equine and bovine micelles, although bovine micelles appear more ordered and equine micelles 'looser', while human micelles are considerably looser than equine micelles (Jasińska & Jaworska, 1991). Such a loose open structure may affect the susceptibility to hydrolysis by pepsin. Jasińska & Jaworska (1991) reported that human micelles are much more susceptible to pepsin hydrolysis than either equine or bovine micelles.

The sub-structure of equine casein micelles has not been studied in detail but some information may be derived from comparison with bovine milk. Based on data from an earlier study, the calcium-binding capacity of equine casein far exceeds the amount of calcium bound (~ 13 mmol L^{-1}, Section 3.5.2), and therefore, it may be assumed that like bovine micelles,

equine micelles contain nanoclusters of calcium phosphate. Both equine α_{s1}-casein (residues 79-83) and β-casein (residues 23-27) contain a phosphorylation cluster, which is required for the formation of nanoclusters (De Kruif & Holt, 2003); both proteins also contain distinct hydrophobic regions through which solvent-mediated protein-protein interactions may occur. The ratio of micellar calcium: micellar inorganic phosphate is 3.76:1 in equine milk, but 2.2:1 in bovine milk (Section 3.2) and might indicate that either a smaller proportion of micellar calcium is incorporated into nanoclusters in equine milk, or that equine nanoclusters contain a higher proportion of casein-bound phosphate.

The presence of κ-casein in equine milk was an issue of debate for several years, with several authors (Visser *et al*., 1982; Ono *et al*., 1989; Ochirkhuyag *et al*., 2000) reporting its absence but others (Kotts & Jenness, 1976; Malacarne *et al*., 2000; Iametti *et al*., 2001; Egito *et al*., 2001) demonstrating its presence, albeit at a low concentration. In Chapter 8, using proteomic techniques, it was definitively shown that equine milk contains κ-casein which did not appear to be susceptible to hydrolysis by calf chymosin. Unlike bovine κ-casein, equine κ-casein does not have a distinctly hydrophilic C-terminal domain and the level of glycosylation which, if high, would enhance the ability of κ-casein to stabilize the micelle (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996), has not been established (Section 2.3.5). It is therefore unclear if the C-terminal of equine κ-casein is capable of protruding from the micellar surface to sterically stabilize it. Ochirkhuyag *et al*. (2000) and Doreau & Martin-Rosset (2002) concluded that the steric stabilization of equine casein micelles by κ-casein may be aided by non-phosphorlated β-casein on the surface of the micelle, thus compensating for the low κ-casein content. In model systems however, the OP– and 1P- forms of human β-casein have been reported not to show any stabilizing ability for 2P or 4P forms in the presence of Ca^{2+} ions (Sood & Slattery, 2002). Azuma *et al*. (1985) reported that highly phosphorylated human β-casein can form casein micelles with κ-casein and the highly phosphorylated β-casein is, in turn, stabilized by the co-operative function of lesser phosphorylated β-caseins and κ-casein.

In Chapter 3 it was shown that the colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles. Equine casein micelles are more stable to coagulation by acid, rennet and, at certain pH values, heat, but are less stable to ethanol-induced coagulation. It was concluded that several of these observations could be attributed to the exceptionally high level of ionic calcium and low level of protein in equine milk compared to bovine milk, coupled with the large size of equine casein micelles and the

exceptionally low level of κ-casein that has been reported for equine milk. κ-Casein was definitively found in equine milk (Chapter 8) and the challenge next was to observe the structure of the equine casein micelle in detail.

Advances in electron microscopy has made it possible to observe directly the microstructure of micelles (Shimmin & Hill, 1964, 1965; Knoop *et al*., 1979; Schmidt, 1982b). Surface images can be obtained by scanning electron microscopy (SEM) without metal coating (Dalgleish *et al*., 2004), and cross-sections of the internal structure can be seen by using transmission electron microscopy (TEM) of freeze-fractured cryo-protected casein micelle suspensions (Heertje *et al*., 1985; Karlsson *et al*., 2007). Total (surface and internal) images can be obtained by TEM of freeze-dried surface-immobilized casein micelles without embedding in resin and sectioning (McMahon & McManus, 1998) and by cryo-TEM of thin vitrified films of casein micelle suspensions (Marchin *et al*., 2007). Micrographs can be difficult to interpret and the sample preparation involved can induce changes to the microstructure of the micelles (McMahon & McManus, 1998; Karlsson *et al*., 2007). The challenge in electron microscopy is to produce micrographs that exhibit minimal variation of the casein micelle from its native form (McMahon & McManus, 1998; McMahon & Oommen, 2008).

The purpose of this study was to examine and compare the micelles of equine and bovine milk using a variety of microscopy techniques and to study the effects of trisodium citrate, rennet and acidification on the micelles of both milk systems. By combining several microscopy methods in one study it was hoped that the limitations of any one method would be minimized and a clear idea of equine casein micelle structure, in particular, would emerge. One of the techniques, cryo-scanning transmission electron microscopy (cryo-STEM), used involves the preparation and imaging of casein micelles with the least disruption of micellar structure reported to date.

9.2. Materials and Methods

9.2.1. Milk supply and sample preparation

Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from 5 milkings over 24 h, from a herd of multiparous New Forest and New Forest/Arabian mares in mid-lactation, physically separated by day from their foals. The milk was defatted by centrifugation at 1,000 x g using a Sorvall® RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 20^{o}C for 20 min followed by filtration through

glass wool to remove fat particles. Raw whole bulk bovine milk was obtained from a local dairy farm and was defatted by centrifugation at 2,000 g for 20 min at 20^{o}C, followed by filtration through glass wool. Sodium azide (0.5 g L^{-1}) was added to the skimmed milks to prevent microbial growth.

All chemicals used were of reagent grade, obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Chemicals used for TEM were of electron microscopy grade, purchased from Agar Scientific Ltd. (Stansted, UK).

High purity, low melting point agar (80-85^{o}C; Fluka Biochemika) was purchased from Sigma-Aldrich Chemie GMbH (Buchs SG, Germany).

Imaging was done on several independent samples of equine or bovine milk and representative micrographs were selected and viewed from a large number of fields for each sample examination. Magnifications of samples are presented as values multiplied by K, where K is 10^3.

9.2.2. Determination of average casein micelle size in equine milk

The size distribution and average size of casein micelles in equine milk diluted 1:250 with lactose-free synthetic milk ultrafiltrate (SMUF; Jenness & Koops, 1962), was determined at 20^{o}C by fixed-angle dynamic light scattering (DLS), using a Malvern Zetamaster 3000 instrument (Malvern Instruments, Malvern, Worcestershire, UK) equipped with a 632.8 nm He-Ne laser at a scattering angle of 90^{o}. Skimmed equine milk was diluted with SMUF and filtered through Whatman No. 40 paper immediately prior to analysis to eliminate any effects of dilution. Analysis was carried out in triplicate.

9.2.3. Dissociation of equine and bovine casein micelles by trisodium citrate

Raw skimmed equine milk was centrifuged at 100,000 g for 60 min at 20^{o}C using an Optima LE-80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA), equipped with a Beckman 50.2 Ti rotor (12 place); the pelleted micelles were resuspended in equine milk dialysate which was prepared by exhaustive dialysis of a 66 g L^{-1} lactose solution against 2 x 20 volumes of equine milk for 48 h at 20^{o}C. The protein content of the suspension was adjusted to 25 g L^{-1}, which approximates the casein concentration of bovine milk. Sodium azide (0.5 g L^{-1}) was added as preservative. Skimmed bovine milk was used without modification.

It was reported in Section 6.5.3, that ≥ 0.04 M trisodium citrate caused extensive dissociation of both equine and bovine casein micelles. In this study,

0.04 M trisodium citrate was added to skimmed bovine milk and to equine casein suspended in dialysate. The samples were vortexed for 1 min, after which gluteraldehde (250 g L^{-1}) was added in a ratio of 9:1, v/v, milk:gluteraldehyde to give a final gluteraldehyde concentration of 25 g L^{-1}. Samples were negatively stained and imaged using TEM.

9.2.4. Renneting equine and bovine milks

Skimmed equine and bovine milks were adjusted to pH 6.5 and tempered at 30^{o}C for 20 min in thin-walled glass tubes in a thermostatically controlled waterbath. Ten μL mL^{-1} of a 1:10 (v/v) aqueous dilution of fermentation-produced chymosin (Maxiren 180, 180 international milk-clotting units (IMCU) per mL, DSM Food Specialities, Delft, The Netherlands) were added and the tubes were oscillated gently in the waterbath. Rennet coagulation time was recorded as the first sign of flocculation, i.e., visible curd flocs on the wall of the tube, and was ~ 15 min for bovine milk and ~ 6 h for equine milk. Gluteraldehyde was added to prevent further micellar aggregation (Green *et al*., 1978a) and to fix the samples for microscopy. For negative staining and examination by TEM, gluteraldehyde was added at a ratio of 9:1 milk:gluteraldehyde to a final gluteraldehyde concentration of 25 g L^{-1}. For cryo-scanning electron microscopy (cryo-SEM), chymosin activity was stopped by heating the samples to ~ 65^{o}C.

9.2.5. Acidification of equine milk

Skimmed equine milk was acidified to pH ~ 4.2 by the addition of 30 g L^{-1} glucono-δ-lactone at 30^{o}C (~ 120 min). For thin sectioning, the samples were flooded with 2.5 % gluteraldehyde in 0.165 M phosphate buffer, pH 7.2. For cryo-SEM imaging, samples were prepared immediately after the pH reached ~ 4.2.

9.2.6. TEM

9.2.6.1. Negative staining

Fresh skimmed equine milk was fixed with electron microscopy (EM)-grade, aqueous gluteraldahyde in a ratio of 9:1, v/v, milk:gluteraldehyde to a final gluteraldehyde concentration of 25 g L^{-1} by the addition of 100 μL of 250 g L^{-1} gluteraldehyde to 900 μL milk in an Eppendorf tube. Ten μL of each fixed sample was mixed with 10 μL of 20 g L^{-1}

phosphotungstic acid (PTA), pH 7.2, and held for 5 min. A Formvar-coated copper grid (400 mesh; Agar Scientific, Stansted, UK) was immersed in the mixture for 5 min after which it was dabbed around the periphery with filter paper to remove excess sample. The sample was allowed to air-dry at room temperature under a clean up-turned Petri-dish and was imaged the following day.

For TEM, fixed samples were diluted 1:50 with 0.01 M $CaCl_2$ before staining (Harwalker *et al*., 1989). Samples dissociated with 0.04 M trisodium citrate were examined undiluted.

9.2.6.2. Thin section transmission electron microscopy

Samples of equine and bovine milk were fixed with gluteraldehyde to a final concentration of 25 g L^{-1} gluteraldehyde in 0.165 M phosphate buffer, pH 7.3, for ~ 2 h with gentle agitation. Milk samples were then encapsulated in agar gel to prevent disintegration during subsequent preparation steps, according to the method of Green *et al*. (1978a), taking care to eliminate air bubbles and froth. Once solidified, the agar blocks were cut carefully into ~ 1 mm^3 cubes. Specimens were washed in 0.165 M phosphate buffer (pH 7.3) for 30 min, and post-fixed in 20 g L^{-1} osmium tetroxide in the same buffer for 2 h, followed by another wash in 0.165 M phosphate buffer. Specimens were dehydrated in an ascending ethanol series (10 to 100%) over a 4 h period, finishing with 30 min in propylene oxide, and embedded in Araldite resin.

For each specimen, semi-thin (0.5μm) sections were obtained from polymerized blocks using an ultramicrotome (Reichert-Jung Ultracut E, Leica, Wetzlar, Germany). Semi-thin sections were stained with toluidine blue and examined using an Olympus BX40 light microscope (Olympus Microscopy, Southend-on Sea, Essex, UK) to ensure that sections contained sufficient sample for examination. Thin sections (70-90 nm) from selected areas of the trimmed blocks were made and collected on Formvar-coated copper grids (100 mesh; Agar Scientific). Thin sections were double stained with 20 g L^{-1} uranyl acetate and Reynolds lead citrate stain.

TEM was carried out using a transmission electron microscope (Joel 2000FXII, Joel Ltd., Tokyo, Japan), at an accelerating voltage of 80 kV. Digital acquisition of areas of interest from TEM images was carried out using a Megaview-III digital camera and AnalySIS software (Olympus Soft Imaging Solutions GmBH., Munster, Germany).

9.2.7. STEM, cryo-SEM and cryo-STEM

STEM, cryo-SEM and cryo-STEM were performed using a Zeiss Supra 40VP field emission scanning electron microscope (FE-SEM) with a Gemini® Multi-mode STEM parallel solid-state quadrant diode detection system (Carl Zeiss AG, Darmstadt, Germany) fitted with a Gatan Alto 2500 cryo-preparation system (Gatan UK, Abingdon, Oxon). The instrument is also equipped with a specially made cryo-STEM sample holder, as described by Gee *et al*. (2010). Images were captured using an SE2 secondary electron detector.

9.2.7.1. STEM

Scanning transmitted electron microscopy (STEM) was performed on equine and bovine milk samples diluted 1: 100 (v/v) with lactose-free synthetic milk ultrafiltrate (SMUF; Jenness & Koops, 1962). The samples were negatively stained using 1% PTA on carbon film (400 mesh; Agar Scientific).

9.2.7.2. Cryo-SEM

Samples of equine or bovine milk were prepared for cryo-SEM by mounting them into copper rivets and plunging them into nitrogen slush (-207^{o}C) or by metal mirror cryofixation. For metal mirror cryofixation, ~ 10 μL of milk sample were placed into a gold planchette which was mounted onto a cushioned carrier. Samples were then placed into the impacter (Alt327 Impact Freezing Device, Gatan, Abingdon, Oxon) and dropped onto a liquid nitrogen-cooled block (polished metal mirror). Samples prepared by freezing in nitrogen slush or by metal mirror cryofixation were transferred under vacuum to the preparation chamber, freeze-fractured with a cold blade, etched at -95^{o}C for 1 min and then sputtered-coated with gold (9 mA for 60 s). Sampled were then transferred under vacuum to the cold stage of the microscope which was maintained at -125^{o}C and imaged using FE-SEM at 2.00 kV.

9.2.7.3. Cryo-STEM

To retain fine micellar structural detail of both equine and bovine casein micelles, the cryo-method of sample preparation and imaging of Gee *et al*. (2010) was used. Sample preparation involved placing a small drop (~ 2 μL) of raw skimmed equine or bovine milk into a carbon-coated copper TEM grid (400 mesh; Agar Scientific). The edge of the grid was blotted gently with filter paper. To prevent evaporation, the grid and sample was cryo-fixed immediately by plunging it directly in to liquid nitrogen slush (-210^{o}C) in the cryo-preparation chamber.

Bright and dark-field STEM images were acquired at -135^{o}C at an accelerating voltage of 25 kV using the Gemini® multi-mode STEM detector and a working distance of 5 to 7 mm.

9.3. Results and Discussion

9.3.1. Average size of casein micelles

The average casein micelle size in bulk equine milk measured by fixed-angle dynamic light scattering was 275.50 ± 2.1 nm which agreed with a value reported in a previous study (Section 3.6.6) and by Welsch *et al*. (1988) and Buchheim *et al*. (1989) and is considerably larger than the average size of bovine casein micelles, ~155-180 nm (Section 3.6.6; Lin *et al.*, 1971, Schmidt *et al.*, 1973; Holt *et al.*, 1978; Walstra & Jenness, 1984; de Kruif, 1998 and Glantz *et al*, 2010).

9.3.2. Transmission electron microscopy

9.3.2.1. Negative staining

TEM images of negatively stained equine and bovine casein micelles are shown, at various magnifications in Figures 9.1. The images confirmed that the casein micelles in equine milk appeared remarkably spherical and uniform in shape (Figure 9.1 A and B). In contrast, bovine casein micelles were more difficult to image clearly and appeared more packed into the field viewed (Figure 9.1 C, D). Bovine micelles were less uniform in shape than equine micelles and had diffuse, ragged, edges (Figure 9.1 D). A very distinctive halo-like effect was evident surrounding the equine casein micelles which was less pronounced around bovine micelles. McMahon & Oommen (2008) reported the presence of a halo around bovine casein micelles and concluded that it was due to a reduced concentration of non-colloidal protein bound to the surface of the carbon grid in the areas close to each micelle. Equine casein micelles appeared to have a different internal density to bovine micelles (Figures 9.1 A and 9.1 D, respectively). It has been reported that micelles imaged using TEM can exhibit central regions which are darker than the periphery and that these areas do not represent a change in electron density but represent thicker areas of sample with greater scattering power (McMahon & Oommen, 2008).

Using imaging software, equine and bovine micelle size was measured from the images in Figures 9.1 B and 9.1 D. A representative size measurement of an equine casein micelle is shown at a magnification of 100 K in Figure 9.2. Equine casein micelles ranged in size from

~ 157 to 350 nm in diameter with an average diameter of ~ 280 nm and were larger than those in bovine milk, which ranged in size from 117 to 167 nm. These values are in agreement with the sizes calculated by photon correlation spectroscopy, above. Measurements of equine casein micelles obtained from electron micrographs agree well with the results of Buchheim *et al.* (1989) who reported that equine casein micelles were considerably larger than bovine casein micelles. In this study, the equine casein micelles were found to be considerably more polydisperse than bovine micelles which was also reported in Section 3.3.6 where the radius of equine and bovine casein micelles was measured by fixed angle dynamic light scattering (DLS). In DLS analysis, a higher dependence of the radius of equine micelles on the scattering wave vector than that of bovine casein micelles was reported (Section 3.3.6) which has been attributed to a more polydisperse size distribution of casein micelles in equine milk (Finsy, 1994). The negative staining technique used here and later with STEM, is a simple and rapid method of studying the morphology and structure of casein micelles and provided good images of equine and bovine casein micelles under a variety of experimental conditions as well as being a means of accurately measuring the size of equine and bovine casein micelles. The negative stain gathers around a structure and in the empty spaces within a structure, providing very high contrast. The major drawback of the technique is that negative staining can result in artefacts on the sample grid due primarily to uneven staining. The deposition of heavy atom stains can also result in structural artefacts such as flattening of a sample. Dilution of the equine and bovine samples with 0.01 M $CaCl_2$ prior to fixation allowed the imaging of micelles with apparently little disintegration of structure, which was also observed by Harwalker *et al*. (1989).

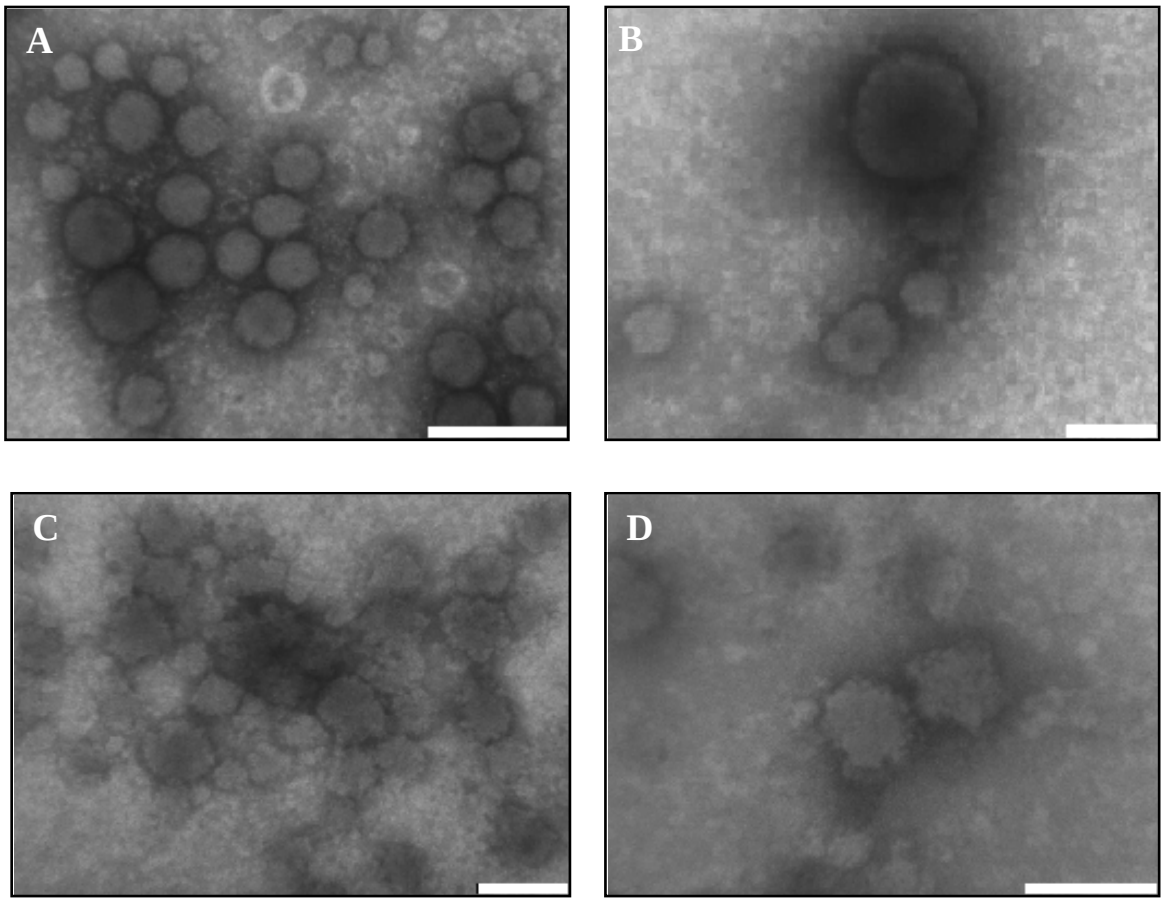

Figure 9.1. Transmission electron micrographs of the casein micelles in equine (A and B) and bovine milk (C and D) diluted 1:100 (v/v) with 0.01 M $CaCl_2$ and negatively stained using 20 g L^{-1} PTA. Magnifications: **A**, 60 K. **B**, 100 K. **C**, 60 K. **D**, 100 K. Scale bars: 500 nm (A), and (C) and 200 nm (B) and (D).

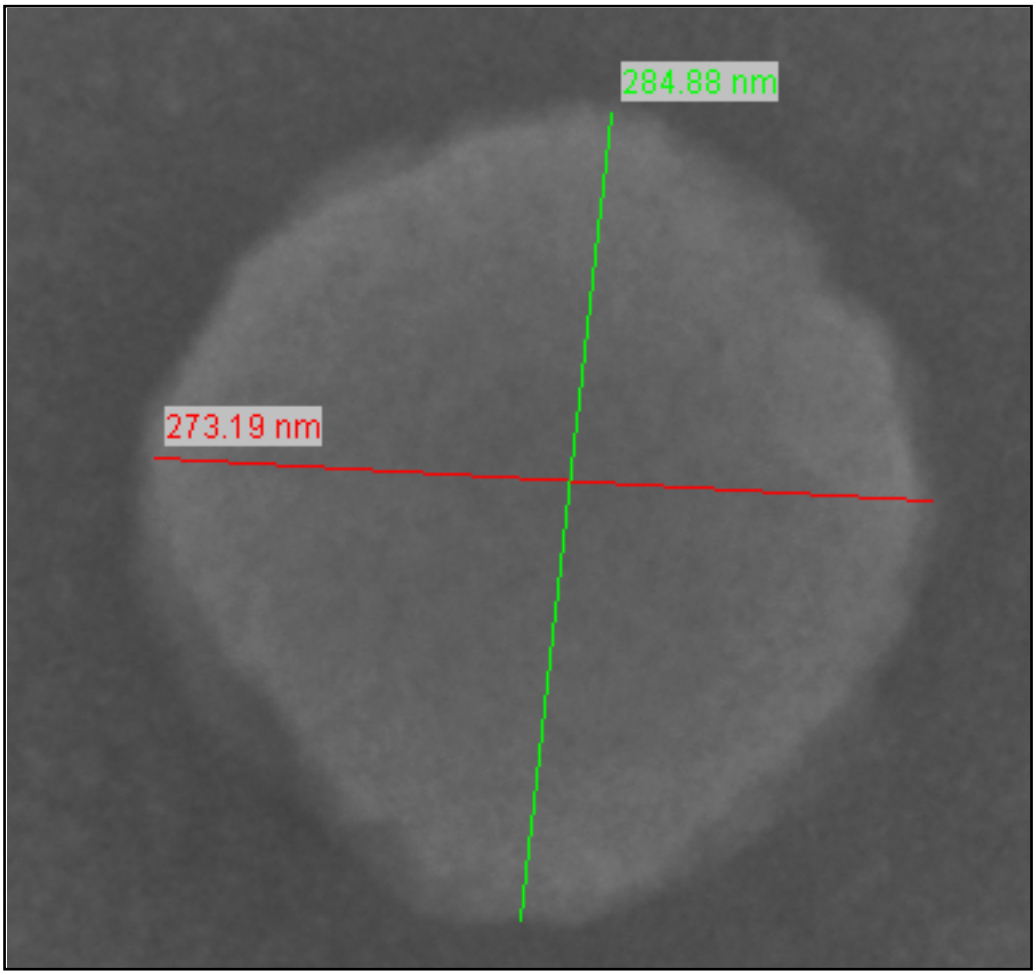

Figure 9.2. Measurement of an equine casein micelle using transmission electron microscophy. Equine milk was diluted 1:100 (v/v) with 0.01 M $CaCl_2$ and stained with 20 g L^{-1} PTA. Magnification of 100 K.

The dissociation of equine and bovine casein micelles with 0.04 M trisodium citrate as determined by imaging using TEM is shown in Figure 9.3 at a magnification of 50 K. Equine micelles suspended in dialysate at a casein concentration of 25 g L^{-1}, which is similar to that of bovine milk, dissociated extensively and, in both milks, few micelles remained intact in the presence of citrate (Figure 9.4 A, B). Trisodium citrate chelates calcium leading to a decrease in free calcium and solubilisation of colloidal phosphate with loss of micelle integrity (Pyne, 1962; Morr, 1967; Walstra, 1990; Pouliot *et al*., 1994). It is likely that the calcium phosphate nanoclusters in equine milk have a similar role in maintaining micellar stability as those of bovine milk.

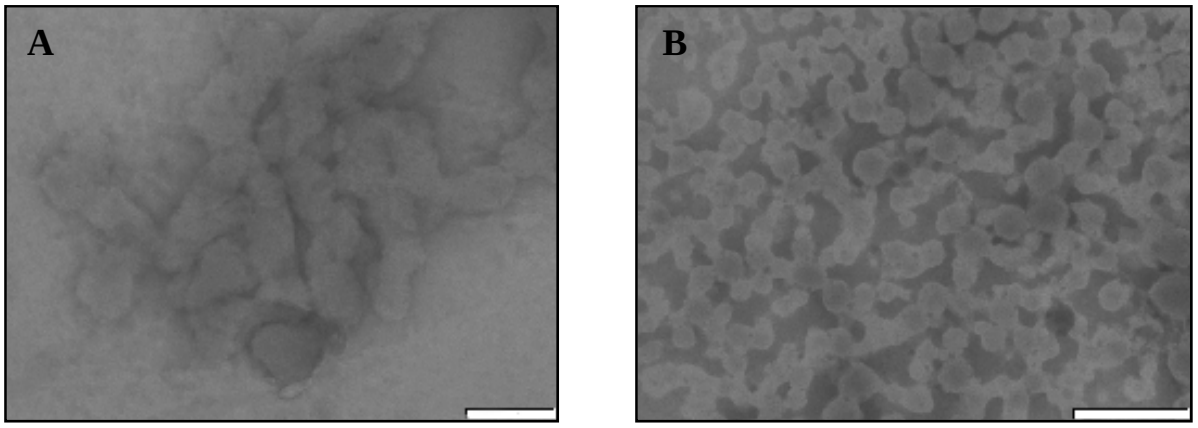

Figure 9.3. Transmission electron micrographs of equine (A) and bovine (B) casein micelles dissociated with 0.04 M trisodium citrate and negatively stained using 20 g L^{-1} PTA. Magnification of 50 K. Scale bars: 200 nm (A) and 500 nm (B).

Equine and bovine milks renneted with chymosin and imaged by TEM with negative staining are shown in Figure 9.4. It was reported in Section 5.4.13 that equine milk requires a considerable length of time to form flocs on renneting with Maxiren 180 and as a result the sample was renneted for 6 h in this study at pH 6.5. It has also been reported that renneting with equine chymosin at pH of 6.2 did not form a firm coagulum (Section 5.4.14). Electron micrographs showed the absence of structure when equine milk was renneted (Figure 9.4 A), although the presence of some associated micelles was evident but little overall fusion. Whether the equine casein micelles are simply touching or partially fused is unclear. The low protein content of equine milk coupled with a very low content of κ-casein would account for the inability of equine milk to form a gel network. In comparison, bovine milk renneted at pH 6.5 for 20 min, the point at which gelation commenced, showed extensive network formation (Figure 9.4 B).

Electron microscopy of renneted bovine milk has shown that chymosin action leads to the formation of chains and clumps of micelles that network into partially-fused micelles (Rüegg & Blanc, 1972; Kimber *et al*., 1974; Green *et al*., 1978b) which becomes more extensive with time as the average size of the aggregates increases (Green *et al*., 1978b). By the time the milk coagulates, most micelles are in contact with others and a network forms, which is depicted in Figure 9.4 B.

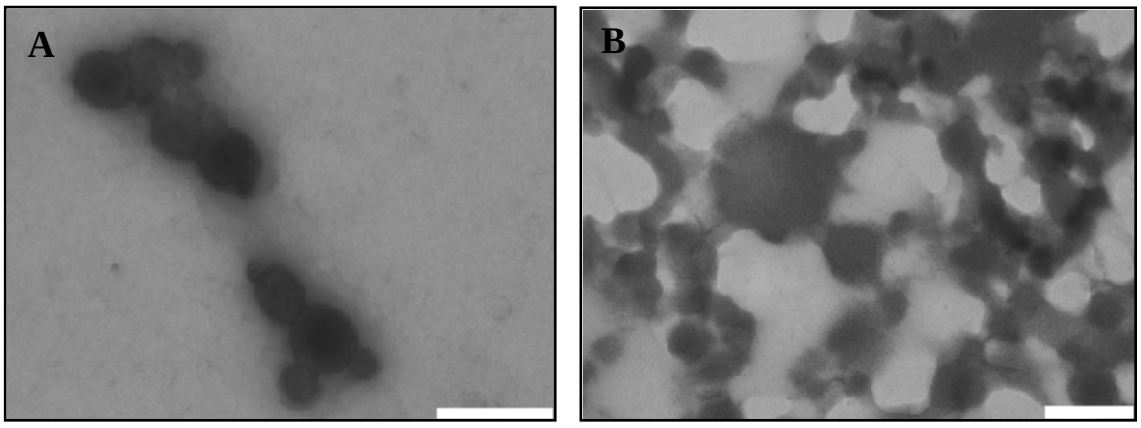

Figure 9.4. Transmission electron micrographs of equine (C) and bovine (D) milk renneted at pH 6.5 with 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 at 30^{o}C for 20 min (bovine) or 6 h (equine) and negatively stained using 20 g L^{-1} PTA. Magnification of 50 K. Scale bar is 200 nm in both images..

9.3.2.2. Thin-sectioning TEM

TEM micrographs of equine and bovine casein micelles stained in thin sections with uranyl acetate and lead citrate are shown in Figure 9.5 at a magnification of 200 K. Thin sectioning of equine and bovine milk samples allowed imaging at high magnification with improved detail of the equine micelle surface (Figure 9.5 A). The shape and size of the micelle were similar to the images from negative staining using PTA but the surface appeared ragged with protrusions into the serum phase. The bovine casein micelle were small (Figure 9.5 B) compared to the equine micelle and had a similar spherical shape, with pronounced diffuse edges which agreed well with observations of bovine micelles in thin sections by Green *et al.* (1978a) and Needs *et al* (2000), who reported approximately spherical structures with rough edges and a granular internal structure although, in this study, equine micelles appeared more regular in shape compared to those of bovine milk, the latter were less symmetrical, in agreement with Marchin *et al*. (2007), Martin *et al*. (2007) and Knudsen & Skibsted (2010). Figure 9.5 C is an image of one of the smallest equine casein micelles recorded and allowed imaging at a magnification of 300 K. In comparison to larger micelles, the smaller equine micelle appeared similar in shape and size to a typical bovine micelle and had very similar contours with diffuse edges. It has been reported (Martin *et al*., 2006) that larger bovine casein micelles tend to be more structured than smaller ones and that seems to be the case for equine casein micelles also.

Thin sectioning of samples can be problematic as sample preparation involves chemical fixation, dehydration and embedding of samples in plastic resin, all of which increase the likelihood of microstructural artifacts (Schmidt, 1982b; Gastaldi *et al*., 1996; Karlsson *et al*., 2007; McMahon & Oommen, 2008). Electron-dense areas were evenly distributed and visible throughout the equine and bovine casein micelles, probably due to the presence of calcium phosphate clusters, as observed in other studies (Knoop et al., 1979; Holt *et al*., 2003; Marchin *et al.,* 2007; Knudsen & Skibsted, 2010). This is in agreement with the model proposed by McMahon & Oommen (2008) from cryo-TEM examination where the micelle structure is described as being composed of aggregated calcium phosphate nanoclusters formed by the binding of the phosphorylated regions of the caseins to small domains of calcium phosphate. In the absence of κ-casein, protein/calcium phosphate nanoclusters would continue to aggregate without limit, but as κ-casein is not involved in the formation of nanoclusters, it stabilizes the surface of micellar aggregates (McMahon & Oommen, 2008).

Images of acidified equine milk following thin section preparation are shown in Figure 9.6A and B. Similar to renneted equine milk observed by negative staining with TEM, the micelles of equine milk did not form a casein network when acidified (Figure 9.6 B). Acidification caused more contact between micelles but not enough contacts to form a gel network. The close-up image (Figure 9.6 B) of equine casein micelles in contact showed that micelles appeared to have come into contact following acidification and fused to some degree. It was reported in Section 4.4.1 that equine casein micelles aggregated when acidified but the coagulum formed was very weak. It is likely that the poor coagulability of equine milk by either acid or rennet addition, which was reported in Chapters 4 and 5, respectively, and confirmed here in electron micrographs showing lack of casein network formation after either treatment, can be explained, at least in part, by the low casein concentration of equine milk and concomitant inability of equine micellar aggregates to span the whole volume and form a network. Increasing the casein content of equine milk to a similar level to that of bovine milk before acidification or renneting had little effect on the strength of coagula formed (see Sections 4.4.6 and 5.4.10, respectively). Another feature of equine casein micelles examined by TEM was that while it was possible to acquire high resolution images of bovine micelles with an accelerating voltage of 200 kV, the equine micelles disintegrated easily under the electron beam, and as a result all analysis was conducted at 80 kV. In agreement with Auty *et al*. (2005), sample preparation with aqueous gluteraldehyde for negative staining or buffered gluteraldehyde in the case of thin-section preparation showed little difference whether the gluteraldehyde was buffered or not.

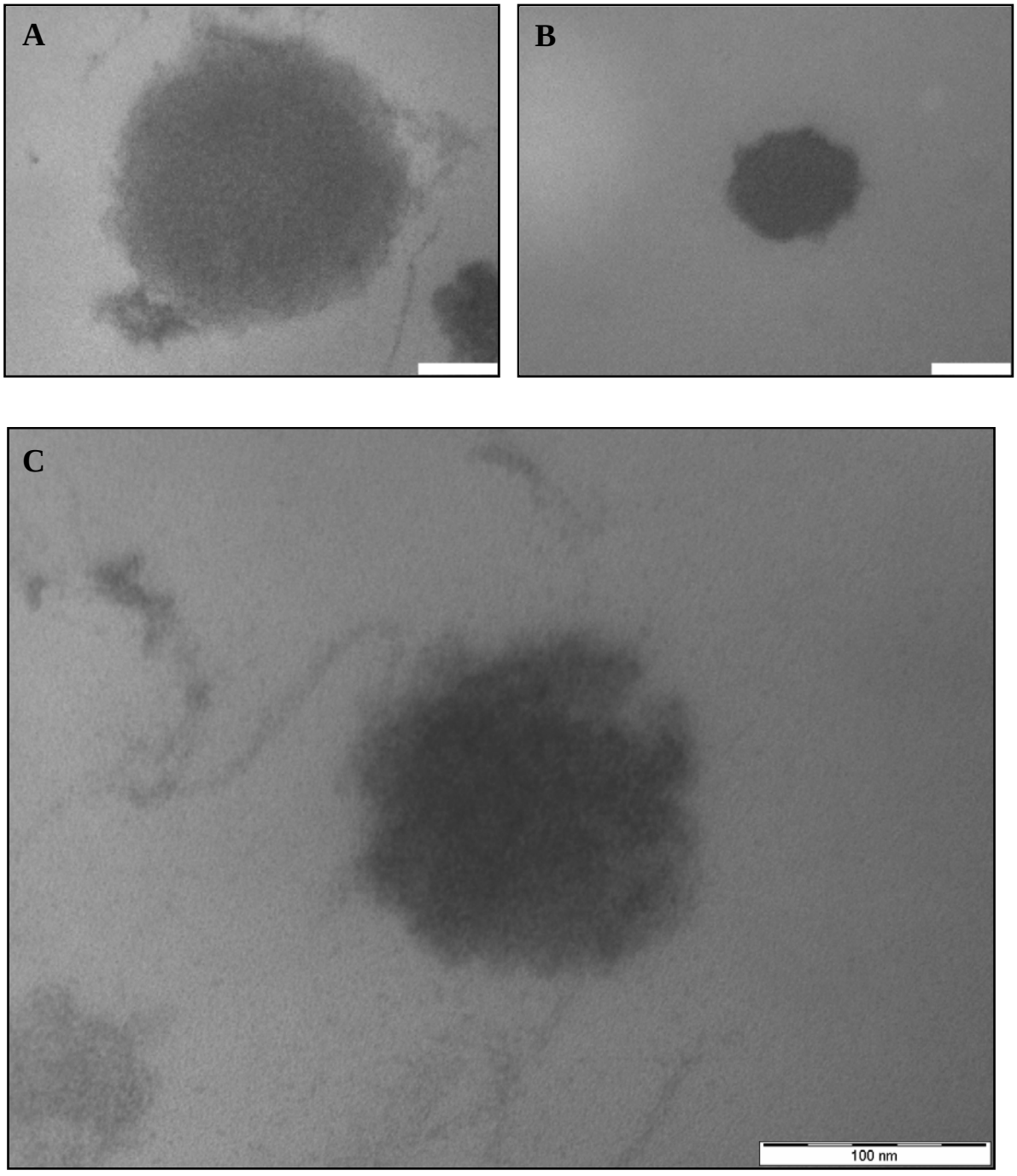

Figure 9.5. Transmission electron micrographs of casein micelles in thin sections of equine (A and C) and bovine (B) milk double contrasted with 20 g L^{-1} uranyl acetate and Reynolds lead citrate stain. Magnification of 200 K, A and B and 300 K, C. Scale bar is 100 nm in all images.

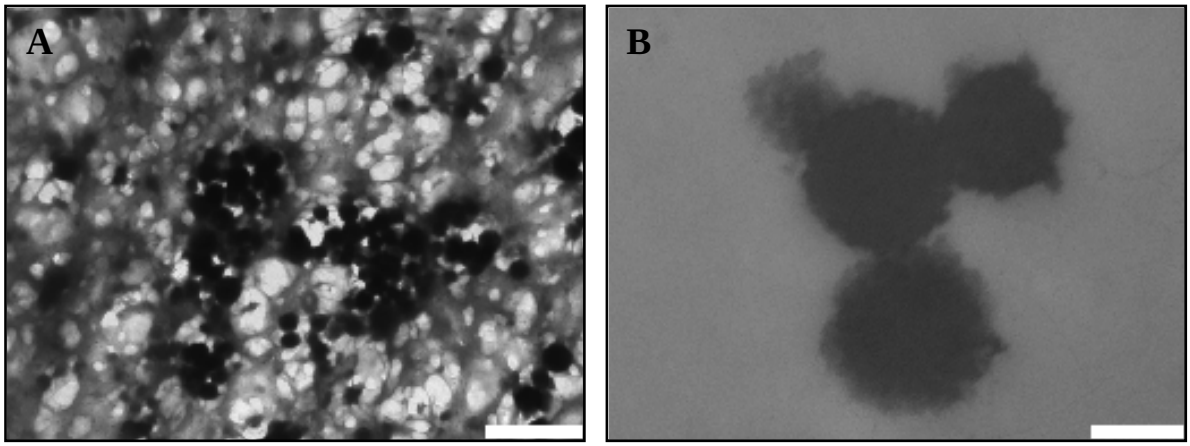

Figure 9.6. Transmission electron micrographs of thin sections of equine milk acidified to pH 4.2 with 30 g L^{-1} glucono-δ-lactone at 30^{o}C for ~ 120 min. Samples were double contrasted with 20 g L^{-1} uranyl acetate and Reynolds lead citrate stain. Magnifications of A, 20 K and B, 100 K. Scale bars of: 1000 nm (A) and 200 nm (B).

9.3.3. Scanning transmission electron microscopy

STEM images of equine and bovine casein micelles at various magnifications are shown in Figure 9.7. Equine milk appeared to have significantly fewer, larger, and more clearly defined spherical micelles (Figure 9.7 A,) than bovine milk (Figure 9.7 C), were micelles were more packed into the sample space and were more irregular in shape. Images of individual equine and bovine casein micelles (Figure 9.7, B and D, respectively) acquired here using STEM with PTA negative staining compared well with those obtained from TEM. With magnification appropriately adjusted, it was possible to view very clearly a representative micelle from each sample. Both equine and bovine micelles had a halo-like effect at the surface (Figure 9.7 B and D, respectively) which was more pronounced around the equine micelle. The bovine micelle had a more diffuse, irregular outline than the equine micelle and, as expected, was considerably smaller.

One image of an equine casein micelle immobilized onto poly-L-lysine-coated TEM grids and freeze-dried followed by examination using TEM has been reported by McMahon & Oommen (2008) although it lacks the structural clarity and fine detail achieved in this study using thin sectioning with TEM or STEM with negatively stained micelles.

9.3.4. Cryo-scanning electron microscopy

Cryo-scanning electron micrographs of freeze-fractured samples of equine and bovine milk (Figure 9.8) showed the large micelle size and relatively few micelles present in equine milk compared to bovine milk (Figure 9.8 A, C, respectively). The cryo-SEM technique produced images of the equine micelle system and structures not previously reported. In figures 9.8 A and D the micelles of both equine and bovine milk are compared and showed the very closely packed, smaller, micelles of bovine milk compared to the larger and less densely packed equine micelles (Figure 9.8 B).

The cryo-SEM images were acquired using an SE2 detector which was preferentially used over an InLens detector for topographic information. The large size and low concentration of equine micelles allowed a clear view of individual micelles and the arrangement of micelles.

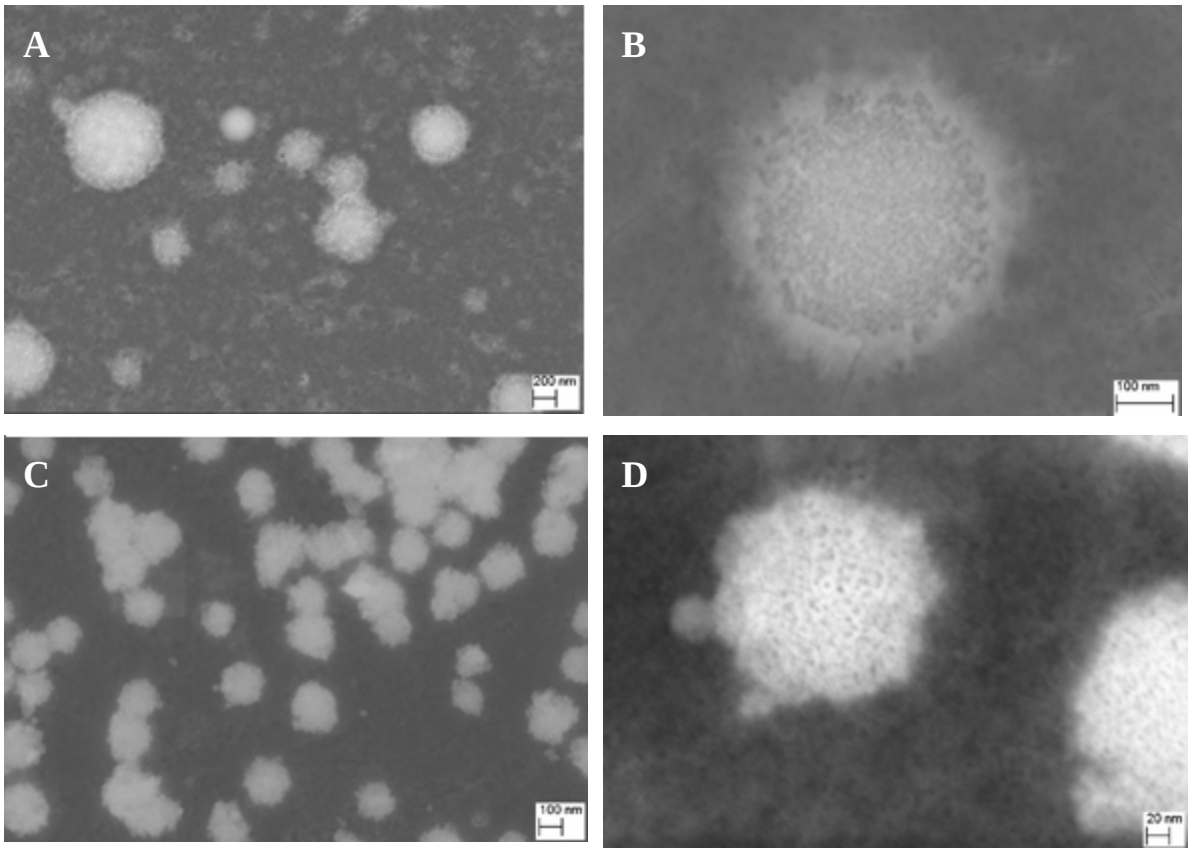

Figure 9.7. Scanning transmission electron micrographs of equine (A, B) and bovine (C, D) milks diluted 1:100 (v/v) with SMUF and stained with 10 g L^{-1} PTA, pH 6.6. Magnifications of 50 K (A), 500 K (B), 100 K (C) and 250 K (D).

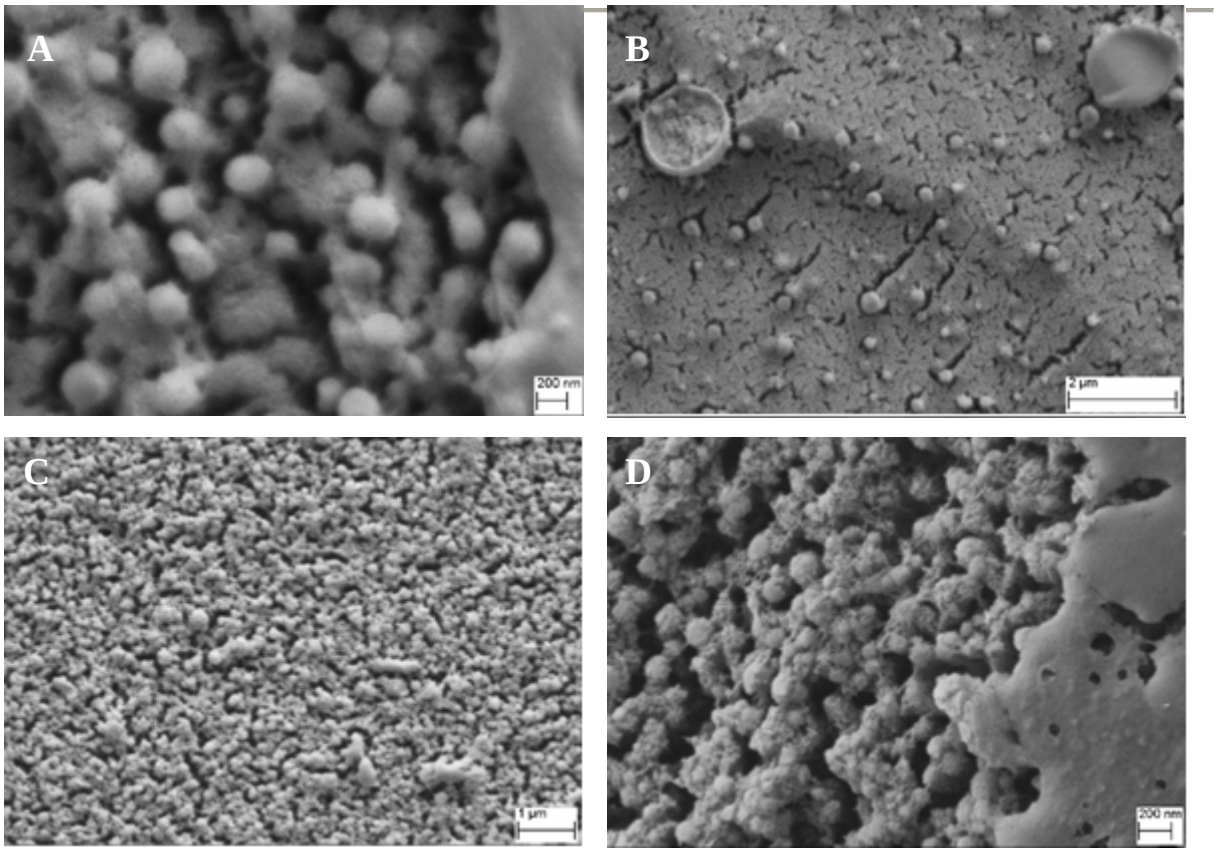

Figure 9.8. Cryo-scanning electron micrographs of freeze-fractured equine (A, B) and bovine (C, D) milk samples. Magnifications of 64 and 23 K, A and B, respectively and 23 and 71 K, C and D, respectively.

Dalgleish *et al*. (2004) obtained micrographs using field emission SEM of casein micelles absorbed on carbon planchets and chemically fixed with gluteraldehyde and osmium tetroxide; the resulting images showed tubular structures on the micelle surface and it was concluded that the micelle core could also be organised into tubular structures. In this study, using cryo-preparative techniques, which retains the finer detail of micelle surface morphology, no such structures were evident at the surface of either bovine or equine micelles.

Cryo-SEM images of renneted equine (Figure 9.9 A and B) and bovine (Figure 9.9 C and D) milk showed the differences between the two samples after chymosin addition better than any other microscopy technique. Equine milk had no network formation and only occasional small clusters of fused micelles were visible (Figure 9.9 A and B) after renneting. These images of renneted equine milk show that it is unlikely that a casein network could form on renneting or acidifying equine milk due to the spatial distribution of the micelles. In contrast, images of renneted bovine milk taken at two magnifications (Figure 9.9 C and D) showed that a casein network had formed. In this study, the clarity of renneted bovine milk has been improved significantly by using a cryo-preparative technique compared to similar images reported by Kalab & Harwalkar (1973), who prepared samples using gluteraldehyde as fixative before SEM.

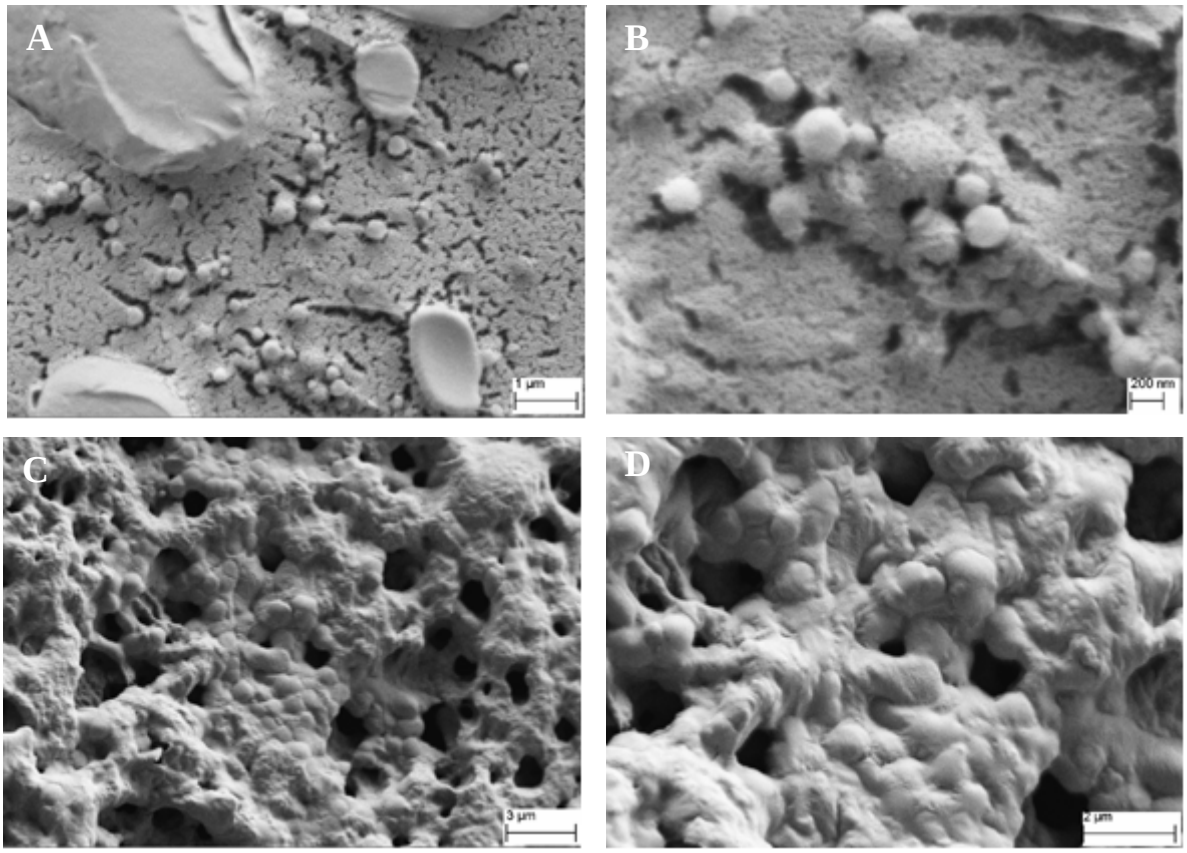

Figure 9.9. Cryo-scanning electron micrographs of freeze-fractured equine (A and B) and bovine milk (C and D) renneted with 10 μL mL^{-1} of a 1:10 (v/v) dilution of Maxiren 180 at 30^{o}C, pH 6.5 for 6 h at magnifications of 27 K (A), 70 K (B), 10 K (C), and 70 K (D).

9.3.5. Cryo-scanning transmission electron microscopy

The cryo-STEM images (Figure 9.10) showed that equine and bovine casein micelles are spherical and regular in shape with diffuse edges, the result for bovine milk is in agreement with a previous report (Gee *et al*., 2010). Individual equine and bovine micelles under dark or light field imaging (9.10 B and C) showed a uniform electron density and there was no evidence of a micelle sub-structure or electron-dense 'sub-particles' in either case, which is in agreement with the images obtained by McMahon & McManus (1998) using a cryopreparation technique with TEM. Furthermore, the dark and light contrasts gave more detail on the outer surface layer of the casein micelles. Freezing the samples on carbon film before cryo-STEM analysis (Figure 9.10 B, C and D) was used to attain an even distribution of micelles over the film surface for imaging. The presence of carbon film helps to stabilize a sample prior to imaging but carbon film has topographical features itself and in all cases samples were frozen without film to confirm that the images presented (Figure 9.10 B, C and D) were true images. Due to the nature of milk, i.e., an extremely high water content, it is difficult to obtain clear and representative images of micelles that do not contain artifacts. The images obtained using cryo-STEM maintained the micelle structure because the samples were frozen very quickly using metal mirror fixation. Freezing of equine and bovine micelles using cryo-STEM has resulted in images that are in agreement with the observed structure of these micelles using alternative techniques in this study.

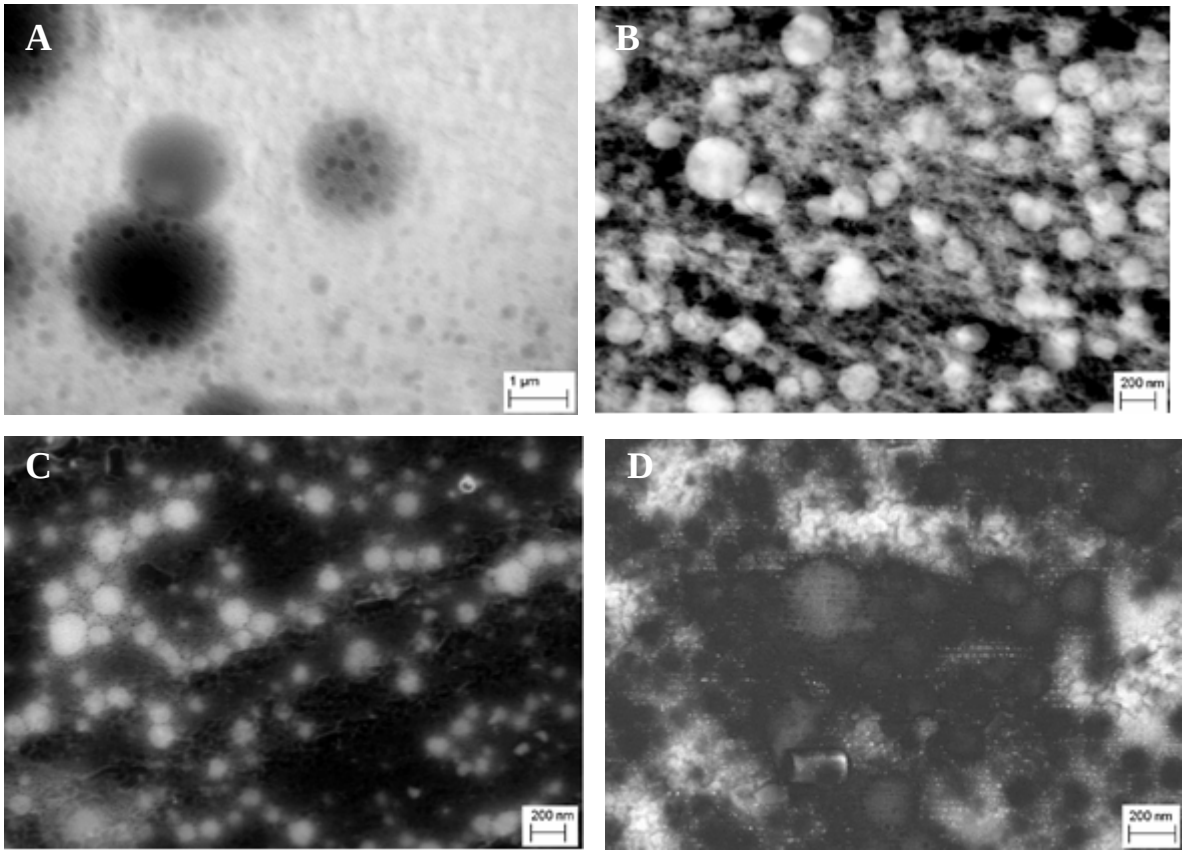

Figure 9.10. Cryo-scanning transmission electron micrographs of equine milk (A and B) and bovine milk (C and D). Without carbon film A, and frozen onto carbon film 400 B, C, and D at magnifications of 25 K (A), 75 K (B), 75 K (C) and 100 k (D).

9.4. Conclusions

The instrumentation available for electron microscopy has improved significantly, allowing greater image resolution and finer detail in the micrographs of casein micelles (McMahon & McManus, 1998). However, the conformational flexibility of casein micelles has meant that their structure changes easily during sample preparation for electron microscopy (McMahon & Oommen, 2008). This study used most electron microscopy techniques, including the latest cryo-STEM technique, which involved minimal sample preparation (Gee *et al*., 2010) and preserved the casein micelle as close to its native state as has been possible to date.

The need for gluteraldehyde fixation, which has been reported to cross-link proteins and potentially change their structure (McMahon & Oommen, 2008), was avoided for both the cryo-SEM and cryo-STEM techniques.

Simpler techniques, if undertaken carefully, such as negative staining and TEM imaging can yield detailed images of casein micelles. The high resolution obtained by cryo-SEM allowed very clear images of equine and bovine casein micelles in their native state and when treated with either acid or chymosin. The sizes found here for equine casein micelles using various imaging techniques were similar.

To date, the outer structure of the surface of the casein micelle is largely unknown. The model of Holt (Holt & Horne, 1996; DeKruif & Holt, 2003) suggests that there is no well-defined structured hairy layer but instead there is a layer of decreasing protein density at the micelle surface. This was evident in the case of equine and bovine casein micelles, particularly those obtained at high magnification using TEM and also agrees with the lack of a defined surface layer in the polymer condensation model of Horne (1998).

The sub-micelle model of casein micelle structure is not universally accepted and mounting evidence suggests that well-defined casein sub-micelles do not exist and that the structure is more open and flexible. The evidence obtained in this study supports this view and using several microscopy techniques with magnifications of up to 300 k, no sub-micelles were evident in equine or bovine casein micelles. The images presented in this study from TEM of thin section samples and STEM clearly show electron-dense areas linked by less dense areas which is probably calcium phosphate nano-clusters linked together by chains of caseins which agrees with the casein micelle models proposed from EM images by Marchin *et al* (2007) and McMahon & Oommen (2008) of an interlocked lattice in which casein-calcium phosphate aggregates and casein polymer chains act together to maintain micelle integrity. These

models, in turn, agree with the model described by de Kruif & Holt (2003), of a homogeneous network of casein polymers containing nanoclusters of calcium phosphate.

Equine micelles contain little κ-casein and the micelles are large and, given the results of this study, it is most likely that other proteins at the micelle surface assist in maintaining micellar stability. Equine β-casein contains a distinctly hydrophilic N-terminal but this also contains the centre of phosphorylation so it is unclear whether this protein could, at least in part, aid the steric stabilization of the equine casein micelles or whether it is located predominately in the interior of the micelle.

The casein systems of different species follow the same basic structural pattern but the components differ which probably confers unique characteristics on the milks, customizing them to suit the varied requirements of the newborn of each species, concurrently producing stable colloidal structures which effectively transport calcium phosphate to the neonate. The range of functionalities of casein in the milks of different species or within the same species may vary. It has been reported that the overlap in functionality of α_{s1}-casein with both β- and α_{s2}-caseiins, in bovine milk is important in maintaining micellar stabilty (McMahon & Oommen, 2008). In caprine milk, α_{s1}-casein variant F, which lacks a phosphoserine cluster, is believed to have a role in regulating micelle size by limiting the effect of the aggregation process of casein during micelle formation (Pierre *et al*., 1999) and may act as a chain terminator with an apparent surface location, like κ-casein (Tziboula & Horne, 1999). Coagulation of milk is important from a nutritional point of view, as clotting of the caseins in the stomach, and the type and strength of the resultant coagulum, strongly affect protein digestibility. Equine casein micelles come into contact and fuse to a certain extent when renneted or acidified but do not form a gel network, as demonstrated in this study; the floccs formed enhance digestibility and ensure fast release of nutrients from equine milk which are important for the development of the foal.

9.5. References

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CHAPTER 10

Digestion of Equine β-Casein and Examination of Peptides for the Presence of Casomorphins

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Abstract

The components of purified equine β-casein were separated by capillary zone electrophoresis (CZE) which demonstrated the multiphosphorylated isoforms of the protein, with phosphorylated residues ranging from 3P to 7P. The *in vitro* digestion of equine β-casein by simulated gastrointestinal digestion (SGID) was investigated to confirm that the opioid β-casomorphins, BCM-5 or BCM-7, or analogous peptides were not present in the digested fractions using HPLC coupled with electrospray ionization and mass spectrometry (HPLC-ESI/MS). Extensive hydrolysis of equine β-casein was evident after hydrolysis by pepsin and Corolase PP™.

Keywords: Equine β-casein; β-casein variants A^1 and A^2; β-casomorphins, BCM-7, BCM-5; capillary zone electrophoresis (CZE); simulated gastrointestinal digestion (SGID); HPLC/ESI MS.

Introduction

About 80% of the protein of bovine milk is casein which is primarily a source of essential amino acids and bioactive peptides, as well as a carrier of calcium and phosphate, for neonates (Shekar *et al*., 2006). In human milk, casein constitutes 20-30% of total milk protein (Hambræus, 1984) and in equine milk ~52% of the total protein is casein (Section 3.6.1). The casein fraction of many milks consists of 4 gene products: α_{s1}-, α_{s2}-, β- and κ-caseins, of which the first 3 are calcium sensitive; the approximate proportions of each casein in bovine milk are 38, 11, 38 and 13%, respectively (Mercier, 1981) and in equine milk are 18, 1.4, 80 and 1.7%, respectively (Uniacke-Lowe *et al*., 2010). In other species, not all of these types of casein are present and the relative and absolute concentrations of the different caseins are not the same (Dalgleish, 2011). In human milk, > 85% of the casein is β-casein and there is little (Rasmussen *et al*., 1995) or no α_s-casein (Hambræus & Lönnerdal, 2003). The protein sequences of the individual caseins are not well-conserved between species (Ginger & Grigor, 1999).

Milk contains an array of bioactive constituents, including minor proteins and peptides secreted in their active form by the mammary gland as well as many more latent bioactive peptides encrypted within a protein sequence and released by proteolysis of the milk protein precursor (Meisel, 1998, 2004; Clare & Swaisgood, 2000; Phelan *et al*., 2009). Milk-derived peptides may be multifunctional, i.e., peptide sequences may have two or more different bioactivities (Meisel, 2004). Bioactive peptides are released in the stomach during protein digestion and the number and size of the peptides decreases between the stomach and the distal end of the duodenum but several long peptides, including casinomacropeptide (CMP) and an antihypertensive peptide sequence (residues 24-35) of α_{s1}-casein have been detected in blood plasma (Chabance *et al*., 1998). Some peptides are capable of modulating specific physiological functions: anti-hypertensive, opioid, metal-binding, anti-bacterial and immunomodulatrory activities have been reported for casein-derived peptides (Abd El-Salam *et al*., 1996; Dziuba & Minkiewicz, 1996; Brody, 2000; Malkoski *et al*., 2001; Baldi *et al*., 2005; Silva & Malcata, 2005; Thomä-Worringer *et al*., 2006; Michaelidou, 2008) and whey-protein derived peptides (Nagaoka *et al*., 1991; Mullally *et al*., 1996; Pellegrini *et al*., 2001; Hernández-Ledesma *et al*., 2005; Chatterton *et al*., 2006; Yamauchi *et al*., 2006; Hernández-Ledesma *et al*., 2008).

A detailed discussion on bioactive peptides is outside the scope of this article; general reviews include; Donnet-Hughes *et al*. (2000), Shah (2000), Malkoski *et al*. (2001), Fitzgerald & Meisel (2003), Pihlanto & Korhonen (2003), Silva & Malcata (2005), Fitzgerald & Murray

(2006), Lopéz Expósito & Recio (2006), Lopéz *et al*. (2006), Michaelidou & Steijns (2006), Thöma-Worringer *et al*. (2006), Dziuba & Darewich (2007), Shahidi & Zhong (2008), Haque *et al*. (2009) and Phelan *et al*. (2009).

There are approximately13 genetic variants of bovine β-casein: A^1, A^2, A^3, A^4, B, C, D, E, F, H^1, H^2, I, G (Ng-Kwai-Hang & Gausclaude, 2002). The most common variants in dairy cattle breeds are A^1 and A^2, B is less common and A^3 and C are rare (Ng-Kwai-Hang & Gausclaude, 2002; Farrell *et al*., 2004). The A^4 allele has only been reported in Bali and Korean cattle (Ng-Kwai-Hang & Gausclaude, 2002; Kamiński *et al*., 2007). β-Casein A^1 is the most frequent variant found in Holstein-Friesian cattle, whereas variant A^2 is most common in Guernsey and Jersey cattle (Kamiński *et al*., 2007).

β-Casomorphins (BCMs), are a group of peptides originating from β-casein with a chain length of 4-11 amino acids all starting with tyrosine at position 60 of β-casein (Kostyra *et al*., 2004). BCMs are found in analogous positions in sheep, water buffalo and human β-casein (Fiat & Jollès, 1989; Teschemacher *et al*., 1990; Meisel & Schlimme, 1996; Meisel, 1997). After ingestion of milk or milk products, BCMs are formed in the stomach of infants by gastric enzymatic breakdown of β-casein (Chang *et al*., 1985). BCMs have unique structural features that impart a high and physiologically significant affinity for the binding sites of endogeneous opioid receptors (Brantl *et al*., 1981; Miesel & Fitzgerald, 2000). Once formed, BCMs are resistant to proteolysis because of their proline-rich sequences and can reach significant levels in the stomach (Sun *et al*., 2003). BCMs are absorbed from the gastrointestinal tract and can cross the blood-brain barrier of newborns and young infants due to an immature central nervous system (Sun *et al*., 1999; Sun & Cade, 1999; Sun *et al*., 2003). Indirect evidence suggests that adults who consume bovine casein produce BCMs in the gastrointestinal tract but are reported not to have circulating BCMs (Svedberg *et al*., 1985; Teshemacher *et al*., 1986).

In bovine β-casein, residue 67 is proline in variant A^2 but is histidine in variant $A^1$and B (Groves, 1969; Jinsmaa & Yoshikawa, 1999) (Figure 10.1). Structural differences between variants A^1 and A^2 β-caseins result in each releasing its own set of bioactive peptides on digestion by gastrointestinal enzymes.

The one amino acid difference at position 67 allows cleavage by digestive enzymes of the peptide chain next to His_{67} but not next to Pro_{67} and, in the former case, BCM-7 is formed. BCM-7 represents amino acids 60 to 66 of β-casein (Kamiński *et al*, 2007) and its release is believed to prevent the release of many peptides with important bioactive properties (Figure

10.1). BCM-7 has been isolated and identified in fresh bovine and human milk as well as in dried infant formulae (Sun *et al*., 2003; De Noni, 2008) and in other dairy products (De Noni & Cattaneo, 2010). BCM-7 is one of the first examples of a bioactive peptide derived from a food protein (Brantl *et al*., 1979) and it can be converted to BCM-5 by proteolysis in the gastrointestinal tract (Meisel *et al*., 1989).

BCM-7 is reported to play a significant role in the aetiology of certain human diseases and can potentially affect numerous opioid receptors in the nervous, endocrine and immune systems (Bell *et al*., 2006). Epidemiological evidence suggests that the consumption of BCM-7 is associated with increased risk of ischaemic heart disease (McLachlan, 2001; Laugesen & Elliott, 2003; Chin-Dusting *et al*., 2006), atherosclerosis (Tailford *et al*., 2003; Venn *et al*., 2006), Type 1 diabetes mellitus (Thorsdottir *et al.*, 2000; Elliott *et al*., 1999), sudden infant death syndrome (Sun *et al*., 2003), autism and schizophrenia (Cade *et al*., 2000; Reichelt & Knivsberg, 2003). Recently, the European Food Safety Authority collected and reviewed the available scientific evidence related to BCM-7 and analogous peptides to assess the existence and robustness of an association between such bioactive peptides and noncommunicable diseases (EFSA, 2009). The EFSA report concluded that, based on available scientific literature, a cause and effect relationship between the oral intake of BCM-7 or related peptides and the aetiology or course of any suggested non-communicable disease cannot be established with certainty. Furthermore, the report concluded that the presence of BCMs or related peptides in unprocessed milk from healthy cows or commercial infant formulae has not been demonstrated conclusively although it acknowledges that the formation of certain BCMs in infant formulae after simulated gastrointestinal digestion has been demonstrated.

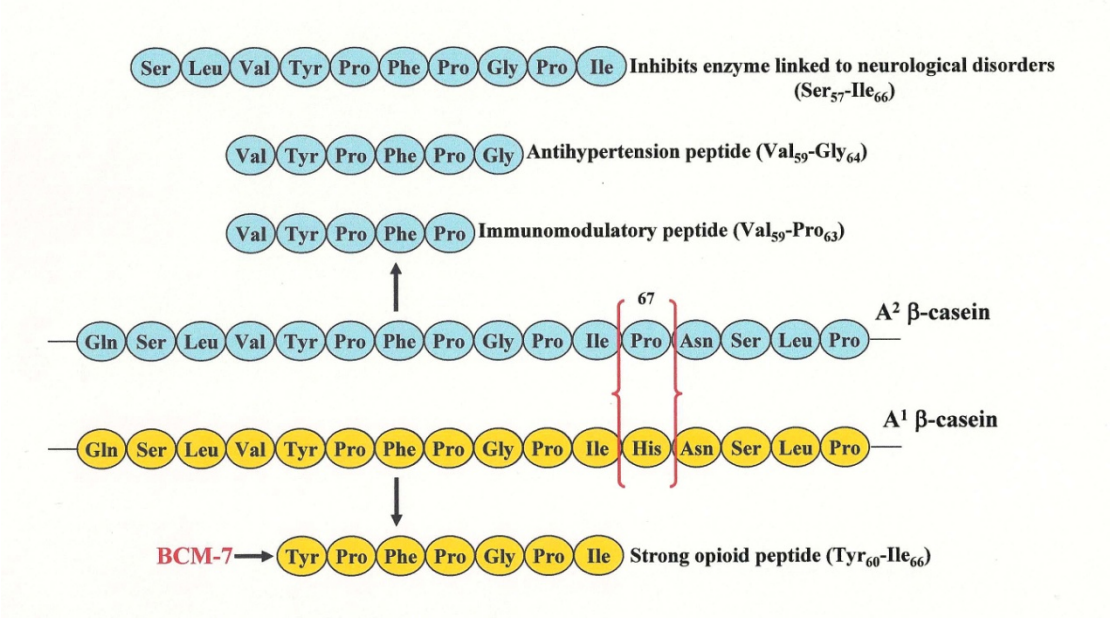

Figure 10.1. Bioactive peptides release from bovine β-casein variants A^1 and A^2, including β-casomorphin-7 (Tyr_{60}-Ile_{66}) released from the A^1 variant .

Research on the bioactive peptides derived from equid milk is very limited. It has been reported that peptides from the hydrolysis of equine β-casein may have a positive action on human health (Doreau & Martin-Rosset, 2002). Chen *et al*. (2010) reported the presence of four novel ACE-inhibitory peptides in koumiss which may enhance the beneficial effects of koumiss (fermented equine milk) on cardiovascular health. Peptides with trophic or protective activity have been identified in asinine milk (Salimei, 2011).

The amino acid sequence of equine β-casein, derived from the cDNA, was reported by Lenasi *et al*. (2003), and revised by Girardet *et al*. (2006) with the insertion of 8 amino acids (Glu_{27} to Lys_{34}). The theoretical molecular mass of this 226 amino acid polypeptide is 25, 511.4 Da. Bovine and human β-casein contain 209 and 211 amino acid residues, respectively. Two smaller variants of equine β-casein, which probably result from casual exon-skipping during transcription, were reported by Miranda *et al*. (2004). Unlike bovine β-casein variants A^1 or A^2, equine β-casein has a tyrosine residue at position 67 of the polypeptide chain which has implications for the type of bioactive peptides produced. The purpose of this study was to separate the components of equine β-casein by capillary zone electrophoresis and to study the digestion of equine β-casein in a simulated gastro-intestinal digestor to confirm that BCM-5 or BCM-7 or analogous peptides were not present in the digests using HPLC coupled with electrospray ionization and mass spectrometry.

10.2. Materials and Methods

10.2.1. Milk supply

Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, the Netherlands) from a bulk supply collected from 5 milkings over 24 h, from a herd of multiparous New Forest and New Forest/Arabian mares in mid-lactation, physically separated from their foals by day. The milk was defatted by centrifugation at 1,000 x g using a Sorvall® RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 20°C for 20 min followed by filtration through glass wool to remove fat particles. Sodium azide (0.5 g L^{-1}) was added to the skimmed milk to prevent microbial growth. All chemicals used were of reagent grade, obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

10.2.2. Preparation of β-Casein

Equine milk was fractionated according to the method of Visser *et al*. (1982) with the modifications outlined in Section 6.4.4. The temperature-sensitive (TS-casein) fraction of equine casein, obtained by this protocol at pH 4.6, had the highest concentration of equine β-casein with little or no contamination by other proteins when assessed by urea-PAGE (Figure 10.2). The TS-casein pellet was freeze-dried and stored at -80^{o}C until used. The β-casein sample used in this study was pooled from 3 separate fractionations.

10.2.3. Pure BCMs and enzymes

Pure β-casomorphins BCM-5 and BCM-7 were purchased from Bachem (Bubendorf, Switzerland). Porcine pepsin (P7000) was obtained from Sigma-Aldrich. Corolase PP™, a porcine pancreatic protease preparation which contains trypsin, chymotrypsin and numerous amino- and carboxy-peptidases, was purchased from Röhm GmbH (Darmstadt, Germany).

10.2.4. Capillary zone electrophoresis of equine milk

Capillary zone electrophoresis (CZE) was used to assess the heterogeneity of undigested equine milk β-casein. The method used was that of Recio & Olieman (1996) using a Beckman P/ACE™ 5000 capillary electrophoresis system (Beckman Coulter Inc., Fullerton, CA, USA).

10.2.5. Simulated gastro-intestinal digestion

Simulated gastro-intestinal digestion (SGID) was carried out on equine β-casein as described by Schmidt *et al*. (1995) with the modifications of De Noni (2008). The sample was hydrolysed initially by pepsin at pH 2.0 (1:50, enzyme: substrate ratio) and aliquots were taken after 30, 60 and 90 min and the pepsin inactivated by raising the pH to 7.5 with 1 M NaOH. The remaining sample-enzyme mixture was brought to pH 7.5 with 4 M NaOH and Corolase PP™ (1:25, enzyme: substrate ratio) was added. Aliquots were taken after 60 and 150 min and heated to 95^{o}C for 10 min to inactivate the enzymes. All hydrolysates were stored at -20^{o}C until analysed by high performance liquid chromatography with electrospray ionization and tandem mass spectrometry (HPLC-ESI/MS).

10.2.6. HPLC-MS/MS analysis

The peptides formed on simulated gastro-intestinal digestion of equine β-casein were separated as described by De Noni (2008) using HPLC-ESI/MS. To determine if BCM-5 or BCM-7 were present in the hydrolysates of equine casein, purified standards of these peptides were included in the analysis. The presence of BCM-5 and BCM-7 was determined by MS/MS of mono-protonated precursor ions of these peptides, following the conditions described by De Noni (2008).

10.3. Results and Discussion

Capillary zone electrophoresis (CZE) of equine β-casein is shown in Figure 10.3. CZE separates compounds based on their size to charge ratio and can separate different forms of a protein with very high resolution compared to the separation by HPLC. CZE showed the heterogeneity of equine β-casein which was shown by Urea-PAGE in Section 3.7.13. The peaks on the CZE electrophoretogram correspond to casein isoforms with different levels of phosphorylation.

The different degrees of phosphorylation was clearly demonstrated by submitting the intact equine β-casein to HPLC/ESI mass spectrometry (Figure 10.4). The deconvoluted spectrum, which extracted mass spectral signals from closely co-eluting components, showed the presence of different phosphorylated forms of equine β-casein containing from 4 to 7 phosphorylated residues. The degree of phosphorylation observed in this study is within the range (from 3 to 7 residues) reported by Girardet *et al*. (2006) and Matéos *et al*. (2009). The study by Matéos *et al*. (2009) reported only trace amounts of the 3P form of equine β-casein in Haflinger equine milk. From an analytical point of view, the ESI-MS response of the 3P form should not be different from those obtained for the 4 to 7P forms. Therefore, it is unclear whether New Forest and New Forest/Arabian bulk mare's milk contained the 3P form of equine β-casein or if it was present in amounts outside the detectable range of the instrumentation. Bovine β-casein, which contains 4 or 5 phosphorylated serine residues, is usually found fully phosphorylated (Swaisgood, 2003).

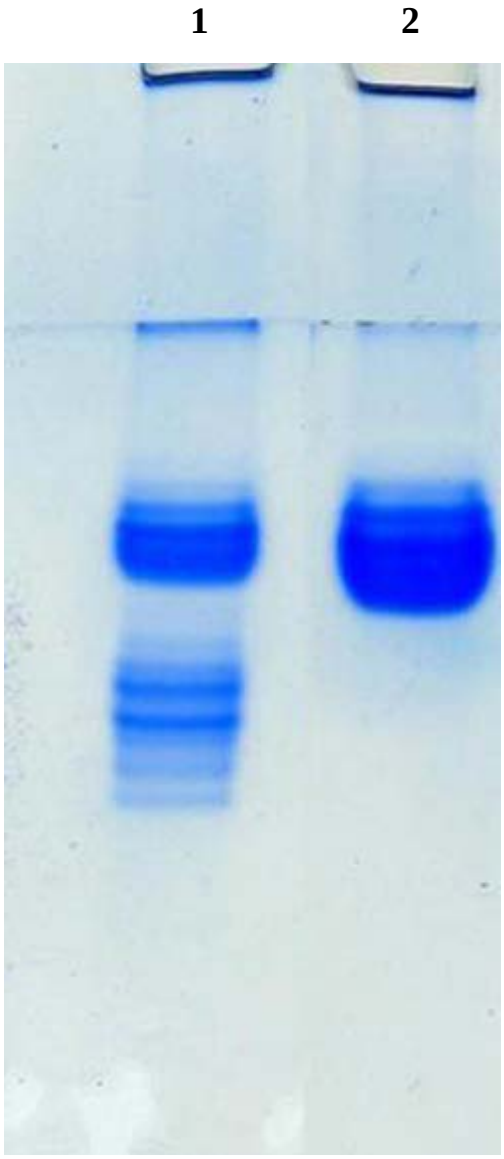

Figure 10.2. Urea polyacrylamide gel electrophoretograms (12.5% T, 4% C, pH 8.9) of fractionated equine casein. *Lanes*: **1**, whole equine casein prepared by precipitation at pH 4.6. 2, temperature- sensitive (TS) equine casein prepared by precipitation at pH 4.6 by a modification of the method of Visser *et al*. (1982).

Human β-casein has up to 6 phosphorylation levels, i.e., 0, 1, 2, 3, 4 or 5 phosphorylated serine residues (Groves & Gordon, 1970), and the 2P and -4P isoforms constitute ~ 70% of the total β-casein fraction (Sood *et al*., 1985; Sood & Slattery, 2000). The high degree of phosphorylation of equine β-casein has several important consequences. Phosphate groups attached to equine β-casein may provide mechanical strength to micelles through Ca^{2+}-binding (Mercier, 1981) which also serve to provide large amounts of calcium for growth and development of the foal (Donovan, 2008).

Due to the low κ-casein content of equine milk it has been suggested that the β-casein with a low level of phosphorylation contributes to the stability of equine casein micelles (Ochirkhuyag *et al*., 2000). Azuma *et al*. (1985) reported that highly phosphorylated human β-casein can form casein micelles with κ-casein and the highly phosphorylated β-casein is, in turn, stabilized by the co-operative function of lesser phosphorylated β-caseins and κ-casein. In the absence of α_{s1}-casein, non-phosphorylated and singly phosphorylated human β-casein can form a micelle framework, in a similar manner to α_{s1}-casein of bovine milk, and along with CCP the more highly phosphorylated forms of human β-casein are incorporated into the structure (Sood *et al*., 1997). In another study Sood & Slattery (2002) reported that mixing similar ratios of 4P to either 0P or 1P and of the 2P to 1P form showed that neither the 0P nor 1P forms had any stabilizing effect on the more highly phosphorylated forms. An earlier study (Yoshikawa *et al*., 1981) reported that dephosphorylated β-casein can behave like κ-casein and is able to form micelles with α_{s1}-casein while chemically phosphorylated κ-casein is unable to stabilize casein micelles.

Unlike bovine κ-casein, equine κ-casein does not have a distinctly hydrophilic C-terminal domain and the level of glycosylation, which, if high, would enhance the ability of κ-casein to stabilize the micelle (Minkiewicz *et al*., 1993; Dziuba & Minkiewicz, 1996) has not been established (Section 2.3.5).

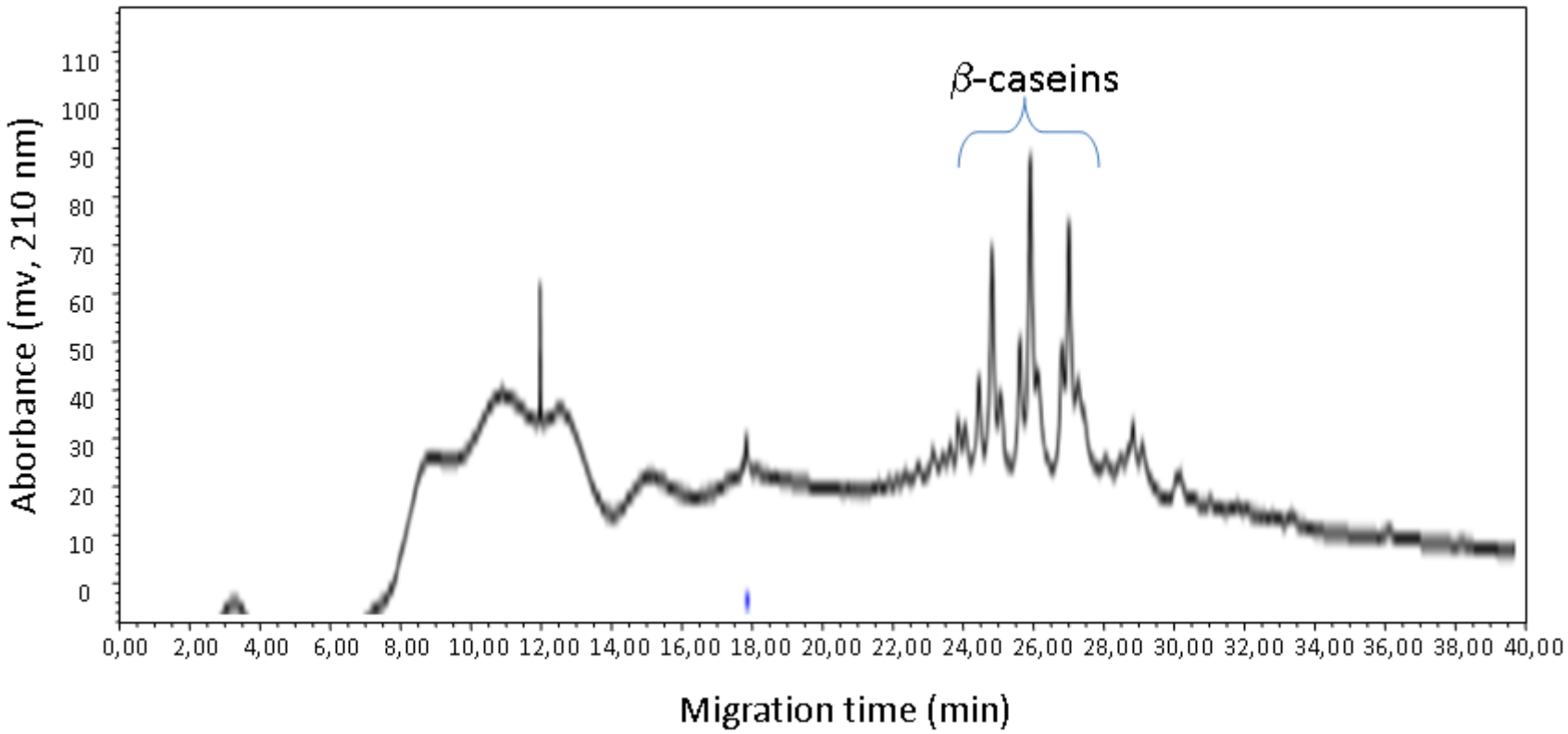

Figure 10.3. Capillary zone electropherogram of undigested equine β-casein.

It is therefore unclear if the C-terminal of equine κ-casein is capable of protruding from the micellar surface to sterically stabilize it. Human κ-casein is more highly glycosylated than bovine κ-casein which gives it increased stabilizing ability and the glycosylated moieties can protect a larger micelle surface area leading to a smaller average micelle size for a given amount of κ-casein (Sood *et al*., 2003). Furthermore, the κ-casein content of human milk at ~1 g L^{-1} (Picciano, 2001) represents 22- 25% (Sheng & Fang, 2009) of total casein compared to an estimated 2% of total casein for equine κ-casein.

The HPLC chromatograms of equine β-casein after hydrolysis by pepsin for 30, 60 and 90 min and Corolase PP™ for and 60 and 150 min are shown in Figure 10.5. Some peptides in hydrolysed equine β-casein eluted close to the retention times of bovine BCM-7 and BCM-5, but MS/MS showed that these peptides were not BCM-5 or BCM-7.

Extensive degradation of equine β-casein was evident after hydrolysis with pepsin and Corolase PP™. After 30 min several peptides were present, which is in agreement with Inglingstad *et al*. (2010), who compared the degradation of human, bovine, caprine and equine proteins *in vitro* by human gastric and duodenal juice and found that while bovine and caprine proteins were degraded slowly by gastric juice, equine caseins were extensively degraded with only ~ 30% (α_s- and β-caseins) remaining after 30 min. Human milk casein has been reported to be extensively degraded by gastric juice but not to the same extent as equine casein (Inglingstad *et al*., 2010). It is possible that the large micelles in equine milk (Section 3.7), coupled with the low level of κ-casein, makes equine casein highly susceptible to gastric enzymes (see Clark, 1992; Lodes *et al.*, 1996).

The rate of protein degradation in milk is also related to the strength of the coagulum formed in the stomach. Coagulation of milk in the stomach delays the degradation of proteins and allows for their better assimilation by the body. Degradation of bovine casein is slow but extensive and while β-lactoglobulin is relatively resistant to gastric proteolysis, α-lactalbumin is readily hydrolysed when the gastric pH is ~ 3.5 (Savalle *et al*., 1988). The structure of the coagulum formed in the stomach depends on the casein content of ingested milk; high-casein milk yields firm, tough clots. Species that nurse their young at frequent intervals, e.g., horses and humans, produce milk

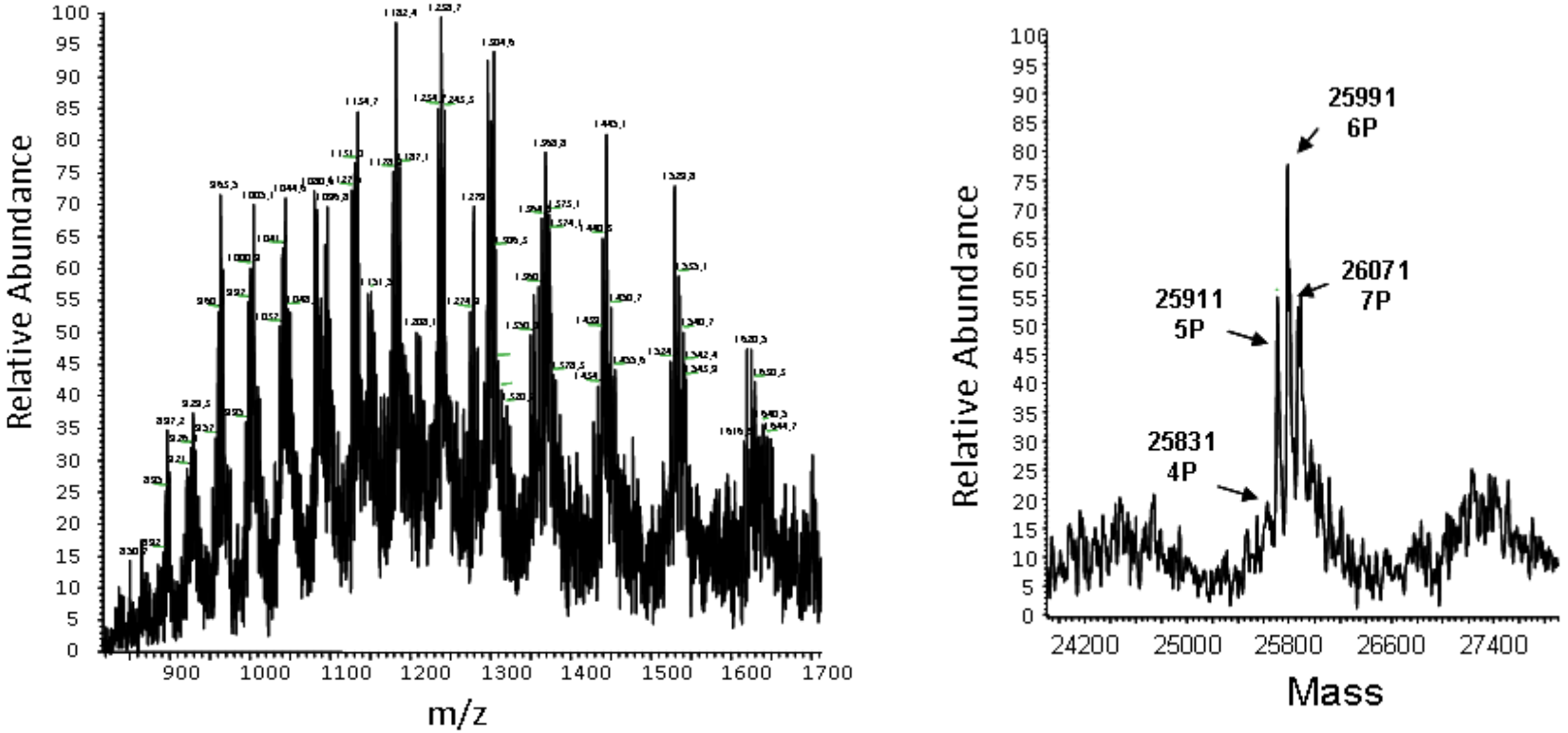

Figure 10.4. HPLC/ESI full-scan mass spectrum of equine β-casein (left) and related deconvoluted spectrum (right). Peaks indicated in the right box refer to phosphorylated (P) forms of equine β-caseins.

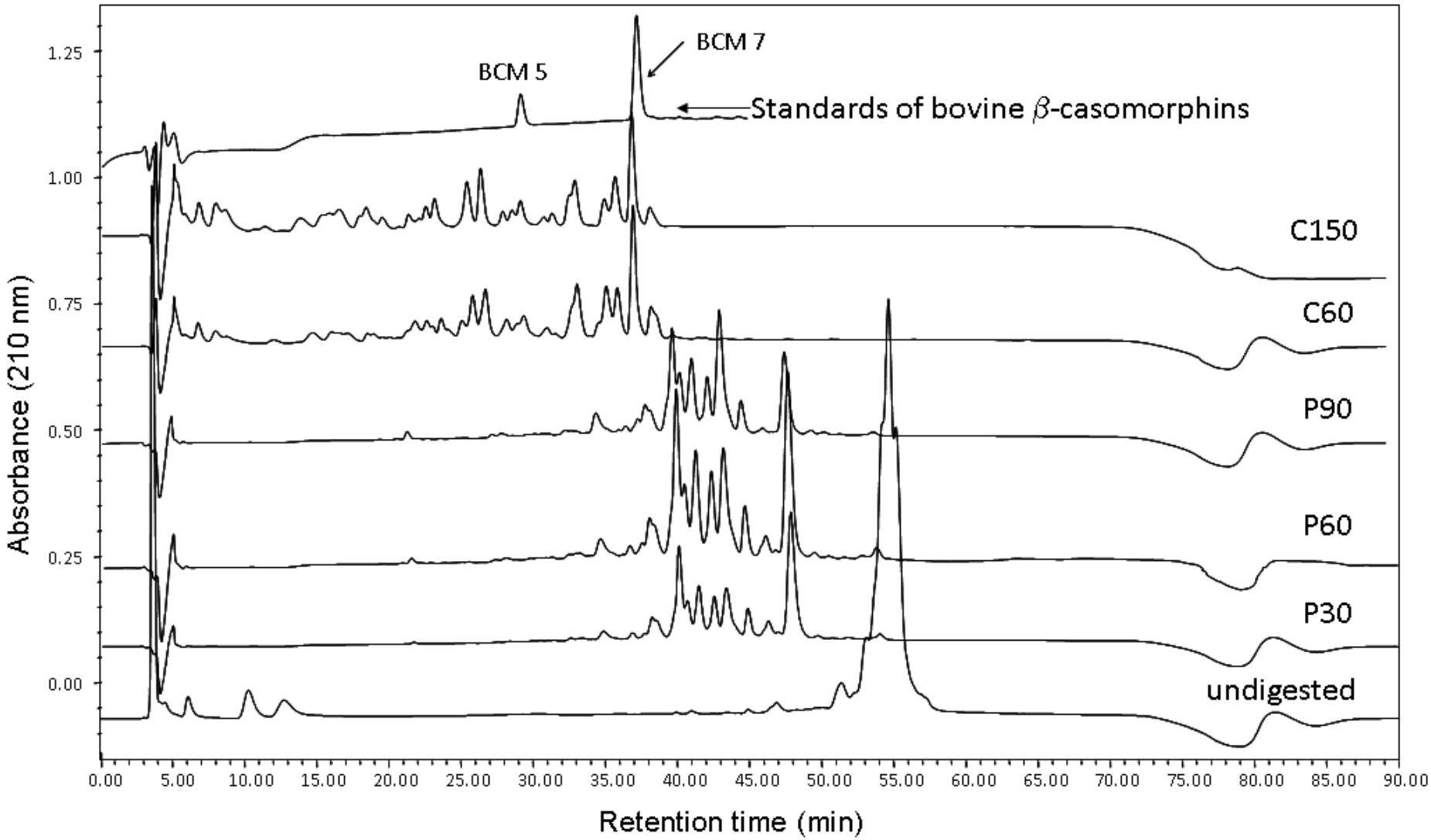

Figure 10.5. HPLC/UV chromatogram of equine β-casein taken at different stages during simulated gastro-intestinal digestion. Sampling time (min) after addition of pepsin (P) or Corolase PP™ (C).

with a low casein content and the coagulum formed in the stomach is soft and is degraded quickly (Oftedal, 1980). The physico-chemical differences between human and bovine caseins result in the formation of different types of curd in the stomach (Hambræus, 1984) and, because the protein profile of equine milk is quite similar to that in human milk, equine milk may be more appropriate in human nutrition than bovine milk (Turner, 1945; Kallila *et al*., 1951).

10.4. Conclusions

Equine milk has important nutritional and therapeutic properties which are beneficial in the diet of the elderly, convalescents, newborn and as a substitute for bovine milk for people with cow's milk allergy (see Uniacke-Lowe *et al*., 2010). The protein content of equine milk is lower than that of bovine milk but similar to human milk and its casein: whey protein ratio, close to that of human milk, is very different from that in bovine milk. Equine milk forms a soft, easily digested, coagulum in the stomach. As expected, this study showed that equine β-casein does not yield BCM-7 or BCM-5 when subjected to *in-vitro* simulated gastric intestinal digestion which is highly significant from a nutritional and medical point of view. Because these peptides have been implicated in many diseases, efforts are now being made in several countries to exclude bovine milk with β-casein variant A^1 from the human diet and in the manufacture of infant formulae.

The absence of BCM-7 and BCM-5 in hydrolysates of equine milk may explain, at least in part, why equine milk is suitable in the diet of those allergic to bovine and other species milks. BCM-7 acts on μ-opioid receptors which causes the release of histamine (Kurek *et al*., 1992, 1995; Kostyra *et al*., 2004). BCM-7 has been blocked from stimulating the release of histamine by naloxone (an opiate receptor antagonist) but only when naloxone concentrations were 100 fold those of BCM-7 (Kurek *et al*., 1992). The release of histamine is of clinical significance for individuals who have allergies or digestive problems in that the identification of a dietary component that is known to signal the release of histamine could lead to recommendation that this component be eliminated from their diet.

10.5. References

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CHAPTER 11

General Discussion

The milk of all mammals contains the same principal components: water, fats, carbohydrates, proteins, salts and vitamins, but these constituents differ significantly both quantitatively and qualitatively between species, although species from the same taxonomic family e.g., equids, produce milk of similar composition. Equine milk is similar in composition to human milk but considerably different from that of the major dairy mammals, e.g., cow, buffalo, sheep, goat, camel, llama and yak. Inter-species differences in milk composition reflect very divergent patterns of nutrient transfer to the young and presumably reflect adaptations in maternal rearing of off-spring to physiological constraints and environmental conditions (Oftedal & Iverson, 1995). While the protein content of mature equine milk is lower than that of bovine milk, there is a strong qualitative resemblance, the principal classes of proteins, i.e., caseins and whey proteins, are similar in both types of milk. However, the caseins are the predominant class of proteins in bovine milk (~80% of total milk protein) but equine milk contains less casein and more whey proteins. Traditionally, equine milk has been an important food for humans in central Asia and the former Soviet Union (Ørskow, 1995) where the only significant product from equine milk is the fermented product, koumiss, which is widely consumed in Russia, Mongolia and Kazakhstan for its therapeutic value for the treatment of a wide variety of illnesses but the variable microbiology of these products has made it difficult to confirm any theoretical basis for the health-giving claims (Tamine & Robinson, 1999). Because equine milk resembles human milk in many respects and due to its claimed therapeutic properties, it is becoming increasingly important in Western Europe, especially in France, Italy, Hungary and the Netherlands. About fifteen years ago, equine milk was produced only in isolated small holdings in parts of Eastern Europe and Mongolia but now there are large-scale operations in France, Belgium, Germany, Austria and the Netherlands.

The characteristics of equine milk of interest in human nutrition include an exceptionally high concentration of polyunsaturated fatty acids, low cholesterol content, high lactose and low protein levels (Solaroli *et al.*, 1993; Salimei *et al*., 2004), as well as high levels of vitamins A, B and C. The high lactose content of equine milk gives good palatability and improves intestinal absorption of calcium which is important for bone mineralization in children (Iacono *et al*., 1992). The renal load of equine milk, based on levels of protein and inorganic substances, is equal to that of human milk, making it suitable in infant nutrition. Furthermore, lysozyme and lactoferrin are considerably higher in equine milk than bovine milk and are known primarily for their anti-bacterial properties.

The physico-chemical differences between human and bovine caseins result in the formation of different types of curd in the stomach (Hambræus, 1984) and because the protein profile of

equine milk is quite similar to that of human milk, equine milk may be more appropriate in human nutrition than bovine milk. The digestibility of equine and human milk (by *in-vitro* experiments) has been reported to be quite similar and both milks are easier to digest than bovine milk (Kallila *et al*., 1951). Human milk forms fine, soft flocs in the stomach with an evacuation time of 2 to 2.5 h, whereas bovine milk forms compact hard curds with a digestion time of 3 to 5 h. Equine milk has been reported to be a suitable substitute for bovine milk in the diet of children with cows' milk allergy (CMA) (Businco *et al.*, 2000), which is defined as a set of immunologically-mediated adverse reactions which occur following the ingestion of bovine milk and affects ~ 2.5% of children during the first 3 years of life (Hill & Hosking, 1996). Lara-Villoslada *et al. (*2005) found that the balance between casein and whey proteins is important in determining the allergenicity of bovine milk proteins in humans and that modification of this balance could reduce the allergenicity of bovine milk; a readjustment of the casein:whey proteins ratio to 40:60 was found to make bovine milk significantly less allergenic. Because equine milk has a casein:whey proteins ratio close to that in human milk, this may be one of the factors contributing to its success in treating patients with CMA.

Prior to this study, the fractionation and characterization of individual equine milk proteins had been reasonably well researched, although the issue of the presence or not of κ-casein remained contentious, as did the overall composition and structural details of equine casein micelles. It had been reported that equine caseins exhibited greater heterogeneity and a higher level of post-translational modifications than those of bovine milk (Miranda, 2004) but no research into the implications of the presence of many isoforms of the different caseins had been reported. This study aimed to address the lack of research on the physico-chemical properties of equine milk while comparing such properties to those of bovine milk and to examine the structure and factors stabilizing equine casein micelles, i.e., to definitively demonstrate the presence or not of κ-casein in equine milk and investigate other factors that may contribute to the stability of equine casein micelles. A detailed study of the colloidal stability of equine casein micelles was necessary to improve and develop the potential of equine milk in the health and nutritional markets.

A detailed compositional analysis of equine milk, including the quantification and distribution of its nitrogenous components and principal salts is reported (***Chapter 3)*** with comparative analysis of bovine milk. The protein content of equine milk, 22.98 g L^{-1}, was similar to that reported by others for milk from mares in mid-lactation (Ullrey *et al.*, 1966; Mariani *et al.*, 2001; Smolders *et al.*, 1990; Zicker & Lönnerdal, 1994) and significantly lower than that in

bovine milk (34.1 g L^{-1}). The lactose content of equine milk, at 66 g L^{-1}, is considerably higher than that of bovine milk (49.1 g L^{-1}) and is in agreement with values reported by Solaroli *et al.* (1993), Malacarne *et al.* (2002) and Park *et al.* (2006). The casein:whey protein ratio in this study was 1.45:1 while that of human milk, to which equine milk is frequently compared, has been reported as ~ 0.5-0.66:1 (Shamsia, 2009). Skimmed equine milk contained 19.35 mmol L^{-1} calcium, whereas its ultracentrifugal supernatant contained 6.3 mmol L^{-1}, suggesting that in equine milk, ~65% of total calcium is in the micellar phase, which agrees with Holt & Jenness (1984). The concentration of total calcium was considerably higher than that reported for Italian Saddle mare's milk (Martuzzi *et al.*, 1997) and mares of the Bardigiano breed (Martuzzi *et al.*, 1998), but is within the range reported by Anderson (1991) for the Quarter Horse breed. Bovine milk had ~ 29 mmol L^{1} total Ca which was within the range reported by Tsioulpas *et al.* (2007). Both equine and bovine milk had significantly higher levels of total calcium than the values reported for human milk (~ 7.5-8.0 mmol L^{-1}, Neville *et al.*, 1995; Silanikove *et al.*, 2003). The concentration of ionic calcium, at the natural pH of equine milk (pH 7.27), was 2.66 mmol L^{-1}, which agrees well with the value predicted by Holt & Jenness (1984) and considerably higher than that obtained for bovine milk, 1.76 mmol L^{-1}. Provision of calcium to the neonate is essential for mammals during lactation (Oftedal, 1980). The concentration of calcium is directly related to the growth rate of the neonate and is relatively high in equine milk, while the concentration in human and primate milk is low at 7-8 mmol L^{-1} (Lewis, 2010). Calcium in milk is found complexed with protein which must be broken down to release the calcium in a soluble and, probably, ionized form before it is absorbed; lactose in milk enhances calcium bioavailability (Allen, 1982) and therefore, the high lactose content of equine milk favours the absorption of calcium. The results of this study suggest that a large proportion of equine calcium is bound directly to casein, increasing its bioavailability (Guégan & Pointillart, 2000), and not in the form of micellar nanoclusters. It is proposed that the high calcium level in equine milk coupled with a low concentration of colloidal inorganic phosphate implies that the foal relies on casein-bound calcium for a substantial amount of its bioavailable calcium. Following ingestion of milk, casein-bound calcium could be made available relatively quickly in the foal's stomach through proteolysis of casein in conjunction with the acid-induced solubilisation of calcium from casein and calcium phosphate.

Chapter 3 included the measurement of equine casein micelle size by two techniques; using multi-angle DLS, the hydrodynamic radius of bovine and equine micelles was found to be 88

and 127 nm, respectively, and by photon correlation spectroscopy (fixed-angle DLS) mean micelle diameters were 179 and 277 nm for equine and bovine milks, respectively, which compared well with the DLS results. The heat-, ethanol-, rennet- and acid-induced coagulation of skimmed equine milk were also examined (***Chapter 3)*** and in detail later (***Chapters 4, 5 and 6***). Heat-induced coagulation of equine milk at 140°C occurred rapidly at pH 6.3-6.9, but the coagulation time at pH 7.0-7.3 was >45 min. The heat stability of 2-fold concentrated milk increased gradually from pH 6.7, up to a maximum at pH 7.1. Skimmed equine milk displayed a sigmoidal ethanol stability-pH profile, but the stability of equine milk was lower than that of bovine milk. Equine casein micelles were not coagulable by calf chymosin at pH 6.6. Some micellar flocculation, but no gelation, was observed on acidification of equine milk.

The failure of calf chymosin to coagulate suspensions containing 25 g L^{-1} equine casein in equine serum, approximating the casein content of bovine milk, at pH 6.6 was found to be related to the equine micelles, rather than to the composition of the equine milk serum, since equine casein micelles suspended in bovine milk serum also failed to coagulate, whereas bovine casein micelles suspended in equine milk serum, coagulated in a manner similar to those suspended in bovine serum. Equine κ-casein has been reported to be susceptible to rennet-induced hydrolysis at the Phe_{97}-Ile_{98} bond (Egito *et al.*, 2001), but its hydrolysis is considerably slower than that of bovine κ-casein (Kotts & Jenness, 1976). The inability of calf chymosin to hydrolyse significant amounts of equine κ-casein within a relatively short time is probably, at least partially, responsible for the inability of chymosin to induce noticeable flocculation of equine milk even after renneting for 36 h.

Urea-PAGE showed the greater microheterogeneity of the α_s- and β-caseins of equine milk than the corresponding bovine proteins, indicating a higher level of post-translational modifications of caseins in equine milk than those of bovine milk (Miranda, 2004), the significance of which is discussed throughout this study. The low casein content of equine milk and differences in micellar casein composition and stability compared to bovine micelles could account for the differences in ethanol stability and in the ethanol-induced temperature-dependent dissociation of the micelles of both milks reported in Chapter 6. The high Ca^{2+} concentration in equine milk may also be a factor contributing to difference in the stability of both milks, as it is generally accepted that Ca^{2+} play an important role in many of the physico-chemical and functional properties of milk, especially stability to ethanol and heat (Demott, 1968; Geerts *et al*., 1983; Augustin, 2000; Jeurnink & de Kruif, 1995; Faka *et al.*, 2009.

The results presented in ***Chapter 3*** provided a first step towards gaining a fundamental understanding of the physico-chemical properties of equine milk, which is required for the successful exploitation of the full potential of equine milk, or products derived therefrom, as a hypo-allergenic food product. The colloidal stability of equine casein micelles differs considerably from that of bovine casein micelles, which may have significant implications for the conversion of equine milk into dairy products. Differences in protein composition and in micellar structure found in this study between equine and bovine milk will markedly influence the properties of coagula produced from acidified or renneted equine milk and hence influence the digestibility and bioavailability of milk nutrients. It appears that equine milk, like human milk, forms very soft, fine floccules which may be more suitable in infant nutrition due to ease of digestion, as reported by Kalliala *et al.* (1951) and Solaroli *et al.* (1993).

The rheological properties of acidified unheated equine milk, with comparative analysis of unheated bovine milk were studied (***Chapter 4)*** using small deformation rheological measurements and creep recovery tests. The influence of acidification by glucono-δ-lactone (GDL) on the storage modulus, G', of equine and bovine milk and an equine sample with an increased casein concentration of 25 g L^{-1}, showed that even when the casein concentration in equine milk was increased, little structure developed in the sample following acidification. Differences in acid-induced flocculation between equine and bovine casein micelles may be related to differences in the mechanism by which they are sterically stabilized. It is unlikely that pre-treatment of equine milk by heating (> 70^{o}C) would increase the gel strength and prevent syneresis of acidified equine milk. Equine β-lactoglobulin lacks a free thiol group which, in the case of heated bovine milk, forms disulfide links with other proteins having a reactive thiol groups or through thiol group-disulfide bridge exchange reactions.

Using visual examination, particle size measurements, light and confocal microscopy and rheological characterization, the rennetability of equine milk was investigated (***Chapter 5)***. Equine milk was coagulated very slowly by calf chymosin at pH 6.6, but decreasing the pH to 6.2 increased the rate of aggregation of equine protein although at both pH values no gel-like structure formed. Concentrating the casein in equine milk three and five fold had little effect on the coagulation process and the final G' remained low. A crude chymosin preparation from foal's stomach coagulated equine milk quickly but without the formation of a strong coagulum. The equine chymosin extract had good activity on bovine milk, although a weaker curd formed than that formed from bovine milk renneted with calf chymosin. Mixing bovine

and equine milks at a ratio of 3:1 is suggested as a means of producing some novel products from equine milk, while maintaining some of the unique properties of the original milk. Equine κ-casein is reported to be hydrolysed slowly by calf chymosin at the Phe_{97}-Ile_{98} bond (Egito *et al*., 2001), without gel formation, and it appears that either the chymosin-sensitive bond of equine κ-casein is located in the micelle in a manner which renders it inaccessible to chymosin, or that the equine casein micelle is rendered colloidaly stable by constituents other than κ-casein. The high level of glycosylation reported for equine κ-casein may also affect the ability of chymosin to hydrolyze equine κ-casein .

While the main focus of the research reported in ***Chapter 6*** was to examine the dissociation of equine casein micelles by various treatments, the detection of glycoprotein in equine milk was also examined. The dissociation of equine casein micelles at their natural concentration or with the casein increased to 25 g L^{-1}, approximating that of bovine milk, by trisodium citrate or urea followed a similar pattern to the dissociation of micelles in skimmed bovine milk . The effect of temperatures of 4 or 20^0C, on the dissociation of equine caseins was also studied using ultracentrifugation and showed less dissociation of equine β-casein at 4 or 20^0C compared to bovine β-casein, while a considerable proportion of equine α_s-casein dissociated at both temperatures. Temperature-sensitive (TS) and cold-precipitated (CP) fractions of equine casein were prepared at pH 4.2 or 4.6, both of which stained positively for glycoproteins. Equine casein micelles were separated by differential centrifugation which also allowed the positive identification of glycoprotein in equine milk. Equine casein micelles were less stable to ethanol than bovine micelles and high ethanol concentrations coupled with a high temperature caused irreversible changes to the micelles. When heated to 80^oC with a final ethanol concentration of 50% (100%, v/v) followed by rapid cooling on ice, bovine milk formed a firm gel but equine milk did not.

The effects of high pressure or heat on equine whey proteins is reported in ***Chapter 7.*** Equine α-lactalbumin (α-la) and β-lactoglobulin (β-lg) were less susceptible to high pressure-induced denaturation than their bovine counterparts. Equine α-la and β-lg showed less denaturation under high pressure than their bovine counterparts in the presence of 25 g L^{-1} equine or bovine micellar casein, respectively, or when the micellar casein phases were reversed. The resistance of equine β-lg to high pressure denaturation is attributed to its lack of a free thiol group and its consequential inability to participate in sulphydryl-disulphide interchange reactions. Equine α-la had a similar denaturation pattern to its bovine counterpart when heated at 60 to 95^oC but equine β-lg was more stable to heat than equine α-la or bovine β-lg.

The lack of a free sulphydryl group in equine β-lg is probably responsible for the different responses of equine and bovine milk to heat or high pressure. High pressure treatment of equine milk could be used as a method of preservation while keeping the whey proteins and casein micelles relatively intact although, for infants with milk protein allergy, the presence of undenatured β-lg may be problematic.

Heat treatment has been used to modify the functional properties of bovine whey proteins (e.g., gelation and emulsification) and to reduce the allergenicity of β-lg (Hill, 1994; Ehn *et al*., 2004), although, if a temperature of 90^{o}C is used, allergenicity may be increased due to the exposure of internal antigenic sites of the protein (Kleber *et al*., 2004). Recently, the sale of raw equine milk has commenced in health food shops and some pharmacies in western Europe but wider acceptance of the product will require heat treatment as a means of preservation. This study has shown that heat treatment denatures β-lg in equine milk and is a suitable method for preserving the milk, reducing its allergenicity and maintaining high levels of active lysozyme (Jauregui-Adell, 1975; Bonomi *et al*., 1994). The use of ultra high temperature (UHT) in-container sterilization of equine milk warrants investigation as such a method of preservation may make equine milk available to a wider market, however, the effects of such treatment on the proteins, specifically whey proteins, of equine milk have not been investigated to date.

Recently, more sequencing information in databases and the increased resolution and sensitivity of separation techniques has meant that it is becoming much easier to separate and identify proteins in the milk of different species. Proteomics and associated technologies, which are very powerful for the detection and characterization of different components in complex protein mixtures, were employed in ***Chapter 8***. The aim of this chapter was to separate and characterize the protein fraction of equine milk, principally to determine if equine milk contained κ-casein. Previous studies (Chapters 3 and 5) had demonstrated the poor coagulability of equine milk by calf chymosin at pH 6.5 and the weak coagulum formed when renneted with equine chymosin. Therefore, this chapter used a proteomic approach to examine the rennetability of equine milk by calf chymosin using 2-DE followed by mass spectrometry with comparative analysis of a renneted bovine milk sample. A set of analytical procedures was used, including different electrophoretic methods, reverse phase high performance liquid chromatography (RP-HPLC), MALDI-TOF MS analysis and nano-LC-MS/MS analysis. By dephosphorylating fractions collected from C4 RP-HPLC of clarified equine milk followed by mass spectrometry, κ-casein was identified. Analysis of protein-

containing spots obtained from 2-DE also positively identified κ-casein in equine milk. Renneting equine and bovine milks with calf chymosin at pH 6.5, and using 2-DE and mass spectrometry to analyse protein-containing spots which increased or decreased in intensity over time, revealed that the κ-casein of equine milk, unlike that of bovine milk, was unaffected by calf chymosin, whereas equine β-casein was hydrolysed reasonably quickly with several β-casein-containing spots decreasing in after rennet addition. Analysis of renneted equine and bovine milks by C18 RPHPLC also showed a different pattern in peptide formation between the two milks.

The purpose of the study reported on in ***Chapter 9*** was to compare the casein micelles in equine and bovine milk using a variety of microscopy techniques and to image the effects of dissociation by trisodium citrate, addition of rennet and acidification on both milk systems using electron microscopy. By combining several microscopy methods in one study it was hoped that the limitations of any one method would be minimized and a clear idea of equine casein micelle structure would result. Over the last twenty years or so, the instrumentation available for electron microscopy has been improved significantly, allowing greater image resolution and finer detail in the micrographs of casein micelles (McMahon & McManus, 1998). However, the conformational flexibility of casein micelles has meant that their structure changes easily during sample preparation for electron microscopy (McMahon & Oommen, 2008). This study used most electron microscopy techniques, including the latest cryo-STEM technique which involved minimal sample preparation (Gee *et al*., 2010) and preserved the casein micelles as close to the native state as has been possible to date. The study demonstrated that techniques such as negative staining with TEM imaging which is relatively easy to perform, can yield detailed images of casein micelles if undertaken carefully. Measurements of casein micelles from electronmicrographs were close to those reported on in ***Chapter 3*** obtained from multi- and fixed-angle laser light scattering and micelle size reported for equine casein micelles using various electron microscopy techniques were largely similar. The high resolution obtained by cryo-SEM gave very clear images of equine and bovine casein micelles in their native state and when treated with either acid or chymosin. The outer layer of the surface of the casein micelle is largely unknown and the model of Holt (Holt & Horne, 1996; DeKruif & Holt, 2003) suggests that there is no well-defined structured hairy layer but instead there is a layer of decreasing protein density at the micelle surface. This was evident in the case of equine and bovine casein micelles in this

study, particularly in images obtained at high magnification using TEM. The results agreed with the lack of a defined surface layer in the polymer condensation model of Horne (1998).

The sub-micelle model of casein micelle structure first introduced by Morr 1967 and modified by Slattery & Evard (1973) is not universally accepted and mounting evidence suggests that well-defined casein sub-micelles do not exist and that the structure is more open and flexible. The results of this study support this theory and using several microscopy techniques with magnifications of up to 300 K, no sub-micelles were evident within equine or bovine casein micelles. Equine micelles contain little κ-casein and the micelles are large and, given the evidence presented (***Chapter 8*** and ***9)***, it is likely that other proteins, probably β-casein, contribute to micellar stability. Whether it is unphosphorylated or phosphorylated β-casein that stabilize the micelle is unknown. Phosphorylated β-casein would be hydrophilic and, in theory, capable of stabilizing casein micelles but, on the other hand, it may precipitate in the presence of calcium.

Coagulation of milk is important from a nutritional point of view, as clotting of the caseins in the stomach, and the type and structure of the resultant coagulum, strongly affects protein digestibility. Equine casein micelles aggregate when renneted or acidified as demonstrated in this study and the lack of network formation and weak coagulum formed is optimal for the physiological well-being and development of the foal.

In the study reported on in ***Chapter 10*** the components of purified equine β-casein were resolved by capillary zone electrophoresis (CZE) and demonstrated the heterogeneity of the protein, with phosphorylated residues, ranging from 4P to 7P. The *in vitro* digestion of equine β-casein by simulated gastrointestinal digestion (SIGD) was investigated to confirm that the opioid β-casomorphins, BCM-5 or BCM-7, or analogous peptides were not present in the digests analysed using HPLC coupled with electrospray ionization and mass spectrometry (HPLC-ESI/MS). The absence of BCM-5 or BCM-7 is significant from a nutritional and medical point of view. Because these peptides have been implicated in many diseases, efforts are now being made in several countries to exclude bovine milk with β-casein variant A^1, from which BCM-7 and BCM-5 are produced, from the human diet and in the manufacture of infant formulae. Extensive hydrolysis of equine β-casein by pepsin and Corolase PP™ was evident.

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Proposed Future Research Work

Based on the results of this study, several areas for future research are proposed

1. A thorough investigation of the calcium-binding by equine casein should be carried out as it appears to be different from bovine milk and may be similar to calcium-binding in human milk. It has been indicated in the present study that the foal, similar to the human neonate, may rely on casein-bound calcium, which is readily bioavailable, for a significant proportion of its calcium.

2. Unlike equine milk, asinine milk formed a weak gel when renneted with calf chymosin at pH 6.6, suggesting differences between *Equidae* species in the formation of a coagulum in the foal's stomach. Asinine casein micelles have been reported to be considerably smaller than those of equine milk. Given that both equine and asinine species are phylogenetically related, it is expected that the respective milks would have similar physico-chemical characteristics; a thorough analysis of the physico-chemical properties of asinine milk is warranted. Similarly, the milk of the zebra, which is another *Equidae* species, has been poorly researched and, subject to sample availability, warrants full compositional and physico-chemical analysis.

3. The degree of glycosylation of equine κ-casein is unknown, although reports suggest that it is highly glycosylated and no unglysosylated κ-casein has been reported. Investigation of the level of glycosylation is warranted as it may affect hydrolysis by chymosin, as well has having important biological significance.

4. Renneting equine milk with a crude preparation of equine chymosin at pH 6.2 produced a weak coagulum with a G' of ~ 10 Pa, the examination of which by electron microscopy is warranted to determine if the casein micelles form clusters or aggregates with more linkages than those formed when calf chymosin is used.

5. In ***Chapter 6***, a crude chymosin extract was prepared from foal's stomach. Further purification and characterization of this extract is required, as well as the quantification of any pepsin that may be present. A study of the renneting of equine milk with purified equine chymosin using the proteomic techniques described in ***Chapter 8***, which included 2-DE and

mass spectrometry, to determine if equine chymosin hydrolyses equine κ-casein and to determine the extent to which it hydrolyses equine β-casein.

6. Results in ***Chapter 6*** provide more evidence that caseins in the milk of some species may be preferentially hydrolysed and coagulated by chymosin of that species. There are many combinations of species milks and chymosins that could be analysed to provide further information on this aspect.

7. ***Chapters 5*** and ***6*** included electrophretograms (Figures 5.18 & 6.4) which showed several very slow and one fast-migrating band which have not been identified and may be unique to equine milk. Identification and characterization of these proteins by nano-LC-MS/MS analysis may extend the portfolio of proteins in equine milk.

8. The functionality of κ-carrageenan in dairy products has been well known for many years as it interacts synergistically with milk proteins, primarily casein micelles, to enhance the viscosity and gelation properties of bovine milk (de Vries, 2002; Spagnuolo *et al*., 2005). It has reported that negatively-charged κ-carrageenan interacts with a positively-charged region (residues 97-112) of bovine κ-casein, thus adsorbing onto the surface of casein micelles (Dalgleish & Morris, 1988), although the surface of the micelle is negatively charged and stabilized by the 'hairy layer' (glycomacropeptide portion of κ-casein) which would make it difficult for penetration by κ-carrageenan to reach the positive region of κ-casein (Verbeken *et al*., 2004); therefore, a second theory proposes that κ-carrageenan forms a weak gel within which the casein micelles are entrapped (Bourriot *et al*., 1999; Spagnuolo *et al*., 2005). It is proposed that addition of κ-carrageenan to equine milk could be investigated to determine if there is any interaction between κ-carrageenan and the equine casein micelle surface *via* κ-casein, which may enhance the gelation properties of equine milk, in addition to which more information on the effect of κ-carrageenan on milk systems in general may be revealed, especially as it is proposed in this study and by others that the surface charge of equine casein micelles may be different to that of bovine micelles.

9. Di Cagno *et al*. (2004) fortified equine milk with bovine sodium caseinate, pectin and threonine and the resultant products had good microbiological, rheological and sensory characteristics. More detailed rheological studies on the fortification of equine milk with

pectin, the gelation of which is favoured by the high ionic calcium concentration in equine milk, are warranted to improve the range of products produced from equine milk. In addition, blends of equine and bovine milk could be created to expand the range of fresh dairy products or fermented products from equine milk, which would retain some of the unique compositional characteristics of equine milk. Addition of the milk of other species could also be investigated, especially the addition of, for example, buffalo milk or sheep milk which have a higher protein content than bovine milk and may allow the development of cheese containing a high proportion of equine milk

10. The effect of high pressure (HP) treatment on equine milk lysozyme warrants investigation as the high lysozyme content of equine milk and its thermal stability is one of the unique characteristics of equine milk. The stability of bovine lysozyme to HP has not been reported on although the stability of several enzymes in bovine milk to HP have been reported on, e.g., alkaline phosphatase, plasmin, lipase and xanthine oxidase.

11. The use of ultra high temperature (UHT) in-container sterilization as a means of preserving equine milk warrants investigation, specifically, the effect of UHT treatment on the principal whey proteins and lysozyme of equine milk.

12. There are several commercially-produced replacement milks on the market for feeding orphaned foals. A study on the composition and properties of these milk-replacers is necessary, in the light of the results in this study, to optimize the composition and physico-chemical properties.

13. It was noted in Chapter 4 that pre-heating equine milk prior to acidification is unlikely to strengthen a curd formed because equine β-lg does not contain a free thiol group and therefore probably won't associate at the micelle surface acting as bridging material between micelles – the effects of pre-heating equine milk on subsequent acid gel formation should be investigated.

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Appendix

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