EFFECT OF HEAT TREATMENT ON IN-VITRO PROTEIN DIGESTIBILITY OF COW MILK

THESIS SUBMITTED TO THE

NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY CHEMISTRY

BY

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B. Tech (Dairy Technology)

DIVISION OF DAIRY CHEMISTRY

NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

KARNAL-132001 (HARYANA), INDIA

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This is to certify that the thesis entitled "**EFFECT OF HEAT TREATMENT ON IN-VITRO PROTEIN DIGESTIBILITY OF COW MILK"** submitted by **MR. VINAY KASHYAP** towards the partial fulfilment of the requirement for award of the degree of **MASTER OF TECHNOLOGY** in **DAIRY CHEMISTRY** of the **ICAR- NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY),** KARNAL (Haryana) is a bonafide research work carried out by her under my supervision and guidance. The work embodied in this thesis is original and no part has been submitted in part or full for the award of any diploma or degree of this or any other university.

Date: 20/11/2021

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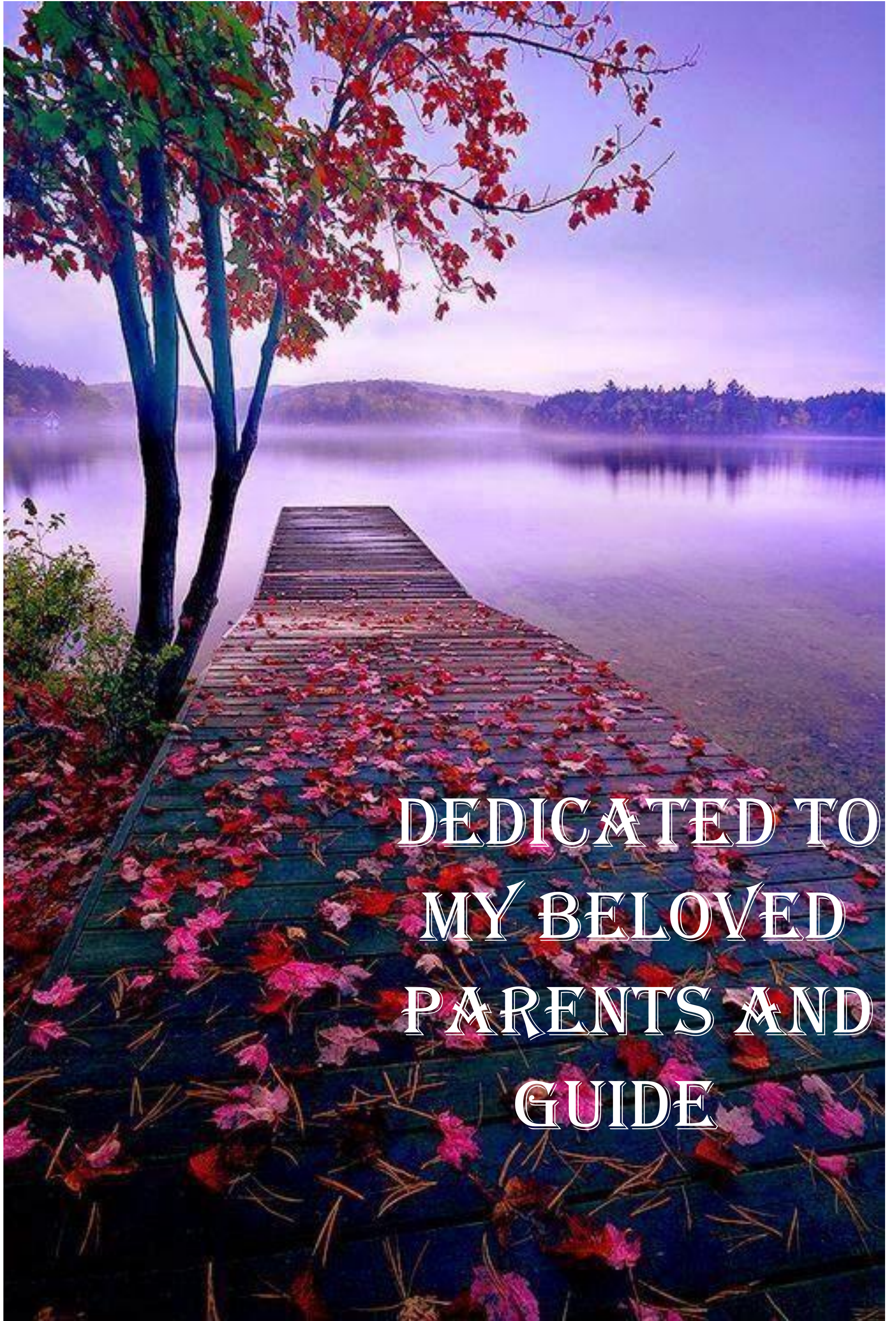
DEDICATED TO
MY BELOVED
PARENTS AND
GUIDE

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LIST OF ABBREVIATION

α-CN	:	α –casein
β-CN	:	β-casein
κ-CN	:	κ-casein
α-LA	:	α-lactalbumin
β-LG	:	β-lactoglobulin
ABTS	:	2,2'azinobis (3-ethylbenzothiazoline)-6-sulfonic acid
ACN	:	Acetonitrile
APS	:	Ammonium persulfate
CMT	:	California Mastitis Test
CN	:	Casein
Da	:	Dalton
DTT	:	Dithiothreitol
GI	:	Gastrointestinal
Gdn	:	Guanidine
HCl	:	Hydrochloric acid
HTST	:	High Temperature Short Time
M	:	Molar
N	:	Normal
NaDodSO4	:	Sodium dodecyl sulphate
NaOH	:	Sodium hydroxide
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffer saline
RP-HPLC	:	Reversed Phase-High Performance Liquid Chromatography
SGD	:	Simulated Gastric Digestion
SGF	:	Simulated Gastric Fluid

SGID	:	Simulated gastrointestinal digestion
SID	:	Simulated intestinal digestion
SIF	:	Simulated intestinal fluid
OPA	:	O-phthadialdehyde
TFA	:	Trifluoroacetic acid
TAC	:	Total Antioxidant Capacity
SCC	:	Somatic cell count
SWM	:	Sahiwal whole milk
SBWM	:	Sahiwal Boiled whole milk
KFWM	:	Karan Fries whole milk
KFBWM	:	Karan Fries Boiled whole milk
MWM	:	Murrah whole milk
MBWM	:	Murrah Boiled whole milk
G0	:	Gastric digestion sample at 0 minute
G5	:	Gastric digestion sample at 5 minute
G10	:	Gastric digestion sample at 10 minute
G15	:	Gastric digestion sample at 15 minute
G30	:	Gastric digestion sample at 30 minute
G60	:	Gastric digestion sample at 60 minute
G90	:	Gastric digestion sample at 90 minute
G120	:	Gastric digestion sample at 120 minute
I0	:	Intestinal digestion sample at 0 minute
I5	:	Intestinal digestion sample at 5 minute
I15	:	Intestinal digestion sample at 15 minute
I30	:	Intestinal digestion sample at 30 minute
I60	:	Intestinal digestion sample at 60 minute
I90	:	Intestinal digestion sample at 90 minute
I120	:	Intestinal digestion sample at 120 minute

Abstract

Milk is one of the main sources for high quality protein. Heat treatment is the most widely used processing technology in the dairy industry. The present study aimed to find effect of heat treatment on digestibility and antioxidant activity of milk protein from cow milk. Cow and buffalo milk samples were heated at different time temperature combination (63°C/30 min, 80°C/10 min and boiled /10 min) and assessed for their protein profile over RP-HPLC and SDS-PAGE. Milk compositional analysis indicate the relatively higher level of fat and SNF content in milk from Sahiwal breed compared to crossbred KF. On extensive heat treatment by boiling concentration resulted in increase in milk total solid level and significant increase in the protein content of cow milk from Sahiwal and KF corresponding to 6.69 ± 0.25% and 5.04 ± 0.06%, respectively. Maximum denaturation of the whey protein was observed in boiled milk samples due to extensive heat treatment compared to pasteurized milk both for cow and buffalo milk, as determined using SDS-PAGE and RP-HPLC. Highest antioxidant activity of milk was observed in heated milk compared to unheated milk both in bovine and buffalo milk. Buffalo milk (631.24 Vit C mg/l) showed highest antioxidant activity followed by cow milk from Karan fries (547.97 Vit C mg/l) and Sahiwal (456.03 Vit C mg/l) after boiling treatment. Following in vitro GI digestion of cow milk from Sahiwal and KF breed, it was observed that relatively greater increase in the release of free amino group in milk from Sahiwal breed (8.45 mmole of serine/g of protein) as compared to that from KF(5.9 mmole of serine/g of protein). Compared to control, a significant increase in release of free amino groups was observed on boiling treatment of cow milk, resulted in 9.72 mmole of serine/ g of protein for Sahiwal breed and 6.99 mmole of serine/ g of protein for KF breed. Based on Tricine SDS-PAGE, the result indicated that the breakdown of protein was faster in heated milk than unheated bovine milk. Further relatively greater increase in antioxidant activity was observed in boiled milk as compared to that for control following in vitro digestion. Thus the result indicates that the extensive boiling of cow milk (Sahiwal & KF Breed) resulted in increase in digestibility and antioxidant activity.

सारांश

दूध उच्च गुणवत्ता वाले प्रोटीन के मुख्य स्रोतों में से एक है। डेयरी उद्योग में हीट ट्रीटमेंट सबसे व्यापक रूप से इस्तेमाल की जाने वाली प्रसंस्करण तकनीक है। वर्तमान अध्ययन का उद्देश्य गाय के दूध से दूध प्रोटीन की पाचनशक्ति और एंटीऑक्सीडेंट गतिविधि पर गर्मी उपचार के प्रभाव का पता लगाना है। गाय और भैंस के दूध के नमूनों को अलग-अलग समय तापमान संयोजन (६३ डिग्री सेल्सियस/३० मिनट, ८० डिग्री सेल्सियस/१० मिनट और उबलते /10 मिनट) पर गर्म किया गया और आरपी-एचपीएलसी और एसडीएस-पेज पर उनके प्रोटीन प्रोफाइल के लिए मूल्यांकन किया गया। दुग्ध संघटनात्मक विश्लेषण, संकर नस्ल केएफ की तुलना में साहीवाल नस्ल के दूध में वसा और एसएनएफ की मात्रा के अपेक्षाकृत उच्च स्तर का संकेत देते हैं। उबलते सांद्रता द्वारा व्यापक गर्मी उपचार के परिणामस्वरूप दूध के कुल ठोस स्तर में वृद्धि हुई और साहीवाल और केएफ से गाय के दूध की प्रोटीन सामग्री में क्रमशः 6.69 ± 0.25% और 5.04 ± 0.06% के अनुरूप वृद्धि हुई। गाय और भैंस दोनों के दूध के लिए पाश्चुरीकृत दूध की तुलना में व्यापक गर्मी उपचार के कारण, एसडीएस-पेज और आरपी-एचपीएलसी का उपयोग करके निर्धारित दूध के नमूनों में मट्ठा प्रोटीन का अधिकतम विकृतीकरण देखा गया। गोजातीय और भैंस के दूध दोनों में गर्म दूध की तुलना में दूध की उच्चतम एंटीऑक्सीडेंट गतिविधि गर्म दूध में देखी गई। भैंस के दूध (६३१.२४ विटामिन सी मिलीग्राम/लीटर) ने उच्चतम एंटीऑक्सीडेंट गतिविधि दिखाई, इसके बाद करण फ्राइ (५४७.९७ विटामिन सी मिलीग्राम/ली) और साहीवाल (४५६.०३ विटामिन सी मिलीग्राम / एल) से गाय का दूध उबालने के बाद मिला। साहीवाल और केएफ नस्ल से गाय के दूध के इन विट्रो जीआई पाचन के बाद, यह देखा गया कि साहीवाल नस्ल (8.45 मिमी सेरीन / प्रोटीन का ग्राम) से दूध में मुक्त अमीनो समूह की रिहाई में केएफ की तुलना में अपेक्षाकृत अधिक वृद्धि हुई। (5.9 मिमी सेरीन / प्रोटीन का ग्राम)। नियंत्रण की तुलना में, गाय के दूध के उबलते उपचार पर मुक्त अमीनो समूहों की रिहाई में उल्लेखनीय वृद्धि देखी गई, जिसके परिणामस्वरूप साहीवाल नस्ल के लिए 9.72 मिमी सेरीन / ग्राम प्रोटीन और केएफ नस्ल के लिए 6.99 मिमी सेरीन / प्रोटीन का ग्राम मिला। ट्राईसीन एसडीएस-पेज के आधार पर, परिणाम ने संकेत दिया कि गर्म दूध में प्रोटीन का टूटना गर्म दूध की तुलना में तेजी से होता है। इन विट्रो पाचन में निम्नलिखित नियंत्रण की तुलना में उबले हुए दूध में एंटीऑक्सीडेंट गतिविधि में अपेक्षाकृत अधिक वृद्धि देखी गई। इस प्रकार परिणाम इंगित करता है कि गाय के दूध (साहीवाल और केएफ नस्ल) के व्यापक उबालने से पाचनशक्ति और एंटीऑक्सीडेंट गतिविधि में वृद्धि हुई है।

CHAPTER –1

Introduction

INTRODUCTION

Milk may be defined as the whole, fresh, clean, lacteal secretion obtained by the complete milking of one or more healthy milch animal, excluding that obtained within 15 days before or 5 days after calving or such periods as may be necessary to render the milk practically colostrum- free and containing the minimum prescribe percentage of milk fat and Solid-not-fat (De, 1980). Milk is a complete food contain fat, protein, carbohydrate, minerals, vitamins etc. Milk being variable biological fluid, its composition varies with breeds, species, stage, age and health of animal, feed and fodder, environmental causes. (Mathur *et al.,* 2008)

Milk is the major source of high quality protein. It is an important source of nutrition and composed with 87% water, 3.4% protein, 4.1% fat, 4.7% lactose and 0.7% minerals. Casein (CN) are the predominant protein components in milk (80%). Casein can have four different forms which can be separated in the four groups: αs1-, αs2-, β-and κ-CN, in the approximate proportions of 32.5%, 8.5%, 32.6% and 10.7%, respectively. Whey protein represent about 20% of the total protein content in milk. β-lactoglobulin, α-lactalbumin , immunoglobulins and serum albumin are the major whey proteins (Walstra *et al.,* 2006)

The world's largest cattle population (190.0 million) resides in India (Ministry of Agriculture, 2014), among these 79% are of Indigenous origin (*Bos indicus*). India possesses 50 acknowledged indigenous breeds of cattle and 17 that of buffalo (http://www.nbagr.res.in/registeredbreed.html). In recent years, a lot of interest has been shown by the public for milk from indian breeds of cattle and has been related to better health properties. Cattle of indigenous breeds not only contribute to milk production but are also used as draught animals for agricultural operations and transport. Indigenous cattle are quality of heat tolerance and ability to withstand extreme climatic condition, resistance to diseases.

Milk protein has high nutritional properties due to its high level of essential amino acids and high bioavailability. This high bioavailability of milk proteins compared to protein from plant sources is due to its high digestibility, which is partly due to the absence of anti-nutritional factors and a variety of processing (Schaafsma, 2012). Heat treatment

is the important processing treatment in dairy plant to establish Specific product quality, to establish technological purpose etc. these processing operations alter the milk properties (Walstra *et al.,* 2006). Dairy industry use different time-temperature combination for processing of milk & milk products like HTST, UHT and sterilization etc. which change the milk digestibility and other properties. (Tunick *et al.,* 2016). Dairy processing treatment can change the structure of milk proteins, Depending on the type of treatment under which it has been processed. The main protein modifications occurring during processing are denaturation, aggregation, dephosphorylation, glycation, oxidation, cross linking of protein and recemization of protein and chemical modifications of its amino acids. These processing-induced changes of protein may change digestion. (Lieshout *et al.,* 2019)

Heating of milk (>80°C) causes partial or full denaturation of the whey proteins (Walstra, 2006; Wouters, 2006; Geurts, 2006) due to heat treatment, β-lactoglobulin and k-casein join together and form aggregate on the casein micelles. Denaturation of protein due to heating affect the digestibility of milk protein, casein is easily digested in the stomach, due to open structure while native whey proteins are more resistant to hydrolysis and are still intact after gastric digestion due to specific conformation of whey protein (Tunick *et al.,* 2016). Denatured β-lactoglobulin becomes more digestible by pepsin due to unfolding and the as a result increased accessibility of protein cleavage sites (Lieshout *et al.,* 2019)

The present study is formulated to assess the effect of heat treatment on the digestive and antioxidant properties of milk from different breeds of cow.

Hence, the project has been proposed with the following objectives:

1. Evaluation of in-vitro protein digestibility of cow milk under different heating condition.

CHAPTER –2

Review of Literature

REVIEW OF LITERATURE

2.1 Milk Composition

Milk is a very complex food which provide a number of nutritional benefits. Milk is a complete fluid comprising many components in different states of dispersion. It is uses as a raw material for production of different types of dairy & food products.

2.1.1 Differences in the composition of milk proteins in different species and breeds:

Kapadiya *et al.*(2016) did the comparison of surti goat milk with cow and buffalo milk for gross composition, nitrogen distribution it was found that range of protein content in goat milk was 3.18-3.49%, whereas cow and buffalo milk protein content was 3.19-3.62% and 4.11- 4.74% respectively

Patel *et al.* (2018) compared indigenous cattle (Tharparkar), crossbred cattle and buffalo (Murrah) for fat content and observed that that indigenous cattle have higher fat% than crossbred cattle but less than buffalo milk and similar pattern was observed for protein% (3.00%, 2.81%, and 3.16%, respectively).

Similar study was done by Islam *et al.* (2014) in which they compared two indigenous cattle milk, crossbred cattle milk and buffalo milk for the total protein content. They observed that the highest protein content was of the indigenous cattle followed by Buffalo and lowest content was found in cross breed milk.

Devarasetti *et al.* (2018) studied the composition of native breeds (Sahiwal, Punganur and ongole) and cross breed (HF and Jersey). They found that protein content in HF, Jersey, Sahiwal, Ongole and Punganur was 3.16%, 3.17%, 3.32%, 3.51%, and 3.45%, respectively.

On study by Das, (2019) related to the effect of 2 seasons (period I – Aug-Sept and period II - Dec-Jan) on the protein composition of milk from different indigenous breeds of cattle and buffalo revealed that there was significant difference in all the breeds of bovine i.e., in Sahiwal (3.39 ± 0.27%; 4.02 ± 0.32%), Gir (3.49 ± 0.19%;

4.11±0.47%), Tharparkar (3.56±0.27%; 4.20±0.07%) and Karan Fries (3.11±0.21%; 3.63±0.16%) with respect to crude protein% and no significant difference was observed in buffalo milk i.e. in Murrah and Nili ravi (4.27±0.41%; 4.46±0.42%).

2.2 Protein Profile

On the C 18 coloumn on RP-HPLC at 220 nm for milk of different breeds of cow (Gir, Tharparkar, Sahiwal) and Buffalo (Murrah), a single peak was reported for α_{s2} casein, whereas multiple peaks of α_{s1} casein, β-casein and k-casein were reported observed. Multiple peaks of k-casein were observed in all breeds. Karan Fries demonstrated multiple peaks for all fraction such as those for α_{s1} casein, β-casein and k-casein. Multiple peaks of β-casein were found in the murrah breed. The levels of different fragments of casein were found to be 52-60% of α_s casein, 23-34% of β-casein and 11-17% of k-casein (Das, 2019)

Bobe *et al.* (1998) isolated and quantified by RP-HPLC using skim milk sample at 220 nm. Different genetic variant of the protein fragments such as $K\text{-}CN_{AA}$, $K\text{-}CN_{AB}$,$K\text{-}CN_{BB}$ double peaks for α_{s2} casein, β-casein variants A1A2, BA2, A1A3, β lactoglobulin variants AA, BB, AB were determined.

Maurmayr *et al.* (2013) detected and quantified α_{s1}, α_{s2}, β, k-caseins, α-lactalbumin, β- lactoglobulin and lactoferrin from Brown Swiss cow's milk by RP-HPLC at 214 nm. The proteins eluted in the following order: k casein, α_{s2}, α_{s1}, lactoferrin, α lactalbumin, β-casein and β-lactoglobulin. Peaks of the genetic variants were identified.

Buffoni *et al.* (2011) conducted isolation and quantified milk protein fraction and genetic variants using RP-HPLC at 214 nm. Buffalo genetic variant of α_{s1} and K-cn were detected. Bonfatti *et al.,* (2008) verified RP-HPLC method for the isolation and quantification of bovine milk protein genetic variants. The peaks of the eluted genetic variants in this study were k-casein A & B, α_{s1} casein, α_{s2} casein, β casein B, A1, A2, α- lactalbumin, β- lactoglobulin.

The detection wavelength was 214 nm in most cases, the methanol and acetonitrile used as solvent. The reducing agents used in most cases were Gdn-HCl, DTT and 2- mercaptoethanol.

2.3 Digestion of Protein:

Digestion is the process of breaking down food structures to allow the release of individual nutrients that can be absorbed through the wall of the gastrointestinal tract. (Boland *et al.,* 2014). Digestion of protein is very important because protein are important nutrients that body uses to build cellular structure that carry out vital function and to formation of bioactive compound. Protein is a major source of bioactive compound which is release after digestion. Protein digestibility is defines as the ratio of NPN after digestion – NPN before digestion / total nitrogen before digestion - NPN before digestion.

2.3.1 In-vitro digestion of milk:

In-vitro digestion is currently the most popular and widely employed to study the structural change, intestinal behaviour of food, digestibility and release of food components under simulated gastrointestinal conditions. In-vitro studies are advantageous over in-vivo. In-vitro studies gives results more rapidly, simple, reproducible, less expensive, less labour intensive and do not have ethical restrictions, large number of samples can be analysed. In-vitro model is closely correlated with in-vivo studies. Animal models are also widely used, although their use generally involves animal death or surgical approaches in which cannulas are placed into digestive organs to access the contents of the gastrointestinal tract. The relevance of animal models for understanding food digestion in humans is also regularly questioned. In vivo (human or animal) intervention trials can be difficult to undertake, unsuitable and expensive or are not justifiable on ethical grounds. For these reasons, in vitro models have been used for many decades to simulate the digestion of food (Brodkorb *et al.,* 2019)

Egger *et al.* (*2019)* compared the static and dynamic in-vitro digestion systems for milk proteins. They used SMP as model food. SMP was digested in vitro according to the harmonized protocol (INFOGEST, 2014; Minekus *et al.,* 2014) and with the dynamic model DIDGI (Menard *et al.,* 2014).They analysed protein hydrolysis by gel electrophoresis, peptide profiling by mass spectroscopy and free amino acids with HPLC. This result showed that similar kinetic behaviour obtain in the static and dynamic digestion during protein hydrolysis. Intact caseins disappeared during digestion the first 30 minutes in the static system and after 60 min of dynamic

digestion, whereas β-lactoglobulin was persistent in both systems during the whole gastric phase.

A standard static in-vitro digestion method suitable for food was developed by Minekus *et al.,* 2014). Three fluids (SSF, SGF, SIF) at different pH to simulate the three phases of digestion were prepared with different electrolyte solution. All the reactions were carried out in a shaking water bath at 37°C. For stopping the gastric digestion, the samples collected at different intervals which is neutralized by adding sodium bicarbonate or by adding protease inhibitor or else snap freezing of the samples can be done using the liquid nitrogen followed by freeze drying of samples.

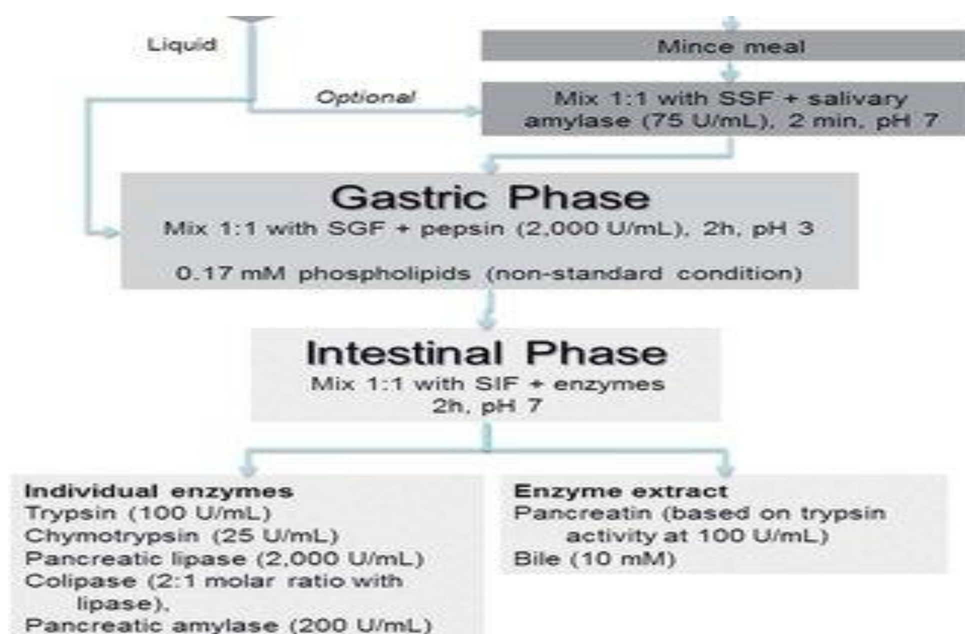


Figure 2.1 A Standardized static in vitro digestion method

Milk and yogurt were used as the liquid and semisolid matrixes respectively while investigating the gastrointestinal digestion of liquid and semi-liquid dairy foods Rinaldi *et al.* (2014). They conducted the chemical analysis of undigested matrixes and the digested matrixes. Protein profiles were analysed by SDS PAGE and proteins were quantified using Pierce bicinchoninic acid BCA protein assay kit. Gas chromatography used for Free fatty acids analyse. Electrophoresis pattern of digested mixtures showed that casein digestion began at the gastric phase and casein

hydrolysis was slower for pasteurized milk than sterilized milk and yogurt. It was shown that the severity of the heat treatment, had more impact on milk's protein digestion than the matrix structure and that the differences in both caseins and whey proteins digestions were mainly governed by the gastric digestion phase.

A new in vitro dynamic system to simulate infant digestion was validated which involves the comparison between in vitro dynamic model and in vivo digestion conducted on piglets and the result was found to be correlated with each other (Menard *et al.,* 2013). Computer program and mathematical model was designed on the basis of the data obtained from in vivo data. Protein breakdown was observed using SDS-PAGE and residual immune reactivity was determined using inhibition ELISA. This result showed that the kinetics of hydrolysis of protein during in-vitro and in- vivo digestion were similar.

Dynamic in-vitro digestion of goat milk and cow milk infant formulae in human gastric simulator was studied (Ye *et al.,* 2019). Infant formulae was subjected to in-vitro digestion. The evolution and decomposition of protein was observed using SDS-PAGE, Mastersizer, Confocal laser scanning microscopy. This result showed that smaller flocs of aggregated protein and oil droplets were formed by goat infant formulae than cow milk infant formulae indicating faster protein digestion of goat milk infant formulae which depends on protein composition.

2.4 Effect of heat treatment on digestion of milk protein

Digestion of protein is affected by denaturation, aggregation, dephosphorylation, glycation, oxidation, cross linking of protein, recemization of protein which is formed by heat.

2.4.1 Impact of denaturation & aggregation on digestibility of protein

Casein is almost completely digested in the stomach due to open structure while native whey proteins are more resistant to hydrolysis and are still intact after gastric digestion. (Tunick *et al.,* 2016). Heated denatured β-lg becomes more digestible by pepsin due to unfolding and the consequential increased accessibility of protein cleavage sites. (Guo *et al.,* 1995; Kitabatake and Kinekawa 1998; Sánchez-Rivera *et al.,* 2015; Wang *et al.,* 2018). The less consistent coagulum as a result of heating caused by: 1) interaction between denatured whey proteins and casein micelles,

thereby blocking micelle aggregation 2) denatured whey proteins and κ-casein forming soluble complexes, incorporating whey proteins in the coagulum, and 3) changing the ion equilibrium due to decrease the concentration of calcium in the serum (Mulet-Cabero *et al.,*2018) The increased hydrolysis is confirmed in commercial milk samples where intact whey proteins, and β-lactoglobulin in specially, in (in-can) sterilized or UHT-treated milk are better digested by pepsin than in pasteurized milk (Rinaldi *et al.,* 2014; Tunick *et al.,* 2016; Wada and Lonnerdal, 2014)

2.4.2 Impact of glycation on protein digestion

Glycation is the covalent attachment of a sugar to a protein. Heat treatment of milk causes glycation via maillard reaction. The maillard reaction involves interaction between a lactose and lysine to form a glycosamine. (Mehta and Deeth, 2015). Glycation decreases protein digestibility due to direct and indirect blocking of Lysine residues in milk proteins when they react with reducing sugars, in the maillard reaction. The blocked or glycated lysines decrease the biological availability of the lysine to metabolic processes and also hinder hydrolysis of the protein by digestive enzymes (Lieshout *et al.,* 2019). Lysine is a essential amino acid, amadori compound, lactulosyl-lysine, is biologically unavailable and, hence, the maillard reaction reduce the biological value of the protein. (Mehta and Deeth, 2015). The bioavailability of lysine in milk and dairy products is reduced when it is glycated and the digestibility of the milk proteins (by enzymes) decreases (Guyomarc'h *et al.,* 2000).

Mulet-cabero *et al.* (2018) perform the effect of process induced changes in milk on in-vitro gastric digestion. For this, three types of treatment were studied: pasteurization, UHT treatment and homogenization. The gastric behaviour of these treated milk samples were studied by semi dynamic gastric digestion model. They measured protein hydrolysis by using OPA method and identification of protein by SDS-PAGE. They found that UHT Homogenized milk showed highest proteolysis. The consistency of the coagulum from the heated samples was more fragmented compared to non- heated sample and also showed that the, the softer coagulum obtained from higher heat treatment temperature and the higher rate of protein hydrolysis at the end of digestion.

Another study was carried out to assess the effect of heat treatment and homogenization on protein and fat globules during simulated dynamic gastric digestion

of milk. (Ye *et al., 2016)* Untreated, homogenized and heated homogenized milk (90°C, 20 min) used as sample. SDS-PAGE, confocal laser scanning microscopy used for assay of sample. This study showed that structure of clots & dried weight of clots formed during the digestion, were different for heated and unheated milk, which lead to different rates of protein hydrolysis and fat release from clots with different rates. The clot obtained from unheated milk showed a closely knitted network with numerous small pores interspersed throughout the matrix, and larger voids was observed in heated milk clot. pH decreased with increasing digestion time, the structures of the clots tightened and became less permeable to serum and solutes. In heated milk, casein hydrolysis was much higher than that in the unheated milk. In heated milk whey proteins, β-lg and α-la, were readily hydrolysed by pepsin. But in unheated milk, largely intact during the whole digestion period.

In a study regarding the effect of homogenization and heat on the in-vitro digestion of milk, raw unprocessed milk, raw skim milk, raw whole milk, homogenized milk, HTST pasteurized, HTST pasteurized skim milk, homogenized cum HTST pasteurized milk, UHT processed skim milk, and homogenized cum UHT processed milk used as sample. All these samples were subjected to in-vitro digestion.100 ml of processed samples (RW, RS, H, P, HP, HU, SP, or SU milk) was mixed with 50 mL of a simulated gastric fluid (SGF), which was prepared by dissolving 2.0 g of NaCl/l, 7 mL of HCl/l, and 3.2 mg pepsin/l at pH 1.2 and acidifying to pH 1.5 with 6 M HCl in a 37°C shaking water bath at 95 rpm. 4- 5 ml aliquots were collected after 0, 5, 15, 30, and 60 min of digestion for analyses. Simulated intestinal fluid (SIF) was prepared with 6.8 g of K_2HPO_4/l and 77 mL of 0.2 M NaOH/l and pH adjusted to 6.8 (Gallier *et al.,* 2012) Protein hydrolysis was analysed by SDS-PAGE & Confocal laser scanning microscopy and particle size was determined by laser-scattering particle size distribution analyzer. They observed that protein digestion has been increased by homogenization and skimming of milk. Under gastric conditions, skim milk formed larger particles than whole milk whereas UHT reduced the particle size. (Tunick *et al.,* 2016)

Systematic review regarding effect of processing factor on protein digestion and overall physiological outcomes given by Lieshout *et al.* (2019). It has been reported heating of milk proteins can modify several amino acids. In vitro and animal studies demonstrate that glycation decreases protein digestibility, and hinders amino

acid availability, especially for lysine. Other chemical modifications, including oxidation, racemization, dephosphorylation and cross-linking also impact protein digestion, which may result in decreased amino acid bioavailability and functionality. On the other hand, protein denaturation does not affect overall digestibility, but can facilitate gastric hydrolysis, especially of β-lactoglobulin. Protein denaturation can also change gastric emptying of the protein.

Similarly, one more Systematic review has been done to understand the effect of heat treatment on milk protein digestion (Dupont and Tome, 2020.) It has been concluded that heat treatment modifies the three dimensional structure of the whey protein markedly resulting in an "Opening" of the globular structure and making the whey proteins more sensitive to the action of digestive enzymes. Casein, with their loose and highly flexible structure, are not strongly modified by heat treatment. Heat treatment at high temperature results in an increased resistance of the casein to simulated digestion which has been formation of thermally induced aggregates between casein and between casein and whey protein.

Another study reports the effect of raw, pasteurized and homogenized milk using ex vivo digestion on lipolysis and proteolysis. Islam *et al.* (2016). Protein analysis were measured by SDS-PAGE. It was found that pasteurization upon lipolysis is very less and proteolysis. Homogenization increased the gastric proteolysis of specifically β-lg but also of α-la However, after 5 min duodenal digestion all the proteins were degraded in all milks. Thus, both lipolysis and proteolysis were increased by homogenization of the milk, making fatty acids from lipids and peptides from β-lactoglobulin more readily available to the human body.

Further, Peram *et al.* (2013) did the in vitro gastric digestion of heat induced aggregates of β-lactoglobulin in simulated gastric fluid and investigated using SDS-PAGE, native PAGE, 2-Dimensional electrophoresis, size exclusion chromatography. They observe native β-lactoglobulin is resistant to digestion by pepsin but heated β-lactoglobulin is digested more rapidly due to heating causes the unfolding of protein molecules, thereby increasing the accessibility of exposed hydrophobic amino acid to pepsin.

Table 2.1 Literature overview of studies investigations the impact of heat treatment on milk protein digestibility

Reference	Method	Protein source	Study with denaturation/ aggregation method	Outcome measure	Effect on digestibility & kinetics
Static in -vitro					
Carbonaro *et al.* (1997)	In vitro static enzymatic digestion with porcine pancreatic trypsin, porcine intestinal peptidase	Whole milk, whey protein extracts	Raw milk, pasteurized milk (15 sec at 72, 75,78 ,80 °C) UHT treated milk (3&5 sec at 145C) sterilized milk (145 °C/3-5sec. followed by 118 °C /12 min	In vitro protein digestibility, whey protein solubility,	Digestibility increased from raw to pasteurized to UHT or sterilized whole milk. The digestibility of whey protein extracts decreased with increasing heat treatment.
Dupont *et al.* (2010)	Static in vitro infant GI digestion	Ultra-low heat SMP	Heating at 80 °C, 20 s heating at 85°C,180 s heating at 105°C, 60 s	SDS-PAGE, Western blotting and LC-MS-MS	β-lg was still present after digestion in all samples, with the highly processed sample showed high resistance of intact casein to digestion.
Dupont *et al.* (2010)	Static in vitro infant GI digestion	Whole milk	Raw milk pasteurized milk (82 °C/30 sec.) sterilized milk (10 min / 120 C)	SDS-PAGE, Western blotting and LC-MS-MS	Pasteurization milk & sterilization increased gastric hydrolysis of β -lg, intact β present in raw & pasteurized milk after intestinal phase of digestion.
Peram *et al.* (2013)	Static in-vitro digestion	β-lg	β -lg heated at 90°C for 0-120 min	SDS-PAGE	Heating increased initial hydrolysis of β -lg with a correlation between hydrolysis & heating duration.

Rahaman, *et al.* (2017)	Static in-vitro digestion	β-lg	β -lg samples prepared with different pH 3,5 & 7 tem. (room Tem., 120 °C)	Degree of hydrolysis, peptide formation (SDS-PAGE), antigenicity assay	Unheated β -lg was resistant to peptic digestion, but was susceptible to pancreatic digestion. Heating increased gastric& pancreatic digestion & resulted in lower Antigenicity
Rinaldi *et al.* (2014)	Static in-vitro digestion	Dairy products	Commercial pasteurized milk, Commercial sterilized milk	Protein content & solubility, peptide analysis (SDS-PAGE), free amino acids.	Faster digestion of caseins & whey in gastric phase in sterilized milk compared to pasteurize milk. β -lg and α- la degraded faster in duodenal phase.
Singh *et al.* (2014)	Static in-vitro digestion	WPI	WPI gels heated at 90 °C for 10 min at pH 6.8 and 4.6	Protein and peptide analysis (SDS-PAGE & RP-HPLC)	In the gastric phase, whey proteins in gels are faster hydrolysed than in native solution.
Lamothe *et al.* (2017)	Static in-vitro GI digestion	Whole milk heated to 50°C	Pasteurized milk (65°C/30 min), milk homogenized and heated at 65°C for 30 min	Matrix degradation, protein hydrolysis	Heating at 95°C increase hydrolysis in first 30 min of gastric digestion compared to 65°C, proteolysis was similar in intestinal phase.
Guo *et al.* (1995)	Static in-vitro GI digestion with pepsin	β-lg	β-lg heating at 70 to 100°C for 5 or 10 minutes at pH 6.8 or pH 3.0	Protein hydrolysis (SDS-PAGE)	Native β-lg resistant to peptic digestion, but heating at > 80°C increased proteolysis with increasing temperature

(Semi)-dynamic in vitro studies					
Mulet-cabero *et al.* (2019)	Semi dynamic in vitro gastric adult digestion	Bovine milk	raw milk, raw homogenized Milk, pasteurized milk, pasteurized homogenized milk UHT milk, UHT homogenized milk	Gastric behaviour, nutrients emptied and protein digestion (SDS-PAGE)	Firmer coagulum obtained from raw milk & open coagulum from heated &UHT milk. Pasteurized & raw milk showed β -lg present during gastric digestion, UHT showed higher protein release in the early stages of digestion.
Sanche -Rivera *et al.* (2015)	Dynamic in vitro gastric digestion	SMP	Non-heated skimmed milk prepared from SMP Heated (90°C 10 min) skimmed milk	Protein & peptide analysis SDS-PAGE LC -MS/MS immunoreact -ivity	Heating increased resistance to pepsin hydrolysis for casein, while β -lg became more susceptible to pepsin hydrolysis.
(Wang and Zhao, 2017)	In vitro dynamic gastric digestion	WPI	Whey protein isolate heated whey protein isolate (90 °C for 20 min)	Curd weight, pH, protein hydrolysis (SDS-PAGE)	No aggregation in native WPI over whole digestion, while aggregation occurred in heated WPI in the stomach. For unheated WPI, β -lg remained intact during whole digestion and decreased gradually with time. β -lg for heated WPI was much less and disappeared.

2.5 Degree of hydrolysis

Extent of hydrolysis of protein during the digestion is a useful way to monitor its availability. Degree of hydrolysis is defined as the percentage of total cleaved bonds during its hydrolysis (Alder-Nissen, 1986). There are various methods used for determine the degree of hydrolysis: OPA, TNBS, pH stat, osmometry and soluble nitrogen content. The OPA method is based on spectrophotometric assay which determines the Colour compound formed during the reaction of the primary amines and OPA. During the reaction the pH is adjusted to alkaline condition and termination of the reaction is done by lowering the pH. This reaction is light sensitive, hence the reaction is carried out in dark condition.

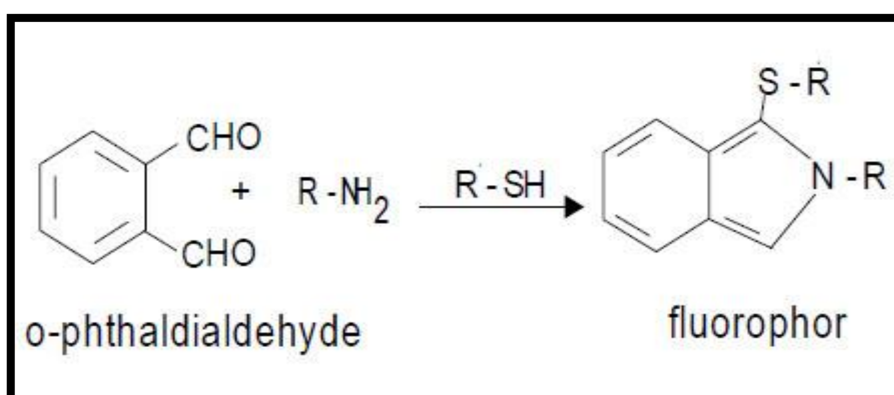


Figure 2.2 Reaction of OPA Assay

Nehir e*t al.* (2015) performed the in vitro digestibility of goat milk and kefir with a new standardised static digestion method and analysed the bioactivities of the resultant peptides. They used SDS-PAGE and OPA method for analyse the hydrolysis the protein and antioxidant properties of milk observed using phosphomolybdenum reduction assay. This result showed that SDS analysis of milk and kefir showed a similar protein profile like fermentation. No visible band after in vitro digestion. Most high MW component (>26.6 kDa) relatively susceptible to pepsin degradation and completely degraded within 1 h of hydrolysis. OPA method showed that degree of hydrolysis in goat milk obtained for gastric phase at 120 min is 27.3% and 59.4% for beginning of intestinal phase (135 min), serially degree of hydrolysis at the end of digestion is 80.4%, antioxidant activity of goat milk is 352 ± 357 µM (equivalent to ascorbic acid)

2.6 Antioxidant activity

When excess of free radicals are generated in the body they will cause oxidation of cellular protein, DNA, membrane lipids, enzymes and stops the cellular respiration which in turn leads to atherosclerosis, diabetes, rheumatoid arthritis, and oxidative DNA damages which causes cancer. Some of the milk derived peptides have the antioxidant properties and free radical scavenging capacities (Mohanty *et al.,* 2015).

Chen *et al.* (2003) assayed antioxidant capacity of bovine milk assayed using spectrophotometer and amperometric method. Here unpasteurized skim milk and homogenized milk with different level of fat content and ABTS, FRAP method used for study of antioxidant capacity of milk. They found that antioxidant capacity increase with increase pH and seven fold higher in milk than whey.

In vitro gastrointestinal digestion studies on antioxidant activity of skim milk from various indeginous breeds of cattle and buffaloes showed that lowest activity was reported for murrah sample while the highest activity was observed for karan fries compared to othr breeds (Gir, Sahiwal and Tharparkar). The antioxidant activity was reported to increase as the digestive tract progresses. (Das, 2019)

2.6.1 Effect of Heat Treatment on Antioxidant activity of milk

Effect of heat treatment of milk on antioxidant activity of milk is depending on the intensity of the thermal treatment applied, milk antioxidant activity may increase as a consequence of thermal treatments, due to protein unfolding and exposure of thiol groups, potentially acting as hydrogen donors. (Taylor & Richardson, 1980; Walstra & Jenness, 1984; Tong *et al.,* 2000). Heat treatment of milk can promote an increase in its pro-oxidant activity, probably as a consequence of both the loss of natural antioxidants and the formation of novel oxidative molecules in the early stages of the maillard reaction. But formation of high molecular weight compounds during advance stage of maillard reaction contribute to increase in antioxidant activity of milk. (Calligaris *et al.,* 2003)

Tagliazucchi *et al.* (2018) studied the bioactivity and peptide profiling of in-vitro digested cow, goat, sheep, and camel skimmed milks. Here, in vitro method was used for the study of the bioactive peptides produced from the milk which was initially firstly analyzed for pH, fat, lactose (phenol-sulphuric acid method), total nitrogen and non-

casein nitrogen (by micro-Kjeldahl). Three treatments were given to the sample with SSF, SGF, and SIF. After that, the sample was subjected for in vitro digestion, they measured degree of hydrolysis of the protein using the TNBS assay using leucine as standard, antioxidant activity by ABTS assay and ACE inhibitory activity by spectrophotometric assay. Among the 4 milks goat milk has exhibited highest digestibility. Sheep milk was found to be best source of ACE inhibitory peptides. Cow milk was observed to be best source of DPP IV inhibitory peptides, antioxidant peptides and amino acids. Degree of hydrolysis in skim milk is more than compare to whole milk and whole milk shown higher antioxidant property than skim milk.

Antioxidant activity of heated cow and buffalo milk was studied by Taj khan *et al.* (2017). For this, cow and buffalo milk was given to different heat treatments i.e. 65°C for 30 min. & boiling for 1 min. and perform total antioxidant capacity, reducing power, DPPH free radical scavenging activity this results showed that buffalo milk showed highest total antioxidant capacity than cow milk. Pasteurization & boiling did not showed significant effect of reducing power of cow and buffalo milk and DPPH activity is high for raw milk than pasteurized & boiled cow and buffalo milk.

Similarly, one more study has been done to understand the effect of heat treatment on antioxidant and pro-oxidant activity of milk by Calligaris *et al.* (2014). Here, milk is exposed to heated in an oil bath at 80°C, 90°C and 120°C for up to 24 h. They found that at each heating temperature, initial increase and a subsequent decrease in pro-oxidant activity. The latter was associated with an increase in the antioxidant properties. Short heat treatments can be potentially responsible for a depletion in the overall antioxidant properties of milk. By contrast, only the application of severe heat treatments, associated with the formation of brown melanoidins, which increase in milk antioxidant properties.

CHAPTER –3

Materials & Methods

3.1 Selection of animals

In this study, the animals from bovine and buffalo were selected. Among the bovine, the breeds include Sahiwal and crossbred i.e. Karan Fries and Murrah for buffalo. Only the samples of boiled milk along with unheated samples from cow milk (Sahiwal & KF) were assessed for detailed protein composition and its vitro GI digestion.

3.2 Collection of milk samples

The mixed pooled samples were collected from cattle and buffalo in evening. All the samples were collected from Livestock Research Centre, NDRI, Karnal

3.3 Test for mastitis milk

Mixed pooled samples were collected from different breeds of cattle and buffalo was subjected for mastitis test using California mastitis kit (CMT)

3.3.1 Protocol for CMT

1. 1-2 ml of milk was added into each well of the paddle
2. Tilted the paddle so equal amount of milk are in each well
3. Added equal volume of the test solution
4. Paddle was shaken to ensure mixing of the milk and test solution
5. Observed the viscosity and colour change

Mastitis free samples was only considered for in- vitro digestion and other analysis.

3.4 Skimming of milk

Raw milk was centrifuged at 5,000 rpm for 20 min. The cream layer was removed and the skimmed portion obtained was stored at -20°C

3.5 Processing Treatment

Whole milk and skim milk from Cow (Sahiwal, Karan Fries) and buffalo breeds was subjected to different heat treatment. 500 ml whole milk and skim milk was heated to 63°C/30 min, 80°C /10 min in conical flask was performed in water bath (Model: BS-11, Lab companion, Jeio Tech Inc., U.S.A.) with continuously stirring (100 rpm) and boiling for 10 min was performed in open stainless steel pan using heater with continuous stirring.

3.6 Scheme for sample storage

The samples collected were preserved in three ways:

1. Whole milk preserved with 0.02% bronopol stored at 4°C were used for Compositional analysis.
2. Skimmed milk stored at -20°C were used for determination of different nitrogen fractions of the milk samples.

3.7 Compositional analysis of the sample

The whole milk and skim milk samples stored at 4°C were assessed for compositional analysis.

3.7.1 Determination of fat

The fat content of milk samples was determined by the Gerber method as described in IS: 1224: 18 (Part I) (1977). 10 ml of Gerber sulphuric acid was taken in a gerber milk butyrometer using an automatic measure. 10.75 ml of the well mixed sample of milk was pipetted in the butyrometer slowly along the neck of the butyrometer. 1 ml of iso-amyl alcohol was added using an automatic measure. The butyrometer was closed firmly with the stopper and shaken carefully to mix the contents and centrifuged at 1100 rpm for 4 min. The butyrometer was then placed in water bath at 65^{0}C for 5 min. Difference between the scale reading corresponding to the lowest point of the fat meniscus and the surface of separation of fat and aqueous phase was read as the fat % of milk sample.

3.7.2 Determination of SNF and TS

The SNF and TS content in milk was determined using the method as described in IS: 10083 (1992). Lactometer reading was recorded at 27ºC otherwise corrected lactometer reading was calculated.

%SNF =CLR / 4 + 0.2 F + 0.29

%TS = CLR / 4 + 1.2 F + 0.29

Where,

CLR = Corrected Lactometer Reading (or Lactometer reading at 27ºC)

F = fat % determined by Gerber method

3.7.3 Determination of distribution of nitrogen fraction

The different nitrogen fractions of the skim milk sample: TN% was determined using IDF protocol, NPN% and NCN% was determined using Rowland scheme (Manual- Chemical Analysis of Milk Proteins, Kumar *et al.,* 2012).

3.7.3.1 Apparatus:

Waterbath, Analytical balance, Digestion block assembly, Distillation unit, Digestion tube, conical flasks, Funnal and Burette.

3.7.3.2 Reagents:

i. Kjeldahl catalyst mixture: It consists of 3.5 g potassium sulfate and 0.105 copper sulphate.

ii. Sulfuric acid

iii. Sodium hydroxide solution (40%)

iv. Boric acid solution: Dissolve 40 g of boric acid in 1 litre of hot water in a 1000ml volumetric flask. Allow the contents to cool to 20ºC and adjust the mark with water.

v. Indicator solution: Dissolve 0.25 g of methylene blue and 0.375 g of methyl red in 300 ml of 95% ethanol

vi. Hydrochloric acid (0.1 N)

vii. Sucrose

3.7.3.3 Procedure:

(a) Estimation of total nitrogen

Warm the test sample, between 38 ºC to 40 ºC in the water bath. Cool the sample to room temperature, while gently mixing the test sample immediately prior to weighing the test portion. To a clean and dry digestion tube, add 5 g of digestion mixture. Weigh 2 g of sample to the nearest 0.1 mg into the tube. Carefully add 10 ml of sulfuric acid along the sides of the digestion tube. Gently mix the contents of the tube and then leave to stand for 10 minutes.

b) Estimation of Non-casein-nitrogen NCN

In a 100 ml volumetric flask, take 10 g of milk and add 60 ml of water and bring the temperature to 40°C. Add 1 ml of acetic acid, mix it then after 2 minutes add 1 ml of sodium acetate solution. Make up the volume to 100 ml with distilled water and allow it to stand for 30 minutes. Filter the content through Whattman No. 42. Discard the first few drops and take 20 ml of filtrate and carryout the nitrogen estimation.

c) Estimation of non-protein-nitrogen NPN

In a 50 ml volumetric flask, take 10 g of milk. Add 15% TCA into the flask upto the mark. Mix and filter the content through Whattman No. 42. Take 20 ml filtrate and carryout the nitrogen estimation.

3.7.3.4 Digestion

Set the digestion block at a low initial temperature so as to control foaming approximately 180°C. Digest the samples until white fumes develop. Then increase the temperature of the digestion block to 411°C and continue digestion until the digest become clear. Remove the tubes from the digestion block and allow it to cool to room temperature.

3.7.3.5 Distillation

Place the tube in its place and run the program for automatic distillation. Put a conical flask at the outlet. Adjust the distillation unit to dispense 45 ml of sodium hydroxide solution, 50 ml of water and 55 ml of boric acid. Distilled off the ammonia liberated by the addition of sodium hydroxide solution and collect the distillate.

3.7.3.6 Titration

Titrate the contents of conical flask using 0.1 N HCl and record the volume required to reach the first appearance of the violet colour.

3.7.3.7 Blank test

Carry out the blank test following the procedure described above except 2 ml of water and about 0.2 g of sucrose instead of test portion.

3.7.3.8 Calculation

Calculate the nitrogen content by using the following equation:

A. $Total\ nitrogen\% = 1.4007(V_s - V_b)N/m$

B. $Non\ casein\ nitrogen\% = 1.4007(V_s - V_b)N\ /m\ x\ DF$

C. $Non\ protein\ nitrogen\% = 1.4007(V_s - V_b)N\ /m\ x\ DF$

Where,

V_S= ml of HCl required for the titration of the sample

V_b= ml of HCl required for the titration of the blank

N=Normality of the HCl

m=amount of the sample taken

True nitrogen= A-C

Casein nitrogen= A-B

Whey protein nitrogen= B-C

Calculate the protein content using the following equation:

$Protein\% = Nitrogen\% \; x \; 6.38$

3.8 Separation and quantification of fractions of Milk protein using RP-HPLC

Protein Profiling of the skim milk samples was done using the RP-HPLC. The method of samples preparation was followed from the protocol given by Vincent *et al.,* (2016) and the protocol for protein separation given by *Bobe et al.* (1998) over C18 column was followed.

3.8.1 Materials:

Bis Tris (B-9754), Gdn-HCl (G-4505), Sodium citrate tribasic dihydrate (C8532), DTT (D-0632), and standards of α-casein (C6780), β-casein (C6905), and κ-casein (C0406) obtained from Sigma, Trifluoroacetic acid (302031), Acetonitrile, Acetic acid. All the chemicals and standards were obtained from Sigma Aldrich Chemicals.

3.8.2 Reagents

a) 0.1 M Bis-Tris buffer (pH 7.0)

b) Reagent A: 6 M Gdn HCl, 5.37 mM Sodium citrate tribasic dehydrate, 20 mM DTT prepared in 0.1 M Bis-Tris buffer.

c) Reagent B: Dilute reagent A in 1:1 ratio in water

d) 50% Acetic acid

e) Solvent A: H_2O: ACN: TFA (900:100:1)

f) Solvent B : H_2O: ACN: TFA (100:900:1)

3.8.3 Procedure

Profiling conditions:

a) Injection volume of the sample: 20 µl

b) Flow rate: 0.5 ml/min

c) Detection wavelength: 214 nm

3.8.4 Sample Preparation

For sample preparation, 0.5 ml reagent B (6M Gdn HCl, 5.37 mM Sodium citrate tribasic dehydrate, 20 mM DTT prepared in 0.1 M Bis-Tris buffer, buffer diluted with 1:1 with distilled water) added in 0.5 ml milk sample. Samples were dissolved by vortex for 1 min and then the incubation was done for 50 min at room temperature. After the incubation period, 20µl acetic acid (50%) was added to samples in such a way that the final concentration reach up to 1%. After that, samples are vortexed for 1 minute and incubation was done at room temperature for 10 min and then the samples was injected for profiling.

C18 column of Dionex Acclaim 300 (4.6x150 mm, 3 µm) in UHPLC of Dionex Ultimate 3000 (Thermo Fisher Scientific) was used for the profiling.

Table 3.1 Gradient conditions for peptide profiling over C18 column

Time(minutes)	%B solvent
0	27
0	27
2	32
18	38
50	45
51	99
64	99
65	27
76	27

The protocol by Bobe *et al.* (1998) was followed after little modifications for the profiling of the samples. C18 column was used at 30 °C for the profiling of the samples. Initially, the column is equilibrated at 27% solvent B, then a linear gradient is given in which 32% of solvent B is achieved in next 2 min following to reach 38% solvent B in further next 18 min for the elution of the sample. After that, gradual washing of the column was done by increasing the concentration of solvent B to reach 45% in 32 min, then to 99% solvent B in next 1 min which was maintained to isocratic gradient for next 13 min. After that the concentration was brought down to equilibration

concentration of solvent B i.e., 27% of solvent B in 1 min which was maintained for next 11 min for the re-equilibration of the column. Total run time was standardized to 76 min. In case of second gradient, which was used for 63°C/30 min and 80°C for 10 min treated sample % of solvent B % was 50% at 50 min instead of 45% solvent B in gradient.

3.9 In vitro gastro intestinal digestion

Fresh raw pooled milk sample was collected from each breed: Sahiwal and Karan Fries milk screened for mastitis. Only mastitis free milk samples were selected for further analysis. Whole milk was stored at 4ºC whole milk and boiled (10 min) milk from cow milk was taken for in vitro digestion

3.9.1 Enzymes

Pepsin from porcine gastric mucosa (EC 3.4.23.1; catalog no. P7012; ≥2,500 units/mg of solid), pancreatin from porcine pancreas (Catalog no. P1750; 4 x USP), and bile salts (Catalog no. B8756) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich unless specified otherwise. Milli-Q water (water purified by treatment with a Sartorium arium pro VF Ultrapure Water System) was used for the preparation of all solutions.

Whole milk was digested in vitro according to the harmonized protocol (Minekus *et al.,* 2014).

3.9.2 Simulated gastrointestinal digestion

For in vitro gastric digestion, 50 ml milk sample was mixed with 37.5 ml of simulated gastric fluid (SGF) electrolyte stock solution. 8 ml porcine pepsin stock solution of 25,000 U/ml made up in SGF electrolyte stock solution, 25 µl of 0.3 M CaCl2, 0.2 ml of 1 M HCl to reach pH 3.0 and 3.475 ml of water and then incubated at 37ºC in a shaking water bath (Model: BS-11, Lab companion, Jeio Tech Inc., U.S.A.) at 100 rpm. 8 ml aliquots were collected after 0, 5, 10, 15, 30, 60, 90 and 120 min of digestion for further analysis. The enzymes were inactivated by adding 1 N NaOH and store at -20°C

After the 2 hours of gastric digestion of the milk samples, in vitro intestinal digestion was carried out. 35 ml of gastric chyme was mixed with 19.25 ml of simulated intestinal fluid (SIF) electrolyte stock solution (Table 3.3), 8.75 ml of a pancreatin solution 800 U/ml made up in SIF electrolyte stock solution based on trypsin activity (pancreatin from porcine pancreas), 4.37 ml fresh bile (160 mM fresh bile), 70 µl of 0.3 M CaCl2, 0.15 ml of 2 M NaOH to reach pH 7.0 and 2.3 ml of water and then incubated at 37ºC in a shaking water bath at 100 rpm. 8ml aliquots were collected at 0, 5, 15, 30, 60, 90 and 120 min of simulated intestinal digestion for further analyses. The enzymes were inactivated by adding 1 N NaOH and store at -20°C

Table 3.2: Preparation of stock solutions of simulated digestion fluids

Constituent	Stock conc.		SGF (pH - 3)		SIF (pH - 7)	
	g/l	mol/l	Vol. of stock (ml)	Conc.in SGF (mmol/l)	Vol. of stock (ml)	Conc. in SIF (mmol/l)
KCl	37.3	0.5	6.9	6.9	6.8	6.8
$KHPO_4$	68	0.5	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	12.5	25	42.5	85
NaCl	117	2	11.8	47.2	9.6	38.4
$MgCl_2(H2O)_6$	30.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.5	0.5	-	-
$CaCl_2(H20)_2$	44.1	0.3	-	0.15(0.075)	-	0.6(0.3)

*In brackets is the corresponding Ca^{+2} concentration in the final digestion mixture

The volumes are calculated for a final volume of 500 ml for each simulated fluid. Instead, stock solution was made up with distilled water to 400 ml instead. The addition of enzymes, bile salts, Ca^{+2} solution etc. and water will result in the correct electrolyte concentration in the final digestion mixture. $CaCl_2$ $(H_20)_2$ is not added to the electrolyte stock solutions as precipitation may occur. Instead, it is added to the final mixture of simulated digestion fluid.

3.10 Measurement of soluble Protein in digested samples

Protein content determined by method described by Lowry *et al.* (1951) with slight modifications.

3.10.1 Materials:

Copper sulphate, sodium potassium tartrate, sodium carbonate and sodium hydroxide were purchased from Hi Media. Folin & Ciocalteau's phenol reagent and bovine serum albumin was obtained from Sigma (St. Louis, MO, USA)

3.10.2 Reagents:

a) Copper sulphate solution (1% w/v): 1.0 g of copper sulphate was dissolved in distilled water and the volume was made up to 100 ml.
b) Sodium potassium tartrate solution (2% w/v): 2.0 g of sodium potassium tartrate was dissolved in distilled water and the volume was made up to 100 ml.
c) Sodium hydroxide solution (0.2M): 8.0 g of sodium hydroxide pellet was dissolved in distilled water and the volume was made up to 100 ml.
d) Sodium Carbonate solution (4% w/v):4.0 g of sodium carbonate was dissolved in distilled water and volume was made up to 100 ml
e) Alkaline reagent: 49 ml of (c), 49 ml of (d), 1 ml of (a), 1 ml of (b) were mixed. This reagent was prepared fresh immediately before use.
f) Folin's reagent (1N): Equal volume of Folin's reagent (2N) was diluted with equal volume of distilled water to make it 1N. This reagent was prepared immediately before use.

3.10.3 Sample preparation:

In vitro digested milk sample was centrifuge at 13000 rpm for 20 min. The curd particles was removed and the supernatant portion taken for soluble protein estimation. Digested milk sample was 20 times diluted with water.

3.10.4 Procedure:

0.5 ml of digested diluted milk sample was added to 5.0 ml of alkaline reagent. The contents were mixed rapidly and allowed to stand for 10 min at room temperature. Thereafter, 0.5 ml of 1N Folin's reagent was added to above contents and mixed

immediately. Incubation was done for 30 min at room temperature. The intensity of blue colour developed was measured by taking absorbance at 660 nm in UV-Vis spectrophotometer (Model: UV-2700, Shimadzu, Tokyo, Japan). Blank samples having 20 mM phosphate buffer (pH 7.5) in place of casein was processed under identical conditions. A calibration curve of bovine serum albumin from the concentration of 50 to 500 µg/mL was prepared.

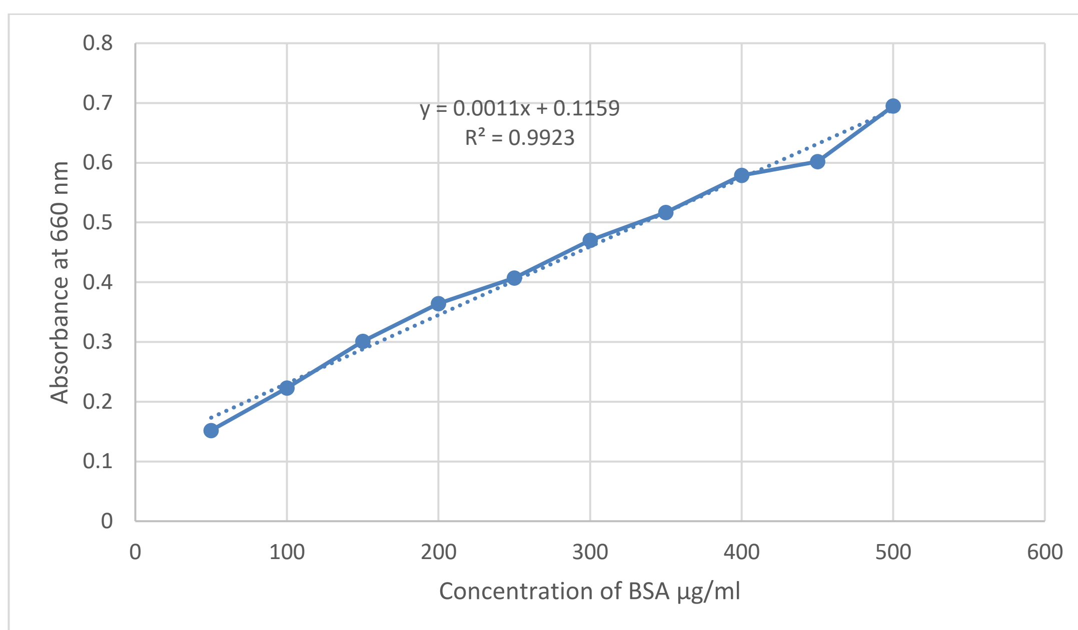


Figure 3.1 Standard curve of BSA for measurement of Protein

3.11 Assessment of protein hydrolysis during the digestion

DH (Degree of Hydrolysis) is defined as the percentage of the total number of peptide bonds in a protein which have been cleaved during hydrolysis (Adler-Nissen, 1986). The degree of hydrolysis was determined by o-phthaldialdehyde (OPA) as described by Nielsen *et al.* (2001) with slight modifications.

3.11.1 Materials:

L-serine, sodium tetraborate decahydrate, o-phthaldialdehyde, β-mercaptoethanol, were procured from Hi Media and sodium dodecyl sulphate was obtained from Sigma-Aldrich.

3.11.2 Reagents

a) Sodium tetraborate 0.1M: 3.814g Sodium tetraborate decahydrate was dissolved in distilled water and make up to 100ml with distilled water.
b) Sodium dodecyl sulfate (SDS), (20% w/v):10g Sodium dodecyl sulfate was dissolved in 50ml distilled water.
c) O-pthaldialdehyde solution: 80mg of OPA was dissolved in 2 ml of methanol.
d) β -mercaptoethanol
e) OPA reagent: 50 mL of 0.1M sodium tetraborate, 5 ml of 20% SDS, 2 ml of OPA solution, 200 μL of β -mercaptoethanol were mixed and volume was made up to 100 ml in an amber colored volumetric flask.
f) L-serine standard (0.0916 meq/L):10 mg serine was dissolved in 50 ml deionized water and made up the volume to 100 ml.

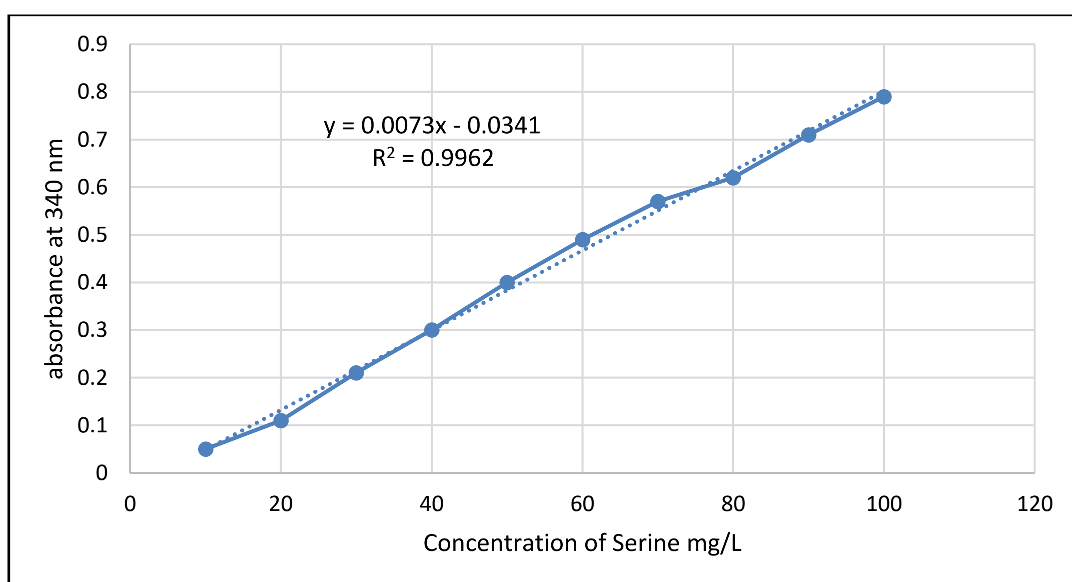


Figure 3.2 Standard curve of serine for OPA Assay

3.11.3 Procedure:

The assay was carried out by mixing 0.4 ml diluted samples (100 times with distilled water) to 3.0 ml OPA reagent. After vertex for 5 sec, incubated for exactly 2 minutes at room temperature. Then absorbance of mixture was read at 340 nm in UV spectrophotometer (Model: UV-2700, Shimadzu, Tokyo, Japan).

A calibration curve of L- serine (10- 100 mg/L) was also prepared in distilled water.

3.12 Antioxidant Activity

ABTS radical scavenging activity was determined using ABTS+ radical cation decolorization assay developed by Re *et al.* (1999) with some modifications described by Tagliazucchi *et al.* (2007).

3.12.1 Materials:

2,2- Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (A1888) and Vitamin C (L-Ascorbic acid)(A7506) were purchased from Sigma (St. Louis, MO, USA). Potassium persulfate, sodium chloride, potassium chloride, disodium hydrogen orthophosphate and potassium dihydrogen orthophosphate were purchased from Hi Media (Mumbai. India).

3.12.2 Reagents:

a) Potassium persulfate solution (140 mM)
b) 1.892 gm of potassium persulfate was dissolved in double distilled water and the volume was made up to 50 ml freshly prepared before use.
c) ABTS stock solution [2,2'-Azinobis (3-ethylbenzo-thiazoline)-6-sulfonic acid] diammonium salt (7 mM): 19.2 mg of ABTS was dissolved in 5 ml of double distilled water and was added with 88 µl of 140 mM potassium persulfate solution (2.45 mM final concentration). The mixture was stirred in an Amber color bottle in dark for 12-16 h for production of sufficient free radicals.
d) Phosphate buffered saline (PBS, pH 7.4)
e) PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl. 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml distilled water, adjusted Ph to 7.4 with 1 N HCl and made the volume up to 1 liter with distilled water and filtered through 0.45µ filter.
f) ABTS working solution
g) 1 ml of ABTS stock solution was diluted with phosphate buffer saline till it gave an absorbance of 0.70±0.02.

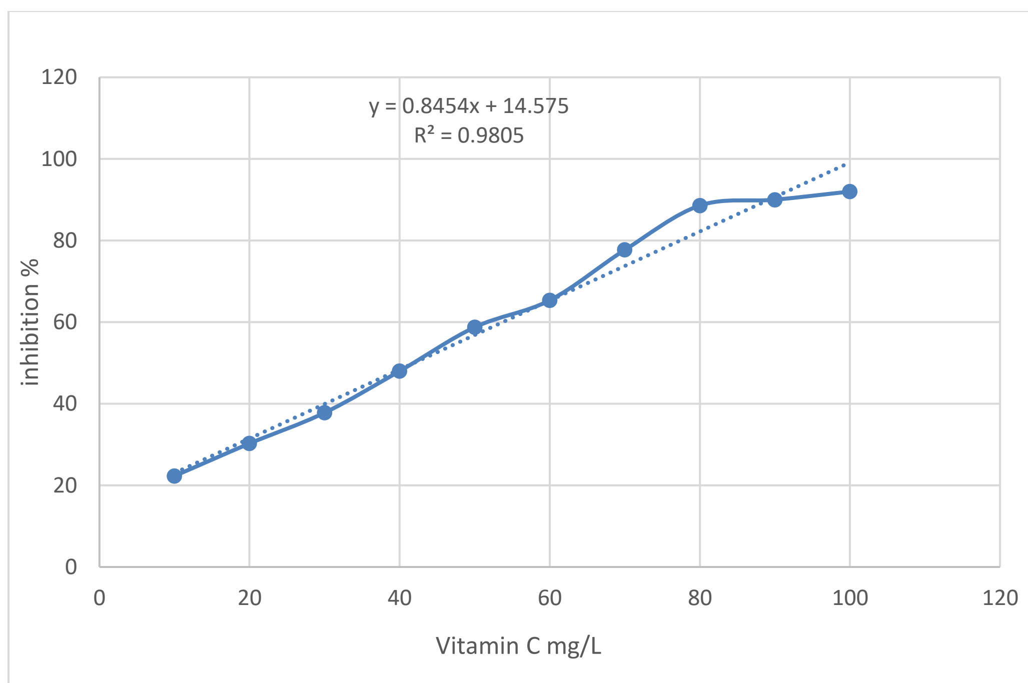


Figure 3.3 Standard Curve of Vitamin C for ABTS assay

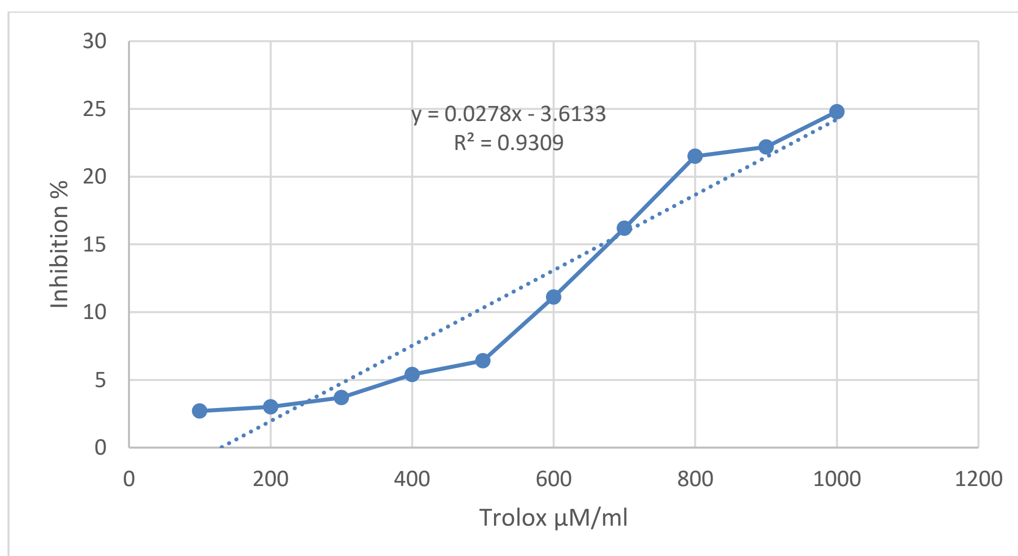


Figure 3.4 Standard curve of Trolox for ABTS Assay

3.12.3 Preparation of standard curve

180 µl of ABTS working solution and 20 µl PBS was added to a well of 96 well micro plate and initial absorbance was recorded at 750 nm using microplate reader (Model: Infinite F200 Pro, Tecan, Austria). Calibration curve of Vitamin C, concentration ranging from 10-100 mg/l was prepared by appropriate dilution of

Vitamin C solution. 20 µl of standards were added to 180 µl of ABTS working solution. The contents were mixed for 5 seconds and change in absorbance at 750 nm was recorded after 10 min. The standard curve was prepared by plotting of Vitamin C (X-axis) v/s %inhibition (Y-axis).

3.12.4 Preparation of sample

For milk sample: milk sample was 5 times diluted with distilled water

For in vitro digested sample: In vitro digested milk sample was centrifuge at 13000 rpm for 20 min. The curd particles was removed and the supernatant portion taken for antioxidant assay. Digested samples diluted with 50 times with distilled water.

3.12.5 Procedure:

Samples were giving 50% inhibition of the blank absorbance. After the addition of 20 µl of samples dissolved in PBS (pH 7.4) to each well, 180 µl of diluted ABTS°+ solution (absorbance adjusted to 0.70 ± 0.02) was added and the absorbance was measured after 10 min spectrophotometrically at 750 nm using a plate reader (Model: infinite 200, Tecan, Austria). Appropriate distilled water blank was run in each assay for digested milk samples and milk samples.

3.12.6 Calculation

Based on the % inhibition of absorbance of sample, vitamin C equivalent was determined from standard curve (Figure 3.3) using the following equation:

y = 0.8454x + 14.575 for Vit C

y = 0.0278x - 3.6133 for Trolox

Where,

y: %inhibition= $(Abs750_{control} - Abs750_{sample} \big/ Abs750_{control}) * 100$

x: mg/l concentration of Vit C

The results were expressed as mmole of Vit C/ g of peptide after converting the obtained results into the mmole values for digested sample. Milk samples were expressed as mg/l of vit C and µM/l of trolox. The calculations were done as described in appendix.

3.13 Tricine Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (Tricine SDS-PAGE)

Tricine Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (Tricine SDS-PAGE) was carried out to detect low molecular weight peptides in hydrolysates as per the method of Schagger and Jagow (1987) and modified by Pardo and Natalucci, (2002).

3.13.1 Reagents:

a) 49.5% Acrylamide Solution (3% C): 48 g of acrylamide and 1.5 g of bis-acrylamaide was dissolved and volume made up to 100 ml with distilled water. The solution was filtered and stored at 4 °C.
b) Gel buffer: 36.34 g of Tris base and 0.3 g of SDS was dissolved in 60 ml of distilled water. pH was adjusted to 8.45 with concentrated HCl and the volume was made to 100 ml with double distilled water.
c) 20% SDS solution: 20 g of SDS was dissolved in 75 ml of distilled water and volume was made up to 100 ml. Stored at room temperature in plastic container.
d) 1 M Tris HCl, pH 6: 12.11 g of Tris base was dissolved in 60 ml of water and pH 6.8 was adjusted with concentrated HCl. Volume was made up to 100 ml with water and stored at 4 °C.
e) 10% W/V APS: 100 mg of APS was dissolved in 1 ml of distilled water. It was always prepared fresh.
f) Anode Buffer (0.2 M Tris, pH 8.9): 12.11 g of Tris base was dissolved in 300 ml of distilled water and pH was adjusted to 8.9 with concentrated HCl and total volume made up to 500 ml and was stored at 4 °C.
g) Cathode buffer (0.1 M Tris, 0.1 M Tricine, SDS): 6.0 g of Tris base, 8.96 g of Tricine, and 0.6 g SDS was dissolved in 300 ml of distilled water and volume was adjusted to 500 ml. The pH of the solution was around 8.2±0.2. The buffer was stored at 4 °C.
h) 2X Sample buffer: 2.4 g of SDS, 6 g of Glycerol, 1.2 ml β-Mercaptoethanol and 0.01 g of Coomassie Brilliant Blue- G250 and volume was adjusted to 20 ml. The buffer was stored at 4 °C till further use.

i) Fixing solution (50% Methanol and 10% Acetic acid): 500 ml of 95 % methanol was added to 300 ml distilled water and 100 ml of reagent grade glacial acetic acid and volume was adjusted to 1000 ml with water.
j) Staining solution: 0.4 g of Coomassie Brilliant Blue- G250 (0.1%) was dissolved in 200 ml of 40 % (v/v) methanol and 200 ml of 20 % acetic acid was added in water. The staining solution was filtered and stored at room temperature.
k) Destaining solution (10% Methanol and 7% Acetic acid): 70 ml of glacial acetic acid was added to 100 ml of methanol and volume was adjusted to 1000 ml with water.
l) Gel solutions: Gel solution was prepared as described in below table just before use.

Table 3.3 Composition of stacking and separating gel

S.no.	Reagents	Unit	Stacking gel	Separating gel	Separating Gel
			4%, 15 ml	16.5%, 30 ml	14.5% 30 ml
1	49.5% Acrylamide	ml	1	10	8.78
2	Gel buffer	ml	3	10	10
3	Glycerol	ml	-	3	3
4	10% APS	µl	60	60	60
5	TEMED	µl	20	20	20
6	Distilled water	ml	8.37	6.67	8

3.13.2 Procedure

Solutions for making gels were prepared according to Shagger and Jagow (1987). For SDS-PAGE profile of heated milk samples, 14.5% T, 3% C separating gel concentration was used while for digested milk samples the value for separating gel concentration corresponding to 16.5% T, 3% C. The stacking gel was same for both types of samples (4% T, 3% C). The electrophoresis process was carried out in

Electrophoresis unit (Model-GENECO-V-MAXI-DUAL, GENAXY) whose glass plate dimension was 24 cm x 19 cm. Anode and Cathode buffer used was 2 L and 300 ml, respectively. The gel mixtures were gently poured in the casting modules of electrophoresis unit. After filling the separating gel, it was carefully overlaid with distilled water to allow a truly flat surface and protect the top of the gel mixture from the atmospheric oxygen. After polymerization, the distilled water was gently removed by paper and then gently poured the staking gel and immediately comb was inserted in stacking gel before polymerization. After the polymerization of the stacking gel, comb was removed and the wells were washed with cathode buffer.

3.13.3 Sample preparation

3.13.3.1 Sample preparation for milk samples

Milk samples were diluted 5 times with distilled water for unheated milk samples and 10 times with distilled water for heated milk samples. The milk samples diluted in 1:3 with SDS sample buffer (glycerol, 20% SDS, β -Mercaptoethanol, 1.0 M Tris-HCl, pH 6.8, Bromophenol Blue) and heated at 100°C for 5 min and brought down to room temperature prior to subjecting for separation by SDS-PAGE.

3.13.3.2 Sample preparation for digested milk samples

Sample for the digested milk samples for Sahiwal subjected to vacuum concentration while for KF used without subjected the concentration, further samples was diluted with sample buffer (1:3). only the heated digested samples were diluted in 1:1 with water before diluted with SDS sample buffer (glycerol, 20% SDS, β -Mercaptoethanol, 1.0 M Tris-HCl, pH 6.8, Bromophenol Blue) and heated at 100°C for 5 min and brought down to room temperature prior to subject for separation by SDS-PAGE (4% stacking gel and 16.5% separating gel). A molecular weight marker (Range 3500 to 43400 Da, Protein Molecular Weight Marker, GENEI) was included. The standards and samples were run under the cathode buffer (0.1 M Tris, 0.1 M Tricine, SDS, pH 8.2).

3.13.4 Electrophoresis conditions

Electrophoresis was performed in refrigerator condition using a voltage stepped procedure, 20 µl of sample was added in each well and voltage was kept constant 120 V until the samples completely leaves the stacking gel and then the

voltage was increased to 240 V. Voltage was maintained constant (200-260 V) until the tracking dye reached the bottom of the separating gel.

3.13.5 Fixing, staining, and destaining of gels

After the complete run of electrophoresis, the gels were kept in a fixing solution for 4 hrs followed by staining with CBB for 4 hrs and later was destained using destaining solution.

CHAPTER –4

Results and Discussion

Milk composition is influenced by various factors including genetic, species, breeds, management, feeding and environment (Bonizzi *et al.,* 2009). Also reported that indigenous and cross breeds of cattle and buffalo showed different composition Patel *et al.* (2018)

In this study, the milk samples from healthy animals of cattle (Sahiwal and Karan Fries crossbred) and buffalo (Murrah) were subjected to heat treatment (63°C/30 min, 80°C/10 min and boiled for 10 min) and assessed for protein profiling over SDS PAGE and RP-HPLC. In vitro gastrointestinal digestion of cow milk sample (before and after heat treatment) was assessed for protein hydrolysis and antioxidant activity.

4.1 Compositional analysis of cow milk

Result on compositional analysis of milk from Sahiwal and Karan Fries breed of cattle is presented in Table 4.1 relatively higher level of fat, SNF and TS content was observed in milk from Sahiwal breed compared to that from crossbred Karan Fries. The level of mean fat, SNF and TS content of milk from crossbred KF was observed to be 4.4 ± 0.14%, 8.29 ± 0.09% and 12.7 ± 0.07%, respectively, while the corresponding values for milk from Sahiwal cattle showed 5.1 ± 0.14%, 9.21 ± 0.16% and 14.3 ± 0.12% respectively. On comparing the results on variation of Fat, SNF, TS content, the highest values were observed in buffalo milk, compared to cow milk from Sahiwal cattle and lowest in crossbreed. Similar results have been reported in earlier studies (Patel *et al.,* 2013; Kapadiya *et al.,* 2016; Das, 2019). Results as presented in Table 4.2, the average protein content in pooled cow milk was observed to be relatively higher in milk from Sahiwal breed cattle compared to that from Karan Fries crossbreeds. The mean value for the true protein, casein and whey protein corresponding to 3.13%, 2.49% and 0.63% respectively for cow milk (Sahiwal) compared to respective value of 2.98%, 2.27% and 0.63% for milk from Karan Fries. 20% higher content of protein was observed in buffalo milk compared to cow milk corresponding to 4.25%. Further no difference was observed in protein composition on separation of fat in both cow & buffalo milk. The results obtained on compositional analysis of milk from cow (Sahiwal and KF) and buffalo (Murrah) are similar to that

reported earlier for compositional analysis of cow and buffalo milk, (Das, 2019) Das, 2019 reported that the crude protein, true protein, casein and whey protein content was 3.39 ± 0.27%, 3.16 ± 0.27%, 2.52 ± 0.24% and 0.64 ± 0.1%, respectively for Sahiwal breeds milk and for Karan fries breeds milk was 3.11 ± 0.21%, 2.91 ± 0.21%, 2.24 ± 0.21% and 0.64 ± 0.06%, respectively.

Table 4.1 Effect of heat treatment on composition of cow and buffalo milk

Breeds		TS %	Fat %	SNF %
		Mean	Mean	Mean
Cow Milk (Sahiwal)	Whole milk	14.3 ± 0.12	5.10 ± 0.14	9.21 ± 0.16
	Boiled whole milk	21.9 ± 0.45	7.20 ± 0.14	14.7 ± 0.10
	Skim milk	9.60 ± 0.07	0.18 ± 0.02	9.48 ± 0.09
	Boiled skim milk	14.7 ± 0.09	0.22 ± 0.02	14.5 ± 0.05
Cow Milk (KF)	Whole milk	12.7 ± 0.07	4.40 ± 0.14	8.29 ± 0.09
	Boiled whole milk	19.65 ± 0.13	6.17 ± 0.10	13.5 ± 0.09
	Skim milk	8.84 ± 0.09	0.12 ± 0.03	8.72 ± 0.09
	Boiled skim milk	14.27 ± 0.10	0.27 ± 0.03	14.0 ± 0.03
Buffalo Milk	Whole milk	17.69 ± 0.28	7.60 ± 0.14	10.9 ± 0.01
	Boiled whole milk	25.5 ± 0.21	10.1 ± 0.14	15.4 ± 0.21
	Skim milk	10.27 ± 0.21	0.17 ± 0.1	10.1 ± 0.09
	Boiled skim milk	16.3 ± 0.07	0.3 ± 0	16.0 ± 0.24

Data are presented as mean ± SD, n = 2

4.2 Effect of heat treatment:

Milk (500 ml) was subjected to heat concentration by subjecting to boiling treatment with continuous stirring over open pan for 10 min. the result as presented in Table 4.1 showed that 1.5- 2.0 fold increase in total solid content was observed. Further it was observed that compared to 80% level of casein in unheated milk, the denaturation & aggregation of whey protein with casein on extensive heat treatment resulted in increase in concentration of casein to 95.8% true protein content.

Table 4.2 Effect of heat treatment on milk protein composition

Traits	SNF (%)	Crude protein (%)	True protein (%)	Casein (%)	Whey protein (%)	NCN (%)	NPN (%)
Cow (Sahiwal)							
Whole milk	9.21 ± 0.16	3.39 ± 0.12	3.13 ± 0.14	2.49 ± 0.12	0.63 ± 0.1	0.14 ± 0.04	0.04 ± 0.006
Boiled whole milk	14.7 ± 0.10	6.69 ± 0.25	6.18 ± 0.1	5.92 ± 0.10	0.26 ± 0.1	0.12 ± 0.03	0.08 ± 0.003
Skim milk	9.48 ± 0.09	3.48 ± 0.12	3.22 ± 0.15	2.58 ± 0.12	0.63 ± 0.14	0.14 ± 0.028	0.04 ± 0.009
Boiled skim milk	14.5 ± 0.05	6.41 ± 0.19	5.9 ± 0.12	5.64 ± 0.15	0.26 ± 0.12	0.12 ± 0.021	0.08 ± 0.003
Cow (Karan Fries)							
Whole milk	8.29 ± 0.09	3.1 ± 0.07	2.9 ± 0.11	2.27 ± 0.12	0.63 ± 0.1	0.13 ± 0.01	0.03 ± 0.003
Boiled whole milk	13.4 ± 0.09	5.04 ± 0.06	4.57 ± 0.12	4.33 ± 0.14	0.23 ± 0.1	0.11 ± 0.02	0.07 ± 0.007
Skim milk	8.72 ± 0.09	3.44 ± 0.05	3.24 ± 0.1	2.60 ± 0.12	0.63 ± 0.15	0.13 ± 0.028	0.03 ± 0.007
Boiled skim milk	14.0 ± 0.03	5.14 ± 0.07	4.7 ± 0.18	4.43 ± 0.1	0.23 ± 0.13	0.11 ± 0.03	0.066 ± 0
Buffalo (Murrah)							
Whole milk	10.9 ± 0.01	4.25 ± 0.07	3.93 ± 0.16	3.16 ± 0.12	0.76 ± 0.12	0.17 ± 0.01	0.05 ± 0.01
Boiled whole milk	15.4 ± 0.21	6.15 ± 0.07	5.57 ± 0.14	5.25 ± 0.15	0.31 ± 0.1	0.14 ± 0.02	0.09 ± 0.007
Skim milk	10.1 ± 0.09	4.32 ± 0.1	3.95 ± 0.1	3.17 ± 0.10	0.78 ± 0.17	0.18 ± 0.021	0.05 ± 0.009
Boiled skim milk	16.0 ± 0.24	6.37 ± 0.1	5.82 ± 0.1	5.47 ± 0.15	0.35 ± 0.11	0.14 ± 0.026	0.08 ± 0.008

Data are presented as mean± SD , n = 2

4.3 Effect of heat treatment on SDS- PAGE profile of milk

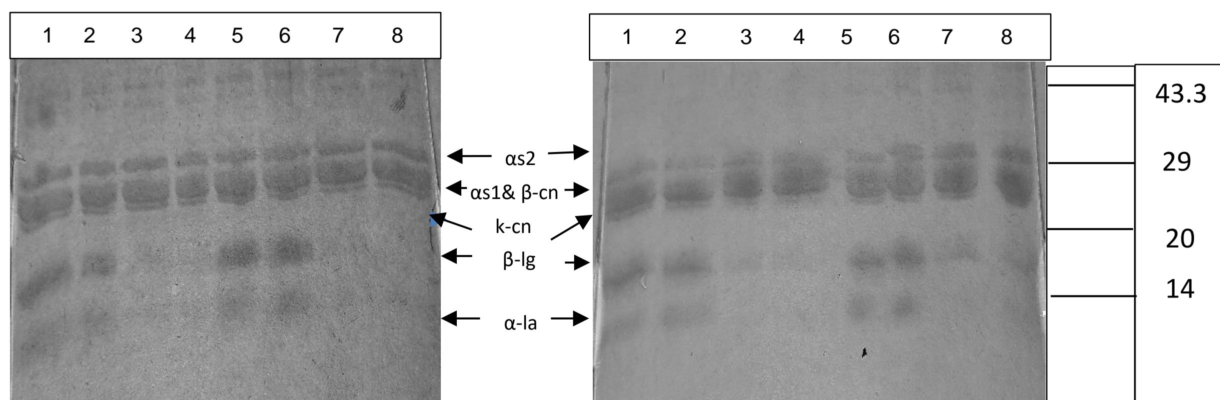


Figure 4.1a Sahiwal **Figure 4.1b** Karan Fries

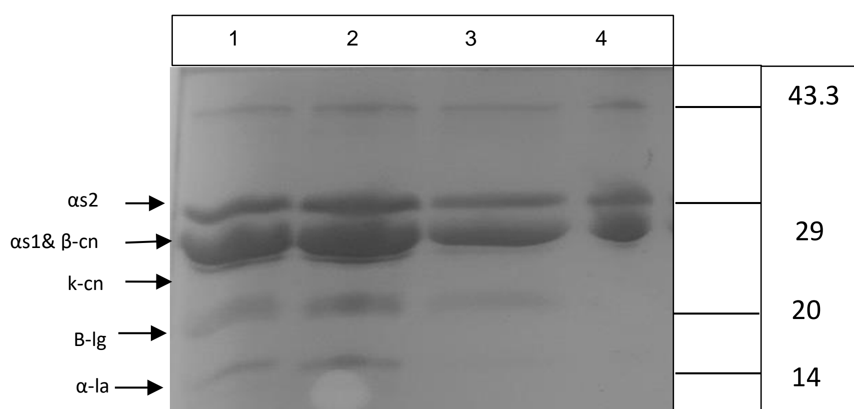


Figure 4.1c Murrah

Lane 1: Skim Milk; Lane 2: Skim Milk (63°C/30 min); Lane 3: Skim Milk (80°C/10 min); Lane 4: Skim Milk (Boiled/10 min); Lane 5: Whole Milk; Lane 6: Whole milk (63°C/30 min); Lane 7: Whole Milk (80°C/10 min); Lane 8: Whole Milk (Boiled/10 min). Molecular marker in kDa

Figure 4.1 Effect of heat treatment on SDS-PAGE Profile of milk

4.3.1 Effect of heat treatment on electrophoretic profile of milk

Milk from different breeds of cattle and buffalo was subjected to heat at 63°C/ 30 min, 80°C/ 10 min and boiled for 10 min and assessed for SDS-PAGE shown in Figure 4.1 it was observed that casein and whey protein band was obtained in control, 63°C/ 30 min, 80°C/ 10 min and boiled for 10 min treated sample. The intensity of whey

protein was reduced at 80°C/ 10 min and boiled milk. Similar results were observed in different heat treated skim milk sample from different breeds of cattle and buffalo.

Unheated milk is composed of casein micelles and native whey protein. Lane 1 indicate the native milk protein (Figure 4.1) casein micelle in milk are large colloidal particles with a size ranging from 20-500 nm mainly linked by non- covalent interaction. On treatment with SDS these are disrupted, result in monomers and disulphide linked polymers. In the presence of 2-merceptoethanol, the casein polymers are disrupted into monomers. On heat treatment of milk (lane 2) pasteurization (63°C/30 min) does not affect electrophoretic mobility as compared to unheated milk, on raising temperature of milk to 80°C & boiling for 10 min the intensity of bands corresponding to whey protein diminished with increasing severity of heat treatment. The change in electrophoretic mobility in due to disulphide interaction between whey protein and K-casein (Figure 4.1). Further the results obtained were similar to that reported earlier (Pinho *et al.,* 2012).

4.4 Profiling of milk proteins by HPLC

Separation of proteins in reversed-phase HPLC analysis was performed based on differences in hydrophobicity of the proteins. The chromatograms as presented in Figure 4.2 shows that the sequence of the elution of the peaks on C18 is k-CN followed by αs2-CN, αs1-CN, β-CN, α-la and β-lg respectively based on their increasing order of hydrophobicity.

4.4.1 Milk protein profile from Cow milk (Sahiwal & KF breed)

The results as presented in Figure 4.2 chromatogram shows the elution profile of different fractions of protein. It was observed that 3 peaks corresponding to k-CN, 1 peak corresponding to αs2-casein, one peak each for αs1-casein and β-CN. α-la, showed 1 peaks and β-lg showed 2 peaks. Further the peaks corresponding to k-CN were eluted at the retention times 7.5, 8.5, 9.5 min. The peaks corresponding to αs2-casein at 12.5 min, αs1-casein fractions eluted at 23.5 min. While that for β-casein was eluted at 31 min. α-la eluted at 42 min and β-lg eluted at 43 and 45 min.

For milk from KF crossbred cattle the chromatogram (Figure 4.3) shows the elution profile of different fractions of casein and whey proteins (i.e.,αs1-casein,αs2-

casein, β-casein, κ-casein, α-la and β-lg) shows similar retention time as observed for milk from Sahiwal cattle.

4.4.2 Effect of heating:

On quantification of individual protein fraction was done by RP-HPLC. Cow milk (Sahiwal) was heated to 63°C/ 30 min, 80°C/ 10 min and boiled for 10 min. Results as presented in Table 4.4 showed that αs1-CN, αs2-CN, β-CN and k-CN in control milk is 39%, 15.6%, 36.2% and 10.1% respectively. It was found that peaks of α-la and β-lg is reduced in all the heated samples. Reduction of peaks of whey protein is more in boiled milk sample compare to that heated at 80°C/ 10 min and 63°C/ 30 min. Boiled milk showed similar percentage of protein fraction but amount of individual fraction was increased after heat treatment due to two fold concentration of total solids. It was observed that level of αs1-CN (1.04 g /100 ml), αs2-CN (0.42g /100 ml), β-CN (0.97g /100 ml) and k-CN (0.24g /100 ml) in control milk, which is increased to αs1-CN (2.17 g /100 ml), αs2-CN (0.78g /100 ml), β-CN (1.99g /100 ml) and k-CN (0.46g /100 ml) after boiling for 10 min. Whey protein denaturation was higher in boiled milk comparing to 63°C/ 30 min, 80°C/ 10 min treated sample. Level of whey protein in the milk was 0.49 g /100 ml which after boiling decreased to 0.39 g /100 ml after boiling.

During heat treatment whey proteins unfold and polymerise via disulfide bond forming aggregates with different hydrophobicity (Curda *et al.,*1997). As the whey proteins denaturation takes place above 70°C, thus the results indicated the peaks corresponding beta-lactoglobulin and alpha-lactalbumin diminished compared to samples heated at 80°C and boiling treatment as compared to control (unheated milk). No change was observed in pasteurized milk samples (63°C /30 min). Reduction of peaks of whey protein was higher in extensively boiled milk sample compared to that heated at 80°C/10min. The results obtained in this study corroborates the earlier finding by Pinho *et al.* (2012) wherein it was reported that on RP-HPLC analysis of boiled cow milk heated for 5 min and reported no peak corresponding to beta-lactoglobulin and alpha-lactalbumin due to the fact that whey protein denaturation results in change in their polarity.

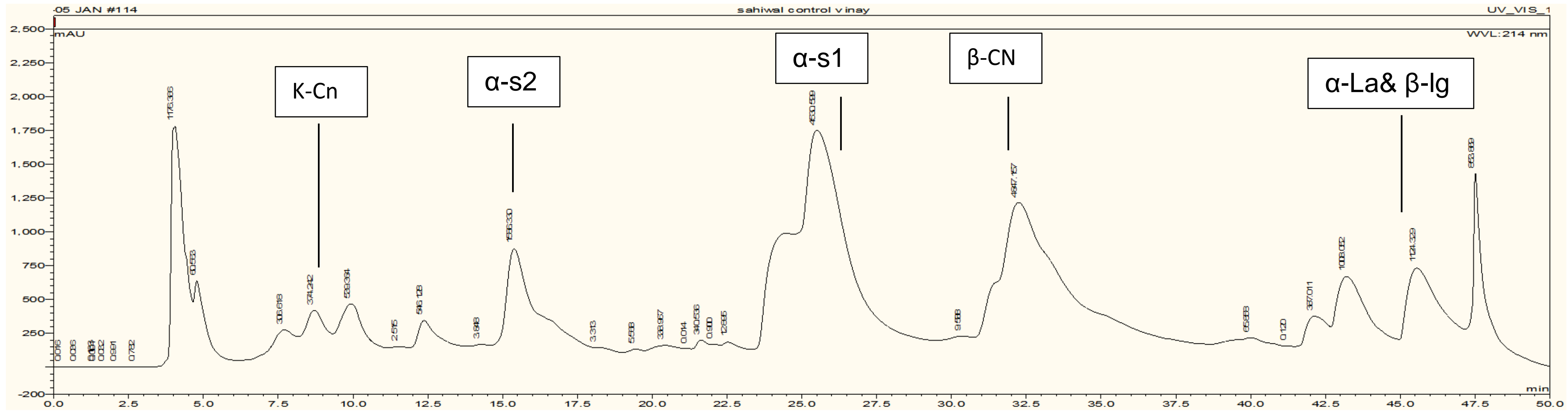
Table 4.3 Retention time of protein fraction over RP-HPLC from cow and buffalo milk

Casein	k-CN			α-CN			β-CN		α-la & β-lg
No. of peaks	1	2	3	αs2	αs1		1	2	1
	Retention time (min)								
Standard	8.4	9.37	11.6	-	-	22.7	26.7	-	-
Cow (Sahiwal) Skim Milk									
Control	7.5	8.5	9.5	12.5	15	24	31	-	41.5
63°C./30 min	6	7.5	8.5	13	-	20.5	26.5	-	33
80°C./10 min	6	7.5	8.5	13	-	21	26.5	-	34
Boiled/10 min	6.5	8	9.5	12.5	14.5	23.5	31	-	42
Cow (Karan Fries) Skim Milk									
Control	6	8	10	16	20	23.5	30.5	34.5	42.5
63°C./30 min	7.5	8.5	11	13.5	-	22.5	28	-	36
80°C./10 min	7.5	8.5	11	13.5	-	22.5	28	-	36
Boiled / 10 min	6.5	8	10	15.5	20.5	23	30.5	-	-
Buffalo skim milk									
Control	-	8	11	18	20	25.5	33.5	-	42
63°C./30 min	-	8	9.5	17.5	-	22.5	29	-	35
80°C./10 min	-	7.5	9.5	17.5	-	22.5	28.5	-	34.5
Boiled/10 min	-	8	11	15.5	20	23	30.5	-	-

Table 4.4 Quantification of protein fraction in heated and unheated skim milk from Sahiwal breed

	αs1	**αs2**	**β-CN**	**ķ-CN**	**WP**
	Protein fraction (g/100 ml)				
Unheated	1.04	0.42	0.97	0.24	0.49
Heated					
63°C/30 min	1.02	0.45	1.03	0.31	0.46
80°C/10 min	1.36	0.45	1.16	0.3	0.37
Boiled/10 min	2.17	0.78	1.99	0.46	0.39

Figure 4.2a Chromatogram of Cow milk (Sahiwal) Without Heat treatment on C_{18} Column



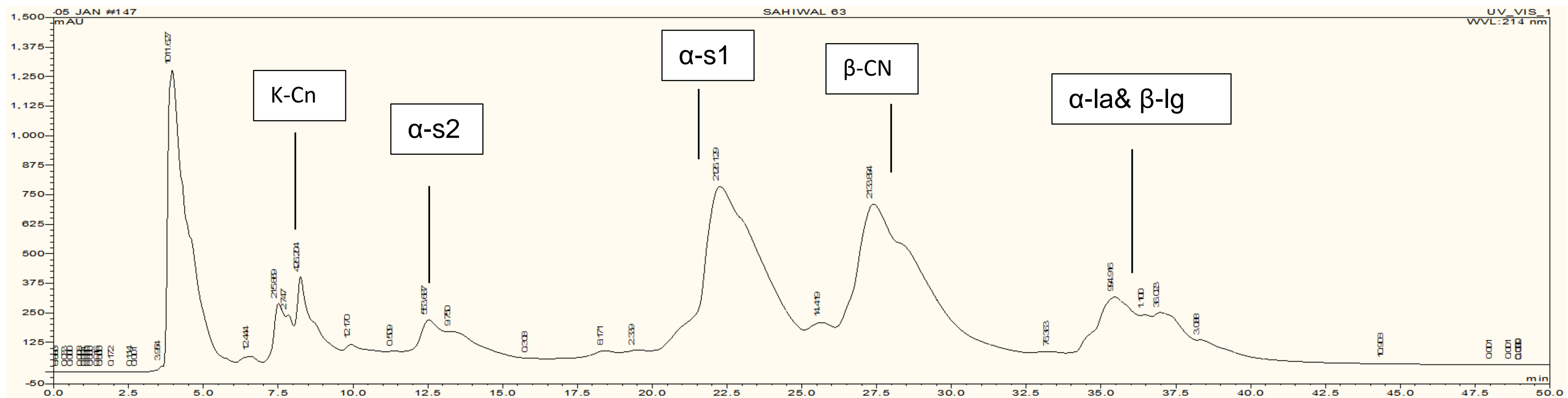
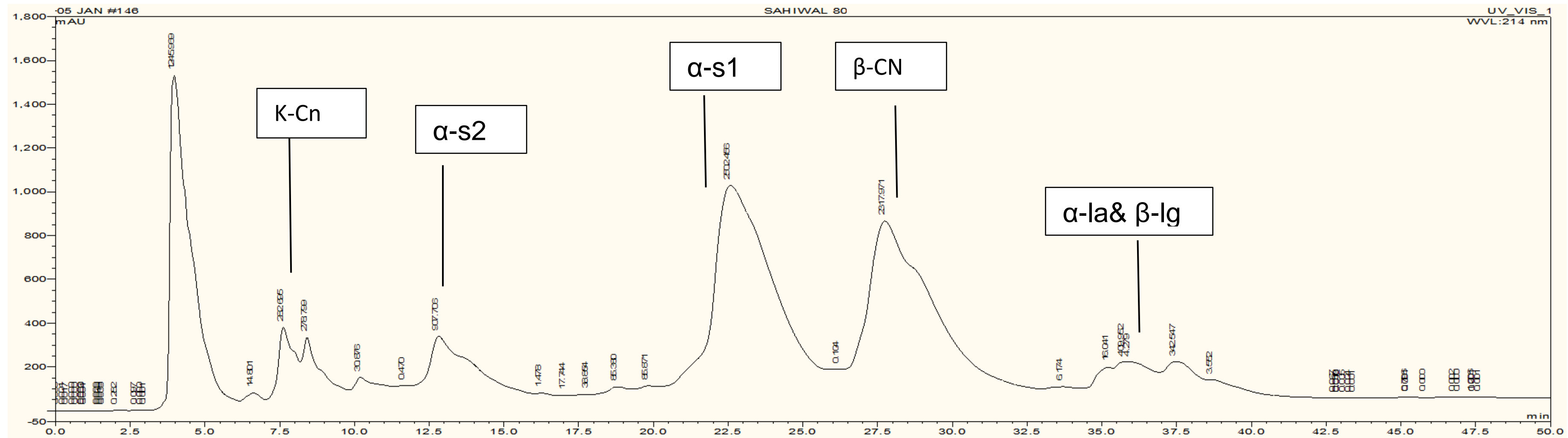


Figure 4.2b Chromatogram of Cow Milk (Sahiwal) 63°C/30 min on C_{18} Column

Figure 4.2c Chromatogram of Cow Milk (Sahiwal) 80°C/10 min on C_{18} Column



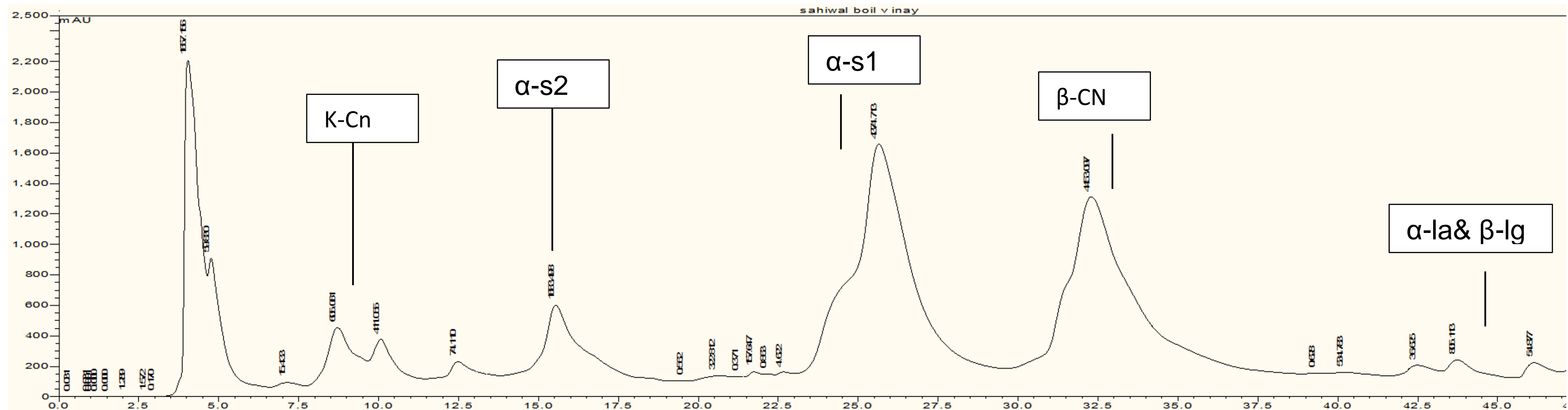
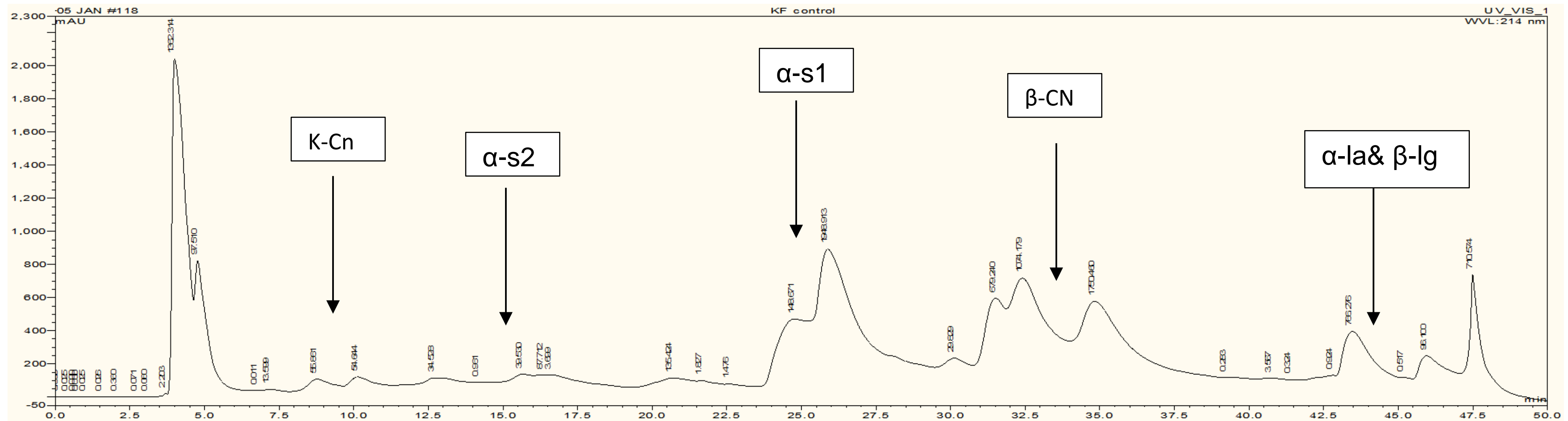


Figure 4.2d Chromatogram of Cow Milk (Sahiwal) Boiled for 10 min on C_{18} Column

Figure 4.3a Chromatogram of Cow Milk (Karan Fries) Without Heat treatment on C_{18} Column



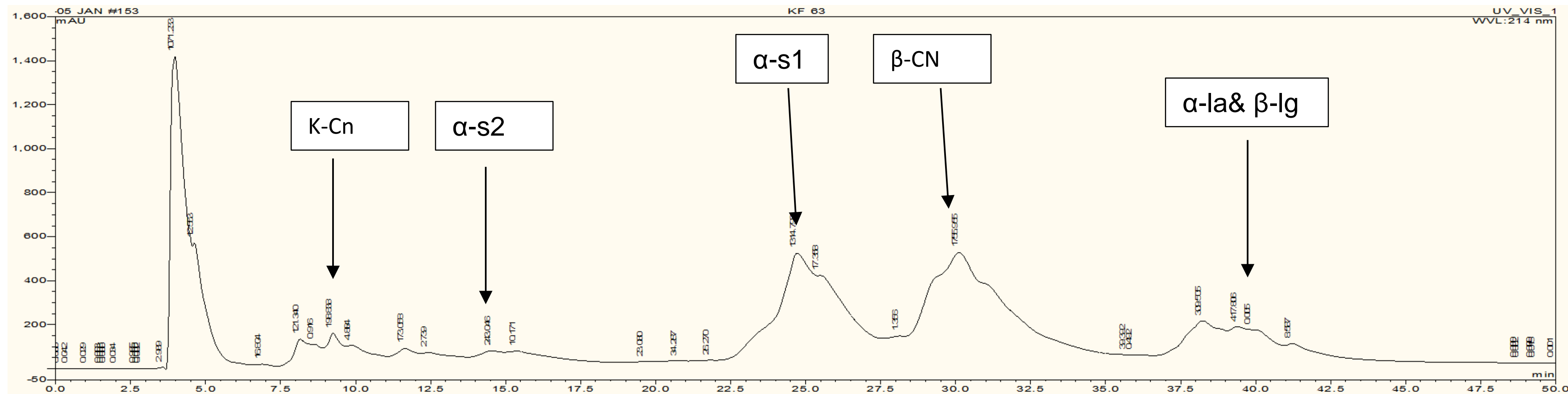
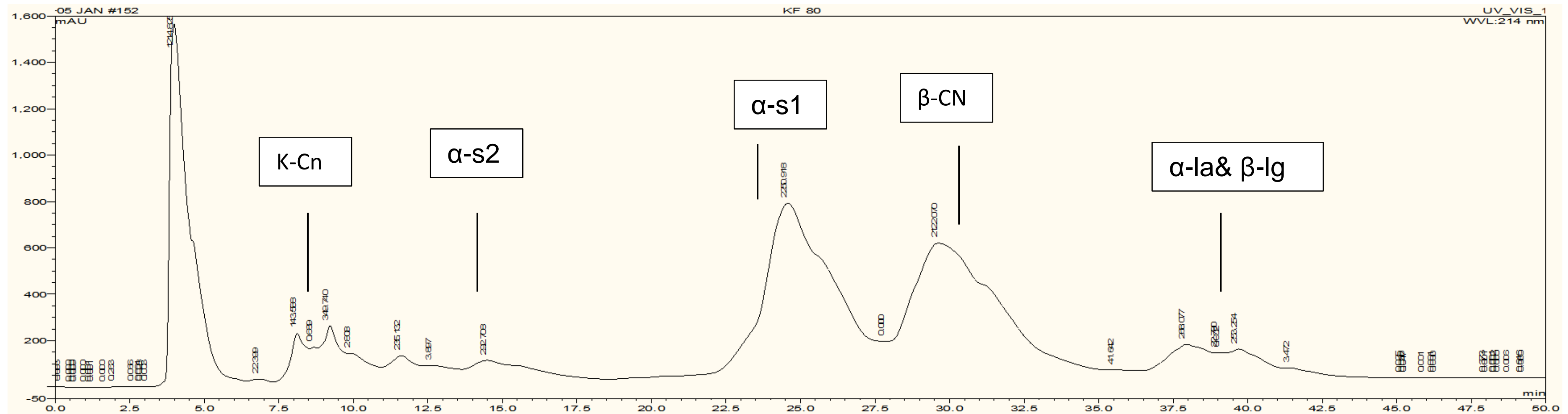


Figure 4.3b Chromatogram of Cow Milk (Karan Fries) 63°C/30 min on C_{18} Column

Figure 4.3c Chromatogram of Cow Milk (Karan Fries) 80°C/10 min on C_{18} Column



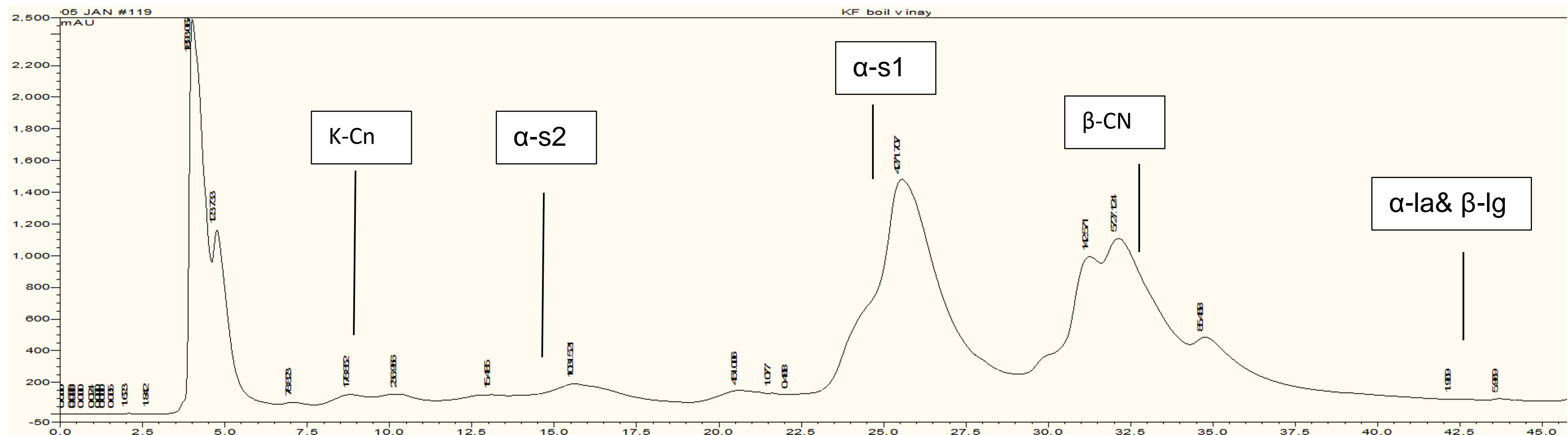
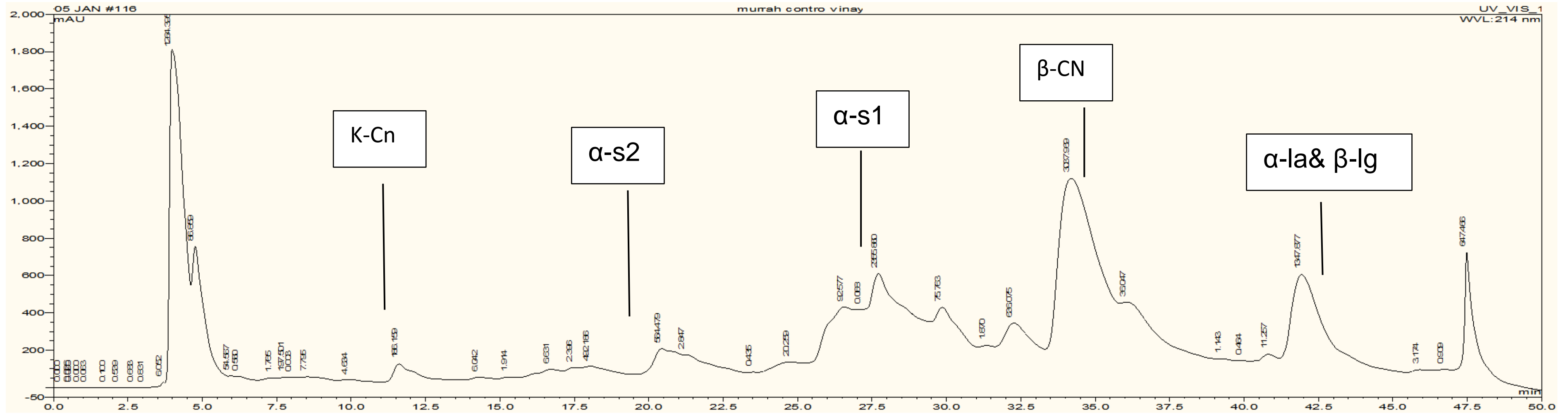


Figure 4.3d Chromatogram of Cow Milk (Karan Fries) Boiled for 10 min on C_{18} Column

Figure 4.4a Chromatogram of Buffalo milk (Without Heat treatment) on C_{18} Column



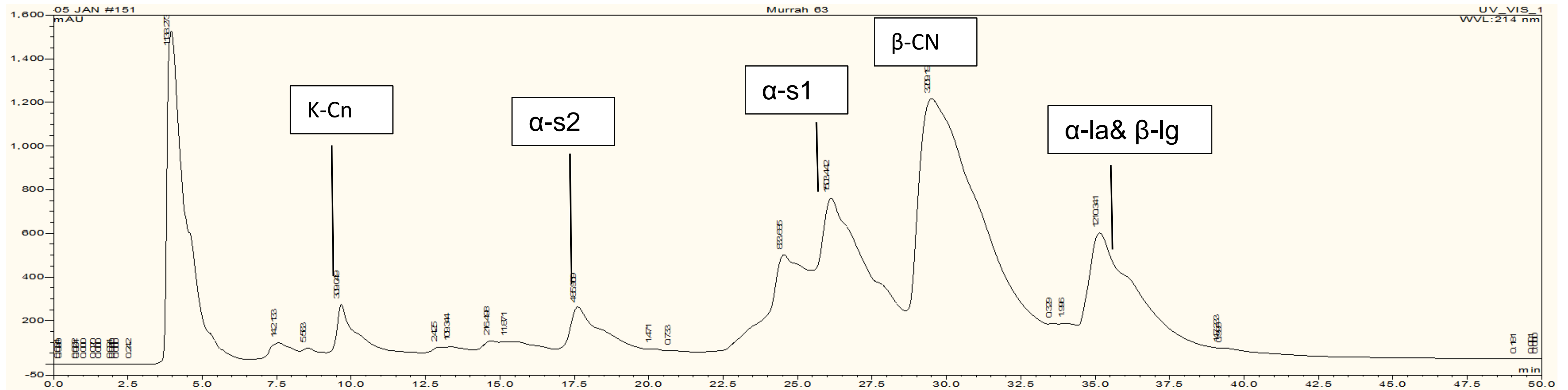
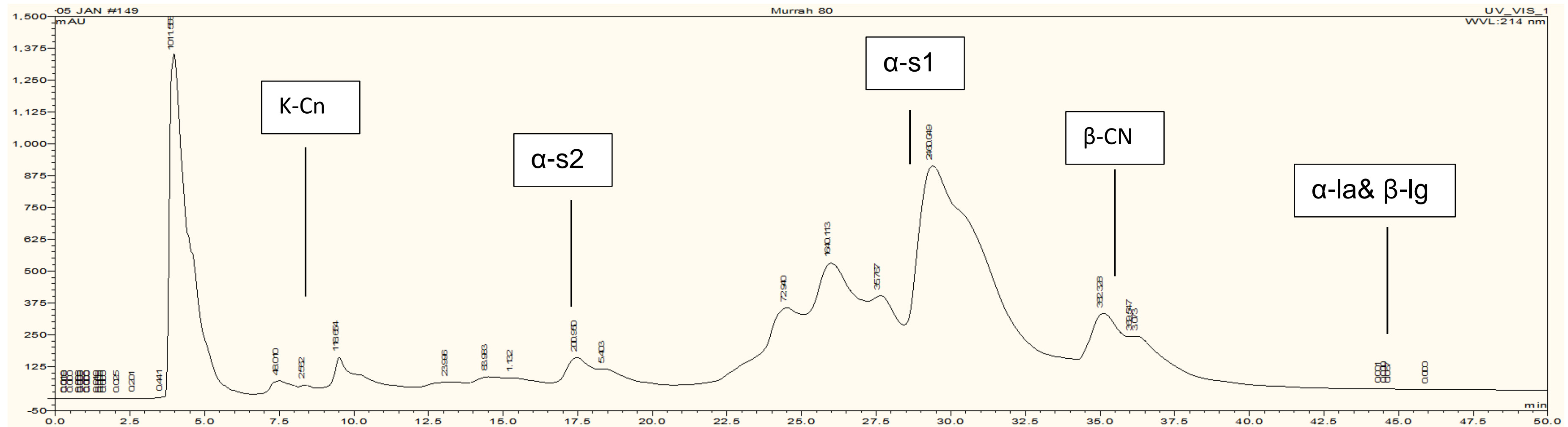


Figure 4.4b Chromatogram of Buffalo milk (63°C/30 min) on C_{18} Column

Figure 4.4c Chromatogram of Buffalo milk (80°C/10 min) on C_{18} Column



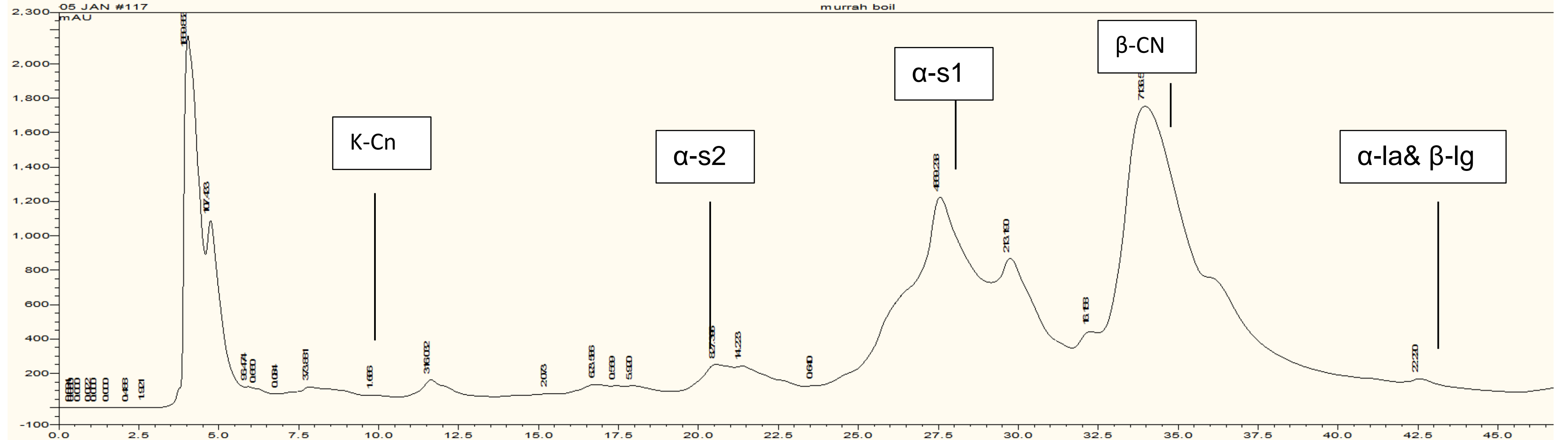


Figure 4.4d Chromatogram of buffalo milk (Boiled for 10 min) on C_{18} Column

4.5 Antioxidant activity of cow and buffalo milk

Skim milk from different breeds of cattle and buffalo was subjected to heat treatment to 63°C/ 30 min, 80°C/ 10 min and boiled for 10 min and diluted with 5 times with water. TAC was determined using ABTS method.

Table 4.5 Antioxidant capacity of heated and unheated milk from Cow (Sahiwal & Karan Fries) and Buffalo (Murrah)

	TAC (Total Antioxidant Capicity)	Control	63°C/30 min	80°C/10min	Boiled/ 10 min
Cow skim milk (Sahiwal)	Vit C (mg/l)/ Trolox (µM/L)	372.68 (7034.7)	397.97 (7394.7)	442.8 (8185.8)	456.03 (8545.5)
Cow skim milk (Karan Fries)	Vit C (mg/l)/ Trolox (µM/L)	332.6 (6243.3)	379.6 (7070.7)	407.36 (7559.9)	547.97 (10038.3)
Buffalo Skim Milk	Vit C (mg/l)/ Trolox (µM/L)	346.95 (6495.1)	368.38 (6872.8)	406.13 (7538.3)	631.24 (11652.6)

4.5.1 Effect of heat treatment on Antioxidant activity of cow and buffalo milk

Result shown in Table 4.5 indicate that TAC of milk is higher in Sahiwal milk (372.68 Vit C mg/l) followed by buffalo milk (346.95 Vit C mg/l) and Karan fries milk (332.6 Vit C mg/l) it was observed that heat treatment increase the antioxidant activity of milk, antioxidant activity increased after heat treatment was higher in boil milk comparing to 63°C/30 min and 80°C/10min. buffalo milk showed higher antioxidant activity (631.24 Vit C mg/l) after 10 min of boiling compared to Karan Fries milk (547.97 Vit C mg/l) and Sahiwal milk (456.03 Vit C mg/l). The increase in antioxidant might be due to generation of -SH group on denaturation of whey proteins.

4.6 In vitro simulated gastrointestinal digestion

The pooled cow milk of Sahiwal and KF cross breed was subjected to boiling for 10 min and assessed for simulated in vitro gastro-intestinal digestion (gastric digestion

2 hours and intestinal digestion 2 hours). Samples were collected after 0, 5, 10, 15, 30, 60, 90 and 120 min of gastric and intestinal digestion and analysed for quantification of soluble protein, protein hydrolysis, and antioxidant activity.

4.7 Quantification of soluble protein from in-vitro digested milk of cow milk

Following in vitro digestion of both heated and unheated cow milk (from Sahiwal and KF breed cattle), quantification of soluble milk protein was done using Lowry method.

Table 4.6 Changes in soluble protein concentration during in-vitro GI digestion of cow milk

Phase	SWM (Sahiwal Whole milk)	SBWM (Sahiwal Boiled Whole milk)	KFWM (Karan Fries Whole milk)	KFBWM (Karan Fries Boiled Whole milk)
Gastric (G)	Concentration of soluble protein mg/ml			
G 0	28.3	12.3	20.4	17.5
G 5	21.5	11.8	21.6	14.2
G 10	16.4	12.02	18.3	12.5
G 15	18	9.62	17.67	11.3
G 30	20.6	8.02	12.5	8.8
G 60	14.4	7.18	12.3	11.1
G 90	21.6	6.85	12.1	8.3
G 120	14.1	10	11.7	7.67
Intestinal (I)				
I 0	-	8.21	11.5	13
I 5	-	7.09	11.5	10.5
I 15	-	7.4	10.6	8.42
I 30	12.9	9.24	9.4	8.49
I 60	16.2	8.6	9	8.7
I 90	14.8	8.8	8.2	7.8
I 120	8.1	9.38	6.8	8.1

4.8 Evaluation of protein hydrolysis during simulated in- vitro digestion by OPA method

Digestibility of heated and unheated whole milk from different breeds of cattle were analysed using OPA method. The degree of hydrolysis of heated and unheated milk during in-vitro digestion was determined using OPA method was expressed in terms of millimoles equivalent of serine residue per gram of protein.

Table 4.7 Effect of heat treatment on digestion of cow milk

Phase	SWM	SBWM	KFWM	KFBWM
	millimoles serine / g of protein			
G0	0.95	2.86	1	1.2
G5	1.29	3.1	1.04	1.56
G10	1.7	3.25	1.2	1.89
G15	1.6	4.1	1.29	2.17
G30	1.69	5.1	1.97	3.3
G60	2.44	5.9	2.02	2.5
G90	1.65	6.6	2	3.6
G120	2.54	4.6	2.2	4.3
I0	-	6.3	2.7	2.62
I5	-	7.7	2.85	3.3
I15	-	7.48	3.1	4.17
I30	4.26	7.03	3.8	4.47
I60	3.6	10.1	4.01	4.65
I90	4.3	10.03	4.65	6.54
I120	8.45	9.72	5.9	6.99

The results as presented in Table 4.7 shows that during gastric phase release of free amino groups to different extent was observed at 0 time among heated and unheated whole milk, higher free amino group released in SBWM (Sahiwal Boil Whole Milk) and lowest serine released observed in SWM (Sahiwal Whole Milk). Released of serine during gastric phase is slow, but after gastric phase serine released goes faster. At

the end of the intestinal phase (120 min), the amount of free amino groups released was observed to be significantly higher in heated milk compared to control (unheated milk). The values of serine released on digestion of heated milk and unheated milk corresponded to 9.72 and 8.45 millimoles serine per gram of protein, respectively.

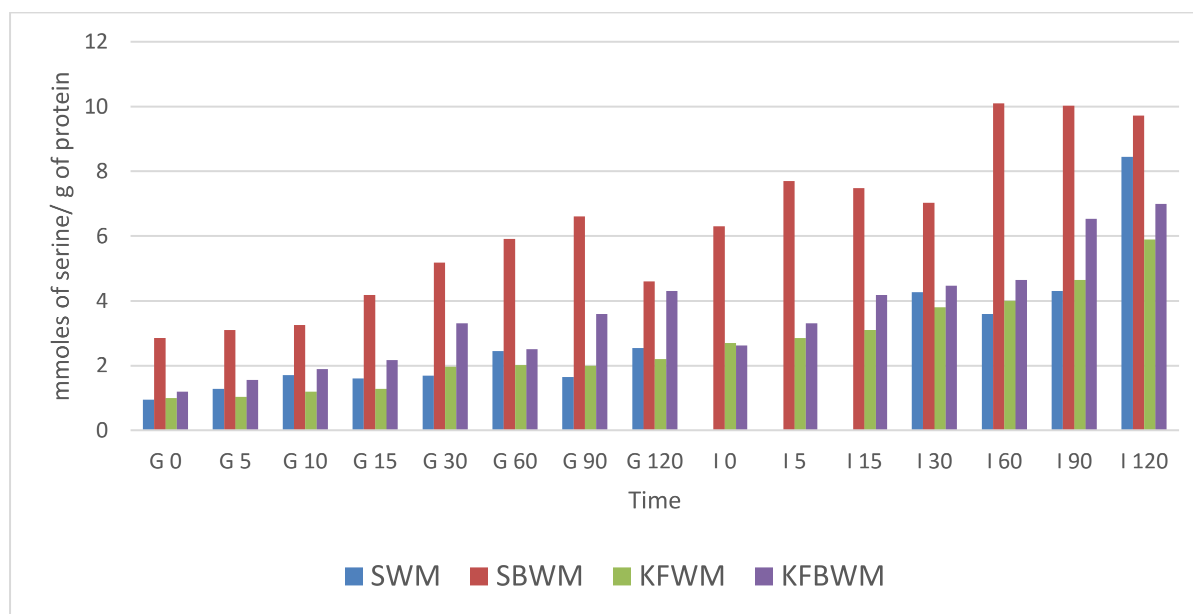


Figure 4.5 Hydrolysis of protein in SWM, SBWM, KFWM and KFBWM

Similar trend was observed for the digestion of cow milk from KF crossbred during gastric and intestinal phase of digestion. The value of millimoles of serine/g of proteins for KFBWM (Karan Fries Boil Whole Milk) corresponded to 6.99 being relatively higher than control milk KFWM (Karan Fries Whole Milk) i.e., 5.9. On comparison of results for release of free amino group on digestion of cow milk from Sahiwal and KF breed, it was observed that the value for both control and heated milk was higher in milk from Sahiwal breed compared to KF crossbred.

4.9 Evaluation of protein digestion by SDS-PAGE

SDS-PAGE was performed for monitoring the effect of heat on hydrolysis of milk protein. SDS-PAGE of raw and boiled whole milk from different breeds of cattle subjecting to gastric and intestinal digestion is shown in Figure. The vitro-gastric digestion was initiated by addition of pepsin and ended at 120 minute followed by pancreatin treatment for 120 minutes.

Based on gel shown in Figure 4.6a is indicate that casein and whey protein is intact during gastric digestion. But casein is degraded at 90 min of gastric digestion in SWM, breakdown of casein fraction to low molecular weight peptides (<6.5 kDa) is visible after 90 min of gastric digestion. α-la and β-lg which remained intact during gastric phase but intensity of band is slightly decrease at 90 min of gastric digestion, intact whey protein is completely degraded in intestinal phase. No band was visible after intestinal phase. In results as presented in Figure 4.6b it was observed that boiled whole milk casein shown faded band after 5 min of gastric digestion. β-lg is intact in entire gastric phase but faded band obtained for α-La after 15 min of gastric digestion. It was observed that faded band found for low molecular weight fraction (6.5 kDa) at 10 and 15 min of gastric digestion.

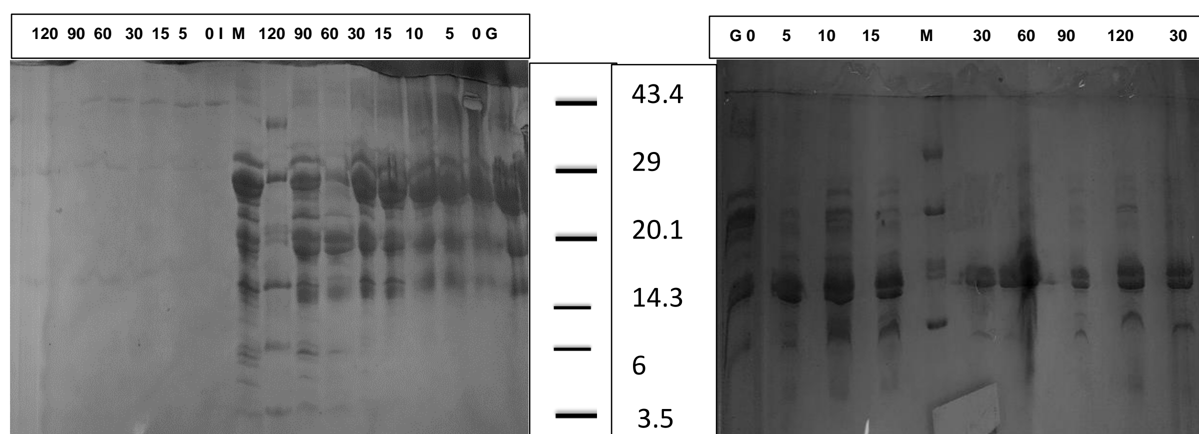


Figure 4.6a SWM

Figure 4.6b SBWM

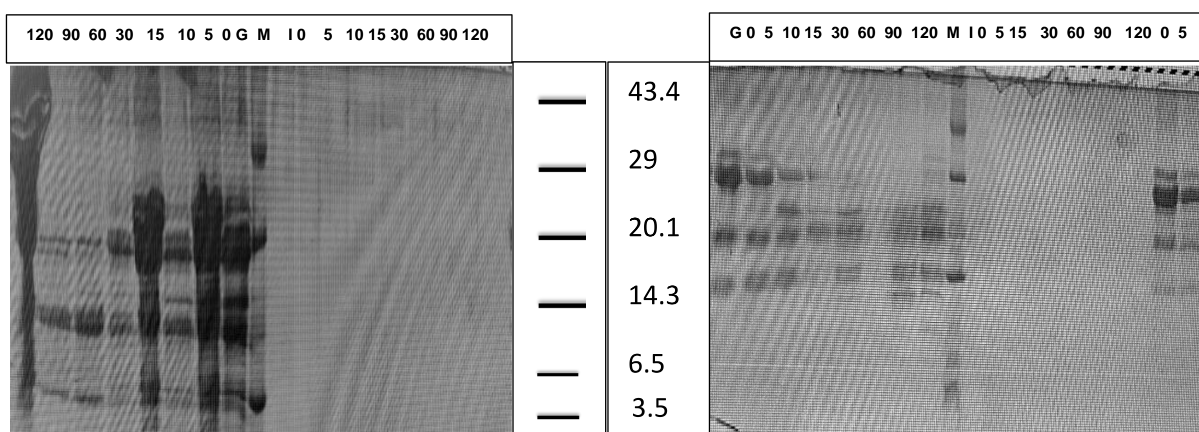


Figure 4.6c KFWM

Figure 4.6d KFBWM

Figure 4.6 Evaluation of protein digestion during in-vitro gastrointestinal digestion of the milk by SDS-PAGE

Gel obtained from whole milk from Karan Fries breeds is shown that intense band obtained at 5, 10 and 15 min of gastric digestion but intensity of band was decreased after 15 min of gastric phase. β-lg and α-la is intact during gastric digestion. Intact whey protein is completely degraded in intestinal phase. No visible band found in intestinal phase of digestion.

4.10 Evaluation of digested samples for antioxidant activity by ABTS Assay

The gastric and intestinal digest sample was subjected to centrifugation and supernatant portion were assessed for antioxidant activity using ABTS method.

The results as presented in Table 4.8 indicate the antioxidant activity was significantly different at different time intervals. At 0 minute of gastric digestion showed the higher antioxidant activity in boiled whole milk compare to control. It was observed that antioxidant activity is higher in intestinal phase compared to gastric phase. At the end of digestion, highest ABTS radical scavenging activity was shown by SBWM (1.1 mmole Vit C/g of protein) followed by SWM (0.95 mmole Vit C /g of protein). Boiled milk sample showed relatively higher antioxidant activity compare to control milk.

In case of heated and unheated whole milk of Karan Fries breeds of milk sample showed the higher antioxidant activity in KFWM comparing to KFBWM at 0 minutes of gastric digestion. It was observed that gradual increase of antioxidant activity in both KFWM & KFBWM with time. KFBWM (2.08 mmole Vit C /g of protein) showed higher antioxidant activity than KFWM (1.45 mmole Vit C /g of protein) at 120 minutes of intestinal digestion. Highest antioxidant activity observed at I15 in KFBWM (2.3 mmole Vit C /g of protein)

Comparing the antioxidant activity of Karan Fries and Sahiwal breeds milk, it has been observed that Karan Fries milk was showing higher antioxidant activity followed by Sahiwal.

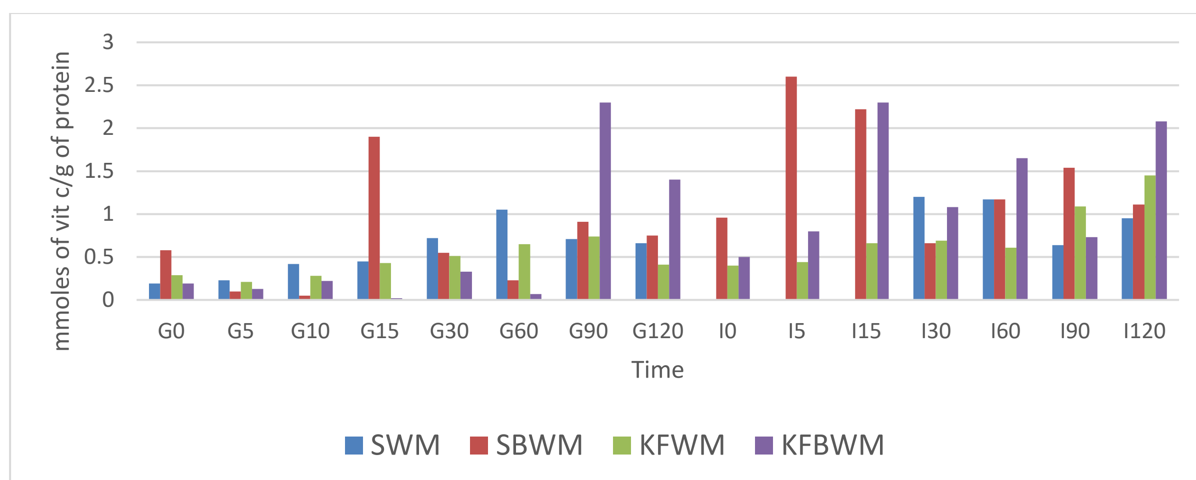


Figure 4.7 Antioxidant activity of digested cow milk by ABTS Assay

Table 4.8 ABTS radical scavenging activity during in vitro gastrointestinal digestion of cow milk

Phase	SWM	SBWM	KFWM	KFBWM
	milimoles of Vit C/ g of protein			
G0	0.19	0.58	0.29	0.19
G5	0.23	0.1	0.21	0.13
G10	0.42	0.05	0.28	0.22
G15	0.45	1.9	0.43	0.02
G30	0.72	0.55	0.51	0.33
G60	1.05	0.23	0.65	0.07
G90	0.71	0.91	0.74	2.3
G120	0.66	0.75	0.41	1.4
I0	-	0.96	0.4	0.5
I5	-	2.6	0.44	0.8
I15	-	2.2	0.66	2.3
I30	1.2	0.66	0.69	1.08
I60	1.17	1.17	0.61	1.65
I90	0.64	1.54	1.09	0.73
I120	0.95	1.1	1.45	2.08

CHAPTER – 5

Summary and Conclusion

SUMMARY AND CONCLUSION

- Comparing the cow and buffalo milk, buffalo milk having higher Fat, SNF and TS compared to cow milk. i.e. buffalo milk (7.6 ± 0.14%; 10.9 ± 0.01% and 17.69 ± 0.28%) on comparison of cow milk higher level of fat, SNF and TS content in Sahiwal breed compared to that from crossbreed Karan Fries. i.e. Sahiwal (5.1 ± 0.14%; 9.21 ± 0.16% and 14.3 ± 0.12%) and KF (4.4 ± 0.14%; 8.29 ± 0.09% and 12.69 ± 0.07%) respectively.
- Similarly crude protein content was higher in buffalo milk compared to cow milk i.e. buffalo milk (4.25 ± 0.07%), Sahiwal (3.39 ± 0.12%) and KF (3.1± 0.07%)
- Whey protein was found to be majorly 0.63% for cow milk and 0.76% for buffalo milk
- NCN% in cow milk is generally ranging from 0.13% - 0.14% for cow milk and 0.17% - 0.18% for buffalo milk.
- NPN% level in cow milk ranging from 0.03% - 0.04% in cow milk and 0.05% in buffalo milk.
- Based on chromatogram obtained from RP-HPLC, it was observed that denatured whey protein was higher in boiled milk than 63°C/30 min and 80°C/10 min in both cow and buffalo milk. Also whey protein denaturation was evidenced with Tricine SDS-PAGE profile of milk.
- Based on protein profiling level of different fraction of αs_1-cn (39%), αs_2 (15.6%), β-cn (36.2%) and k-cn (10.1%) was found in Sahiwal breed milk.
- It was observed that heat treatment increase the antioxidant activity of milk. Antioxidant activity was higher in buffalo milk (631.24 Vit C mg/l) compared to cow milk (Sahiwal - 456.03 Vit C mg/l and KF - 547.97 Vit C mg/l) after Boiling.
- Based on the simulated in-vitro GI digestion, it was observed that relatively greater increase in the release of free amino group in milk from Sahiwal breed (8.45 mmole of serine/g of protein) as compared to that from KF(5.9 mmole of serine/g of protein). Compared to control, a significant increase in release of free amino groups was observed on boiling treatment of cow milk, resulted in 9.72 mmole of serine/ g of protein for Sahiwal breed and 6.99 mmole of serine/ g of protein for KF breed, respectively.
- Based on the study of antioxidant activity using ABTS method, it was observed that greater increase in antioxidant activity was observed in boiled milk (1.1 mmole Vit C/g of protein for Sahiwal breed milk and 2.08 mmole Vit C / g of protein for KF breed milk) a

compared to that for unheated milk (0.95 mmole Vit C/g of protein for Sahiwal breed milk and 1.45 mmole Vit C/g of protein for KF breed milk) following in vitro digestion.

Conclusion

- Present study indicates that boiling of cow milk (Sahiwal & KF Breed) resulted in increase in digestibility and antioxidant activity

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Annexure

Annexure

Calculation of concentration of serine released

Example : Sahiwal whole milk sample (G0)

Dilution factor = 100

OD sample = 0.191

OD blank /control = 0.019

y- (OD sample – OD blank)

x- Concentration of serine

$y = 0.0073x - 0.0341$ (standard curve equation)

$x = (y+0.0341)/0.0073$

$x = \{(0.172+0.0341)/0.0073\} * df$

$x = 28.23 * 100$

x = 2823.28 mg/L

Since the molecular weight of serine is 105.09 g/mol

$x = 2823.28 / 105.09$

= 26.86 mM serine

Since protein concentration used was 28.3 g/L

$x = 26.86/28.3$

x = 0.95 millimoles serine / g of protein

Calculations for ABTS activity

Example: Sahiwal whole milk

Dilution factor = 50

A750sample = 0.483

A750control = 0.70

%inhibition = ($A750control - A750sample/\ A750control$) $*$ 100

= {(0.7-0.483)/0.7}*100 = 31 %

y = 0.8454x + 14.575 (standard curve equation)

y - % inhibition

x – Concentration of Vit C

x = {(y-14.575)/0.8454}*df

x = {(31-14.575)/0.8454}*50

x = 971.43 mg/L

Since molecular weight of vit C is 176.14 g/mol

x = 971.43/176.14

= 5.51 mM

Since the peptide concentration in the sample was 28.3 g/L

x = 5.51/28.3

x = 0.19 millimole Vit C / g of protein

x = 194.8 micromoles Vit C / g of protein