ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PRODUCTION OF A CASEINOMACROPEPTIDE CONCENTRATE FROM SWEET WHEY AND USAGE IN A MILK-LIKE DRINK

Ph.D. THESIS

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Department of Food Engineering

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To those who search light in darkness with hope

& to my grandma.

FOREWORD

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ABBREVIATIONS

ANOVA **:** Analysis of variance
CMP **:** Caseinomacropeptide
DF **:** Diafiltration
HPLC **:** High Pressure Liquid Chromatography
kDa **:** KiloDalton
MW **:** Molecular weight
Phe **:** Phenylalanine
Ip **:** Isoelecteric point
PKU **:** Phenylketonuria
RSM **:** Response Surface Methodology
Trp **:** Tryptophan
Tyr **:** Tyrosine
UF **:** Ultrafiltration
WPC **:** Whey protein concentrate
α-la **:** α-lactalbumin
β-lg **:** β-lactoglobulin

SYMBOLS

^{o}C	: Degree of Celsius
L	: Liter
mL	: milliliter
mbar	: millibar
gr	:gram
nm	: Nanometer
d	: Day
t	: Time
h	: Hour
$Lm^{-2}h^{-1}$	: Liter per meter square hour
η_{app}	: Apparent viscosity

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PRODUCTION OF A CASEINOMACROPEPTIDE CONCENTRATE FROM SWEET WHEY AND USAGE IN A MILK-LIKE DRINK

SUMMARY

Caseinomacropeptide (CMP) is a valuable polypeptide with structural and health-improving functional properties. CMP is part of κ-casein that is transferred into sweet whey after rennet coagulation of milk in cheese manufacturing. CMP is composed of fractions including glycosylated gCMP and its aglycon aCMP at almost equal proportions in whey. gCMP has a sugar moiety mostly sialic acid bound which was found to be responsible for some of its activities. CMP does not contain any aromatic amino acids such as phelnylalanine and tyrosine which makes it an alternative protein source for phenylketonuria patients. CMP has structural functionality including foaming, gelling and emulsifying. Bioactive properties such as antimicrobial, antioxidant, immune-modulating, satiety providing and prebiotic activities were also reported for CMP. These properties make CMP a special protein source to be utilized as a food ingredient. CMP is present about 1.2-1.5 gL^{-1} in sweet whey that makes up 15-20% of whey proteins. There are various methods to separate CMP from other sweet whey components for its isolation which are combinations of heat treatment, acid treatment, ultrafiltration (UF) and ion-exchange chromatography. In this study, CMP was isolated from sweet whey by a two-stage process where it was separated from other whey proteins by a heat-acid treatment firstly and then further purified by UF. Effects of process parameters in both stages on recovery and purity of CMP were determined to select best conditions. Final CMP concentrate was utilized as an ingredient in development of a rice drink that can be consumed for phenylketonuria patients.

In the first part of this study, separation of CMP from other whey proteins by heat and acid treatments was investigated. Especially, the impact of order of the heat and acid treatments on isolation of CMP from sweet whey and its composition was explored. Acidification of sweet whey to pH values in the range of 3.0-5.0 and heat treatment at 90°C for 1 h in different order were applied to precipitate and separate whey proteins. The aim was to obtain the highest amount of CMP the lowest amounts of other whey proteins in the supernatant. Dry matter, crude protein, non-nitrogen protein, ash, lactose, bound sialic acid, phenylalanine and tyrosine contents of the supernatants from treated whey samples were determined. CMP, α-lactalbumin and β-lactoglobulin in the supernatants were quantified by RP-HPLC. Order of heat and acid treatments and pH had no effect on amounts of lactose and ash in treated samples. The lowest crude protein and dry matter contents in treated samples were found at pH values 4.0, 4.5 and 5.0 regardless of the order of heat and acid treatments. Heat-acid treatment at pH 4.5 and 5.0 were found to yield the highest CMP content and the lowest concentration of α-lactalbumin and β-lactoglobulin, respectively. Concentrations of Phe and Tyr were the lowest at pH values of 4.0, 4.5 and 5.0 in both treatments. Bound sialic acid was recovered at the highest extent in the treated sample that was heated and then acidified to pH 5.0.

In the second part of the study, the pre-treated whey which was heated and then acidified to pH 5.0 that yielded the highest CMP and bound sialic acid contents was further purified by ultrafiltration and then diafiltration. The aim was to reduce water, lactose and ash contents in the retentate without loss of CMP. UF was performed by using a tubular unit with a feed volume of 9 L. Two PES membranes with MWCO of 9 and 25 kDa were used at pressure of 5 and 3 bar, respectively. Preliminary experiments were performed to obtain sufficient flow rate and hence flux for permeate from the UF unit which was crucial to achieve a high volumetric concentration of retentate. pH of pre-treated whey was changed to values in the range of 3-9 with acid and alkali addition for this purpose. As the pH of pre-treated whey was increased flow rate of permeate was increased. The highest relative mean flux was obtained at pH 9 with the membrane with MWCO of 9 kDa and at a pH of 7.0 with the 25 kDa membrane. A volumetric concentration factor of 5 was possible with both membranes. The flow rate of permeate obtained from 25 kDa membrane was higher than that from 9 kDa membrane, the process took shorter time accordingly. While one diafiltration cycle was possible without significant loss of CMP with 25 kDa membrane, four diafiltration cycles could be performed with 9 kDa membrane. Higher recovery and purity of CMP were obtained with 9 kDa membrane compared to that with 25 kDa membrane. Retentates from both UF/DF runs with two membranes were freeze-dried to obtain CMP concentrates. A CMP concentrate with 9.7% CMP, 2.8% α-lactalbumin, 1.5% β-lactoglobulin, 20.2% protein, 61.8% lactose, 18.1% ash, 0.85% bound sialic acid, 0.29% Phe and 0.34% Tyr in dry matter was obtained from pre-treated whey by using 9 kDa membrane. Although CMP content of the concentrate obtained by using 25 kDa membrane was lower (5.8%), it could be preferred because of lower ash content (7.5%) and shorter process time.

In the last part of the study, a rice drink fortified with the CMP concentrate was developed. A base formula containing rice flour (3-8%, w/w) and xanthan gum (0.01-0.05%, w/w) as the structuring components that were heated at different temperatures (80-90^{o}C) for 15 min was developed according to three-factor at three levels CCD by the response surface methodology. All samples were pre-hydrated in water bath 50^{o}C for 20 min, heated, homogenized and cooled. Particle size, zeta potantial, color values, rheological properties (apparent viscosity, flow behaviour index, consistency and thixotropy), sedimentation and phase seperation of the drinks were measured as responses. None of the factors were found to have significant effect on sedimentation. While rice flour and temperature affected all responses significantly, there was no effect of level of xanthan gum on apparent viscosity and particle size. Selected response variables phase separation, particle size, apparent viscosity and zeta potential were targeted to be minimized in optimization of the base-formula and heating temperature. Optimum base formula of rice drink was estimated to contain 3% rice flour and 0.05% xanthan gum that was heated at 80°C. The desirability of the values of estimated response from the optimized model with respect to the targeted values of responses was found 84%.

Rice drink prepared at the optimum conditions was sweetened with 2.5% (w/w) sucrose to improve its acceptability. Descriptive sensory analysis was performed to compare appearance, flavor and texture of sweetened and unsweetened rice drinks with those of low fat milk. The color, mouthfeel, consistency, particle size, graininess and overall acceptability of rice drinks were found to be significantly different than those of low fat milk. Although the highest scores were given to low fat milk, sweetened rice drink was found to be closer to low fat milk compared to unsweetened rice drink.

Isolated CMP concentrate (7%, w/w) was added to sweetened rice drink and then physical properties of the drink was measured. The amount of CMP to be added was calculated by considering the average daily amount of Phe allowed for the diet of children at age of 1-10 years. CMP resulted in reduction of zeta potential, increase in particle size and slight sedimentation in rice drink. It also enhanced apparent viscosity and color values of the drink.

TATLI PEYNİRALTI SUYUNDAN KAZEİNOMAKROPEPTİD KONSANTRESİNİN ÜRETİMİ VE SÜTE BENZER BİR İÇECEKTE KULLANIMI

ÖZET

Kazeinomakropeptid (KMP), yapısal ve sağlığa faydalı fonksiyonel özellikleri olan değerli bir polipeptiddir. KMP κ-kazeinin bir parçası olup peynir yapımında rennet ile koagülasyon sonucunda tatlı peyniraltı suyuna geçer. Glikolize gKMP ve bunun aglikonu aKMP olmak üzere peyniraltı suyunda aynı oranlarda bulunan iki fraksiyondan oluşur. gKMP yapısında bazı fonksiyonel özelliklerinden sorumlu olan bağlı bir şeker grubu sıklıkla siyalik asit bulundurur. KMP peptid zincirinde fenilalanin ve tirozin gibi aromatik amino asitler bulunmaması nedeniyle fenilketonüri hastaları için alternatif bir protein kaynağı sayılmaktadır. KMP jelleşme, köpük ve emülsiyon oluşturma gibi yapısal fonksiyonlara sahiptir. KMP'in antimikrobiyal, antioksidan, bağışıklık-düzenleyici, tokluk sağlayıcı ve prebiyotik aktivite gibi biyoaktif özellikleri bildirilmiştir. Bu özellikleri KMP'nin özel bir protein kaynağı ve gıda bileşeni olarak tercih edilmesine sebep olmaktadır. KMP tatlı peyniraltı suyunda 1,2-1,5 gL^{-1} oranında bulunur ve peyniraltı suyu proteinlerinin % 15-20'sini oluşturur. KMP'nin tatlı PAS'nun diğer bileşenlerinden ayrılarak izolasyonu için farklı yöntemler bulunmaktadır. Bu yöntemler ısıl işlem, asidifikasyon, ultrafiltrasyon ve iyon-değişimi kromatografisidir. Bu çalışmada, KMP öncelikle ısı-asit uygulaması ile diğer peyniraltı suyu proteinlerinin ayrılması ve daha sonra ultrafiltrasyon ile ileri saflaştırma olmak üzere iki aşamalı bir proses ile tatlı peyniraltı suyundan izole edilmiştir. Proses parametrelerinin KMP'nin geri kazanımı ve saflığına olan etkileri en iyi koşulların seçilmesi amacıyla belirlenmiştir. Elde edilen KMP konsantresi fenilketonüri hastaları tarafından tüketilebilecek bir pirinç içeceğinin geliştirilmesinde bir bileşen olarak kullanılmıştır.

Tezin ilk bölümünde, ısı ve asit uygulamaları ile KMP'nin diğer peyniraltı suyu proteinlerinden ayrılması araştırılmıştır. Bu amaçla, PAS yağsız süttozundan %10 kuru madde içerecek şekilde rennet enzimi kullanılarak üretilmiştir. Elde edilen PAS'a 3,0-5,0 aralığındaki pH değerlerine asidifikasyon ve 90°C'de 1 saat ısıl işlem uygulaması d-farklı sırada uygulanarak peyniraltı suyu proteinleri çöktürülmüştür. Burada hedef, KMP'yi en yüksek ve diğer peyniraltı suyu proteinlerini en düşük oranda içeren bir üst faz elde etmektir. İşle görmüş peyniraltı suyundan elde edilen üst fazlarda kül, kuru madde, toplam protein, protein olmayan azot, laktoz, bağlı siyalik asiti fenilalanin ve tirozin içerikleri ölçülmüştür. Üst fazlardaki KMP, α-laktalbumin and β-laktoglobulin miktarları RP-HPLC yöntemi kullanılarak belirlenmiştir. Isı ve asit uygulamalarının farklı sırada uygulanması ve pH'nın örneklerdeki laktoz ve kül miktarlarına etkisi saptanmamıştır. En düşük kuru madde ve protein içeriği pH 4,0, 4,5 ve 5,0'te gözlenmiştir. pH 4,5 ve 5,0'te asit-ısı ve ısı-asit uygulamalarının en yüksek KMP miktarı ve en düşük α-laktalbumin ve β-laktoglobulin miktarı sağladığı bulunmuştur. En düşük fenilalanin ve tirozin içeriği asit-ısı ve ısı-asit uygulamalarında

pH 4,5 ve 5,0'te tespit edilmiştir. İzole edilmiş KMP'deki bağlı siyalik asidin pH 5,0'de ısı-asit uygulaması ile en yüksek oranda geri kazanıldığı gözlenmiştir.

Tezin ikinci kısmında, en yüksek KMP ve bağlı siyalik asit içeriğine sahip ısı ve pH 5'e kadar asit uygulanması ön-işlemlerinden geçmiş peyniraltı suyu ultrafiltrasyon ve daha sonra diafiltrasyon yoluyla saflaştırılmıştır. Burada amaç, retentattaki KMP'i kaybetmeden su, laktoz ve mineral içeriğini azaltmaktır. UF 9 L besleme hacminde borulu bir ünite kullanılarak yapılmıştır. Farklı molar kütle limitine (9 ve 25 kDa) sahip olan iki PES membran sırasıyla 5 ve 3 bar basınçta kullanılmıştır. UF işleminde yüksek hacimsel konsantrasyon faktörüne ulaşabilmek için yeterli düzeyde permeat akış hızı ve dolayısıyla akı elde etmek gerekmektedir. Bu amaçla, ön denemelerde yeterli düzeyde akış hızı sağlanması için ön işlem görmüş peyniraltı suyunun pH değeri 3-9 arasındaki değerlere asit veya baz eklenerek ayarlanmıştır. Ön işlem görmüş peyniraltı suyunun pH değeri arttıkça permeat akış hızı artmıştır. Molar kütle limiti 9 kDa olan membran kullanıldığında en yüksek akı pH 9 değerinde elde edilmiştir. Molar kütle limiti 25 kDa olan membran kullanıldığında ise pH 7'de yeterli düzeyde akı elde edilebilmiştir. Her iki membranla da 5 hacimsel konsantrasyon faktörüne ulaşılabilmiştir. Akı 25 kDa-membran ile 9 kDa membrana göre daha fazla olduğundan proses te daha kısa sürmüştür. Molar kütle limiti 25 kDa olan membranla bir diafiltrasyon döngüsü yapılabilirken diğer membranla dört diafiltrasyon döngüsü yapılabilmiştir. KMP'in geri kazanımı ve saflığının 9 kDa membran kullanıldığında daha yüksek olduğu bulunmuştur. Elde edilen retentatlar dondurarak kurutularak CMP konsantratları elde edilmiştir. Ön-işlem görmüş peyniraltı suyu 9 kDa membran kullanıldığında kuru maddede %9,7 KMP, %2,8 α-laktalbumin, % 1,5 β-laktoglobulin, %20,2 protein, %61,8 laktoz, %18,1 kül, % 0,85 bağlı siyalik asit, % 0,29 fenilalanin and % 0,34 tirozin içeren bir KMP konsantresi elde edilmiştir. Molar kütle limiti 25 kDa olan membran kullanıldığında elde edilen KMP konsantresinin KMP içeriği daha düşük (%5,8) olmasına rağmen daha düşük kül içeriğine (%7,5) sahip olması ve daha kısa proses süresinden dolayı tercih edilebilir.

Tezin üçüncü bölümünde, KMP ile fortifiye edilmiş pirinç içeceği geliştirilmiştir. Yapı oluşturan bileşenler olarak pirinç unu (%3-8, w/w) ve ksantan zamkı (% 0,01-0,05, w/w) içeren ve farklı sıcaklıklarda (80-90^{o}C) 15 dk ısıl işleme tabi tutulan bir içecek üretiminin geliştirilmesi için yanıt yüzey metodolojisine göre üç seviyede üç faktör içeren Merkezi Kompozit Deney Tasarımı kullanılmıştır. Formüle edilen pirinç içeceğinin partikül büyüklüğü, zeta potansiyeli, çökme oranı, renk (L, a ve b) değerleri, reolojik özellikleri ve faz ayrımı yanıtlar olarak ölçülmüştür. Ksantan zamkının partikül büyüklüğü ve görünür viskozite üzerinde etkili olmadığı saptanmıştır. Her faktör için istatistiksel olarak bağımsız etkenlerin bu faktörler üzerindeki etkisi incelmiştir. Sıcaklık ve pirinç unu oranı zeta potansiyel, partikül büyüklüğü, faz ayrımı, görünür viskozite, tiksotropi ve renk üzerinde etkili faktörler olarak bulunmuştur. Çökme oranı üzerinde incelen faktörlerin önemli düzeyde bir etkisi bulunmamıştır.

Formülasyon ve ısıl işlem sıcaklığının optimizasyonunda, minimum partikül büyüklüğü, zeta potansiyeli, faz ayrımı ve görünür viskozite hedef olarak seçilmiştir. Elde edilen yanıt-faktörler ilişkisini gösteren modellere göre, optimum formülasyonun %3 pirinç unu ve %0,05 ksantan zamkı içerdiği ve ısıl işlem sıcaklığının 80°C olduğu tahmin edilmiştir. Optimizasyondan tahmin edilen faktörler ile elde edilebilecek yanıtların hedef değerlerine göre kabul edilebilirliği %84 olarak belirlenmiştir. Optimizasyonun validasyonu için optimum üretim koşullarında içecek hazırlanmış ve

yanıtlar ölçülmüştür. Görünür viskozite haricinde tüm yanıtlar tahminlenen değerlere benzer çıkmıştır.

Optimum koşullarda hazırlanmış pirinç içeceğine kabul edilebilirliğinin artırılması amacıyla % 2,5(w/w) oranında şeker eklenmiştir. Tanımlayıcı duyusal analiz yapılarak şekerli ve şekersiz pirinç içeceğinin renk, tat ve yapı özellikleri az yağlı sütün özellikleri ile karşılaştırılmıştır. Partikül boyutu, kumsuluk, kıvam, renk, ağız hissi ve genel kabul edilebilirlik değerlendirmelerinde her iki pirinç sütü de az yağlı süte oranla panelistlerden daha yüksek puan almışlardır. İçeceklerin rengi az yağlı süte göre daha kremsi olarak belirtirmiştir. Tatlılık açısından, şekersiz pirinç içeceği ve az yağlı süt aynı puan almışken şekerli içecek daha yüksek puan almıştır. Yağlılık ve acımsı tatta örnekler arasında istatistiksel olarak farklılık bulunmamıştır. Genel kabul edilebilirlik açısından, şekerli pirinç sütünün puanının şekersizden daha yüksek ancak az yağlı sütten daha düşük olduğu görülmüştür.

Şeker eklenmiş pirinç sütü formülasyonuna %7 oranında KMP konsantresi eklenere pirinç içeceği üretilmiş ve fiziksel özellikleri ölçülmüştür. Eklenen KMP miktarının belirlenmesinde içeceğin toplam fenilalanin içeriğinin, fenilketonüri hastası 10 yaştan daha küçük bir çocuğun diyetinde kullanılabilecek maksimum düzeyden az olması gözönünde bulundurulmuştur. KMP içeceğin yüzey yükünde azalma, partikül boyutunda artma ve düşük düzeyde çökmeye sebep olmuştur. Aynı zamanda içeceğin görünür viskozitesinde ve renk değerlerinde artışa sebep olmuştur.

1. INTRODUCTION

Dairy food ingredients are widely used in different food products to improve flavor, color, structure, nutritive value. Beside this, functional and special dietary foods that are needed for consumers on a certain diet can be developed by using dairy ingredients with structural and bioactive functional properties. Sweet whey is by-product of cheese making process which has been utilized to produce such ingredients including protein concentrates and isolates. Utilization of sweet whey in this way serves to both reduction in environmental pollution and its valorization via producing functional food ingredients. One protein fraction of whey with functional benefits is caseinomacropeptide (CMP) which is released by rennet coagulation of κ-casein. CMP is composed of glycosylated (gCMP) and non-glycosylated fractions (aCMP). gCMP has bound heterogeneous carbohydrate chains, which are mostly sialic acid. Some bioactivity of CMP was reported to be associated with gCMP fraction (Neelima et al., 2013). CMP lacks aromatic amino acids (Phe, Trp, and Tyr) that makes it unique for the diet of phenylketonuria patients. CMP is present at a concentration of 1.2-1.5 gL^{-1} in sweet whey which constitutes 15-20% of total proteins. Isolation of CMP involves separation of other whey components especially major whey proteins. Isolation is based mostly on two methods including membrane filtration and ion exchange chromatography. Pre-treatment of sweet whey with acidification and heat treatment prior to these methods is preferable (more suitable, advisable) to increase recovery and purity of CMP isolate. are applied to increase the purity and recovery of the product. The composition and level of gCMP with bound sialic acid of isolated CMP varies widely based on whey source and isolation technology applied (Neelima et al, 2013; Martín-Diana et al, 2002).

The aim of this study was to develop an isolation method for CMP from sweet whey by ultrafiltration based on the methods reported in the literature (Ergül and Karkaya, 2013; Holst and Chatterton, 2002; Martín-Diana et al. 2002; Kawasaki et al, 1993b, 1994; Lieske et al, 2004a). The methods involved pre-treatment of sweet whey by acidification and heat treatment to separate CMP from other whey proteins and then

ultrafiltration/diafiltration to further purify CMP. Effects of process parameters on composition and bound sialic acid of CMP were investigated. CMP concentrate was obtained after freeze-drying of retentate from UF/DF. Final concentrate powder was utilized in development of a rice drink fortified with CMP suitable for diet of phenylketonuria patients.

1.1 Purpose and Scope of the Thesis

The purpose of this study is produce and a CMP concentrate that could be used as a food ingredient from sweet whey by means of a two-stage process applying heat-acid treatment and ultrafitration/diafiltration. Effects of process parameters in both stages on composition and bound sialic acid content of CMP concentrate were analyzed to increase recovery, purity and gCMP content. A rice drink was developed according to response surface methodology varying levels of two structuring components, rice flour and xanthan gum, and heating temperature. Physical properties of rice drink were measured as responses. Optimum amounts of rice flour and xanthan gum and heating temperature were determined according to the desired levels of responses. Optimized rice drink was fortified with the CMP concentrate to prepare a drink consumable by phenylketonuria patients and consumers with allergy to milk proteins.

Following studies were carried out in the thesis:

1. Sweet whey was treated by heat treatment and acidification for separation of CMP from other whey proteins

2. CMP was isolated by ultrafiltration/diafiltration from pre-treated whey and then final retentate was freeze-dried to obtain a concentrate

3. A rice drink was formulated with CMP concentrate for development of a drink for phenylketonuria patients.

1.2 Hypothesis

A CMP concentrate can be produced from sweet whey which is a waste from cheese making by a low cost, high yield and environmentally friendly process. This CMP concentrate could be used for development of food products for phenylketonuria patients and others with allergy to milk proteins.

2. LITERATURE REVIEW

2.1 Caseinomacropeptide

Caseinomacropeptide (CMP) is obtained from sweet whey which is a by-product of cheese manufacture (Manso and Lopez-Fandino, 2004). Whey causes great polluting effect on water and environment because of its high biological oxygen demand (BOD) (Singh et al, 2004). On the other hand, whey is a dilute solution composed of low molecular weight solutes such as lactose, salts and other minor components and variety of proteins with functional benefits such as emulsification, gelation and foaming properties (Fernández, et al, 2011).

There are two types of whey, acid and sweet, depending on the process it is obtained from. Acid whey is the solution remaining after acid coagulation of milk. Sweet whey is the solution left over after renneting coagulation of milk in cheese making. Both wheys contain about 6% dry matter (Table 2.1). Whey proteins are about 0.7% (w/v) of the mass of whey, include β-lactoglobulin (β-lg), α-lactalbumin (α-la) and other minor proteins, bovine serum albumin (BSA), immunoglobulins (Ig), lactoferrin (LF), lactoperoxidase (LP), enzymes, proteose-peptones and CMP (only found in sweet whey) (Jovanović et al, 2005). CMP makes up 15-25% of total whey proteins (Farías et al, 2010).

Table 2.1 : Composition of acid and sweet bovine whey.

Components (%)	Acid whey	Sweet whey
pH	4.6-5.0	6.2-6.7
Total dry matter	5.0-6.0	6.0-6.5
Lactose	5.8-4.3	4.5-5.0
Ash	0.5-0.7	0.5-0.7
Whey proteins	0.8-1.0	0.8-1.0
β–Lactoglobulin	50	50
α–Lactalbumin	20	20
Caseinomacropeptide	-	15
Proteose-Peptones	20	5
Immunoglobulins	8	8
Serum albumin	6	6

CMP is part of κ-casein with three phosphorylation and several glycosylation sites that is released only when rennet cleaves κ-casein at the peptide bond between Phe105-Met106. The insoluble fragment of κ-casein, para-κ-casein (fragment 1–105) remains in the curd, while the soluble fragment caseinomacropeptide (CMP, fragment 106–169) is released to the whey. CMP concentration was found to be 2 $mgmL^{-1}$ in colostrum and 1.2 $mgmL^{-1}$ in milk (Korhonen, 2013). It is present at a concentration of 1.2-1.5 $mgmL^{-1}$ in sweet whey (Thomä et al, 2006; Thomä-Worringeret al, 2006; Rojas and Torres, 2013). The hydrodynamic diameter of CMP was reported around 6-7 nm (De Kruif, 1999). Two genetic variants A and B of CMP from a total of 11 genetic variants of κ-casein was detected in sweet whey (Thoma et al, 2006). CMP is a disordered peptide (random coil) without a secondary or tertiary structure with high conformational flexibility (Neelima et al, 2013). It is rich in the amino acids Threonine (Thr), proline (Pro), glutamine (Glu), serine (Ser) and contains no aromatic amino acids phenylalanine (Phe), tryptophan (Try) and tyrosine (Tyr). It also contains low amounts of glycine (Gly), histidine (His) and arginine (Arg), only one methionine (Met) residue (Etzel, 2004; Thomä-Worringer et al, 2006). Amino acid composition of CMP is given in Table 2.2.

Table 2.2 : Amino acid content of caseinomacropeptide.

Amino acid	Theoretical CMP* Martín-Diana et al, (2002)	Martín-Diana et al, (2002)	Etzel (2004)
Asp	8.1	12.4	5.1
Thr	16.1	15.7	16.7
Ser	9.8	5.3	7.8
Glu	19.8	20.2	19.3
Gly	1.2	1.6	0.9
Ala/ Val	14.8	11.6	15.3
Met	0	0.6	2.0
Ile/ Leu	11.8	10.8	1.7**
Tyr	0	0	0
Phe	0	2.7	0
Lys	6.4	7.6	5.8
His	0	1.1	0
Arg	0	1.3	0
Pro	11.1	8.2	11.7
Cys	0	0	0

*Calculated based on 20% of CMP content in whey protein.
** Only Leucine.

CMP has two fractions including glycosylated fraction called glycomacropeptide (gCMP) which is about 50% of total CMP, and non-glycosylated fraction (aCMP) (Fig. 1-2). Five different mucin-type carbohydrate chains composed of N-acetylneuraminyl (NeuAc), galactosyl (Gal) and N-acetylgalactosamine (Gal-NAc) in gCMP structure differentiate it from aCMP (Mollé and Léonil, 1995). gCMP is rich in N-acetylneuraminic (NANA) acid or sialic acid (Fig. 2.3). N-acetylglucosamine (GlcNAc) and fucose (Fuc) have been identified as part of six other saccharides in cow's colostrum (Saito et al, 1991; Fiat and Jolles, 1989). The carbohydrate content and composition of CMP varies according to composition of whey source and isolation method (Neelima et al, 2013; Martín-Diana et al, 2002). Post-translational modifications result in a heterogeneous CMP, 14 glycosylated forms of variant A and 10 components of variant B differing in the NANA and/or phosphorus content have been identified (Vreeman et al, 1986; Mollé and Léonil, 2005, 1995).

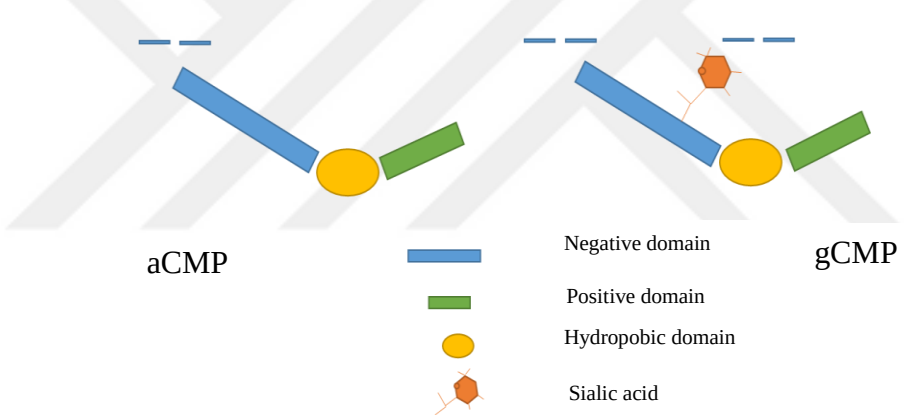


Figure 2.1 : Molecular structure of non-glyco CMP (aCMP) and glycol CMP (gCMP) (Martinez et al., 2013).

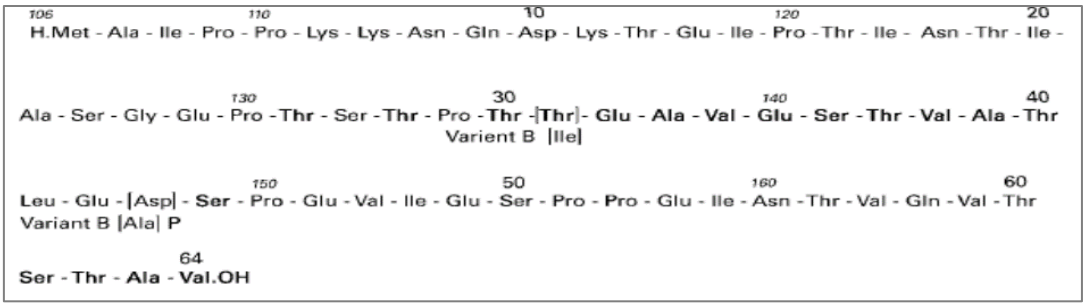


Figure 2.2 : Primary structure of CMP variants A and B (Eigel et al., 1984).

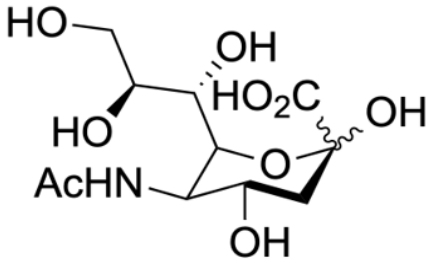


Figure 2.3 : Molecular structure of sialic acid (N-acetylneuraminic acid, Neu5Ac) (Varki and Schauer, 2009).

2.2 Biological, Nutritional and Structural Functional Properties of CMP

CMP has different structural functional properties such as gelation, emulsification and foaming (Kreuß et al, 2009a; Kreuß et al, 2009b) and also biological activity (Sharma et al, 2013). Several biological activities has been reported for CMP such as prebiotic activity (Janer et al, 2004; Hernandez-Hernandez et al, 2011), inhibition of *Cholera* toxin (Kawasaki et al, 1992), prevention of intestinal infection (Nakajima et al, 2005). Glyco and peptide parts were found responsible for its properties. Each of the gCMP fractions with different carbohydrate chains might be responsible for different biological functions (Li and Mine, 2004). Other properties such as reduction of gastric secretion, bifido-factor and anti-cariogenic effect also were reported (Azuma et al, 1984; Neeser et al, 1994; Aimutis, 2004). Nutritional properties as a food ingredients for PKU and hepatic patients have been linked to peptide part (LaClair et al, 2009; Lim et al, 2007). Modulation of immune response and stimulation of cholecystokinin release (Beucher et al, 1994; Burton-Freeman, 2008) were found to relate to both peptide as well as glycan part (Requena et al, 2010).

2.2.1 Biological and nutritional properties

Bioactive peptides have been defined as specific protein fractions that have a positive impact on body functions or conditions and may influence body health (Sánchez and Vázquez, 2017). CMP is also a bioactive peptide with many reported health-improving effects. Many studies have attempted to establish the potential role of CMP in regulation of intestinal functions (Aleinik et al, 1984; Azuma et al, 1984; Beucher et al, 1994; Korhonen and Pihlanto, 2003; Neeser et al, 1994; Sawin, et al, 2015). It was reported that content and structure of its carbohydrate residues, mainly sialic acid plays an important role for recognition of bacterial cell wall and viruses. High content of sialic acid was also reported to support learning ability and brain development

(Fernando and Woonton, 2010; Wang et al, 2001). Thermal process at weak acidic pH was reported to reduce glycosidic content of CMP but some residual free oligosaccharides might still retain the ability to bind pathogenic organisms or toxins based (Abe et al, 1991; Siegert et al, 2010).

Different biological activities such as regulation of the action of the digestive tract, anticariogenic, antithrombotic or antihypertensive activity were reported for CMP (Brody, 2000; Li and Mine, 2004). Some other reported bological properties of CMP are listed below:

Binding of *Cholera* toxin and *Escherichia coli* enterotoxins (Kawasaki et al, 1992; Oh et al, 2000) and protection of cells from infection by influenza virus (Kawasaki et al, 1993a). A product with a brand named biopure CMP produced from WPI was claimed as functional bioactive peptide due to prevention of dental caries, influence on the clotting of blood and protection against viruses and bacteria (Korhonen and Pihlanto, 2003).

Promotion of bifidobacterial growth which play role in modulating the gut microflora (Minkiewicz et al, 1996; Patel et al, 201). Robitaille (2013) claimed that CMP was an efficient additive for probiotic (*Lactobacillus rhamnosus* RW-9595-M and *Bifidobacterium thermophilum* RBL67) growth and the glyco fraction was not necessarily needed to show this ability.

Inhibiton of the adhesion of cariogenic bacteria such as *Streptococcus mutans*, *S. sanguis* and *S. sobrinus* to the oral cavity (Neeser, 1991; Aimutis, 2004). It can be used as a dental anti-plaque and anti-caries agent as shown in a patent no 4.99.420 by Neeser (1991). Also CMP in a combination with xylitol were used to prevent caries and also remineralize teeth in a patent WO1998052524A1 by Zhang and Gaffar (2001).

Reduction of gastric acid and digestive secretions (Yvon et al, 1994; Brody, 2000). *In vivo* experiments (dogs and rats) showed that CMP reduced acid and motions at the gastric fundus (Vasilevskaya et al, 1977; Stan and Chernikov, 1982). On the other hand, Guilloteau et al. (1994) found that intravenous injection of gCMP caused no inhibition of gastric secretions or changes in digestive hormone levels in blood plasma of preruminant calves. In an experiment on human (male and female), 0.4% and 2.0% CMP solution were served and no effect of CMP on satiety or motivation to eat were reported (Gustafon et al, 2001). The influence of CMP on weight gain was analyzed

in rats treated with whey protein isolate (control), whey protein isolate +10% CMP, whey protein isolate + 20% CMP and casein by Mcintosh et al. (2005). They found a significant reduction in weight gain of rats treated with CMP compared to those of the control (Mcintosh et al, 2005). In another study with overweight women, no different effect of CMP on energy intake was reported by Poppitt et al. (2013). Despite these contradictory reports, th commercial products containing CMP were sold in the market for the purpose of appetite control and weight management (Korhonen, 2013). CMP was also recognized for enhancing the absorption of minerals such as calcium, iron and zinc due to its large negative charge (Kelleher et al, 2003).

CMP also exerts immunomodulating and lymphocyte proliferation activities. It can even induce apoptosis of certain lymphocytes including tumor lymphocytes (Matin and Otani, 2000; Monnai and Otani, 1997). CMP also displayed anti-inflammatory activity in rats with hapten-induced colitis, probably through the inhibition of the activation of immune cells (Daddaoua et al, 2005). In a recent study, CMP and its papain (EC 3.4.22.21, with activity of 2 Umg^{-1}) hydrolysate were found to have antioxidant activity (Cheng et al, 2015). CMP hydrolysate had higher free radical-scavenging capacity, ferrous ion (Fe^{2+})-chelating ability, and ferric reducing (FRAP) activity when compared to native CMP at the concentration of 2 $mgmL^{-1}$ (Cheng et al, 2015).

CMP is rich in essential amino acids but it is the only known natural peptide that lacks aromatic amino acids Tyr, Trp and Phe. Therefore, it can provide an alternative protein source for phenylketonuria (PKU) patients. The main target of industry is to manufacture CMP with sufficient purity to ensure that CMP is free from Phe. CMP was evaluated for safety, acceptability and efficacy for the nutritional management of PKU by Lim et al. (2007). A variety of foods and beverages can be made with CMP to improve the taste, variety and convenience of the PKU diet. Sensory studies made with individuals with PKU demonstrated that CMP foods were acceptable alternatives to amino acid medical foods (Ney and Etzel, 2017; Ergül and Karakaya, 2013).

2.2.2 Structural functional properties

CMP is a surface-active peptide, it can be used as food ingredient due to its structural functionality (Martinez et al, 2013). pH was found to be important in controlling aggregation of CMP and its functional properties. CMP was found to form aggregates

depending on pH (<4.5) at room temperature and this structure was partially pH-reversible (Kreuß et al, 2009b; Farías et al, 2010). In addition, hydrophobically formed dimers at pH 3.5 were found to be resistant to pH changes once formed (Farías et al, 2010; Martinez et al, 2012). No helix or sheet structure is present in structure of CMP. CMP has a high negative charge with a high conformational flexibility (Uversky, 2002). There are differences in functionality between non-glyco and glyco fractions of CMP. aCMP was found to have stronger emulsifying and foaming activity and this was explained by decrease in surface activity and hydrophobicity by glycosylation (Kreuß et al, 2009a; Siegert et al, 2010). The strong negative charge as well as steric and hydrophilic repulsion of the glycan residues results in a decrease of adsorption of glyco fraction of CMP at the interface which limit the formation of a stable surface layer or peptide network (Kreuß et al, 2009b).

2.2.2.1 Emulsifying activity

Emulsifying activity of CMP was shown by Chobert et al. (1989). Bovine CMP exhibited maximum emulsifying activity at alkaline pH and a minimum in the pH range of 4.5-5.5, but emulsions lost stability after 24 h of storage. According to Chobert et al. (1989) the solubility of CMP was not dependent on pH in the range of 1-10 but the emulsifying activity of it was effected by pH. Emulsions of CMP were found to be more stable to pH changes compared to that of WPC (Martin-Diana et al, 2005). Emulsifying activity of CMP is affected by the hydrophilic nature of CMP which reduces the emulsifying activity as compared to that of native amphipathic κ-casein, and also by glycan moiety (Kreuß et al, 2009b). Kreuß et al. (2009b) reported that aCMP had a higher emulsifying activity index (150.7 m^2g^{-1}) compared to that of gCMP (98.5 m^2g^{-1}). In addition, the stability of emulsions of aCMP was 1.4 times higher than that of gCMP. This was explained by hydrophilic and electrostatic effects created by gCMP which prevents efficient adsorption of gCMP molecules at the oil/water interface (Kreuß et al, 2009a). pH influenced both formation and stability of the emulsions formed by CMP. Above the pI, negatively charged peptide adsorbs on oil-water interface creating repulsion between individual oil droplets. At the pI, the oil droplets coalesce due to lack of repulsion. Below the pI, there is repulsive positive charge which stabilizes the oil droplets (Kreuß et al, 2009a).

Modification of CMP with other molecules can change its functionality. Emulsification activity of CMP can be modified by conjugating it with other

molecules. The conjugation of CMP with lactose by Maillard reaction increased its emulsifying activity while the solubility of it was not changed significantly (Moreno et al, 2001). The conjugation of CMP with medium and long chain fatty acid (effects on foaming and emulsifying activities was investigeted by Wong et al. (2006). As the regardless of chain length, solubility of CMP was reduced. Long chain fatty acids also reduced foam formation and stability but its emulsifying activity was enhanced (Wong et al, 2006).

2.2.2.2 Gelation

CMP can form gel depending on pH, temperature and time. First stage of gel formation is formation of dimer by hydrophobic interactions, then polymers are formed at the second stage by electrostatic interactions (Martinez et al, 2009). CMP concentration required for gel formation mainly depends on applied pH (Farías et al, 2010). CMP can form a gel even at room temperature by pH-dependent self-assembly (Kreuβ et al, 2008; Kreuβ et al, 2009; Farías et al, 2010). It has been reported that self-assembled aggregates formed at room temperature under pH 4.5. CMP aggegates were relatively pH-reversible, but hydrophobically formed dimers are resistant to pH changes (Farías et al, 2010; Martinez et al, 2012). At pH higher than 4 polymer forms of CMP with MW of 45-50 kDa was reported (Kawasaki et al, 1993b).

CMP is a heterogeneous glyco and phospho-peptide (Moreno et al, 2001). pI of CMP depends on its amino acid chains, glycosylation degree and phosphate content (Neelima et al, 2013). It was reported to be 3.15 for aCMP and 4.15 for gCMP (Kreuß et al, 2009b). Burton and Skudder (1987) found that CMP formed a gel at a minimum concentration of 9.3% CMP at pH 4.5 or below at 20°C after 18-24 h, but not at 90°C (Burton and Skudder, 1987). In another study, it was shown that CMP solution at a concentration of 12.5% gelled at 80°C at pH 7.0 (Ahmed and Ramaswamy, 2003). However, Wang (2007) was studied gel forming of CMP and indicated that for gel forming low pH and high temperature were needed where hydrophobic interaction was strong. Martín-Diana et al. (2004) made fermented milk by using goat's milk supplemented with CMP, they reported that the addition of CMP increased elasticity and a more orderly and structured gel was formed when compared with gels made with whey protein concentrate.

CMP can form dimers and tetramers depending on pH at and above room temperature (Farías et al, 2010). Self-assembly and gelation of CMP is mostly effected by pH but temperature in combination with pH can affect gel forming (Martinez et al, 2011). Farías et al. (2010) studied CMP gel formation at 25°C with 3-10% protein concentration. They found that a gel can be formed at a concentration of 3.5% at pH 3-3.5 and at pH 4 a gel was formed at a concentration in the range of 3-10% . Martinez et al. (2012, 2013) studied CMP gel forming capacity with 12% CMP concentration, at temparatures 20 and 70°C. At room temperature, gel forming time was reduced in response to decreasing pH from 3.5 to 2 when compared to 70°C. The most firm gels were formed at 70°C at pH 3 and 3.5.

Assembly of CMP and β-lg in aqueous solutions showed a strong impact on gelation (Martinez et al, 2010). The influence of CMP on the gel forming by sodium caseinate (NaCas) at different ratios was also studied. The presence of CMP promotes interactions between aggregates of NaCas so made a firmer protein network (Morales et al, 2015). Loria et al. (2018) analysed the effect of pH (5.0–9.0) and temperature (5–60°C) on flow properties of CMP aqueous solutions (1-15%). Newtonian flow behavior particularly at pH values 5.0–6.0 and non-Newtonian shear thinning behavior at pH 7.0–9.0 were observed that was explained by the electrostatic repulsion and the peptide-water interaction. When CMP and gelatine were mixed, lower concentration of both substances were needed to form gel (Morales et al, 2016). This synergistic effect on gelation can enhance use of CMP in food industry for the preparation of foods with gel structure (Morales et al, 2016; Morales et al, 2019).

2.2.2.3 Foaming

CMP has been shown to have good foaming capacity but low foam stability in various studies (Marshall, 1991; Martinez et al, 2013). The foam made with 10% CMP in different food products was found to have a higher overrun but lower foam stability compared with that with egg white in the same foodstuffs. The CMP foam in food systems like meringues was not stable and resulted in a low meringue density upon cooling (Marshall, 1991).

Martinez et al. (2013) studied the interfacial and foaming properties of CMP with gelatin mixed systems at pH 6.5 and 3.5. A synergistic effect between CMP and gelatin was observed on foam forming ability and stability of system. This was explained by

high surface activity provided by CMP and stability provided by gelatin. Martinez et al. (2012) studied the foaming properties of CMP and β-lg mixed systems at pH 6.5 and 3.5. they observed that there was a synergistic effect between CMP and β-lg in mixed foams at pH 3.5 due to interactions between these biopolymers in the solution.

Kreuß et al. (2009a) found that the foam rigidity and stability with aCMP was the higher than to those with gCMP and WPI. They explained this result by higher overrun obtained with a smaller flexible proteins compared to larger and globular proteins. Foam with aCMP was the most stable and lower pH effect on aCMP was reported, in contrast, gCMP only formed a stable foam at above pI (Kreuß et al, 2009a). Martinez et al. (2012) reported that the foam stability of with CMP (4%) against the drainage and collapse strongly increased by incorporation of propylene glycol alginate (PGA) at a concentration of 0.50%.

2.3 Isolation and Purification Methods of CMP

CMP has been isolated from sweet whey by heat treatment, ethanol precipitation, dialysis and trichloracetic acid (TCA) precipitation for research purposes (Ceppa et al, 2007; Li et al, 2010; Lieske and Kornad, 1996; Saito et al, 1991; Shammet et al, 1992). Li and Mine (2004) compared three isolation methods of CMP including separation with TCA and precipitation by ethanol from whey protein isolate (WPI). The authors found that pre-treatment with TCA recovered only gCMP and eliminated all other proteins with a low recovery of CMP (6.7%). Ethanol precipitation had better recovery results with 20.4% of CMP, of which 75.7% was the glycosylated form (Li and Mine 2004).

For industrial production of CMP, various ultrafiltration processes with heat treatment or microfiltration have been developed (Bouhallab et al, 1992; Tanimoto et al, 1991; Kawakami et al, 1992, Kawasaki et al, 1993b; Lieske et al, 2004a; Holst and Chatterton, 2002; Martín-Diana et al, 2002). Purity of CMP can be further increased by selective adsorption by chromatographic techniques including gel permeation, ion-exchange and hydrophobic interaction chromatography (Ayers et al, 2003; Kaeasaki et al, 1993; Abd El-salam, 2006; Ceppa et al, 2007; Li et al, 2010).

In all isolation process, level of Phe was used to evaluate purity of CMP isolates. Other factors that should be considered are cost, industrial simplicity and suitability of

selected process for large scale. Commercially available CMP isolates contain approximately 0.4% Phe (Bio-Pure GMP^{TM}, Davisco Food Inc., Minnesota, US), 1.1 and 0.2% in protein (Lacprodan® CGMP-10 and Lacprodan® CGMP-20, Arla Food Amba., Visby, Denmark).

2.3.1 Methods based on membrane filtration

Early studies for isolation of CMP describe UF technique in a patent EP 0393850 A2 by Tanimoto et al. (1990) and combination of UF with cation exchange chromatography was described in the patent US 527828 8A by Kawasaki and Dosako (1994). Kawazaki et al. (1993) isolated CMP by ultrafiltration of whey at a pH less than 4 with a membrane with a MWCO of 10-50 kDa followed by concentration of the permeate with a UF membrane with the same MWCO membrane at pH 7.0. Kawazaki et al. (1993) obtained a purified product with phenylalanine content of 0.6% (w/w) and 81% purity by this method. The authors claimed that the dissociated form of CMP permeates through the UF membrane with a MWCO of 20–50 kDa at pH 3.5, but the majority of other whey proteins remained in the retentate. They suggested that after pH adjustment to 7.0, the permeate containing CMP can be concentrated by means of the same UF membrane. The authors concluded that this technique allowed a good separation of CMP from other whey proteins, but due to the low permeation rate of CMP at least two diafiltration steps were necessary to obtain fractions with a high degree of purity and yield.

Arla Foods with patent no US 6462181 B1 by Holst and Chatterton (2002) modified method of Tanimato et al. (1990). They used cold membrane filtration at 15ºC instead of 50ºC and starting material had higher protein content 7.5% instead of 2% and MWCO of UF membrane was different (20 and 5 kDa). The final retentate had 79.5% protein content with 90% of CMP.

Martín-Diana et al. (2002) used a combination of heat treatment and UF for isolation of CMP from sweet whey and WPC (2%, w/v). They heated the whey at 90°C for 1 hour for complete denaturation and aggregation of whey proteins at pH 3.5. The denatured proteins were removed by centrifugation at 5200xg at 4°C for 15 min and the supernatant containing CMP was concentrated by UF with a membrane with MWCO of 10 kDa after pH adjustment to 7. They reported that CMP at pH 7 has a molar massof 33 kDa. The authors recovered CMP from sweet whey from bovine,

ovine and caprine milk with 69%, 76% and 71%, respectively. Recovery from WPC was reported as 73%. Purity of CMP isolate obtained from whey was found to be higher ranging from 85% to 90% than 75.4% obtained from WPC (Martín-Diana et al, 2002).

Performance of UF process depends on composition of feed, properties of the components and membrane. Naue et al. (1995) reported that the charges of both molecules and membrane affect transmission of particles. Large sized proteins such as β-lg and α-la were reported to have less influence than small proteins on membrane fouling during UF. Large sized proteins were absent in the fouling layer after long term UF of Cheddar cheese whey (Wen-qiong et al, 2017).

Javanmard et al. (2012) used two step UF process based on the method of Kawasaki et al. (1993b) with 50-10 kDa UF membranes to seperate CMP from whey protein concentrate (10% protein, w/w). The purity of CMP according to Phe content at pH 4 was 372 ppm which was higher than those obtained at pH 3.5 and 4.5. The recovery was found to be higher at pH 4.5 than those at other pH and they claimed that this was due to presence of polymeric form of CMP with a molar mass of 45 kDa compared to monomeric form of with a molar mass of 10 kDa at other pH.

Ergül and Karakaya (2013) modified the method of Martín-Diana et al. (2002) by changing pH of sweet whey from 7 to 5.2 at the heat treatment step for separation of CMP from other whey proteins. The isolate of CMP had a purity of 57.56% in powder with a recovery of 71.2% . They reported 330 mg Phe per 100 g and 83.8 mg Tyr per 100 g in the isolate. Diafiltration improves separation efficiency and the purity of product from UF process where the retentate is diluted with water and re-ultrafiltrated. (Chu, 1996, Naue et al, 1995).

2.3.2 Methods based on combined UF and IEC

Ion exchange is used in large scale production in patents U.S. No. 5.290.107 (Kawakami et al., 1992) and U.S. No. 5.278.288A (Kawasaki and Dosako, 1994). However, the recovery of CMP using these methods is uneconomically low, at most 18%. Combination of two ion exchange columns was applied in the patent US5968586A by Etzel (1999). Nakano and Ozimek (1999) reported that a preparation of CMP isolated from sweet whey by anion exchange chromatography contained traces of Phe, Tyr, Arg and His. Ozimek (2000) found that the optimum pH value ranged

from 2.5-4.0 for CMP purification by an anion-exchange chromatography with DEAE-Sephacel. Ayers et al. (2003) studied the effect of pH on the adsorption of CMP from whey protein concentrate (WPC) on to an anion exchanger and found the optimum pH range of 4–5 for purification of CMP containing with less than 0.5% (w/w) Phe. This Phe concentration was stated to be lower than detectable level of Phe according to the standard ISO 13903: 2005 (Animal feeding stuffs-Determination of amino acids content) (Christensen and Holst, 2018). A method involving heat treatment of sweet whey followed by ethanol precipitation and anion-exchange chromatography was applied by Saito et al. (1991). Davisco got the patent WO2002/080961, by using bovine deproteinized whey to produce CMP by micrifiltraion and heating with a purity of at least 91% determined by SDS electrophoresis (Davis et al, 2002).

Smithers et al. (1991) developed a method for isolation of CMP from cheese or rennet casein whey by selective adsorption at pH 5 by using an anion exchange chromatography. Unbound proteins were washed out of the exchanger using water and the CMP eluted with a mild acid. The eluate was microfiltered and then concentrated by ultrafiltration and dried to obtain a CMP powder with greater than 90% purity. Xu et al. (2000) used Amberlite IRA-93 (a weak anion exchange resin) that selectively adsorbs CMP from cheese whey at pH 4.7. According to the researchers, this isolation method yielded three fractions, CMP, Ig and WPI.

Kreuß and Kulozik (2009) studied separation of a gCMP fraction of aCMP at pilot plant scale by a direct capture anion exchange membrane adsorption process. Separations were performed using two different starting material; CMP isolate solutions and skim milk whey. Strong membrane fouling was observed at a pH of 4.1 which is near to pI of CMP. A pH of 6.5 was preferred at which CMP has negative charge and therefore protein-membrane repulsion occurred which avoids the fouling process. Fresh skim milk whey yielded a lower purity 91% when compared to the CMP isolate with 97% purity in gCMP fraction and this is because of difference in binding capacity of gCMP.

Li et al. (2010) modified chitosan by crosslinking with β-cyclodextrin for isolation of gCMP. It was shown that, at pH near 3, the β-cyclodextrin modified chitosan bead presented higher selectivity (90.23%) and better adsorption capacity than native chitosan beads for the separation of gCMP from the sweet whey.

2.3.3 Methods based on combined UF and enzyme

To improve isolation process of CMP, polymerization of CMP with transglutaminase was investigated by Tolkach and Kulozik (2005). They used a whey protein concentrate with 6% total protein content as the feed. CMP was cross-linked by the transglutaminase enzyme with dosage of 3 Ug^{-1} protein. Aggregates of CMP formed by the enzyme was separated from other proteins by microfiltration with pore size of 0.1 μm and diafiltrated using a membrane with a MWCO of 25 kDa (Tolkach and Kulozik, 2005). In another study by Bo-zhi et al. (2009) a similar method was used where CMP was obtained after 80 min transglutaminase cross-linking at a substrate concentration of 8% and enzyme dose of 7 Ug^{-1} at pH 6.5. CMP was separated by 6 microfiltration cycles to reach a purity of 70% (Bo-zhi et al., 2009). Bonnaillie et al. (2014) introduced a method to fractionate 10% WPI solution proteins by using supercritical CO_2. Fractionation was carried at temperature of 70°C, pH 4.9 for 305 min. After ultrafiltration, the filtrate contained 94% CMP with a 46% recovery and after two diafiltration steps, a CMP solution with purity of 80.4% and recovery of 82% was obtained.

2.3.4 Methods based on modified memberanes

Bhushan and Etzel (2009) developed a charged UF membrane to fractionate CMP from cheese whey. They used a solution of CMP and β-lg alone and binary mixture at pH values of 3, 4, 6 and 7.5 at different pressures. A positively charged UF membrane was prepared by regenerated cellulose membranes of 30 kDa MWCO that were reacted with (3-bromopropyl) trimethylammonium bromide. Charged UF membrane increased the selectivity of the separation compared to uncharged membranes by over 600% especially at acidic pH values of 3 and 4. Binary systems had a higher selectivity that single protein in buffer. In another study, Arunkumar and Etzel (2014) used wide-pore negatively charged UF membrane of 300 kDa MWCO for completely retaining all whey proteins at neutral pH. They obtained an isolate from Swiss cheese whey. They suggested that the 100 kDa MWCO negatively charged membrane had a higher recovery of CMP (86%) than the 10 kDa unmodified membrane (67%). However, 300 kDa negatively charged membrane yielded lower recovery (16%) than compared to that from 10 kDa unmodified membrane.

Table 2.3 : Industrial methods of isolation of caseinomacomacropeptid and its yield, purity and sialic acid contents.

Isolation method	Starting material	Recovery (%)	Purity (%)	Sialic acid (%)	Reference
Anion-exchange	Cheese whey WPC	80-85	>88	12-17	Ayers et al. (2003)
Two-stage ultrafiltration	WPC	80-85	82	-	Holst and Chatterton (2002)
Ultrafiltration and cation-exchange	WPC	95	98	-	Christensen and Holst (2018)
Ultrafiltration	WPC	<88	78-80	-	Tanimato et al. (1991)
Ultrafiltration	Cheese whey	-	79-88	-	Kawazaki and Dosako (1994)
Ion-Exchange	Sweet whey	92	96	-	Doultani et al. (2003)
Anion exchange membrane	Sweet whey	100	97	91	Kreuß and Kulozik (2009)
Ultrafiltration	WPC and sweet whey	69-76	75-90	34-4	Martín-Diana et al. (2002)
Cation and Anion-exchange	Sweet whey	-	82	-	Etzel (1999)
Anion exchange and immobilized metal affinity column (IMAC)	Sweet whey	-	85	-	Etzel (2001)

Bipolar membrane electroacidification (BMEA) was used to produce high purity bovine milk casein (Bazinet et al, 1999) and soybean isolates and to fractionate soybean 11S and 7S fractions (Bazinet et al, 2000). Bazinet et al. (2004) applied this method to obtain β-lg-enriched-fraction with 98% purity from 5% and 10% whey protein isolate (WPI). In future this method can also be tested to produce CMP.

2.4 Analysis Methods for CMP

Determination of CMP includes analysis of two fractions: glycosylated (gCMP) and non-glycosylated (aCMP). Sialic acid is accepted as an indirect index of glyco fraction. Quantitative determination of total CMP includes chromatographic methods such as RP-HPLC, LC-MS, size exclusion chromatography and also electrophoretic methods,

e.g. SDS-PAGE and capillary electrophoresis techniques (Mole and Leoni, 2008). For the analysis of milk powder adulterated with whey, European Commission adapted HPLC method for CMP detection (Commission Regulation EC No.213/2001) (Oancea, 2009).

In 1975, the thiobarbituric acid assay has been developed by Warren (1959) for un-bound sialic acids detection in tissue hydrolysates. Two procedures, the thiobarbituric acid and the alkali-Ehrlich methods were developed by Aminoff (1961). Method of Aminoff (1961) was used by Martín-Diana et al. (2002) for determination of sialic acid content of isolated CMP from different milk whey. Sialic acid content of CMP isolated from bovine, ovine and caprine milk whey varied from 1.5 to 5.7%.

Van Hooydonk and Olieman (1987) searched for all the forms of CMP by precipitation of other proteins with 80 gL^{-1} TCA and detection by HPLC. Fukuda et al (2004) proposed the standardization of the spectrophotometric (470 nm) acidic ninhydrin method for quantitative determination of bound and free sialic acid in milk and whey. By using method of Fukuda et al. (2004), Furlanetti and Prata (2003) calculated free and total gCMP content in bovine milk during lactation. Near to colostral period and at the end of lactation period the content of free gCMP was determined to be higher.

Visser et al. (1986) applied HPLC for separation of bovine caseins. Fractionation was carried out by HPLC on hydroxyapatite (HAP-HPLC), high-performance anion-exchange choromatography (HP-IEC) and two types of reversed-phase (RP-HPLC) columns. They recommended the technique in rapid purification of small quantities of material.

Lopez-Fandino et al. (1993) described an HPLC method for the isolation and characterization of hydrolysis products from rennet-treated caseins from sheeps milk and compared with that from ovine CMP. Martín-Diana et al. (2002) used this method for CMP detection. In this method, CMP was monitored by RP-HPLC equipped UV detector by using a C18 column at 214 nm.

Ferreira et al. (2003) developed an RP-HPLC method using Chrompack P300 RP that contains polystyrene divinylbenzene copolymer column to quantify CMP content in whey and WPC powder. Two forms of CMP were separable at 214 nm. Recoveries of 93 and 98% were obtained. Thomä et al. (2006) determined CMP fractions by a RP-HPLC using PLRP-S column and peaks of CMP were detected at 226 nm. Both forms

of CMP were eluted between 12-20 min. The elution order was gCMP A+B< aCMP A < gCMP A+B< aCMP B. Immunochemical assays (ELISA) to analyze CMP (Mikkelsen et al, 2005) and gCMP (Immunostick gCMP) based on monoclonal antibodies against gCMP have been developed (Chávez et al, 2012; Oancea, 2009).

2.5 Application of CMP in Foods

Fruit gels fortified with CMP were prepared in addition to κ-carrageenan (Marshall, 1991). Fruit gels at pH 4.5 with CMP were found to be more firmness and have better appearance compared with fruit gels made wiyhout CMP (Marshall, 1991).

Lim et al. (2007) studied palatable, low-Phe foods and beverages that made with CMP to provide an alternative low-Phe protein source for the PKU diet. Their results demonstrated that the functional properties of CMP were especially well-suited for use in beverages and semi-solid foods such as pudding. They suggested that CMP could be used to make a beverage that is more palatable than the amino acid formulas used as the primary source of protein in the PKU diet (Lim et al, 2007). Ergül and Karakaya (2013) formulated different pudding by the addition of different levels of CMP isolate (10, 12 or 15%). Based on sensory and viscosity analysis, the puddings containing 15% CMP and 15% dried blueberries was chosen as the best formulation. (Ergül and Karakaya, 2013). Karakaya et al. (2016) produced a salad dressing with germinated seed, sprouts of lentils and cowpea in combination with CMP. This salad was claimed to have desirable sensory characteristics and beneficial health effects. Pena et al. (2018) investigated the palatability of CMP containing foods for PKU individuals. Chocolate and strawberry aromas were found to be the most acceptable flavoured products with CMP.

CMP is valuable peptide with special application for PKU patients in food industry based on its bilogical, nutritional and structural properties. Therefore, a cost-effective production method with high yield, recovery and purity has been searched. Membrane and filtration methods are used widely in industry to isolate CMP from whey. Pre-treatment of whey before membrane technology can improve product purity.

2.6 Phenylketonuria

Phenylketonuria (PKU) is an inborn metabolic deficiency caused by lack of phenylalanine hydroxylase (PAH, EC 1.14.16.1). Lack or low activity of the enzyme which hydroxylase dietary essential amino acid Phe to Tyr or due to mutation of essential co-factor tetrahydrodiopterin (BH4) that also reducesthe conversion of Phe to Tyr causes accumulation of Phe or its major metabolites in blood (Blau et al, 2010; Ney et al, 2017). PKU is classified according to the severity of hyperphenylalaninemia (HPA) based on concentrations of Phe in blood. Normal concentration of Phe in blood is 50–110 $\mu molL^{-1}$ and it increases to 120–600 $\mu molL^{-1}$ in HPA, 600–1200 $\mu molL^{-1}$ in moderate HPA, above 1200 $\mu molL^{-1}$ in classic PKU (Blau et al., 2010). PKU was first described in 1934 by the Norwegian physician Asbjörn Fölling (Centerwall and Centerwall, 2000). In the middle of 50s, a patient with PKU was treated with a low PKU diet (Centerwall and Centerwall, 2000; Volckey et al, 2014).

If PKU left untreated, it results in mental retardation, seizures and eczema (Centerwall and Centerwall, 2000). Early diagnosis and treatment of PKU patients are required to prevent its irreversible effects (Pena et al, 2015). In treated PKU individuals the, main aim is to maintain Phe concentration under control to prevent neurological damage. Therefore, concentration of Phe in blood should be checked like glucose in diabetic patients (Evat et al, 2015; Soltanizadeh and Mirmoghtadaie, 2014; Van Calcer et al, 2012). According to recommendations for maintenance of Phe concentration at unharmful level, two groups are important: new born children-12 years old and those older than 12 years (Van Calcer et al, 2012). In European countries and the US, it is considered that a newborn child with a Phe concentration in plasma higher than 400–600 M should be started on a low-Phe diet.

The low-Phe diet in classical PKU 500 mg Phe per day (MacLeod and Ney, 2010). To achieve this principal treatment, a low Phe diet (commonly permitting less than 10 g natural protein per day) in combination with vitamins, minerals, trace elements and some free synthetic amino acid supplements are used (Evans et al, 2016). A daily diet is mostly based on the level of PKU, age and gender. For example, a daily total Phe consumption for a female older than 19 years must not exceed value from 220 to 770 $mgday^{-1}$ and 290 to 1200 mg day^{-1} for males (Soltanizadeh and Mirmoghtadaie, 2014). In order to achieve better compliance, the formulation of low protein foods for PKU

diet should satisfy consumers with a good taste and easy access for ordering and buying (Pena et al, 2015).

The incidence of PKU which is an autosomal recessive disorder in different countries is dependent on mostly the rates of consanguineous marriage. In Turkey, almost 4 in 100 of population is HPA and 1 in 4000 newborn babies are diagnosed with PKU . According to the estimations, 15000 and 50000 PKU individuals worldwide are living with a treatment (Van Calcar and Ney, 2012). The lowest frequency of PKU is in Japan (1 in 125.000) and Finland (1 in 200.000) and the highest is in Turkey (1 in 4000) (Durukan and Donat, 2017). Coskun et al. (1993) reported 471 Turkish HPA cases of which 408 (89.3%) had classical form and 49 (10.7%) had moderate form. Özlap et al. (1995) reported that the ratio of PKU/non-PKU hyperphenylalaninemia was 2.0. Nation wide newborn screening programs for PKU was started in 1986 for early detection and prevention (Özalp et al, 1995; Erdem and Tekşen, 2013; Giżewska et al. 2016).

CMP which naturally lacks aromatic amino acids has attracted attention of food scientists and dieticians for formulation and development of foods for PKU diet (Zaki et al, 2016). CMP has the advantage of having lower osmolality than the regular amino acid formula used in diet for PKU (Solverson et al, 2012). CMP can be isolated from cheap whey which make it a suitable alternative for manufactures. Phe is measured as a purity index of CMP isolates. Commercial CMP isolate powders with approximately 4 and 2 mg Phe per g are available from Arla Foods (Viby, Denmark) and Davisco (Le Sueur, Minnesota, USA), respectively. CMP has been used in a variety foods and bevereges as it is known safe and pure for the nutritional management of PKU (Lim et al. 2007). Moreover, sensory evaluations made with individuals with PKU showed that foods and bevereges fortified with CMP can be acceptable by consumers (Lim et al, 2007; Ney et al, 2008). Therefore, use of CMP in foods can be an alternative source of protein for individuals with PKU.

2.7 Plant-based Milk-like Drinks

Allergy towards animal proteins especially in infants and children has been a driving force development of plants-based milks as alternatives to cow's milk. Moreover, recently increasing consumer demand for plant-based foods has directed food industry towards plant-based alternatives (Bastıoğlu et al, 2016; Bath et al, Sethi et al, 2016;

Zhang et al, 2007). Several studies have focused on the production of plant-based milk from nuts and seeds such as almond, peanut, sesame and rice (Latifah and Warganegara, 2018; Lukaszuk et al, 2007). These alternative beverages are prepared with grains or nuts in addition to gums (Lindahl et al, 1997; Sethi et al, 2016). Grains are used as protein source for vegan diets, for indiviuals with allergy to milk proteins and lactose and special diets for PKU individuals and those who need to consume low-protein foods.

Preparation of plant-based milks requires milling of raw materials (nuts, cereal and grain) to achieve uniform particle size. The particle size of the cereals and grains has a large dependence upon the homogenization method (Durand, 2003). In addition, the stability of the emulsified beverage also depends upon droplet size and aggregation (Shaherardekani et al, 2011). Acceptibility of plant-based milk drinks depends on food culture of each contury.

Rice milk is made from both brown and white rice. Total solid content of rice milk is adjusted around 11-13% by mixing ingredients, a rice water slurry containing 25-40% dry weight rice (this slurry was treated by heat and liquefied, preferably by treatment with an alpha-amylase enzyme), 1-5% vegetable oil (safflower, sunflower or canola), 0-1% stabilizer and 1% salt (Mitchell et al, 1988). This mixture is heated to 65-70^{o}C for 15-30 min, homogenized and then cooled to 0-18^{o}C. Additional flavors like brown rice syrup and vanilla can be added and then heated and homogenized and then after production it can be cooled and packed (Mitchell et al, 1988). Additional ingredients like vitamin D2 and B12 and also minerals like calcium can also be added to fortify the drink (Bath et al, 2017; Manfredi et al, 2017, Mitchell et al, 1988). Different stabilisers including xanthan, gellan, carrageenan, guar and locust bean gums are used in commercially available plant-based milk drinks. Carboxymethyl cellulose was also used as a thickener at a level of 0.4% in rice milk beverage by Russel and Delahunty (2004).

Starch, protein and gums are important components in structure development and stability of rice milk. Rice contained 80% starch with a composition of 70% long-chain amylose and 30% amylopectin (Reddy and Bhotmange, 2014; Lin et al, 2009). Hot pasting temperature of rice starch was 72^{o}C and peak viscosity was found 4.4 Pa.s. Viscosity of rice starch after isolation at concentrations of 1-4% was varied between 0.002-0.15 Pa.s (Kesarwani et al, 2016). Stability of rice starch gels mainly depends

on protein and amylose content, cooking temperature and rice cultivation ground (Kesarwani et al, 2016; Reddy and Bhotmange, 2014).

Rice consists of four protein fractions: globulin (12%), glutelin (80%), albumin (5%) and prolamin (3%). Glutelin of rice has high molar mass and soluble in dilute acid. It is composed of subunits bound by disulfide linkages, while rice globulin is composed of low molecular components (Ju et al, 2001). In addition, rice bran protein contains around 37% albumin, 32% globulin, 27% glutelin and 2% prolamin (Sagar et al, 2016). These proteins have different denaturation temperatures. The denaturation temperatures of albumin, globulin, and glutelin were reported as 73.3, 78.9, and 82.2^{o}C, respectively (Ju et al, 2001). For rice flour, denaturation temperature was reported to be 80.5^{o}C (Issara and Rawdkuen, 2016). It was reported that heating at 73^{o}C to 85^{o}C was critical in cooking quality of rice and the formation of structure (Sagar et al, 2016). Ju et al. (2001) found that heat treatment at 80^{o}C for 10 min resulted in a change in surface hydrophobicity of rice probably by unfolding or denaturing all globulin molecules.

Adding salt to a protein-based solution has two effects on protein solubility. At low levels, it increases solubility by preventing the electrostatic protein–protein interactions (salting-in). At higher concentration, the protein solubility is decreased due to competition between the protein and salt (salting-out). Effects of NaCl at a concentration range of 0-1 M on solubility, emulsifying capacity and foam capacity of melon seed flour were investigated by Nnadozie et al. (2015).

Rice milk is recommended to be a better alternative than other plant-based milks for babies which have allergy to cow's milk (Fanaro and Vigi, 2002). Unlike some other plant-based milks, rice milk contains little or no fiber except rice milk made from rice bran (Manfredi et al, 2017). Moreover, rice milk is a dietary drink with low fat and different carbohydrate and protein composition compared to milk (Manfredi et al, 2017).

Xanthan does not form gels when used singly and it was known as a non-gelling gum. Its application in combination with starch was studied in sweet and sour sauces (Saha and Bhattacharya, 2010). Oat and potato starch were combinated with xanthan gum. Oat-starch samples showed better thickening effect (Gibinski et al, 2006). This was explained by this fact that potato starch and xanthan gum are both anionic polysaccharides and hence are less intermolecular interaction between them (Gibinski

et al, 2006). As a desirable thickener xanthan-starch combination was used in dessert sauces (Sikora et al, 2007).

3. METHODS AND MATERIALS

3.1 Materials

Non-fat milk powder (Pinar Inc., Izmir, Turkey) and microbial rennet (180 IMCU mL-1, MYS Fermente, Maysa Gida, Istanbul, Turkey) were used for preparation of sweet whey. The composition of non-fat milk powder according to the manufacturer was 31-36% protein, 2.7% ash, 2.1% moisture and 52-56% lactose. A commercial CMP isolate (Lacprodan®CGMP-10, Arla Foods, Viby, Denmark) with CMP, protein, ash and lactose content of approximately 64%, 80%, 5% and 2% was used. was used as a standard for HPLC analysis. Bovine whey proteins α-la and β-lg (Sigma-Aldrich, Istanbul, Turkey) were also used as standards for HPLC analysis. A laboratory-scale RO/UF unit with a capacity of 15 L (FT18, Armfield Ltd, Ringwood, Hampshire, England) was used. Two PES UF membranes with 9 (ES209) and 25 kDa (ES625) were used for UF with membrane area 0.024 m^2, and filter length 2×0.3 m (FT18-8) (Table 3.1). Sunflower oil (Biryağ, Edirne, Turkey), white rice flour (Ülker Bizim, Istanbul, Turkey), sea salt (Doğa evi, Istanbul, Turkey) were purchased from a local supermarket. Xanthan gum was provided from (Eykim Gıda ve Kimya San. Dış Tic. Ltd., Istanbul, Turkey). For all samples distilled water was used.

Table 3.1 : Specification of PES tubular membranes.

Membrane code	Material	MWCO (kDa)	pH range	Maximum presure (bar)	Maximun temperature (°C)
ES625	PES	25	1.5-12	15	80
ES209	PES	9	1.5-12	30	80

3.2 Sweet Whey Preparation

Non-fat milk powder was reconstituted in distiluted water to 10% dry matter content then stirred at 40°C for 30 min. After cooling to 32°C, rennet was added at a level of 0.02% (w/w). To obtain a coagulum, the mixture was held in a water-bath at 32°C for 2 h. Coagulum was cut into 1 cm-cubes and then heated to 38ºC for releasing whey

as much as possible. Whey was separated from the coagulum by filtration through cheese-cloth.

3.3 Acidification and Heat Treatment of Whey

To check the effect of order of heat and acid treatments on separation of CMP from other whey proteins, mainly α-la and β-lg, the method of Martín-Diana et al. (2002) was used with some modifications. Sweet whey at pH 6.5 was separated into two batches. For heat-acid treatment, one batch was heat-treated at 90°C for 1 h and cooled to room temperature. After cooling, it was divided into parts with the same volume and the pH of each of them was adjusted to different pH values of 3.0, 3.5, 4.0, 4.5 and 5.0 by the addition of 1N HCl. For acid-heat treatment, the other batch was divided into parts of the same volume and acidified to the same pH values and then the samples were heat treated at 90°C for 1 h. To remove precipitated proteins, samples were centrifuged at 6000xg for 20 min at 4°C. The supernatant from each sample was collected and analysed for gross composition, protein composition, sialic acid, phenylalanine and tyrosine.

3.4 Composition Analyses of Pre-treated Whey

Crude protein (CP) and non-protein-nitrogen (NPN) contents of whey samples were determined by Kjeldahl method (AOAC, 2002 a,b). Crude protein was calculated by using a factor of 6.38. The Munson-Walker gravimetric method was used for determination of lactose content (AOAC, 1990a). The amount of ash was analysed by using the gravimetric method (AOAC, 1990b). The dry matter content of the samples was analysed by the vacuum oven method at 70°C (AOAC, 1995).

3.5 Determination of CMP and Whey Proteins in Samples by RP-HPLC

CMP and whey proteins in whey samples was analyzed by using a modified method based on the methods of Ferreira and Oliveira (2003) and Thoma et al. (2006). CMP was quantified by RP-HPLC (Waters Separation Module 2695, MA, USA) using PLRP-S 300Å 8 mm column (Agilent Technologies, CL, USA) and Waters 2996 PDA detector (Waters, Milford, MA, USA). Gradient elution was applied by using solvents A (0.1% TFA in water, v/v) and B (0.055%, v/v, TFA in acetonitrile: water (80:20,

v/v)). The gradient started with 10% of solvent B and increased to 49% over 17 min, 50% in 3 min, 70% in 3 min, 100% in 1 min and then kept at 100% for 3 min before returning to the initial conditions. Flow rate was 1 $mLmin^{-1}$. Peaks were detected at 226 nm. Column temperature was kept at 40°C. Injection volume was 20 µL. Standard stock solutions of β-lg (5 $mgmL^{-1}$), α-la (2 $mgmL^{-1}$) and CMP (2.5 $mgmL^{-1}$) were used for the preparation of calibration curves.

3.6 Determination of Sialic Acid of Samples

Whey samples (2 mL) were dialysed against deionised water at 4°C for 24 h by using a 3.5 kDa dialysis membrane (Nakano and Ozimek, 1999). Dialysis membranes were pre-treated at 3 steps: 1. 10 min boiling in 1 mM EDTA + 10 mM $NaHCO_3$ solution, 2. Washing with distilled water, 3. 10 min boiling in 1mM EDTA solution and 4. Washing with distilled water. Amounts of free and total sialic acid in dialysates were analysed according to the method of Aminoff (1961). The total amount of sialic acid was determined after the release of bound sialic acid by heating the acidified solution of dialysates at 80oC for 1 h (Nakano and Ozimek, 1999). For this purpose, 0.5 mL of dialysed samples was mixed with 11.2 µL 4.5N H_2SO_4 and then heated at 80°C for 1 h. Samples were cooled and then neutralized with 50 µL of 0.5 N NaOH. Free sialic acid in 0.5 mL samples was reacted with 0.25 mL of 25 mM Na-periodate (in 0.125N H_2SO_4) at 37°C for 30 min. The samples was mixed with 0.2 mL 2% Na-arsenite (in 0.5 N HCl) and vortexed until yellow-brown color disappeared. The sample was mixed with 2 mL of 0.1 M TBA (in water, pH 9 with 4N NaOH), vortexed and then was held in boiling water bath for 7.5 min. After cooling, 3 mL of acidified 1-Butanol (95:5, v/v, 1-butanol:HCl) was added to the sample. The sample was centrifuged at 2500xg for 5 min. Absorbance of supernatant was read at 549 nmby using spectrophotometer. The molar extinction coefficient of 70.7×10^3 was adapted from Aminoff (1961) for calculation of concentration from absorbance value. Bound sialic acid in the samples was determined by subtracting the amount of free sialic acid from the total amount of total sialic acid. As free sialic acid can be lost to water during dialysis, amount of free sialic acid does not reflect the actual concentration in the sample. Therefore, only the concentration of bound sialic acid was reported.

3.7 Determination of Phenylalanine and Tyrosine in Samples

Proteins in samples were hydrolyzed by adding 6N HCl (500 mL per g protein) at 110ºC for 24 h. To prevent oxidation of amino acids during hydrolysis, 50 µL of phenol was added to the samples. The solvent was evaporated by rotary vacuum evaporator at 65ºC (Andrensek et al. 2003). Two milliliters of mobile phase (0.0125 M $_{KH2PO4}$ (pH 3.5): acetonitrile, 98:2) was added to the samples after evaporation. Phenylalanine and tyrosine contents of hydrolysates were determined by the RP-HPLC method of Prodolliet and Bruelhart (1993). Column was C18 ZORBAX Eclipse XDB-C18 150 x 4.6mm 5µm Particles 80 Å Pore (Model, Agilent technology, USA, 5µm (250 mm×4.6 mm) and flow rate was 0.8 $mLmin^{-1}$. Mobile phase was prepared 0.0125 M KH_2PO_4 at pH 3.5: acetonitrile (98:2). Detection was made at 214 nm. Standard solutions of the amino acids at concentrations ranging from 0.05 to 1 $mgmL^{-1}$ for Phe an 0.05-0.9 $mgmL^{-1}$ for Tyr were used for preparation of calibration curves.

3.8 Ultrafiltration and Diafiltration of Pre-treated Whey

Whey that was heat-treated at 90ºC for 1 h and then acidified to pH 5 was used for ultrafiltration and diafiltration trials to separate lactose and minerals from CMP. This whey was called as pre-treated whey throughout the thesis. pH of pre-treated whey was adjusted to pH values of 5, 4, 3 with 6N HCl and 7 and 9 with 8N NaOH. for preliminary experiments in UF unit for flow rate adjustement. After re-adjustment of pH, samples were stored at 4ºC over one night. Deionized water at room temperature was used for measurement of flow rate of water through membrane in UF unit for 30 min at 3 bar. After removal of water from the unit, pre-treated whey at a volume of 9 L and at a temperature of 50ºC was put into the feed tank. Transmembrane pressure was adjusted to 3 bar for the membrane with MWCO of 25 kDa and 5 bar for the membrane with MWCO of 9 kDa. The feed was held at 50ºC by a circulating water bath. Flow rate of permeate was measured after 5 min and then every 30 min until the end of run. UF was continued until 1.8 L of retentate was remained in the feed tank. After removal of retentate, the unit was cleaned with following chemicals for 10 min each in the order shown: Water, 0.25 N HCl, water, 0.25 N NaOH, water, 100 ppm active chlorine supplying disinfectant and water (SUMA, JohnsonDiversey, Fort Mill, SC, USA).

Diafiltration was carried out with the same unit at the same conditions 1 d after UF. Water at 50ºC was added to the retentate at 50ºC from UF at a volumetric ratio of 1:1. Each run of DF was continued until the initial volume of retentate was obtained in the feed tank. Flow rate of permeate was measured during DF run. The same volume of water at 50ºC was added and DF was repeated. Cleaning procedure was repeated after DF run each day. The scheme of isolation procedure of CMP was given in Fig 3.1.

A sample of retentate was taken from feed tank after UF and each DF cycle and stored at 4ºC for analysis of composition. Dry matter, protein, ash, lactose, CMP, α-la, β-lg, phenylalanine, tyrosine and sialic acid contents of retentates were measured as outlined in sections 3.4-3.7.

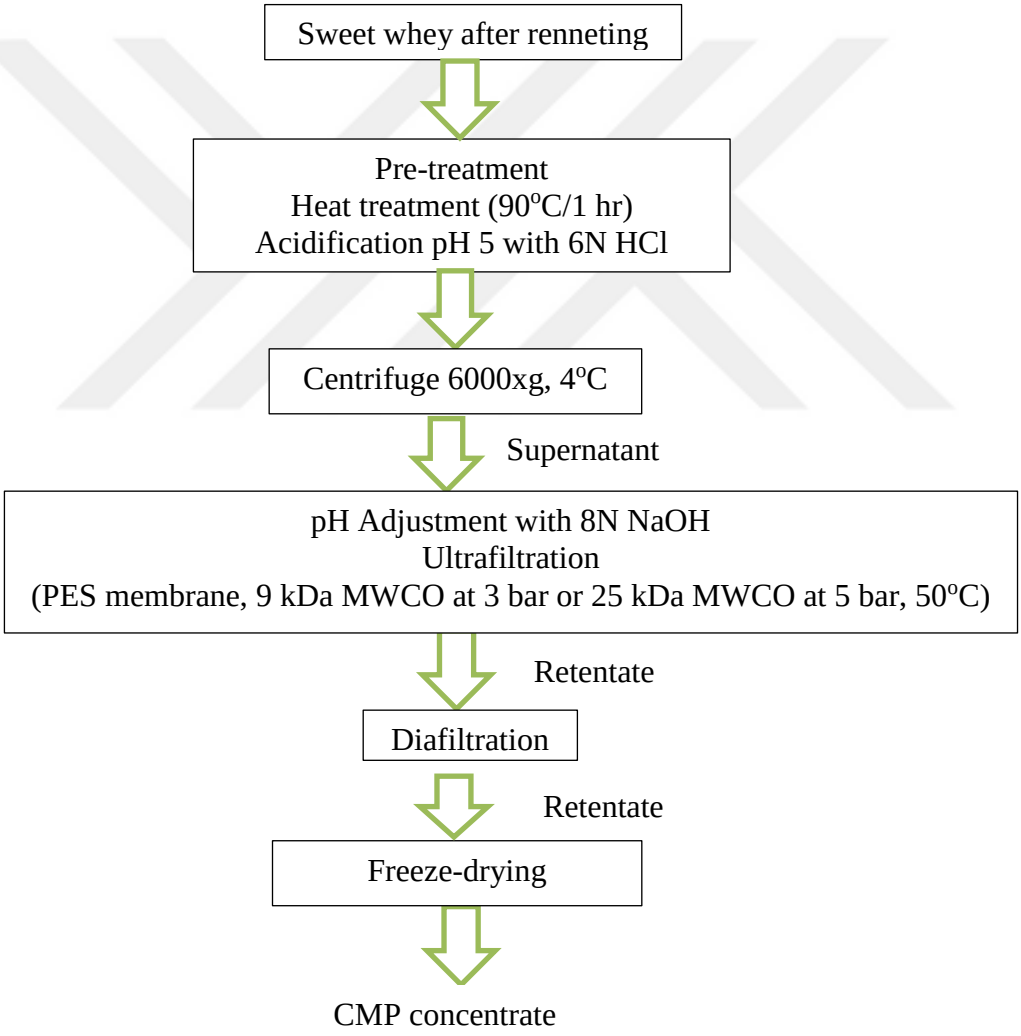


Figure 3.1 : Scheme of the isolation of caseinomacropeptide from sweet whey.

3.9 Freeze-drying of Retentate to Obtain CMP Powder

The retentates from UF/DF process were dried by using a freeze-dryer (FreeZone 4.5 Liter Benchtop Freeze-Dryer, Labconco Corporation, Fort Scott, Kansas, USA) at a temperature of -50°C and pressure of 0.018 mbar for 48 h.

3.10 Determination of Permeat Flux, Rejection, Recovery and Purity for Components in Whey by Isolation Process

Flux of permeate was calculated from measured flow rate by using the membrane area of 0.024 m^2 and expressed as $Lm^{-2}h^{-1}$. Relative flux was calculated by dividing the flux value to that of water at 20°C under the same pressure. Mean flux was calculated by taking average of flux values during an UF or DF run. Volumetric concentration factor (VCF) was calculated from the ratio of volume of pre-treated whey to that of retentate for each UF run.

Flux of permeate can calculated based on below formula as $\dot{V}_p$ is shown permeate flow (Lh^{-1}) and A is the area of membrane (m^2):

$$Flux\ (Lm^{-2}h^{-1}) = \frac{\dot{V}_p}{A} \tag{3.6}$$

Rejection of components including CMP, α-la, β-lg, ash and lactose by the membrane in UF process was calculated based on mass of component (m_i) according to formula given below:

$$\text{Rejection}\ (\%) = \frac{\mathrm{m_{i\ retentate}}}{\mathrm{m_{i\ feed}}} \times 100 \tag{3.7}$$

The recovery of CMP and sialic acid from feed by the isolation process was calculated by formula given below:

$$Recovery\ (\%) = \frac{m_{i\ retentate}}{m_{i\ whey}} \times 100 \tag{3.8}$$

Purity of CMP was expressed in both dry matter and protein by using compositional data. Concentrations of phenylalanine and tyrosine were also used to evaluate purity of isolated CMP.

3.11 Formulation of Rice Drink by Response Surface Method (RSM)

Rice flour was chosen as a base ingredient for the formulation of drink as it contains lower protein compared to other cereals and nuts. Rice drinks were prepared from xanthan gum (0.01-0.05%, w/w), rice flour (3-8%, w/w), sun flower oil (1%, w/w), sea salt (0.1%, w/w) and distilled water. For determination of optimum levels of rice flour and xanthan gum and heating temperature, an experimental design with three factors at three levels and three blocks was established according to central composite design in RSM (Table 3.2). Color values, phase separation, sedimentation, apparent viscosity, rheological properties, particle size and zeta potential of rice drinks were measured as responses. Effects of factors on responses were evaluated and predictive models for each response were developed by nonlinear regression analysis. Predictive models were used for optimization of production of rice drink.

Table 3.2 : All runs of the central composite design (CCD) with all independent variables.

Run	Block	Rice flour (% w/w)	Xanthan gum (%w/w)	Temperature (^{o}C)
1	1	3	0.01	80
2	1	3	0.05	90
3	1	8	0.01	90
4	1	8	0.05	80
5	1	5.5	0.03	85
6	2	8	0.05	90
7	2	8	0.1	80
8	2	3	0.05	80
9	2	5.5	0.03	85
10	2	3	0.01	90
11	3	5.5	0.01	85
12	3	8	0.03	85
13	3	5.5	0.03	90
14	3	3	0.03	85
15	3	5.5	0.03	80
16	3	5.5	0.05	85
17	3	5.5	0.03	85

To prepare the drink, white rice flour, sea salt and sunflower oil were dispersed in deionized water (50^{o}C) using a heater and magnetic stirring hotplate . Mixing was carried at 300 rpm for 20 min at 50^{o}C to achieve full hydration. Xanthan gum was added to sample and the solution was mixed at 500 rpm for 15 min at one of the temperatures based on experimental design. After mixing all the ingredients, samples

were homogenized by a probe homogenizer (Ultra-Turrax T18 Basic, IKA, Staufen, Germany) at 7200 rpm for 10 min.

3.12 Phase Separation

Colloidal stability of samples was determined through the phase separation analysis by quantifying the volume of the separated phases. About 20 mL of samples were poured into tubes and stored at 4°C. Measurements were carried out at first day and after storage for one week and reported as % separated phase in total volume.

3.13 Sedimentation

Sedimentation measurements are performed by a turbidimeter (Turbiscan™ LAB, Formulaction, l'Union, France). Backscattering of light at 880 nm by the sample in a glass tube was measured. Sedimentation in samples were measured from change in light scattering as mm. Sedimentation was expressed as % sediment layer in total height (40 mm) of the sample in glass tube. Measurements at first and seventh day were recorded. Samples were kept at 4°C during storage.

3.14 Rheological Properties

The rheological behavior of samples was characterized by using a rheometer (Rheostres1, Thermo, Karlsruhe, Germany) equipped with a cone/plate sensor (C35/2, Thermo, Karlsruhe, Germany). The shear stress (τ) was measured as a function of shear rate ($\dot{\gamma}$) in the shear rate range of 0-300 s^{-1} for 2.5 minutes. The Power law model (Equation (3.9)) was fitted to the experimental data to determine flow behavior index (n) and consistency coefficent (k) by using a non-linear regression procedure. Apparent viscosity of the samples s (η_{app}) were taken from data at 50 s^{-1}.

$$\tau = k\,\dot{\gamma}^{n} \tag{3.9}$$

3.15 Color

Color values of the samples were measured using a chromameter (CR-400, Konica Minolta Inc, Chiyoda-Tokyo, Japan). Color was characterized by the Hunter lightness index (L), redness index (a) and yellowness index (b). A sample (20 mL) was poured

into a special glass container and Hunter indices were read. Calibration of the device was done by using standard white plate.

3.16 Particle Size and Zeta Potantial

The zeta potential and particle size of samples were measured by using a dynamic light scattering instrument Malvern Zetasizer Nano ZS (Malvern Panalytical, Worcestershire, UK). Deionized water was used as dispersant. Refractive infex of a commercial rice drink (1.35) was introduced to the device as a reference value. Measurements were carried out in a clear disposable zeta cell at room temperature. Particle size was expressed as zeta average value given by the instrument. Zeta potential was expressed in mV.

3.17 Sensory Analysis of Rice Drink

Sensory analysis was performed according to the descriptive sensory analysis methodology.Unsweetened, sweetened (2.5% w/w sucrose) rice drink and a commercial light milk (1.5% fat, İçim, Istanbul, Turkey) were presented to ten experienced panelists. The panelists evaluated and scored color, odor, mouthfeel, graininess and consistency, bitterness, sweetness, oily and overall acceptability of the samples on a 6-point scale rating from 0 (dislike extremely) to 5 (like extremely). The panelists cleaned their mouth with water between tasting of the samples. The sensory evaluation was performed 1 d after storage of prepared rice drink samples at 4°C. Two replicated sensory analyes were performed at different times.

3.18 Fortifying Formulated Rice Drink with Isolated CMP Concentrate

Isolated CMP concentrate was added to rice drink for fortification. The amount of CMP concentrate powder that could be added to the formula was determined by considering maximum level of phenylalanine (200-500 $mgday^{-1}$) that could be consumed by a child with PKU between 1-10 years of age (MacLeod and Ney, 2010). By adding 7% CMP concentrate powder to 100 mL of rice drink, Phe content reached to 25 mg. Physical properties of rice drink with CMP concentrate were measured based on methods described in sections 3.15-3.19.

3.19 Statistical Analysis

Effects of examined factors on measured properties were evaluated by analysis of variance (ANOVA). Means were compared by using Tukey's test at a level of significance of 0.05 (IBM® SPSS® Statistics 24, Armonk, NY, USA).

Response surface method was performed by using Design Expert software (Version 11.0, StatEase®, MN, USA).

Data of sensory analysis were evaluated by one-way analysis of variance (ANOVA). Means were compared using Tukey's test at a level of significance of 0.05 (IBM® SPSS® Statistics 24, Armonk, NY, USA).

4. RESULTS AND DISCUSSION

4.1 Isolation of CMP from Sweet Whey by Acidification and Heat Treatment

4.1.1 Composition of acidified and heat-treated sweet whey

Sweet whey was collected after rennet treatment of reconstituted skim milk. The composition of sweet whey was presented (Table 4.1). To investigate the effect of pH and heat treatment on separation of CMP from other whey proteins, two differents treatments where the order of heating and acidification was changed. For acid-heat-treated sampels, the whey was divided to 5 different batches with the same volume and its pH was reduced to values between 5.0 and 3.0 and then heated to 90^{o}C for 1 h. For heat-acid-treated samples, another 5 batches were first heated to 90^{o}C for 1 h and then pH was reduced to values between 5.0 and 3.0. Precipated whey proteins by the treatments were seperated by centrifuge and the supernatant was collected as CMP sample.

Table 4.1 : Composition of sweet whey.

Component	Amount
pH	6.5 ± 0.1
Dry matter (%)	7.34 ± 0.06
Lactose (%)	5.35 ± 0.15
Ash (%)	0.57 ± 0.02
Crude protein (%)	0.97 ± 0.00
Non-protein N (%)	0.06 ± 0.00
True protein (%)	0.62 ± 0.05
CMP ($mgmL^{-1}$)	1.60 ± 0.08
α-lactalbumin ($mgmL^{-1}$)	2.70 ± 0.60
β-lactoglobulin ($mgmL^{-1}$)	4.38 ± 0.36
Bound sialic acid (µg NANA mL^{-1})	172.8 ± 3.5
Tyr (mg $100mL^{-1}$)	36.6 ± 6.3
Phe (mg $100mL^{-1}$)	23.4 ± 0.6

In both heat-acid and acid-heat treatments, by decreasing pH to 3.0, it was observed that dry matter and protein contents increased (Table 4.2). More of dry matter was removed at pH 3.5 by heat-acid treatment compared to that by acid-heat treatment. NPN content of the isolates decreased as the pH was reduced. NPN content could relate to the change in the amount of CMP in the isolates, in the lack of proteose peptone (NPN = CMP+PP) (Javanmard et al, 2012; Holst and Chatterton, 2002). More protein was present in isolates that are acid-heat treated at pH of 3.0 and 3.5 and heat-acid treated at pH 3.0 than those of the others. When heat treatment was applied before acidification, more protein was precipitated at pH 3.5 compared to those in corresponding acid-heat treated sample. This could be explained by more effective denaturation and aggregation of whey proteins by heat-acid treatment. Lactose and ash contents were similar in both treatments

Table 4.2 : Composition of acid-heat and heat-acid-treated sweet whey samples.

Treament order	pH	Dry matter (%)	Lactose (%)	Ash (%)	Non-protein N (%)	Crude protein (%)	True protein (%)
Acid-heat	3.0	7.25 [a]	4.95 [a]	0.55 [a]	0.043 [c]	0.91 [a]	0.63 [a]
	3.5	7.16 [ab]	5.14 [a]	0.55 [a]	0.043 [c]	0.85 [a]	0.57 [a]
	4.0	6.88 [c]	5.11 [a]	0.52 [a]	0.044 [c]	0.53 [b]	0.24 [b]
	4.5	6.88 [c]	5.07 [a]	0.53 [a]	0.046 [bc]	0.53 [b]	0.23 [b]
	5.0	6.90 [c]	5.19 [a]	0.56 [a]	0.046 [bc]	0.53 [b]	0.20 [b]
Heat-acid	3.0	7.20 [a]	5.06 [a]	0.57 [a]	0.044 [c]	0.88 [a]	0.60 [a]
	3.5	6.88 [c]	4.77 [a]	0.58 [a]	0.047 [bc]	0.59 [b]	0.32 [b]
	4.0	6.85 [c]	5.09 [a]	0.54 [a]	0.054 [ab]	0.51 [b]	0.17 [b]
	4.5	6.96 [bc]	5.10 [a]	0.54 [a]	0.058 [ab]	0.54 [b]	0.17 [b]
	5.0	6.92 [c]	5.15 [a]	0.54 [a]	0.055 [a]	0.59 [bc]	0.24 [b]

[a] Values are means ± SD. Means followed by the same letter are not significantly different ($p < 0.05$).

[b] Least significant difference at $p \leq 0.05$ according to Tukey's HSD.

4.1.2 Protein composition pre-treated sweet whey samples containing CMP

Isoelectric and heat precipitation were applied to separate other whey proteins from CMP in the isolation method. Heat precipitation, based on the heat-resistance of CMP, has been applied for elimination of other whey proteins (Saito et al, 1991). Two major whey proteins, α-la and β-lg, were followed to determine efficiency of the treatments for isolation of CMP. Whey proteins are completely denatured after 5 minutes of heating at 90°C (Maćej, 1983). α-La is known as the most heat stable whey protein due to lack of free thiol groups and irreversible denaturation occurs at >90°C

(Wijayanti et al, 2014; Jovanović et al, 2005). Its aggregation depends on the type of acid, pH and calcium ions in whey during heating (Jovanović et al, 2005; Fernández et al, 2012).

Comparison of amounts of α-la and β-lg showed that the most of the whey proteins were precipitated at pH 5.0 (Table 4.3). There was a decrease in whey proteins as the pH was increased from 3.0 to 5.0 in both treatments. pI value of α-la is reported between 4.2-4.5 and that of β-lg is reported as 5.2 (Jovanović et al, 2005). Most of these proteins were removed from the samples at pH values above 4 without regard to the order of acid and heat treatments. During heating below proteins' pI, there is electrostatic repulsion between positively charged proteins.

Table 4.3 : Protein content of acid-heat and heat-acid-treated sweet whey samples by RP-HPLC*.

Treatment order	pH	CMP ($mgmL^{-1}$)	α-Lactalbumin fraction** ($mgmL^{-1}$)	β-lactoglobulin A-B ($mgmL^{-1}$)	Tyr (mg $100mL^{-1}$)	Phe (mg $100mL^{-1}$)
Acid-heat	3.0	1.20 ^{c}	2.66 ^{a}	3.53 ^{a}	32.02 ^{a}	13.6 ^{a}
	3.5	1.28 ^{bc}	2.24 ^{b}	2.51 ^{b}	-	-
	4.0	1.37 ^{ab}	1.46 ^{c}	0.53 ^{cd}	5.1 ^{c}	2.6 ^{b}
	4.5	1.51 ^{a}	1.18 ^{cd}	0.24 ^{d}	7.6 ^{c}	4.1 ^{b}
	5.0	1.50 ^{a}	0.95 ^{de}	0.18 ^{d}	4.0 ^{c}	3.3 ^{b}
Heat-acid	3.0	1.39 ^{ab}	0.71 ^{ef}	1.09 c	29.0 ^{a}	12.6 ^{a}
	3.5	1.39 ^{ab}	0.67 ^{ef}	0.67 ^{cd}	-	-
	4.0	1.38 ^{ab}	0.45 ^{f}	0.33 ^{d}	14.0 ^{b}	6.4 ^{b}
	4.5	1.46 ^{a}	0.44 ^{f}	0.25 ^{d}	4.0 ^{c}	4.0 ^{b}
	5.0	1.52 ^{a}	0.51 ^{f}	0.41 ^{d}	4.0 ^{c}	4.0 ^{b}

* Values are means of three replicated trials; values sharing the same superscript letter are not statistically different from one another ($p>0.05$).

** α-Lactalbumin was coeluted with other components in the sample as a wide peak.

In HPLC chromatograms, there was broadening of peak of of α-la possibly due to a co-elution with some other proteins or change in its conformation after heat and acid treatments, but there was a clear reduction by the treatments compared to that in whey (Fig. 4.1). Removal of α-la was less by acid-heat treatment than that obtained by heat-acid treatment at all pH values. In acid-heat treatment, at lower pH calcium was released from calcium phosphate and this possibly increased solubility and heat stability of α-la in the acid-heat treatment. Calcium ions increase solubility of α-la making it less hydrophobic (Bramaud et al, 1997). Lucena et al. (2007) reported that

most of α-la was precipitated at pH 3.5-4.5 from whey acidified with HCl at a temperature 55^0 C. Heating at 90°C at near pI of whey proteins results in more precipitation of whey proteins (Lieske et al, 2004b).

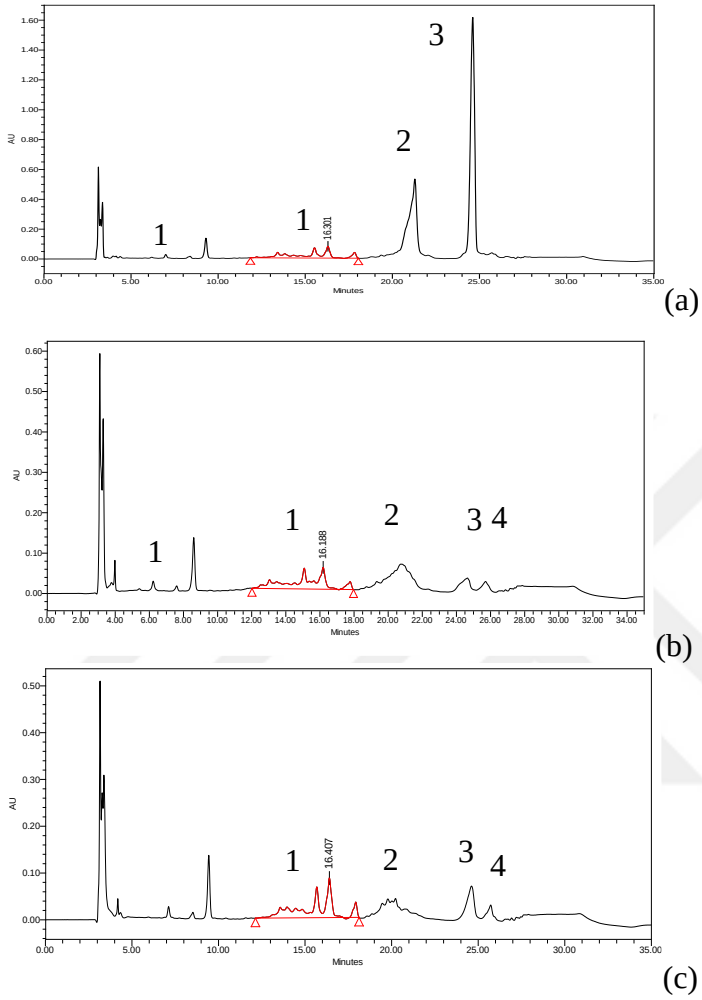


Figure 4.1 : RP-HPLC chromatograms of treated whey samples. CMP was quantified as total CMP including both aCMP and gCMP (red line). (a) Sweet whey, (b) Acid-heat-treated sample at pH 5, (c) Heat-acid-treated sample at pH 5 (5AH). 1: Total CMP (aCMP+gCMP), 2: α-la, 3-4: β-lg A-B.

β-lg was removed at a similar degree in both treatments when pH was at and above 4.0 regardless of order of acid and heat treatments. But it was precipitated more by heat-acid treatment than that was obtained by acid-heat treatment at pH values below 4.0. β-lg is not thermally stable as it contains free thiol groups, it can form covalent linkages with itself and other thiol containing proteins like BSA after unfolding by heat denaturation. Therefore, application of heat at near neutral pH before acidification led to more precipitation of β-lg than acid-heat treatment at pH values below 4.0. β-lg remains mostly in the soluble phase at low calcium concentrations at the same temperature (Bramaud et al, 1997; Lucena et al, 2007). Hydrophobic interactions also

occur between whey proteins as well as CMP depending on pH which affects charges of proteins and electrostatic interactions between them. β-lg was reported to be denaturated more efficiently at temperature above 65°C (Bonnaillie et al, 2014).

By using RP-HPLC, CMP was quantified as total CMP including both fractions aCMP and gCMP. The peaks of gCMP were not baseline resolved and in some cases, not separated distinctively from aCMP forms in the chromatograms (Fig 4.1). Therefore, sum of all the peaks were reported as total CMP (Table 4.3). This was in aggrement with earlier studies where acid-heat treatment was applied to isolate CMP (Thomä et al, 2006; Lieske et al, 2004a; Fernández et al, 2011). CMP losses were less at pH 4.5-5.0 than those at pH lower than 4.5 in both treatments. As these pH values are close to pI of CMP, isoelectric precipitation of CMP was observed below pH 4.5. At pH 3.5 using citric acid and lactic acid as precipitating agent 27% and 73% of CMP and at pH 4.0 15% and 27% were reported to be precipitated, respectively (Fernández et al, 2011). When HCl was used at pH 4.0, 20% of CMP was precipitated by Fernández et al. (2011). CMP can form dimers by hydrophobic interactions and can form aggregates by electrostatic interactions below pH 4.5 (Martinez et al, 2011). The loss in CMP in this study was not to a high extent possibly due to weak interactions between CMP molecules and its high solubility in water. Precipitation of proteins at the isolectric point is irreversible because their secondary and tertiary structures are affected. But CMP lacks secondary and tertiary structure, so it is highly stable even at low pH values. CMP was reported to behave similarly to β-lg with respect to pH where less than 20% of it was precipitated under the same conditions in aggrement with this study (Fernández et al, 2011).

According to the results, CMP was lost to a higher extent in acid-heat treated samples at pH 3.0-3.5 compared to those in corresponding heat-acid treated samples. This could be due to more electrostatic interactions between CMP molecules and possibly CMP and other whey proteins prior to heat treatment causing precipitation (Martinez et al, 2010). The loss in CMP was explained by its entrapment between large protein molecules such as α-la at pH 3.5 by Fernández et al. (2011). In this study, the entrapment of CMP could be lower than that reported by Fernández et al. (2011). This can be explained by the fact that the beginning material was WPC with a protein content of 33 and 65% in their study.

4.1.3 Determination of Phenylalanine and Tyrosine in pre-treated sweet whey samples

CMP is a uniqe peptide that contains no Phe and Tyr despite other major whey proteins. Indeed, concentrations of Phe and Tyr in sample has been used as an index of purity of isolated CMP (Ayers et al, 2003). The lowest amounts of Phe and Tyr were determined at pH 5.0, in both heat-acid and acid-heat treatments. These results were in accordance with the measured concentrations of α-la and β-lg at pH 5.0 (Table 4.3). In addition, this trend could be due to presence of heat-stable and soluble proteose peptone and non-protein nitrogen fractions which contained these amino acids.

4.1.4 Determination of Sialic acid

CMP has two fractions including glycosylated and non-glycosylated fractions. However, aCMP and gCMP could not be detected separately with high accuracy by RP-HPLC method. As sialic acid is present in gCMP, it was aimed to determine sialic acid content of samples to indicate the amount of gCMP fraction indirectly. The concentration of bound sialic acid in sweet whey was found to be 172.8 $mgmL^{-1}$ (2.35 mg in mg dry matter) which was in the range of values reported by Nakano and Ozimek (1999). Based on results, around 73% of the bound sialic acid content of whey was recovered in the sample at pH 5 obtained by heat-acid treatment (Table 4.4). So it can be concluded that pH 5 has the most glycosylated fraction of CMP. Recoveries of bound sialic acid in acid-heat treated sample at pH 5.0 and heat-acid treated one at pH 4.5 and 5.0 were found to be statistically similar to each other. About 70-80% of sialic acid was lost at pH 3.0. Acid-heat treatment caused more loss when compared to heat-acid treatment. The findings for acid-heat treatment were in agreement with those of earlier studies (Lieske et al, 2004b; Saito et al, 1991; Siegert et al, 2012). Lieske et al. (2004c) reported 89.6% recovery of bound sialic acid by acid-heat treatment (95^{o}C for 5 min) at pH 5.2. The recovery of sialic acid was found at pH 5.0 was found to be lower than this value. This could be due to longer duration of heat treatment in this study.

The highest purity of CMP (less amount of Phe and Tyr) and recovery of bound sialic acid were found at pH values of 4.5 or 5.0 in heat-acid and 5.0 in acid-heat treatments. CMP was found more stable to heating and then acidification to pH 5 compared to other treatments. A pH of 5.0 can be applied in heat-acid treatment to selectively

isolate CMP from sweet whey with high degree of bound sialic acid. The results of this study was used in further purification of CMP from sweet whey heat-treated and then acidified to pH 5.0 with ultrafiltration in next step.

Table 4.4 : Sialic acid content of acid-heat and heat-acid-treated sweet whey samples.

Treatment order	pH	Bound sialic acid (µg NANA mL^{-1})	Recovery of bound sialic acid from whey (%)
Acid-heat	3.0	36.3 [c]	21
Acid-heat	4.5	49.3 [c]	29
Acid-heat	5.0	105.9 [ab]	61
Heat-acid	3.0	46.8 [c]	27
Heat-acid	4.5	77. 3 [bc]	45
Heat-acid	5.0	125.2 [a]	73

[a] Values are means ± SD. Means followed by the same letter are not significantly different ($p < 0.05$).
[b] Least significant difference at $p \leq 0.05$ according to Tukey's HSD.

4.2 Optimization of Process Conditions in Ultrafiltration

4.2.1 Preliminary investigations to increase flux in UF process

Removal of other whey proteins, mainly α-la and β-lg, were aimed by pre-treatment, heating and acidification. Residual amount of these proteins remained in the pre-treated whey. Pre-treament was efficient to reduce whey proteins with no elimination of CMP from whey. To increase the purity and obtain a CMP concentrate, UF was carried out. UF was recommended to carry out at a temperature from 50-52°C in order to reduce viscosity, while avoiding calcium phosphate precipitation, which occurs at 55°C (Akoum et al, 2005).

The pH of pre-treated whey after heating and acidification was re-adjusted to different pH values before used as feed in UF process. Feed at pH 7.0 obtained after heat and acid (90°C/1 h at pH 3.5) treatments was concentrated by using an UF unit equipped with a PES membrane with a MWCO of 10 kDa according to the literature (Martín-Diana et al, 2002). The aim in concentration was to retain CMP completely in the retentate while most lactose, minerals and water were removed in the permeate. However, an efficient permeate flow rate could not be obtained at pH 5.0 (Table 4.5).

Therefore, pH of feed was adjusted to 5 to solubilize proteins according to Ergül and Karakaya (2013). This change did not yield a sufficient flow rate either. Then, pH was reduced 3.0 and 4.0 to solubilize any calcium precipitating on the membrane. This did not solve the problem. Another PES membrane with a MWCO of 25 kDa was tried with feed at pH of 5 and 7. While pH 5 resulted in a low flow rate, pH of 7 yielded in a high enough flow rate in the UF unit. However, there was loss of protein with this membrane since it had larger pores than the membrane with MWCO of 9 kDa. As increasing pH was effective on flux, pH of feed was increased to 9.0 and another trial was conducted with the membrane with 9 kDa MWCO. Sufficient flux was obtained in this case. From these trials, the highest flux of permeate was obtained when pH values of feed were 7 with 25 kDa membrane and 9.0 with 9 kDa membrane.

Table 4.5 : Relative mean flux at different pH values through PES membranes with different MWCO.

pH of feed	MWCO of membrane (kDa)	Relative mean flux ($Lm^{-2}h^{-1}$)
3.0	9	0.6
4.0	9	0.4
5.0	9	0.5
	25	0.7
7.0	9	0.7
	25	0.8
9.0	9	0.8

4.2.2 Ultrafiltration and diafiltration of pretreated whey

There are more than one factor that can help UF/DF processes to be effective with high enough flow rate including pH of whey, area of membrane, temperature, pressure, membrane type, physicochemical properties of components of whey. This was also reported in previous studies (Butylina et al, 2006; Rektor and Vatai, 2004). Three main factors resulting in differences in fluxes are: 1) interactions between the membrane and feed, 2) effects of mass diffusivity and 3) the viscosity of the feed (Baldasso et al, 2011). The whey permeate flux was smaller than the water flux. It can be due to

concentration-polarization effect. The increase of flux by pressure is limited by an increased polarization layer, i.e., the increase of transmembrane pressure is counter-balanced by the increase in total resistance. Baldasso et al. (2011) reported thatat different DF step, the permeate flux was below the initial flux in UF due to the fouling formed in the protein-concentration stage. But in this study, as the DF was started the next day and membrane was washed and cleaned, the permeate flux was close to that in UF step.

The most important factor to optimize UF process is flux or flow rate. The more flow rate, the more volumetric concentration factor (VCF) is obtained in the retentate. Membrane can be blocked in two ways: 1) Surface blocking and 2) Membrane pore blocking (Zhu et al, 2016). Molecules larger than MWCO results in membrane surface blocking if the conditions, pH and temperature results in coagulation of components in the feed. Mesurements of permeate flux was started after 10 min as a beginning time.

Proteins interact with membrane surface lead to membrane fouling. This interaction can happen by hydrogen bonding, electrostatic interaction and Van der Waals forces (Lee et al, 2003). This interaction between proteins and membrane surface depends on protein surface charge. At different pH values proteins contain different charge. Positive protein attract to negative layer of membrane. This can be lead to fouling (Cheryan, 1986). Besides electrostatic interactions between membrane and whey proteins during the UF, aggregation and flocculation of whey proteins on the surface of membrane can result in flux decay. Normaly the permeate flux slows by passing time and increasing in VCF (Smith, 2003). The flux decay was described according to higher osmotic pressure, concentration of solutes in polarized layer and increasing of resistance to permeation (Bacchin et al, 2006; Cheryan, 1986; Rektor and Vatai, 2004).

The mechanism of separation by PES membrane was based on surface interactions, solution pH and fouling mechanisms. Interactions can occur between surface layer of membrane and charged proteins and ions in whey as the PES membrane has a negatively charged active layer (Cheryan, 1986). Charge of a protein depends on pH of the solution it is in. Charge of a protein or peptide depends on its isoelectric point. Isoelecteric points of major whey proteins, α-la and β-lg are 4.4 and 5.2, respectively (Ding et al, 2013). Ip of aCMP is 3.15 and that of gCMP is 4.15 (Kreuβ et al, 2009).

Most whey proteins are negatively charged at pH 7 and 9, therefore, they were rejected by the membrane. Wu et al. (2015) was reported that large whey proteins can be positive at pH lower than 5 and negative at pH higher than 5 by measuments of zeta potential of feed. As pH 5 is close to Ip of major whey proteins, they have a tendency to aggregate and possibly precipitate on the membrane surface. Molecular weight of whey proteins, their shapes and flexibility were reported as factors that help the proteins to pass the deposit layer and membrane. The pore size of membrane and its geometry of the pores can effect the filtration process (Akoum et al, 2005; Heidebrecht et al, 2018).

UF/DF 9 L of feed was carried out until 1.8 L of retentate was obtained which corresponded to VCF of 5 (Table 4.6). Mean flux of permeate was 46.4 $Lm^{-2}h^{-1}$ at pH 7 with 25 kDa membrane and 29.2 $Lm^{-2}h^{-1}$ at pH 9 with 9 kDa membrane. As the water flux was different in each case, the relative mean flux values were calculated. The retentate was collected and held at 4°C overnight. The membrane and the unit were cleaned. On the next day, water at the same volume as the retentate was added to the retentate and DF was performed with the cleaned membrane. As the flux was very high and there was loss of protein to the permeate, DF was carried out once in the case of the membrane with MWCO of 25 kDa (Fig 4.2). Four DF was possible in the case of the membrane with MWCO of 9 kDa. Total process time was different in each case as the flux was different. As the flux was higher through 25 kDa membrane, process time was considerably shorter than that with the 9 kDa membrane.

Table 4.6 : Ultrafiltration and diafiltration at pH 7 and 9 values with PES membranes of 25 and 9 kDa MWCO.

pH of feed	Membrane MWCO (kDa)	Dry matter of whey (%)	Number of diafiltration	UF-DF time (h)	Relative mean flux ($Lm^{-2}h^{-1}$)	Volumetric concentration factor
7	25	7	1	4-1	0.82	5
9	9	7	4	5-8	0.81	5

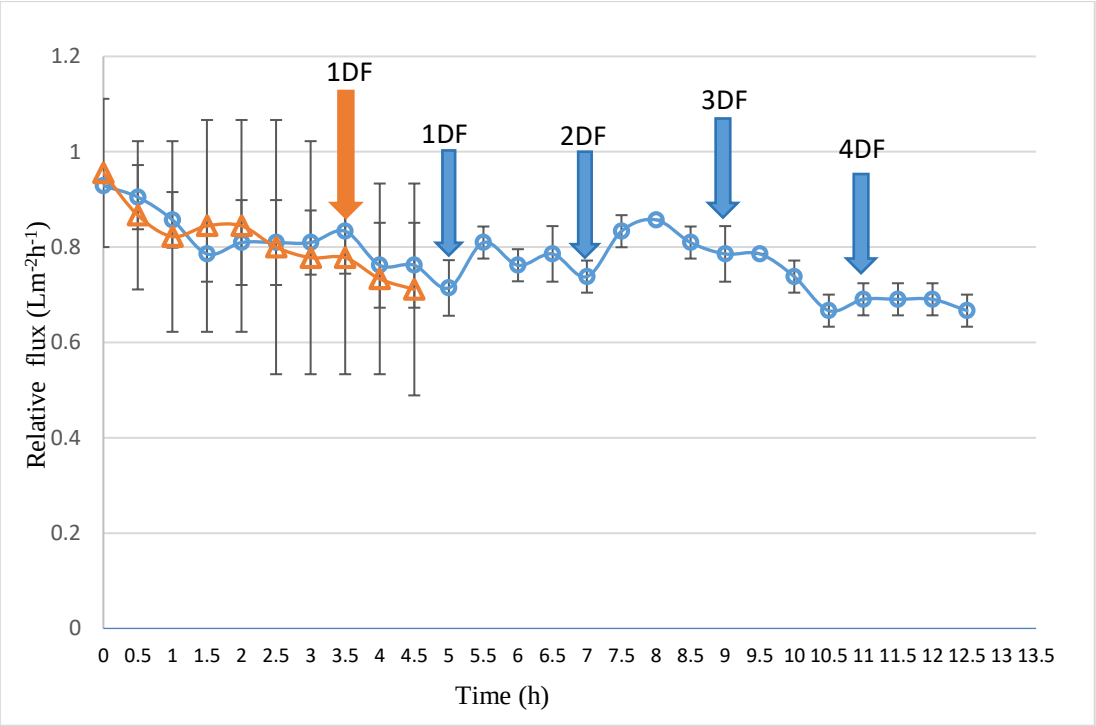
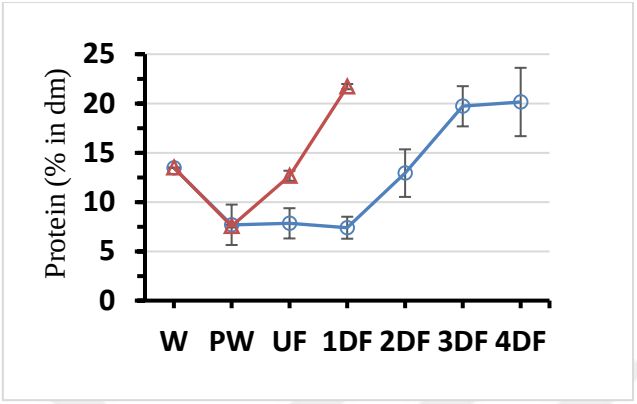


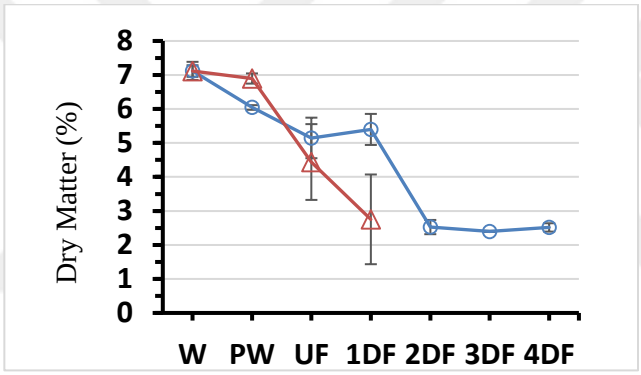
Figure 4.2 : Relative flux decay during ultrafiltration and diafiltration at pH 9 with the 9 kDa MWCO membrane (Δ) and pH 7 with 25 kDa MWCO (○) at 3 and 5 bar and 50°C.

Samples taken from the retentates obtained from UF and DF were analysed for gross and protein composition to evaluate the effect of concentration on components, especially proteins and CMP (Fig. 4.3). Protein content of retentate increased while its lactose content was reduced by the process. Ash content was not changed in UF but it increased as the DF was repeated. The minerals accumulated during DF cycles possibly were held by proteins, CMP and membrane surface. CMP content was increased while other whey proteins remained at the same concentration after 3 DF runs. However, there was a slight increase at 4th DF cycle in concentration of other whey proteins, this could be due to inceasing total crude protein by washing out more lactose. As molar masses of whey proteins (14.2 kDa α-la, 18.3 kDa β-lg) are larger than 9 kDa, this membrane rejected these whey proteins. In addition, β-lg typically forms non-covalent dimers with a MW of 36 between pH 5.3 and 8.0 (Bonnaillie et al, 2014). Molar mass of CMP monomer was reported as 6-11 kDa which was also rejected by this membrane (Holland et al, 2006). In addition, CMP can polymerize depending on pH, it can form aggregates of 20-50 kDa at pH 7 and 35 kDa at pH 8 (Mikkelsen et al, 2005; Rojas and Torres, 2013) that could lead to more rejection of it. Aggregation phenomenon depending on pH can also affect efficiency of separation process by UF/DF. If more of lactose and ash were removed, the concentration of CMP

would be increased more in the retentate. However, the UF system used in this study did not allow removal of more lactose, losses in CMP was observed when DF was continued. Pre-treatment of the whey was essential to eliminate other whey proteins and increase purity of CMP before UF.



(a)



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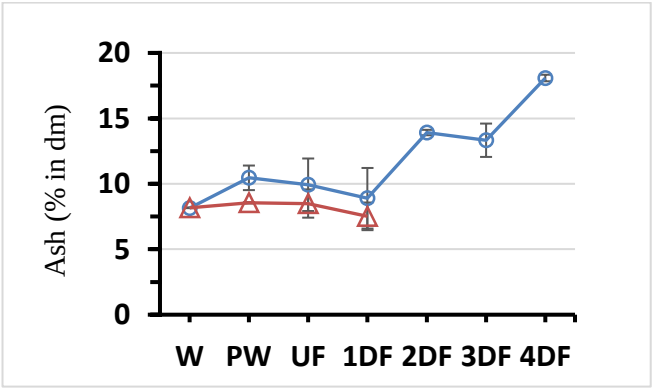
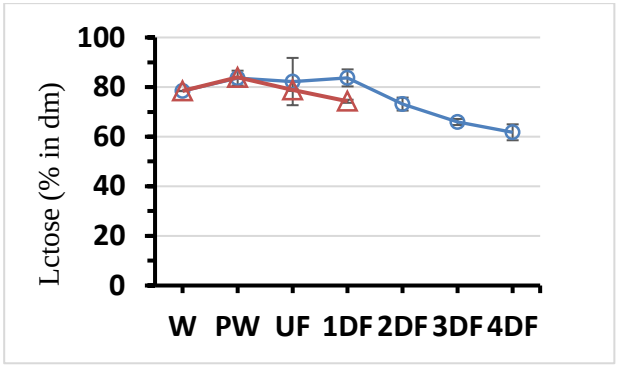


(c)

Figure 4.3 : Variation of concentrations (in dry matter) of components of whey in retentate (a) crude protein, (b) dry matter, (c) ash, (d) lactose, (e) CMP, (f) α-lactalbumin and (g) β-lactoglobulin during ultrafiltration and diafitration process at pH 9 with the 9 kDa MWCO membrane (□) and pH 7 with 25 kDa MWCO (□) at 5 bar and 50oC. W: Whey, PW: Pre-treated whey.



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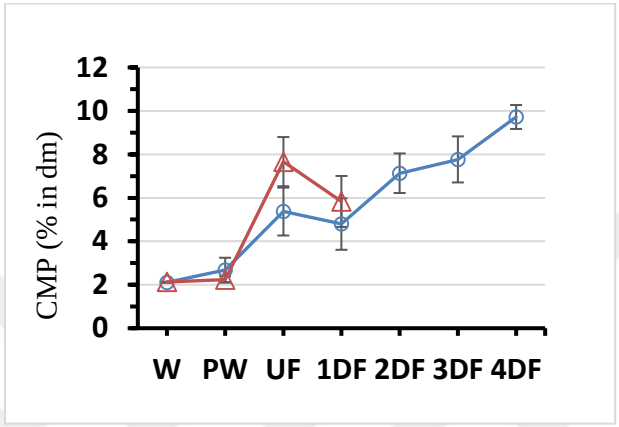

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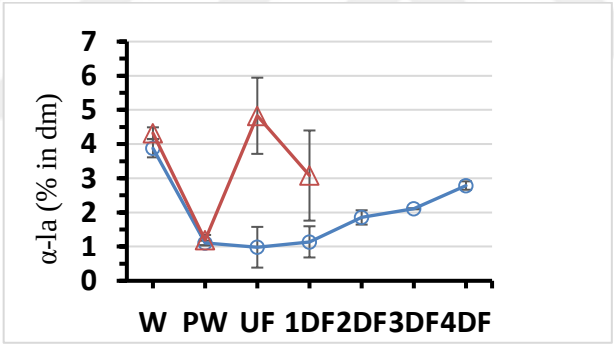
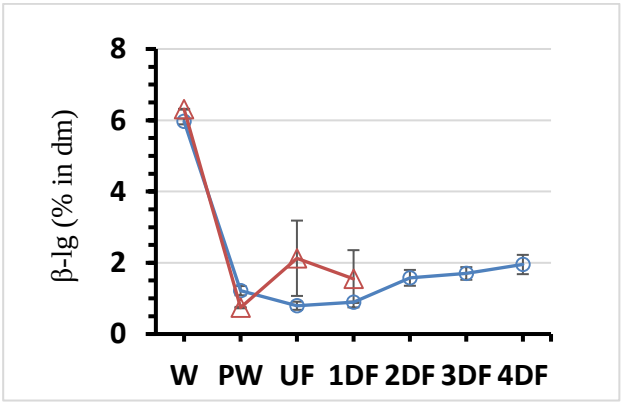


(f)



(g)

Figure 4.3 (Continued) : Variation of concentrations (in dry matter) of components of whey in retentate (a) crude protein, (b) dry matter, (c) ash, (d) lactose, (e) CMP, (f) α-lactalbumin and (g) β-lactoglobulin during ultrafiltration and diafitration process at pH 9 with the 9 kDa MWCO membrane (□) and pH 7 with 25 kDa MWCO (□) at 5 bar and 50oC. W: Whey, PW: Pre-treated whey.

Rejections of components of feed by PES membranes were calculated and data was given in table 4.8. In general, more rejection of proteins lead to more protein content in retentate. Higher rejections of protein components were obtained by 9 kDa membrane compared to those by 25 kDa. Fouling and pore blocking of membrane by protein components and salts reduced rejection and increased retention of lactose and ash especially in the case of 9 kDa membrane. Higher CMP rejection was obtained with 9 kDa membrane. While rejection of ash followed the same trend as CMP, rejections of α-la, β-lg and lactose were not different in two cases.

Table 4.7 : Rejection of different components during UF/DF with two different PES membranes.

pH of feed	Membrane MWCO (kDa)	CMP (%)	α-lactalbumin (%)	β-lactoglobulin (%)	Ash (%)	Lactose (%)
7	25	37	24	18	12	12
9	9	62	22	20	24	14

4.2.3 Composition analysis of CMP concentrate obtained by the process

UF and DF were applied to obtain CMP concentrate with higher purity from pre-treated whey. The aim was to force more lactose and ash passing to the permeate. Crude protein content of isolated CMP concentrate at pH 7 and pH 9 was found 15% and 21% in dry matter, respectively. These values are lower than those reported by Martín-Diana et al. (2002) (78.9%), Lieske et al. (2004a) (89.3%), Ergül and Karakaya (2013). Therefore, product produced from this study was named as concentrate not an isolate. A commercial CMP powder produced based on a patent by Holst and Chatterton (2002) was used as a reference contained protein at a level of 80%. CMP content of this product was reported as 64% which was 6-10 times higher than that in CMP concentrate isolated in this study. If more lactose and ash can be removed from this concentrate, concentration of CMP can be increased.

Theoretically, most of the lactose and ash could be removed using more than one DF step (Baldasso et al, 2011; Rektor and Vatai, 2004, Butylina et al, 2006). The lowest level of lactose in dry matter (62%) was found at pH 9 with 9 kDa MWCO membrane with 4 DF cycles. The highest amount of crude protein (22%) was found in the UF/DF process at pH 9 with 9 kDa membrane. Martín-Diana et al. (2002) found that 76.8%

protein, 8.2% lactose and 11.1% ash in CMP isolate obtained from pre-treated whey (pH 3.5, 90ºC/1h) after UF/DF process at pH 7. Ergül and Karakaya (2013) reported 72.8% protein, 2.3% lactose and 2.8% ash in CMP isolate from pre-treated whey (pH 5.2, 90ºC/1h) after UF/DF at pH 5.2 according to the method of Martín-Diana et al. (2002). Similar process was applied in this study with some modifications but lower protein and higher lactose contents were achieved in the final concentrate. The ash content was found higher with UF with 9 kDa membrane in this study than those reported with 10 kDa membrane in the literature. In this study, pore blocking of membrane by protein monomers, small peptides and minerals and lesser efficiency of the membrane and UF unit used might be the reason for lower degree of separation. Moreover, pH can be an effective factor on minerals to bound with whey proteins and concentated in retentate. On the other hand, the range of ash in commercial whey protein concentrate and nonfat milk powder can vary between 4 to 9% which covers the ash content of CMP concentrate obtained from 25 kDa membrane.

Table 4.8 : Composition of isolated CMP concentrate obtained from UF/DF*.

Sample	Membrane MWCO (kDa)	Dry matter (%)	In dry matter		
			Crude protein (%)	Ash (%)	Lactose (%)
Whey	-	7.1 ± 0.1	13.5 ± 0.1	8.1 ± 0.1	78.4 ± 0.1
Pre-treated whey pH 5	-	5.9 ± 1.0	7.6 ± 0.2	8.5 ± 0.0	83.9 ± 0.2
Feed pH 7	25	2.8 ± 0.9 [a]	21.7 ± 0.4 [a]	7.5 ± 1.5 [b]	74.3 ± 6.1 [a]
Feed pH 9	9	2.5 ± 0.9 [a]	20.2 ± 3.5 [a]	18.1 ± 1.6 [a]	61.8 ± 3.7 [a]

*Values are means ± SD (n=3). Means marked by different letters are significantly different ($P < 0.05$).

CMP content of the concentrate from 9 kDa membrane was higher compared to that from 25 kDa membrane. On the other hand, other whey proteins were at similar concentrations in both concentrates. Concentrations of two aromatic amino acids, Phe and Tyr, as indirect purity indices were determined in the CMP concentrates. Since CMP lacks these amino acids, their presence and concentration indicate level of purity of the concentrate. Levels of Phe and Tyr in two concentrates were similar. In this study, the main step that can effect the purity of isolated CMP concentrate was efficient elimination of α-la and β-lg by pre-treatment of sweet whey before UF. Therefore, this result was expected. Sialic acid contents of obtained concentrates were found similar.

Table 4.9 : Protein composition and sialic acid content of isolated CMP concentrate on dry basis obtained from UF/DF.

Sample	MWCO (kDa)	CMP (%)	α-lactalbumin (%)	β-lactoglobulin (%)	Phenylalanine (%)	Tyrosine (%)	Sialic acid (%)
Whey	-	2.1 ± 0.0	2.9 ± 1.03	6.0 ± 0.1	0.33 ± 0.03	0.51 ±0.07	0.24 ±0.04
Pre-treated whey pH 5	-	2.2 ± 0.2	1.2 ± 0.2	0.74 ± 0.03	0.08 ±0.01	0.08 ±0.01	0.25 ±0.03
Feed pH 7	25	5.8 ± 0.2 [b]	3.1 ± 0.9 [a]	1.7 ± 0.5 [a]	0.26 ± 0.04 [a]	0.32 ± 0.01 [a]	0.85 ± 0.13 [a]
Feed pH 9	9	9.7 ± 0.9 [a]	2.8 ± 0.6 [a]	1.8 ± 0.5 [a]	0.29 ± 0.03 [a]	0.34 ± 0.02 [a]	0.85 ± 0.10 [a]

* Values are means ± SD (n=3). Means marked by different letters are significantly different ($P < 0.05$).

4.2.4 Recovery of CMP from whey and purity of CMP concentrate by the process

The amount of CMP in dry matter was found to be higher in the concentrate produced by 9 kDa membrane compared to that obtained with 25 kDa membrane (table 4.11). Recovery of CMP frpm whey was found 58% at pH 9 with 10 MWCO membrane by the process. Martín-Diana et al. (2002) reported recovery of CMP as 69% and Ergül and Karakaya (2013) found 71% by a similar process. The efficiency of PES membrane and UF unit could result in lower CMP in the concentrate in this study. Even though the CMP contents of concentrates from two membranes were not similar, their sialic acid contents were similar. This shows that more of CMP isolated with 25 kDa membrane was glycosylated compared to that in the isolate obtained with 9 kDa membrane. The amount of sialic acid in crude protein (4%) in this study is close to that reported by Martín-Diana et al. (2002) (4.4%).

Table 4.10 : Recovery of CMP and sialic acid after UF/DF from sweet whey.

pH of feed	MWCO (kDa)	Recovery of dry matter (%)	Recovery of CMP (%)	Recovery of sialic acid (%)
7	25	15	33	43
9	9	12	58	44

The purity of isolated CMP concentrate at pH 9 with 9 kDa membrane was compared to the values reported in the literature. Concentration of CMP in isolate and in protein was reported as 75.56%, 57.66% and 64% and 87.4%,72.8% and 46.2% by Martín-Diana et al. (2002), Ergül and Karakaya (2013) and Arla Foods (Lacprodan® CGMP-10, Arla Foods), respectively. In this study, the highest concentrations of CMP in dry matter and in protein were 9.7%. and 46.2%. Concentrations of Phe and Tyr in protein was found as 1.38% and 1.62% in the concentrate, respectively in this study. The concentrate contained less Phe in protein than the isolate produced by Martín-Diana et al. (2002) (2.8%). Despite less content of protein and CMP in the final concentrate, its purity in terms of Phe and Try in protein was close to that of a commercial CMP isolate

(Lacprodan® CGMP-10, Arla Foods, 0.8 and 1.1% in protein Phe and Tyr, respectively).

Experiments in this part put forward two ways of concentration of CMP from pre-treated sweet whey with acceptable recovery. These methods yielded two products with varying amounts of CMP with acceptable purity. The concentrates can be used in suitable formulations as they contained lower amounts of Phe and Tyr compared to those present in commercial protein powders.

4.3 Optimization of Rice Drink by RSM

4.3.1 Model fiting

Structure of rice drink was formed primarily by starch and xanthan gum and secondarily by rice proteins. Therefore, important factors on structure formation and stability of drink, amounts of rice flour and xanthan gum, and temperature of heat treatment were investigated for determination of optimum level of ingredients and process temperature (Table 4.11).

Table 4.11 : Model equations that relate independent and response variables.

Response	Factors [a]	p-value	Model fit	Model equation [a]
Zeta potential(ZP, mV)	A B C BC	0.0518 0.0034 0.1569 0.0452	R^2 =0.73 F = 6.75 P= 0.0067	ZP = -5.68-1.13A+1.95B+0.78C+1.31BC
Particle size (d, nm)	B C BC C^2	0.7887 0.1399 0.0168 0.0515	R2 =0.61 F = 3.94 P = 0.036	$\frac{1}{\sqrt{d}}$ = 0.025+0.0002B+0.0013C-0.0026BC-C^2
Sedimentation (%)	A B C	0.1334 0.3384 0.2685	R2 =0.31 F = 1.66 P= 0.2321	S= 0.216-0.93A+0.58B-0.67C
Phase separation (PS, %)	A B C AB AC BC	0.0001 0.0001 0.0023 0.0011 0.259 0.8227	R2 =0.96 F = 31.65 P= 0.0001	$\sqrt{PS}$ = 1.77-1.77A-1.28B- 0.82C+1.03AB+0.57AC-0.05BC
Apparent Viscosity ($\eta_{app@50s^{-1}}$,Pa.s)	B C	0.0003 0.0253	R2 =0.73 F = 16.19 P= 0.0004	ln (η)= -2.58+1.32B+0.6629C
Flow behavior index (n)	A B AB	0.1675 0.0031 0.0125	R2 =0.70 F = 8.43 P= 0.0034	n= 0.5459-0.0332A 0.0847B+0.0748AB
Consistency coefficient (K, $Pa.s^n$)	A B C AB BC B^2	0.0054 0.0001 0.0001 0.0378 0.0001 0.0001	R2 =0.99 F = 92.07 P= 0.0001	K= 0.5775+0.3923A+1.90B+0.9164C+0.288AB+0.8694BC+1.53B^2
Thixotropy (Th, Pas^{-1})	A B C AB AC BC	0.6310 0.0001 0.0001 0.8027 0.0095 0.4400	R2 =0.98 F = 68.28 P= 0.0001	$\frac{1}{\sqrt{Th}}$= 0.0974-0.0013A-0.048B-0.025C-0.0008AB-0.0102AC+0.0024BC
Lvalue	A B C	0.1857 0.0001 0.0626	R2 =0.97 F = 117.92 P= 0.0001	L= 53.12+0.5A+6.72B+0.75C
a value	A B C	0.4863 0.0001 0.0024	R2 =0.93 F = 51.40 P= 0.0001	a= -0.63+0.015A-0.25B-0.08C
b value	A B C	0.0142 0.0001 0.0001	R2 =0.95 F = 83.18 P= 0.0001	b= -1.75+0.14A+0.62B-0.4C

[a] Coded Factors A: Xanthan gum, B: rice flour and C: temperature.

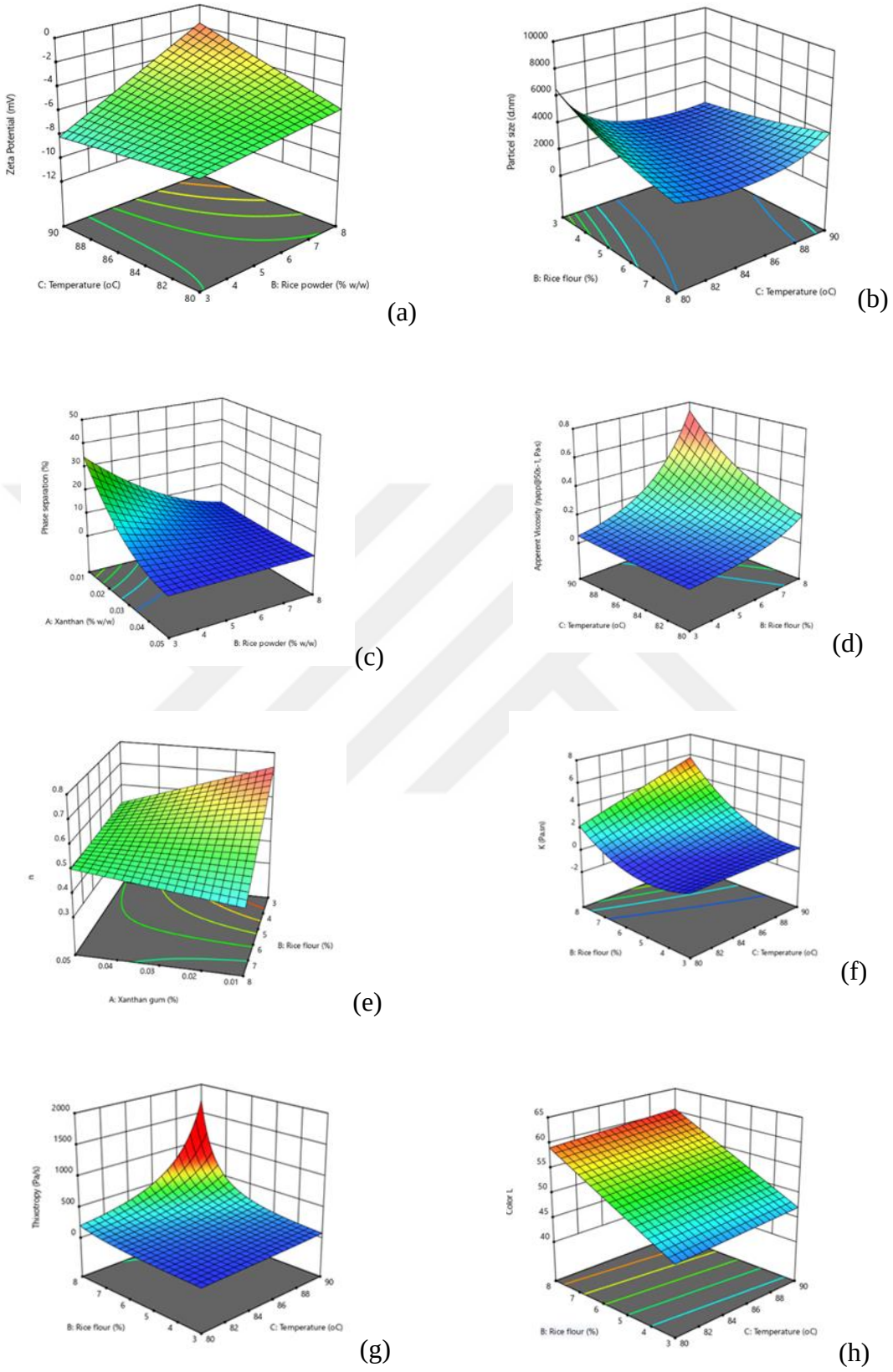


Figure 4.4 : 3D plots showing the combined effect of rice flour, xanthan gum and temperature on (a) Zeta potential (b) Particle size, (c) Phase separation, (d) Apparent viscosity, (e) Flow behavior index, (f) Consistency coefficient, (g) Thixotropy (h) Color L, (i) Color a, and (j) Color b.

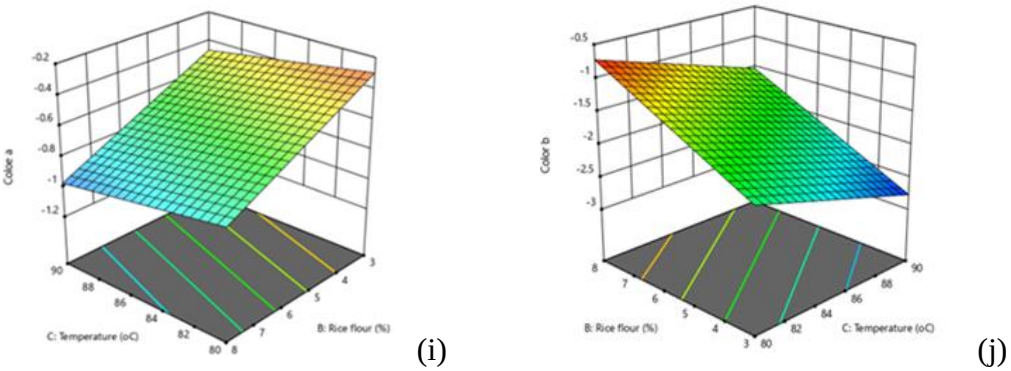


Figure 4.4 (Continued) : 3D plots showing the combined effect of rice flour, xanthan gum and temperature on (a) Zeta potential (b) Particle size, (c) Phase separation, (d) Apparent viscosity, (e) Flow behavior index, (f) Consistency coefficient, (g) Thixotropy (h) Color L, (i) Color a, and (j) Color b.

4.3.2 Physical properties analyses of formulated rice milk

4.3.2.1 Zeta potential and particle size

Temperature and level of rice flour affected the particle size of the drink. Both a low temperature and a low level of rice flour increased the particle size. There was more effect of level of rice flour as the temperature was reduced. These could be due to protein denaturation and aggregation occurring more at high level of rice flour and temperature. There was no effect of xanthan gum on particle size. Pre-homogenization and using a high pressure homogenizer for more than one time were suggested to obtain fine emulsion (Gharibzahedi et al, 2018). Homogenization at high pressure can be applied to form finer particles and a homogeneous structure in rice milk.

Zeta potential of rice drink was affected by level of rice flour and interaction between rice flour and temperature. As the level of rice flour and temperature were increased, zeta potential approached zero, which means that the neutralisation of charged groups occurs as a consequence of the change in rice flour and temperature interaction. When the level of rice flour was raised, increase in temperature reduced negative charge. However, the trend was opposite at the lowest level of rice flour. The charged particles such as proteins, ions and ionic gums such as xanthan gum in a dispersion can affect zeta potential. Aggregation of proteins above temperature of denaturation possibly play a significant role on value of zeta potential. Zeta potential of light milk was

measured as -2.8 ± 0.4 mV which means a lower charge compared to that of rice drink. Usually zeta potential values lower than -25 mV do not ensure the stability of dispersed systems (Roland et al, 2003). The rice protein charge will decrease, thus promoting a reduction in the zeta potential and repulsive forces among the dispersed particles. This effect will lead to the phenomenon of sedimentation in the system. Particle sedimentation will be responsible for the increase in number of particle size. This result was coherent with pI range of rice proteins (4.1–4.8) (Ju et al, 2001).

4.3.2.2 Sedimentation and phase separation

There was sedimentation in some of the samples, but there was no clear trend according to the factors. Although sedimentation was slightly reduced as level of rice flour decreased and level of xanthan gum and temperature increased. The sedimentation was the primary mode of destabilization in the rice milk. The rice and oat milks were reported to have significant fractions of large particles that were denser than water compared to other plant-based milks (Duranda et al, 2003). A straight line at zero delta back scattering across the sample length indicates a stable sample in which no changes occurred. In this case, the delta backscattering level at the bottom of the sample increased due to an increasing amount of particles moving to the bottom of the sample. This was coupled with clarification (a decrease in backscattering) at the top of the sample, reflecting decrease in particle concentration at the top of the sample.

Phase separation was reduced by increasing values of all factors examined. In addition, a significant interaction between the levels of rice flour and xanthan gum was observed ($p < 0.05$). There was no phase separation at high levels of rice flour or xanthan gum. Effect of xanthan gum on phase separation was reduced at high levels of rice flour. On the other hand, the effect of heating temperature on phase separation decreased at high levels of xanthan gum.

4.3.2.3 Rheological properties

Formulated rice drinks exhibited non-Newtonian behavior with flow behavior index less than 1. This could be due to the presence of partial gelatinization of starch (Lee at al, 2019). The value of n greatly depends on the extent of starch granule swelling. Heating of rice drink at different temperatures would result in final products with different level of swelling of starch granules.

The consistency coefficient of rice milk was influenced by levels of rice flour and xanthan gum and temperature ($p < 0.05$) (Table 4.11). The level of rice flour had quadratic effect on consistency. There were interactions between effects of the levels of rice flour and temperature and between the levels of rice flour and xanthan gum. Gelatinization temperature of rice starch was reported to occur in the range of 62-82^{o}C (Waters et al, 2006). Proteins in rice flour can also be denatured and aggregated depending on temperature. The denaturation temperatures of different protein fractions of rice varied from 73^{o}C to 82^{o}C (Ju et al, 2001). High temperature (more than 80^{o}C) can induce unfolding, denaturation and aggregation of rice proteins. This could increase consistency in addition to starch gelatinization and xanthan hydration. Consistency increased at a greater extent as the the level of rice flour and temperature of heating were increased (Bernat et al, 2015). There was a slight effect of xanthan gum on consistency.

A thixotropic fluid can be described as a shear thinning system where the viscosity decreases not only with increasing shear rate but also with time at a constant shear rate. Change in thixotropy of the samples followed a similar trend to that of consistency coefficient. The area between upward and downward flow curves can be measured to determine thixotropic flow behaviour. Thixotropy values of samples ranged between 26 to 790 Pas^{-1}. Thixotropy of a commercial rice drink was measured 48 $Pa.s^{-1}$. Rice flour, temperature and interaction of xanthan gum with temperature were found as affective factors on thixotropy of the samples.

For apparent viscosity, there was no significant effect of xanthan gum on apparent viscosity where temperature and rice flour were effective factors. Apparent viscosity of commercial rice drinks were reported 3.2 mPa.s on average (Russell and Delahunty, 2004). Apparent viscosity of a commerical rice drink sold in a local market was measured as 50 mPa.s. Apparent viscosity of the samples changed in a wide range in this study. As the rice flour content was increased, apparent viscosity of the drink became too high to be acceptable as a drink. Developed rheological parameters can be used for estimation of apparent viscosity according to the power-law model.

4.3.2.4 Color

Color is one of the important qualities of foods which can effect acceptability of product by consumers. Rice flour was dominant on all color values of rice drink. Color

a value was affected by rice flour and temperature and color b value was affected by all factors. While rice flour intensified lightness, increase in temperature and xanthan gum incremented red and yellow tones. Low fat milk color values were measured as a of -3.08 ± 0.02, b of 4.16 ± 0.04 and L of 76.24 ± 0.1. L value of rice drink was lower than that of light milk and its b value was negative in contrast to those of low fat milk. These values showed that rice drink is less light with more red and yellow tones than low fat milk.

4.3.3 Optimization and validation

Predicted models that relate physical properties of rice drink with significant factors were developed and used in optimization (Table 4.12). Optimization was carried out for predicting the optimum level of factors to obtain minimum phase separation, particle size and apparent viscosity and zeta potential in the measured range in rice drink. An upper limit for apparent viscosity was set as 0.0216 Pa.s which was the apparent viscosity of chocolate milk. Targeted conditions were achieved at a level of 3% (w/w) for rice flour, 0.05% (w/w) for xanthan gum and temperature of 80^{o}C according to the models for selected responses. Predicted response values under the optimum conditions for zeta potential, phase separation, particle size and apparent viscosity of the rice drink were -9.3 mV, 0.21%, 4394 nm and 0.007 Pa.s, respectively. The desirability of predicted model was found 84%.

Table 4.12 : Optimum levels of independent variables and corresponding predicted and measured response values.

Sample	Rice flour (% w/w)	Xanthan gum (% w/w)	Temperature (^oC)	Zeta potential (mV)	Particle size (nm)	Sedimentation (%)	Phase separation (%)	Apparent viscosity ($Pa \cdot s$, @$50s^{-1}$)	Flow behavior index (n)	Thixotropy (Pas^{-1})	Color L	Color a	Color b
Predicted	3	0.05	80	-9.3	4394	0	0.2	0.007	0.6	30.3	46.2	-0.3	-1.8
Validated	3	0.05	80	-10±0.5	5072±120	0±0.01	0±0.01	0.025±0.002	0.5±0.004	42±1.1	45±0.01	-0.25±0.3	-1.6±0.2

Optimized conditions were validated by dublicated trials. Measured values zeta potential, phase separation, particle size and apparent viscosity of rice milk with the optimum formulation were given in table 4.12. While, estimated and measured values for zeta potential, phase separation and particle size were similar, there was a difference between predicted and experimental value of apparent viscosity. These results suggested that the response models had high capability to predict the factors including combination of rice flour, xanthan gum and temperature for a stable rice drink based on.

4.3.4 Sensory analysis of the formulated rice drink

Sensory analysis was performed to determine acceptability of the rice drink compared to low fat milk. Unsweetened rice drink received lower overall acceptability compared to low fat milk. When sugar was included in the formula, the acceptability of rice drink increased. There were significant differences between low fat milk and the rice drinks in terms of color, particle size and graininess ($p < 0.05$). However, there was no difference in oiliness and bitterness between the samples. Consistensy and mouthfeel of sweetened rice drink was found significant different from light milk and similar to unsweetened. Sweetened rice drink was got the highest score compared to the other two drinks.

Table 4.13 : Sensory evaluation of the different formulated rice drinks and the light milk (1.5% fat) [a,b].

Sample	Consistensy	Particle size	Graininess	Color	Mouthfeel	Sweetness	Bitterness	Oiliness	Over all acceptability
Light milk	1.3±0.9[b]	0.3±0.7[b]	0.2±0.7[b]	2.8±1.3[a]	1.4±0.9[b]	1.6±1.1[b]	0.3±0.6 [a]	1.4±0.9 [a]	3.7± 1.0[a]
Sweetened rice drink	2.35±1.1[a]	2.35±1.4[a]	3.2±1.4[a]	0.9±0.9 [b]	2.55±1.4 [a]	2.9±0.9[a]	0.9±1.2 [a]	1.7±1.4 [a]	2.35±1.1 [ab]
Unsweetened rice drink	1.9±1.6[ab]	1.6± 1.4[a]	2.6±1.3[a]	1.4±0.3[b]	2.3±1.6[ab]	0.4±0.8[c]	0.9±1.2 [a]	1.9±1.7 [a]	1.9±1.2 [b]

* Values are means ± SD (n=3). Means marked by different letters are significantly different ($P < 0.05$).

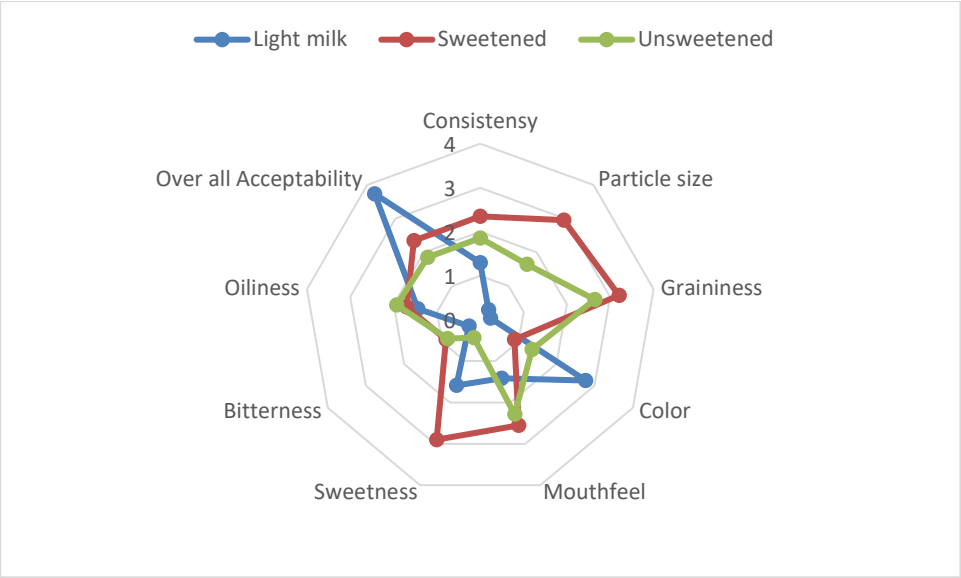


Figure 4.5 : Sensory properties of sweetened, unsweetened rice drinks and light milk.

4.3.5 Physical properties of rice drink with CMP concentrate

Sweetened rice drink was fortified with CMP concentrate at a level of 7%. There was no influence of CMP concentrate on lightness of rice drink. However, CMP concentrate changed color values of a and b making these closer to those of low fat milk possibly because of riboflavin. Consistency coefficient and flow behavior index values of the samples with and without CMP were similar but they were higher than those of low fat milk. Addition of CMP led to sedimentation in rice drink possibly caused by precipitation of proteins or salts. Particles were enlarged by CMP more likely by large proteins and peptides. Measured zeta potential value indicated that negative charge of particles was reduced. Fortification by CMP could introduce positively charged components such as protein fractions and ions into rice drink.

Table 4.14 : Physical properties of light milk, unsweetened, sweetened and CMP fortified sweetened rice drink (mean ± SD).

Sample	Color			Phase seperation (%)	Rheology properties				Sedimentation (%)	Particle size (d.nm)	Zeta potainal (mV)
	L	a	b		K ($Pa.s^n$)	n	Thixotropy (Pas^{-1})	Appearent viscosity at $50s^{-1}$ (Pa.s)			
Light milk	76.2 [a]	-3.1 [c]	4.2 [a]	0.0 [c]	0.013 [b]	0.7 [a]	40.7 [b]	0.003 [b]	0 [b]	323 [c]	-2.8 [a]
Unsweetened	45.0 [c]	-0.3 [a]	-1.6 [b]	0.5 [a]	0.10 [a]	0.5 [a]	23.1 [c]	0.025 [a]	0 [b]	4497 [b]	-11.2 [c]
Sweetened	45.0 [c]	-0.3 [a]	-1.6 [b]	0.5 [a]	0.13 [a]	0.6 [a]	24.5 [c]	0.023 [a]	0 [b]	5072 [b]	-10.8 [c]
Sweetened CMP	50.0 [b]	-1.7 [b]	4.3 [a]	0.3 [b]	0.18 [a]	0.6 [a]	47.2 [a]	0.034 [a]	0.8 [a]	8337 [a]	-6.4 [b]

*Values are means ± SD (n=3). Means marked by different letters are significantly different ($P < 0.05$).

Based on statistical analysis between rice drink fortified with CMP and sweetened rice drink there were significant differences ($p < 0.05$) in color b, particle size and zeta potantial. It seems adding CMP powder has no major effect on rice drink properties.

5. CONCLUSION AND RECOMMENDATION

A two-stage isolation method for production of a CMP concentrate from sweet whey was developed. In the first stage, heat (90^{o}C/1 h) and acid treatments were applied to remove other proteins from whey. Order of heat and acid treatments and pH value were found to be effective on protein composition, bound sialic acid content and purity of CMP isolate. Application of heat and then acidification to pH 5.0 is recommended for effective removal of other whey proteins without losing CMP and its bound sialic acid.

In the second stage, ultrafiltration and diafiltration with a unit with two tubular membranes were applied to concentrate and purify CMP from pre-treated whey. A sufficient flow rate or flux of permeate was critical in reaching a high concentration after the membrane process. pH of the pre-treated whey was changed to obtain a high enough permeate flow rate. In addition, MWCO of membrane was also effective on flow rate by the membrane process. As a result, two PES membranes with 9 and 25 kDa were used at pH 9 and 7, respectively, for UF/DF. A volumetric concentration factor of 5 in UF was reached in both trials with these membranes, but the process time was shorter in the trial with 25 kDa membrane due to higher permeate flow rate. Diafiltration was required to wash more of lactose and minerals from the retentate obtained by UF. One DF cycle was possible with 25 kDa membrane as there was a loss in CMP compared to four DF cycles with 9 kDa membrane. However, composition and recovery of CMP were found to be different in two trials. Higher CMP, ash and lactose contents were determined in the retentate obtained by the membrane with MWCO of 9 kDa compared to the membrane with MWCO of 25 kDa. While 58% of CMP in whey was recovered by using the membrane with MWCO of 9 kDa, 33% was recovered in the case of the membrane with MWCO of 25 kDa. α-La, β-lg, Phe and Tyr contents in dry matter of retentate which are related with purity of CMP were found similar in both cases. Both membranes can be used in processing of whey depending on the desired composition and process time. Other membranes made of different materials and other UF systems can be evaluated to improve concentration.

The CMP concentrate produced by UF/DF process with 9 kDa membrane was freeze-dried to a powder. This powder was used for fortification of a rice drink. First, production method of rice drink was developed by response surface methodology. Effects of levels of white rice flour (3-8% w/w), xanthan gum (0.01-0.1% w/w) and temperature (80-90^{o}C) on physical properties of rice drink were evaluated. These factors were optimized to produce a rice drink similar to milk-like drinks in terms of physical properties and stability. As a result of optimization, the levels of rice flour, xanthan gum and temperature were found 3%, 0.05% and 80^{o}C, respectively.

Sweetened (2.5%, w/w, sucrose) and unsweetened rice drink were compared with low fat milk for color, flavor and texture attributes by sensory analysis. Sweetened rice drink got higher scores in sensory analysis and was more acceptable and resembling more to low fat milk compared to unsweetened one. CMP was added to sweetened rice drink at level suitable for PKU patients. CMP affected color b value, particle size, apparent viscosity, zeta potential and sedimentation of the drink. However, these changes were not too excessive to influence overall acceptability of the drink. Better homogenization at high pressure could be applied to reduce particle size and increase stability of the drink with CMP concentrate. Furthermore, addition of fruit concentrate, vanilla or cacao powder can improve flavor and acceptability.

CMP can be isolated by the developed two-stage process at low cost with sufficient recovery and purity. The CMP concentrate produced can be used as a functional ingredient in various food formulations for not only PKU patients but also consumers with allergy to milk proteins. It could also be used to produce functional food products for health-conscious consumers. On the other hand, rice drink without CMP can be consumed as plant-based drink by vegetarian individuals.

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APPENDICES

APPENDIX A: Sensory analysis forms

APPENDIX B: Response surface methology data

APPENDIX A: Sensory analysis forms

Name: Date:

Descriptive Analysis for milk-like drinks
Explanation:
1) Taste the samples according to the presented order of the samples.
2) After finish evaluation of a sample, drink the water and clean your mouth with water before you taste other sample.
3) After examining the sample, please enter the code number in the appropriate box on the given scale. A box can contain more than one sample code.

Review each coded sample in terms of the following features.

Texture

Attribute	0 Extremely low	1 Low	2 A little	3 Average	4 High	5 Extremely high
Consistensy						
Particle size						
Graininess						
Color						
Odour						
Describe:						
Consistensy						

Taste

Attribute	0 Extremely low	1 Low	2 A little	3 Average	4 High	5 Extremely high
Mouthfeel						
Sweetness						
Biterness						
Oiliness (milk fat)						
Other taste Describe:						

Acceptibility

Age: ____

Please select your prefernce based on the texture and taste analysis of the samples.

Sample code	0 Extremely low	1 Low	2 A little	3 Average	4 High	5 Extremely high
123						
497						
865						

Texture Characteristic

Attribute	Descriptions
Consistency	Consistency felt in the mouth or sample flow rate from spoon. Examples:Water: 0 – Honey: 5
Particle size	Size of visible particles. Examples: Flour: 0 – Sugar crystal: 5
Graininess	The presence of a visible particle or clot in the sample Examples: Milk: 0 – Yogurt drink: 5
Color	Examples: White: 0 – Cream: 5
Odour	Describe: Examples: Water: 0 – High level: 5

Taste Characteristic

Attribute	Descriptions
Mouthfeel	Examples:Water: 0 – Pudding: 5
Sweetness	Examples:Non: 0 – Sugar: 5
Bitterness	Examples:Non: 0 – Coffee: 5
Oiliness (milk fat)	Examples:Non: 0 – Butter: 5
Other taste	Description:

APPENDIX B: Response surface methodology data

Table B.1 : Matrix of the central composite design (CCD) and experimental data obtained for the response variables (mean ± SD).

Run	Block	Rice flour (%)	Xanthan gum (%)	Temperature (°C)	Color			Phase separation (%)	Rheology				Sedimentation (%)	Partical size (d.nm)	Zeta potantioal (mV)
					L	a	b		Consistency coefficient (K, $Pa.s^n$)	Flow behavior index (n)	Apparent viscosity ($Pa \cdot s$, @$50s^{-1}$)	Thixotropy (Pas^{-1})			
1	1	3	0.01	80	46.36±0.2	-0.47±0.02	-2.25±0.02	50±0.001	0.02±0.0002	0.7±0.002	0.007±0.00001	43±1.0	6.4±0.05	9063±501	-3.1±0.1
2	1	3	0.05	90	53.46±0.2	-0.73±0.01	-1.78±0.07	5±0.01	0.56±0.01	0.6±0.004	0.040±0.002	95±2.5	0±0.15	2845±32.7	-4.9±0.5
3	1	8	0.01	90	59.68±0.1	-1.06±0.0	-1.63±0.06	0±0.0	5.0±0.04	0.45±0.003	0.415±0.001	769±0.5	5.8±0.12	4313±303.7	-11±0.01
4	1	8	0.05	80	47.97±0.18	-0.51±0.02	-0.72±0.05	0±0.0	0.43±0.003	0.43±0.0005	0.305±0.002	180±0.5	3.1±0.00	2331±409.6	-9.5±1.7
5	1	5.5	0.03	85	58.92±0.1	-0.81±0.01	-0.72±0.05	0±0.0	2.75±0.0.07	0.52±0.002	0.085±0.0003	141±0.6	6.0±0.05	2034±63.6	-6.2±1.6
6	2	8	0.05	90	53.55±0.01	-0.52±0.01	-1.65±0.03	5±0.01	0.5±0.01	0.6±0.002	0.470±0.001	789±2.2	0±0.05	2660±189.8	-5.5±1.1
7	2	8	0.1	80	44.01±0.01	-0.32±0.01	-2.85±0.01	25±0.01	0.07±0.003	0.7±0.003	0.022±0.00001	161±1.5	3.3±0.00	2257±51.9	-7.15±0.3
8	2	3	0.05	80	44.91±0.07	-0.24±0.01	-1.64±0.03	0.05±0.01	0.16±0.02	0.5±0.0004	0.017±0.0001	26±1.4	0±0.0	4497±432.2	-11.2±1.5
9	2	5.5	0.03	85	58.25±0.02	-0.68±0.01	-0.65±0.01	10±0.01	1.05±0.0005	0.6±0.0003	0.085±0.0001	88±1.2	3.0±0.2	3623.7±435.9	-6.6±1.1
10	2	3	0.01	90	60.32±0.04	-0.9±0.01	-1.24±0.05	0±0.0	6.1±0.2	0.44±0.0008	0.020±0.003	48±0.9	0.0±0.0	2772.7±373.3	-3.5±1.3
11	3	5.5	0.01	85	54.34±0.2	-0.52±0.01	-1.53±0.05	0±0.0	0.94±0.03	0.55±0.005	0.613±0.001	102±1.1	0.8±0.05	2022.7±55.1	-5.23±1.6
12	3	8	0.03	85	46.51±0.1	-0.35±0.01	-2.28±0.02	10±0.01	0.1±0.006	0.65±0.007	0.415±0.0008	758±2.1	0.0±0.0	2304±82.0	-9.1±2.4
13	3	5.5	0.03	90	53.00±0.1	-0.61±0.01	-1.84±0.01	10±0.01	0.3±0.03	0.63±0.01	0.15±0.0002	270±2.3	0.3±0.02	2083.7±260.0	-6.3±1.0
14	3	3	0.03	85	52.9±0.07	-0.57±0.01	-1.37±0.05	5±0.01	0.15±0.01	0.7±0.009	0.016±0.0000001	49±1.2	0±0.0	4343.3±356.3	-5.2±1.3
15	3	5.5	0.03	80	56.98±0.27	-0.82±0.01	-2.18±0.13	0±0.0	1.7±0.15	0.5±0.02	0.040±0.000001	62±2.3	0±0.1	3682.3±217.1	-3.1±0.8
16	3	5.5	0.05	85	59.73±0.04	-0.97±0.01	-1.27±0.05	0±0.0	4.9±0.3	0.5±0.01	0.120±0.0001	127±1.2	4.0±0.0	3358.7±138.3	-3.2±0.8
17	3	5.5	0.03	85	53.63±0.045	-0.69±0.01	-2.19±0.03	0±0.0	1.4±0.1	0.5±0.01	0.140±0.0001	168±2.1	2.3±0.1	1891.7±185.8	-4.93±0.9

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