Recovery and application of whey proteins in conventional and nonconventional food systems

Dissertation submitted to the Faculty of Science and Technology, University of Coimbra, to the Degree of Doctor in Philosophy (PhD) in Chemical Engineering, specialization in Chemical Processes

Marta Helena Fernandes Henriques

2012

Work financially supported by

FCT Fundação para a Ciência e a Tecnologia

MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

under the program Bolsas de Doutoramento

(SFRH/ BD/ 36645/ 2007)

Dedico este trabalho aos meus pais:

Maria Helena Fernandes e Luis Henriques

Obrigada por tudo…

Abstract

Whey is the liquid that separates from the curd during cheese or casein manufacture. It is the main by-product of the cheese industry (≈9 L of whey per 1 kg of cheese), representing a serious environmental problem (BOD = 35-45 g/L). Its disposal or waste management involves high investments and operational costs that provide no profits for the cheese producers. However, whey is an important source of proteins with a high functional, nutritional and biological value, thus justifying its commercial use. Conventional practices for the valorisation of whey comprise the manufacture of dried products such as whey powder, whey protein concentrate (WPC) and isolate (WPI) and lactose, using concentration processes such as reverse osmosis and evaporation, or ultrafiltration followed by spray-drying of the retentates and/or permeates. However, the high costs associated with the dehydration step mean that these technologies cannot be used in the medium/small scale industries which represent 79% of the dairy sector in Portugal and a significant volume of the business (37%).

This thesis focuses on the development of innovative solutions for the valorisation of whey, in particular whey proteins, in medium/small scale cheese production. Batch ultrafiltration (UF) and diafiltration (DF) were used to produce liquid whey protein concentrates (LWPC) and dehydrated whey protein concentrates (WPC) of bovine and ovine origin. The products obtained were applied to food products, namely fresh cheese and set yogurt, to partially replace conventional ingredients, and as a raw material in lactic gels to produce innovative dairy products. Nonconventional applications were also explored, such as the production of films and coatings with bioactive properties.

The effects of the production process configurations and operational conditions on the characteristics of liquid and dry whey protein concentrate products were evaluated. Bovine LWPC with protein contents ranging from 61% to 87% (dry basis) were obtained. Diafiltration performed in volume reduction mode produced the best results in terms of protein content (20% higher than in the UF process) and purity. The ovine products (LWPC and WPC) had higher protein yields (62-84%, dry basis), making them more attractive for valorisation.

It is common practice in the industrial manufacture of cheese and yogurt to increase the total solids content by applying skimmed milk powder, caseinates or protein concentrate powders in order to increase production yields and overcome syneresis problems. However, a less expensive alternative is to use LWPC, although this has not yet been explored in yogurts and or fully investigated in cheese. The production of fresh cheese and set yogurt incorporating various levels of LWPC proved to be an asset, not only by increasing production yields but also by improving the functional and nutritional properties of the final products. With regard to fresh cheese, the incorporation of LWPC increased product stability during storage and spontaneous syneresis reduction. No differences were found in texture between the conventional and tested products, although sensorial attributes were affected by the amount of LWPC that was incorporated, which indicates that this is an essential parameter to control. Only high levels of incorporation penalise product acceptance.

The good performance of bovine LWPC in yogurt, the higher protein content of ovine LWPC and the fact that this type of whey is available from national producers led to an evaluation of their use in yogurts. Yogurts produced from bovine milk incorporating (bovine and ovine) LWPC and ovine yogurts were compared to conventional bovine yogurts. It was found that the incorporation of LWPC in set yogurts helps to reduce hardness, gumminess and viscosity, and consequently increases syneresis. These results indicate that a more open gel structure with a lower water retention capacity was produced. Moreover, the modest syneresis values obtained for all the formulations tested (typical of yogurts with an high total solids content) revealed the high functionality (gelation capacity) of LWPC. The protein content in ovine yogurts was significantly higher them in bovine yogurts with or without the incorporation of LWPC. On a sensorial level, no differences were found between the conventional bovine yogurts and those incorporating LWPC, indicating that regardless of the origins of the LWPC (bovine or ovine) the sensorial properties of set yogurts were not affected. However, differences were detected for ovine yogurts, which received a poor classification in the preference test.

One very attractive market opportunity, which has not yet been explored, is the use of LWPC as a raw material in the manufacture of innovative products. Its high protein content and the well-established functional and nutritional value of its proteins are good indicators *per se* of its high added value. The physicochemical, textural and rheological properties of thermal gels produced from LWPC were evaluated in terms of the type of LWPC (skimmed or unskimmed, produced by ultrafiltration, or skimmed produced by diafiltration), protein concentration (5% and 7%, dry basis) and pH (4 and 7). Non-defatted LWPC, high protein concentrations and lower pH values lead to stronger thermal gels. LWPC produced by diafiltration at pH 7 with a 5% protein concentration did not produce solid structures, probably due to the lower intermolecular interactions prevailing under these conditions. All acid gels produced by bacterial fermentation (yogurt type) and by glocono-δ-lactone (GDL) acidification (dessert type) showed viscoleastic behaviour, regardless to skimmed milk powder (SMP) fortification. The yogurt type gels were the weakest gels. It was concluded that, depending on the gelation process adopted and the manufacturing conditions, products with specific chemical and rheological properties could be obtained. This versatility enables the desired product to be manufactured with the appropriate characteristics.

Increasing environmental concerns about replace synthetic polymers with natural ones is stimulating research into new materials, particularly those obtained from nonconventional sources such as agro-residues. Whey proteins may be included in this premise. The research carried out so far in this field has used WPI as the base material and the solvent casting method to produce edible films and coatings. However, this research studied the performance of WPC as a less expensive alternative to WPI and the application of UV irradiation to promote film modification. The chemical composition of the film and the modification method affected the physicochemical, optical and barrier properties in specific ways. Using WPC with 50% protein and 40% lactose resulted in the production of films with attractive functional properties. The colour of the films was slightly penalised by the presence of lactose, but the film transparency remained within the range of values obtained for commercial synthetic films (oriented polypropylene (OPP) and polyethylene (PE)). UV irradiation enabled films to be produced with increased hydrophobicity and less moisture content. However, their greater solubility may limit their applicability under high humidity conditions. The molecular structure observed by FTIR revealed the presence of weaker chemical bonds between the formulation components using UV modification, which leads to less thermally stable films. The mechanical properties were not

affected by the modification method, although their modest tensile strength and Young´s modulus in comparison to the synthetic films may restrict their applications.

Edible coatings with antimicrobial activity were applied to semi-cured cheeses serving as a food model. Under these conditions, both the coating modification method and antimicrobiological coating activity were evaluated by monitoring the physicochemical, microbiological and sensorial properties of the cheese during ripening. Uncoated cheeses and cheeses coated with commercial polyvinyl alcohol (PVA) coatings served as negative and positive controls. The type of modification influenced the performance of the edible whey protein coatings. In comparison with commercial coatings, the antimicrobial coatings produced using both heat denaturation and UV irradiation showed similar or better results with regard to preventing cheese dehydration and the microbiological control of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and moulds. No sensorial differences were detected between cheeses with antimicrobial edible whey protein coatings and those with commercial coatings. The results are very promising in terms of effectively replacing commercial coatings with natural ones.

The work presented in this thesis showed the great potential for using whey protein in less complex processes in the medium/small cheese industries to partially solve the environmental problem of whey at relatively low operational and investment costs. The methodologies adopted enabled highly functional and nutritional products to be manufactured for use in food products or in nonconventional applications with proven success. However, the whey valorisation solutions studied here still require further research and development. The processing conditions required to produce and store LWPC for further use need to be defined more accurately and this will be addressed in future work. Moreover, other issues, including the fermentation of the ultrafiltration/diafiltration permeate during the production of innovative products such as probiotics, also need to be considered. With regard to whey protein based films and coatings, there is a need for additional research into upscaling to an industrial level, as well as improving the physical and bioactive properties of films and coatings. Nevertheless, the work has contributed towards the sustainability of the whey processing industries.

Resumo

O soro lácteo ou lactossoro é a designação dada ao subproduto resultante do processamento bioquímico do leite no fabrico do queijo, ou da caseína ácida, aquando da separação da coalhada. É o principal subproduto da indústria queijeira (≈9 L de soro por 1 kg de queijo) e constitui um grave problema ambiental (CBO_5 = 35-45 g/L), pelo que a sua eliminação directa no meio ambiente ou o seu tratamento como efluente industrial pressupõem elevados custos tanto ambientais como operacionais. Por outro lado, é também a sua composição bioquímica, nomeadamente em proteínas, que lhe confere um elevado valor funcional, nutricional e biológico e que conduz naturalmente ao seu potencial de valorização. As soluções convencionais existentes e amplamente praticadas actualmente para o tratamento do soro lácteo passam pela produção e comercialização de produtos desidratados tais como: o soro em pó, concentrados e isolados de proteínas e lactose. Nestes casos são utilizados processos de concentração (osmose inversa e evaporação) ou de separação por ultrafiltração, seguidos de secagem dos respectivos retidos e permeados. A grande limitação destes processos diz respeito à etapa de desidratação, que acarreta elevados custos a nível de capital e de funcionamento, para além de exigir uma dimensão de produção considerável. Estes constrangimentos condicionam a sua aplicação nas pequenas e médias empresas (PMEs), que representam efectivamente a maioria das empresas do sector em Portugal (79%) e contribuem com um volume de negócio bastante significativo (37%).

Neste trabalho pretendeu-se estudar e comprovar a eficácia da aplicação de soluções inovadoras para a valorização do soro lácteo, particularmente das suas proteínas, recorrendo à utilização de processos de separação selectiva pouco dispendiosos, passíveis de serem aplicados às PMEs do

sector. Os produtos resultantes da tecnologia aplicada, depois de caracterizados, foram aplicados como coadjuvantes ou matéria de base em produtos alimentares (queijos frescos, iogurtes e sobremesas lácteas) ou usados em aplicações menos convencionais, nomeadamente na produção de filmes e revestimentos com propriedades antimicrobianas.

Recorrendo à flexibilidade dos processos descontínuos de ultrafiltração (UF) e diafiltração (DF) foram produzidos concentrados de proteínas líquidos (CLPS) e desidratados (CPS) de duas origens distintas (bovina e ovina). Foi também avaliado o efeito das condições operatórias e da configuração dos processos nas características dos produtos obtidos. Da aplicação da UF e da DF resultaram CLPS bovinos com conteúdos proteicos entre 43 e 66% (base seca) e CLPS ovinos com 61 e 87% (base seca). Apenas a aplicação da DF por redução de volume (DFvrm) proporcionou a obtenção de produtos quer líquidos quer desidratados mais puros. Neste caso, e face aos produtos da UF, o conteúdo em proteínas foi 20% superior, embora a composição em α-Lactalbumina tenha sido penalizada. Os produtos de origem ovina demonstraram rendimentos superiores em proteínas (62-84%, base seca), o que os torna mais atractivos em termos de processamento.

A utilização de coadjuvantes desidratados (leite em pó, caseinatos ou concentrados de proteínas) no fabrico de queijos ou iogurtes, como forma de aumentar o teor em sólidos é prática comum na indústria. Já, a utilização de concentrados líquidos de proteínas soro (CLPS), principalmente na produção de iogurtes, não está minimamente explorada. A produção de queijos frescos e iogurtes sólidos com vários níveis de incorporação de CLPS de origem bovina revelou-se uma mais valia, não só pelo aumento do rendimento produtivo como também nas propriedades funcionais dos alimentos avaliados. Relativamente aos queijos frescos, a incorporação dos CLPS permitiu o aumento da estabilidade do produto e a redução da sinérese espontânea durante o armazenamento. Apesar de não se encontrarem diferenças significativas ao nível da textura (dureza, mastigabilidade e gomosidade) entre os produtos convencionais e os testados, a proporção de incorporação revelou ser um parâmetro determinante, nomeadamente pelas alterações sensoriais que pode provocar nos alimentos. A nível sensorial não foram encontradas diferenças entre os produtos com baixos níveis de incorporação e os convencionais, enquanto que para os queijos frescos as concentrações mais elevadas de CLPS (50% (m/m)) penalizaram a sua aceitação.

Os bons resultados obtidos para o caso dos iogurtes incorporados com CLPS de origem bovina e os rendimentos proteicos mais elevados para os CLPS de origem ovina, aliados à disponibilidade deste tipo de soro nas queijarias nacionais, conduziram à avaliação da sua performance em iogurtes sólidos. Em Portugal, a utilização de leite de ovelha está praticamente cingida ao fabrico de queijo e requeijão, o que limita a flexibilidade das indústrias quando por razões económicas, estes produtos têm menos procura. De forma a dar resposta a estas questões, foram produzidos iogurtes com leite bovino e com incorporação de CLPS (de ambas as origens - bovina e ovina), iogurtes com leite ovino e posteriormente comparados com os iogurtes convencionais de origem bovina. O conteúdo proteico dos iogurtes de origem ovina foi significativamente distinto do dos iogurtes de origem bovina (com ou sem incorporação de CLPS). Não se verificaram variações significativas no aspecto dos iogurtes ao longo do longo do tempo. A incorporação de CLPS proporcionou uma diminuição de dureza, adesividade, gomosidade e viscosidade nos iogurtes e por conseguinte um maior índice de sinérese. Estes resultados indicam que a estrutura do gel produzido é mais aberta e com menor capacidade de retenção da água. No entanto, os baixos índices de sinérese (0,5 e 5,0%) obtidos para todas as formulações, característicos de produtos com elevados teores de sólidos são reveladores da boa performance funcional dos CLPS. Em termos sensoriais não foram detectadas diferenças entre os iogurtes bovinos convencionais e os incorporados com CLPS. No entanto, no caso do iogurte de origem ovina essas diferenças foram identificadas acabando por ser o mais penalizado no teste de preferência. Os resultados revelaram também que independentemente da origem dos CLPS (bovina ou ovina) estes podem ser usados no fabrico de iogurtes substituindo total ou parcialmente o uso dos coadjuvantes convencionais desidratados sem que esta diferença seja perceptível pelo consumidor.

Nos dois estudos apresentados anteriormente referiu-se a aplicação dos CLPS como substitutos dos coadjuvantes usados no fabrico industrial de produtos lácteos já existentes no mercado. No entanto, encarar os CLPS como matéria de base na preparação de produtos inovadores representa sem dúvida uma oportunidade de mercado extremamente atractiva e ainda não explorada. Os elevados conteúdos proteicos e as características nutricionais e funcionais das suas proteínas contribuem por si só para a obtenção de produtos de elevado valor acrescentado. Nesta perspectiva, e apesar de serem reconhecidas as propriedades gelificantes das proteínas de soro na forma desidratada a sua performance a partir dos CLPS não tinha sido até agora avaliada. As propriedades físico-químicas, de textura e reológicas dos géis térmicos e ácidos produzidos a partir dos CLPS foram avaliados em função do tipo de CLPS (desnatado (D) e não desnatado

(ND) obtidos por ultrafiltração; e desnatado obtido por diafiltração (DF)), da concentração proteica nas formulações (5 e 7%, base seca) e do pH (4 e 7). Os géis produzidos termicamente a partir de CLPS não desnatado, a pH 4 e concentração de proteína mais elevada apresentaram maior dureza, elasticidade e capacidade de retenção de água. Por sua vez o CLPS produzido por diafiltração, para 5% de proteínas e a pH 7 não formou géis, provavelmente devido ás fracas interacções intermoleculares nestas condições. Todos os géis ácidos, produzidos quer por fermentação láctica (tipo iogurte) quer por acidificação com glocona-δ-lactona (GDL) (tipo sobremesa) demonstraram um comportamento viscoelástico, independentemente da incorporação de leite em pó. O processo de acidificação por fermentação forma géis mais fracos. Dependendo do processo de gelificação e das condições de produção é possível obter produtos com composição e propriedades reológicas apropriadas para aplicações específicas.

A crescente preocupação ambiental na substituição dos polímeros sintéticos por polímeros naturais tem impulsionado a investigação na procura de novos materiais, de fontes menos convencionais, nomeadamente agro-resíduos. As proteínas do lactossoro enquadram-se portanto nestas premissas. Os estudos que têm sido desenvolvidos usam os isolados de proteínas de soro (IPS) e utilizam o processo de destabilização proteica por via térmica e consequente gelificação durante a evaporação do solvente para a formação dos filmes e revestimentos.

O trabalho desenvolvido sobre este tópico, e apresentado nesta tese, pretendeu demonstrar as potencialidades dos concentrados de proteínas de soro desidratados (CPS) como alternativas menos dispendiosas aos IPS. A modificação por radiação ultravioleta (UV) foi o método alternativo usado na produção dos filmes. A composição química dos filmes e os diferentes métodos de modificação afectaram as propriedades físico-químicas, ópticas e de permeabilidade de forma diferenciada. A utilização de CPS com aproximadamente 50% de proteínas e 40% de lactose, resultou em materiais com boas propriedades funcionais. A cor dos filmes foi ligeiramente afectada pela presença da lactose, que potenciou o desenvolvimento de uma cor amarela. No entanto, os valores de transparência enquadram-se na gama dos valores obtidos para os filmes sintéticos disponíveis no mercado como é o caso do polipropileno orientado (OPP) e do polietileno (PE). A utilização da radiação UV permitiu produzir filmes mais hidrofóbicos, com menores teores de humidade e menos permeáveis ao vapor de água. No entanto, apresentaram uma solubilidade em água bastante elevada, o que pode limitar a sua aplicação em ambientes húmidos. A estrutura molecular dos filmes produzidos com recurso à radiação UV demonstrou a presença de ligações químicas mais fracas entre os componentes da formulação o que os tornou

menos estáveis termicamente. As propriedades mecânicas dos filmes não foram afectadas pelos métodos de polimerização, mas a sua baixa resistência à tracção e módulo de Young quando comparados com os filmes sintéticos pode condicionar a sua utilização nalgumas aplicações.

As limitações apresentadas pelos filmes à base de proteínas de soro quando potencialmente usados em condições de elevada humidade, conduziram ao estudo da sua utilização como revestimentos em produtos alimentares onde este factor não fosse condicionante. Neste contexto, foram produzidos revestimentos com actividade antimicrobiana (acido láctico e natamicina) e aplicados a queijos semi-curados. A eficiência do revestimento antimicrobiano e dos vários métodos de produção utilizados foi avaliada pela monitorização das propriedades físico-químicas, microbiológicas e sensoriais dos queijos revestidos durante o seu tempo de cura (45 dias). Estas propriedades foram comparadas com as dos queijos onde foi aplicado o revestimento comercial à base de poliacetato de vinilo (PVA) e sem revestimento. Os diferentes métodos utilizados foram determinantes nas propriedades dos queijos. Os revestimentos produzidos simultaneamente por desnaturação térmica e modificação por radiação UV foram aqueles que apresentaram resultados físico-químicos e microbiológicos idênticos ou superiores aos dos revestimentos comerciais, tanto na prevenção da desidratação como no controlo do desenvolvimento de *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae*, bolores e leveduras. Em termos sensoriais, apesar dos queijos com revestimento comercial serem os mais valorados, não foram identificadas diferenças significativas relativamente aos queijos com revestimentos antimicrobianos. Os resultados produzidos permitem concluir que os revestimentos à base de proteínas de soro com propriedades antimicrobianas e produzidos por desnaturação térmica em associação com a modificação por radiação UV são uma alternativa efectiva aos revestimentos comerciais.

O trabalho desenvolvido nesta tese demonstrou que a valorização do soro lácteo por processos de baixa complexidade e a baixos custos apresenta um enorme potencial para implementação em pequenas e médias queijarias. As metodologias utilizadas permitiram produzir CLPS com boa performance a nível funcional quando aplicados no fabrico de queijos frescos, iogurtes e sobremesas lácteas. Esta solução permite não só reduzir os custos na aquisição dos coadjuvantes desidratados convencionais, como também diversificar a gama de produtos a comercializar, nomeadamente produtos totalmente inovadores no mercado à base proteínas de soro com características nutricionais muito valorizadas. Esta estratégia permite resolver simultaneamente o

problema ambiental associado à produção do soro nestas unidades industriais, assim como fortalecer a sua estratégia de mercado. Por outro lado, permitiu também demonstrar a diversificação da utilização das proteínas de soro em aplicações não convencionais, nomeadamente na produção de filmes e revestimentos com propriedades antimicrobianas. A sua aplicação a queijos semi-curados permitiu verificar que estes revestimentos podem ser fortes alternativas aos revestimentos comerciais actualmente utilizados.

Acknowledgements

This work would not have been possible without the contribution of several persons and entities, not just in a technical or logistical level but also on a personal level. And as *the whole equals the sum of the parts* I want to leave here my sincere gratitude to:

- my PhD supervisor, Professor Maria Helena Gil with her enthusiasm, inspiration, and great efforts to explain things clearly and simply.
- my PhD co-supervisor Professor Carlos Dias Pereira, that provided me encouragement, sound advice, good teaching, good company and lots of good ideas.
- Foundation for Science and Technology - FCT, for financial support under PhD Scholarship program (SFRH / BD / 36645/2007) supported by program POCI 2010.
- Faculty of Science and Technology of the University of Coimbra - FCTUC, for the opportunity to develop and defend this thesis under its academic affiliation.
- Escola Superior Agrária de Coimbra, Polytechnic Institute of Coimbra - ESAC-IPC, in particular to the Department of Food Science and Technology (DCTA) for the excellent pilot plant infrastructure that allowed me to develop the most significant part of the practical work presented here.
- Department of Chemical Engineering of FCTUC - DEQ for the infrastructures support available, mainly equipments and analytical methods for products characterization.

- Queijaria Serqueijos and Queijaria Flor da Beira for providing the bovine and ovine whey used in this work.
- all DCTA collaborators: David Gomes, Jorge Viegas, Paula Oliveira, Jorge Arede, Lourdes Pires, Adélia Vaz e Helena Silvestre by the joy, motivation and support that always gave me throughout these years.
- all colleagues from DCTA who helped me during this journey.
- all DEQ collaborators, investigators, teachers and friends that always helped me when I knocked at their doors.
- Hélder Ramos, Daniela Rodrigues, Patrícia Lameiras, Gustavo Santos and Ana Rodrigues for their dedication and prompt response to the various challenges that were being proposed.
- all the persons who have meant a lot to me during the most difficult moments of this journey, who helped me to grow in a personal and professional level, thank you all.

Lastly, and most importantly, I wish to thank my parents, Luis e Maria Helena, my grandmother Maria and my brothers Miguel, Emanuel and Marcelo by showing me that no matter what may happen they will always be there for me.

List of Publications

Published papers that resulted from the research work presented in this thesis:

- **Henriques MHF**, Gomes DMGS, Pereira CJD and Gil MHM (2012) Effects of liquid whey protein concentrate on functional and sensorial properties of set yogurts and fresh cheese. Food and Bioprocess Technology, 1-12, doi:10.1007/s11947-012-0778-9
- **Henriques MHF**, Gomes DMGS, Rodrigues D, Pereira CJD and Gil MHM (2011) Performance of bovine and ovine liquid whey protein concentrate on functional properties of set yoghurts. Procedia Food Science, 1, 2007-2014.
- **Henriques MHF**, Gomes DMGS, Pereira CJD and Gil MHM (2011) Characterization of bovine and ovine WPC obtained by different membrane configuration processes, Journal of Chemistry & Chemical Engeneering, 5, 316-324.

Papers and book chapters submitted for publication that resulted from the research work presented in this thesis:

- **Henriques MHF**, Gomes DMGS and Pereira CJD (2012) Valorisation of whey in small and medium dairy industries. Production and incorporation of liquid whey protein concentrates in fresh cheese and evaluation of the physicochemical and sensorial properties" Submitted to the book: Cheese: Production, Chemistry and Sensory Properties, Nova Science Publishers, Inc., December.

- **Henriques MHF**, Santos G, Rodrigues A, Gomes DMGS, Pereira CJD and Gil MHM (2012) Replacement of conventional cheese coatings by natural whey protein edible coatings with antimicrobial activity. Submitted to: LWT - Food Science and Biotechnology, July.
- **Henriques MHF**, Gomes DMGS and Pereira CJD (2012) The use of alternative ingredients in the production of traditional dairy foods: their performance and acceptability. Submitted to the book: Trilogy on Traditional Foods, ISEKI Food Series, vol 10-12, March.

Portuguese patent submitted for registration that resulted from some research work presented in this thesis:

- Henriques MHF, Gomes DMGS, Pereira CJD, Gil MHM (2012) Produção e utilização de concentrados líquidos de proteínas de soro no fabrico de iogurtes e outros produtos lácteos. Escola Superior Agrária de Coimbra – ESAC.

  Phase 1: State of the Art (concluded) - Clarke, Modet & Co (2012) Report: State of the Art. Portugal, February.

  Phase 2: Patentability opinion (concluded) - Clarke, Modet & Co (2012), December.

  Phase 3: Temporary Portuguese patent request (running)

List of abbreviations and acronyms

1.5-15LWPC	Yogurt with 1.5% fat, incorporated with 15% of LWPC
1.5-30LWPC	Yogurt with 1.5% fat, incorporated with 30% of LWPC
1.5-C	Conventional yogurt with 1.5% fat
25LWPC	Fresh cheese incorporated with 25% of LWPC
50LWPC	Fresh cheese incorporated with 50% of LWPC
9-30LWPC	Yogurt with 9% fat, incorporated with 30% of LWPC
9-C	Conventional yogurt with 9% fat
AIC	Akaike information criterion
ANOVA	Analysis of variance
ATR	Attenuated total reflectance
a_w	Water activity
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BIC	Bayesian information criterion
BOD	Biochemical oxygen demand
BOD_5	Biochemical oxygen demand after 5 days
BSA	Bovine serum albumin
C	Conventional fresh cheese
CLPS	Concentrado líquido de proteínas de soro
CMP	Caseinmacropeptide
COD	Chemical oxygen demand
CPS	Concentrado de proteínas de soro desidratados
Cys	Cysteine residue
D	Defatted/Desnatado
DF	Diafiltration/Diafiltração
DFFsdm	Filtrate from diafiltration in sequential dilution mode
DFR	Filtrate from diafiltration

DFR	Retentate from diafiltration
DFRP	Retentate powder from diafiltration
DFRPsdm	Retentate powder from diafiltration in sequential dilution mode
DFRPvrm	Retentate powder from diafiltration in volume reduction mode
DFRsdm	Retentate from diafiltration in sequential dilution mode
DFsdm	Diafiltration in sequential dilution mode /diafiltração por diluição sequencial
DFvrm	Diafiltration in volume reduction mode /diafiltração por redução de volume
DSW	Skimmed whey for diafiltration
DTG	Derivative decomposition rate temperature
DTG_{max}	Derivative maximum decomposition rate temperature
E	Elongation
E'	Elastic or Young's modulus
ELV	Emission limit values
ESAC	Escola Superior Agrária de Coimbra
EU	European Union
EU27	European Union of 27 members
EVOH	Ethylene-vinyl alcohol copolymer
FAO	Food and Agriculture Organization of the United Nations
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared spectrometry
GDL	Glucono-δ-lactone
Gly	Glycerol
GMP	Glycomacropeptide
GRAS	Generally recognised as safe
HD	Heat denaturation
HDPE	High-density polyethylene
Ig	Immunoglobulin
INE	Instituto Nacional de Estatística
IPPC	Integrated pollution prevention and control
IPS	Isolados de proteínas de soro
LB	Conventional bovine yogurt
LB-LWPCb	Bovine yogurt incorporated with bovine liquid whey protein concentrate
LB-LWPCo	Bovine yogurt incorporated with ovine liquid whey protein concentrate
LDPE	Low-density polyethylene
Lf	Lactoferrin
LMw	Low molecular weight
LO	Conventional ovine yogurt
LPO	Lactoperoxidase
LWPC	Liquid whey protein concentrate
LWPCb	Bovine liquid whey protein concentrate
LWPCo	Ovine liquid whey protein concentrate
M	Moisture
MF	Microfiltration
MFGM	Milk fat globule membrane
Mw	Molecular weight
ND	Non-defatted/Não desnatado
NF	Nanofiltration
NPN	Non-protein nitrogen

OP	Oxygen permeability
OPP	Oriented polypropylene/polipropileno orientado
p.e.	People equivalent
PDO	Protected designation of origin
PE	Polyethylene/polietileno
PEG200	Polyethylene glycol 200 /polietilenoglicol 200
PEG400	Polyethylene glycol 400 /polietilenoglicol 400
PET	Polyethylene terephthalate /teraftalato de polietileno
PG	Propylene glycol /propilenoglicol
PGI	Protected geographical indication
Phot	Photoinitiator
PMEs	Pequenas e médias empresas
PMMA	Polymethyl methacrylate /polimetacrilato de metilo
PS	Polystyrene /poliestireno
PVA	Polyvinyl acetate/poliacetato de vinilo
PVC	Polyvinyl chloride /policloreto de vinilo
PVCH	Polyvinyl cyclohexane
PVDC	Polyvinylidene chloride
PVOH	Polyvinyl alcohol
PVP	Poly 2-vinylpyridine
RH	Relative humidity
RO	Reverse osmosis
S	Solubility
SA	Serum albumin
SDS	Sodium dodecyl sulphate
SE	Solvent evaporation
SMM	Skimmed milk membrane
SMP	Skimmed milk powder
SNF	Solids non-fat
Sob	Sorbitol
ST	Storage time
Suc	Sucrose
T	Transparency
TA	Titratable acidity
TCA	Trichloroacetic acid
T_d	Decomposition temperature
TEC	Trade effluent control
TGA	Thermogravimetric analyses
Th	Thickness
TN	Total nitrogen
TPA	Texture profile analysis
TPX	Poly 4-methyl-1-pentene
TS	Tensile strength
Ts	Total solids
UF	Ultrafiltration/Ultrafiltração
UFF	Filtrate from ultrafiltration
UFR	Retentate from ultrafiltration
UFRP	Retentate powder from ultrafiltration

UHT	Ultra high temperature
US	United States
USA	United States of America
USDA	United States Department of Agriculture
USW	Skimmed whey for ultrafiltration
UV	Ultraviolet
UV-VIS	Ultraviolet-Visible light
UWTD	Urban wastewater treatment directive
VCF	Volumetric concentration factor
WFD	Water framework directive
WPC	Whey protein concentrate
WPC35	Whey protein concentrate with 35% of protein
WPC80	Whey protein concentrate with 80% of protein
WPI	Whey protein isolate
WVP	Water vapour permeability
Xy	Xylitol
YM	Young´s modulus
ΔE*	Colour difference
α-La	α-Lactalbumin
β-Lg	β-Lactoglobulin

Contents

List of figures

List of tables

Chapter 1

Motivation, scope and organisation of the thesis

Abstract

This chapter presents the motivation and the scope of this thesis. It addresses the subject of the efficient and integral utilization of whey to recover valuable constituents and the reasons that have led to its specific evaluation as a raw material with potential for valorisation. It then describes the objectives of the doctoral work that has been developed and, finally, the organisation of the thesis.

1.1 Motivation and scope of the thesis

The food industry produces a varied and significant amount of residue which, in general, constitutes a serious environmental concern. Currently, waste streams are only partially valorised as animal feed, by being transformed into biomass fuel, or in composting, whereas the majority is managed as waste. Its specific composition, in particular the high organic content, requires

adequate treatment to comply with legislation on the minimisation and disposal of waste. Moreover, it is not unreasonable to assume that future legislation on industrial waste will become even more demanding, thus increasing the associated costs of waste management. One of the possibilities for minimising these costs involves recovering valuable compounds with potential for valorisation from the aforementioned effluents. In addition, the correct use of discarded natural materials may prove to be a profitable form of recycling and creating value in the entire chain-production pipeline. In recent years, new clean technologies have been proposed for the more efficient use of agro-industrial residue, not only in terms of its re-use in agriculture or the food industry, but also in the production of common and innovative products for other sectors and applications. One of the most interesting areas involves the use of these residues to recover valuable natural constituents, with a particular emphasis on their functional properties and bioactive substances, in which whey proteins play an important role (Abd-El-Salam et al., 2009). These compounds may help develop unexplored niche markets resulting from the interconnection between traditional areas – the food, pharmaceutical and cosmetics industries – and new business areas, such as functional foods, nutraceuticals and nutricosmetics.

Whey is the main effluent from the cheese industries and is produced in extremely high volumes (9 litres of whey per kilogram of cheese). This by-product has a high nutritional and functional value since it retains approximately 55% of milk solids, of which lactose and soluble proteins are the most abundant components. Both these components are also responsible for the environmental impact caused by whey disposal, representing a biological oxygen demand (BOD) of 35-45 g/L and a chemical oxygen demand (COD) of 60-80 g/L. This evidence, together with the pressure from antipollution regulations, challenges the dairy industry to view whey surplus as a resource rather than simply as waste. The increase in milk production worldwide and consumption demands in the cheese market in the last few years (Smithers, 2008) have stimulated global cheese production, which has a growth rate of 1-2% per year (FAO, 2012; Gerosa and Skoet, 2012). This trend ranks the European Union (EU27) as the main cheese producer worldwide with a 43% share of the market, representing an annual cheese production rate of 9.2 million tons and about 83 billion litres of whey. Within this scenario, a 2-3% increase in lactose and whey derived products was observed in the EU27 in 2011, with whey powder, whey protein concentrates (WPC) and isolates (WPI) as well as whey protein fractions representing a global market value of approximately 6.3 billion euros (3ABC, 2012). In Portugal, cheese production has remained quite stable over the last five years – at 77 000 tons per year (INE, 2010 and 2012),

corresponding to an average of 691 million litres of whey that could be valorised. It is estimated that only 21% of liquid whey production in Portugal has a known destination, meaning that the potential for whey valorisation is enormous and also imperative. The national dairy sector comprises 2% large companies, 7% and 30% medium and small scale companies and 61% micro companies (MADRP, 2007). Although the large companies dominate the volume of business (69%) and may have the appropriate dimensions and resources to treat whey by applying conventional drying technologies, the medium and small companies represent an equally important part (29%) of the market but lack of appropriate solutions for whey disposal. It is also important to note that ovine cheese production, normally associated with cheeses with a protected designation of origin (PDO), is very important in Portugal (representing about 25% of total cheese production), and the majority of these production plants are small and micro enterprises. The direct use of whey is one of the oldest practices for managing this product. It can be supplied to farmers as a biofertiliser or livestock feed, or used in the production of whey cheeses (e.g. *requeijão*). However, not all cheese whey production can be processed in this way due to the high volumes of whey generated. Improvements to membrane technologies, designed to make it easier to fractionate whey components, especially whey proteins with potentially useful functional properties, have contributed towards a significant increase in the manufacture of products derived from whey protein, such as WPC and WPI. Nevertheless these alternatives still imply a final drying stage which limits their application in medium/small scale cheese factories.

This work proposes new approaches to the use of whey in medium and small-scale cheese industries. The techniques it presents are very effective in recovering whey proteins, ensuring that their structural and functional properties are retained for further conventional and nonconventional applications in the food industry. However, the profiles of the whey protein products depend on several factors, such as the type of feedstock whey (acid or sweet), the source of the milk (bovine or ovine) and, in particular, the processing technologies (separation, concentration and isolation techniques) and processing conditions adopted during production. Variations in the final products can be avoided by the manufacturers controlling the processing parameters in order to ensure consistent quality and fully meet the intended specifications for future use.

1.2 Objectives of the thesis

The main objective of the research activities developed during this doctoral training period was to develop promising alternatives (differentiated from the traditional ones) for whey valorisation in small/medium-sized cheese factories which have no economic and technological means of treating this by-product using conventional methodologies. It focused in particular on the valorisation of whey proteins. Two lines of investigation regarding the application of whey protein concentrate products were followed: conventional applications which, in this thesis, refer to direct incorporation in food applications, and nonconventional applications, with particular emphasis on the development of packaging materials. Since the intention was to develop an entire solution process (from the source - whey - to the final application), all the steps were executed, investigated and in some cases optimised, and several other objectives were therefore identified:

- Production of bovine and ovine liquid and dry whey protein concentrates (LWPC and WPC, respectively) by ultrafiltration/diafiltration, using whey from local small/medium cheese producers. Characterisation of both liquid and dry whey protein concentrate products and optimisation of processing conditions and configurations.
- Production of fresh cheese and set yogurts incorporating various levels of bovine LWPC. Evaluation of their effect on the physicochemical, functional and sensorial properties of both food products during storage.
- Production of set yogurts using ovine LWPC and comparison of the functional and sensorial properties of set yogurt produced by incorporating bovine LWPC and yogurts manufactured using conventional industrial practices (from bovine and ovine milk).
- Production of dairy gels using LWPC as the primary raw material in their formulation, studying the influence of several processing parameters: protein concentration, pH and the gelation method (thermal protein denaturation or acid gelation by bacterial fermentation or by glucono-δ-lactone (GDL) hydrolyses) in gel rheological properties.
- Production of edible whey protein films by UV modification using WPC. Evaluation of their physicochemical, optical, barrier, molecular, thermal, mechanical and surface properties. Comparison with edible whey protein films produced by the conventional solvent casting method.

- Production of edible whey protein coatings with antimicrobial activity using different modification methods and their application to semi-cured cheeses. Evaluation of coating performance during ripening based on the physicochemical, textural, microbiological and sensorial characteristics of cheese.

1.3 Organisation of the thesis

The thesis is the result of a PhD research project entitled "Recovery and application of whey proteins in conventional and nonconventional food systems". The thesis is divided into five parts (A, B, C, D and E).

Part A provides an introduction to the main subject covered in this thesis. Chapter 1 presents the issue of the efficient and integral utilisation of whey in order to recover valuable constituents. The agro-residue is described with reference to the global and national situation and the reasons that have led to its evaluation as an agro-residue with potential for valorisation are presented. The state-of-the-art knowledge relating to the valorisation of whey components, in particular whey proteins, is described and questions associated with whey origins, whey protein recovery processes, principal characteristics (such as functional, nutritional and biological perspectives) and their possible applications and uses are presented.

Part B comprises Chapter 2, which describes the production and characterisation of whey protein concentrates (WPC). It addresses whey origin and its initial composition in order to evaluate the influence of different membrane methodologies on the quality and composition of intermediate liquid products and final dry products.

Part C consists of three chapters that include all the research carried out into the conventional applications of whey proteins. Whey proteins were incorporated into food systems in the form of a liquid whey protein concentrate (LWPC) and its performance accessed. Chapters 4 and 5 describe the testing of the direct use of liquid whey protein concentrates in traditional food applications such as fresh cheese and yogurt, evaluating the textural, rheological and sensorial characteristics of the products. The effect of LWPC origin (bovine or ovine) in yogurt is also evaluated, as well as its performance as a replacement for conventional ingredients. Chapter 6 investigates LWPC, not as a food additive but as a primary raw material for the production of dairy gels (thermal or acid gels), as a novel food system.

Part D concerns research into films and coatings as nonconventional applications of WPC dry products and includes three chapters. Chapter 7 presents an introduction to the topic, with reference to the composition of films and coatings, formation methods, properties and opportunities for application. Chapter 8 studies the influence of the different formulations and modification methods on the physicochemical, molecular, optical, barrier, surface and mechanical properties of whey protein films. The perspectives for WPC coatings with antimicrobial properties applied to traditional ripened cheeses as a food model system are presented in Chapter 9. The performance of WPC coatings produced by different methods is evaluated in cheese storage by addressing the physicochemical composition, microbiological quality and sensorial characteristics of cheese.

Finally, Part E presents the major conclusions that may be drawn on the basis of the work developed within this thesis (Chapter 10) and, Chapter 11, offers suggestions for future work directly related to the research that has been carried out, the optimisation of processes and products, the resolution of industrial problems and their implementation.

References

3ABC - 3A Business Consulting (2012) Global Opportunities for Whey and Lactose Ingredients 2010-2014 <http://www.3abc.dk/>

INE - Instituto Nacional de Estatística, I.P. (2010) Estatísticas Agrícolas 2009. Lisboa, Portugal ISBN 978-989-25-0085-4

INE - Instituto Nacional de Estatística, I.P. (2012) Estatísticas Agrícolas 2011. Lisboa, Portugal ISBN 978-989-25-0155-0

MADRP - Ministério da Agricultura do Desenvolvimento Rural e das Pescas (2007) Leite e Lacticínios: diagnóstico sectorial. Gabinete de Planeamento e Políticas. Portugal.

Abd El-Salam MH, El-Shibiny S and Salem A (2009) Factors Affecting the Functional Properties of Whey Protein Products: A Review. Food Reviews International, 25(3), 251-270.

Gerosa S and Skoet J (2012) Milk availability - Trends in production and demand and medium-term outlook. ESA Working paper No. 12-01, February, Food and Agriculture Organization of the United Nations. <www.fao.org/economic/esa>

FAO - Food and Agriculture Organization of the United Nations (2012) <http://faostat.fao.org/default.aspx?lang=en>

Smithers GW (2008) Whey and whey proteins - from 'gutter-to-gold'. International Dairy Journal, 18, 695-704.

Part A.

Introduction

Chapter 2

Whey and whey proteins

Abstract

The aim of this chapter is to characterise whey according to its origins and describe the current scope of the international and national whey markets. The chemical composition of whey, its nutritional value and the functional properties attributed to whey proteins will be described briefly and the reasons that led to their specific evaluation as raw material with potential for valorisation will be presented. In addition the chapter also provides an important account of whey valorisation processes, the technologies applied, the final products obtained and their applications in the food industry, with particular emphasis on whey protein concentrates.

2.1 Whey

Whey is the liquid that separates from the curd during the manufacture of cheese and casein and was, until recently, treated as sewage by small and medium-scale commercial cheese producers. However, since whey generates a substantial biochemical oxygen demand, environmental

concerns and regulation have become important issues. A series of different technologies have been applied to whey valorisation components, particularly proteins and lactose, in an attempt to solve the problem of whey disposal.

2.1.1 Whey production

World dairy markets have held up better than many industries and markets since the global financial crisis and recession of 2008-2009 (Hoogwegt Groep, 2012). However, worsening economic and financial concerns may limit growth in the world cheese and whey trade. It is estimated that 33% of milk production worldwide is used in cheese manufacture averaging about 19×10^6 ton per year (Table 2.1). Milk production is considered the most important agricultural activity in the European Union (EU), representing approximately 18% of the total value of agricultural production. The EU leads the world trade in milk and milk products, with a share of around 25.8% of the market (Blaskó, 2012).

The EU27 is by far the largest producer and exporter of cheese, representing 43% and about 32% of the market, respectively (FAO, 2012; Blaskó, 2012). Cheese production in the EU27 rose by around 2.4% between 2008 and 2011, driven both by increased milk production and the domestic demand for cheese. The 2012 growth forecast for cheese production is 1.3% (Table 2.1) and cheese exports are expected to rise by about 3% to 640 000 tons in 2012, with Russia and the United States as the main markets (Hoogwegt Groep, 2012). Assuming that the production of 1 kg of cheese can generate approximately 9 litres of liquid whey (Jelen, 2003), Table 2.1 shows the estimated whey available for valorisation. As the largest manufacturers of cheese in the world, the EU27 and the United States are also the largest manufacturers of whey and whey derivatives. The major players in the whey and lactose ingredient industry are the leading dairy and cheese companies in the world, such as Lactalis, FrieslandCampina, Fonterra, Arla Foods, Glanbia, Murray Goulburn and Hilmar. Specialist whey and lactose ingredient companies such as Meggle, Euroserum, Milei and Davisco also play a significant role, particularly in various speciality products. The world's two largest dairy companies - Nestlé and Danone - are not actual producers, although they are major users of whey and lactose ingredients. According to 3A Business Consulting (2012) in Denmark, production of whey and lactose products in the EU27 is estimated to have increased between 2% and 3% in 2011. Whey powder, whey proteins and whey protein fractions represent a global market value of approximately 6.3 billion euros and are forecast to rise to 8.1 billion euros in 2014. Lactose, pharmaceutical lactose, permeate and lactose

derivatives were worth more than 2 billion euros in 2010, and the market value for 2014 is estimated at nearly 2.5 billion euros. The production trend for whey protein concentrate (WPC80) and whey protein isolate (WPI) is more positive (perhaps up as much as 6%) than for the lower protein content whey products (WPC35).

Table 2.1 Production of milk, cheese, dry whey, whey protein concentrates (WPC), whey protein isolates (WPI) and liquid whey production estimation Worldwide and in the United States (US), Europe (EU27) and Portugal (2007-2012).

(×10³ ton)	2007	2008	2009	2010	2011	2012*
Worldwide						
Milk production[1]	685.000	700.000	709.000	721.000		
Cheese production[2]	19.038	19.066				
*Liquid Whey** (×10⁶ L)*	*171.342*	*171.594*				
US						
Milk production (cow's)[2]	84.189	86.177	85.881	87.461		
Cheese production[3]	4.956	5.055	5.037	5.222	5.299	
*Liquid Whey** (×10⁶ L)*	*44.608*	*45.492*	*45.334*	*46.995*	*47.687*	
Dry whey[3]	541	501	501	507		
WPC[3]	221	208	208	214		
WPI[3]	22	26	26	31		
EU27[4]						
Milk production	147.942	148.534	147.620	148.565	150.000	151.000
Cheese production	9.248	9.084	9.083	9.220	9.300	9.420
*Liquid Whey** (×10⁶ L)*	*83.232*	*81.756*	*81.747*	*82.980*	*83.700*	*84.780*
Dry whey		1.680	1.670	1.580	1.620	1.640
Portugal						
Milk production[5]	2.029	2.077	2.048	2.002	2.007	
Cheese production[5]	80	77	73	76	77	
*Liquid Whey** (×10⁶ L)*	*716*	*693*	*663*	*688*	*694*	
Dry whey[2]	4.4	8.6	6.3	6.3		

*Forecast, ** estimated as 9-fold the cheese production (1) Krijger (2011); (2) FAO (2012); (3) USDA (2007, 2010, 2011, 2012); (4) EDA (2012); (5) INE (2010, 2012)

The dairy industry in Portugal represents approximately 11% of the total generated by the agri-food and beverage sectors. Milk production has not varied significantly over the past 5 years, with a production volume of 2 million tons per year (Table 2.1). Cow's milk represents 95% of overall milk production and the remaining 5% is distributed between sheep and goat milk production, at 4% and 1% respectively (Figure 2.1a). Comparing these figures with the 1% production of sheep's milk worldwide (Gerosa and Skoet, 2012), the importance of this product in the national dairy economy is obvious. This trend was also verified in some Mediterranean countries (France, Italy, Spain and Greece) where sheep's and goat's milk is significant in the dairy markets, especially in the production of fermented milk products and cheeses (Fox, 2001) in

contrast with the amount of dairy products derived from cow's milk (> 95%) on a global level (Park and Haenlein, 2006). In Portugal, sheep's and goat's milk are used exclusively for cheese production (ovine, caprine and mixed cheeses), with the relative weighting of sheep's cheese very significant in terms of total cheese production (about 25%). However, a reduction in goat products is expected, in line with a decline or levelling of milk production or a slight increase in sheep products. Between 2007 and 2011 an average of 76 752 tons of cheese were produced in Portugal (Table 2.1). About 56 506 tons of cheese are obtained from cow's milk, 13 841 from sheep's milk, 1 633 ton from goat's milk and 4 773 ton from a mixture of various types of milks (Figure 2.1b). This results in approximately 690 million litres of liquid whey being produced annually (Table 2.1). According to data from the FAO (2012), the average dry whey production in Portugal between 2007 and 2010 was about 6 400 tons, corresponding to 95 million litres of treated liquid whey (assuming 97% of total solids in dry whey and 6.5% total solids in liquid whey). National statistics (INE, 2010, 2012) report that around 2 million litres are used as animal feed and that whey exports amount to about 16 400 tons, although there is no reference to the form in which it is sold. Most probably these data refers to dry whey and, if it is the case, corresponds to approximately, 245 000 tons of liquid whey assuming a content of 6.5% total solids in this product. Hence, one could assume that approximately 49% of the whey produced in Portugal is dried.

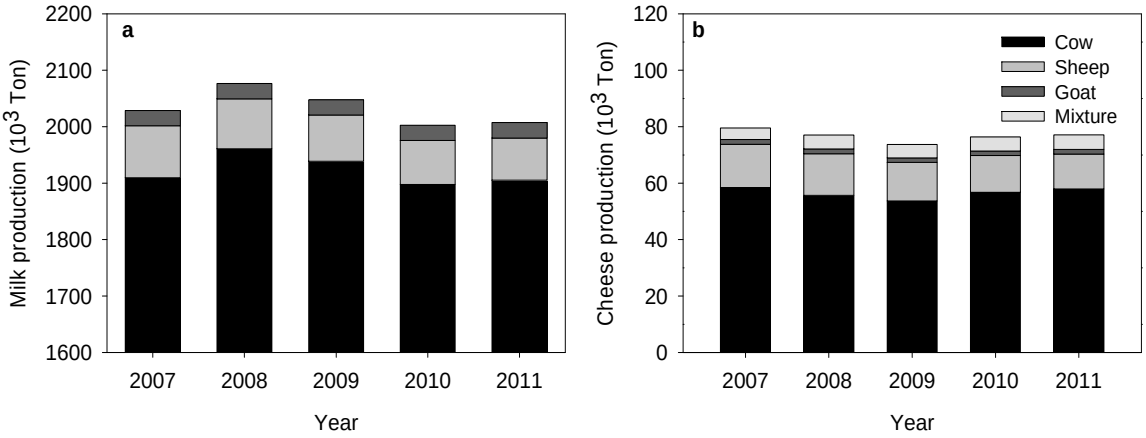


Figure 2.1 Portuguese milk (a) and cheese (b) production by milk's type (2007-2011) (adapted from INE, 2010 and 2012).

The export of whey previously concentrated by reverse osmosis, (VFC=3) (information supplied verbally by the industry), to Spain through direct contact with medium-scale cheese producers is

a common practice. However there is no information about the volume annually exported. This scenario indicates that little more than 50% of liquid whey production in Portugal has a known destination, meaning that the potential for whey valorisation is enormous and also imperative.

The whey market in Portugal was characterised by Tecninvest in 2001. However, no national strategy has yet been adopted, mainly due to the particular characteristics of the Portuguese dairy sector, namely: (i) the large number of small cheese producers; (ii) the wide variations in whey composition, associated with whey origins and manufacturing practices (iii) the distribution of cheese producers within national territory, which raises problems for the transportation of whey.

In Portugal the large and medium-sized dairy industries represent only 9% of the total dairy producers. Micro and small industries predominate at 91% (Figure 2.2a). However, their volume of business only accounts for 11% of the sector (Figure 2.2b) due to their size (with an average annual production of 13.6 tons), poor technological resources and low level of product differentiation. Their area of business is the niche market for traditional and regional (ovine and caprine) cheeses, thus avoiding competition with the medium-sized and large cheese producers associated with bovine cheese types (Casper et al., 1998).

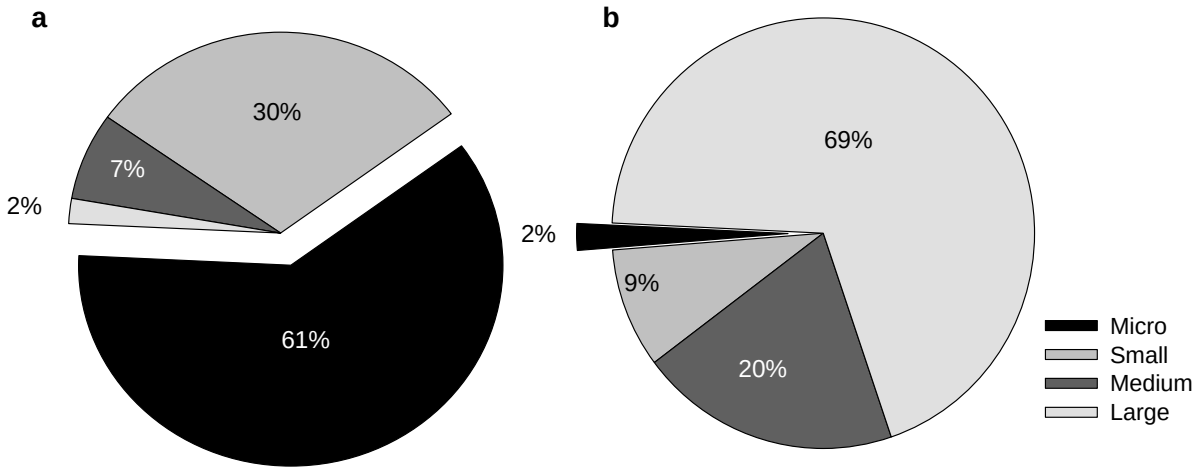


Figure 2.2 Portuguese dairy company's distribution by: (a) scale size and (b) volume of business (adapted from MADRP, 2007).

Table 2.2 lists several traditional Portuguese cheeses produced by 107 cheese manufactures and classified as PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication). 85% of these traditional cheeses are made from sheep's and goat's milk and only 5%

from bovine milk. However, cheeses classified as PDO or PGI only represent 8% of the cheeses made from the milk of small ruminants (sheep and goats).

Table 2.2 Traditional Portuguese cheeses. Classification, cheese type, dairy companies and annual production (adapted from MADRP, 2007).

Traditional Portuguese cheeses	Classification	Cheese type	Dairy companies*	Production (kg/year)*
Serra da Estrela	PDO	Sheep	35	99.488
Rabaçal	PDO	Sheep/Goat	4	950
Azeitão	PDO	Sheep	5	111.030
Castelo Branco	PDO	Sheep/Goat	3	46.725
Amarelo da Beira Baixa	PDO	Sheep/Goat	6	64.400
Picante da Beira Baixa	PDO	Goat	2	28.000
Cabra Transmontano	PDO	Goat	3	14.850
Terrincho	PDO	Sheep	2	37.718
Évora	PDO	Sheep	16	93.019
Nisa	PDO	Sheep	10	129.000
Serpa	PDO	Sheep	8	67.257
Pico**	PDO	Cow	-	-
São Jorge	PDO	Cow	8	750.000
Mestiço de Tolosa	PGI	Sheep/Goat	5	13.000
Total			107	1.455.437

* data from 2005; ** data not available; PDO - Protected Denomination of Origin; PGI - Protected Geographic Indications.

The national distribution of cheese dairy companies (according to volume of production) reflects milk production and distribution in the country (Figure 2.3), meaning that the large companies are located on the coast (the favoured milk production areas) where cow's milk is the main product. Small and medium-sized companies are established in areas of low production where milk from small ruminants is of major importance to the sector. The Azores, which is one of the biggest producers of cow's milk (30%), also adds to the territorial dispersion of cheese and consequently of whey production.

Given this scenario, it is not possible to devise a comprehensive strategy for whey treatment in Portugal. Only particular solutions implemented directly in the cheese producing industries (especially the small and medium-sized companies with fewer financial resources) and adapted to their specific situations might solve the problem of whey and simultaneously add value to the existing products or eventually contribute to their diversification.

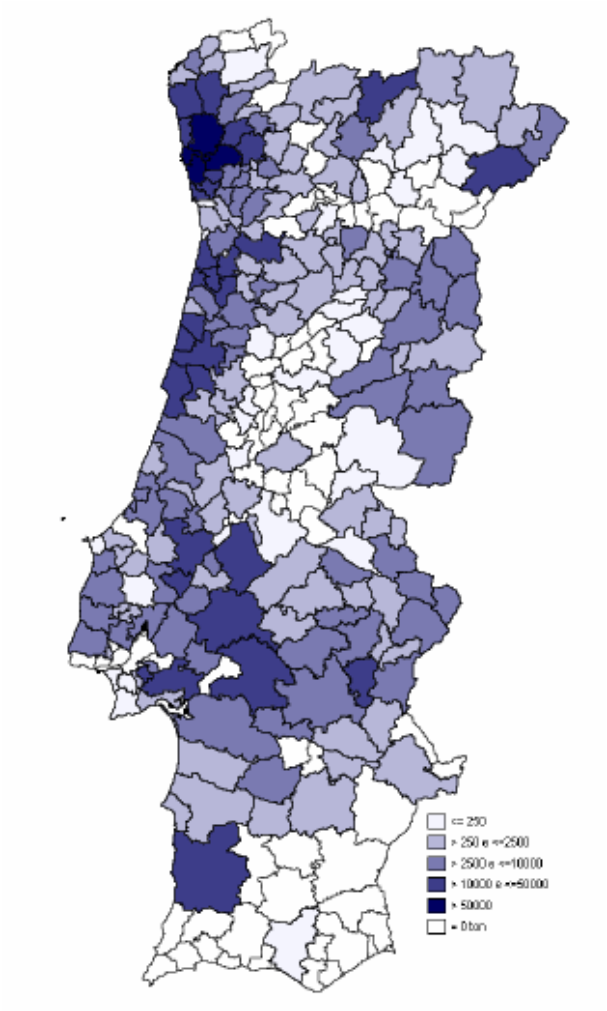


Figure 2.3 Portuguese milk production and transformation by region (adapted from MADRP, 2007). Colour scale: white (0 ton); light colour (≤ 250 ton) dark colour (> 50000 ton).

2.1.2 The environmental problem of whey

The processes of setting emission limit values (ELVs) for wastewater and treated effluent discharged into surface water have evolved over time in the EU Member States and are now to a large extent similar. With regard to technology-based standards for wastewater discharge into surface water, two EU Directives are of prime significance: the Urban Wastewater Treatment Directive (UWTD) and the Integrated Pollution Prevention and Control (IPPC) Directive. Both are referenced in the Water Framework Directive (WFD) (Frost, 2009).

The UWTD establishes the minimum treatment standards to be met by urban wastewater treatment plants and industrial activities that generate similar effluents. The minimum level of treatment to be applied and the ELVs to be achieved depend on the nature of the surface water

affected by the discharge. With regard to the application of the UWTD to industry, Article 11 stipulates that discharges of industrial waste water into collecting systems and urban wastewater treatment plants is subject to prior regulation and/or specific authorisation by the competent authority or appropriate body, i.e. it requires industrial discharges to sewers to be subjected to trade effluent control (TEC). Article 13 refers to biodegradable industrial wastewater from plants that belong to certain food and animal feed industrial sectors (which include milk-processing) representing a wastewater load of 4 000 people equivalent (p.e.) or more.

In Portugal, the UWTD was transposed into national legislation (DL 236/98) in 1998. However, the threshold of 4 000 p.e. was not taken into consideration and all discharges of industrial wastewater must, whatever their size, be subject to prior regulation and/or specific authorisation. DL 236/98 set emission limit values (ELVs) for wastewater and treated effluent discharged into surface water (e.g. BOD 40 mg/L and COD 159 mg/L). If industrial wastewater is released into municipal sewage systems, specific minimum ELVs are prescribed by local authorities (e.g. BOD of 800 mg/L and COD 1 000 mg/L (AC, 2012)), depending on the performance of the existing urban wastewater treatment plant, in order to avoid overloading the system (Smithers, 2008). Although some particular regulations have been issued for specific activities in the agro-food sector (e.g. pig farming) the dairy industry must follow the general recommendations.

Liquid whey is a very biodegradable substrate (≈99%) and an effective pollutant with a biological oxygen demand (BOD) of 35-45 g/L and low alkalinity. 4 000 L of whey, the output of a small cheese factory, has the polluting strength equivalent to 1 900 people (Marwaha and Kennedy, 1988). The disposal of whey by dumping in waterways was a common practice before environmental regulations took hold. However, in the current scenario whey cannot be regarded as an effluent, forcing cheese producers to be responsible for its management. This implies that cheese manufacturers must make their own decisions with regard to whey treatment, transformation and reutilisation or shift the responsibility to another company appointed to solve the problem. The easiest solution for whey is to use it as food for livestock, but this is inadequate due to the perishable nature of whey and the large quantities that are produced. A further possibility implies whey treatment which, in turn, requires investments. However, disposing of whey by these means provides no valuable product, and is costly and labour intensive for the cheese manufacturer, who generally bears all the direct transport and handling costs (Guimarães et al., 2010).

Nowadays whey is evolving into a sought-after product because of the lactose, minerals and proteins it contains, as well the functional properties it can impart to food (Onwulata and Tomasula, 2004). This may be an extremely attractive prospect for producers, who may develop integrated solutions for the cheese whey problem by viewing its great potential as a source of added value to incorporate into products.

Currently in Portugal four big companies have the capacity to dry whey: Lactogal, Fromageries Bel, Insulac and Pronicol. The first has two industrial drying plants in Leça da Palmeira and Avis, the second has one drying plant in Oliveira de Azeméis, the third has one in São Miguel (in the Azores) and the last one in Terceira - Hangra do Heroísmo also in the Azores. Only these companies are large enough and sufficiently well equipped to invest in the expensive drying technology. Some medium/small companies have made some investments in membrane technologies, especially reverse osmosis, but this is only used to concentrate whey, which is then sold at very low prices. In my opinion, the lack of human resources with know-how in this area is the main factor that limits the profitability of this already installed technology.

Whey composition

There are two types of whey: sweet and acid whey (Table 2.3), differentiated by their mineral composition, protein fraction, lactose content and acidity (Jelen, 1992, 2003). Sweet whey or rennet whey is the result of an enzymatic coagulation of caseins during cheese manufacturing at a pH $\geq$ 5.6 and represents about 75% of the total whey produced. Acid whey is obtained during cottage or quark cheese or casein manufacture under acid coagulation conditions (pH $\leq$ 5.1). In general, sweet whey has more protein and lactose but less calcium and phosphorous than acid whey. Variations in non-protein nitrogen compounds can also be observed; sweet whey generally has higher concentrations of peptides and amino acids than acid whey due to proteolysis by rennet (Schmidt et al., 1984; Zall, 1992; Pintado et al., 2001).

Apart from the type of coagulation, whey composition primarily depends on milk origin (e.g. bovine, ovine or caprine), although seasonal cycles of lactation or feed changes may also be responsible for some variations (Casper et al., 1998). Table 2.3 shows the average composition of (acid and sweet) bovine whey and ovine whey. The components of whey ranked in decreasing order by relative amount on a dry weigh basis are lactose (70-75%), nitrogenous compounds (protein, peptides and amino acids) accounting for 8-11%, and minerals (10-15%). It has been observed that ovine whey has significantly higher levels of total solids, lactose, lipids and

especially proteins (Casper et al., 1998; Pintado et al., 1999, 2001; Pereira et al., 2002) than bovine whey due to the composition of ovine milk, which may make it more attractive for valorisation.

Table 2.3 Acid and sweet bovine and ovine whey composition.

Composition (g/L)	Acid bovine whey (pH 4.6 - 4.8) (1, 2)	Sweet bovine whey (pH 5.5 - 6.4) (1, 3)	Sweet ovine whey (4, 5, 6)
Total solids	63.0-70.0	63.0-70.0	74.6-83.84
Lactose	44.0-46.0	46.0- 52.3	47.5-59.8
Protein	6.0-8.0	6.0-10.0	10.5-18.71
Lipids	2.3-2.4	1.5-6.0	6.5-12.5
Ash	7.5	6.0-9.0	4.3-5.65
Lactates	6.4	2.0	-
Calcium	1.2-1.6	0.4-0.6	0.49
Phosphorous	2.0-4.5	1.0-3.0	-
Chloride	0.90-1.2	1.0-1.2	-
Magnesium	0.10-0.11	0.07-0.08	-
Citrate	0.2-10	1.2-1.7	-
Sodium	0.4-0.51	0.4-0.53	-
Potassium	1.4-1.6	1.4-1.6	-
Sulfate	0.5	0.7	-

(1) Jelen (2003); (2) Riera et al. (1996); (3) Morr and Ha (1993); (4) Comendador et al. (1996); (5) Casper et al. (1998); (6) Pintado et al. (2001).

Lactose

Lactose is a disaccharide consisting of galactose and glucose, linked by a β–1.4 glycosidic bond. It is the principal carbohydrate in the milk of all mammals and its concentration varies widely between species (Fox and McSweeney, 1998). In whey, 4.4-4.9% is lactose and represents about 75% of the total solids content. The concentration of lactose in whey is quite stable, although the production of lactic acid via lactose fermentation during acid whey production contributes towards lowering its content. Lactose solubility and sweetness is limited, which restricts its application in food products (Jelen, 1992) and for this reason lactose hydrolysis is often used to overcome this drawback (Walzem et al., 2002). It has also been cited as responsible for the yellowness in whey and whey products due to its role in chemical Maillard reactions with whey proteins.

Whey proteins

About 80% of milk proteins (26 g/kg) are caseins. These proteins are a specific group of milk phosphoproteins (α_{S1}, α_{S2}, β e K) that precipitate at pH 4.6. The soluble proteins that remain in

solution (6.3 g/kg) under these conditions are called whey proteins. It is a relatively heterogeneous group that includes five main proteins: β-lactoglobulin, α-lactalbumin, serum albumin, imunoglobulins, and low molecular peptides. Table 2.4 shows the average protein composition in bovine milk and whey, and ovine whey. With regard to the relative distribution of whey proteins, β-lactoglobulin is the major protein followed by α-lactalbumin. These two proteins represent about 70-80% of the total whey proteins and the remaining 20-30% corresponds to other proteins that include serum albumin, immunoglobulins, proteose peptones, soluble caseins and a variety of minor proteins (e.g. enzymes, lactoferrin).

Table 2.4 Protein composition of bovine milk, bovine whey and ovine whey (Morr and Ha, 1993; Casper et al., 1998; Kilara and Vaghela, 2004)

Origin	Bovine			Ovine
	% of protein		Composition in whey	% of protein
	Milk	Whey	(g/L)	Whey
β-Lactoglobulin	7-12	48-50	3	74
α-Lactalbumin	2.5	13-19	0.7	14.8
Immunoglobulins	1.9-3.3	8	0.6	7.3
Serum albumin	0.7-1.3	6	0.3	4.1
Minor proteins and peptides*	2-6	20	1.4	-

*Included β-casein fragments (proteose-peptone fraction), fragment of k-casein (glycomacropeptide - GMP) and endogenous peptides in the protein nitrogen fraction.

β-lactoglobulin

β-lactoglogulin (β-Lg) is a globular protein with 162 amino acids per monomer and a molecular weight (Mw) of 18.3 kDa. Although seven genetic variants exist, the A and B forms are the most common. These two forms are distinguished by the substitution of aspartic acid in variant A by glycine in variant B. Monomeric β-Lg (when pH is not in the 3-8 range) contains one free SH group, which is normally buried internally in the native molecule, and two S-S bonds. S-S bonds are formed between Cys 160 and 66, and Cys 119 and 106, and the free thiol is in the Cys-121 position (Morr and Ha, 1993). β-Lg is a highly structured protein: optical rotary dispersion and circular dichroism measurements show that in the pH range 2-6, β-Lg consists of 10-15% α-helix, a 43% β-sheet and 47% unordered structure, including β-turns (Fox and McSweeney, 1998). However, the conformation and association of β-Lg are pH and temperature sensitive. Dimers of approximately 36.7 kDa are formed at pH range 5.1-7.5 and tetramerise in the 3.5-5.2

range to form octamers of approximately 147 kDa. β-Lg undergoes time and temperature-dependent denaturation reactions above 65 ºC, which result in general molecular expansion, exposure of the internal SH group, and hydrophobic and -NH_2 groups (Pérez-Gago and Krochta 2002).

Since other principal whey proteins have a biological function, it has long been felt that β-Lg might have a biological function, although this is not clear or well-established. It appears that β-Lg may act as a carrier for retinol (vitamin A) (de Wit, 1989) but its capacity to bind many other hydrophobic molecules suggests that its ability to bind retinol may be incidental, especially in species lacking β-Lg, such as humans (Fox and McSweeney, 1998). β-Lg also binds free fatty acids and thus stimulates lipolysis (since lipases are inhibited by free fatty acids), which may perhaps be its physiological function. β-Lg, as the major whey protein, tends to dominate in the thermal behaviour of total whey protein systems such as whey protein concentrates (Mehra and O'Kennedy, 2008).

α-lactalbumin

α-Lactalbumin (α-La) is the second most abundant whey protein, accounting for about 13-19% of the total whey protein (3.5% of total milk proteins) (Table 2.4). With a molecular weight of 14 kDa, it is a globular protein that contains 123 amino acids which include eight cysteine residues that can form four S-S bonds. α-La is a compact globular protein with a low organised secondary structure content that consists of 26% α-helix, 14% β-structure and 60% unordered structure (Alexandrescu et al., 1993; Fox and McSweeney, 1998). Its capacity to bind calcium maintains the protein globular structure and stabilises it against denaturation (it is the most heat stable whey protein) (Kinsella and Whitehead, 1989). Conformational changes occur in α-La depending on the pH range. At pH 4 the molecule loses the Ca^{2+} that is tightly bound at a higher pH. At pH values between 4 and 5 it appears to occur as two forms with different thermal stabilities. At pH 6.5, it begins to unfold at 62 ºC but on cooling the molecule reverts to its native configuration. This reversibility is lost if the native S-S bonds are broken by heat-induced thiol-disulfide interchange reactions between α-La and β-Lg (de Wit, 1989).

One of the most interesting characteristics of α-La, as a biological function, is its role in lactose synthesis. The concentration of lactose in milk is directly related to the concentration of α-La, which has been isolated in several species, including cows, sheep, goats and humans, and for this

reason the milk of marine mammals, which does not contain α-la, contains no lactose. Since lactose is the principal constituent in milk affecting osmotic pressure, its synthesis must be controlled (Brew and Grobler, 1992).

Immunoglobulins

The immunoglobulins (Igs) are a heterogeneous family of glycoproteins of 150-1 000 kDa. Four classes of Igs have been identified in milk - IgG_1, IgG_2, IgA and IgM - although their content and distribution depends on species, breed, age, health and lactation period. All appear to be glycoproteins with a similar structure, namely monomers or polymers of a four-chain molecule consisting of two light polypeptide chains (20 kDa) and two heavy chains (50-70 kDa) linked together by disulfide bonds (Wong, 1999). Up to 80% of the Igs in whey is IgG. These molecules denature at a higher temperature than β-Lg or α-La (de Wit, 1989). However, they are thermally unstable in the presence of other whey proteins, which may be related to the activity of thiol groups of β-Lg and BSA (Morr and Ha, 1993; Onwulata, 2008). Igs may also be responsible for fat globule agglutination, which can be avoided by thermal treatment or by homogenisation (Marnila and Korhonen, 2003).

Igs are cited as responsible for the natural antimicrobial properties of milk, acting as antibacterial agents and also modulating the immune functions in the body (Onwulata, 2008).

Serum albumin

It has been demonstrated that in all the properties investigated bovine serum albumin from whey is identical to blood serum albumin, except in its electrophoretic behaviour at pH 4.0 (Wong, 1999). Serum albumin (SA) represents approximately 4-6% of total whey protein (Table 2.4). It is the longest single-chain whey protein, with a molecular weight of 66 kDa and 582 amino acids. The molecule contains 17 disulphides, one free sulphydryl group and no phosphorous (Kilara and Vaghela, 2004). All the disulphides involve cysteine (35) residues that are relatively close together in the polypeptide chain, which is therefore organised in a series of relatively short loops. The molecule is elliptical in shape and is divided into three domains.

At pH values lower than 4 and higher than 8, it appears partially denatured (Park and Haenlein, 2006). The main conformational changes at its isoelectric point are an increase in volume and viscosity and a decrease in solubility by ionic strength enhancement (Riera et al., 1996). It is prone

to precipitation at around 40-45 ºC due to increased hydrophobic binding between the chains (de Wit and Klarenbeek, 1984).

In blood, SA serves various functions but it probably has little significance in milk, although it does bind metals and fatty acids and the latter characteristic may enable it to stimulate lipase activity (Fox and McSweeney, 1998).

Minor proteins and peptides

About 1.1% of total milk protein consists of proteose peptone. The proteose peptone fraction is composed of four major components called 3, 5, 8-fast and 8-slow fractions, whilst other minor components are also recognised. It precipitates in 12% trichloroacetic acid (TCA) and includes small essential and aromatic amino acids (Walstra et al, 2006). However, it remains in solution after milk heat treatment at 95 ºC for 20 minutes and acidification to pH 4.7 (Veisseyre, 1988). Proteose peptone component 3 is found only in whey and is not associated with casein. It contains over 17% carbohydrate and has a molecular weight of 20 kDa. The antibody to proteose peptone component 3 will cross-react with the fat globule membrane and it has been suggested that this component is derived from membrane. On a biological level, proteose peptone component 3 improves bacteriological defence and promotes the growth of bifidobacteria. Proteose peptone component 5 has a molecular weight of 13 kDa and is associated with both the whey and casein fractions of milk. The molecule contains phosphorus and has been shown to contain the N-terminal 107 amino acids of β-casein resulting from the proteolytic cleavage that yields the g-caseins. Proteose peptone component 8-fast with a molecular weight of 3.9 kDa represents the N-terminal 28 amino acids released from the cleavage of β-casein. The other major proteose peptone component, 8-slow, has a molecular weight of 9.9 kDa and has not yet been shown to derive from the proteolysis of any milk proteins. At neutral pH a considerable amount of proteose peptone is included in casein micelles and they are also very surface active due, in part, to their low molecular weights and also to the carbohydrate associated with component 3. All the proteose peptone fractions are found only in acid whey, due to the extent of proteolysis in sweet whey (Walstra et al., 2006).

The k-casein bond that is sensitive to chymosin hydrolysis has been identified as the bond between the phenylalanine residue at position 105 and the following methionine residue. The hydrolytic products are k-paracasein (residues 1-105) and glycomacropeptide (GMP) (residues 106-169), sometimes also called caseinmacropeptide (CMP) (Wong, 1999; Walzem et al., 2002).

GMP is not present in acid whey unless enzymes are added during cheese production, but in sweet whey its proportion can reach 20% of the protein faction (Jelen, 2003). Under lower pH values (< 4) its molecular weight varies between 6 and 8 kDa, but polymerisation at a higher pH may increase its molecular weight to approximately 50 kDa (Riera et al., 1996). It has been shown that GMP supplements in diets increase levels of cholecystokinin (a hormone regulating energy and food intake), inhibit platelet aggregation and support beneficial intestinal bacteria (i.e. bifidobacteria). It has also been noted that GMP may help to prevent dental cavities and protect against viruses and bacteria.

In addition to the major protein fractions indicated above, some minor proteins have been also identified and isolated in whey, which deserve special attention due to their recognised biological value as bioactive agents.

Lactoferrin (Lf) appears to be distributed between the casein, whey and probably the fat globule membrane fraction of the milk. It is a polypeptide with a molecular weight range of 80 to 92 kDa and two binding sites for ferric ions which polymerises easily in the presence of calcium (Morr and Ha, 1993). Its high isoelectric point (8.4-9.0) increases its affinity to other macromolecules such as SA and β-Lg making aggregates. Under UHT treatments it loses its biological activity but not under pasteurisation. The role of Lf is to sequester iron (an essential nutrient for bacterial growth, making it unavailable) and interact with microbial cell wall components and cellular receptors through its highly positively charged N-terminus (Nuijens et al., 2005; Dangaran and Krockta, 2008). This protein has recognised antibacterial, antiviral, and antioxidant properties and modulates iron metabolism and immune functions (Walzem et al., 2002; Marshall, 2004). It therefore has potential as a natural antimicrobial agent in products such as personal health items, pharmaceuticals, and special dietary formulas (Smithers et al., 1996).

Lactoperoxidase (LPO) is a broad-specificity peroxidase present in high concentrations in bovine milk but in low levels in human milk. It is a heme protein containing about 0.07% Fe, with an absorbance peak (Soret band) at 412 nm ($A_{412}/A_{280}\approx0.9$). The pH optimum is around 8.0, its molecular weight is 77.5 kDa and it consists of two identical subunits. Two principal forms (A and B) occur, each of which exhibits micro-heterogeneity with regard to amide groups (glutamine and/or asparagine) and carbohydrate content, giving a total of 10 variants (Fox and McSweeney, 1998). LPO, which is positively charged at neutral pH, can be isolated from whey by ion-exchange chromatography which has been scaled up for industrial application. It is an effective

antimicrobial agent, and consequently applications are being found in food preservation, cosmetics and therapeutics. Its use in milk and milk products is related to its effective antimicrobial activity, which reduces bacterial microflora. It is fairly heat resistant and used as an indicator of overpasteurisation of milk. Furthermore, lactoperoxidase can be used as an anti-tumour and anti-viral agent, as well as in dental and wound treatments. It inhibits bacterial growth by catalysing thiocynates and other halides and reducing hydrogen peroxide (de Wit, 1998; Dangaran and Krockta, 2008).

Composition of lipids

The amount of lipids in whey varies widely (Table 2.3), ranging from 1.5 to 12.5 g/L depending on the origin of the milk (bovine, ovine, etc), cheese type (acid or sweet coagulation) the efficiency of the cheese manufacturing process and the practices used (i.e. use of skimmed milk or whole milk) (Jelen, 1992). Two milk membrane systems also contribute to protein and lipids in whey: skimmed milk membrane (SMM) and milk fat globule membrane (MFGM). These membrane materials consist of high molecular weight protein complexes with phospholipids, cholesterol and triglycerides that have been termed phospholipoprotein complexes (Morr and Ha, 1993). These non-polar complexes are recovered in whey protein concentrates (WPC) where they have been shown to inhibit whey protein functionality and are believed to contribute towards flavour instability. MFGM contains 25-60% protein (on a dry basis). The remainder of the MFGM consists of 58-80% neutral lipids and 20-44% phospholipids. Boyd et al. (1999) reported that the fatty acid distribution in sweet and acid whey is 63-67% saturated fatty acids, 28-30% monounsaturated fatty acids and 3.2-4.7% polyunsaturated fatty acids. The most abundant saturated fatty acids are palmitic and stearic acids, both in total lipid fractions and in triglycerides fractions. The same was verified in the phopholipid fraction where behenic acid (C22:0) and lignoceric acid (C24:0) appeared in considerable amounts. Oleic acid predominates in monounsaturated fatty acids and linoleic in polyunsaturated fatty acids. In commercial WPC produced by ultrafiltration it was found that 48-53% of the lipids are triglycerides, 6-15% diglycerides, 7-15% free fatty acids and 27-28% phospholipids (Morr and Foegeding, 1990).

Minerals

Ash is the third major constituent in bovine whey and the fourth in ovine whey (Table 2.3). It also contains significant and varying concentrations of inorganic minerals, namely Ca^{2+}, Mg^{2+}, citrate and phosphate (PO_4^{3-}) ions derived from casein micelles prior to draining from the cheese

vat (Morr and Ha, 1993). Acid whey contains small curd particles and higher concentrations of these four minerals than sweet whey. Due to its high salinity, salty whey is more difficult to process and has higher disposal costs than sweet whey. However, the addition of sodium or calcium chloride during cheese manufacture can also contribute to variations in the mineral content of whey (Jelen, 1992). During whey processing the mineral content can change considerably due to the use of electro-dialyses, nanofiltration or ion exchange processes (Walzem, 2004). In ultrafiltration, minerals that bind to proteins (i.e. calcium, phosphorus and magnesium) are concentrated in the retentate, although soluble minerals (i.e. sodium, potassium and citrate) pass through the membrane and are part of the permeate (Renner, 1992).

Vitamins

Considerably high proportions of hydrosoluble milk vitamins remain in whey: 40-70% vitamin B12, 55-75% B6 and pantothenic acid, 70-80% of riboflavin and biotin, and 80-90% of thiamine, nicotinic, folic and ascorbic acids. The amount of vitamin C decreases during cheese manufacture and for this reason whey is not a rich source of this vitamin. Vitamin A is the most abundant liposoluble vitamin in whey (Walzem, 2004). During ultrafiltration these vitamins (A, D, E and K) are concentrated with lipids and remain in the retentate. Soluble vitamins pass to the permeate, with the exception of folic acid and vitamin B12 (Renner, 1992).

2.2 Whey valorisation

Whey valorisation has been the subject of much research (Guimarães et al., 2010; Onwulata and Huth, 2008). The aim has been to solve the environmental problem of whey disposal whilst simultaneously producing energy (biogas, ethanol) and added value compounds with high nutritional and functional properties (Siso, 1996). Figure 2.4 summarises the whey valorisation processes and final products according to their complexity and costs, respectively. Three main alternatives are identified: direct whey utilisation, whey concentration and drying, and fractionation and/or isolation of whey components.

Siso (1996) reported that approximately 50% of the total world cheese whey production is treated and transformed into various animal feed and food products. About half of this amount is used directly in liquid form, 30% as powdered whey, 15% as lactose and its by-products and the remainder as whey protein concentrates (Spălățelu, 2012). It has been estimated that this percentage is likely to increase, due to continuing research in the field of whey utilisation together

with the pressure brought to bear on cheese and casein producers by stricter legislation on effluent disposal (Guimarães et al., 2010).

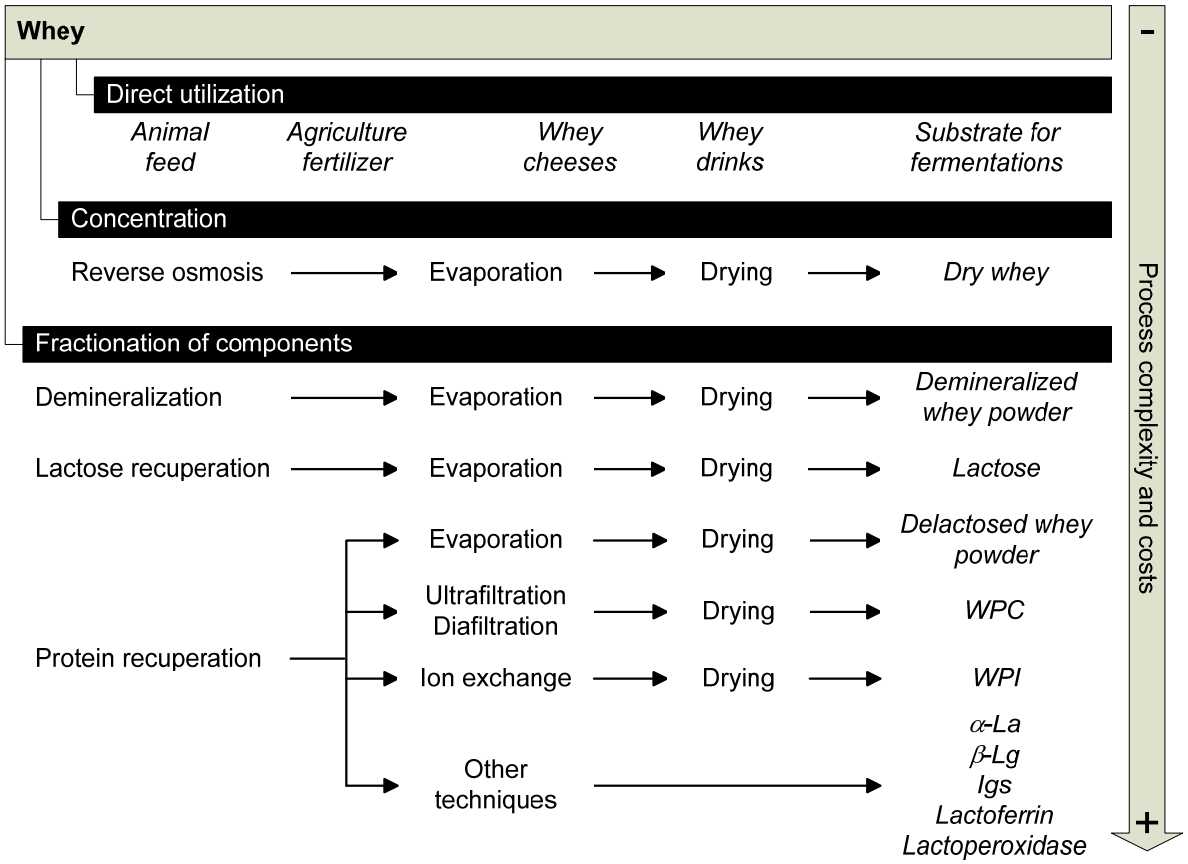


Figure 2.4 Processes of whey valorisation and final products.

2.2.1 Direct utilisation

Direct utilisation of whey is the traditional and oldest practice for this dairy by-product. Liquid whey can be supplied to farmers for use either as a biofertiliser or to provide proteins and lactose for feeding farm animals. However, it should be noted that the transport of untreated liquid whey is very expensive and only applicable when farms are located near the cheese industry in order to minimise microbial development (Smithers, 2008). In order to use it as a fertiliser, although this may depend on soil characteristics and precipitation, the amount should not exceed 7 m^3/ha/d to avoid excess acidity and sanitary problems (Radford et al., 1986; Westergaard, 2004).

Currently, the production of whey cheeses seems to be the easiest method for recovering valuable components from whey. These products have different names according to the country or region in which they are produced. *Mysost Primost, Gjestost* and *Grubransdalsost* are produced in Norway, *Schottenziergr, Hudelziger* and *Mascarpone* in Switzerland, *Serac, Brousse, Broccio* and *Greuil* in France, *Manouri, Mysithra* and *Anthotyros* in Greece, *Ricotta* in Italy, *Requesón* in Spain and *Requeijão* in Portugal. The most popular products are *Ricotta* (Pintado et al., 2001) and *Mysost* (Jelen, 2003). Their sensorial properties and chemical composition differ significantly according to the origins of the whey and the manufacturing practices (Pintado et al., 2001). *Requeijão* is obtained by heating the whey to approximately 90-100 ºC for 15-30 min, with or without the addition of 10-20% (v/v) ovine/caprine milk. Pintado et al. (1996) has characterised the product and reported on the optimisation of the manufacturing process. However, the residual whey, called *Sorelho*, resulting from the manufacture of *Requeijão* still contains approximately 60% of the original whey dry matter. Lactose and minerals largely contribute to the dry mass, but residual fat and non-thermally precipitated nitrogen components are still present. The composition of this product does not allow for direct disposal without treatment (Pereira et al., 2002).

Whey drinks, as well as whey cheeses, are the most obvious direct application of whey (Jelen, 2003). Although they have not met with much success among consumers, there are some exceptions. *Rivella*, a Swiss carbonated drink produced since 1950, is commercialised in three distinct forms: original (33% whey at acid pH 3.7), light and aromatised (with green tea extracts) (de Wit, 2003; Smithers, 2008). The disagreeable flavour of crude whey is cited as the main drawback to these whey-based products and the use of modified whey in combination with fruit juices or liquid yogurts is gaining acceptance (Jelen, 2003).

Since lactose represents more than 70% of total whey solids, one of the major applications of liquid whey involves its use as substrate for fermentations. The classic examples are ethanol and single cell protein production in yeast-based bioprocesses, although the production of antibiotics, organic acids, amino acids, carbohydrates, enzymes is also cited (Castillo et al., 1996; Pesta et al., 2007; Guimarães et al., 2010). These applications will be discussed in more detail in Section 2.2.3 since some of these processes do not directly involve whey, but use whey permeates or purified lactose as a source of lactose. However the decision to use already purified lactose, whey permeates or whey as raw materials will depend on the intended final product and also on economic considerations.

2.2.2 Concentration

As previously mentioned (Section 2.1.1), dry whey is the whey end-product that is most widely produced and traded worldwide and represents more than half of the treated whey. Dry whey or powdered whey is a very stable dehydrated product with a moisture content ranging from 3.5% to 5%. It is also easy to handle and transport and has attractive nutritional and functional properties. The simplest and most traditional technology used in its production is liquid whey concentration by multi-effect evaporation systems until the total solid content reaches 40-60%, followed by lactose crystallisation (avoiding hygroscopic problems) and atomisation in spray-dryers. The temperature during evaporation may not exceed 70ºC and must be strictly controlled during drying to avoid protein denaturation and loss of functionality. It is also possible apply reverse osmosis as a pre-concentration step before evaporation (Figure 2.4). However the extent of this technique is limited by the viscosity of the retentate, and for this reason it cannot be used as an alternative to evaporation (Pearce, 1992).

Dry whey is mainly used for animal feed but smaller quantities are also used in foods such as ice-cream, baked goods, cakes, sauces and milk derivatives (Siso, 1996). However, due to its excessively saline taste, its use in human foods is not favoured. For this reason, demineralised whey powder (which will be discussed in the next section) is generally produced and commercialised for food purposes instead of whole powdered whey.

2.2.3 Fractionation of components

Liquid whey is not a balanced source of nutrients regardless of its origins (cheese or casein production). Its high lactose and mineral content and low protein concentration, which do not allow for the expression of functional properties, limits its application in several products. Increasing whey functionality involves separating its components (Figure 2.4), and their concentration, purification and/or elimination. Six major classes of dry whey-derived products can be obtained from the fractionation of whey: demineralised whey (Table 2.5), delactosed whey, lactose or whey permeate (Table 2.5), whey protein concentrates (e.g. WPC35, WPC80 – Table 2.5), whey protein isolates (WPI) and individual proteins (e.g. β-Lg, α-La, SA, Igs, Lf and LPO).

A wide range of whey fractionation techniques has been developed and used (Table 2.6). Three main groups can be identified: membrane fractionation, precipitation or complexation with reagents, and physical and chromatographic separation. Although methods based on

complexation and precipitation lead to high purity components (e.g. proteins), they suffer from problems related to residual reagent contamination. Membrane methods are the most attractive methods since they are clean technologies.

Table 2.5 Composition of whey derived most common dry products in the market.

Composition (%)	Sweet whey* (1)	Whey permeate (1)	WPC35 (1)	WPC80 (1)	WPI (2, 3, 4)
Moisture	3.5-5.0	3.0-5.0	3.0-4.5	3.5-4.5	4.0-5.5
Protein	11.0-14.5	3.0-8.0	34.0-36.0	80.0-82.0	95.0-96.0
Carbohydrates (Lactose)	63.0-75.0	65.0-85.0	48.0-52.0	4.0-8.0	0.5-2.0
Fat	1.0-1.5	1.5 Maximum	3.0-4.5	4.0-8.0	0.4-8.0
Ash	8.2-8.8	8.0-20.0	6.5-8.0	3.0-4.0	1.0-6.0

*demineralized; (1) Neuhaus (2008); (2) Huffman and Harper (1999); (3) Walstra et al. (2006); (4) Foegeding and Luck (2003).

Table 2.6 Techniques used in the manufacture of whey protein derived products (adapted from Schmith et al., 1984).

Techniques	Operations / complexation reagents
Membrane fractionation	Microfiltration Ultrafiltration/Diafiltration Nanofiltration Reverse osmosis Electrodialysis
Precipitation or complexation with reagents	Metaphosphates Carboxymethylcellulose (CMC) Polyacrylic acids Iron
Physical and chromatographic separation	Centrifugation/Ultracentrifugation Ion-exchange chromatography Gel filtration Foam concentration Heat precipitation Supercritical extraction

Membrane filtration technologies are pressure-driven molecular separation processes used to obtain concentration, fractionation, clarification and/or even sterilisation of a liquid. Separation is determined by the membrane characteristics (the molecular weight cut-off value) and the molecular size of the individual components present in the liquid (SPX, 2010). The volume and/or composition of a liquid as the feed is divided into two new liquids with an altered chemical composition: the retentate (concentrated by the membrane, e.g. proteins), and the permeate (which passes through the membrane, e.g. lactose, water and minerals). Figure 2.5 illustrates the fractionation of milk and whey components according to molecular size and

membrane separation process. Four filtration processes can be applied in whey component fractionation: microfiltration (MF), ultrafiltartion (UF), nanofiltration (NF) and reverse osmosis (RO).

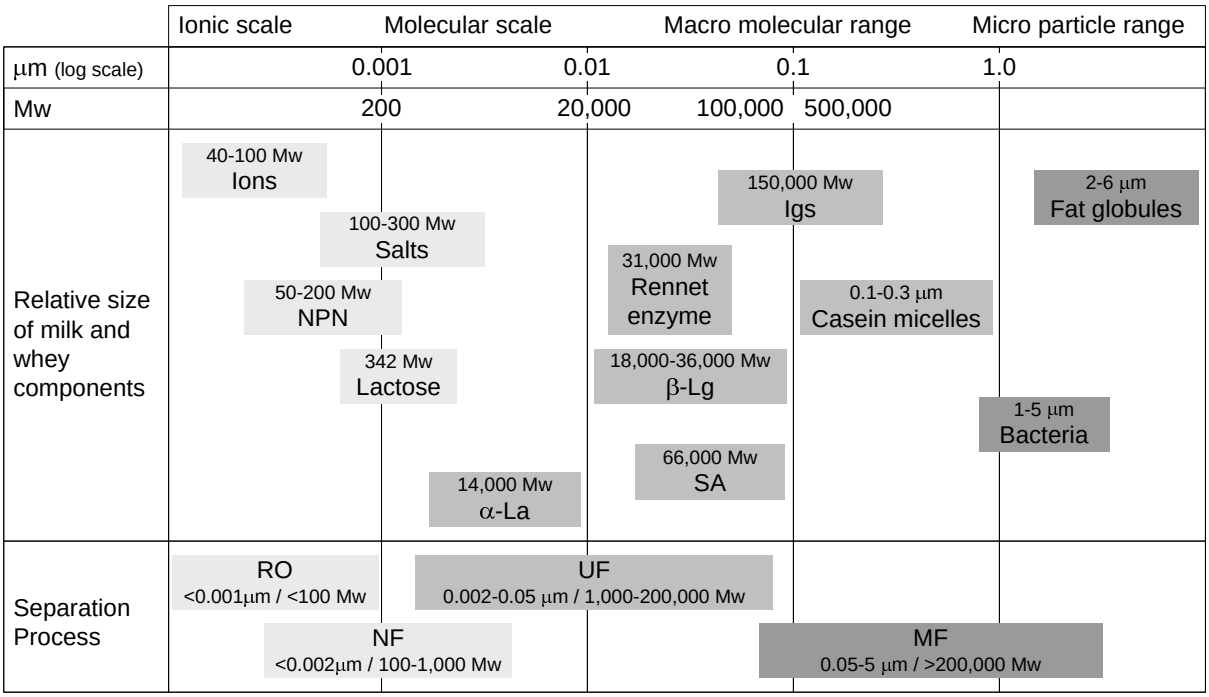


Figure 2.5 The filtration spectrum for milk and whey components fractionation (adapted from SPX, 2010).

Microfiltration (0.05-5 μm) may be used to eliminate fat globules and casein micelles that remain in the whey in order to improve whey protein functionality or to eliminate bacterial cells (Maubois, 1997; Pereira et al., 2002).

Ultrafiltration (0.002-0.05 μm) is the most important membrane technique in whey processing. This process allows for the concentration of whey proteins in the retentate and the elimination of lactose, salts, non-protein nitrogen compounds (NPN) and ions in the permeate. The relative protein concentration can also be controlled by the selection of UF membranes with specific cut-offs. When products with a higher purity are necessary, diafiltration (DF) is applied. In diafiltration, water is added to the UF retentate to increase the "washing out" of dissolved substances such as lactose and minerals into the permeate.

Nanofiltration is very similar to the reverse osmosis process. NF membranes, however, are slightly more open (< 0.002 μm) than in conventional RO (< 0.001 μm). NF allows for the passage of monovalent ions such as Na^+, K^+ and Cl^-, whereas divalent ions like Mg^{2+} and Ca^{2+} are

almost completely rejected by the membrane. In this way the NF process demineralises the feed, typically by 30% to 40%. In RO practically all the total solid components are rejected by the membrane, allowing only water to pass through. Since almost all ions (apart from OH^- and H^+) are also rejected by the membrane, the osmotic pressure in the retentate will increase, requiring high pressure pumps to overcome osmotic pressure, 4-8 MPa (Glover, 1985). It is commonly used in whey concentration by water and mineral elimination (Rosenberg, 1995). Low molecular components such as organic acids and NPN components are not fully rejected by the membrane, especially when they are uncharged (non-ionic), typically in acidic environments. This is the reason why COD levels in the permeate are higher when processing acidic products (e.g. acid whey) in comparison to sweet products (e.g. sweet whey). The maximum achievable solids by RO are in the 17% to 25% range for whey and UF permeates (SPX, 2010).

Demineralised whey powder

As mentioned in the previous section, the use of whey powder in food implies reducing its salt content. In general, dry whey contains 15% of minerals (dry basis). It is necessary to reduce this by 90-95% for use in infant formulas, whereas a reduction 50-70% is satisfactory for ice cream in order to avoid a salty flavour (Burling, 2003; Jelen 2003). The principal techniques applied to whey demineralisation are electrodialysis, diafiltration and ion exchange chromatography. However, nanofiltration (Figure 2.5) may also be applied as a treatment prior to total demineralisation (Kelly, 2003).

Lactose and delactosed whey powder

Lactose can be obtained from whey during concentration or from whey permeate produced after ultrafiltration (Spălăţelu, 2012).

The concentration of whey by evaporation (to around 65% solids) allied to lower lactose solubility leads to the formation of lactose crystals, which can be separated by crystallisation followed by centrifugation. The whey that results from this process can be dried and is therefore called delactosed whey powder. It is a very attractive product for use in foods for lactose-intolerant individuals. The lactose crystals are washed and dried in a fluidised bed dryer to attain an edible grade, whereas refining is necessary to obtain a pharmaceutical grade of lactose. This involves re-dissolving the lactose crystals and treating the solution with activated carbon, which absorbs a number of solutes including riboflavin and a variety of proteins (e.g. proteose peptones). The carbon is removed by flocculation and filtration and then discarded. After

crystallisation, the crystals are separated by centrifugation and drying and a high purity white pharmaceutical grade lactose is obtained. The crystals are milled or sifted to yield products with specific particle size distributions (Kellam, 2012).

During the processing of whey for the production of WPC, high volumes of a lactose-rich stream (permeate) are also obtained. The permeate remains a major pollutant since it retains the lactose, which represents more than 70% of total whey solids (Guimarães et al., 2010). If the intention is to produce dry lactose, the previous crystallisation and purification processes can be applied to the permeate. However, the use of UF permeate as a substrate for fermentations (Zadow, 1984; Yang and Silva, 1995; Siso, 1996; Audic et al., 2003; Pesta et al., 2007; Spălăţelu, 2012) or to produce hydrolysed lactose syrup is well developed (Gänzle et al., 2008). The fermentation bio-products include biogas (methane), organic acids (acetic, propionic, lactic, citric and gluconic), amino acids (glutamic, lysine, and threonine), vitamins (B12 and B2, or cobalamins and riboflavin, respectively), polysaccharides (xanthan gum, dextran, phosphomannan, pullulan, and gellan), oils (lipids), enzymes (β-galactosidase and polygalactorunase) and other compounds (fructose-diphosphate, 2,3-butanediol, calcium magnesium acetate, ammonium lactate, butanol, and glycerol).

WPC and WPI

Proteins are the second most abundant constituent of whey after lactose, and are considered its most valuable fraction. Whey processing for protein recovery has benefited from developments in membrane technology. Nowadays whey ultrafiltration and diafiltration are standard operations in the dairy industry (Pouliot, 2008), allowing for their recovery (Figure 2.5) without significant loss of functional properties and with a low salt content that makes them fit for human consumption. The protein content in the final products determines whether they are classified as whey protein concentrates (WPC) or whey protein isolates (WPI). The protein concentration in WPC ranges from 30% to 80%, whereas WPI requires a protein concentration of over 90%. WPC is typically obtained by ultrafiltration (WPC with protein content < 50%) or diafiltration (WPC with protein content > 50%), followed by drying (Figure 2.4). However, the use of ion-exchange resins offers an effective method for the preparation of high-quality whey protein products, referred to as WPI. Although the functional properties of WPI are superior to those of WPC on an equiprotein basis (due to lower levels of lipids, lactose and salts), production is rather

limited, due to higher production costs (Huffman and Harper, 1999; Foegeding et al., 2002; Foegeding and Luck, 2003; Onwulata, 2008).

The fractionation or isolation method used in the manufacture of WPC and WPI products is the principal factor responsible for their composition, but this obviously depends on the whey origin (e.g. milk type or coagulation type). A wide range of compositions has been reported for WPC and WPI prepared by the various techniques: 30-95 % protein, 1-80% lactose, 1-18% ash, and 1-9% fat (Huffman and Harper, 1999; Foegeding and Luck, 2003; Walstra et al., 2006; Neuhaus, 2008). Table 2.5 shows the composition of typical WPC products (WPC35 and WPC80) and WPI available on the market.

Individual proteins

Several techniques may be used for the fractionation of whey proteins into individual proteins (Table 2.6). Although membrane separation processes can be applied to individual protein separation by careful selection of membrane type and cut-off, they are more suitable for concentration purposes. Techniques such as precipitation or complexation with reagents that make use of protein chemical reactivity, as well as physical and chromatographic separations that are related to the ionic nature, molecular weight and denaturation capacity of proteins are the methods most commonly applied to fractionation (Etzel, 2004). Despite the thermal denaturation required for protein separation and isolation, the loss of functionality and biological activity restricts their use in foods when the intention is to improve their functionality or the biological effects of whey proteins. In this situation the addition of whey proteins to food does no more than enhance the final protein content (Pearce, 1992; Castillo et al., 1996). Huffman and Harper (1999) have reported that the industrial-scale techniques that usually produce enriched fractions of α-La are also appropriate for the recovery of β-Lg. However, the optimised protein concentration, pH, temperature and acid/Ca^{2+} molar ratio can selectively separate α-La (Lucena et al., 2006, 2007). Lactoferrin, Lactoperixidase and GMP are commercially fractionated by ion exchange chromatography, although supercritical extraction by CO_2 is attracting interest (Bonnaillie and Tomasula, 2008). Individual whey proteins are the most expensive whey-derivative products available on the market. This is due to the fractionation techniques applied during recovery/isolation and the complexity of the manufacturing processes. Their use is restricted to pharmaceutical applications, due to their recognised biological properties (Madureira et al., 2007).

2.2.4 WPC and WPI functionality affected by processing

Although WPC and WPI may be used in food products solely for their high nutritional properties they have also gained acceptance as functional ingredients (Morr and Ha, 1993; Singh, 2003; Estrada, 2010). Most of the key functional properties identified in whey proteins may be classified as hydration-related and surface-related properties. The first group includes solubility, dispersability, swelling, viscosity, gelation, adhesion, precipitation and water retention. The second group includes foaming, emulsification and adsorption at air-water and oil-water interfaces. However, diffusion, denaturation and protein-protein, protein-ion and protein-ligand binding properties are also of interest, although the lack of consistency in the gross chemical composition and functionality of commercial WPC and WPI may limit their acceptance by food processors (Cerbulis et al., 1982; Morr and Foegeding, 1990; Smithers et al., 1996). Nowadays, a real challenge in the area of WPC/WPI functionality as food ingredients is to predict how manufacturing practices may affect protein functionality, and on this basis, select WPC/WPI products with the optimum functionality for each product application.

The protein functionality of WPC and WPI is affected, to a greater or lesser extent, by the process variables presented in Table 2.7, namely whey source and composition, heat treatments, fractionation and isolation techniques, cheese manufacturing practices, and storage and sanitation conditions.

Whey source and composition

In the earlier sections it was noted that ovine whey had whey protein concentrations that were uniquely different from those of bovine whey. The comparatively high β-Lg levels in ovine speciality cheese whey may allow for the production of dry whey products with specific enhanced functional characteristics and applications (Casper et al., 1998). The functional properties of WPC and WPI of bovine origin have been widely explored (Kinsella 1976; Kilara, 1984; Morr and Ha, 1993; de Wit, 1998; Ha and Zemel, 2003; Abd-El-Salam et al., 2009). However, few studies have been developed regarding the functional properties of WPC of ovine or caprine origins (Pizzichini et al., 1995; Comendador et al., 1996; Casper et al., 1999; Días et al., 2004; Estrada, 2010). For this reason, until further research on this topic effectively proves the potential for caprine and ovine whey valorisation, the options available to speciality cheese producers for whey disposal are few and costly.

Table 2.7 Processing variables that may affect the functional properties of WPC and WPI (adapted from Schmidt et al., 1984).

Process variables	
Whey source and composition	Milk/whey origin: bovine, ovine or caprine Milk/whey composition
Heat treatment	Milk pasteurization; whey heat treatment Heat during isolation, evaporation and concentration
Fractionation and isolation	Technique used Process factors (e.g. foaming, pumping)
Cheese manufacturing practices	Type of cheese Rennet (or rennet substitutes); starter culture Process modification (e.g. addiction of $CaCO_3$, direct acid)
Storage factors	Whey storage conditions Whey protein product storage conditions
Sanitation factors	Microbial load Peroxide addition

Heat treatments

Heat is the factor with the most measurable effects on whey protein functionality. From a practical point of view, protein denaturation in WPC or WPI reflects the effects of individual heat treatments, if applied, during the manufacturing stages, especially the processes performed before protein fractionation and/or isolation (milk and/or whey pasteurisation) or after (heating during evaporation, concentration or dehydration). The structural unfolding of proteins may occur at moderate temperatures (60-70 ºC). However, higher temperatures lead to protein aggregation as a function of compositional factors such as total solids, pH, lactose and mineral content. The aggregation process involves disulfide linkage mediated by calcium ions, although other types of molecular interactions such as hydrogen and hydrophobic bonding also play a role. The order of heat stability for whey proteins measured by loss of solubility is α-La > β-Lg > SA > Igs (Fox, 2001). However, the dynamic changes in composition during WPC or WPI manufacture in each stage of the process makes it difficult to achieve real heat denaturation, if it occurs. It has been reported that an increase in total solids content is related to a reduction in β-Lg heat denaturation and an acceleration of α-La denaturation (Dalgleish et al., 1997; Havea et al., 2002). Higher lactose content generally decreases the rate of protein denaturation and the presence of calcium induces heat protein aggregation (Puyol et al., 2001).

Fractionation and isolation

Membrane processing techniques have a direct effect on WPC composition, as discussed in previous sections. However, indirect effects of protein denaturation may also occur, depending

on the process design and operation (i.e. degree of foaming, pumping, mixing and aeration). Membrane fouling appears to be a problem in ultrafiltration. In order to minimise this drawback some whey pre-treatments may be adopted, such as clarification and centrifugation to remove protein aggregates and lipid components, heating and pH adjustment to induce protein complexation and demineralization, and changes in flow velocity during concentration (Schmidt et al., 1984).

Cheese manufacturing practices

Factors that affect cheese yield (such the milk heating treatment and manufacturing conditions) would generally be expected to alter whey composition and consequently WPC composition. However there are certain other factors whose influence can affect WPC, such as the rennet or the starter cultures used in cheese making. Some proteolytic activity, caused by residual enzymes, can directly alter the composition of non-protein nitrogen compounds and also have a direct impact on protein conformation. The whey proteins most susceptible to proteolytic attack by rennet are bovine serum albumin and immunoglobulin (Pintado and Malcata, 2000). β-Lg and α-La are highly resistant to proteolysis. The use of starter cultures (lactic acid bacteria) in cheese does not seem to have a significant impact on WPC or WPI composition. In this case, whey can be affected but only to a small extent. Increasing the bacterial metabolism and the amount of starter inoculum may increase the calcium and phosphate in whey. The use of buffered culture media for the propagation of starter cultures can also affect the ionic composition.

Storage and sanitation factors

Whey storage conditions (e.g. temperature and time) should be a consideration in avoiding spoilage microorganisms. These microorganisms are, in general, highly proteolytic, and if they are present in high concentrations WPC functionality can be compromised.

Although the main problems that need to be addressed in the commercial processing of whey protein ingredients are associated with protein denaturation during production and the high variability of the composition of products classified as whey protein concentrates or isolates, the introduction of certain process modifications may alter or improve protein functionality. These methods include thermocalcic precipitation and microfiltration to remove phopholipoproteins (Karleskind et al., 1995; Pereira et al., 2002; Díaz et al., 2004), selective demineralisation to control the ionic environment (Jonhs and Ennis, 1981), proteolytic enzyme hydrolysis of heat denaturated whey protein in order to improve solubility (Morr and Ha, 1993), controlled heat-

induced polymerization (Foegeding et al., 2002, Nicorescu et al., 2008), and the use of high hydrostatic pressures to change protein structure and functionality (Liu et al., 2009).

2.3 Applications and opportunities of whey protein products

Table 2.8 shows the wide range of whey protein product applications. They are grouped into two major categories: conventional and nonconventional applications. The conventional applications include food product applications, the main purpose of which is to take advantage of the functional properties of whey protein, health product applications for people with specific nutritional needs, and use in medicinal/pharmaceutical products. The nonconventional applications correspond to non-food products, although in some cases this includes the possibility of their subsequent use in food products. In this area three different categories of products are identified: cosmetics and care products, microencapsulation systems, and films and coatings.

Table 2.8 Conventional and nonconventional applications of whey proteins products: WPC, WPI and individual whey protein fractions (adapted from Fox and McSweeney, 1998; Díaz et al., 2009).

Conventional applications		
Products	**Used in**	**Effect**
Bakery products	• Bread, cakes, muffins, croissants	Nutritional, emulsifier, egg replacer
Dairy products	• Yoghurt, Quark, Ricotta cheese	Yield, nutritional, consistency, curd cohesiveness
	• Cream cheeses, cream cheese spreads, sliceable/squeezable cheeses, cheese fillings and dips	Emulsifier, gelling, sensory properties
Beverages	• Soft drinks, fruit juices, powdered or frozen orange beverages	Nutritional
	• Milk-based flavoured beverages	Viscosity, colloidal stability
Dessert products	• Ice-cream, frozen juice bars, frozen dessert coatings	Skim-milk solids replacement, whipping properties, emulsifying, body texture
Confectionary	• Candy mixes, meringues, sponge cakes	Whipping properties, emulsifier
Pasta	• Macaroni, pasta and imitation pasta	Nutritional, texture, freeze-thaw stability, microwaveable
Meat products	• Frankfurters, luncheon meats	Pre-emulsion, gelation
	• Injection brine for fortification of whole meat products	Gelation, yield
Seafood products	• Shrimp, salmon	Structuring, texturing, nutritional, yield
Textured products	• Puffed snack foods, protein-enriched snack-type products, meat extenders	Structuring, texturing, nutritional

Table 2.8 (Continued)

Conventional applications		
Products	**Used in**	**Effect**
Convenience foods	• Gravy mixes, soup mixes, sauces, canned cream soups and sauces, dehydrated cream soups and sauces, salad dressings, microwaveable foods, low lipid convenience foods	Whitening agents, dairy flavor, flavor enhancer, emulsifier, stabilizer, viscosity controller, freeze-thaw stability, egg yolk replacement, lipid replacement
Functional and nutraceutical products	• Special dietary preparations for dieting patients/people, athletes, astronauts	High protein levels
	• Infant foods: whey protein hydrolysates used in hypoallergic formulae preparations,	Nutritional fortification; 'humanized' infant formulae; low-lactose infant formulae; specific mineral balance infant food
Medicinal/pharmaceutical products	• Intravenous foods	Patients suffering from metabolic disorders, intestinal disorders for postoperative patients
	• Special food preparations	Patients suffering from cancer, pancreatic disorders of anaemia
	• Specific drug preparations	β-caseinomorphins used in sleep or hunger regulation or insulin secretion; sulphonated glycopeptides used in treatment of gastric ulcers
	• Pharmaceutical products, wound treatment preparations	Diseases prevention or treatment
Nonconventional applications		
Products	**Used in**	**Effect**
Miscellaneous products	• Toothpastes, cosmetics	Improve product's properties, application of natural compounds
Microencapsulation agents	• Microespheres, nanoparticles	Controlled drug release, oral delivery vehicles for bioactive compounds
Films and coatings	• Food and drug packaging	Barrier and mechanical properties
	• Food coating, synthetic plastic coating	Visual appearance, barrier properties, antimicrobial and antioxidant activity, carrier of nutraceuticals

Food preparations represent the main end uses of WPC and WPI. They are applied to: bakery products and baking mixes, namely confectionary, chocolate, cakes and pastries; processed dairy products such as cheese, yogurts, ice cream and desserts; meat products and sausages; seafood products; processed fruits and vegetables; texturised products, crackers and snack foods; coffee whiteners; convenience food, including salad dressings, gravies and sauces, soups, mayonnaise,

pasta and pie fillings; fruit beverages. In all these examples the intention is to make use of the functional properties of whey protein that can be added to foods. WPC with a lower protein content tends to be used more in the lower-value food products, such as dairy and bakery products, and higher concentration WPC is generally used in higher-value food products, such as meat and seafood (Tunick, 2008). WPI and individual whey protein fractions are mainly associated with healthcare products recommended for their nutritional value, such as infant formulas, baby food, special dietary supplements and sports drinks, medicinal and pharmaceutical products, intravenous foods, specific drug preparations and wound treatment preparations.

Non-food applications of whey proteins are the most recent and less widely explored and, for this reason, more promising applications. The cosmetics and care products industry is extremely attractive due to the recognised added value of their products. In both cases the incorporation of particular fractions of whey proteins may improve specific properties and/or replace synthetic products with natural ones. Microencapsulation systems also show great potential for use within the pharmaceutical and food industry. The ability of whey proteins to form gels, emulsions, foams, microcapsules and nanoparticles makes them very attractive matrixes for encapsulating a wide range of sensitive products by protecting them from undesirable environmental conditions, or for their controlled delivery. In terms of food product applications, whey proteins have been tested for the encapsulation of: bifidobacteria (Adhikari et al., 2003; Picot and Lacroix, 2004; Krasaekoopt et al., 2006); flavourings (Baranauskiene et al., 2006), essential oils (Bylaitë et al, 2001; Parris et al., 2005), antioxidants (Chang et al., 2005); caffeine (Gunasekaran et al., 2007); and enzymes (Kailasapathy and Lam, 2005). As drug delivery systems, studies have been carried out on theophylline (Lee and Rosenberg, 2001), folic acid (Madziva et al., 2006) and retinol (Beaulieu et al., 2002). Albumin nanoparticles have been extensively investigated with regard to their preparation methods and release properties (Langer et al., 2003; Loo et al., 2004). Bovine serum albumin has been used as natural matrix material for delivery devices (Brannon-Peppas, 1995). The use of whey proteins in the production of films and coatings with attractive mechanical and barrier properties as alternatives to traditional plastics is attracting the interest of consumers and companies (Dangaran and Krochta, 2008). They have been the subject of a considerable amount of research and will also be one of the main topics of investigation in this thesis (Chapters 7, 8 and 9). The food industry is paying attention to their use and testing how they may meet their packaging needs. It can therefore be observed that whey proteins have high potential in a wide range of areas. However, all the applications presented make use of

dehydrated whey protein products, which is incompatible with their valorisation in small/medium scale industries due to the costs associated with the drying stage. Alternatives such as the use of whey protein concentrate without dehydration may be part of the solution.

2.4 Conclusions

Few big companies have the capacity to implement different technologies for the efficient recovery and upgrading of whey. A challenge exists in terms of incorporating medium and small whey producers (the reality in the Portuguese dairy industry) into management strategies that include recovery, processing and upgrading. The recovery, fractionation and transformation of whey may acquire a significant economic value for speciality cheese producers (ovine and caprine cheeses), thus reconciling the interests of the productive sector with the social demands of environmental protection within the framework of sustainable development.

References

3ABC – 3A Business Consulting (2012) Global Opportunities for Whey and Lactose Ingredients 2010-2014. <http://www.3abc.dk/>

Abd El-Salam MH, El-Shibiny S and Salem A (2009) Factors Affecting the Functional Properties of Whey Protein Products: A Review. Food Reviews International, 25(3), 251-270.

AC - Águas de Coimbra (2012) ESPIT032 A-01 – Valores limites de emissão para águas residuais industriais.

Adhikari K, Mustapha A and Grun IU (2003) Survival and metabolic activity of microencapsulated *Bifidobacterium longum* in stirred yogurt. Journal of Food Science, 68, 275-280.

Alexandrescu AT, Evans PA, Pitkeathly M, Baum J and Dobson CM (1993) Structure and dynamics of the acid-denatured molten globule state of α-Lactalbumin: A two-dimensional NMR study. Biochemistry, 32, 1707-1718.

Audic JL, Chaufer B and Daufin G (2003) Non-food applications of milk components and dairy coproducts: a review. Lait, 83, 417-38.

Baranauskiene R, Venskutonis PR, Dewettinck K and Verhe R (2006) Properties of oregano (*Origanum vulgare* L.), citronella (*Cymbopogon nardus* G.) and marjoram (*Majorana hortensis* L.) flavors encapsulated into milk protein-based matrices. Food Research International, 39(4), 413-425.

Beaulieu L, Savoie L, Paquin P and Subirade M (2002) Elaboration and characterization of whey protein beads by an emulsification/cold gelation process: application for the protection of retinol. Biomacromolecules, 3(2), 239-248.

Blaskó B (2012) World importance and present tendencies of dairy sector. Applied Studies in Agribusiness and Commerce - APSTRACT, Agroinform Publishing House, Budapest, 119-123. < http://ageconsearch.umn.edu/bitstream/104683/2/20_Blasko_PHD_Apstract.pdf>

Bonnaillie LM and Tomasula PM (2008) Whey protein Fractionation. In: CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

Boyd LC, Drye NC and Hansen AP (1999) Isolation and characterization of whey phospholipids. Journal of Dairy Science, 82, 2550-2557.

Brannon-Peppas L (1995) Recent advances on the use of biodegradable microparticles and nanoparticles in controlled drug delivery. International Journal of Pharmaceutics, 116, 1-9.

Brew K and Grobler JA (1992) α-Lactalbumin In PF Fox (Ed.) Advanced dairy chemistry. vol. 1: Proteins, Elsevier Applied Science, London, pp. 191-229.

Burling H (2003) Whey processing: demineralization. In: H Roginki, JW Fuquay and PF Fox (Ed.) Encyclopedia of Dairy Sciences, vol. 4, London, Academic Press (Elsevier Science).

Bylaitë E, Rimantas VP and Maþdþierienë R (2001) Properties of caraway (*Carum carvi* L.) essential oil encapsulated into milk protein-based matrices. European Food Research and Technology, 212(6), 661-670.

Casper JL, Wendorff WL and Thomas DL (1998) Seasonal Changes in Protein Composition of Whey from Commercial Manufacture of Caprine and Ovine Specialty Cheeses. Journal Dairy Science, 81(12), 3117-3122.

Casper JL, Wendorff WL and Thomas DL (1999). Functional properties of whey protein concentrates from caprine and ovine specialty cheese whey, Journal Dairy Science, 82, 265-271.

Castillo M, Jordán MJ, Abellán A, Laencina J and López MB (1996) Tecnología de aprovechamiento del lactosuero. Revista Española de Lechería, Marzo, 24-30.

Cerbulis J, Woychik JH and Wondolowski MV (1972) Composition of commercial wheys. Journal Agricultural Food Chemistry, 20(5), 1057-1059.

Chang P-S, Lee JH and Lee JH (2005) Development of a New Colorimetric method determining the yield of microencapsulation of α-tocopherol. Journal of Agricultural and Food Chemistry, 53(19), 7385-7389.

Comendador FJ, Nardo N, Bertone A, Maurizi A and Quaglia G (1996). Effetto dell'ultrafiltrazione sulle proprietá funzionali della scotta ovina. Scienza e Tecnica, Lattiero-Casearia, 47(2), 103-112.

Dalgleish DG, van Mourik L and Corredig M (1997) Heat-Induced Interactions of Whey Proteins and Casein Micelles with Different Concentrations of α-Lactalbumin and β-Lactoglobulin. Journal of Agricultural and Food Chemistry, 45(12), 4806-4813.

Dangaran K and Krochta JM (2008) Whey Protein Films and Coatings. In: CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

de Wit JN (1989) Functional properties of whey proteins. In PF Fox (Ed.), Developments in dairy chemistry. New York: Elsevier Applied Science, USA.

de Wit JN (1998) Nutritional and functional characteristics of whey proteins in food products. Journal of Dairy Science, 81, 597-608.

de Wit JN (2003) Dairy ingredients in non-dairy foods. In H. Roginski, J.W. Fuquay and P.F. Fox, Encyclopedia of dairy sciences (pp. 718-727), London: Academic Press (Elsevier Science).

de Wit JN and Klarenbeek G (1984) Effects of various heat treatments on structure and solubility of whey proteins, Journal of Dairy Science, 67, 27012710.

Decreto-Lei n.º 236/98 de 1 de Agosto. Ministério do Ambiente. Diário da República - I série A.

Díaz O, Pereira CD and Cobos A (2004) Functional properties of ovine whey protein concentrates produced by membrane technology after clarification of cheese manufacture by-products. Food Hydrocolloids, 18(4), 601-610.

Díaz O, Pereira CD and Cobos A (2009) Aplicaciones de los concentrados y aislados de proteínas de lactosuero de la industria alimentaria. Alimentaria - Investigacion, Tecnologia e Seguridad, 400, 108-115.

EDA - European Dairy Association (2012) About the European Dairy Industry - Statistics. <http://www.euromilk.org/eda/index.aspx>

Estrada BS (2010) Aprovechamiento de suero de quesería de origen caprino mediante la obtención de concentrados de proteínas séricas y subprodutos de clarificatión. Estudio de sus propreidades tecnológicas. Tesis Doctoral, Faculdad de Ciencias, Campus de Lugo - Universidad de Santiago de Compostela, Lugo Espanha.

Etzel MR (2004) Manufacture and use of dairy protein fractions. Journal of Nutrition, 134(4s), 996s-1002s.

FAO - Food and Agriculture Organization of the United Nations (2012). <http://faostat.fao.org/default.aspx?lang=en>

Foegeding EA and Luck PJ (2003) Milk proteins: Whey protein products. In: H Roginski, JW Fuquay and PF Fox (Eds.) Encyclopedia of dairy sciences. London: Academic Press (Elsevier Science).

Foegeding EA, Davis JP, Doucet D and McGuffey MK (2002) Advances in modifying and understanding whey protein functionality. Trends in Food Science & Technology, 13(5), 151-159.

Fox PF (2001) Milk proteins as food ingredients. International Journal of Dairy Technology, 54(2), 41-55.

Fox PF and McSweeney PLH (1998) Dairy Chemistry and Biochemistry. Blackie Academic & Professional, 1st edition, London, UK ISBN 0412720000.

Frost RC (2009) EU practice in setting wastewater emission limit values. <http://www.wgw.org.ua/publications/ELV%20-%20EU%20practice.pdf>

Gänzle MG, Haase G and Jelen P (2008) Lactose: crystallization, hydrolysis and value-added derivatives. International Dairy Journal, 18, 685-94.

Gerosa S and Skoet J (2012) Milk availability - Trends in production and demand and medium-term outlook. ESA Working paper No. 12-01, February, Food and Agriculture Organization of the United Nations. <www.fao.org/economic/esa>

Glover FA (1985) Ultrafiltration and reverse osmosis for the dairy industry. National Institute for Research in Dairying, Reading, England, Technical Bulletin nº 5, pp. 100-119.

Guimarães PMR, Teixeira JA, Domingues L (2010) Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnology Advances, 28, 375-384.

Gunasekaran S, Ko S and Xiao L (2007) Use of whey proteins for encapsulation and controlled delivery applications. Journal of Food Engineering, 83(1), 31-40.

Ha E and Zemel MB (2003) Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review). The Journal of Nutritional Biochemistry, 14(5), 251-258.

Havea P, Singh H and Creamer LK (2002) Heat-Induced Aggregation of Whey Proteins: Comparison of Cheese WPC with Acid WPC and Relevance of Mineral Composition. Journal Agriculture Food Chemistry, 50, 4674-4681.

Hoogwegt Groep B.V. (2012) Hoogwegt Horizons, Arnhem, The Netherlands. <http://www.hoogwegtus.com/en/pdf/Horizons%20January%202012.pdf>

Huffman LM and Harper WJ (1999) Maximizing the value of milk through separation technologies. Journal of Dairy Science, 82, 2238-2244.

INE - Instituto Nacional de Estatística, I.P. (2010) Estatísticas Agrícolas 2009. Lisboa, Portugal ISBN 978-989-25-0085-4.

INE - Instituto Nacional de Estatística, I.P. (2012) Estatísticas Agrícolas 2011. Lisboa, Portugal ISBN 978-989-25-0155-0.

Jelen P (1992) Whey: composition, properties, processing and uses. In: YH Hui (Ed.) Encyclopedia of food science and technology, New York: John Wiley & Sons, Wiley-Interscience Publication, pp. 2835-2845.

Jelen P (2003) Whey processing: Utilization and products. In H Roginski, JW Fuquay and PF Fox (Ed.), Encyclopedia of Dairy Sciences, New York: Academic Press, USA.

Jonhs JEM and Ennis BM (1981) The effect of replacement of calcium with sodium in acid whey on the functional properties of whey protein concentrates. Journal Dairy Science and Technology, 15, 75.

Kailasapathy K and Lam SH (2005) Application of encapsulated enzymes to accelerate cheese ripening. International Dairy Journal, 15, 929-939.

Karleskind D, Laye I, Mei F-I and Morr CV (1995) Chemical pretreatment and microfiltration for making delipidized whey protein concentrates. Journal of Food Science, 60(2), 221-226.

Kellam S (2012) The manufacture of lactose. In: H Wansbrough (Ed.) III-Dairy-F-Lactose. Lactose New Zealand Ltd. <http://nzic.org.nz/ChemProcesses/dairy/3F.pdf>

Kelly PM (2003) Membrane separation. In: H Roginki, JW Fuquay and PF Fox (Ed.) Encyclopedia of Dairy Sciences, vol. 4, London, Academic Press (Elsevier Science).

Kilara A (1984) Standardization of Methodology for Evaluating Whey Proteins. Journal Dairy Science, 67(11), 2734-2744.

Kilara A and Vaghela MN (2004) Whey proteins. In: RY Yada (Ed.) Proteins in food processing. Cambridge: Woodhead Publishing Limited, pp. 72-99.

Kinsella JE (1976) Functional properties of food proteins: a review. Critical Reviews in Food Science and Nutrition, 7, 219-280.

Kinsella JE and Whitehead DM (1989) Proteins in Whey: Chemical, Physical and Functional Properties. Advanced Food Nutrition Res., 33, 343-438.

Krasaekoopt W, Bhandari B and Deeth HC (2006) Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage. LWT - Food Science and Technology, 39(2), 177-183.

Krijger A (2011) The World Dairy Situation 2011. Bulletin of the International Dairy Federation, Dutch Dairy Board (PZ), Zoetermeer, Netherlands.

Langer K, Balthasar S, Vogel V, Dinauer N, Briesen HV and Schubert D (2003) Optimization of the preparation process for human serum albumin (HSA) nanoparticles. International Journal of Pharmaceutics, 257, 169-180.

Lee SJ and Rosenberg M (2001) Microencapsulation of theophylline in composite wall system consisting of whey proteins and lipids. Journal of Microencapsulation, 18(3), 309 - 321.

Liu X, Ning J and Clark S (2009) Changes in structure and functional properties of whey proteins induced by high hydrostatic pressure: A review. Frontiers of Chemical Engineering in China, 3(4), 436-442.

Loo C, Lin A, Hirsch L, Lee MH, Barton J, Halas N, et al. (2004) Nanoshell-enabled photonics-based imaging and therapy of cancer. Technology in Cancer Research & Treatment, 3, 33-40.

Lucena ME, Alvarez S, Menéndez C, Riera FA and Alvarez R (2007) alpha-Lactalbumin precipitation from commercial whey protein concentrates. Separation and Purification Technology, 52, 446-453.

Lucena ME, Alvarez S, Menéndez C, Riera FA and Alvarez R. (2006) Beta-lactoglobulin removal from whey protein concentrates production of milk derivatives as a base for infant formulas. Separation and Purification Technology, 52, 310-316.

MADRP - Ministério da Agricultura do Desenvolvimento Rural e das Pescas (2007) Leite e Lacticínios: diagnóstico sectorial. Gabinete de Planeamento e Políticas, Portugal.

Madureira AR, Pereira CI, Gomes AMP, Pintado ME and Malcata FX (2007) Bovine whey proteins - Overview on their main biological properties. Food Research International, 40, 1197-1211.

Madziva H, Kailasapathy K and Phillips M (2006) Evaluation of alginate-pectin capsules in Cheddar cheese as a food carrier for the delivery of folic acid. LWT - Food Science and Technology, 39(2), 146-151.

Marnila P and Korhonen H (2003) Immunoglobulins. In: H Roginski, JW Fuquay and PF Fox, Encyclopedia of dairy sciences. London: Academic Press (Elsevier Science), pp. 1950-1956.

Marshall K (2004) Therapeutic applications of whey protein. Alternative Medicine Review, 9, 136-156.

Marwaha SS and Kennedy JF (1988) Review: Whey-pollution problem and potential utilization. International Journal Food Science and Technology, 23, 323-336.

Maubois JL (1997) Current uses and future perspectives of MF technology in the dairy industry. Bulletin of the IDF, 320, 37-40.

Mehra R and O'Kennedy BT (2008). Separation of β-Lactoglobulin from whey: Its physico-chemical properties and potential uses. In CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

Morr CV and Ha EYW (1993) Whey protein concentrates and isolates: Processing and functional properties. Critical Reviews of Food Science and Nutrition, 33, 431-476.

Morr CV and Foegeding EA (1990) Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report. Food Technology, 44, 100-112.

Neuhaus T (2008) Wheys to softness, fluffiness and moistness in bakery goods. In: US Dairy Export Council (Ed.) USDECNews, March. <http://usdec.files.cms-plus.com/Japan/USDECNews_Mar08_Jap_7.pdf >

Nicorescu I, Loisel C, Vial C, Riaublanc A, Djelveh G, Cuvelier G and Legrand J (2008) Combined effect of dynamic heat treatment and ionic strength on denaturation and aggregation of whey proteins - Part I. Food Research International, 41(7), 707-713.

Nuijens JH, van Berkel PHC and Schanbacher FL (2005) Structure and biological actions of lactoferrins. Journal of Mammary Gland Biology Neoplasia, 1(3), 285-295.

Onwulata CI (2008) Milk whey processes: current and future trends. In: CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

Onwulata CI and Huth PJ (2008) Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

Onwulata CI and Tomasula PM (2004) Whey texturization: Away forward. Food Technology, 58(7), 50-54.

Park YW and Haenlein GFW (2006) Handbook of Milk of Non-Bovine Mammals. Blackwell Publishers, Ames, Iowa and Oxford, England.

Parris N, Cooke PH and Hicks KB (2005) Encapsulation of essential oils in zein nanospherical particles. Journal of Agricultural and Food Chemistry, 53(12), 4788-4792.

Pearce RJ (1992) Whey protein recovery and whey protein fractionation. In J.G. Zadow, Whey and lactose processing. Barking: Elsevier Science Publishers.

Pereira CD, Díaz O and Cobos A (2002) Valorization of by-products from ovine cheese manufacture: clarification by thermocalcic precipitation/microfiltration before ultrafiltration. International Dairy Journal, 12(9), 773-783.

Pérez-Gago MB and Krochta JM (2002) Formation and properties of whey protein films and coatings. In: A. Gennadios (Ed.), Protein-based films and coatings. Boca Raton FL, USA: CRC Press, pp. 159-180.

Pesta G, Meyer-Pittroff R and Russ W (2007) Utilization of whey. In: Oreopoulou V, Russ W (Eds.) Utilization of by-products and treatment of waste in the food industry. Springer.

Picot A and Lacroix C (2004) Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. International Dairy Journal, 14(6), 505-515.

Pintado ME and Malcata FX (2000) Hydrolysis of ovine, caprine and bovine whey proteins by trypsin and pepsin. Bioprocess Engineering, 23, 275-282.

Pintado ME, Lopes da Silva JÁ and Malcata FX (1996) Characterization of Requeijão and technological optimization of its manufacturing process. Journal of Food Engineering, 30, 363-376.

Pintado ME, Lopes da Silva JÁ and Malcata FX (1999) Comparative Characterization of Whey Protein Concentrates from Ovine, Caprine and Bovine Breeds. Lebensmittel-Wissenschaft und-Technologie, 32(4), 231-237.

Pintado ME, Macedo AC and Malcata FX (2001) Review: Technology, chemistry and microbiology of whey cheeses. Food Science and Technology International, 7(2), 105-116.

Pizzichini M, Zeddita G, Comendador FJ, Bertone A, Maurizi A, Nardo N and Quaglia G (1995). Recupero di sieroproteine e acqua depurata dalla scotta ovina com tecnologie di membrana. Scienza e Tecnica Lattiero-Casearia, 46, 361-379.

Pouliot Y (2008) Membrane processes in dairy technology-from a simple idea to worldwide panacea. International Dairy Journal, 18(7), 735-740.

Puyol P, Perez MD and Horne DS (2001) Heat-induced gelation of whey protein isolates (WPI): effect of NaCl and protein concentration. Food Hydrocolloids, 15(3), 233-237.

Radford JB, Galpin DB and Parkin MF (1986) Utilization of whey as a fertilizer replacement for dairy pasture. New Zealand Journal of Dairy Technology, 21, 65-72.

Renner E (1992) Nutritional aspects. In: JG Zadow (Ed.) Whey and lactose processing Barking: Elsevier Science Publishers. pp. 449-471.

Riera FA, Álvarez R, Argüello MA and Cabero M (1996) Fraccionamento y aprovechamiento de proteínas del suero lácteo. I. Propriedades fisico-químicas y biológicas de las proteinas. ILE, abril, 46-61.

Rosenberg M (1995) Current and future applications for membrane processes in the dairy industry. Trends in Food Science and Technology, 6, 12-19.

Schmidt RH, Packard VS and Morris HA (1984) Effect of processing on whey protein functionality. Journal of Dairy Science, 67, 2723-2733.

Singh H (2003) Functional properties. In H Roginski, JW Fuquay and PF Fox (Eds.) Encyclopedia of dairy sciences. London: Academic Press (Elsevier Science).

Siso GMI (1996) The biotechnological utilization of cheese whey: a review. Bioresource Technology, 57, 1-11.

Smithers GW (2008) Whey and whey proteins - from 'gutter-to-gold'. International Dairy Journal, 18, 695-704.

Smithers GW, Ballard FJ, Copeland AD, de Silva KJ, Dionysius DA, Francis GL, Goddard C, Grieve PA, Graeme HM, Mitchel IR, Pearce RJ and Regester GO (1996) New opportunities from the isolation and utilization of whey proteins. Journal of Dairy Science, 79, 1454-1459.

Spălățelu C (2012) Biotechnological valorization of whey. Innovative Romanian Food Biotechnology, 10, 1-8.

SPX - Flow Technology (2010) White Paper. The APV SepStream System. Pre-engineered and cost effective membrane systems for multi dairy applications. Primarily for lower and medium scale capacities. Denmark.
<http://www.apv.com/pdf/WhitePaper/SepStream_System_22001_03_06_2010_GB.pdf >

Tecninvest (2001) Mercado do soro lácteo em Portugal. ANIL - Associação Nacional dos Industriais de Lacticínios.

Tunick MH (2008) Whey Protein Production and Utilization: A Brief History. In: CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

USDA – United States Department of Agriculture (2007) Dairy products 2006 summary. National Agriculture Statistics Service, USA.

USDA – United States Department of Agriculture (2010) Dairy products 2009 summary. National Agriculture Statistics Service, USA.

USDA – United States Department of Agriculture (2011) Dairy products 2010 summary. National Agriculture Statistics Service, USA.

USDA – United States Department of Agriculture (2012) Dairy products 2011 summary. National Agriculture Statistics Service, USA.

Veisseyre R (1988) Lactología técnica: composición, recogida, tratamiento y transformación de la leche. Zaragosa: Acribia S.A.

Walstra P, Wouters JTM and Geurts TJ (2006) Dairy science and technology. USA: CRC Press Taylor & Francis Group.

Walzem RL (2004) Nutritional properties of whey products. In J Page, D Meyer, B Haines, V Lagrange and A Kenney (Eds.) Reference manual for U.S. whey and lactose products. Chapter 6. Nutritional properties of whey, lactose and milk minerals products, pp. 53-57.

Walzem RL, Dillard CJ and German JB (2002) Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. Critical Reviews in Food Science and Nutrition, 42, 353-375.

Westergaard V (2004) Milk powder technology. Evaporation and spray drying. Copenhagen, Denmark. <http://www.niro.com/>

Wong NP (1999) Fundamental of dairy chemistry. 3th ed. Aspen Publishers, Inc. Gaithersburg, Maryland. ISBN: 0-8342-1360-5.

Yang ST and Silva EM (1995) Novel products and new technologies for use of a familiar carbohydrate, milk lactose. Journal Dairy Science, 78, 2541-62.

Zadow JG (1984) Lactose - properties and uses. Journal Dairy Science, 67, 2654-79.

Zall RR (1992) Sources and composition of whey permeate. In: JG Zadow (Ed.) Whey and lactose processing. Barking: Elsevier Science Publishers.

Part B.

Whey protein concentrates

Chapter 3

Production and characterisation of whey protein concentrates

Abstract

The objective of this study was to valorise bovine and ovine cheese whey obtained from small and medium cheese manufacturing plants by producing liquid and dry whey protein concentrates (LWPC and WPC). The flexibility afforded by batch ultrafiltration (UF) and diafiltration (DF) enabled liquid bovine WPC to be produced with a 43-66% protein content (dry basis) and liquid ovine WPC with 61-87% (dry basis). Diafiltration performed in sequential dilution mode (DFsdm) did not significantly improve the composition of WPC liquid products in comparison to the results achieved by conventional UF. However, by using DF in volume reduction mode (DFvrm) the protein content was increased by more than 20% in comparison to conventional

UF. The ovine products presented higher protein levels (62-84% dry basis), which made their manufacture an attractive proposition. Protein profiles varied according to whey origin, the concentration process and UF membrane cut-off. Using batch DFvrm, it was possible to obtain richer protein products that were free of low Mw compounds and thermally more stable.

3.1 Introduction

Whey is a rich source of valuable compounds with high nutritional and functional properties, due, in particular, to its protein content. Whey proteins have been highlighted for their biological functions and as carriers for ligands and trace elements (de Wit, 1998).

In Portugal as well as in some Mediterranean countries such Spain and Italy, a small part of the whey production is further processed in order to obtain products such as *Requeijão*, *Requesón* and *Ricotta*, respectively. In these countries, the products are typically made from sheep and goat whey, although cow's milk whey can also be used, with lower production yields. *Requeijão* is obtained by heating the whey to approximately 90-100 ºC for 15-30 minutes, with or without the addition of 10-20% (v/v) ovine/caprine milk (Pintado and Malcata, 1996). This process seems to be the easiest way to recover valuable products from whey. However, the high volume of cheese whey generated cannot be processed in this manner due to the shorter shelf life of the final products (about 5 days in refrigerated conditions) and the difficulty in selling the full amount during this period. In small and medium-scale cheese production plants (which represent the majority of dairy companies in Portugal) the disposal of whey is still considered the most significant environmental problem. Large scale production plants solve this by using complex and expensive treatments because the production volumes justify the investments. However, the solution for small producers can be achieved by using batch or modified batch systems that involve lower capital costs, less automation and additional operational flexibility.

The use of membrane technology, especially ultrafiltration (UF), in the production of whey protein concentrates (WPC) is not new (Singh, 1995). The valorisation of this by-product solves the environmental issues and adds nutritional value to existing products. The direct incorporation of treated liquid whey protein concentrate (after concentration, heat treatment and homogenisation) to partially replace milk in the manufacture of cheese or yoghurt can contribute towards increasing functional properties and the overall process yields. However, WPC powders are easier to use and more suitable for conservation and transport. In food products, WPC powders have been used in bakery and confectionary products (Strouts, 2004), in beverages as

stabilisers and acidity regulators (de Wit et al., 2003) and as functional ingredients and milk replacers in dairy products (Remeuf et al., 2003). The application of WPC in food products to increase nutritional and functional properties (e.g., foamability, emulsifying capacity and gelation) is not random, and depends on their origin and chemical composition (Casper et al., 1999). Bovine whey, mainly produced by large-scale cheese production plants, is normally transformed into whey protein concentrates and whey protein isolates (WPI). The most common commercial WPC products available on the market are of bovine origin with 35% and 80% proteins or WPI with over 90% protein. The composition of these products depends entirely on the technology and process configuration used. The production of WPC of bovine origin has been studied comprehensively (Kinsella 1976; Morr and Ha, 1993; Kilara, 1984) although only a few researchers (Pizzichini et al., 1995; Comendador et al., 1996; Pereira et al., 2002; Díaz et al., 2004) have attempted to establish the causal effects by relating the operating processes involved in production to the biochemical and protein profile of WPC from ovine whey. One reason for this situation may be that the production of (ovine and caprine) whey from small ruminants amounts to only a very small fraction of the total whey produced in the developed world (Gerosa and Skoet, 2012). Nevertheless, in Portugal, the manufacture of ovine cheese represents a very attractive fraction (25%) of the total cheese production (INE, 2010 and 2012). Moreover, the proteins of ovine whey possess comparatively higher nutritional and functional values than those of bovine whey (Casper et al., 1999; Díaz et al., 2004), so there is also a dietary and technological reason for obtaining detailed knowledge of this product. In order to establish a suitable process for whey treatment (both of bovine and ovine origins) and valorisation in small to medium-scale cheese production plants, different configurations of ultrafiltration and diafiltration processes were used. The performance of each process was evaluated on the basis of the biochemical composition of the products obtained (liquid and dry WPC products), particularly with regard to their protein content and distribution.

3.2 Material and methods

3.2.1 Whey origin

Bovine and ovine whey was provided by medium-sized cheese producing industries (processing about 5-15×10^4 L of milk per day) which, depending on the milk origin and cheese variety (fresh, ripened or semi-ripened cheese), may have involved a more or less complex cheese manufacturing process (Figure 3.1). In Portugal, bovine milk is normally used in the manufacture

of both fresh and ripened cheese, whereas ovine milk is usually reserved for ripened cheese. Both cheese varieties can also be produced using a mixture of milk from both origins.

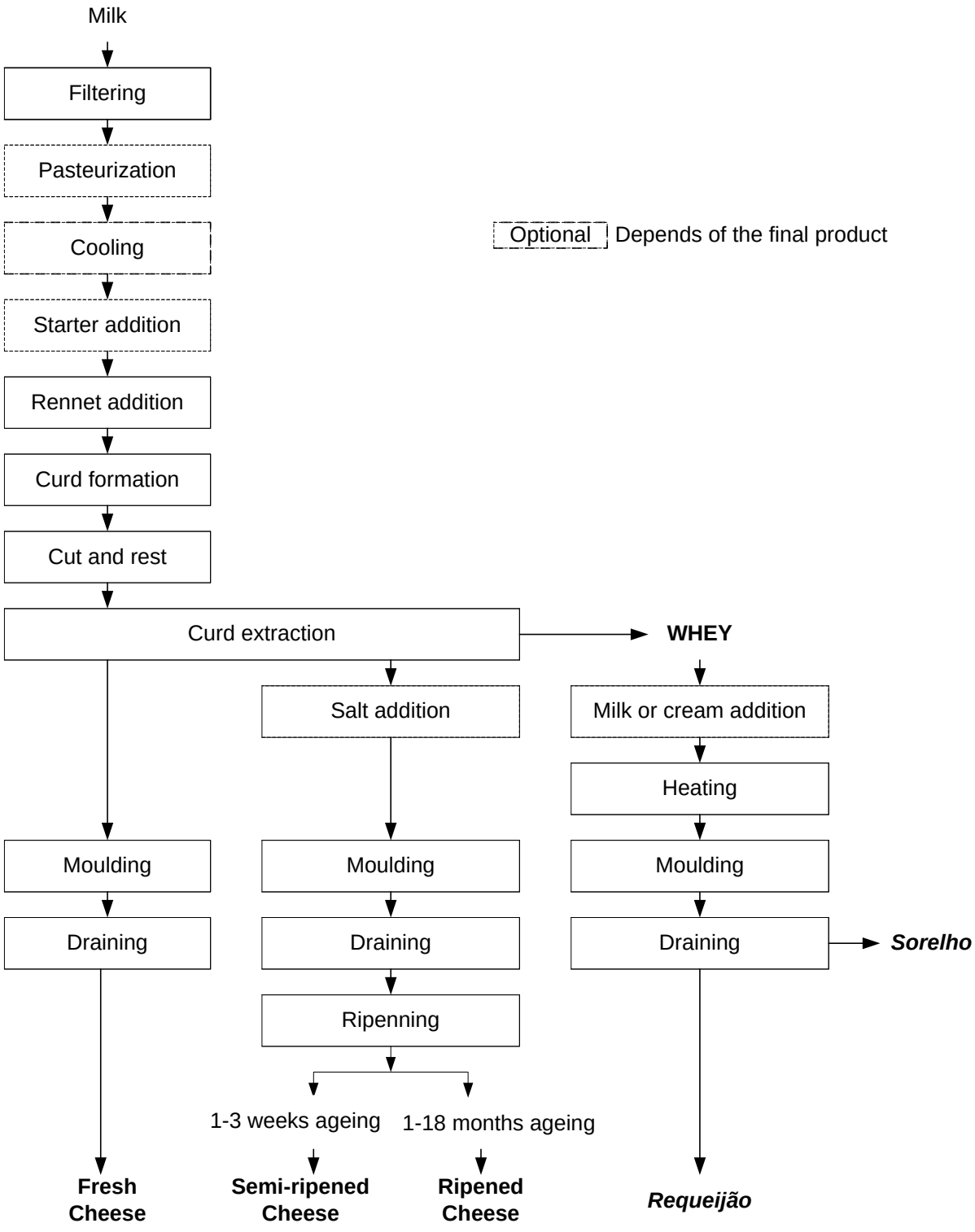


Figure 3.1 Process diagram for making cheese and producing bovine and/or ovine whey.

In the production of bovine whey, the bovine milk was initially filtered and pasteurised (73 ºC/30 s). The pasteurisation was responsible for eliminating native microflora and the addition of lactic bacteria was required. Subsequently, animal rennin was added to promote curd formation (approx. 30 min at 30-34 ºC). After the curd was completely formed, it was cut and put to rest. The whey resulted from the extraction of the curd, which was then moulded and drained to form the cheese. When a maturation step is performed, the final product is a semi-ripened or ripened cheese, according to the ageing time. The bovine whey was supplied by Queijaria Serqueijos SA (Portugal).

The ovine whey was provided by Queijaria Flor da Beira (Portugal) and produced by a similar process to the one described for bovine whey, but in this case the milk was not pasteurised and coagulation occurred in approximately 45 min, at 28-30 ºC using a vegetal enzyme (cardosin from *Cynara cardunculus*).

3.2.2 LWPC and WPC manufacture

The preparation of pasteurized bovine whey and raw ovine whey was similar. After the cheese was produced, the whey was immediately refrigerated and taken in metal recipients at room temperature (15±2 ºC) to the pilot plant at the Escola Superior Agrária de Coimbra, where it was refrigerated until processing. The resting time between leaving the production plant and arriving at the pilot plant was less than 3 hours, in order to minimise product deterioration. Approximately 200 L of each product were used in each trial.

On reception, the whey (W) products were skimmed (SW) in a Westfalia™ separator type ADB in order to reduce the fat content in the final products and simultaneously improve the flux of the concentration step in the ultrafiltration equipment. Each type of whey was concentrated using conventional ultrafiltration (UF) or diafiltration (DF). Both processes were performed in ultrafiltration batch equipment (Figure 3.2) using an organic UF membrane DSS (model 20K 3838-30) with a 5.5 m^2 effective filtration area and a 20 kDa cut-off. This consisted of a feed tank, where the fluid to be processed was initially placed and continuously recycled after passing through the UF membrane, two centrifugal pumps, one responsible for fluid circulation in the system and the other to feed the membrane, and a plate and frame heat exchanger to maintains a constant temperature during the operation.

Figure 3.2. Batch ultrafiltration equipment, Escola Superior Agrária de Coimbra - Oficina Tecnológica de Lacticínios (OTL).

Independently of the whey origin, if the WPC was obtained using a conventional UF process, the intermediate liquid products were termed UFR for retentates and UFF for filtrates. If the DF process was applied, the intermediate liquid products were termed DFR and DFF for retentates and filtrates respectively. The final dry products obtained using each technique were termed UFRP and DFRP for ultrafiltration and diafiltration processes respectively. These products resulted from the freeze drying of each retentate, using Labconco model Lyph-Lock apparatus. Figure 3.3 shows the production process diagram for both liquid and dry WPC products.

In order to obtain WPC with a protein concentration higher than 65% in dry matter, water is normally added during ultrafiltration to create a diafiltration operation (Schwartz, 2003) whose main purpose is to remove large amounts of salts and lactose (Scott, 1986; Barba et al., 2000). Diafiltration is essentially a dilution process performed in conjunction with a concentration process. The discontinuous DF process (Figure 3.4) was performed in two operational modes: (1) sequential dilution mode (DFsdm); and (2) volume reduction mode (DFvrm). In the first case, the skimmed whey was diluted with water to double its initial volume and then concentrated until

the retentate volume was 2.5% of the volume after dilution (volumetric concentration factor - VCF of 40). The concentrated product obtained was called DFRsdm. The diafiltration performed in volume reduction mode (DFvrm) involved an initial concentration step with a VCF of 20, followed by dilution of the retentate to the initial whey volume (200 L), and a second concentration step with the same VCF.

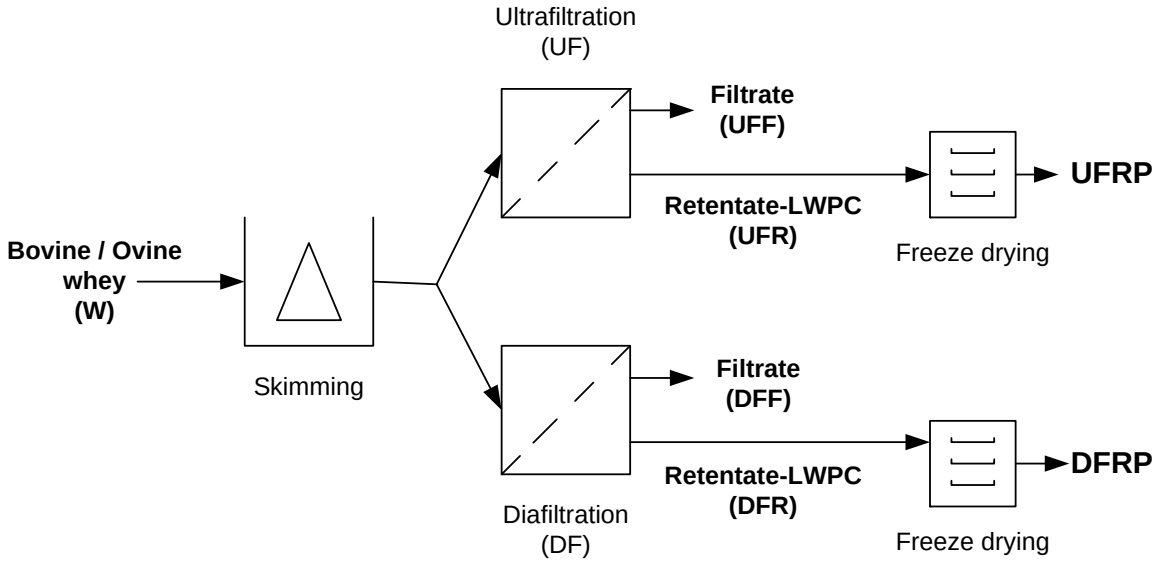


Figure 3.3 Process diagram for whey protein concentrates production by ultrafiltration (UF) and diafiltration (DF).

During the concentration process at 35±3 ºC, the transmembranar pressure was approximately constant (nearly 4 bar) and the filtrate flux decreased from 9 L/m^{2}h to 0.9 L/m^{2}h. At the end of each process the equipment was washed and cleaned according to the manufacturer's recommendations.

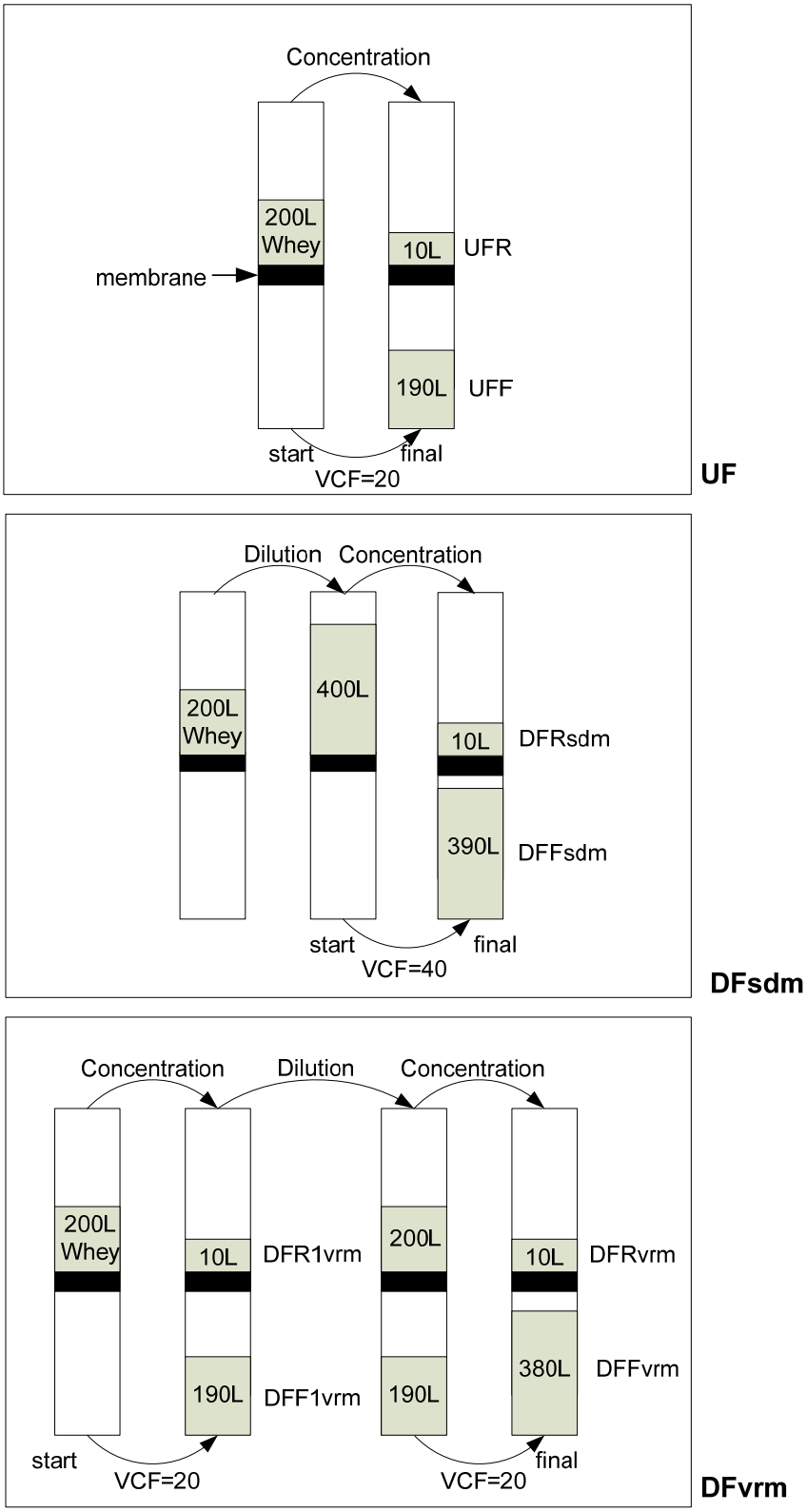


Figure 3.4 Schematic representation of conventional ultrafiltration (UF), diafiltration sequential dilution mode (DFsdm) and diafiltration volume reduction mode (DFvrm).

3.2.3 Compositional analysis

The chemical composition of the intermediate liquid products and the dry products was evaluated on a wet and dry basis respectively. Both types of liquid products (bovine and ovine) were analysed after production and characterised in terms of dry matter (AOAC, 1980a), ash content (AOAC, 1995), fat content (NP 469:2002; ISO 1736:2000), total proteins (ISO 8968-1:2001), lactose (AOAC, 1980b) and calcium (AOAC, 2005) content, pH, and titratable acidity (Appendix A). The freeze dried WPC samples were also evaluated for phosphorous content (AOAC, 1996), true protein and non-protein nitrogen (NPN) compounds (Díaz et al., 2004) (Appendix B). The percentage of individual whey proteins (β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), Serum Albumin (SA) and Imunoglobulins (IgG)) was determined by fast protein liquid chromatography (FPLC) (Appendix B). The protein standards were purchased from Sigma Chemical (St. Louis, USA). Samples of dry products were dissolved in ultrapure water (10 mg/mL) and filtrated with a 0.22 μm filter. Several concentrations of each standard protein were prepared using the same procedure to construct standard curves. All the chromatographies were performed using a gel filtration Superose 12 HR 10/30 (Amersham Biosciences) column. Proteins were chromatographed at room temperature at a flow-rate of 0.4 mL/min using a Beckman System Gold Liquid Chromatograph (Amersham Biosciences). The mobile phase was NaCl 0.15 M in a sodium phosphate buffer 100 mM (pH 7.0) with 0.2 g/L of NaN_3. Detection was carried out at 280 nm in a UV-MII detector. The sample volume injected into to the column was 0.2 mL, with a total running time of 80 minutes. Quantification was based on peak areas of whey proteins and external standards for each protein. All determinations were made in triplicate.

3.2.4 Thermal analysis

Thermogravimetric analyses (TGA) were performed with a Thermogravimetric Analyzer Q500 V20.10 from TA Instruments (USA). WPC samples (5-10 mg) were pre-weighed in platinum pans using a empty pan as a reference, placed in the balance system and heated from 25 ºC to 600 ºC at 10 ºC/min, under a nitrogen atmosphere (60 mL/min). The decomposition temperatures (T_d), derivative maximum decomposition rate temperature (DTG_{max}), and corresponding weight losses, as well as the residual mass at 600 ºC, were all determined using Universal Analysis software (TA Instruments). All assays were duplicated.

3.2.5 Statistical analysis

The biochemical composition data from the liquid and dry products was analysed by one-way ANOVA and the means were compared using the Unequal N HSD test with significance at $p < 0.05$. Statistical analyses were implemented using the Statistica 8 package for Windows (StatSoft Inc., USA).

3.3 Results and discussion

The biochemical composition of whey, liquid whey protein concentrate products (LWPC) (Table 3.1 and 3.2) and retentate powders (Table 3.3) produced by conventional UF was compared with those obtained by the DF processes (performed in the same batch pilot plant) for both whey product origins, particularly with regard to their protein distribution and contents (Table 3.4 and 3.5; Figure 3.5).

3.3.1 Composition of liquid products

The values for the general composition of bovine (Table 3.1) and ovine whey (Table 3.2) were in accordance with those reported by Morr and Ha (1993), Comendador et al. (1996), Casper et al. (1998), Pintado et al. (2001), Ozer et al. (2002) and Jelen (2003). The ovine whey was richer than the bovine whey in all its components, especially protein content, which was basically double (on average 1.43%) the values obtained for bovine whey (0.75%). This may be an attractive aspect of the valorisation of ovine whey in medium/small scale dairy factories.

Both bovine and ovine whey were considered sweet, due to their pH values in the 6.0-6.7 range (Wendorff, 2001; Ozer et al., 2002). However, the titratable acidity was lower in the case of bovine whey (0.12-0.13% of lactic acid) in comparison to the slightly higher values obtained for ovine whey (0.14-0.17% of lactic acid). This behaviour has already been found in ovine milk and is normally associated with a greater amount of dry matter. Another reason for this difference could be the absence of milk pasteurisation during ovine cheese manufacture, which allows for the presence of higher amounts of microorganisms responsible for whey fermentation and lactic acid production. It was possible to observe that the acidity variation in some products was not followed by pH values, particularly the high acidity obtained in filtrates and the lower acidity in retentates. The buffer effect of proteins present in larger amounts in retentates may explain this behaviour. The acidity values also decreased in filtrates produced by diafiltration; in this case water dilution is responsible for the decrease in lactic acid concentration.

Independently of the process used (UF or DF), the biochemical composition of intermediate liquid products as well as final products differs significantly ($p < 0.05$) in relation to the original product. The exception was the composition of skimmed whey (USW/DSW), which was very similar to the composition of the original whey (Table 3.1 and Table 3.2). In this case, the decrease in fat content due to centrifugation, and consequently the decrease in dry matter, were the only differences observed. The centrifugation step seems to be very efficient in reducing fat content below 0.01% and preventing fouling problems in the ultrafiltration membrane.

Using conventional ultrafiltration (UF) it was possible to double the dry matter in the retentate in relation to the original whey, in both bovine and ovine products. The ash content was very similar in the original and skimmed whey, but the values were lower in filtrates and higher in retentates ($p < 0.05$). After UF, bovine and ovine retentates had only 0.08% and 0.06% of fat respectively, since around 87% and 97% was removed from the original bovine and ovine whey during centrifugation. The protein content in conventional UF retentates represented 43% and 61% of the dry matter for bovine and ovine products respectively, in comparison to 11% and 18% in the original whey (Table 3.1 and Table 3.2). Despite the reduction in lactose content from 62-65% (in the whey dry matter) to 28% (in the retentate dry matter) for product of both origins, this still represents a high content if the intention is to produce enriched protein powders. In both cases the calcium content increased in retentates and decreased in filtrates in comparison to the initial whey content.

As previously mentioned, two operational modes of DF were implemented. The DF performed in sequential dilution mode (DFsdm) had the main advantage of increasing the filtrate flux rate by diluting the initial sample. However, high capacity tanks were needed to hold the entire volume after dilution. On the other hand, the DF performed in volume reduction mode (DFvrm) solved the space problem but increased the number of steps in the process and decreased the filtrate flux rates. The two modes of DF applied in this study produced very different results with regard to the biochemical composition of the final products.

Table 3.1 Biochemical composition of bovine whey, intermediate liquid products and LWPC (UFR; DFRsdm and DFR2vrm) during conventional ultrafiltration, diafiltration in sequential dilution mode (DFsdm) and diafiltration in volume reduction mode (DFvrm) (%, wet basis).

Process	Product	pH	Acidity (% lactic acid)	Dry Matter$^{\alpha}$ (%)	Ash$^{\alpha}$ (%)	Fat$^{\alpha}$ (%)	Protein$^{\alpha}$ (%)	Lactose$^{\alpha}$ (%)	Calcium$^{\beta}$ (%)
UF	UW	6.0	0.13 e	6.69±0.01 h	0.53±0.01 ef	0.30±0.03 b	0.75±0.01 b	4.14±0.06 g	0.04±0.01 ab
	USW	6.1	0.12 e	5.88±0.02 f	0.50±0.04 ef	< L.D.	0.77±0.02 b	4.15±0.01 g	0.04±0.01 ab
	UFF	4.8	0.76 h	4.02±0.02 c	0.36±0.01 d	< L.D.	0.05±0.01 a	3.46±0.06 e	0.03±0.01 ab
	UFR	6.0	0.08 cd	12.87±0.02 l	0.58±0.01 g	0.08±0.03 a	5.50±0.10 f	3.64±0.04 f	0.08±0.01 cd
DFsdm	DW	6.0	0.13 e	6.69±0.01 h	0.53±0.01 ef	0.30±0.03 b	0.75±0.01 b	4.14±0.06 g	0.04±0.01 ab
	DSW	6.5	0.13 e	5.89±0.02 f	0.50±0.04 ef	< L.D.	0.77±0.02 b	4.15±0.01 g	0.04±0.01 abc
	DFFsdm	5.1	0.36 g	2.16±0.01 b	0.18±0.01 c	< L.D.	0.02±0.01 a	1.86±0.11 b	0.01±0.01 a
	DFRsdm	6.5	0.04 b	7.56j±0.03	0.37±0.01 d	0.09±0.01 a	3.66±0.12 d	2.40±0.05 c	0.04±0.01 abc
DFvrm	DW	6.6	0.12 e	7.00±0.01 i	0.54±0.03 fg	0.73±0.09 c	0.75±0.01 b	4.74±0.05 h	0.10±0.01 d
	DSW	6.6	0.11 de	6.10±0.09 g	0.51±0.01 ef	< L.D.	0.75±0.01 b	4.81±0.06 h	0.08±0.01 d
	DFF1vrm	6.7	0.17 f	5.20±0.09 e	0.48±0.02 e	< L.D.	0.05±0.01 a	3.39±0.01 e	0.06±0.01 bcd
	DFR1vrm	6.6	0.09 c	8.66±0.02 k	0.48±0.05 e	0.12±0.03 a	4.16±0.03 e	3.03±0.03 d	0.09±0.03 d
	DFF2vrm	6.3	0.09 c	0.34±0.04 a	0.06±0.01 a	< L.D.	0.06±0.01 a	0.24±0.01 a	0.02±0.01 a
	DFR2vrm	6.5	0.01 a	4.50±0.12 d	0.12±0.02 b	0.11±0.03 a	2.98±0.01 c	0.004±0.001 a	0.09±0.02 d

Mean values and standard deviation (±); for α (n=3) and β (n=2); L.D. - Low Det. a, b, c, … means within the same column without the same superscript differ significantly ($p < 0.05$).

(UW/DW) bovine whey; (USW/DSW) skimmed bovine whey; (UFR/UFF) conventional ultrafiltration retentate/filtrate; (DFRsdm/DFFsdm) sequential dilution mode diafiltration retentate/filtrate; (DFR1vrm/DFF1vrm) volume reduction mode diafiltration retentate/filtrate from the 1st step; (DFF2vrm/DFR2vrm) volume reduction mode diafiltration retentate/filtrate from the 2nd step.

Table 3.2 Biochemical composition of ovine whey, intermediate liquid products and LWPC (UFR; DFRsdm and DFR2vrm) during conventional ultrafiltration, diafiltration in sequential dilution mode (DFsdm) and diafiltration in volume reduction mode (DFvrm) (%, wet basis).

Process	Product	pH	Acidity (% lactic acid)	Dry Matter$^{\alpha}$ (%)	Ash$^{\alpha}$ (%)	Fat$^{\alpha}$ (%)	Protein$^{\alpha}$ (%)	Lactose$^{\alpha}$ (%)	Calcium$^{\beta}$ (%)
UF	UW	6.7	0.14 bc	8.68±0.02 h	1.15±0.02 f	0.83±0.03 d	1.57±0.02 c	5.61±0.02 l	0.18±0.02 ef
	USW	6.7	0.14 bc	8.03±0.03 g	1.17±0.01 f	< L.D.	1.56±0.02 c	5.50±0.02 k	0.19±0.01 f
	UFF	5.2	0.93 g	5.32±0.03 d	0.94±0.01 d	< L.D.	0.07±0.01 a	4.30±0.03 g	0.11±0.01 cd
	UFR	6.8	0.09 b	20.20±0.03 k	1.31±0.01 g	0.06±0.03 a	12.39±0.12 g	5.79±0.02 m	0.27±0.03 g
DFsdm	DW	6.4	0.14 bc	7.72±0.04 f	1.02±0.02 e	0.68±0.03 c	1.35±0.05 b	4.66±0.01 ij	0.17±0.01 ef
	DSW	6.4	0.12 bc	6.90±0.02 e	1.01±0.02 e	< L.D.	1.27±0.04 b	4.69±0.02 j	0.18±0.01 ef
	DFFsdm	5.3	1.03 h	2.71±0.01 b	0.50±0.01 b	< L.D.	0.06±0.01 a	3.84±0.02 e	0.07±0.01 bc
	DFRsdm	5.5	0.23 d	13.89±0.15 j	0.47±0.02 b	0.11±0.01 a	8.91±0.12 f	2.53±0.04 c	0.10±0.01 cd
DFvrm	DW	6.0	0.17 cd	7.68±0.07 f	1.02±0.01 e	0.83±0.05 d	1.38±0.05 b	4.56±0.07 hi	0.10±0.01 cd
	DSW	6.0	0.16 c	6.87±0.04 e	1.04±0.01 e	< L.D.	1.38±0.03 b	4.56±0.04 h	0.10±0.01 cd
	DFF1vrm	5.9	0.40 f	5.09±0.01 c	0.91±0.01 d	< L.D.	0.08±0.01 a	4.00±0.04 f	0.08±0.01 bc
	DFR1vrm	5.9	0.11 bc	13.12±0.20 i	0.82±0.05 c	0.26±0.06 b	8.36±0.04 e	3.10±0.04 d	0.14±0.02 de
	DFF2vrm	5.6	0.34 e	0.61±0.01 a	0.11±0.01 a	< L.D.	0.05±0.01 a	0.46±0.01 b	0.02±0.01 a
	DFR2vrm	6.1	0.01 a	8.52±0.02 h	0.17±0.01 a	0.25±0.05 b	7.44±0.06 d	0.004±0.001 a	0.05±0.01 ab

Mean values and standard deviation (±); for α (n=3) and β (n=2); L.D. - Low Det. a, b, c, … means within the same column without the same superscript differ significantly ($p < 0.05$).

(UW/DW) bovine whey; (USW/DSW) skimmed bovine whey; (UFR/UFF) conventional ultrafiltration retentate/filtrate; (DFRsdm/DFFsdm) sequential dilution mode diafiltration retentate/filtrate; (DFR1vrm/DFF1vrm) volume reduction mode diafiltration retentate/filtrate from the 1st step; (DFF2vrm/DFR2vrm) volume reduction mode diafiltration retentate/filtrate from the 2nd step.

In the DFsdm operation a small increment in the protein contents, in comparison to the conventional UF, was achieved (48% and 64% in dry matter for bovine and ovine products, respectively) and practically no lactose reduction was observed. These results show that DF in sequential dilution mode does not offer any advantages to conventional UF, since it was more time-consuming, required larger tanks and more water consumption and offered no improvements to the chemical composition of the products. Using DFvrm the final protein concentration obtained was 66% and 87% of the dry matter in the bovine and ovine retentates respectively, and the lactose present in the original whey was practically removed (99.2%). These observations indicate significant advantages for this process instead of diafiltration performed by sequential dilution mode (DFsdm) and UF itself.

It is also important to note that the reduction in the dry matter content of retentates in DF (especially DFvrm) compared to UF (Table 3.1 and Table 3.2) was not due exclusively to lactose and mineral reduction but also to some protein loss in the filtrates. This means that the products achieved by means of DF were richer in proteins but the overall amount of protein recovered from whey was lower. This was probably due to greater losses of α-Lactalbumin and lower molecular weight (Mw) nitrogen compounds in the filtrate, owing to the relatively high Mw cut-off for the membrane (20 kDa) and the high foaming capacity of retentates, leading to protein losses in the balance tank during the concentration process.

The ash content for products of both origins (bovine and ovine) increased in the retentates during UF but decreased when DF was performed. Water dilution enabled larger amounts of minerals to be eliminated, especially in the case of DFvrm, which showed a reduction from 0.48% and 0.82% in the 1st step (DFR1vrm) to 0.12% and 0.17% in the 2nd diafiltration step (DFR2vrm) for the bovine and ovine products respectively.

Fat content increased in the retentates due to its accumulation during the concentration process. Higher values were observed in DFvrm than in DFsdm, mainly because the fat content in the original whey used in the first case was also higher. Throughout DFvrm the amount of fat obtained in the retentate in the 1st step remained approximately constant in the 2nd step ($p>0.05$), demonstrating that lipids were efficiently retained in the UF membrane.

There was a different trend for calcium composition in DFsdm in comparison to UF. In this case, the calcium content in the retentate (DFRsdm) was similar (Table 3.1) or lower (Table 3.2) because it was diluted and removed in the filtrate (DFFsdm). With DFvrm, there were very

similar concentrations of calcium in liquid products, which only differed significantly ($p < 0.05$) in the retentates and filtrates from the 2nd diafiltration stage (DFR2vrm and DFF2vrm, respectively).

3.3.2 Composition of dry products

After lyophilization of the UF and DF retentates (Figure 3.3) the products obtained were designated UFRP, DFRPsdm and DFRPvrm according to whether the process was performed by ultrafiltration, diafiltration in sequential dilution mode or volume reduction mode, respectively. Table 3.3 shows the final chemical composition of the dry products of both origins and Table 3.4 provides a more detailed evaluation of their protein composition.

Table 3.3 Chemical composition of dry bovine and ovine WPC obtained by: conventional ultrafiltration (UFRP), diafiltration in sequential dilution mode (DFRPsdm) and diafiltration in volume reduction mode (DFRPvrm) (%, dry basis).

Composition (%)	UFRP	DFRPsdm	DFRPvrm
Bovine			
Dry Matter$^{\alpha}$	93.21±0.12 a	91.18±0.02 b	93.54±0.08 c
Ash$^{\alpha}$	4.44±0.10 b	4.47±0.12 b	2.68±0.01 a
Fat$^{\alpha}$	0.73±0.11 a	0.71±0.05 a	4.58±0.28 b
Protein$^{\alpha}$	49.88±0.72 a	50.19±0.56 a	71.11±1.27 b
Lactose$^{\alpha}$	41.79±0.26 b	44.61±0.42 c	5.47±0.04 a
Calcium$^{\beta}$	0.56±0.02 a	0.54±0.01 a	1.12±0.03 b
Phosphorus$^{\beta}$	0.34±0.02 b	0.40±0.02 c	0.19±0.01 a
Ovine			
Dry Matter$^{\alpha}$	97.53±0.11 c	92.70±0.02 b	91.50±0.02 a
Ash$^{\alpha}$	6.09±0.12 c	3.39±0.12 b	1.87±0.08 a
Fat$^{\alpha}$	0.98±0.06 b	0.89±0.05 a	6.36±0.24 c
Protein$^{\alpha}$	61.53±0.91 a	68.29±0.56 b	83.67±1.59 c
Lactose$^{\alpha}$	28.28±0.25 c	21.26±0.42 b	5.70±0.77 a
Calcium$^{\beta}$	1.31±0.02 c	0.72±0.01 b	0.57±0.04 a
Phosphorus$^{\beta}$	0.83±0.02 c	0.70±0.02 b	0.15±0.01 a

Mean values and standard deviation (±); α (n=3); β (n=2); a, b, c, … means within the same row without the same superscript differ significantly ($p < 0.05$); DFRPvrm: retentate powder from the 2nd diafiltration step.

The moisture content of the different dry products varied between 6.40-8.82% for bovine and 2.47-8.5% for ovine WPC. These values agree with some reported data (Rinn et al., 1990; Morr and Foegeding, 1990) but are higher than those stated by Casper et al. (1999) and Evans et al. (2009) for commercial WPC. One possible reason for this could be related to the drying method

used, which was spray drying for commercial products instead of freeze drying. However, the higher amount of moisture in the manufactured products does not imply any loss of quality.

Despite the fact that the lactose content in ovine whey was higher than in bovine whey (Table 3.2 and Table 3.1) the use of conventional UF allowed for the production of ovine dry products (UFRP) with a lower lactose concentration. This behaviour was maintained when using diafiltration, which accords with the findings of Casper et al. (1999). However, in the case of DFvrm, the lactose content in bovine and ovine dry products does not differ significantly ($p < 0.05$), with values of 5.47% and 5.70% respectively.

Table 3.4 Protein composition (g/100 g powder) of bovine and ovine WPC obtained by conventional ultrafiltration (UFRP), diafiltration in sequential dilution mode (DFRPsdm) and diafiltration in volume reduction mode (DFRPvrm) (dry basis).

Product	UFRP	DFRPsdm	DFRPvrm
Bovine			
Total nitrogen (TN)$^{\alpha}$	7.82±0.11 a	7.87±0.07 a	11.68±0.22 b
Non-protein nitrogen (NPN)$^{\beta}$	0.8±0.004 b	0.72±0.00 ab	0.63±0.08 a
NPN compounds (NPN×3.60)$^{\beta}$	2.88±0.02 b	2.60±0.02 ab	2.28±0.28 a
True protein ((TN – NPN)×6.45)	45.28±0.11 a	46.08±0.07 a	67.80±1.28 b
IgG	1.44±0.15 a	1.05±0.76 a	5.56±1.02 b
Serum Albumin$^{\alpha}$	1.27±0.01 a	1.79±1.92 a	3.48±0.32 a
β-Lactoglobulin$^{\alpha}$	35.61±0.09 a	36.27±2.19 a	51.73±1.35 b
α-Lactalbumin$^{\alpha}$	6.95±0.06 a	6.96±0.27 a	7.03±0.01 a
Ovine			
Total nitrogen (TN)$^{\alpha}$	9.64±0.14 a	10.70±0.09 b	13.12±0.25 c
Non-protein nitrogen (NPN)$^{\beta}$	1.27±0.02 a	1.55±0.04 b	1.59±0.06 b
NPN compounds (NPN×3.60)$^{\beta}$	4.62±0.09 a	5.60±0.14 b	5.71±0.21 b
True protein ((TN – NPN)×6.45)	53.99±0.17 a	59.01±0.16 b	74.37±1.51 c
IgG$^{\alpha}$	4.52±0.16 b	3.71±0.27 a	6.94±0.03 c
Serum Albumin$^{\alpha}$	2.73±0.13 b	1.76±0.19 a	4.01±0.03 c
β-Lactoglobulin$^{\alpha}$	40.68±0.07 a	45.98±0.70 b	56.85±0.03 c
α-Lactalbumin$^{\alpha}$	6.05±0.01 a	7.56±0.25 c	6.56±0.03 b

Mean values and standard deviation (±); α (n=3); β (n=4); a, b, c, … means within the same row without the same superscript differ significantly ($p < 0.05$); DFRPvrm: retentate powder from the 2nd diafiltration step. nd - not detected.

The percentage of fat in bovine and ovine dry products was very similar whether UF or DFsdm was used, and ranged from about 0.71% to 0.98% (Table 3.3). These values fall within the range of those obtained by Rinn et al. (1990), who used centrifugal clarification and microfiltration to reduce the content of lipids, but are higher than those found by Casper et al. (1999) and Pereira et al. (2002), who used thermocalcic precipitation followed by microfiltration, ranging from

0.2-0.41% for ovine and 0.3% for bovine WPC. However commercial bovine WPC, as documented by Evans et al. (2009), has higher amounts of fat (2.5-3.52%). As expected in DFvrm products, the percentage of fat in the solids content of the dry products increased as a result of eliminating the lactose (4.58% and 6.36% for bovine and ovine products, respectively). These values fall within the range found in some commercial bovine WPC, as reported by Morr and Foegeding (1990).

As expected, ash decreased in products of both origins according to UF, DFsdm and DFvrm (Table 3.3) with the exception of bovine DFRPsdm, which was the same as UFRP.

The type and amount of salts in WPCs determine their functional properties and performance in final applications (Resch and Daubert, 2002). The mineral composition of WPC dry products was evaluated only for calcium and phosphorus, two of the major mineral components in these products. The mineral composition in bovine WPC products, obtained by UF and DFsdm, agrees with the data reported by Morr and Foegeding (1990) and Casper et al. (1999), but in the case of calcium, only when compared with the data of Evans et al. (2009). The percentage of calcium and phosphorous in ovine WPC was significantly higher than that of the bovine products, as well as in comparison to values mentioned by Pereira et al. (2002) and Casper et al. (1999) for products of the same origin. In diafiltrated products, Ca^{2+} and PO_4^{2-} reduction was not as pronounced as expected, although a decrease was observed in ovine WPC. In this case, if the total amount of calcium and phosphorous is compared to the ash content, a significant difference can be observed. The possible high proportion of sodium chloride in ovine cheese manufacture added to the milk prior to coagulation, as mentioned by Pereira et al. (2002), could be responsible for this difference.

WPC ovine powders are richer in protein than their bovine counterparts (Table 3.3). The bovine WPC produced by conventional UF and DFsdm shows a similar protein content (50%), although if DFvrm is applied the protein concentration improves to 71%. In the case of ovine WPC, the total protein content amounted to 62% for conventional UF, 68% for DF in sequential dilution mode and 84% for DF in volume reduction mode. True protein presented the same behaviour as total protein in all products, whereas NPN compounds slightly decreased in bovine products using diafiltration and increased in ovine products (Table 3.4 and Figure 3.5). The values obtained for bovine NPN compounds agree with the values reported by Morr and Foegeding (1990) but are higher than the data reported by Casper et al. (1999). The reported amount of

NPN compounds in ovine products (Casper et al., 1999; Díaz et al., 2004) is higher than in bovine products, which also concurs with the results achieved in this study. However, their concentration in the WPC produced was even higher than the figures reported by those authors. Muir et al. (1993) have stated that ovine milk contains substantially higher amounts of NPN compounds than bovine milk and this can be extended to whey and consequently to WPC. It was mentioned that NPN compounds in liquid whey can be associated with the thermally induced fragmentation of whey proteins and the formation of ammonia (Bajpai and Gupta, 1979). According to Pintado and Malcata (1996) ovine whey proteins have a lower thermal stability than bovine whey proteins. This lack of stability in ovine whey proteins could be responsible for the increase in NPN in products with higher amounts of total proteins in the DFRPvrm > DFRPsdm > UFRP sequence, since this behaviour was exactly the opposite in bovine WPC (Table 3.4 and Figure 3.5). However, further research is needed to explain and justify this trend.

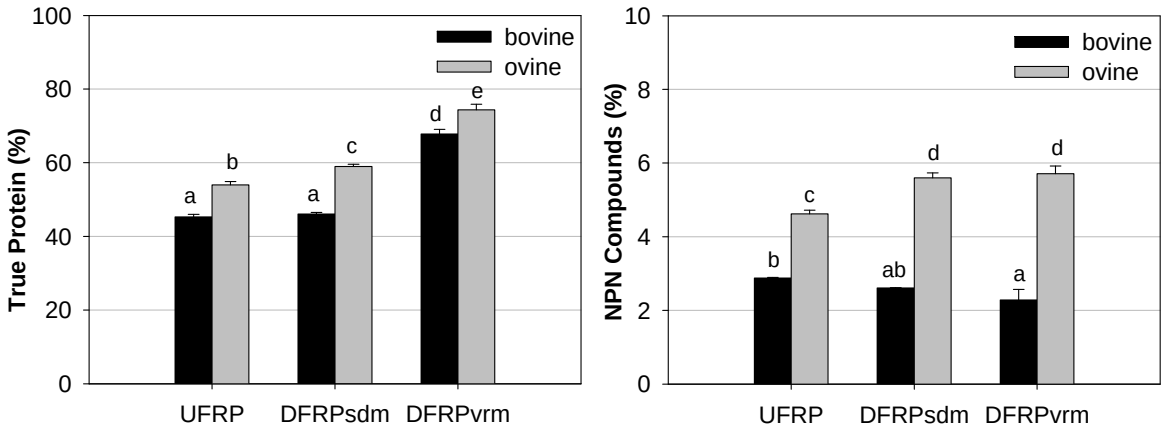


Figure 3.5 True protein and NPN compounds content in bovine and ovine WPC powders (%, dry basis) obtained by conventional ultrafiltration (UFRP), diafiltration in sequential dilution mode (DFRPsdm) and diafiltration in volume reduction mode (DFRPvrm). Mean values ± standard deviation; (n=3); a, b, c, … means within the same component without the same superscript differ significantly ($p < 0.05$).

Table 3.4 also shows the individual protein composition in bovine and ovine WPC. In this case the values reported are related to the true protein content of each product. β-Lactoglobulin was the major protein in all WPC products regardless of whey origin and production mode, followed by α-Lactalbumin. Although the concentration of β-Lactoglobulin was higher in ovine products (40.68-56.85 g/100g), the α-Lactalbumin was higher in bovine products in comparison to its

ovine product content (6.92-7.03 g/100 g). IgG and SA had the highest concentration in the case of ovine products, which agrees with previously reported values indicating that WPC originating from small ruminants (caprine and ovine) contains greater amounts of these proteins.

3.3.3 Protein profile of WPC

A protein characterisation of WPC was performed in order to assess whether alternative processes lead to differences in protein profiles. A comparison of the FPLC profiles of the three types of bovine WPC powders is shown in Figure 3.6. All the main proteins in the original whey, such as β-Lactoglobulin, α-Lactalbumin, IgG, and Serum Albumin (SA), are present. A peak was also observed at approximately 50 min (peak 6) only for products obtained by conventional ultrafiltration (UFRP) and diafiltration in sequential dilution mode (DFRPsdm). This was identified as urotic acid, which usually appears at this retention time (Pintado and Malcata, 1996). Although uric acid was also identified in original bovine and ovine whey and detected at longer retention times then the urotic acid in the FPLC analysis, it was not observed in any type of WPC produced. Its low Mw meant that it was eliminated during the UF and DF processes. In all the bovine samples a significant peak was observed at approximately 20 min (peak 1), which was mentioned in previous works as casein fractions, lipoproteins or soluble whey protein aggregates (Parris et al., 1993), IgM, with a Mw of 900 kDa (Dannenberg and Kessler, 1988), or other of high Mw proteins belonging to the IgG family (Andrews et al., 1985).

In ovine UFRP, DFRPsdm and DFRPvrm, β-Lg and α-La were clearly recognised in all products. In the case of ovine products, an extra peak was observed at approximately 40 min. (peak 7, Figure 3.7). Similar behaviour was reported by Hill and Kakuda (1990), Law et al. (1993) and Pintado and Malcata (2000), who referred to this as fractions of low Mw (ca. 3-4 kDa) mainly composed of non-protein material that was not included in the proteose-peptone (PP) fraction or hydrolyzed with trypsin and pepsin. The presence of various compounds with higher Mw in peak 1 (Figure 3.7) was more evident in ovine products, except in the case of DFRPsdm where only a single peak was observed.

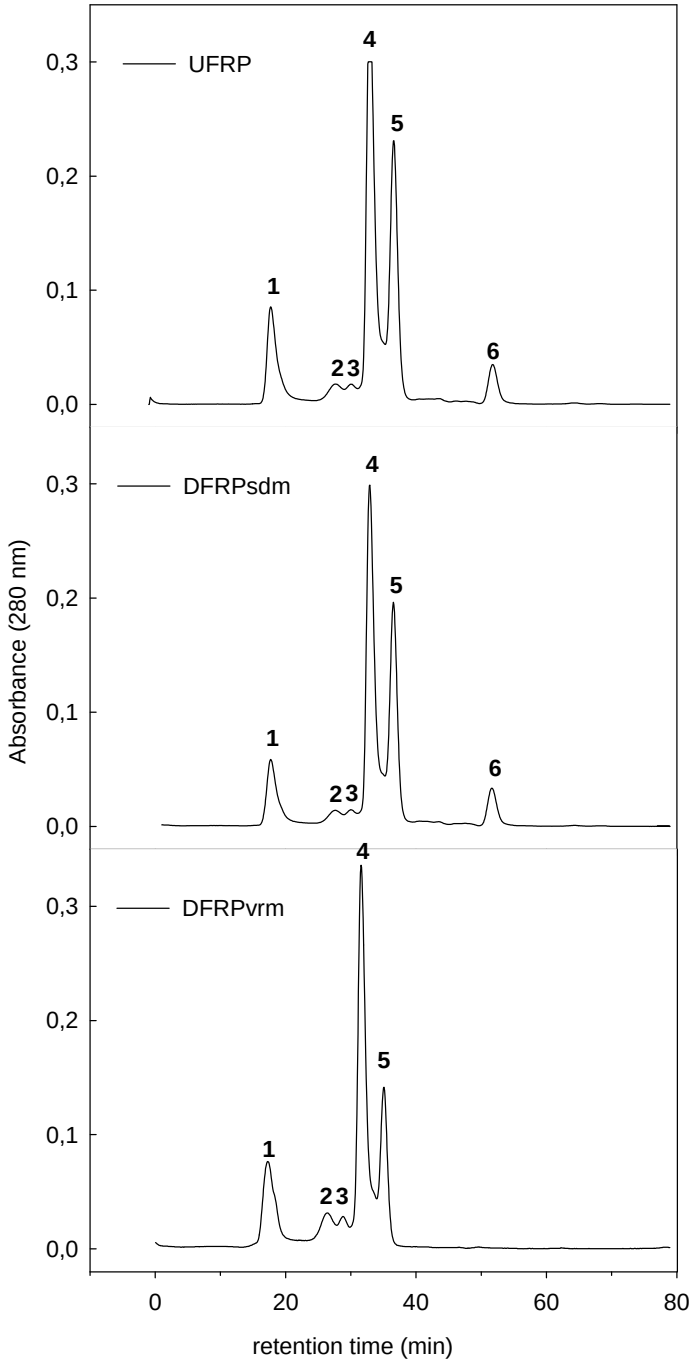


Figure 3.6 FPLC chromatograms (280 nm) of the major components in bovine WPC obtained by: conventional ultrafiltration (UFRP); diafiltration in sequential dilution mode (DFRPsdm); diafiltration in volume reduction mode (DFRPvrm). (1) caseins; lipoproteins or soluble protein aggregates; (2) IgG; (3) SA; (4) β-Lg; (5) α-La; (6) LMW peptides.

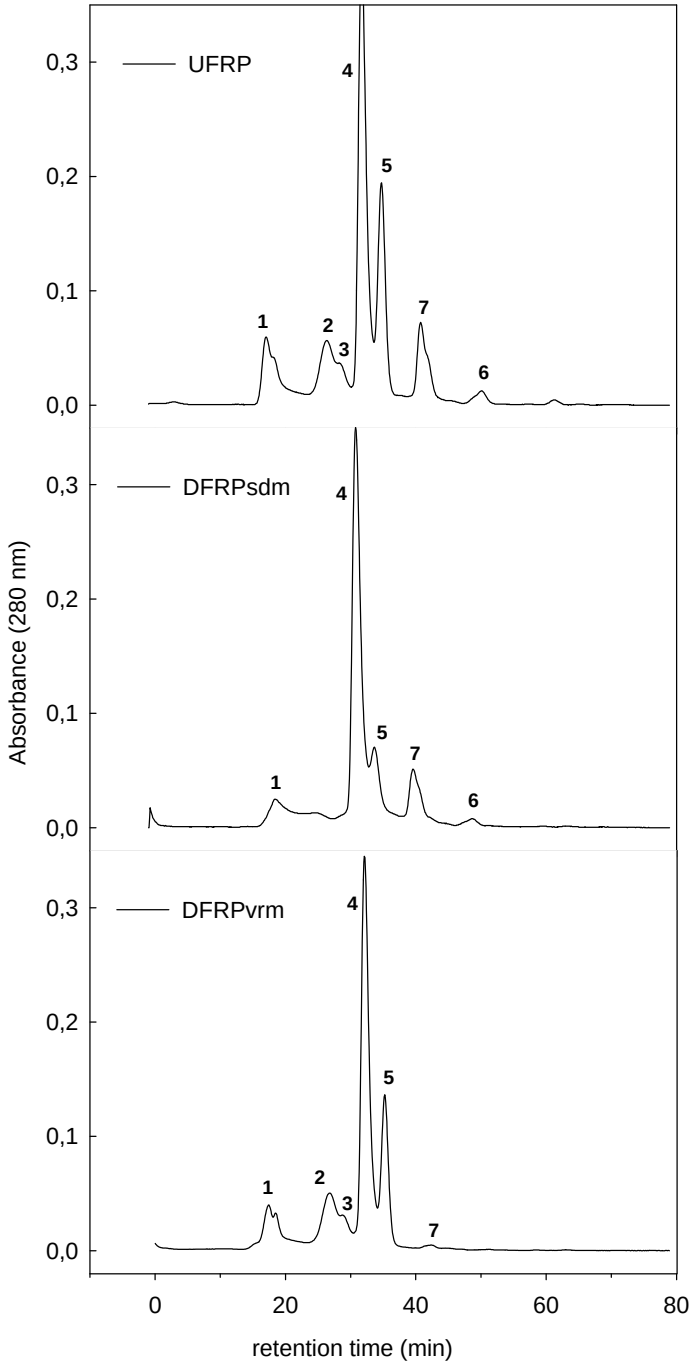


Figure 3.7 FPLC chromatograms (280 nm) of the major components in ovine WPC obtained by: conventional ultrafiltration (UFRP); diafiltration in sequential dilution mode (DFRPsdm); diafiltration in volume reduction mode (DFRPvrm). (1) caseins, lipoproteins or soluble protein aggregates; (2) IgG; (3) SA; (4) β-Lg, (5) α-La; (6) and (7) LMW peptides.

In products of both origins in which DFvrm was used lower Mw compounds (peaks 6 and 7) were eliminated from the final WPC. These products were more purified than the ones produced by diafiltration with sequential dilution (DFRPsdm) and by conventional UF. However, the removal of smaller nitrogen compounds also affected the α-La content in both products, since this protein has the lowest Mw (14.2 kDa) of whey proteins and the membrane used during concentration had a higher cut-off (20 kDa). As a consequence, the relative percentage of higher Mw proteins such as SA and IgG was improved in DFRvrm products (Table 3.5).

Table 3.5 Relative protein distribution[1] in bovine and ovine whey protein concentrate powders obtained by conventional ultrafiltration (UFRP), diafiltration in sequential dilution mode (DFRPsdm) and diafiltration in volume reduction mode (DFRPvrm).

Product	UFRP	DFRPsdm	DFRPvrm
Bovine			
IgG	3.19±0.33 a	2.28 ±0.33 a	8.20 ±1.50 b
Serum Albumin	2.81±0.01 a	3.89±1.48 a	5.13±0.48 a
β-Lactoglobulin	78.65±0.19 a	78.72±3.57 a	76.30±1.99 a
α-Lactalbumin	15.36±0.12 b	15.11±0.31 b	10.38±0.01 a
β-Lg/α-La	5.12±0.03 a	5.21±0.12 a	7.35±0.20 b
Ovine			
IgG	8.38±0.30 b	6.28±0.46 a	9.33±0.04 b
Serum Albumin	5.06±0.24 b	2.99±0.32 a	5.39±0.04 b
β-Lactoglobulin	75.35±0.13 a	77.93±1.18 b	76.45±0.04 ab
α-Lactalbumin	11.21±0.01 b	12.80±0.42 c	8.82±0.04 a
β-Lg/α-La	6.72±0.01 b	6.09±0.29 a	8.67±0.03 c

[1]Mean percentages of true protein and standard deviation (±); (n=3); a, b, c means within the same row without the same superscript differ significantly ($p < 0.05$)

Harper (1991) reported that losses of α-La in the permeate may reach 20% during ultrafiltration and diafiltration, depending on the extent of the processing and the true membrane pore size. It was mentioned that a membrane with 10 kDa cut-off is usually employed in whey concentration in order to retain all the proteins (Tunick, 2008). However, fouling problems were frequent and more time-consuming. On the other hand, a membrane with 30 kDa retains SA, lactoferrin and IgG but not α-La and β-Lg (Bonnaillie and Tomasula, 2008). The use of a 20 kDa cut-off membrane may be responsible for the retention of the larger proteins and some permeation to α-La, leading to an increase in the β-Lg/α-La ratio (Table 3.5) in diafiltration processes. The main characteristic of ovine whey was the low α-La percentage compared to bovine and caprine whey (Pintado and Malcata, 1996; Casper et al., 1998; Moatsou et al., 2005). The relative

proportion of β-Lg and α-La in unprocessed whey decreases according to ovine and bovine origins and this behaviour is maintained in ovine and bovine WPC (Table 3.5).

It is widely reported (Joshep and Mangino, 1988; de Wit, 1989; Gault and Fauquant, 1992; Capitani et al., 2007) that bovine β-Lg shows better foaming and gelation properties than α-La, whereas the latter shows better emulsifying properties. According to Casper et al. (1999), ovine WPC with higher amounts of β-Lg has significantly better functional properties than that of bovine or caprine origin. Therefore the differences in the β-Lg/α-La ratio may offer, not only in bovine but particularly in ovine WPC (Table 3.5), the potential for the production of products with enhanced foaming and gelation properties (Casper et al., 1999; Díaz et al., 2004).

3.3.4 Thermal stability of WPC

Thermogravimetric analysis and derivative thermogravimetry (DTG) are used to show how water evaporates from a system or to show the mechanism by which a material loses weight as a result of controlled heating. Figure 3.8 and Figure 3.9 show the thermogravimetric profile (TGA) and derivative thermogravimetric curves (DTG) respectively of bovine and ovine WPC powders produced using the various methods. The decomposition temperatures, estimated weight losses and residual mass are presented in Table 3.6. The decomposition reactions (TGA/DTG curves) suggest four and three mass-loss stages for bovine and ovine products, respectively. However, the first stage of thermal decomposition for all the dried powder samples was related to dehydration within the 40-120ºC temperature range, with 6.5-8.8% of water weight loss, confirmed by the moisture content of the samples presented in Table 3.6.

It was observed that, independently of the whey origin, products achieved by ultrafiltration (UF) or diafiltration in sequential dilution mode (DFsdm) present similar thermal profiles. These results were, to some extent, expected, since both these products have a relatively close chemical composition (Table 3.6). On the other hand, WPC produced by diafiltration in volume reduction mode (DFvrm) is thermally more stable (Figure 3.8 and 3.9).

They start to decompose (Table 3.6) at high temperatures (T_d), namely 218.57 ºC and 208.49 ºC, for bovine and ovine products respectively, with associated weight losses of 10.67% and 10.94%, corresponding to dehydration in the samples. Although the initial decomposition temperature of bovine WPC was higher than that of the ovine product, the decomposition maximum derivative temperature (DTG_{max}) is of the same order of magnitude (308.91 ºC and 311.80 ºC).

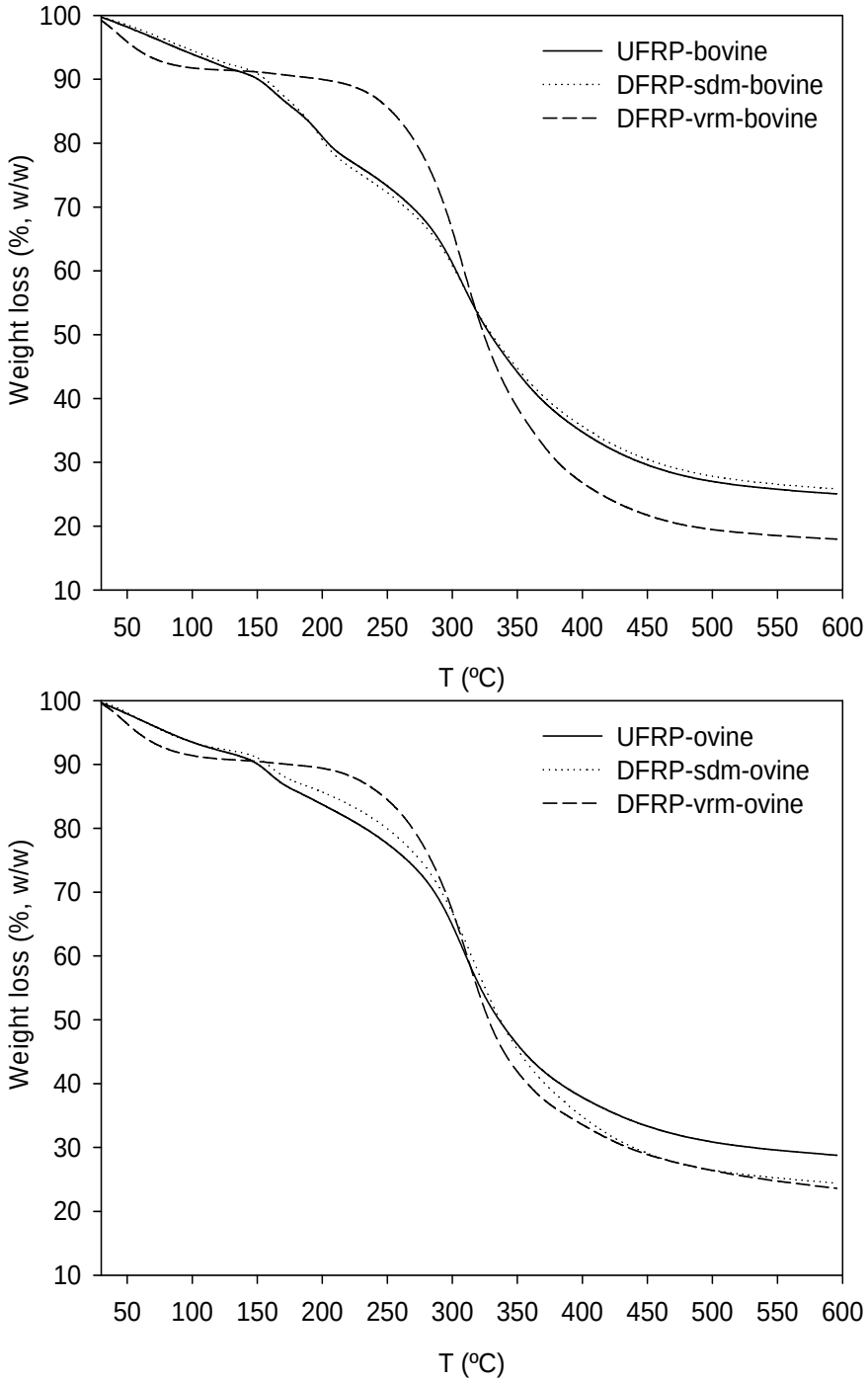


Figure 3.8 TGA thermograms of bovine and ovine WPC powders produced by conventional ultrafiltration (UFRP), diafiltration in sequential dilution mode (DFRPsdm) and diafiltration in volume reduction mode (DFRPvrm).

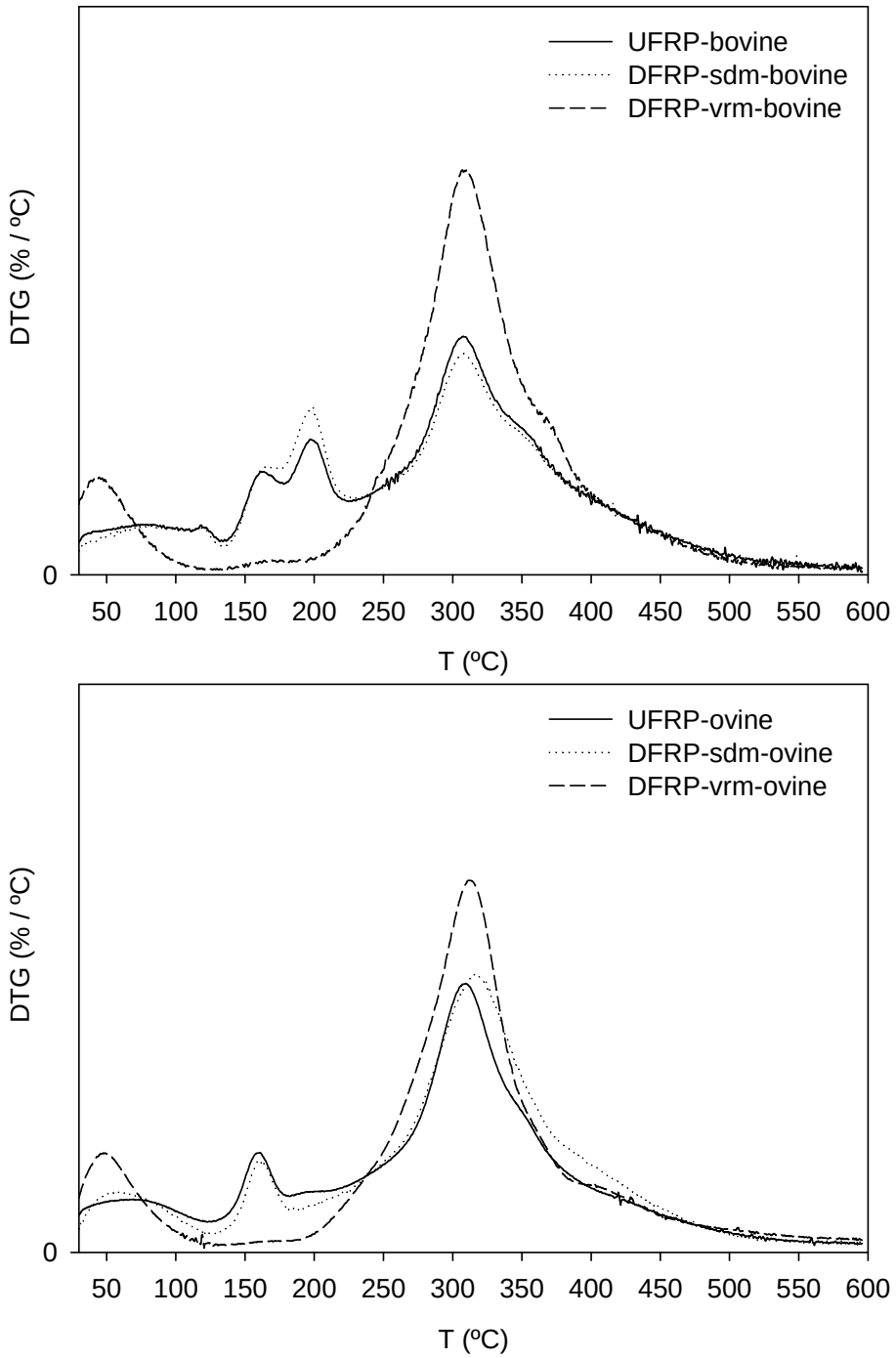


Figure 3.9 Derivative thermogravimetric curves (DTG) of bovine and ovine WPC powders produced by conventional ultrafiltration (UFRP), diafiltration in sequential dilution mode (DFRPsdm) and diafiltration in volume reduction mode (DFRPvrm).

At this temperature, weight losses range between 39.69-40.26%. At 600 ºC samples obtained by DFRPvrm presented a lower residual mass (17.97% and 23.61%, for bovine and ovine products respectively). The small amounts of lactose in both cases (5.47% and 5.70%, for bovine and ovine products respectively - Table 3.3) compared with the values reported for UFRP and DFRPsdm products (41.79% and 44.61% for bovine products and 28.28% and 21.26% for ovine products - Table 3.3) may explain the differences in thermal stability between products. It has been reported that α-monohydrate lactose starts to decompose at 144.47 ºC (Brittain and Blaine, 2012) and for this reason the initial decomposition temperatures in products with higher amounts of lactose are lower and closer to this value. Comparing bovine and ovine products produced by UF and DFsdm (Figures 3.8 - 3.9 and Table 3.6), a distinct decomposition profile was observed in the second stage of the thermal decomposition range (120-225 ºC). In bovine products two well-distinguished decomposition episodes were observed, the first at 163.6-167.13 ºC and the second at 196.79-198.27 ºC. Ovine products presented a single decomposition episode in this region (at 159.68-161.08 ºC). These differences are related to distinct decomposition structures that are only present in bovine products when the purity is relatively low (UFRP and DFRP). However, further research is needed in order to identify the nature of these structures.

Table 3.6 Thermogravimetric analysis (TGA) of bovine and ovine WPC powder produced by conventional ultrafiltration (UFRP), diafiltration in sequential dilution mode (DFRPsdm) and diafiltration in volume reduction mode (DFRPvrm), in terms of decomposition temperature (T_d), derivative maximum decomposition rate temperature (DTG_{max}), weight loss and residual mass.

Thermal property	Bovine-WPC			Ovine-WPC		
	UFRP	DFRPsdm	DFRPvrm	UFRP	DFRPsdm	DFRPvrm
T_d (ºC)	163.50 198.27	167.13 196.79	218.57	159.68	161.08	208.49
Weight loss (% w/w)	11.94 18.35	11.84 18.53	10.67	11.49	10.49	10.94
DTG_{max} (ºC)	307.29	307.63	308.91	309.00	316.03	311.80
Weight loss (% w/w)	41.74	42.40	39.69	39.21	40.61	40.26
Residual mass (% w/w)	25.07	25.84	17.97	28.80	24.43	23.61

3.4 Conclusions

By using batch ultrafiltration or diafiltration processes in small and medium-scale cheese production plants, it is possible to solve the environmental problem posed by whey disposal whilst also helping to increase the overall process yield through the production of liquid or dry WPC. DFsdm does not improve the protein composition in comparison to conventional

ultrafiltration. The increase in process complexity and material requirements introduced by diafiltration is only justified in the case of DFvrm, as it is possible to obtain more purified products without lower Mw compounds. However, this process penalises the amount of α-Lactalbumin and consequently the overall amount of proteins in WPC. The use of a 10 kDa cut-off membrane should avoid this drawback. It was observed that thermal behaviour is heavily dependent on sample preparation and the amount of impurities. The decomposition temperature for all the freeze-dried powders was in the range of 150-400 ºC, showing their high thermal stability.

References

Andrews AT, Taylor MD and Owen AJ (1985) Rapid analysis of bovine milk proteins by fast protein liquid chromatography, Journal of Chromatography, 348, 177-185.

AOAC (1980a) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 16.032. Total Solids, Method I - Official Final Action, 13th ed., Washington DC, USA.

AOAC (1980b) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 16.057. Lactose, Gravimetric Method - Official Final Action. 13th ed., Washington DC, USA.

AOAC (1995) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 930.30. Ash of dried milk, 16th ed., Gaithersburg, MD, USA.

AOAC (1996) Official Methods of Analysis of the Association of Official Analytical Chemists, Phosphorus in dried milk: Colorimetric procedure, In P. Cunliffe (Ed.). Official Methods of Analysis, 16th ed., Vol. II, Maryland, USA.

AOAC (2005) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 991.25. Calcium, magnesium and phosphorus in cheese - AAS method. 16th ed., Gaithersburg, MD, USA.

Bajpai AK and Gupta MP (1979) Studies on heat induced destabilization of caprine whey proteins. Indian Journal Dairy Science, 32, 62-67.

Barba D, Beolchini F and Veglio F (2000) Minimizing water use in diafiltration of whey protein concentrates, Separation Science and Technology, 35, 951-965.

Bonnaillie LM and Tomasula PM (2008) Whey protein fractionation in Whey processing, functionality and health benefits, Onwulata CI and Huth PJ (Ed.), John Wiley & Sons, ISBN: 978-0-813-80903-8

Brittain HG and Blaine RL (2012) α-Monohydrate phase in lactose by DSC. TA Instruments, T293, 1-3.

<http://www.tainstruments.co.jp/application/pdf/Thermal_Library/Applications_Briefs/TA293.PDF>

Capitani C, Perez OE, Pacheco B, Teresa M and Pilosof AMR (2007) Influence of complexing carboxymethylcellulose on the thermostability and gelation of α-Lactalbumin and β-Lactoglobulin, Food Hydrocolloids, 21, 1344-1354.

Casper JL, Wendorff WL and Thomas DL (1998) Seasonal Changes in Protein Composition of Whey from Commercial Manufacture of Caprine and Ovine Specialty Cheeses. Journal Dairy Science, 81, 3117-3122.

Casper JL, Wendorff WL and Thomas DL (1999) Functional properties of whey protein concentrates from caprine and ovine specialty cheese whey, Journal Dairy Science, 82, 265-271.

Comendador FJ, Nardo N, Bertone A, Maurizi A and Quaglia G (1996) Effetto dell'ultrafiltrazione sulle proprietá funzionali della scotta ovina. Scienza e Tecnica, Lattiero-Casearia, 47(2), 103-112.

Dannenberg F and Kessler H (1988) Reactions kinetics of the denaturation of whey proteins in milk, Journal of Food Science, 53, 258-263.

de Wit JN (1989) Functional properties of whey proteins, in: P.F. Fox, Editor, Developments in Dairy Chemistry, Functional Milk Proteins, Applied Science Publishers, London, pp. 285-322.

de Wit JN (1998) Nutritional and functional characteristics of whey proteins in food products, Journal of Dairy Science, 81, 597-608.

de Wit JN, Roginski H, Fuquay JW and Fox PF (2003) Dairy ingredients in non-dairy foods, Encyclopedia of dairy sciences, Academic Press (Elsevier Science), London, pp. 718-727.

Díaz O, Pereira CD and Cobos A (2004) Functional properties of ovine whey protein concentrates produced by membrane technology after clarification of cheese manufacture by-products, Food Hydrocolloids, 18, 601-610.

Evans J, Zulewska J, Newbold M, Drake MA and Barbano DM (2009) Comparison of composition, sensory, and volatile components of thirty-four percent whey protein and milk serum protein concentrates, Journal Dairy Science, 92, 4773-4791.

Gault P and Fauquant J (1992) Aptitude à la gélification thermique de la b-Lactoglobuline: influence du pH, de l'environnement ionique et la présence des autres protéines du lactosérum, Lait, 72, 491-510.

Gerosa S and Skoet J (2012) Milk availability - Trends in production and demand and medium-term outlook. ESA Working paper No. 12-01, February, Food and Agriculture Organization of the United Nations. <www.fao.org/economic/esa>

Harper WJ (1991) Whey and whey protein composition, in: Proc. Ctr. Dairy Res. Am. Dairy Products Inst. Whey Protein Workshop, Madison, WI. Wisconsin Ctr. Dairy Res., Madison.

Hill AR and Kakuda Y (1990) Size exclusion chromatography of caprine whey proteins, Milchwissenshaft, 45, 207-210.

INE - Instituto Nacional de Estatística, I.P. (2010) Estatísticas Agrícolas 2009. Lisboa, Portugal. ISBN 978-989-25-0085-4.

INE - Instituto Nacional de Estatística, I.P. (2012) Estatísticas Agrícolas 2011. Lisboa, Portugal. ISBN 978-989-25-0155-0.

ISO 1736:2000 Dried milk and dried milk products-determination of fat content: Gravimetric method. <http://jds.fass.org/cgi/reprint/88/8/2689.pdf>

ISO 8968-1:2001 Milk-determination of nitrogen content Part 1: Kjeldahl method.

Jelen P (2003) Whey processing: Utilization and products in Encyclopedia of dairy sciences, Roginski H, Fuquay JW and Fox PF (Ed.), Academic Press (Elsevier Science), London, pp. 2739-2745.

Joshep MSB and Mangino ME (1988) The effects of milk fat globule membrane protein on the foaming and gelation properties of β-Lactoglobulin solutions and whey protein concentrates, The Australian Journal of Dairy Technology, 43, 9-11.

Kilara A (1984) Standardization of methodology for evaluating whey proteins. Journal of Dairy Science, 67, 2734-2744.

Kinsella JE (1976) Functional properties of food proteins: a review. Critical Reviews in Food Science and Nutrition, 7, 219-280.

Law AJR, Leaver L, Banks JM and Home DS (1993) Quantitative fractionation of whey proteins by gel permeation FPLC, Milchwissenschaft, 48, 663-666.

Moatsou G, Hatzinaki A, Samolada M and Anifantakis E (2005) Major whey proteins in ovine and caprine acid wheys from indigenous greek breeds International Dairy Journal, 15, 123-131.

Morr CV and Foegeding EA (1990) Composition and functionality of commercial whey and milk protein concentrates and isolates: A status report, Food Technology, 44, 100-112.

Morr CV and Ha EYW (1993) Whey protein concentrates and isolates: processing and functional properties. Critical Reviews in Food Science and Nutrition, 33, 431-476.

Muir DD, Horne DS, Law AJR and Steele W (1993) Ovine milk. 1. Seasonal changes in composition of milk from a commercial Scottish flock. Milchwissenschaft, 48, 363-366.

NP 469:2002 Norma Portuguesa. Leites. Determinação da matéria gorda (técnica de Gerber). 3ª ed., Lisboa, Portugal.

Ozer B, Atasoy F and Akin S (2002) Some properties of urfa cheese (a traditional white-brined Turkish cheese) produced from bovine and ovine milks. International Journal of Dairy Technology, 55, 94-99.

Parris N, Anema SG, Singh H and Creamer LK (1993) Aggregation of whey proteins in heated sweet whey, Journal of Agriculture and Food Chemistry, 41, 460-464.

Pereira CD, Diaz O and Cobos A (2002) Valorization of by-products from ovine cheese manufacture: Clarification by thermocalcic precipitation/microfiltration before ultrafiltration, International Dairy Journal, 12, 773-783.

Pintado ME and Malcata FX (1996) Effect of thermal treatment on the protein profile of whey from ovine and caprine milk throughout lactation, International Dairy Journal, 6, 497-518.

Pintado ME and Malcata FX (2000) Hydrolysis of ovine, caprine and bovine whey proteins by trypsin and pepsin, Bioprocess Engineering, 23, 275-282.

Pintado ME, Macedo A and Malcata FX (2001) Review: technology, chemistry and microbiology of whey cheeses. Food Science and Technology International, 7, 105-116.

Pizzichini M, Zeddita G, Comendador FJ, Bertone A, Maurizi A, Nardo N and Quaglia G (1995) Recupero di sieroproteine e acqua depurata dalla scotta ovina com tecnologie di membrana. Scienza e Tecnica Lattiero-Casearia, 46, 361-379.

Remeuf F, Mohammed S, Sodini I and Tissier JP (2003) Preliminary observations on the effects of milk fortification and heating on microstructure and physical properties of stirred yogurt, International Dairy Journal, 13, 773-782.

Resch JJ and Daubert CR (2002) Rheological and physicochemical properties of derivatized whey protein concentrate powders, International Journal of Food Properties, 5, 419-434.

Rinn JC, Morr CV, Seo A and Surak JG (1990) Evaluation of nine semi-pilot scale whey pretreatment modifications for producing whey protein concentrate, Journal of Food Science, 55(2), 510-515.

Schwartz L (2003) Diafiltration: a fast, efficient method for desalting, or buffer exchange of biological samples. PALL Life Sciences.
<http://site.pall.com/pdf/02.0629_Buffer_Exchange_STR.pdf>

Scott R (1986) Cheesemaking practice. 2nd ed, Elsevier Applied Science Publishers, London.

Singh RK (1995) Bioseparation Processes in Food, Ift Basic Symposium Series. vol. 10, Taylor & Francis, Inc. ISBN: 082479608X.

Strouts B (2004) An overview and latest developments, in: J. Page, D. Meyer, B. Haines, V. Lagrange, A. Kenney (Eds.), Reference Manual for U.S. Whey and Lactose Products, chapter 10, Bakery applications for whey and lactose products, pp. 98-99.

Tunick MH (2008) Whey Protein Production and Utilization: A Brief History in Whey processing, functionality and health benefits, Onwulata CI and Huth PJ (Ed.), John Wiley & Sons. ISBN: 978-0-813-80903-8.

Wendorff WL (2001) Freezing qualities of raw ovine milk for further processing. Journal Dairy Science, 84, E74-E78.

Part C.

Whey protein conventional applications

Chapter 4

Effects of LWPC on the functional and sensorial properties of set yogurts and fresh cheese

Abstract

The production of liquid whey protein concentrates (LWPCs) and their incorporation in fresh cheese and set yogurt is proposed as a solution for the immediate reutilisation of whey produced by small and medium-scale dairy plants, thereby avoiding expensive processing steps (e.g., evaporation and drying) for the recovery of this by-product. LWPCs were therefore incorporated into these products in order to compare the functional and sensorial properties of the modified

products with the conventional ones. The use of LWPC in fresh cheese increased water holding capacity as well as product stability during storage. Fresh cheese hardness, chewiness, and gumminess decreased during storage in a more pronounced way in products containing LWPC and, the fat content significantly influenced all the physicochemical properties tested in set yogurts. Incorporating LWPC into set yogurts does not produce appreciable differences to the visual properties of products with a medium fat content, but these differences become significant in full-fat yogurts. Adhesiveness and springiness were not significantly affected by storage time or the amount of LWPC incorporated into medium-fat yogurts. Higher hardness and gumminess values were obtained for full-fat yogurts, but these parameters decreased with the incorporation of LWPC. Syneresis was reduced using LWPC, but increased with storage time. During storage, the differences in viscosity between yogurts incorporating LWPC and conventional products were only maintained in the case of creamy yogurts. The sensory panel detected differences between conventional and modified products in the case of fresh cheeses but no significant differences were detected in yogurts. LWPCs can be a good alternative to the conventional dry products used in the manufacture of fresh cheese and set yogurt, since they reduce milk consumption and increase the total solids content. Additionally, their incorporation in milk creates end products with attractive physicochemical and sensorial characteristics and lower production costs.

4.1 Introduction

The industrial production of cultured dairy products such as fresh cheese and yogurt involves the standardisation of solids in milk in order to prevent the spontaneous separation of whey (currently called syneresis), which is seen as a drawback in these products (Lucey, 2004; Amatayakul et al., 2006). This operation is normally performed in a mix tank with proper agitation, in which the various, normally dry, (conventional) ingredients are added to the milk. Skimmed milk powder is often used in fresh cheese production (Lucey, 2002). Although products such as whole milk powder, caseinates, or whey protein concentrate (WPC) are also used in the manufacture of yogurts (Tamine and Robinson, 1999), there is no reference to the use of liquid whey protein concentrates (LWPCs). The latter procedure allows for the internal reutilisation of whey in small and medium-scale dairy plants, thus reducing the need to acquire dry ingredients from external sources.

Whey protein concentrate powders (WPC) are largely used as attractive food ingredients in a wide range of food applications (Diaz et al., 2009), especially due to their specific functional and nutritional properties. The production of bovine WPCs or whey protein isolates (WPI) is usually associated with large-scale production plants. For economic reasons, smaller dairy industries cannot apply the same strategy, which may create difficulties for the disposal or reutilisation of whey. However, increasing environmental constraints are obliging the industries to look at whey not as a residue but as a valuable by-product for internal valorisation. This perspective can both improve production yields and add nutritional and functional value to existing products.

The inclusion of an ultrafiltration/diafiltration (UF/DF) section in small and medium-scale dairy plants is considered economically feasible for producing LWPC and LWPI which, in turn, can be usefully incorporated in traditional products, namely cheeses. Several authors refer to this possibility (Abrahamsen, 1979; Banks and Muir, 1985; Baldwin et al., 1986; Korolczuk and Mahaut, 1991a,b; Mahaut and Korolczuk, 1992; Smithers et al., 1996; Jameson and Lelievre, 1996; Pérez-Munuera and Lluch, 1999; Hinrichs, 2001). The main topics and conclusions of their work concern: (1) the importance of whey protein denaturation in order to guarantee its contribution to the protein matrix of the cheese; (2) the importance of the dimensions of the denatured aggregates; (3) the increased water holding capacity of cheese curds; (4) the lower acidification of modified products as a result of the higher buffering capacity of whey proteins; (5) differences in flavour in the modified products; (6) the optimal amount of LWPC that can be incorporated. LWPCs are rarely used in the production of yogurts. Most of the published studies only evaluate the influence of WPC powders on the chemical, functional, rheological, textural and sensorial characteristics of liquid or set yogurts (Guzmán-González et al., 1999; Sodini et al., 2005, 2006; Damin et al., 2009; Herrero and Requena, 2006; Cais-Sokolinska and Pikul, 2006).

Although the use of LWPCs in small and medium-sized dairy plants appears to be quite promising, little attention has been paid to their use and performance in terms of the functional and sensorial characteristics of the resulting new products in research related to the dairy industry, which is therefore the purpose of this study.

4.2 Material and methods

4.2.1 LWPC manufacture

Bovine cheese whey was supplied by Queijaria Serqueijos SA (Portugal). It was obtained immediately after production, and transported to the pilot plant in 50 L jars. On reception, the whey was first filtered, analysed, and then processed at 24-30 °C to obtain the required amount of LWPC using an UF pilot plant equipped with an organic DSS™ membrane, 20K 3838-30 model, 5.5 m^2 filtering area and 20 kDa cut-off. During ultrafiltration with a volumetric concentration factor of 20, the transmembrane pressure was held at 3.7-4.0 bar. After concentration, the retentate was submitted to a thermal treatment (90 °C/60 s) to achieve whey protein denaturation, cooled to 65 °C and homogenised at 100 bar in order to reduce the diameter of the thermally induced aggregates to a value bellow 10 μm. This procedure was undertaken to avoid disturbance to the casein matrix caused by large particles, as pointed out by Walstra and van Vliet (1991) and Lebeuf et al. (1998). Before it was incorporated into milk batches for fresh cheese or yogurt production, the LWPC was analysed and frozen at -15 °C.

4.2.2 Fresh cheese manufacture

In the production of fresh cheese, three formulations were tested. For each formulation, bovine standardised milk was partially skimmed, pasteurised, and mixed with: (1) 4 g/100 g of skimmed milk proteins (with about 50 g of proteins/100 g of powder) (Tecnilac, Portugal) – the conventional process - (C); (2) 25 g/100 g (25LWPC), and (3) 50 g/100 g (50LWPC) of bovine LWPC. In each trial, the fat content was normalised at 3.3 g/100 g. Batches of each formulation were pasteurised at 72 °C for 30 s and then quickly cooled to the coagulation temperature (33 °C). Coagulation was performed for 20 min in the presence of a 200 mg/L $CaCl_2$ solution (51 g/100 mL) (Enzilab, Portugal) and 70 mg/L of rennet (> 96% chymosin) (Tecnilac, Portugal) previously diluted in tap water. After cutting the curd and draining off the whey, fresh cheeses (of approx. 100 g) were moulded in plastic packages and maintained at 4±2 °C during storage (7 days).

4.2.3 Set yogurt manufacture

Two trials of yogurts were produced using skimmed bovine milk, with posterior fat normalisation to 1.5 and 9 g/100 g with cream, medium-fat and full-fat yogurts respectively. Three formulations with 1.5 g/100 g of fat (medium-fat yogurts) were normalised in terms of dry matter content (15

g/100 g) and prepared: (1) with skim milk powder (conventional yogurt) (1.5-C), (2) with 15 g/100 g (1.5-15LWPC), and (3) 30 g/100 g (1.5-30LWPC) added LWPC.

In the case of the full-fat yogurts (9 g/100 g of fat), the dry matter content was set at 19 g/100 g and 30 g/100 g of LWPC was incorporated. The conventional product was termed (9-C) and the test product (9-30LWPC). All the ingredients for each formulation were mixed, homogenised at 200 bar and pasteurised at 92 °C/30 seconds. Before filling and packaging, the mixture was stirred for 20 min at 43 °C and inoculated with a mixed culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Ezal YO–MIX 601). The fermentation step was performed in 125 mL polystyrene cups at a constant temperature of 43±1 °C until the yogurt pH reached 4.6±1. The yogurts were then stored at 4±2 °C after cooling for 1 h.

4.2.4 Chemical analyses

The biochemical composition of whey, LWPC, milk, skimmed milk protein, and cream was evaluated using the Portuguese Standards Methods (IPQ, 1990) and the Official Analytical Methods (AOAC, 1997). Samples of each formulation were collected using the Portuguese specific standard procedure for dairy products (NP 4146:1991). In the case of the fresh cheese and set yogurt analyses, three samples were selected at random. However, if the parameter in question demanded homogeneous samples (such as cheese chemical composition), a homogenizer (Masticator IUL Instruments) was used.

The pH was determined with a pH meter (HI 9025 HANNA Instruments) immediately after production and later during storage. Titrable acidity, expressed as a percentage of lactic acid, was determined by titration using a 0.1 N NaOH solution following the technique described in NP 470:1983 for milk, AOAC (1997) for whey, LWPC and cheese, and NP 701:1982 for yogurts.

Total solids were determined by oven drying the samples according to NP 580:1970 for milk, AOAC (1997) for LWPC, NP 3544:1987 for cheese, and NP 703:1982 for yogurt. Ash content was determined by the incineration of dry samples in an electric muffle furnace (Nabertherm, model LE 4/11/R6) using the AOAC methods (1997).

The fat content of the milk, LWPC and product formulations was determined by the Gerber method (NP 469:2002), adapted to each product and specific for yogurt (NP 1923:1987). In the case of fresh cheese, the Van Gulik method was used (NP 2105:1983).

The total N content was determined using the Kjeldahl method, in accordance with AOAC (1997). The total protein was estimated by multiplying the total nitrogen content of the samples by a factor of 6.38.

The lactose concentration was determined by the difference between the dry mass of the sample and the sum of the remaining components (ash, fat and protein). All the analyses were carried out in triplicate.

4.2.5 Physicochemical analyses of fresh cheese and set yogurt

Colour

Colour was determined in triplicate with a Minolta Chroma Meter colorimeter, model CR-200B, using the L*a*b* CIELAB system calibrated with a white standard dish (CR-A47).

Syneresis

Spontaneous cheese syneresis was calculated as the ratio between the mass of serum lost spontaneously over 6 days of storage and the original mass of the cheese in normal cooling conditions (< 6 °C). This analysis was performed using six fresh cheese samples chosen randomly. The yogurt syneresis index (water retention capacity) followed the method described by Gauche (2007). Triplicate 20 g samples were centrifuged at 350×g in a refrigerated centrifuge (Hettich, model Rotanta 460R) for 10 min at 5 °C. The supernatant was collected and weighed. The syneresis index (percent) was the proportion of the supernatant mass in the total mass sample, multiplied by 100.

Viscosity

The evaluation of yogurt viscosity was performed in triplicate at controlled temperature (5±1 °C) for 10 min (30 s intervals) in a rotational Brookfield Viscometer, model DV II, with a concentric cylinder RV (spindle 3) set at a constant angular velocity (2.5 rpm). Prior to determining viscosity, the refrigerated set yogurt samples were manually homogenised for 1 min and set to rest for 5 min in the fridge (5±1 °C). The viscosity of the samples was the mean value of the viscosity measurements during the 10 min of analysis.

Texture analysis

A Stable Micro Systems Texture analyser, model TA.XT Express Enhanced, was used to perform textural analysis (n=3) and the results were calculated using Specific Expression PC Software. For

fresh cheese, a texture profile analysis was run with a penetration distance of 15 mm at 1 mm/s test speed, using an acrylic cylindrical probe with a diameter of 12.5 mm and a height of 38.1 mm. For set yogurts, the penetration distance was 20 mm at 2 mm/s using a stainless steel cylindrical probe with a diameter of 25.4 mm and a height of 38.1 mm. The following parameters were quantified (Phadungath, 2010): hardness (the peak force measured during the first compression cycle), adhesiveness (the negative force area for the first bite, representing the necessary work to pull the compression plunger away from the sample), cohesiveness (the ratio of the positive force area during the second compression to that during the first compression), springiness (the height that the sample recovers during the time elapsing between the end of the first cycle and the start of the second cycle), and gumminess (the product of hardness and cohesiveness). Additionally, chewiness (the product of gumminess and springiness) was also quantified for fresh cheese.

Sensorial Analysis

Sensorial analysis was based on preference and triangular tests performed by an untrained panel in order to detect differences between products with conventional formulations and those incorporating LWPC. A panel of 31 individuals was used for the preference test and for the two triangular tests performed on fresh cheese, and a panel of 35 for the three trials with set yogurts (two for medium-fat yogurts and one for full-fat yogurts). The triangular tests were based on binomial distribution (ISO 8586-2:1994), with a confidence level at $p < 0.05$.

4.2.6 Statistical analyses

Statistical analysis of the data was carried out using the ANOVA package included in Statistica 8.0 (Hill and Lewicki, 2007). One-way ANOVA tests were performed to compare the means for the gross composition of bovine whey and liquid whey protein concentrates shown in Table 4.1, and the gross composition of medium-fat and full-fat yogurts, presented in Table 4.4. The comparison was made using a multiple comparison Tukey HSD test with a confidence level of 95%. Two-way ANOVA with interaction was employed to determine the effects of both storage time and LWPC incorporation on the biochemical composition (Figure 4.1), colour (Table 4.2), and texture (Table 4.3) of fresh cheese. The impact of three independent variables (storage time, incorporation of LWPC, and fat content) on the colour, texture, syneresis, and viscosity of yogurt was statistically analysed with N-way ANOVA (data not shown). Subsequently, whenever the impact of fat content was observed to be significant for all the parameters evaluated, the effect of

storage time and LWPC incorporation was tested separately for medium-fat and full-fat yogurts, using two-way ANOVA with interaction. The results obtained are shown in Table 4.5 (colour analysis), Table 4.6 (texture analysis), Figure 4.3 and Table 4.7 (syneresis), and Figure 4.4 and Table 4.7 (viscosity). All analyses were performed using Tukey's HSD post tests with a 95% confidence level, corresponding to a critical $p = 0.05$.

4.3 Results and discussion

4.3.1 Characterisation of LWPC

During approximately 30 min of ultrafiltration, 190 L of bovine whey were concentrated to recover 9 L of retentate. As expected, the LWPC had a higher dry matter, fat and total protein content than the original whey (Table 4.1). However, the approximately sevenfold increase in protein and fat was not in accordance with the volumetric concentration factor. This difference can be explained by the following factors: protein losses in the permeate throughout concentration due to the relatively high molecular weight (Mw) membrane cut-off; loss of fat adhering to the membrane and equipment surfaces; water incorporation in the retentate during its recovery from the dead volume of the batch ultrafiltration equipment. As expected, the amounts of ash, lactose, and lactic acid in the whey were maintained in LWPC ($p > 0.05$), with the approximately 2.2-fold increase in LWPC total solids content due chiefly to the increase in protein and fat contents.

The drawbacks identified in protein and fat recovery which led to lower production yields in LWPC can be overcome by reducing the Mw membrane cut-off and increasing the volumes processed by minimising the aforementioned dilution effect.

Table 4.1 Gross chemical composition of bovine whey[1] and liquid whey protein concentrate (LWPC[1]): total solids, ash, fat, protein, lactose and titratable acidity (TA).

Composition (g/100 g)	Whey	LWPC
Total solids	6.34±0.28 a	14.37±0.48 b
Ash	0.50±0.03 a	0.41±0.03 a
Fat	0.60±0.04 a	4.45±0.64 b
Protein	0.72±0.09 a	4.95±0.35 b
Lactose	4.53±0.12 a	4.56±0.46 a
TA (% lactic acid)	0.11±0.02 a	0.16±0.02 a

[1]means of two batches. a, b means within the same rows without the same superscript are statistically different at $p = 0.05$.

4.3.2 Composition and physicochemical properties of fresh cheese

Fresh cheese is a very perishable product with a limited shelf life (about 7 days). Figure 4.1 compares the biochemical composition of conventional fresh cheese and alternative products during storage. As expected, it can be observed that all the samples show increases in total solids, fat, protein, and ash contents as a result of the loss of humidity during the storage period. On the first day of storage, the total solids, fat and protein concentrations were statistically different ($p < 0.05$) in the conventional and alternative products. The mineral content only differed during the assessment period but not between products. At the end of the storage time, no differences were found between both types of products with regard to total solids content. The increase in protein content during storage was not statistically significant (with the exception of 50LWPC), although differences were found between products ($p < 0.05$), with lower values for fresh cheese incorporating LWPC. After 7 days of storage, the lactic acid content (TA) was significantly lower for products with LWPC. The lower acidity of modified products can be attributed to the higher buffering capacity of the whey proteins incorporated into the cheese matrix. For sensorial purposes, this aspect may be very interesting, since one of the main defects of fresh cheese in the final days of storage is evidence of a sour taste.

The yields achieved in this work for cheeses incorporating LWPC were lower than those obtained for the conventional product (Figure 4.2). The reasons identified for this may be the nature and relative proportion of proteins present in each formulation (caseins in the conventional products and whey proteins in the test products). However, the use of LWPC in fresh cheese increased the water holding capacity. As a consequence, the products with LWPC showed greater stability during storage time and the differences in cheese yield between the first and seventh day were less evident in these products than in the conventional ones. These results agree with those published by Jorge et al. (2006) for ripened cheese with 10 and 20 g/100 g of LWPC incorporation. Jameson and Lelievre (1996) studied the effects of whey protein incorporation on the characteristics of cheeses and concluded that they were greatly influenced on a chemical, biochemical, and biophysical level, according to whether the proteins were in their native state or denatured. Abrahamsen (1979) reported increases of 1% to 17% in ripened cheese yields, with denatured whey proteins incorporated at 2.5 to 10 g/100 g in comparison to ripened cheese with no protein addition. Banks and Muir (1985) referred to increases of 7% for Cheddar cheese. Punidadas et al. (1999) concluded that the homogenisation of denatured whey proteins

improves fat retention because it promotes fat dispersion in the cheese matrix, favouring interactions between proteins and lipids.

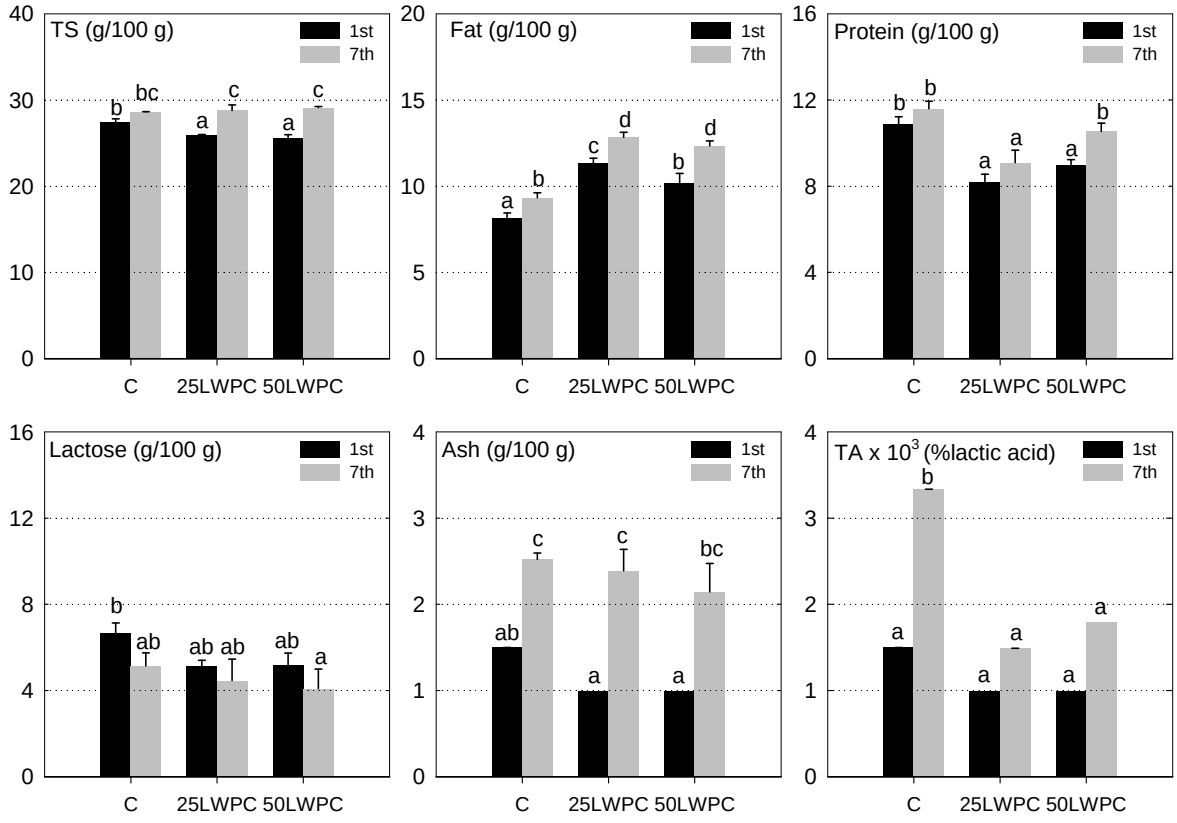


Figure 4.1 Biochemical composition of fresh cheese during storage (1st and 7th day). Total solids (Ts), fat, protein, lactose, ash and titratable acidity (TA) for: C - conventional fresh cheese; 25LWPC and 50LWPC - fresh cheese incorporated with LWPC (25 and 50 g/100 g respectively). Different letters indicate significant differences ($p < 0.05$).

Table 4.2 shows the colour parameters for fresh cheese during the storage time and the results of the corresponding statistical analysis. During the 7 days of storage, all samples underwent a slight darkening (lower L* values). However, increasing the LWPC incorporation reduced cheese darkening, thus improving the appearance of the product (Table 4.2a). Significant differences were also observed in a* and b* values, both during storage and with the incorporation of LWPC (Table 4.2b), thus demonstrating the importance of these two factors to the appearance of fresh cheese.

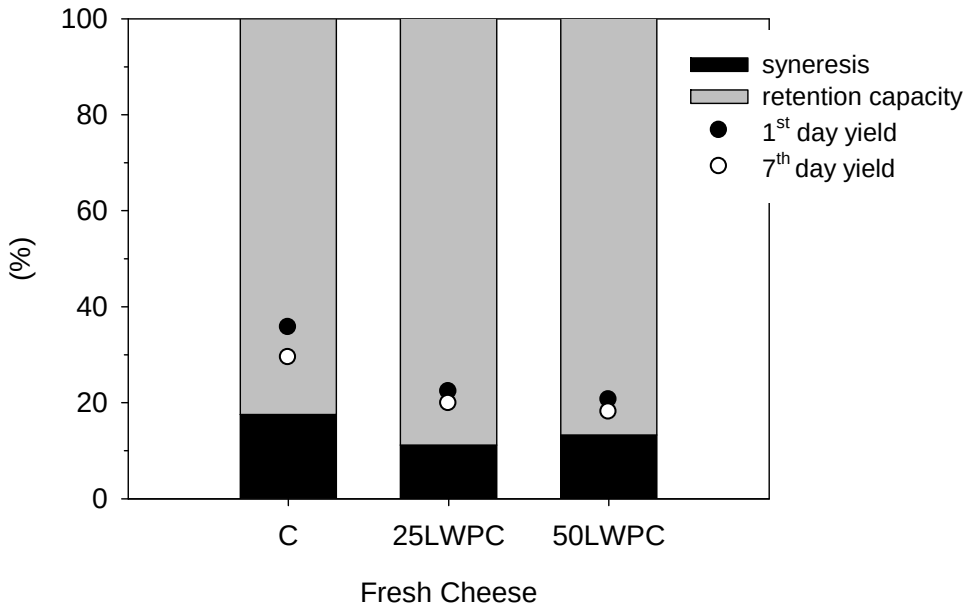


Figure 4.2 Spontaneous syneresis, retention capacity and cheese yield at 1st and 7th storage day for conventional and LWPC incorporated fresh cheeses. 25LWPC and 50LWPC - cheese incorporated with LWPC (25 and 50 g/100 g respectively).

Table 4.2 Colour parameters (L*, a*, b*) of fresh cheese during storage (a); p values of Two-Way ANOVA with factors interaction, for colour parameters in fresh cheese (b).

a) Colour parameters	Storage time (days)	Fresh cheese[1]		
		C	25LWPC	50LWPC
L*	1	94.73±1.12 bc	95.00±0.10 bc	95.30±0.30 c
	7	92.30±0.82 a	93.47±0.15 ab	94.67±0.06 bc
a*	1	-3.70±0.10 a	-2.97±0.15 b	-3.50±0.20 a
	7	-1.63±0.06 d	-2.33±0.06 c	-2.73±0.15 b
b*	1	10.20±0.17 b	10.03±0.15 b	10.33±0.06 b
	7	12.77±0.06 c	10.30±0.10 b	8.87±0.06 a

b) Factors	Colour parameters		
	L*	a*	b*
Storage time (ST)	*0.000*	*0.000*	*0.000*
LWPC incorporation	*0.003*	*0.000*	*0.000*
ST×LWPC incorporation	0.062	*0.000*	*0.000*

[1] C - conventional fresh cheese; 25LWPC and 50LWPC - fresh cheese with 25 and 50 g/100 g LWPC incorporation respectively. a, b, c different letters indicate significant differences ($p < 0.05$) for each colour parameter. ($p < 0.05$ indicates that factors produce statistically significant differences in dependent variables).

The textural results obtained are presented in Table 4.3a. Hardness, chewiness and gumminess were similar in the control and tested samples and decreased during storage (Table 4.3b). The

results obtained agree in general with those cited by Punidadas et al. (1999), who concluded that reduced-fat cheeses made with homogenised whey proteins were similar to the control in terms of physical properties. Adhesiveness was not affected by time, whereas springiness and cohesiveness were statistically different for both factors evaluated ($p < 0.05$).

Despite some texture parameters were not significantly different in the conventional and modified products differences were detected by the sensory panel for fresh cheese products with a confidence level of 5% in the triangular test. It was obtained 21 right answers for the tests between conventional cheese (C) and 25LWPC and 24 right answers for 50LWPC, in both cases higher than the minimum of 16 right answers required to validate each test. In the preference test between conventional cheese (C) and cheese containing 25 g/100 g of LWPC (25LWPC), the panellists gave the second formulation a more positive evaluation, stating that this cheese type was softer. It is believed that the proportion of whey proteins and caseins in formulations is the key factor to improving both the sensorial characteristics and production yields of cheese. With appropriate modifications to the production technique, both objectives can be optimised.

4.3.3 Composition and physicochemical properties of set yogurt

The gross composition, TA, and pH of bovine medium-fat and full-fat yogurt are listed in Table 4.4. Full-fat yogurts present a lower total protein concentration. Their higher total solids content is due exclusively to the percentage of fat in the formulation. No significant differences ($p > 0.05$) were observed in total solids, fat, and pH between each type of conventional yogurt (1.5-C and 9-C) and the samples incorporating whey proteins (1.5-15LWPC, 1.5-30LWPC, and 9-30LWPC). The protein concentration increased in the alternative products, but the ash content was similar in all samples. During storage, pH and titrable acidity varied significantly ($p < 0.05$). The yogurts which presented a lower acidity were the ones that had higher levels of protein, presumably due to their buffering capacity. Salaün et al. (2005) and Kailasapathy et al. (1996) mention that substituting part of the skim milk powder with whey proteins increases buffering capacity at pH 4 and decreases buffering capacity between pH 5 and 6.

Table 4.3 Texture parameters (hardness, adhesiveness, springiness, chewiness, gumminess and cohesiveness) of fresh cheese during storage (a); p values of Two-Way ANOVA with factors interaction, for texture parameters in fresh cheese (b).

a) Texture parameters	Storage time (days)	Fresh Cheese[1]		
		C	25LWPC	50LWPC
Hardness (N)	1	1.26±0.12 b	1.38±0.08 b	1.73±0.12 c
	7	1.06±0.06 ab	0.88±0.16 a	0.82±0.18 a
Adhesiveness (N.s)	1	-2.91±0.12 c	-5.17±0.17 a	-2.03±0.06 d
	7	-2.64±0.16 c	-4.49±0.09 b	-2.65±0.10 c
Springiness	1	0.99±0.01 b	0.99±0.01 b	0.99±0.00 b
	7	0.92±0.01 a	0.99±0.01 b	0.99±0.00 b
Chewiness (N)	1	0.65±0.06 cd	0.61±0.03 bcd	0.78±0.05 d
	7	0.50±0.03 abc	0.47±0.09 ab	0.38±0.08 a
Gumminess (N)	1	0.66±0.06 cd	0.62±0.03 bcd	0.79±0.05 d
	7	0.55±0.04 abc	0.47±0.09 ab	0.39±0.08 a
Cohesiveness	1	0.53±0.01 cd	0.45±0.01 a	0.45±0.01 a
	7	0.52±0.01 c	0.54±0.01 d	0.47±0.01 b

b) Factors	Texture parameters					
	Hardness	Adhesiveness	Springiness	Chewiness	Gumminess	Cohesiveness
Storage time (ST)	*0.000*	0.082	*0.000*	*0.000*	*0.000*	*0.000*
LWPC incorporation	0.145	*0.000*	*0.000*	0.455	0.273	*0.000*
ST×LWPC incorporation	*0.001*	*0.000*	*0.000*	*0.006*	*0.004*	*0.000*

[1] C - conventional fresh cheese; 25LWPC and 50LWPC – fresh cheese with 25 and 50 g/100 g LWPC incorporation respectively. [a,…d] Different letters indicate significant differences ($p < 0.05$) for each texture parameter. ($p < 0.05$ indicates that factors produce statistically significant differences in dependent variables).

The statistical evaluation provided evidence of the influence of fat content on the physicochemical properties of yogurt, leading to the conclusion that this factor produces statistically significant differences in the properties evaluated. Hence, a factorial-ANOVA analysis was performed for each kind of yogurt (medium-fat and full-fat), in which the independent variables were the storage time and the amount of LWPC incorporated in the samples.

Table 4.4 Gross chemical composition of medium-fat and full-fat yogurts: total solids, ash, fat, protein, lactic acid and pH.

Composition (g/100 g)	Yogurts[1]				
	1.5-C	**1.5-15LWPC**	**1.5-30LWPC**	**9-C**	**9-30LWPC**
Total solids	13.98±0.12 a	13.76±0.08 a	13.61± 0.06 a	17.81±0.2 b	18.27±0.48 b
Ash	0.98±0.01 c	0.93±0.01 b	0.90± 0.01 a	0.94±0.01 b	0.96±0.01 c
Fat	1.43±0.03 a	1.38±0.06 a	1.41±0.03 a	8.81±0.08 b	9.00±0.05 c
Protein	4.82±0.12 c	5.12±0.17 d	5.43±0.44 d	3.21±0.15 a	3.58±0.11 b
Lactic acid	0.89±0.01 b	0.91±0.01 c	0.87±0.01 a	0.89±0.01 b	0.96±0.01 d
pH	4.43±0.03 a	4.38±0.03 a	4.38±0.03 a	4.37±0.03 a	4.40±0.00 a

[1]medium-fat yogurts: 1.5-C (conventional), 1.5-15LWPC and 1.5-30LWPC (with 15 and 30 g/100 g LWPC incorporation respectively); full-fat yogurts: 9-C (conventional) and 9-30LWPC (with 30 g/100 g LWPC incorporation). a, b, c, … means within the same rows without the same superscript are statistically different at $p = 0.05$.

In comparing conventional medium-fat yogurts with the ones produced with LWPC no significant differences in L* and a* colour parameters were found (Table 4.5). However, higher L* values were observed in full-fat yogurts and in this case the amount of whey proteins significantly influenced the L* and b* values (Table 4.5b). The storage time did not influence the b* value for medium-fat yogurts or L* value for full-fat yogurts ($p > 0.05$).

The textural analysis (Table 4.6) did not reveal any significant differences ($p > 0.05$) in medium-fat yogurt formulations in terms of adhesiveness and springiness (Table 4.6b). However, hardness, gumminess and cohesiveness differed statistically according to storage time and incorporation of whey proteins. Higher values for hardness, adhesiveness, and gumminess were observed in full-fat yogurts than in medium-fat yogurts. The presence of LWPC in a 9-30LWPC formulation led to a reduction in these textural parameters in comparison to the conventional product 9-C (Table 4.6a).

Table 4.5 Colour parameters (L*, a*, b*) of yogurts during storage (a); p values of Two-Way ANOVA with factors interaction, for colour parameters in yogurts (b).

a) Colour parameters	Storage time (days)	Medium fat yogurts[1]			Full fat yogurts[2]	
		1.5-C	1.5-15LWPC	1.5-30LWPC	9-C	9-30LWPC
L*	1	92.97±0.47 a	93.17±0.40 a	93.20±0.79 a	96.03±0.06 A	95.40±0.26 A
	10	92.80±0.69 a	92.10±0.36 a	92.40±0.70 a	95.87±0.12 A	95.47±0.61 A
	20	92.83±1.50 a	90.53±1.86 a	92.33±0.40 a	95.83±0.25 A	95.17±0.38 A
a*	1	-4.10±0.17 a	-4.17±0.06 a	-4.07±0.06 ab	-3.93±0.23 A	-3.67±0.15 AB
	10	-3.77±0.06 ab	-4.00±0.10 ab	-3.90±0.10 ab	-3.50±0.10 AB	-3.40±0.10 AB
	20	-3.67±0.21 b	-3.90±0.26 ab	-3.87±0.15 ab	-3.27±0.15 B	-3.23±0.06 B
b*	1	7.70±0.10 a	8.27±0.15 a	7.50±0.10 a	9.47±0.06 B	9.13±0.21 AB
	10	7.73±0.15 a	8.43±0.35 a	7.57±0.15 a	9.37±0.12 AB	8.97±0.12 A
	20	7.80±0.78 a	8.33±0.90 a	7.87±0.21 a	9.17±0.12 AB	8.93±0.15 A

b) Factors	Colour parameters		
	L*	a*	b*
Medium fat yogurts			
Storage time (ST)	*0.044*	*0.001*	0.689
LWPC incorporation	0.118	0.061	*0.006*
ST×LWPC incorporation	0.243	0.683	0.939
Full fat yogurts			
Storage time (ST)	0.518	*0.000*	*0.025*
LWPC incorporation	*0.004*	0.072	*0.000*
ST×LWPC incorporation	0.757	0.375	0.577

[1] 1.5-C (conventional), 1.5-15LWPC and 1.5-30LWPC (with 15 and 30 g/100 mL LWPC incorporation respectively); [2] 9-C (conventional) and 9-30LWPC (with 30 g/100 mL LWPC incorporation). Different letters indicate significant differences ($p < 0.05$). a, b small letters: differences amongst each colour parameter for medium fat yogurts. A, B capital letters: differences amongst each colour parameter for full fat yogurts. ($p < 0.05$ indicates that independent variables produce statistically significant differences in dependent variables).

Table 4.6 Texture parameters (hardness, adhesiveness, springiness, gumminess and cohesiveness) of set yogurts during storage (a); p values of Two-Way ANOVA with factors interaction, for texture parameters in yogurts (b).

a) Texture parameters	Storage time (days)	Medium fat yogurts[1]			Full fat yogurts[2]	
		1.5-C	1.5-15LWPC	1.5-30LWPC	9-C	9-30LWPC
Hardness (N)	1	0.92±0.005 a	0.99±0.005 ab	1.09±0.013 ab	2.59±0.015 B	1.89±0.029 A
	10	1.13±0.189 ab	1.09±0.042 ab	1.18±0.018 b	3.33±0.250 C	2.03±0.196 A
	20	1.08±0.148 ab	1.12±0.096 ab	1.18±0.018 b	3.17±0.327 C	1.78±0.150 A
Adhesiveness (N.s)	1	-1.59±0.21 a	-2.08±0.22 a	-2.47±0.12 a	-5.88±0.09 B	-5.19±0.10 BC
	10	-2.34±0.49 a	-2.27±0.35 a	-2.56±0.40 a	-7.88±0.84 A	-4.45±0.43 C
	20	-2.63±0.68 a	-2.16±0.21 a	-2.41±0.55 a	-7.03±0.11 A	-4.40±0.04 C
Springiness	1	0.96±0.01 a	0.97±0.01 a	0.98±0.01 a	0.97±0.01 A	0.97±0.01 AB
	10	0.98±0.01 a	0.97±0.01 a	0.97±0.02 a	0.98±0.01 AB	0.99±0.01 B
	20	0.97±0.02 a	0.98±0.01 a	0.98±0.01 a	0.99±0.01 AB	0.97±0.01 A
Gumminess (N)	1	0.42±0.01 ab	0.40±0.01 a	0.47±0.01 ab	1.19±0.01 B	0.83±0.00 A
	10	0.50±0.02 ab	0.46±0.03 ab	0.51±0.02 ab	1.45±0.01 C	0.90±0.02 A
	20	0.49±0.01 ab	0.48±0.03 ab	0.53±0.02 b	1.42±0.02 C	0.82±0.02 A
Cohesiveness	1	0.46±0.01 b	0.41±0.01 a	0.43±0.01 ab	0.46±0.01 A	0.44±0.00 A
	10	0.45±0.02 ab	0.42±0.03 ab	0.44±0.02 ab	0.44±0.01 A	0.45±0.02 A
	20	0.45±0.01 ab	0.43±0.03 ab	0.45±0.02 ab	0.45±0.02 A	0.46±0.02 A

b) Factors	Texture parameters				
	Hardness	Adhesiveness	Springiness	Gumminess	Cohesiveness
Medium fat yogurts					
Storage time (ST)	*0.007*	0.129	0.169	*0.002*	0.255
LWPC incorporation	*0.045*	0.205	0.286	*0.030*	*0.002*
ST×LWPC incorporation	0.732	0.184	0.259	0.905	0.625
Full fat yogurts					
Storage time (ST)	*0.008*	*0.045*	*0.015*	*0.009*	0.183
LWPC incorporation	*0.000*	*0.000*	0.757	*0.000*	0.696
ST×LWPC incorporation	*0.020*	*0.000*	*0.008*	0.055	*0.043*

[1] 1.5-C (conventional), 1.5-15LWPC and 1.5-30LWPC (with 15 and 30 g/100 mL LWPC incorporation respectively); [2] 9-C (conventional) and 9-30LWPC (with 30 g/100 mL LWPC incorporation). Different letters indicate significant differences ($p<0.05$). [a, b] Small letters: differences amongst each texture parameter for medium fat yogurts. [A, B] Capital letters: differences amongst each texture parameter for full fat yogurts. ($p < 0.05$ indicates that factors produce statistically significant differences in dependent variables).

Figure 4.3 compares the syneresis of the different types of yogurts during storage. A significant reduction in syneresis, which increased during storage, was evident with the use of LWPC in both medium and full-fat yogurts (Table 4.7). The syneresis indexes obtained in this study for full-fat yogurts (9-C and 9-30LWPC) are significantly lower than those reported by Guzmán-González et al. (1999), Sodini et al. (2005, 2006) and Li and Guo (2006). Only the values obtained for medium-fat yogurts (1.5-C, 1.5-15LWPC, and 1.5-30LWPC) are in the same order of magnitude as those presented by Li and Guo (2006). The wide range of syneresis outcomes found in the literature may result from two types of factors: those related to the evaluation method and those related to operating conditions during yogurt production. With regard to the first factor, Amatayakul et al. (2006) concluded that determining the syneresis index by different methods leads to completely different values and therefore also assesses different behaviours. Whereas the siphon method evaluates the spontaneous separation of whey on the surface of the gel (Lucey et al., 1998), the drainage and centrifugal methods evaluate the separation of whey from gels that may have suffered partial or total breakdown of the solid structure due to the action of gravitational and centrifugal forces respectively (Harwalkar and Kalab, 1986; Guzmán-González et al., 1999). The other factor is related to yogurt manufacture, including the various procedures or operational parameters adopted during production (temperature, pH and agitation), the type of yogurt (set or liquid), the origin of the milk base (cow, sheep or goat) (Salaün et al., 2005), embedded products (milk powder, whey protein concentrates), the form in which they are incorporated (powder, liquid, with heat denaturation prior or subsequent to the milk mixture) (Lee and Lucey, 2010), and the biochemical composition of the formulations (total solids, protein content, and presence of polysaccharides) (Shah, 2003).

The values for yogurt syneresis can be observed in Figure 4.3. As previously mentioned, full-fat yogurts presented the lowest syneresis values (0.7% to 6.3%), despite lower amounts of protein (3.21 and 3.58 g/100 g). This can be explained by the higher total solids content (18 g/100 g on average), which is in the same order of magnitude as the yogurts produced by Cais-Sokolinska and Pikul (2006), Li and Guo (2006), for fortified yogurt with powdered milk, and Gomes (2010) for creamy yogurts. This observation is also supported by data from Amatayakul et al. (2006) and Jaros et al. (2002), who observed a reduction in syneresis in line with an increase in total solids, indicating that above certain levels the influence of other factors on this parameter is no longer so obvious, particularly with regard to the quantity, shape, and type of protein added to the yogurt.

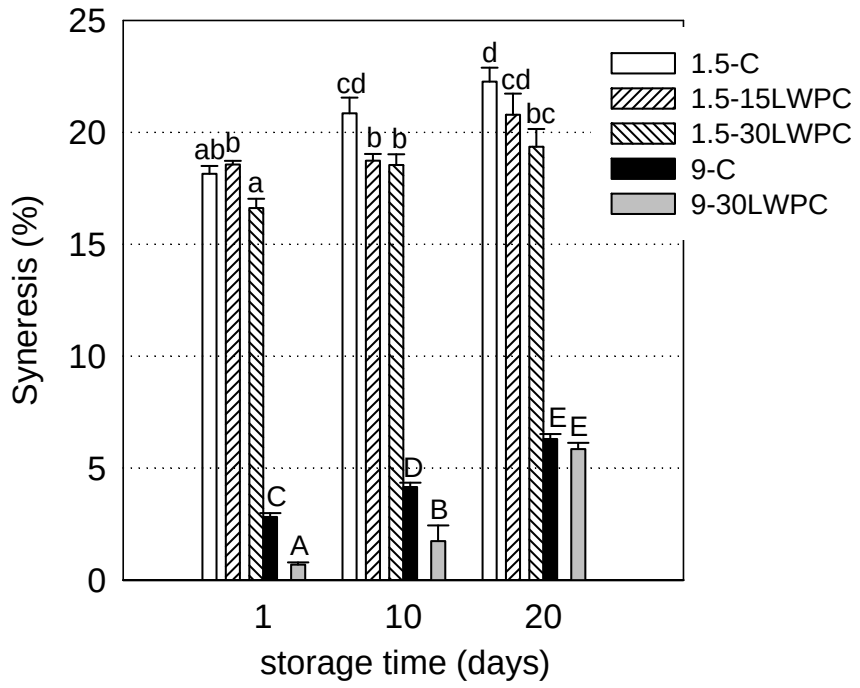


Figure 4.3 Yogurt syneresis during storage. Medium-fat yogurts: 1.5-C (conventional), 1.5-15LWPC and 1.5-30LWPC (with 15 and 30 g/100 g LWPC incorporation respectively); full-fat yogurts: 9-C (conventional) and 9-30LWPC (with 30 g/100 g LWPC incorporation). Different letters indicate significant differences ($p < 0.05$). Small letters: differences amongst syneresis for medium-fat yogurts. Capital letters: differences amongst syneresis for full-fat yogurts.

The small increase in total protein content in LWPC enriched yogurts (Table 4.4) may be responsible for the decrease in syneresis. However, it may not be exclusively responsible for these differences, since the total protein content of the medium-fat yogurts (4.82 to 5.43 g/100 g) tested in this research is similar to the values used in previous studies which showed higher syneresis (Guzmán-González et al., 1999; Katsiari et al., 2002; Amatayakul et al., 2006; Sodini et al., 2006). Li and Guo (2006) mention the excellent water retention properties of whey proteins when denatured prior to their addition to milk. They noticed that in this case, the bonds between denatured whey proteins and caseins are more effective, promoting the formation of a protein network with smaller pores and a greater ability to reduce syneresis.

It is believed that the main reasons for the different behaviours are, firstly, the level of total solids, followed by the protein concentration and ratio between the different protein types (casein and whey protein) present in the formulations and, finally, the form of whey protein added to the milk base (denatured or otherwise). Yogurt viscosity (Figure 4.4) increased with the fat content and the amount of LWPC incorporated (Table 4.7). However, at the end of the storage time (20th

day) no statistical differences were observed in the viscosity of yogurts containing 1.5 g of fat/100 g (with or without LWPC). In the case of creamy yogurts, the differences in viscosity between the conventional and tested products observed on the tenth day remained until the end of the storage time.

These results disagree with those presented by Modler and Kalab (1983) and Sodini et al. (2005), who concluded that enriched products (with milk powder) or with higher levels of casein tend to produce more viscous gels with a higher water retention capacity than products fortified with whey protein. It is important to note that in these cases the whey protein was incorporated in powder form and protein denaturation occurred in the presence of milk caseins during yogurt pasteurisation. As already stated, Li and Guo (2006) found that previously denatured whey proteins (such as LWPC) added to milk caseins favour the formation of bridges between them, leading to a narrow-pored mixed casein/whey protein network, thus resulting in improved viscosity, consistency, and lower syneresis (Lee and Lucey, 2010). Hence, it may be concluded that replacing WPC in powder form with LWPC is a less expensive alternative which simultaneously reduces syneresis and increases yogurt viscosity.

No sensorial differences were detected between the conventional and modified yogurts in the two types of products (medium-fat and full-fat). These results also indicate that the incorporation of LWPC at levels lower than 30 g/100 g is a suitable way of increasing the overall process yield without the consumer being aware of any differences in formulation.

Table 4.7 p values of Two-Way ANOVA with factors interaction, for syneresis and viscosity in yogurts.

Factors	Dependent variables	
	Syneresis	**Viscosity**
Medium fat yogurts		
Storage time (ST)	*0.000*	*0.000*
LWPC incorporation	*0.000*	*0.004*
ST×LWPC incorporation	*0.011*	*0.000*
Full fat yogurts		
Storage time (ST)	*0.000*	*0.003*
LWPC incorporation	*0.004*	*0.000*
ST×LWPC incorporation	*0.000*	*0.016*

($p < 0.05$ indicates that factors produce statistical significant differences in dependent variables).

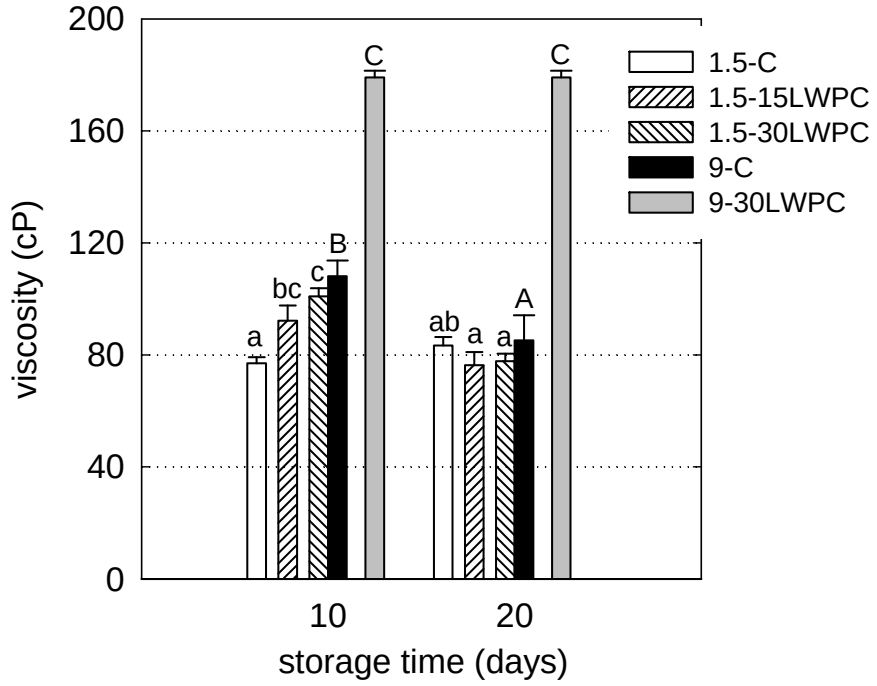


Figure 4.4 Yogurt viscosity during storage. Medium-fat yogurts: 1.5-C (conventional), 1.5-15LWPC and 1.5-30LWPC (with 15 and 30 g/100 g LWPC incorporation respectively); full-fat yogurts: 9-C (conventional) and 9-30LWPC (with 30 g/100 g LWPC incorporation). Different letters indicate significant differences ($p < 0.05$). Small letters: differences amongst viscosity for medium-fat yogurts. Capital letters: differences amongst viscosity for full-fat yogurts.

4.4 Conclusions

It was concluded that incorporating LWPC in dairy products may be very interesting, not only in terms of the overall process yield but also the functional properties of the products. Fresh cheese with LWPCs showed lower spontaneous syneresis and greater stability over time. This research also showed that the amount of LWPC incorporated into fresh cheese formulations is a very important parameter in terms of sensorial evaluation. Although no statistical differences ($p < 0.05$) were detected in the textural properties (hardness, chewiness and gumminess) of the conventional and innovative products, higher amounts of LWPCs (50 g/100 mL) reduced their acceptability according to the panellists.

The fat content in yogurts influenced the functional LWPC performance in formulations. In medium-fat yogurts the use of LWPC did not produce any significant differences in the colour

(L*, a*) and texture parameters, either over time or in the different formulations. However, increasing LWPC in full-fat yogurts reduced hardness and gumminess. In both types of yogurts, syneresis increased during storage and decreased with the incorporation of LWPC, presenting very low values for full-fat yogurts. Viscosity was improved with the addition of LWPCs especially in full-fat products. In the case of yogurts, no sensorial differences were detected between the conventional and tested products.

The lower production costs and operational complexity involved in the manufacture of LWPCs, as well as their effect on the functional properties of fresh cheese and yogurt, enables them to be used in small to medium-sized dairy production plants. This avoids or reduces the need to acquire other conventional dry products such as skimmed or whole milk powder or WPC traditionally used in the manufacture of these dairy products.

References

Abrahamsen RK (1979) Cheese making from milk fortified with ultrafiltrated whey protein concentrate. Milchwissenschaft, 34(29), 1-4.

Amatayakul T, Sherkat and Shah NP (2006) Syneresis in set yogurt as affected by EPS starter cultures and levels of solids. International Journal of Dairy Technology, 59, 216-221.

AOAC (1997) Official Methods of Analysis of Association of Official Analytical Chemists. 16th ed. Volume II. 33 Dairy Products USA.

Baldwin KA, Baer RJ, Parsons JG, Seas SW, Spurgeon KR and Torrey JS (1986) Evaluation of yield and quality of cheddar cheese manufactured from milk with added whey protein concentrate. Journal of Dairy Science, 69, 2543-2550.

Banks JM and Muir DD (1985) Effect of incorporation of denatured whey protein on the yield and quality of cheddar cheese. Journal Society of Dairy Technology, 38, 27-32.

Cais-Sokolinska D and Picul J (2006) Use of colour measurement to evaluate yoghurt quality during storage. Italian Journal of Food Science, 1(18), 63-71.

Damin MR, Alcântara MR, Nunes AP and Oliveira MN (2009) Effects of milk supplementation with skim milk powder, whey protein concentrate and sodium caseinate on acidification kinetics, rheological properties and structure of nonfat stirred yogurt. LWT - Food Science and Technology, 42, 1744-1750.

Díaz O, Pereira CD and Cobos A (2009) Aplicaciones de los concentrados y aislados de proteínas de lactosuero de la industria alimentaria. Alimentaria - Investigacion, tecnologia e seguridad, 400, 108-115.

Gauche C (2007) Polimerização de proteínas do soro de leite por transglutaminase e propriedades físicas de iogurte elaborado após tratamento enzimático. Pós-Graduação em Ciência dos Alimentos. Florianópolis, Brasil.

Gomes D (2010) Incorporação de concentrados líquidos de proteínas de soro em produtos lácteos. Escola Superior Agrária de Coimbra. Master thesis, College of Agriculture - Polytechnic Institute of Coimbra, Coimbra, Portugal.

Guzmán-González M, Morais F, Ramos M and Amigo L (1999) Influence of skimmed milk concentrate replacement by dry dairy products in a low fat set-type yoghurt model system. I: Use of whey protein concentrates, milk protein concentrates and skimmed milk powder. Journal of the Science of Food and Agriculture, 79, 1117-1122.

Harwalkar VR and Kalab M (1986) Relationship between microstructure and susceptibility to whey separation in yoghurt made from reconstituted nonfat dry milk. Food Microstructure, 5, 287-294.

Herrero AM and Requena T (2006) The effect of supplementing goat's milk with whey protein concentrate on textural properties of set-type yoghurt. International Journal of Food Science and Technology, 41, 87-92.

Hill T and Lewicki P (2007) Statistics Methods and Applications. StatSoft, Tulsa, OK.

Hinrichs J (2001) Incorporation of whey proteins in cheese. International Dairy Journal, 11, 495-503.

IPQ (1990) Leite e Produtos lácteos. Edição Normas Portuguesas, Lisboa, Portugal.

ISO 8586-2:1994 Sensory analysis – General guidance for the selection, training and monitoring of assessors (Part 2: experts). 1st edition.

Jameson GW and Lelievre J (1996) Effects of whey proteins on cheese characteristics. Bulletin of the IDF, 313, 3-8.

Jaros D, Rohm H, Haque A and Kneifel W (2002) Influence of the starter cultures on the relationship between dry matter content and physical properties of set-style yogurt. Milchwissenschaft, 57, 325-326.

Jorge C, Lobete N and Pereira C (2006) Adição de proteínas de soro desnaturadas no fabrico de queijo. Leite I+D+T. N.º 0, 2-4.

Kailasapathy K, Supriadi D and Hourigan JA (1996) Effect of partially replacing skim milk powder with whey protein concentrate on buffering capacity of yoghurt. Australian Journal of Dairy Technology, 51, 89–93.

Katsiari MC, Voutsinas LP and Kondyli E (2002) Manufacture of yoghurt from stored frozen sheep's milk. Food Chemistry, 77, 413-420.

Korolczuk J and Mahaut M (1991a) Effect of whey proteins and heat treatment of milk on the consistency of UF fresh cheese. Milchwissenschaft, 46, 435-437.

Korolczuk J and Mahaut M (1991b) Consistency of acid fresh cheese. Role of whey proteins. Milchwissenschaft, 46, 153-156.

Lebeuf Y, Lacroix C and Paquin P (1998) Effet de l'incorporation des protéines du lactosérum dénaturées et microparticules dans le cheddar jeune. Le Lait, 78 (3), 303-318.

Lee WJ and Lucey JA (2010) Formation and Physical Properties of Yogurt. Asian-Australasian Journal of Animal Sciences, 23(9), 1127-1136.

Li J and Guo M (2006) Effects of polymerized whey proteins on consistency and water-holding properties of goat's milk yogurt. Journal of Food Science, 71(1), 34-38.

Lucey JA (2002) Formation and Physical Properties of Milk Protein Gels. Journal of Dairy Science, 85, 281-294.

Lucey JA (2004) Cultured dairy products: an overview of their gelation and texture properties. International Journal Dairy Technology, 57, 77-84.

Lucey JA, Munro PA and Singh H (1998) Whey separation in acid milk gels made with glucono-delta-lactone: effects of heat treatment and gelation temperature. Journal of Texture Studies, 29, 413-426.

Mahaut M and Korolczuk J (1992) Effect of whey protein addition and heat treatment of milk on the viscosity of UF fresh cheese. Milchwissenschaft, 47, 157-159.

Modler HW and Kalab M (1983) Microstructure of yogurt stabilized with milk proteins. Journal of Dairy Science, 63, 430-437.

NP 1923:1987 Iogurte Determinação do teor de matéria gorda Técnica de Gerber. Processo corrente. Comissão Técnica - 32, 1ª edição, Portugal.

NP 2105:1983 Queijos. Determinação do teor de matéria gorda. Técnica de Van Gulik. Processo corrente. Comissão Técnica - 32, 1ª edição, Portugal.

NP 3544:1987 Queijos e queijos fundidos. Determinação do resíduo seco e do resíduo seco isento de matéria gorda. Comissão Técnica - 32, 1ª edição, Portugal.

NP 4146:1991 Leite e produtos lácteos. Métodos de colheita de amostras. Comissão Técnica – 32, 1ª edição, Portugal.

NP 469:2002 Leites. Determinação da matéria gorda (técnica de Gerber). Processo corrente. Comissão Técnica - 32, 3ª edição, Portugal.

NP 580:1970 Leite. Determinação do resíduo seco e do resíduo seco isento de matéria gorda. Processo corrente. Comissão Técnica - 32, 1ª edição, Portugal.

NP 701:1982 Iogurtes. Determinação da acidez. Comissão Técnica - 32, 2ª edição, Portugal.

NP 703:1982 Iogurtes. Determinação do resíduo seco e resíduo seco isento de matéria gorda. Comissão Técnica - 32, 2ª edição, Portugal.

Pérez-Munuera ME and Lluch MA (1999) Study of some typical Spanish cheeses by scanning electron microscopy. Main microstructural modifications caused by freezing. Food Science and Technology International, 5(6), 515-521.

Phadungath C (2010) Basic Measurements for food texture. Available at: http://science.srru.ac.th/org/food_sci/result_civil_file/BasicMeasurementforFoodTexture.pdf. Accessed 16 August 2011.

Punidadas P, Feirtag J and Tung MA (1999) Incorporating whey proteins into mozzarella cheese. International Journal of Dairy Technology, 52, 51-55.

Salaün F, Mietton B and Gaucheron F (2005) Buffering capacity of dairy products. International Dairy Journal, 15, 95-109.

Shah N P (2003) Yogurt: The product and its manufacture. In: Caballero B, Trugo LC and Finglas PM (ed) Encyclopedia of Food Science and Nutrition. vol. 10, 2nd ed. London: Academic Press, London.

Smithers GW, Ballard FJ, Copeland AD, de Silva KJ, Dionysius DA, Francis GL, Goddard C, Grieve PA, Graeme HM, Mitchel IR, Pearce RJ and Regester GO (1996) New opportunities from the isolation and utilization of whey proteins. Journal of Dairy Science, 79, 1454-1459.

Sodini I, Mattas J and Tong PS (2006) Influence of pH and heat treatment of whey on the functional properties of whey protein concentrates in yoghurt. International Dairy Journal, 16, 1464-1469.

Sodini I, Montella J and Tong PS (2005) Physical properties of yogurt fortified with various commercial whey protein concentrates. Journal of the Science of Food and Agriculture, 85, 853-859.

Tamine AY and Robinson RK (1999) Yoghurt: science and technology. 2nd ed., FL:CRC Press, Boca Raton.

Walstra P and vanVliet T (1991) On the fractal nature of particle gels. In: Dickinson E (Ed.) Food polymers, gells and colloids. Royal Society of Chemistry, London.

Chapter 5

Performance of bovine and ovine LWPC: functional properties of set yogurts

Abstract

The effects of liquid whey protein concentrates (LWPC) on the biochemical, physical and sensorial properties of set yogurt were studied. Bovine and ovine LWPC were used to partially replace skimmed milk powder (SMP) in bovine yogurt formulations. The properties of the modified yogurts were evaluated during their shelf life and compared with conventional bovine and ovine yogurts. The protein content of ovine yogurt differed significantly ($p < 0.05$) from the bovine yogurts (with or without LWPC supplementation). Higher values for hardness, adhesiveness and gumminess were observed in the conventional yogurts, although cohesiveness, resilience and springiness did not vary between formulations. During their shelf life a decrease in

the luminosity of the products was observed, but there were no significant differences in colour between the formulations. Low syneresis indexes, ranging from 0.5% to 5.0%, which are typical in the range of yogurts with high levels of solids, were obtained for the yogurts produced. The decrease in viscosity led to an increase in syneresis, indicating that the gel structure was more open and did not retain water so efficiently. Ovine yogurts showed lower syneresis and higher viscosity values, whereas the yogurts enriched with LWPC showed the opposite. On a sensory level, no differences ($p < 0.001$) were found between conventional bovine yogurt and yogurts with added LWPC. However, in the case of ovine yogurt (LO) significant differences were identified, and this product was strongly penalised in the preference test. The results revealed that LWPC (regardless of the source) can be used in set yogurt formulations, increasing protein and total solids content by total or partial replacement of the conventional adjuvant (SMP - skimmed milk powder). The use of these products is very attractive, given that they do not require very complex processing conditions, production costs are lower and whey disposal is more effective.

5.1 Introduction

The European Commission has been working on a draft directive (AGRI/38743/2003) to establish quality standards and requirements for the composition, labelling and control of yogurt and yogurt-like products. Although this directive is still in the draft stages, the Codex Alimentarius is part of European Commission legislation and the Codex Standard for fermented milks (Codex Stan 243-2003) serves to regulate the markets, facilitating trade between EU Member States and non-EU countries importing their products into the Union. Currently, each member state has its own standards for products labelled as "yogurt", such as the Real Decreto 179/2003 in Spain or Décret No 88-1203 in France. In Portuguese legislation (Portaria No 742, 1992), yogurt is the food product obtained from the fermentation of milk or milk derivatives using only *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. This specific microflora should be viable and abundant in the final product ($\geq 10^7$/g of product). Some facultative ingredients are permitted, such as milk powder (whole, partially or totally skimmed), buttermilk, concentrated whey, whey powder, whey protein concentrates, milk proteins, sugars and sweeteners.

The conventional and commercial methods used to improve the texture of yogurts consist of increasing the solids content in milk (mostly proteins) or, in the case of stirred yogurts, the application of stabilisers such as pectin (Lucey and Singh (1998), cited by Li and Gou, 2006).

Increasing the protein content in yogurts involves fortifying the milk, either by concentration (using evaporation or ultrafiltration) or the addition of skimmed milk powder (SMP). More recently, whey protein concentrate (WPC) has also been used, due to its availability and low cost. Although WPC is often applied as an attractive food ingredient in a wide range of food applications, the direct reincorporation of liquid whey protein concentrates (LWPC) in dairy products, as a less expensive alternative, is seldom discussed. The effect on textural and physicochemical properties of yogurts of replacing SMP with WPC has been studied by several authors (Sodini et al., 2005; Amatayakul et al., 2006; Sodini et al., 2006; Damin et al., 2009), but in some cases their conclusions were contradictory. The reasons cited for this are the significant variations in the functionality of WPC as a result of the whey processing conditions, especially heating (de Wit and Klarenbeek, 1984; Mangino et al., 1987; de la Fuente et al., 2002; Pelegrine and Gasparetto, 2005; López-Fandiño, 2006; Nicorescu et al., 2008), and the whey source (Mangino et al., 1987; Casper et al., 1999; Ji and Haque; 2003). In Portugal, bovine and ovine cheese production totals approximately 60×10^3 and 15×10^3 ton/year respectively (Agrotec, 2009; INE, 2010 and 2012), and the latter product is normally associated with Protected Geographical Indication labels (MADRP, 2007). Based on the figures for cheese production, the overall volume of whey produced annually is estimated to be approximately 660 000 tones. In Mediterranean countries as well as in Portugal, the majority of producers are micro, small and medium-scale dairy industries (MADRP, 2007) who simultaneously face environmental problems relating to whey disposal, difficulty in succeeding in the market due to the fact that they specialise in just one product, and low production yields. The use of membrane technologies, namely ultrafiltration (UF) and diafiltration (DF), enables whey proteins to be extracted and concentrated from whey for reincorporation in production, thus solving the environmental problems and adding value to existing products.

No information is available on LWPC functionality in yogurt. The aim of this research was to evaluate the effects of partially substituting SMP with LWPC of bovine and ovine origin on the physicochemical, textural, rheological and sensorial properties of set yogurts, and to test the acceptability of ovine yogurt as an alternative product in Portugal.

5.2 Materials and methods

5.2.1 Materials

Bovine and ovine whole milk was supplied by a local dairy industry, pasteurised (91-92ºC for 25-30 seconds), cooled to 4 ºC and stored until the yogurts were formulated and produced. Ovine whole milk only, from Bordaleira Serra da Estrela and Churra Mondegueira breeds, was skimmed in a Westfalia™ separator type ADB in order to reduce its fat content prior to pasteurisation and storage. Cream was used to normalise the fat content in the final yogurt formulations. Skimmed milk powder (SMP) was supplied by Enzilab (Portugal). Bovine and ovine cheese whey was supplied by Queijaria Serqueijos SA (Portugal) and Queijaria Flor da Beira SA (Portugal), respectively.

5.2.2 LWPC manufacture

Bovine and ovine cheese whey obtained immediately after production was transported to the pilot plant at the Escola Superior Agrária de Coimbra in 50 L jars. On reception, the whey was filtered, analysed and processed. LWPC was produced by whey concentration at 24-30 ºC in a batch ultrafiltration pilot plant, using an organic membrane DSS™ 20K 3838-30 model, with a 5.5 m^2 installation area and a 20 kDa cut-off. After concentration, the retentate was submitted to thermal treatment (90 ºC/60 s) to precipitate the denatured whey proteins. The mixture was then homogenised at 100 bar to achieve a particle diameter lower than 10 μm in order to avoid disturbance of the casein matrix (Walstra and van Vliet, 1991; Lebeuf et al., 1998). The products obtained were termed LWPCb and LWPCo for bovine and ovine whey origins respectively. Before it was incorporated into the milk batches for yogurt production, the LWPC was analysed and frozen at -15 ºC.

5.2.3 Set yogurt manufacture

Four yogurt formulations (Table 5.1) with 16% total solids were produced. Conventional ovine yogurts (LO) were produced exclusively with ovine skimmed milk. The fat content of the bovine milk formulations was normalised with cream, and the protein content was adjusted using, respectively: (i) SMP (conventional bovine yogurt (LB)); (ii) 7.3% of bovine LWPC + 4.4% of SMP (LB-LWPCb) and (iii) 7.3% of ovine LWPC + 4.8% of SMP (LB-LWPCo). The different incorporation levels for bovine milk, SMP and LWPC were calculated by considering their gross

composition (Table 5.2) in order to obtain similar fat and protein contents in the yogurts produced.

Table 5.1 Set yogurt formulations.

Composition (% w/w)	Formulations[1]			
	LO	LB	LB-LWPCo	LB-LWPCb
Ovine skimmed milk	100.0	-	-	-
Bovine milk	-	93.8	86.7	88.0
Skimmed milk powder (SMP)	-	5.0	4.8	4.4
Cream	-	1.2	1.2	0.3
LWPCb	-	-	-	7.3
LWPCo	-	-	7.3	-

[1] LO and LB: conventional ovine and bovine yogurt; LB-LWPCo and LB-LWPCb: yogurts with ovine and bovine LWPC incorporation, respectively.

All the ingredients for each formulation were mixed, homogenised at 200 bar and pasteurised at 92 ºC/30 min. Before filling and packaging, the mixture was stirred for 20 min at 43 ºC and inoculated with a mixed culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Ezal YO-MIX 601). The fermentation step was performed in 50 mL polystyrene cups at a constant temperature of 43±1 ºC until the yogurt pH reached 4.6±1. The yogurts were then stored at 4±2 ºC. After one day of cool storage the biochemical composition and functional properties of some yogurt samples were evaluated, whilst the remaining samples were evaluated on the 7th, 14th and 21st day.

5.2.4 Chemical analyses of yogurt base ingredients and set yogurts

The biochemical composition (pH, titrable acidity (TA), total solids, ash, fat and protein) of milk, skimmed milk powder, cream, whey, LWPC and yogurt was evaluated using the Portuguese Standards Methods (IPQ, 1990) and the Official Analytical Methods (AOAC, 2005). Each product sample was collected using the Portuguese specific standard procedure for dairy products (NP 4146:1991). Three random samples were selected for set yogurt analyses.

5.2.5 Physical and sensorial evaluation of yogurts

Yogurt colour was determined with a Minolta Chroma Meter colorimeter, model CR-200B, using the L*a*b* CIELAB system. The syneresis index followed the method described by Gauche (2007). Yogurt viscosity was evaluated over 10 min (at 2 min intervals) in a rotational Brookfield Viscometer, model DV II, with a concentric cylinder RV (spindle 3) at a constant angular velocity

(5 rpm). A Stable Micro Systems Texture Analyzer, model TA.XT Express Enhanced was used to perform textural analysis and the results were calculated using Specific Expression PC Software. In the case of refrigerated yogurt samples, a TPA was run with a penetration distance of 20 mm at 5 mm/s test speed, using an acrylic cylindrical probe with a diameter of 12.7 mm and a height of 35 mm. For sensorial analysis, preference tests and triangular tests were performed by untrained panels in order to detect differences between products with conventional formulations and those incorporating LWPC. The triangular tests were based on Binomial distribution with a confidence level at $p < 0.001$ (ISO 8586-2:1994). In the preference test, the panel evaluated yogurts in terms of their appearance, mouthfeel and flavour.

5.2.6 Statistical analysis

A statistical analysis of the data was carried out using the ANOVA package included in Statistica 8 software. Means were compared using the Tukey HSD test. Differences were considered significant at $p < 0.05$.

5.3 Results and discussion

5.3.1 Composition of yogurt base ingredients

The composition of yogurt base ingredients such as bovine and ovine milk, SMP, cream and liquid whey protein concentrates (LWPC) used for yogurt production is shown in Tables 5.2 and 5.3.

Characterisation of bovine and ovine milk, SMP and cream

The chemical composition (Table 5.2) of the ovine milk used in the production of set yogurts was typical of the Bordaleira Serra da Estrela and Churra Mondegueira breeds (Rodrigues et al., 2000) and the standard milk composition normally used in the manufacture of sheep's yogurt in the dairy industry. With regard to the amount of non-fat solids (SNF), protein and ash, these breeds produce a richer milk than the milk of the Boutsiko breed, widely used in the production of Greek yogurt (Katsiari et al., 2002) and also the average sheep milk composition reported by Pandya and Ghodke (2007). The purpose of standardising the fat content (3.5-4.0%) in ovine milk was to maintain a similar fat content to that of conventional bovine yogurts. The figures for the composition of bovine milk were lower than the average for cow's milk reported by Pandya and Ghodke (2007). These differences can be attributed to factors concerning production breed and seasonality.

Table 5.2 Gross chemical composition: total solids, fat, solids non-fat (SNF), ash, protein, lactose, titratable acidity (TA) and pH of bovine and ovine milk, skimmed milk powder (SMP) and cream used for yogurt formulations.

Composition (%)	Ovine milk[1,*]	Bovine milk[1]	SMP[1]	Cream
Total solids	16.17±0.00	11.65±0.06	91.90±0.45	50.59
Fat	3.70±0.10	3.39±0.00	0.13±0.06**	43.00
Solids non-fat (SNF)	12.47±0.06	8.26±0.03	91.77±0.26	7.59
Ash	1.03±0.003	0.67±0.01	7.60±0.04	nd
Protein	7.51±0.21	3.19±0.08	32.17±0.51	2.80
Lactose	3.92±0.21	4.39±0.08	4.39±0.08**	nd
Titratable acidity (% lactic acid)	0.282±0.005	0.185±0.003	0.167±0.005**	nd
pH	6.66±0.03	6.83±0.01	6.83±0.01**	nd

[1]average of three determinations. *fat standardized milk. **determined in a 10% (w/v) water solution. nd – not determined.

Characterisation of bovine and ovine LWPC

Table 5.3 shows the chemical composition of bovine and ovine whey and LWPC after ultrafiltration. The ovine whey was significantly ($p < 0.05$) richer than the bovine whey in all its components. The protein, mineral and fat contents (dry weight basis) in ovine whey (18.6%, 13% and 19.0%, as opposed to 12.8%, 7% and 11.2% in bovine whey) indicate that ovine products can be more attractive in terms of producing higher yields. In LWPC, it was observed that the ovine product presented lower amounts of all the components, except minerals. The reason for this is the volume concentration factor applied in the UF step, which was lower in this case (VCF = 13) than in the case of bovine LWPC production (VCF = 20). Even so, the figures for the protein content are still higher (36.0%, dry weight) than the bovine LWPC (31.3%).

Table 5.3 Gross chemical composition of bovine and ovine whey[1] and liquid whey protein concentrate (LWPC[1]): total solids, fat, protein, ash and titratable acidity (TA).

Composition (%)	Whey		LWPC	
	Bovine	Ovine	Bovine	Ovine
Total solids	6.92±0.04 a	7.60±0.02 b	19.53±0.20 b	14.09±0.02 a
Fat	0.78±0.00 a	1.45±0.00 b	7.82±0.001 b	4.06±0.002 a
Protein	0.89±0.00 a	1.41±0.01 b	6.12±0.24 b	5.09±0.05 a
Ash	0.50±0.02 a	1.01±0.01 b	0.61±0.004 a	0.93±0.01 b
TA (% lactic acid)	0.11±0.003 a	0.13±0.017 a	0.24±0.04 b	0.22±0.01 a

[1]means of two batches. a, b means within the same rows for the same product without the same superscript are significantly different ($p < 0.05$).

Tritratable acidity was also higher in the case of ovine whey. The main reason for this may be the type of compounds formed during enzymatic hydrolysis of casein by cardosin (*Cynara cardunculus*), normally used in the manufacture of Portuguese ovine cheeses, as opposed to the rennet extract (> 96% of quimosin) used in bovine cheese production. The TA reduction in ovine LWPC may be due to the higher buffer capacity of ovine proteins.

5.3.2 Composition and physicochemical properties of yogurt during storage

The biochemical composition of the four different types of yogurts prepared according to the formulations presented in Table 5.1 is shown in Table 5.2. The ovine yogurts (LO) only differed from the conventional bovine yogurts (LB) in terms of protein content. In this case, the amount of protein (6.06%) was significantly higher than in the bovine yogurts, but in the same order of magnitude as similar products (Katsiari et al., 2002).

Table 5.4 Gross chemical composition of yogurts: total solids, ash, fat, protein, titrable acidity (TA) and pH after production.

Composition (%)	Yogurt[1]			
	LO	LB	LB-LWPCo	LB-LWPCb
Total solids	15.97±0.06 b	16.03±0.001 b	15.52±0.06 a	15.58±0.01 a
Ash	1.04±0.06 ab	1.06±0.01 b	1.04±0.01 ab	0.97±0.03 a
Fat	3.40±0.00 a	3.53±0.23 ab	3.80±0.00 b	3.80±0.00 b
Protein	6.06±0.24 b	4.28±0.29 a	5.11±0.23 a	4.46±0.03 a
TA (% lactic acid)	1.16±0.03 c	1.04±0.02 b	0.99±0.01 a	1.04±0.00 b
pH	4.61	4.61	4.67	4.62

[1]LO and LB: conventional ovine and bovine yogurt; LB-LWPCo and LB-LWPCb: yogurts with ovine and bovine LWPC incorporation, respectively. a, b, c means within the same rows without the same superscript are significantly different ($p < 0.05$).

The TA value for the yogurts which incorporated ovine LWPC (LB-LWPCo) after manufacture was significantly lower ($p < 0.05$) than the other yogurt formulations. During storage, the TA increased in all products (Figure 5.1), although this was more pronounced in the conventional ones, especially LB, indicating that the higher buffer capacity of LWPC minimizes yogurt acidification during storage. Amatayakul et al. (2006) also reported that the higher amounts of solids (such as in conventional yogurts) available during fermentation could increase microbiological activity and consequently lead to a higher production of lactic acid. This increase in titratable acidity during storage agreed with the findings of Katsiari et al. (2002) for sheep's milk yogurts, and Abrahamsen and Holmen (1980, 1981) for cow's and goat's milk yogurts. It was also observed that conventional ovine yogurts (LO) had lower TA values than conventional

bovine yogurts (LB), demonstrating the higher buffer capacity of ovine proteins (Diáz et al., 2004).

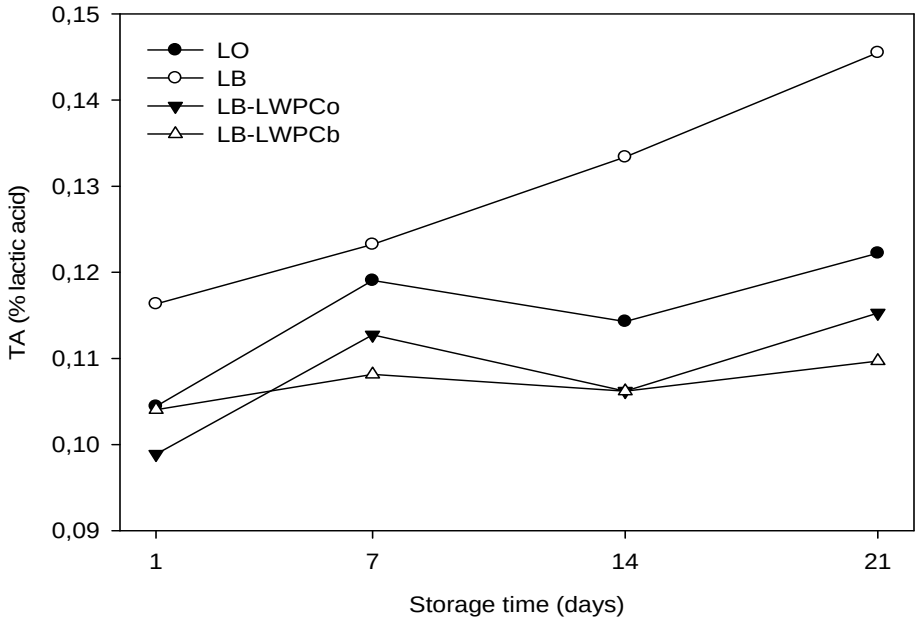


Figure 5.1 Titratable acidity (TA) of yogurts during storage. LO and LB: conventional ovine and bovine yogurt; LB-LWPCo and LB-LWPCb: yogurts with ovine and bovine LWPC incorporation respectively.

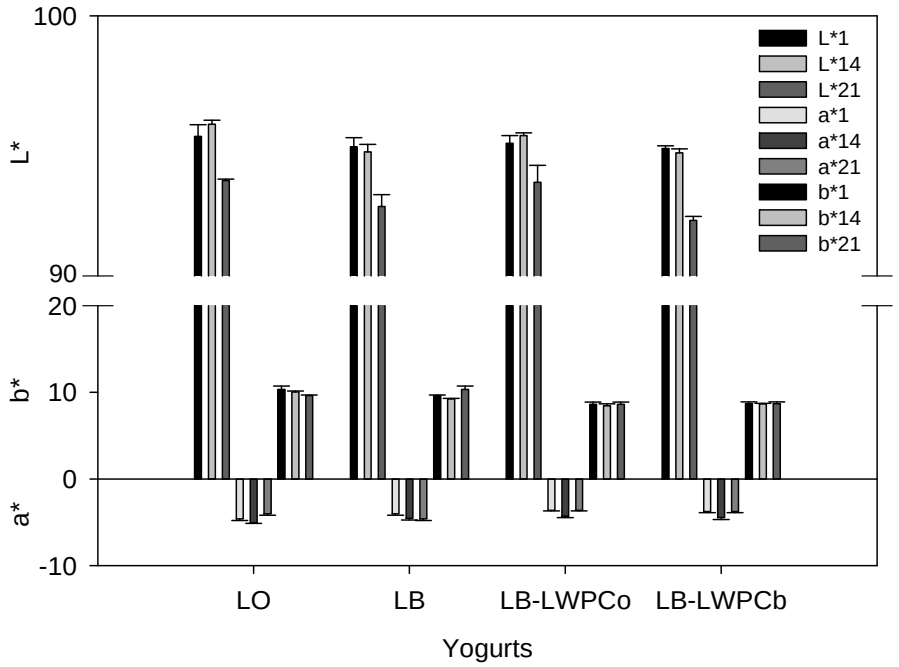


Figure 5.2 Colour coordinates L*, a* and b* of yogurts after 1, 14 and 21 days of storage. LO and LB: conventional ovine and bovine yogurt; LB-LWPCo and LB-LWPCb: yogurts with ovine and bovine LWPC incorporation respectively.

No significant differences in colour were found between LO and LB yogurts and those produced by incorporating LWPC. However, during storage the L* value decreased in all the formulations (Figure 5.2). These results are accord with the findings of Cais-Sokolinska and Pikul (2006) and Gomes (2010), who concluded that the luminosity of yogurts tends to decrease during storage.

The textural analysis did not differ during the shelf life of each type of formulation, neither between formulations for cohesiveness, springiness and resilience. The results for cohesiveness indicate that the nature and magnitude of yogurt cross-links remain the same regardless of the formulation. The yogurt springiness observed (near the unit) showed that yogurts recovered their initial position after the first deformation independently of the amount and type of proteins (whey proteins or caseins). Hardness, adhesiveness and gumminess were significantly higher ($p < 0.05$) in the ovine yogurts (LO), followed by the bovine yogurts (LB) and, finally, the products with LWPC (Figure 5.3, Figure 5.4 and Figure 5.5). These results were also observed by de Wit et al. (1986), who confirmed a decrease in hardness with the use of WPC with a high protein denaturation level. Other authors concluded that gels produced by incorporating previously denatured WPC in milk caseins resulted in less homogeneous gels with a more open structure than those produced by the denaturation of whey proteins in the presence of caseins (Schorsch et al., 2001). They suggested that the large whey protein aggregates produced during the pre-denaturation step cannot coat the caseins properly, thus penalising gel formation. The LWPC origin (bovine or ovine) did not influence the textural parameters. No differences were observed in texture in the different types of yogurts during storage. Hardness only increased significantly between the 1st and the 21st days of storage in the case of conventional ovine yogurt (LO) (Figure 5.3).

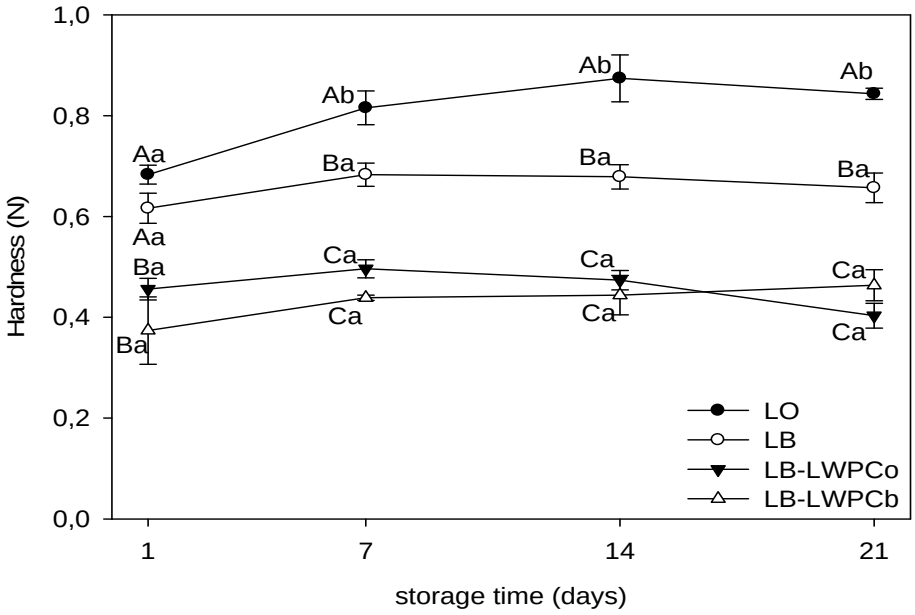


Figure 5.3 Yogurt hardness during storage. A, B, C means with different capital letters differ significantly ($p < 0.05$) between formulations at the same day of storage. a, b means with different small letters differ significantly ($p < 0.05$) during storage for each formulation.

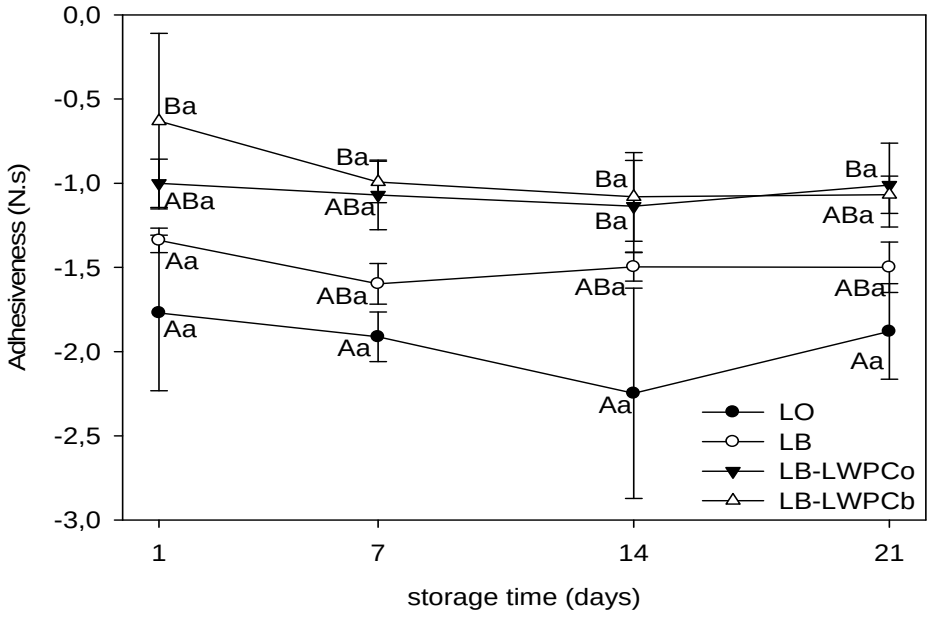


Figure 5.4 Yogurt adhesiveness during storage. A, B means with different capital letters differ significantly ($p < 0.05$) between formulations at the same day of storage. a, b means with different small letters differ significantly ($p < 0.05$) during storage for each formulation.

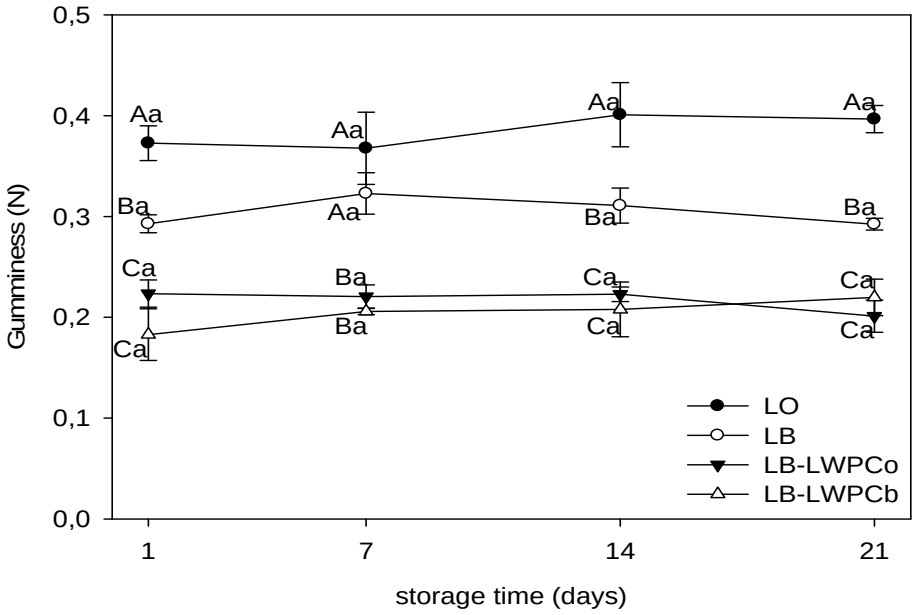


Figure 5.5 Yogurt gumminess during storage. A, B, C means with different capital letters differ significantly ($p < 0.05$) between formulations at the same day of storage. a, b means with different small letters differ significantly ($p < 0.05$) during storage for each formulation.

Figure 5.6 represents the relationship between the syneresis index and apparent yogurt viscosity in all the products tested. The high amount of solids (15.5-16.0%) in the formulations tested is partly responsible for the low syneresis indexes observed (0.5-5.0%). However, the use of LWPC decreased the water holding capacity by increasing yogurt syneresis. This behaviour can be explained by the nature and proportion of proteins in each formulation and the way in which they were incorporated (as previously noted, mainly caseins in conventional products and denatured whey proteins in tested ones). Yogurt viscosity varied in inverse proportion to syneresis, enabling three distinct groups of products to be identified (Figure 5.6). The first group (I) includes ovine yogurts (LO) and is characterised by low syneresis values and higher viscosity. This behaviour can be explained by their higher protein concentration and therefore the possibility of building a more cohesive polymer network. Set yogurts prepared with LWPC (LB-LWPCo and LB-LWPCb) showed the lowest values for viscosity and the highest syneresis index (group II). The final group (III) includes conventional bovine yogurts (LB), which presented intermediate values for both parameters.

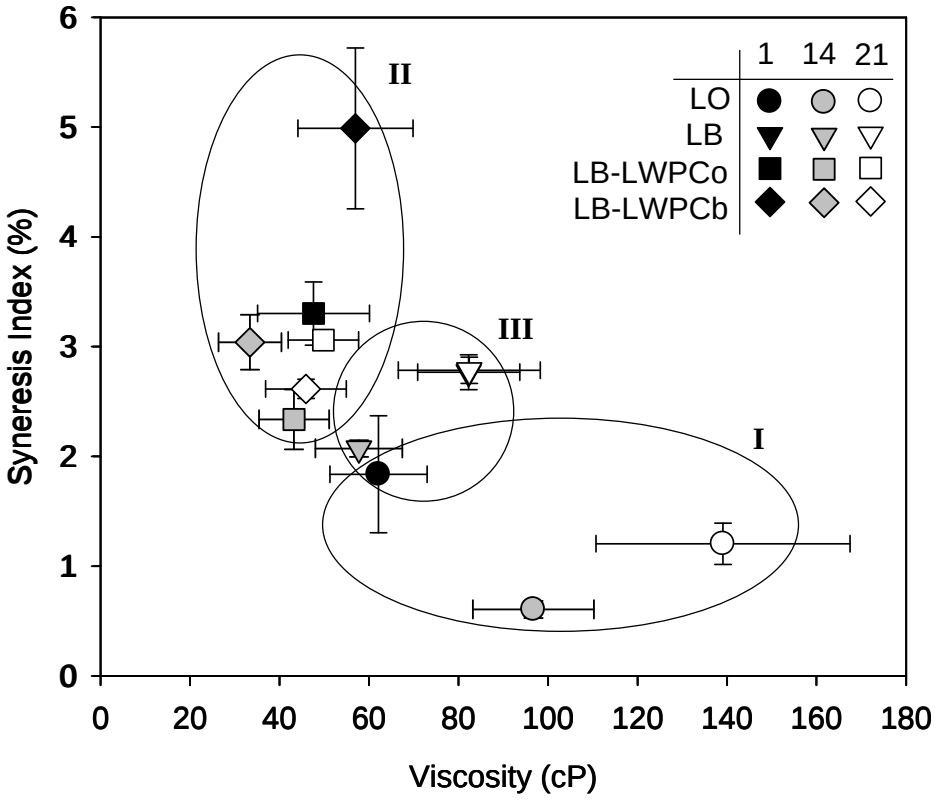


Figure 5.6 Set yogurt syneresis index (%) vs apparent viscosity (cP) after 1, 14 and 21 days of storage. LO and LB: conventional ovine and bovine yogurt; LB-LWPCo and LB-LWPCb: yogurts with ovine and bovine LWPC incorporation respectively. I - yogurts with low syneresis and high viscosity; II - yogurts with high syneresis and low viscosity: III - yogurts with intermediate syneresis and viscosity.

Although the total protein content does not differ significantly in the two latter groups of yogurts (group II and III) (Table 5.4), the proportion of caseins and whey proteins in the formulations was different (Table 5.1). As reported by Guzmán-González et al. (1999), these differences are responsible for the decrease in viscosity and increase in syneresis in yogurts enriched with WPC, which therefore have higher ratios of denatured whey proteins. The results of this research also agree the data published by Modler et al. (1983) and Sodini et al. (2005), who concluded that products enriched with SMP or higher casein contents tend to produce more compact and more viscous gels with a higher water retention capacity than those enriched with whey proteins. No particular trend in either property was observed during storage.

Despite the similar biochemical composition of LO and LB yogurts the sensory panel distinguished between ($p < 0.001$) both products. However, no significant differences were found in LB based products (Table 5.5). The yogurts that incorporated LWPC (LB-LWPCo and

LB-LWPCb) were preferred and the ovine yogurts (LO) less appreciated. These results showed that the yogurts with textural properties, viscosity and syneresis closer to conventional products (LB) were appreciated more. A lack of familiarity with ovine yogurts on the part of Portuguese consumers reflects the fact that these products were less well accepted. Despite their classification as homogeneous and uniform in terms of appearance, with a creamy and velvety mouthfeel and absence of any characteristic smell or flavour, the panel members stated that they had a thick, pasty texture and recommended that they could be used as a dessert. The panellists were unanimous in their characterisation of bovine yogurts with or without LWPC, citing their homogeneity, lightness and uniformity.

Table 5.5 Set yogurt sensorial tests.

Tests	Triangular test		Preference test (preference percentage)
	right answers	result ($p < 0.001$)	
LO/LB	16/19	differ	LB (73.7%)
LB/LB-LWPCb	7/19	do not differ	LB-LWPCb (42.0%)
LB/LB-LWPCo	4/16	do not differ	LB (50.0%)
LO/LB-LWPCb	12/16	differ	LB-LWPCb (87.5%)
LO/LB-LWPCo	14/16	differ	LB-LWPCo (81.3%)

LO and LB: conventional ovine and bovine yogurt; LB-LWPCo and LB-LWPCb: yogurts with ovine and bovine LWPC incorporation respectively.

5.4 Conclusions

It was concluded that conventional SMP can be partially replaced by LWPC (regardless of its origin) in set yogurts and that this can be very attractive, not only in terms of the overall process yield for micro, small or medium-sized dairy industries, reducing effluents and adding value to existing products, but also with regard to their functional properties. Further work envisages optimising the LWPC denaturation step, as well as improving the sensory properties of ovine milk yogurts.

References

Abrahamsen RK and Holmen TB (1981) Goat's milk yogurt made from non-homogenized and homogenized milks, concentrated by different methods. Journal of Dairy Research, 48, 457-463.

Abrahamsen, RK and Holmen TB (1980) Yogurt from hyperfiltrated, ultafiltrated and evaporated milk and from milk with added milk powder. Milchwissenschaft, 35, 399-402.

AGRI/38743/2003 Proposal for a Council Regulation laying down quality characteristics and requirements for the composition, labelling and control of yogurt and yogurt-like products. Commission of the European Communities, 3.7.2003, Brussels.

Agrotec (2009) Desafio da competitividade na fileira do leite. Anil – Feira Internacional de Lisboa, 6 de Março de 2009. [Consult. 22 Fevereiro 2011]. <http://www.anilact.com/documentos/agrotec09mcm>.

Amatayakul T, Sherkat F and Shan NP (2006) Syneresis in set yogurt as affected by EPS starter cultures and levels of solids. International Journal of Dairy Technology, 59, 216-221.

AOAC (2005) Official Methods of Analysis of Association of Official Analytical Chemists. 18th ed. Volume II, 33, Dairy Products, Published by AOAC International, Gaithersburg, Maryland, USA.

Cais-Sokolinska D and Pikul J (2006) Use of color measurement to evaluate yogurt quality during storage. Italian Journal of Food Science, 1(18), 63-71.

Casper JL, Wendorff WL and Thomas DL (1999) Functional Properties of Whey Protein Concentrates from Caprine and Ovine Specialty Cheese Wheys. Journal Dairy Science, 82, 265-271.

Codex Stan 243-2003. Codex Standard for Fermented Milks. Milk and milk products 2nd edition.

Codex Alimentarius - International Food Standards (2012) World Health Organization / Food and Agriculture Organization of the United Nations. <http://www.codexalimentarius.org>

Damin MR, Alcântara MR, Nunes AP and Oliveira MN (2009) Effects of milk supplementation with skim milk powder, whey protein concentrate and sodium caseinate on acidification kinetics, rheological properties and structure of nonfat stirred yogurt. LWT - Food Science and Technology, 42, 1744-1750.

de la Fuente MA, Hemar Y, Tamehana M, Munro PA and Singh H (2002) Process-induced changes in whey proteins during the manufacture of whey protein concentrates. International Dairy Journal, 12, 361-369.

de Wit JN and Klarenbeek G (1984) Effects of Various Heat Treatments on Structure and Solubility of Whey Proteins. Journal Dairy Science, 67, 2701-2710.

de Wit JN, Klarenbeek G and Adamse M (1986) Evaluation and functional properties of whey protein concentrates and whey protein isolates. 2. Effects of processing history and composition. Netherland Milk Dairy Journal, 40, 41-56.

Décret nº 88-1203 du 30 Décembre 1988. Laits fermentés et au yaourt ou yoghourt. France.

Diáz O, Pereira CD and Cobos A (2004) Functional properties of ovine whey protein concentrates produced by membrane technology after clarification of cheese manufacture by-products. Food Hydrocolloids, 18(4), 601-610.

Gauche C (2007) Polimerização de proteínas do soro de leite por transglutaminase e propriedades físicas de iogurte elaborado após tratamento enzimático. Pós-Graduação em Ciência dos Alimentos, Florianópolis, Brasil.

Gomes D (2010) Incorporação de concentrados líquidos de proteínas de soro em produtos lácteos. Instituto Politécnico de Coimbra, Escola Superior Agrária de Coimbra, Tese de Mestrado, Portugal.

Guzmán-González M, Morais F, Ramos M and Amigo L (1999) Influence of skimmed milk concentrate replacement by dry dairy products in a low fat set-type yogurt model system. I: Use of whey protein concentrates, milk protein concentrates and skimmed milk powder. Journal of the Science of Food and Agriculture, 79, 1117-1122.

IPQ (1990) Leite e Produtos lácteos. Edição Normas Portuguesas. Lisboa. Portugal.

ISO 8586-2:1994 Sensory analysis – General guidance for the selection, training and monitoring of assessors (Part 2: experts). 1st edition.

Ji T and Haque ZU (2003) Cheddar whey processing methods and source: 1. Effect on composition and functional properties of whey protein concentrate. International Journal of Food Science and Technology. 38, 453-461.

Katsiari MC, Voutsinas LP and Kondyli E (2002) Manufacture of yogurt from stored frozen sheep's milk. Food Chemistry, 77, 413-420.

Lebeuf Y, Lacroix C and Paquin P (1998) Effet de l'incorporation des protéines du lactosérum dénaturées et microparticules dans le cheddar jeune. Le Lait, 78(3), 303-318.

Li JC and Guo MR (2006) Effects of polymerized whey proteins on consistency and water-holding properties of goat's milk yogurt. Journal of Food Science, 71, C34-C38.

López-Fandiño R (2006). Functional Improvement of Milk Whey Proteins Induced by High Hydrostatic Pressure. Critical Reviews in Food Science and Nutrition, 46, 351 - 363.

Lucey JA and Singh H (1998) Formation and physical properties of acid milk gels: a review. Food Research International, 30(7), 529-542.

Mangino ME, Liao YY, Harper NJ, Morr CV and Zadow JG (1987) Effect of heat processing on the functionality of whey protein concentrates. Journal of Food Science, 52(6), 1522-1524.

Modler HW and Kalab M (1983) Microestructure of yogurt stabilized with milk proteins. Journal of Dairy Science, 63, 430-437.

Nicorescu I, Loisel C, Vial C, Riaublanc A, Djelveh G, Cuvelier G and Legrand J (2008) Combined effect of dynamic heat treatment and ionic strength on denaturation and aggregation of whey proteins - Part I. Food Research International, 41, 707-713.

NP 4146:1991 Leite e produtos lácteos. Métodos de colheita de amostras. Comissão Técnica - 32, 1ª edição, Portugal.

Pandya AJ and Ghodke KM (2007) Goat and sheep milk products other than cheeses and yoghurt. Small Ruminant Research, 68, 193-206.

Pelegrine DHG and Gasparetto CA (2005) Whey proteins solubility as function of temperature and pH. Lebensmittel-Wissenschaft und-Technologie, 38, 77-80.

Portaria n.° 742/92 de 24 de Julho. Produção, comercialização e consumo de iogurtes e leites fermentados, Diário da Republica - I série B.

Real Decreto 179/2003 de 14 de febrero 2003. Norma de Calidad para el yogur o yoghourt. Espanha.

Rodrigues RC, Almeida JC, Pereira CD, Gomes DS, Madanelo JP, Oliveira MJ and Fonseca ML (2000) Queijo Serra da Estrela processos tradicionais e inovações tecnológicas. DRABL - Direcção Regional de Agricultura da Beira Litoral, Edição co-financiada pelo FEOGA.

Schorsch C, Wilkins DK, Jones MG and Norton IT (2001) Gelation of caseine whey mixtures: effect of heating whey proteins alone or in the presence of caseine micelles. Journal Dairy Research, 68, 471-481.

Sodini I, Mattas J and Tong PS (2006) Influence of pH and heat treatment of whey on the functional properties of whey protein concentrates in yogurt. International Dairy Journal, 16, 1464-1469.

Sodini I, Montella J and Tong PS (2005) Physical properties of yogurt fortified with various commercial whey protein concentrates. Journal of the Science of Food and Agriculture, 85, 853-859.

Walstra P and van Vliet T (1991) On the fractal nature of particle gels. In E. Dickinson (Ed.) Food polymers, gells and colloids. Royal Society of Chemistry, London.

INE - Instituto Nacional de Estatística, I.P. (2010) Estatísticas Agrícolas 2009. Lisboa, Portugal ISBN 978-989-25-0085-4.

INE - Instituto Nacional de Estatística, I.P. (2012) Estatísticas Agrícolas 2011. Lisboa, Portugal ISBN 978-989-25-0155-0.

MADRP - Ministério da Agricultura do Desenvolvimento Rural e das Pescas (2007) Leite e Lacticínios: diagnóstico sectorial. Gabinete de Planeamento e Políticas. Portugal.

Chapter 6

LWPC as a primary raw material for dairy gels

Abstract

The aim of this chapter is to study the gelation properties of liquid whey protein concentrates (LWPC) as raw material for dairy food applications. Two methods of gelation were used: conventional heat-induced gelation and acid-induced gelation. In the former, the nature of LWPC thermal gelation is determined by three major conditions: (1) the degree of LWPC processing (non-defatted - ND, defatted - D and diafiltrated - DF), (2) protein concentration and (3) pH. Acid-induced gels were produced with non-defatted LWPC by bacterial fermentation (yogurt type) and glucono-δ-lactone acidification (dessert type) with or without fortification with skimmed milk powder (SMP). All the gels produced showed viscoelastic behaviour. Non-defatted LWPC leads to stronger heat-induced gels with a more cohesive microstructure, a higher water retention capacity and rheological properties (G' and G" modulus). It was found that gel

properties were not improved in products with lower non-protein compounds. The increase in protein concentration positively influences protein interactions. However, the pH is responsible for the equilibrium between attraction and repulsion forces in the gel components that influence gel hardness and water retention capacity. The fermented systems (yogurt type acid gels) produced weaker gel structures than the equivalent chemically acidified gels (dessert type acid gels). The sequence in which protein denaturation is performed and the acidification processes may be responsible for these differences. It was also observed that molecular rearrangement continues during cold storage and that fortification with SMP favoured gelation.

6.1 Introduction

As mentioned in Chapter 2 (section 2.3), whey protein concentrates (WPC) have become well established as food ingredients and applications have been found across a wide range of food products (Fox and McSweeney, 1998; Díaz et al., 2009). Their nutritional and functional properties have been extensively reviewed and reported in the literature (Livney et al., 2003). Although dehydrated whey protein products with a high protein content have been studied extensively, no attention has been paid to the functionality of liquid whey protein concentrates (LWPC). Among the functional properties of whey proteins, gelation is cited as one of the most interesting hydration-related properties (Mulvihill, 1992). Gels consist mostly of fluid, but have the remarkable ability to behave as a solid whilst retaining many properties characteristic of the fluid component (Geara, 1999). Gelation is a two-step process involving protein denaturation and aggregation (Morr and Ha, 1993; Zayas, 1997; Sullivan et al., 2008). During heat treatment the intramolecular bonds of proteins responsible for their secondary and tertiary structure (hydrogen and disulphide bonds) break down and expose the hydrophobic groups and cysteine residues which are located in the inner region of native proteins, whilst the primary structure of the protein is maintained. This phenomenon is called protein denaturation. Protein aggregation or polymerization corresponds to the formation of a three-dimensional network by the establishment of intermolecular bonds between the exposed groups of denatured proteins. Traditionally, protein gelation has been achieved by heating, but some chemical processes form protein gels in a way analogous to heat-induction. In addition to heat, a chemical method, namely acidification, leads to modifications in protein-protein and protein-medium interactions (Totosaus et al., 2002). Acid-induced gels are common in the production of a variety of food products such as surimi, mayonnaise and gelatine-like desserts (Bryant and McClements, 1998).

Lowering the pH produces enough protein denaturation to cause interactions and the formation of a network structure. In this type of gelation the acidification process moves towards the protein isoelectric point, which can be achieved by applying a starter culture containing lactic acid bacteria (e.g. yogurt fermentation) or chemically by the addition of glucono-δ-lactone (GDL). The isoelectric points (IP) of whey proteins are 5.2 for β-Lg, from 4.2 to 4.5 for α-La, from 4.7 to 4.9 for BSA and between 5.5 and 6.8 for Ig (Bryant and McClements, 1998; Hong and Creamer, 2002).

Several intrinsic and extrinsic factors affecting the gelation of whey proteins, as well as the type and properties of gels, have been investigated and mentioned in the literature (Totosaus et al., 2002). These include the protein type (molecular weight and amino-acid composition) (Kim et al., 1987; Singh, 2003), its hydrophobicity and free sulphydryl groups (Mangino, 1992), its concentration (Mangino, 1984; Boye et al., 1995, 1997; Zayas, 1997; Blecker et al., 2000; Singh, 2003), the type and amount of salts present in the formulation (Kuhn and Foegeding, 1991; Brandenberg et al., 1992; Mulvihill and Kinsella, 1988; Bryant and McClements, 2000), lipids (Joseph and Mangino, 1988), lactose (Brandenberg et al., 1992; Singh, 2003) and the conditions involved in the process (pH, temperature, heating and cooling rate) (Mangino, 1984; Paulsson et al., 1990; Boye et al., 1997; Zayas, 1997; Damodaran, 1996). Among these factors attention has focussed on protein concentration and pH. The cross-linking of macromolecules of an arbitrary initial size distribution is required for gelation and is proportional to the protein concentration. There must also be a minimal concentration of the protein itself, below which a continuous three-dimensional structure cannot be formed. However, the minimum protein concentration needed to form a gel also correlates with the pH (in particular with the IP of proteins). The more the pH moves away from the isoelectric point of proteins, the more charged they become and a greater electrostatic repulsion occurs between molecules, preventing the interactions required to form a gel matrix (Kinsella et al., 1994; Zayas, 1997). This implies that at pH 4.5 (close to the IP of whey proteins) the minimum whey protein concentration is 4%, at pH 6 between 6-8% and at pH 7 (far from the IP) the amount of proteins increases to 8-12% (Morr and Foegeding, 1990).

The gel structure determines the water holding capacity, permeability, texture, and appearance of the gel (Bryant and McClements, 2000). A knowledge of the rheological or mechanical properties of the system is very important, since the physical attributes of foods, including the lack of visual

whey separation and the perceived viscosity, are crucial aspects of quality and overall sensory acceptance on the part of consumers (Cavallieri et al., 2007; Lee and Lucey, 2010).

The objective of this study was to investigate the gelation properties of liquid whey protein products (LWPC) manufactured according to various processing levels, namely non-defatted (ND), defatted (D) and diafiltrated (DF). Two types of gels were produced: thermal-induced gels and acid-induced gels. In the preparation of the thermal gels, protein denaturation was achieved exclusively by heat treatment, and the influence of protein concentration and pH were evaluated in terms of gel water retention capacity, rheological properties (small and large-strain properties) and microstructure. Acid gels were obtained by lactic acid fermentation (yogurt type gels) or acidification by GDL (dessert type gels) with or without the incorporation of skimmed milk powder (SMP). Interactions between proteins in the gel network structure were studied during storage by measuring the water retention capacity, texture and rheological behaviour of gels.

6.2 Material and methods

Bovine cheese whey was supplied by Queijaria Serqueijos SA (Portugal). Glucono-δ-lactone (GDL) was supplied by Enzilab (Porto, Portugal) and the mixed culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* was supplied by Ezal YO-MIX 601. All the chemicals used were of analytical grade.

6.2.1 LWPC manufacture

Figure 6.1 shows the production process for liquid whey protein concentrates (LWPC) according to the final products obtained. 600 L of bovine cheese whey obtained immediately after production were transported in metallic recipients (50 L) at ambient temperature to the pilot plant at the Escola Superior Agrária de Coimbra. The resting time between leaving the production plant and arriving at the pilot plant was less than 2 hours, in order to minimise product deterioration. On reception, the cheese fines were removed by filtration using a cloth filter. 300 L of whey was skimmed in a Westfalia™ separator type ADB in order to reduce the fat content in the final products. The remaining whey (300 L) was used directly for ultrafiltration. Each type of whey (non defatted (ND) and defatted (D)) was concentrated using conventional ultrafiltration (UF) with a volumetric concentration factor (VCF) of 20. The process was performed with an ultrafiltration batch equipment using an organic UF membrane DSS (model 20K 3838-30) with a 5.5 m^2 effective filtration area and a 20 kDa cut-off at 25-35 ºC. In the

diafiltration process, which the main purpose was to reduce the presence of lactose, half of the retentate from ultrafiltration (7.5 L) of defatted whey was diluted with water (142.5 L) and then concentrated until the final volume of 15 L (VCF = 10). After diafiltration the retentate was subjected to reverse osmosis (RO), resulting in a 5 L final volume retentate (VCF = 3). The LWPC products obtained using the conventional UF process were named ND if the whey was not defatted, D if the whey was defatted and ultrafiltration only was performed, and DF if the diafiltration process was applied. Before preparing the thermal gel the LWPC products were analysed and frozen at -15 ºC.

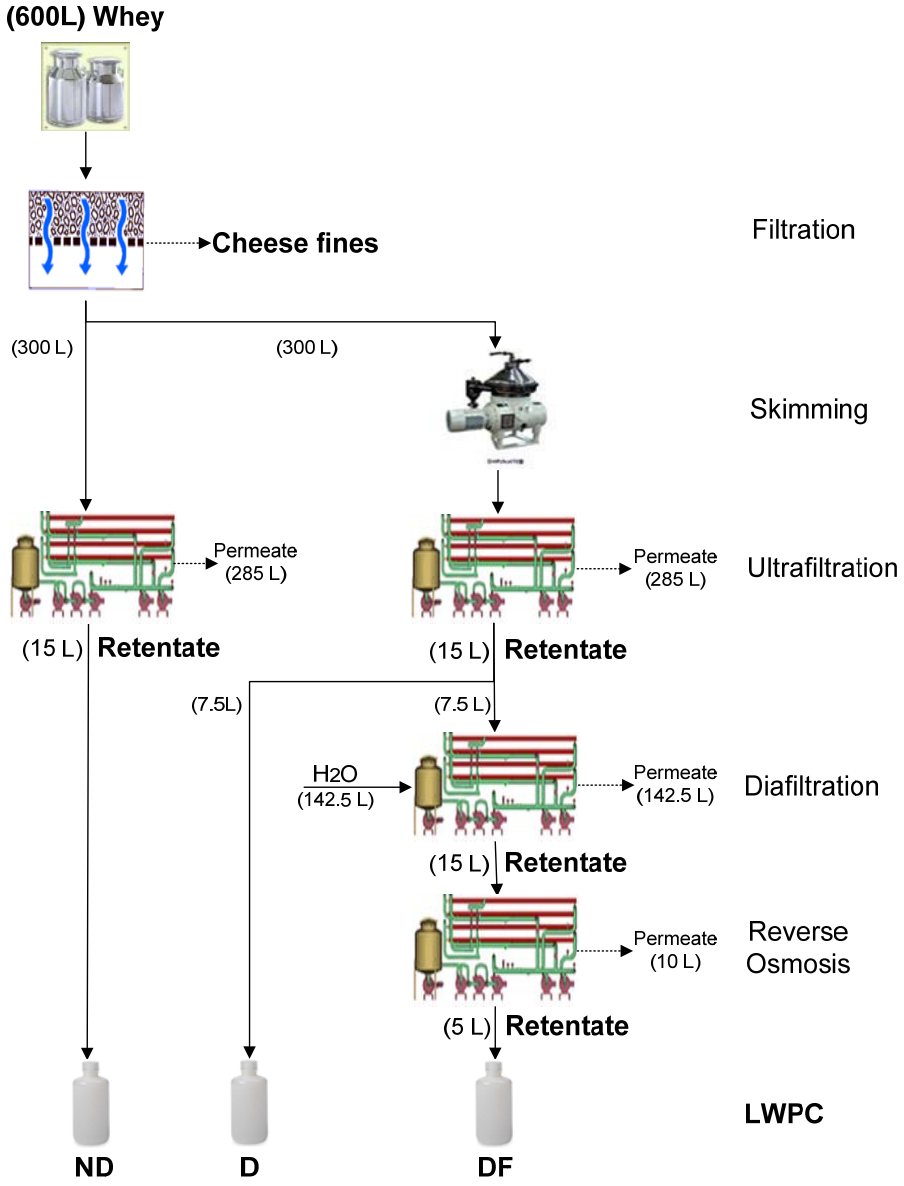


Figure 6.1 Liquid whey protein concentrates (LWPC) production process and final products obtained: non-defatted (ND), defatted (D) and diafiltrated (DF).

6.2.2 Gel preparation

The gelation properties of the various types of LWPC were evaluated in gels produced exclusively by heat-induced protein denaturation. Acid gels of ND LWPC, used as food model applications for yogurt and dessert types respectively, were produced by lactic fermentation or acidification by GDL.

For thermal gel preparation (Figure 6.2), each type of LWPC (ND, D, DF) was first thawed in a refrigerated chamber (5 ºC) and dissolved with distilled water to obtain final protein concentrations of 5% and 7%. The pH of each solution was measured and adjusted to 4 and 7 using 0.1 N NaOH or 0.1 N HCl prior to total dilution with water. 100 mL of each solution was poured into plastic cups, sealed with a plastic rack and heated in a water bath at 80 ºC for 30 min. After heat treatment the solutions were rapidly cooled in an ice-bath to ambient temperature and then placed in a refrigerated chamber (5 °C). The gels were prepared in triplicate and stored for 14 days.

Two types of acid gels were produced: yogurt and dessert type gels (Figure 6.3). Both acid gels were prepared from non-defatted LWPC with or without the addition of skimmed milk powder (SMP). In these cases the LWPC was refrigerated after production and left overnight prior to manufacturing the acid gel. The chemical composition of the LWPC used in the manufacture of the products was 20.5 % of total solids, 7.1% of fat, 9.6% of protein and 0.85% of ash. The titratable acidity was 0.52% expressed as lactic acid. For yogurt type acid gel production, the LWPC was first pasteurised at 90 ºC for 5 min (Tamime and Robinson, 2007) and then cooled down to 60 ºC. The heat treatment of milk was also used to destroy unwanted microorganisms, thus providing less competition for the starter culture. The product was divided into two parts and SMP was added to one part, to obtain 5% incorporation in the final formulation. The remaining fraction was not enriched with SMP and, after cooling to 60 ºC, both products were homogenised at 100 bar.

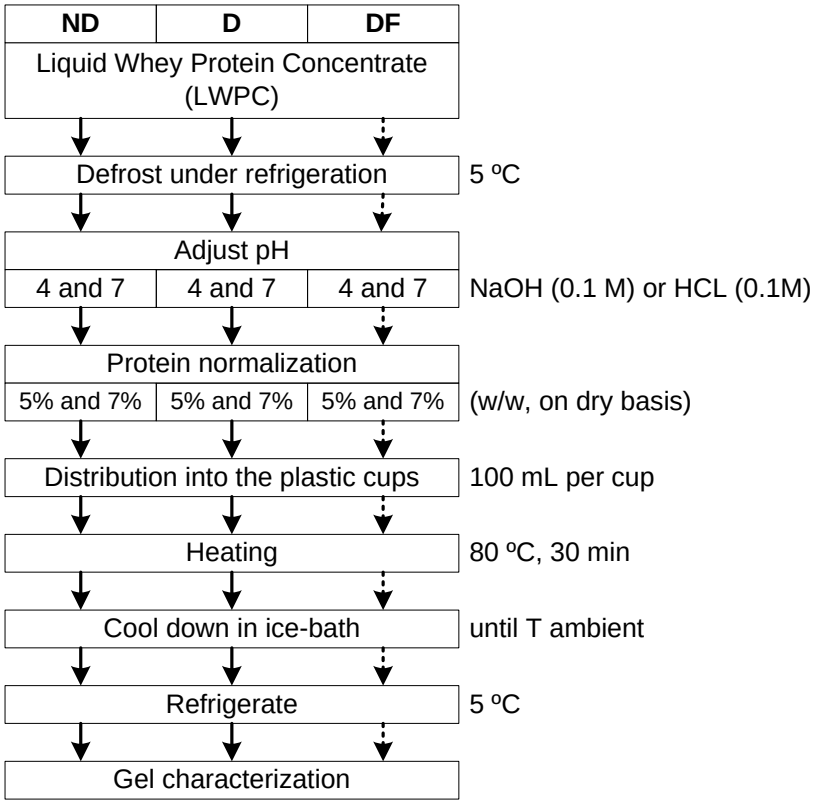


Figure 6.2 Process diagram of thermal gels produced from LWPC: non-defatted (ND), defatted (D) and diafiltrated (DF).

In the case of yogurts, before fermentation at 44 ºC in a incubator chamber (Jenogand, model Y - 1000) the mixture was inoculated with a commercial mixed culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Ezal YO-MIX 601), stirred and distributed into polystyrene cups (100 mL capacity). LWPC yogurt fermentation was monitored by assessing the pH until the products reached pH 4.6. After one day of cold storage (5 ºC) the biochemical composition of the yogurt type acid gels was determined. The physicochemical and rheological properties of the samples were evaluated on the first and last day of storage (21st day) in order to investigate product stability. In the case of the acid gel desserts, 5% sucrose was added to the LWPC and two separate formulations were produced (Figure 6.3); one without SMP and one the incorporating 5%. Both mixtures were heated to 60 ºC and homogenised at 100 bar. GDL was then added to a level of 1.5% (w/w, protein basis) and the mixture was gently stirred and distributed into 100 mL glass cups. Acidification by GDL hydrolysis to gluconic acid took place under the same conditions as the yogurt type gel fermentation (44 ºC). After the gel pH reached 4.6 a heat treatment of 90 ºC was applied for 30 min in order to simultaneously promote pasteurisation of

the product and increase the gel strength by protein thermal denaturation. Samples were then refrigerated in the same conditions as the yogurt type acid gels and their properties were evaluated using the same methods, on the 1st and 21st days after production.

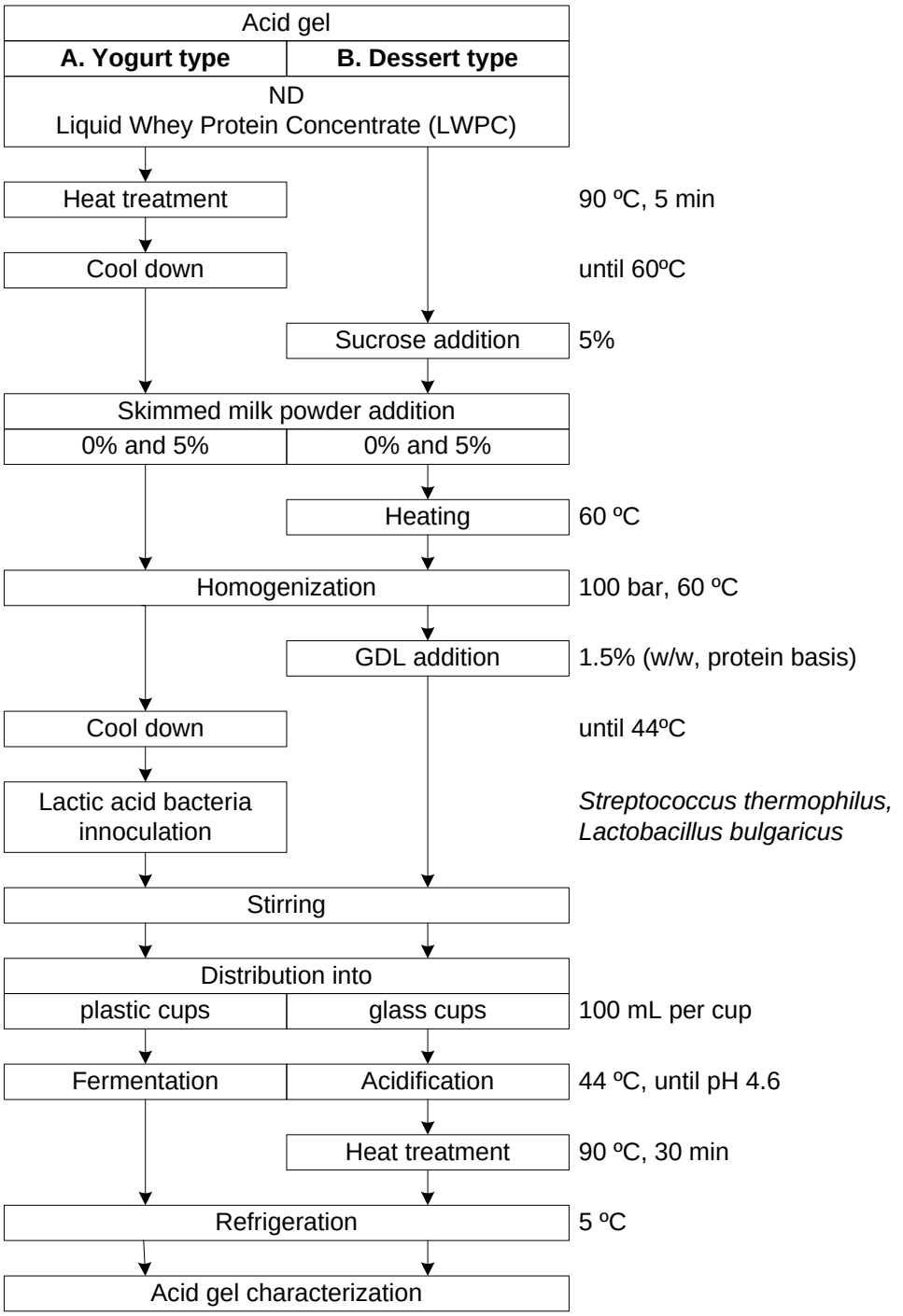


Figure 6.3 Process diagram of acid gels produced from ND - LWPC: (A) yogurt type and (B) dessert type.

6.2.3 LWPC and gel compositional analyses

After production, the three types of LWPC (D, ND and DF), the thermal gels and acid gels were analysed in triplicate and quadruplicate, respectively, and characterised in terms of dry matter, fat, total proteins, ash and lactose. The pH of the samples was directly determined with a pH meter (PHM61 Laboratory) immediately after production. The titratable acidity of the LWPC, expressed as a percentage of the lactic acid, was determined by titration using 0.1 N NaOH in an automatic titrator (Metrohm 665 Dosimat) with the technique described in NP 470:1983 used for milk. Total solids were determined by oven drying the samples for 6 hours (Memmert Universal Schurtzart Din 40050-IP20) and the mass of the samples was determined in a Bosch S2000 analytical balance (AOAC, 1980). The total nitrogen content was determined using the Kjeldahl method according to ISO 8968-1:2001. The total protein was estimated by multiplying the total nitrogen content of the samples by a factor of 6.38 (Appendix A). The fat content of LWPC was determined by the adapted Gerber method (NP 469:2002) and the fat content of gels by the Gerber method used for cheese (NP 2105:1983), in both cases using a Gerber K. Schneider & CO AG centrifuge. The ash content was calculated by incinerating dried samples at 550 ºC in an electric muffle furnace (Nabertherm, model LE 4/11/R6) according to AOAC (1995). The remaining solids (most of which was lactose) were determined by the difference between the total solid content of the samples and the sum of the ash, fat and protein contents.

6.2.4 Gel physicochemical analyses

Colour

Colour was determined in triplicate on the surface of the gels located at the bottom of the plastic cups used to produce them, with a portable HP-2132, Zhejiang Top Instruments Co, Ltd colorimeter using C illuminant in the CIEL*a*b* system calibrated with a white standard dish ($L^*_{standard}$ = 97.03; $a^*_{standard}$ = -0.67; $b^*_{standard}$ = 5.57). The colour of the gels was expressed by the three individual coordinates of the CIEL*a*b* system.

Water retention capacity

Water retention capacity was determined using the method described by Gauche (2007). Duplicate samples of LWPC gels were centrifuged at 350 rpm in a refrigerated centrifuge (a Hettich Rotanta 460R model) for 10 min at 5 ºC. The supernatant was collected and weighed. The water holding capacity (%) was reported as the held water values calculated as the ratio of the weight of water held in the gels after centrifugation to the weight of the gels.

Textural analysis

A Stable Micro Systems Texture analyser, TA.XT Express Enhanced model, was used to carry out a textural analysis of the gels and the results were calculated using Specific Expression PC Software. A texture profile analysis (TPA) was run with a penetration distance of 5 mm at 1 mm/s test speed, using an acrylic cylindrical probe with a diameter of 0.6 mm and a height of 3.5 mm. The following parameters were quantified: hardness, adhesiveness, cohesiveness, springiness and gumminess (the product of hardness and cohesiveness) (Phadungath, 2010).

Small-strain rheological properties

A controlled stress rheometer (Rheostress 1, ThermoHaake) was used to determine the rheological properties of the gels, in oscillatory mode. The measuring system consisted of a cone and plate geometry, C60/Ti - 0.052 mm (35 mm diameter and of 1º angle). Stress sweep tests were conducted at a frequency of 1 Hz to investigate the rheological linear viscoelastic behaviour of gels. The rheological properties, elastic modulus (G'), viscous modulus (G"), complex viscosity (η^*) and the damping factor (tan δ) of gels were evaluated in the range of 0.05 - 1.5 Hz at 3 Pa. All the measurements were taken in triplicate at 15 ºC.

Large-strain rheological properties - compression test

Lubricated compression tests were performed using a TA.XT Express Enhanced universal texture analyser equipped with an acrylic cylindrical probe (5 mm diameter). Cylindrical gel samples (17 mm diameter and 15 mm height) were placed on a flat base and compressed to 10 mm. The pre-test speed and test speed were fixed at 5 mm/s and 1 mm/s, respectively. Each test was replicated twice at room temperature (≈24 ºC). Experimental stress-strain curves were generated from the force displacement data, from which strain (ε_h) and stress (σ) were calculated according to equations 6.1 and 6.2, respectively.

$$\varepsilon_h(t) = \ln\left(\frac{h_0}{h_0 - h(t)}\right) \tag{6.1}$$

$\varepsilon_h(t)$ - applied strain; h_0 - initial height of the specimen (mm); $h(t)$ - sample compression distance at any time during the compression test (mm).

$$\sigma(t) = \frac{F(t)\, h(t)}{A_0\, h_0} \tag{6.2}$$

σ (t) - applied stress (Pa); F (t) - applied force perpendicular to the area of the material at any time during the compression test (N); A_0 - initial cross-sectional area of the sample (m^2).

Young's modulus (E') was calculated as the slope of the initial linear region of the stress-strain curves, and the stress at rupture (σ_{rup}) the maximum stress value obtained during the compression test, which could be correlated to the hardness of the samples.

Microstructure

The samples used to analyse the microstructure of the gels were prepared 24 hours after gelation. Gels were cut into cylinders (5 mm diameter and 10 mm length) and preserved in a 10% (v/v) trichloromethane solution prior to analysis. Samples were dehydrated for 20 min, each in 30%, 50%, 70%, 90% and 100% ethanol. After dehydration, ethanol was replaced by 100% ketone and samples were critical point dried using CO_2 in Bal-Tec CDP 020 equipment. Dried samples were mounted with gold-pallatium conducting paint (300 Å, at 1200 V and 10 mA) using a Jeol equipment (model JFC-1100, Akishima, Tokyo, Japan). Microstructure observations were made in duplicate on a Jeol scanning electron microscope (model JSM-T220 A, Akishima, Tokyo, Japan) at an accelerating voltage of 15 kV.

6.2.5 Statistical analysis

A statistical analysis of the data was carried out using the ANOVA package included in the Statistica 8 software. Means were compared using the Tukey HSD test. Differences were considered significant at $p < 0.05$.

6.3 Results and discussion

Table 6.1 shows the gross chemical composition of non-defatted, defatted and diafiltrated liquid protein concentrates (LWPC). The total solids content is significantly different ($p < 0.05$) between products. Diafiltrated products had lower values and non-defatted products higher total solids content. As expected, the amount of fat in defatted (D) and diafiltrated (DF) LWPCs was similar and significantly lower than in the non-defatted (ND) LWPC, as a result of fat removal during centrifugation (Figure 6.1). The protein concentration differed significantly ($p < 0.05$) between products and was lower in the case of ND and higher in DF. With regard to ash and lactose, lower values were obtained for DF LWPC. The use of diafiltration allows greater amounts of lactose and minerals to be eliminated and protein to be concentrated in the retentate.

Titratable acidy (TA) increases as the number of process unit operations increase, since the time required for the entire process allows for the acidification of products by bacterial microflora, and consequently a pH reduction. For this reason, titratable acidity increases from ND to DF products.

Table 6.1 Gross chemical composition of non-defatted (ND); defatted (D) and diafiltrated (DF) LWPC: total solids, fat, protein, ash, lactose. pH and titratable acidity (TA).

(% wet basis)	LWPC		
	ND	D	DF
Total solids	18.68±0.08 c	17.39±0.14 b	15.08±0.04 a
Fat	8.03±0.35 c	0.43±0.06 a	0.60±0.00 a
Protein	6.95±0.01 a	9.25±0.14 b	11.93±0.03 c
Ash	0.26±0.01 a	0.71±0.04 b	0.18±0.02 a
Other solids*	3.40±0.61 b	7.06±0.02 c	2.37±0.03 a
pH	6.43±0.03 c	5.25±0.05 b	4.62±0.03 a
TA (% lactic acid)	0.28±0.00 a	0.73±0.01 b	1.09±0.01 c

means within the same letter in the same line do not differ significantly ($p > 0.05$). *of which the major component is lactose (calculated by the difference between the total solids content and the sum of fat, protein and ash contents)

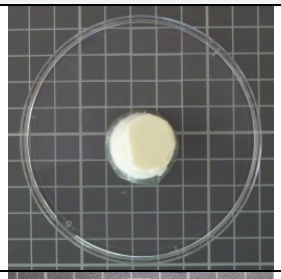
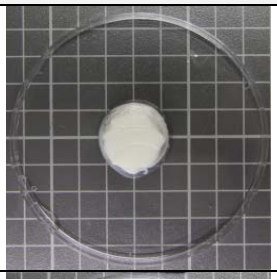
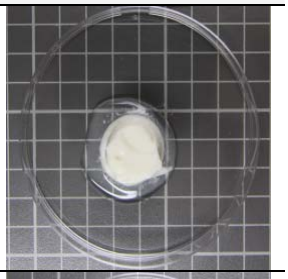
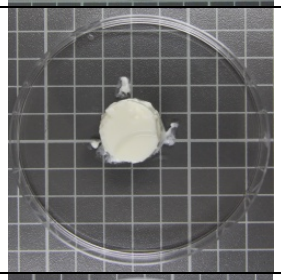
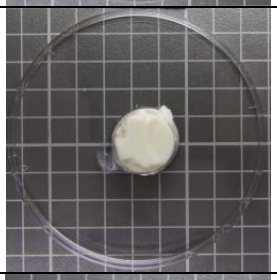
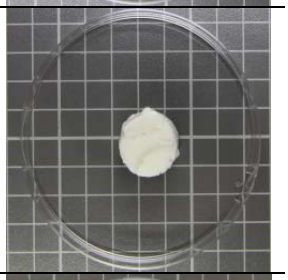
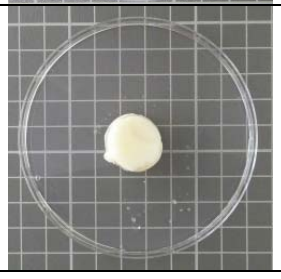
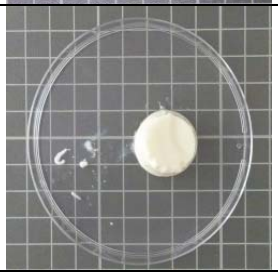
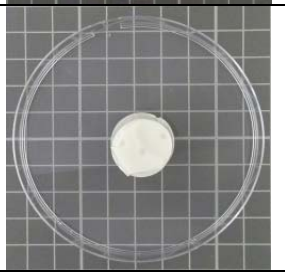
6.3.1 Gelation properties of LWPC

The gelation properties of WPC powders are well established in the literature but there is no information on the gelation of LWPC directly after production. In order to investigate gelation properties, heat-induced gels were therefore produced (Table 6.2). It was observed that liquid whey protein concentrates have attractive gelation properties as well as WPC powders. Depending on the protein concentration and pH, it was possible to obtain different structures, ranging from liquid ones, with no capacity to form a gel, to viscoelastic structures.

At pH 7 and 5% protein it was observed that no gel was formed for the DF product and the consistency of ND and D products was very poor (Table 6.2). The lower protein concentration (4.76%, 4.77% and 4.51%) observed in these three cases (ND, D and DF) and the higher pH value may be responsible for this behaviour, although the total solids content of the gels varied between 11.83% and 6.14% (Table 6.3). It is well established that a minimum protein concentration is needed for gelation (Robin et al., 1993; Sullivan et al., 2008). If the protein concentration is lower, intramolecular forces predominate and proteins have no capacity to form an ordered structure, whereas by increasing the amount of proteins in the solution the

intermolecular interactions (protein-protein and water-protein) are favoured and gelation occurs (Zayas, 1997). Lower protein concentration values (6-8%) are cited for gelation using WPC (Tang et al., 1994, 1995; Oakenfull et al., 1997), and higher concentrations (at least 10%) were needed for WPI gelation (Tang et al., 1994, 1995; Ju and Kilara, 1998b; Casper et al., 1999). At pH 7, our results also corroborate these findings, since products with lower levels of non-protein compounds (such as the diafiltrated products (DF)) behave like a liquid, indicating that a higher protein concentration is needed to form a gel structure.

Table 6.2 Appearance of non-defatted (ND), defatted (D) and diafiltrated (DF) LWPC thermal gels as function of pH and protein concentration.

pH	% protein	LWPC type		
		ND	D	DF
4	5			
	7			
7	5			
	7			

At pH 4, all gels were formed, regardless of the amount of protein. Under these conditions, the lower total solids content of defatted (D) and diafiltrated (DF) products is due to their lower levels of fat, lactose and mineral contents but the protein levels are high enough to promote gelation.

Table 6.3 Non-defatted (ND), defatted (D) and diafiltrated (DF) LWPC thermal gels gross composition: total solids, protein, fat, ash and lactose.

pH	Gel type	Protein (%)	Composition (%, wet basis)				
			Total solids	Protein	Fat	Ash	Other solids*
4	ND	5	14.35±0.63	5.55±0.10	1.50±0.05	0.43±0.00	6.87±0.50
		7	19.96±0.77	7.64±0.60	2.20±0.10	0.85±0.01	9.26±0.20
	D	5	6.76±0.18	5.16±0.04	0.10±0.01	0.31±0.16	1.19±0.10
		7	13.70±0.08	7.65±0.09	0.20±0.05	0.79±0.22	5.06±0.50
	DF	5	10.62±0.49	5.14±0.25	0.10±0.06	0.95±0.34	4.44±0.30
		7	9.41±0.13	7.34±0.06	0.10±0.01	0.43±0.11	1.54±0.25
7	ND	5	11.83±0.37	4.76±0.07	1.50±0.06	0.45±0.00	5.12±0.50
		7	19.69±1.19	9.27±0.78	1.45±0.02	0.81±0.24	8.16±0.40
	D	5	9.27±0.24	4.77±0.10	0.01±0.00	0.21±0.01	4.28±0.35
		7	14.05±0.39	7.14±0.36	0.20±0.01	0.82±0.00	5.89±0.50
	DF	5	6.14±0.10	4.51±0.00	0.20±0.04	0.20±0.04	1.23±0.10
		7	9.73±0.09	7.46±0.00	0.30±0.01	0.52±0.20	1.45±0.08

*of which the major component is lactose (calculated by the difference between the total solids content and the sum of fat, protein and ash contents).

Rheological properties of the thermal gels

The rheological properties of the heat-induced LWPC gels were evaluated (Figure 6.4 and 6.5) using an oscillatory rheometer for small-strain rheological properties and (Figure 6.6) a mechanical compression test for large-strain rheological properties.

The elastic modulus values (G') were higher than the viscous modulus G" in all the samples with the exception of 7DF5[1] (Figure 6.4). This behaviour indicates typical viscoelastic systems (Sullivan et al., 2008) and confirms that, given the conditions of the sample 7DF5 (composition and pH), no gel formation occurred, since G' < G". Tang et al. (1995) noted that the viscoelastic characteristic found for whey protein gels (the typical behaviour of LWPC gels) may be considered the most attractive characteristic with regard to the use of whey protein in food

[1] The first number - 4 - indicates the pH, the letters - DF - the type of LWPC, and the last number - 5 - the protein content (5%).

applications. It should be noted that these characteristics are achieved when the protein aggregation step is slower than the protein denaturation step, thus providing enough time for the orderly rearrangement of proteins.

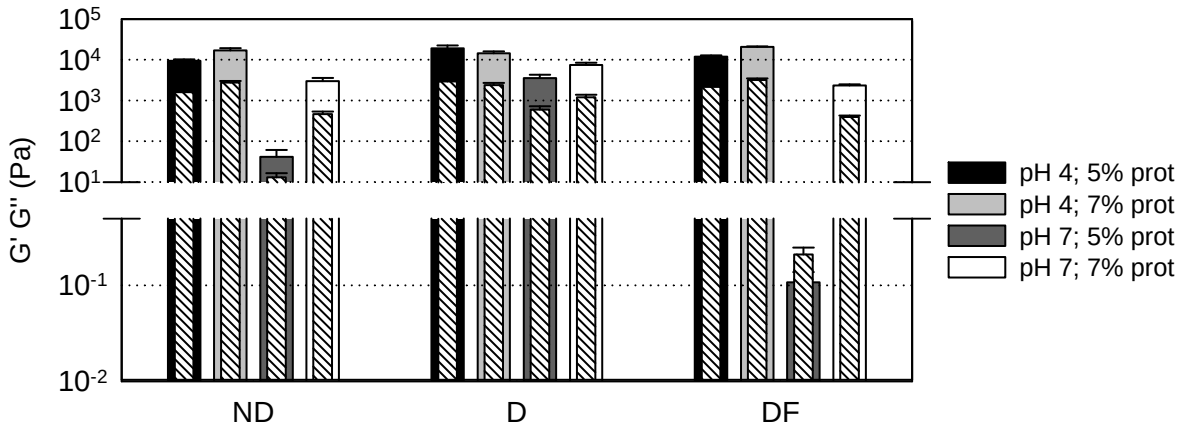


Figure 6.4 Elastic modulus (G' – filled bars) and viscous modulus (G" – striped bars) of ND, D and DF LWPC thermal gels as a function of pH (4 and 7) and protein concentration (5% and 7%).

The weaker gels were observed at pH 7, regardless of the LWPC product used. The lower protein levels (< 5%), in the case of 7DF5, did not allow for the formation of a solid matrix. The increase in protein concentration led to an increase in G' in the ND and DF gels and also to the formation of a firmer gel, which corroborates the results obtained by several authors (Lupano et al., 1992; Singh, 2003; Bordenave-Juchereau et al., 2005). An exception was found in the case of the D gel at pH 4, with no significant changes in the elastic and viscous modulus when the protein concentration was increased. The results for G' and G" were in the same range of magnitude as the values found by other authors for gels produced with bovine WPC (Pintado et al., 1999; Casper et al., 1999; Lorenzen and Schrader, 2006; Heino et al., 2007; Estrada, 2010). However, they are lower than the values found for caprine WPC (Pintado et al., 1999; Estrada, 2010) or ovine WPC gels (Pintado et al., 1999; Casper et al., 1999; Diáz et al., 2006). These authors stated that one possible reason for the formation of weaker gels may be the presence of lower amounts of β-Lg and the higher ash and lactose content in bovine WPC. The presence of fat in the gel formulation is also cited as a negative influence on gelation (Morr 1992; Blecker et al., 2000). It was noted that the intermolecular forces established between the fat globules and proteins reduce its ability to react with other protein molecules in the formation of the gel network. In this research, no differences in the firmness of the gels were observed between non-

defatted (ND) and defatted (D, DF) products, especially with regard to the proximity of the isoelectric point of whey proteins (pH 4), which was also verified by Estrada (2010) for caprine gels. The complex viscosity (η*) (Figure 6.5) follows the same behaviour as G' and G", and the damping factor (tan δ) also indicates the fluid behaviour of the sample 7DF5 (tan $\delta > 2.0$), the weak gel structure of the ND sample at pH 7 and 5% protein and the stronger gel structure of the remaining samples (tan $\delta < 0.25$) (Figure 6.5). It was observed that reducing the non-protein components in the formulation (e.g. lactose and ions) by applying diafiltration did not improve the rheological characteristics of the gels. These results contradict the findings of Schmidt et al (1984) and Zayas (1997) who cited diafiltration as a good method for improving gel hardness and cohesiveness due to the elimination of lactose. However, the higher protein levels used by them (10%), as opposed to 7% in this research, may justify the differences, since the protein interaction in the former case is more effective.

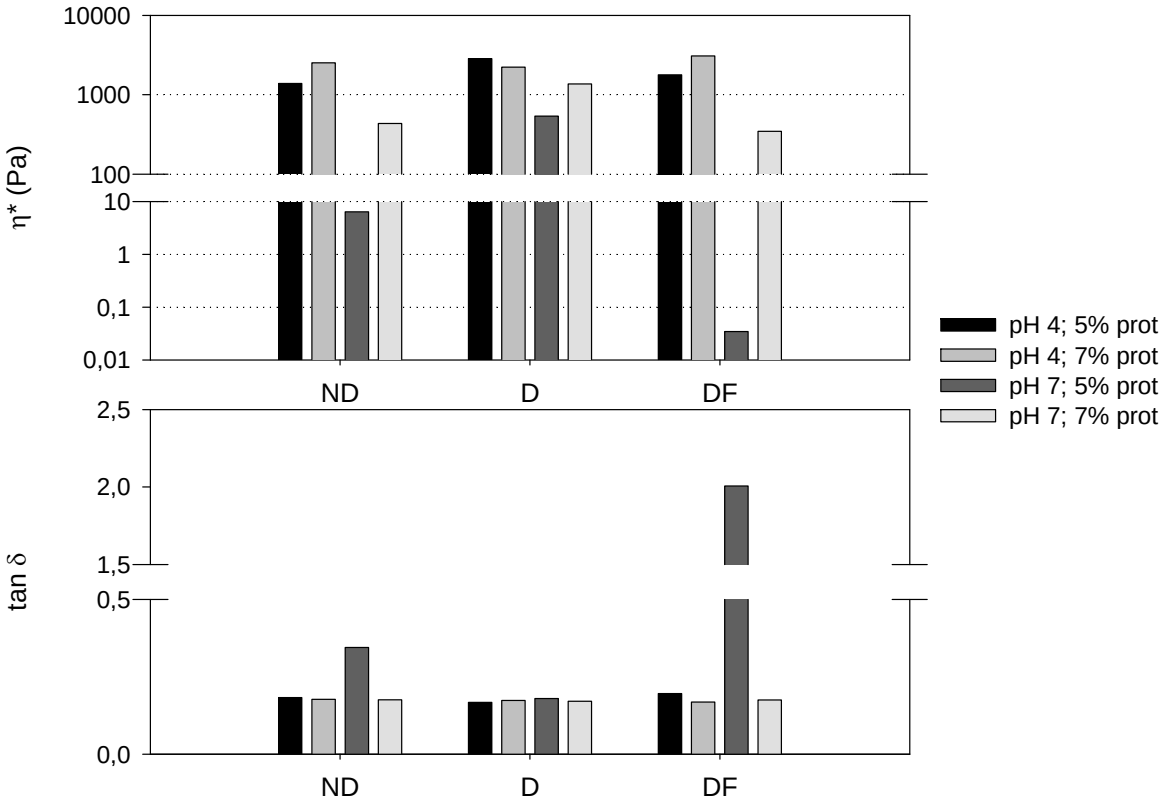


Figure 6.5 Complex viscosity (η*) and damping factor (tan δ) of ND, D and DF LWPC thermal gels as a function of pH (4 and 7) and protein concentration (5% and 7%).

In the evaluation of large-strain rheological gel properties, only the samples that showed a self-supporting structure (i.e. that were able to maintain their form sufficiently for the compression experiments) were suitable for testing. For this reason, the products at pH 7 and 5% protein were not appropriate for determining mechanical properties (Figure 6.6). A similar trend was observed with regard to stress at rupture (σ_{rup}) and Young's modulus (E') for both the ND and D gels (increasing in line with the protein content and pH). The hardness determined by the texture profile analysis (TPA) test had a similar behaviour to E'. In the DF gels this trend was not verified at pH 7, probably due to the stronger repulsion forces between proteins at this pH level. The ND gels showed the highest σ_{rup} and E' values, and the D gels the lowest.

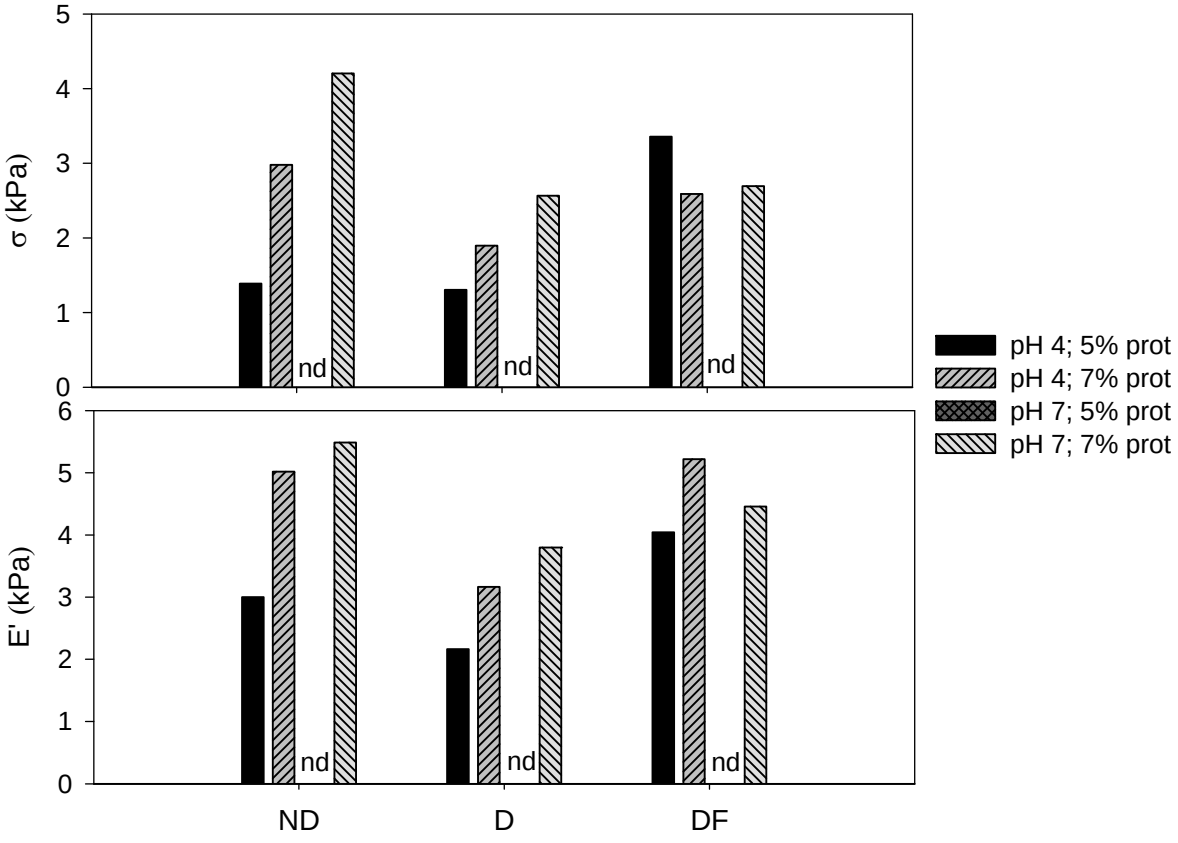


Figure 6.6 Stress at rupture (σ_{rup}) and Young's modulus (E') of ND, D and DF LWPC thermal gels as a function of pH (4 and 7) and protein concentration (5% and 7%). nd – not determined.

At pH 4 (close to the IP of whey proteins), the gels were less firm, regardless of the gel type. In these conditions the intramolecular bonds are more effective than the intermolecular interactions

and the gel matrix structure becomes weaker. Increasing the protein amount or the pH favours the intermolecular forces. At pH 7 our observations for large-strain rheological properties (σ_{rup}, E' and hardness) are not consistent with those observed in small-strain rheological gel properties (G' and G"). G' and G" decreased, whereas E' and hardness under pH 7 were higher than the values for pH 4 and 5% of protein. At higher pH values, although the protein concentration was higher (7%), the matrix network responds on a molecular level like a weak material, whereas on a macroscopic level it acts like a stronger gel. This may be due to higher protein denaturation, which enhances intermolecular rather than intramolecular forces.

Water retention capacity of thermal gels

No significant differences ($p < 0.05$) were observed in water retention capacity during storage and for this reason Table 6.4 represents the average values of each gel type according to pH and protein concentration. The ND gels had a higher water retention capacity than the D and DF gels. It has been noted that the presence of lipids may diminish hydrophobic protein-protein interactions, since they also participate in this type of interaction with proteins (Mulvihill and Kinsella, 1987; Morr, 1992; Zayas, 1997). However, Sodini et al. (2002) have pointed that when fat is present in gel formulation in significant amounts it may also participate in the formation of the gel structure, contributing towards reducing syneresis. This phenomenon can be improved by homogenisation during gel production, because this reduces the size of the fat globules, increasing their surface area and enhancing their interaction with proteins (Tamime and Robinson, 2007). From our results, it was observed that lipids, which are present in higher levels in non-defatted products, may establish hydrophobic interactions with proteins that lead to the formation of a gel structure with higher capacity to retain water, which confirms the results obtained for rheological properties (G' and G").

Table 6.4 Water retention capacity of ND, D and DF LWPC thermal gels as a function of pH (4 and 7) and protein concentration (5% and 7%). nd – not determined (liquid sample).

Gel type	pH	4		7	
	Protein (%)	5	7	5	7
ND		97.72±0.89 a	99.50±0.47 b	nd	98.80±1.59 ab
D		94.61±0.80 a	96.21±0.64 a	nd	96.06±0.51 a
DF		92.60±0.92 a	98.24±0.73 b	nd	97.92±0.35 b

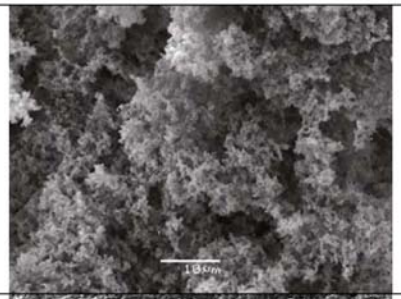
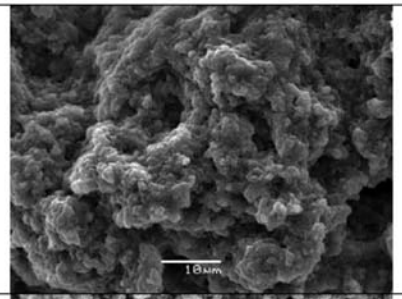
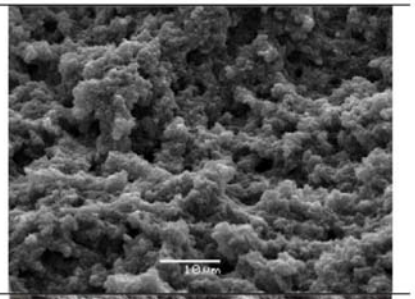
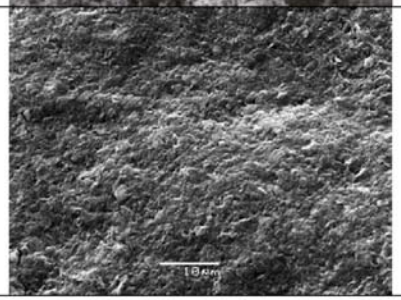
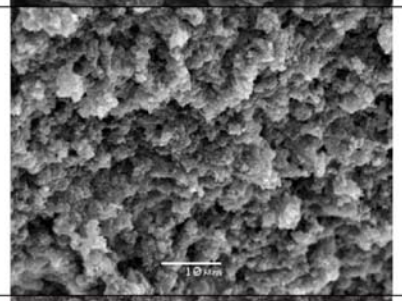
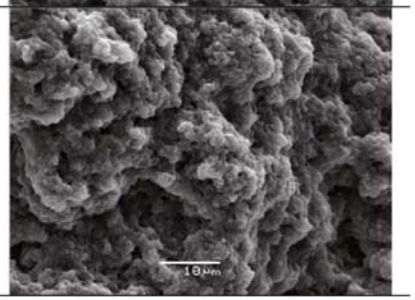
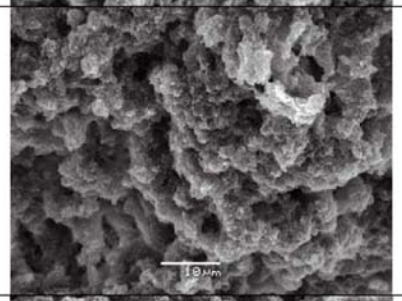
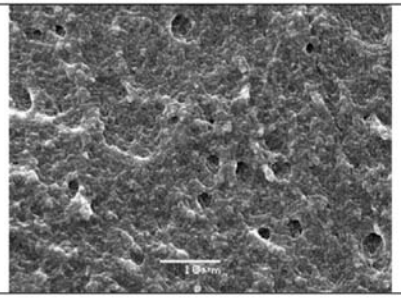
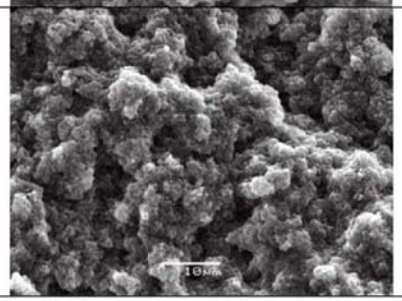
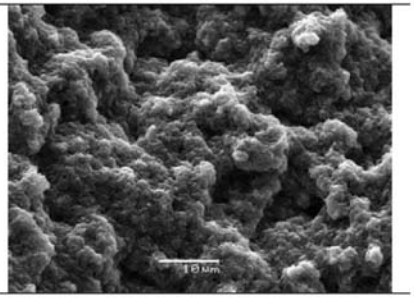
means within the same letter for the same gel type (same line) do not differ significantly ($p > 0.05$).

An increase in protein concentration leads to a decrease in syneresis. The number of hydrophobic groups and cysteine residues available to react increases with protein concentration and the formation of a more effective gel matrix is possible (Blecker et al., 2000; Singh, 2003). At lower protein concentrations protein intramolecular interactions predominate and there is a greater tendency to form protein aggregates instead of a gel structure (Robin et al, 1993; Zayas, 1997). In this case, the aggregates do not have the capacity to retain water and syneresis increases. It was also observed that close to the isoelectric point of whey proteins (pH 4), the water retention capacity decreased, as the protein hydrophobic intramolecular forces are more effective than the protein intermolecular forces (SH- bonds), leading to a more open matrix expelling more water, as observed by de Wit, 1989 and Kitabake and Doi, 1993. At pH 7 and lower protein concentrations (5%) no gel was formed, regardless of the type of LWPC. In this case, the attractive forces between proteins were not sufficient to produce a solid network, and the continuous phase was still a liquid. Only under higher protein contents is the balance between protein-protein interactions (attractive forces) and protein-water interactions (repulsive forces) more effective, meaning that the gel structure has the capacity to retain water more efficiently.

Thermal gel microstructure

It is well recognised that the structure of foods greatly affects their various properties, including texture, functionality and appearance. The microstructure of thermal gels produced by LWPC is shown in Table 6.5. It was noted that the gels, prepared exclusively by whey proteins, consist of a coarse particulate network of protein particles linked together in clusters, chains and strands. This type of organisation is also identified in the microstructure of whey protein gels produced by WPC (Aguilera, 1995) and acid milk gels (Harwalkar and Kalab 1980; Kalab et al., 1983). The network has pores or void spaces in which the aqueous phase is confined. In fat containing products (ND), especially at higher protein levels (7%), a continuous gel matrix was observed that obscures the finer details of the aggregates, clusters and strands (Table 6.5), although small pores can be identified. The presence of higher mineral contents in the case of gels with higher protein levels (Table 6.3) may also contribute towards protein aggregation and the diminution of pores (Hongsprabhas et al., 1999). In the ND gels with lower protein contents (5%) at pH 4, a reticulate gel microstructure was observed, demonstrating their fragility and lower water retention capacity. It was also observed that the diameter of the pores varies considerably, with larger pores found in gels made by defatted (D) and diafiltrated (DF) LWPC products.

Table 6.5 Scanning electron micrographs (x 2000) showing effects of pH and protein concentration on LWPC thermal gels microstructure. Bar length is 10 µm.

pH	% protein	LWPC type: ND	LWPC type: D	LWPC type: DF
4	5			
	7			
7	5	-		-
	7			

In both cases, regardless of the protein concentration, no significant differences were found in the granulated microstructure, and the presence of small protein structures (aggregates with a spherical shape), linked together in bigger structures (clusters), was evident. The former, are formed by intramolecular protein interactions, and the latter by intermolecular interactions among aggregates (Vardhanabhuti et al, 2001; Yamul and Lupano, 2003; Diáz et al, 2006). If the pH is increased, no differences are observed in the gel microstructure. It is not clear from SEM analysis that small pores were formed at pH 7 in order to produce a more homogeneous (less granulated) gel network which could justify the previous results obtained for WRC, indicating that pH values far from the isoelectric point of proteins lead to the production of a gel network with a higher capacity to retain solvents (Vardhanabhuti et al, 2001; Chantrapornchai and McClements, 2002).

6.3.2 The performance of LWPC in acid gel production

As a result of the good gelation properties obtained in the case of heat-induced LWPC gels, especially the non-defatted (ND) products, two types of acid-induced gels were produced (yogurt type and dessert type LWPC) in order to evaluate their performance under acidic conditions.

Table 6.6 shows the acid gel biochemical composition for LWPC yogurts and desserts with 5% of SMP and without incorporation (0%). As expected, the products with SMP had significantly higher total solids than the unfortified products. The fat content did not vary in each product type ($p > 0.05$) with the addition of SMP. Although the protein content increased in products with 5% SMP, due to the incorporation of caseins, no significant differences ($p > 0.05$) were observed either in the formulations or the product types. Lactic desserts had significantly higher levels of other solids (mainly carbohydrates) due to the addition of sucrose (5%, wet basis) in the formulation. Solvent evaporation during the longer heat treatment (30 min) and the fermentation process in the case of yogurts, which converted lactose into lactic acid, may also have contributed to this difference.

The titratable acidity (Table 6.7) achieved for LWPC yogurt type acid gels (1.30-1.88%) was higher than for the dessert gels (1.05-1.58%). This may indicate that acidification by bacterial fermentation is more effective than by GDL hydrolysis to gluconic acid. Higher acidity values were obtained in products based exclusively on LWPC or fortified with SMP in comparison to conventional yogurts or yogurts which incorporated LWPC (see Chapters 4 and 5). This was to some extent expected, since the LWPC products used as a raw material for yogurt production

have higher acidity levels (≈0.52% lactic acid) than the milk used in the production of conventional yogurts (≈0.185% lactic acid for bovine milk – Chapter 5). These findings may indicate that yogurt or desserts produced from LWPC may probably be more microbiologically stable during storage.

Table 6.6 LWPC yogurt type and dessert type acid gels gross composition: total solids, fat, protein, ash and lactose for 0% and 5% skimmed milk powder (SMP) incorporation.

Composition (%)	Yogurt type		Dessert type	
	0%	5%	0%	5%
Total solids	22.13±0.98 a	26.10±0.15 b	28.93±0.77 c	32.08±0.07 d
Fat	5.50±0.49 b	4.63±0.41 ab	4.53±0.65 a	4.93±0.73 ab
Protein	7.17±0.68 a	8.94±1.33 a	6.29±2.43 a	8.88±1.44 a
Ash	0.96±0.04 a	1.63±0.34 a	1.38±0.10 a	3.51±0.19 b
Other solids*	7.94±0.59 a	10.37±1.73 ab	16.17±1.03 c	14.99±1.68 bc

means within the same rows without the same superscript are statistically different at $p < 0.05$. *Calculated by the difference between the total solids content and the sum of fat, protein and ash contents, in which carbohydrates are the major components.

Table 6.7 Titratable acidity (TA), water retention capacity (WRC) and colour parameters (L*, a*, b*) of LWPC yogurts and desserts with 0% and 5% of SMP incorporation, during storage.

Parameter	Time (days)	Yogurt type		Dessert type	
		0%	5%	0%	5%
TA (% lactic acid)	1	1.38±0.17 aA	1.46±0.18 aA	1.05±0.01 aA	1.58±0.02 bA
	21	1.30±0.08 aA	1.88±0.11 bB	1.19±0.08 aB	1.21±0.14 aB
pH	1	4.62±0.02 bB	4.48±0.03 aB	4.32±0.02 aA	4.62±0.01 bA
	21	4.46±0.02 bA	4.39±0.01 aA	4.39±0.02 aB	4.64±0.04 bA
WRC (%)	1	65.74±1.62 bA	53.11±1.16 aA	70.49±4.58 aB	99.52±0.58 bA
	21	64.04±1.03 bA	56.59±2.94 aB	64.12±4.87 aA	100.00±0.00 bA
Colour					
L*	1	96.26±0.41 aA	96.98±0.17 aA	88.58±1.70 aA	92.16±3.61 aA
	21	95.98±0.23 aA	97.65±0.42 bA	94.67±1.77 bA	82.52±6.83 aA
a*	1	-0.45±0.24 aA	-0.30±0.22 aA	0.91±0.15 aA	0.13±0.35 aA
	21	-0.59±0.41 aA	-0.73±0.48 aA	0.39±0.36 aA	1.86±1.11 aB
b*	1	7.19±0.36 aA	7.08±0.16 aA	10.34±0.44 aA	11.08±0.70 aA
	21	7.33±0.23 aA	7.33±0.09 aA	9.92±0.16 aA	12.31±1.20 bA

means without the same letter differ statistically at $p < 0.05$. Lowercase letters represent differences between products (0 and 5% of SMP) and uppercase letters differences over time (1st and 21th day).

Some authors have stated that protein composition is one of the most significant factors influencing the duration of yogurt bacterial fermentation (Puvanenthiran et al., 2002). They have noted that by increasing the whey protein/casein fraction the time of bacterial fermentation also increases as a result of the higher buffer capacity of the whey proteins. Our results (Table 6.7)

concur with this, since the yogurts with higher amounts of whey proteins (0% of SMP) showed higher pH values for the same fermentation time. A significant decrease ($p < 0.05$) in pH was also observed during storage for both types of yogurts, which may indicate that fermentation continues during storage. A distinctive type of behaviour was found in desserts. Higher pH values were observed in products incorporating SMP, which corroborate the results published by Lucey et al. (1999) for acid gels produced by GDL. This divergence in the pH of acid gels produced by bacterial fermentation or by GDL acidification may be due to the time at which the heat treatment was performed during our acid gel production. In the case of yogurts, whey protein denaturation occurs during the pasteurisation (90 ºC, 5 min) of LWPC, prior to the addition of SMP and fermentation. However, in desserts produced chemically by GDL, acid-induced gelation occurs when the whey proteins are still in their native form, since the heat treatment (90 ºC, 30 min) is only applied at the end of the production process (Figure 6.3). It was also observed that during storage the pH of desserts hardly varies.

Table 6.7 shows the water retention capacity (WRC) of the systems studied. Specific WRC behaviour can be observed in the LWPC acid gels according to their nature (yogurt or dessert), SMP incorporation and storage time. The desserts showed a higher water retention capacity (64.12-100%) than yogurts (53.11-65.74%), which can be attributed firstly to their higher total solids content (Table 6.6) and also to the heat treatment after acidification. In this case, protein denaturation under acidic conditions significantly improved this property. In yogurts, the acidification occurs progressively during fermentation but only after whey protein denaturation. Cavallieri et al. (2007) distinguished two kinds of interaction in the protein network of the acid gels: interaction between part of the protein and water by hydrogen bonding and interaction between the majority of proteins by electrostatic and hydrophobic interactions and even by hydrogen bonding. It was also observed that yogurts fortified with 5% SMP had a lower WRC. This may be due to the fact that the presence of native caseins (that have not been denatured, as with whey proteins) may create some discontinuity in the whey protein network by reducing the capacity to retain water. Sodini et al. (2004), Puvanenthiran et al. (2002) and Amatayakul et al. (2006) mentioned that higher amounts of whey proteins in yogurt lead to a higher level of protein interaction and to the formation of a compact gel structure (Bhullar et al., 2002; Aziznia et al., 2008) that is responsible for reducing syneresis. On the other hand, when whey protein and casein denaturation occurred simultaneously (as in the case of desserts with 5% SMP) the WRC benefited in comparison to desserts with no caseins (0% of SMP). This demonstrates that whey

proteins and caseins are able to interact when denatured simultaneously, and this in turn favours the formation of bridges leading to a narrow-pore mixed casein/whey protein network. As the pore size decreases, lower syneresis values are obtained (Li and Guo, 2006).

With regard to colour (Table 6.7), higher luminosity values (L*) were found for yogurt type gels, which are in the range of 95.98-97.95. The slightly darker colour for desserts (82.52-94.67) was probably due to the intense heat treatment performed in this case (90 ºC, 30 min) and the higher solids content as a result of solvent evaporation. The colour coordinate a* was the most affected amongst the products. It changed from negative values (green direction) in the case of yogurts to positive values (red direction) in the case of desserts. During heating, the Maillard reactions lead to the development of a light brown colour that explains these results, as well as the increase in the b* coordinate (yellow direction). The colour parameters of yogurt type acid gels are in the same range of magnitude as the values mentioned by Martín-Diana et al. (2004) and Vargas et al. (2008), and in some cases have higher luminosity values than those reported by Estrada (2010). However, in the case of desserts the chromatic coordinates were particularly penalised.

The texture parameters of acid gels are presented in Table 6.8. With regard to hardness, it was observed that desserts present higher values than yogurts, and the incorporation of SMP in the formulation also contributes to this. These results concur with the higher water retention observed for desserts with 5% SMP (Table 6.7), indicating that harder gel structures have the ability to prevent syneresis more efficiently. Bell (1995) has also found that bovine and caprine fermented systems produce weaker gel structures then their equivalent chemically acidified gels (GDL acidification). Yogurts with 5% SMP showed lower hardness values after production (1st day), although during storage they became harder, meaning that protein polymerization continues under refrigeration conditions. The fortified desserts showed the highest values for adhesiveness, chewiness and gumminess. No differences ($p > 0.05$) were observed in springiness and cohesiveness, either between products (SMP incorporation) or during storage. The values for springiness (near to the unit) indicate the viscoelastic behaviour of the LWPC acid gels.

Figure 6.7 represents the elastic and viscous modulus (G' and G") of acid-induced gels (yogurts and desserts) as a function of incorporating SMP, storage time and frequency (rad/s). Figure 6.8 shows their complex viscosity (η^*).

Table 6.8 Texture parameters: hardness, adhesiveness, springiness, chewiness, gumminess cohesiveness and resilience of LWPC yogurts and desserts with 0% and 5% of SMP incorporation, during storage.

Parameter	Time (days)	Yogurt type		Dessert type	
		0%	5%	0%	5%
Hardness (N)	1	0.10±0.00 aA	0.09±0.01 aA	0.11±0.02 aA	0.59±0.12 bA
	21	0.11±0.00 aA	0.15±0.01 bB	0.18±0.02 aA	0.44±0.16 bA
Adhesiveness (N.s)	1	-0.31±0.04 aA	-0.13±0.02 bB	-0.30±0.12 bA	-2.42±0.52 aA
	21	-0.35±0.03 aA	-0.33±0.04 aA	-0.63±0.24 aA	-1.35±0.71 aA
Springiness	1	0.96±0.01 aA	0.97±0.01 aA	0.97±0.02 aA	0.97±0.01 aA
	21	0.96±0.01 aA	0.97±0.00 aA	0.95±0.02 aA	0.97±0.03 aA
Chewiness	1	6.52±0.19 aA	6.14±0.21 aA	6.78±1.13 aA	35.23±6.79 bA
	21	7.69±0.10 aB	10.55±0.44 bB	10.23±1.47 aA	26.76±10.58 aA
Gumminess	1	6.82±0.18 aA	6.33±0.23 aA	7.01±1.23 aA	36.29±6.92 bA
	21	8.01±0.16 aB	10.91±0.49 bB	10.78±1.53 aA	27.45±10.13 bA
Cohesiveness	1	0.70±0.01 aA	0.71±0.03 aA	0.62±0.04 aA	0.60±0.03 aA
	21	0.69±0.03 aA	0.72±0.05 aA	0.57±0.02 aA	0.60±0.03 aA
Resilience	1	0.05±0.00 aA	0.09±0.01 bB	0.04±0.01 aA	0.02±0.01 aA
	21	0.05±0.00 aA	0.05±0.01 aA	0.02±0.01 aA	0.03±0.01 aA

means without the same letter differ statistically at $p < 0.05$. Lowercase letters represent differences between products (0 and 5% of SMP) and uppercase letters differences over time (1st and 21th days).

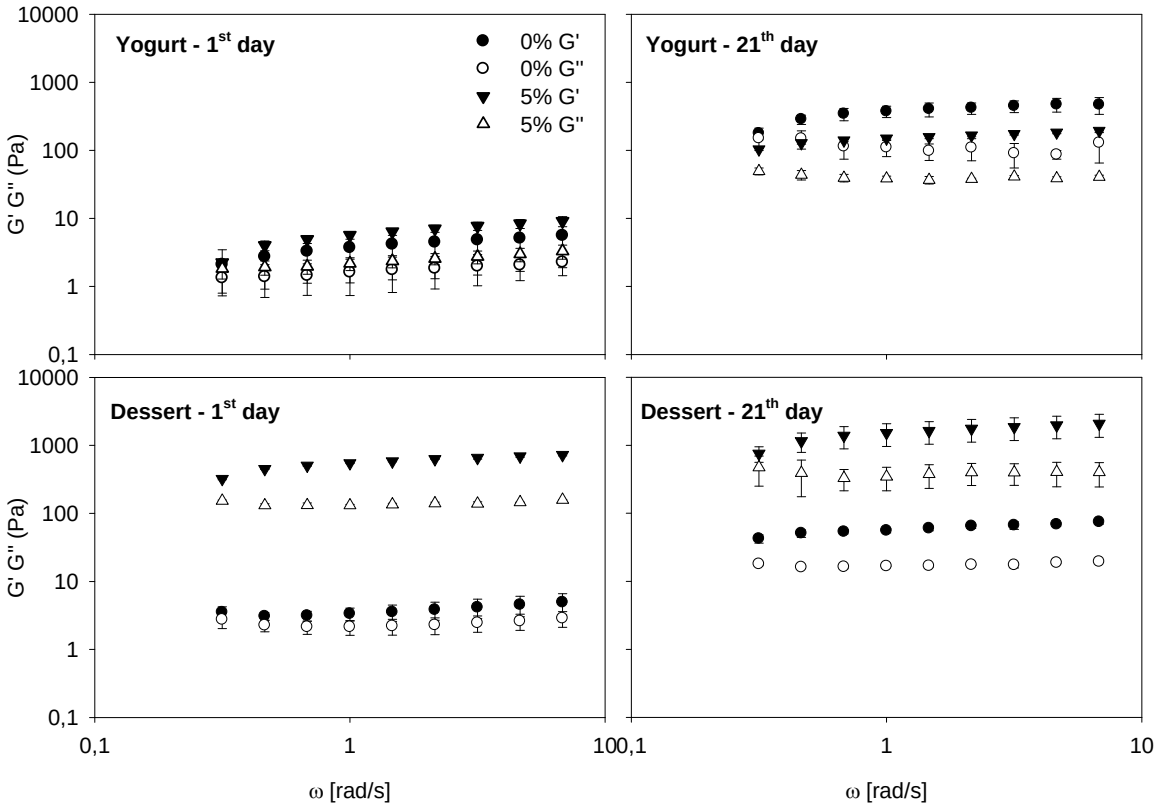


Figure 6.7 Elastic modulus (G') of LWPC yogurts and desserts with 0% (●) and 5% (▼) of SMP incorporation; viscous modulus (G") of LWPC yogurts and desserts with 0% (○) and 5% (Δ) of SMP incorporation, during storage.

It was observed that in all cases the elastic modulus (G') is higher than the viscous modulus (G") demonstrating the gel structure and the viscoelastic behaviour of both products. An increase in G' and G" was observed over time. This indicates that protein polymerization and molecular structure rearrangements continue to occur during refrigeration, thus making the gels stronger, as also noted by Morr and Ha (1993). In yogurts no significant differences were observed between products one day after production, with or without SMP fortification. However, at the end of the storage period the yogurts with SMP had lower G' and G" values. Once again, the presence of casein micelles that were not denatured in the presence of whey proteins may increase protein heterogeneity and disturb the protein network by producing weaker gels. In the case of desserts, a very stable product was obtained using SMP (Figure 6.7). These results corroborate the higher hardness values (Table 6.8) and WRC (Table 6.7) achieved for these acid gels, indicating that the incorporation of 5% SMP allowed for the production of stronger gel structures immediately after production when the whey proteins and caseins simultaneously undergo denaturation. Lee and Lucey (2010) compared gels made from unheated milk with acid gels made from heat treated milk (at ≥ 78 ºC for 30 min) and found higher G' values for the latter due to the increase in the covalent cross-linking of proteins after denaturation, especially in caseins. In desserts produced from whey proteins only (0% SMP), an increase in G' and G" was observed over time. However, in this case the final values for G' and G" after 21 days of storage were lower than those obtained for the yogurts, which is also confirmed by the lower viscosity values (Figure 6.8).

It has been noted that the rate of protein thermal denaturation influences gel rheological characteristics (Damodaran, 1996; Singh, 2003). In general, firmer gels are produced when the velocity of thermal denaturation is lower and proteins have enough time to react with each other and establish a well-ordered network. The thermal treatment in dessert type acid gels follows these assumptions (Figure 6.3), since it occurs over 30 min in glass cups that have a higher heat capacity and may promote slow heat transfer. In the case of the yogurt type acid gels, the thermal treatment is rapid (5 min) and the proteins do not have enough time to rearrange, thus leading to weaker structures. Dannenberg and Kessler (1988) and Sodini et al. (2006) have also stated that the extent of whey protein denaturation during heat treatment affects functionality in terms of the viscosity of acid milk gels. From our results, in the case of SMP incorporation only, it was observed that the more intense the heat treatment was (in the case of desserts), the higher the complex viscosity (η^*) values were (Figure 6.8). Without the presence of caseins (0% SMP) the viscosity of the desserts was in the same range of magnitude as the yogurt viscosity, which may

indicate that under these conditions the heat treatment does not seem to be so influential. Over time, the viscosity of the samples increased, although for yogurts with or without SMP did not vary as greatly, on either the 1st or the 21st day of storage, as it did in the case of desserts.

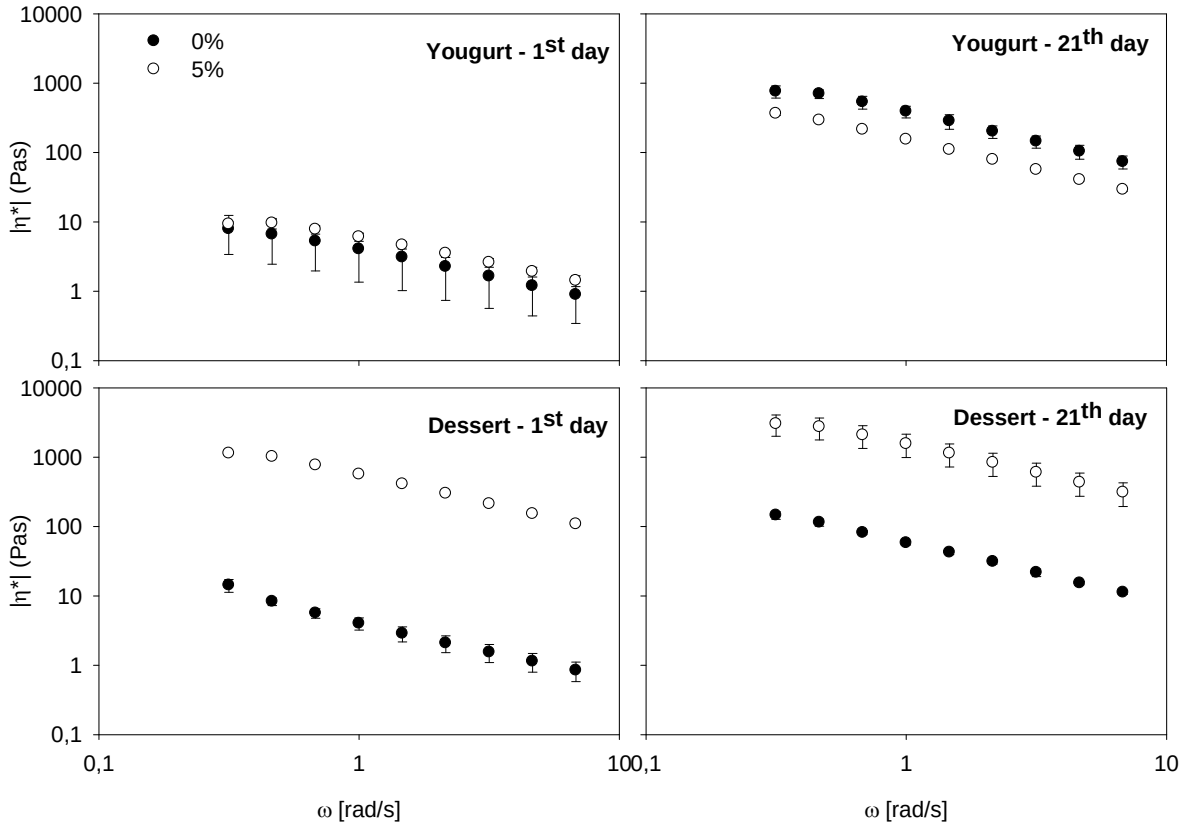


Figure 6.8 Complex viscosity (η*) of LWPC yogurts and desserts with 0% (●) and 5% (○) of SMP incorporation during storage.

6.4 Conclusions

LWPC is a suitable primary raw material for producing heat-induced gels and acid-induced gels. This may have an important impact on food characteristics, such as improved flavour, colour and costs, particularly in the case of heat sensitive foods that may be subjected to non-thermal gelation methods. Depending on the production process used to manufacture LWPC and gels, specific products were achieved in terms of chemical and rheological properties, which can be selected according to the desired food application. Non-defatted LWPC produced stronger heat-induced gels with a continuous microstructure network, whereas defatted and diafiltrated LWPC

produced gels in which the microstructure had a more open matrix network formed by aggregates. Increasing the protein concentration favours the water retention capacity and viscoelastic behaviour of gels. However, the pH had a significant influence on gelation by determining the balance between the forces of attraction and repulsion among proteins. The acid-induced gels produced by lactic bacteria were weaker than the ones chemically acidified by GDL. Cold storage and the fortification of acid gels with SMP improved the rheological properties, G', G" and viscosity.

These applications allow for the production of high value and highly nutritional dairy products as a low cost alternative to the use of powdered products. The use of more than one gelation mode is another important option that can be applied in the food industry to meet the modern consumer's demand for both safety and satisfaction.

References

Aguilera, J. M. 1995 Gelation of whey proteins. Food Technology 49 (10) 83-89

Amatayakul T, Sherkat F and Shah NP (2006) Syneresis in set yogurt as affected by EPS starter cultures and levels of solids. International Journal of Dairy Technology, 59(3), 216-221.

AOAC (1980) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 16.032. Total Solids, Method I - Official Final Action, 13th ed., Washington DC, USA.

AOAC (1995) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 930.30. Ash of dried milk, 16th ed., Gaithersburg, MD, USA.

Aziznia S, Khosrowshahi A, Madadlou A and Rahimi J (2008) Whey protein concentrate and gum tragacanth as fat replacers in nonfat yogurt: chemical, physical, and microstructural properties. Journal of Dairy Science, 91(7), 2545-2552.

Bell IVE (1995) Preliminary studies on the gelation processes of fermented and GDL-acidified bovine and caprine milk systems. International Journal of Dairy Technology, 48(4), 112-116.

Bhullar YS, Uddin MA and Shah NP (2002) Effects of ingredients supplementation on textural characteristics and microstructure of yoghurt. Milchwissenschaft, 57, 328-332.

Blecker C, Paquot M and Deroanne C (2000) Gelling properties of whey proteins after enzymic fat hydrolysis. Journal of Food Science, 65, 561-563.

Bordenave-Juchereau S, Almeida B, Piot JM and Sannier F (2005) Effect of protein concentration, pH, lactose content and pasteurization on thermal gelation of acid caprine whey protein concentrates. Journal of Dairy Research, 72(1), 34-38.

Boye JI, Alli I, Ismail AA, Gibbs BF and Konishi Y (1995) Factors affecting molecular characteristics of whey protein gelation. International Dairy Journal, 5(4), 337-353.

Boye JI, Alli I, Ramaswamy H and Raghavan VGS (1997) Interactive effects of factors affecting gelation of whey proteins. Journal of Food Science, 62, 57-65.

Brandenberg AH, Morr CV and Weller CL (1992) Gelation of commercial whey protein concentrates: effect of removal of low-molecular-weight components. Journal of Food Science, 57(2), 427-432.

Bryant CM and McClements DJ (1998) Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey, Trends Food Science Technology, 9, 143-151.

Bryant CM and McClements DJ (2000) Influence of NaCl and CaCl2 on cold-set gelation of heat-denatured whey protein. JFS: Food Chemistry and Toxicology, 65(5), 801-804.

Casper JL, Wendorff WL and Thomas DL (1999) Functional properties of whey protein concentrates from caprine and ovine specialty cheese whey, Journal Dairy Science, 82, 265-271.

Cavallieri ALF, Costa-Netto AP, Menossi M and Cunha RLD (2007) Whey protein interactions in acidic cold-set gels at different pH values. Le Lait, 87(6), 535-554.

Chantrapornchai W, McClements DJ (2002) Influence of NaCl on optical properties, large-strain rheology and water holding capacity of heat-induced whey protein isolate gels. Food Hydrocolloids, 16, 461-6.

Damodaran DG (1996) Functional properties. In: S Nakai and HW Modler, Food proteins, properties and characterization. New York: VCH Publishers Inc.

Dannenberg F and Kessler H-G (1988) Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 1. Syneresis. Milchwissenschaft, 43, 632-635.

de Wit JN (1989) Functional properties of whey proteins. In: PF Fox (Ed.), Developments in dairy chemistry. New York: Elsevier Applied Science, USA.

Diáz O, Pereira CD and Cobos A (2006) Rheological properties and microstructure of haet-induced gels of oven whey protein concentrates obtained from clarified cheese whey. Milchwissenschaft, 61(2), 193-196.

Díaz O, Pereira CD and Cobos A (2009) Aplicaciones de los concentrados y aislados de proteínas de lactosuero de la industria alimentaria. Alimentaria - Investigacion, Tecnologia e Seguridad, 400, 108-115.

Estrada BS (2010) Aprovechamiento de suero de quesería de origen caprino mediante la obtención de concentrados de proteínas séricas y subprodutos de clarificatión. Estudio de sus propreidades tecnológicas. Tesis Doctoral, Faculdad de Ciencias, Campus de Lugo - Universidad de Santiago de Compostela, Lugo Espanha.

Fox PF and McSweeney PLH (1998) Dairy Chemistry and Biochemistry. Blackie Academic & Professional, 1st edition, London, UK ISBN 0412720000.

Gauche C (2007) Polimerização de proteínas do soro de leite por transglutaminase e propriedades físicas de iogurte elaborado após tratamento enzimático. Pós-Graduação em Ciência dos Alimentos, Florianópolis, Brasil.

Geara C (1999) Study of the Celation of Whey Protein Isolate by FTIR Spectroscopy and Rheological Measurements. Master thesis. Department of Food Science and Agricultural Chemistry. Montreal: Macdonald Campus of McGill University.

Harwalkar VR and Kalab M (1980) Milk gel structure. XI. Electron microscopy of glucono-δ-lactone-induced skim milk gels. Journal of Texture Studies, 11, 35-49.

Heino A, Ussi-Rauva J, Rantamaki P and Tossavainen O (2007) Functional properties of native and cheese whey protein concentrate powders. International Journal of Dairy Technology, 60(4), 277-285.

Hong Y-H and Creamer LK (2002) Changed protein structures of bovine [beta]-lactoglobulin B and [alpha]-lactalbumin as a consequence of heat treatment. International Dairy Journal, 12(4), 345-359.

Hongsprabhas P, Barbut S, and Marangoni AG (1999) The structure of cold-set whey protein isolate gels prepared with Ca^{+2}. Lebensmittel-Wissenschaft und-Technologie, 32, 196-202.

ISO 8968-1:2001 Milk-determination of nitrogen content Part 1: Kjeldahl method.

Joseph MSB and Mangino ME (1988) The effect of milk fat globule membrane on the foaming and gelation properties of β-Lactoglobulin solutions and whey protein concentrates. Australian Journal of Dairy Technology, 43, 9-11.

Ju ZY and Kilara A (1998b) Effects of preheating on properties of aggregates and of cold-set gels of whey protein isolate. Journal of Agricultural and Food Chemistry, 46, 3604-3608.

Kalab M, Allan-Wojtas P and Phipps-Todd BE (1983) Development of microstructure in set-style non fat yoghurt – a review. Food Microstructure, 2, 51-66.

Kim YA, Chism GW and Mangino ME (1987) Determination of the beta-lactoglobulin, alfa-lactalbumin and bovine serum albumin of whey protein concentrates and their relationship to protein functionality. Journal of Food Science, 52, 124-127.

Kinsella JE, Rector DJ and Phillips LG (1994) Physicochemical properties of proteins: texturization via gelation, glass and film formation. In: R Yada, RL Jackman and JL Smith (Eds.) Protein Structure-Function Relationship in Foods. Cambridge: Blackie Academic.

Kitabake N, Doi E (1993) Denaturation and texturization of food protein. In: Kokini JL, Ho CT Karwe MV (Eds.) New York: Marcel Dekker, Inc.

Kuhn PR and Foegeding EA (1991) Mineral salt effects on whey protein gelation. Journal of Agricultural and Food Chemistry, 39(6), 1013-1016.

Lee WJ and Lucey JA (2010) Formation and Physical Properties of Yogurt. Asian-Australian Journal Animal Science, 23(9), 1127-1136.

Li J and Guo MR (2006) Effects of polymerized whey proteins on consistency and syneresis of probiotic goat`s milk yogurt. Journal Food Science, 71, C34-38.

Livney YD, Corredig M, Dalgleish DG (2003) Influence of thermal processing on the properties of dairy colloids, Current Opinion in Colloid & Interface Science, 8, 359-364.

Lorenzen PC and Schrader K (2006) A comparative study of the gelation properties of whey protein concentrate and whey protein isolate. Le Lait, 86, 259-271.

Lucey JA, Munro PA and Singh H (1999) Effects of heat treatment and whey protein addition on the rheological properties and structure of acid skim milk gels. International Dairy Journal, 9, 275-279.

Lucey JA, Tamehana M, Singh H and Munro PA (1998c) Effects of interactions between denaturated whey proteins and casein micelles on the formation and rheological properties of acid skim milk gels. Journal of Dairy Research, 65, 555-567.

Lupano CE, Dumay E and Cheftel JC (1992) Gelling properties of whey protein isolate: influence of calcium removal by dialysis or diafiltration at acid or neutral pH. International Journal of Food Science and Technology, 27, 615-628.

Mangino ME (1984) Physicochemical aspects of whey protein functionality, Journal of Dairy Science, 67(11), 2711-2722.

Mangino ME (1992) Gelation of whey protein concentrates. Food Technology, 46(1), 114-117.

Martín-Diana AB, Janer C, Peláez C and Requena T (2004) Effect of milk fat replacement by polyunsaturated fatty acids on the microbiological, rheological and sensorial properties of fermented milks. Journal of the Science of Food and Agriculture, 84(12), 1599-1605.

Morr CV (1992) Improving the texture and functionality of whey protein concentrate. Food Technology, 46(1), 110-113.

Morr CV and Foegeding EA (1990) Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report. Food Technology, 44, 100-112.

Morr CV and Ha EYW (1993) Whey protein concentrates and isolates: Processing and functional properties. Critical Reviews of Food Science and Nutrition, 33, 431-476.

Mulvihill DM (1992) Production, functional properties and utilization of milk protein products. Advances Dairy Chemistry, 1, 369-404.

Mulvihill DM and Kinsella E (1988) Gelation of β-lactoglobulin: effects of sodium chloride and calcium chloride on the rheological and structural properties of the gels. Journal of Food Science, 53, 231-236.

Mulvihill DM and Kinsella JE (1987) Gelation characteristics of whey proteins and beta-lactoglobulin. Food Technology, 41(9), 102-111.

NP 2105:1983 Queijos. Determinação do teor de matéria gorda. Técnica de Van Gulik. Processo corrente. Comissão Técnica - 32, 1ª edição, Portugal.

NP 469:2002 Norma Portuguesa. Leites. Determinação da matéria gorda (técnica de Gerber). 3ª ed., Lisboa, Portugal.

NP 470:1983 Norma Portuguesa. Leites. Determinação da acidez. Lisboa, Portugal.

Oakenfull D, Pearce J and Burley RW (1997) Protein gelation. In: S. Damodaran and A. Paraf (Eds.) Food Proteins and Their Applications. New York: Marcel Dekker.

Paulsson M, Dejmek P and van Vliet T (1990) Rheological Properties of Heat-Induced β-Lactoglobulin Gels. Journal of Dairy Science, 73, 45-53.

Phadungath C (2010) Basic measurement for food texture. Texture Technologies Corp - Texture Profile Analysis. < http://www.texturetechnologies.com/texture_profile_analysis.html>.

Pintado ME, Lopes da Silva JÁ and Malcata FX (1999) Comparative Characterization of Whey Protein Concentrates from Ovine, Caprine and Bovine Breeds. Lebensmittel-Wissenschaft und-Technologie, 32(4), 231-237.

Puvanenthiran A, Williams RPW and Augustin MA (2002) Structure and visco-elastic properties of set yoghurt with altered casein to whey protein ratios. International Dairy Journal, 12, 383-391.

Robin O, Turgeon S and Paquin P (1993) Functional properties of milk proteins. En Y.H. Hui, Dairy science and technology handbook. Vol. 1. Principles and properties. New York: VCH Publishers Inc.

Schmidt RH, Packard VS and Morris HA (1984) Effect of processing on whey protein functionality. Journal Dairy Science, 67, 2723-2733.

Singh H (2003) Functional properties. In: Roginski H, Fuquay JW and Fox PF (Ed.), Encyclopedia of dairy sciences. London: Academic Press (Elsevier Science).

Sodini I, Lucas A, Oliveira MN, Remeuf F and Corrieu G (2002) Effect of milk base and starter culture on acidification, texture, and probiotic cell counts in fermented milk processing. Journal of Dairy Science, 85(10), 2479-2488.

Sodini I, Mattas J and Tong PS (2006) Influence of pH and heat treatment of whey on the functional properties of whey protein concentrates in yoghurt. International Dairy Journal, 16(12), 1464-1469.

Sodini I, Remeuf F, Haddad S and Corrieu G (2004) The relative effect of milk base, starter, and process on yogurt texture: a review. Critical Reviews Food Science Nutrition, 44, 113-137.

Sullivan ST, Khan SA and Eissa AS (2008) Whey proteins: functionality and foaming under acidic conditions. In: CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

Tamime AY and Robinson RK (2007) Yoghurt: science and technology. Cambridge, England: Woodhead Publishing Limited.

Tang Q, McCarthy OJ and Munro PA (1994) Oscillatory rheological comparison of the gelling characteristics egg white, whey protein concentrates, whey protein isolates and β-lactoglobulin. Journal of Agricultural and Food Chemistry, 42(10), 2126-2130.

Tang Q, McCarthy OJ and Munro PA (1995) Oscillatory rheological study of the effects of pH and salts on gel development in heated whey protein concentrate solutions. Journal of Dairy Research, 62, 469-477.

Totosaus A, Montejano JG, Salazar JA and Guerrero I (2002) A review of physical and chemical protein-gel induction. International Journal of Food Science and Technology, 37, 589-601.

Vardhanabhuti B, Foegeding EA, McGuffey MK, Daubert CR and Swaisgood HE (2001) Gelation properties of dispersions containing polymerized and native whey protein isolate. Food Hydrocolloids, 15(2), 165-175.

Vargas M, Pastora C, Chiralta A, McClementsb DJ and González-Martíneza C (2008) Recent advances in edible coatings for fresh and minimally processed fruits. Critical Reviews in Food Science and Nutrition, 48(6), 496-511.

Yamul DK and Lupano CE (2003) Properties of gels from whey protein concentrate and honey at different pHs. Food Research International, 36(1), 25-33.

Zayas JF (1997) Functionality of proteins in food. Berlin: Springer-Verlag.

Part D.

Whey protein nonconventional applications

Chapter 7

Whey protein-based films and coatings

Abstract

Increasing concerns about reducing the use of limited resources and substituting them with renewable alternatives in order to help resolve environmental issues have been the stimulus for the production of edible and biodegradable films that can improve product quality and reduce waste disposable problems (Weber et al., 2002). Whilst many biopolymers such as wheat, gluten, soy protein, starch, cellulose and casein have been studied as edible films and coatings, this chapter reviews the existing literature on whey protein-based film and coatings. The main topics are: (1) definitions of edible and biodegradable films and coatings and their composition; (2) the identification of some available methods for the production of protein films and coatings and the

properties used to evaluate their characteristics; (3) a summary of their barrier, mechanical and optical properties; (4) a discussion of the existing and potential applications for whey protein films and coatings as active food systems.

7.1 Definitions and functions

The use of the term *edible film* or *edible coating* depends on the research or application in question. Films are thin continuous sheets formed from polymer matrixes which, owing to their cohesiveness and stand-alone physical integrity to, are normally produced without any specified use. Their thickness typically ranges from 0.05 to 0.25 mm. Depending on their thermal and surface properties they can be used as covers, wraps, or separation layers to produce bags, casings or capsules. The main purpose of edible films is to control the mass transfer of gases (e.g. O_2, CO_2), aromas, water and oil from or into food, thus preserving its quality and increasing its shelf life. Edible coatings involve the formation of a film directly on the surface of the object they are intended to protect or enhance in some manner. For this reason, coatings are typically thinner then films and remain on the product throughout its use and consumption, whereas films should be removed before use. Protein coatings can enhance the nutritional value of foods, not only by increasing their protein content but through the incorporation of nutritional supplements. Additionally, they can improve the appearance and quality of a product and make it more appealing to consumers by adding gloss or colour or preventing microbiological development. Whereas films are produced to study their mechanical, barrier and surface properties, coatings are studied as one type of application of edible films (Krochta, 2002; Dangaran and Krochta, 2008).

Protein based films and coatings can be *edible* and/or *biodegradable* depending on their formulation (Li and Chen, 2000), the formation method or modification treatments they undergo. If the compounds and additives involved in the formulation are food-grade (e.g., proteins, plasticizers, salts, enzymes, antioxidants, acids and solvents) and the film formation is only achieved by heating, pH modification, enzymatic treatment and water/solvent removal, the film or coating produced is edible. Edible films and coatings are both biodegradable. However, if the proteins react with other chemicals before or during the formation of the film or coating (e.g. chemical cross-linking), or when non-edible compounds are added to the formulation, they are no longer edible (Krochta, 2002). The challenge for biodegradable food packaging development is to ensure that biodegradation only begins after the film or coating has safely and effectively served its purpose within the prescribed time for product consumption.

In order to meet the desired requirements, films or coatings must be carefully selected (formulation) on the basis of the chemical composition of the food product matrix, the storage conditions and the characteristics to be maintained or improved. It is possible to identify four specific functions (Figure 7.1) for films and coatings used in food systems: safety, appearance and sensorial improvement, reduction or prevention of mass transfer, and nutritional enhancement.

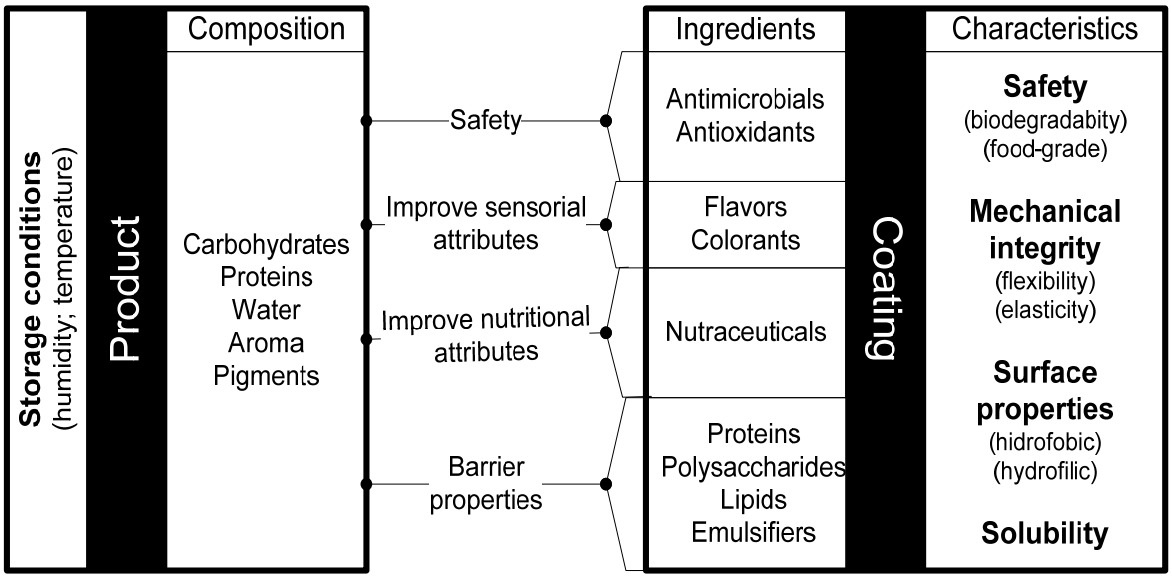


Figure 7.1 Functions and attributes of edible coatings applied to food products.

Safety, mechanical integrity (flexibility and elasticity), good surface properties and coating solubility are the main features that should be considered when selecting coatings for particular applications.

7.2 Composition of films and coatings

Proteins, polysaccharides and lipids are the main materials available for producing edible films and coatings; although the first two are the most widely used and considered the basis of formulations. It is common to find certain plasticizers used to reduce brittleness, surface-active agents to aid film or coating adhesion and flavours and colourings to improve sensorial attributes. In some cases, especially active food packaging, other compounds are also included, such as antioxidants, antimicrobials, nutraceuticals, fatty acids and preservatives.

Proteins

Proteins are polymer chains of amino acids bonded together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. Although the amino acids are similar in terms of containing an amino group ($-NH_2$) and a carboxyl group (-COOH) attached to a central carbon atom, they have a specific side group that gives then a unique character. The side group can be non-polar (hydrophobic), polar (hydrophilic) and positively or negatively charged at pH 7 (Cheftel et al., 1985). It is possible to distinguish various structures in proteins. The primary structure is related to the sequential order of amino acids. The secondary structure, based on hydrogen bonding, van der Walls, electrostatic, hydrophobic and disulfide cross-link interactions, allows the protein to assume different structures in the polymer chain. The relative organisation of the secondary structure of proteins based on the same type of interactions, forms globular, fibrous or random protein structures that correspond to the tertiary structure. Finally, the quaternary structure occurs when whole proteins interact with each other to provide unique structural or biological activity (Krochta, 2002). Various chemical and physical agents such as lipid interfaces, acids, alkalis, metal ions, heat, pressure and irradiation can modify the secondary, tertiary and quaternary structure of proteins (Cheftel et al., 1985). Protein configuration, protein interactions and film properties are optimised through a combination of such agents.

The proteins used to produce films and coatings may be derived from animal or vegetal sources. The former include collagen, gelatin, myofibrillar proteins, keratin, egg white protein, casein and whey proteins. Corn zein, wheat gluten, soy protein, peanut and cotton protein are examples of vegetal materials. Although collagen and gelatin have been widely employed in meat products (such as casings and wraps) the use of proteins as the basis for edible films and coatings has not been studied as comprehensively as polysaccharides. More recently, whey proteins have attracted increasing interest, not only because they can be obtained from renewable resources (dairy industry by-products) but also due to their recognised oxygen barrier properties (Baldwin et al., 1995).

The main factor responsible for the characteristics of edible protein-based films and coating is the various chemical compositions of whey protein products (WPC and WPI), mainly with regard to the protein amounts and types discussed in Chapters 2 and 3. According to the type and sequence of membrane processing technology, the proportions of whey proteins are not preserved either in WPC or WPI, which is generally richer in β-Lg and α-La but proportionally poorer in imunoglobulins, lactoferrin and other minor proteins. However, the presence of fat,

lactose, salts (NaCl, KCl and $CaCl_2$), non-nitrogen compounds and vitamins can also influence their performance as edible coating materials (Banerjee and Chen, 1995). One extra advantage cited for whey proteins is their intrinsic bioactive properties, due to the presence of enzymes such as lactoperoxidase and lactoferrin.

Polysaccharides

The most common polysaccharide film-forming materials studied include starch and its derivatives, cellulose derivatives, gums (e.g. arabic gum, guar gum and xanthan gum), agar, alginate, carrageenan, chitosan/chitin, pectin, gellan and pullulan (Liu, 2005; Lacroix and Le Tien, 2005). Their wide variety of structures allows different packaging alternatives to be developed during food processing. Films and coatings based on polysaccharides offer good mechanical strength and integrity and perform well in terms of gas, aroma and oil barriers. However, their hydrophilic nature is responsible for poor moisture barrier efficiency.

Proteins can be combined with polysaccharides to modify and enhance the mechanical and barrier properties of films and coatings (Le Tien et al., 2000; Krochta, 2002; Erdohan and Turhan, 2005; Gounga et al., 2007). The use of polysaccharides in protein film-forming solutions increases their viscosity. This behaviour is used in coating formation in order to improve adherence between the food and the coating.

Lipids

Lipids and resin materials are not polymers and they do not form cohesive stand-alone films. Their poor film-forming ability and weak mechanical properties restrict their use as coatings to a few applications, such as fruit and meat products (Ramos and Malcata, 2011). However, they can be included in the film matrix to provide some desirable characteristics such as gloss or nutritional value or to increase moisture barriers by reducing water vapour permeability, owing to their hydrophobicity (Morillon et al., 2002; Fernández et al., 2007). The most popular lipids included in edible coatings are waxes (e.g., bees wax, carnauba wax, candelilla wax), triglycerides (e.g., milk fat fractions), acetylated monoglycerides, oils (vegetable and mineral oil), fatty acids and surfactants. The edible resins are shellac and terpene resin. It is also common the production of composite films with a lipid layer supported by a protein or polysaccharide layer (Krochta, 2002).

Plasticizers

When proteins or polysaccharides are used as the base material for manufacturing films and coatings, plasticizers are usually required because the final film structures are normally stiff and brittle (Ramos, 2011). The result of adding plasticizer to films or coatings is a decrease in protein chain-to-chain interaction, thus increasing free volume and chain movements (Daniels, 1989). As a result of this reduction, the protein glass transition temperature (Tg) is lower, film elongation (stretchiness) and flexibility increase (lowering of the film elastic modulus), and film strength decreases. The most negative impact associated with the use of plasticizers is the reduced capacity of the film to act as a barrier to moisture, aroma, oxygen, antioxidants, bacteriocines, oils and other solutes (McHugh et al., 1994; Bodnar et al., 2007). There are two types of plasticizers: internal and external plasticizers. Internal plasticizers form part of the polymer molecule by copolymerization in the polymer structure or by reaction with the polymer basis, whereas external plasticizers are substances that are added to polymers. In this case they interact with polymers and produce swelling without any chemical reaction taking place (Sothornvit and Krochta, 2005).

The most common plasticizers used in whey protein-based edible films are monosaccharides or disaccharides (e.g. sucrose), polyols (glycerol, sorbitol, polyethylene glycol and propylene glycol), lipids (e.g. fatty acids) and, of course, water. However, water can easily be lost by dehydration at low relative humidity (Sothornvit and Krochta, 2005). The film moisture content is also affected by the relative humidity (RH) of the surroundings and has a significant effect on film properties. The use of hydrophilic plasticizers attracts additional moisture to the film, in particular increasing water activity, which can reduce the shelf life of food products (Krochta, 2002).

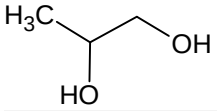
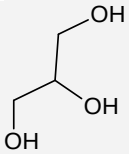
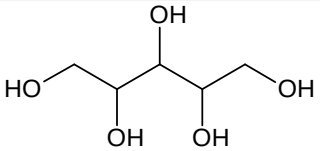
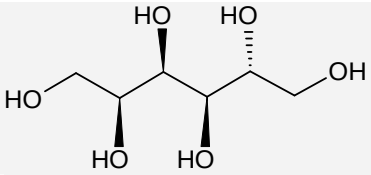
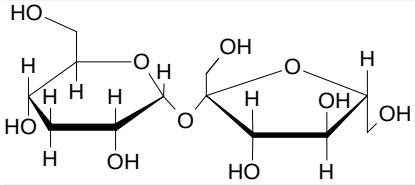
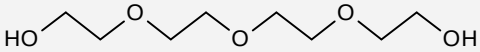
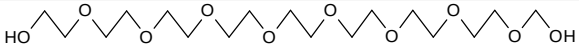
The plasticizers used in edible whey protein films and coatings referred to in the literature are presented in Table 7.1. Their various chemical structures, molecular weight and number of hydroxyl groups (involved in hydrogen bonds with biopolymers) are responsible for the different solubility, mechanical and barrier properties of the films, which will be discussed later.

It is also possible to incorporate lipids into protein-based films and this may provide a plasticizer effect (Shellhammer and Krochta, 1997a; Anker et al., 2002; Reinoso et al., 2007). The most commonly used fatty acids are presented in Table 7.2. Myristic, palmitic, stearic, arachidic, behenic and lauric acids have been studied as plasticizers in WPI films (Sherwin et al., 1998) and

WPI-lipid emulsions films (McHugh and Krochta, 1994). Normally, larger chain length fatty acids reduce moisture permeation through the film.

Combining a variety of plasticizers is another possible way of obtaining average film properties. For example, it is possible to increase flexibility and reduce water vapour permeability by using a polyol and a fatty acid simultaneously.

Table 7.1 Plasticizers commonly used in whey protein edible films and coatings, molecular weight (Mw), chemical structure and applications (adapted from Sothornvit and Krochta, 2005).

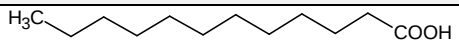
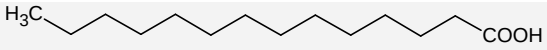
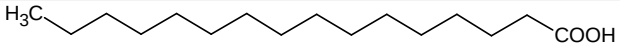
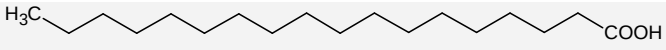
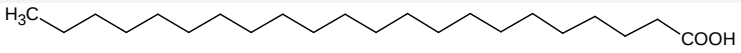
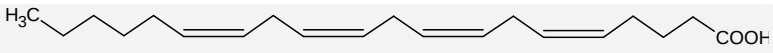
Plasticizer	Mw (g/mol)	Chemical structure	Ref.
Propylene glycol (PG)	$C_3H_8O_2$ 76		1, 6
Glycerol (Gly)	$C_3H_8O_3$ 92		1, 2 3, 4 5, 6 7
Xylitol (Xy)	$C_5H_{12}O_3$ 152		4
Sorbitol (Sob)	$C_6H_{14}O_6$ 182		1, 2 3, 4 5, 7
Sucrose (Suc)	$C_{12}H_{22}O_{11}$ 342		1, 2 3, 6
Polyethylene glycol 200 (PEG 200)	$H(OCH_2\text{-}CH_2)_8OH \approx 200$		1, 2 3
Polyethylene glycol 400 (PEG 400)	$H(OCH_2\text{-}CH_2)_8OH \approx 400$		1, 6

(1) Sothornvit and Krochta, 2001; (2) Lin and Krochta, 2003; (3) Hong and Krochta, 2003; (4) Shaw et al., 2002; (5) Kim and Ustunol, 2001a, 2001b; (6) Lee et al., 2002a; (7) McHugh and Krochta, 1994.

Edible whey protein films and coatings (produced mainly from WPI) usually require a plasticizer content ranging from 10% to 60% (w/w). However, these levels depend on the desired properties of the films and the choice of plasticizer (McHugh and Krochta, 1994b; McHugh et

al., 1994; Sothornvit and Krochta, 2005). When WPCs are used as protein source, lower amounts of plasticizers are required (Banerjee and Chen, 1995). In this case, the non-protein compounds (e.g. lactose and fat) present in the formulation act as plasticizers.

Table 7.2 Fatty acids used in whey protein edible films and coatings (adapted from Sothornvit and Krochta, 2005).

Fatty acid	Mw (g/mol)	Chemical structure	Ref.
Lauric	$C_{12}H_{24}O_2$ 200		1, 2
Myristic	$C_{14}H_{28}O_2$ 228		1, 2
Palmitic	$C_{16}H_{32}O_2$ 256		1, 2
Stearic	$C_{18}H_{36}O_2$ 284		1, 2
Behenic	$C_{22}H_{44}O_2$ 340		2
Arachidic	$C_{20}H_{40}O_2$ 312		1

(1) Sherwin et al., 1998; (2) McHugh and Krochta, 1994a.

Other additives

Some proteins are sufficiently surface-active to form well dispersed composite films or provide good surface wetting and adhesion. However, when the molecules do not have this property an emulsifier is necessary. Surfactants such as sodium dodecyl sulphate (SDS) may also be used to enhance mechanical properties (Fairley et al., 1996). Edible films and coatings have the potential to enhance food safety, nutrition and quality by incorporating antimicrobial compounds (Seydim and Sarikus, 2006; Min et al., 2008; Lee et al., 2008; Zinoviadou et al., 2009), providing antioxidants (Lee et al., 2003; Pérez-Gago et al., 2006; Min and Krochta, 2007), nutraceuticals such as essential fatty acids, flavours (Lanciotti et al., 2004) and colorants.

7.3 Film and coating formation

Film formation and properties depend on two types of interaction; cohesion (attraction forces between polymer molecules) and adhesion (attraction forces between the film and substrate). In this section more attention will be paid to the first phenomenon in which polymer properties such as molecular weight, polarity and chain structure are relevant (Sothornvit and Krochta, 2005).

Edible protein films are produced by two different mechanisms, namely wet and dry processing. The wet process, also called the solvent casting method, is the most widely used method. In this case polymers are dispersed or solubilised into a liquid phase (usually water or aqueous ethanol solutions), cast, and dried by solvent evaporation. The wet process is preferred for applying coatings in liquid form directly onto food products by dipping, brushing, or spraying. In the dry process, which is now attracting attention, films are produced in lower water contents by extrusion then compression-moulded, using the thermoplastic properties of polymers.

7.3.1 Solvent casting

When films and coatings are produced by solvent casting, proteins are firstly dissolved into the solvent. With a few exceptions (e.g. keratin and corn zein) the majority of proteins used in film production are water soluble (Table 7.6). Plasticizers, lipids, polysaccharides or emulsifiers are added during this phase and homogenisation takes place. This is followed by a pH adjustment, if necessary, or the induction of protein cross-linking (by heating, irradiation or chemical and enzymatic treatments) in order to enhance film formation. Finally, the protein coating or film is formed by applying the prepared formulation to the desired product surface or casting, respectively, and allowing the solvent to evaporate. The formation of food coatings is achieved by solvent drying, after dipping, spraying or enrobing the food in the film-forming solution. In film production, the film-forming solution should be released from the casting surface after drying.

In terms of equipment, the solvent casting of whey proteins can be performed on various scales. From a research point of view, a very simple methodology is used as it is effective and cost-efficient. Usually, the film-forming solutions are manually spread into levelled teflon plates or Petri dishes and left to dry at ambient conditions. On a larger scale, whey protein films are mechanically produced in batch or continuous coaters at fixed thicknesses (Dangaran and Krochta, 2008).

Protein network formation

As described in Chapter 2, native whey proteins are globular proteins containing most of the hydrophobic and SH groups protected in the interior of the molecule. However, they have a good film-forming ability and can form transparent solvent-cast films in which cohesion is governed through electrostatic interactions, hydrogen bonds and van der Walls forces that occur

between protein chains as the water evaporates (Pérez-Gago et al., 1999). In this case a more randomised film structure is achieved.

In order to improve the protein film network, the method most widely studied and applied is heat denaturation and the subsequent cross-linking of whey proteins chains (Dalgleish et al., 1997; Puyol et al., 2001; Hong and Creamer, 2002). Heating modifies the three-dimensional protein structure, exposing the SH groups and hydrophobic groups (Shimada and Cheftel, 1998) and promoting S-S bonding and hydrophobic interactions (McHugh and Krocha 1994b).

A film-forming solution should be neither a solution nor a gel. Extended structures formed by the unfolding of protein molecules are required for film formation. Amorphous three-dimensional structures formed by non-covalent interactions among protein chains stabilise the films (Cuq et al., 1998). The distance between polymer molecules (concentration) will determine the solution and the gel stage. The polymer concentrations have to be optimised to reach the ideal transition point between solution and gel for the film-forming solution (Hettiarachchy and Eswaranandam, 2005).

It is possible to produce WPI films by heating 8-12% (w/w) protein solutions at temperatures between 75 ºC and 100 ºC. Lower protein levels (5% (w/w)) can also form good whey protein-edible films if the amount of solids per casting surface is kept constant. (Pérez-Gago et al., 1999). With protein contents higher than 12% (w/w), gelation occurs during heating and no film is obtained. The optimal conditions for preparing WPI films and coatings were established to be heat denaturation of 10% (w/w) protein solutions at approximately 90 ºC for 30 min (McHugh and Krocha, 1994b). The heat denaturation and subsequent cross-linking of β-Lg is well documented (de Wit and Klarenbeek, 1983; Dalgleish et al., 1997; Galani and Apenten, 1999). Above 40 ºC, at neutral pH the β-Lg dimer dissociates. At around 60-65 ºC the thiol group of cysteine 121 is exposed, due to α-helix conformational changes. Finally, polymerization takes place through the formation of new disulfide bond intra or intermolecularly between free cysteine residues if the temperature remains higher than 60 ºC and the pH rises above 6.8. In addition to cross-linking, non-covalent aggregation (hydrophobic, ionic and van der Wall interactions) between the new exposed groups also occurs, increasing as the pH decreases towards the isoelectric point of β-Lg (Kinsella and Whitehead, 1989).

The cross-linking of whey proteins can also be induced chemically or enzymatically. The main disadvantage in using formaldehyde, glutaraldehyde, tannic and lactic acid to cross-link whey

proteins through lysine residues is the fact that the final films will no longer be edible, as well as the toxic properties of the cross-linking agents (Galietta et al., 1998; Lee and Rosenberg, 1999). Alternatively, by using transglutaminase (a food-grade enzyme) to promote cross-linking, the final films remain edible. This enzyme is Ca^{2+} dependent and uses the acyl transferase mechanism to link the γ-carboxyamide (acyl donor) of a glutamine residue to the γ-amine (acyl acceptor) of lysine residues along protein chains (Mahmoud and Savello, 1992). α-La, β-Lg and α-La/β-Lg mixtures showed a molecular increase after enzymatic treatment, demonstrating an increase in cross-linking.

Irradiation can also be used to induce protein cross-linking and has been applied successfully in casein (Mezgheni et al., 1998; Lacroix et al., 2002). The proposed mechanism is radical polymerization through tyrosine and the formation of bityrosine linkages between protein chains. According to the amino acid distribution of whey proteins, poor tyrosine residues can be observed and in such conditions, irradiation alone cannot be effective. However, the use of other proteins such as casein, or chemicals such as photoinitiators, can increase the molecular weight and change protein film properties. Although this process is mentioned in the literature (Lacroix et al., 2002; Dangaran and Krochta, 2008), no significant work has been done to use this clean technology in the production of whey proteins films and coatings. In Chapter 8, the use of UV irradiation will be discussed in more detail and applied to the modification and production of whey protein films.

Drying conditions

Drying conditions and methods can significantly affect the physical properties and morphology of the films (Denavi et al., 2009; Soazo et al., 2011). These may involve ambient conditions, hot air, infrared or microwave energy. Two drying periods were identified: the constant rate period, during which the major phenomenon is the mass transfer between the film surface and the air, and the falling rate period, when the mass transfer of the solvent is limited by diffusion through the film. Several factors affect the air drying rate, such as air temperature and relative humidity (RH), exposed surface area, air velocity and drying period (Alcantara et al., 1998). Infrared drying requires an energy source and acceptor and also depends on the temperature and shape of the heating and receiving materials, the emissivity of the emitting body and the adsorption of the receiving body (Brennan and Crowell, 1990). Microwaves can penetrate more quickly into films, leading to faster solvent diffusion then air or infrared drying.

7.3.2 Extrusion and compression moulding

Extrusion and compression moulding are common industrial techniques used to form films and containers. Their adaptation to the production of whey protein-based films allows for the mass manufacture of products such as water-soluble pouches and cups for individual servings of various dry ingredients and foods (Balagtas et al., 2003).

Thermoplastic extrusion would be an attractive way to produce protein casings and films, avoiding the need to add and then removing the solvent. Although some researchers suggest that some proteins have thermoplastic behaviour, this property has not been explored much in edible film production (Hernandez et al., 2005, 2006). Solvent evaporation, in the solvent casting method, is time consuming (especially for aqueous based film-forming solutions involving lower drying rates than organic solvents) and energy expensive, due to the cost and maintenance of the drying oven. On the other hand, extrusion is a faster and energy cheaper method that may reduce biopolymer production costs to levels competitive with synthetic polymers. Hernandez et al. (2007) successfully produced extruded, homogeneous, transparent and flexible whey protein sheets using glycerol as a plasticizer (46-52% on a dry basis). The extruder configuration (six heating zones, with a length to diameter ratio of 30:1) and operation conditions (temperatures ranging from 20 ºC to 130 ºC in the heating zones and a screw speed of 250 rpm) allowed for heating denaturation and cross-linking whey protein sheets produced with similar or enhanced mechanical properties compared to solvent casting heat-denatured films. The elongation of the extruded films was not affected by the amount of plasticizer (Hernandez et al., 2006) and had higher values than solvent casting films. The tensile strength was also higher. These authors found that whey protein extruded sheets displayed thermoplastic behaviour that enabled them to be compression-moulded to form thinner films or to be heat-sealed. Depending on the operation temperatures and type of plasticizer, Sothornvit et al. (2003) reported that compression-moulded whey protein films plasticized with water were more brittle and insoluble than those plasticized with glycerol. In the latter, elongation can be increased (from 85% to 94%) by increasing the glycerol contents from 40% to 50%, and tensile strength decreased (from 8 to 4 MPa). Despite these promising results, the properties of extruded and compression-moulded whey protein films need to be better understood (Dangaran and Krochta, 2008; Hernandez and Krochta, 2008).

7.4 Properties of films and coatings

Edible films and coatings are the most interesting systems with the potential to provide the optimal combination of moisture, gases (e.g. O_2, CO_2) and aroma permeability, allied to mechanical integrity and appearance (e.g. colour and transparency) for food or drug products. The same criteria used to evaluate conventional food packaging materials are applied to assess the properties of edible whey protein films and coatings. However, some modifications need to be applied to the classic methods used to evaluate synthetic material properties, mainly due to the marked influence of temperature and RH on the final properties of whey protein films. Barrier properties or permeability (e.g. water vapour, oxygen, carbon dioxide, aroma, oil and light), mechanical properties (e.g. tensile strength, elastic modulus and elongation), surface properties, solubility and optical properties (e.g. colour and transparency) are the most common properties considered when comparing films.

7.4.1 Barrier properties

The challenge in optimising the barrier properties of whey protein-based films and coatings is selecting the protein type (e.g. β-Lg; WPC or WPI), additives (for instance plasticizers or fatty acids) and their proportions in the formulation, the film-formation conditions (temperature and RH) and methods. During this process, other desirable characteristics should be achieved, such as strength, elasticity and solubility, in order to produce the best solution for the desired applications (Krochta, 2002; Morillon et al., 2002).

Diffusion is the primary mechanism that describes the transmission of a substance through a film driven by gradient concentration or pressure between the two film surfaces, mathematically described by Fick's first law (Dangaran and Krochta, 2008). However, during mass transport through a film, two more phenomena occur: permeate adsorption into one side of the film and desorption on the other side of the film. Permeability depends on the diffusion coefficient and permeate solubility in the film matrix (Han and Scanlon, 2005). Various factors have been identified as influencing both the diffusion coefficient and solubility, such as temperature, protein and solute chemistry, glass transition temperature, and the presence of crystalline areas since diffusion occurs in amorphous regions, as well as type and plasticizer content (Hernandez et al., 2000).

The polar nature of proteins determines the barrier properties of protein films. For non-polar substances such as oxygen, aromas and oils, permeability through the films is low, but an increase

in compound polarity leads to an increase in permeability. The use of plasticizers with polar behaviour, such as water, enhances this property.

Water vapour permeability

Water vapour permeability (WVP) is the most widely studied transport property for edible films. Table 7.3 compares the WVP of whey protein films of various compositions and synthetic polymers. In general, according to their hydrophilic nature, whey protein films and coatings have a higher WVP than the synthetic packaging polymers (e.g. polyvinylidene chloride (PVDC), low-density polyethylene (LDPE), polyvinyl chloride (PVC) and cellophane) and edible waxes that are used as moisture barriers in food and drug products. For this reason they are considered only moderate barriers to moisture at best (Dangaran and Krochta, 2008).

One of the critical factors affecting food sensorial quality and shelf life is its moisture content - water activity (a_w) - which also depends on the interactions between water molecules and other ingredients present in food. This property governs many of the chemical (lipid oxidation, Maillard reaction), enzymatic (enzymatic browning) and microbial reactions during food storage, as well as some textural properties (Singh and Singh, 2005; Ramos, 2011). Controlling WVP in edible whey protein films leads to a reduction in a_w changes and consequently modifications to food properties.

The effects of plasticizer content and relative humidity during film formation both have an increasing influence on WVP (Table 7.3). With similar plasticizer contents WVP decreases as the molecular weight of the plasticizer increases due to its lower hygroscopic behaviour (McHugh el al., 1994; McHugh and Krochta, 1994b; Parris et al., 1995; Shaw et al., 2002). Because water acts as a plasticizer, moisture sorption evaluation is important for whey protein films. Coupland et al. (2000) concluded that WPI plasticized with higher glycerol contents and higher RH has greater flexibility. With increasing moisture content, plasticization and swelling of the polymer matrix provides additional binding sites and results in enhanced water sorption at high a_w (Lim et al., 1998).

Fatty acids and other lipids are also incorporated to reduce the effect on increased permeability in whey protein films, as previously mentioned. This is achieved by producing protein laminated films by forming a bilayer of protein and lipid or forming emulsions and composite films by homogenisation of the lipids in the protein-plasticizer film forming solution (Anker et al., 2002; Reinoso et al., 2007). Although laminated films have solid layers of lipids or wax which are

excellent barriers to water, Shellhammer and Krochta (1997b) showed that bilayer composite films tend to delaminate due to the high surface energy between the two components. Another disadvantage is the time consuming two-step technique that is difficult to apply for practical applications (Dangaran and Krochta, 2008). On the other hand the well known emulsifying properties of whey proteins make the emulsification method particular useful for producing lower WVP films with good mechanical properties (Pérez-Gago and Krochta, 2002). McHugh and Krochta (1994a) suggested that decreasing the lipid particle size also decreases WVP. One possible explanation for this is the increase in path tortuosity with the increase in the number of lipid particles for the same lipid amount. Water does not diffuse through lipids, but around them. Pérez-Gago and Krochta (2001) also found that there is a connection between particle size and the amount of lipids. The increase in the protein-lipid interface, promoted by the increased amount of lipids and the consequent protein immobilisation, lowers the free volume for water vapour to diffuse. To reduce the water vapour transmission rate at 100% relative humidity, Gällstedt and Hedenqvist (2002) coated whey protein-based films with an alkyd, a beeswax compound or a nitrocellulose lacquer. The results showed that the beeswax and the nitrocellulose compound provided appropriate water vapour barriers.

Although the addition of lipids and waxes can reduce WVP in whey protein films and coatings, their effect on mechanical properties must also be considered. In terms of applications, whey protein may be more appropriate for food products with a lower or moderate moisture barrier, avoiding condensation on the surface of the product. Another aspect to consider is the opacity that lipid creates in food products, which may result in an unattractive appearance.

Methylcellulose-whey protein films were also evaluated for WVP by Erdohan and Turhan (2005). These films showed lower WVP than those produced simply with methylcellulose and whey proteins, making them a suitable film material for moisture-sensitive food products.

Table 7.3 Water vapour permeability (WVP) and oxygen permeability (OP) according to plasticizer type and amount, at different relative humidity on whey protein-based films (adapted from Pérez-Gago and Krochta, 2002; Krochta, 2002; Sothornvit and Krochta, 2005).

Protein : plasticizer (w : w)	Test conditions T(ºC) / RH(%)	WVP (g mm/m² d kPa)	OP (cm³ µm/m²d kPa)	Ref.
β-Lg:Gly				
(1.70:1 - 3.20:1)	-	-	20-43	1, 2
β-Lg:PG				
(2.06:1 - 3.87:1)	-	-	17-27	1
β-Lg:Sor				
(0.86:1 - 1.62:1)	-	-	3-8	1, 2
β-Lg:Suc				
(0.46:1 - 0.86:1)	-	-	<0.05	1, 2
β-Lg:PEG 200				
(0.78:1 - 1.47:1)	-	-	110-700	1, 2
(1:1)	25 / 0-62	134.64	-	3
β-Lg:PEG 400				
(0.39:1 - 0.73:1)	-	-	1050-2220	1, 2
(1:1)	25 / 0-63	129.6	-	3
β-Lg:Gly				
(1.5:1)	25 / 0-68	132.0	-	4
(2.3:1)	25 / 0-75	93.6	-	4
(5.7:1)	25 / 0-84	55.2	-	4
WPI:Gly				
(1:1 - 2:1)	-	116-144	-	5
WPI:Xy				
(1:1 - 2:l)	-	84-89	-	5
WPI:Sor			-	
(1:1 - 2:l)	-	84-112		5
WPI:PEG 200				
(1:1)	-	134.64	-	3
WPI:PEG 400				
(1:1)	-	129.6	-	3
WPI:Gly				
(1:1)	25 / 0-59	153.6	-	3
(1.5:1)	25 / 0-68	132.0	-	4
(1.6:1)	25 / 0-11	4.8	-	3
(1.6:1)	25 / 0-50	38.8	-	3
(1.6:1)	25 / 0-65	120.0	-	3
(2:1)	23 / 55-73	291	-	10
(2.3:1)	23 / 0-50	-	76.1*	6
(2.3:1)	25 / 0-76	91.2	-	4
(2.3:1)	23 / 0-34	-	20	12
(2.3:1)	23 / 0-46	-	55	12
(2.3:1)	23 / 0-56	-	132	12
(5.7:1)	25 / 0-84	57.6	-	4
(5.7:1)	23 / 0-50	-	18.5*	6

Table 7.3 (Continued)

Protein : plasticizer (w : w)	Test conditions T(ºC) / RH(%)	WVP (g mm/m² d kPa)	OP (cm³ μm/m²d kPa)	Ref.
WPI:Sor				
(1:1)	23 / 0-50	-	8.3*	6
(1:1)	25 / 0-10	4.8	-	3
(1:1)	25 / 0-50	21.6	-	3
(1:1)	25 / 0-65	55.2	-	3
(1:1)	25 / 0-75	84.0	-	3
(1:1)	23 / 50-72	211	-	11
(1.5:1)	23 / 0-50	-	6.0*	6
(1.6:l)	25 / 0-79	62.4	-	3
(2.3:1)	23 / 0-50	-	4.3*	6
(3.5:1)	23 / 0-40	-	0.7*	6
(3.5:1)	23 / 0-70	-	43.3*	6
PVDC	28 / 0-100	0.0192	-	7
	23 / 0-50	-	0.4-5.1*	9
LDPE	28 / 0-100	0.0312	-	7
	23 / 0-50	-	1870*	9
PVC	28 / 0-100	0.6168	-	7
EVOH	-	0.001	0.2	13
Cellophane	38 / 0-90	7.2696	-	8
	23 / 0-50	-	16*	8

*(g mm/m² h kPa); (1) Sothornvit and Krochta, 2000a, 2000b and 2000c; (2) Sothornvit and Krochta, 2001; (3) McHugh et al., 1994; (4) Maté and Krochta, 1996a; (5) Shaw et al., 2002; (6) McHugh and Krochta, 1994a; (7) Shellhammer and Krochta, 1997b; (8) Taylor, 1986; (9) Salame, 1986; (10) Banerjee and Chen, 1995; (11) Anker et al., 1998; (12) McHugh and Krochta, 1994c; (13) Hernandez et al., 2000.

Oxygen permeability

Oxygen permeability is the second most commonly studied transport property in edible polymer films after WVP (Pérez-Gago and Krochta, 2002; Hong and Krochta, 2006). The OP values of protein-based films (Table 7.3) are closer to those obtained for the best oxygen barriers, such as ethylene-vinyl alcohol copolymer (EVOH) and PVDC at low and intermediate RH. In these conditions they have lower values than polyethylene (not a good oxygen barrier) and similar values to polyesters (Krochta, 2002). Protein films also have lower OP than cellulose-based films. This may be related to their higher linear structure compared to polysaccharides (ring chains) and their polar nature, which leads to a higher cohesive energy density and a lower free volume among polymer chains (Miller and Krochta, 1997). The increase in RH leads to an increase in OP (McHugh and Krochta, 1994a, 1994c; Hong and Krochta, 2006), so if the intention is to use them in oxygen sensitive products the key factor is to work at low RH and reduce the use of plasticizers (higher protein:plasticizer ratio). The effect of various plasticizers on the OP of β-Lg

and WPI was assessed by Sothornvit and Krochta (2000a, 2000b and 2000c) and McHugh and Krochta (1994b), respectively. Plasticizer shape, size and the number of oxygen atoms in the plasticizer molecules were shown to have a significant impact on the plasticizer effect in terms of OP efficiency. Sucrose and sorbitol had the best oxygen barrier efficiency in β-Lg based films. The larger size of sorbitol compared to glycerol (Table 7.1) and its lower hygroscopicity results in lower OP (Sothornvit and Krochta, 2001). The use of a plasticizer that is a solid at room temperature, such as sorbitol, significantly lowers oxygen permeability in comparison to glycerol in equivalent amounts to the plasticizers. One possible explanation for this is the possibility of this molecule creating crystalline domains within the film, lowering permeability (Rogers, 1985). An Arrhenius relationship was also found between whey proteins and temperature (Maté and Krochta, 1996a; Hong and Krochta, 2006).

Aroma and oil permeability

Whey protein films have been identified as good aroma and oil barrier films. However, there has been little research into quantifying the aroma permeability of protein films. Hansen and Heins (1992) found that whey protein (β-Lg) limits the flavour perception of benzaldehyde, citral and D-limonene. These results were also confirmed by Miller and Krochta (1997) with regard to the compound permeability if the latter in whey protein films. They showed that D-limonene (citrus flavour) permeability is 250 to 15000 times lower than on vinylidene chloride copolymer and similar to the results for EVOH copolymer films.

The data on oil permeability is limited. De Mulder-Johnston (1999) and Chan and Krochta (2001) showed respectively that whey protein films and whey coatings on paper provide excellent grease resistance. In the first case, no visible oil permeation through WPI-based edible films containing 0-60% of beeswax was detected over 19 weeks. In the case of whey protein coatings, Chan and Krochta (2001) found that denatured whey protein coatings were better oil barriers than native protein ones, and had a similar performance to polyvinyl alcohol (PVOH). The type of plasticizer used in whey protein films and coatings also influences oil penetration. Glycerol (at 1.3 M) and PEG 200 in whey protein coatings are good alternatives as oil barriers (Lin and Krochta (2003).

7.4.2 Mechanical properties

The mechanical properties normally used to characterise films are: tensile strength (TS), elongation (E), elastic or Young's modulus (E') and toughness or resilience (the product of TS and E). The magnitude of TS and E are greatly affected by temperature and RH in the case of

whey protein films, which implies proper previous sample conditioning (Olivas and Barbosa-Canovas, 2005). Table 7.4 summarises the mechanical properties of edible whey protein based films and coatings. Mechanical properties can reflect the type and extent of protein-protein interactions, the presence of crystalline regions and the free volume in whey protein films (Dangaran and Krochta, 2008). TS is defined as the pulling force per film cross-section area required to break the film. Whey protein films showed lower TS than polysaccharides and synthetic films, due to their strong protein interactions, resulting in stiff and brittle films with little elongation. It was possible to reduce this by changing the state of the protein or by adding plasticizers.

The degree of whey protein denaturation, which is affected by processing temperature and shear, also affects tensile properties (Pérez-Gago and Krochta, 1999). Higher protein denaturation leads to an increase in protein-protein interactions, via disulphide bonds, and stronger and stiffer films.

The key principle of plasticizers in the film matrix is to interrupt protein-protein interactions and increase the free volume in the film, making it more flexible. The use of plasticizers has a significant effect on mechanical properties, usually increasing E (the degree to which the film can stretch before breaking) and decreasing TS and E' (film stiffness, as determined by the pulling force/area to the degree of film stretch ratio). McHugh and Krochta (1994b) confirmed this behaviour by increasing the glycerol content. However, when using sorbitol, the elongation was much lower although the films were stronger (i.e. TS was higher) (Table 7.4). The crystalline domains of sorbitol at room temperature can act as cross-linkers, thus increasing film strength although they are not flexible in themselves. Shaw et al. (2002) observed even lower elongation values for xylitol. Sothornvit and Krochta (2001) produced very brittle β-Lg films plasticized with polylene glycol (PG) and no change in mechanical properties was observed. The low polarity of this compound compared to other plasticizers tested is possibly the reason for the lower interactions with β-Lg. According to the overall effect on film mechanical properties, the plasticizers used may be ranked as follows: PEG 400, PEG 200, sucrose, sorbitol and glycerol. It was found that the molecular weight and number of oxygen atoms are responsible for plasticizer efficiency. No significant differences were found in tensile properties when comparing β-Lg based films with WPI based films for the same type of plasticizer (Maté and Krochta, 1996a).

The main drawback to whey protein films is the lack of toughness compared to synthetic polymer films (Table 7.4). Nevertheless, their mechanical properties are adequate and can be

improved for some product applications. Until the mechanical properties are improved, their most viable application is their use in pouches for smaller amounts of foods or coatings.

Table 7.4 Mechanical properties of whey protein-based edible films: Tensile strength (TS), elastic or Young´s modulus (YM) and elongation (E) (adapted from Pérez-Gago and Krochta, 2002; Krochta, 2002; Sothornvit and Krochta, 2005; Dangaran and Krochta, 2008)

Protein/plasticizer (w/w)	TS (MPa)	YM (MPa)	E (%)	Ref.
β-Lg:Gly				
(1.70:1 - 3.20:1)	4.98-16.01	150.1-705.6	11.36-76.46	1, 2
β-Lg:PG				
(2.06:1 - 3.87:1)	13.2-21.8	1476.4-1922.3	-	1
β-Lg:Sor				
(0.86:1 - 1.62:1)	2.71-10.06	99.6-383.8	24.75-65.85	1, 2
β-Lg:Suc				
(0.46:1 - 0.86:1)	1.74-9.71	64.1-340.8	30.33-89.41	1, 2
β-Lg:PEG 200				
(0.78:1 - 1.47:1)	1.80-6.46	67.4-255.2	41.67-77.09	1, 2
β-Lg:PEG 400				
(0.39:1 - 0.73:1)	0.72-2.88	28.7-177.2	25.53-32.31	1, 2
WPI:Gly				
(1:1 - 2:1)	1-3.5	20-110	35-48	3
WPI:Xy				
(1:1 - 2:l)	0.5-8.5	80-275	2-15	3
WPI:Sor				
(1:1 - 2:l)	2.5-9	75-325	12-22	3
WPI:Gly				
(1.5:1) native whey protein	3	100	7	8
(1.5:1)	7	199	41	8
(2:1)	3	-	21	5
(2.3:1)	14	475	31	4
(5.7:1)	29	1100	4	4
WPI:Sor				
(1:1)	15	475	8.7	4
(1.2:1)	2-3	-	28-53	6
(1.5:1)	18	625	5	4
(2:1)	6	-	23	5
(2.3:1)	14	1040	3	4
LDPE	19-44	280-410	600	7
HDPE	22-31	1000-1600	10-1200	7
PP	31-38	1170-1730	100-600	7
PS	45-83	2620-3380	1-4	7

(1) Sothornvit and Krochta, 2000a, 2000b; (2) Sothornvit and Krochta, 2001; (3) Shaw et al., 2002; (4) McHugh and Krochta, 1994b; (5) Banerjee and Chen, 1995; (6) Anker et al., 1999; (7) Hernandez et al., 2000; (8) Pérez-Gago and Krochta, 1999.

7.4.3 Surface properties

Relatively little attention has been paid to the surface properties of whey protein-based films and coatings, which may be essential to their effectiveness in food applications. In order for edible coatings to function properly, it is essential that there is adequate adhesion (interactions between coating solutions and coated surfaces) and cohesion (interactions between the molecules of the coating solutions) (Krochta and Mulder-Johnston, 1997). Controlling coating solution wettability as well as the behaviour of coatings in relation to water and organic solvents allows for the optimisation of both coating formulation and polymerization methods, leading to an enhanced packaging material design for specific applications (Park, 1999; Han et al., 2005). As already mentioned, the hydrophilic nature of whey proteins makes the biopolymer films wettable by water, leading to a decrease in water vapour barrier properties that substantially influences the film structure (Guilbert et al., 1997; Bialopiotrowicz, 2003).

The wettability of a solid surface can be determined by measuring the contact angle (θ) produced by a drop on a solid surface (Figure 7.2), governed by the Young equation (7.1). This equation establishes the equilibrium relationship between the contact angle and the three interfacial tensions (γ_{LV}, γ_{SV} and γ_{LS}) (Kiery and Olson, 2000; Ornebro et al., 2000).

$$\gamma_{LV} \cos\theta = \gamma_{SV} - \gamma_{SL} \tag{7.1}$$

According to this equation, if the contact angle between a liquid and a solid is nearly zero, the liquid spreads over the solid easily and the surface is wettable. If, on the other hand, the contact angle is greater than 90° the surface is non-wettable and the drop will ball up and run off the surface easily.

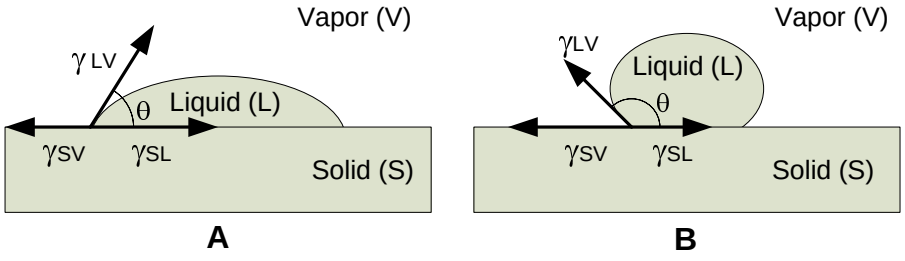


Figure 7.2 Contact angle (θ) in: (A) wetting surfaces; (B) non-wetting surfaces.

In general, films with higher θ values exhibit higher surface hydrophobicity (Tang and Jiang, 2007) and the quantitative differentiation between hydrophobic and hydrophilic surfaces is based on whether $\theta > 65°$ or $\theta < 65°$, respectively (Vogler, 1998).

Contact angles can be determined with a digital microscope connected to a personal computer. Analysis of the shape of a drop of the test liquid placed on a solid is the basis for goniometry, also referred to as the sessile drop technique. Polymer films are placed on a well-levelled platform, and the microscope is positioned horizontally to capture the side-view image (Figure 7.2). The side-view image is obtained and converted into a binomial edge-enhancing picture using conventional photo-editing software. The contact angles of the digital images are measured using line-slope calculation software (Han and Krochta, 1999).

The surface free energy is a thermodynamic parameter that describes wetting, and the condition for wetting or spreading of a liquid on a solid surface is determined by the spreading coefficient (S_{LS}).

$$S_{LS} = \gamma_{SV} - \gamma_{LV} - \gamma_{SL} \tag{7.2}$$

If $S_{LS} > 0$, according to equation 7.2 the liquid will spread out on the surface of a solid, but if $S_{LS} < 0$ it will form a droplet with a finite contact angle. This means that wetting depends on the surface tension of the liquid and the total free energy required for the creation of the new interface (Ornebro et al, 2000). If the total free energy between the two materials decreases, good adherence is expected, whereas wetting prevention implies greater total free energy between the film and water.

Zisman and co-workers found that cos θ is usually a function of γ_L for homologous liquids according to equation 7.3.

$$\cos\theta = 1 - \beta(\gamma_L - \gamma_C) \tag{7.3}$$

where β is the Zisman constant, γ_L the liquid surface tensions from various liquids and γ_C the critical surface tension that can be calculated by extrapolation to $\cos\theta = 1$ from the Fox-Zisman plot ($\cos\theta$ vs γ_L) with various liquids of different γ_L values. McGuire and Kirtley (1988) concluded that γ_C could be related to the true surface energy of a solid because it is solely a function of its surface properties. From this perspective γ_C becomes a characteristic property, providing information about the wetting behaviour of the surface and enabling its nature to be

predicted. However it is important to note that γ_C is not γ_S; the latter is probably larger than the former (Toussaint and Luner, 1993; Lawton, 1995).

The critical surface energy of some fruits, vegetables and conventional plastic films is presented in Table 7.5. They all show lower surface energies, indicating their hydrophobic nature. The values for apple (Park, 2003), garlic (Hershko and Nussinovith, 1998), grapefruit and orange (Hagenmaier and Baker, 1993), tomato and carrot (Casariego et al., 2008) indicate that the skin of most fruit covered with a layer of wax interacts with liquids primarily through dispersion (van der Wall forces), which is characteristic of poor adhesion with hydrophilic coating solutions.

Whey protein coating solutions and films, on the other hand, have higher critical surface energy (as is the case with most hydrophilic edible coatings) that may seriously compromise the application of biopolymer films as packaging materials if the intention is to decrease water vapour permeability. However, if the purpose is to reduce oil permeability they perform well (De Mulder-Johnston, 1999; Chan and Krochta, 2001; Lin and Krochta, 2003).

Plasticizers are known to reduce cohesion of the film-forming polymers (Guilbert et al., 1996), so they may even increase the critical surface energy of the films. Hong et al. (2004) studied the influence of various plasticizers in WPI-PP and WPI-PVC composite films on critical surface energy. They found that sorbitol, PEG and sucrose decrease critical surface energy, but glycerol and PG, according to their hydrophilic behaviour, increase the critical surface energy of coated plastics, thus increasing wettability and reducing the water contact angle. The selection of the plasticizer also should take into account how the surface properties are affected, otherwise they could compromise the adherence or barrier properties of the coating. If the plasticizer makes the film more hydrophilic, possible applications are foods with lower moisture content under storage conditions with lower RH levels.

The use of surfactants (tween, lecithin or Span 20) in edible whey protein films and coatings also increases their critical surface energy. However, if the intention is to improve the adhesion between whey protein coating solutions and the food product or other packaging material (paper or synthetic polymers) to produce coatings or composite films, surfactants may be a good choice (Lin and Krochta, 2006; Lin and Krochta, 2003).

The increase in the hydrophobic character of film-forming solutions was studied by Fernández et al., (2007) using saturated and unsaturated lipids (stearic, oleic and linoleic fatty acids). The use of unsaturated fatty acids reduces the surface tension of the film-forming solution, which leads to an

improvement in adhesion between the film-forming whey protein solutions and the intended applications. Stearic acid does not produce changes in the surface tension of solutions, probably due to its solid state at 23 ºC.

Table 7.5 Surface energies for some fruits and vegetables and synthetic polymers (adapted from Park, 2003 and Han et al., 2005)

Products	Surface energy (mJ/m^2)
Fruits and Vegetables	
apple	18.7
grapefruit	23.0
orange	20.0
garlic	18.3
tomato	17.4
carrot	24.1
Synthetic polymers	
polystyrene (PS)	30.0±1.0
polyethylene (PE)	32.0±1.6
poly ethylene terephthalate (PET)	38.0±2
poly 2-vinylpyridine (PVP)	50.0±2.0
poly 4-methyl-1-pentene (TPX)	21.5±0.1
poly methyl methacrylate (PMMA)	40.0±0.2
poly vinylcyclohexane (PVCH)	29.0±1.0

7.4.4 Solubility

Protein film formers that are soluble in water produce films with differing solubility, as a result of film formation conditions and treatments. Table 7.6 lists the solubility properties of some native proteins. As previously mentioned, native and heat-denatured whey protein films are transparent and have similar water vapour permeability at their natural pH. However, they possess different solubility and mechanical properties. The extent of covalent S-S bonds formed during film drying and the unfolded structure of heat-denatured whey proteins lead to the production of more water insoluble and mechanically stronger films which can withstand higher deformations (Pérez-Gago and Krochta, 2002). In contrast, the globular structure and lower energy bonding of native whey proteins account for their complete solubility in water and poor mechanical properties (Krochta et al., 1994; Pérez-Gago et al., 1999).

Floris et al. (2008) studied the mechanism governing the solubility of whey protein films and coatings. The building blocks of these films were aggregated whey proteins and the film and coating solubility was related to the continuity of the network of disulfide cross-links between aggregates and dynamic rearrangements of disulfide bonds occurring via the thiol-disulfide

exchange reaction. These authors found that the use of a neutral solution containing SDS allows for the total dissolution of the films and hypothesised that SH/S-S exchange reactions are responsible for this phenomenon. The chemical blocking of thiol groups prohibited the rearrangement of disulfide cross-links, resulting in the reduced solubility of the protein coating. Kim and Ustonol (2001c) studied the solubility and equilibrium moisture contents of plasticized whey-protein and whey-protein emulsion films using sorbitol and glycerol as plasticizers, and butterfat and candelilla wax as lipids. They found that the solubility and equilibrium moisture contents of the films were reduced by lipid incorporation in plasticized films.

Table 7.6 Solubility properties of native proteins before film and coating formation (Krochta, 2002).

Protein	Solvent			
	Water	Acid solutes	Alkaline solutes	Ethanol
Collagen		X		
Gelatine	X			
Fish myofibrillar protein		X	X	
Keratin				X
Egg white protein			X	
Casein	X			
Whey protein	X			
Corn zein				X
Wheat gluten		X	X	X
Rice bran protein		X	X	
Soy protein	X		X	
Peanut protein			X	
Cotton seed protein			X	

7.4.5 Optical properties

Optical properties are intrinsically related to the sensorial perception of the consumers, especially when the films or coatings are intended to be used as food packaging. However, consumers must be attracted by the appearance of the food product not only at the time of purchase but also when they are consumed (Kunte et al., 1997). Colour, transparency (opacity) and gloss are the most important optical properties for films and coatings.

The most popular scales and methodologies used to evaluate colour are the Hunter Lab and CIE L*a*b*, which are recognised in a three dimensional form. The L axis is vertical, in which 100 is the maximum L value (perfect reflecting diffuser material) and the minimum is 0, corresponding to black. The *a* and *b* axes have no specific numerical limits. Positive *a* (+a) is red and negative (-a) is green. Positive *b* (+b) is yellow and negative (-b) is blue. Delta values (ΔL, Δa and Δb) are

associated with this colour scale, denoting how much the sample and standard differ from one another in L, *a* and *b* coordinates. The total colour difference ΔE can also be calculated (equation 7. 4) as a single numerical value that takes into account the differences between the L, *a* and *b* values of the sample and the standard. It is generally known that ΔE values less than 3.0 cannot be easily detected by the human eye (Hong et al., 2004).

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \tag{7.4}$$

The Hunter Lab scale is not used as frequently nowadays because the CIE L*a*b* released in 1976 has become more popular, although they are both uniform colour scales.

Gloss is physically defined as the amount of light scattered by a material and represents a highly desirable appearance attribute in packaging applications. Light scattering is normally associated with material surface roughness, since in many cases the bulk contribution is small. Light transmission (in UV and visible light (UV-VIS)), as well as transparency are normally measured using a spectrophotometer at specific wavelengths (from 200 to 800 nm). Transparency is usually measured at 600 nm and calculated by the Han and Floros (1997) equation.

Films composed of whey proteins have suitable overall optical properties and the potential to improve the visual quality of foods, especially their appearance, due to their high gloss and transparency (Trezza and Krochta, 2001; Caner, 2005). They vary from transparent to translucent, which enhances their potential benefits and applications in food and non-food commodities (Khwaldia et al., 2004). However, the use of some additives such as waxes (e.g. candelilla wax) or fatty acids (saturated or unsaturated) may produce opaque films which are valued less in some applications (Kim and Ustonol, 2001a; Fernández et al., 2007). Hong et al. (2004) concluded that protein concentration did not affect the gloss values of WPI-coated plastic films, and that high gloss values are maintained after several months of storage at 23 ºC and 75% RH. Glycerol, sorbitol, PP, PEG and sucrose do not influence the gloss or the colour of coated PP and PVC films. Trezza and Krochta (2000) found that whey protein isolate (WPI) coatings had lower yellowing rates during prolonged storage than whey protein concentrate (WPC) and the same rates as shellac coatings. The results indicated that WPI coatings can be used in place of shellac coatings when low-colour development is desired. WPC coatings can be used to tailor the colour development of food.

7.5 Applications and opportunities

On the basis of their intrinsic properties, whey protein films and coatings have been studied and developed for certain specific applications. Table 7.7 summarises these applications and the film properties involved (Balagtas et al., 2003). Taking into account the function of whey protein coatings in terms of food or packaging materials, it is possible to categorise 6 different groups of applications:

- Moisture barrier coatings on foods

  These can be used to prevent the movement of moisture from one component to another, preventing sogginess, unwanted chemical reactions and microbial growth in order to extend shelf life (e.g. in fresh cut fruit, eggs or breakfast cereals). However this is not the most promising application for whey protein based films and coatings, due to their higher hydrophobicity and poor moisture barrier.

- Oxygen barrier coatings on foods

  Coating foods that are prone to oxidation (rancidity) will prolong their shelf life (e.g. snack peanuts, nuts for confectionery bars) (Lee and Krochta, 2002; Lee et al., 2002b). Only non-toxic edible films and coatings can be ingested with the food, thus eliminating the use of all synthetic plastics in these applications.

- Oxygen barrier coatings on plastics

  Most plastics that are good moisture barriers are poor oxygen barriers. Thus, they are usually coated to provide a good oxygen barrier (Hong and Krochta, 2003) and to allow for contact between the packaging and the food.

- Grease barrier coatings on paper and paperboard

  These coatings are used in packaging for products such as fast food and pet food (Chan and Krochta, 2001).

- Gloss coatings on foods

  The most widely used glaze is shellac, also called confectioners glaze. Confectionery manufacturers are looking for an alternative glaze that will not be subject to the restrictions that accompany the use of shellac. The specific application that has been most thoroughly investigated is whey protein gloss coatings for chocolate-panned

confectionery (Lee et al., 2002a). Gloss-coatings on other non-panned confectionery have not yet been investigated, but could potentially be viable. Increasing the gloss on fruit, especially minimal processed fruit, is also of great interest.

- Active films and coatings

  Flavour, appearance, chemical protection and microbial safety are probably the most attractive properties for enhancing in foods and for this reason practically all the applications of whey protein films and coatings presented in Table 7.7 aim to make use of their carrier properties. Flavours and colorants, antioxidants, nutraceutical and antimicrobial compounds are the active agents usually applied.

In terms of the first five functions described above, whey protein films and coatings can be classified as passive barriers that compete directly with traditional packaging materials, whose main purpose is to offer mechanical and barrier protection. However, this does not indicate any economic potential for biopolymers, due to their high production costs in comparison to synthetic materials, at least in the short-term (Weber et al., 2002).

The added value of bio-based edible films and coatings, particularly whey protein-based films and coatings, as opposed to traditional packaging, is firstly their edible nature and inherent biodegradability, secondly their capacity to incorporate functional compounds that offer further protection for systems, creating the so-called active packaging (Brody et al., 2001), and, last but not least, their intrinsic bioactive properties. The active packaging category is the most promising application for whey protein-based edible films and coatings. By definition, active packaging interacts directly with the food or headspace of the product in a positive way to extend shelf life or create certain characteristics that cannot be obtained otherwise without raising concerns about toxicity (Regalado et al., 2006). Table 7.8 shows examples of bioactive compounds grouped according to their most widely recognised functions, although some of them can simultaneously fulfil various purposes. They can include flavours and colorants, natural oxygen scavengers (antioxidants), nutraceuticals and antimicrobial agents.

7.5.1 Flavours and colorants

Colour and flavour are the main attributes used by consumers to make sensorial judgments on food quality and for this reason it is essential that they are improved or preserved in foods. Furthermore, these types of enhancers applied to fruit, meat and fish products may also contain

nutritional and health benefits such as carotenoids, anthocyanins and betacyanins (Stintzing et al., 2001 and 2003; Butera et al., 2002; Galati et al., 2003; Olivas and Barbosa-Canovas, 2005). In some applications the use of flavorants (sodium or calcium salts based on amino acids and nucleotides) aims to mask packaged food off-flavours. These include citric, maleic, tartaric, lactic and acetic acids, which also play a role in preservation (Pintado et al., 2009; Hettiarachchy and Eswaranandam, 2007). Kim and Ustunol (2001a) evaluated the sensorial attributes of WPI candelilla wax emulsion. They concluded that these films were opaque, slightly sweet and adhesive. Even though some reports have been produced on the inclusion of flavours and colorants in whey protein films and coatings, little information is available on the inclusion of the natural substances listed in Table 7.8 designed for contact with food.

7.5.2 Antioxidants

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are popular synthetic antioxidants used in food packaging. These compounds are vaporised into the package headspace and then absorbed on the surface of the food. Although they are considered safe, there are recommendations for consumers to avoid or replace them with natural ones (Dangaran and Krochta, 2008). Table 7.8 lists some examples of antioxidant agents, including crude extracts from plants that have demonstrated high antioxidant activity and could be incorporated into whey protein films and coatings as natural antioxidants. However, there are few examples of their applications. Most natural antioxidants are obtained from plant extracts (e.g. oils, fruits, spices, seeds, leaves) and may have higher antioxidant capacities than synthetic ones (Davidson and Zivanovic, 2003; Okonogi et al., 2007).

Ascorbic acid is found to have an antioxidant function that enhances the oxygen barrier function in whey protein coatings when applied to roasted peanuts (Lee and Krochta, 2002; Min and Krochta, 2007) and apple slices (Lee et al., 2003; Pérez-Gago et al., 2006). Min and Krochta (2007) found significantly lower levels of peroxide compounds using ascorbic acid in WPI-coated nuts compared to uncoated and WPI only coated nuts. The use of ascorbic acid films in fresh cut apples prevents the enzymatic browning that occurs due to the oxidative reactions of phenolic compounds by polyphenoloxidase and products of reactions (o-quinones) to various polymerized products (Lee et al., 2003). Cysteine and 4-hexylresorcinol were also tested in fresh-cut apples by Pérez-Gago et al. (2006) and the results showed that the latter is the least effective at reducing browning. Lee et al. (2000) demonstrated the efficiency of oxalic acid in enzymatic browning.

Table 7.7 Applications of whey protein coatings (adapted from Dangaran and Krochta, 2008; Krochta 2002).

Application	Function	Film property	Ref.
Fresh cut products Apples Potatoes	Carry protective antioxidants and texture enhancer Reduce water loss Improves flavour	Active film Moisture barrier Aroma barrier	Olivas and Barbosa-Canovas (2005); Le Tien et al. (2001); Pérez-Gago et al. (2003, 2006); Baldwin et al.(1996); Lee et al. (2003);
Plums	Reduce water loss Improves texture and appearance	Moisture barrier Oxygen barrier Gloss	Reinoso et al. (2007)
Peanuts	Protect from lipid oxidation Carry protective antioxidants Extend shelf life	Oxygen barrier Active film	Maté and Krochta (1996b, 1998); Lee and Krochta (2002); Lin and Krochta (2006); Min and Krochta (2007)
Eggs	Prevent weight loss Extend shelf life	Moisture barrier Gas barrier	Caner (2005)
Breakfast cereals Cereal-based products (bran with raisins)	Glazed appearance Stickiness prevention Reduce water transference between products Improves flavour	Gloss Moisture barrier Active film	Chen (1995)
Cheese (ripened)	Antimicrobial carrier Prevent weight loss Improve texture and appearance	Active film Moisture barrier Colour and gloss	Franssen (2002); Ramos (2011)
Bakery product Muffins Cookies	Reduce water absorption by fibres Improve texture	Moisture barrier	Onwulata (2008)
Chocolate	Add smooth finish Add gloss Add colour		Lee et al. (2002a) Dangaran and Krochta (2003)
Meat products Pork Roasted turkey Sausages Freeze dried chicken Fresh beef	Enzyme carrier Antimicrobial carrier Handling protection Improve appearance	Active film	Samelis et al. (2001); Min et al. (2006); Cagri et al. (2002); Alcantara (1996); Zinoviadou et al. (2009)
Fish products Smoked salmon Frozen salmon	Enzyme carrier Antioxidant carrier Antimicrobial carrier Improve appearance	Active film	Min et al. (2008) Stuchell and Krochta (1995); Neetoo et al. (2008)
Synthetic polymers PP PVC PE	Reduce oxygen permeability Optical properties Antimicrobial carrier	Oxygen barrier Colour and gloss Active film	Hong et al. (2004); Hong and Krochta (2006); Lee et al. (2008)
Paper and paperboard	Improve mechanical properties Reduce oxygen permeability Prevent oil migration	Mechanical properties Oxygen barrier Oil barrier	Chan and Krochta (2001); Lin and Krochta (2003); Gällstedt el al. (2005)

Vitamin E, chemically α-tocopherol, has been widely marketed as a viable and better alternative to BHT as a food-grade odour and taste remover as well as a stronger antioxidant additive in package materials (Brody et al., 2001; Han and Krochta, 2007).

7.5.3 Antimicrobials

Food safety is an important issue worldwide and many technologies based on temperature changes, reduction of water activity, pH control and irradiation have been developed. However, in combination with antimicrobial agents incorporated into films and coatings this allows for the use of less severe treatments and leads to more specific growth control of the pathogenic microorganisms in each particular application. Several factors should be considered in developing antimicrobial films or coatings, such as the effect of the antimicrobial agent on the mechanical and physical properties of the films and coatings, the spectrum of antagonic microorganisms, the antimicrobial mechanism, migration into the food, and toxicological issues, as well as their effect on food product composition (Regalado et al., 2006).

Antimicrobial agents may be grouped as bacteriocins, fungicides, enzymes, organic acids, salts, oil extracts and polysaccharides. Evidence of the antimicrobial properties of the compounds listed in Table 7.8 and concentrations used to control various target food-born microorganisms is widely available in the literature. Organic acids, for example, are protective compounds when sprayed onto foods surfaces, but can quickly diffuse into the food interior leaving the surface susceptible to bacterial contamination. Little information on about the antimicrobial activity of bioactive agents when incorporated into whey protein films, and in this case it is mainly evaluated *in vitro* using the film disk agar diffusion essay (Lee et al., 2003; Min et al., 2005; Cagri et al., 2001; Seydim and Sarikus, 2006; Ko et al., 2001). There is even less antimicrobial evaluation of active whey protein coatings in real food applications (Table 7.7). Only a few studies have been carried out into cheese, meat or fish products (Alcantara, 1996; Stuchell and Krochta, 1995; Samelis et al., 2001; Cagri et al., 2002; Franssen, 2002; Min et al., 2006 and 2008; Zinoviadou et al., 2009; Ramos, 2011). In these food products (solids and semisolids) microbiological growth normally starts on the surface, due to post processing steps and handling. The use of appropriate coatings that can retain antimicrobial compounds on the surface can reduce the amount of antimicrobial compounds used, as well as eliminate the need to compensate for the amount moving into the products. The inhibition of *L. monocytogenes* in hot dogs was more effective using WPI casings with *p*-aminobenzoic acid than sorbic acid. However, the opposite was observed in Bolonha and

summer sausages in the case of *L. monocytogenes, E. coli* and *Salmonella typhimurium,* which could be influenced by the natural pH of each product (Cagri et al., 2001 and 2002). These findings indicated that organic acids in their undissociated form (pH 5.2) are more effective and may be very attractive as antimicrobial agents for use in coated food products such as cheeses or fermented meat with lower pH values.

Using lysozyme and lactoperoxidase in WPI films extended the shelf life of smoked salmon in terms of aerobic microorganisms (yeasts and moulds) and L. *monocytogenes* through their inhibition or even reduction (Min et al., 2005; Min et al., 2008; Neetoo et al., 2008). Lysosyme hydrolyses linkages in peptidoglycan cell walls causing cell lysis and lactoperoxidase systems oxidize thiocyanate to hypothiocyanate, which then oxidizes sulphydryl groups in microbial enzymes (Dangaran and Krochta, 2008). Nisin is a natural bacteriocin that has also been investigated in WPI films (Ko et al., 2001; Pintado et al., 2009). A reduction in *L. monocytogenes* at pH 3 and 6,000 IU/g in WPI-nisin films was observed.

Concerning the effect of the composition of whey protein films and coatings on the diffusion of bioactive compounds in food, Franssen et al. (2004), Ozdemir and Floros (2003) and Min et al. (2006) determined the diffusion coefficient of potassium sorbate, natamicym (antimicotic used by the cheese industry), lysosyme and lactoperoxidase, respectively. The type of plasticizer used in the film forming solution (glycerol or sorbitol) and the molecular weight of the bioactive compound are responsible for the different diffusion coefficients.

Finally, it is not possible to disregard the nutritional value provided by whey proteins as source of edible films and coatings, coupled with their intrinsic biological activities such as anticarcinogenic and immunomodulatory properties (Morris and FitzGerald, 2008) and bioactive properties, such as antimicrobial (due to the presence of lysozyme, lactoferrin and lactoperoxidase proteins) and antioxidant properties (due to the presence of cysteine) (Lacroix and Cooksey, 2005).

Table 7.8 Active ingredients used or potentially used in whey protein edible films and coatings (adapted from Ramos et al., 2012).

Active agent	Active compound (E-number)	Ref.
Colorant		
	Turmeric (E-100)	Schwartz and Winterhalter (2004)
	Riboflavin (E-101)	Cai et al. (1998); Cai et al. (2001)
	Carmine (E-120) Betacyanins (E-163)	Butera et al. (2002); Galati et al. (2003); Stintzing et al. (2001, 2003)
	Caramel (E-150)	Greenfield (2005)
	Chlorophylls (E-141)	Cai et al. (2001)
	Carotenoids (E-150) Anthocyanins (E-163)	Duhard et al. (1997); Stintzing and Carle (2004)
Antioxidant/extract		
	Ascorbic acid, vitamin C (E-300)	Saleem et al. (2004); Kim et al. (2006); Desai et al. (2008)
	α-tocopherol, vitamin E (E-307)	Kweon et al.(2001); Sang-Myung et al. (2003); Saleem et al. (2004)
	Cysteine 4-hexylresorcinol	Pérez-Gago et al. (2006)
	Gallic acid	Baratto et al. (2003)
	Propyl gallate (E-310)	Sultanova et al. (2001)
	Butylated hydroxyanisole, BHA (E-320)	Sang-Myung et al. (2003); Wang et al. (2007)
	Butylated hydroxytoluene, BHT (E-321)	Pourmorad et al. (2006)
	Cafeic acid	Kim et al. (2006); Sai-Mokbel and Suganuma (2006)
	Ferulic acid Chlorogenic acid	Kweon et al. (2001)
	Oxalic acid	Lee et al. (2000)
	Quercetin	Kim et al. (2006)
	Rosmarinic acid	Tepe et al. (2006)
	Chitosan	Pasanphan (2008)
	Green tea; Black tea; Sage; Peppermint; Thyme; Absinthium; Roselle; Olive leaves; Shrubby blackberry	Aynur and Nehir (2008)
Antimicrobial		
Bacteriocin	Nisin (E-234)	Daeschel et al. (1992); Siragusa et al. (1999); Coma et al. (2001); Ko et al. (2001); Janes et al. (2002); Limjaroen (2003); Neetoo et al. (2008); Lee et al. (2008)
Fungicides	Natamycin (E-235)	Brik (1981); Ramos (2011)
Enzymes	Glucose oxidase (E-1102)	Kim et al. (1990); Shin et al. (1993); Murray et al. (1997); Wohlfahrt et al. (1999); Massa et al. (2001); Simpson (2006)
	Lactoperoxidase	Bjorck et al. (1975); Zapico et al. (1991, 1995); Min et al. (2005); Santos et al. (2008);
	Lysosyme (E-1105)	Appendini and Hotchkiss (2002); Min et al. (2008)
	Lactoferrin	Al-Nabulsi and Holley (2005)

Table 7.8 (Continued)

Active agent	Active compound (E-number)	Ref.
Organic acids	Lactic acid (E-207)	Hettiarachchy and Eswaranandam (2007); Pintado et al. (2009); Ramos (2011)
	Citric acid (E-330) Tartaric acid (E-334) Malic acid (E-296)	Lee et al. (2003); Hettiarachchy and Eswaranandam (2007); Pintado et al. (2009)
	Acetic acid (E-260)	Samelis et al. (2001); Pintado et al. (2009)
	Lauric acid	Padget et al. (2000); Řiháková et al. (2001)
	Formic acid and fumaric acids	Pintado et al. (2009)
	Sorbic acid (E-200)	Limjaroen (2003), Cagri et al. (2001)
Salts	Sodium lactate (E-325)	Shelef et al. (1997); Juneja and Thippariddi (2004); Neetoo et al. (2008)
	Sodium propionate (E-281)	Keeney and Broyles (1943)
	Calcium Propionate (E-282)	Droby et al. (2003); Mills et al. (2004)
	Sodium benzoate (E-211)	Combina et al. (1999); Neetoo et al. (2008)
	Potassium benzoate (E-212)	Samelis et al. (2001)
	Sodium sorbate (E-201)	Combina et al. (1999)
	Potassium sorbate (E-202)	Han and Floros (1997, 1999); Combina et al. (1999); Limjaroen (2003); Neetoo et al. (2008); Cagri et al. (2001)
Oil extract	Oregano	Burt and Reinders (2003); Dadalioglu and Evrendilek (2004); Zinoviadou et al. (2009); Seydim and Sarikus (2006)
	Garlic	Pranoto et al. (2005); Seydim and Sarikus (2006)
	Rosemary	Smith-Palmer et al. (1998); Hammer et al. (1999); Pintore et al. (2002); Seydim and Sarikus (2006)
	Cynara scolymus	Zhu et al. (2005)
Polysaccharides	Chitosan	Uchida et al. (1989); Sekiguchi et al. (1994); No et al. (2002)

7.6 Conclusions

Whey protein films and coatings are attractive alternatives to petroleum-based packaging materials. They simultaneously solve two environmental problems, whey disposal and the treatment of packaging waste, due to the fact that they are biodegradable and edible.

They are considered excellent oxygen and oil barrier films with very attractive visual properties. Their hydrophilic nature is responsible for their poor moisture barrier properties, although the use of some specific compounds may improve this, as well as their mechanical properties.

WPI has been the raw material used in most of the research into whey protein-based films and coatings. The use of WPC, as a less expensive alternative, is even more economically attractive in terms of packaging applications. However, further research is necessary.

Increasing the shelf life of products by improving food safety is probably the most promising application for whey protein films and coatings as bioactive packaging. However, the great challenge involved in applying this technology is the development of reliable modification and polymerization methods that could be implemented on an industrial basis.

References

Alcantara CR (1996) Barrier and mechanical properties of whey-protein-isolate-based films: film drying rate and coated-food integrity studies. Ph.D. Thesis, University of California, Davis, USA.

Alcantara CR, Rumsey TR and Krochta JM (1998) Drying rate effect on the properties of whey protein films. Journal Food Process Engineering, 21, 387-405.

Al-Nabulsi AA and Holley RA (2005) Enhancing the antimicrobial effects of bovine lactoferrin against *Escherichia coli* O157:H7 by cation chelation, NaCl and temperature. Journal of Applied Microbiology, 100, 244-255.

Anker M, Berntsen J, Hermansson AM and Stading M (2002) Improved water vapor barrier of whey protein films by addition of an acetylated monoglyceride. Innovative Food Science & Emerging Technologies, 3, 81-92.

Anker M, Stading M and Hermansson AM (1998) Mechanical properties, water vapor permeability, and moisture contents of β-Lactoglobulin and whey protein films using multivariate analysis. Journal Agriculture Food Chemistry, 46, 1820–1829.

Anker M, Stading M and Hermansson AM (1999) Effect of pH and the gel state on the mechanical properties, moisture contents, and glass transition temperatures of whey protein films. Journal Agriculture Food Chemistry, 47, 1878-1886.

Appendini P and Hotchkiss JH (2002) Review of antimicrobial food packaging. Innovative Food Science & Emerging Technologies, 3, 113-126.

Aynur B and Nehir E (2008) Determination of in vitro antidiabetic effects, antioxidant activities and phenol contents of some herbal teas. Plant Foods for Human Nutrition, 63, 27-33.

Balagtas JV, Hutchinson FM, Krochta JM and Sumner DA (2003) Anticipating market effects of new uses for whey and evaluating returns to research and development. Journal of Dairy Science, 86, 1662-1672.

Baldwin EA, Nisperos-Carriedo MO and Baker RA (1995) Edible coatings for lightly processed fruits and vegetables. Journal of Horticultural Science & Biotechnology, 30, 35–38.

Baldwin EA, Nisperos MO, Chen X and Hagenmaier RD (1996) Improving storage life of cut apple and potato with edible coating. Postharvest Biology and Technology, 9, 151-163.

Banerjee R and Chen H (1995) Functional properties of edible films using whey protein concentrate. Journal of Dairy Science, 78, 1673-1683.

Baratto CM, Tattini M, Galardi C, Pinelli AR, Visioli F and Basosi R (2003) Antioxidant activity of galloyl quinic derivatives isolated from P. lentiscus leaves. Free Radical Research, 37, 405-412.

Bialopiotrowicz T (2003) Wettability of starch gel films. Food Hydrocolloids, 17, 141-147.

Bjorck L, Rosen CG, Marshall V and Reiter B (1975) Antibacterial activity of the lactoperoxidase system in milk against *pseudomonas* and other Gram-negative bacteria. Applied Microbiology, 30, 199-204.

Bodnar I, Alting AC and Verschueren M (2007) Structural effects on the permeability of whey protein films in an aqueous environment. Food Hydrocolloids, 21, 889-895.

Brennan JG and Cowell ND (1990) Food Engineering Operations, 3rd ed., New York, Elsevier, pp. 700.

Brik H (1981) Natamycin. In: Florey K (Ed.) Analytical Profiles of Drug Substances, Academic Press, New York, USA, pp. 513-561.

Brody AL, Strupinsky ER and Kline LR (2001) Active Packaging for Food Applications, CRC Press LLC, USA, ISBN1-58716-045-5

Burt SA and Reinders RD (2003) Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters Applied Microbiology, 36, 162-167.

Butera D, Tesoriere L, di Gaudio F, Bongiorno A, Allegra M and Pintaudi AM (2002) Antioxidant activities of Sicilian prickly pear (*Opuntia ficus-indica*) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin. Journal Agriculture Food Chemistry, 50, 6895-6901.

Cagri A, Ustunol Z and Ryser ET (2001) Antimicrobial, mechanical, and moisture barrier properties of low pH whey protein-based edible films containing aminobenzoic or sorbic acids, Journal Food Science, 66(6), 865-870.

Cagri A, Ustunol Z and Ryser ET (2002) Inhibition of three pathogens on bologna and summer sausages using antimicrobial edible films. Journal Food Science, 67, 2317-2324.

Cai Y, Sun M and Corke H (1998) Colorant properties and stability of Amaranthus β-cyanin pigments. Journal Agriculture Food Chemistry, 46, 4491-4495.

Cai Y, Sun M, Schliemann W and Corke H (2001) Chemical stability and colorant properties of betaxanthin pigments from *Celosia argentea*. Journal Agriculture Food Chemistry, 49, 4429-4435.

Caner C (2005) The effect of edible eggshell coatings on egg quality and consumer perception. Journal of the Science of Food and Agriculture, 85, 1897-1902.

Casariego A, Souza BWS, Vicente AA, Teixeira JA, Cruz L and Díaz R (2008) Chitosan coating surface properties as affected by plasticizer, surfactant and polymer concentrations in relation to the surface properties of tomato and carrot. Food Hydrocolloids, 22, 1452-1459.

Chan M and Krochta JM (2001) Grease and oxygen barrier properties of whey protein isolate coated paperboard. Solutions (TAPPI), 84(10), 57.

Cheftel JC, Cuq JL and Lorient D (1985) Amino acids, peptides, and proteins. In OR Fennema (Ed.), Food Chemistry, New York, Marcel Dekker, pp. 245-369.

Chen H (1995) Functional properties and applications of edible films made of milk proteins. Journal Dairy Science, 78, 2563-2583.

Coma V, Sebti I, Pardon P, Deschamps A and Pichavant FH (2001) Antimicrobial edible packaging based on cellulosic ethers, fatty acids, and nisin incorporation to inhibit *Listeria innocua* and *Staphylococcus aureus*. Journal of Food Protection, 64, 470-475.

Combina M, Dalcero AM, Varsavsky E and Chulze S (1999) Effects of food preservatives on *Alternaria alternata* growth and tenuazonic acid production. Food Additives and Contaminants, 16, 433-437.

Coupland JN, Shaw NB, Monahan FJ, O'Riordan ED and O'Sullivan M. (2000) Modeling the effect of glycerol on the moisture sorption behavior of whey protein edible films. Journal Food Engineering, 43, 25-30.

Cuq B, Gontard N and Guilbert S (1998) Proteins as agricultural polymers for packaging production. Cereal Chemistry, 75(1), 1-9.

Dadalioglu, I. and Evrendilek, G. (2004) Chemical compositions and antibacterial effects of essential oils of Turkish oregano (*Origanum minutiflorum*), bay laurel (*Laurus nobilis*), Spanish lavender (*Lavandula stoechas* L.), and fennel (*Foeniculum vulgare*) on common foodborne pathogens. Journal of Agricultural and Food Chemistry, 52, 8255-8260.

Daeschel MA, McGuire J and al-Makhlafi H (1992) Antimicrobial activity of nisin absorbed to hydrophilic and hydrophobic silicon surfaces. Journal of Food Protection, 55, 731-735.

Dalgleish DG, Senaratne V and Francois S (1997) Interactions between alpha-Lactalbumin and beta-Lactoglobulin in the early stages of heat denaturation. Journal of Agricultural and Food Chemistry, 45, 3459-3464.

Dangaran KL and Krochta JM (2003) Aqueous whey protein coatings for panned products. Manufacture Confectionary, 83, 61-65.

Dangaran KL and Krochta JM (2008) Whey protein films and coatings. In: CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

Daniels CA (1989) Polymers: Structure and Properties. Technomic Publishing, Lancaster, PA. ISBN 13:9780877625520.

Davidson PS and Zivanovic S (2003) The use of natural antimicrobials. In: P Zeuthen and L Bøgh-Sørensen (Eds.) Food Preservation Techniques, Woodhead Publishing Ltd, Cambridge, pp. 5-30.

De Mulder-Johnston (1999) Thermal Analysis of, and oil migration through films from, whey protein isolate. PhD Thesis, University of California, Davis, USA.

de Wit JN and Klarenbeek G (1983) Effects of various heat treatments on structure and solubility of whey proteins. Journal Dairy Science, 67, 2701-2710.

Denavi G, Tapia-Blácido DR, Añón MC, Sobral PJA, Mauri AN and Menegalli FC (2009) Effects of drying conditions on some physical properties of soy protein films. Journal of Food Engineering, 90, 341-349.

Desai PV, Wadekar RR, Kedar GH and Patil KS (2008) Free radical scavenging activity of aqueous extract of roots of *Baliospermum montanum* Muell-Arg. International Journal of Green Pharmacy, 2, 31-33.

Droby S, Wisniewski M, el-Ghaouth A and Wilson C (2003) Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product Aspire. Postharvest Biology and Technology, 27, 127-135.

Duhard V, Garnier JC and Mégard D (1997) Comparison of the stability of selected anthocyanin colorants in drink model systems. Agro-Food-Industry Hi-Tech, 8, 28-34.

Erdohan ZÖ and Turhan KN (2005) Barrier and mechanical properties of methylcellulose–whey protein films. Packaging Technology and Science, 18, 295-302.

Fairley P, Monahan FJ, German JB and Krochta JM (1996) Mechanical Properties and Water Vapor Permeability of Edible Films from Whey Protein Isolate and Sodium Dodecyl Sulfate. Journal of Agricultural and Food Chemistry, 44, 438-443.

Fernández L, de Apodaca ED, Cebrián M, Villarán M and Maté J (2007) Effect of the unsaturation degree and concentration of fatty acids on the properties of WPI-based edible films. European Food Research and Technology, 224, 415-420.

Floris R, Bodnár I, Weinbreck F and Alting AC (2008) Dynamic rearrangement of disulfide bridges influences solubility of whey protein coatings. International Dairy Journal, 18, 566-573.

Franssen LR (2002) Antimicrobial properties and diffusion modeling of preservative-containing whey protein films and coatings on Cheddar cheese. Ph.D. Thesis, University of California, Davis.

Franssen LR, Rumsey TR and Krochta JM (2004) Whey protein film composition effects on potassium sorbate and natamycin diffusion. Journal of Food Science, 69, C347-C350.

Galani D and Apenten RKO (1999) Heat-induced denaturation and aggregation of beta-lactoglobulin: Kinetics of formation of hydrophobic and disulphide-linked aggregates. International Journal Food Science Technology, 34, 467-476.

Galati EM, Mondello MR, Giuffrida D, Dugo G, Miceli N and Pergolizzi S (2003) Chemical characterization and biological effects of Sicilian *Opuntia ficus indica* (L.) Mill. Fruit juice: antioxidant and antiulcerogenic activity. Journal Agricultural Food Chemistry, 51, 4903-4908.

Galietta G, di Gioia L, Guilbert S and Cuq B (1998) Mechanical and thermomechanical properties of films based on whey proteins as affected by plasticizer and crosslinking agents. Journal Dairy Science, 81, 3123-3130.

Gällstedt M and Hedenqvist MS (2002) Oxygen and water barrier properties of coated whey protein and chitosan films. Journal of Polymers and the Environment, 10, 1-4.

Gällstedt M, Brottman A and Hedenqvist MS (2005) Packaging-related properties of protein- and chitosan-coated paper, Packaging Technology and Science, 18, 161-170.

Gounga ME, Xu SY and Wang Z (2007) Whey protein isolate-based edible films as affected by protein concentration, glycerol ratio and pullulan addition in film formation. Journal of Food Engineering, 83, 521-530.

Greenfield AB (2005) A Perfect Red: Empire, Espionage, and the Quest for the Color of Desire. Harper Collins Publishers, New York, USA.

Guilbert S, Cuq B and Gontard N (1997) Recent innovations in edible and biodegradable packaging materials. Food Additives & Contaminants, 14, 741-751.

Guilbert S, Gontard N and Gorris LGM (1996) Prolongation of the shelf-life of perishable food products using biodegradable films and coatings, Food Science and Technology, 29, 10-17.

Hagenmaier, RD and Baker RA (1993) Reduction in gas exchange of citrus fruit by wax coatings. Journal of Agriculture and Food Chemestry, 41, 283-287.

Hammer KA, Carson CF and Riley TV (1999) Antimicrobial activity of essential oils and other plant extracts. Journal Applied Microbiology, 86, 985-990.

Han JH and Floros JD (1997) Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. Journal of Plastic Film and Sheet, 13, 287-298.

Han JH and Floros JD (1999) Modeling antimicrobial activity loss of potassium sorbate against baker's yeast after heat process to develop antimicrobial food packaging materials. Food Science Biotechnology, 8, 11-14.

Han JH and Krochta JM (1999) Water vapor permeability and wetting properties of whey protein coating on paper. Transactions ASAE, 42(5), 1375-1382.

Han JH and Krochta JM (2007) Physical Properties of Whey Protein Coating Solutions and Films Containing Antioxidants. Journal of Food Science, 72, E308-E314.

Han JH and Scanlon MG (2005) Mass transfer of gas and solute through packaging materials. In: JH Han (Ed.), Innovations in Food Packaging. London, UK, Academic Press. ISBN: 0-12-311632-5.

Han JH, Zhang Y and Buffo R (2005) Surface chemistry of food, packaging and biopolymer materials. In: JH Han (Ed.), Innovations in Food Packaging. London, UK, Academic Press. ISBN: 0-12-311632-5.

Hansen AP and Heinis JJ (1992) Benzaldehyde, citral, and d-limonene flavour perception in the presence of casein and whey proteins. Journal Dairy Science, 75, 1211-1215.

Hernandez RJ, Selke SEM and Culter JD (2000) Plastics packaging: properties, processing, applications, and regulations. Cincinnati, OH: Hanser, pp. 425.

Hernandez VM (2007) Thermal properties, extrusion and heat-sealing of whey protein edible films. PhD Thesis, University of California, Davis, Davis, CA.

Hernandez VM and Krochta JM (2008) Thermoplastic processing of proteins for film formation - A review. Journal of Food Science, 73(2), R30-R39.

Hernandez VM, McHugh TH, Berrios J, Olson D, Pan J and Krochta JM (2006) Glycerol content effect on the tensile properties of whey protein sheets formed by twin-screw extrusion. IFT Annual Meeting and Food Expo, Orlando. FL.

Hernandez VM, Reid DS, McHugh TH, Berrios J, Olson D and Krochta JM (2005) Thermal transitions and extrusion of glycerol-plasticized whey protein mixtures. IFT Annual Meeting and Food Expo, New Orleans, LA.

Hersko V and Nissinovitch A (1998) The behavior of hydrocolloid coatings on vegetative materials. Biotechnology Prog, 14, 756-765.

Hettiarachchy NS and Eswaranandam S (2005) Edible films and coatings from soybean and other protein sources. In: F Shahidi (Ed.), Bailey's Industrial Oil and Fat Products - Industrial and non edible products from oil and fats. volume 6, 6th edition, John Wiley & Sons. ISBN:978-1-60119-121-2.

Hettiarachchy NS and Eswaranandam S (2007) Organic acids incorporated edible antimicrobial films. US Patent No. US7160580B2.

Hong SI and Krochta JM (2003) Oxygen barrier properties of whey protein isolate coatings on polypropylene films. Journal of Food Science. 68(1), 224-228.

Hong SI and Krochta JM (2006) Oxygen barrier performance of whey-protein-coated plastic films as affected by temperature, relative humidity, base film and protein type. Journal of Food Engineering, 77, 739-745.

Hong SI, Han JH and Krochta JM (2004) Optical and surface properties of whey protein isolate coatings in plastic films as influenced by substrate, protein concentration, and plasticizer type. Journal of Applied Polymer Science, 92, 335-343.

Hong YH and Creamer LK (2002) Changed protein structures of bovine β-lactoglobulin B and α-lactalbumin as a consequence of heat treatment. International Dairy Journal, 12, 345-359.

Janes ME, Kooshesh S and Johnson MG (2002) Control of *Listeria monocytogenes* on the surface of refrigerated, ready-to-eat chicken coated with edible zein film coatings containing nisin and/or calcium propionate. Journal of Food Science, 67, 2754-2757.

Juneja VK and Thippareddi H (2004) Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. International Journal Food Microbiology, 93, 155-163.

Keeney EL and Broyles EN (1943) Sodium propionate in the treatment of superficial fungous infections. Bulletin of the Johns Hopkins Hospital, 73, 479.

Khwaldia K, Perez C, Banon S, Desobry S and Hardy J (2004) Milk proteins for edible films and coatings. Critical Reviews in Food Science and Nutrition, 44, 239-251.

Kiery LJ and Olson NE (2000) The physicochemical surface characteristics of *Lactobacillus casei*. Food Microbiology, 17, 277-291.

Kim HJ, Kim EJ, Seo SH, Shin CG, Jin C and Lee YS (2006) Vanillic acid glycoside and quinic acid derivatives from *Gardeniae fructus*. Journal Natural Products, 69, 600-603.

Kim KK, Fravel DR and Papavizas GC (1990) Glucose oxidase as the antifungal principle of talaron from *Talaromyces flavus*. Canadian Journal Microbiology, 36, 760-764.

Kim SJ and Ustunol Z (2001a) Sensory attributes of whey protein isolate and candelilla wax emulsion edible films. Journal Food Science, 66(6), 909-911.

Kim SJ and Ustunol Z (2001b) Thermal properties, heat sealability and seal attributes of whey protein isolate/lipid emulsion edible films. Journal Food Science, 66(7), 985-990.

Kim SJ and Ustunol Z (2001c) Solubility and moisture sorption isotherms of whey-protein-based edible films as influenced by lipid and plasticizer incorporation. Journal Agricultural Food Chemistry, 49, 4388-4391.

Kinsella JE and Whitehead DM (1989) Proteins in whey: chemical, physical, and functional properties. Advances Food Nutrition Resources, 33, 343-427.

Ko S, Janes ME, Hettiarachchy NS and Johnson MG (2001) Physical and chemical properties of edible films containing nisin and their action against *Listeria monocytogenes*. Journal of Food Science, 66, 1006-1011.

Krochta J and Mulder-Johnston C (1997) Edible and biodegradable polymer films challenges and opportunities. Food Technology, 51, 61-74.

Krochta JM (2002) Proteins as raw materials for films and coatings: definitions, current status, and opportunities. In: A Gennadios (Ed.), Protein-based film and coatings. USA, CRC Press LLC, ISBN1-58716-107-9.

Krochta JM, Baldwin EA and Nisperos-Carriedo M (1994) Edible coatings and films to improve food quality. Technomic Publishing Co. Basel, Switzerland.

Kunte LF, Gennadios A, Cuppett SL, Hanna MA and Weller CL (1997) Cast films from soy protein isolates and fractions. Cereal Chemistry, 74, 115-118.

Kweon M, Hwang H and Sung H (2001) Identification and antioxidant activity of novel chlorogenic acid derivatives from bamboo (*Phyllostachys edulis*). Journal of Agriculture and Food Chemistry, 49, 4646-4655.

Lacroix M and Crooksey K (2005) Edible films and coatings from animal-origin proteins. In: JH Han (Ed.), Innovations in Food Packaging. London, UK, Academic Press. ISBN: 0-12-311632-5.

Lacroix M and Le Tien C (2005) Edible films and coatings from non starch polysaccharides In: JH Han (Ed.), Innovations in Food Packaging. London, UK, Academic Press. ISBN: 0-12-311632-5.

Lacroix M, Le TC, Ouattara B, Yu H, Letendre M, Sabato SF, Mateescu MA and Patterson G (2002) Use of gamma-irradiation to produce films from whey, casein, and soya proteins: structure and functional characteristics. Radiation Physics and Chemistry, 63, 827-832.

Lanciotti R, Gianotti A, Patrignani F, Belletti N, Guerzoni ME and Gardini F (2004) Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. Trends in Food Science and Technology, 15, 201-208.

Lawton JW (1995) Surface energy of extruded and jet cooked starch. Starch/Stärke, 47, 62-67.

Le Tien C, Letendre M, Ispas-Szabo P, Mateescu MA, Delmas-Patterson G, Yu HL and Lacroix (2000) Development of Biodegradable films from whey proteins by cross-linking and entrapment in cellulose. Journal of Agricultural and Food Chemistry, 48, 5566-5575.

Le Tien C, Vachon C, Mateescu MA and Lacroix M (2001) Milk protein coatings prevent oxidative browning of apples and potatoes. Journal Food Science, 66, 512-516.

Lee CY, Son SM and Moon KD (2000) Kinetic study of oxalic acid inhibition on enzymatic browning. Journal Agriculture Food Chemistry, 48, 2071-2074.

Lee JW, Son SM and Hong SI (2008) Characterization of protein-coated polypropylene films as a novel composite structure for active food packaging application. Journal of Food Engineering, 86, 484-493.

Lee JY, Park HJ, Lee CY and Choi WY (2003) Extending shelf-life of minimally processed apples with edible coatings and antibrowning agents. Lebensmittel-Wissenschaft und-Technologie, 36, 323-329.

Lee SJ and Rosenberg M (1999) Preparation and properties of glutaraldehyde cross-linked whey protein-based microcapsules containing theophylline. Journal of Controlled Release, 61, 123-136.

Lee SY and Krochta JM (2002) Accelerated shelf-life testing of whey-protein coated peanuts analyzed by static headspace gas chromatography. Journal Agriculture Food Chemistry, 50, 2022-2028.

Lee SY, Dangaran KL and Krochta JM (2002a) Gloss stability of whey-protein plasticizer coating formulations on chocolate surface. Journal Food Science, 67(3), 1121-1125.

Lee SY, Trezza TA, Guinard JX and Krochta JM (2002b) Whey-protein-coated peanuts assessed by sensory evaluation and static headspace gas chromatography. Journal Food Science, 67, 1212-1218.

Li J and Chen H (2000) Biodegradation of whey protein-based edible films. Journal of Polymers and the Environment, 8, 135-143.

Lim LT, Mine Y and Tung MA (1998) Transglutaminase cross-linked egg white protein films: tensile properties and oxygen permeability. Journal Agriculture Food Chemistry, 46(10), 4002-4029.

Limjaroen P (2003) Development of a food packaging coating material with antimicrobial properties. Journal Plastic Film Sheeting, 19, 95-109.

Lin SY and Krochta JM (2003) Plasticizer effect on grease barrier and color properties of whey-protein coating on paperboard. Journal Food Science, 68(1), 229-233.

Lin SY and Krochta JM (2006) Fluidized-Bed system for whey protein film coating of peanuts. Journal of Food Process Engineering, 29, 532-546.

Liu Z (2005) Edible films and coatings from starches. In: JH Han (Ed.), Innovations in Food Packaging. London, UK, Academic Press. ISBN: 0-12-311632-5.

Mahmoud R and Savello P (1992) Mechanical properties of and water vapor transferability through whey protein films. Journal Dairy Science, 75, 942-946.

Massa S, Petruccioli M, Brocchi G, Altieri C, Sinigaglia M and Spano M (2001) Growth inhibition by glucose oxidase system of enterotoxic *Escherichia coli* and *Salmonella derby*: in vitro studies. World Journal of Microbiology & Biotechnology, 17, 287-291.

Maté JI and Krochta JM (1996a) Comparison of oxygen and water vapor permeabilities of whey protein isolate and β-Lactoglobulin edible films. Journal of Agricultural and Food Chemistry, 44, 3001-3004.

Maté JI and Krochta JM (1996b) Whey protein coating effect on the oxygen uptake of dry roasted peanuts, Journal Food Science, 61, 1202-1206.

Maté JI and Krochta JM (1998) Oxygen uptake model for uncoated and coated peanuts. Journal Food Engineering, 35, 299-312.

McGuire J and Kirtley SA (1988) Surface characterization for prediction of food particle behavior at interfaces: Theoretical considerations and limitations. Journal Food Engineering, 8, 273-286.

McHugh TH and Krochta JM (1994a) Water vapor permeability properties of edible whey protein-lipid emulsion films. Journal of the American Oil Chemists' Society, 71(3), 307-3 11.

McHugh TH and Krochta JM (1994b) Sorbitol- vs glycerol-plasticized whey protein edible films: integrated oxygen permeability and tensile property evaluation. Journal of Agricultural and Food Chemistry, 42, 841-845.

McHugh TH and Krochta JM (1994c) Milk protein based edible films and coatings. Food Technology, 48(1), 97-103.

McHugh TH, Aujard JF and Krochta JM (1994) Plasticized whey protein edible films: water vapor permeability properties. Journal Food Science, 59(2), 416-419.

Mezgheni E, D'Aprano G and Lacroix M (1998) Formation of sterilized edible films based on caseinates: Effects of calcium and plasticizers. Journal of Agricultural and Food Chemistry, 46, 318-324.

Miller KS and Krochta JM (1997) Oxygen and aroma barrier properties of edible films: A review. Trends in Food Science & Technology, 8, 228-237.

Mills AAS, Platt HW and Hurta RAR (2004) Effect of salt compounds on mycelial growth, sporulation and spore germination of various potato pathogens. Postharvest Biology and Technology, 34, 341-350.

Min S and Krochta JM (2007) Ascorbic acid-containing whey protein film coatings for control of oxidation. Journal of Agricultural and Food Chemistry, 55, 2964-2969.

Min S, Harris LJ and Krochta JM (2005) Antimicrobial effects of lactoferrin, lysozyme and the lactoperoxidase system, and edible whey protein films incorporating the lactoperoxidase system against *Salmonella enterica* and *Escherichia coli* O157:H7. Journal of Food Science, 70(7), M332-M338.

Min S, Harris LJ and Krochta JM (2006) Inhibition of *Salmonella enterica* and *Escherichia coli* O157:H7 on roasted turkey by edible whey protein coatings incorporating lactoperoxidase system. Journal of Food Protection, 69, 784-793.

Min S, Rumsey TR and Krochta JM (2008) Diffusion of the antimicrobial lysozyme from a whey protein coating on smoked salmon. Journal of Food Engineering, 84, 39-47.

Morillon, V, Debeaufort F, Blond G, Capelle M and Voilley A (2002) Factors affecting the moisture permeability of lipid-based edible films: a review. Critical Reviews in Food Science and Nutrition, 42, 67-89.

Morris PE and FitzGerald RJ (2008) Whey Proteins and Peptides in Human Health. In: CI Onwulata and PJ Huth (Ed.), Whey processing, functionality and heath benefits. USA, John Wiley & Sons, Inc. ISBN 978-0-813-80903-8.

Murray FR, Llewellyn DJ, Peacock WJ and Dennis ES (1997) Isolation of the glucose oxidase gene from *Talaromyces flavus* and characterisation of its role in the biocontrol of *Verticillium dahliae*. Current Genetics, 32, 367-375.

Neetoo H, Ye M and Chen H (2008) Potential antimicrobials to control *Listeria monocytogenes* in vacuum-packaged cold-smoked salmon pâté and fillets. International Journal of Food Microbiology, 123, 220-227.

No HK, Park NY, Lee SH and Samuel P (2002) M. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. International Journal Food Microbiology, 74, 65-72.

Okonogi S, Duangrat C, Anuchpreeda S, Tachakittirungrod S and Chowwanapoonpohn S (2007) Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. Food Chemistry, 3, 839-846.

Olivas GI and Barbosa-Cánovas GV (2005) Edible coatings for fresh-cut fruits. CRC Critical Reviews Food Science, 45, 657-670.

Onwulata CI (2008) Baking properties of milk-coated wheat bran. Journal of Food Processing and Preservation, 32, 24-38.

Ornebro J, Nylandert T and Eliasson AC (2000) Critical review - Interfacial behaviour of wheat proteins. Journal Cereal Science, 31(2), 195-221.

Ozdemir M and Floros JD (2003) Film composition effects on diffusion of potassium sorbate through whey protein films. Journal of Food Science, 68, 511-516.

Padget T, Han IY and Dawson PL (2000) Effect of lauric acid addition on the antimicrobial efficacy and water permeability of corn zein films containing nisin. Journal of Food Processing and Preservation, 24, 423-432.

Park HJ (1999) Development of advanced edible coatings for fruits. Trends in Food Science and Technology, 10, 254-260.

Park HJ (2003) Edible coatings. In: P Zeuthen, LB Sorensen (Eds.) Food preservation techniques, Woodhead Publishing, CRC Press.

Parris N, Coffin DR, Joubran RF and Pessen H (1995) Composition factors affecting the water vapor permeability and tensile properties of hydrophilic films. Journal of Agriculture and Food Chemistry, 43, 1432-1435.

Pasanphan W (2008) Chitosan conjugated with deoxycholic acid and gallic acid: a novel biopolymer-based additive antioxidant for polyethylene. Journal Applied Polymer Science, 109, 38-46.

Pérez-Gago MB and Krochta JM (1999) Water permeability of whey protein emulsion films as affected by pH. Journal of Food Science, 64, 695-698.

Pérez-Gago MB and Krochta JM (2001) Lipid particle size effect on water vapour permeability and mechanical properties of whey protein/beeswax emulsion films. Journal of Agriculture and Food Chemistry, 49, 996-1002.

Pérez-Gago MB and Krochta JM (2002) Formation and properties of whey protein films and coatings. In: A. Gennadios (Ed.), Protein-based films and coatings. Boca Raton FL, USA: CRC Press, pp. 159-180.

Pérez-Gago MB, Nadaud P and Krochta JM (1999) Water vapor permeability, solubility, and tensile properties of heat-denatured versus native whey protein films. Journal Food Science, 64, 1034-1037.

Pérez-Gago MB, Serra M and del Río MA (2006) Color change of fresh-cut apples coated with whey protein concentrate-based edible coatings. Postharvest Biology and Technology, 39, 84-92.

Pérez-Gago MB, Serra M, Alonso M, Mateos M and Del Rio MA (2003) Effect of solid content and lipid content of whey protein isolate-beeswax edible coatings on color change of fresh-cup apples. Journal Food Science, 68, 2186-2191.

Pintado CM, Ferreira MASS and Sousa I (2009) Properties of whey protein-based films containing organic acids and nisin to control *Listeria monocytogenes*. Journal of Food Protection, 72(9), 1891-1896.

Pintore G, Usai M, Bradesi P, Juliano C, Boatto G and Tomi F (2002) Chemical composition and antimicrobial activity of *Rosmarinus officinalis* oils from Sardinia and Corsica. Flavour Fragrance Journal, 17, 5-19.

Pourmorad F, Hosseinimehr SJ and Shahabimajd N (2006) Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. African Journal Biotechnololy, 5, 1142-1145.

Pranoto Y, Salokhe VM and Rakshit SK (2005) Physical and antibacterial properties of alginate-based edible film incorporated with garlic oil. Food Research International, 38, 267-272.

Puyol P, Perez MD and Horne DS (2001) Heat-induced gelation of whey protein isolates (WPI): effect of NaCl and protein concentration. Food Hydrocolloids, 15, 233-237.

Ramos OS, Fernandes JF, Silva SI, Pintado ME and Malcata FX (2012) Edible films and coatings from whey proteins: a review on formulation, and on mechanical and bioactive properties. Critical Reviews in Food Science and Nutrition, 52(6), 533-52.

Ramos OS (2011) Development and characterization of bioactive edible, whey protein films and coatings, to improve quality and safety of food products. PhD Thesis, Instituto de Tecnologia Química e Biológica – Universidade Nova de Lisboa, Lisboa, Portugal.

Ramos OS and Malcata FX (2011) Edible and biodegradable packaging for food storage. In: GE Cohen and CM Levin (Eds.), Food storage. Nova Science Publishers.

Regalado C, Pérez-Pérez C, Lara-Cortés E and García-Almendanez B (2006) Whey protein based edible food packaging films and coating. In: Guevara-González RG and Torres-Pacheco I (Eds.) Advances in agricultural and food biotechnology. Research Signpost, Mexico, pp 237-261, ISBN 81-7736-269-0.

Reinoso E, Mittal G and Lim LT (2007) Influence of whey protein composite coatings on plum (Prunus Domestica L.) fruit quality. Food and Bioprocess Technology, 1(4), 314-325.

Řiháková Z, Plocková M, Filip V and Šmidrkal J (2001) Antifungal activity of lauric acid derivatives against *Aspergillus niger*. European Food Research and Technology, 213, 488-490.

Rogers CE (1985) Permeation of gases and vapours in polymers. In J. Comyn (Ed.) Polymer Permeability, London, England: Kluwer Academic Publishers, pp. 11-74.

Sai-Mokbel SM and Suganuma T (2006) Antioxidant and antimicrobial activities of the methanol extracts from pummelo (*Citrus grandis* Osbeck) fruit albedo tissues. European Food Research and Technology, 224, 29-47.

Salame M (1986) Barrier Polymers. In: M. Bakker (Ed.) The Wiley Encyclopedia of Packaging Technology, New York, John Wiley & Sons, pp. 48–54.

Saleem M, Kim HJ, Jin C and Lee YS (2004) Antioxidant caffeic acid derivatives from leaves of *Parthenocissus tricuspidata*. Archives of Pharmacal Research, 27, 300-304.

Samelis J, Sofos JN, Kain ML, Scanga JA, Belk KE and Smith GC (2001) Organic acids and their salts as dipping solutions to control *Listeria monocytogenes* inoculated following processing of sliced pork bologna stored at 4 ºC in vacuum packages. Journal of Food Protection, 64, 1722-1729.

Sang-Myung L, Min-Kyun N, Ren-Bo A, Byung-Sun M and Hyeong-Kyu L (2003) Antioxidant activity of two phloroglucinol derivatives from *Dryopteris crassirhizoma*. Biological and Pharmaceutical Bulletin, 26(9), 1354-1356.

Santos JA, Gonzalez C, García-López ML, García-Fernaández MC and Otero A (2008) Antibacterial activity of the lactoperoxidase system against *Aeromonas hydrophila* in broth, skim milk and ewes' milk. Letters in Applied Microbiology, 19, 161-164.

Schwartz M and Winterhalter P (2004) Novel aged anthocyanins from Pinotage wines: Isolation, characterization and pathway of formation. In: AL Waterhouse and JA Kennedy (Eds.) Red Wine Color: Revealing the Mysteries, American Chemical Society symposium series 886, Oxford University Press, Washington DC, USA, pp. 179-197.

Sekiguchi S, Miura Y, Kaneko H, Nishimura SI, Nishi N, Iwase M and Tokura S (1994) Molecular weight dependency of antimicrobial activity by chitosan oligomers. In: K Nishinari, E Doi (Eds.) Food Hydrocolloids: Structures, Properties, and Functions, Plenum, New York, USA, pp. 71-76.

Seydim AC and Sarikus G (2006) Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. Food Research International, 39, 639-644.

Shaw NB, Monahan FJ, O'Riordan ED and O'Sullivan M (2002) Physical properties of WPI films plasticized with glycerol, xylitol, or sorbitol. Journal Food Science, 67(1), 164-167.

Shelef LA, Mohammed S, Tan W and Webber ML (1997) Rapid optical measurements of microbial contamination in raw ground beef and effects of citrate and lactate. Journal of Food Protection, 60, 673-676.

Shellhammer TH and Krochta JM (1997a) Whey protein emulsion film performance as affected by lipid type and amount. Journal Food Science, 62(2), 390-394.

Shellhammer TH and Krochta JM (1997b) Water vapor barrier and rheological properties of simulated and industrial milkfat fractions. Trans. ASAE, 40, 1119-1127.

Sherwin CP, Smith DE and Fulcher RG (1998) Effect of fatty acid type on dispersed phase particle size distributions in emulsion edible films. Journal Agriculture Food Chemistry, 46(11), 4534-4538.

Shimada K and Cheftel JC (1998) Sulfhydryl group disulfide bond interchange during heat induced gelation of whey protein isolate. Journal of Agriculture Food Chemistry, 37, 161-168.

Shin KS, Youn HD, Han YH, Kang SO and Hah YC (1993) Purification and characterisation of δ-glucose oxidase from white rot fungus *Pleurotus ostreatus*. European Journal of Biochemistry, 215, 747-752.

Simpson C (2006) Isolation, purification and characterization of a novel glucose oxidase from *Penicillium canescens Tt42*. Master thesis, Rhodes University, Grahamstown, South Africa.

Singh RK and Singh N (2005) Quality of packaged foods. In: JH Han (Ed.), Innovations in Food Packaging. London, UK, Academic Press. ISBN: 0-12-311632-5.

Siragusa GR, Cutter CN and Willett JL (1999) Incorporation of bacteriocin in plastic retains activity and inhibits surface growth of bacteria on meat. Food Microbiology, 16, 229-235.

Smith-Palmer A, Stewart J and Fyfe L (1998) Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters Food Microbiology, 26, 118-122.

Soazo M, Rubiolo AC and Verdini RA (2011) Effect of drying temperature and beeswax content on physical properties of whey protein emulsion films. Food Hydrocolloids, 25, 1251-1255.

Sothornvit R and Krochta JM (2000a) Oxygen permeability and mechanical properties of films from hydrolyzed whey protein. Journal of Agriculture and Food Chemistry, 48(9), 3913-3916.

Sothornvit R and Krochta JM (2000b) Plasticizer effect on oxygen permeability of beta-lactoglobulin (β-Lg) films. Journal of Agriculture and Food Chemistry, 48(12), 6298-6302.

Sothornvit R and Krochta JM (2000c) Water vapor permeability and solubility of films from hydrolyzed whey protein. Journal Food Science, 65(4), 700-703.

Sothornvit R and Krochta JM (2001) Plasticizer effect on mechanical properties of beta-lactoglobulin (β-Lg) films. Journal Food Engineering, 50(3), 149-155.

Sothornvit R and Krochta JM (2005) Plasticizers in edible films and coatings. In: JH Han (Ed.), Innovations in Food Packaging. London, UK, Academic Press. ISBN: 0-12-311632-5

Sothornvit R, Olsen CW, McHugh TH and Krochta JM (2003) Formation conditions, water vapor permeability, and solubility of compression-molded whey protein films. Journal Food Science, 68(6), 1985-1989.

Stintzing FC and Carle R (2004) Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. Trends in Food Science and Technology, 15, 19-38.

Stintzing FC, Schieber A and Carle R (2001) Phytochemical and nutritional significance of cactus pear. European Food Research Technology, 212, 396-407.

Stintzing FC, Schieber A and Carle R (2003) Evaluation of colour properties and chemical quality parameters of cactus juices. European Food Research Technology, 216, 303-311.

Sultanova N, Makhmoor T, Abilov ZA, Parween Z, Omurkamzinova VB, ur-Rahman A and Choudhary MI (2001) Antioxidant and antimicrobial activities of *Tamarix ramosissima*. Journal Ethnopharmacology, 78, 201-205.

Stuchell YM and Krochta JM (1995) Edible coatings on frozen ding salmon: Effect of whey protein isolate and acetylated monoglycerides on moisture loss and lipid oxidation. Journal Food Science, 60, 28-31.

Tang CH and Jiang Y (2007) Modulation of mechanical and surface hydrophobic properties of food protein films by transglutaminase treatment. Food Research International, 40, 504-509.

Taylor CC (1986) Cellophane. In: M. Bakker (Ed.), The Wiley Encyclopedia of Packaging Technology, New York, John Wiley & Sons, pp. 159-163.

Tepe B, Sokmen M, Akpulat HA and Sokmen A (2006) Screening of the antioxidant potentials of six Salvia species from Turkey. Food Chemistry, 95, 200-204.

Toussaint AF, Luner P (1993) The wetting properties of grafted cellulose films. Journal Adhesion Science Technology, 7(6), 635-648.

Trezza TA and Krochta JM (2000) Color stability of edible coatings during prolonged storage. Journal Food Science, 65(7), 1166-1169.

Trezza TA and Krochta JM (2001) Specular reflection, gloss, roughness and surface heterogeneity of biopolymer coatings. Journal Applied Polymer Science, 79, 2221-2229.

Uchida Y, Izume M and Ohtakara A (1989) Preparation of chitosan oligomers with purified chitosanase and its application. In: G. Skjåk-Bræk, T. Anthonsen, P Sandford (Eds.) Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications. Elsevier, London, UK, pp 373-382.

Vogler EA (1998) Structure and reactivity of water at biomaterial surfaces. Advances in Colloid and Interface Science, 74, 69-117.

Wang TC, Ti MC, Lo SC and Yang CC (2007) Free radical-scavenging activity of aqueous extract of *Pteris multifida* Poiret. Fitoterapia, 78, 248-249.

Weber CJ, Haugaard V, Festersen R and Bertelsen G (2002) Production and applications of biobased packaging materials for the food industry. Food Additives Contaminants, 19, 172-177.

Wohlfahrt G, Witt S, Hendle J, Schomburg D, Kalisz HM and Hecht HJ (1999) 1.8 and 1.9 Å resolution structures of the *Penicillium amagasakiense* and *Aspergillus niger* glucose oxidases as a basis for modelling substrate complexes. Acta Crystallographica, 55, 969-977.

Zapico P, Gaya P, de Paz M, Nunez M and Medina M (1991) Influence of breed, animal and days of lactation on lactoperoxidase system components in goat milk. Journal of Dairy Science, 74, 783-787.

Zapico P, Gaya P, Nuñez M and Medina M (1995) Activity of goat's milk lactoperoxidase system on *Pseudomonas fluorescens* and *Escherichia coli* at refrigeration temperatures. Journal of Food Protection, 58, 1136-1138.

Zhu X, Zhang H, Lo R and Lu Y (2005) Antimicrobial activities of *Cynara scolymus* L.: leaf, head, and stem extracts. Journal Food Science, 70, M149-M152.

Zinoviadou KG, Koutsoumanis KP and Biliaderis CG (2009) Physico-chemical properties of whey protein isolate films containing oregano oil and their antimicrobial action against spoilage flora of fresh beef. Meat Science, 82, 338-345.

Chapter 8

The development of whey protein concentrate films by UV modification

Abstract

This chapter describes and characterises edible films made from whey protein concentrates (WPC) produced by UV modification (UV) and compares them with films produced by solvent evaporation (SE) only, films produced by heat denaturation using the conventional solvent casting method (HD) and films that combine UV modification and heat denaturation (HD+UV). It aims to assess the influence of film formulation (WPC, glycerol and photoinitiator contents) on the thickness, moisture, solubility, barrier and optical properties of WPC films using a response surface methodology. The edible WPC films produced had thicknesses of less than 180 μm. The presence of lactose and the use of UV irradiation may be responsible for a slight yellowness in

the films due to the development of Maillard reactions, although some transparency values were within the range for synthetic films (e.g. oriented polypropylene, and polyethylene). It was verified that by using different methods and film compositions it was possible to produce WPC films with similar water vapour permeability and moisture content. UV modification therefore leads to the production of films with lower water vapour permeability and moisture content. Heat denaturation improved film insolubility, making WPC films more suitable for higher moisture applications.

The effect of the film production method on the molecular structure, thermal, mechanical and surface properties of the said films was also determined by setting the film chemical composition at 10% (w/v solution) for WPC, 10% (w/w, WPC basis) for glycerol content and 3.5% (w/w, WPC basis) for photoinitiator. As well as the film production method, the chemical composition of the WPC base material and plasticizer also affected the molecular structure of the film matrix. Film thermo-stability was improved by applying heat treatment. However, the film surface properties showed that the hydrophobic nature of the film was improved by UV modification. The film tensile properties were not significantly affected by the film production method. In comparison to synthetic polymers (e.g. polystyrene), the elongation values were similar, but the tensile strength and Young modulus values were significantly lower, which limits their application as a packaging material. Nevertheless their use in edible food coatings remains viable and promising.

8.1 Introduction

Given that 265 million tons of plastics are used annually throughout the world, 39% of which are used for packaging (Plastics of Europe, 2010 and 2011), it is evident that the potential market for biobased packaging materials is enormous if they can be produced with the proper processability and functionality and at a competitive price. The markets for biobased food-packaging materials are expected to start out as niche markets, in which the unique properties of the biobased materials, their availability, source, manufacturing process and specific applications offer advantages to the packaging concept (Weber et al., 2002). The development of edible/biodegradable packaging with non-toxic properties that might replace synthetic polymers, thus reducing the environmental impacts, is of great interest nowadays (Li and Chen, 2000). A wide variety of polymers produced from renewable sources such as polysaccharides, proteins, lipids and their composites, derived from plant and animal feedstocks have been investigated

(Fernández et al., 2007; Naushad and Stading, 2007; Guerrero et al., 2010). Although these materials have found specific applications as effective barriers (Osés et al., 2009; Miller and Krochta, 1997), their use as packaging film depends largely on the intended purpose (Cha and Chinnan, 2004).

The use of whey protein to manufacture films and coatings has received a great deal of attention, since such films are edible, biodegradable, possess interesting mechanical and barrier properties (Fang et al., 2002; Gennadios, 2004; Hernandez-Izquierdo and Krochta, 2008; Ghanbarzadeh and Oromiehi, 2009) and allow for the upgrading of cheesemaking effluents. The interesting properties attributed to whey proteins have accordingly been applied to the manufacture of transparent, flexible, colourless and odourless films for foodstuff applications.

Research into edible whey protein films has focused on the use of whey protein isolate (WPI) with a protein content greater than 90%. Nevertheless, several whey protein concentrates (WPCs) ranging from 35-80% are available at a considerably lower price, although the presence of other constituents such as lipids, minerals and lactose that comprise the bulk of the non-nitrogenous components in WPC is cited as responsible for the functional changes in WPC-based edible films. These differences may have a marked influence on the intermolecular bonds in films manufactured from these materials, and consequently their barrier, mechanical and thermal properties, as a result of distinct molecular structures (Khwaldia et al, 2004; Floris et al., 2008). Therefore it is necessary to take into account the composition of the film-forming solution, the method of film production, and the incorporation of plasticizers to reduce brittleness, allowing for easier removal from the forming support and plastic behaviour for the film. Glycerol is recognised as the plasticizer that produce the best results in whey protein films among polyols (e.g. sorbitol, polyethylene glycol), mono- and di- oligosaccharides (Gounga et al., 2007) and lipids (e.g. oleic, linoleic and stearic acid) (Fernández et al., 2007). The plasticizer molecules reduce intermolecular forces along the polymer chains, thus improving the flexibility, extensibility, toughness and tear resistance of the film. However, their barrier properties and mechanical resistance are compromised (Karbowiak and Voilley, 2006; Coupland et al., 2000).

Edible whey protein films are mainly produced by the well-known solvent casting method (Chapter 7 - Section 7.3) in which protein cross-linking is promoted by thermal denaturation. Extensive work has been published on solvent casting and drying polymerization as well as on the influence of processing conditions (such as pressure, temperature and time), due to their

direct effects on the denaturation of proteins via the unfolding of their globular structure which promotes interaction and entanglement between protein chains (Ghanbarzadeh and Oromiehi, 2009; Denavi et al., 2009). However, in order to enhance film formation, other cross-linking processes, such as chemical, enzymatic or even irradiation processes, have also been documented. Very little information exists on the application of UV irradiation in the production of WPC based films (Hettiarachchy and Eswaranandam, 2005). For that reason this research therefore intends to investigate in greater detail the cross-linking mechanism induced by UV irradiation and applied to WPC films.

8.1.1 UV modification

UV irradiation technology uses the energy of photons from radiation sources in the short wavelength region of the electromagnetic spectrum (200-400 nm) to form reactive species which trigger a fast chain growth reaction. This is based on a fast, room temperature process with low energy consumption, requiring little equipment space (Schwalm, 2006). The more expensive and high energy e-beam and X-ray photons are sufficient to cleave C-C or C-H bonds and therefore do not need photoinitiator species to form the radicals required as initiators for polymerization. In the case of UV exposure (in the wavelength region of 300-400 nm), the cleavage of C-C bonds should be possible although photoinitiators are commonly used, since the direct cleavage processes are not efficient enough.

The chemistry involved in radical UV induced cross-linking (Odian, 2004) starts with the absorption of a photon by the photoinitiator molecule (Figure 8.1), which results in the excitation of an electron into higher singlet states. If the inactivation of this exited molecule does not take place, the process can follow the intersystem crossing an electron spin inversion by producing the excited triplet state (Schwalm, 2006). From the triplet state two main reactions can lead to initiating species (free radicals) which can start radical polymerization; the intramolecular scission of an α-bond, or the intermolecular abstraction of a hydrogen atom, depending on the photoinitiator type. Intramolecular scission is the most effective process in the formation of radicals, since hydrogen abstraction is a bimolecular type reaction in which diffusion is controlled and may be accompanied by several deactivation reactions. Thus, only the initiation step is different to thermal initiated radical polymerization, whereas the polymerization reactions pursued follow almost exactly the same rules.

Photoinitiators are molecules that absorb photons on irradiation with light and form reactive species from their excited state, which then initiate consecutive reactions. For this reason, it is essential that they are selected to match with the output spectrum of the UV light source. They may be classified as cations, anions or radical photoinitiators, the latter representing more than 90% of the commercially used initiators. Almost all radical photoinitiators contain the benzoyl(phenyl-CO-) structure element. The two most important classes are the α-cleavable (type I) and the non-cleavable (hydrogen abstraction - type II) photoinitiators. The α-cleavage-type photoinitiators are very versatile, exhibiting higher efficiency in comparison to hydrogen abstraction types due to the unimolecular cleavage reaction and consequently they are the most widely used (Schwalm, 2006). In this study, the radical photoinitiator Irgacure® 2959 (2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone) supplied by Ciba Specialty Chemicals (CSC, 2002), was used to perform UV whey protein modification for coating production. It is an α-cleavable photoinitiator commercialised in powder form and recommended for the production of clear coatings (Figure 8.1). This photoinitiator is especially recommended for waterborne formulations and its main characteristics are the GRAS (generally recognised as safe) classification which enables it to be used in food products, the very low odour, low volatility and, most importantly, the presence of terminal hydroxyl groups which may react into polymer backbone and do not generate benzaldehyde after cleavage (NICNAS, 2002).

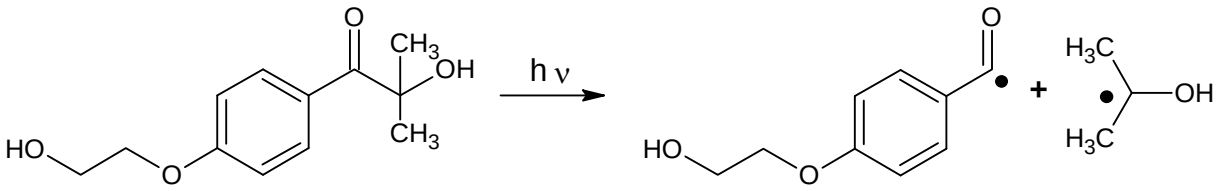


Figure 8.1 Initiation step of the α-cleavage (type I) photoinitiator Irgacure 2959® from Ciba Specialty Chemicals.

The typical photoinitiator loadings (as recommended by the supplier) are in the range of 1-5%, and 2-3% are often reasonable. High concentrations are only necessary to overcome the effects of oxygen inhibition (Schwalm, 2006). However, the loading should not be too high in order to avoid filter effects that prevent light penetrating to the bottom coating layers, resulting in through cure problems.

The goal of this study was to examine the various physical properties of the WPC films as a cheaper alternative to WPI films and to provide data to support a comparative characterisation of films obtained from bovine WPC (50% protein) using different production methods, with various levels of WPC as a protein source, glycerol as a plasticizer and a photoinitiator (when UV modification is applied). Films were produced by UV modification and compared with films produced by solvent evaporation and by the conventional solvent casting method. The combination of UV modification and heat treatment was also tested in order to evaluate the extent to which the film properties were improved. Firstly, the effects of film chemical composition were assessed in terms of the thickness (Th), moisture (M), solubility (S), water vapour permeability (WVP) and optical properties (i.e. colour difference (ΔE*) and transparency (T)) of films using the experimental design procedure referred to in 8.2.5 for each film production method. In a second phase, films with a similar chemical composition were compared and characterised with regard to their molecular structure (by FTIR), thermal, mechanical and surface properties in order to understand the relationships between these properties, the nature of the protein feedstock, the effect of the plasticizer and the film production method.

8.2 Materials and methods

8.2.1 Materials

Bovine whey protein concentrate powder (UFRP), produced as described in Chapter 3 at the Escola Superior Agrária, was the base protein feedstock used to produce edible films. Glycerol was supplied by Panreac (Barcelona, Spain) and used as a plasticizer. The photoinitiator marketed as Irgacure® 2959 was supplied by Ciba Special Chemicals and used for coatings preparation by UV modification (CSC, 2002). All the other chemicals were reagent-grade or better, and were used without further purification.

8.2.2 Chemical analyses

The composition of the bovine whey protein concentrate (UFRP) was determined as described in Chapter 3 (3.2.3) and Appendix B and shown in Table 3.3.

8.2.3 Film production

Four different types of WPC-based films were produced according to each of the corresponding film production methods schematised in Figure 8.2. Edible films produced only by solvent

evaporation (SE) were used as a control for films produced by heat denaturation (HD), UV modification (UV) or a combination of both treatments (HD+UV). Three WPC levels were tested (5%, 7.5% and 10% (w WPC/v solvent)). However, according to the film production method used, different levels of plasticizer (0%, 5%, 7.5%, 10%, 20% and 30% (w glycerol/w WPC)) and photoinitiator (2%, 3.5% and 5% (w phot/w WPC)) were applied (Figure 8.2). Table 8.1 summarises the film formulations according to the film production method for each WPC concentration.

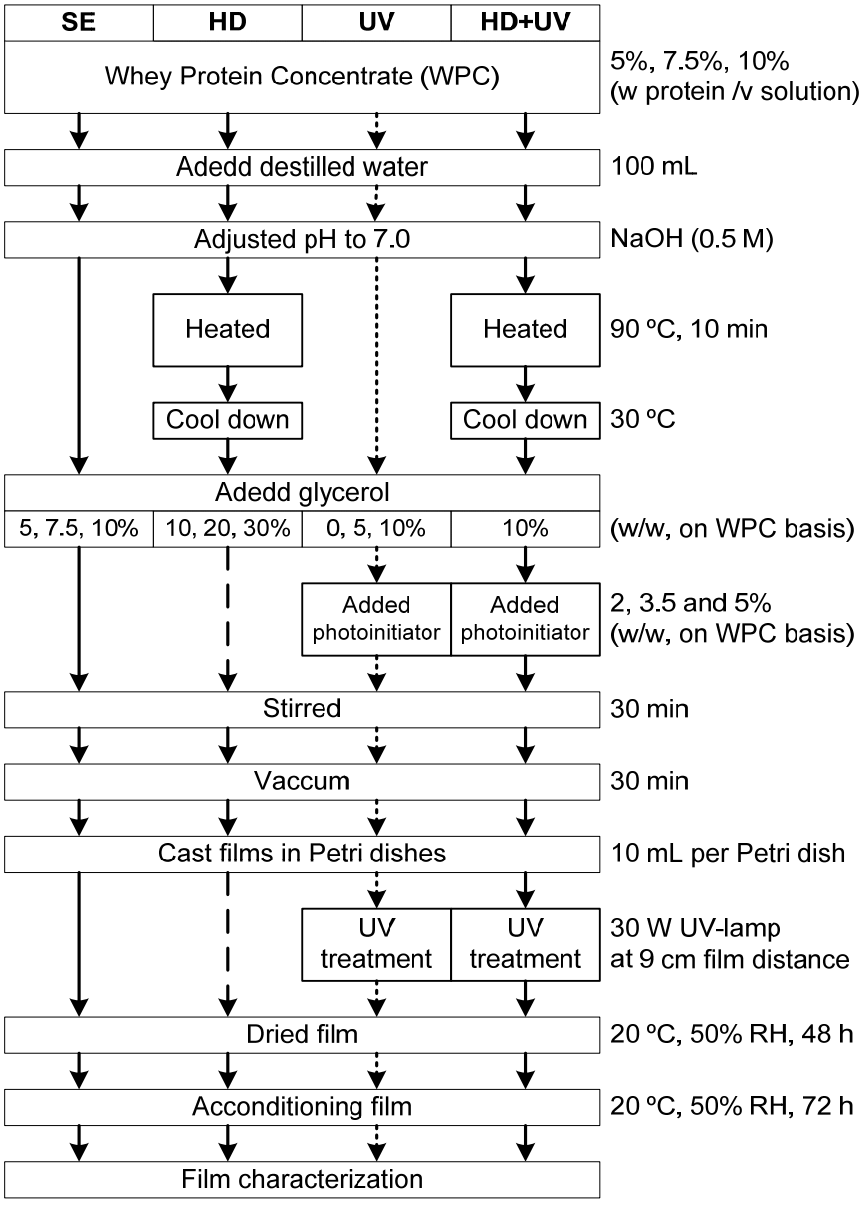


Figure 8.2 WPC-based edible films produced by the methods: solvent evaporation (SE); heat denaturation (HD); UV irradiation (UV); heat denaturation in combination with UV irradiation (HD+UV).

Solvent evaporation technique (SE)

Film-forming solutions were prepared by slowly dissolving bovine WPC powder (5%, 7.5% and 10% (w/v)) in deionised water (Figure 8.2). The solutions were adjusted to pH 7.0 using 0.5 M NaOH (Merck) with continuous stirring. Glycerol was added, at three different levels (5%, 7.5% and 10% (w/w), on a WPC basis) to plasticize the films. The resulting solutions were magnetically stirred until complete dissolution and homogenisation was obtained. The films were produced by measuring exactly 10 mL of each film-forming solution and pouring this onto level Petri dishes (9 cm diameter) in order to control the film thickness. The spreading solutions were allowed to dry in a storage chamber (Fitoclima S600) at a controlled temperature and humidity (20 ºC and 50% RH) for 48 h. Once formed, the films were peeled off and conditioned under the same conditions for at least 72 h prior to testing.

Heat denaturation method (HD)

After WPC aqueous film-forming solutions (5%, 7.5% and 10% (w/v)) were prepared and the pH adjusted (pH = 7.0) with 0.5 M NaOH, they were heated in a water bath at 90 °C for 10 min (Figure 8.2). This step is essential to the formation of intermolecular bonds, which in turn help establish a cross-linked polymeric network structure for obtaining a flexible film that can retain its structural integrity under high moisture environments. However, at this point only protein denaturation is required, without gel formation. After cooling at approximately 30 ºC, glycerol was added on a 10%, 20% and 30% (w/w) WPC basis. In order to remove the air incorporated during stirring, a vacuum was applied for 30 min. The film production and conditioning was as described previously for the solvent evaporation method, maintaining the same ratio (volume of film-forming solution by film area (10 mL/Petri dish)).

UV modification method (UV)

In the UV modification method (Figure 8.2), the preparation and pH correction of the 5%, 7.5% and 10% (w/v) WPC solutions followed the same procedure described for the SE and HD methods. For each WPC concentration, three amounts of plasticizer (0%, 5% and 10% (w/w) WPC basis - Table 8.1) were added to the initial WPC solutions and homogenised, prior to the UV irradiation step. At this point, three different levels of photoiniator (Irgacure 2959) were tested: 2%, 3.5% and 5% (w/w) WPC basis. UV irradiation (using a 30 W UV-lamp, at 9 cm distance from the film) was performed for 10 min, after placing 10 mL of the film-forming solutions into the Petri dishes. This procedure enabled a thin solution layer to be produced,

enhancing the effect of UV-irradiation by the formation of free radicals responsible for starting polymerization. After UV exposure, the film formation and conditioning were similar to the methods previously described (20 ºC, 50% RH for 48 h and 72 h, respectively).

Table 8.1 WPC edible films formulations according to the film production methods: solvent evaporation (SE); heat denaturation (HD); UV irradiation (UV) and heat denaturation in combination with UV irradiation (HD+UV).

Film production method	WPC (% w WPC/ v solution)	Glycerol (% w gly/w WPC)	Photoinitiator (% w phot/w WPC)
SE	5.0, 7.5, 10	5.0	-
SE	5.0, 7.5, 10	7.5	-
SE	5.0, 7.5, 10	10	-
HD	5.0, 7.5, 10	10	-
HD	5.0, 7.5, 10	20	-
HD	5.0, 7.5, 10	30	-
UV	5.0, 7.5, 10	-	2.0
UV	5.0, 7.5, 10	-	3.5
UV	5.0, 7.5, 10	-	5.0
UV	5.0, 7.5, 10	5	2.0
UV	5.0, 7.5, 10	5	3.5
UV	5.0, 7.5, 10	5	5.0
UV	5.0, 7.5, 10	10	2.0
UV	5.0, 7.5, 10	10	3.5
UV	5.0, 7.5, 10	10	5.0
HD+UV	5.0, 7.5, 10	10	2.0
HD+UV	5.0, 7.5, 10	10	3.5
HD+UV	5.0, 7.5, 10	10	5.0

Heat denaturation and UV modification method (HD+UV)

The aim of using this method was to evaluate the combined effect of heat denaturation and UV modification in the production of edible whey protein films (Figure 8.2). In this procedure, protein denaturation was applied (at 90 ºC for 10 min) immediately after adjusting the pH of each WPC solution (5%, 7.5% and 10% (w/v)). After cooling to approximately 30 ºC the photoinitiator (2%, 3.5% and 5% (w/w)) and glycerol (10% (w/w)), both on a WPC basis, were added and homogenised. The amount of plasticizer was set at 10%, since lower plasticizer values create films that are too brittle in the case of HD cross-linking, whilst higher values lead to very sticky UV films which are also very difficult to handle. The solutions were then submitted to a vacuum for 30 min in order to remove the air incorporated during stirring and finally distributed in Petri dishes (10 mL in each). The UV treatment was applied using the same 30 W UV-lamp

placed 9 cm from the film for 10 min, and similar ambient conditions were applied in the storage chamber (20 ºC and 50% RH) during film formation.

8.2.4 Film characterisation

Thickness

The film thickness was determined using a manual micrometer Model - Arco Aço Fundido, Pantec Metrologia Dimensional (0-25±0.01 mm). The mean thickness of each film was assessed from 8 independent measurements at different positions selected at random.

Moisture content

The moisture (M) content of the films was determined after drying in an oven at 105 ºC, under forced air circulation for 1 h. Small film specimens of approximately 0.2 g were cut and placed on Petri dishes, which were weighed before and after oven drying. The moisture content reported on a wet basis according to equation 8.1 was determined as a fraction of the initial film weight lost during drying.

$$\%\ moisture = \frac{m_i - m_f}{m_i} \times 100 \tag{8.1}$$

where m_i is the initial specimen weight (g) and m_f the film weight after drying (g).

Solubility in water

Film solubility (S) in water is defined as the percentage of the film dry matter solubilised after immersion in water for one hour (equation 8.2). Approximately 0.2 g (dry basis) of the film were weighed and immersed in 20 mL of deionised water under continuous agitation. After one hour the sample was vacuum filtered through a pre-weighed porous crucible (5-8 μm pore diameter). The crucible, containing the undissolved pieces of film, was dried at 105 ºC for 24 h to determine the weight of dry matter not dissolved in water. The weight of the dry matter dissolved in water is the difference between the initial dry matter of the samples and the remaining undissolved dry matter recovered in the crucible.

$$\%\ solubility = \frac{m_0 - m_1}{m_0} \times 100 \tag{8.2}$$

where m_0 is the initial dry matter of the film sample (g) and m_1 is the dry matter of the non dissolved film. Solubility determination was performed in triplicate.

Water vapour permeability

Film water vapour permeability (WVP) was assessed gravimetrically (Gounga et al., 2007). The film was sealed onto a circular plastic permeation cup (with a mouth area of 11×10^{-4} m^2 and a depth of 6 cm) containing silica gel (desiccant RH 0%) with a plastic O ring to hold the film in place. The cups were then placed in a controlled-environment room at 80% RH and 20 ºC. The cups were weighed at certain intervals within a 24 h period and a linear regression analysis of weight gain versus time was performed. The slope of the line in g/d provided the water vapour transmission rate. WVP (expressed as g mm m^{-2} d^{-1} kPa^{-1}) was calculated according to:

$$WVP = \frac{C \times \Delta x}{A \times \Delta P} \times 100 \qquad (8.3)$$

where C is the weight gain of the cup per day (g/d), Δx is the film thickness (mm), A is the area of exposed film (m^2) and ΔP = 1.364 kPa is the differential vapour pressure across the test film for the aforementioned temperature and humidity conditions. At least 3 replicates were produced from each film type.

Optical Properties

Film colour was determined with a Minolta Chroma Meter colorimeter Model CR-200b, previously calibrated with a standard white plate of known parameters ($L^*_{standard}$ = 97.03; $a^*_{standard}$ = -0.67; $b^*_{standard}$ = 5.57), using D_{65} illuminant in the colour space CIEL*a*b*. The three coordinates of CIEL*a*b* are defined in section 7.4.3 and the colour of the films was expressed as the total colour difference (ΔE) calculated according to equation 7.4. Three samples were measured for each condition tested and three readings were taken for each piece of film.

The film transparency was measured as described by Gounga et al. (2007) at 600 nm using a UV-VIS spectrophotometer (Hach Lange, DR 5000) and calculated as described by Han and Floros (1997), using equation 8.4. The film samples were cut into strips (3 x 1 cm) and placed in the reading cell of the spectrophotometer while the empty reading cell was used as a control.

$$Transparency = \frac{A_{600}}{\Delta x} \qquad (8.4)$$

where A_{600} is the absorbance at 600 nm and Δx the film thickness (mm). At least three strips of each film type were tested.

Thermogravimetry

Thermogravimetric analyses (TGA) were performed using a Thermogravimetric Analyzer Q500 V20.10 from TA Instruments (USA). Film samples (5-10 mg) were pre-weighed in platinum pans using a empty pan as reference, placed in the balance system and heated from 25 ºC to 600 ºC at 10 ºC/min, under a nitrogen atmosphere (60 mL/min). The initial decomposition temperatures (Tdi), derivative maximum decomposition rate temperature (DTGmax) and corresponding weight losses, as well as residual mass, were all determined using Universal Analysis software (TA Instruments). All the assays were duplicated.

FTIR-ATR analysis

The spectra of the films were determined using Fourier transform infrared spectrometry (FTIR) with a Jasco spectrometer (model 4000, UK), in attenuated total reflectance (ATR) mode. The spectra were recorded in absorbance mode from 550 to 4000 cm^{-1}, using 32 scans at 4 cm^{-1} resolution. Three spectra regions were preferentially selected (i.e. 800-1200 cm^{-1}; 1600-1700 cm^{-1} and 3000-3600 cm^{-1}), owing to their relevance in protein based films. For each region analysed a linear baseline was subtracted and the absorbance was normalised to the peak maximum (Lefèvre et al., 2005). The initial values of the peak positions were then determined by Fourier deconvolution after several trials to produce proper fits. All the data was processed with the nonlinear fitting software Fityk v 0.8.5. For each film production method two film samples were analysed and three replicates from each film sample were collected.

Mechanical properties

Film tensile properties, tensile strength (TS), elongation at break (E) and elastic or Young's modulus (YM) were measured using a universal testing machine (Chatillon, TCD-1000) equipped with fixed grips with a 50N-static load cell. Film samples were cut into strips (40x10 mm). The initial grip separation was set at 10 mm and the crosshead speed at 30 mm min^{-1}. TS, E and YM were determined using the Nexygen 4.1 Lloyd Instruments software. At least 5 strips of each film sample were analysed.

Surface properties

The surface energy of solids cannot be measured directly. Instead, the contact angle is measured when various test liquids are placed on the solid material. The critical surface energy of the films was determined using the Owens-Wendt-Rabel-Kaelbe (OWRK) model due to the hydrophilic nature of the films. This model requires the measurement of the optical contact angle (θ) on the film surface using the sessile drop technique for various solvents within a wide range of polarity. The Contact Angle System OCA (from Dataphysics, Filderstadt, Germany) with SCA 20 version 2 software was used to determine the contact angle of distilled water, formamide (99% Sigma-Aldrich), ethylene glycol (99.8% Sigma-Aldrich) and propylene glycol (99.5% Sigma-Aldrich) whose surface tensions, previously determined in the same conditions, were 72.8 $mN.m^{-1}$, 58.13 $mN.m^{-1}$, 48.28 $mN.m^{-1}$ and 35.40 $mN.m^{-1}$ respectively.

For static contact-angle measurements of films, small strips (5 x 1 cm) of the film were cut and placed on a glass microscope slide to ensure a flat viewing surface. The glass slide was placed onto the stage, where a 10 μL-droplet of water was deposited on the film surface with a 500 μL precision syringe, using a needle with a diameter of 0.75 mm. The 5 s image of the drop was recorded with a video camera, and its profile was numerically solved and fitted to a Laplace-Young equation. Once the drop was measured, the slide was moved to allow another drop to be placed on the sample surface. Ten replicated measurements of θ were obtained. This exact process was then repeated using a 7 μL droplet size for the remaining chemicals. The critical surface energy of WPC edible films was then calculated by the software using the contact angle calculated for the selected solvents for each film type.

8.2.5 Experimental design

The effect of film composition (WPC, glycerol (Gly) and photoinitiator (Phot) contents) on the thickness (Th), moisture (M), solubility (S), water vapour permeability (WVP), colour difference (ΔE) and transparency (T) of the films was studied for all the film production methods. The values of the Gly and Phot independent variables for each level of WPC (5.0%, 7.5% and 10%) are shown in Table 8.1. The results were used to:

(i) infer the relevance of the various factors in each specific output, using an Analysis of Variance based approach. N-way ANOVA with interactions was used and all the computational results were obtained with Statistica 8.0 software;

(ii) build predictive models using an Analysis of Regression based strategy. The models served to build surface responses which were later used to infer the optimal conditions for each film production method. The Least Squares paradigm was used to regress the data, with the linear regression procedures of Mathematica 8 Kernel for Windows (Wolfram Research, Inc.).

Regarding goal (ii), the data was fitted to a second order equation (equation 8.5) as a function of the dependent variables.

$$\begin{aligned} Yi = {} & b0 + \\ & b1\ WPC + b2\ WPC^2 + \\ & b3\ Gly + b4\ Gly^2 + \\ & b5\ Phot + b6\ Phot^2 + \\ & b7\ WPC\ Gly + b8\ WPC\ Phot + b9\ Gly\ Phot \end{aligned} \tag{8.5}$$

where bn are constant regression coefficients, Yi dependent variables (Th, M, S, WVP, ΔE and T) and WPC, Gly and Phot are the independent variables. For films produced by SE and HD in which the photoinitiator was not present in the formulation, the coefficients b5, b6, b8 and b9 take the value of zero. In the case of films produced by HD+UV, the coefficients b3, b4, b7 and b9 are also zero, since the glycerol content was fixed at 10%.

Three statistical criteria were used to evaluate and compare the quality of all the fitted models: rsquared, AIC (Akaike information criterion) and BIC (Bayesian information criterion). Both the AIC and BIC criterion aim to balance the accuracy and complexity of the model by penalising the number of parameters in each model (Royston and Sauerbrei, 2008) and were used with the basic aim of producing parsimonious models, by selecting the most relevant regressors. BIC then applies a much larger penalty for complex models, and hence leads to simpler models than AIC. BIC also tries to find the true model among the set of candidates which fits the purposes. For these two reasons, fitted models were ranked using the BIC criterion and the best fitted model was considered the one that minimises BIC.

Finally, the best fitted models for film moisture content (M) and water vapour permeability (WVP) were used to evaluate the extent to which different film production methods can produce films with similar properties.

8.2.6 Statistical analysis

A statistical analysis of the thermal, mechanical (tensile strength (TS), elongation (E) and Young's modulus (YM)) and surface properties of films produced from a predefined chemical composition using all the different methods was carried out with the ANOVA package included in Statistica 8.0 (Hill and Lewicki, 2007), through one-way analysis of variance. The difference of means between pairs was resolved via confidence intervals, using a Tukey HDS test. The significance level was set at $p < 0.05$.

8.3 Results and discussion

8.3.1 Effect of chemical composition on WPC films properties

Data for the thickness (Th), moisture (M), solubility (S), water vapour permeability (WVP), transparency (T) and colour difference (ΔE^*) of whey protein films obtained according to the experimental design for each film production method was fitted to a second order mixture equation (8.5) as a function of dependent variables. Table 8.2 shows the p values for the analysis of variance (ANOVA) corresponding to the interactions of film properties with film formulation (WPC, Gly and Phot contents). Table 8.3 summarises the best fitted models for each film property according to the BIC criterion. In comparing the AIC and BIC values obtained for the selected models (Table 8.3) it was observed that they were of the same order of magnitude, indicating that both criteria produced similar results and conclusions in best model fitted selections. In this case, the analysis produced using BIC was also valid for AIC. It was also noted that some of the models were quite poor at explaining output variability, which may be due to: (i) the various factors not significantly influencing the output, meaning that other factors should be included; (ii) the nonlinear relation between inputs and outputs, which is not captured by linear regression forms.

Analysis of variance and regression analysis

According to the analysis of variance (Table 8.2), it was verified that WPC is the only statistically significant regressor in the formulation ($p < 0.05$), producing thickness variations by increasing the total solids and protein contents in the films. On the other hand, the glycerol and photoinitiator contents did not contribute to the film thickness ($p > 0.05$). The same behaviour was found for glycerol in films produced by the solvent casting method (Shaw et al., 2002; McHugh et al., 1994; Schou et al., 2005; Gounga et al., 2007). These authors did not observe any

statistically significant differences in film thickness by increasing the glycerol content from 40% to 60% (protein basis) in films produced with WPI.

The use of WPC instead of WPI allowed glycerol to be reduced as a plasticizer because the presence of lactose in the former acts in the same way. In the results of this research, it was also observed that the amount of glycerol used in each film formulation was extremely dependent on the film production method applied. The use of heat denaturation implied that the glycerol content should not be less than 10%, otherwise the films were too brittle. In applying UV irradiation it was verified that glycerol levels of over 10% penalised film handling by making it rather sticky.

Table 8.2 p values (significant at 95% ($p < 0.05$)) of 2,3-way ANOVA corresponding to interactions of (Th), moisture (M), solubility (S), water vapour permeability (WVP), transparency (T) and colour difference (ΔE*) with factors WPC, Gly and Phot of films produced by the SE, HD, UV and HD+UV methods.

SE	Factors	Th	M	S	WVP	T	ΔE*
	WPC	0.00000	0.00000		0.00000	0.00000	0.00564
	Gly		0.02193		0.00000	0.00000	0.00000
	WPC.Gly			0.00119	0.02272	0.00000	
HD							
	WPC	0.00000	0.00386	0.00175	0.00000	0.00000	0.00021
	Gly		0.00000	0.00000	0.00000	0.00000	0.00000
	WPC.Gly			0.01017	0.00000	0.00000	0.00000
UV							
	WPC	0.00000	0.00000		0.00000	0.00000	0.00000
	Gly		0.00000		0.00000	0.00000	0.00000
	Phot	0.08706*	0.00000		0.00000	0.00000	0.02105
	WPC.Gly			0.00040	0.00000	0.00000	0.00340
	WPC.Phot			0.09194*	0.00054	0.00000	
	Gly.Phot			0.00476	0.00000	0.00000	0.00009
HD+UV							
	WPC	0.00001	0.00001	0.00000	0.00001	0.00000	0.00326
	Phot		0.00565			0.00000	
	WPC.Phot					0.00000	

* Significant at 90% ($p < 0.10$). Missing values are not statistically significant at 90% ($p > 0.10$).

The best fitted models found for film thickness (Th) explain a small fraction of the variability of the output, with R^2 values lower then 0.5 (Table 8.3) regardless of the film production method used. These results confirmed the information from the analysis of variance previously discussed.

Table 8.3 Best fitted model for thickness (Th), moisture (M), solubility (S), water vapour permeability (WVP), transparency (T) and colour difference (ΔE*) according to the BIC criterion, of WPC films produced by SE, HD, UV and HD+UV polymerization.

Ind. Var.		WPC	WPC²	Gly	Gly²	Phot	Phot²	WPC.Gly	WPC.Phot	Gly.Phot	R²	AIC	BIC
coefficients	b0	b1	b2	b3	b4	b5	b6	b7	b8	b9			
SE													
Th	-0.00419	0.01121				-	-		-	-	0.376	-297.1	-290.3
M	17.6294	-1.18176	0.13629		0.03165	-	-	-0.04271	-	-	0.879	46.6	54.3
S	99.4574					-	-		-	-	0.000	-1.2	1.3
WVP	-19.4719		0.50243	10.7090	-0.37971	-	-	-0.36274	-	-	0.953	125.1	132.1
T	0.77978	-0.06941		0.36452	-0.01380	-	-		-	-	0.940	-61.6	-53.7
ΔE*	73.1154	2.5657	-0.15722	-6.2239	0.49558	-	-		-	-	0.892	115.5	125.0
HD													
Th	0.05737		0.00081			-	-		-	-	0.448	-306.4	-299.6
M	12.9478					-	-	0.01078	-	-	0.777	37.6	41.6
S	77.1421	-4.14145	0.17256	0.9908	-0.02690	-	-	0.05519	-	-	0.891	98.0	107.1
WVP	119.883	-27.9413	2.26564			-	-	0.18927	-	-	0.929	211.1	218.1
T	9.5337	-1.85099	0.09760	-0.1244		-	-	0.01836	-	-	0.743	16.1	25.6
ΔE*	65.9777	0.37616		-1.2285	0.05827	-	-		-	-	0.971	150.1	158.2
UV													
Th	0.01352	0.01342									0.260	-709.6	-699.5
M	10.1363	0.69344	-0.03515	0.19297	-0.00932		-0.02030				0.829	27.8	44.5
S	97.8826										0.000	266.7	271.5
WVP	4.50543		0.16588	4.65000		17.0839	-1.92742			-0.77293	0.832	497.6	514.4
T	4.10113					-1.5032	0.24472				0.344	166.7	177.4
ΔE*	63.0999		0.01478		-0.02601			0.05492			0.460	392.0	405.4
HD+UV													
Th	0.02486	0.01142		-	-			-		-	0.309	-273.1	-266.2
M	12.0431	0.29762		-	-			-	-0.02415	-	0.741	18.0	23.2
S	120.517	-12.8998	0.74403	-	-			-		-	0.896	103.5	108.7
WVP	28.272	4.4326		-	-	4.2859		-	-0.65997	-	0.735	146.7	153.3
T	4.3292	0.50329	-0.05623	-	-	-2.5672	0.28193	-	0.07850	-	0.767	10.2	21.3
ΔE*	61.9618		0.00766	-	-			-		-	0.294	35.7	40.4

Solubility in water is a very important property for edible films and coatings in a wide spectrum of food applications. In some cases, they should have lower solubility in order to maintain their integrity and also the integrity of the product to which they are applied. However, if the goal is complete dissolution when the food product is consumed (i.e. coatings used in specific dehydrated doses of food that are intended to dissolve in water or other types of food), higher levels of solubility are required (Guilbert and Biquet, 1989).

It was observed that the best fitted models (Table 8.3) for film solubility in water (S) in SE and UV films are constant (99.46% and 97.88%, respectively) and for that reason independent of the film chemical composition. In both cases, the film solubility showed very high values of nearly 100%, which means that the predominant molecular bonds were not water insoluble disulfide bonds (S-S). Pérez-Gago et al. (1999) confirmed that ionic and hydrogen bonds are responsible for the higher solubility of films produced by native whey proteins (without heat treatment). Conversely, the presence of S-S bonds in films in which polymerization implies heat denaturation (HD and HD+UV) decreased film water solubility (ranging between 60.72-72.03% and 59.33-71.14%, respectively). It was also observed that the WPC content in film formulation had a negatively high influence on solubility, confirmed by the values of the b1 coefficient (Table 8.3). This observation proves that by increasing the protein content, the amount of disulfide bonds also increase and the film water solubility decreases. Similar results for partial insolubility were reported by McHugh and Krochta (1994), Fairley et al. (1996a, b) and Gounga et al. (2007) for WPI films and by Mckibben and Krochta (2000) and Ramos (2011) for WPC films. They claimed that the stronger intermolecular bonds (i.e. disulfide bonds as a result of the heat treatment) between protein molecules in the film matrix play an important role in this finding. It was also noted that the higher network stability of WPI as opposed to WPC films accounts for their lower levels of small peptides, monomers and non-protein material (i.e. lactose), which are the soluble materials (Yoshida and Antunes, 2004). In films produced by heat denaturation (HD) it was observed that the glycerol content significantly influenced ($p < 0.05$) film solubility (Table 8.2 and 8.3). It was found that by increasing the amount of glycerol from 10% to 20% (w/w, protein basis) a significant increase ($p < 0.05$) was verified, whereas no significant changes ($p > 0.05$) occurred when glycerol was increased from 20% to 30% (w/w). These observations may be attributed to the hygroscopic nature of glycerol, which attracts and holds water, favouring its absorption into the film surface which therefore becomes more soluble in water (Sobral et al., 2005; Kokoszka et al., 2010). Very similar results to those obtained for HD film solubility were

found for WPC films produced by a combination of heat denaturation and UV irradiation (HD+UV). Once again, these lower solubility values can be explained by the dominance of the stronger covalent disulfide bonds over ionic and hydrogen bonds. It was also verified that the amount of photoinitiator did not produce any statistical difference ($p > 0.05$) in solubility (Table 8.2), as in the case of UV films, whereas an increase in WPC reduces solubility.

Film optical attributes are of primary importance because they directly influence consumer acceptability. Films or coatings with higher transparency values are required when the intention is to enhance the intrinsic properties of coated or packaged products. The transparency values obtained for WPC films ranged between 1.67-2.70, 1.02-2.47, 1.31-3.32 and 0.97-2.47 (Abs_{600}/mm) for films produced by SE, HD, UV and HD+UV, respectively. Similar results, but with a lower upper level, were obtained by Ramos (2011) for WPC (80% protein) plasticized with glycerol at 40%, 50% and 60% and produced by heat denaturation – i.e. 1.11-1.29 (Abs_{600}/mm). Films made from WPI as protein feedstock showed greater transparency, i.e. 3.01-3.43 (Abs_{600}/mm) (Ramos, 2011) and 3.41 (Abs_{600}/mm) (Gounga et al., 2007). In comparison with the transparency of synthetic films (Shiku et al., 2003), the results of this research were inferior to those for LDPE (low-density polyethylene) and PVDC (polyvinylidine chloride) films (3.05 and 4.59 (Abs_{600}/mm), respectively) but in the same order of magnitude as OPP (oriented polypropylene) and PE (polyester) at 1.67 and 1.51 (Abs_{600}/mm), respectively. The lowest transparency values were obtained for films produced using both heat denaturation and UV irradiation (HD+UV). Possibly the combined effect of temperature (90 ºC) and UV irradiation in WPC films, which have high amounts of lactose, may promote chemical reactions (i.e. Maillard reactions) and consequently a reduction in transparency. The influence of film chemical composition on the transparency and colour difference (ΔE^*) of WPC films produced by the different film production methods was evaluated. From the results of this research, the analysis of variance (Table 8.2) shows that film transparency was significantly affected at 95% confidence level ($p < 0.05$) by all independent variables. Colour difference followed the same behaviour for all the films produced with the exception of HD+UV films which do not depend on photoinitiator content ($p > 0.1$). However, the best models achieved for optical properties T and ΔE^* were not well correlated for films produced by UV modification (Table 8.3) with R^2 values of 0.344 and 0.460, respectively, nor for colour difference in films produced by HD+UV, with an R^2 of 0.294. For SE films, T was slightly penalised by increasing the WPC in the formulations (negative b1 values) but improved when glycerol was increased. In HD films it was verified that

higher transparency values were obtained with simultaneously lower values of WPC and glycerol or, alternatively, higher values for both variables. This may indicate that a compromise must be reached between the protein and the plasticizer in the formulation in order to increase film transparency by heat denaturation. A similar effect was observed for HD+UV film transparency, but in this case a trade-off should be established between the protein and photoinitiator contents.

The colour differences in WPC films, were very high compared to the values obtained by Ramos (2011) for WPI or even WPC films, regardless of the film production method used. The development of intense yellow films (with higher b* values) was the main cause of these differences, which may be attributed to the presence of higher amounts of contaminants (lactose, fat and phospholipids (Lorenzen and Schrader, 2006). It was also claimed, in the case of films produced by heat denaturation, that increasing the glycerol content in the film formulation leads to a reduction in colour difference, since it is a colourless component which probably has a dilution effect on proteins and other components (Sobral et al., 2005; Ramos, 2011). This behaviour was not observed in the results for HD films in this research. However, the increase in glycerol enhanced the reflection of light on the film surface, thus producing increased L* values (data not shown) and the effect of higher b* values was predominant in the total film colour difference. Lower colour differences were obtained for films produced by simultaneous heat treatment and UV irradiation (HD+UV) although they were relatively higher than those reported for WPI films. The higher intense colour of films produced by WPC can be a minor defect or even an advantage for practical uses. If the intention is to use them as packaging film, the addition of colouring agents or lamination with opaque layers can work efficiently (Hong et al., 2004; Hong and Krochta, 2006). If, on the other hand, the intention is to apply them as food coatings (i.e. for cheese) a more intense colour can mask certain imperfections by giving them a more homogeneous appearance.

Water vapour permeability (WVP) is one of the most important transport properties studied in edible polymers and films, due to its influence on food product quality and shelf life. However, film moisture content also influences the migration of water between foods and their surroundings or vice-versa and can influence WVP control (Anker et al., 1998; Cagri et al., 2001; Morillon et al., 2002). According to the analysis of variance (Table 8.2), it was verified that all the independent variables (WPC, Gly and Phot) are statistically significant regressors in the formulation ($p < 0.05$) producing moisture and water vapour permeability variations. Fitted models of film moisture content (M) and water vapour permeability (WVP) as a function of film

chemical composition confirmed these results by showing high R^2 values for all the film types ($R^2 > 0.741$ for moisture and $R^2 > 0.735$ in the case of WVP) (Table 8.3). This behaviour was to a certain extent expected, since these two properties are highly dependent and directly related; moisture measures the ability of film to retain water and WVP determines the water transport phenomenon through the film. However, it is also important to mention that the molecular structure of protein films (the type and extent of cross-linking), resulting from the method used for film production, plays a determining role in these properties. Information about these two properties of edible whey protein films produced by the conventional solvent casting method is available, although it is limited with regard to how they relate to each other, and in particular how they are influenced by the film production method. This study aimed to fill this gap by providing additional information on this topic.

Optimisation based on surface response

On the basis of the plan of experiments carried out, an approach commonly designated as response surface methodology (Montgomery, 2008) was used to optimise the properties of the films from the data obtained. The best fitted models for film moisture content and water vapour permeability (Table 8.3) were used for each film production method and their restrictions in relation to film chemical composition (Table 8.1), in order to produce expectations for response variables. The surfaces were then used to geometrically find the optimum corresponding to the most efficient production conditions. In short, the aim was to optimise the level of WVP.

Figure 8.3 presents the WVP obtained with different M for each film production method. It was observed that a higher film moisture content leads to higher WVP. On the basis of the results of this research, film moisture ranged from 12.21% to 18.09% and WVP from 33.14 to 123.82 g.mm/(m^2.d.kPa). However, it was observed that the film production method significantly affects the moisture and water vapour permeability of WPC films. Films produced by solvent evaporation (SE) had a higher moisture content, although the maximum WVP (63.69 g.mm/(m^2.d.kPa)) was lower than the value obtained for HD films. Films produced by UV modification (UV) had lower moisture and WVP values, whereas films produced by heat denaturation (HD) showed higher permeability. Although the nature of the molecular interactions between film components (as a result of the type of film production method) could be the main reason for these results, the differences can also be explained by the chemical composition of each film, especially the glycerol content, since the WPC levels were the same in

all cases (5%, 7.5% and 10%, w/w). With UV films the glycerol content varied between 0-10% (w/w) and for HD films between 10-30% (w/w) (Table 8.1). The interception area between UV and HD film areas in Figure 8.3 probably corresponds to films produced with the same level of glycerol (10%, w/w). This was confirmed by the results obtained for HD+UV films (in which the glycerol content was set at 10%), since part of the area corresponding to this film was also located in the previous interception area (Figure 8.3).

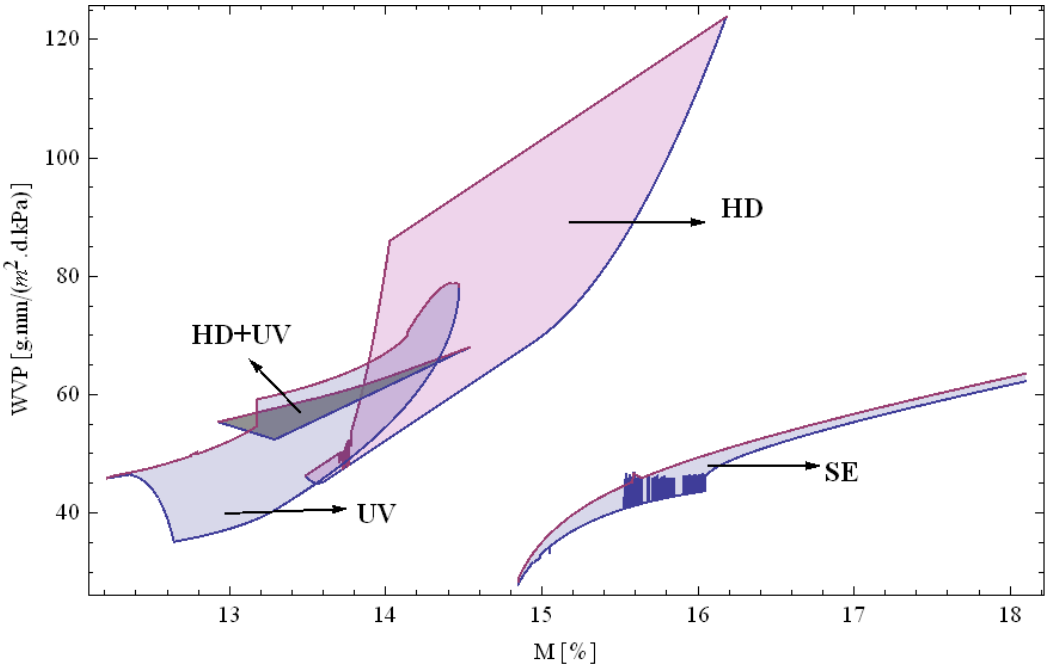


Figure 8.3 Limits of water vapour permeability (WPV) and moisture content (M) according to their best fitted models of WPC films produced by SE, HD, UV and HD+UV film production methods.

Given the WVP and moisture achieved for films produced by simultaneous heat denaturation and UV irradiation (HD+UV), it is important to mention that the lower values fell within the UV film range and the upper values were located in the HD region, demonstrating the influence of both types of film production methods on these film properties.

As previously mentioned, the amount of glycerol contributed towards increasing the WVP and moisture content in WPC films. The influence of glycerol on edible protein-based film WVP has been extensively investigated (Lieberman and Guilbert, 1973; Cuq et al., 1997; Shaw et al., 2002; Kokoszka et al., 2010; Ramos, 2011). It is well established that glycerol reduces the internal hydrogen bonding of proteins, thus enhancing water clustering and increasing the free volume

between molecules in the film matrix, which contributes towards facilitating film permeability by increasing water diffusivity. Film moisture is also favoured, due to the higher availability of hydroxyl groups which increase the tendency for water binding via hydrogen bonds.

It was observed that both moisture and WVP increased in line with the increasing WPC content in the edible film matrix. The hygroscopic nature of WPC, which attracts and holds water molecules, is responsible for moisture absorption. However, this phenomenon also depends on the chemical composition of the protein feedstock, in particular the amount of non-protein components present. Studies comparing WPI and WPC films with similar protein contents (Mckibben and Krochta, 2000; Hong and Krochta, 2006; Ramos 2011) revealed that by increasing the non-protein components (e.g. lactose) in the film matrix, the film moisture content also increased since they act as plasticizers. The higher lactose content (42%) in WPC used in this study may have contributed towards significantly increasing the WVP in these films, since this compound has a relatively low molecular weight, exerting a plasticizer effect on the protein polymer matrix that allows for water migration (Hong and Krochta, 2006; Ghanbarzadeh and Oromiehi, 2008). However, the WVP values were within the WVP range obtained by McHugh et al. (1994), Banerjee and Chen (1995), Maté and Krochta (1996) and Shaw et al. (2002) for films produced with β-Lg or WPI plasticized with glycerol and even lower than the ones obtained for β-Lg or WPI films plasticized by PEG 200 or PEG 400 (McHugh et al., 1994).

With regard to the influence of photoinitiator on film moisture content and WVP, it was observed that this also depended on the WPC and glycerol proportions in the film formulation. The cross-factor (WPC.Phot) that appeared in the best fitted models for M and WVP in the case of HD+UV films (constant glycerol content) shows this dependence (Table 8.3) on WPC concentration. In the case of UV films (where the influence of glycerol also was tested), the moisture content in films was decreased by increasing the amount of photoinitiator (negative b6), but WVP was also glycerol dependent (cross-factor Gly.Phot - Table 8.3). These findings reveal that at higher protein or glycerol levels the photoinitiator contributed to a strongly cross-linked network via the non-covalent and covalent bonds responsible for an effective decrease in moisture and WVP in WPC-based films.

In this study it was also clear that the production of WPC films with certain specific properties was not process dependent. An appropriate chemical composition, according to the technology available, allows the same final result to be achieved, thus considerably increasing the

technological possibilities of solving the same problem. However, it is important to understand the extent to which the technology can compromise or favour other film properties which are also decisive in terms of the intended film application.

8.3.2 Effect of the film production method on WPC film properties

The influence that the film production method has on protein film thermal properties, molecular structure, mechanical and surface properties was assessed using WPC films with a similar chemical composition (10% (w/v solution) WPC, 10% (w/w, WPC basis), glycerol and 3.5% (w/w, WPC basis) photoinitiator for films produced using UV irradiation.

Thermal properties

TGA thermograms of WPC films are depicted in Figure 8.4 according to the film production method. The decomposition temperatures (T_d), derivative maximum decomposing rate temperature (DTG_{max}), corresponding weight losses and residual mass are shown in Table 8.4.

The initial weight losses displayed by the WPC films can be related to the loss of free water absorbed by the films, since glycerol degradation starts at up to approximately 130 ºC (Nuthong et al., 2009; Su et al., 2010). Figure 8.4 shows an overlap in the thermal profiles of the WPC films produced by solvent evaporation (SE) and heat denaturation (HD), as well as in those produced by UV modification (UV) and the heat denaturation and UV modification methods (HD+UV) until approximately 150 ºC. Above this temperature, both films subjected to thermal denaturation (HD e HD+UV) behave similarly and the UV film follows the thermal behaviour of the SE film. This may indicate that the molecular protein structure network and its interaction with glycerol plays an important role in the thermal stabilisation of the films. The TGA thermograms also showed that films produced by heat denaturation (HD and HD+UV) showed a single T_d in the temperature range of 120º-250ºC, which is an indication of compatibility between the components in the formulation (protein and plasticizer). On the other hand, SE and UV films showed two T_d values that can be associated with lower molecular bonding between proteins and glycerol or other major WPC compounds (e.g. lactose), which may indicate that the first T_d value corresponds to the plasticizer and the second to the protein network (Table 8.4). Films produced by UV modification start to decompose at lower temperatures (145.6 ºC), followed by films produced without heat denaturation or UV modification (152.6 ºC), probably due to the presence of the thermosensitive photoinitiator that was not effectively involved in radical formation and protein modification. However, the second T_d, observed for the UV film (185.3 ºC) was similar

to the T_d found for HD and HD+UV films, indicating that UV modification promoted effective protein bonding. In the SE film, the second T_d value (174.1 ºC) was significantly lower, demonstrating its fragile structure and lower thermal stability. HD and HD+UV films started to decompose at significantly higher temperatures (185.9 - 187.4 ºC), indicating that the heat treatment performed during the protein denaturation step leads to the production of WPC films with superior thermal stability. However, no statistical differences between T_d of HD and HD+UV films were found ($p > 0.05$), the associated weight loss was significantly ($p < 0.05$) higher in the former (22.8% against 20.2%).

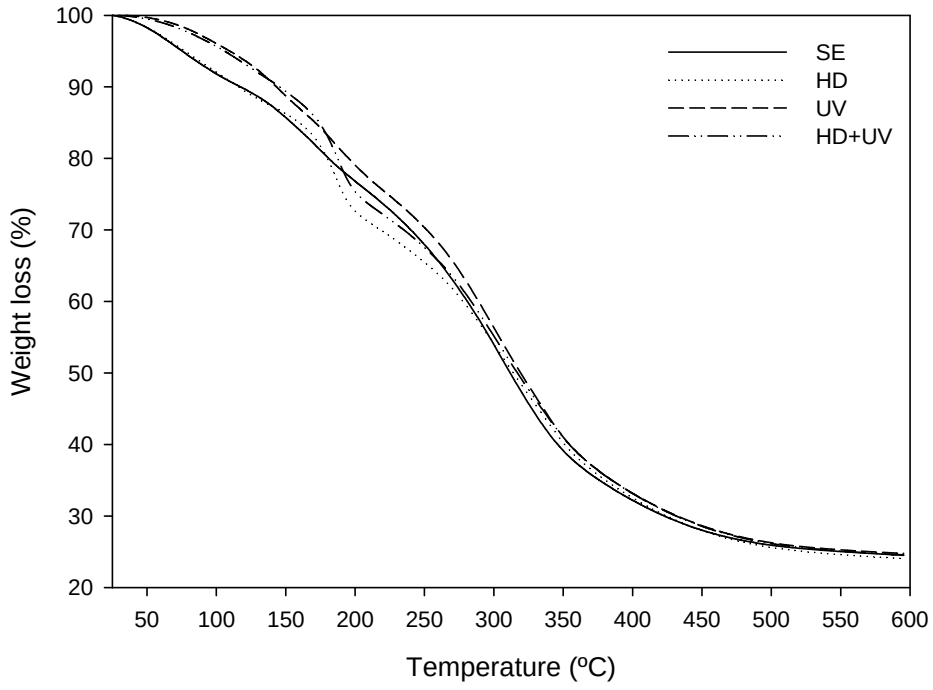


Figure 8.4 TGA thermograms of WPC-based films produced by the SE, HD, UV and HD+UV methods showing the weight loss as function of temperature.

A sharp weight loss between 250º and 500 ºC was also observed, mainly associated with the degradation of the major protein components and the plasticizer incorporated into the film matrix. This pattern was also documented for sodium caseinate and gelatin films (Barreto et al., 2003).

There are no statistical differences between the DTG_{max} of the films, nor for their corresponding weight loss. The DTG_{max} for all the films produced was observed in a very narrow range of 300.3º to 304.5 ºC, and the weight loss between 45.3% and 47.5%. Furthermore, WPC films

exhibited a similar mass loss during the overall heating scan (Table 8.4), except for the UV films whose residual mass was statistically higher ($p > 0.05$). The T_d and DTG_{max} values obtained in this study are much lower them the ones reported by Ramos (2011) for WPI and WPC80-based films produced by heat denaturation. He concluded that lower decomposition temperatures were obtained by using WPC instead of WPI and by increasing the glycerol content in the film matrix. The results of this study also confirm that decreasing the protein content in WPC (50% protein, in this case) leads to less thermal stable films and the presence of lactose acts as a plasticizer by decreasing degradation temperatures.

Table 8.4 Thermogravimetric analysis (TGA) of WPC-based films produced by the SE, HD, UV and HD+UV methods, in terms of initial decomposition temperature (T_d), derivative maximum decomposition rate temperature (DTG_{max}), weight loss and residual mass (average ± standard deviation).

Thermal Property	WPC-film			
	SE	HD	UV	HD+UV
T_d (ºC)	152.6±0.9 b 174.1±0.4 c	185.9±1.7 d	145.6±0.4 a 185.3±0.9 d	187.4±2.1 d
Weight loss (% w/w)	15.3±0.3 b 19.3±0.9 c	22.8±1.1 d	10.8±0.0 a 18.2±0.3 c	20.2±0.1 c
DTG_{max} (ºC)	300.3±4.3 a	304.5±1.7 a	301.2±0.4 a	303.3±0.9 a
Weight loss (% w/w)	46.6±0.9 a	47.5±0.7 a	45.3±1.9 a	46.1±0.0 a
Residual mass (% w/w)	24.3±0.0 a	24.0±0.3 a	24.68±0.1 b	24.5±0.0 a

a, b, c… means within the same row, labelled with the same letter do not statistically differ from each other ($p > 0.05$).

FTIR-ATR analysis

The FTIR spectra of WPC films produced by the various film production methods are shown in Figure 8.5a. In almost the entire frequency range an overlapping of WPC film spectrum was observed, with the exception of the region between 1600-1800 cm^{-1} (Figure 8.5b) and 3000-3600 cm^{-1} (Figure 8.5a). It is possible to identify six spectral regions where the main WPC film peaks are located. The peaks observed are in the range of (i) 800-1200 cm^{-1}, attributed to the glycerol absorption bands (Figure 8.6a); (ii) the region 1200-1350 cm^{-1}, related to amide III (simultaneous vibration of N-H in plane bending and C-N stretching); (iii) 1400-1550 cm^{-1}, associated with the N-H bending vibration of amide II; (iv) 1600-1700 cm^{-1}, governed by the stretching vibration of C=O and C-N of amide I; (v) 2850-2980 cm^{-1}, corresponding to C-H stretching; and (vi) 3000-3600 cm^{-1}, attributed to the free and bound N-H and O-H groups (Richardson, 1981).

Three particular spectral regions were selected for discussion, on the basis of their relevance to a better understanding of how the film production method can affect molecular interactions amongst whey proteins and glycerol. The first selected region (800-1200 cm^{-1}) was attributed to the presence of glycerol (Figure 8.6), the second region (1600-1700 cm^{-1}) was characteristic of amide I involved in the extended β-sheet structure (Allain et al., 1999), and the third region (from 3000 to 3600 cm^{-1}) is related to free and bounded O-H and N-H groups.

The glycerol spectra (Figure 8.6a) shows five characteristic peaks in the range of 800-1200 cm^{-1} and a broad adsorption band at approximately 3300 cm^{-1}. The peaks assigned to the backbone C-C bonds were observed at 855, 920 and 990 cm^{-1}, and the band associated with the stretching of the C-O bond in C1 and C3 was observed at 1034 cm^{-1}, whereas the one associated with stretching in C2 was perceived at 1101 cm^{-1}. These values, with exception of the first peak frequency, were lower them those observed by Guerrero et al. (2010) for pure glycerol (i.e. 850, 925, 995, 1045 and 1117 cm^{-1}). In the FTIR film spectra it was very difficult to observe the peaks at 855 and 920 cm^{-1}. With the SE and UV films some visual recognition was possible (Figure 8.6a, b). However, in the case of HD and HD+UV films this was only detected by applying spectra deconvolution. The band intensity in this region may be explained by the extension of molecular bonding between hydroxyl groups of glycerol and proteins. If the number of free O-H groups increases, thus becoming available to bind to water molecules that may contribute to increasing the water content in films, the band intensity is higher. Conversely, if they are involved in molecular bonding with proteins, the band intensity decreases. On the other hand, in this spectrum region two more peaks were detected at 1112 and 1155 cm^{-1} for all the films produced, with no statistical differences ($p > 0.05$) between them. These findings may indicate that the bond types did not depend on the film production method and probably correspond to the presence of higher amounts of lactose in the formulations, since they were not observed by Ramos (2011) in films produced by WPC-80 and WPI. Solís-Oba et al. (2011) found that well-defined signals in the 930-1190 cm^{-1} region correspond to lactose. The absorption zone of amide I (1600-1700 cm^{-1}, Figure 8.5b), mainly governed by the stretching vibration of C=O and C-N groups, is sensitive to the secondary protein structure (Pereira et al., 2010).

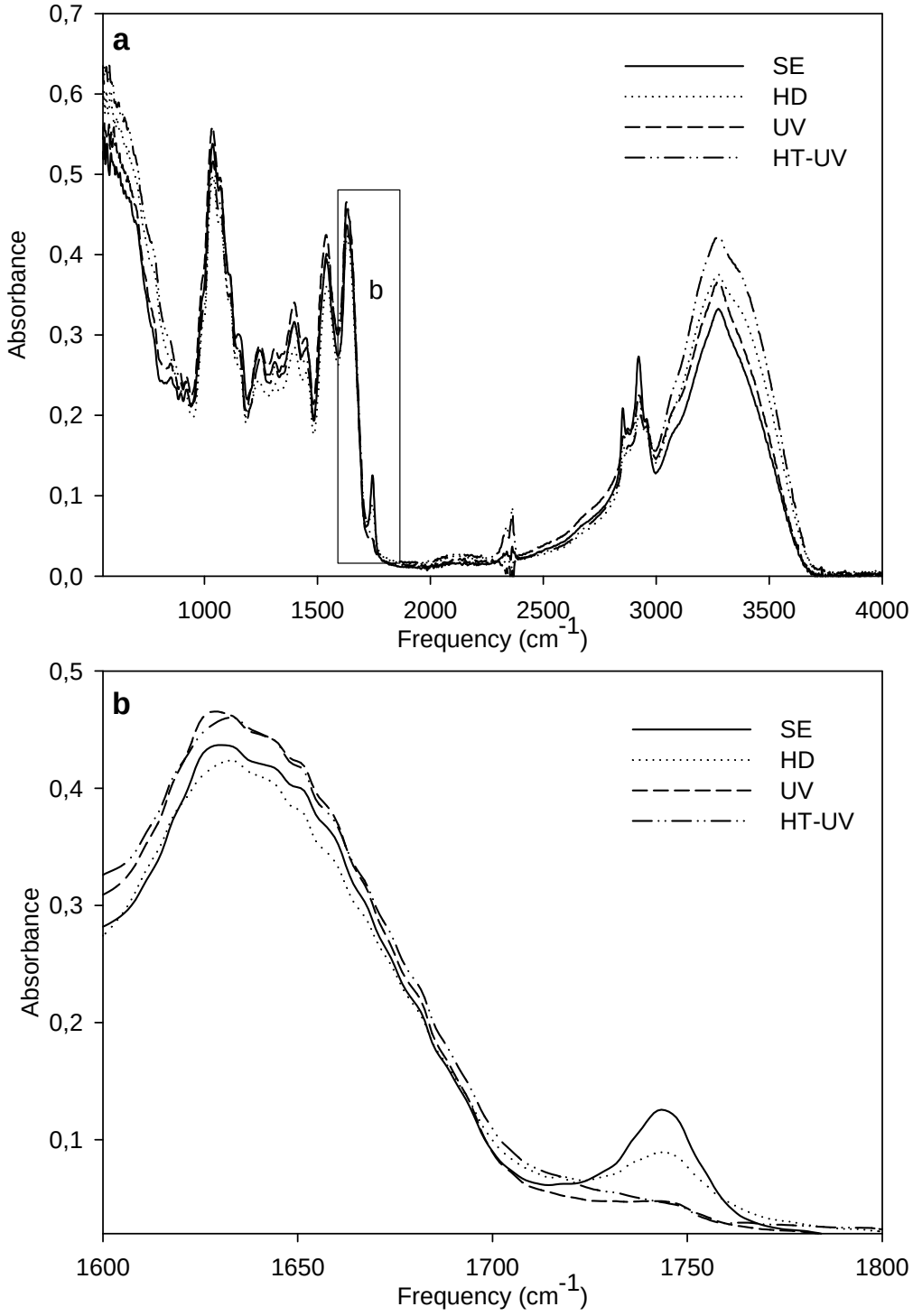


Figure 8.5 (a) FTIR absorbance spectra of WPC-based films produced by the SE, HD, UV and HD+UV methods. (b) FTIR absorbance spectra in the range 1680-1800 cm^{-1}.

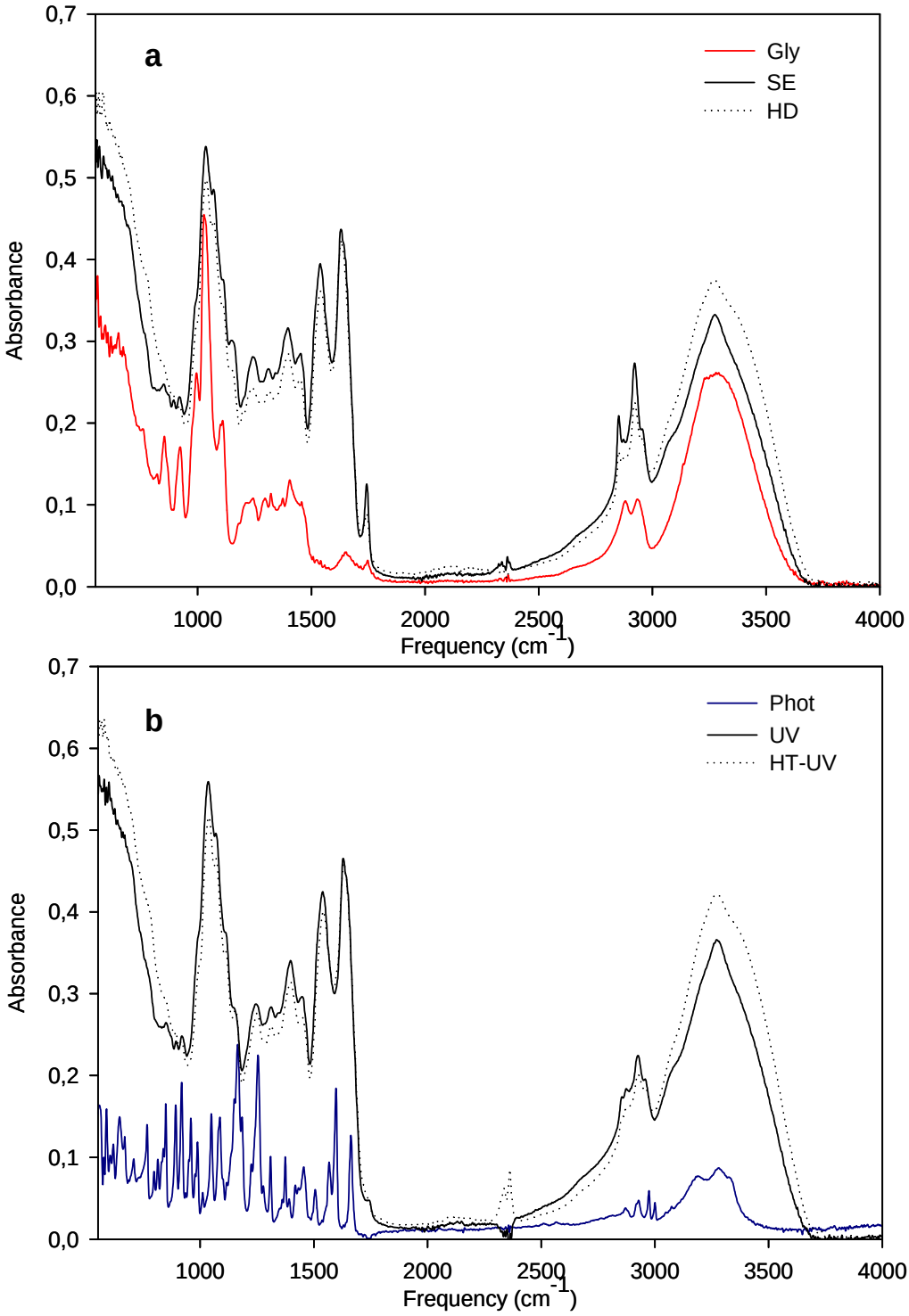


Figure 8.6 Comparison between FTIR spectra of: (a) glycerol and WPC films produced by the SE and HD methods; (b) photoinitiator and WPC films produced by the UV and HD+UV methods.

In the literature it was mentioned that FTIR spectrum bands found at 1616-1618 cm^{-1} and 1681-1690 cm^{-1} were associated with intermolecular antiparallel β-sheets (Lefèvre et al., 2005); the characteristic bands of amide groups involved in the extended β-sheet structure were observed at 1625-1627 and 1630-1635 cm^{-1} (Allain et al., 1999); the range between 1642-1646 cm^{-1} and 1651-1952 cm^{-1} was attributed to unordered and α-helix structures respectively (Allain et al., 1999; le Tien et al., 2000); whereas the bands in the 1667-1673 cm^{-1} range correspond to turns (Goormaghtigh et al., 1990).

α-Lactalbumin and β-Lactoglobulin are generally organised in α-helix (small fraction), β-sheet structures and random conformations, which can be considered an "unordered" structure (le Tien et al., 2000). Their predominance in whey protein films is decisive to the FTIR bands observed at frequencies between 1600 and 1700 cm^{-1}. In applying deconvolution to this region, distinct bands with different intensities were observed for each film production method (Table 8.5). It is possible to conclude that the main molecular changes during WPC film formation occur in this region and are highly dependent on the type of polymerization.

Table 8.5 Frequency and band intensity of Fourier self-deconvolution FTIR absorbance spectra under attenuated reflectance (ATR) mode, in the 1600-1800 cm^{-1} region, of WPC films produced by the SE, HD, UV and HD+UV methods.

SE		HD		UV		HD+UV		Molecular bond type
Freq (cm^{-1})	Intensity (%)	Freq (cm^{-1})	Intensity (%)	Freq (cm^{-1})	Intensity (%)	Freq (cm^{-1})	Intensity (%)	
1612.1	2.9	1608.9	1.7	1612.6	3.3			side-chain vibrations
		1617.9	9.2			1616.9	6.0	intermolecular antiparallel β-sheet
1627.1	25.6			1625.4	18.0	1626.5	11.5	intramolecular
		1630.2	18.5			1635.5	29.5	β-sheet
1646.5	31.0	1642.8	16.8	1643.5	41.1	1644.0	15.7	random coil structures
1652.0	0.3	1651.5	0.3	1651.9	0.3	1651.6	0.3	α-helix
1658.8	1.1	1656.7	16.5	1659.0	0.6	1658.7	7.7	
1667.7	20.4			1668.2	29.8	1667.2	14.2	turns
		1671.4	27.3			1673.2	12.1	
1681.3	4.1	1681.5	0.3	1681.4	0.8	1681.7	0.3	intermolecular antiparallel β-sheet
1691.7	2.3	1692.0	1.4	1691.1	3.0	1690.6	2.8	
1725.4	0.7	1724.0	0.5					
1744.0	11.4	1745.2	7.5	1743.9	3.2			

According to Table 8.5, it was observed that WPC films produced by SE and UV modification did not show peaks for intermolecular antiparallel β-sheet structures in the lower frequency range (1616-1618 cm^{-1}), indicating that only heat denaturation favoured these kind of molecular interactions (HD and HD+UV). However, the small intensity band observed for all the films in the higher frequency range (1681-1683 cm^{-1}) shows they are present to a greater extent in SE. The relative amount of intermolecular antiparallel β-sheet structures in films produced by UV modification was very poor, indicating that UV irradiation did not contribute to this kind of intermolecular interaction.

Intramolecular β-sheet structures were present in all the films produced in relative high proportions (18-41%). However, it is important to mention that the band achieved for β-sheet structure in HD films was located at a higher frequency (1630 cm^{-1}) than the UV films (1625 cm^{-1}), which maintained the original SE films band (1627 cm^{-1}). By using both heat denaturation and UV irradiation, the total proportion of the intramolecular β-sheet structures increased (41%) and two distinct bands were observed as a result of the association of both film production mechanisms. A high content of β-sheets structures were commonly found in aggregated proteins, especially those in which denaturation was extensive. Moreover, aggregation is always followed by the frequent formation of intermolecular antiparallel β-sheets (Fabian et al., 1999). Our results confirmed this behaviour in films produced by thermal denaturation, but not in the case of UV treatment, which means that the type of β-sheets found in UV films (at lower frequencies) were not responsible for intermolecular antiparallel β-sheet induction. These findings once again indicate that the polymerization mechanisms were distinct, leading to different film molecular structures as determined by thermal analysis.

Unordered film molecular structures represented the most intense band for UV films, accounting for a relative band of 41.1%, followed by 31.0% in SE films, whereas in HD and HD+UV films they only represented 16.8% and 15.7%, respectively. This observation may also corroborate the fact that an increase in intermolecular cross-linking promotes greater molecular rearrangement due to the consequent reduction of unordered structures.

The α-helix structure had the smallest band intensity for SE and UV film types, representing only 1.4% and 0.9%, respectively. However, relatively high proportions of this structure were found for films produced by heat, which means that this depended on the film production method and

was favoured by thermal denaturation. In comparison with the results obtained by Ramos (2011) for films based on WPI (16.5%) and WPC80 (15%) produced by thermal treatment, the results of this research were in the some order of magnitude.

Regardless of the film production method used, turns were found in relative high amounts in WPC films as well as in β-sheet or even unordered structures. In HD films they represented the highest amount of molecular structures, at 27%. An increase in band frequencies was observed when the film was heat treated, as well as in the case of β-sheet structures.

The most significant differences between the FITR spectra of the films were found in the 1700-1800 cm^{-1} frequency range (Figure 8.5b), corroborated by deconvolution of this region and peak identification, as shown in Table 8.5. No bands were found when UV irradiation was applied in combination with heat treatment (HD+UV), whereas a small band was observed when the UV method was performed separately, and two bands were detected by deconvolution of the SE and HD spectra. These molecular differences suggest that the polymerization mechanism was responsible for the prevalence of some molecular bonds as opposed to others. The characteristic infrared absorption band at the frequency range 1685-1750 cm^{-1} was associated with the stretching of the C=O bond of aldehydes or ketones (Workman and Springsteen (1998), which means that UV irradiation was responsible for the cleavage of this bond. As in the UV mechanism initialization step in which the photoinitiator is cleaved at this molecular site (Figure 8.1), compounds that have the same molecular structure are also sensitive to UV irradiation.

The third spectral region (from 3000 to 3600 cm^{-1}) was characterised by a broad absorption band for all the protein films, attributed to free and bound O-H and N-H groups (le Tien et al., 2000). Figure 8.5a shows that the band intensity increased from the film produced by SE, to the UV, HD and finally, the highest intensity band, namely the one produced by HD+UV. It was also observed that the presence of glycerol (Figure 8.6a) in the film formulation can be responsible for the intensity of this band, since it also presented a wide band at this spectral region corresponding to free O-H groups. Conversely, the modest band observed for the photoinitiator (Figure 8.6b) indicates that it did not contribute to this band in the protein film spectrum. Several studies of proteins in these regions mention that the band corresponding to N-H generally appears at 3254 cm^{-1} (Bandekar, 1992) and this band shift could be due to the presence of other components in the film formulation (le Tien et al., 2000). On applying deconvolution in this

region, three peaks were detected (Table 8.6). The first in the 3081-3091 cm^{-1} range, was the least intense (1.96-2.88%). The second, representing more than 69% of the band, was probably associated with the N-H groups (3261-3277 cm^{-1}), and the third, contributing 17.66-28.16%, was located at 3445-3461 cm^{-1}.

Table 8.6 Frequency and band intensity of Fourier self-deconvolution FTIR absorbance spectra under attenuated reflectance (ATR) mode, in the 3000-3600 cm^{-1} region, of WPC films produced by the SE, HD, UV and HD+UV methods.

SE		HD		UV		HD+UV	
Freq (cm^{-1})	Intensity (%)	Freq (cm^{-1})	Intensity (%)	Freq (cm^{-1})	Intensity (%)	Freq (cm^{-1})	Intensity (%)
3084.9	2.88	3091.2	2.41	3081.5	2.75	3085.2	1.96
3276.6	79.45	3261.0	69.44	3276.6	81.42	3261.9	72.18
3460.9	17.66	3445.2	28.16	3464.2	15.84	3447.0	25.86

Some authors who have studied WPI and WPC films produced by thermal denaturation claim that the lower the band intensity is in this spectrum region, the higher the degree of protein cross-linking networking will be, with chains closer to each other, promoted by more frequent hydrogen bonding and fewer free O-H groups available for hydration (Fairley et al., 1996a, 1996b; McHugh and Krochta 1994, Ramos 2011). However, our results contrast with these observations, since the films with a lower moisture content which are less soluble and more thermostable (HD+UV and HD) presented a broad global band (Figure 8.5a). This can only be explained as a result of the film production method used for film formation and its role in terms of the extent and nature of the molecular interactions between the various compounds present in the formulation. Considering individual peak frequencies and intensities (Table 8.6), it was found that the second and third peak of the UV film are very similar to the ones observed for film produced by solvent evaporation (SE), corresponding to weaker and less thermo-stable films. The shift to lower frequency values by applying heat protein denaturation was most probably a result of the increase in intermolecular protein cross-linking via disulfide bonds to replace hydrogen bonds, leading to more readily available O-H and N-H free groups. On the other hand, in SE and UV films the molecular interactions between proteins could be predominantly hydrogen bond types and for this reason O-H and N-H free groups were less available, contributing to a narrow global band in this specific FTIR spectrum region. According to Lefèvre and Subirade (2000) and Gilbert et al. (2005), the shift to higher frequency values suggests weaker cross-linking via hydrogen bonds, observed in the case of HD and HD+UV films.

Consequently, these results suggest that UV irradiation can cross-link proteins and lead to a change in protein conformation. These changes could be related to the tendency of the proteins to adopt a more stable structure after cross-linking (Lacroix et al., 2002).

Mechanical properties

Figure 8.7 shows the mechanical properties of WPC films produced by the different film production methods. No statistical differences ($p > 0.05$) were observed in tensile strength (TS), Young's modulus (YM) and elongation at break (E) between the films produced. This may indicate that the influence of the type of polymerization on the mechanical properties of WPC films under the test conditions is not decisive, with film composition playing a more important role. However, it is important to mention that films produced by UV modification are a little stronger and more flexible than their counterparts, owing to their higher mechanical resistance (i.e. higher TS) and extensibility (i.e. higher E).

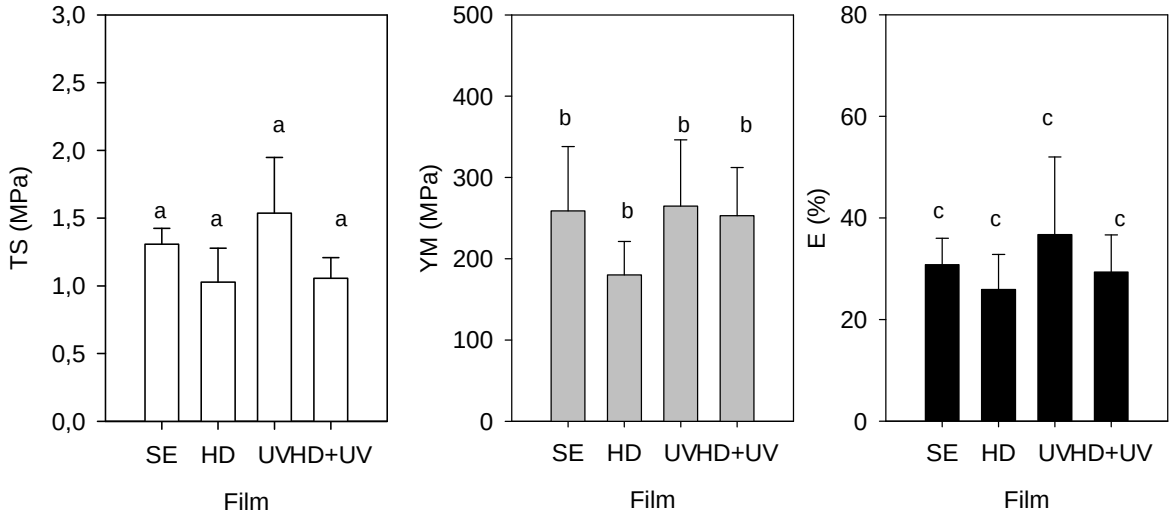


Figure 8.7 Tensile strength (TS), Young's modulus (YM) and elongation at break (E) of WPC-based films produced by the SE, HD, UV and HD+UV methods. a, b, c values labelled with the same letter, in the same mechanical property do not statistically differ from each other ($p > 0.05$).

Comparing the tensile properties of the films produced by solvent evaporation (SE) with the ones produced by Pérez-Gago and Krochta (1999) from native whey proteins (WPI) plasticized by glycerol, our values are poor in TS (1.3 MPa against 3 MPa) but more promising in terms of YM (258.7 MPa against 100 MPa) and especially E (30.8% against 7%). These results indicate that the lower protein content in films based on WPC instead of WPI was the main reason for

the production of weaker films. Nevertheless, the presence of higher amounts of lactose acts as an additional plasticizer and increases film extensibility and stiffness (i.e. higher YM).

The values recorded for TS, YM and E for WPC-based films (Figure 8.7) are within the range of tensile properties for films produced with β-Lg plasticized by sucrose, PEG200 and PEG400 (Sothornvit and Krochta, 2000a, 2000b and 2001) and those based on WPI with higher ratios of glycerol or xylitol (30-50%, protein basis) (Shaw et al., 2002). However, they show higher E and lower TS and YM than the WPI films produced by McHugh and Krochta (1994) by applying protein heat denaturation to a lower glycerol content (15%) or sorbitol as plasticizers. It is claimed that an increase in the plasticizer content increases film flexibility and elongation, since it constrains the establishment of hydrogen bonds between the protein chains, thus increasing intermolecular spacing and chain mobility (Barreto et al., 2003). However, it has also been noted that such an increase is limited. Above certain levels of plasticizer, or components with the same function, film elongation was not improved as a result of saturating the matrix with plasticizer. In the films in this study, despite the smaller amounts of glycerol added to the film matrix, the presence of a relatively higher lactose content in the WPC produced the same effect.

It has also been documented that drying conditions (temperature and relative humidity) during film formation affect tensile strength and elongation (Denavi et al., 2009), making it more difficult to compare film mechanical properties even with the similar protein or plasticizer contents. The WPC films produced showed elongation values within the range of the synthetic polymers HDPE, PP and PS (Hernandez et al., 2000). However, TS and YM were much lower, signifying that their application as packaging is not feasible given these conditions. Nevertheless their use as edible food coatings is still viable and promising.

Surface properties

The critical surface energy of WPC films produced by the various film production methods is presented in Table 8.6. It was observed that significantly lower values of critical surface energy were obtained for films produced by solvent evaporation and UV modification. Their higher dispersive component demonstrated a higher hydrophobic character in comparison to films produced by heat denaturation and its combination with UV irradiation (HD+UV). These findings are in agreement with the lower WVP observed in SE and UV films. However, synthetic polymeric films such as PP, LDPE, PS and PET have a lower critical surface energy (Bicerano, 2002) than the ones produced, indicating that they are more appropriate for use in environments

or food products with a higher moisture content. The higher polar component observed for all the films produced confirms this (Table 8.6). Nevertheless, UV modification demonstrates that film wettability can be reduced by increasing the water contact angle for WPC film. From this study it was obvious that neither the molecular interactions between film components resulting from the application of heat treatment nor those involving UV irradiation after protein denaturation contributed towards improving film hydrophobicity.

Table 8.7 Critical surface energy of WPC-based films produced by the SE, HD, UV and HD+UV methods.

Film production method	CSE (mJ/m²)	Components		R^2
		polar	dispersive	
SE	54.55±3.61 a	51.79±3.55 a	2.76±0.64 a	0.998
HD	80.91±5.05 b	80.9±5.05 b	0.01±0.04 b	0.984
UV	56.6±2.17 a	53.79±2.13 a	2.80±0.44 a	0.996
HD +UV	99.74±4.14 c	99.67±4.14 c	0.07±0.09 b	0.979

a, b, c means within the same column, labelled with the same letter do not statistically differ from each other ($p > 0.05$).

Hong and Krochta (2004) found that sorbitol, PEG and sucrose decrease critical surface energy, but glycerol and PG, according to their hydrophilic behaviour, increase the critical surface energy of coated plastics, by increasing wettability and reducing the water contact angle. This may indicate that the lower levels of glycerol and higher lactose concentration in the WPC films in this study could also contribute towards decreasing their critical surface energy.

8.4 Conclusions

This study has shown that WPC-based films with modest levels of proteins (≈50%) and relatively higher levels of lactose (≈42%) have acceptable film functionality. Film thickness, solubility, moisture content, WVP and optical properties (transparency and ΔE*) can be modified as a function of the chemical composition of the film forming solution (WPC, glycerol and photoinitiator contents) and the polymerization technology used for film production.

It was observed that the use of WPC instead of WPI significantly reduced the amount of glycerol needed in the film formulation. However, the application of the conventional heat denaturation method implies a higher level of plasticizer then the UV modification method. WPC film transparency was similar to some synthetic films available on the market (OPP and PE), but a slightly yellow colour developed as a result of the higher amount of lactose in the film

formulation. Heat denaturation allowed for the production of partially insoluble films, whereas films produced by UV modification showed higher solubility, which is a limiting factor in terms of their application in higher moisture environments.

Film moisture content and water vapour permeability correlated well with film chemical composition, with a second order model for all the WPC films. It was concluded that a lower film moisture content and WVP were obtained by using UV modification, since this technique implies the addition of lower levels of glycerol. However, it was also proved that with the appropriate adjustments, specific methodologies lead to the production of WPC films with similar properties.

Thermal analysis revealed that WPC films produced by heat denaturation presented greater thermal stability and compatibility between film components, which was not improved by the use of UV irradiation. Moreover, FTIR analysis revealed that UV irradiation was efficient in inducing cross-links in edible protein films although the molecular protein structure was affected, as well as interactions with glycerol. No modifications were observed in the mechanical properties of WPC films by the application of the various film production methods. Similar elongation values were obtained for the films produced and synthetic ones, although their lower tensile strength and Young's modulus restricts their applications. WPC films produced by UV modification exhibited a lower critical surface energy which revealed their higher hydrophobic character, as opposed to films produced by the conventional solvent casting method. These results indicate that UV treatment can be a very attractive method for reducing WVP in whey protein based films.

At present, the cost of WPI means that its application as a food coating may be limited. However, this study proved that the lower cost of WPCs may improve their economic viability as coatings, due to their appropriate film properties.

References

Allain AF, Paquin P, Subirade M (1999) Relationships between conformation of β-lactoglobulin in solution and gel states as revealed by attenuated total reflection Fourier transform infrared spectroscopy. International Journal of Biological Macromolecules, 26, 337-344.

Anker M, Stading M and Hermansson AM (1998) Mechanical properties, water vapor permeability, and moisture contents of β-Lactoglobulin and whey protein films using multivariate analysis. Journal Agriculture Food Chemistry, 46, 1820-1829.

Bandekar J (1992) Amide modes and protein conformation. Biochimica et Biophysica Acta, 1120, 123-143.

Banerjee R and Chen H (1995) Functional properties of edible films using whey protein concentrate. Journal of Dairy Science, 78, 1673-1683.

Barreto PLM, Pires ATN and Soldi V (2003) Thermal degradation of edible films based on milk proteins and gelatin in inert atmosphere. Polymer Degradation and Stability, 79, 147-152.

Bicerano J (2002) Prediction of Polymer Properties. Revised and expanded 3th edition. New York: Marcel Dekker. ISBN: 0-8247-0821-0.

Cagri A, Ustunol Z and Ryser ET (2001) Antimicrobial, mechanical, and moisture barrier properties of low pH whey protein-based edible films containing aminobenzoic or sorbic acids. Journal Food Science, 66(6), 865-870.

Cha DS and Chinnan MS (2004) Biopolymer-based antimicrobial packaging: a review. Critical Reviews in Food Science and Nutrition, 44, 223-237.

Coupland JN, Shaw NB, Monahan FJ, Dolores O'Riordan E and O'Sullivan M (2000) Modeling the effect of glycerol on the moisture sorption behavior of whey protein edible films. Journal of Food Engineering, 43, 25-30.

CSC - Ciba Special Chemicals (2002) National Industrial Chemical Notification and Assessment Scheme. Full public report: 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure 2959).

Cuq B, Gontard N, Aymard C and Guilbert S (1997) Relative humidity and temperature effects on mechanical and water vapor barrier properties of myofibrillar protein-based films. Polymer Gels and Networks, 5, 1-15.

Denavi G, Tapia-Blácido DR, Añón MC, Sobral PJA, Mauri AN and Menegalli FC (2009) Effects of drying conditions on some physical properties of soy protein films. Journal of Food Engineering, 90, 341-349.

Fabian H, Fälber K, Gast K, Reinstädler D, Rogov VV, Naumann D, Zamyatkin DF and Filimonov VV (1999) Secondary structure and oligomerization behavior of equilibrium unfolding intermediates of the lambda Cro repressor. Biochemistry, 38, 5633-5642.

Fairley P, Monahan F, German J and Krochta M (1996a) Mechanical properties and water vapor permeability of edible films from whey protein isolate and sodium dodecylsulfate. Journal of Agricultural and Food Chemistry, 44, 438-443.

Fairley P, Monahan F, German J and Krochta M (1996b) Mechanical properties and water vapor permeability of edible films from whey protein isolate and N-ethylmaleimide or cysteine. Journal of Agricultural and Food Chemistry, 44, 3789-3792.

Fang Y, Tung MA, Britt IJ, Yada S and Dalgleish DG (2002) Tensile and barrier properties of edible films made from whey proteins, vol. 67, pp. 188-193.

Fernández L, de Apodaca ED, Cebrián M, Villarán M and Maté J (2007) Effect of the unsaturation degree and concentration of fatty acids on the properties of WPI-based edible films. European Food Research and Technology, 224, 415-420.

Floris R, Bodnár I, Weinbreck F and Alting AC (2008) Dynamic rearrangement of disulfide bridges influences solubility of whey protein coatings. International Dairy Journal, 18, 566-573.

Gennadios A (2004) Edible films and coatings from proteins. In R Yada (Ed.), Proteins in food processing. Cambridge, UK: Woodhead Publishing.

Ghanbarzadeh B and Oromiehi AR (2008) Studies on glass transition temperature of mono and bilayer protein films plasticized by glycerol and olive oil. Journal of Applied Polymer Science, 109, 2848-2854.

Ghanbarzadeh B and Oromiehi AR (2009) Thermal and mechanical behavior of laminated protein films. Journal of Food Engineering, 90, 517-524.

Gilbert V, Rouabhia M, Wang H, Arnould A-L, Remondetto G and Subirade M (2005) Characterization and evaluation of whey protein based biofilms as substrates for in vitro cell cultures. Biomaterials, 26, 7471-7480.

Goormaghtigh E, Cabiaux V and Ruysschaert JM (1990) Secondary structure of the membrane-bound form of the pore-forming domain of colicin A - an attenuated total-reflection polarized Fourier-transform infrared spectroscopy study. European Journal of Biochemistry, 193, 409-420.

Gounga ME, Xu S-Y and Wang Z (2007) Whey protein isolate-based edible films as affected by protein concentration, glycerol ratio and pullulan addition in film formation. Journal of Food Engineering, 83, 521-530.

Guerrero P, Retegi A, Gabilondo N and de la Caba K (2010) Mechanical and thermal properties of soy protein films processed by casting and compression. Journal of Food Engineering, 100, 145-151.

Guilbert SG, Biquet B (1989) Les films et enrobages comestibles. In G. Bureau, J. Multon (Eds.) L'emballage des denrées alimentaires de grande consummation. Paris: Tech et Doc, pp. 320-350.

Han JH and Floros JD (1997) Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. Journal of Plastic Film and Sheet, 13, 287-298.

Hernandez RJ, Selke SEM and Culter JD (2000) Plastics packaging: properties, processing, applications, and regulations. Cincinnati, OH: Hanser, pp. 425.

Hernandez-Izquierdo VM and Krochta JM (2008) Thermoplastic processing of proteins for film formation - a review. Journal of Food Science, 73, 30-39.

Hettiarachchy NS and Eswaranandam S (2005) Edible films and coatings from soybean and other protein sources. In: F Shahidi (Ed.), Bailey's Industrial Oil and Fat Products - Industrial and non edible products from oil and fats. vol. 6, 6th edition, John Wiley & Sons. ISBN:978-1- 60119-121-2.

Hill T and Lewicki P (2007) Statistics Methods and Applications. StatSoft, Tulsa, OK.

Hong S, Han JH and Krochta JM (2004) Optical and Surface Properties of whey protein isolate coatings on plastic films as influenced by substrate, protein concentration, and plasticizer type. Journal of Applied Polymer Science, 92, 335-343.

Hong SI and Krochta JM (2006) Oxygen barrier performance of whey-protein-coated plastic films as affected by temperature, relative humidity, base film and protein type. Journal of Food Engineering, 77, 739-745.

Karbowiak T, Hervet H, Léger L, Champion D, Debeaufort F and Voilley A (2006) Effect of plasticizers (water and glycerol) on the diffusion of a small molecule in iota-carrageenan biopolymer films for edible coating application. Biomacromolecules, 7, 2011-2019.

Khwaldia K, Perez C, Banon S, Desobry S and Hardy J (2004) Milk proteins for edible films and coatings. Critical Reviews in Food Science and Nutrition, 44, 239-251.

Kokoszka S, Debeaufort F, Lenart A and Voilley A (2010) Water vapour permeability, thermal and wetting properties of whey protein isolate based edible films. International Dairy Journal, 20, 53-60.

Lacroix M, Lea TC, Ouattara B, Yu H, Letendre M, Sabato SF, Mateescu MA and Patterson G (2002) Use of γ-irradiation to produce films from whey, casein and soya proteins: structure and functional characteristics. Radiation Physics and Chemistry, 63, 827-832.

le Tien C, Letendre M, Ispas-Szabo P, Mateescu MA, Delmas-Patterson G, Yu HL and Lacroix, M (2000) Development of biodegradable films from whey proteins by cross-linking and entrapment in cellulose. Journal of Agricultural and Food Chemistry, 48, 5566-5575.

Lefèvre T and Subirade M (2000) Molecular differences in the formation and structure of fine-stranded and particulate β-lactoglobulin gels. Biopolymers, 54, 578-586.

Lefèvre T, Subirade M, Pézolet M (2005) A molecular description of the formation and structure of plasticized globular protein films. Biomacromolecules, 6, 3209-3219.

Li J and Chen H (2000) Biodegradation of whey protein-based edible films. Journal of Polymers and the Environment, 8, 135-143.

Lieberman ER and Gilbert SG (1973) Gas permeation of collagen films as affected by cross-linkage, moisture, and plasticizer content. Journal of Polymer Science, 41, 33-43.

Lorenzen PC and Schrader K (2006) A comparative study of the gelation properties of whey protein concentrate and whey protein isolate. Le Lait, 86, 259-271.

Maté JI and Krochta JM (1996) Comparison of oxygen and water vapor permeabilities of whey protein isolate and β-Lactoglobulin edible films. Journal of Agricultural and Food Chemistry, 44, 3001-3004.

McHugh TH and Krochta JM (1994) Sorbitol- vs glycerol-plasticized whey protein edible films: integrated oxygen permeability and tensile property evaluation. Journal of Agricultural and Food Chemistry, 42, 841-845.

McHugh TH, Aujard JF and Krochta JM (1994) Plasticized whey protein edible films: water vapor permeability properties. Journal Food Science, 59(2), 416-419.

Mckibben, J and Krochta JM (2000) Properties of whey protein concentrate films of varying protein contents compared to whey protein isolate films. 78C-16, IFT Annual Meeting: IFT Food Expo; <http://ift.confex.com/ift/2000/techprogram/paper_3608.htm>

Miller KS and Krochta JM (1997) Oxygen and aroma barrier properties of edible films: A review. Trends in Food Science & Technology, 8(7), 228-237.

Montgomery D (2008) Introduction to Statistical Quality Control. John Wiley & Sons, 5th ed., New York. ISBN: 0471661228.

Morillon V, Debeaufort F, Blond G, Capelle M and Voilley A (2002) Factors affecting the moisture permeability of lipid-based edible films: a review. Critical Reviews in Food Science and Nutrition, 42, 67-89.

Naushad ME and Stading M (2007) In situ tensile deformation of zein films with plasticizers and filler materials. Food Hydrocolloids, 21, 1245-1255.

NICNAS – National Industrial Chemicals Notification (2002) Full Public Report 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure 2959), File No: NA/991.

Nuthong P, Benjakul S and Prodpran T (2009) Characterization of porcine plasma protein-based films as affected by pretreatement and crosslinking agents. International Journal of Biological Macromolecules, 44, 143-148.

Odian G (2004) Principles of polymerization, 4th Edition, John Wiley & Sons, USA. ISBN 0-471-27400-3.

Osés J, Fernández-Pan I, Mendoza M and Mate JI (2009) Stability of the mechanical properties of edible films based on whey protein isolate during storage at different relative humidity. Food Hydrocolloids, 23, 125-131.

Pereira RN, Souza BWS, Cerqueira MA, Teixeira JA and Vicente AA (2010) Effects of electric fields on protein unfolding and aggregation: influence on edible films formation. Biomacromolecules, 11, 2912-2918.

Pérez-Gago MB, Nadaud P and Krochta JM (1999) Water vapor permeability, solubility, and tensile properties of heat-denatured versus native whey protein films. Journal Food Science, 64, 1034-1037.

Plastics Europe (2010) Plastics - the Facts 2010. An analysis of European plastics production, demand and recovery for 2009. Brussels.

Plastics Europe (2011) Plastics - the Facts 2011. An analysis of European plastics production, demand and recovery for 2010. Brussels.

Ramos OS (2011) Development and characterization of bioactive edible, whey protein films and coatings, to improve quality and safety of food products. PhD Thesis, Instituto de Tecnologia Química e Biológica - Universidade Nova de Lisboa, Lisboa, Portugal.

Richardson JS (1981) The anatomy and taxonomy of protein structure. Advanced Protein Chemistry, 34, 168.

Royston P and Sauerbrei W (2008) Multivariable Model-Building: A pragmatic approach to regression analysis based on fractional polynomials for modelling continuous variables. England, John Wiley & Sons, Ltd. ISBN 978-0-470-02842-1.

Schou M, Longares A, Montesinos-Herrero C, Monahan FJ, O'Riordan D and O'Sullivan M (2005) Properties of edible sodium caseinate films and their application as food wrapping. Lebensmittel-Wissenschaft und-Technologie, 38, 605-610.

Schwalm R (2006) UV Coatings - Basics, Recent Developments and New Applications, Elsevier Science. ISBN-13: 978-0444529794.

Shaw NB, Monahan FJ, O'Riordan ED and O'Sullivan M (2002) Physical properties of WPI films plasticized with glycerol, xylitol, or sorbitol. Journal Food Science, 67(1), 164-167.

Shiku YH, Hamaguchi PY and Tanaka M (2003) Effect of pH on the preparation of edible films based on fish myofibrillar proteins. Fisheries Science, 69, 1026-1032.

Sobral PJA, dos Santos JS, García FT (2005) Effect of protein and plasticizer concentrations in film forming solutions on physical properties of edible films based on muscle protein of Thai Tilapia. Journal of Food Engineering, 70, 93-100.

Solís-Oba M, Teniza-García O, Rojas-López M, Delgado-Macuil R, Díaz-Reyes J and Ruiz R (2011) Application of infrared spectroscopy to the monitoring of lactose and protein from whey after ultra and nano filtration process. Journal of the Mexican Chemical Society. 55(3), 190-193.

Sothornvit R and Krochta JM (2000a) Oxygen permeability and mechanical properties of films from hydrolyzed whey protein. Journal Agriculture Food Chemistry, 48(9), 3913-3916.

Sothornvit R and Krochta JM (2000b) Plasticizer effect on oxygen permeability of beta-lactoglobulin (β-Lg) films. Journal Agriculture Food Chemistry, 48(12), 6298-6302.

Sothornvit R and Krochta JM (2001) Plasticizer effect on mechanical properties of beta-lactoglobulin (β-Lg) films. Journal Food Engineering, 50(3), 149-155.

Su JF, Huang Z, Zhao YH, Yuan XY, Wang XY and Li M (2010) Structure and properties of carboxymethyl cellulose/soy protein isolate blend edible films crosslinked by Maillard reactions. Carbohydrate Polymers, 79, 145-153.

Weber CJ, Haugaard V, Festersen R and Bertelsen G (2002) Production and applications of biobased packaging materials for the food industry. Food Additives and Contaminants, 19(4 supp 1), 172-177.

Workman J and Springsteen A (1998) Applied Spectroscopy: a compact reference for practitioners. Academic Press. ISBN 978-0-12-764070-9.

Yoshida CMP and Antunes AJ (2004) Characterization of whey protein emulsion films. Brazilian Journal of Chemical Engineering, 21, 247-252.

Chapter 9

The performance of edible whey protein coatings with antimicrobial activity applied to cheese

Abstract

This work assesses the efficacy of edible whey protein coatings with antimicrobial properties produced by heat denaturation and modification by UV irradiation and applied to ripened cheese as alternatives to commercial cheese coatings. Edible whey protein coatings were produced from whey protein concentrate (WPC - 10% w protein/w coating solution), glycerol as a plasticizer (50% w/w, protein basis), guar gum (0.7% w/w), tween 20 (0.2% w/w), sunflower oil (10% w/w), lactic acid (6 g/L) and natamycin (0.125 g/L) as antimicrobials. Two methods of coating

production were used separately and in combination: the heat denaturation method (HD) and the innovative UV modification method (UV). Their effectiveness was evaluated by measuring the physicochemical, microbiological and sensorial properties of coated cheeses during 45 days of storage and comparing these properties with those of uncoated cheeses and cheeses with commercial coatings (made from polyvinyl alcohol (PVA) and natamycin as an antimicrobial agent).

The coating production method influences the physicochemical, microbiological and sensorial attributes of cheese. Coatings produced by heat denaturation only did not significantly improve coating efficiency, whereas UV modification in combination with thermal treatment (HD+UV) enhanced cheese attributes. With regard to physicochemical evaluation, after 45 days of storage no significant differences were found ($p > 0.05$) between the cheese samples with commercial coatings and those with edible coatings (produced exclusively by UV modification or in combination with heat denaturation) in terms of weight loss, fat, protein and salt content, as well as a_w, pH and hardness, which indicates that the antimicrobial edible coatings applied could be used as an alternative to their commercial counterpart(s). Microbiological analysis proved that the edible antimicrobial coatings prevented the growth of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and moulds, thus demonstrating their ability to ensure the safety of cheese. In fact, the coatings produced by HD+UV inhibited or reduced microbial growth, probably as a result of the synergetic effect of antimicrobial and UV light. The commercial coatings proved to have the best performance against yeasts and moulds, due to the higher amounts of natamycin (2.5 g/L against 0.125 g/L in the WPC-based coatings). With regard to sensorial analysis, the cheese with the commercial coating was evaluated best by panellists in terms of general acceptance at the end of the ripening period. However, there were no significant differences in cheeses with antimicrobial edible coatings and commercial coatings.

The best coating performance was obtained with the antimicrobial whey protein coating produced by the HD+UV method, which prevented water loss more efficiently, led to a physicochemical cheese composition similar to the commercial coating, inhibited microbial growth and offered a good visual appearance. These results indicate that the antimicrobial whey protein-based edible film produced by the HD+UV method is an effective alternative to commercial coatings.

9.1 Introduction

Food safety depends on the hygienic characteristics of foodstuffs during production, storage and commercialisation. Several physical and chemical methods for preserving food quality, such as high pressure, sterilisation, irradiation, ultrasound and acidification have been developed with this goal in mind. However, none of these methods are self sufficient without the use of an appropriate package as the final step in the preservation process. Plastic films are valued for their efficiency in protecting and reducing mass transfer between the food and its surroundings. Nevertheless, their increasing use and non-biodegradability have led to disposal problems. Edible films and coatings are natural and biodegradable products that help protect the environment whilst reducing agro-industry residue.

Characteristics which facilitate the handling and carriage of food can be improved by the use of edible films and coatings, enhancing sensorial attributes such as colour, transparency, roughness or stickiness. Although many functions of edible packaging are identical to those of synthetic packaging, such as gas, vapour and solute permeability, they appear to offer a complementary parameter for the quality of fresh or treated products, given their non-toxic character and carrier capacity (Olivas and Barbosa-Canovas, 2005; Min et al., 2006; Min and Krochta, 2007; Zinoviadou et al., 2009). One novel function attributed to edible coatings is their action as carriers of active ingredients and additives (e.g. flavours, pigments, antioxidants and antimicrobial agents), thus protecting and improving food quality. Whey protein films and coatings have proved to be poor moisture barriers because of their hydrophilic nature, but present very interesting oxygen barrier properties, comparable to synthetic polymers (Pérez-Gago and Krochta, 2002; Krochta, 2002; Hong and Krochta, 2006), and better mechanical characteristics than polysaccharide-based edible films (Miller and Krochta, 1997). Improving edible whey protein films and coatings with antioxidants and antimicrobial ingredients to enhance food safety and shelf life is their most promising application (Lee et al., 2003; Manab et al., 2011).

Reducing the rancidity of foods with polyunsaturated fatty acids by controlling oxygen transfer rates or using antioxidant compounds (ascorbic acid, cysteine and 4-hexylresorcinol) incorporated into whey protein coatings has proved to be very efficient (Lee and Krochta, 2002; Pérez-Gago et al., 2006; Min and Krochta, 2007). It is also possible to improve the visual appearance of fresh cut fruit and vegetables by using edible whey protein coatings with antioxidants to reduce browning (Lee et al., 2003; Olivas and Barbosa-Canovas, 2005). The antimicrobial performance of whey protein coatings was tested in some applications, namely fresh or processed meat (Min et

al., 2006; Cagri et al., 2002; Zinoviadou et al., 2009), fish (Stuchell and Krochta, 1995; Min et al., 2008; Neetoo et al., 2008) and cheese (Franssen, 2002; Ramos, 2011). The presence of antimicrobial agents in the coating applied to the surface of these food products may reduce or even prevent the growth of pathogenic and spoilage microorganisms through its gradual diffusion and controlled release onto the food surface and throughout the food itself. This mechanism allows for the use of antimicrobials at lower initial levels and ensures a constant background of these compounds during storage (Min and Krochta, 2005). Common antimicrobials incorporated into food packaging include organic acids (lactic, propionic, citric, tartaric, malic, acetic, lauric formic and sorbic acids), enzymes (glucose oxidase, lactoperoxidase, lysosyme and lactoferrin), bacteriocins (nisin), fungicides (natamicyn), salts (sodium or calcium sorbates, benzoates and propionates); polysaccharides (chitosan) and oil extracts (oregano, garlic, rosemary).

Antimicrobial selection depends primarily on the food application and the target pathogenic microorganisms that are to be eliminated or whose growth is to be controlled. Wide spectrum antimicrobials or very specific ones can therefore be selected. Among the organic acids, propionic acid is considered a wide spectrum antimicrobial and it has proved to have a good antifungal performance against moulds and success in combating gram-negative and gram-positive bacteria (Ray, 2004). However, its incorporation into edible whey protein based films as an antimicrobial compound produces extremely fragile films with significant changes in film mechanical properties, thus compromising and limiting its inclusion (Ramos, 2011) due to its lower dissociation constant and the presence of a single binding group that establishes a lower density network within the protein polymer chains (Manab et al., 2011). The addition of lactic acid in edible films and coatings has been proved to offer higher levels of efficiency (Hettiarachchy and Eswaranandam, 2007; Pintado et al., 2009; Ramos, 2011). It is frequently added to food for preservation purposes by reducing or eliminating pathogenic gram-positive bacteria. However, it may not be considered a wide spectrum antimicrobial, due to the significant absence of antimicrobial activity against yeast and moulds (Ray, 2004). Natamicyn, a specific antimicrobial compound, is a successful antimycotic polyene that prevents the growth of yeasts and moulds on cheese surfaces or slices (Franssen et al., 2004; Amefia et al., 2006) and sausages (Cagri et al., 2002). It is considered GRAS (Generally Recognised as Safe) by the U.S. Food and Drug Administration and assigned number E-235-natural preservative in the European Union.

The formation of protein coatings on food products may involve dipping, spraying, enrobing or panning the food with the coating formulation and they all require drying the solvent from the protein coating formulation after application to food (Krochta, 2002). Thermal protein denaturation is the most commonly used method for the production of whey protein coatings (Dalgleish et al., 1997; Puyol et al., 2001; Hong and Creamer, 2002), whilst protein cross-linking can also be induced chemically (Galietta et al., 1998; Lee and Rosenberg, 1999), enzymatically (Mahmoud and Savello, 1992) or by means of irradiation.

The used of UV or γ-irradiation presents some advantages. It is a clean, well-known process for sterilising goods (Thakur and Singh, 1994) and less expensive than using enzymes. γ-irradiation was used by Mezgheni et al. (1998), Vachon et al. (2000) and Lacroix et al. (2002) to produced edible films from caseinates and whey proteins. They reported that γ-irradiation induced cross-links in calcium caseinate films and the combination of irradiation and thermal treatments increased the puncture strength of both calcium caseinate and whey protein films. The proposed mechanism was radical polymerization through tyrosine and the formation of bityrosine linkages between protein chains. As in the case of γ-irradiation, the efficiency of UV-irradiation in terms of the properties of films and coatings depends on several factors: the amino acid composition and molecular protein structure; the time when irradiation is applied to the film solution (e.g. before dissolving the raw protein, before heating, before casting, before or after drying) and also the pH of the film-forming solution. Hettiarachchy and Eswaranandam (2005) studied the use of UV-irradiation applied to soy protein films. They found that if the UV treatment was performed before heating, no significant differences were found in comparison to the control with no UV treatment. However, if it was applied after heating, the drastic conformational changes in protein molecules during the thermal treatment may affect the cross-links formed during UV-irradiation and significantly increase film tensile strength. It was also pointed out that the high levels of tyrosine and phenylalanine in the soy protein involved in the cross-linking reactions induced by UV irradiation are responsible for this behaviour. The lack of tyrosine residues in whey proteins may be the main reason why there has been no significant research into the implementation of this clean technology in the production of whey protein-based films and coatings. However, this drawback can be overcome with the use of chemicals such as photoinitiators, which create radicals under UV-irradiation that start the polymerization reaction.

Our assumption for this research was that the combination of both active compounds (lactic acid and natamycin) and UV-irradiation would generate edible whey protein coatings with improved cohesion and antimicrobial properties, thus making them suitable for coating cheese and perhaps providing an alternative to the existing commercially available (PVA-based) cheese coatings. The effect of the WPC-based edible coating production method and its antimicrobial activity can be assessed by a physicochemical, microbial and sensorial evaluation of coated cheeses over 45 days of ripening, and by comparison with uncoated cheeses or those with a commercial coating.

9.2 Material and methods

9.2.1 Materials

Ovine freeze-dried WPC produced by ultrafiltration, as described in Chapter 3, with the chemical composition presented in Table 3.3 and obtained by the chemical methods previously described in 3.2.3 and Appendix B, was used as a protein source for whey protein-based coating formulations. Glycerol (99% purity) supplied by JM Vaz Pereira Ltd (Lisbon, Portugal) was used as a plasticizer. Guar gum was added as a natural thickener and emulsifier (supplied by Formulab Aditivos Alimentares, LDA, Maia, Portugal) in order to increase coating viscosity. Sunflower oil (Olimambo) was provided by Cidacel S.A. (Lousã, Portugal) and incorporated in order to reduce water vapour permeability by increasing coating hydrophobicity. The surfactant tween 20 was supplied by Fluka Chemika (Madrid, Spain) and added to the formulation to overcome the tendency towards phase separation between polymer mixtures of WPC and polysaccharide gums, as well as acting as an emulsifier to assist in oil dissolution when preparing the coating solutions. The photoinitiator 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) was used only for coatings produced by UV modification and was supplied by Ciba Specialty Chemicals The active antimicrobial compounds were lactic acid (90% purity) supplied by JM Vaz Pereira Ltd (Lisbon, Portugal) and natamycin (50% purity) supplied by Enzilab - Comércio de Aditivos e Tecnologias Alimentares, Lda. (Porto, Portugal).

The commercial coating (Readom D), provided by Enzilab, was composed of polyvinyl acetate (as the base material) and approximately 2.5 g/L natamycin as the active component (indicated by the supplier).

Microbial analyses of *Staphylococcus* spp. were performed in a Baird-Parker Agar Base, BPA (HIMEDIA) supplemented with egg yolk and telurite emulsion (Fluka Chemika), *Pseudomonas*

spp. in Pseudomonas Agar F, PAF (DIFCO Laboratories), *Enterobacteriaceae* in Violet Red Bile Glucose Agar, VRBGA (HIMEDIA) with Triptona Soya Agar, TSA (HIMEDIA) and yeast and moulds in Rose-Bengal Chloramphenicol Agar, RBC (Merck). Peptone water was obtained from Merck (Darmstadt, Germany) and used for the sample decimal dilutions. All the other chemicals were reagent-grade or better, and were used without further purification.

9.2.2 Cheese

Cylindrical and semi-hard bovine cheeses of approximately 120 g were produced in the Escola Superior Agrária (ESAC) facilities (in Coimbra, Portugal) and used as food model material for whey protein-based coating applications. Standardised (3.5±0.1% fat) bovine milk was heated and maintained at (30±0.5 °C) during coagulation (45 min) in the presence of a 0.04% $CaCl_2$ solution (36% (w/w), Betegeux), 10 ppm of Mesófilo Plus Ferment (Ferlac), 25 ppm of Lysozyme and 20 ppm of rennet (> 92% chymosin, Tecnilac) previously diluted in tap water. The curd was moulded in plastic moulds and stored at 5±1°C and 80±3% relative humidity for 1 day before the coating was applied.

9.2.3 Production and application of coating

The coating formulation developed by Ramos (2011) was the basis for the coating formulation used in this research, with some specific adaptations. The protein base material, initially WPI, was replaced by WPC with the same protein content (10% (w/w)) and the amount of natamycin acting as an antimicrobial was reduced from 0.25 g/L to 0.125 g/L, in order to evaluate the antimicrobial effect of UV-irradiation better. All the remaining coating ingredients were maintained at the optimised concentration achieved by Ramos (2011): glycerol was added at 50% (w/w, protein basis), guar gum (0.7 (w/w)) raised the viscosity in the coating matrix to values similar to those of commercial coatings, sunflower oil and tween 20 (10% (w/w) and 0.2 (w/w), respectively were selected on the basis of their spreading coefficient in order to obtain polarity values similar to the cheese surface and, finally, the antimicrobial agent lactic acid (6 g/L, in the coating solution) produced the best results for the growth limitation or inhibition of pathogenic bacteria. Three different types of whey protein-based antimicrobial coatings were made for application to cheese, based on the coating production methods: heat denaturation (HD), UV modification (UV) and a combination of both methods (HD+UV). Cheeses coated with the whey protein coatings were compared to the negative control (uncoated cheeses) and positive control (cheeses with commercial coatings).

Heat denaturation method (HD)

The production of WPC coatings using the HD technique implies that the thermal treatment, and consequently protein denaturation, may occur in the coating solution before the coating is applied to the cheese surface. Glycerol at 50% (w/w, on a protein basis) and the amount of WPC needed to adjust the whey protein content in the coating solution to 10% (w/w) were homogenised in deionised water until completely dissolved. The solution was then heated in a water bath at 80±2 ºC for 20 min under continuous agitation and cooled down to room temperature (±30 ºC). Guar gum (0.7 (w/w)), sunflower oil (10% (w/w)) and tween 20 (0.2 (w/w)) in coating solution basis were added while stirring for approximately 20 min at room temperature. Afterwards, the antimicrobial agents, lactic acid and natamycin were added in order to obtain 6 g/L and 0.125 g/L respectively in the final coating matrix. The pH was adjusted to 7.0 using 1 M NaOH. The coating solution was homogenised using a T25 Ultra-Turrax® (IKA from Staufen, Germany) at 10 000-13 000 rpm for approximately 2 min before being applied to the cheese.

UV modification method (UV)

In the UV method, protein polymerization only occurs after the coating solution is applied to the cheese surface and after UV exposure. The WPC-glycerol solution was prepared as described in the previous method, and the same concentrations of guar gum, sunflower oil and tween 20 were added without any previous heat treatment. During this phase, the photoinitiator at 3.5% (w/w, on a protein basis) was also added to the solution under continuous stirring. Antimicrobial compounds (acid lactic and natamycin) were incorporated into the edible coating matrix in order to obtain 6 g/L and 0.125 g/L respectively, and the pH was adjusted to 7.0 using 1 M NaOH. The coating solution was then homogenised with a T25 Ultra-Turrax® under the same conditions described previously. After the coating was applied to the cheese surface, the cheeses were exposed for 10 min on each side to direct UV-irradiation using UV lamps (G8 T5 - 8W, PHILIPS®) set approximately 23 cm away from the cheeses.

Heat denaturation and UV modification method (HD+UV)

The combination of both techniques implies the protein denaturation of the coating solution by heat treatment before the coating is applied, and protein modification after the coating is applied to the cheese surface, by UV-irradiation. The coating composition and preparation steps were similar to the previous methods.

The latter and the commercial coating (Readom D) were directly applied to the cheese surface one day after manufacture. The coatings were applied manually by brushing until all the cheese surfaces were covered, with the residual coating being allowed to drip off. The cheeses were then stored in an appropriate chamber for 45 days at 11 ºC and 85% RH, and were turned from time to time until the coating was essentially dry. The coated cheeses were then compared with their uncoated counterparts.

9.2.4 Rheological analyses of coating solutions

Before they were applied to the cheese, the rheological behaviour of the coating solutions was evaluated, on the basis of their apparent and Brookfield viscosity.

Apparent viscosity

The apparent viscosity of the coating solutions was determined using a controlled stress rheometer (Rheostress 1 - RS1, Haake, Thermo Fisher Scientific, Germany) with a parallel plate sensor (TP20 Ti, 20 mm diameter, 115 μm gap). Flow tests were carried out at 20±0.1 ºC with upward and downward linear shear rate ramps between 0.1 and 10 s^{-1}. For each thixotropic cycle, the shear rate was increased over a period of 100 s, held at the upper limit for 20 s and then decreased again over a period of 100 s. Thixotropy was recorded as the resultant area between the upward and downward shear stress (σ) curves as a function of shear rate (γ). Three measurements were taken for each coating solution. The upward shear rate ramp was used to determine the consistency index (k) and the power law factor (n) according to the Power Law or Ostwald model (equation 9.1).

$$\eta = k\left(\gamma\right)^{n-1} \tag{9.1}$$

where η is the apparent viscosity (Pa.s) and γ is the shear rate (1/s) (Blair et al., 1939). According to the power-law factor, three different types of fluids can be identified based on their flow behaviour: n<1 pseudoplastic; n=1 Newtonian and n>1 dilatant fluids (less common).

Brookfield viscosity

More empirical measurements of viscosity were obtained using a rotational Brookfield viscometer (model DV II) at room temperature (± 25 °C) and constant angular velocity. Depending on the consistency of the coating solution, two different dish spindles were used to measure the Brookfield viscosity. For solutions with lower viscosities, data was collected from a 220 mL

coating solution held in a 250 mL beaker by a RV-1 spindle (56 mm diameter) at 20 rpm. Measurements were taken with a gap size of about 6 mm between the spindle and the walls of the reservoir. More viscous solutions were measured in a 100 mL beaker with 90 mL of each coating sample using the RV-5 spindle (21 mm diameter) also rotating at 20 rpm. In this case a gap size of about 14 mm was observed. In both cases the Brookfield viscosity was recorded for 5 min (at 60 s intervals) and a mean value was then calculated.

9.2.5 Physicochemical analyses of cheese

Physicochemical analyses of the cheeses were assessed in triplicate, 1, 15, 30 and 45 days after the coatings were applied.

Weight loss

Each cheese was individually weighed with a Mettler Toledo PB102 (Switzerland) analytical balance at the beginning and during the storage period. The percentage of the relative weight loss (ΔW) was calculated as:

$$\% \, \Delta W = \frac{I_{wo} - F_{wi}}{I_{wo}} \times 100 \tag{9.2}$$

where I_{wo} is the initial cheese weight and F_{wi} is the final cheese weight at time i. One reading per cheese sample was obtained.

Moisture content

The cheese moisture content was gravimetrically determined by the difference between samples before and after total solid content evaluation according to the Portuguese standard method (NP 3544:1987). 5-6 g of the cheese samples were weighted into a Petri dish, previously dried and weighted using an analytical balance (Geber Bosh S200, Germany) with a precision of ± 0.0001 g. The samples were dried overnight in an oven (Memmert, Western Germany) at 110 ºC until constant weight. The moisture content in cheese is obtained by applying equation 9.3.

$$\% \ moisture = \frac{m_i - m_f}{m_i} \times 100 \tag{9.3}$$

where m_i represents the initial weight of the cheese samples and m_f is the weight of the cheese samples after drying. Three readings per cheese sample were obtained.

Protein content

The Kjeldahl method (ISO 8968-1:2001) was used to evaluate the total nitrogen content, using distillation apparatus (Kjeltec System 1002 Tecator). To evaluate protein, the amount of total protein in the cheese was calculated by multiplying the percentage of the total nitrogen present in the samples by a factor of 6.38 as described in Appendix A (section A.3). Three readings per cheese sample were obtained, on the first day of storage and after 45 days.

Fat content

The fat content was determined using the Van Gulik method (NP 2105:1983). On the basis of this technique, 3 g of cheese previously homogenised in an IUL Instruments Masticator, was weighed with precision into the Van Gulik butyrometer and 15 mL of sulphuric acid (95% w/w) was added. The complete dissolution of the cheese sample in the butyrometer (taking about one hour) was performed in a water bath (approx. 65 ºC) under frequent agitation to help the cheese dissolve completely. After dissolution, 1 mL of isoamyl alcohol was added and the mixture immediately agitated for 3 s. Sulphuric acid was then added until it reached 35% on the butyrometer scale. The butyrometer was then sealed and vigorously agitated. The centrifugation step was performed in a Gerber centrifuge for 10 min at 1200 rpm and 65 ºC. The fat content (% w/w) was read immediately after centrifugation using the butyrometer scale. One reading was obtained for each cheese sample.

Salt content

Total chloride in cheese was determined using the official method 935.43 (AOAC, 1997a). 2 g of finely comminuted and thoroughly mixed cheese sample were weighed and put into a 300 mL Erlenmeyer flask. 25 mL of 0.1 N $AgNO_3$, 10 mL of HNO_3 (65% w/w) and 50 mL of distilled water were added. The mixture was boiled until total sample digestion and at this point a $KMnO_4$ solution was added in small portions (3 mL) while the solution was boiled until dark brown. The boiling continued until the colour disappeared and small portions of $KMnO_4$ continued to be added (up to a total volume of 15 mL) until the solution retained the dark colour for several minutes before clearing. After the solution was cooled down, it was filtrated and distilled water was added, reaching a final volume of 200 mL. 100 mL of the filtrated solution was titrated with 0.1 N KSCN using 2 mL of ferric alum indicator (reagent grade $FeNH_4(SO_4)_2.12.H_2O$ saturated aqueous solution). The percentage of sodium chloride in the cheese samples was determined by the application of equation 9.4

$$\% \ NaCl = (12.5 - n) \times 0.00585 \times 100 \quad (9.4)$$

where *n* is the volume of KSCN (0.1 N) used in titration. Two readings per cheese sample were obtained.

pH and titratable acidity

pH was measured using a pH meter (PHM61 Laboratory pH Meter, Denmark) equipped with a probe for reading solids. The probe was inserted directly into the cheese sample. Three readings were taken per cheese sample.

Titratable acidity was determined according to the 920.124 method (AOAC, 1997b) for cheese samples. 10 g of cheese were weighed into a beaker. Distilled water at 40 ºC was added until 105 mL, and mixed with the cheese sample in order to dissolve the components. After dissolution the mixture was left to decant and filtrate. 25 mL of the filtered solution was titrated with NaOH (0.1 N) using phenolphthalein as an indicator. Each millilitre of sodium hydroxide solution (0.1 N) used in titration indicates the presence of 0.009 g of lactic acid in 2.5 g of cheese (equation 9.5).

$$\% \ lactic \ acid = 0.009 \times V \times 40 \quad (9.5)$$

where *V* is the volume of NaOH used in titration. Two readings were taken per cheese sample.

Water activity

Water activity (a_w) was measured using a hygrometer (Rotronic Hygroskop BT, Zurich, Switzerland) coupled with a DMS 100H device and equipped with a WA-14TH probe connected to a thermostatic bath for temperature stabilisation at 20ºC. The plastic probe dish for water activity measurement was filled with the cheese sample (taken from both the bulk and the cheese surface) and placed on the sample holder device. When a_w became stable its value was recorded. One reading was taken per cheese sample.

Texture

A Stable Micro Systems Texture Analyzer, model TA.XT Express Enhanced, was used to perform textural analysis and the resulting data was processed using Specific Expression PC Software. The cheese texture profile analysis (TPA) was performed with a penetration distance of 15 mm at 2 mm/s test speed, using an acrylic cylindrical probe with a diameter of 5 mm and a height of 38.1 mm. Three penetrations were performed per cheese in specific locations. This type

of test identified cheese hardness, defined as the maximum peak force during compression (first bite), often referred to as firmness (Uprit and Mishra, 2003).

Colour

Cheese colour was determined by a portable colorimeter HP-2132, Zheijang Top Instruments Co, Ltd., previously calibrated with a standard white plate of known parameters ($L^*_{standard}$ = 97.03; $a^*_{standard}$ = -0.67; $b^*_{standard}$ = 5.57), using a C illuminant in the colour space CIEL*a*b*. The colour of the cheeses was expressed by the three individual coordinates of CIEL*a*b* (defined in section 7.4.5) and by the total colour difference (ΔE). Three cheese samples were measured for each type of coating and ripening time, and three readings per cheese were taken.

9.2.6 Cheese microbiological analyses

Microbiological development on the cheese surface was evaluated by the enumeration of viable cells, 1, 15, 30 and 45 days after the application of the coatings. 10 g of cheese was aseptically removed from the upper surface area of each cheese into a stomacher bag and was diluted to 1:10 (w/v) in sterile 1% (w/v) sodium citrate (Merck) and blended in a stomacher (Masticator IUL Instruments) for 1.5 min at 260 rpm. Decimal dilutions were subsequently prepared with 0.1% (w/v) peptone water and plated, in triplicate, on the corresponding media.

Staphylococcus spp. were enumerated on a Baird-Parker Agar Base, BPA supplemented with egg yolk and telurite emulsion, as originally proposed by Baird-Parker (1969) and *Pseudomonas* spp. were counted on a Pseudomonas Agar F, PAF. Both media were incubated aerobically at 37 ºC for 48 hours. *Enterobacteriaceae* were counted on a Violet Red Bile Glucose Agar, VRBGA with Triptona Soya Agar, TSA after incubation at 30 ºC for 48 hours. Yeasts and moulds were determined after 5 days of incubation at 25 ºC on a Rose-Bengal Chloramphenicol Agar, RBC. Except for the enumeration of *Enterobacteriaceae* on VRBGA (for which the pour plate technique was used) the surface plating technique described by Miles et al. (1938) was followed for the samples and growth media.

9.2.7 Sensorial analyses of cheese

The sensorial evaluation of cheeses at the end of the ripening period (45 days) aimed to assess the influence of the WPC-based coatings on the colour, odour, hardness and flavour of the cheeses tested, as well as to compare them with the uncoated cheeses and those with commercial coatings.

Sensorial tests were carried out in the ESAC sensory room by a trained panel of 12 members of both sexes who were familiar with Portuguese regional cheeses. Two tests were performed by each panellist; the first to evaluate the overall appearance of the cheese, in which the whole cheese was analysed, and the second to evaluate the cheese characteristics, using cheese slices of approx. 1 cm thickness placed on individual plastic Petri dishes. Before the sensorial analysis session, the whole and sliced cheese samples were equilibrated at room temperature (20±2 ºC) and coded using random three-digit codes, prior to being presented randomly to the panellists. Between samples, the panellists were given toasts and water to clean their palates.

A 5-point scale was used by the panellists to evaluate all the attributes used to classify whole and sliced cheese samples. For whole cheese evaluation, the sensorial attributes assessed were: shape (1= not characteristic and 5= ideal); rind colour (1= white and 5= dark yellow); colour homogeneity (1= heterogenic and 5= homogeneous); hardness (1= very soft and 5= very hard). For cheese slice evaluation the sensorial attributes were: difference between the paste and ring colour (1= imperceptible and 5= intense); odour (1= imperceptible and 5= intense); consistency (1= very soft and 5= very hard); flavour (1= imperceptible and 5= intense) and overall acceptability (1= least acceptable and 5= most acceptable). In order to define the coating attributes better, the panellists were asked to include useful information in the "observations" section included on each evaluation card.

9.2.8 Statistical analyses

A statistical analysis of the data was carried out using the analysis of variance (ANOVA) package included in StatSoft Statistica 8.0 (Hill and Lewicki, 2007). The Tukey-HSD post-hoc test, with a 95% confidence level, was applied to assess differences between the physicochemical, microbiological and sensory properties of cheeses coated with WPC-based coatings, commercial coatings and uncoated cheeses.

Two-way ANOVA with interaction was employed to determine the effects of both storage time (at 1, 15, 30 and 45 days) and coating type on the physicochemical properties (moisture, fat, and salt contents; weight loss; pH; water activity; texture and colour) and microbiological properties (*Staphylococcus* spp.; *Pseudomonas* spp.; *Enterobacteriaceae* and yeast and moulds) of ripened cheeses.

One-Way ANOVA tests were performed to compare the means for the rheological properties of the coating solutions (thixotropy, apparent and Brookfield viscosity) and the cheese sample

attributes (scored on a 5-point scale) used in the sensorial evaluation of the whole and sliced cheese.

9.3 Results and discussion

The physicochemical, microbiological and sensorial properties of the semi-hard cheeses coated with the antimicrobial edible WPC coating matrix developed by the different coating production methods were compared with cheese with a commercial coating (positive control) and uncoated cheese (negative control) during the 45 days of ripening.

9.3.1 Rheological properties of coatings

The apparent and Brookfield viscosity of the WPC-based coatings produced by the different coating production methods (HD, UV and HD+UV) were analysed and compared with the commercial coating matrix viscosity before it was applied to the cheese surface in order to understand the extent to which these properties influenced the coating phenomenon. The results are shown in Figure 9.1 and Table 9.1.

Over the entire range of shear rates similar flow behaviour was observed in all the coating solutions produced (Figure 9.1), although distinctions between individual batch viscosities were clearly visible over the shear rate range tested. The shape of the flow curves of the coating solutions exhibited shear-thinning behaviour, characteristic of pseudoplastic flow, showing a decreasing viscosity with increasing shear rate. Most of the weakly aggregating dispersion systems showed the same behaviour (de Rooij et al., 1993) in which apparent viscosity continuously decreases with an increasing shear rate, due to the breakdown of aggregates. Eventually, when all the aggregates are broken up and only colloidal particles are present, hydrodynamic forces dominate all other forces and the sample became Newtonian, i.e. has constant viscosity.

Coating matrixes subjected to heat denaturation (HD+UV and HD) had the highest apparent viscosity values and both the commercial coating solution and the coating solution used for the production of the UV coating type showed the lowest viscosity values, especially in the case of the former. The main reasons for these differences may be related to the amount and nature of the polymeric base material (e.g. whey proteins or PVA), coating additives and the method used to perform coating polymerization, which dictates the starting point of the polymerization process. It is important to note that in the UV coating solution, the polymerization process may only begin after application to the cheese surface and UV irradiation exposure. The lower

viscosity obtained in this case corroborates this, demonstrating that until this stage only colloidal particles are present in the coating solution, with no indication that polymerization has started. On the other hand, the application of a thermal process to the whey protein coating solutions promotes protein denaturation and the beginning of cross-linking before application of the coating, leading to the highest viscosity values.

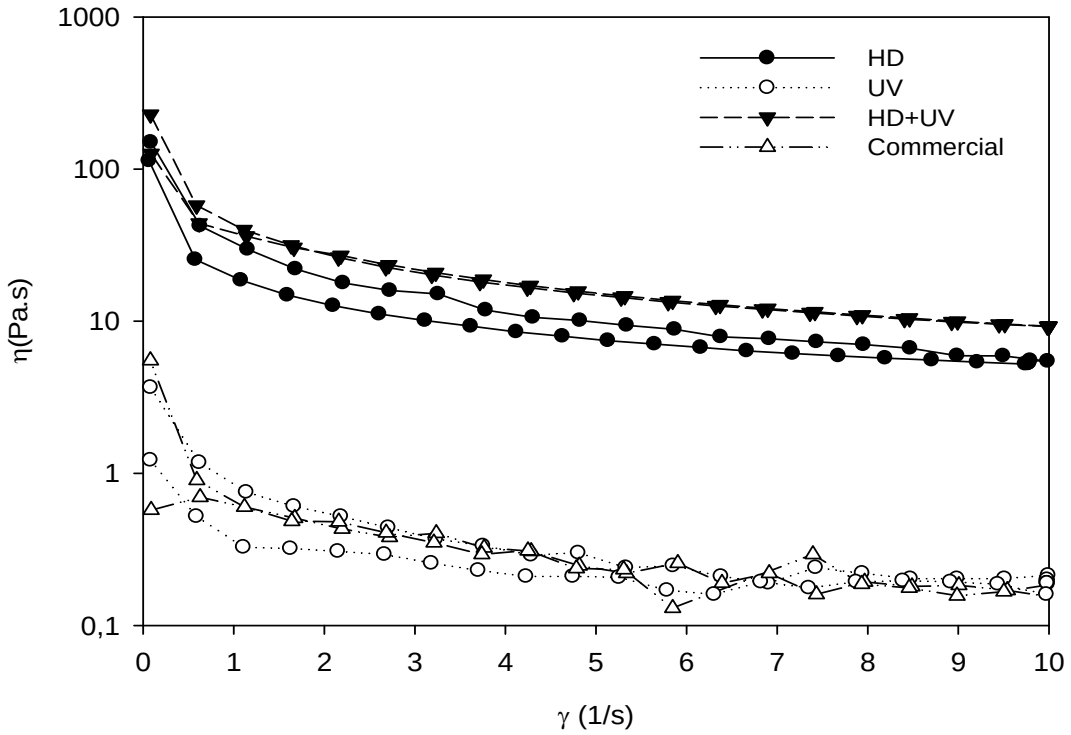


Figure 9.1 Apparent viscosity (η) as a function of shear rate of the antimicrobial WPC coating solutions produced by (●) HD; (○) UV and (▼) HD+UV coating production methods compared with (Δ) commercial coating solution, before its application to the cheese surface.

Thixotropic or "hysteresis" loops were generated for the coating samples tested, as shown in Figure 9.2. The difference in the area under the curves between the upward and downward curves was calculated as thixotropy in Pa/s and plotted in Table 9.1. It was observed that the HD coating solution showed the highest thixotropic behaviour. The presence of the photoinitiator in the HD+UV formulation may probably contribute to a more effective and accurate molecular rearrangement of denatured proteins after heat treatment, thus explaining the distinct rheological behaviour in both samples.

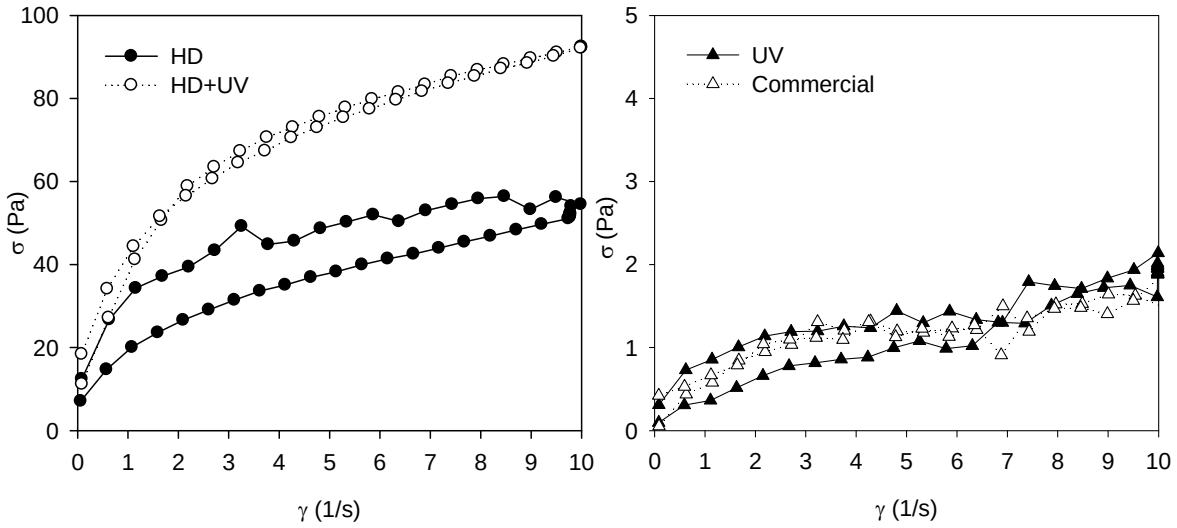


Figure 9.2 Thixotropic loops of the antimicrobial WPC coating solutions produced by (●) HD, (▲) UV and (○) HD+UV coating production methods compared with the (Δ) commercial coating solution, before its application to the cheese surface.

The power law or Ostwald model (equation 9.1) was applied in order to describe the flow behaviour of coating solutions on the cheese surfaces by determining the consistency index (k) and the power law factor (n). The consistency index (k) obtained for each coating solution (Table 9.1) was in agreement with the aforementioned observations. Significantly higher values ($p < 0.05$) were obtained for HD+UV and HD coating solutions (36.68 $Pa.s^n$ and 29.30 $Pa.s^n$, respectively) as opposed to 0.81 $Pa.s^n$ for the UV and 0.65 $Pa.s^n$ for the commercial coating solution. According to the power law factor (n), no significant differences were observed between samples and the pseudoplastic flow behaviour was confirmed ($n < 1$) for all the coating solutions. Pseudoplastic or shear-thinning fluids have a lower apparent viscosity at higher shear rates, and are usually solutions of large, polymeric molecules in a solvent with smaller molecules. It is generally supposed that the large molecular chains tumble at random and affect large volumes of fluid under low shear, but gradually align themselves in the direction of increasing shear and produce less resistance.

In comparing the Brookfield viscosity of coating samples (Table 9.1) it was possible to find significant differences ($p < 0.05$) between the HD and HD+UV formulations, with higher values for the latter. The similar ($p > 0.05$) lower Brookfield viscosity achieved for the UV and

commercial coating solutions may indicate that they may behave in the same manner during coating application.

Table 9.1 Thixotropy, consistency index (k) and power law factor (n) of the power law model and Brookfield viscosity of the antimicrobial WPC coating solutions produced by HD, UV and HD+UV coating production methods compared with the commercial coating solution.

Coating type	Thixotropy (Pa/s)		Power law model (equation 9.1)				Brookfield viscosity (Pa.s)	
			k (Pa.s^n)		n			
HD	43.30±0.35	c	29.30±13.39	b	0.35±0.13	a	0.99±0.02	b
UV	3.35±0.70	a	0.81±0.08	a	0.37±0.02	a	0.01±0.00	a
HD+UV	24.08±12.7	b	36.68±0.32	b	0.44±0.01	a	3.17±0.13	c
Commercial	4.40±0.84	a	0.65±0.13	a	0.41±0.08	a	0.02±0.00	a

a, b, c means with different letters within a column differ significantly ($p < 0.05$); (average ± standard deviation)

On the basis of these findings it was possible to predict that during coating application the more viscous solutions would exhibit higher adherence to the cheese surface than the less viscous coating solutions, which could result in very distinct coating thickness among cheeses if a dipping process was used. In order to produce coatings with a similar thickness it was decided to apply the coating solutions manually with a brush until all the cheese surfaces were covered.

9.3.2 Cheese physicochemical profile

The physicochemical properties of cheese were assayed on days 1, 15, 30 and 45 of storage and are displayed in Figure 9.3 (weight loss, water activity (a_w), moisture, fat, protein and salt (NaCl) contents), Table 9.2 (titratable acidity and pH) and Figure 9.4 (hardness), with the aim of finding a suitable alternative to commercial coatings.

Our analyses provided information on how cheese weight loss over 45 days was affected by the presence of a coating, its type and the influence of the coating production method used. Figure 9.3a shows an increase in weight loss in all cases throughout storage. This increase is statistically higher ($p < 0.05$) during the first 30 days, with the exception of the commercial coating, in which weigh loss is less pronounced after 15 days. No differences in weight loss ($p > 0.05$) were observed among the coated cheeses. However, the WPC coating produced by the HD+UV method and the commercial coating had the best performance. Cheeses with edible WPC coatings produced by the HD+UV method showed statistically ($p < 0.05$) lower values than uncoated cheeses, which presented a higher weight loss.

Water activity (a_w) was the main factor affecting cheese stability during ripening, revealing relatively high values (0.87 – 0.92), which were to some extent expected since this type of cheese generally has a_w close to unity (Singh et al., 2003). This property remained practically constant during the entire storage period in all the tested cheeses, with no significant differences ($p > 0.05$) during the first 30 days. In the uncoated cheeses and cheeses coated with WPC produced by the HD and UV method only (Figure 9.3b) a significant decrease ($p > 0.05$) was observed between the 30th and 45th day of the study. Water loss (Figure 9.3c) was the main factor responsible for the decrease, although other causes may have contributed to this. Sousa et al (2001), for instance, noted that protein degradation via releasing carboxyl and amino groups through water binding during ripening contributes to a decrease in water activity.

In terms of moisture (Fig. 9.3c), the values decreased significantly ($p < 0.05$) during storage, displaying moisture losses of approx. 33.4% to 40.0% for coated cheeses. The moisture loss profile was different between cheeses during the ripening period, with significant differences ($p < 0.05$) between samples on the 15th and 45th day. In uncoated cheese and cheese with HD coating, moisture loss was less pronounced during the first 15 days, whereas the remaining samples showed the opposite trend. At the end of the ripening period, cheeses with WPC coatings produced by the UV and HD+UV method had the highest levels of humidity, followed by the commercially coated cheese and, finally, the HD coated and uncoated cheese. The fastest decrease observed in moisture at the beginning of the storage period was in cheeses coated with WPC when UV modification was used, and its similarity to the moisture profile of commercial coated cheese may suggest analogous water availability conditions for controlling microbial growth that are crucial in the first days of ripening. These results prove that the presence of a coating, its nature and coating production method influence the water transfer phenomenon between cheeses and their surroundings during ripening. Moreover, after 45 days, the edible whey protein antimicrobial coatings reduced the weight loss and moisture loss of cheese by 5.7% and 3.3% respectively if the coating was prepared by HD, 7.7% and 9.8% when only UV modification was used and 11.2% and 9.9% using coatings prepared by HD+UV, whereas the use of commercial coatings reduced this by 10.4% and 8.5%. The performance of the edible whey protein coatings was therefore better or similar to the commercial ones, with the exception of the HD coating.

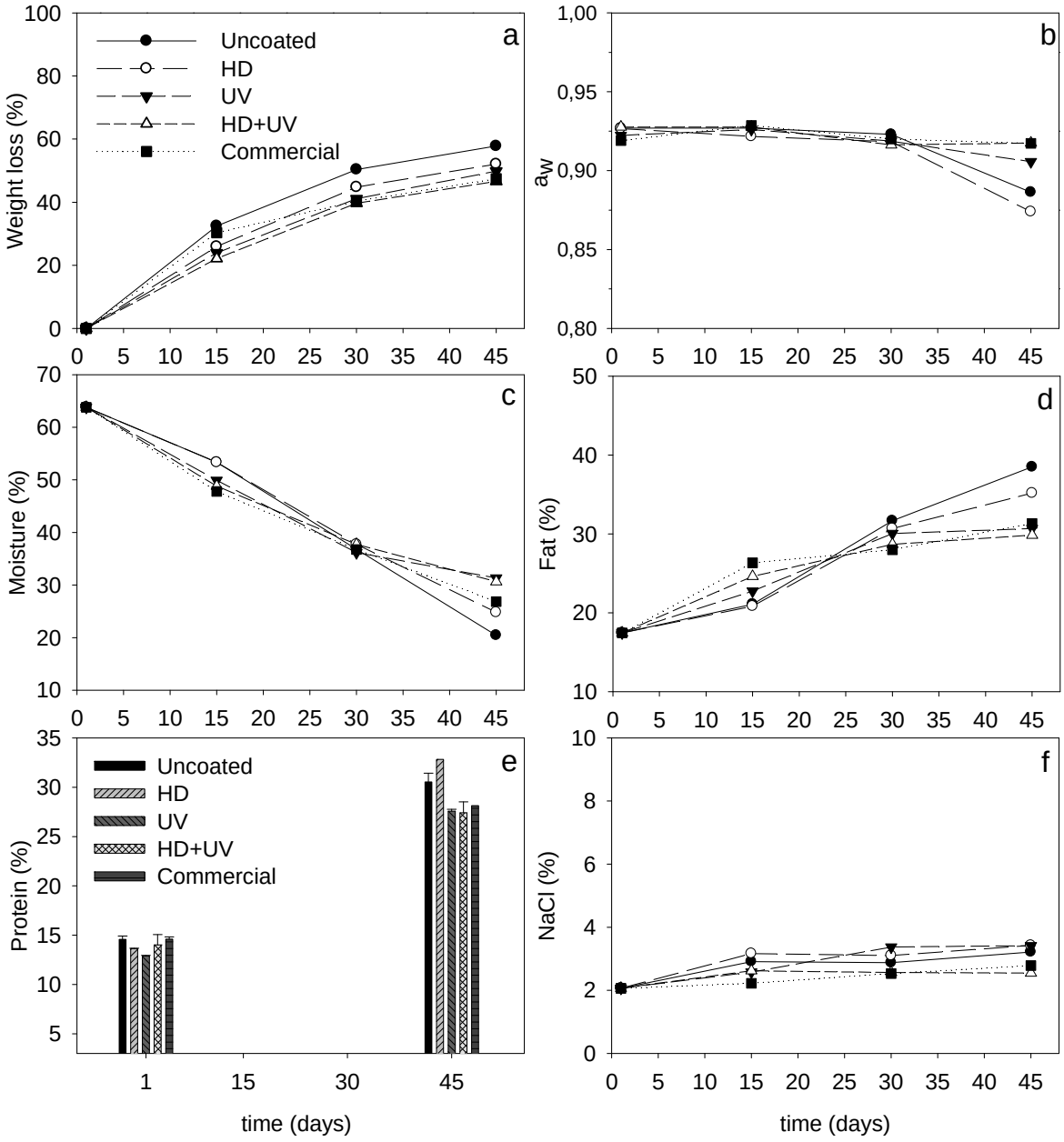


Figure 9.3 Weight loss, water activity (a_w), moisture, fat, protein and salt (NaCl) contents of cheese samples coated with antimicrobial whey protein edible coatings produced by (○) HD, (▼) UV and (Δ) HD+UV coating production methods compared with (●) uncoated cheese and (■) cheese with commercial coating, during 45 days of ripening at 11 ºC and 85% RH.

Fat content (Figure 9.3d) increased ($p < 0.05$) during the ripening period in all the cheese samples, whether coated or not. However, this evolution was coating type dependent and varied

in inverse proportion to the cheese moisture content. Dried cheeses have a higher percentage of fat.

Protein content (Figure 9.3e) (evaluated on the first and last day of the study only) significantly increased ($p < 0.05$) in all the cheese samples, mainly due to the reduction in water content. The protein nature of the edible WPC coatings did not affect the protein composition of cheese, as no differences ($p > 0.05$) were observed between cheeses after coating. However, the use of a coating and the method used to produce it influenced the final protein composition of cheeses. Similar lower protein contents were obtained for cheese coated with commercial coating and cheese coated with WPC produced by UV and HD+UV methods. When only the HD method was applied, the higher protein content achieved was in the same order of magnitude as that of the uncoated cheese.

The salt content (Figure 9.3f) was not affected by the presence of a coating. Moreover, the performance of the WPC coatings was similar to that of the commercial coating and no statistical differences were observed between cheeses with either coating. During ripening a significant ($p < 0.05$) increase in NaCl content was observed. The cheese with a WPC coating produced by HD+UV had the lowest increase (0.43%) and very similar values to commercially coated cheese. Variations were more pronounced in WPC coated cheese produced by HD (1.36%).

Table 9.2 shows cheese titratable acidity (expressed as a percentage of lactic acid) and pH variation during ripening. During the first 15 days, titratable acidity increased significantly ($p < 0.05$) in both the coated and uncoated cheese samples. Cheese with a WPC coating produced by the HD+UV method had significantly higher values (1.40% of lactic acid) than its counterparts. The activity of indigenous lactic acid bacteria cultures that metabolise lactose to lactate is responsible for the production of acids which result in an acidity increase and consequent pH reduction. The pH decrease observed between the 1st and the 45th day of storage (Table 9.2) showed some variations and did not follow the opposite behaviour of titratable acidity. However, significant differences were obtained when comparing pH values between samples on the 30th day of storage only ($p < 0.05$).

Table 9.2 Titrable acidity (% lactic acid) and pH of cheese samples coated with antimicrobial whey protein edible coatings produced by HD, UV and HD+UV coating production methods compared with uncoated cheese and cheese with commercial coating, during 45 days of ripening at 11 ºC and 85% RH.

	Coating type	Ripening time (days)			
		1	15	30	45
Lactic acid (%)	Uncoated	0.42±0.09 aA	0.97±0.03 aB	0.95±0.02 aB	1.08±0.15 aB
	HD	0.42±0.09 aA	0.96±0.07 aB	0.98±0.16 aB	1.13±0.40 aB
	UV	0.42±0.09 aA	1.04±0.05 aB	1.05±0.02 abB	1.16±0.16 aB
	HD+UV	0.42±0.09 aA	1.29±0.06 bB	1.54±0.05 cC	1.40±0.09 bBC
	Commercial	0.42±0.09 aA	1.06±0.07 aB	1.17±0.03 bB	1.10±0.14 aB
pH	Uncoated	5.19±0.23 aB	4.62±0.04 aA	4.69±0.10 abA	4.76±0.19 aA
	HD	5.19±0.23 aB	4.56±0.04 aA	4.37±0.06 aA	4.69±0.07 aA
	UV	5.19±0.23 aB	4.49±0.02 aA	5.03±0.05 cB	4.81±0.06 aAB
	HD+UV	5.19±0.23 aC	4.49±0.01 aA	4.93±0.03 bcB	4.70±0.05 aAB
	Commercial	5.19±0.23 aB	4.73±0.03 aA	4.62±0.03 abA	4.57±0.02 aA

A, B, C means with different capital letters differ significantly ($p < 0.05$) during ripening time for the same coating type (line). a, b, c means with different small letters differ significantly ($p < 0.05$) between coating types at the same ripening day (column).

Figure 9.4 presents the hardness values for cheese samples throughout storage, which increased significantly ($p < 0.05$) in all cases. It was observed that the presence of a coating, its nature and the coating production method considerably influenced hardness. Some authors (Cerqueira et al., 2009; Ramos, 2011) state that cheeses with a lower moisture content are harder, which could justify the results during the first 15 days of ripening, when the cheeses with a higher moisture content (uncoated, HD and UV) (Figure 9.3c) had lower hardness values (Figure 9.4). However, the moisture content for the different cheese samples was similar on the 30th day of storage (Figure 9.3c), although hardness was significantly different ($p < 0.05$) at this point, and at the end of ripening (45th day). The most dehydrated cheeses (uncoated and HD) had the lowest hardness values, whereas cheeses that had significantly higher humidity (UV, HD+UV and commercial) had higher hardness values. It is important to note that determining cheese hardness does not depend exclusively on cheese bulk consistency but is also influenced by rind consistency, in which the polymeric material used in coating formulation and the type of chemical interactions occurring during the formation of the coating play an important role.

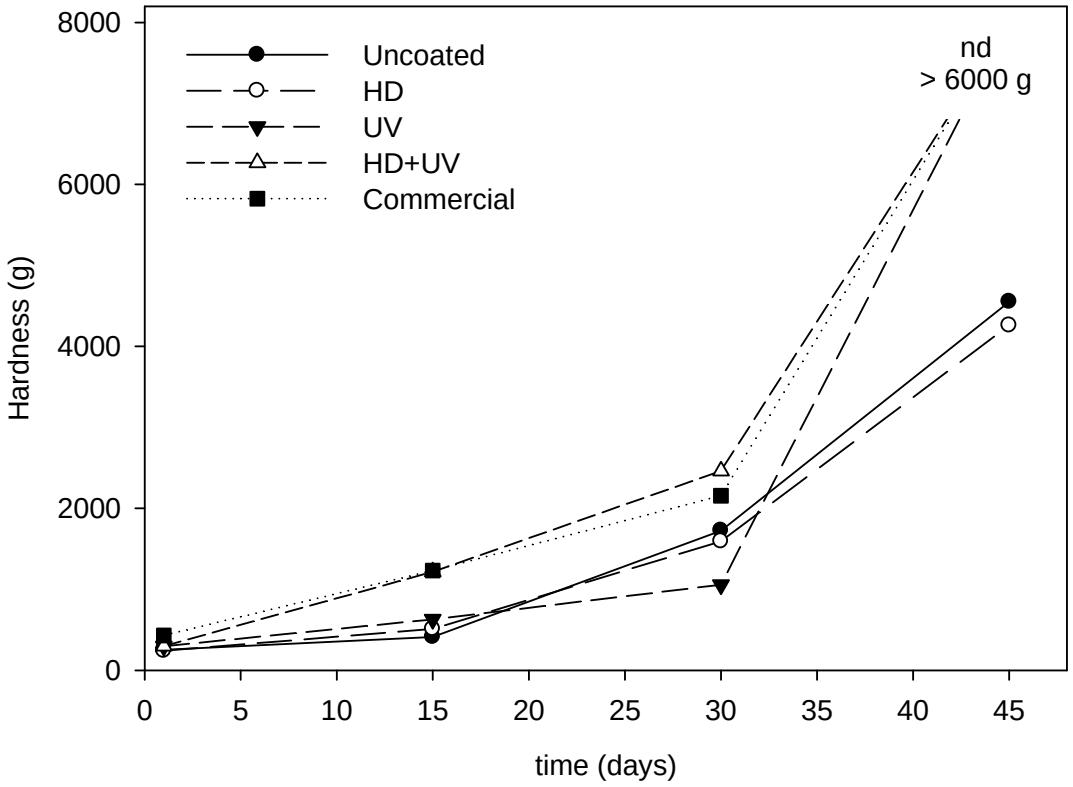


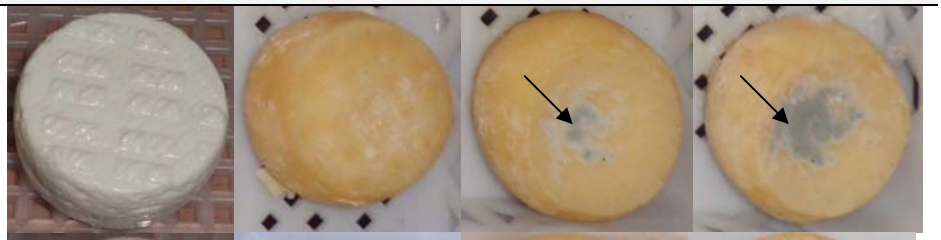
Figure 9.4 Hardness of cheese samples coated with antimicrobial whey protein edible coatings produced by (○) HD, (▼) UV and (Δ) HD+UV coating production methods compared with (●) uncoated cheese and (■) cheese with commercial coating, during 45 days of ripening at 11 ºC and 85% RH.

The similarity in hardness profiles obtained for cheeses coated with commercial coatings and with the WPC coating produced by the HD+UV method (Figure 9.4) may indicate that the nature of the molecular and chemical interactions are the same, despite the different polymeric base materials (PVA for the commercial coating). The faster drying and coating formation during the first days of ripening was evident in both cheeses, allowing a harder crust to form which prevented further dehydration in the cheese bulk.

9.3.3 Cheese appearance

The appearance of the cheeses coated with antimicrobial WPC-based coatings produced by the three different coating methods (HD, UV and HD+UV) was compared with uncoated and commercially coated cheeses during the 45 days of ripening (Table 9.3).

Table 9.3 Appearance of bovine cheeses of ca. 120 g, coated with antimicrobial whey protein edible coatings produced by HD, UV and HD+UV coating production methods compared with uncoated cheese and cheese with commercial coating, during 45 days of storage at 11 ºC and 85% RH. Arrow – presence of moulds.

Coating	Ripening time (days)			
	1	15	30	45
Uncoated				
HD				
UV				
HD+UV				
Commercial				

During application of the coating it was observed that coating solutions with a lower viscosity, especially the UV coating solution, resulted in less adherence to the cheese surface and consequently significant drainage from it. Heat denaturation (HD and HD+UV) was responsible for increasing the viscosity of coating solutions, contributing towards good adherence. The coating procedure adopted (spreading the coating solution onto the cheese surface with a brush) was reasonably efficient for coatings with higher viscosities, since it allowed for the production of

thinner, less sticky coatings with higher drying rates during storage. However, using this method with lower viscosity coatings may compromise coating efficiency as a result of the extremely thin coatings produced. For this reason, a dipping procedure could be a better alternative for improving coating thickness in these cases.

The identical and homogeneous appearance of the various cheese surfaces (top, bottom and lateral surfaces) was assessed one day after application of the coating (Table 9.3) with no visual differences observed between coated and uncoated cheese, or among the different types of coated cheeses (commercial and WPC-based coatings). The most relevant visual changes in cheese throughout ripening occurred during the first 15 days (Table 9.3), when the initial white colour of the cheese and its wet appearance changed to a dry appearance and a light yellow colour.

It was not easy to detect colour differences between uncoated and coated cheese by visual inspection, but with regard to microbial growth, the uncoated cheese presented a significant amount of mould on its surface after 30 days of storage, which increased over the 45 days. Some mould was also observed on the WPC coatings produced by HD or UV modification but was much less extensive (Table 9.3). Possible reasons for this include the lower antimicrobial efficiency of these coatings or probably the insufficient amount of coating deposited on the cheese surface. Visually the presence of moulds was not detected in the commercial coated cheese and cheese with the antimicrobial WPC coating produced by the HD+UV method during the 45 days. In both cases a good appearance was achieved.

The colour analysis based on L*, a*, b* coordinates (Figure 9.5) and colour difference (ΔE*) (Figure 9.6) confirmed that all the cheese samples changed colour during storage ($p < 0.05$), with statistically significant differences ($p < 0.05$) recorded between them.

The most pronounced colour change occurred during the first 15 days of storage ($p < 0.05$). Lightness (L*) decreased significantly from near 95 (white samples) to approximately 70; the a* values changed from negative (green) to positive values (red) and b* increased in the positive axis direction from approx. 10 to 20 (yellow direction). In the 30 days following this period, the colour changes were not so pronounced although the presence of coating and the coating production method had a more important influence on L* (Figure 9.5) and ΔE* values (Figure 9.6).

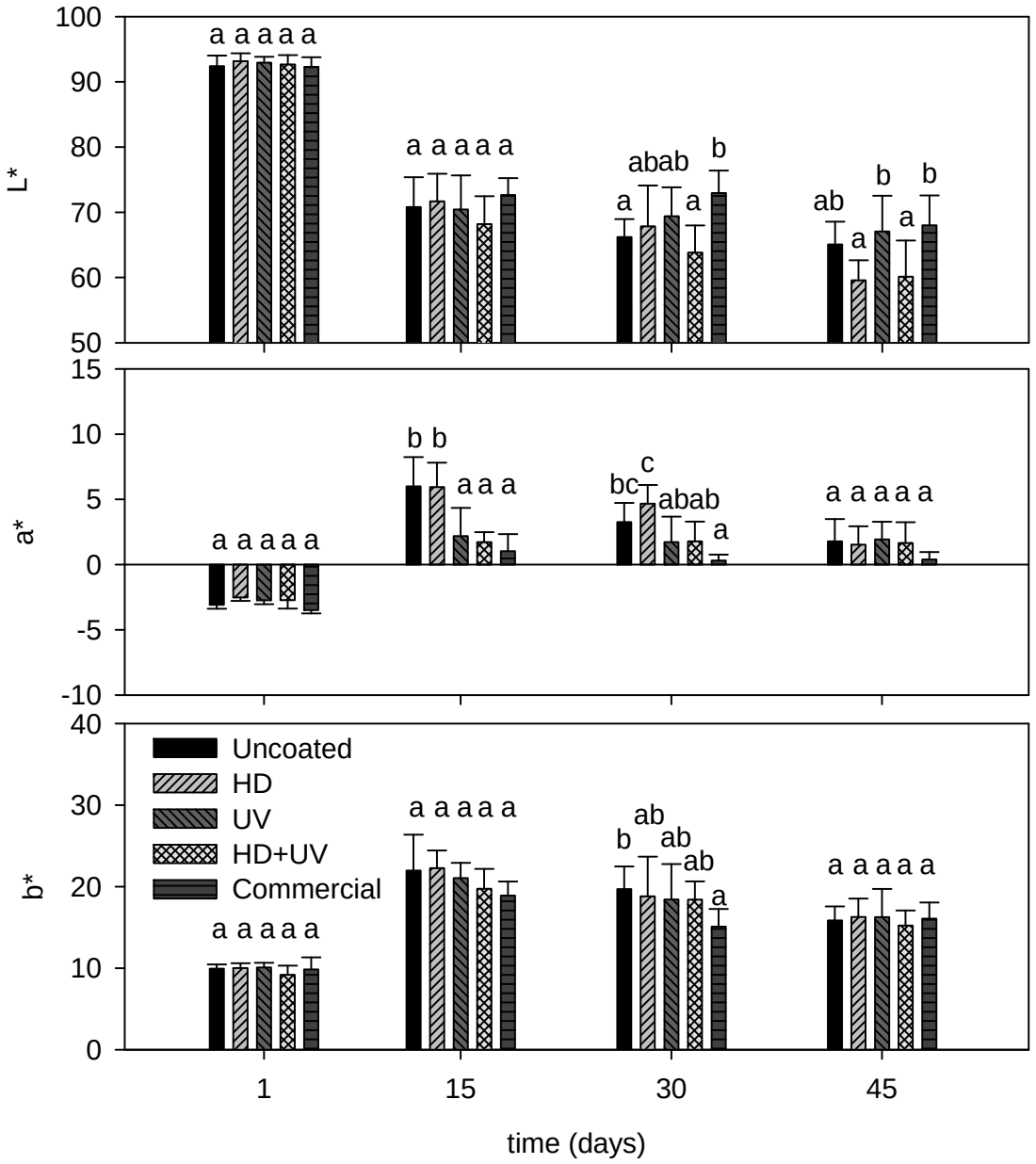


Figure 9.5 Colour coordinates L*, a* and b* of cheese coated with antimicrobial whey protein edible coatings produced by HD, UV and HD+UV coating production methods compared with uncoated cheese and cheese with commercial coating, during 45 days of storage at 11 ºC and 85% RH. a, b means with different small letters differ significantly ($p < 0.05$) between coating types at the same ripening day.

It was observed that using heat denaturation to produce coatings (HD and HD+UV) leads to darker cheeses than uncoated ones. One possible reason for this is that the higher amounts of lactose (28.28%) present in WPC when exposed to thermal treatments starts chemical Maillard browning reactions. On the other hand, cheeses with commercial coatings and WPC coatings

produced only by UV modification presented lower colour differences than uncoated cheeses. This colour change in uncoated cheese can be attributed in part to cheese oxygen and light oxidation, which is lower in coated cheeses due to reduced oxygen permeability and increased coating opacity (Cerqueira et al., 2009). The cheese dehydration rate during ripening, which was lower for the aforementioned cheeses (commercial and UV), may also be associated with less dry, and therefore less dark rind cheese at the end of the study. Cagri et al. (2004) have also mentioned that coatings incorporating lactic acid and its acidulant feature have the ability to reduce colour change. However, our results do not show this behaviour in the comparison of WPC based coatings (which had lactic acid as an antimicrobial agent) and their commercial counterparts, probably due to the use of WPC instead WPI. The presence of higher lactose concentrations in WPC and its tendency to become darker during time probably masked the lactic acid effect (Mckibben and Krochta, 2000).

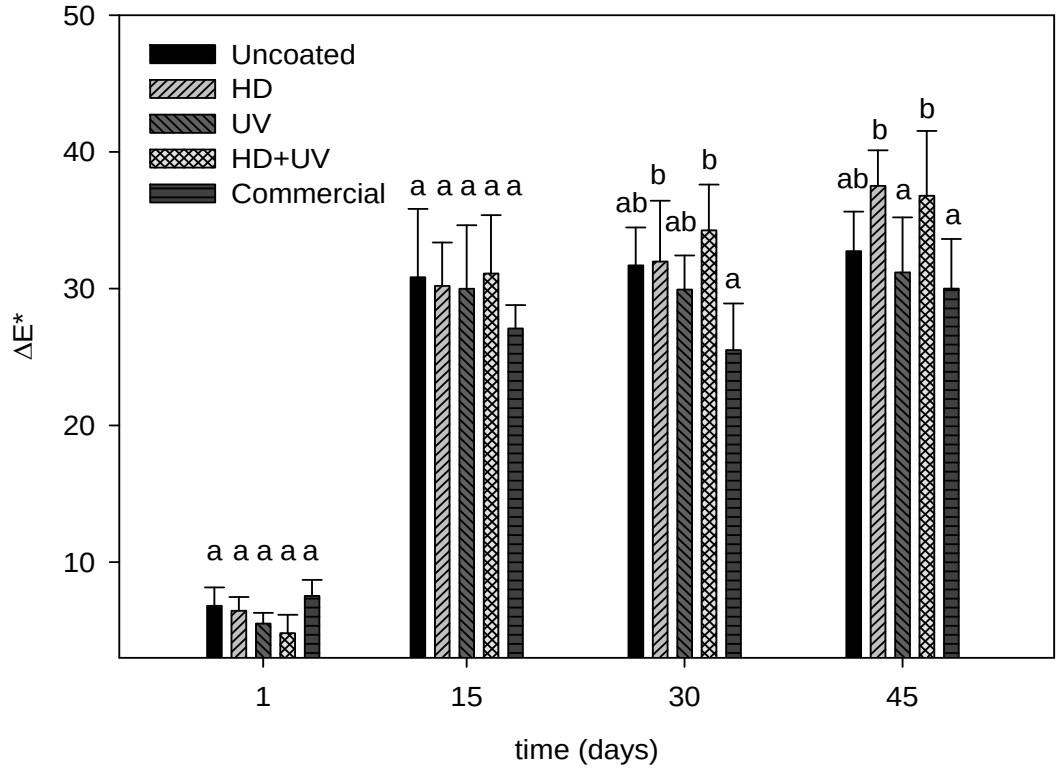


Figure 9.6 Colour difference (ΔE*) of cheese coated with antimicrobial whey protein edible coatings produced by HD, UV and HD+UV coating production methods compared with uncoated cheese and cheese with commercial coating, during 45 days of storage at 11 ºC and 85% RH. a, b means with different small letters differ significantly ($p < 0.05$) between coating types at the same ripening day.

9.3.4 Cheese microbiological profile

The antimicrobial performance of the various WPC-based coatings with lactic acid and natamycin as bioactive agents was ascertained using a set of spoilage/pathogenic microflora frequently found on the cheese surface, namely *Staphylococcus* spp. working as a gram-positive bacterium model, *Pseudomonas* spp. and *Enterobacteriaceae* as a gram-negative bacterium model and, finally, yeast and moulds. The microbiological results of the cheese samples during ripening were presented in Figure 9.7. These results showed that all the types of pathogenic or contaminant microorganisms evaluated were detected on the cheese surface. Gram-positive bacteria (*Staphylococcus* spp.) were found in lower levels (< 6.5 log(CFU/g)) than gram-negative bacteria (*Pseudomonas* spp. and *Enterobacteriaceae*) or even yeast and moulds. A microbiological analysis also indicated that after 45 days of storage, there are statistical differences ($p < 0.05$) between uncoated cheese and cheeses with the different antimicrobial coatings.

The best results for microbial growth control or inhibition were found in cheese coated with the antimicrobial WPC-based edible coating produced by the HD+UV method, regardless of the microorganism evaluated. The microbial inhibition of *Staphylococcus* spp. and growth control of *Enterobacteriaceae* (Figure 9.7) was very clear in this coating type, in contrast to the performance of the remaining coatings in terms of gram-positive and gram-negative bacteria. The results obtained for yeast and moulds revealed a similar performance ($p > 0.05$) for HD+UV and commercial coatings. In both cases there was no evidence of the growth of yeasts and moulds, in comparison to uncoated cheeses or cheeses with UV and HD coatings. Overall, a significant reduction ($p < 0.05$) in these types of microorganisms was recorded after 30 days of storage. This outcome was to some extent expected in the commercial coating, since it includes natamycin as an active compound which has a well-established success rate in preventing growth of yeast and moulds on cheese surfaces (Amefia et al., 2006). However the effective protection against this type of microorganisms afforded by the application of the HD+UV coating, which had a relatively low amount of natamycin (0.125 g/L against 2.5 g/L in the case of the commercial coating), was extremely attractive as an efficient substitute. According to these results, the first postulated hypothesis for this behaviour was the possible germicidal effect of UV-irradiation promoted during the UV modification process.

UV irradiation has been studied as an efficient method for bacterial growth inactivation. The germicidal effects are mainly due to DNA mutations induced through absorption of UV light by DNA molecules (Setlow and Carrier, 1966; Wang et al., 2005), called UV photoproducts, which

can affect cell survival. It has also been noted that gram-positive bacteria show a higher resistance than gram-negative bacteria to exposure to UV irradiation (Kim et al., 2002; Sharifi-Yazdi and Darghahi, 2006). However it is not clear whether this difference in susceptibility to UV radiation is caused by the difference in the cell-wall structure between the two types of bacteria, since gram-positive bacteria have many layers of peptidoglycan that form thick, rigid cell walls, whilst gram-negative bacteria have only a single layer or a few layers of peptidoglycan (Blatchley et al., 2001).

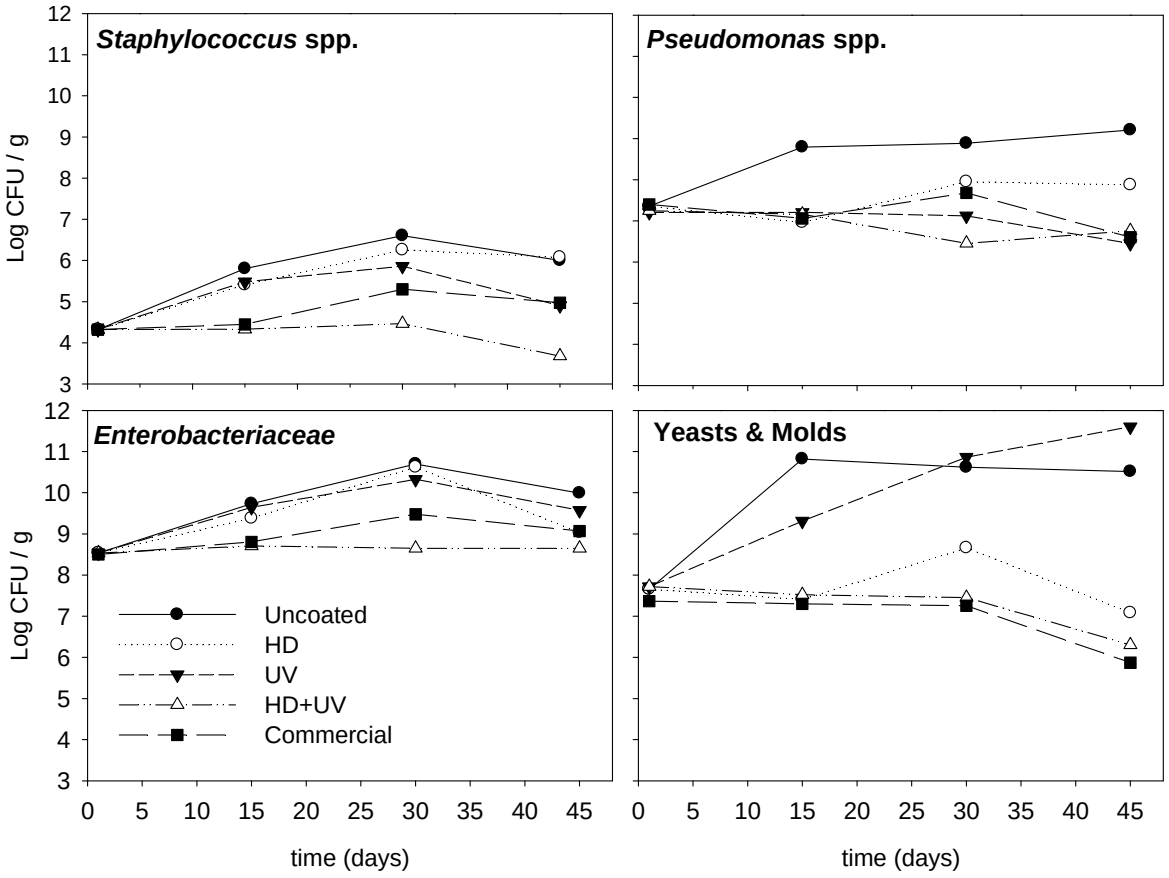


Figure 9.7 Viable cell counts (log(CFU/g)) of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and yeasts and moulds of cheese samples coated with antimicrobial whey protein edible coatings produced by (○) HD, (▼) UV and (Δ) HD+UV coating production methods compared with (●) uncoated cheese and (■) cheese with commercial coating, during 45 days of storage at 11 ºC and 85% RH.

The germicidal effect of UV irradiation was not clear from the microbiological results (Figure 9.7). Antimicrobial efficiency in the UV coating, in which only UV irradiation was applied, was extremely poor with regard to preventing the growth of *Staphylococcus* spp.,

Enterobacteriaceae and in particular yeast and moulds. One possible explanation for this may be the very short exposure time to UV irradiation (only 10 min at the beginning of the ripening period), which may be not sufficient for the germicidal effect, but essential to begin the UV process. Another possibility that may be cited is the extremely low thickness of this coating type, as a result of its reduced adhesion to the cheese surface during coating, which limited the effectiveness of the coating.

9.3.5 Cheese sensorial profile

The results observed in terms of the sensory analysis of cheese samples coated with antimicrobial edible whey protein coatings and with a commercial coating (or none) are presented in Table 9.4. Sensory assessment was performed for the external attributes (whole cheese evaluation) and also for the internal cheese attributes (sliced cheese). After the external evaluation, all the cheese samples were manually washed and dried at ambient temperature in order to eliminate any contaminant from the cheese surface so that they could be used for internal sensory evaluation.

With regard to external cheese evaluation (Table 9.4), no differences were observed ($p > 0.05$) between cheeses in terms of shape and rind colour by visual inspection. Sensorial differences were only found for colour homogeneity and hardness ($p < 0.05$). It was observed that the commercial coated cheese had the lowest score for colour homogeneity and the cheese with the HD coating was classified as the most uniform. These results were consistent with the ΔE^* values obtained above (Figure 9.6) for colour measurement, which indicated that the commercial coating was the lightest and the HD coating the darkest at the end of the ripening period. In some applications, cheeses with darker coatings could benefit in terms of homogeneity, since smaller defects in the cheese surface may be masked, making them more attractive to consumers. The cheeses tested were classified as hard by the panellists, since they score this attribute with values higher than 4 on a 5-point scale (very hard). These results corroborate the behaviour of the cheeses according to the hardness measurements, which produced values of over 3000 g (Figure 9.4). The 45 days of ripening led to higher levels of dehydration in the cheeses (about 40%), indicating that the ripening period was too long for the small cheeses (120 g) used in this study. During ripening it was observed that the ideal texture for cheese occurs after 30 days.

With regard to the internal cheese evaluation, no statistically significant differences ($p > 0.05$) were found between the cheese samples in terms of the colour difference between paste and rind, odour, consistency and flavour. Nevertheless, it was observed that the cheese with the

commercial coating had the lowest classifications for the first three attributes. In fact, the lighter rind colour in the commercial cheese (Figure 9.5 and 9.6) led to a lower colour difference between the rind and paste. Cheese coated with edible WPC coatings had a more intense odour, probably due to the presence of the whey protein in the coating. However, this difference does not significantly influence the flavour of the cheese ($p > 0.05$). Ramos (2011) reported that cheeses coated with edible whey protein coating solutions had a bitter flavour and high astringency, but this was not observed in our study.

Finally, in terms of overall acceptability, the uncoated cheese was found the least acceptable by the panellists and the cheeses coated with edible coatings showed a statistically similar ($p > 0.05$) acceptability to the commercial coated cheese.

Table 9.4 Whole cheese and sliced cheese sensorial evaluation in a 5 point scale (average ± standard deviation) between cheese coated with antimicrobial whey protein edible coatings produced by HD, UV and HD+UV coating production methods compared with uncoated cheese and cheese with commercial coating, after 45 days of storage.

Sensorial test	Coating type				
Attributes	Uncoated	HD	UV	HD+UV	Commercial
Whole cheese					
shape	3.21±0.78 a	3.58±0.72 a	3.46±0.96 a	2.90±0.87 a	2.77±0.89 a
rind colour	2.94±0.59 a	3.43±0.81 a	3.46±0.68 a	2.85±0.40 a	3.82±0.69 a
colour homogeneity	3.30±0.85 ab	4.02±0.64 b	3.07±0.92 a	2.91±0.75 a	2.56±0.91 a
hardness	4.33±0.34 ab	4.13±0.43 ab	4.59±0.27 b	4.03±0.59 a	4.43±0.52 ab
Sliced cheese					
differences between paste and ring colour	3.75±0.83 a	3.44±0.70 a	3.47±0.66 a	3.30±0.80 a	2.96±0.82 a
odour	3.06±0.83 a	3.19±0.82 a	3.19±1.13 a	3.23±1.11 a	2.69±0.78 a
consistency	3.90±0.67 a	3.93±0.47 a	3.49±0.56 a	3.65±0.64 a	3.43±0.66 a
flavour	3.17±0.83 a	3.23±0.90 a	3.19±0.63 a	3.43±0.87 a	3.22±0.39 a
overall acceptability	2.60±0.88 a	2.88±0.93 ab	2.72±1.02 ab	3.30±0.89 ab	3.73±0.72 b

a, b means with different small letters in the same line differ significantly ($p < 0.05$) between coating types.

9.4 Conclusions

The distinct rheological behaviour of the whey protein coating solutions, as a result of the coating production methods applied, influences the coating adhesion on the cheese surface. More viscous coating solutions with better adhesion were produced when heat denaturation was applied. The coating production method had a significant influence on the performance of WPC-edible coating in terms of the physicochemical, microbiological and sensorial characteristics of cheeses.

The production of coatings only by heat denaturation does not improve cheese attributes. However, the application of UV irradiation enhanced the whey protein coating properties, especially in terms of protein cross-linking during coating formation. In this case, the germicidal effect of the UV-light was not so clear and more investigation is needed. The microbiological results proved that the antimicrobial edible coatings (with lactic acid and natamycin as antimicrobial agents) prevented the growth of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and moulds, demonstrating their ability to ensure the safety of cheese. The HD+UV coating proved to have a similar or better antimicrobial performance than the commercial coating. The sensory analysis correlated well with the physicochemical measurements for both colour and hardness. Although the commercial coated cheese was accepted best by the panellists at the end of the ripening period, it was not significantly different to the cheeses with the antimicrobial edible coating.

Antimicrobial edible coating solutions based on WPC proved a less expensive and suitable alternative to edible coatings based on WPI, as well as commercial coatings, since the cheese samples with either coating displayed similar ($p > 0.05$) values in terms of physicochemical, microbiological and sensorial properties, in particular the edible whey protein coating produced by heat denaturation and UV modification (HD+UV). UV treatment of WPC-based coatings may offer improved functionality and provide opportunities for extending the use of this technology in the food industry, and therefore warrants research attention.

References

Amefia AE, Abu-Ali JM and Barringer SA (2006) Improved functionality of food additives with electrostatic coating. Innovative Food Science and Emerging Technologies, 7, 176-181.

AOAC (1997a) Official Methods of Analysis of Association of Official Analytical Chemists. 16th ed. Volume II. Official Method 935.43, Chloride (Total) in Cheese.

AOAC (1997b) Official Methods of Analysis of Association of Official Analytical Chemists. 16th ed. Volume II. Official Method 920.124, Acidity in Cheese.

Baird-Parker AC (1969) The use of Baird-Parker's medium for the isolation and enumeration of *Staphylococcus aureus*. In: DA Shapton and GW Gould (Eds.) Isolation Methods for Bacteriologists, London, UK: Soc Applied Bacteriology Technical Series, No. 3, Academic Press, pp. 1-8.

Blatchley ER, Halaby TN, Dumoutier N, Laine JM and Levi Y (2001) Bacterial responses to ultraviolet irradiation. Water Science Technology, 43(10), 179-186.

Cagri A, Ustunol Z and Ryser ET (2002) Inhibition of three pathogens on bologna and summer sausages using antimicrobial edible films. Journal Food Science, 67, 2317-2324.

Cagri A, Ustunol Z and Ryser ET (2004) Antimicrobial edible films and coatings. Journal of Food Protection, 67, 833-848.

Cerqueira MA, Lima AM, Souza BWS, Teixeira JA, Moreira RA and Vicente AA (2009) Functional polysaccharides as edible coatings for cheese. Journal of Agricultural and Food Chemistry, 57, 1456–1462.

Dalgleish DG, Senaratne V and Francois S (1997) Interactions between alpha-Lactalbumin and beta-Lactoglobulin in the early stages of heat denaturation. Journal of Agricultural and Food Chemistry, 45, 3459-3464.

Franssen LR (2002) Antimicrobial properties and diffusion modeling of preservative-containing whey protein films and coatings on Cheddar cheese. Ph.D. Thesis, University of California, Davis.

Franssen LR, Rumsey TR and Krochta JM (2004) Whey protein film composition effects on potassium sorbate and natamycin diffusion. Journal of Food Science, 69, C347-C350.

Galietta G, di Gioia L, Guilbert S and Cuq B (1998) Mechanical and thermomechanical properties of films based on whey proteins as affected by plasticizer and crosslinking agents. Journal Dairy Science, 81, 3123-3130.

Hettiarachchy NS and Eswaranandam S (2005) Edible films and coatings from soybean and other protein sources. In F. Shahidi (Ed.), Bailey's Industrial Oil and Fat Products (6th ed.). Fayetteville: John Wiley & Sons, Inc.

Hettiarachchy NS and Eswaranandam S (2007) Organic acids incorporated edible antimicrobial films. US Patent No. US7160580B2.

Hill T and Lewicki P (2007) Statistics Methods and Applications. StatSoft, Tulsa, OK.

Hong SI and Krochta JM (2006) Oxygen barrier performance of whey-protein-coated plastic films as affected by temperature, relative humidity, base film and protein type. Journal of Food Engineering, 77, 739-745.

Hong YH and Creamer LK (2002) Changed protein structures of bovine β-lactoglobulin B and α-lactalbumin as a consequence of heat treatment. International Dairy Journal, 12, 345-359.

ISO 8968-1:2001 (2001). Milk-determination of nitrogen content Part 1: Kjeldahl method.

Kim T, Silva JL and Chen TC (2002) Effects of UV irradiation on selected pathogens in peptone water and on stainless steel and chicken meat. Journal of Food Protection, 65(7), 1142-1145.

Krochta JM (2002) Proteins as raw materials for films and coatings: definitions, current status, and opportunities. In: A Gennadios (Ed.), Protein-based film and coatings. USA, CRC Press LLC, ISBN1-58716-107-9.

Lacroix M, Le TC, Ouattara B, Yu H, Letendre M, Sabato SF, Mateescu MA and Patterson G (2002) Use of gamma-irradiation to produce films from whey, casein, and soya proteins: structure and functional characteristics. Radiation Physics and Chemistry, 63, 827-832.

Lee JY, Park HJ, Lee CY and Choi WY (2003) Extending shelf-life of minimally processed apples with edible coatings and antibrowning agents. Lebensmittel-Wissenschaft und-Technologie, 36, 323-329.

Lee SJ and Rosenberg M (1999) Preparation and properties of glutaraldehyde cross-linked whey protein-based microcapsules containing theophylline. Journal of Controlled Release, 61, 123-136.

Lee SY and Krochta JM (2002) Accelerated shelf-life testing of whey-protein coated peanuts analyzed by static headspace gas chromatography. Journal Agriculture Food Chemistry, 50, 2022-2028.

Mahmoud R and Savello PA (1992) Mechanical properties of and water vapor transferability through whey protein films. Journal Dairy Science, 75, 942-946.

Manab A, Sawitri ME, al Awwaly KU and Purnomo H (2011) Antimicrobial activity of whey protein based edible film incorporated with organic acids. African Journal of Food Science, 5, 6-11.

Mckibben, J and Krochta JM (2000) Properties of whey protein concentrate films of varying protein contents compared to whey protein isolate films. 78C-16, IFT Annual Meeting: IFT Food Expo; http://ift.confex.com/ift/2000/techprogram/paper_3608.htm

Mezgheni E, D'Aprano G and Lacroix M (1998) Formation of sterilized edible films based on caseinates: Effects of calcium and plasticizers. Journal of Agricultural and Food Chemistry, 46, 318-324.

Miles A, Misra SS and Irwin JO (1938) The estimation of the bactericidal power of the blood. Journal of Hygiene, 38, 732-749.

Miller KS and Krochta JM (1997) Oxygen and aroma barrier properties of edible films: A review. Trends in Food Science & Technology, 8, 228-237.

Min S and Krochta JM (2007) Ascorbic acid-containing whey protein film coatings for control of oxidation. Journal of Agricultural and Food Chemistry, 55, 2964-2969.

Min S, Harris J and Krochta JM (2005) Antimicrobial effects of lactoferrin, lysozyme and the lactoperoxidase system, and edible whey protein films incorporating the lactoperoxidase system against *Salmonella enterica* and *Escherichia coli* O157:H7. Journal of Food Science, 70(7), M332-M338.

Min S, Harris LJ and Krochta JM (2006) Inhibition of *Salmonella enterica* and *Escherichia coli* O157:H7 on roasted turkey by edible whey protein coatings incorporating lactoperoxidase system. Journal of Food Protection, 69, 784-793.

Min S, Rumsey TR and Krochta JM (2008) Diffusion of the antimicrobial lysozyme from a whey protein coating on smoked salmon. Journal of Food Engineering, 84, 39-47.

Neetoo H, Ye M and Chen H (2008) Potential antimicrobials to control *Listeria monocytogenes* in vacuum-packaged cold-smoked salmon pâté and fillets. International Journal of Food Microbiology, 123, 220-227.

NP 2105:1983 Queijos. Determinação do teor de matéria gorda. Técnica de Van Gulik. Processo corrente. Comissão Técnica - 32, 1ª edição, IPQ, Portugal.

NP 3544:1987 Queijos e queijos fundidos. Determinação do resíduo seco e do resíduo seco isento de matéria gorda. Comissão Técnica - 32, 1ª edição, IPQ, Portugal.

Olivas GI and Barbosa-Cánovas GV (2005) Edible coatings for fresh-cut fruits. CRC Critical Reviews Food Science, 45, 657-670.

Pérez-Gago MB and Krochta JM (2002) Formation and properties of whey protein films and coatings. In: A. Gennadios (Ed.), Protein-based films and coatings, Boca Raton FL, USA: CRC Press, pp. 159-180.

Pérez-Gago MB, Serra M and del Río MA (2006) Color change of fresh-cut apples coated with whey protein concentrate-based edible coatings. Postharvest Biology and Technology, 39, 84-92.

Pintado CM, Ferreira MASS and Sousa I (2009) Properties of whey protein-based films containing organic acids and nisin to control *Listeria monocytogenes*. Journal of Food Protection, 72(9), 1891-1896.

Puyol P, Perez MD and Horne DS (2001) Heat-induced gelation of whey protein isolates (WPI): effect of NaCl and protein concentration. Food Hydrocolloids, 15, 233-237.

Ramos OS (2011) Development and characterization of bioactive edible, whey protein films and coatings, to improve quality and safety of food products. PhD Thesis, Instituto de Tecnologia Química e Biológica – Universidade Nova de Lisboa, Lisboa, Portugal.

Ray B (2004) Fundamental Food Microbiology. 3rd ed., Boca Ratton FL, USA: CRC Press.

de Rooij RD, Potanin AA, van den Ende D and Mellema J (1993) Steady shear viscosity of weakly aggregated polystyrene latex dispersions. Journal of Chemistry and Physics, 99, 9213-9223.

Setlow RB and Carrier WL (1966) Pyrimidine dimers in ultraviolet-irradiated DNA's. Journal of Molecular Biology, 17(1), 237-254.

Sharifi-Yazdi MK and Darghahi H (2006) Inactivation of pathogenic bacteria ising pulsed UV-Light and its application in water disinfection and quality control. Acta Medica Iranica, 44(5), 305-308.

Singh TK, Drake MA and Cadwallader KR (2003) Flavor of Cheddar Cheese: A Chemical and Sensory Perspective. Comprehensive Reviews in Food Science and Food Safety, 2, 166-189.

Sousa MJ, Ardö Y and McSweeney PLH (2001) Advances in the study of proteolysis during cheese ripening. International Dairy Journal, 11, 327-345.

Stuchell YM and Krochta JM (1995) Edible coatings on frozen ding salmon: Effect of whey protein isolate and acetylated monoglycerides on moisture loss and lipid oxidation. Journal Food Science, 60, 28-31.

Thakur BR and Singh RK (1994) Food irradiation chemistry and applications. Food Reviews International, 10, 437-473.

Uprit L and Mishra HN (2003) Microwave convective drying and storage of soy-fortified paneer. Transactions of the American Institute of Chemical Engineers, 81, 89.

Vachon C, Yu HL, Yefsah R, Alain R, St-Gelais D and Lacroix M (2000) Mechanical and structural properties of milk protein edible films cross-linked by heating and gamma irradiation. Journal Agriculture and Food Chemistry, 48, 3202-3209.

Wang T, Macgregor SJ, Anderson JG and Woolsey GA (2005) Pulsed ultra-violet inactivation spectrum of Escherichia coli. Water Resources, 39(13), 2921-2925.

Zinoviadou KG, Koutsoumanis KP and Biliaderis CG (2009) Physico-chemical properties of whey protein isolate films containing oregano oil and their antimicrobial action against spoilage flora of fresh beef. Meat Science, 82, 338-345.

Part E.

Final remarks

Chapter 10

Conclusions

The main objective of the work described in this thesis was the valorisation of whey proteins and their use in conventional and nonconventional food systems. Firstly, whey protein products were produced and characterised, in order to investigate how process configuration and process conditions may affect their composition. Secondly, whey protein concentrates were tested as a means of partially or totally replacing conventional food ingredients in the manufacture of food products or in the production of edible films and coatings with improved properties.

Using batch ultrafiltration or diafiltration it is possible to solve the environmental problem posed by whey disposal, whilst also contributing towards increasing the overall process yield, through the production of liquid or dry whey protein concentrates (WPC). Two modes of diafiltration were applied, although diafiltration in sequential dilution mode (DFsdm) does not improve the protein composition in comparison to conventional ultrafiltration. The increase in process complexity and material requirements introduced by diafiltration is only justified in the case of diafiltration in volume reduction mode (DFvrm), since it is possible to achieve more purified products without lower Mw compounds. It was observed that thermal behaviour is heavily dependent on sample preparation and the amount of impurities. The decomposition temperature for all the freeze-dried powders was in the range of 150-400 ºC, indicating their high thermal stability.

Incorporating liquid whey protein concentrates (LWPC) into dairy products can be a very interesting procedure, not only in terms of the overall process yield but also the functional properties of the products. Fresh cheese with LWPCs showed lower spontaneous syneresis and

higher stability over time. In medium fat yogurts the use of LWPC did not produce any significant differences in colour (L*, a*) and texture parameters, either over time or between formulations. However, increasing LWPC in full-fat yogurts reduced hardness and gumminess. In both types of yogurts, syneresis increased during storage and decreased with the incorporation of LWPC, presenting very low values for full-fat yogurts. Viscosity was improved with the addition of LWPCs, especially in full-fat products. The amount of LWPC incorporated into fresh cheese formulations is a very important parameter in terms of sensorial evaluation, as it may penalise their acceptability. In the case of yogurts, no sensorial differences were detected between the conventional and the tested products. The lower production costs and less complex operations involved in the manufacture of LWPCs, as well as their functional properties in the food products tested, allow for its implementation in small to medium-sized dairy production plants. The procedure avoids or reduces the acquisition of additional conventional dry products such as skimmed/whole milk powder or WPC, traditionally used in the manufacture of these dairy products. It was also concluded that partial substitution of conventional SMP by ovine LWPC in set yogurts does not produce any significant differences in comparison to the use of bovine LWPC, either on a physiochemical and textural or a sensorial level. These findings prove that speciality ovine cheese producers can apply the same technologies to the valorisation of ovine whey with the same final results with regard to functional properties. Further work envisages optimising the LWPC denaturation step, as well improving the sensory properties of ovine milk yogurts, which were penalised in the sensorial evaluation. The use of LWPC as a raw material in the manufacture of innovative products proved to be an attractive opportunity. Its high protein content and well-established functional and nutritional values are good indicators of high performance. The high protein concentration and lower pH values lead to stronger thermal gels. However, LWPC produced by diafiltration, with a lower protein concentration (5%) at pH 7, did not produce solid structures, probably due to the lower intramolecular interactions occurring under these conditions. The acid-induced gels produced by lactic bacteria were weaker than the ones chemically acidified by GDL. Cold storage and the fortification of acid gels with SMP improved the rheological properties. It was concluded that, depending on the gelation process and manufacturing conditions, specific products can be produced in terms of chemical and rheological properties, which can then be selected according to the desired food application.

Whey protein films and coatings are attractive alternatives to petroleum-based packaging materials, given that they solve both the whey disposal and the packaging waste processing

problems due to their biodegradability and edibility. They are considered excellent oxygen and oil barrier films with very attractive visual properties. Their hydrophilic nature is responsible for the poor moisture barrier properties, although the use of some specific compounds may improve this, as well as their mechanical properties. WPI is the raw material that has been used in most of the research into whey protein-based films and coatings. The use of WPC, as a less expensive alternative, is even more economically attractive in terms of its use in packaging applications. The study developed on this topic shows that WPC-based films with modest levels of proteins (≈50%) and relatively high levels of lactose (≈42%) have acceptable film functionality. Film thickness, solubility, moisture content, WVP and optical properties (transparency and ΔE*) can be modified as a function of the polymerization technology used for film production and the chemical composition of the film forming solution (WPC, glycerol and photoinitiator contents). It was observed that the use of WPC instead of WPI significantly reduced the amount of glycerol needed in the film formulation. However, the application of heat denaturation (the conventional solvent casting method) implies a higher level of plasticizer then the UV modification method. WPC film transparency was similar to that of some synthetic films available on the market (OPP and PE), but a slightly yellow colour developed as a result of the higher amount of lactose in the film formulation. Heat denaturation allowed for the production of partially insoluble films, whereas films produced by UV modification showed greater solubility, which may limit their application in higher moisture environments. The film moisture content and water vapour permeability were well correlated to the film chemical composition with a second order model for all the WPC films. It was concluded that a lower film moisture content and WVP can be obtained by using UV modification, since this technique implies the addition of lower levels of glycerol. However, it was also proved that with the appropriate adjustments in formulation, it is possible to produce WPC films with similar properties from different methodologies. The thermal analysis revealed that WPC films produced by heat denaturation presented greater thermal stability and compatibility between film components, which was not improved by the used of UV irradiation. Moreover, FTIR analysis revealed UV irradiation was efficient in inducing cross-links in edible protein films although the protein molecular structure and interaction with glycerol was negatively affected. No modifications were observed in the mechanical properties of WPC films by the application of the various polymerization methods. Although the elongation of the films produced was similar to synthetic film, their lower tensile strength and Young's modulus restrict the range of applications. WPC films produced by UV polymerization had a

lower critical surface energy, which revealed their higher hydrophobic nature, in comparison with films produced by the conventional solvent casting method. These results indicate that UV treatment can be a very attractive method for reducing WVP in whey protein based films. At present the cost of WPI limits its application as a food coating. However, this study also proved that the lower cost of WPCs may enhance their economic feasibility as coatings, given their suitable film properties.

Increasing the shelf life of products by improving food safety is probably the most promising application for whey protein films and coatings as bioactive packaging. However, the great challenge involved in applying this technology is the development of reliable polymerization methods that can be implemented on an industrial scale. The distinct rheological behaviour of the whey protein coating solutions proved to be a decisive factor in the coating's adhesion to the cheese surface, as a result of the protein polymerization method applied. It was observed that the more viscous coating solutions produced by heat denaturation provided better adhesion. The physicochemical, microbiological and sensorial characteristics of the cheeses depended greatly on the characteristics of the WPC-coating resulting from the polymerization method used. The use of heat denaturation only to produce coatings did not improve the attributes of the cheese. However, the application of UV-irradiation enhanced the properties of whey protein coatings, especially in terms of protein cross-linking during coating formation. The microbiological results proved that the antimicrobial edible coatings (with lactic acid and natamycin as antimicrobial agents) prevented the growth of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and moulds, thus demonstrating their ability to ensure the safety of cheese. The coating produced by heat denaturation and UV-irradiation (HD+UV) offered a similar or better antimicrobial performance than the commercial coating. The cheese with a commercial PVA coating was best accepted by the panellists at the end of the ripening period, but no significant differences were found in comparison to cheese with antimicrobial edible coatings. WPC showed that it can be used as a less expensive and suitable alternative to edible coatings based on WPI, as well as commercial coatings, since the cheese samples with either coatings displayed similar ($p > 0.05$) values in terms of their physicochemical, microbiological and sensorial properties, in particular for the edible whey protein coating produced by heat denaturation and UV polymerization (HD+UV). UV treatment of WPC-based coatings may offer improved functionality and provide opportunities for extending the use of this technology in the food industry.

Chapter 11

Suggestions for future research

The studies pursued in this PhD thesis are innovative with regard to:

- the direct incorporation of liquid whey protein concentrates (LWPC) into foods or their use in the production of innovative dairy products;
- the production of whey protein films and coatings using WPC instead of WPI, applying UV irradiation as a modification technique.

However, opportunities for further research in whey proteins exist, including the optimisation of the production process and storage conditions for LWPC and studying the incorporation of LWPC in non-dairy food products, such as bakery products, as a gluten substitute in the production of gluten-free foods. With regard to whey protein based films and coatings, there is a need for additional research into upscaling to an industrial level, and efficient drying techniques that do not damage the surface of the film or compromise food safety. Improvements to the physical properties of films and coatings through the inclusion of cross-linkers, surfactants, or emulsifiers, as well as to their bioactive properties through the use of nutraceuticals, antioxidants, flavours, colorants and antimicrobials, still need to be developed and confirmed.

If the major goal is to fully valorise whey components, opportunities for further research also exist. Figure 11.1 shows that this thesis has only explored solutions concerning the valorisation of whey protein fractions, although large volumes of the permeate, a lactose-rich stream, are also

produced during whey processing. The permeate still has an extremely high pollution load, since it retains lactose, which represents more than 70% of the total whey solids. Some researchers have proposed producing bioethanol by fermenting the lactose from the whey permeate. However, this solution involves high investment and maintenance costs, which can only be resolved by large scale production. Nevertheless, other aspects of fermentation can be explored in small scale production, such as the production of probiotics or fermented drinks. In Figure 11.1, kefir fermentation is proposed for the ultrafiltration permeate, after concentration by reverse osmosis. This technology allows for the closure of the entire whey valorisation cycle, leaving water as the only effluent.

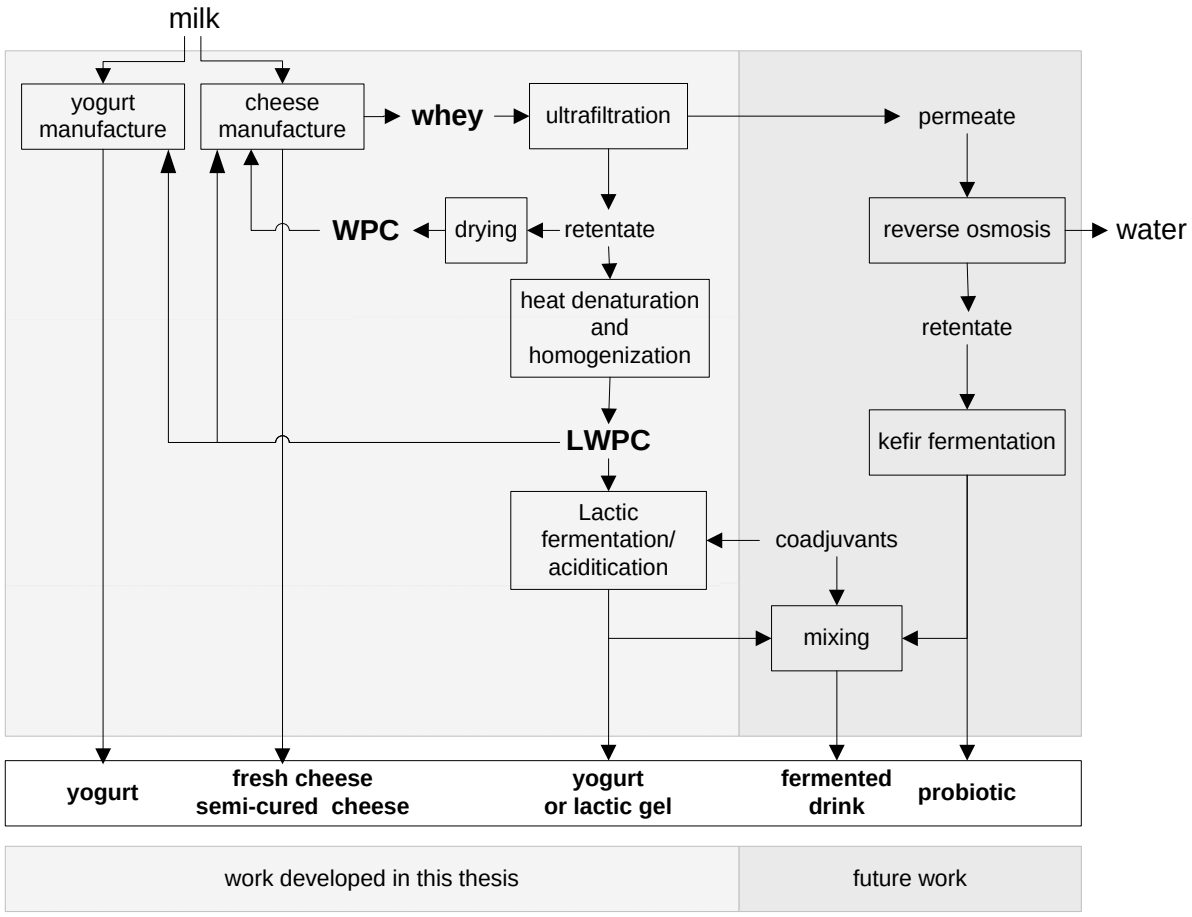


Figure 11.1 Total valorisation of whey in small and medium-sized cheese factories. Work developed in this PhD thesis and suggestions for future research.

Appendix

Analytical methods

A

Chemical analyses of whey and liquid whey protein concentrate

A.1 Dry matter

The dry matter was evaluated by oven drying the samples at 105 ºC for 12 hours, until a constant weight was obtained (AOAC, 1980a). This method involves assessing the water loss in a 10 g sample (weighed in an analytical balance). The dry matter content is calculated using the equation (A1).

$$(\%)\ DM = 100 - \frac{(m_i - m_d)}{(m_a)} \times 100 \quad \text{(A1)}$$

where: m_i – mass of the initial sample + crucible (g)
m_d – mass of desiccated sample + crucible (g)
m_a – mass of the initial sample (g)

A.2 Ash

The ash content was determined by the calcination of previously desiccated samples in a muffle at 550 ºC for 6 hours, in accordance with the standard method (AOAC, 1995). The percentage of ash on a wet basis is calculated as:

$$(\%)\ Ash = \frac{m_c - m_0}{(m_a)} \times 100 \quad \text{(A2)}$$

where: m_c – mass of ashes + crucible (g)
m_0 – mass of crucible (g)
m_a – mass of initial sample (g)

A.3 Protein

The Kjeldahl method (ISO 8968-1:2001) was used to evaluate the total nitrogen content, using distillation apparatus (Kjeltec System 1002 Tecator). The digestion of samples with sulphuric acid in the presence of a catalyst leads to the total destruction of organic matter and the complete conversion of nitrogen into ammonium sulphate ($(NH_4)_2SO_4$).

Approximately 50 mL of liquid samples (weighed analytically) were placed in a digestion tube. Two catalyst tablets (3.5 g de K_2SO_4 + 0.4 g de $CuSO_4$) and 25 mL of concentrated sulphuric acid (95-97%, Sigma-Aldrich) were added. The digestion tubes were then placed in the digestion unit where they remained for approximately 2 hours at 450 ºC. Under these conditions complete digestion was achieved and the samples were totally clarified.

After digestion, 50 mL of distillate water and 50 mL of NaOH (40% (w/v), Eka Chemicals) were added to each test tube and the resulting solution distilled. The ammonium formed (NH_3) was distilled using heating vapour and recovered in a boric acid solution (50 mL of (H_3BO_3) at 4% (w/v), Fisher Chemical) until a final volume of 250 mL was obtained. This volume was titrated with HCl (0.1 N, Panreac) in the presence of the indicator Tashiro (mixture of methyl red - methylene blue). Equation (A3) enables the percentage of total nitrogen in each sample to be determined.

$$(\%)TN = \frac{(14.007 \times N \times (V - V_0))}{m_a} \times 100 \quad \text{(A3)}$$

where: V - volume of HCl solution used in sample titration (L)
V_0 - volume of HCl solution used in blank titration (L)
N - normality of the HCl solution (N)
m_a – mass of the initial sample (g)

The total protein content was obtained by multiplying the percentage of total nitrogen content (%TN) with a factor of 6.38.

A.4 Lactose

The lactose content was determined gravimetrically using the procedure described by Munson and Walker (AOAC, 1980b).

Gravimetrical method

The basis of this method was the reducing capacity of lactose in the presence of Felling Liquor.

25 mL of the liquid sample (previously weighed) were placed in a 250 mL volumetric flask with 100 mL of distillate water. After homogenisation of the solution a 2.5 mL potassium ferricyanide solution ($K_3(Fe(CN)_6$) at 15% (w/v), May & Baker Ltd), 2.5 mL zinc sulphate solution (($ZnSO_4$) at 30% (w/v), Merck) and distillate water were added to fill the vessel. The final solution was then filtered.

A 25 mL copper(II) sulphate pentahydrate solution (($CuSO_4.5H_2O$, Riedel deHaën) at 7% (w/v), Riedel-de Haën) was put in a precipitation vessel. A 25 mL tartaric/alkali solution (350 g Seignette Salt + 100 g of NaOH in one litre of distillate water) and 50 mL of the filtrate obtained in the previous step were added to the copper(II) sulphate pentahydrate solution. The vessel was heated to enable the solution to reach boiling point after 4 min and kept boiling for 2 min. The precipitate was left to settle and the remaining liquid (still warm) was filtrated using a crucible filter (5-8 μm) with a vacuum pump. The glass was washed with tepid distillate water, and the cuprous oxide precipitate obtained with 10 mL of absolute ethanol ((CH_3CH_2OH) at 99.5%, Panreac) and 10 mL of diethyl ether ($C_4H_{10}O$, Fisher Chemicals).

The crucible filter was weighed after drying in an oven at 102 ± 2 ºC for 30 min, and the mass of cuprous oxide was determined.

The percentage of hydrated lactose was calculated using equation (A4), considering that the mass of lactose (m_1) corresponds to the mass of cuprous oxide (Cu_2O) using the table in Annex I.

$$(\%)\,Lactose = f \times \frac{m_1}{m_0} \times 100 \quad \text{(A4)}$$

where: f - dilution factor
m_1 - mass of lactose (mg)
m_0 - mass of sample (mg)

A.5 Fat

Fat was determined using the Gerber method (NP 469:2002), widely used to quantify fat in dairy products. Since the fat present in the sample is not selectively attacked by sulphuric acid (H_2SO_4) as the organic matter, it is possible to separate it by centrifugation in the presence of isoamyl alcohol ($(CH_3)_2CHCH_2CH_2OH$), Merck), which is responsible for modifying the surface tension. In this method it is essential to ensure the correct temperature of the analysis. The following were placed in succession in the Gerber butyrometer: 10 mL of concentrate sulphuric acid, 11 mL of the sample and 1 mL of isoamyl alcohol. The butyrometer was sealed with the rubber stopper and shaken until the caseine residues were complete homogenised. The butyrometer was centrifuged in the Gerber centrifuge for 10 min at 65 °C. The fat percentage was read directly from the butyrometer scale.

A.6 Titratable acidity

Titratable acidity can be defined as the amount of acids in the sample that reacts with an alkali of a known concentration. In derived dairy products, titratable acidity (TA) can be determined using the equation (A5) in ºThorner[1].

$$(^{o}\text{Thorner})\; TA = \frac{V_{NaOH}}{0.1} \quad \text{(A5)}$$

where: V_{NaOH} - volume of the sodium hydroxide solution used in titration (mL)

The method involves the titration of 10 mL of the sample with NaOH (0.1 N) in the presence of a phenolphthalein indicator (NP 470:1983).

A.7 Calcium

The Ca^{2+} content was determined in accordance with the official AOAC (2005) method, using atomic absorption spectrometry (Perkin. Elmer Analyst 300).

[1] ºThorner indicates the amount, in milliliters (0.1 mL), of a sodium hydroxide solution (0.1 N) needed to neutralize 10 mL of milk.

An initial concentration step by evaporation was performed on the liquid samples, using a water bath. After concentration, sample calcination was carried out to ensure preservation of the mineral content. The ashes obtained had to be dissolved in 10 mL of nitric acid (HNO_3, Panreac) and evaporated in a vapour bath. The residue was filtered into a 100 mL volumetric flask and topped up with distillate water. Prepare a following solution using 50 mL of the previous solution, 10 mL of lantanium solution[2] ($LaCl_3$) and distillate water up to the 100 mL volumetric flash capacity.

Sample absorbance was read at 422.7 nm. The calcium concentration in the solutions tested was calculated directly by the spectrometer using the calibration curve previously determined with standard calcium carbonate solutions ($CaCO_3$, 0-5 ppm), and entered into the spectrometer software. The calculations were made in duplicate. The calcium content in the sample was calculated using the equation A6.

$$(\%)\,Calcium = f \times \frac{m_1}{m_0} \times 100 \qquad \text{(A6)}$$

where: f - dilution factor
m_1 - calcium concentration (mg/100 mL)
m_0 - sample weight (mg)

A.8 pH

The pH of the whey and intermediate liquid samples (LWPC) was evaluated in triplicate using a PHM 61 potentiometer immediately after their production.

References

AOAC (1980a) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 16.032. Total Solids, Method I - Official Final Action, 13th ed., Washington DC, USA.

AOAC (1980b) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 16.057. Lactose, Gravimetric Method - Official Final Action. 13th ed., Washington DC, USA.

AOAC (1995) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 930.30. Ash of dried milk, 16th ed., Gaithersburg, MD, USA.

[2] Added to the sample in order to control interference by other metals.

AOAC (2005) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 991.25. Calcium, magnesium and phosphorus in cheese - AAS method. 16th ed., Gaithersburg, MD, USA.

ISO 8968-1:2001 Milk-determination of nitrogen content Part 1: Kjeldahl method.

NP 469:2002 Norma Portuguesa. Leites. Determinação da matéria gorda (técnica de Gerber). 3ª ed., Lisboa, Portugal.

NP 470:1983 Norma Portuguesa. Leites. Determinação da acidez. Lisboa, Portugal.

B

Chemical analyses of whey protein concentrate and dairy powders

B.1 Dry matter

The method described in Appendix A (section A.1) was used to quantify the dry matter content of freeze dried products. In this case, the sample amount was about 1 g.

B.2 Ash

The ash content of freeze dried products was quantified in accordance with the method described in Appendix A (section A.2). The results of these samples were expressed on a dry basis.

$$(\%)\,Ash = \frac{m_c - m_0}{(m_d)} \times 100 \qquad \text{(B1)}$$

where: m_c - mass of the ashes + crucible (g)
m_0 - mass of the crucible (g)
m_d – mass of the desiccated sample (g)

B.3 Protein

The protein content of the freeze dried samples was determined using the Kjeldahl method (ISO 8968-1:2001) described in Appendix A (section A.3), although in this case the sample had to be approximately 1 g (weighed analytically).

Non-protein nitrogen compounds and true protein determination

Non-protein nitrogen is defined as the soluble nitrogen in 12% (w/v) trichloroacetic acid (CCl_3COOH). The nitrogen content was also determined using the Kjeldahl method.

During preparation of the sample, the proteins were separated by precipitation. Six grams of freeze dried samples were dissolved in 100 mL of distillate water and an equal volume of trichloroacetic acid (24% (w/v), Merck), leading to a final acid concentration of 12% (w/v). Protein denaturation and precipitation occurs in these conditions. The solution was then filtered with a vacuum pump in order to remove the precipitated proteins. It was possible to quantify nitrogen of non-protein origin by applying the Kjeldahl method to the filtrate solution (Rizvi and Josephson, 1974).

The non-protein nitrogen compounds were quantified by multiplying the non-protein nitrogen by a factor of 3.60. In the case of true protein determination, the equation (B2) was applied.

$$(\%)\,\text{True Protein} = (\%TN - \%NPN) \times 6.45 \qquad \text{(B2)}$$

where: $\%TN$ – percentage of total nitrogen in the sample
$\%NPN$ – percentage of the non-protein nitrogen

The conversion factors recommended by Karma and Van Boekel (1986) are 3.60 and 6.45 for the non-protein nitrogen compounds and sweet whey proteins respectively. The results are expressed as a relative percentage of the dry weight.

Protein compositional analyses

The content of α-lactalbumin, β-lactoglobulin, serum albumin (SA) and Imunoglobulins (IgG) was determined by FPLC (Fast Protein Liquid Chromatography) (Amersham Biosciences). In this case, analytical grade reagents were used. Protein standards were purchased from Sigma Chemical (St. Louis, USA). Samples of dry products were dissolved in ultrapure water (10 mg/mL) and filtrated with a 0.22 μm filter. Several concentrations of each standard protein were prepared using the same procedure for the construction of standard curves. All the chromatography was performed with a gel filtration Superose 12 HR 10/30 (Amersham Biosciences) column. Proteins were chromatographed at room temperature at a flow-rate of 0.4 mL/min. The mobile phase was a NaCl solution (0.15 M) in a sodium phosphate 100 mM (pH 7.0) buffer with 0.2 g/L of NaN_3. Detection was determined at 280 nm in a UV-MII detector. The sample volume injected into the column was 0.2 mL and the total running time was 80 min. Quantification was based on peak areas of whey proteins and external standards for each protein. All determinations were made in triplicate.

B.4 Fat

The fat content of freeze dried products was evaluated gravimetrically after extraction by the Rose-Gottlieb (ISO 1736:2000) and Soxhlet methods (AOAC, 1990).

Rose-Gottlieb method

In the Rose-Gottlieb method (ISO 1736:2000), two grams of the sample were dissolved in distillate water (100 mL). 10 mL of the solution obtained was transferred to an extraction tube and 10 mL of ethanol (99.5%, Panreac) was added. The mixture was shaken thoroughly for one minute. 25 mL of petroleum ether was then added and shaken vigorously for about half a minute. After the mixture had settled, the upper ethereal layer was clear and completely separated. The clear layer was decanted into a suitable vessel (flask, glass bowl, aluminium dish, etc.) that had been weighed previously. The remaining liquid in the extraction tube was extracted twice using 15 mL of the solvent each time. The ethereal extract was added to the same container and completely evaporated at reduced pressure in a rotative evaporator (Heidolph, Model HBR2-

51703). The flask was dried in an oven at 102±2 ºC for two hours, cooled in a desiccator and weighed. The fat content can be calculated using equation (B3).

Soxhlet method

With the Soxhlet method (AOAC, 1990), 2 g of the sample was placed in a cylindrical moulded filter paper and inserted into the internal tube of the Soxhlet extractor apparatus. The fat present in the sample had to be extracted continuously with petroleum ether for 20 hours. Following extraction, the ether was recovered and completely evaporated, as described for the Rose-Gottlieb method. The residue was dried in an oven for 2 hours, cooled and weighed. The calculation of the percentage of fat in the sample is:

$$(\%)\ Fat = \frac{m_1 - m_0}{(m_a)} \times 100 \qquad \text{(B3)}$$

where: m_1 – mass of the flask + fat (g)
m_0 – mass of the flask (g)
m_a – mass of initial sample in dry basis (g)

B.5 Lactose

The lactose content of WPC powders was evaluated gravimetrically using the AOAC (1980) method described in Appendix A (section A.4), and the colorimetric method proposed by Marrier and Boulet (1959) applied to milk and whey products.

Gravimetric method

To use the gravimetric method for the evaluation of the lactose content of this kind of sample two grams were needed. The weighed sample was dissolved in distillate water in a 100 mL volumetric flask. In each analysis, 25 mL of the mother solution was required, using the procedure described in section A.4. Although this method for lactose determination is easily reproducible, it involves many consecutive steps and is time consuming. As an alternative, a colorimetric method was used, namely the phenol-sulphuric acid method (Marrier and Boulet, 1959), which was easier to execute and produced results in a few minutes.

Colorimetric method

0.10-0.15 g of the freeze dried sample was weighed and 1 L of aqueous solution was prepared in a volumetric flask. The dissolution had to be complete. 2 mL of the previous solution was placed in a test tube and 100 μL of an aqueous phenolic solution ((C_6H_5OH) at 89% (w/v), Merck) was

added. After shaking, 6 mL of concentrated sulphuric acid was added. According to the authors' recommendations 11 mg of phenol plus 0.74 mL of acid should be present for each mL of the final solution. After further homogenisation in a vortex, the solution was left to rest for 10 min at ambient temperature. The absorbance was read at 490 nm against a blank with distillate water that had been subjected to the same procedure. The lactose content was determined using the calibration curve previously calculated for standard monohydrated lactose solutions in a range of 10-70 mg/L. The calibration equation (B4) achieved a correlation coefficient of 99.4%.

$$^{1}\,(g/L)\ Lactose = 0.094 A_{490} - 0.0018 \quad \text{(B4)}$$

where: A_{490} - sample absorbance at 490 nm

B.6 Calcium

Calcium content determination as done has described in appendix A (section A.7) and expressed in dry basis.

B.7 Phosphorous

A spectrophotometric method adapted from the AOAC (1996) method was used to evaluate the phosphorous content. The ashes obtained by incineration of the samples were dissolved in 10 mL of nitric acid (65%, Panreac) and heated for 30 min in a steam bath. The residue was filtrated and transferred to a 100 mL volumetric bowl filled with distillate water. 30 mL of the colorimetric reagent molibdovanadate (2.5 g of ammonium monovanadate, 20 mL of nitric acid (22% (w/v)) and 50 g of ammonium heptamolybdate in one litre of water) were added to 20 mL of the previous solution. The mixture was left to rest for 15 min for the colour to develop. The absorbance was then read at 400 nm on a Hitachi U-2000 spectrophotometer.

The phosphorous concentration was determined using the calibration curve (B5) previously obtained with a standard solution of phosphoric acid (H_3PO_4). The results were expressed in relation to the dry basis.

$$^{2}\,(\mu g/mL)\ PO_4^{2-} = 55.452 A_{400} - 0.468 \quad \text{(B5)}$$

where: A_{400} - absorbance of the test solution read at 400 nm

[1] (g/L) represents the amount of lactose (g) in the volume of the solution (L).
[2] (mg/mL) represents the amount of phosphorous (mg) in the volume of the solution (mL).

References

AOAC (1980) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 16.057. Lactose, Gravimetric Method - Official Final Action. 13th ed., Washington DC, USA.

AOAC (1990) Official Methods of Analysis of the Association of Official Analytical Chemists, Method 963.15. Fat in cacao products, Soxhlet extraction method, 15th ed., Arlington, VA.

AOAC (1996) Official Methods of Analysis of the Association of Official Analytical Chemists, Phosphorus in dried milk: Colorimetric procedure, In P. Cunliffe (Ed.). Official Methods of Analysis. 16th ed., Vol. II, Maryland, USA.

ISO 1736:2000 Dried milk and dried milk products-determination of fat content: Gravimetric method. <http://jds.fass.org/cgi/reprint/88/8/2689.pdf>

ISO 8968-1:2001 Milk-determination of nitrogen content Part 1: Kjeldahl method.

Karman AH and Boekel MAJS (1986) Evaluation of the Kjeldahl factor for conversion of the nitrogen content of milk and milk products to protein content. Netherlands Milk and Dairy Journal, 40, 315-336.

Marrier JR and Boulet M (1959) Direct Analysis of Lactose in milk and serum. Journal of Dairy Science, 42(8), 1390-1391.

Rizvi SH and Josephson RV (1974) Determinations of nonprotein content of cheese whey. Journal of Dairy Science, 58(10), 1521-1523.