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USE OF MICROPARTICULATED WHEY PROTEIN (MWP) IN THE ITALIAN DAIRY INDUSTRY

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LIST OF ABBREVIATIONS

1-VR	Coefficient of determination of cross validation
a_{30}	Curd firmness 30 minutes after rennet addition
aa	Amino acids
ACN	Acetonitrile
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
CL	CoAgulite
CMP	Caseinomacropeptide
CN	Casein
CV	Coefficient of variation
Cys	Cysteine
d	days
D	Detrending
DTT	Dithiothreitol
FT	Fourier transform
GndHCl	Guanidine hydrocloride
GSH	Glutathione
HSI	Hyperspectral Image
k_{20}	Curd firming time
LF	Lactoferrin
LSM	Least square means
MCP	Milk coagulation properties
MF	Microfiltration
MIRS	Mid infrared spectroscopy
MWP	Microparticulated whey protein
NF	Nanofiltration
NIRS	Near infrared spectroscopy
PFR	Protein-to-fat ratio
PLS	Partial least square
PP	Proteose peptone
R^2	Coefficient of determination
RCT	Rennet coagulation time
RMSE	Root Mean square error
RO	Reverse osmosis
RPD	Ratio performance deviation
RP-HPLC	Reversed phase high pressure liquid chromatography
RSD	Relative standard deviation
SD	Standard deviation
SEC	Standard error calibration
SE_{CV}	Standard error cross validation
SNV	Standard normal variate
TFA	Trifluoroacetic acid
UF	Ultrafiltration
WP	Whey proteins

WPC	WP concentrate
WPI	WP isolate
α-LA	Alpha-Lactoalbumin
β-LG	Beta-Lactoblobulins

ABSTRACT

ABSTRACT

The thesis is composed of four contributes, dealing with different approaches developed to understand the different aspects of whey proteins (WP) recovery, and their use across cheese manufacturing. The general aim of the research project was to investigate and propose strategies for the utilization of whey and whey products in cheese produced by an Italian dairy industry. The Soligo dairy cooperative (Soligo, Treviso, Italy) was partner and supported the project.

In this scenario, the objectives of the first contributes was to develop a gold-method for WP quantification in whey, by Reversed Phase -HPLC, and to evaluate the potential of mid infrared spectroscopy (MIRS) in WP prediction. Whey proteins included α-Lactoalbumin (α-LA), β-Lactoglobulin A and B (β-LG), bovine serum albumin, caseinomacropeptides, proteose peptone and total WP identified. Repeatability and reproducibility tests, in validation procedures, were performing by calculating the relative standard deviation (RSD) within and across days for retention times and peak areas. Samples of whey (n = 187) were analysed according to the reference methods and MIRS spectra were stored (900 - 4000 cm^{-1}); statistical analysis was carried out through partial least squares regression and random cross-validation procedure. Retention times were stable, with RSD ranging between 0.03% and 0.80%. The RSD of peak area in repeatability and reproducibility tests ranged from 0.25% to 8.48% depending on the considered proteins and their relative abundance; indeed, better coefficients of determination in validation were obtained for fractions present in whey in large amounts, as β-LG (0.58), total identified WP (0.58), α-LA (0.56), while minor WP were predicted with minor appreciable accuracy. Results from this study propose a high-throughput and high-resolution method for WP quantification in whey and show the potential of MIRS for their prediction.

The objective of the second contribute was to investigate the effect of increasing concentrations of microparticulated whey proteins (MWP; from 0.0 to 9.0%, vol/vol) on milk coagulation properties (MCP), namely rennet coagulation time (RCT), curd-firming time, and curd firmness 30 min after rennet addition (a_{30}). Three bulk milk samples, collected and analyzed during 3 days, were added with 6 concentrations of MWP (vol/vol): 1.5%, 3.0%, 4.5%, 6.0%, 7.5%, and 9.0%. Moreover, a sample without MWP was used as control. Milk coagulation properties were measured using Formagraph (Foss Electric A/S, Hillerød, Denmark). The increment of the amount of MWP added to milk led to longer RCT. In particular, significant differences were found between RCT of the control samples (13.5 min) and RCT of samples added with 3.0% (14.6 min) or more of MWP. Similar trend was observed for curd-firming time, which showed the shortest time in the control samples and the

longest in samples with 9.0% (21.4 min) of MWP. No significant differences were detected for a_{30} across concentrations of MWP. Adjustments in cheese processing should be made when recycling MWP, in particular during coagulation process by prolonging the time of rennet activity before cutting of the curd.

Aim of the third study was to evaluate the effect of MWP, using standardized milk with different protein-to-fat ratios (PFR; high, standard and low levels of fat) and increasing MWP concentrations (from 0.0 to 4.0 %, vol/vol) on milk coagulation process, cheese yield and composition of 30 cheese samples carried out through a mini cheese-making technique. The increment of PFR affected RCT. Moreover, cheese yield decreased as the level of fat decreased, and it was higher in low-fat cheese (high PFR) with 4.0% MWP compared with low-fat cheese with 3.0% MWP. No differences were found for cheese yield in standard and high fat cheese (standard and low PFR) across MWP concentrations. The stable composition of low-fat Caciotta suggests the possibility to include MWP as fat replacer to maintain the yield.

The fourth contribute aimed at the investigating the effectiveness of Hyper Spectral Image (HSI) technique to detect MWP in low-fat Caciotta cheese, produced with increased concentration of MWP (2.0%, 3.0%, 4.0% vol/vol). Hyperspectral image is an emerging technology successfully employed in food inspection, by combining the advantages of conventional digital image and spectroscopy to obtain both spatial and spectral information from an object. Twelve mini-cheese making were performed using standardized milk in low fat condition (3.5% of protein and fat). Protein levels were adjusted with 2.0%, 3.0% or 4.0% MWP vol/vol. For each day of cheese making a control thesis without MWP was performed (0.0% MWP). After one month of ripening a slice of each cheese was analysed for the acquisition of near infrared image in range wavelengths from 1,100 to 1,600 nm, for a total of 140 wavelengths measured. Several spatial and spectral pre-processing were tested: two times spatial binning, and standard normal variate plus second derivate were select as optimal. Principal component analysis reported an explained variability of 7% across treatments. Cluster analysis evidenced an increment in component presence by increasing MWP percentage in treatments. Moreover, a score plot reported a destine classification of samples contains MWP and control without. The results confirm the ability of HIS in MWP detection, and this information can be used to construct further classification models able to discriminate cheese adulteration for MWP addition.

RIASSUNTO

L'obiettivo generale di questa tesi di dottorato, composta da quattro contributi sperimentali, è stato la valorizzazione del siero derivante dalla caseificazione e l'utilizzo delle sieroproteine (WP) nel processo di caseificazione di formaggi freschi.

Il caseificio di Soligo (Soligo, Treviso, Italy) è stato partner del progetto e ha supportato le attività sperimentali fornendo tutte le materie prime.

In questo scenario, l'obiettivo del primo contributo è stato lo sviluppo di un metodo HPLC a fase inversa (RP - HPLC) per la quantificazione di WP, e la possibilità di poter utilizzare tale metodo come riferimento per la predizione di WP utilizzando la spettroscopia del medio infrarosso (MIRS). Le WP predette sono state: α-Lattoalbumina (α-LA), β-Lattoglobulina A and B (β-LG), albumina di siero bovino, caseinomacropeptides, proteoso peptone e WP totali identificate. I test di ripetibilità e riproducibilità, per la validazione del metodo RP-HPLC, sono stati calcolati come deviazione standard relativa (RSD) entro e tra giorni, per il tempo di ritenzione e l'area dei picchi. Per lo sviluppo dei modelli di calibrazione MIRS i campioni di siero (n = 187) sono stati analizzati con il metodo di riferimento (HPLC) e sono stati archiviati gli spettri MIRS (900 - 4000 cm^{-1}); le analisi statistiche sono state effettuate utilizzando le partial least square regression e una procedura di validazione interna random. Il tempo di ritenzione è risultato stabile, con RSD tra 0.03% e 0.80%. Mentre, l'area variava da 0.25% a 8.48%, in modo dipendente dalle concentrazioni delle WP. Inoltre, i migliori coefficienti di determinazione dei modelli di predizione MIRS sono stati evidenziati per le WP presenti in elevate quantità, come le β-LG (0.58), WP totali (0.58), e α-LA (0.56). I risultati di questo primo studio hanno evidenziato un metodo RP-HPLC ad alta risoluzione e che potrà essere utilizzato come metodo di riferimento per lo sviluppo di modelli di predizione MIRS.

L'obiettivo del secondo contributo sperimentale è stato la valutazione dell'aggiunta di microparticolato di siero proteine (MWP; da 0.0 a 9.0%, vol/vol) sulle proprietà di coagulazione del latte (MCP), conosciute come tempo di coagulazione (RCT), tempo di rassodamento, e consistenza del coagulo dopo 30 minuti dall'aggiunta del caglio (a_{30}). Tre campioni di latte di massa sono stati analizzati in tre giorni; entro giornata, le aliquote di latte sono state addizionate con concentrazioni crescenti di MWP (vol/vol): 1.5%, 3.0%, 4.5%, 6.0%, 7.5%, and 9.0%. Inoltre, è stato preparato un doppio controllo senza MWP. Le proprietà di coagulazione sono state misurate con lo strumento Formagraph (Foss Electric A/S, Hillerød, Denmark). L'aumento di concentrazione di MWP ha portato ad un prolungamento di RCT. In particolare, differenze significative sono state ottenute tra il

controllo (13.5 min) e RCT nei campioni con 3.0% (14.6 min) o più di MWP. Un andamento simile è stato ottenuto per il tempo di rassodamento. Nessun effetto è stato osservato per a_{30} tra le concentrazioni di MWP. I risultati hanno dimostrato che l'utilizzo di MWP durante la coagulazione del latte richiede un prolungamento della fase di coagulazione prima del taglio della cagliata.

Il terzo contributo mirava alla valutazione dell'effetto dell'aggiunta di MWP (da 0.0 a 4.0%, vol/vol), utilizzando latte standardizzato con diversi rapporti proteina-grasso (PFR: elevato, standard e basso livello di grasso) sul tempo di coagulazione, composizione e resa del formaggio. Trenta campioni di formaggio sono stati ottenuti attraverso una tecnica di mini caseificazione. L'aumento di PFR ha influenzato il tempo di coagulazione. La resa di caseificazione è diminuita al diminuire dei livelli di grasso, ed è risultata più elevata quando sono stati utilizzati livelli del 4% di MWP nei formaggi a basso contenuto di grasso. Mentre, non è stata evidenziata nessuna differenza nella resa di caseificazione nel formaggio prodotto in condizioni standard e ad elevato contenuto di grasso considerando le diverse concentrazioni di MWP usato. È, inoltre, risultata stabile la composizione chimica dei formaggi magri, questo suggerisce la possibilità di includere MWP come sostituto del grasso per mantenere composizione e resa.

L'obiettivo dell'ultimo contributo sperimentale è stato la valutazione dell'utilizzo dell'analisi d'immagine iperspettrale (HSI) per la rivelazione dell'aggiunta di MWP (da 2.0% a 4.0%, vol/vol) in prodotti caseari a basso contenuto di grasso. L'analisi d'immagine iperspettrale è una tecnica relativamente recente applicata con successo nell'indagine di composizione degli alimenti; HSI, infatti, combina la tradizionale analisi d'immagine con le informazioni spettrali dell'oggetto analizzato. Dodici campioni di formaggio sono stati ottenuti con una tecnica di mini caseificazione, e prodotti utilizzando latte magro (3.5% grasso) mentre i livelli proteici (3.5%) sono stati aggiustati con concentrazioni crescenti di MWP (da 2.0% a 4.0% vol/vol). Inoltre è stato effettuato un controllo senza MWP (0.0% MWP). Dopo un mese di stagionatura i campioni sono stati analizzati archiviando le lunghezze d'onda da 1,100 a 1,600 nm, per un totale di 140 valori misurati. Le informazioni spettrali sono state trattate statisticamente attraverso le standard normal variate e derivata seconda prima dell'analisi PCA. L'analisi delle componenti principali ha riportato una variabilità spiegata del 7% tra i trattamenti con MWP ed il controllo. Lo score plot ha mostrato una diversa classificazione dei campioni contenenti MWP rispetto ai campioni di controllo senza l'aggiunta di MWP. Questi risultati confermano la possibilità di utilizzare la tecnologia HSI per la rilevazione di MWP nei prodotti lattiero caseari, e possono essere

utilizzati per lo sviluppo di modelli di predizione capaci di discriminare i prodotti nei quali è avvenuta l'aggiunta di MWP.

INTRODUCTION

GENERAL INTRODUCTION

Dairy sector is one of the most important in agriculture (Cassandro et al., 2008). Simultaneously, at the increased dairy production, large amount of wastewater is generated. In particular, cheese manufacturing is responsible of the most polluting effluent, the whey. In the past, it was considered a waste with heavy environmental impact for its physiochemical properties; indeed whey shows a relatively high organic content, consequently high biological and chemical oxygen demand (Prazeres et al., 2012).

Whey is a green-yellowish liquid resulting from the precipitation of casein during milk coagulation in cheese production (Siso, 1996). Whey contains 100% of lactose, ash, and 20% of milk proteins not retained in cheese (whey proteins, **WP**) for a 50% of total solids (Smithers et al., 1996). To avoid direct discharge of whey in waters or its use as animal feeding several whey treatments have been developed as reported in the review of Prazeres et al. (2012). The most common are biological and physiochemical treatments (thermal precipitation and membrane separation). Different combinations of the technologies must be considered to provide multiple alternatives taking into account the technical and economic potential of each individual cheese factory (Prazeres et al., 2012). An example is the product produced by controlled aggregation of WP by heating and shearing after the membrane concentration; this products is named microparticulated whey proteins (**MWP**). Microparticulated whey proteins (**MWP**) are colloidal particles of native whey protein and protein aggregates with different soluble properties (Renard et al., 2002). Aggregates diameter size ranged from 0.005 to 100 µm depending on microparticulation process condition. The desirable dimension varied by the final application; for instance, in cheese manufacturing, particles should be between 0.1 and 10 µm to avoid casein network destruction during the coagulation (Kulozik et al., 2001).

Gelling properties of WP were exploited in several non-food industrial applications. One of the main no-food applications has resulted in the production of biopolymer based films (Ramos et al., 2013). Other applications of WP ranging in pharmaceutical and cosmetic industries, in which WP were exploited as microencapsulations systems (Xue et al., 2007; Bae et al., 2008).

Applications in human nutrition are important outlets for WP sector. Confectionery and bakery products contains WP ingredient for its properties in enhancing their flavor. Moreover water binding, textural and retention of freshness properties of WP incorporation with other emulsifiers are optimized for the production of wheat bread and soups. In the last years WP have become appropriate ingredients in dairy industries for their nutritive and

functional properties, moreover they can be used as a substitute of milk ingredient. The main application is the use of WP as fat replacer. Indeed, increasing consumer demand for reduced-fat products was reflected also in dairy products.

In the present thesis, thanks to the collaboration with Soligo Dairy Cooperative (Soligo, Treviso, Italy) the application of MWP during the coagulation and cheese production has been studied.

Whey from cheese making

The whey is a complex watery solution retaining 50% of milk total solid solubilized as summarized in Table 1 (Smithers et al., 1996). The amount of solids is 7% composed by: 10-12% of proteins, and the rest being mainly lactose 74%, minerals 8%, milk fat 3% and lactic acid. Several factors influence whey composition as the composition and quality of milk, and the cheese manufacturing technique (e.g. coagulation time and temperature).

Lactose is a disaccharide composed by glucose and galactose. It is present in aqueous solution in two forms: α and β with different solubility, this last is the most soluble at standard ambient conditions, while at high temperature (>90°C) α-lactose is much soluble. Lactose is an important source of dietary energy and enhances the intestinal absorption of calcium from food.

Moreover, whey contained whey proteins, soluble proteins with high concentration of branched-chain amino acids, as leucine, isoleucine, and valine. In particular, leucine is an important factors in tissue growth and repair. Whey proteins are also rich in the sulfur containing amino acids cysteine (**Cys**) and methionine. With a high concentration of these amino acids, immune function is enhanced through intracellular conversion to glutathione (**GSH**; Marshall, 2004).

Table 1. Average composition of bovine milk and whey (Smithers et al., 1996).

Component	Concentration, % w/vol	
	Milk	Whey
Casein Proteins	2.8	0.0
Whey Proteins	0.7	0.7
Fat	3.7	0.05
Ash	0.7	0.7
Lactose	4.9	4.9
Total	12.8	6.35

Whey treatments and Microparticulation Process

Dairy industry is divided into several sectors, in which inevitably entails the production of whey and contaminant wastewater. Without an appropriate treatment, these effluents represents serous environmental hazard (Rivas et al., 2011). Ice-cream, butter and cheese production effluents are the most important source of organic contaminants in dairy sector. Among these, cheese manufacturing is responsible of the most significant environmental impact for the high value of organic matter that is for around 99% biodegradable (Ergüder et al., 2001). From the previous sentence it is obvious that cheese whey cannot be directly discharged to the environment without treatments and valorization.

In the past, cheese factories used their effluents for land application or even as animal feeding, nowadays whey management is becoming a new sector of dairy industry for the important challenge due to the strict legal requirements and for the source of organic material that whey represents (Farizoglu et al., 2007). In this scenario, whey management to reduce pollutant potential can be carried out following two ways: recovery organic material and its valorisation or not. Biological and physiochemical treatments are the options. In the first case the technology can be used both for the valorization, by recovering proteins and lactose, or for the reduction of organic material contents without valorization. In the second case, application as coagulation, precipitation and filtration is applied only for the valorization step.

Biological treatments are performed by using microorganisms in aerobic or anaerobic condition. Aerobic processes do not permit the valorization of whey organic material, for the limited selected specie and for the excessive energy requirements for oxygen supply (Cordi et al., 2006). While, high organic removal efficiency is obtained by selecting acclimated anaerobic microorganisms thought anaerobic digestion. Moreover, the valorization of whey components is possible by exploiting microorganisms able to use lactose to produce glucose, galactose, ethanol, methane and hydrogen. These materials can be successively adopted in food (i.e. glucose and galactose) and no-food (i.e methane and hydrogen) industry (Prazeres et al., 2012). Biological process provided a valorization only for carbohydrates, but the increasing knowledge on the proteins importance in human diet provided a new field of research focused to find new source of proteins. For instance, physicochemical treatments of whey are meant to the recovery of valuable products present in whey as proteins. Precipitation of proteins by thermal or chemical treatments have a disadvantage in the reuse of remaining effluents; this problem can be avoided by using membrane separation. Indeed, among physiochemical treatments, membrane separation has been extensively used in industrial scale, firstly, for its high efficiency in contaminant removal and secondly for the quality and purity of protein or lactose recovered.

Filtration by membrane is a pressure-driven separation process using semi-permeable materials, the material retained by the membrane is called retentate, while material filtered over the membrane is called permeate. The size of membrane pores and the pressure used indicate type of filtration and characterized the retentate and permeate produced. The main filtration system are: reverse osmosis (**RO**) which uses the highest pressure and membranes with the smallest pores (1 - 5 Å), and RO generally retains all compounds and only water cross into the permeate; nanofiltration (**NF**) with similar RO pores size but lower pressure, in this manner only monovalent ions are removed into the permeate; whereas microfiltration (**MF**) has the lowest operating pressures and membranes with the largest pores and it allows the passage of many larger compounds; ultrafiltration (**UF**) is intermediate in pressure used and membrane pore size, and it retains lipids and proteins, while sugar and minerals pass into the permeate. Whey protein concentrate (**WPC**) and isolate (**WPI**) are retained by UF and NF system in the range from 80-85% and 87-100%, respectively (Smith et al., 2013).

Different combinations of the technologies must be considered to provide multiple alternatives taking into account the technical and economic potential of each individual cheese factory (Prazeres et al., 2012). An example is the first product produced by controlled aggregation of WP by heating and shearing after the membrane concentration; this product has been release in 1990. This whey protein ingredient, called Simplesse®, opened an interest in the use of microparticulation process (Singer, 1996). Microparticulated whey proteins (**MWP**) are colloidal particles of native WP and protein aggregates with different soluble properties (Renard et al., 2002). Aggregates diameter size ranged from 0.005 to 100 µm depending on microparticulation process condition. The desirable dimension varied by the final application; for instance, in cheese manufacturing, particles should be between 0.1 and 10 µm to avoid casein network destruction during the coagulation (Kulozik et al., 2001).

Different processes in MWP production have been patented, the main are three: traditional technique, extrusion cooking and microfluidization. In traditional MWP production technique, an heat treatment (85-120°C) and high shearing are simultaneous, while in the extrusion cooking heating and shearing phases are divided. A different method is the microfluidization in which, after an initial heating phase, a homogenization phase at high pressure (35-75 MPa) is applied to MWP production (Dissanayake and Vasiljevic, 2009).

Initial WP composition and microparticulation parameters influenced the characteristics of MWP (Table 2). Concerning WP composition, higher protein level accelerates the denaturation reaction and the resulting MWP shows small aggregates (Kulozik et al., 2001); moreover low presence of lactose results in a small microparticles, while in high concentration of lactose large aggregates are formed (Spigel et al., 1999), while an opposite

trend is reported for calcium presence (Spiegel and Huss, 2002). Concerning, processing parameters, heating condition reports a large aggregates formation in low (<85°C) or high temperature (100-130°C), while small aggregates results in intermediate temperature condition (85-100°C; Spiegel et al., 1999). The improvement of mechanical shear force affects aggregates formation by decreasing their size (Spiegel et al., 1999).

Table 2. Chemical and processing parameters influencing the characteristics of microparticulated whey protein.

Parameters	**Extent of variation**	**Effect**
Chemical composition		
Protein concentration	Low	Big aggregates
	High	Small aggregates
Lactose	1.5-7.5%	Small aggregates (5-15 µm)
	13.5-20.0%	Large aggregate (30 µm)
Calcium	0.05%	Large aggregate (>170 µm)
	0.33%	Small aggregates (10 µm)
Processing parameters		
Temperature	<85°C	Large aggregates (0.1-50 µm)
	85-100°C	Small aggregates (0.1-20 µm)
	>100-130°C	Large aggregates (20-100 µm)
Shear rate	50-250 s^{-1}	Large aggregates (20-50 µm)
	250-450 s^{-1}	Medium aggregates (10-30 µm)
	>450 s^{-1}	Small aggregates (<10 µm)

Whey Proteins and MWP uses

Whey proteins include β-lactoglobulins (**β-LG**), α-lactalbumin (**α-LA**), bovine serum albumin (**BSA**), caseinmacropeptide (**CMP**), immunoglobulins (**Ig**), lactoferrin (**LF**), lactoperoxidase and very small amount of other enzymes and proteins, as proteose-peptone (**PP**).

The most abundant WP is β-LG following by α-LA, CMP, and BSA. In Table 3 are shown the whey protein fractions and their biological functions as proposed by Marshall (1998).

Table 3. Composition and biological function of whey proteins in bovine milk
(Marshall, 1998).

Whey protein	Biological function
Major	
β-LG	Pro vitamin A transfer
α-LA	Lactose synthesis
BSA	Fatty acid transfer
IgG	Passive immunity
Bioactive	
LF	Bacteriostatic agents
LP	Antibacterial agent
Enzymes	Health indicators
PP	Opioid activity

β-LG: β-Lactoglobulin, α-LA α-Lactoalbulin, BSA bovine serum albumin, IgG immunoglubulin G, LF lactoferrin, LP lactoperoxidase, PP proteose-peptone.

β-Lactoglobulin is composed by 162 amino acids (**aa**) and its molecular weight is 18.3 KDa. In primary structure β-LG contains cysteine (**Cys**), a sulfur aa responsible of the formation of two disulfide bounds (Cys66-Cys160 and Cys106-Cys119) and a free thiol group in tertiary structure (Schokker et al., 2000). In whey β-LG is presents in dimer conformation for the presence of non-covalent bound (Sakai et al., 2007), while during heat treatments (>40°C) a reversible dissociation of dimer into monomers occurs (Schokker et al., 2000). Irreversible denaturation of β-LG happens at temperature higher than 90°C with the formation of oligomers and high molecular aggregates (Busti et al., 2005). The abundance of β-LG in

WPC highly dominate the characteristics of the resulting MWP (Spiegel and Huss, 2002). The other main protein is α-LA, a small protein (124 aa and14.2 KDa) stabilized by four disulphide bonds. Heating temperature across 85 and 120°C cause irreversible denaturation of α-LA.

The application of WP in non-food and food industry is competitive for the cheap source that WP represents, besides of the good solubility and functional properties of the major WP (Gonzalez Siso, 1996).

Gelling properties of whey proteins were exploited in several non-food industrial applications. One of the main non-food applications has resulted in the production of biopolymer based films. Whey proteins were used to produce edible films that present major quality and extended the shelf-life of foods by functioning as a barrier to moisture, oxygen carbon dioxide, lipids, aromas and other volatile compounds between foods and their surroundings.

Whey proteins based films exhibited good mechanical and excellent oxygen barrier compared to other protein or polysaccharide-based film (Ramos et al., 2013). Other applications of whey proteins ranging also in pharmaceutical and cosmetic industries, WP were exploited as microencapsulations systems as wall for cosmetics or pharmaceutical products (Xue et al., 2007; Bae et al., 2008) or in dental caries protective agents (Grenby et al., 2001).

Applications in human nutrition are important outlets for WP sector. Confectionery and bakery products contains WP ingredient for its properties in enhancing of flavour and tenderizing qualities. Moreover water binding, textural and retention of freshness properties of WP incorporation with other emulsifiers are optimized for the production of wheat bread and soups. The high nutritional quality of WP and the presence of specific growth factors make of this ingredient an important source for infant formula and elderly foods. Besides, WP ingredients provide the desired WP to casein ratio in infant formula for the perfect reproduction of human milk composition (de Wit, 1998). The nutritional value of WP has also encouraged their use in a variety of drinks. High protein beverages have been obtained with the usage of flavour-masking to addressing typical astringency sensor of whey (Beecher et al., 2006).

In the last years WP have become an appropriate ingredient in dairy industries for their nutritive and functional properties, moreover they can be used as a substitute of milk ingredient. The main application is the use of WP as fat replacer. Indeed, increasing consumer demand for reduced- fat products was reflected also in dairy products. The main problem from the reduction of milk fat is the loss of consistency (decrement of total solid) and the

reduction of yield. At this regard, the replacement of fat with MWP introduces a new structural element in product matrix. The positive effect on sensorial and textural properties of low-fat ice cream, yogurt and cheese was confirmed in several studies (Haque and Ji, 2003; Akalm et al., 2008; Sahan et al., 2008; Torres et al., 2012).

Antioxidant activity of whey

Whey has a potent antioxidant activity, because of high Cys rich proteins content. Indeed, Cys intervene in glutathione synthesis (**GSH**), a potent intracellular antioxidant. Glutathione is tri-peptide composed of glycine, glutamate, and Cys, this last contains a thiol group that serves as an active reducing agent in preventing oxidative stress. Several studies confirmed the use of WP, as source of cysteine, to increase intracellular glutathione levels, and in the treatment of cancer by stimulate immunity, and detoxify potential carcinogens.

Indeed, in vitro and animal studies, confirms the ability of WP in reducing colon cancer incidence (Hakkak et al., 2000), and in particular BSA has demonstrated inhibition of growth in human breast cancer cell (Laursen et al., 1990), while Yoo et al. (1998), demonstrated as lactoferrin has the ability to inhibit metastasis of primary tumors in mice. Moreover, WP are associated with the prevention of osteoporosis due to its bioavailable calcium content. Several in vivo studies determined WP had the ability to increase femoral bone strength in young ovariectomized rats (Takada et al., 1997).

Total antioxidant activity of WP was assessed in human in different pathological conditions. In an effort, to increase Cys, and ultimately glutathione, several studies have been conducted on the use of the WP in HIV-positive individuals (Micke et al., 2002), and in patient infected with hepatitis B or C. Moreover, WP has demonstrated a protective effect on protective effect on the gastric mucosa. This effect is related to the sulfhydryl component, particularly Cys and its link with glutamic acid in the production of glutathione. In addition, WP improved cognitive function and coping ability in highly stressed individuals (Markus et al., 2002).

Finally, whey has made a significant commercial impact in the weight-loss industry for its protein content alone. The essential and non-essential amino acids in whey act as substrates for protein synthesis and may improve body mass index in individuals participating in exercise programs (Burke et al., 2001).

Economic aspects of cheese making and whey management

Whey produced after cheese-manufacturing represent 85% of the initial milk volume, and cheese factories cannot easily stored the large amount of whey generates. To the environmental point of view whey treatments reduced pollutant potential, but from an economic point of view whey treatments required a capital investment. In particular, the main characteristics that affect the initial investment are: the size and the type of whey treatment plant (Gilles et al., 1977; Peter, 2005). After the initial investments, fixed costs must be considered, as membrane cost and their cleaning, mineral fraction and fat removal, and energy. These investments are covered with the simultaneously production of WPC, with more of 80% of WP, and permeate with high level of lactose (Peter, 2005). In particular this operation is possible by using UF membrane. As reported by Peter (2005) a quarter of cheese price can be returned at dairy farmers in whey payments. Moreover, higher income can be obtained by investing in system for organic material recovery from whey.

The transformation of whey over the past approximately 50 years, founded on advances in science and technology, has resulted in increasingly sophisticated products. Concomitant increases in the value of these products in an increasingly sophisticated marketplace have resulted in enhanced wealth to dairy manufacturers. An illustration of this transformation, highlighting WP products and ingredients, is depicted in Figure 1, as reported in the review "Whey and whey proteins-from Gutter-to-gold" of Smithers (2008).

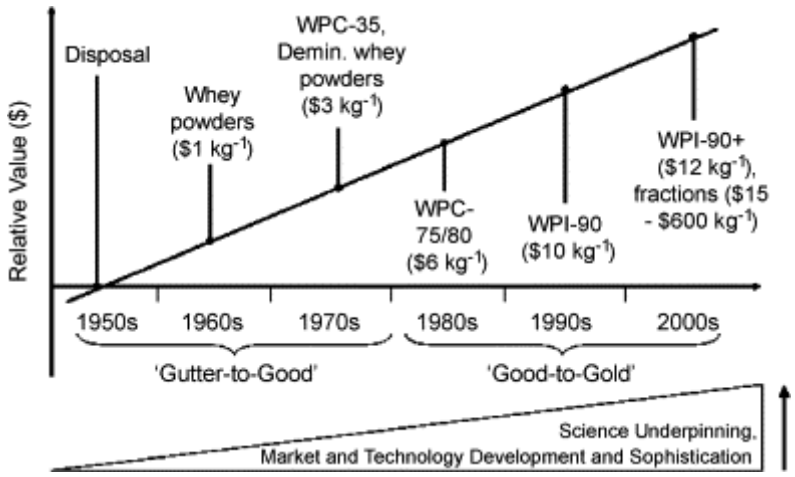

Figure 1. Schematic representation of relative increase in value of WP and peptide products as proposed by Smithers (2008)

Whey composition determination: analytical solution

Different relationship between whey components causes changes in characteristics of products, because of heterogeneous nature of lactose and proteins, and their concentration (Alomirah and Alli, 2004). The linkage between industrial request and whey composition appears more interesting, indeed increasingly studies focused on analytical determination of lactose and whey protein.

Applications of lactose in foods and pharmaceuticals sector have required methods for its determination. The earlier methods for lactose quantification were conducted using X-ray diffraction, nuclear magnetic resonance spectroscopy and differential scanning calorimeter (Buma et al., 1967; Earl and Parrish, 1983; Figura and Epple, 2005) with the disadvantage to quantify low level crystallinity. To solve this problem spectroscopy method are becoming more exhaustively. Near infrared (**NIR**) and Raman spectra have been used for lactose determination, in whey and whey permeate powder, quantifying the degree of crystalline, amorphous, polymorphs crystal forms (Gombàs et al., 2005; Nørgared et al., 2005; Smith et al., 2005). Moreover, Lei et al. (2010) proposed Fourier Transform Infrared Spectroscopy (**FT-IR**) to detect crystallized lactose in milk powder, providing information about the source and typology of additives used.

Spectroscopy methods, NIR, Raman and FT-IR, are fast, simply, precise and non-destructive techniques. Furthermore, spectroscopy applications provides different advantages in industrial sector, to test lactose quality in lactose based preparation, or in pharmaceutical, food and dairy additive, simultaneously to other quality parameters (Kirk et al., 2007).

Technological properties of whey depend on the composition of WP that exhibits individual functionality. The properties of whey peptides and protein fractions resulted interesting in food and pharmaceutical industries requiring methods of their detection. Majority studies on whey protein determination were conducted exploiting chromatographic method (Table 4). In particular high performance liquid chromatography (**HPLC**) became one of the main techniques for its versatility and high resolution. Main chromatographic techniques were reversed-phase (**RP**), size exclusion, and ione exchange. Principles of these HPLC separations were based on interaction between sample and stationary phase, in particular hydrophobic interactions, molecular size separation, interaction between ions, respectively. Applications of chromatographic methods were exploited for separation, quantification and isolation of WP for their healthy and functional properties, utilizable in several industries. A review of analytical methods is summarized in Table 4.

Chromatographic method were proposed to maximize resolution of separation of all protein fractions and to check the purity of WP in whey (Pedersen et al., 2003; Doultani et al.,

2004; Thoma et al., 2006), WPC and WPI (Eldagd et al. 2000; Alomirah et al., 2004 Spelman et al., 2005; Kiokias et al., 2007; Innocenti et al., 2011; Bund et al., 2012). Furthermore chromatographic method has been used to isolate single protein fractions for their health properties as LF (Palmano et al., 2002; Thoma et al., 2006), CMP (Ferreira et al., 2003; Li et al., 2004; Kreuß et al., 2008).

High performance liquid chromatography has been applied to evaluate processing treatment to produce WPC or WPI from whey (Moatsou et al., 2003; Roufik et al., 2005; Bhattacharjee et al., 2006; Bouaouina et al., 2006; Anandharamakrishnan et al., 2008; Dissanayake et al., 2009; San Martin et al., 2011). Moreover, HPLC have been used to detect fraudulent additions on milk or whey (Moatsou et al., 2003; Moatsou et al., 2005), while Allelein et al. (2012) have proposed this method to understand linkage between a whey protein and a polysaccharide in food.

Finally, mass spectrometry technique provided masses of the molecules on samples, and it has been used to confirm the results of separation methods, identifying protein and peptide sequencing (Bound et al., 2012), quantitation of proteins (Czerwenka et al., 2007) and peptides (Mollè et al., 2006), finally adulteration of dairy products (Cozzolino et al., 2002; Chen et al., 2004).

The need for fast, cheap, and high-throughput methods of chemical analysis has also led to the application of mid infrared spectroscopy (MIRS) in food sector. The spectroscopic technique is based on the study of the interaction between matter and electromagnetic waves. Electromagnetic radiation comprises different regions according to the following wavelengths: the xray region (0.5-10 nm), UV region (10-350 nm), visible region (350-800 nm), near-infrared region (800-2,500 nm), mid-infrared region (2,500-25,000 nm), microwave region (100 μm-1 cm), and radio frequency region (1 cm-1 m).

Currently, MIRS is used to determine milk quality traits, as recently reviewed by De Marchi et al. (2014). In particular, MIRS models are often used in official milk-recording schemes to predict protein, casein, fat, lactose, and urea contents. Besides these traditional traits, MIRS has been used to predict other milk chemical characteristics: fatty acids composition (Rutten et al., 2009; Soyeurt et al., 2011; De Marchi et al., 2011), milk proteins composition (De Marchi et al., 2009a; Bonfatti et al., 2011; Rutten et al., 2011), milk coagulation properties (MCP; Dal Zotto et al., 2008; De Marchi et al., 2009b), milk acidity (De Marchi et al., 2009b). In addition, several laboratories involved in routine milk-recording systems have been storing spectral data to predict a posteriori several traits.

Table 4. Analytical methods for whey protein separation, identification, quantification.

Chromatographyc method	Whey material	Protein Fractions	Reference
RP-HPLC	Acid, Cheese Whey	α-LA, β-LG, BSA	Eldgar et al., 2000
		LF	Palmano et al., 2002
		CMP	Ferreira et al., 2003
	Mozzarella Whey, WPC, WPI	α-LA, β-$LG_{A\,B}$, BSA	Alomirah et al., 2004
	Caprine, Bovine Whey	α-LA, β-LG, BSA	San Martin et al., 2012
		α-LA, β-LG, CMP, GMP	Thoma et al., 2006
	WPC / WPI	α-LA+BSA, β-LG	Innocenti et al., 2011
		α-LA, β-$LG_{A\,B}$	Kiokias et al., 2007
		α-LA, β-$LG_{A\,B}$, BSA	Spelman et al., 2005
		α-LA, β-LG	Anandharamakrishnan et al., 2008
	Whey based Infant Formula	Se, WP	Bermejo et al., 2001
Size Exclusion HPLC	Pure WP	α-LA, β-$LG_{A\,B}$, BSA	Pedersen et al., 2003
	Whey different breed	α-LA, β-LG, BSA	Moatsou et al., 2005
	WPC / WPI	α-LA, β-LG, BSA	Roufik et al., 2005
		α-LA, β-LG, BSA, no glycated WP	Bound et al., 2012
		GMP	Li et al., 2004
		α-LA, β-LG, BSA	Dissanayake et al., 2009
		β-LG, BSA Hydrolizate	Spelman et al., 2005
		α-LA, β-LG, BSA	Bouaouina et al., 2006
Ione Exchange	Pure WP	CMP, GMP	Kreuß et al., 2008
	Rennet Whey	α-LA , β-$LG_{A\,B}$, LF, Lactoperoxidase	Ye et al., 2000
	Mozzarella Whey	α-LA , β-LG, BSA, IgG, LF, Lactoperoxidase	Doultani et al., 2004
	WPI	Glycated/no Glycated WPI	Allelein et al., 2012
Mass Spectrometry	Goat, Cow Buffalo WP	α-LA, β-$LG_{A\,B}$	Chen et al., 2004
		β-LG	Czerwenka et al., 2007
		α-LA, β-LG	Cozzolino et al., 2002
	WPC / WPI	Glycated proteins	Bound et al., 2012
		CMP, GMP	Mollè et al., 2005
		β-LG	Spelman et al., 2005

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OBJECTIVES

OBJECTIVES

The thesis is made up of four contributes, dealing different with approaches developed to understand the different aspects of whey proteins (WP) recovery, and their utilization during cheese manufacturing. The general aim of the research project was to investigate and to propose strategies for whey valorisation working with cheese products produced by an Italian dairy industry.

The Soligo dairy cooperative (Soligo, Treviso, Italy) was born 131 years ago by the action of Prof. Giuseppe Toniolo and nowadays one of the most important dairies of the north-east of Italy, with more than 200 farms. The cooperative has financed the project.

In this scenario, the objectives of the four contributes of thesis were:

I. to develop a gold-method based on Revesed Phase -HPLC for WP quantification in whey , and to evaluate the potential of Mid Infrared Spectroscopy in WP prediction;

II. to investigate the effect of increasing concentrations of microparticulated whey proteins (MWP; from 0.0 to 9.0%, vol/vol) on milk coagulation properties (MCP);

III. to evaluate the effect of increasing MWP concentrations (from 0.0 to 4.0 %, vol/vol) and different protein-to-fat ratios (high, standard and low levels of fat) and on milk coagulation process, cheese yield and composition;

IV. to investigate the effectiveness of Hyper Spectral Image (HSI) technique in the detection of MWP in low-fat Caciotta cheese, produced with increased concentration of MWP (2.0%, 3.0%, 4.0% vol/vol);

I

ORIGINAL PUBLICATIONS

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Quantification of Whey Proteins by Reversed-Phase High-Performance Liquid Chromatography and their prediction by Mid-infrared Spectroscopy

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ABSTRACT

In dairy industry membrane filtration is used to reduce the amount of whey waste, simultaneously to whey proteins (WP) recovery, generating WP concentrate and microparticulated WP, widely used in food industry. This study aimed to develop a Reversed Phase - High-Performance Liquid Chromatography protocol for WP quantification and to evaluate the ability of mid-infrared spectroscopy (MIRS) in predicting these traits in dairy conditions. Whey proteins included α-Lactoalbumin (α-LA), β-Lactoglobulin A and B (β-LG), bovine serum albumin, caseinomacropeptides, proteose peptone and total WP identified. References analysis was conducted on C8 column and measured with UVB detector (214 nm). Repeatability and reproducibility tests were performing by calculating the relative standard deviation (RSD) within and across days for retention times and peak areas. Samples of whey (n = 187) were analysed according to the reference methods and MIRS information were stored (900 - 4000 cm^{-1}); statistical analysis was carried out through partial least square regression and random cross-validation procedure.

Retention times were stable, with RSD ranging between 0.03% and 0.80%. The RSD of peak area in repeatability and reproducibility tests ranged from 0.25% to 8.48% depending on the considered proteins and their relative abundance; indeed, better coefficients of determination in validation were obtained for fractions present in whey in large amounts, as β-LG (0.58), total identified WP (0.58), α-LA (0.56), while minor WP were predicted with minor appreciable accuracy.

This study proposes a high-throughput and high-resolution method for WP quantification in whey and shows the potential of MIRS for their prediction.

Key words: whey proteins quantification, mid-infrared spectroscopy, RP-HPLC

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INTRODUCTION

The increasing consumer demand for products with healthy, high nutritional properties led the dairy industries to recognize the value of whey proteins (**WP**; Smithers, 2008). In several food products, WP can be incorporated to maintain the structure and nutritional value (Lo and Bastian, 1998). Moreover the WP recycling offers the possibility of improvement of economic revenue for the dairy industry. Indeed, recently dairy industry has been exploited different processes for whey treatments (Prazeres et al., 2012). Ultrafiltration (**UF**) of whey to produce WP concentrate (**WPC**) simultaneously to physical process as heating (Singer et al., 1990) and high pressure treatment (Dissanayake and Vasiljevic, 2009) results as one of the most important applications to obtain microparticulated whey protein (**MWP**). The controlled aggregation of WP during MWP production provides colloidal particles with several technological and healthy properties in the products where it is used. The main WP involved in the aggregation are β-Lactoglobulins (**β-LG$_A$** and **β-LG$_B$**) and α-Lactalbumin (**α-LA**); other minor WP are bovine serum albumin (**BSA**), various peptides as caseinomacropeptide (**CMP**) formed by chymosin cleavage of κ-casein, and proteose peptone (**PP**) resulting from proteolysis of β-casein by indigenous milk proteinases. The WP composition can strongly affect the aggregation during the production of MWP (Guyomarc'h et al., 2009). Indeed, different chromatographic methods were designed for the WP characterization, such as ion-exchange chromatography (Doultani et al., 2004), size exclusion (Dissanayake and Vasiljevic, 2009; Torres, et al., 2011), and reversed phase high performance liquid chromatography (**RP-HPLC**; Anandharamakrishnan et al., 2008; Innocente et al., 2011).

One of the main goals of the dairy industry is the rapid characterization of WP intended to UF and microparticulation process. Moreover, mid-infrared spectroscopy (**MIRS**) combined with chemometric analysis has been recently proposed as fast, non-destructive, and cheap technique to predict several chemical and technological traits of milk (De Marchi et al., 2014). Nowadays, the implementation of MIRS in dairy industry should be of interest not only on milk but also on whey products, since rapid and cheap assessment of quality whey produced after the cheese making is requested.

The aims of this study were (i) to develop a gold-method for WP quantification in whey, by RP-HPLC, and (ii) to evaluate the potential of MIRS in WP prediction.

MATERIALS AND METHODS

Reagents and Samples Collection

Guanidine hydrochloride (**GdnHCl**), Bis-Tris Buffer, Sodium Citrate, DL-Dithiothreitol (**DTT**), trifluoroacetic acid (**TFA**), acetonitrile (**ACN**) and standard protein (α-LA, β-LG_A, β-LG_B and BSA) were purchased by Sigma (Sigma-Aldrich, St. Louis, MO, USA). Pure water was obtained by Milli-Q plus (<18.3 MΩ-cm) system in laboratory.

Whey of different cheese making productions without any preservative was collected from a dairy industry (Soligo, Treviso Italy); A total of 187 whey samples were collected from May to August 2014.

Chromatographic conditions, Repeatability and Reproducibility tests for RP-HPLC method

Whey protein fractions were quantified by RP-HPLC method. The chromatographic system used was Agilent 1260 Series (Agilent Technologies, Santa Clara, CA, USA) equipped by a quaternary pump, a diode array detector, and an auto-sampler with an injection loop of 100 µl. Standard proteins were used for instrument calibration and the purified proteins were α-LA, β-LG_A and β-LG_B, and BSA. Caseinomacropeptide and PP were purified from bulk bovine milk as proposed by Mollè et al. (2006), and Paquet et al. (1988), respectively. Spectrophotometric Bradford assay has been performed for their quantification at 595 nm following the manufacturer instruction (Sigma-Aldrich, St. Louis, MO, USA). Pure proteins and whey samples have been prepared as described by Bobe et al. (1998).

The equipment was controlled by the Agilent Chem-Station for LC Systems software (Agilent Technologies, Santa Clara, CA, USA) which sets solvent gradient, data acquisition and data processing. Separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP; Agilent Technologies, Santa Clara, CA, USA) with Poroshell packing (5µm, 300 A˚, 2.1×75 mm).

The gradient elution was carried out with a mixture of two solutions, A and B. Solution A consisted of 0.1% TFA and 5.0 % ACN in water, and solution B was 0.1% TFA in ACN. The gradient started with 5.0% of solution B, after 30 s the gradient was 15.0% of B, and at the first min it was 18.0%. Afterward, the gradient was: from 18.0% to 27.5% in 1 min, from 27.5% to 30.5% in 1 min, from 30.5% to 31.0% in 15 s, from 31.0% to 32.0% in 45 s, from 32.0% to 33.8% in 35 s, and from 33.8% to 50.0% in 1.10 min. From 5.45 min to 7 min (end of the run), the gradient was brought back to initial conditions (5.0% of B). Between sample injections, the column was re-equilibrated under the starting conditions for 2 min. The total analysis time per sample was 9.0 min. The flow rate was 2.5 $ml*min^{-1}$, the

column temperature was kept at 70°C and the detection was at a wavelength of 214 nm. The injection volume consisted of 2 μl.

Ten samples of whey were used for the evaluation of chromatographic method: repeatability and reproducibility tests were performed as proposed by Bonfatti et al. (2008). Data were expressed as relative standard deviation (**RSD**) for peaks area and retention time within and across days.

Calibration of the chromatographic system has been made using the external standard method. Standard solution was analyzed in duplicate. Retention time has been registered for each pure protein, it has been used for protein identification in samples. Calibration curve for each protein was obtained by regression of the peak area and injected amount.

Spectral collection and chemometric analysis

Spectra information were collected by MilkoScan FT2 (Foss, Hillerød, Denmark) within 4 hours from collections in a spectrum range from 900 to 4000 cm^{-1} for a total of 1,060 absorbance data.

Statistical analysis was carried out by WinISI II (Infrasoft International Inc., State College, PA) and through partial least square (**PLS**) regression. A random cross-validation was performed dividing the calibration dataset in 5 groups, using one of them to check the results (prediction) and the remaining four to construct the calibration model. The model was repeated as many times as there were groups available; in such a way that all of them passed through the calibration and the prediction set.

To optimize the accuracy of the calibration models outliers samples were selected using the Mahalanobis distance (Global H > 5.0) and samples exceeding 2.5 (T outlier value) times the standard error of cross validation (**SEcv)**. Different spectral treatments were compared before PLS analysis. Spectral scattering correction were none, standard normal variate (**SNV**), standard normal variate + detrending (**SNV + D**) while mathematical pre-treatments (no derivative, first and second derivatives) on different gaps and smoothing segments (from 1 to 10) were performed.

For each WP the best equation was selected according to standard error of calibration (**SEC**), coefficient of determination of calibration ($\mathbf{R^2}$), standard error of cross-validation, coefficient of determination of cross-validation (**1-VR**), and ratio performance deviation (**RPD**), calculated as standard deviation / SE_{CV}. Ratio performance deviation values should be as high as possible: RPD higher than 10 is considered as equivalent to the reference

method (Williams and Sobering, 1993) whereas RPD values above 2.5 are considered adequate for analytical purposes (Sinnaeve et al., 1994).

RESULTS

Separation and quantification

Mobile phase composition and wavelength were selected according to literature protocols for protein quantification in milk. Optimization of gradient, flow rate, and temperature were carried out for WP separation using a column made with a superficially thin layer of porous particle. The optimal method was used in further analysis.

Retention time of standard solutions was used to determine the respective WP fractions. The gradient of mobile phase and the flow rate were set to separate in the following order of elution: CMP, PP, α-LA, BSA, β-LG_B and β-LG_A. The chromatographic profile of CMP and PP showed several peaks according to the different CMP and PP fractions, thus a single integration was used and total quantification was calculated for both the fractions. The α-LA profile reported a double peak related to non-glycosylated and glycosylated fractions, both peaks were included in the integration for quantitative analysis, because α-LA commercial standard contains both fractions. Bovine serum albumin, β-LG_B and β-LG_A eluted in a defined peak.

To evaluate repeatability and reproducibility, whey sample chromatograms for each protein fractions were overlaid, as shown in Fig. 1. Temperature and wavelength were selected to provide improvements in the efficiency, resolution and response factor of the protein peaks.

The protein quantification was performed using calibration curves derived from regression parameters computed for the area and injected amount of the single standard pure proteins. Parameters of calibration curve reported for all the protein fractions showed a $R^2 > 0.99$ (Table 1). The parameters of regression equations for calibration curves, response factors and limit of detection for WP fractions are given in Table 1. The injected amount was different for each WP fractions in agreement with the expected concentration for single WP.

Repeatability and Reproducibility tests

Repeatability and reproducibility tests expressed as relative standard deviation (RSD%) of retention times and peak areas for WP fractions are reported in Table 2. Values of RSD of retention time for repeatability and reproducibility tests evidenced great precision in

retention time for each protein fraction both within and between days. For repeatability test, the RSD ranged from 0.09% to 0.20%, while the RSD for reproducibility test ranged from 0.26% to 0.69%. A slight worsening in retention time accuracy was reported between days compared to the RSD within day (Fig. 1b). Moreover, similar results comparing the RSD of peak area in repeatability and reproducibility tests were reported, with a major accuracy within a day. Similar trend in both tests was observed between the protein fractions. Greater precision was found for CMP, PP and β-LG_A and β-LG_B with a RSD ranged from 0.55% to 0.79% and 0.25% to 1.50% in repeatability and reproducibility test, respectively. Lowest values of RSD were found in areas within and between days of α-LA (2.19% and 3.00%) and BSA (2.66% and 2.65%). The acceptable precision for retention time and area was confirmed by overlaying the chromatograms of the same sample analysed within the same day (Fig. 1a) or between days (Fig. 1b).

Prediction of Whey Proteins

Prediction models were obtained omitting the portion of MIR spectra from 1,550 cm^{-1} to 1,658 cm^{-1} and from 2,955cm^{-1} to 4,000cm^{-1} due to water absorption with low signal to noise ratio. Fig. 2 depicts an example of whey spectra without aforementioned no informative regions.

Numbers of samples selected for the calibration models were different for each WP fraction, according to outliers setting identification. In Table 3 are summarized the average composition of samples selected for the PLS analysis; Means (SD) for α-LA, β-LG, β-LG_A, β-LG_B, BSA, CMP, PP, and total quantified WP were 0.73 (0.11), 4.06 (0.86), 2.79 (0.63), 1.30 (0.24), 0.14 (0.03), 0.47 (0.14), 0.19 (0.06) and 5.62 (1.14), respectively. Coefficient of variation (**CV**) was greater in fractions where less samples were discarded as outliers, in particular for CMP (0.29) and PP (0.32) with 181 and 178 samples selected for calibration, respectively, while the lower CV was observed for α-LA (0.15) in which 18 samples were discarded.

Fitting statistics for MIRS prediction models are reported in Table 4. The number of terms of MIRS models were quite low ranged from 4 to 9 demonstrating a quite strong relationship between spectra information and WP fractions. The best prediction models were obtained for WP fractions presented in large amounts (i.e. β-LG, total identified, α-LA). All of WP fractions were well predicted by MIRS with 1-VR values ranging from 0.50 (PP) to 0.66 (BSA); only β-LG_B prediction models showed poor result (1-VR = 0.33). On average, the differences between calibration (R^2) and validation (1-VR) statistics were very low

evidencing a robustness of MIRS models. Ratio performance deviation ranged from 1.22 (β-LG_B) to 1.70 (BSA).

DISCUSSION

Validation of RP-HPLC method

The eluent solution and detection conditions used in the present study were the same reported by several studies in whey (Spellman et al., 2005) and in milk (Kiokias et al., 2007). A higher flow rate was required by column, thus obtaining a reduction in analysis time. The elution profile was CMP, PP, α-LA, BSA, β-LG_B and β-LG_A. Similar protein order in retention times was proposed for WPC in the study of Innocente et al. (2011) utilizing water and ACN in 0.1% TFA as mobile phases. A typical peaks' position (protein fingerprint) were reported for CMP variants, glycosylated and non-glycosylated for both A and B fractions were obtained, as reported with the separation proposed by Thomä et al. (2006) using a whey from a single cow's milk homozygous for κ-casein AA or BB. The selected wavelength (214 nm) has the advantage in improvement of sensitivity of response factor for PP fractions, as demonstrated in the study of Elgar et al. (2000) that used a column with polystyrene-divinylbenzene matrix, but a more distinct separation between CMP and PP was obtained with the present gradient and column. The α-LA elution pattern was in agreement with several studies (Elgar et al., 2000; Thomä et al., 2006) where a partial overlapping of glycated forms was detected. Small signal for BSA elution was obtained, a single ill-defined peak was reported (Elgar et al., 2000) with a similar behaviour of a commercial standard. Moreover, a single well defined peak was reported for both β-LG variants. The earlier elution of β-LG_B compared to the variant A was reported also in milk (Bobe et al., 1998; Bonfatti et al., 2008) and whey (Elgar et al., 2000). Satisfactory separation between β-LG_A and B variants was obtained with the present method; only few studies proposed an acceptable separation profile for β-LG variants (Thomä et al., 2006), while other studies only a partial separation was commonly reported using similar mobile phase (Elgar et al., 2000; Thomä et al., 2011).

The temperature selected in this study had improved the overall WP separation compared to room temperature, in agreement with the results of other studies in milk (Spellman et al., 2005; Bonfatti et al., 2008).

Variation of RSD values for retention times and peak areas obtained in repeatability and reproducibility tests were similar to those reported in literature within and across days (Bobe et al., 1998; Bordin et al., 2001; Bonfatti et al., 2008). Results indicate that the

repeatability and reproducibility of the method was acceptable, in particular for the most abundant fractions. To improve the accuracy of the method, 2 minutes of post-run was set to equilibrate the column with the initial condition, for a total run time of 9 minutes. To improve precision of the method, a pre-column was used as proposed by Bonfatti et al. (2008). Moreover, a blank (6 M of GdnHCl) was run before samples injection. The cleaning run was in an isocratic elution at 50% solvent B.

Prediction of Whey Proteins by MIRS

In the last ten years, spectroscopic techniques combined with chemometric have become the preferred method for milk and/or dairy product analysis. Mid-infrared spectroscopy is one of these techniques widely used in milk laboratories and the milk applications of MIRS have been reviewed in detail by De Marchi et al. (2014). Concerning the application of MIRS to predict detailed milk protein composition, different works have been published (De Marchi et al., 2009; Rutten et al., 2011) even if only Bonfatti et al. (2011) found promising results with 1-VR values greater than 0.53.

Compared to the past, when it was regarded only as the most contaminant wastewater generated by the dairy sector, nowadays whey starts to be considered also as a resource (Prazeres, et al., 2012). The interest in the valorization of whey components is related to the improvement of economic revenue for dairy industry. Indeed, the recovery of WP and their concentration provides new ingredients widely used in food and no food sector. A rapid determination of WP fractions should be of interest for dairy industry to increase the value of WP utilization.

To our knowledge, no studies have been performed so far to evaluate the effectiveness of MIRS to predict WP fractions in whey. The performance of MIRS prediction models for α-LA and β-LG found in our study is greater than those reported by De Marchi et al. (2009) and Rutten et al. (2011) for whey protein in milk using untreated spectra information; regarding spectra pretreatments, the scientific literature reported contradictory results. Nevertheless, in similar untreated spectra condition, Rutten et al. (2011) used a different (electophoresis) gold method respect to that used in the present study, indeed, as reported by De Marchi et al. (2014) protein fractions were better predicted when the reference method was HPLC than capillary zone electrophoresis. Similar 1-VR values were reported by Bonfatti et al. (2011) for total whey protein in milk, obtained with the same spectral pre-processing (SNV) besides the first order derivative. The soluble properties of WP and the greater concentration of α-LA and β-LG might be the reasons for better

prediction models respect to the other minor WP fractions; indeed, as reported by Rutten et al. (2011) in milk, the limited concentration and the micelle status of caseins reduce the prediction ability.

CONCLUSION

The recovery of WP from whey and their utilization in dairy products is a source of income for dairy industry. This study proposes a RP-HPLC method for the quantification of WP and its application as reference method to predict WP composition using MIR spectroscopy. Reference method was validated by performing repeatability and reproducibility tests. Retention times were stable. Results for area depend on the considered protein and their relative abundance; indeed, the potential of MIRS in predicting WP composition of whey was demonstrated and better MIRS prediction models were obtained for large amounts fractions (i.e. β-LG, Total, α-LA).

In conclusion, RP-HPLC method is suitable for high-resolution analysis for WP quantification in whey and represents an adequate gold method for developing MIRS prediction models in dairy industry.

TABLES AND FIGURES

Table 1. Parameters of regression equations for calibration curves, response factors (RF), limit of detection (LOD) for whey protein fractions

Protein[1]	R^2	[2]Intercept ±SE	Slope	[3]RF ± SD (10^{-3})	[4]LOD (μg)	Injected μg
α-LA	0.996	66.31±40.06	1,170.1	0.86±0.09	0.60	3.12-50.0
β-LG_A	0.994	105.27±33.24	396.14	2.09±0.13	0.70	5.0-160.0
β-LG_B	0.996	-30.08±34.75	594.24	1.89±0.08	0.70	5.0-160.0
BSA	0.997	-4.48±10.16	809.98	1.26±0.16	0.50	1.56-25.0
CMP	0.990	0.02±5.91	1,100.7	0.82±0.003	0.50	1.8-3.6
PP	0.999	-0.02±4.53	1,001.0	0.96±0.03	0.50	2.5-40.0

[1]Protein: α-LA = α-Lactoalbumin; β-LG = β-Lactoglobulins A and B; BSA = Bovin Serum Albumin; CMP = caseinomacropeptides; PP = Proteose Peptone.

[2]SE: Standard Error.

[3]Response factor ± standard deviation (μg*$area^{-1}$) x 10^{-3}.

[4]LOD= 10×(3×SD) where SD is the standard deviation of the background noise.

Table 2. Repeatability and reproducibility tests expressed as relative standard deviation (RSD%) of retention times and peak areas for whey protein fractions

Protein[1]	[2]Repeatability, RSD%		[3]Reproducibility, RSD%	
	Retention time	Area	Retention time	Area
α-LA	0.09	2.19	0.31	3.00
β-LG_A	0.04	0.79	0.19	1.50
β-LG_B	0.06	0.68	0.61	0.54
BSA	0.20	2.66	0.26	2.65
CMP	0.16	0.55	0.27	0.25
PP	0.12	0.57	0.69	0.32

[1]Protein: α-LA = α-Lactoalbumin; β-LG = β-Lactoglobulins A and B; BSA = Bovin Serum Albumin; CMP = caseinomacropeptides; PP = Proteose Peptone.

[2]Ten aliquots of the same whey sample were injected consecutively.

[3]A sequence of 10 whey samples was injected over 4 days.

Table 3. Descriptive statistics of whey protein fractions ($mg*ml^{-1}$) in whey samples selected for the calibration

Protein[1], $mg*ml^{-1}$	[2]N	Mean	[3]SD	Minimum	Maximum
α-LA	169	0.73	0.11	0.43	1.01
β-LG	177	4.06	0.86	1.40	6.76
$\beta\text{-LG}_A$	177	2.79	0.63	0.65	4.72
$\beta\text{-LG}_B$	176	1.30	0.24	0.16	2.04
BSA	178	0.14	0.03	0.05	0.23
CMP	181	0.47	0.14	0.03	0.89
PP	178	0.19	0.06	0.01	0.33
TOTAL IDENTIFIED	176	5.62	1.14	1.92	9.23

[1]Protein: α-LA = α-Lactoalbumin; β-LG = β-Lactoglobulins ($\beta\text{-LG}_A$ + $\beta\text{-LG}_B$); BSA = Bovin Serum Albumin; CMP = caseinomacropeptides; PP = Proteose Peptone; TOTAL IDENTIFIED = α-LA + β-LG + BSA + CMP + PP.

[2]N: number of samples considered to build the equations.

[3]SD: Standard Deviation.

Table 4. Statistics of prediction models for whey protein composition (mg*ml^{-1}) by MIR spectroscopy (900 - 4,000 cm^{-1})

Protein[1], mg*ml^{-1}	[2]Math	[3]Terms	[4]R^2	[5]SEC	[6]1-VR	[7]SE_{cv}	[8]RPD
α-LA	SNV 0,0,1,1	6	0.64	0.07	0.56	0.07	1.51
β-LG	NONE 0,0,1,1	5	0.65	0.50	0.58	0.56	1.54
β-LG_A	NONE 0,0,1,1	5	0.66	0.36	0.60	0.39	1.61
β-LG_B	SNV 0,0,1,1	4	0.47	0.18	0.33	0.20	1.22
BSA	SNV + D 0,0,1,1	4	0.70	0.02	0.66	0.02	1.70
CMP	NONE 1,0,1,1	9	0.65	0.08	0.55	0.10	1.48
PP	NONE 0,0,1,1	5	0.56	0.04	0.50	0.04	1.43
TOTAL IDENTIFIED	SNV + D 0,0,1,1	8	0.66	0.66	0.58	0.74	1.54

[1]Protein: α-LA = α-Lactoalbumin; β-LG = β-Lactoglobulins (β-LG_A + β-LG_B); BSA = Bovin Serum Albumin; CMP = caseinomacropeptides; PP = Proteose Peptone; TOTAL IDENTIFIED = α-LA + β-LG + BSA + CMP + PP.

[2]Math: Mathematical treatment, SNV: Standard Normal Variate; D: Detrending. The first digit of the mathematical treatment is referred to the derivative number (0: none derivative; 1: first derivative), the second number is the gap over which the derivative is calculated, the third is the length of segment smoothed, and the fourth is the second smoothing segment.

[3]Terms: number of modified PLS factors used in calibration.

[4]R^2: coefficient of determination of calibration.

[5]SEC: standard error of calibration.

[6]1-VR: coefficient of determination of cross-validation

[7]SE_{CV}: standard error of cross-validation.

[8]RPD: ratio performance deviation calculated as SD/SE_{CV}.

Figure 1

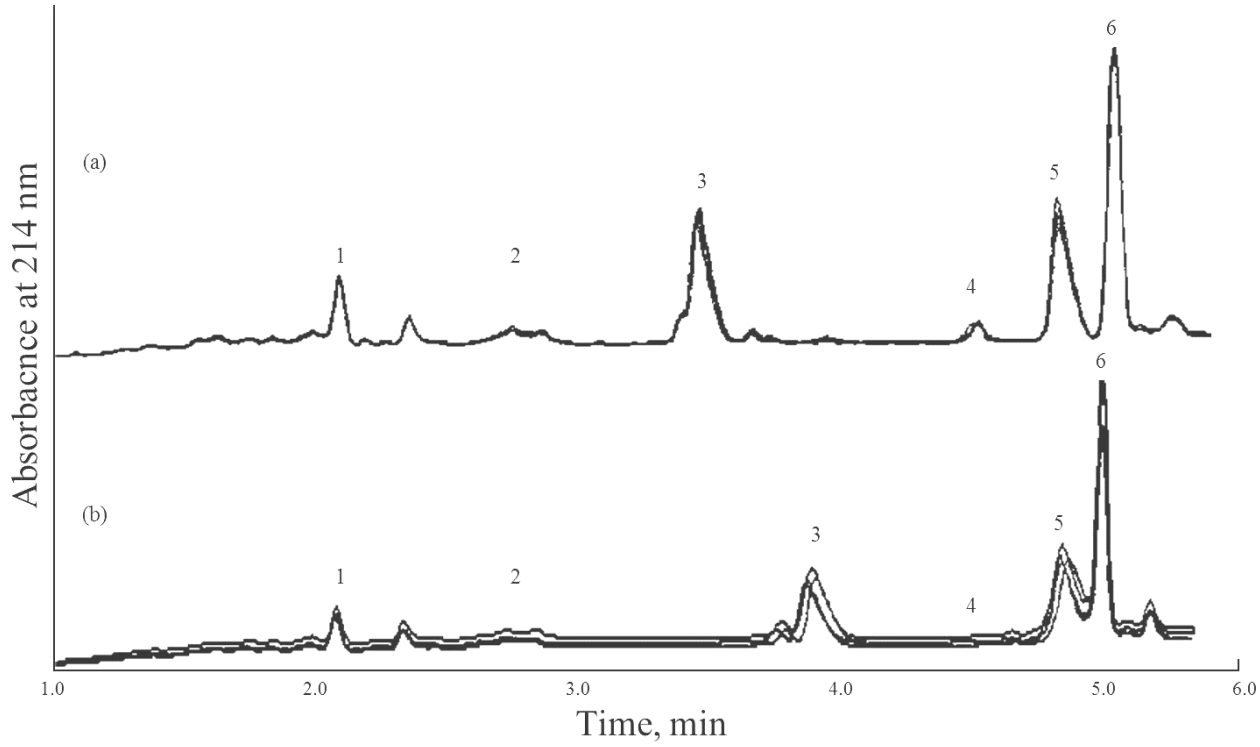

Fig. 1. Overlaid chromatograms relative to whey sample in repeatability (a) and reproducibility tests (b) for the whey protein fractions: Caseinomacropeptides (CMP; 1), Proteose peptones (PP; 2), α-Lactoalbumin (α-LA; 3), Bovine serum albumin (BSA; 4), β-Lactoblobulin B (β-LG_B; 5), β-Lactoblobulin A (β-LG_A; 6).

Figure 2

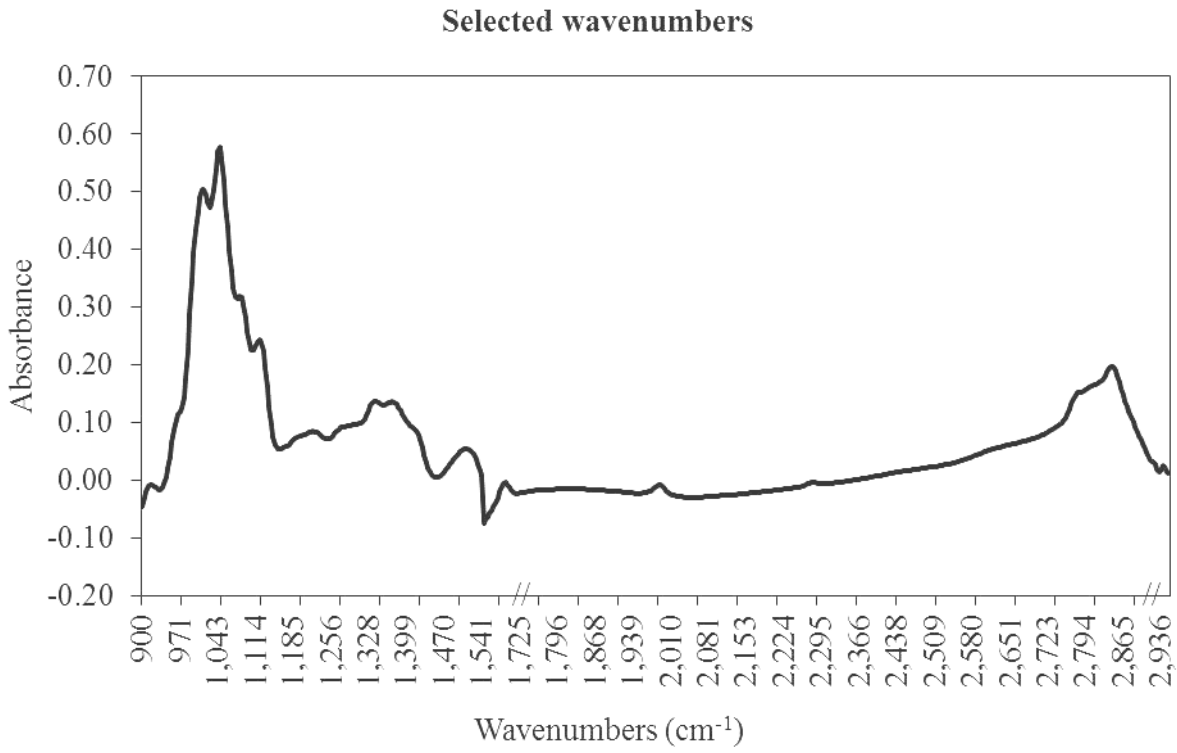

Fig. 2. Example of untreated spectrum with wavenumbers selected for calibration.

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II

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EFFECT OF MICROPARTICULATED WHEY PROTEINS ON MILK COAGULATION PROPERTIES

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Effect of microparticulated whey proteins on milk coagulation properties

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ABSTRACT

The enhancement of milk coagulation properties (MCP) and the re-use of whey produced by the dairy industry are of great interest to improve the efficiency of cheese making process. Native whey proteins (WP) can be aggregated and denatured to obtain colloidal microparticulated WP (MWP). The objective of this study was to assess the effect of MWP on MCP, namely rennet coagulation time (RCT), curd-firming time, and curd firmness 30 min after rennet addition (a_{30}). Three bulk milk samples, collected and analyzed during 3 days, were added with 6 concentrations of MWP (vol/vol): 1.5%, 3.0%, 4.5%, 6.0%, 7.5%, and 9.0%. Moreover, a sample without MWP was used as control. Within each day of analysis, 6 replicates of MCP for each treatment were obtained, changing the position of the treatment in the rack. For control samples, 2 replicates per day were performed. Besides MCP, WP fractions were measured on each treatment during the 3 days of analysis. Milk coagulation properties were measured on 144 samples using Formagraph (Foss Electric A/S, Hillerød, Denmark). The increment of the amount of MWP added to milk led to longer RCT. In particular, significant differences were found between RCT of the control samples (13.5 min) and RCT of samples added with 3.0% (14.6 min) or more of MWP. Similar trend was observed for curd-firming time, which showed the shortest time in the control samples and the longest in samples with 9.0% (21.4 min) of MWP. No significant differences were detected for a_{30} across concentrations of MWP. Adjustments in cheese processing should be made when recycling MWP, in particular during coagulation process by prolonging the time of rennet activity before cutting of the curd.

Key words: microparticulated whey protein, milk coagulation property, dairy industry

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INTRODUCTION

The dairy industry has an important economic role in the primary sector of Italy and the main interest is the transformation of milk into high quality cheeses. In this context, the evaluation of milk coagulation properties (**MCP**) is valuable to improve milk processing (Cassandro et al., 2008; De Marchi et al., 2008). Indeed, good technological properties of milk have been associated with enhanced cheese yield (Riddell-Lawrence and Hicks, 1989; Pretto et al., 2013). Several instruments can be used to measure MCP (O'Callaghan et al., 2002) and among them mechanical tools such as Formagraph (Foss Electric A/S, Hillerød, Denmark) have been widely used to determine MCP. These instruments produce a typical diagram as reported by De Marchi et al. (2009) and provide the measurement of rennet coagulation time (**RCT**, min), curd-firming time ($\mathbf{k_{20}}$, min), and curd firmness 30 min after rennet addition ($\mathbf{a_{30}}$, mm). Recently, mid-infrared spectroscopy combined with chemometric analysis has been proposed as fast, non-destructive, and cheap technique to predict MCP (De Marchi et al., 2013, 2014; Tiezzi et al., 2013).

At present, strong effort is needed to improve the efficiency of the dairy industry. The recovery of whey protein (**WP**) after cheese making process is one of the most interesting applications. Native WP can be extracted from whey through filtration to obtain WP concentrate (**WPC**), which is further aggregated and denatured through a controlled process to produce colloidal microparticulated WP (**MWP**; Spiegel and Huss, 2002). Also, the dairy industry has to face the growing demand of low-fat products; in this context, the incorporation of WPC and MWP as fat replacer into milk to maintain the yield and nutritional value of dairy products can be an efficient solution (Lo and Bastian, 1998). Microparticulated WP has been used to improve overall sensory properties of low-fat dairy products such as ice cream (Yilsay et al., 2006; Karaca et al., 2009), yogurt (Janhøj et al., 2006; Aziznia et al., 2008; Torres et al., 2011), and cheeses (Koca and Metin, 2004; Sahan et al., 2008; Ismail et al., 2011). Substitution of fat with MWP reduces the firmness of low-fat products, because of the water holding capacity of MWP. Nevertheless, excessive addition of WP is likely to interfere with curd formation and adversely affect cheese quality (Guinee et al., 1998).

The aggregation of MWP is influenced by WP composition, the presence of lactose or k-casein (**CN**) (Guyomarc'h et al., 2009), heat treatment, the pH, and ionic strength conditions (Chen et al., 2006; Nicolai and Durand, 2007; Gulzar et al., 2011). There is a paucity of studies investigating the effect of the addition of WP to milk. Ismail et al. (2011) reported shorter RCT of buffalo milk when added with WPC, whereas Guinee et al. (1997)

found impaired renneting properties of bovine milk added with WPC. The interest in the valorisation of whey components and the improvement of MCP during cheese making are of great interest for dairy industry specialized in cheese production. Therefore, the present study investigated the effect of increasing concentrations of MWP (from 0.0 to 9.0%, vol/vol) on MCP.

MATERIALS AND METHODS

Sample Collection and Experimental Design

Three samples of raw bulk milk were collected from 3 farms of the Veneto region (northeast Italy) during 3 sampling dates in October 2012. Milks were stored in a portable refrigerator (4°C) and analysed within 1 h in the milk quality laboratory of the Breeders Association of Veneto region (ARAV, Padova, Italy) for fat, protein, CN, and lactose contents using MilkoScan FT6000 (Foss Electric, Hillerød, Denmark).

Microparticulated WP was collected in the Soligo dairy cooperative (Farra di Soligo, Treviso, Italy) at the beginning of the trial, and it was used for all the sessions. During the days of analysis, aliquots of MWP were stored at -20°C. Microparticulated WP was extracted from total skim sweet whey produced by the dairy cooperative in a working day through the ultrafiltration process (Tetrapak International, Rubiera, Italy), using a tubular semi-permeable polyethersulfone membrane with a surface of 700 m^2, and a cut-off of 10,000 Da (6338 HFK-131; Koch Membrane System, Wilmington, MA, USA) at 10°C. Moreover, microparticulation process was carried out for 10 min at 95°C by shell and tube heat exchanger and homogenization at 40 bar following the manufacturer protocols (Tetrapak International).

Microparticulated WP were added at increasing concentrations (1.5%, 3.0%, 4.5%, 6.0%, 7.5%, and 9.0%; vol/vol) to bulk milk. A sample without MWP (MWP = 0.0%) was used as control. Within each day of analysis, 6 replicates of MCP for each treatment were obtained, changing the position of the treatment in the rack. For the control samples, 2 replicates per day were performed. Besides MCP, WP fractions were measured on each treatment during the 3 days of analysis.

Analysis of Microparticulated Whey Protein

Microparticulated protein fractions were quantified by reversed-phase HPLC (**RP-HPLC**) after solubilization with 6 M Guanidine hydrochloride (Sigma) for 24 h. Reversed-

phase HPLC analysis was carried out in the laboratory of the Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padova (Legnaro, Italy) using Agilent 1260 Series chromatograph instrument (Agilent Technologies, Santa Clara, CA, USA), and separation was performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a Poroshell packing (5μm, 300A˚, 2.1×75 mm). Detection condition was at 214 nm. Gradient elution was carried out with a mixture of two solvents: solution A consisted of 0.1% trifluoroacetic acid and 5% acetonitrile in water, and solution B was 0.1% trifluoroacetic acid in acetonitrile. The gradient started with 95% of solution A, after 1 min the gradient was 82% of A, after 2 min it was 70%, and after 5 min A and B were in equilibrium. From 5 to 9 min (end of the run), the gradient was brought back to initial conditions (95% of A). Quantified WP were: α-Lactoalbumin (**α-LA**), β-lactoglobulin A (**β-LG$_A$**), β-lactoglobulin B (**β-LG$_B$**), bovine serum albumin (**BSA**), lactoferrin (**LF**), caseinomacropeptide (**CMP**), and proteose-peptone (**PP**).

Analysis of Milk Coagulation Properties

Aliquots of daily milk and serial percentage of MWP were mixed within each day of the trial, and kept at 4°C until the beginning of the analysis. A final volume of 10 ml for each sample was heated to 35°C in 10 min; once 35°C was reached, 200 μL of rennet (Hansen standard 190, Pacovis Amrein AG, Bern, Switzerland) diluted 1.6% with distilled water was added to milk (Pretto et al., 2013). A total of 144 measures of MCP were determined using Formagraph (Foss Electric A/S, Hillerød, Denmark). The working principle of Formagraph is based on the swing of a pendulum immersed in the milk and driven by an electromagnetic field. During coagulation the swing of the pendulum becomes smaller, and differences in the electromagnetic field are recorded (O'Callaghan et al., 2002). The output of the instrument consists of 3 measurements of MCP: rennet coagulation time (RCT, min), defined as the interval from the addition of the clotting enzyme to the beginning of coagulation; curd-firming time (k_{20}, min), which is the interval from the beginning of coagulation to the time at which the width of the graph attains 20 mm; and curd firmness (a_{30}, mm), defined as the width of the diagram 30 min after rennet addition.

Whey Samples Collection and Analysis

Whey was collected after manual cross-cut, using a bistoury, of the curd immediately after the end of MCP analysis. Whey from all replicates of the same treatment (n = 21) was combined and WP fractions were identified using RP-HPLC as previously described for

MWP analysis. Quantified WP (mg/ml) were α-LA, β-LG_A, β-LG_B, BSA, LF, CMP, and PP. Microparticulated WP losses (MWP_{losses}, %) were calculated for each fraction as:

$$MWP_{losses} = (WP_{treatment} / WP_{MWP}) * 100$$

where $WP_{treatment}$ is WP fraction in each MWP treatment sample, and WP_{MWP} is WP fraction added to milk through MWP.

Statistical Analysis

Data of MCP, WP fractions, and MWP losses were analyzed through the GLM procedure (SAS Inst. Inc., Cary, NC, USA). The linear model for MCP included the fixed effects of MWP concentration (7 levels), day of analysis (3 levels), replicate (6 levels), and interaction between MWP concentration and day of analysis, and the random effect of residual. The model for WP fractions and MWP losses included the fixed effects of MWP concentration (7 and 6 levels, respectively), and day of analysis (3 levels), and the random effect of residual. A multiple comparison of means was performed for the main effect of MWP, using Bonferroni's test ($P < 0.05$).

RESULTS

Milk Coagulation Properties

Means (SD) of fat, protein, CN, and lactose contents of raw bulk milk used during the whole trial were 2.73% (0.82), 3.70% (0.15), 2.77% (0.04), and 4.87% (0.05), respectively. Rennet coagulation time, k_{20}, and a_{30} averaged 14.7 min, 20.1 min, and 35.5 mm, respectively, and the CV was 16.3% for RCT, 14.8% for k_{20}, and 18.1% for a_{30}, indicating that the variability among MCP was similar (Table 1).

Results from the ANOVA for MCP are summarized in Table 2. The coefficient of determination was 0.80 for RCT, 0.71 for k_{20}, and 0.63 for a_{30}. The concentration of MWP, the day of analysis, and the replicate effects were highly significant ($P < 0.001$) in explaining the variation of RCT and k_{20}. Curd firmness was significantly ($P < 0.01$) affected by day of analysis and replicate, but not by the concentration of MWP ($P = 0.23$). Finally, the influence of fixed interaction effect between MWP and day of analysis was statistically negligible for all MCP ($P > 0.05$; Table 2).

Figure 1 depicts the LSM of MCP across concentrations of MWP. The increment of the amount of MWP added to milk led to longer RCT. In particular, significant differences ($P < 0.05$) were found between RCT of the control samples (13.5 min) and samples added with

3.0% (14.6 min) or more of MWP. Also, milk with 9.0% of MWP exhibited longer RCT (16.0 min) than samples added with 4.5% (14.8 min) or less of MWP ($P < 0.05$). Similar trend was observed for k_{20}, which showed the shortest time to attain 20 mm in the control samples (18.9 min) and the longest in samples with 9.0% (21.4 min) of MWP (Figure 1). Statistically significant differences ($P < 0.05$) were found between control and samples with 6.0% (20.9 min) or more of MWP, and between samples with 1.5% (19.1 min) and those with more than 7.5% (21.2 min) of MWP. Contrarily to RCT and k_{20}, no significant differences ($P > 0.05$) were detected for a_{30} across concentrations of MWP. However, the tendency was for a weaker gel moving from control (36.1 mm) to samples with 7.5% (34.9 mm) and 9.0% (35.1 mm) of MWP (Figure 1).

Whey Protein Fractions and Microparticulated Whey Protein Losses

The average quantity of WP fractions ranged from 0.03 mg/ml for LF to 3.43 mg/ml for β-LG_A, and the CV from 6.1% (β-LG_B) to 55.7% (LF; Table 1). Average losses of MWP varied from 17.37% (PP) to 92.6% (α-LA), and the CV from 3.6% (α-LA) to 24.7% (BSA; Table 1).

Results from the ANOVA for WP fractions and MWP losses are summarized in Table 3. Coefficient of determination of the models for WP fractions ranged from 0.46 (α-LA) to 0.89 (PP), and for MWP losses from 0.58 (LF) to 0.88 (β-LG_B). The day of analysis was significant ($P < 0.01$) in explaining the variability of β-LG_B, BSA, and PP, both in whey and MWP losses, and it was important to explain the variation of LF in whey and β-LG_A in MWP losses ($P < 0.01$). Microparticulated WP effect was important ($P < 0.05$) to explain the variability of CMP and PP fractions, both in whey and MWP losses. Moreover, MWP effect was important ($P < 0.05$) for β-LG_B and BSA fractions in whey, and for α-LA in MWP losses (Table 3).

Least squares means of WP fractions and MWP losses across concentrations of MWP are in Table 4. The percentage of α-LA, β-LG_A, and BSA in whey tended to decrease when moving from zero (control samples) to 9.0% of MWP in milk, but no statistical significance was observed ($P > 0.05$). Lactoferrin showed ($P > 0.05$) an inconsistent trend across MWP. A significant reduction of β-LG_B from 1.75 to 1.58 mg/ml ($P < 0.05$) was observed comparing control samples and samples added with 9.0% of MWP, whereas no differences were detected ($P > 0.05$) for concentrations of MWP between 1.5 and 7.5% (Table 4). Significant increments of CMP and PP were observed in whey when moving from zero to 9.0% of MWP ($P < 0.05$); in particular, CMP increased from 0.76 mg/ml to 1.15 mg/ml, and

PP from 0.22 mg/ml to 0.34 mg/ml. Microparticulated whey losses of α-LA, β-LG_A, β-LG_B, and BSA decreased as the concentration of MWP in milk increased, even if not significantly ($P > 0.05$; Table 4). On the contrary, microparticulated whey losses of LF, CMP, and PP were higher in milk added with high percentage of MWP; in the case of CMP and PP, the losses increased from 17.7% to 25.2%, and from 13.7% to 19.9%, respectively, when moving from control samples to samples with 9.0% of MWP ($P < 0.05$; Table 4).

DISCUSSION

Effect of Microparticulated Whey Proteins on Milk Coagulation Properties

Milk composition is the main factor that influences MCP (Politis and Ng-Kwai-Hang, 1988; Formaggioni et al., 2001). Raw bulk milk used for MCP analysis exhibited higher protein and CN percentages, and lower fat percentage than vat milk from De Marchi et al. (2008). However, mean values for protein and CN percentages of the present work were comparable to findings of Penasa et al. (2014) on milk from multi-breed dairy herds. Protein and CN contents are positively related to milk coagulation ability (Pretto et al., 2013) and are crucial in determining cheese yield (Summer et al., 2002).

Several other factors affect MCP and among them the most important are stage of lactation, breed of the cow (De Marchi et al., 2007; Penasa et al., 2014), CN genotypes (Ikonen et al., 1999; Comin et al., 2008; Penasa et al., 2010), and additive polygenic effects (Ikonen et al., 2004; Cassandro et al., 2008; Tiezzi et al., 2013). The average values of MCP from the present study are quite difficult to compare with those reported in the literature, because of the use of MWP. Control milk coagulated 0.65 min earlier than milk added with 1.5% of MWP, and a progressive deterioration of RCT was observed by increasing the percentage of MWP. A similar pattern was reported by Guinee et al. (1997) who used a commercial WP based ingredient. However, the weak gel formation in Guinee et al. (1997) was probably related to the use of modified pasteurization of milk, which increased WP denaturation. Indeed, increasing the temperature of pasteurization promotes the complex formation between denatured WP and CN, with the concomitance reduction in accessibility of the rennet to the CN (Rynne et al., 2004). Contrary to the present study, the addition of WP to buffalo milk shortened RCT in the work of Ismail et al. (2011). This was probably related to higher acidity content of WP that increased the acidity of milk. The aforementioned effect could be due to type of WP used by Ismail et al. (2011), derived from whey immediately after cheese making. Regarding k_{20}, Guinee et al. (1997) observed a reduction of

the time to attain 20 mm of firmness using MWP, which is opposite to the trend reported in our research.

Microparticulated WP is widely used in low-fat products to maintain the smoothness. The joined effect of MWP addition and heat treatment on MCP decreases the degree of CN aggregation during curd formation and may therefore have potential to improve the texture of low-fat products by imparting a softening effect for the higher moisture content (Yilsay et al., 2006; Sahan et al., 2008; Torres et al., 2011).

Effect of Microparticulated Whey Protein on Whey Protein Fractions and Microparticulated Whey Protein Losses

The identified WP, listed according to their content in whey, were β-LG_A, β-LG_B, CMP, α-LA, PP, BSA, and LF, as reported in other studies (Marshall, 1998; Casal et al., 2006). Protein fractions such as β-LG_A, β-LG_B, and α-LA showed decreasing trend with increasing concentration of MWP in milk. It is known that α-LA and β-LG can adhere to the surface of CN micelles through formation WP-k-CN complexes (Lucey et al., 1998). On the contrary, peptides do not embed in the aggregate microparticles, increasing their loss in whey, as reported in the present work for CMP and PP. It is well documented that whey composition affects aggregation of MWP (Guyomarc'h et al., 2009).

The efficiency of MWP recovery depends on the denaturation degree of WP obtained in MWP process. Denatured proteins obstruct syneresis and thus less water drains off during the cheese making process. Moreover, advantage in the use of denatured WP is that they can be inserted inertly into the pores of the CN network similarly to fat globules, while the addition of undenatured WP are lost in the whey. However, larger particles disturb the homogeneity of the network, and result in a reduction of the firmness (Hinrichs, 2001). Slightly differences were observed for MWP losses, in particular α-LA, and BSA which showed a reduction in WP loss, increasing MWP. The positive effects of β-LG on gelation properties have been recently reported by de Faria et al. (2013) confirming the higher quality of gels produced with high proportion of β-LG compared to materials produced with higher CMP. Similar results were reported by Roufik et al. (2005) including BSA and α-LA.

CONCLUSIONS

The addition of increasing concentrations of MWP to milk led to slightly prolonged RCT and k_{20}. In particular, MWP decreased the rapidity of CN aggregation during curd formation, which in turn led to delayed coagulation. No significant differences were detected

for a_{30} across concentrations of MWP even if the tendency was for a weaker curd moving from control to samples with high levels of MWP. The fractions β-LG_A, α-LA, and BSA showed a major recovery. Adjustments in cheese processing should be made when recycling MWP, in order to optimize the potential of MWP as ingredient in low-fat products. This could be achieved during coagulation process by prolonging rennet activity before cutting of the curd.

TABLES AND FIGURE

Table 1. Descriptive statistics of milk coagulation properties, whey protein fractions and microparticulated whey protein losses

Trait	N	Mean	SD	Minimum	Maximum
Milk coagulation properties[1]					
RCT, min	144	14.7	2.39	10.3	22.3
k_{20}, min	144	20.1	2.98	14.5	28.2
a_{30}, mm	144	35.5	6.44	18.0	48.0
Whey protein fractions, mg/ml					
α-Lactoalbumin	21	0.68	0.09	0.21	0.78
β-Lactoglobulin A	21	3.43	0.35	2.28	4.03
β-Lactoglobulin B	21	1.66	0.10	1.45	1.79
Bovine serum albumin	21	0.13	0.03	0.05	0.02
Lactoferrin	21	0.03	0.02	0.01	0.06
Caseinomacropeptide	21	0.94	0.16	0.73	1.27
Proteose-peptone	21	0.28	0.05	0.17	0.36
Microparticulated whey protein losses[2], %					
α-Lactoalbumin	18	92.6	3.37	85.9	97.9
β-Lactoglobulin A	18	89.1	6.67	75.0	99.6
β-Lactoglobulin B	18	59.6	3.80	54.5	67.1
Bovine serum albumin	18	65.8	16.2	38.2	81.5
Lactoferrin	18	62.4	15.2	48.7	79.1
Caseinomacropeptide	18	22.0	3.28	16.0	28.0
Proteose-peptone	18	17.4	2.81	12.4	21.7

[1]RCT = rennet coagulation time; k_{20} = curd-firming time; a_{30} = curd firmness 30 minutes after rennet addition.

[2]Calculated as: $MWP_{losses} = (WP_{treatment} / WP_{MWP}) * 100$, where $WP_{treatment}$ is WP fraction in each MWP treatment sample, and WP_{MWP} is WP fraction added to milk through MWP.

Table 2. Results from ANOVA (*F*-value and significance) for milk coagulation properties

Trait[1]	Effect				RMSE[3]	R^2
	MWP[2]	Day	Replicate	MWP*Day		
RCT, min	14.3***	157.0***	10.3***	0.47	1.17	0.80
k_{20}, min	6.9***	96.5***	5.9***	0.63	1.77	0.71
a_{30}, mm	0.23	83.8***	3.3**	0.31	4.32	0.63

[1]RCT = rennet coagulation time; k_{20} = curd-firming time; a_{30} = curd firmness 30 minutes after rennet addition.

[2]MWP = concentration of microparticulated whey proteins (vol/vol).

[3]RMSE = root mean square error.

$^{**}P < 0.01$; $^{***}P < 0.001$.

Table 3. Results from ANOVA (*F*-value and significance) for whey protein fractions and microparticulated whey protein losses

Trait	Effect		RMSE[3]	R^2
	MWP[1]	Day		
Whey protein fractions, mg/ml				
α-Lactoalbumin	1.43	0.01	0.10	0.46
β-Lactoglobulin A	1.40	2.90	0.30	0.54
β-Lactoglobulin B	3.49*	28.8***	0.05	0.87
Bovine serum albumin	3.56*	9.51**	0.02	0.77
Lactoferrin	1.10	23.2***	0.01	0.82
Caseinomacropeptide	8.4**	0.14	0.09	0.81
Proteose-peptone	12.4**	13.8**	0.02	0.89
Microparticulated whey protein losses[2], %				
α-Lactoalbumin	3.95*	1.97	2.40	0.70
β-Lactoglobulin A	2.31	23.6**	3.32	0.85
β-Lactoglobulin B	1.80	32.7***	1.71	0.88
Bovine serum albumin	1.73	9.01**	11.0	0.73
Lactoferrin	2.21	1.41	12.8	0.58
Caseinomacropeptide	5.38*	0.12	2.22	0.73
Proteose-peptone	8.84**	9.78**	1.35	0.86

[1]MWP = concentration of microparticulated whey proteins (vol/vol).

[2]Calculated as: $MWP_{losses} = (WP_{treatment} / WP_{MWP}) * 100$, where $WP_{treatment}$ is WP fraction in each MWP treatment sample, and WP_{MWP} is WP fraction added to milk through MWP.

[3]RMSE = root mean square error.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4. Least squares means of whey protein fractions and microparticulated whey protein losses across different concentrations of MWP[1]

Trait	MWP 0.0%	MWP 1.5%	MWP 3.0%	MWP 4.5%	MWP 6.0%	MWP 7.5%	MWP 9.0%
Whey protein fractions, mg/ml							
α-Lactoalbumin	0.75^{a}	0.70^{a}	0.69^{a}	0.70^{a}	0.67^{a}	0.52^{a}	0.65^{a}
β-Lactoglobulin A	3.73^{a}	3.48^{a}	3.49^{a}	3.04^{a}	3.33^{a}	3.34^{a}	3.33^{a}
β-Lactoglobulin B	1.75^{a}	1.66^{ab}	1.66^{ab}	1.65^{ab}	1.66^{ab}	1.61^{ab}	1.58^{b}
Bovine serum albumin	0.16^{a}	0.14^{a}	0.13^{a}	0.10^{a}	0.11^{a}	0.13^{a}	0.11^{a}
Lactoferrin	0.02^{a}	0.04^{a}	0.04^{a}	0.03^{a}	0.03^{a}	0.03^{a}	0.04^{a}
Caseinomacropeptide	0.76^{a}	0.81^{ab}	0.88^{ab}	0.99^{abc}	1.06^{b}	1.12^{bc}	1.15^{c}
Proteose-peptone	0.22^{a}	0.23^{ab}	0.26^{b}	0.29^{bc}	0.32^{bc}	0.32^{bc}	0.34^{c}
Microparticulated whey protein losses[2], %							
α-Lactoalbumin		95.3^{a}	93.8^{a}	95.6^{a}	92.3^{a}	90.1^{a}	88.8^{a}
β-Lactoglobulin A		88.0^{a}	87.3^{a}	84.6^{a}	91.4^{a}	91.6^{a}	91.6^{a}
β-Lactoglobulin B		58.7^{a}	58.7^{a}	59.0^{a}	58.6^{a}	60.7^{a}	61.8^{a}
Bovine serum albumin		76.0^{a}	72.2^{a}	57.3^{a}	58.6^{a}	71.5^{a}	58.9^{a}
Lactoferrin		64.1^{a}	56.5^{a}	71.0^{a}	49.2^{a}	55.2^{a}	78.7^{a}
Caseinomacropeptide		17.7	19.4^{ab}	21.8^{ab}	23.6^{ab}	24.5^{ab}	25.2^{b}
Proteose-peptone		13.7^{a}	15.7^{ab}	17.3^{ab}	18.7^{b}	18.9^{b}	19.9^{b}

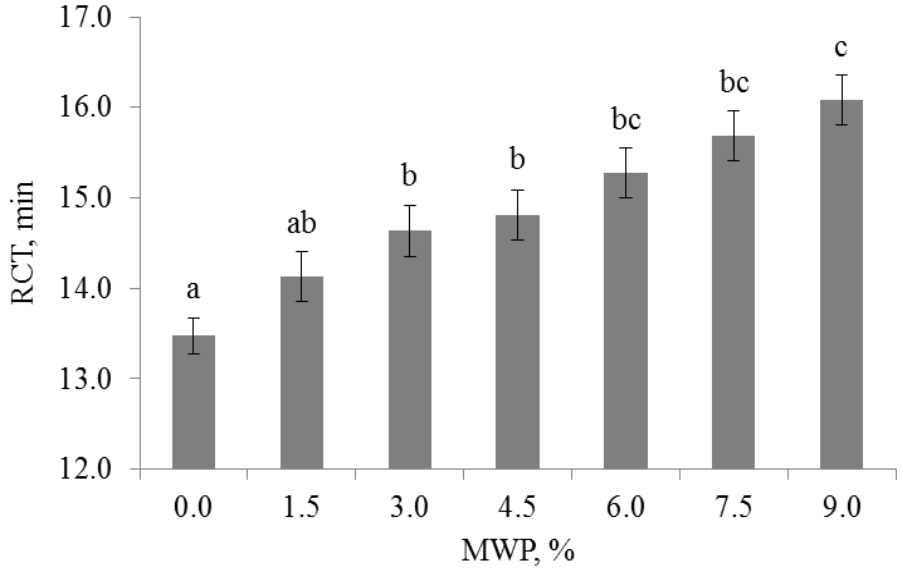
[1]MWP =concentration of microparticulated whey proteins vol/vol.

[2]Calculated as: $MWP_{losses} = (WP_{treatment} / WP_{MWP}) * 100$, where $WP_{treatment}$ is WP fraction in each MWP treatment sample, and WP_{MWP} is WP fraction added to milk through MWP.

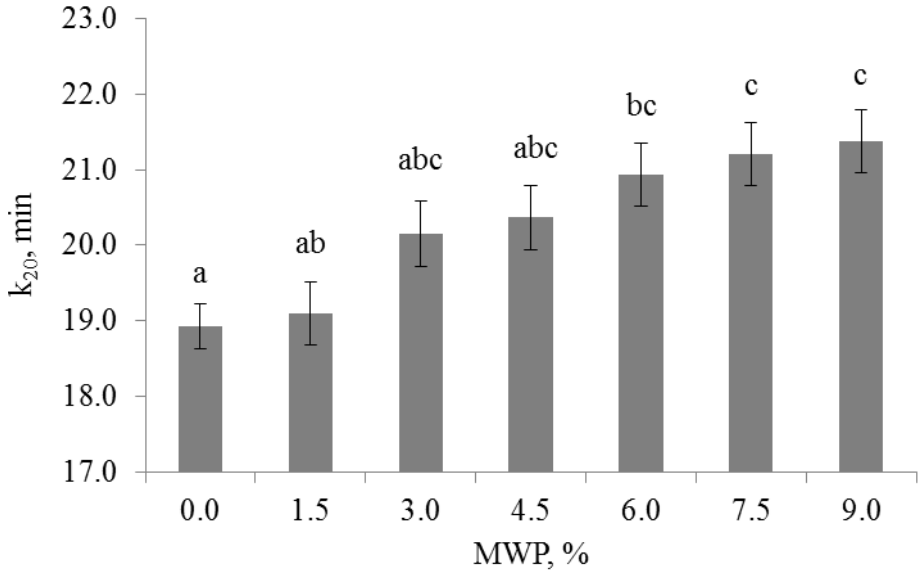
[a-c]Least squares means with different letters within a row are significantly different according to Bonferroni's test ($P < 0.05$).

Figure 1.

(A)


17.0
16.0
15.0
14.0
13.0
12.0
RCT, min
a
ab
b
b
bc
bc
c
0.0
1.5
3.0
4.5
6.0
7.5
9.0
MWP, %

(B)


23.0
22.0
21.0
20.0
19.0
18.0
17.0
k20, min
a
ab
abc
abc
bc
c
c
0.0
1.5
3.0
4.5
6.0
7.5
9.0
MWP, %

(C)

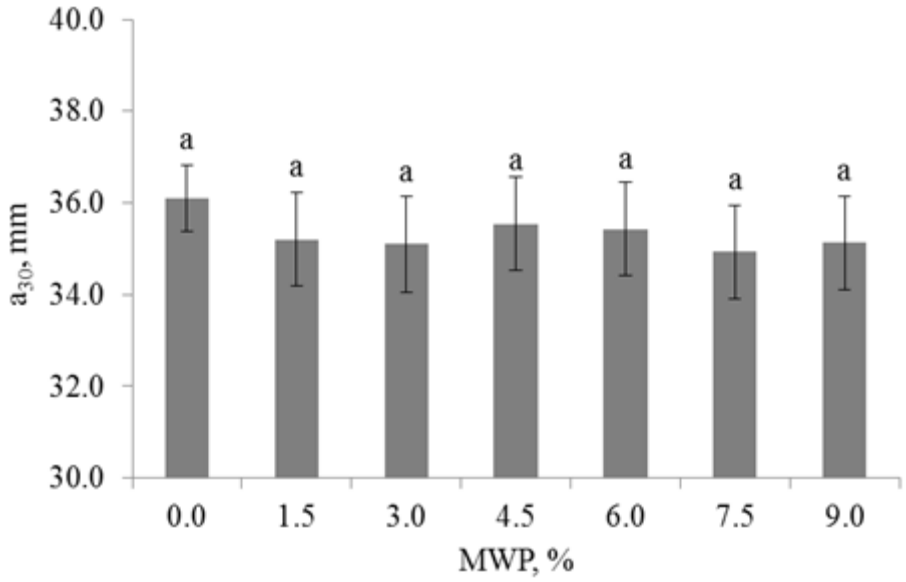


Figure 1. Least squares means (with SE) of (A) rennet coagulation time (RCT, min), (B) curd-firming time (k_{20}, min), and (C) curd firmness 30 minutes after rennet addition (a_{30}, mm) across different concentration of microparticulated whey proteins (MWP: 0.0, 1.5, 3.0, 4.5, 6.0, 7.5, and 9.0% vol/vol). $^{a\text{-}c}$Least squares means with different letters are significantly different according to Bonferroni's test ($P < 0.05$).

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III

Alba Sturaro, Massimo De Marchi, Elisa Zorzi, Martino Cassandro

EFFECT OF MICROPARTICULATED WHEY PROTEIN CONCENTRATION AND PROTEIN-TO-FAT RATIO ON CACIOTTA CHEESE YIELD AND COMPOSITION

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Effect of Microparticulated Whey Protein Concentration and Protein-to-Fat Ratio on Caciotta Cheese Yield and Composition

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ABSTRACT

Dairy industry exploits different processes to increase the value of whey and among them whey protein (WP) recovery is one of the most important applications. Microparticulated whey proteins (MWP), colloidal particles formed by controlled aggregation of WP, are widely used in low-fat products because of their ability to improve textural characteristics. Aim of the present study was to evaluate the effect of MWP concentration (0.0%, - 4.0% vol/vol) and different protein-to-fat ratio (PFR) on milk coagulation process, cheese yield and composition of Caciotta cheese using milk standardized to different protein-to-fat ratios (PFR). Samples of curd and whey were collected and analyzed after cheese making process while samples of cheese were analyzed after 10 days of ripening. Statistical analysis of the data was performed using a generalized linear model. The increment of PFR affected rennet coagulation time. Moreover, cheese yield decreased as the level of fat decreased, and it was higher in low-fat cheese (high PFR) with 4.0% MWP compared with low-fat cheese with 3.0% MWP. No differences were found for cheese yield in standard and high fat cheese (standard and low PFR) across MWP concentrations. The stable composition of low-fat Caciotta suggests the possibility to include MWP as fat replacer to maintain the yield.

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INTRODUCTION

Milk fat is important for texture (Lucey, 2004), flavour (Brauss et al., 1999) and visual characteristics (Guven et al., 2005) of dairy products. In low-fat products, whey proteins (**WP**) can be incorporated to maintain yield and nutritional value (Lo and Bastian, 1998). Because of the increasing consumer demand of products with healthy, high nutritional and environmental friendliness properties, the dairy industry recognized the value of WP (Jayaprakasha and Brueckner, 1999; Smithers, 2008). Dairy industry exploits different processes to whey treatments and in particular to recover protein fractions. Preliminary, filtration of whey to produce WP concentrate or isolate (**WPC**, **WPI**), simultaneously to physical processes as heating (Singer et al., 1990) and high pressure treatment (Dissanayake and Vasiljevic, 2009) are the main steps to obtain microparticulated WP (**MWP**).

Microparticulated WP are colloidal particles formed by controlled aggregation of WP with a particle size ranging from 0.1 to 100 μm (Spiegel and Huss, 2002). Microparticulated WP are widely used in food industry to their ability to improve sensory and textural properties in the products. Preliminary studies about MWP utilization were carried out to understand the best pre-processing of whey to obtain desirable characteristics (Singer et al., 1990; Sanchez and Paquin, 1997). In general, for dairy products as ice cream, yogurt and cheese an increase of pressure in MWP homogenization results in an increment of viscosity and consistency of the products (Lim et al., 2007; Padiernos et al., 2008; Innocente et al., 2009). In most studies registered trademark MWP was used as fat replacer (Lobato-Calleros, et al., 2001; Koca and Metin, 2004; Sahan, et al., 2008), confirming the capacity of MWP to maintain yield in low-fat products (Romeih et al., 2002; Zalazar et al., 2002). Few differences were observed on the sensory characteristics of low-fat semi-hard cheeses (Lobato-Calleros et al., 2001; Koca and Metin, 2004), while during their storage, defects were observed in fat replacer-cheese with more than 30 days (**d**) of ripening (Koca and Metin, 2004). These results indicated that MWP could be used to improve texture and sensory properties of fresh dairy products.

One of the main goal of MWP is the optimization of whey protein-to-fat ratio, because of the great variability in MWP efficiency, depending on denaturation degree of protein during MWP manufacture (Torres et al., 2011). The particle size of denatured WP aggregates plays a crucial role in fat substitution; this property has been extensively used in low-fat dairy products as ice cream (Yilsay et al., 2006; Lim et al., 2007; Innocente et al., 2009; Karaca, et al., 2009;), yogurt (Sandoval-Castilla et al., 2004; Janhoj and Ipsen, 2006; Torres et al., 2011), and cheese (Romeih et al., 2002; Koca et al., 2004; Sahan et al., 2008).

In the ice cream industry MWP has been used to improve overall sensory characteristics of low or free-fat products (Yilsay et al., 2006; AkalÂn et al., 2008; Karaca et al., 2009). Further, MWP behavior as stabilizer and textural agent provides enriched protein products with low levels of fat and stable total solid (Adapa et al., 2000; Patel et al., 2006). Similar chemical and sensory results have been found in yogurt added with increasing concentration of MWP; moreover an increment of matrix density and viscosity level were observed on low-fat yogurt added with MWP (Yazici and Akgun, 2004; Aziznia e t al., 2008; Torres et al., 2011).

First studies on cheese obtained from milk added with WP were conducted 40 years ago; several patents have been developed for the production of different types of cheese including fresh and semi-hard cheeses as reported by Czulak (1985). Recently, patents have been published to produce cheese with high texture (Clinton et al., 2004; Lindstrom et al., 2005; Ma et al., 2009), or low-fat products (Ma et al., 2008; van Arem et al., 2010) with similar coagulum hardness and chewiness of full-fat products (Lobato-Calleros et al., 2007; Jooyandeh, 2009).

There is an increasing interest of Italian dairy industry to exploit MWP in cheese making (Di Cagno et al., 2014). Moreover, a change in the nature of whey, from waste to resource, appears an attractive alternative. Whey resulting from cheese making is the most contaminant wastewater generated in dairy sector, primarily for the large volume produced during cheese manufacturing (Prazeres, et al., 2012). The interest in the valorization of whey components is related to the improvement of economic revenue for dairy industry and so, for Italian farmers.

Aim of the present study was to evaluate the effect of MWP, produced by Italian dairy industry, using standardized milk with different protein-to-fat ratios (**PFR**). The effects of MWP and PFR on milk coagulation process, cheese yield and composition were investigated on Caciotta Italian cheese produced by a mini-cheese making technique.

MATERIALS AND METHODS

Experimental design and mini cheese making procedure

Milk, cream and MWP samples were obtained from the Soligo dairy cooperative (Farra di Soligo, Italy) and milk was used without pasteurization or homogenization treatments, according to a standardize protocol. Cream and MWP were collected in the dairy cooperative at the beginning of the experiment, and they were used during each d of analysis. Aliquots of MWP and cream were stored at -20°C and at 4°C, respectively. Microparticulated

WP was extracted from total skim sweet whey produced by the dairy cooperative in a working day through the ultrafiltration process (Tetrapak International, Rubiera, Italy), using a tubular semi-permeable polyethersulfone membrane with a surface of 700 m^2, and a cut-off of 10,000 Da (Koch Membrane System, Wilmington, MA, USA) at 10°C. Moreover, microparticulation process was carried out for 10 min at 95°C by shell and tube heat exchanger and homogenization at 40 bar following the manufacturer protocols to produce a final MWP products with particle size ranged from 2 to 15 µm (Tetrapak International).

Thirty mini-cheese making were carried out in 2 different trials in July 2013. In *Trial 1* (n = 18) milk was standardized at 3.5% of proteins using 3.0% or 4.0% MWP treatment (in three d for each treatments). The effect of MWP was tested in 3 PFR (0.8, 0.9 and 1.0) conditions, by modifying fat percentage at 4.35%, 3.89%, and 3.5%, to obtain high, standard and low-fat cheese, respectively within each d.

In *Trial 2* (n = 12) standardized milk in low fat condition (3.5% of protein and fat, PFR 1.0) was tested. Protein levels were adjusted with 2.0%, 3.0% or 4.0% MWP. For each d of cheese making a control thesis without MWP was performed (0.0% MWP) in *Trial 2*.

Three replicates were carried out for each treatment and trial.

Ten liters of milk were used for each cheese making. Milk was slowly heated to 35°C by water circulation in a heating jacket using a water bath (SB24, Falc Instrument, Treviglio, Italy). Once milk reached 35°C, starter cultures of freeze-dried formulation of mixed *Streptococcus thermophiles* and *Lactobacillus bulgaricus* (MicroMilk, Crema, Italy) were added to milk as indicated by producer. Temperature was increased up to 38°C in 10 minutes; when milk reached this temperature, 15 ml of commercial liquid rennet of calf (75 chymosin: 25 bovine pepsine; De Longhi Michele and C., Treviso, Italy) in water solution (1:3) was added to vat. Continuous monitoring of milk coagulation was performed directly on vat using CoAguLite (**CL**) sensor (Reflectronics Inc., Lexington, KY, USA; Fagan et al., 2007). The sensor is composed by infrared LED (880 nm) as light source, and two optic fibre (600 µm), the first for light emission, and the other for transmitting backscattered light from milk to the photodetector (Castillo et al., 2000). The CL sensor allowed the selection of the cutting time (six minutes after the T_{max} signal). The cooking phase was performed at 40°C for 15 minutes. After draining, the curd was extracted from vat and put in mold pressed. The mold was stored in thermostat at 37°C for 3 hours until the pH lowered to 5.5. The curd was then immerged in a brine solution (1.14 $kg*l^{-1}$ of NaCl) for 1 hour. Finally, a curd sample was immediately analyzed before press phase and then curd was stored in the ripening cell for 10 d at 4 °C and 85.0 % of relative humidity.

Samples Collection, Reference Analysis and Yield

For each cheese making session, samples of milk, curd, whey and cheese (10 d of ripening) were collected.

Milk was analyzed before and after standardization using MilkoScan FT2 (Foss, Hillerød, Denmark). Composition of curd (at the end of cheese making) and cheese (10 d of ripening) was predicted for fat, protein and dry matter contents using FoodScan Infratec 1255 Food Analyzer (Foss, Hillerød, Denmark). Microparticulated WP and whey sample (50 mL) at the end of cheese making session were collected and fat, protein, lactose and total solid percentages were predicted by MilkScan FT2.

Whey protein fractions in whey sample were quantified by RP-HPLC method. Pure protein and chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Pure proteins were used for calibration, and the relation between peak area and injected amount of protein provided calibration curve (R^2 > 0.99). Pure proteins used for the calibration were: α-Lactoalbumin (**α-LA**), β-Lactoglobulin B (**β-LG_B**), β-Lactoglobulin A (**β-LG_A**), Bovine serum albumin (**BSA**), and lactoferrin (**LF**). Calibration for caseinomacropeptides (**CMP**) and proteoso peptone (**PP**) were performed with a purified fractions obtained as proposed by Mollè et al. (2006) and Paquet et al. (1988), respectively. Caseinomacropeptides and PP purified fractions were quantified by Bradford assay with spectrophotometric determination at 595 nm following the manufacturer instruction (Sigma-Aldrich). Pure proteins and whey samples were prepared as described by Bobe et al. (1998).

Agilent 1260 Series instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump, and a diode array detector was used. Analysis condition, data acquisition and processing were controlled by the Agilent Chem-Station for LC systems software. Separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a Poroshell packing (5μm, 300A˚, 2.1×75 mm). Gradient elution was carried out with a mixture of two solvents: A solution consisted of 0.1% trifluoroacetic acid and 5.0 % acetonitrile in water, and B solution was 0.1% trifluoroacetic acid in acetonitrile. The gradient started with 95.0% of A solution; after the first minute the gradient was 82.0% of A and in the second minute A was at 70.0%. After five minutes A and B solutions were in equilibrium, and at the end of the course the gradient returned at the initial condition. The flow rate was 2.5 ml/min, the column temperature was kept at 70°C and the detection was at a wavelength of 214 nm. The injection volume consisted of 2 μl.

Curd and cheese yield were expressed as percentage, obtained by the ratio of the quantity of curd (at the end of cheese making) and cheese (10 days of ripening) to the amount of standardized milk at the beginning of the cheese making, respectively. Yield of cheese fat,

protein and dry matter (%) were calculated as the ratio of cheese fat, protein and dry matter to milk fat, protein and total solids, respectively.

Statistical analysis

Data of the trials were analyzed separately (*Trial 1* and *2*) through a generalized linear model using the SAS software (SAS Institute Inc., Cary, NC, USA). The model for data of *Trial 1* included the fixed effects of MWP concentrations (2 levels), PFR (3 levels), their interaction (MWP*PFR), day of sampling nested within MWP concentration (6 levels), and the random residual. Significance of MWP effect was tested on the day of sampling nested within MWP variance.

For data of *Trial 2*, the model included the fixed effects of day of sampling (3 levels) and MWP (4 levels), and the random effect of residual.

A multiple comparison of means was performed for the effect of MWP using Bonferroni's correction ($P \leq 0.05$) to determine differences between means of coagulation properties, whey composition, curd and cheese composition and yield.

RESULTS

Characterization of Milk and Microparticulated whey protein used during standardization

Milk before standardization had a stable composition in fat, protein, casein, and lactose contents, with average values (standard deviation, **SD**) of 3.87% (0.07%), 3.48 (0.01), 2.61% (0.04), and 4.82% (0.05) in *Trial 1*, and 3.82% (0.07), 3.30 (0.04), 2.53% (0.03), and 4.86% (0.01) in *Trial 2*, respectively.

Microparticulated WP used for standardization of milk in *Trial 1* had a composition in fat, protein, lactose, total solid and pH of 0.73%, 6.32%, 5.29%, 11.68%, and 6.58, respectively; while in *Trail 2* the MWP composition was 0.75%, 6.42%, 5.42%, 12.09% and 6.60, respectively.

Effect of Microparticulated whey protein and Protein-to-Fat ratio on Milk Coagulation Properties (Trial 1 and Trial 2)

Descriptive statistics of rennet coagulation time (**RCT**: time from rennet addition and the point of maximum coagulation detected by CL) of *Trial 1* and *Trial 2* are summarized in Table 1. Average (SD) of RCT were 13.76 min (1.84) and 15.28 (1.36) for *Trial 1* and *2*, respectively. The CV were 13.4% (*Trial 1*) and 8.9% (*Trial 2*). Results from the analysis of

variance of RCT are reported in Tables 2 (*Trial 1*) and 3 (*Trial 2*). Protein-to-fat ratio and MWP*PFR effects were significant ($P < 0.05$) in explaining the variability of RCT in *Trial 1*. Effects of MWP and d of analysis were not statistically significant for RCT in both trials.

In *Trial 1* least squares means (**LSM**) of RCT for PFR effect in high, standard and low levels of fat were 11.93 min, 14.68 min and 14.66 min, respectively. An elongation of RCT was observed with decreasing fat levels (i.e., increasing PFR). Significant differences ($P < 0.05$) were observed between the high fat levels (PFR 0.8) and the standard and low fat levels (PFR of 0.9 and 1.0, respectively). Least squares means of RCT for MWP effect did not show any statistically significant differences across MWP treatments in both trials.

Effect of Microparticulated whey protein and Protein-to-Fat ratio on Chemical Composition and Yield of cheese (Trial 1 and Trial 2)

Descriptive statistics of curd composition and yield, and cheese composition and yield for *Trial 1* and *Trial 2* are summarized in Table 1. The expected variability of experimental design was confirmed: indeed, greater variability was reported for cheese composition in *Trial 1*, in which different PFR were tested, while slight reduced variability was reported in *Trial 2*, in which only low fat condition was select. Average (SD) composition of cheese in *Trial 1* was 28.87% (1.50), 22.76% (1.77), and 50.37% (2.06), while in *Trial 2* it was 25.08% (1.00), 24.06% (0.73), and 50.85% (1.12) for fat, protein and dry matter, respectively. Mean and SD of curd and cheese yields between the trials were similar. Results from analysis of variance for curd and cheese chemical composition and yield of *Trial 1* and *Trial 2* are reported in Tables 2 and 3, respectively. The coefficient of determination of the model ranged from 0.65 (cheese yield proteins) to 0.90 (curd dry matter) in *Trial 1*, and from 0.26 (curd yield) to 0.85 (curd proteins) in *Trial 2*. Day of cheese making effect was statistically significant ($P < 0.05$) for curd proteins and dry matter, and fat yield in cheese ($P < 0.01$) in *Trial 1*, while this effect was not significant for all the studied traits in *Trial 2*. In *Trial 1*, PFR effect was statistically significant ($P < 0.05$) for curd and cheese fat and yield, and proteins in cheese. Concerning the curd and cheese chemical composition, LSM of cheese fat across PFR levels were 28.48% (PFR 0.8), 26.52% (PFR 0.9), and 25.61% (PFR 1.0), respectively. Statistically significant differences in cheese fat were reported between high level of fat (PFR 0.8) and standard and low levels of fat (PFR 0.9 and 1.0, respectively) according to the experimental design. Moreover, according to the PFR effect, the fat reduction provided a worsening in curd and cheese yield ($P < 0.05$).

Microparticulated WP effect was significant in explaining the variability of curd yield and curd proteins in *Trial 1* (Table 2) and *Trial 2* (Table 3), respectively. An increment of curd yield was reported in *Trial 1* ($P < 0.05$) across MWP treatments (Data not shown); similar trend was reported in *Trial 2*, even if not significant differences were found. In *Trial 2*, curd proteins were influenced by MWP quantity; an increasing trend was observed when MWP was added (Table 4).

Least squares means of cheese composition in *Trial 1* for MWP effect were 26.62% and 27.12%, 22.03% and 23.48%, 51.34% and 49.38% for fat, protein and dry matter, respectively, with 3.0% and 4.0% of MWP. Least squares means of cheese yield ranged from 11.29% (3.0% MWP) to 11.15% (4.0% MWP) in *Trial 1*, and from 11.10% (0.0% MWP) to 11.36% (2.0% MWP) in *Trial 2* (Table 4). Moreover, no differences were observed in fat, protein and dry matter recovery in both trials across MWP treatments.

Effect of Microparticculated whey protein and Protein-to-Fat ratio on Whey Composition and Whey Protein Fractions (Trial 1 and Trial 2)

Descriptive statistics of whey composition and protein fractions for *Trial 1* and *Trial 2* are reported in Table 5. Average (SD) fat, protein, lactose and total solid in *Trial 1* were 0.95% (0.16), 1.00% (0.03), 5.07% (0.06), and 8.10% (0.19), respectively, and in *Trial 2* they were 0.81% (0.16), 0.99% (0.04), 5.14% (0.03), and 7.97% (0.18), respectively. The variation in total solid was explained by the variation in fat percentage. The limited variability of total proteins was also confirmed in the quantification of WP fractions by HPLC method.

Results from analysis of variance for whey composition and WP fractions are reported in Table 6 (*Trial 1*) and Table 7 (*Trial 2*). The day of cheese making was not significant effect for all the studied traits, with the exception of PP and BSA ($P < 0.05$) in *Trial 1* and *Trial 2*, respectively. Moreover, in *Trial 1*, PFR effect resulted significant for LF ($P < 0.05$). Microparticulated WP effect was statistically significant ($P < 0.05$) in explaining the variation of whey composition, in particular fat, protein and total solid in *Trial 1*, and protein ($P < 0.01$) in *Trail 2*. Concerning protein fractions composition, MWP ($P < 0.05$) affected BSA in *Trial 1* and CMP in *Trial 2*. Least squares means of whey composition and protein fractions across MWP concentration are presented in Table 8. A statistically significant ($P < 0.05$) increment of fat (from 0.90% to 1.06%), protein (from 0.98% to 1.03%), and total solids (from 7.98% to 8.21%) by increasing MWP content (from 3.0% to 4.0% MWP) was observed in whey of *Trial 1,* while no differences were reported in whey lactose content. Statistically significant variation of protein losses was observed in the whey generated by MWP cheese production in

Trial 2. The increment of protein loss across MWP concentration were also confirmed in WP fractions in both trials, where BSA and LF increased across MWP treatments in *Trial 1*; moreover, in *Trial 2*, CMP reported the higher value in 4.0% MWP treatmentand the lowest in 0.0% MWP.

Effect of Interaction between Microparticulated whey protein and Protein-to-Fat ratio on Milk Coagulation Properties, Chemical Composition and cheese Yield, and Whey Composition (Trial 1)

Results from analysis of variance for the MWP * PFR interaction effect are reported in Table 2 for RCT, chemical composition and yield of Caciotta cheese, and in Table 5 for whey composition. Interaction effect was highly significant in explaining the variability of RCT ($P < 0.01$) and LF in WP fractions ($P < 0.05$).

Fig. 1 depicts LSM of (A) RCT (min), (B) fat percentage in curd, (C) curd yield, (D) protein percentage in cheese, (E) fat percentage in cheese and (F), and cheese yield for the interaction effect. A worsening of RCT was found by reducing the fat level (i.e., increasing PRF); the best value was reported in high fat condition (PFR 0.80) with 4.0% MWP (10.53 min), while no statistically significant differences were reported comparing standard (14.30 min and 15.23 min) and low (14.13 min and 15.20 min) fat levels across MWP concentration (from 3.0% to 4.0% MWP, respectively).

Concerning curd composition and yield, no differences were found for protein percentage and dry matter and their yield. The interaction effect between MWP and PFR was statistically significant for curd fat and yield as depicted in Fig. 1 (B and C). As expected, differences in curd fat content were reported across PFR levels: high fat values were found for high fat condition (PFR 0.8: 20.36% and 20.43% in 3.0% and 4.0% MWP, respectively), whereas no differences were observed across MWP concentration. Moreover, a worsening in curd yield by reducing fat contents has been obtained, while an increment was observed in 4.0% compared to 3.0% of MWP. The better value was in 4.0% MWP (21.99%) in standard concentration of fat (PFR 0.9), while the worst value was in low-fat product (PFR 1.0) with 3% of MWP (19.76%). Intermediate values were in standard and low fat condition (PFR 0.9 and 1.0, respectively) with 4.0% MWP.

The interaction effect was significant for fat, proteins and cheese yield. A reduction of fat content was detected by increasing PFR level, while no significant differences were observed across MWP concentrations (Fig. 1 E). Moreover, an increasing content in protein percentage was observed by increasing MWP; the highest proteins content was found for PFR

of 1.0 (24.20%) with 4.0% MWP, while the lowest for PFR of 0.8 (20.88%) in 3.0% MWP (Fig. 1 D). A worsening in cheese yield was observed by decreasing the fat and MWP contents (Fig. 1 F). The lowest value of cheese yield was recorded in low fat product with 3.0% MWP (10.52%), while low fat cheese with 4.0% MWP (10.64%) did not report a significant difference in cheese yield compared to standard-fat products (11.0%).

DISCUSSION

Effect of Microparticulated whey protein and Protein-to-Fat ratio on Milk Coagulation Properties

During the last decade, several studies have been focused on milk coagulation traits as reported by Pretto et al. (2013). Recently, Sturaro et al. (2014) evaluated the effect of MWP on coagulation traits using the Formagraph; those authors found that the excessive MWP addition to milk led to slightly prolonged RCT, while no studies have been carried out on the effect of whey protein and milk coagulation traits during cheese production.

Differences between studies on milk coagulation traits are due to the type of coagulant and the different detection instrument used, as reported by Pretto et al. (2011). Indeed, in the present study CL sensor, based on diffuse reflectance technology, was used to monitor the coagulation, as proposed by Payne et al. (1993). Diffuse reflectance technology offers the possibility to measure changes of diffuse reflectance during the coagulation. As reported by several authors, T_{max} recorded by the sensor represents the maximum of the first derivative of the reflectance that is strongly correlated to evolution of casein micelle size during the aggregation (Payne et al., 1993). Nevertheless, coagulation time found in our study is close to that reported by Pretto et al. (2013) for Grana Padano cheese; the latter authors measured milk coagulation traits using the Formagraph. Furthermore, the relationship between milk composition and coagulation traits is well known (Summer et al., 2002); fat reduction prolongs coagulation time (Auldist et al., 2004; Arango et al., 2013) as confirmed in the present study (*Trial 1*).

An increment in gel time was reported by Guinee et al. (1997) using 1.0% w/w of commercial fat replacer in high pressure homogenized milk with a great level of denatured whey protein. In *Trial 1* and *Trial 2* no effect of MWP addition (from 2.0% to 4.0% MWP) was detected for RCT, probably for the low levels in denaturation degree of WP. Indeed, preliminary pasteurization or homogenization treatments were not performed in cheese making standard protocol. A significant increment in coagulation time was reported in pre-processed milk at great temperature and pressure (Guinee et al., 1998; Hayes and Kelly, 2003)

and in concomitance with fat replacer addition (Guinee et al., 1997). Also, Rynne et al. (2004) confirmed the worsening of rennet coagulation traits and an increasing of WP denaturation with growing pasteurization temperature. The stability in RCT in *Trial 1* and *Trial 2* using different concentrations of MWP (from 2.0% to 4.0% MWP) confirmed that the WP concentrate treatment provided an optimal denaturation degree to maintain the coagulation ability.

Effect of Microparticulated whey protein and Protein-to-Fat ratio on Cheese Composition and Yield

Microparticulated WP have been widely used as fat replacer in ice cream (Karaca et al., 2009), yogurt (Yazici and Akgun, 2004), and cheese (Koca and Metin, 2004). To our knowledge, no studies are known about the addition of MWP to high-fat products. In the present study, the average composition of high-fat Caciotta produced adding MWP, was relatively close to semi-ripened Caciotta proposed by Di Cagno et al. (2011), or Emmental cheese (Ikonen and Ruottinen, 1999) without MWP addition. Instead, the majority of studies have been performed to evaluate the efficiency of MWP retention in low-fat cheese (Lobato-Calleros et al., 2001; Lobato-Calleros et al., 2007; Jooyandeh, 2009; Di Cagno et al., 2014). Standard (PFR 0.90) and low (PFR 1.00) fat Caciotta after 10 d of ripening showed a similar fat content of Caciotta at 14 d of ripening proposed by Di Cagno et al. (2011). Compared to the present study, Di Cagno et al. (2014) reported greater protein percentage and moisture level in low-fat Caciotta with 0.50% $w*vol^{-1}$ of MWP; nevertheless, a lower level of fat was used by Di Cagno et al. (2014) in cheese manufacturing. The negative correlation between protein and fat was observed in other studies (Gilles and Lawrence, 1985; Guinee, Auty, and Fenelon, 2000). Moreover, PFR strongly affected cheese yield and the mass balances, as confirmed in several studies (Fenelon and Guinee, 1999; Guinee et al., 2007). Recovery of fat, protein and dry matter with 3.0% or 4.0% MWP were similar, and no effect was reported for MWP addition, but the variability was explained by different PFR. The variation in cheese composition, fat recovery and cheese yield was confirmed using different PFR conditions by Guinee et al. (2007).

Furthermore, MWP at 3.0% or 4.0% were tested in high, standard and low fat conditions. In several studies the increment of MWP is related to an increasing of protein percentage in low-fat cheese (Lobato-Calleros et al., 2000; Lobato-Calleros et al., 2007; Jooyandeh, 2009) confirming our results not only in low-fat Caciotta, but also in high and standard fat conditions. The average composition of Caciotta cheese after 10 d of ripening

exhibited similar moisture and protein percentage compared to full-fat Caciotta after 60 d of ripening reported by Di Cagno et al. (2011). Higher moisture contents in cheese with fat replacer was explained for different types of low fat cheeses, as fresh Iranian White cheese (Jooyandeh, 2009) and Caciotta (Di Cagno et al., 2011, 2014). In the present work, statistically difference was not found for cheese moisture using 3.0% or 4.0% MWP in different fat conditions, but a decreasing trend in dry matter content was observed by increasing MWP. No MWP effect was reported in cheese yield in high (PFR 0.80) and standard fat (PFR 0.90) condition, whereas an improvement in cheese yield was reported using 4.0% MWP in low-fat products (Fig. 1). Average cheese yield was similar to Caciotta with MWP (Di Cagno et al., 2014) but also to other cheeses without MWP addition (Rudan et al., 1998; Lanciotti et al., 2006).

The average chemical composition of low-fat Caciotta manufactured with 2.0%, 3.0% and 4.0% MWP agrees with other cheeses produced with or without fat replacer (Zalazar et al., 2002; Kavas et al., 2004). The increment in protein content adding WP was reported in several studies (Drake et al., 1996; Lobato-Calleros et al., 2000; Lobato-Calleros et al., 2007; Jooyandeh, 2009;). In the present study the improvement in protein percentage was recorded in curd, but not statistically difference was reported in ripened cheese. During ripening a loss of proteins and moisture have occurred. Indeed, contradictory results in dry matter and moisture content were reported in the literature. In the present study, no differences in dry matter were observed, in agreement with several studies on fresh cheeses (Lobato-Calleros et al., 2007; Jooyandeh, 2009), while a moisture increment with MWP addition was reported in other cheeses with longer ripening time (Skeie et al., 2013; Di Cagno et al., 2014). As expected, fat content did not show any statistical differences between control, 2.0%, 3.0% and 4.0% MWP. In low-fat products without fat replacer a worsening in yield was reported in several studies (Romeih et al., 2002; Sahan et al., 2008; Di Cagno et al., 2014;). Schreiber et al. (1998) reported an improvement in cheese yield using 0.5% MWP, simultaneously to an increment in cheese moisture content. In the present study, an increasing yield trend was found in the curd, but not differences in cheese yield and moisture content were observed increasing MWP. Likely, during ripening time an increment of water loss occurred. Microparticulated WP did not influence the recovery of fat, protein and dry matter. The percentage of MWP tested resulted well incorporated into casein micelle without negative effect on chemical composition. Moreover, the absence of pasteurization can be a positive effect, indeed an excessive WP addition is likely to interfere with curd formation as reported by Guinee et al. (1998) because of the formation of the β-Lactoglobulin-K-casein at the micelle surfaces, at temperature over 70°C, which sterically impede fusion of the rennet.

Effect of Microparticulated whey protein on Whey Composition

The ability of MWP to be retained into the pores of the curd strongly depends on the denaturation level of WP and on the ratio between native WP (Walstra and van Vliet, 1986). Moreover, the ability of fat, protein and total solid to recovery within the coagulum depends on WP treatments or cheese manufacturing approaches (Banks et al., 1994; Dybing and Smith, 1998). In this study, a significant increment of proteins in whey was observed but no differences in whey total solid contents were found in low-fat product (*Trial 2*). Mc Mahon et al. (1996) reported an increment of fat and protein losses when MWP was used as fat replacer: this confirms the loss of protein (especially CMP) found in present and previously studies (Sturaro et al., 2014). Caseinmacropeptide is a hydrophobic peptide released in the whey from k-casein during the renneting process. The average concentration of WP fraction agrees with that reported by Madureira et al. (2007).

In the current study not registered trademark MWP was used, but the microparticulation process provided aggregated WP that were incorporated into casein coagula. Previous results obtained with diluted WP solutions showed that the presence of more than 3.50% microparticles accelerated the thermal aggregation of WP, and MWP sized between 1 and 10 μm are integrated inertly into the structure (Banks et al., 1994). The protocol of MWP production allowed the production of aggregates that can be incorporated into curd, confirming the results on milk coagulation properties. The advantages of incorporating WP into cheese are to enhance nutritional value, increase the income of dairy industry, and stabilize the cheese yield, especially in low fat cheese.

CONCLUSIONS

The application of MWP in Caciotta Italian cheese has been investigated demonstrating different efficiency in MWP retained across different PFR levels. A worsening in milk coagulation properties and a reduction of cheese yield has been observed decreasing milk fat content. The interactions between MWP and PFR showed interesting results; in particular no differences in cheese yield were observed in high and standard fat cheeses with 3.0% or 4.0% MWP, while an increment in cheese yield was reported in low fat cheeses with 4.0% MWP. No differences in milk coagulation traits, cheese composition and yield was reported comparing low-fat products with 2.0%, 3.0% and 4.0% MWP and a control low-fat without fat replacer. The stability in low-fat Caciotta composition suggests the possibility to include MWP as fat replacer to maintain the yield; moreover it underlines the possibility for specialized cheese industry to recycling whey by-products.

TABLES AND FIGURES

Table 1. Descriptive statistics of rennet coagulation time (RCT), curd chemical composition and yield, cheese chemical composition and cheese yield in *Trial 1* and *Trial 2*

Trait	*Trial 1*		*Trial 2*	
	Mean	SD[b]	Mean	SD[b]
RCT[a] (min)	13.76	1.84	15.28	1.36
Curd composition and yield (%)				
Fat	19.50	1.28	18.89	0.94
Protein	15.69	1.40	16.21	0.77
Dry matter	35.19	1.58	35.10	1.14
Yield	20.93	0.92	20.55	0.55
Cheese composition (%)				
Fat	26.87	1.50	25.08	1.00
Protein	22.76	1.77	24.06	0.73
Dry matter	50.37	2.06	50.85	1.12
Cheese yield (%)				
Total	11.22	0.67	11.23	0.26
Fat	77.32	2.95	80.40	2.59
Protein	73.15	3.33	77.36	3.05
Dry matter	42.86	3.31	44.46	1.68

[a]RCT = difference between time to enzyme add and time to the maximum R' of light backscatter of Coagulite Instrument.

[b]SD = Standard Deviation.

Table 2. Results from ANOVA (F-value and significance) of rennet coagulation time (RCT), curd chemical composition and yield, cheese chemical composition and yield in *Trial 1*, for data of production nested within microparticulated whey protein (Date(MWP)), microparticulated whey protein (MWP), protein (3.5%) - to - fat ratio (PFR), and interaction between percentage of MWP and PFR (MWP*PFR) effects

Trait	Effect				RMSE[b]	R^2
	Date(MWP)	MWP	PFR	MWP*PFR		
RCT[a] (min)	1.62	0.15	17.92**	8.99**	0.91	0.88
Curd composition and yield (%)						
Fat	2.75	0.69	15.64**	2.38	0.70	0.86
Protein	4.15*	0.01	2.44	1.02	0.10	0.75
Dry matter	15.04**	0.10	3.89	1.63	0.72	0.90
Yield	0.58	14.55*	6.07*	2.20	0.64	0.77
Cheese composition (%)						
Fat	2.40	0.80	22.03**	0.71	0.76	0.88
Protein	2.62	2.92	7.46*	0.82	1.11	0.81
Dry matter	1.88	2.99	0.47	0.68	1.76	0.66
Cheese yield (%)						
Total	2.91	0.21	16.8**	1.00	0.36	0.85
Fat	4.05*	0.02	0.44	1.13	2.33	0.70
Protein	2.07	2.83	0.04	0.32	2.87	0.65
Dry matter	2.38	26.81	1.55	1.20	2.69	0.69

[a]RCT = difference between time to enzyme add and time to the maximum R' of light backscatter of Coagulite Instrument.

[b]RMSE = root mean square error.

*$P < 0.05$; **$P < 0.01$

Table 3. Results from ANOVA (F-value and significance) of rennet coagulation time (RCT), curd chemical composition and yield, cheese chemical composition and yield in *Trial 2*, for data of production (Date) and microparticulated whey protein (MWP) effects

Trait	Effect		RMSE[b]	R^2
	Date	MWP		
RCT[a] (min)	0.65	1.66	1.28	0.51
Curd composition and yield (%)				
Fat	4.65	0.2	1.45	0.62
Protein	3.34	9.26*	0.40	0.85
Dry matter	1.33	0.6	1.16	0.42
Yield	0.02	0.69	0.64	0.26
Cheese composition (%)				
Fat	4.93	2.01	0.71	0.73
Protein	0.29	1.06	0.77	0.38
Dry matter	2.28	4.12	0.78	0.74
Cheese yield (%)				
Total	1.47	0.50	0.26	0.43
Fat	2.49	1.31	2.14	0.80
Protein	0.79	0.48	3.37	0.33
Dry matter	2.60	2.11	1.32	0.66

[a]RCT = difference between time to enzyme add and time to the maximum of R' light backscatter of Coagulite Instrument.

[b]RMSE = root mean square error.

*$P < 0.05$.

Table 4. Least squares means of rennet coagulation time (RCT), curd chemical composition and yield, cheese chemical composition, and cheese yield across different concentrations of MWP[a] in *Trial 2*

Trait	Control	MWP 2.0%	MWP 3.0%	MWP 4.0%
RCT[b] (min)	14.16^{a}	15.70^{a}	14.90^{a}	16.34^{a}
Curd composition and yield (%)				
Fat	19.33^{a}	18.20^{a}	19.50^{a}	18.50^{a}
Protein	15.23^{a}	16.56^{b}	16.18^{ab}	16.87^{b}
Dry matter	35.57^{a}	34.77^{a}	35.68^{a}	35.40^{a}
Yield	20.86^{a}	20.74^{a}	20.20^{a}	20.40^{a}
Cheese composition (%)				
Fat	25.66^{a}	24.60^{a}	25.50^{a}	24.56^{a}
Protein	24.60^{a}	24.23^{a}	23.86^{a}	23.53^{a}
Dry matter	49.73^{a}	51.16^{a}	50.63^{a}	51.90^{a}
Cheese yield (%)				
Total	11.10^{a}	11.36^{a}	11.23^{a}	11.26^{a}
Fat	81.43^{a}	79.80^{a}	81.82^{a}	79.78^{a}
Protein	78.32^{a}	78.74^{a}	76.74^{a}	75.87^{a}
Dry matter	43.06^{a}	45.25^{a}	44.18^{a}	45.48^{a}

[a]MWP = microparticulated whey proteins.

[b]RCT = difference between time to enzyme add and time to the maximum R' of light backscatter of Coagulite Instrument.

[a-b]Least squares means with different letters within a row are significantly different according to Bonferroni's correction ($P < 0.05$).

Table 5. Descriptive statistics of whey composition and protein fractions in *Trial 1* and *Trial 2*

Trait, %	*Trial 1*		*Trial 2*	
	Mean	SD[c]	Mean	SD[c]
Whey components ([a]w*w^{-1})				
Fat	0.95	0.16	0.81	0.16
Proteins	1.00	0.03	0.99	0.04
Lactose	5.07	0.06	5.14	0.03
Total Solid	8.10	0.19	7.97	0.18
Whey protein fractions ([b]w*v^{-1})				
α-lactalbumin	0.08	0.036	0.07	0.067
β-lactoglobulin A	0.30	0.019	0.29	0.259
β-lactoglobulin B	0.14	0.080	0.15	0.139
Bovin serum albumin	0.01	0.002	0.01	0.016
Lactoferrin	0.001	0.0003	0.002	0.0002
Caseinmacropeptide	0.06	0.008	0.05	0.058
Proteose-peptone	0.03	0.003	0.02	0.010

[a]w*w^{-1} = g * 100 g^{-1} of whey; [b]w*v^{-1}= g * 100 ml^{-1} of whey; [c]SD = Standard Deviation.

Table 6. Results from ANOVA (F-value and significance) of whey composition and protein fractions in *Trial 1*, for data of production nested within microparticulated whey protein (Date(MWP)), microparticulated whey protein (MWP), protein (3.5%) - to - fat ratio (PFR), and interaction between percentage of MWP and PFR (MWP*PFR) effects

Trait	Effect				RMSE[c]	R^2
	Date(MWP)	MWP	PFR	MWP*PFR		
Whey components ([a]w*w^{-1})						
Fat	0.79	7.71*	3.48	0.09	0.13	0.67
Protein	0.83	28.19*	1.20	1.58	0.02	0.80
Lactose	0.86	0.97	0.88	0.86	0.06	0.49
Total Solid	0.54	15.94*	1.40	0.04	0.16	0.63
Whey protein fractions ([b]w*v^{-1})						
α-lactalbumin	0.24	0.79	0.51	0.10	0.05	0.23
β-lactoglobulin A	0.60	1.03	0.78	0.88	0.20	0.44
β-lactoglobulin B	0.96	0.05	0.63	0.13	0.08	0.40
Bovin serum albumin	2.99	7.22*	2.45	0.06	0.01	0.83
Lactoferrin	2.13	5.03	7.17*	5.84*	0.001	0.85
Caseinmacropeptide	3.30	0.03	1.54	0.57	0.06	0.69
Proteose-peptone	5.22*	0.08	1.88	1.69	0.02	0.78

[a]w*w^{-1}= g * 100 g^{-1} of whey; [b]w*v^{-1} = g * 100 ml^{-1} of whey.

[c]RMSE = root mean square error.

*P < 0.05; **P < 0.01.

Table 7. Results from ANOVA (F-value and significance) of whey composition and protein fractions in *Trial 2*, for data of production (Date) and microparticulated whey protein (MWP) effects

Trait	Effect		RMSE[c]	R^2
	Date	MWP		
Whey components ([a]w*w^{-1})				
Fat	0.10	2.19	0.14	0.53
Proteins	0.62	11.70**	0.02	0.86
Lactose	0.02	1.50	0.03	0.43
Total Solid	0.14	3.63	0.14	0.65
Whey protein fractions ([b]w*v^{-1})				
α-lactalbumin	2.47	1.89	0.05	0.64
β-lactoglobulin A	0.36	1.02	0.27	0.38
β-lactoglobulin B	1.19	1.16	0.13	0.49
Bovin serum albumin	10.56*	1.73	0.009	0.81
Lactoferrin	0.01	1.22	0.002	0.37
Caseinmacropeptide	1.04	5.56*	0.04	0.76
Proteose-peptone	2.75	1.69	0.08	0.64

[a]w*w^{-1} = g * 100 g^{-1} of whey; [b]w*v^{-1} = g * 100 ml^{-1} of whey.

[c]RMSE = root mean square error.

*$P < 0.05$; **$P < 0.01$.

Table 8. Least squares means of whey composition and protein fractions across different concentrations of microparticulated whey protein (MWP) in *Trial 1* and *Trial 2*

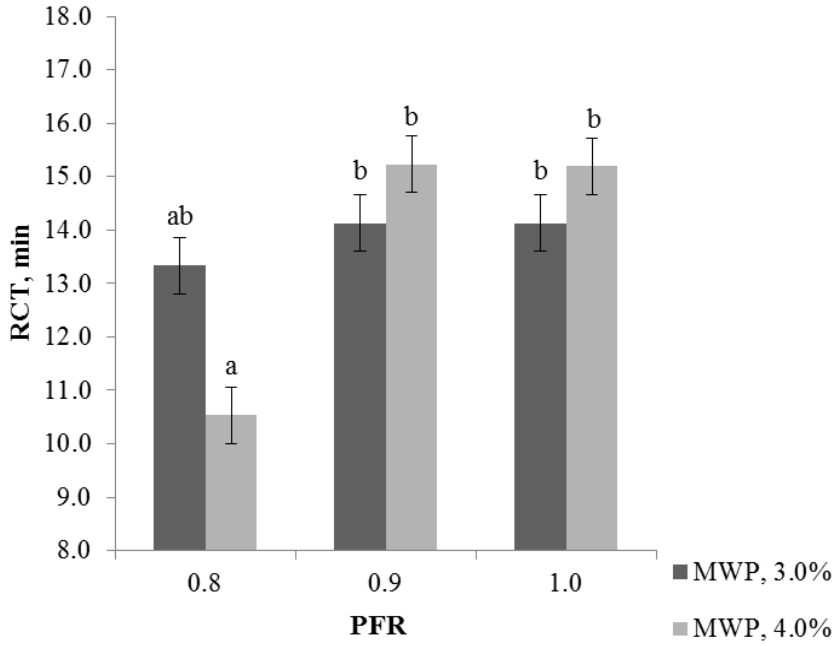
Trait	*Trial 1*		*Trial 2*			
	MWP 3.0%	MWP 4.0%	Control	MWP 2.0%	MWP 3.0%	MWP 4.0%
Whey components ([a]w*w^{-1})						
Fat	0.90^{a}	1.06^{b}	0.64^{a}	0.85^{a}	0.81^{a}	0.94^{a}
Proteins	0.98^{a}	1.03^{b}	0.93^{a}	1.02^{b}	1.02^{b}	1.03^{b}
Lactose	5.06^{a}	5.08^{a}	5.16^{a}	5.11^{a}	5.16^{a}	5.13^{a}
Total Solid	7.98^{a}	8.21^{b}	7.74^{a}	8.00^{a}	7.99^{a}	8.12^{a}
Whey protein fractions ([b]w*v^{-1})						
α-lactalbumin	0.074^{a}	0.075^{a}	0.078^{a}	0.07^{a}	0.069^{a}	0.070^{a}
β-lactoglobulin A	0.290^{a}	0.300^{a}	0.300^{a}	0.29^{a}	0.271^{a}	0.308^{a}
β-lactoglobulin B	0.141^{a}	0.140^{a}	0.160^{a}	0.15^{a}	0.140^{a}	0.153^{a}
Bovin serum albumin	0.0142^{a}	0.0164^{b}	0.013^{a}	0.01^{a}	0.012^{a}	0.013^{a}
Lactoferrin	0.0018^{a}	0.002^{b}	0.002^{a}	0.002^{a}	0.002^{a}	0.002^{a}
Caseinmacropeptide	0.057^{b}	0.059^{b}	0.044^{a}	0.05^{ab}	0.05^{ab}	0.056^{b}
Proteose-peptone	0.025^{a}	0.026^{a}	0.031^{a}	0.02^{a}	0.02^{a}	0.023^{a}

[a]w*w^{-1} = g * 100 g^{-1} of whey; [b]w*v^{-1} = g * 100 ml^{-1} of whey.

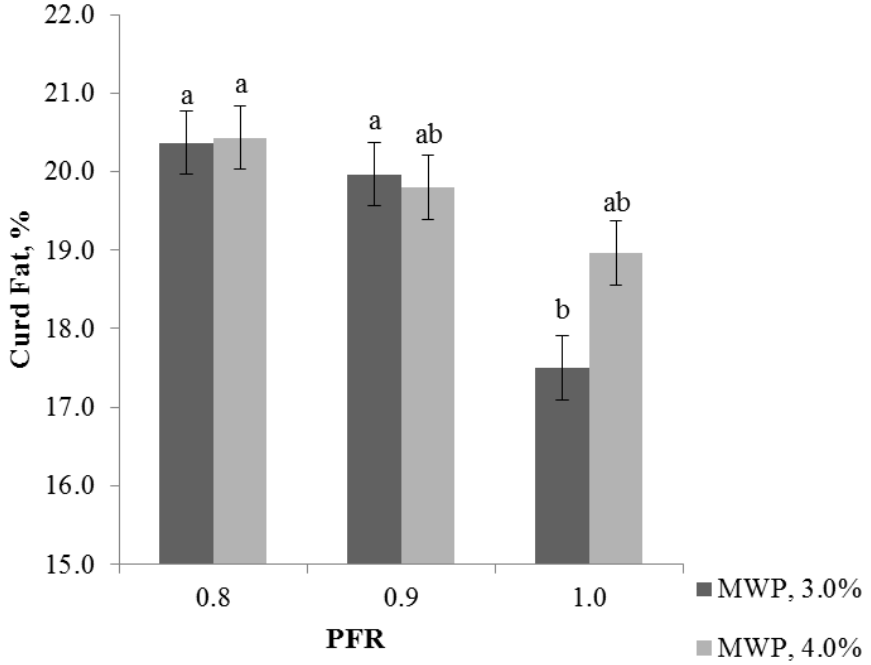
[a-b]Least squares means with different letters within a row are significantly different according to Bonferroni's correction ($P < 0.05$).

Figure 1

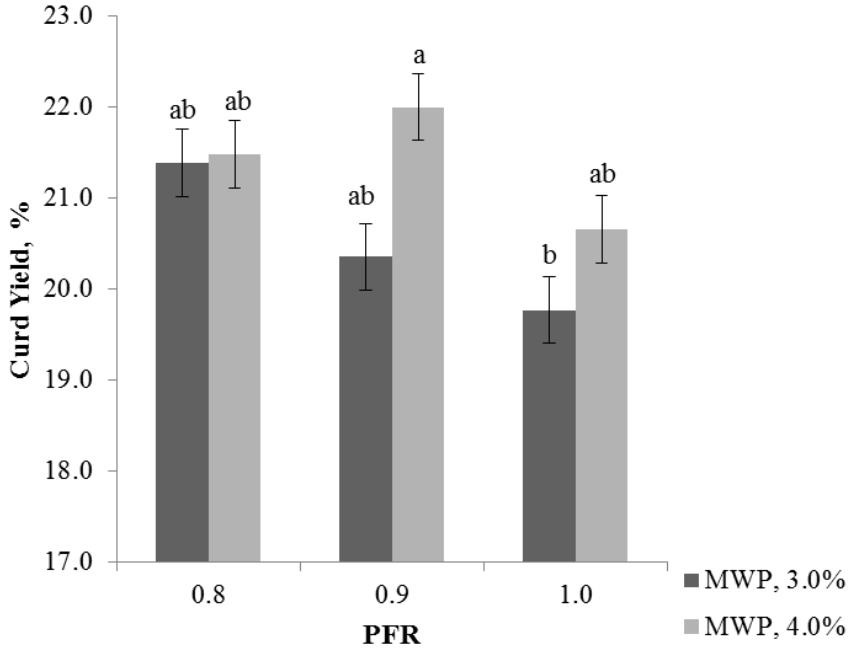
(A)


18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
RCT, min
ab
a
b
b
b
b
0.8
0.9
1.0
PFR
MWP, 3.0%
MWP, 4.0%

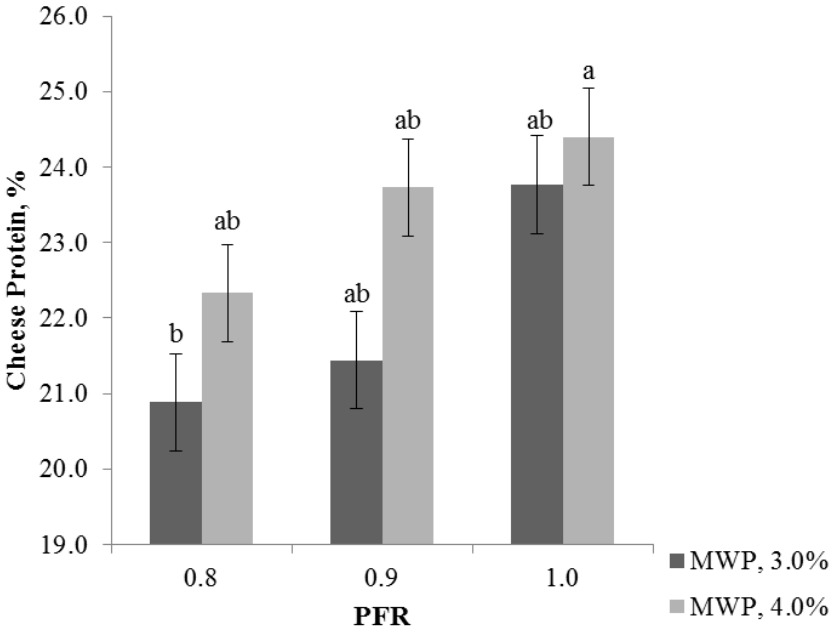
(B)


22.0
21.0
20.0
19.0
18.0
17.0
16.0
15.0
Curd Fat, %
a
a
a
ab
b
ab
0.8
0.9
1.0
PFR
MWP, 3.0%
MWP, 4.0%

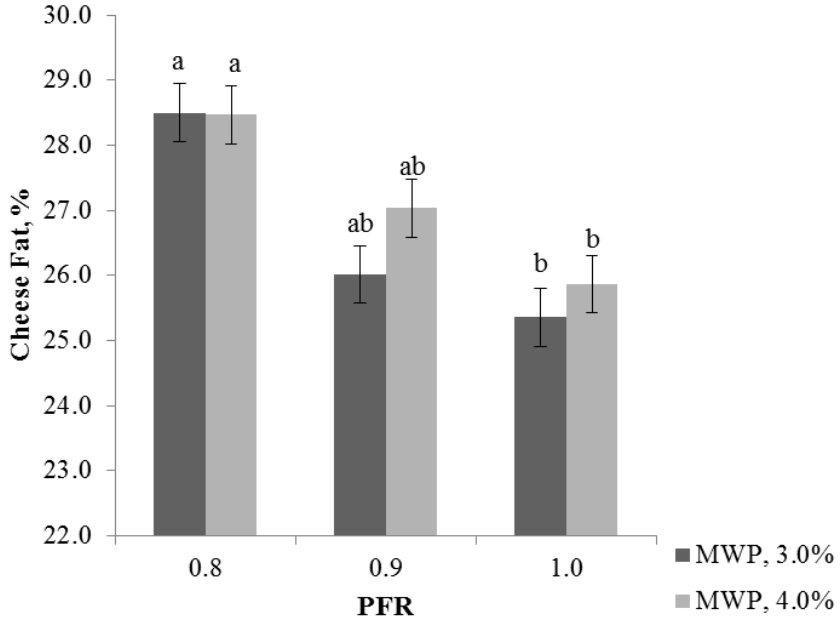
(C)


23.0
22.0
21.0
20.0
19.0
18.0
17.0
Curd Yield, %
ab
ab
ab
a
b
ab
0.8
0.9
1.0
PFR
MWP, 3.0%
MWP, 4.0%

(D)


26.0
25.0
24.0
23.0
22.0
21.0
20.0
19.0
Cheese Protein, %
b
ab
ab
ab
ab
a
0.8
0.9
1.0
PFR
MWP, 3.0%
MWP, 4.0%

(E)



(F)

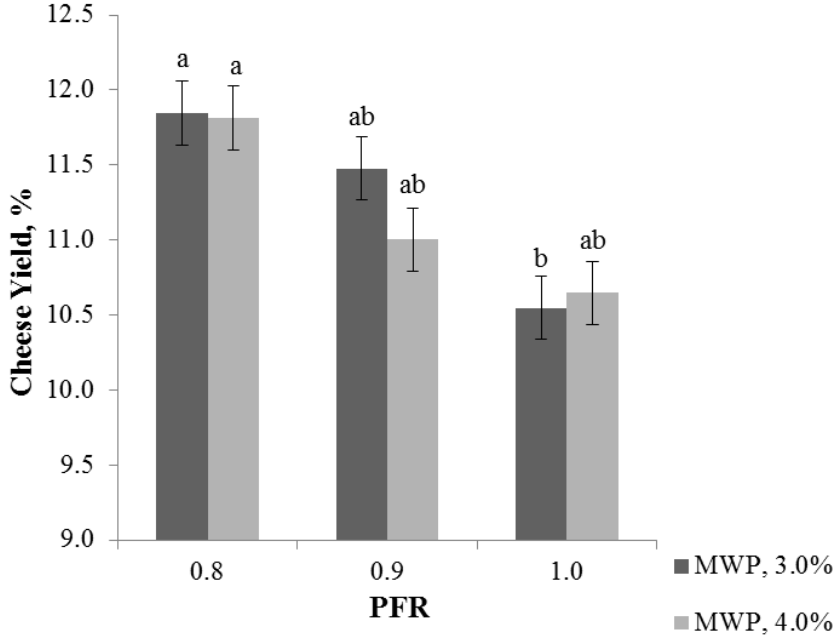


Fig. 1. Least squares means (with standard errors) of (A) rennet coagulation time (RCT, min), (B) fat percentage in curd, (C) curd yield, (D) protein percentage in cheese, (E) fat percentage

in cheese and (F) cheese yield for microparticulated whey protein * protein to fat ratio interaction (MWP*PFR) effect in *Trial 1*. [a-b]Least squares means with different letters are significantly different according to Bonferroni's correction ($P < 0.05$).

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IV

Alba Sturaro, Massimo De Marchi, Josè Manuel Amigo, Richard Ipsen and Martino Cassandro

EFFECTIVENESS OF HYPERSPECTRAL IMAGING FOR MICROPARTICULATED WHEY PROTEINS DETECTION IN LOW-FAT CACIOTTA CHEESE

To be submitted for publication to Journal of Dairy Science

Effectiveness of Hyperspectral imaging for microparticulated whey proteins detection in low-fat Caciotta cheese

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ABSTRACT

In the last decades market offers numerous low-fat products. To avoid undesirable characteristic in low-fat products, ingredients based on microparticulated whey proteins (MWP) are extensively used especially in dairy products. In Italy, the dairy industry is one of the most important sectors of food industry. In this scenario numerous products have protected designation with legal restriction as in the case of external ingredients (e.g. MWP) addition that is not permitted. At strong efforts is need to a rapid detection of MWP addition in traditional products.

Hyperspectral image (HSI) is an emerging technology successfully employed in food inspection, by combining the advantages of conventional digital image and spectroscopy to obtain both spatial and spectral information from an object. The aim of the present study was to test the effectiveness of HSI technique in the detection of MWP in low-fat Caciotta.

Twelve mini-cheese making were performed using standardized milk in low fat condition (3.5% of protein and fat). Protein levels were adjusted with 2.0%, 3.0% or 4.0% MWP vol/vol. For each day of cheese making a control thesis without MWP was performed (0.0% MWP). After one month of ripening a slice of each cheese was analysed for the acquisition of near infrared image in range wavelengths from 1,100 to 1,600 nm, for a total of 140 wavelengths measured.

Several spatial and spectral pre-processing were tested: two times spatial binning, and standard normal variate plus second derivate were select as optimal. Principal component analysis reported an explained variability of 7 % across treatments. Cluster analysis evidenced an increment in component presence by increasing MWP percentage in treatments. Moreover,

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a score plot reported a destine classification of samples contains MWP and control without. The results confirm the ability of HIS in MWP detection, and this information can be used to construct further classification models able to discriminate cheese adulteration for MWP addition.

Key words: microparticulated whey protein, Low-fat products, hyper spectral imaging

INTRODUCTION

In the last decades market offers numerous low-fat products according to consumer's request, indeed an increment of obesity and heart diseases is associated at an unhealthy diet (Patterson et al., 2013). The reduction of fat intake represents a challenge but despite the enormous market potential, the diffusion of low-fat products has been lower than expected; one of reasons might be the inadequate texture and taste attributes of light products, consequently at the absence of fat and its ability in preserving the sensory characteristics. To avoid undesirable characteristic in low-fat products, ingredients based on whey proteins (**WP**) are extensively used, in particular in dairy products (Torres et al., 2012; Di Cagno et al., 2014). Moreover, the recovery of WP from whey after cheese making process is needed to improve the sustainability of the dairy industry (Prazeres et al., 2012). Native WP can be extracted from whey through filtration to obtain WP concentrate (**WPC**), which is further aggregated and denatured through a controlled high heat process to produce colloidal microparticulated WP (**MWP**; Spiegel and Huss, 2002). Microparticulated WP is versatile ingredient able to retain water, indeed it has been used for different purpose, e.g. texturizing and emulsifying agents, and as fat replacer (Torres, 2012). The MWP properties are related to the physiochemical characteristics of WP under different extrinsic factor, as high temperature and pressure, indeed WP in denaturing condition provided intramolecular cross-linkage which constitute a new form of polymer (Graham and Cameron, 1998). Concerning MWP application in cheese as fat replacer, the linkage in MWP must provide a polymer with dimension between 0.1 to 10 µm to not destroy casein network (Kulozik et al., 2001); Indeed, the optimal processing parameter to obtained small aggregates (0.1-20 µm) is a rapid denaturation reaction by heating at temperature between 85-100°C as reported by Spiegel (1999). Furthermore, a moderate MWP addition is required for not affected the rapidity of casein aggregation during curd formation (Sturaro et al., 2014).

In Italy, the dairy industry is the most important sector in food industry, and more than 70% of milk is destined to cheese production (Cassandro et al., 2008). In this scenario, the Caciotta cheese is one of the oldest and most produced Italian cheese; to our knowledge only Di Cagno et al. (2014) used Caciotta cheese as model cheese to produce low-fat type cheese. In the aforementioned study, MWP was used as fat replacer at 0.5% wt/vol, indeed it markedly contributed to the increase in moisture content and, in turn, to the yield of low-fat cheese. Besides the low-fat cheese, Italy produces numerous protected designation products with legal restriction reported in disciplinary of production. Addition of external ingredients

(e.g. MWP) is not permitted. At strong efforts is need to a rapid detection of MWP addition in traditional products.

Considering that hyperspectral image (**HSI**) is an emerging technology successfully employed in food safety inspection and control, as reported by Feng and Sun (2012), in an a comprehensive review of HSI application for the determination of physical, chemical, and biological contamination on food products, MWP may be detected when used in cheese production. Hyperspectral image is a rapid technique, based on spectroscopic image, used in non-destructive quality and safety inspection of food and agricultural products. Hyperspectral image combines the advantages of conventional digital image and spectroscopy to obtain both spatial and spectral information from an object (Gowen et al., 2007). Principles of HSI is the collection of images in several wavelengths and the result is that for each pixel of images, with a spatial position, are associated the spectral information. The resulting hyperspectral cube (hypercube) is a multidimensional data set often complicates the prediction of a dependent variable.

The interest in the valorisation of whey components and the improvement of low-fat products are a great opportunity for dairy industry specialized in cheese production, but a rapid technique for WP detection in products with legal restriction are required. Therefore, the present study aimed to investigate the effectiveness of HSI technique in the detection of low-fat Caciotta cheese produced with increased concentration of MWP (2.0%, 3.0%, 4.0% vol/vol).

MATERIALS AND METHODS

Sample collection, experimental design and mini cheese making procedure

Milk and MWP samples were obtained from the Soligo dairy cooperative (Farra di Soligo, Italy) and milk was used without pasteurization or homogenization treatments, according to a standardize protocol. Microparticulated WP was collected in dairy at the beginning of the experiment, and they were used during each day of analysis. Aliquots of MWP were stored at -20°C. Microparticulated WP was extracted from total skim sweet whey produced by the dairy cooperative in a working day through the ultrafiltration process (Tetrapak International, Rubiera, Italy), using a tubular semi-permeable polyethersulfone membrane with a surface of 700 m^2, and a cut-off of 10,000 Da (Koch Membrane System, Wilmington, MA, USA) at 10°C. Moreover, microparticulation process was carried out for 10 min at 95°C by shell and tube heat exchanger and homogenization at 40 bar following the manufacturer protocols (Tetrapak International).

A total of 3 days of cheese making were carried out using standardized milk in low fat condition (3.5% of protein and fat). For each day protein levels were adjusted with 2.0%, 3.0% or 4.0% MWP vol/vol and a control thesis without MWP (0.0% MWP) was carried out. Each day of cheese making were replicated 3 times in order to have 12 total observations.

Ten liters of milk were used for each cheese making; a mini cheese making protocol was carried out according to that reported by Sturaro et al. (unpublished data). Briefly, ten liters of milk was slowly heated to 35°C by water circulation in a heating jacket using a water bath (SB24, Falc Instrument, Treviglio, Italy). Once milk reached 35°C, starter cultures of freeze-dried formulation of mixed *Streptococcus thermophiles* and *Lactobacillus bulgaricus* (MicroMilk, Crema, Italy) were added to milk as indicated by producer. Temperature was increased up to 38°C in 10 minutes; when milk reached this temperature, 15 ml of commercial liquid rennet of calf (75 chymosin:25 bovine pepsine; De Longhi Michele and C., Treviso, Italy) in water solution (1:3) was added to vat. Continuous monitoring of milk coagulation was performed directly on vat using CoAguLite (**CL**) sensor (Reflectronics Inc., Lexington, KY, USA; Fagan et al., 2007). The cooking phase was performed at 40°C for 15 minutes. After draining, the curd was extracted from vat and put in mold pressed. The mold was stored in thermostat at 37°C for 3 hours until the pH lowered to 5.5. The curd was then immerged in a brine solution (1.14 $kg*l^{-1}$ of NaCl) for 1 hour. Finally, curd was stored in the ripening cell for 10 days at 4 °C and 85.0 % of relative humidity.

After that cheese samples were stored at 4°C under vacuum conditions and send in Food Science department of Copenhagen University.

Image acquisition

A slice of each cheese of 1 cm was cut with a slicer and was analyzed for the acquisition of Near infrared image. Digital image analysis were performed after first months of ripening using Near-Infrared Chemical Imaging (**NIR-CI)** in range wavelengths from 1100 to 1600 nm, for a total of 140 wavelengths measured. Analysis of HIS was carried out in the Laboratory of Food Science Department of Copenhagen University.

Statistical Analysis

Hyperspectral data were analysed in MatLab (The MathWorks, Natick, MA, USA). Each original image (n = 12) was 350x320x 142 x, y, z vectors dimension, respectively. Each slice image was collected in on big image whit cheese made in the same day (3) in row and the same treatment (4) in column. The final hypercube had the dimension 1,050x1,280x142. To

avoid the influence of undesirable phenomena affecting the measurement, like areas of scanned surface (background) pre-processing of spatial and spectral measurements were performed, as proposed by Amigo et al. (2013). After 2 times spatial binning there was a reduction of spatial information of two times, and the resulting image was with dimension 525x640x142 without dead pixel and wavelengths. Spatial binning was performed for all images. None spectral binning was carried out. Moreover, a mask was constructed to delete background pixels by principal component analysis (**PCA**) and by selecting the second component that explained the variability of pixel intensity between background and samples. Pixel discarded were 176,471 in spatial dimension. At last, spectra of hypercube was pre-processed with several method: standard normal variate (**SNV**), smoothing for 10 length segments, mean centering (**MC**); moreover mathematical treatments as derivative of the first (**d1**) and second (**d2**) order was tested. Best preprocessing (SNV) and mathematical treatment (d2) have been considered for further analysis with a windows size of 10 and a polynomial degree of 2. After that, the hypercube reduced the wavelengths dimension at 132.

Different exploratory techniques have been tested in order to estimate the variability across samples. Principal component analysis was used after mean centering of spectra; truncated singular value decomposition was used as algorithm to calculated the model for 4 components. Moreover, cluster analysis was performed as classification techniques, by k-means algorithm by selecting 3 centroids with the maximum distances, furthermore each pixel was classified within a cluster by minimized the distances with the selected centroid. Concluding, unsupervised classification by PCA for general study of the variability in many samples at the same times was applied in order to detect differences in MWP usage. Average values of spectra for each cheese was performed and MWP treatment was tested by PCA analysis as reported by Amigo et al. (2013).

RESULTS AND DISCUSSION

Pre-Processing of Image

Hyperspectral images are composed of thousands of data points: 1,050x1,280 pixel operating at 140 wavebands. This amount of information requires much storage space and the compression of image is advantageous (Amigo et al., 2013). Spatial binning is one of the method use to this respect. In the present work two times spatial binning has been performed to reduce the spatial dimension form 1,050x1,280 to 525x640. Moreover, when sample not cover all scanned area all background must be eliminated to avoid highly noise spectra. Usually a mask can be constructed by selecting region of interest (Amigo et al., 2013).

Principal components method was used in order to select only the pixel of samples. Differences of spectra and image between raw data and image with mask were reported in Fig. 1. Image of cheeses made in the same day in row, and the same treatment in column treatments were represented in the middle of spectral region acquired in Fig. 1 a and b, respectively for raw data and for image with mask. Indeed, in Fig. 1b elimination of background has been obtained. Results were confirmed also for spectra, indeed in Fig. 1a, 20 random spectra reported noise signal, while in Fig. 1b spectra represents only samples.

Concerning spectra pre-processing, the most common practice are the same reported for classical spectroscopy for the suppression of scattering. Pre -processing must be handling with care because of the decrement in spectral resolution (Rinnan et al., 2009); indeed in the present study a reduction of 10 wavelengths was obtained as reported in Fig. 1c. Standard normal variate and second order derivate was considered as best pre-processing for further techniques to explore image.

Exploration of Image and Classification Techniques

Image exploration consists in several methods that permit the extraction of the main source of variability; The most common is PCA applied in different fields of agricultural products, as mushrooms for the detection of bruises (Gowen et al., 2008), cherries for the detection of pits (Qin and Lu, 2005) and in chicken for the detection of fecal contaminants (Park et al., 2002).

Fig. 2 depicts the results of PCA for cheese samples. The explained variance has been 40.5%, 16.31%. 7.0% and 5.2%, respectively for PC1, PC2, PC3 and PC4. Considering map classification within image, the variability across MWP treatments was explained by PC3, with 7.0% of variability. Main spectra regions responsible of variability are the wavelengths numbered as 50 to 60 and from 90 to 100, as reported in Fig. 2 in loadings for PC3.

Hypercube classification enables the identification of regions with similar spectral characteristics (Gowen et al., 2007). In this scenario cluster analysis conforms a number of classification methods that perform the analysis of the image by segmenting the data into specific compounds based on the information of the pixels (Amigo et al., 2008). As reported by aforementioned authors for the cluster analysis the numbers of components is usually perfectly known before, indeed in present work a cluster using three centroids has been select, considering that cheese contains water, fat and proteins. In Fig. 3 are reported the image classification (a), and the three centroids selects for the clustering procedure (b).

Across treatments, an increment in components number three was observed, and this corresponds at the increment of MWP usage in cheese manufacturing.

Application of unsupervised classification of MWP in cheeses

Principal component analysis can be used to detect variability between different samples and not only the surface variability of individual sample. An example was reported by Amigo et al. (2013) by using an image composed in the first row of sweet almonds and the second with bitter almonds. Indeed, score plot of PC1 and PC2 projections reported an evident separation between sweet and bitter almonds.

At the same manner, in the present study the image was organized to detect differences in MWP usage across treatments. Indeed, samples of control cheese without MWP were in the first column, in second, third and fourth columns there were 2.0%, 3.0%, 4.0% of MWP used in cheese manufacturing. In Fig. 4 is reported score plot obtained by mean spectrum for each MWP treatments (MWP 2.0%, 3.0% and 4.0%) and Control (without MWP) for PC2 and PC3, and the explained variability of 15.6% and 6.9%, respectively. The separation from Control to MWP treatments was given by a combination of PC2 and PC3, and it can be related to the chemical reason for this separation. Moreover, this information can be used to construct further classification models able to discriminate cheese with or without MWP addition.

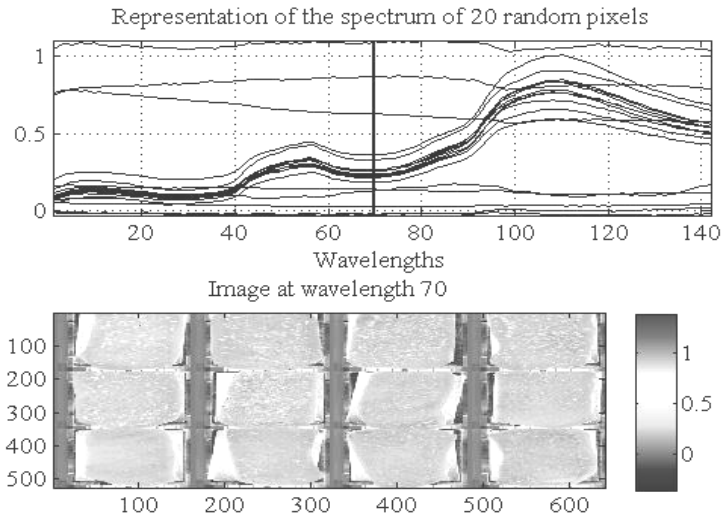
CONCLUSIONS

The addition of increasing concentrations of MWP during cheese manufacturing can be detect rapidly by NIR- Hyperspectral image (HSI) in cheese. In particular, MWP addition, from 2.0% to 4.0% (vol/vol) compared with cheese control without MWP, was explained by PC3 with about 7% of explained variance. Moreover, differences across MWP treatments and control were individuated by clusters analysis and unsupervised classification by PCA. These preliminary results of MWP detection by HSI can be used as information to construct further classification models able to discriminate cheese adulteration for MWP addition.

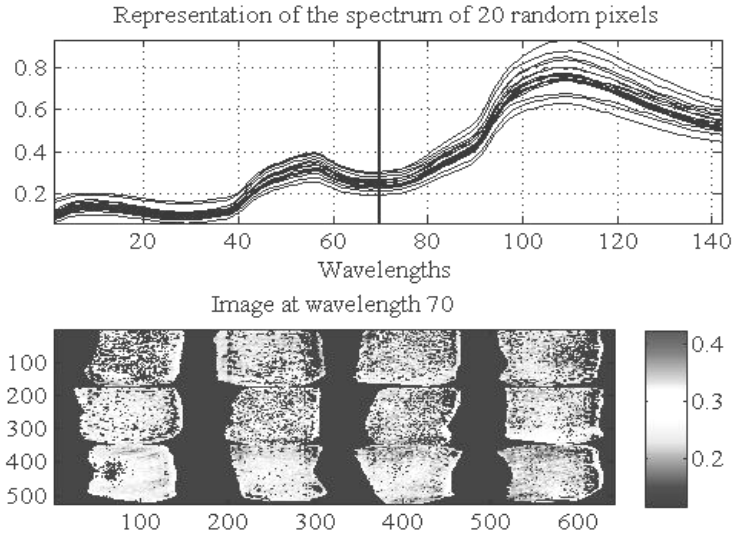
FIGURES

Figure 1

(a)



(b)



(c)

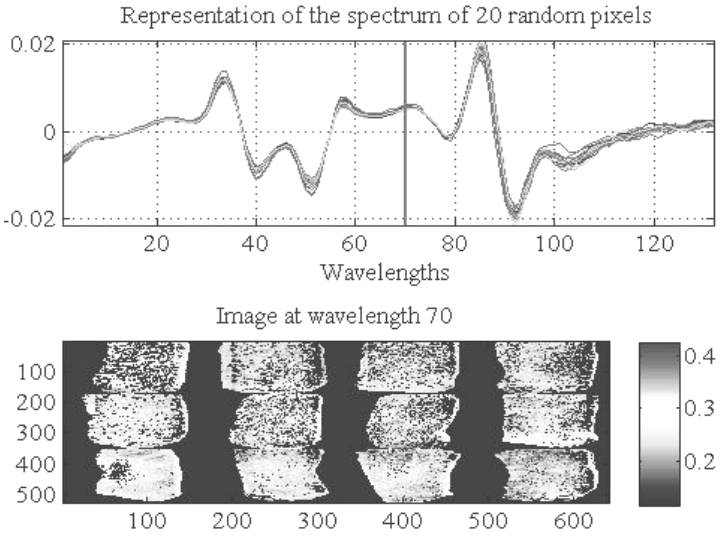
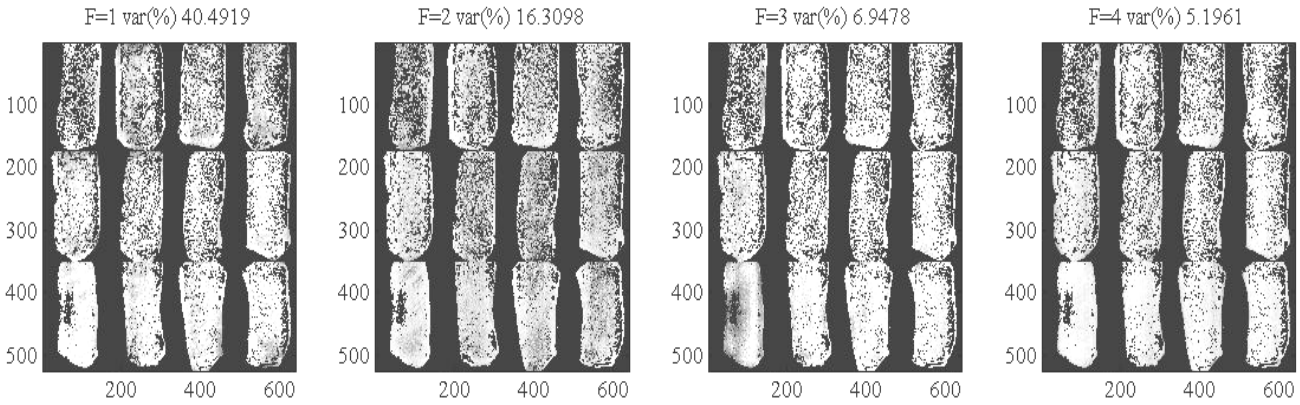


Fig. 1. Raw image of samples cheese, made in the same day in row, and the same treatment in column treatments, in the middle of spectral region acquired, and spectral profile of 20 random pixel of raw image (a); Same image with a mask for delete background and retains only sample spectra (b); Spectra after pre-processing: Standard normal variate and second derivate (c).

Figure 2

(a)



(b)

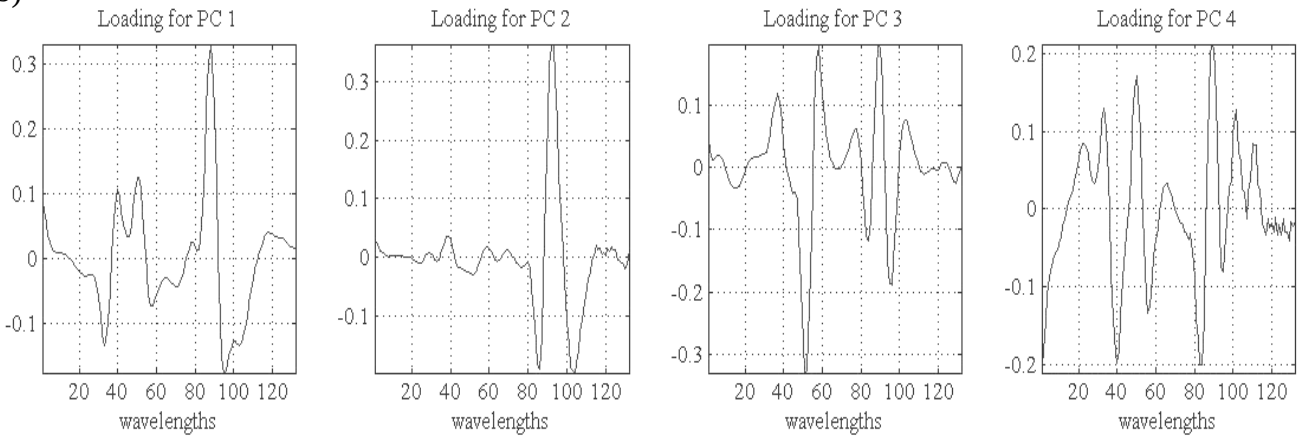
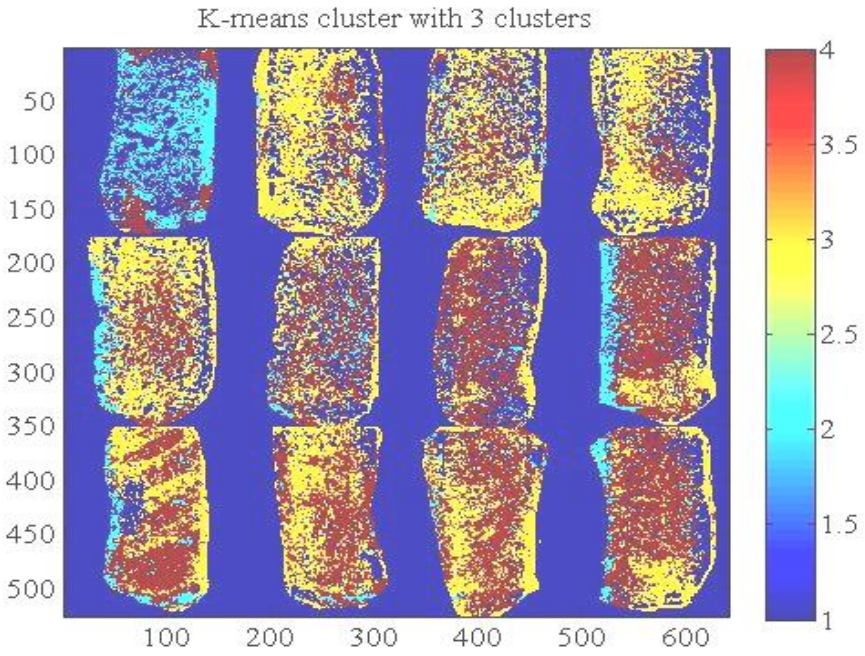


Fig. 2. Results of principal components analysis (PCA) for cheese samples, in the row cheese made in the same day, in column same treatment, and the explained variance (F) for the four principal components (PC; a); Loading of PCA for each PC (b).

Figure 3

(a)



(b)

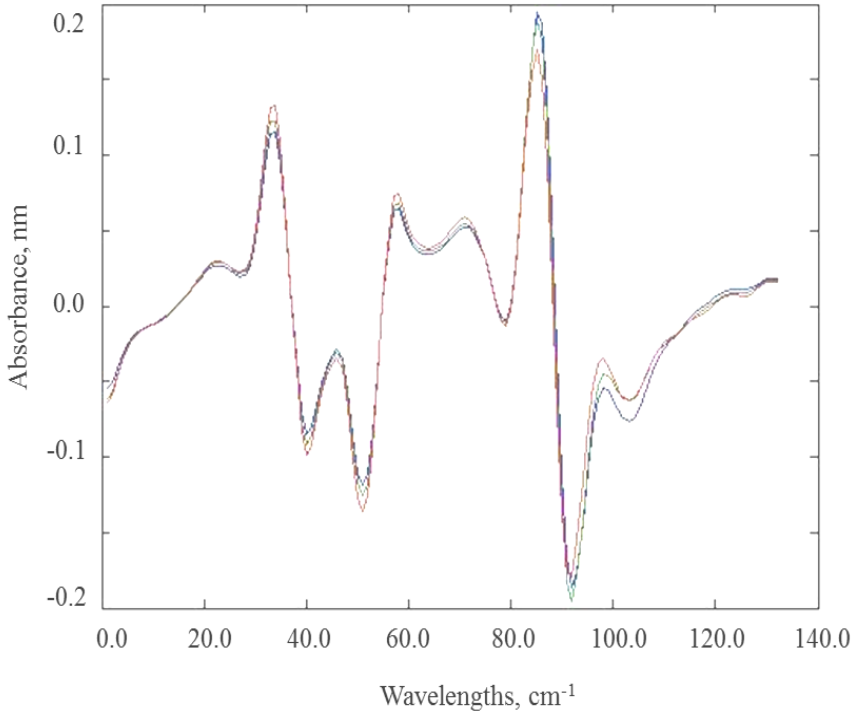


Fig. 3. Cluster analysis using three clusters by selecting centroids with the maximum distances; Clusters distribution in image of cheese made in the same day in raw, and same treatment in column (a); plot of the three centroids (b).

Figure 4

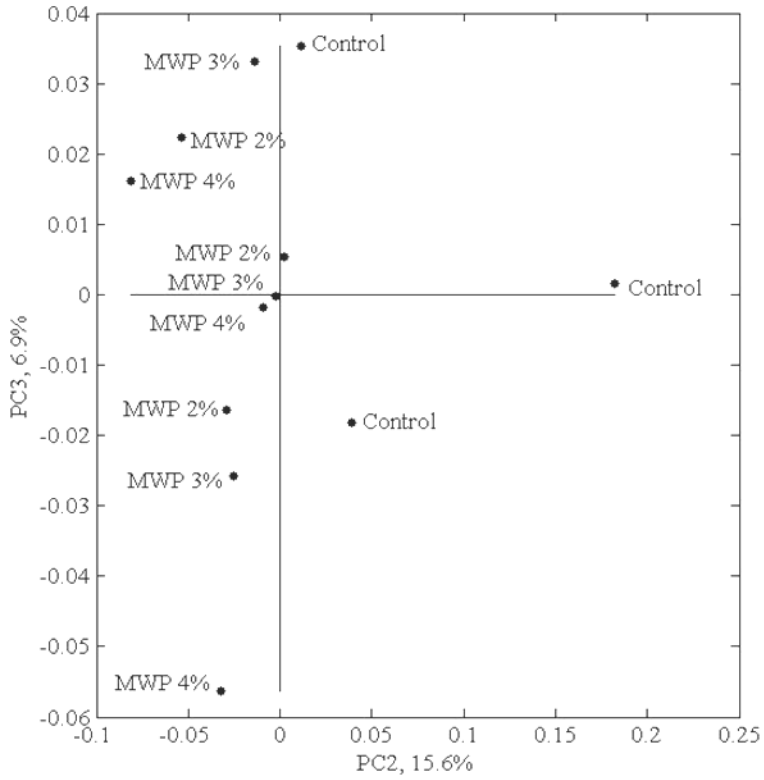


Fig. 4. Score plot of mean spectrum across MWP treatments (MWP 2.0%, 3.0% and 4.0%) and Control (without MWP) for principal component 2 (PC2) and PC3, and the explained variability of 15.6% and 6.9%, respectively.

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CONCLUSIONS

GENERAL CONCLUSION

Results of different studies follow the general objective of the thesis and in particular the recovery of whey proteins (WP) from whey and in the possibility of introduce WP during cheese manufacturing by microparticulated whey proteins (MWP).

Different approaches have been developed and exploited to characterized WP contents in starting material whey but also their detection in final cheese products. Moreover, the aspects of MWP addition in cheese production were tested for different traits, as milk coagulation properties, cheese composition and yield.

In particular, the first contribute, dealing with validated Reversed Phase-HPLC method for WP identification and quantification and its application as reference method to predict WP composition by Mid Infrared Spectroscopy (MIRS). This reference method was validated through repeatability and reproducibility tests. Results of each WP for retention time were stable, while results for peak area depend on the considered protein and their relative abundance. In addition, the potential of MIRS in predicting WP composition of whey confirmed aforementioned results; better MIRS prediction models were obtained for large amounts fractions (i.e. β-Lactoglobulins, Total Proteins identified, α-Lactoalbumin). In conclusion, RP-HPLC method is suitable for high-resolution analysis for WP quantification in whey and represents an adequate gold method for developing MIRS prediction models in dairy industry.

The second contribute focused on the effect of increasing MWP (from 0.0 to 9.0 %, vol/vol) on milk coagulation properties, evidenced a slightly prolonged rennet coagulation time and curd firming time by increasing MWP addition. In particular, MWP decreased the rapidity of casein aggregation during curd formation, which in turn led to delayed coagulation. No significant differences were detected for curd firmness, after 30 min from rennet addition, across concentrations of MWP even if the tendency was for a weaker curd moving from control to samples with high levels of MWP. Moreover, the WP fractions β-Lactoglobulin, α-Lactoalbumin, and bovine serum albumin showed a major recovery. Adjustments in cheese processing should be made when recycling MWP, in order to optimize the potential of MWP as ingredient in low-fat products. This could be achieved during coagulation process by prolonging rennet activity before cutting of the curd.

The third contribute, evaluated the effect of MWP addition (increasing from 0.0 to 4.0 %, vol/vol), in different protein-to-fat ratios (PFR: high, standard and low levels of fat) conditions on milk coagulation process, cheese yield and composition. Results showed a

worsening in milk coagulation properties and a reduction of cheese yield by decreasing milk fat content. Instead, no differences have been detected on cheese yield using 3.0% or 4.0% MWP. The interactions between MWP and PFR showed interesting results; in particular no differences in cheese yield were observed in high and standard fat cheeses with 3.0% or 4.0% MWP, while an increment in cheese yield was reported in low fat cheeses with 4.0% MWP. In low-fat products a slight increment of curd proteins was observed by increasing MWP. The stability in low-fat Caciotta composition suggests the possibility to include MWP as fat replacer to maintain the yield.

The last contribute, dealt with the effectiveness of Hyper Spectral Image (HSI) technique in the detection of MWP in low-fat Caciotta cheese, produced with increased concentration of MWP (2.0%, 3.0%, 4.0%, vol/vol). Differences across MWP treatments and control were individuated by clusters analysis and unsupervised classification by PCA. In particular, MWP addition, from 2.0% to 4.0% (vol/vol) compared with cheese control without MWP, was explained by PC3 with about 7% of explained variance. Moreover, these preliminary results of MWP detection by HSI can be used as information to construct further classification models able to discriminate cheese adulteration for MWP addition.

In general, the results of the present thesis had evidenced, in different ways, the possibility of WP recovery from whey and their utilization in dairy products; whey from cheese manufacturing can be considered as a new source of income for Italian dairy industry. Indeed, the application of MWP in low-fat Italian cheese has been investigated demonstrating a good efficiency in MWP retaining, moreover the stability of composition and yield of products suggests the possibility to include MWP as fat replacer to maintain the yield. Finally, present results underline the possibility for specialized cheese industry to recycling whey by-products.

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