Inline Optimization of Cheese Making Using a Near Infrared Light Backscatter Sensor Technology

Ahmed Rabiea Abdelgawad Eid

Universitat Autònoma de Barcelona

Faculty of Veterinary Medicine, Animal and Food Science Department

Facultat de Veterinària, Departament de Ciència Animal i dels Aliments

Inline Optimization of Cheese Making Using a Near Infrared Light Backscatter Sensor Technology

Optimización en línea de la fabricación de queso mediante una tecnología óptica de dispersión de luz de infrarrojo próximo

Optimització en línea de la fabricació de formatge mitjançant una tecnologia òptica de dispersió de llum d'infraroig proper

Doctoral Dissertation on Food Science

Doctorate en Ciència dels aliments

By

Ahmed Rabiea Abdelgawad Eid

Directed By

Manuel Castillo Zambudio

Bellaterra (Cerdanyola del Vallès), 2016

Inline optimization of cheese making using a near infrared light backscatter sensor technology

Dr. MANUEL CASTILLO ZAMBUDIO, Professor of Food Science, Animal and Food Science Department, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain,

INFORMS

That Mr. AHMED RABIEA ABDELGAWAD EID has performed, under his supervision, the work entitled "INLINE OPTIMIZATION OF CHEESE MAKING USING A NEAR INFRARED LIGHT BACKSCATTER SENSOR TECHNOLOGY", which is presented to obtain the PhD Degree in Food Science.

Bellaterra (Cerdanyola de Vallès), September 27, 2016.

MANUEL CASTILLO ZAMBUDIO

Dr. MANUEL CASTILLO ZAMBUDIO, profesor del Área de Tecnología de los Alimentos del Departamento de Ciencia Animal y de los Alimentos de la Facultad de Veterinaria, Universitat Autónoma de Barcelona, Barcelona, España,

INFORMA

Que el Sr. AHMED RABIEA ABDELGAWAD EID ha realizado, bajo su dirección, el trabajo titulado "OPTIMIZACIÓN EN LÍNEA DE LA FABRICACIÓN DE QUESO MEDIANTE UNA TECNOLOGÍA ÓPTICA DE DISPERSIÓN DE LUZ DE INFRARROJO PRÓXIMO" que presenta para optar al grado de Doctor en Ciencia de los Alimentos.

Y para que así conste, firma el presente documento en Bellaterra (Cerdanyola de Vallès) el día 27 de septiembre de 2016.

MANUEL CASTILLO ZAMBUDIO

Dr. MANUEL CASTILLO ZAMBUDIO, profesor del l'Área de Tecnologia dels Aliments del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària, Universitat Autònoma de Barcelona, Barcelona, Espanya,

FAN CONSTAR

Que el SR. AHMED RABIEA ABDELGAWAD EID ha realitzat, sota la seva direcció, el treball titulat "OPTIMITZACIÓ EN LÍNEA DE LA FABRICACIÓ DE FORMATGE MITJANÇANT UNA TECNOLOGIA ÒPTICA DE DISPERSIÓ DE LLUM D'INFRAROIG PROPER" que presenta per optar al grau de Doctor en Ciencia dels Aliments.

I perque aixi consti, signe el present document en Bellaterra (Cerdanyola de Vallès) el dia 27 de setembre de 2016.

MANUEL CASTILLO ZAMBUDIO

To My Family (Dad, Mom, Sisters and Brothers), I'm proud to be part of you, I Love you

Ahmed

قصيدة مَن اَلديوان اَلشعري اَللإمام اَلشافعي رَحمه اَلله (أحد اَلأئمة اَلأربع)

رَأَيْتُ الْعِلْمَ صَاحِبُهُ شَرِيفٌ		وَإِنْ وَلَدَتْهُ آبَاءٌ لِئَامُ
وَلَيْسَ يَزَالُ يَرْفَعُهُ إِلَى أَنْ		يُعَظِّمَ قَدْرَهُ الْقَوْمُ الْكِرَامُ
وَيَتَّبِعُونَهُ فِي كُلِّ أَمْرٍ		كَرَاعِ الضَّأْنِ تَتْبَعُهُ السَّوَامُ
وَيُحْمَلُ قَوْلُهُ فِي كُلِّ أُفْقٍ		وَمَنْ يَكُ عَالِمًا فَهُوَ الإِمَامُ
فَلَوْلا الْعِلْمُ مَا سَعِدَتْ نُفُوسٌ		وَلا عُرِفَ الْحَلالُ وَلا الْحَرَامُ
فَبِالْعِلْمِ النَّجَاةُ مِنَ الْمَخَازِي		وَبِالْجَهْلِ الْمَذَلَّةُ وَالرَّغَامُ
هُوَ الْهَادِي الدَّلِيلُ إِلَى الْمَعَالِي		وَمِصْبَاحٌ يُضِيءُ بِهِ الظَّلامُ
كَذَاكَ عَنِ الرَّسُولِ أَتَى عَلَيْهِ		مِنَ اللَّهِ التَّحِيَّةُ وَالسَّلامُ
أَخِي لَنْ تَنَالَ الْعِلْمِ إِلاَّ بِسِتَةٍ		سَأُنَبِيكَ عَنْ تَفْصِيلَهَا بِبَيَـانِ
ذكاءٌ وحرصٌ واجتهادٌ وبلغةٌ		وصحبةِ أستاذٍ وطولِ زمانِ

From Imam Shafi's poetry (one of the four great Islamic scholars)

I found that learning is honoring its seeker,		Even if he is born of depraved parents;
It still brings him up,		Until distinguished people value him;
They follow him in every matter,		As the cattle do with its shepherd;
His opinion is always considered,		As the scholar is the leader;
Without learning; neither hearts can feel happiness,		nor the permitted and forbidden can be differentiated;
Learning is a survival of perils,		and ignorance is abjectness and humiliation;
It distances you from disgrace,		but ignorance brings humbleness and meekness;
It's the guide to noble things and,		the light that brightens in darkness;
This is as narrated from the messenger,		peace and prayers of Allah be upon him;
"O my Brother you will never gain knowledge without [possessing] six [qualities];		I will inform you of these in detail and with clarity:
Sharpness [of the mind] eagerness [to learn] sacrifice [in terms of time, etc.] and means [i.e. wealth],		And the company of a teacher and length of time!"

Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

Albert Einstein

Try not to become a man of success, but rather try to become a man of value.

Albert Einstein

Anyone who has never made a mistake has never tried anything new.

Albert Einstein

Science never solves a problem without creating ten more.

George Bernard Shaw

Dedication

This work is dedicated to my beloved country ***Egypt****, my father* ***Dr. Rabiea*** *and my mother* ***Soad****, my sisters* ***Asmaa*** *and* ***Jehad****, my brothers* ***Abdulrahman*** *and* ***Mohamed****. I am very appreciative of their prayers, love, caring, advices and support. Also dedicated to my second country* ***Spain*** *and my teacher* ***Dr. Manuel Castillo****. Without their support, advices and caring this achievement would not be possible.*

The Author of this thesis had a scholarship from *Agencia Española de Cooperación Internacional para el Desarrollo*, Ministry of Foreign Affairs and Cooperation, Spanish Government, Spain

Preface

This PhD thesis entitled "**Inline Optimization of Cheese Making using a Near Infrared Light Backscatter Sensor Technology**" was carried out at Animal and Food Science Department, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona (UAB). The thesis was made under the supervision of **Dr**. **Manuel Castillo Zambudio**, Professor of Food Science, **UAB.** The PhD project was funded by:

1- **AECID** (Agencia Española de Cooperación Internacional para el Desarrollo), Ministry of Foreign Affairs and Cooperation, Spanish government, Spain.
2- **EvalXaRTA** Project. UAB2 306260, **Sistema de predicción del tiempo de corte en queso elaborado con mezcla de leche de vaca, oveja y cabra mediante dispersión de infrarrojo próximo** financed by Xarxa de Referència en Tecnologia dels Aliments de la Generalitat de Catalunya.

The experimental work began in October 1, 2010 and continued until September 30, 2011 with personal financial support given by the Agencia Española de Cooperación Internacional para el Desarrollo, to obtain my Master Degree in Food Science from **UAB**. Then my PhD project began in October 1, 2011 and continued until September 30, 2014 with financial support given by the Agencia Española de Cooperación Internacional para el Desarrollo.

This PhD dissertation is the result of more than 5 years (Master + PhD) of study focused on optimization of cheese making using a NIR light backscatter sensor technology.

The present Dissertation is structured in ten chapters. The first chapter is an introduction to show the relevance of carrying out this research.

The second chapter is a literature overview including the main steps of cheese making with more specific description of milk coagulation, cutting time, curd syneresis and cheese yield, and a brief description of factors affecting milk coagulation, cutting time and syneresis with specific emphasis on the effect of **a)** mixing different types of milk, and **d)** subclinical mastitis infection on milk coagulation and cutting time during the monitorization of milk coagulation and syneresis by NIR light backscatter.

Chapters three and four describe the objectives of this study and work plan, respectively, including the experimental design carried out to achieve these objectives.

Chapter five shows a brief description of the general aspects of material and methods as well as the pieces of equipment used in this experiment.

Chapter six to eight, detail all experiments performed in the thesis to achieve the proposed objectives.

Chapter six studies the effect of temperature, enzyme concentration and milk mixture proportions on gelation and cutting time of cow, sheep and goat milk mixtures using light backscatter and rheology.

Chapters seven and eight evaluate coagulation, cutting time, curd syneresis and cheese yield properties of milk from animals having subclinical intramammary infection using near infrared light backscatter.

Chapter nine highlights the final conclusions of this PhD dissertation.

Chapter ten shows the references used in this study.

Ahmed Rabiea Abdelgawad Eid

2016

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"Knowledge is in the end based on acknowledgment"

It is difficult to thank all people who has been beside me to make this dissertation possible and the most difficult thing is to transform my feelings into words. You really deserve my sincere thanks, thank you all.

First of all, my deepest, greatest and sincere thanks to my **ALLAH** the most Merciful, Great and Clement God**,** because of his uncut givens, being the reason for things happened and for the goods and privileges that he gave me during all my life. He also helped me to achieve this humble work.

From deepest of my heart I would like to thank especially **my beloved parents and my brothers and sisters** for being always beside me, supporting me unconditionally and praying for me. My deepest grateful, sincere appreciation and special thanks to my older brother, my teacher and my supervisor Dr. **Manuel Castillo**, Professor of Food Science at UAB, for believing in me, for encouraging me continuously, and for advising me whenever needed. He provided me indispensable supervision, problem solving assistance, valuable guidance and constructive criticism throughout the investigation. I also thank him for the most important thing, for giving me the family feeling, teaching me that in research, honest, concentrated, and deeply focusing could solve any problem. I really appreciate the support he has given to me.

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Last but not least, I would like to thank all members of my big family at the Dairy Science Department, Fayoum University.

List of Publications Derived from this Dissertation

Refereed Journal Articles

1. Abdelgawad, A. R., Guamis, B., Castillo, M. (2014). **Using a fiber optic sensor for cutting time prediction in cheese manufacture from a mixture of cow, sheep and goat milk**. *Journal of Food Engineering* **125** 157-168.

2. Abdelgawad, A. R., Rovai, M.., Caja, G.., Litner, G., Castillo, M. (2016). **Evaluating coagulation properties of milk from dairy sheeps with subclinical intramamary infection using near infrared light scatter. A preliminary study.** *Journal of Food Engineering.* **168,** 180-190.

3. Abdelgawad, A. R., Rovai, M.., Caja, G.., Litner, G., Castillo, M.: **NIR in-line cheese-vat monitoring of milk coagulation and curd syneresis in sheeps with subclinical mastitis.** *Food Control Journal (Submitted).*

Oral Presentations at International Meetings and Conferences

1. Abdelgawad, A. R., Guamis, B., and Castillo, M. (2013). **Monitoring the Effect of Temperature, Enzyme Concentration and Mixture Proportion on Gelation of Cow, Sheep and Goat Milk Mixtures Using Light Backscatter and Rheology**. ***Proceedings FaBE 2013*** - *International Conference on Food and Biosystems Engineering (FaBE),* Skyathos, Greece, May, 30 – June, 2, 2013.

2. Abdelgawad, A. R., Guamis, B., and Castillo, M. (2013). **Inline Prediction of Clotting and Cutting Times in Cheese Manufacture from Cow, Sheep and Goat Milk Mixtures**. ***Proceedings FaBE 2013*** - *International Conference on Food and Biosystems Engineering (FaBE),* Skyathos, Greece, May, 30 – June, 2, 2013.

Poster Presentations at International Meetings and Conferences

1. Abdelgawad, A. R., Ferragut, V., Guamis, B., and Castillo, M. (2011). **Monitoring milk coagulation of cow, sheep and goat milk mixtures using a light backscatter fiber optic sensor.** *2nd International ISEKI Food Conference.* August, 31 - September 2. 2011, Milan, Italy. (ISBN: 9788890598906)

Conference Papers Derived from Participation at International Conferences

1. Abdelgawad, A. R., Guamis, B., and Castillo, M. (2013). **Monitoring the Effect of Temperature, Enzyme Concentration and Mixture Proportion on Gelation of Cow, Sheep and Goat Milk Mixtures Using Light Backscatter and Rheology**. ***Proceedings FaBE 2013*** - *International Conference on Food and Biosystems Engineering, ISBN: 978-960-9510-11-0,* Vol. (2), pages 30-42.

2. Abdelgawad, A. R., Guamis, B., and Castillo, M. (2013). **Inline Prediction of Clotting and Cutting Times in Cheese Manufacture from Cow, Sheep and Goat Milk Mixtures**. ***Proceedings FaBE 2013*** - *International Conference on Food and Biosystems Engineering, ISBN: 978-960-9510-11-0,* Vol. (2), pages 43-54.

Workshops and training courses

1. **"Mendelay workshop"**

   Organized by Food and Animal Science, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, December 15th, 2011. **Barcelona, Spain.**

2. **"XII Workshop on rapid methods and automation in food microbiology (MRAMA) "**

   Organized by Centre Especial de Recerca Planta de Tecnologia dels Aliments Departament de Ciència animal i dels aliments, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona in collaboration with **Professor Dr. Daniel Y. C. Fung** (Kansas State University, Manhattan, Kansas, USA) in Nov. 25th-28th, 2013, **Barcelona, Spain**.

3. **"Systems of examinations and student assessment"**

   Training Program organized by Faculty and Leadership Development Project (FLDP), Fayoum University, August, 13th -14th. 2013. **Fayoum, Egypt.**

4. **"Academic Course Characterization"**

   Training Program organized by Faculty and Leadership Development Project (FLDP), Fayoum University, August, 3th -4th Jun., 2013. **Fayoum, Egypt.**

5. **"Jornada de Confocal Aplicada a Materiales"**

   Workshop organized by Microscopic service, Universitat Autònoma de Barcelona, Barcelona, España Abril, 5th. 2013, **Barcelona, Spain**

6. **"Microscopio 3D con Tecnología Dual (Confocal e Interferométrico) para Visualización y Caracterización Superficial de Materiales"**

   Organized by Microscopic service, Universitat Autònoma de Barcelona, Barcelona, España Abril, 4th. 2013**, Barcelona, Spain**.

7. **"Credit Hours System"**

   Training Program organized by Faculty and Leadership Development Project (FLDP), Fayoum University, December, 18th -20th, 2012. **Fayoum, Egypt.**

8. **"Effective Communication Skills"**

   Training Program organized by Faculty and Leadership Development Project (FLDP), Fayoum University, August, 7th -9th, 2012. **Fayoum, Egypt.**

9. **"Advanced in UHPH processes (Funentech workshop)"**

   Organized by Food and Animal Science, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, December 15th, 2011. **Barcelona, Spain.**

List of Abbreviations

ALLIC	Laboratori Interprofessional Lleter de Catalunya
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
β	Regression coefficient
C	Control
CEEAH	Ethical Committee on Animal and Human Experimentation
CERPTA	Centre Especial de Recerca Planta de Tecnologia dels Aliments
$CaCl_2$	Calcium Chloride
CCP	Colloidal calcium phosphate
Ch	Cheese weight
Ch_F	Percentage of fat in cheese
Ch_P	Percentage of protein in cheese
Ch_M	Percentage of moisture content in cheese
Ch_{TS}	Percentage of total solids in cheese
ChY_D	Cheese yield dry basis
ChY_W	Cheese yield wet basis
ChY_T	Cheese yield calculated using van slyke equation
CL	CoAguLite™
CN	Casein
CNS	Coagulase-negative *Staphylococci*
CORR	Correlation
CV	Coefficient of variation
CY_W	Curd yield wet basis
CY_D	Curd yield dry basis
CY_T	Curd yield calculated using van slyke equation
D	Days of storage

D_k	Fixed effect of storage days
DF	Degrees of freedom
E_0	Enzyme concentration
F	ANOVA F-statistic, or Fat concentration
FP	Ratio between fat and protein
G´	Elastic or storage modulus
G´´	Viscose or loss modulus
GLM	General linear model
IDF	International Dairy Federation
ILC	Lacaune sheep infected with subclinical mastitis
IMI	Intramammary infection
IR	Index of refraction
JAOAC	Journal Association of Official Analytical Chemists
k_s	The kinetic rate constant for the syneresis process
k_{LFV}	The kinetic rate constant for the LFV sensor response during syneresis.
κ-CN	*κ*-casein
LB	Light backscatter
LC	Lacaune sheep
LED	Light-emitting diode
LFV	Large field of view
LSM	Least-squares mean
M	Molar
M_L	Percentage of lactose in milk
M_F	Percentage of fat in milk
M_P	Percentage of protein in milk
M_{CS}	Percentage of casein in milk
M_{TP}	Percentage of true protein in milk
M_{TS}	Percentage of total solids in milk

Mix	Type of milk mixture
Mix x E_0	Interaction between type of milk mixture and enzyme concentration
Mix x T	Interaction between type of milk mixture and milk temperature
MMC	Mixed milk cheese
MN	Manchega sheep
MT_i	The fixed effect of milk type
N	Number of tests
n	Number of milk batches
NEFA	Non esterified fatty acid
NIR	Near-infrared
NLIN	Nonlinear
NPN	Non protein nitrogen
P	Protein
p-p	Proteose peptones
Q_{10}	Temperature coefficient for the hydrolysis of κ-casein
r	Pearson correlation coefficient
R	Light backscatter ratio (Reflectance ratio)
R_t	The light backscatter ratio at time t
R_0	The light backscatter ratio at cutting time (i.e., at time zero for the syneresis process)
R_∞	The light backscatter ratio at an infinite time
R^2	Coefficient of determination of a linear regression
R'	The first derivative of the light backscatter ratio as a function of time
R''	The second derivative of the light backscatter ratio as a function of time
RCT	Rennet coagulation time
REP	Replication
RH	Relative humidity

$\boldsymbol{R_{max}}$	The value of R at $\boldsymbol{t_{max}}$
$\boldsymbol{R_{2max}}$	The value of R at $\boldsymbol{t_{2max}}$
$\boldsymbol{R_{2min}}$	The value of R at $\boldsymbol{t_{2min}}$
$\boldsymbol{R_{2max2}}$	The value of R at $\boldsymbol{t_{2max2}}$
$\boldsymbol{R_{2min2}}$	The value of R at $\boldsymbol{t_{2min2}}$
$\boldsymbol{R'_{max}}$	The value of the first derivative of R at $\boldsymbol{t_{max}}$
$\boldsymbol{R'_{max2}}$	The value of the first derivative of R at $\boldsymbol{t_{max2}}$
$\boldsymbol{R'_{2max}}$	The value of the first derivative of R at $\boldsymbol{t_{2max}}$
$\boldsymbol{R'_{2min}}$	The value of the first derivative of R at $\boldsymbol{t_{2min}}$
SAOR	Small amplitude oscillatory rheometry
SAS©	Statistical Analysis System
SCC	Somatic cell count
SCM	Subclinical mastitis
SEP	Standard error of predictions
SD	Standard deviation
SGCE	Servei de Granges i Camps Experimentals
$\boldsymbol{t}$	Time
$\boldsymbol{T}$	Temperature
$\boldsymbol{T_i}$	Fixed effect of temperature
$\boldsymbol{tan\delta}$	Tangent δ or loss tangent
$\boldsymbol{t_{clot}}$	Visual clotting time
$\boldsymbol{t_{cut}}$	Visual cutting time
$\boldsymbol{t_{firm}}$	Gel firming time
$\boldsymbol{t_{gel}}$	Rheological gelation time (the time when the gels reached a $G' \geq 1$ Pa)
$\boldsymbol{t_{GE30}}$	Gel assembly time (lag time elapsed between t_{gel} and $t_{G'30}$)
$\boldsymbol{t_{GE60}}$	Gel assembly time (lag time elapsed between t_{gel} and $t_{G'60}$)
$\boldsymbol{t_{G'30}}$	Rheological cutting time (the time when the gels reached a $G' \geq 30$ Pa)

$t_{G'60}$	Rheological cutting time (the time when the gels reached a $G' \geq$ 60 Pa)
t_{max}	The elapsed time from enzyme addition to the first maximum of R' or time from enzyme addition to the inflection point of the light backscatter ratio
t_{max2}	The elapsed time from enzyme addition to the second maximum of R'
$t_{max}X$	t_{max} times X
t_{2max}	The elapsed time from enzyme addition to the first maximum of R''
t_{2min}	The elapsed time from enzyme addition to the first minimum of R''
t_{2max2}	The elapsed time from enzyme addition to the second maximum of R''
t_{2min2}	The elapsed time from enzyme addition to the second minimum of R''
t_{NGE30}	Normalized gel assembly time (normalized against its respective t_{max} value. Calculated by dividing t_{GE30} by t_{max})
t_{NGE60}	Normalized gel assembly time (normalized against its respective t_{max} value. Calculated by dividing t_{GE60} by t_{max})
TS	Total solids
$T \times E_0$	Interaction between milk temperature and enzyme concentration
Type IV SS	Type IV sum of squares
UAB	Universitat Autònoma de Barcelona
UMC	Useful material content
V_0	Initial voltage
$V(w)$	The voltage intensity at every waveband
W	The weight of the whey separated during cheese manufacturing
W_F	The percentage of fat lost in whey during cheese manufacturing
W_P	The percentage of protein lost in whey during cheese manufacturing
w/v	Weight/volume

w/w	Weight/weight
$\boldsymbol{\varepsilon_i}$	Error term
$\boldsymbol{Y}_i$	Dependent variable studied
$\boldsymbol{\mu}$	Overall mean
$\boldsymbol{\delta}$	Delta
$\boldsymbol{\bar{x}}$	Means
$\boldsymbol{\Upsilon}$	Regression coefficient
ΔR	The difference between the light backscatter ratio at time (0) and the light backscatter ratio at (∞**)** time.

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Abstract

Cheese making is the “controlled process of removing water from milk”. This process concentrates the milk protein, fat and other nutrients and increases its shelf life. Cheese manufacture consists of two main steps occurring in the cheese vat, milk coagulation and curd syneresis. Real-time monitoring of milk coagulation, curd firming and syneresis as well as inline prediction of cutting time is essential for cheese making as those factors exert a substantial impact in both cheese yield and quality. Many factors affect the cheese manufacturing process by modifying the quantity, quality, and processing properties of the produced milk.

The general objective of this dissertation was to evaluate the impact of milk mixture (i.e., different proportions of goat, sheep and cow milk) and low quality milk (i.e., milk from animals with subclinical mammary infections) in the prediction of clotting time, cutting time, syneresis rate and several other cheese making indexes based on monitoring milk coagulation and syneresis using NIR light backscatter sensor technologies.

Several optical devices: a lab-scale coagulation tester (CoAguLab), an inline coagulation sensor and an inline large field of view (LFV) syneresis sensor were used to monitor milk coagulation, cutting time, and whey separation during Manchego cheese manufacture.

Optical parameter t_{max} and several other time-based light backscatter parameters were highly correlated with visually- and rheologically-derived clotting and cutting times as well as cheese yield, yield of whey and *SCC*.

It was observed that milk mixtures and animal breed did not have a significant ($P \geq 0.05$) effect on optical and rheological time parameters related to clotting time, while different enzyme concentrations, coagulation temperatures, and subclinical infection had a significant effect on all optical and rheological parameters related to milk clotting time, and gel assembly rate (i.e., aggregation and firming rates). Subclinical mastitis, milk mixtures, temperature, and breed had a significant effect on curd syneresis while cheese yield was affected by subclinical mastitis and breed (note that syneresis effect of enzyme concentration, temperature and milk mixtures was not directly evaluated).

Prediction models using light backscatter parameters alone or in combination with protein/solids concentration were successfully obtained for visually determined clotting and

cutting times, rheologically derived gelation and cutting times, tanδ at cutting, syneresis rate constant and several cheese yield indicators. Our results confirm the usefulness of light backscatter inline monitoring of milk coagulation and curd syneresis for improved process control of those two critical cheese making steps. The results obtained show that the impact of factors such as milk mixtures and subclinical mastitis in cheese manufacture needs to be considered on cheese making process control operations.

Resumen

La elaboración de queso puede considerarse como un "proceso controlado de eliminación de agua de la leche". Este proceso concentra la proteína, grasa y otros nutrientes de la leche, aumentando su vida útil. La fabricación de queso consta de varias etapas, de entre las cuales dos de las más importantes tienen lugar en la cuba quesera: la coagulación de la leche y la sinéresis de la cuajada.

La monitorización a tiempo real de la coagulación de la leche y el endurecimiento del gel láctico, así como la predicción del tiempo de corte es esencial para la producción de queso ya que dichos factores ejercen un impacto sustancial tanto en el rendimiento quesero como en la calidad del queso final. Existen numerosos factores que afectan al proceso de fabricación de queso mediante la modificación de la cantidad, calidad y propiedades tecnológicas de la leche. Si bien la mayoría de dichos factores son bien conocidos, algunos no han sido suficientemente estudiados.

El objetivo general de esta tesis fue evaluar el impacto de la mezcla de leche (i.e., diferentes proporciones de cabra, oveja y vaca) y la leche de baja calidad (i.e., leche de animales con mamitis subclínica) en la predicción del tiempo de coagulación, del tiempo de corte, de la velocidad de desuerado y de varios otros índices de producción quesera, mediante la monitorización de la coagulación y la sinéresis con varias tecnologías de sensores de dispersión de luz de infrarrojo próximo: sensor de coagulación de laboratorio CoAguLAb; sensor de coagulación CoAguLite y sensor de sinéresis LFV. Los dos últimos, instalados en la pared de una cuba de quesería de diez litros a escala de planta piloto.

El parámetro de dispersión t_{max} y varios otros parámetros ópticos de tiempo se correlacionaron significativamente con los tiempos visuales y reológicos de coagulación y de corte así como con los rendimientos de suero y queso; y con el recuento de células somáticas.

Se observó que las mezclas de leche y la raza de los animales no tienen un efecto significativo ($P \geq 0,05$) ni en los indicadores ópticos ni en los reológicos del tiempo de coagulación, mientras que la concentración de enzima, la temperatura de coagulación, y la infección subclínica

tuvieron un efecto significativo sobre todos los índices tanto ópticos como reológicos relacionados con el tiempo de coagulación y la velocidad de ensamblado del gel láctico (i.e., agregación micelar y endurecimiento del gel). La mastitis subclínica, la mezcla de leche, la temperatura y la raza tuvieron un efecto significativo sobre la sinéresis de la cuajada, mientras que el rendimiento quesero se vio afectado por la mastitis subclínica y la raza (nótese que el efecto de la concentración de enzima, la temperatura y la mezcla de leche no fue evaluado directamente).

Se obtuvieron modelos de predicción para los tiempos de coagulación y de corte tanto visuales como reológicos, el ángulo de fase (tgδ) en el momento del corte, la velocidad de sinéresis y varios indicadores de rendimiento quesero. Nuestros resultados confirman la utilidad de la monitorización a tiempo real tanto de la coagulación de leche como del desuerado de la cuajada mediante dispersión de luz de infrarrojo próximo, a fin de mejorar el control de esas dos etapas críticas de elaboración de queso. Los resultados obtenidos demuestran que el impacto de factores tales como la mezcla de leches y la mastitis subclínica debe ser tenido en consideración en las operaciones de control de procesos de la elaboración de queso.

Resumé

L'elaboració de formatge es pot considerar un "procés controlat d'eliminació d'aigua de la llet". Aquest procés concentra la proteïna, el greix i altres nutrients de la llet, augmentant la seva vida útil. La fabricació de formatge consta de diverses etapes d'entre les quals, dues de les més importants, tenen lloc en el tanc de formatgeria: la coagulació de la llet i la sinèresi de la quallada.

La monitorització a temps real de la coagulació de la llet i l'enduriment del gel lacti, així com la predicció del temps de tall, són essencials per la producció de formatge ja que aquests factors exerceixen un impacte substancial tant en el rendiment formatger com en la qualitat del formatge final. Hi ha nombrosos factors que afecten el procés de fabricació de formatge mitjançant la modificació de la quantitat, la qualitat i les propietats tecnològiques de la llet. Si bé la majoria d'aquests factors són ben coneguts, alguns no han estat suficientment estudiats.

L'objectiu general d'aquesta tesi fou avaluar l'impacte de la mescla de llet (i. e., diferents proporcions de llet de cabra, ovella i vaca) i la llet de baixa qualitat (i. e., llet d'animals amb mastitis subclínica) en la predicció del temps de coagulació, del temps de tall, de la velocitat d'eliminació del sèrum i de diversos altres índexs de producció formatgera, mitjançant la monitorització de la coagulació i la sinèresi amb vàries tecnologies de sensors de dispersió de llum d'infraroig proper: sensor de coagulació de laboratori CoAguLAb; sensor de coagulació CoAguLite i sensor de sinèresi LFV. Els dos darrers, instal·lats en la paret d'un tanc de formatgeria de deu litres a escala de planta pilot.

El paràmetre de dispersió t_{max} i diversos altres paràmetres òptics de temps es correlacionaren significativament amb els temps visual i reològics de coagulació i de tall, així com amb els rendiments de sèrum i formatge i amb el recompte de cèl·lules somàtiques.

Es va observar que les mescles de llet i la raça dels animals no tingueren un efecte significatiu ($P \geq 0,05$) en els indicadors òptics ni reològics del temps de coagulació, mentre que la concentració d'enzim, la temperatura de coagulació i la infecció subclínica tingueren un efecte significatiu sobre tots els índexs, tant òptics com reològics, relacionats amb el temps de coagulació i la velocitat d'acoblament del gel lacti (i. e., agregació micel·lar i enduriment del gel). La mastitis subclínica, la mescla de llet, la temperatura i la raça tingueren un efecte significatiu sobre la

sinèresi de la quallada, mentre que el rendiment formatger es veié afectat per la mastitis subclínica i la raça (cal fer notar que els efectes de la concentració d'enzim, la temperatura i la mescla de llet no foren avaluats directament).

S'obtingueren models de predicció pels temps de coagulació i de tall, tant visuals com reològics, l'angle de fase (tgδ) en el moment de tall, la velocitat de sinèresi i diversos indicadors de rendiment formatger. Els nostres resultats confirmen la utilitat de la monitorització a temps real mitjançant dispersió de llum d'infraroig proper tant de la coagulació de llet com de l'eliminació de sèrum de la quallada, amb la finalitat de millorar el control d'aquestes dues etapes crítiques de l'elaboració del formatge. Els resultats obtinguts demostren que l'impacte de factors com la mescla de llets i la mastitis subclínica s'ha de tenir en consideració en les operacions de control de processos de l'elaboració de formatge.

Chapter One

Interest of the study

Chapter one: Interest of the study

Cheese making is a complex manufacturing process involving four main physical-chemical stages: coagulation, syneresis, salting and ripening (Brule and Lenoir, 1989). Cheese making could be considered as a process concentrating milk components, in particular fat and protein contents, which are determinant factors of cheese yield (Banks *et al.*, 1981). During milk gelation, a three-dimensional casein network is formed, which retains fat and moisture (O´Brien and Guinee, 2011). After gelation, the gel is cut into small cubes to induce whey separation, which allows obtaining whey and curd. In most cases the curd is moulded, pressed and salted (fresh cheese) and in many cases fresh cheese is further matured for periods of up to two years to obtain a ripened cheese (Fox *et al.*, 2004). Three different types of coagulation could be distinguished depending on the gelation induction method (Law and Tamime, 2010): acid, rennet, and mixed coagulation. Rennet-induced gelation is the result of two processes involving the attack on the κ-casein on the surface of the casein micelles by the addition of acid proteinases, referred to generically as rennets (eg., chymosin, pepsin) and the clotting of the micelles, which have been destabilized by this enzymatic attack (Picón *et al*., 1994). In acid coagulation, gelation is induced by acidification (using starter cultures, food-grade acids, and/or acidogens –eg., glucono-δ-lactone–) at a temperature of 20-40 ºC, to a pH value close to the isoelectric pH of casein, i.e. ~4.6. Finally, often, a combination of both rennet and acidification is used; this type of mixed coagulation produces curds, which have intermediate properties, combining characteristics from both acid and rennet gels (Law and Tamime, 2010). In this dissertation, only rennet or mixed coagulation has been applied.

Milk clotting and cutting times are two of the main coagulation parameters used to control the milk coagulation process during cheese making. Selection of a proper cutting time is important because it greatly affects moisture, yield, and quality of cheese as well as whey fat losses. Traditionally, in cheese plants, the curd is cut after a predetermined time from the enzyme addition or upon the subjective evaluation of the coagulum firmness by the cheese maker. The selection of cutting time depends on rheological and microstructural properties of gels, such as coagulum firmness and rearrangement capability that, in turn, depend on coagulation factors, milk composition, and milk pretreatment (Nicolau *et al*., 2010). A plethora of factors affect milk coagulation, which in turn modify clotting and/or cutting time. In addition, the way those factors

are modifying coagulation is also variable, as they might affect in a different degree of intensity the chemical reactions involved in milk coagulation (i.e., κ-casein hydrolysis, aggregation of destabilized micelles and gel firming -Castillo *et al*., 2003b-). Milk coagulation factors can be divided in several groups as follows: a) chemical compositional factors, which directly affect milk coagulation (e.g., fat/protein ratio, colloidal calcium phosphate (CCP) content of milk, casein concentration, etc.). Compositional factors are widely influenced by a large number of animal-related, physiological and environmental factors, which indirectly affect milk coagulation (e.g., animal nutrition, genetic variance, seasonal effect, lactation stage, lactation number, milking frequency, subclinical mastitis, etc.); b) milk pre-treatment factors, which modify the existing chemical composition of milk (e.g., refrigeration, pasteurization and homogenization, etc.); c) technological factors, which affect coagulation directly during the process (e.g., milk coagulation temperature and pH, enzyme concentration and type, $CaCl_2$ addition, etc.).

As a result of the large number of factors affecting milk coagulation and its subsequent effect on milk coagulation process control, an ideal milk coagulation and/or gel firming monitoring method has been pursued over the past eight decades. Most of the proposed instruments have been developed for either fundamental research of milk coagulation or development of inline cutting time sensors. Most devices are based on the measurement of a wide range of physical properties. In general, those systems studying rheological properties are destructive and not practical for inline application. The alternative is non-destructive equipment based on the measurement of thermal conductivity, electrical/light waves, vibrations and ultrasound. Thermal and optical techniques are awakening great expectations, but the development of optic fibers is giving advantage to optical methods (Castillo, 2006a). To date, an objective and effective method to determine optimum cutting time is not available, although some existing methods can consistently reproduce the cutting time subjectively selected by the cheese maker (Castillo, 2006a).

A promising inline optical sensor designed to measure changes in light backscatter of infrared light at 880 nm was proposed by Payne *et al*. (1993a). This technology was initially developed for cow milk. However, since then, this technology has been adapted successfully to goat milk (Castillo, 2001) and sheep milk (Nicolau *et al*., 2010). Also, a large field of view sensor (LFV) using 980 nm light for monitoring both milk coagulation and curd syneresis was designed

by Castillo, *et al.* (2005a) and evaluated over a range of cutting times, temperatures and calcium chloride levels (Fagan *et al*., 2007b).

The effect of most coagulation factors on the different steps of milk coagulation is well understood (eg., temperature, pH, calcium, enzyme concentration, protein, etc.) and the influence of such factors on light backscatter coagulation monitoring and both clotting and cutting time prediction algorithms has been already tested. However, near infrared light backscatter sensors have never been used to test coagulation properties of milk mixtures from different animal species (cow, goat and sheep), or milk from animals having subclinical mastitis. Not only the effect of these two factors on the NIR light backscatter coagulation profile is unknown but the suitability of the existing prediction models for clotting and cutting time has not been tested/validated yet.

The production of cheese using mixtures of milk (hereafter *"mixed milk cheese* –**MMC**–*"*) has achieved widespread acceptance in Spain and some other countries such as Italy, France, Greece and Cyprus. A large variety of MMCs are typically manufactured in Spain such as *"Cabrales"*, *"Picón"*, *"Gamonedo"*, *"Iberico"*, etc. MMCs are very important for the Spanish cheese sector, not only for the proportion of sales it represents, but also because of technological differences required for appropriate processing of mixtures of different types of milk. Sheep, goat and cow milk show marked differences in their colloidal structure and chemical composition, which introduces additional difficulties, compared with cheeses made with one type of milk, regarding the control of coagulation and the selection of cutting time.

The health condition of the mammary gland dictates the rate of milk secretion and has a multitude of effects on the quantity, quality, and processing properties of the produced milk, which in turn has a potential effect on cheese manufacture. The terminology “mastitis” means intra mammary infection (**IMI**), which is an inflammatory response of the udder tissue to bacterial, thermal, chemical or mechanical injuries (Omaleki *et al*., 2011). The purpose of the inflammatory reaction is to restore affected udder to the normal function. Mastitis is one of the costliest and more common diseases of dairy herds, exerting a negative impact on dairy ruminant's and public health as well as causing economic losses to dairy farmers due to reduced milk yield (Heringstad *et al*., 2003a). Mastitis can appear both clinical and subclinical, being the latest form the most prevalent one (Maréchal *et al*., 2011). Subclinical mastitis **(SCM)** is the presence of infection in the mammary gland without apparent signs of local inflammation or systemic affection and requires a

diagnostic test for detection (Harmon, 1994a). Mastitis is of special relevance in sheep dairy herds as in some European Mediterranean countries, such as Spain, France Italy, Bulgaria and Rumania, cheese industry plays an important role supporting their large sheep milk production (Martínez *et al*., 2011). In Spain, for instance, most of sheep milk is destined for manufacturing of dairy products, mainly cheese (*Manchego*) (Marqués *et al*., 2011). SCM is characterized by changes in milk composition with no modification of milk color, consistency abnormalities or unhealthy look of udders. In fact, it could be considered the most dangerous form of mastitis since it can go unnoticed. SCM can be detected only by tests such as somatic cell counts, California Mastitis Test or bacteriological tests (Bergonier and Berthelot, 2003; Omaleki *et al*., 2011). The major pathogenic agents causing SCM in sheep are coagulase-negative *Staphylococci* **(CNS)** (Leitner *et al*., 2004; Leitner *et al*., 2011). Sheep milk quality is critical for high quality cheeses and dairy products. Milk from ewes with SCM present modified functional properties and, as a result, inadequate technological suitability (e.g., altered rennet coagulation time, curd firmness, curd syneresis and cheese yield), which in turn impacts negatively on quality of cheese.

As discussed by Abdelgawad *et al.* (2016), failure to detect subclinical infection by the farmers and high prevalence (up to 15 to 40%) of *SCM* in sheep herds causes milk from normal animals and animals having SCM to get mixed in the tank. Assuming the mentioned prevalence of SCM in sheep herds, if all animals in a dairy sheep flock are milked into the milk tank; about 15 to 30% of the milk would be of infected glands (Leitner *et al*., 2008, and 2011).

Industrial implementation of a light backscatter monitoring sensor technology as an inline method of controlling milk coagulation requires a better understanding of the impact of factors evaluated in this study on both coagulation and clotting/cutting prediction algorithms. The lack of knowledge regarding the influence of milk mixtures and subclinical mastitis on light backscatter milk coagulation monitoring technology and prediction of coagulation control parameters justifies this work. It is expected that the results from this PhD dissertation would provide insight in this topic, contributing to validate or adapt the prediction algorithms already exiting under typical coagulation conditions to situations where milk mixtures or subclinical mastitis are inadvertently affecting cheese production.


389
Royal Briard
Fromage
Lait cru de vache
32 % MG
14,25 € la pièce
256
Brie de Nangis
Fromage
Lait cru de vache
40 % MG
20,50 € le kilo

Chapter Two

Literature Review

Chapter two: Literature Review

2.1 Milk composition and structure

Milk is the whole, clean, fresh, lacteal secretion obtained by the complete milking of one or more healthy animals, excluding that obtained within 15 days before or 5 days after calving or such periods as may be necessary to render the milk practically colostrum-free, and containing the minimum prescribed percentage of milk fat and milk solid not fat (Sukesh *et al*., 2010). The milk of certain domesticated animals, and dairy products produced therefrom, are major components of the human diet in many parts of the world. Domesticated goats, sheep and cattle have been used for milk production since about 8000 B.C (Boland *et al*., 2009).

Nutritionally, milk is the most complete single food available. It is free from toxins and anti-nutritional factors. While milk of all species has similar overall characteristics and classes of constituents, these differ in specific terms both qualitatively (i.e. the exact nature of constituents) and quantitatively (i.e. the amount of each constituent / liter). Composition of milk differs between different mammals and between different breeds of the same species as shown in **Table 2.1.**

Table 2.1 Chemical composition (g 100 g^{-1}) of milk of different species of mammals

Milk Type	Fat%	Protein%	Casein%	Lactose%	Ash%	Water%	Total Solids%
Cow	3.9	3.3	2.8	4.7	0.7	87.4	12.6
Goat	4.5	3.3	2.7	4.6	0.6	87.0	13.0
Sheep	7.5	5.6	3.9	4.4	0.9	81.6	18.4

Source: based on data from Park and Haenlein (2006; 2010).

As it is shown in **Fig. 2.1**, milk can be regarded as a colloidal suspension of fat and casein.

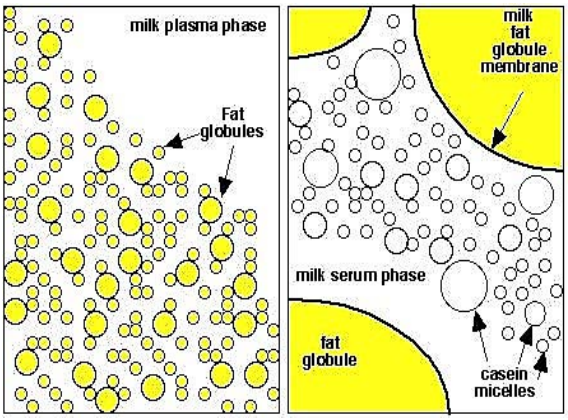


Fig. 2.1 Milk structure. (Reproduced from Walstra, 1979).

Light scattering in milk is caused by particles of relatively large size whose refractive index differs from water. Both fat globules and casein micelles are the primary contributors to the light scattering and color in milk (Castillo *et al.*, 2005b, McSweeney and fox, 2013). **Fig. 2.2**I shows the main milk constituents.

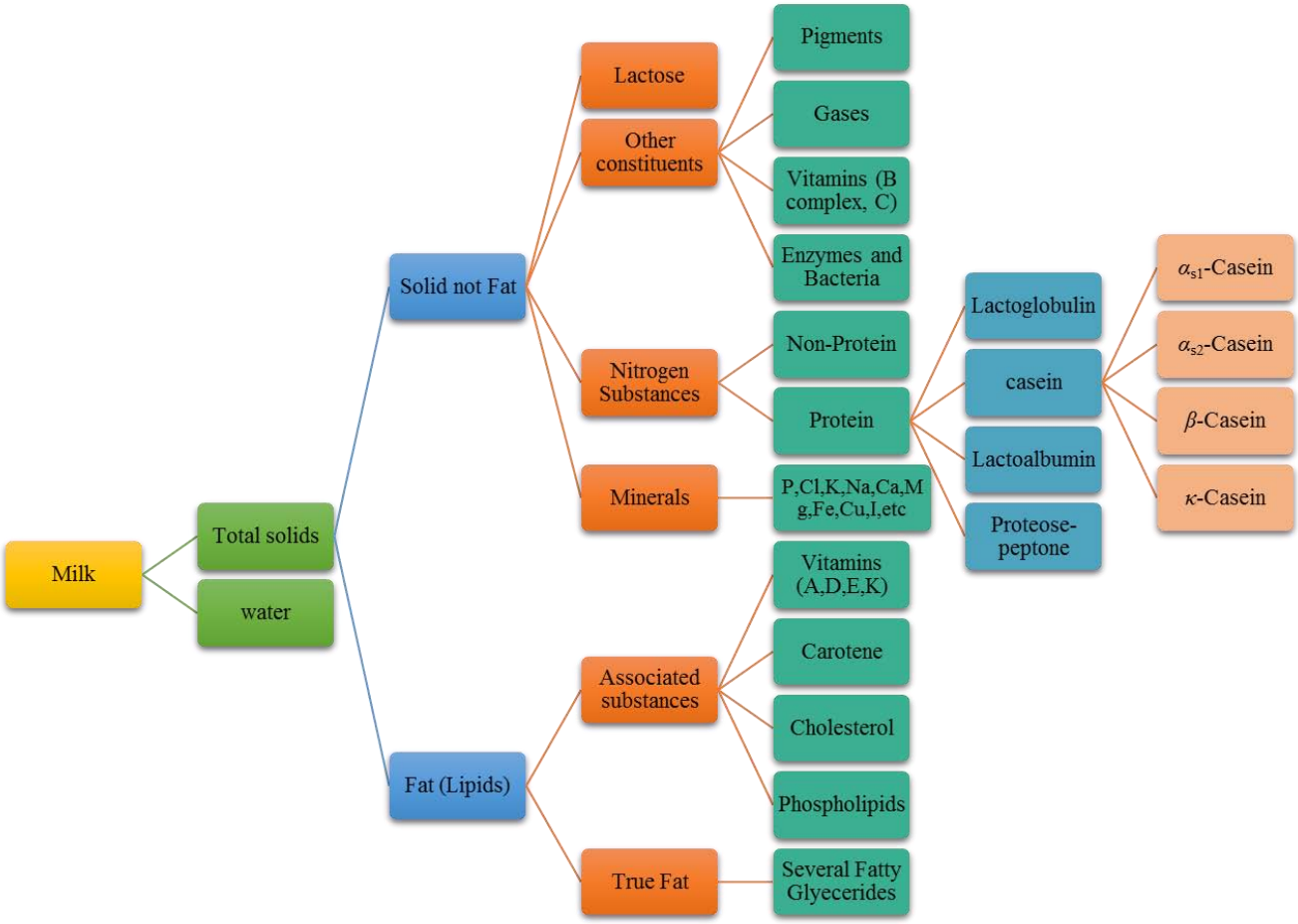


Fig. 2.2 Milk constituents (Reproduced from Ramesh *et al.*, 2008).

In general, the main constituents of milk are water, lactose, protein (casein and whey protein), fat, and salts, ash or minerals (soluble and insoluble); also milk contains minor constituents, such as vitamins, free amino acids, peptides and enzymes (Haug *et al.*, 2007). The casein fraction coexists with the insoluble minerals as a calcium phosphate–casein complex. The water and its soluble constituents (lactose, native whey proteins, some minerals, citric acid and minor components) are referred to as serum (Law and Tamime, 2011). Milk always contains somatic cells, which is low in milk from a healthy udder, but increases if the udder is diseased, usually in proportion to the severity of the disease. Milk also contains gases, some 5 – 6% by

volume in fresh milk from the udder, but on arrival at the dairy the gas content may be as high as 10 % by volume (Bylund, 2003).

2.1.1 Lactose

Lactose is a sugar found only in milk; Lactose is responsible for ~50% of the osmotic pressure of milk, which is equal to that of blood (Walstra and Jenness, 1984), in both cases due to the effect of NaCl from the blood and the resultant need to maintain the osmotic equilibrium (Fox, 2003b). Lactose content of milk varies between 3.6 and 5.5%. **Fig. 2.3** shows what happens when lactose is attacked by lactic acid bacteria. These bacteria contain an enzyme called lactase, which attacks lactose, splitting its molecules into glucose and galactose, which then transformed into lactic acid (Fox and McSweeney, 1998). In cheese making most of the lactose remains dissolved in the whey. (O'Brien, 1997).

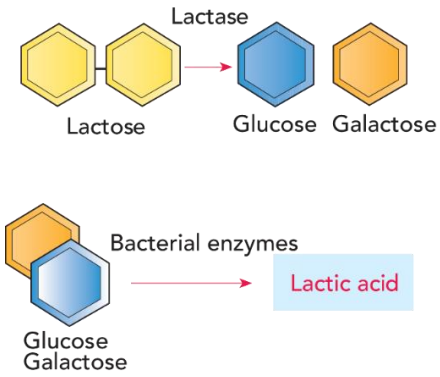


Fig. 2.3 Breakdown of lactose by enzymatic action and formation of lactic acid. (Reproduced from Bylund, 2003)

2.1.2 The milk Protein and enzymes

Proteins are an essential part of our diet. The proteins we eat are broken down into simpler compounds in the digestive system and in the liver (Bylund, 2003). These compounds are then conveyed to the cells of the body where they are used as construction material for building the body's own protein.

Proteins are giant molecules built up of smaller units called amino acids, **Fig. 2.4**. A protein molecule usually contains around 100 – 200 linked amino acids, but both smaller and much larger numbers are known to constitute a protein molecule.

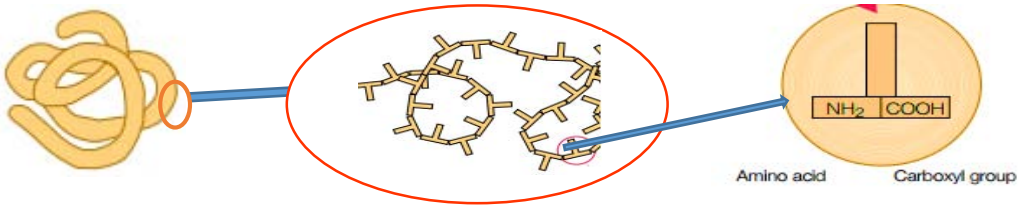


Fig. 2.4 Model of a protein molecule chain of amino acids, the amino and carboxyl groups. (Reproduced from Bylund, 2003).

Milk contains hundreds of types of protein, most of them in very small amounts. The proteins can be classified in various ways according to their chemical or physical properties and their biological functions. Milk contains 3.3% total protein, and total milk protein content and amino acid composition varies with cow breed and individual animal genetics (Fox and McSweeney, 1998). **Table 2.2** shows a list of milk proteins.

Table 2.2 Concentration of proteins in milk.

Protein	Protein types	Conc. In milk g/kg	% of total protein *w/w*
Casein	α_{s1}-casein*	10.0	30.6
	α_{s2}-casein*	2.60	8.00
	β-casein**	10.1	30.8
	κ-casein	3.30	10.1
Whey protein	α-lactalbumin	1.20	3.70
	β-lactoglobulin	3.20	9.80
	Blood Serum Albumin	0.40	1.20
	Immunoglobulins	0.70	2.10
	Total Whey Proteins	6.30	19.3
Fat Membrane Proteins		0.40	1.20

***) Henceforth called αs -casein**
****) Including γ-casein**
Ref: Walstra and Jennis (1984).

Technologically, the milk proteins are probably the most important constituents of milk, due to their unique properties, which allow for rather easy conversion of milk into a wide range of products, such as cheese or yoghurt. Milk proteins can be divided into two major classes that are broadly defined by their chemical composition and physical properties (Singh, 1995). Those soluble and those insoluble at pH 4.6. The pH 4.6-insoluble proteins are called the caseins, whereas the pH 4.6-soluble proteins are referred to as serum proteins or whey proteins. An ~80% of protein in milk are caseins and the maintaining 20% are whey proteins (Fox, 2003c). The caseins are

suspended in milk in a complex called a micelle. **Table 2.3** shows nitrogen fractions of cows, sheep's, and goat's milk.

Table 2.3 Nitrogen fractions of cows, sheep's, and goat's milk.

Fraction	Cow	Sheep	Goat
Total N, g/Kg	32.5	55.5	34
True Whey N, g/Kg	5.7	11	7.4
Casein-N, g/Kg	25.1	43	24
NPN x 6.38, g/Kg	1.7	1.5	2.6

Ref: Potocnik *et al.* (2011)

According to Potocnik *et al.,* (2011) some differences in casein fractions between cow, goat and sheep milk were observed as it is shown in **Table 2.4.**

Table 2.4 Differences in casein fractions between cow, goat and sheep milk.

Fraction	Cow	Sheep	Goat
Casein, g/Kg	25.1	43	24
α_s-casein %	48.46	50.23	21.2-32
β-casein %	35.7	39.95	48-60
κ-casein %	12.63	9.82	12-20
Micelle Size, nm	182	210	260

Ref: Potocnik *et al.* (2011).

Milk also contains different enzymes coming from several sources: the native milk, bacteria that are added intentionally for fermentation, or in somatic cells present in milk. The former are normal constituents of milk and are called original enzymes. The latter, bacterial enzymes, vary in type and abundance according to the nature and size of the bacterial population. Several of the enzymes in milk are utilized for quality testing and control. Among the more important ones are peroxidase, catalase, phosphatase and lipase (Farkye, 2003).

Lipases are enzymes that degrade fats. The major lipase in milk is lipoprotein lipase. It is mainly associated with the casein micelle. Agitation during processing may bring the lipase into contact with the milk fat resulting in fat degradation and off-flavors. Pasteurization will inactivate the lipase in milk and increase shelf life (Bylund, 2003).

Proteases are enzymes that degrade proteins. The major protease in milk is plasmin, which is part of a complex system. Some proteases are inactivated by heat and some are not. Protein degradation can be undesirable and result in bitter off-flavors, or it may provide a desirable texture

to cheese during ripening. Proteases are important in cheese manufacture, and a considerable amount of information is available in the cheese literature (Bylund, 2003).

Alkaline phosphatase is a heat sensitive enzyme in milk that is used as indicator of pasteurization. If milk is properly pasteurized, alkaline phosphatase is inactivated (Bylund, 2003).

Lactoperoxidase is one of the most heat-stable enzymes found in milk. Both Lysozyme and Lactoperoxidase (when combined with hydrogen peroxide and thiocyanate) present antibacterial properties.

2.2 Cheese

Cheese was probably first discovered in the Middle East by nomads (Fox, 2003a). Cheese is a very varied group of dairy products, produced in a great range of flavors and forms throughout the world. From humble beginnings (i.e. simply as a means of conserving milk constituents as well as being highly nutritious and as classical example of a convenience food), it can be used as the main course in a meal, as a dessert or snack, as a sandwich filler, food ingredient or condiment (Hansen, 2010). Cheese production and consumption, which vary widely between countries and regions is increasing in traditional producing countries and is spreading to new areas (Fox *et al.*, 2015). Although most traditional cheeses have a rather high fat content, they are rich sources of protein and in most cases of calcium and phosphorus (Fox *et al.*, 2015). There are probably about 2,000 named cheese varieties, most of which have very limited production. All varieties can be classified based on the method used to coagulate the milk, i.e., rennet coagulation, which represent ~75 % of total production, isoelectric (acid) coagulation and a combination of rennet, acid and/or heat and acid (Fox *et al.*, 2015).

Cheese is a concentrated protein gel, which occludes fat and moisture. Its manufacture essentially involves gelation of cheese milk, dehydration of the gel to form a curd and treatment of the curd (e.g. dry stirring, cheddaring, texturisation, salting, moulding, and pressing). The moulded curd may be consumed fresh (shortly after manufacture, for example within 1 week) or matured for periods of up to 2 years to form a ripened cheese (Fox *et al.*, 2015). Cheese making was done by coming from experience, thus allowing for different flavor and texture characteristics in the cheese (Hansen, 2010).

2.2.1 Overview of cheese making

The technology of cheese making has two overriding goals: firstly, to establish the parameters that make a given cheese desirable (flavor, body, texture, melt and stretch properties); and, second, to develop a manufacturing and ripening protocol that will routinely reproduce these parameters every time this cheese is made (Law and Tamime, 2011). Cheese making is a rather simple process in itself, but it involves complex chemical and physical phenomena. Cheese making involves a number of main stages (**Fig. 2.5**) which are common to most types of cheese.

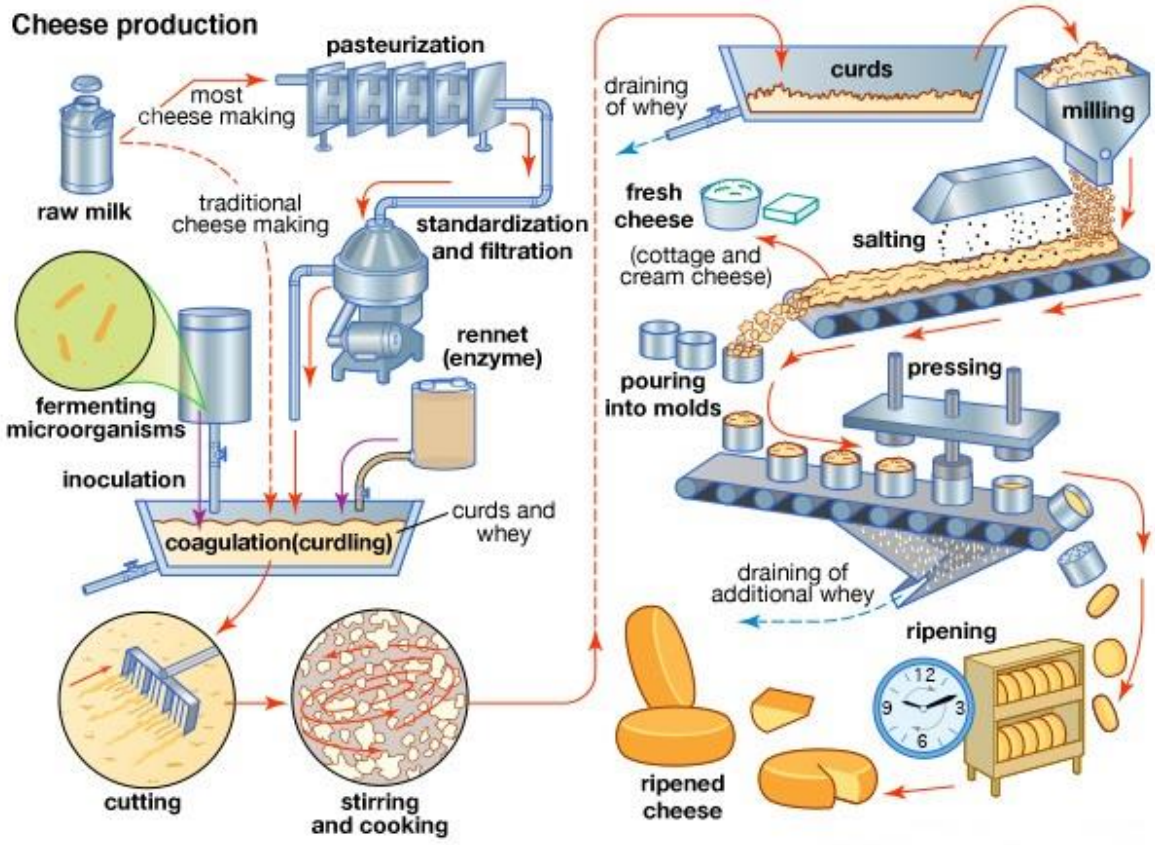


Fig. 2.5 Cheese making steps during cheese manufacturing (Reproduced from encyccheese, 2011).

There are also different stages required to produce specifics varieties. Although the manufacturing protocols can differ for individual varieties, cheese processing can be basically divided into the following steps: milk pre-treatment, coagulation, syneresis, whey drainage, molding, pressing, salting, and ripening or maturation. The manufacturing of cheese is essentially a concentration process, where milk fat and casein are concentrated approximately ten-fold, while the whey proteins, lactose and water soluble salts are removed with the whey. The concentration

process, begins with the coagulation of the major milk protein, casein, and then continues with manufacturing steps mainly designed to control the chemistry of the casein molecules. The physical or rheological characteristics of cheese are governed by interactions between casein molecules (Johnson and Lucey, 2006). After pre-treatment, milk is coagulated by enzymatic and/or acid action, which transforms the milk into a gel. Casein gels consist of a three-dimensional, porous and viscoelastic matrix of casein micelles saturated with an interstitial, viscous fluid called whey. Once the gel reaches an adequate firmness, the gel is cut into curd grains, which induces syneresis and the expulsion of whey. After a certain stirring period, the physical separation of the whey and curd follows. Thus, curd is further processed into cheese by molding, pressing, salting, and ripening.

2.2.2 Milk treatment prior to cheese making

The suitability of milk as a raw material for cheese production depends largely on conditions at the dairy farm (e.g. feeding animals on badly prepared silage can adversely affect the quality of several varieties of cheese) and milk pretreatment at the cheese manufacturing plant (e.g. cold storage and pasteurization). Milk from sick cows or animals undergoing treatment with antibiotics must not be used for cheese making, or any other milk product (Bylund, 2003).

A. Collecting the Milk

The composition of cheese is strongly influenced by the composition of the milk, especially the content of fat, protein, and calcium. The constituents and composition of milk, are influenced by several factors, such as species, breed, nutritional status, health, and stage of lactation. Owing to major compositional abnormalities, milk from animals in the very early or late stages of lactation and those suffering from mastitis should be excluded. Some genetic polymorphs of the milk proteins have a significant effect on cheese yield and quality and there is increasing interest in breeding for desirable polymorphs, especially in the case of sheep and goats (Park and Haenlein, 2013). Cheese milk should be free of chemical taints and free fatty acids, which cause off-flavors in the cheese, and antibiotics, which inhibit bacterial cultures (Fox and McSweeney, 1998).

B. Clarification or Filtration

Raw milk as produced on the farm and transported to the collection center of a dairy plant generally contains varying amounts of visible, invisible impurities. This foreign matter includes straw and hair pieces, dust particles, leukocytes (somatic cells or white blood cells), insects, etc (Bylund, 2003). If not effectively removed, such extraneous insoluble matter can result in deposits in milk handling equipment such as cooler, etc., and, more importantly, cause unsightly appearance. Relatively large pieces of such material e.g. straw, hair and insects, are usually removed by 'straining' (passing the milk through a fine metal–gauge strainer or metallic sieve on the farm, at the collection center or at the processing plant) (Bylund, 2003). This steps of aesthetic improvement of product are particularly useful for overcoming the problem of sediments in fluid milk and liquid milk products in general, and homogenized milk in particular **(Fig. 2.6)**.

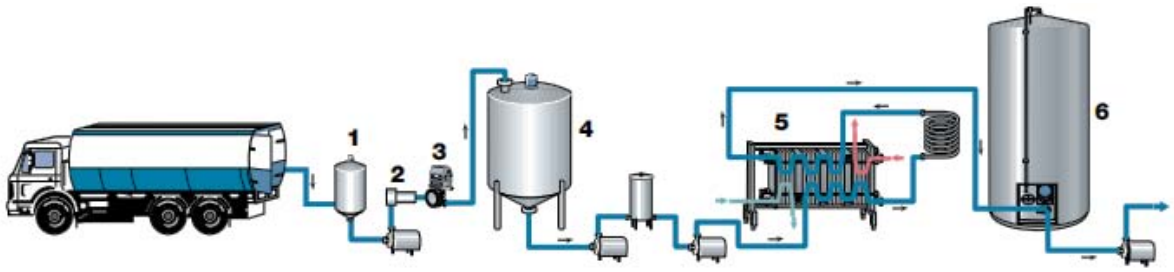


Fig. 2.6 Reception arrangements for cheese milk (Reproduced from Bylund, 2003).

1. Air eliminator 2. Filter 3. Milk meter
4. Intermediate storage tank 5. Cooling 6. Silo tank

C. Cold Storage

It has become an increasingly common practice in many countries that milk for cheese is normally cooled to 4 °C immediately after milking on the farms and may be held at about this temperature for several days on the farm and at the factory. These have led to the storage of milk for longer periods. Apart from the development of an undesirable psychrotrophic microflora, cold storage causes physico-chemical changes (e.g. shifts in calcium phosphate equilibrium and dissociation of some micellar caseins, which will tend to increase the possibility of proteolysis by enzymes derived from psychrotrophic bacteria, somatic cells or the blood), having undesirable effects on the cheese making properties of the milk. Milk stored at temperatures of less than 7 °C

contained a high proportion of soluble casein and clotted during large time (Bylund, 2003). The use of refrigerated milk could resulted in a weaker curd, lower curd yield and greater losses of fat and curd fines into the whey than the use of milk stored at 10-20 °C. Although raw milk is still used in both commercial and farmhouse cheese making, however, in most cases, the cold milk is heat treated before processing (Grandison, 1986) **(Fig. 2.7)**.

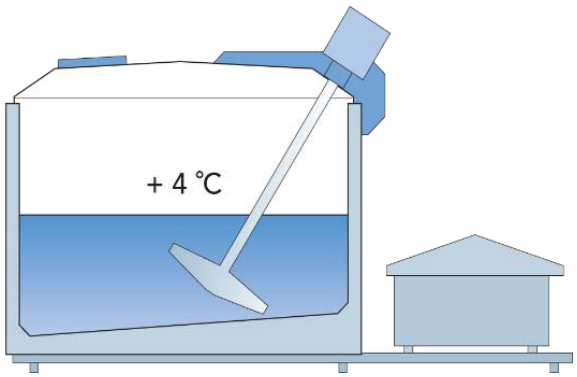


Fig 2.7 Bulk cooling tank with agitator and chilling unit (Reproduced from Bylund, 2003).

D. Standardization of milk

Standardization is the process of changing the solids composition of milk from that which is received from the producer. Milk standardization gives the cheese maker the ability to manipulate the composition of the final cheese by controlling the composition of the starting milk in order to meet the legal definition of the specific variety and to improve yields. The ratio of casein to fat (C/F) determines cheese composition in terms of the amount of fat in the total solids (TS) portion of cheese, i.e. fat-in-dry matter is an important parameter affecting cheese quality (Fox and McSweeney, 1998). Depending on the ratio required, the cheese maker can alter (standardise) milk composition by removing some fat by natural creaming or centrifugation, adding skimmed milk, adding cream (fat), addition of casein via non-condensed or condensed milks (whole milk, nonfat dry milk, or skim milk), evaporated milk, or ultrafiltration (UF) retentate, or just water removal (Fox and McSweeney, 1998). Such additions also increase the total solids content of the milk and hence increase the yield of cheese curd per unit volume (Fox, 2003a) and also the level of fat influences several aspects of cheese, including composition, biochemistry, microstructure, yield, rheological and textural properties, cooking properties, and ripening (Guinee and McSweeney, 2006).

According to Guinee and McSweeney, (2006), the reasons encouraging standardization of milk for cheese making are: **1)** variability in milk composition; particularly casein content will result in differences in clotting rate, coagulum firmness, and rate of pH drop owing to buffering by casein. Variability in casein content of the milk may require that the cheese maker adjust the rate and extent of acid development. Failure to do so may result in production of cheese that does not meet quality standards related to functionality and body characteristics. It is often undesirable, difficult, and not feasible for a cheese maker to make process adjustments during cheese making. Standardization lessens the need for 'on the fly' process adjustments, **2)** standardization of milk composition reduces variability in cheese composition, that is, fat on a dry basis (FDB) and moisture content, and pH, thus standardization can reduce variability in cheese quality, **3)** fortification, or standardization by adding milk solids, improves productivity; that is, more cheese per vat, more cheese per man-hour, and improved productivity of whey processing, **4)** standardization of milk is necessary to produce cheese that meets legal standards in terms of FDB and moisture content (Scott *et al.*, 1998).

E. Heat treatment

During cold storage, the milk protein and milk salts change character, which tends to impair cheese making properties. It has been shown that about 25% of the calcium precipitates as phosphate after 24 hours of storage at +5 °C (Banks *et al.*, 1993). This reduction, however, is temporary; when the milk is pasteurized, the calcium tends to re-dissolve and the coagulating properties of the milk are partially restored. *β*-casein also leaves the complex casein micelle system during cold storage, which further contributes to reduce the cheese making properties.

Pasteurization at 72–76 °C for 15–18 s or at 63-65 °C for 30 min is not a severe enough heat treatment to cause problems in cheese making, and adjustments are not usually necessary in the manufacturing protocol, other than the addition of a small amount of calcium chloride (Johnson *et al.*, 1990). However, high heat treatment of milk prolongs rennet coagulation times and reduces the strength of rennet gels (Dalgleish *et al*., 1992), leading to impaired syneresis (Pearse *et al*., 1985; Marshall *et al*., 1986). Guinee *et al*. (1998) attributed decreased cheese firmness with increased heating severity of milk to increased cheese moisture and decreased protein content. High heat treatment increases retention of whey protein and moisture in cheese and reduces the

level of calcium and phosphorus due to reduced dry matter and increased whey protein content (Banks *et al*., 1993).

F. Pre-acidification

It is known that pH affects the rate of renneting, curd firmness, and syneresis, as well as fat recovery, Ca retention, and, consequently, the final texture of cheese (Watkinson *et al.*, 2001). Furthermore, it has been demonstrated that milk acidification reduces the rennet coagulation time (RCT), whereas gel firmness is inversely related to the pH (Mellema, 2002; Tsioulpas *et al.*, 2007). By acidifying milk (e.g., bacterial fermentation, addition of acid, etc.), many of the physicochemical properties of casein micelles undergo considerable change, especially in the pH range from 5.5 to 5.0. As the pH of milk is reduced, CCP is dissolved, increases the Ca^{2+} activity, and the caseins are dissociated into the milk serum phase (Roefs *et al.*, 1985; Dalgleish and Law, 1988). The extent of dissociation of caseins is dependent on the temperature of acidification: at 30 °C, a decrease in pH causes virtually no dissociation while at 4 °C, about 40% of the caseins is dissociated in the serum at pH -5.5 (Dalgleish and Law, 1988). Aggregation of casein occurs as the isoelectric point (pH 4.6) is approached. Apparently, little change in the average hydrodynamic diameter of casein micelles occurs during acidification of milk to pH 5.0 (Roefs *et al.*, 1985). The lack of change in the size of the micelles on reducing the pH of milk may be due to concomitant swelling of the particles as CCP is solubilized Heat treatment of milk prior to acidification has little effect on the extent of solubilization of CCP (Singh *et al.*, 1996).

G. Calcium chloride ($CaCl_2$)

Calcium plays an essential role in the coagulation of milk by rennet and in the subsequent processing of the coagulum. In a typical cheese production plant, $CaCl_2$ is added before the addition of the coagulant, without affecting final cheese quality (Green *et al*., 1979). Addition of calcium chloride to the cheese milk can alleviate cold-storage and heat-induced impairments of clotting and curd firmness, and improve poor coagulating milk if the cause is low calcium content.

The effect is probably due to a combination of: (a) calcium binding to the casein micelles in such a way that it reduces the repulsive forces between them, thus enhancing hydrophobic interactions and (b) a slight drop in pH promotes the action of the coagulant and increases the rate

of aggregation, speeds up the curd formation and whey separation (McMahon and Brown, 1984; van Hooydonk, 1987; Zoon *et al*., 1988).

Once the milk is ready to coagulate, the coagulation is induced as indicated below by acidification or enzyme addition.

2.2.3 Conversion of milk to cheese curd

Typically, four steps, or groups of steps, are involved in the conversion of milk to cheese curd: coagulation, syneresis (expulsion of whey), moulding/shaping and salting. These steps, which partly overlap, enable the cheese maker to control the composition of cheese, which, in turn, has a major influence on cheese ripening and quality.

2.2.3.1 Milk coagulation

The ability of casein micelles to stay in solution at natural milk pH (~6.7) relies on the net negative charge and hydrophilic character of the C-terminal end of κ-CN at the micelle surface. The essential characteristic step in the manufacture of all cheese varieties involves coagulation of the casein component of the milk protein system to form a gel which entraps the fat, if present (Fox *et al.*, 2000). There are two basis approaches to induce micelle aggregation: by enzymatic action or by acidification, sometimes a combination of those is used. Coagulation may be achieved by:

- Limited proteolysis by selected proteinases;
- Acidification to pH 4:6;
- Acidification to pH values >4.6 in combination with heating.

The outcome of these reactions is to a large extent determined by the amounts and proportions of the various components in milk, with the protein composition contributing significantly in this regard.

A. Acid-induced coagulation

In acid coagulation of milk, casein micelle properties are altered by a lowered milk pH (Lucey and Singh, 1997) without the presence of rennet. This causes CCP to dissociate from the micelles and the negative charges in the casein micelles are neutralized, with aggregation occurring

as the isoelectric point of the casein micelle (pH 4.6) is approached (Fox and McSweeney, 1998). A porous network of loosely linked aggregates is formed. Milk used in manufacture of fermented milk products (e.g., yogurt) is generally subjected to a quite severe heat treatment (90˚C, 5-10 min), with a marked effect on the end product. Temperatures above 60˚C cause denaturation of whey proteins (mainly β-LG), which via disulphide bonds either associate with κ-CN on the casein micelles (Sawyer, 1969; McKenzie *et al*., 1971) or form soluble aggregates (Haque and Kinsella, 1988; Guyomarc'h *et al*., 2003). This results in increased curd firmness (Dannenberg and Kessler, 1988) due to an increased number and strength of bonds of the acid gel, as denatured whey proteins associated with casein micelles interact with each other (Lucey and Singh, 1997). Further, the concentration of protein in the gel network will be increased because of the active participation of denatured whey protein in structure formation.

B. Enzyme-induced coagulation

Enzymatic coagulation of milk is the modification of casein micelles via limited hydrolysis of casein by rennet, followed by calcium-induced micelle aggregation (Fox and McSweeney, 1998). Rennet is traditionally extracted from calf abomasa and is a mixture of the two gastric proteases chymosin and pepsin (Andrén, 2003). Coagulation of milk by rennet probably occurred initially by accident, as warm milk was stored in sacks made from the stomachs of ruminant animals which contained some residual proteinase. Chymosin is the major and the most active component, specifically cleaving the peptide bond Phe_{105}-Met_{106} of κ-CN. According to (Hansen, 2010) and previous literature there seems to be consensus that rennet induced milk coagulation is the result of three underlying stages with different mechanisms (**Fig.2.8**):

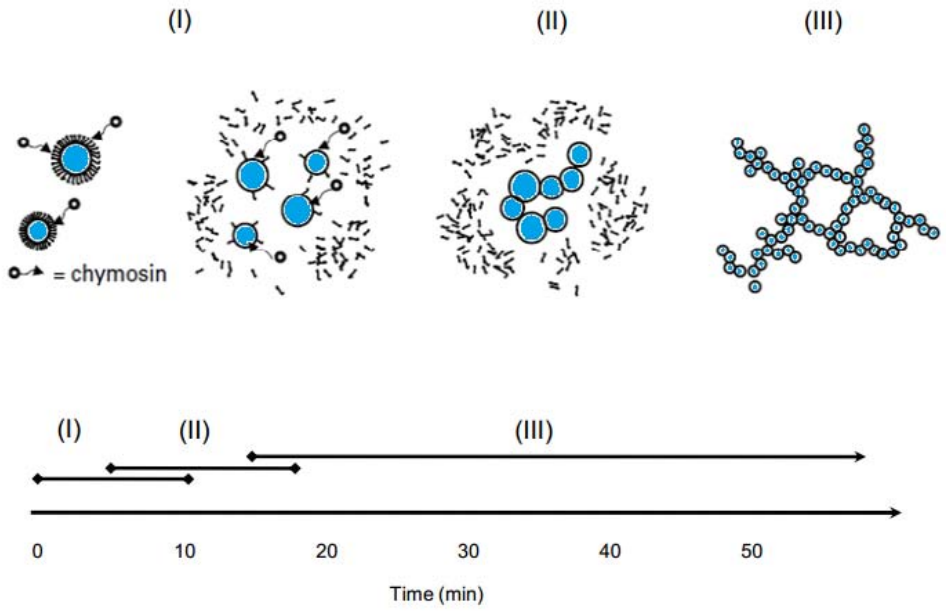


Fig.2.8. Illustration of the rennet coagulation process. (I) *κ*-casein removal by chymosin (II) para-CN aggregation and (III) gel network formation (Reproduced from Hansen, 2010; Dalgleish, 1993).

1) Primary Stage (enzymatic hydrolysis): during the primary phase, enzymatic hydrolysis occurs by enzyme (rennet), which cuts off a specific fragment of one of the caseins, namely, *κ*-casein into para-*κ*-CN and caseino-macropeptide, with the hydrophilic caseino-macropeptide part being released into the whey. This causes loss of a negatively charged group and decreased steric stabilization (Senge *et al*., 1997). When ~80% of *κ*-CN is hydrolyzed (Walstra *et al*., 2006), at the natural pH of milk, colloidal stability of the micelles is reduced enough for the spontaneous, secondary aggregation phase to start. **2) Secondary Stage (casein micelle aggregation):** the next stage is the physical-chemical process of aggregation of casein particles to form a gel. After losing its water soluble tail, *κ*-CN can no longer keep the casein particles separated, so they begin to form a gel as molecular chains and clusters initially connect through hydrophobic bonds. The clusters continue to grow until they form a continuous, three dimensional network followed by further solidification through calcium cross-linking and traps water inside, forming a gel. The aggregation rate depends on the concentration of free para-casein sites implying that this stage is dependent on rate and degree of casein proteolysis. **3) The third stage** refers to an ongoing firmness in the development of the gel network. For more cheeses the gel is cut as soon as it is firm enough to do

so. Coagulation is enhanced by decreasing pH, increasing calcium concentration and temperature (no aggregation below 20˚C). Rennet coagulation is faster (minutes) compared to the slower rate of acid development (hours) required by cultures to coagulate milk for cheeses such as cottage cheese. Rennet milk gels also undergo much greater syneresis than acid milk gels, which helps to produce cheeses with lower moisture levels (Lucey, 2003). Rennet gelation and the factors affecting it are described in more detail below. For better understanding of the biochemical processes behind enzymatic milk coagulation, it was important to study the structure of the casein micelle as this structure and the behavior of the casein micelles in milk are highly related to the coagulation process.

C. Size of casein micelles

Casein micelles and fat globules are the main two particles responsible for light scattering in milk; light scattering intensity depends on particle nature and size (Castillo *et al.*, 2005d). Several studies have shown that smaller micelles are relatively rich in κ-casein and relatively depleted in β-casein, while the contents of α_s-caseins appear to be independent of size (Marchin *et al.*, 2007). In milk, there is an average micelle diameter of 300 nm (Roefs *et al.*, 1985). The average sizes depend on the composition of the milk, but, in general, the average hydrodynamic diameter is from 150 to 350 nm. Analysis of the size distribution has suggested a minimum diameter of approximately 80 nm (de Kruif, 2014), although some particles may be as low as 50 nm in milk reconstituted from skim milk powder (Udabage *et al.*, 2003). Electron microscopy generally yields smaller values for the micellar diameter, probably because sample preparation and dehydration steps can cause significant shrinkage of the micelles (Martin *et al*., 2006). Recently, it has been claimed that a population of so-called mini-micelles with diameters in the 20 to 40 nm range coexists with the larger particles observed by light scattering and separation methods (Muller-Buschbaum *et al*., 2007).

Goat casein micelles have greater average diameter and polydispersity, higher mineral content and lower degree of hydration than cow (Trujillo *et al.*, 1998). Tziboula and Horne (1999) found that milk with high and low α_{s1}-CN presents diameters 210-320nm and 160-280nm respectively.

D. Structure of casein micelles

Casein is the major protein component of bovine milk, comprising approximately 80% of the total milk protein, which is not present in true solution but as a suspension of micelles having hydrophobic core and a hydrophilic surface. The caseins easily form polymers containing several identical or different types of molecules. The polymers are built up of hundreds and thousands of individual molecules and form a colloidal solution, which is what gives skim milk its whitish-blue tinge. These molecular complexes are known as casein micelles.

There are four individual types of casein molecules (**Fig. 2.9**), the α_{s1}-, α_{s2}-, β-, and κ-CN in approximate relative amounts of 4, 1, 3.5, and 1.5, respectively. The four subgroups of casein are all heterogeneous and consist of 2 – 8 genetic variants. These variants differ from each other only by a few amino acids (Caroli *et al*., 2009).

Since the structure of the micelles has been debated for many years, and several reviews have appeared in the past 10 years, giving somewhat divergent points of view (de Kruif and Holt, 2003, Castillo *et al.,* 2005a, Horne, 2006, Fox and Brodkorb, 2008, Horne, 2011, Dalgleish, 2011), a number of casein micelle models have been proposed.

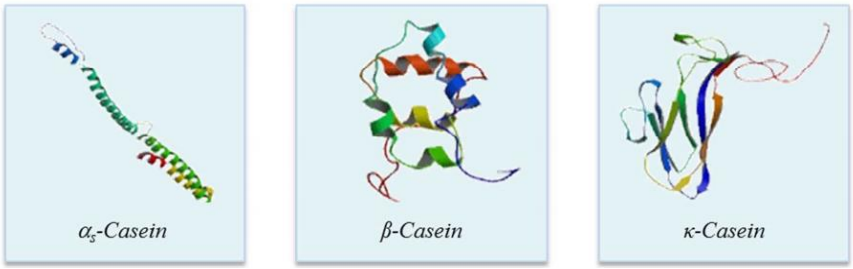


Fig. 2.9. Protein structure of caseins. Reproduced from Requena and Rebouillat (2015).

Fig 2.10 shows one of the most common accepted models (Walstra and Jennes, 1984) for casein micelles, which consist of a complex of sub-micelles (**Fig. 2.11**) of a diameter of 10 to 15 nm.

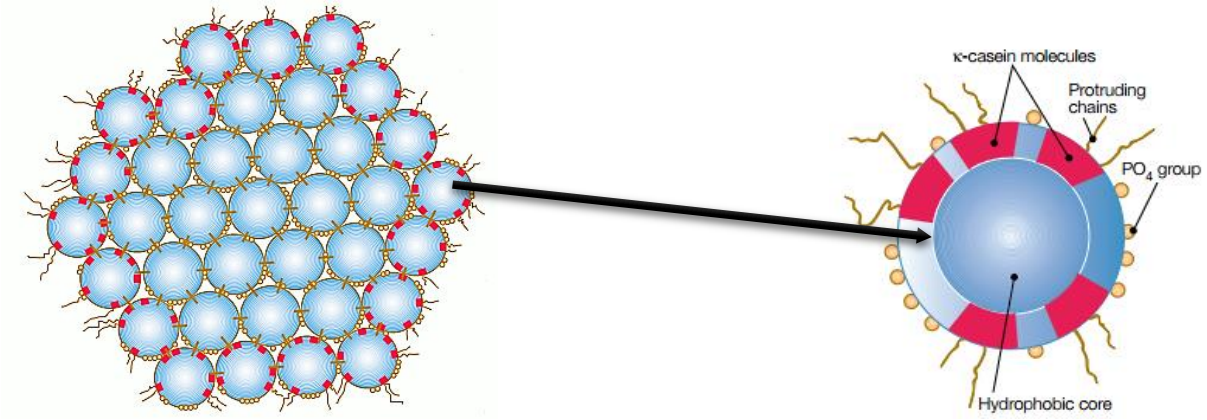


Fig 2.10 Structure and stabilization of casein micelles (Reproduced from Bylund, 2003).

Fig. 2.11. Structure of a casein submicelle. (Reproduced from Bylund, 2003).

Submicelle

Calcium phosphate

κ-casein

Protruding chain

Hydrophobic interactions (PO_4 groups)

The hydrophilic surface is due to the presence of *κ*-CN, which is a long peptide (build up from 169 amino acid units) representing 12-15% of the total casein. The *κ*-CN plays an important role in relation to coagulation because the rennet enzyme chymosin is extremely selective towards Phe_{105}– Met_{106} bond in *κ*-CN. It is precisely this event of *κ*-CN that initializes rennet coagulation (Fox and McSweeney, 1998).

Because most, if not all, of the *κ*-CN is found on the surfaces of the micelles, it is believed to limit the growth of the particles by binding to the surface of growing aggregates formed by the three other caseins and the calcium phosphate (Horne, 2006). Most of the functional properties of the micelles depend on the properties of the surface, rather than those of the interior, and to some extent the micelles can be regarded as hard spheres with a protective coating (de Kruif, 1999). On the other hand, the interior of the micelle becomes important in post-coagulation rearrangements as cheese curd forms. Electron microscopy appeared to suggest that the micellar interior was of a granular appearance consistent with the presence of submicelles (Schmidt, 1982), although later studies have tended to disagree with this assessment (McMahon and McManus, 1998, McMahon and Oomen, 2008).

More recently proposed models for the casein micelle have emerged that refute the notion of discrete sub-micellar structures within the micelle. The first model (**Fig. 2.12**) to depart from the submicelle theory was that of de Kruif and Holt (2003).

During a series of studies on casein-calcium-phosphate interactions Holt *et al.* (1998) discovered that the phosphopeptide fraction of *β*-casein could bind to and stabilize calcium-phosphate aggregates resulting in the formation of nanoclusters of a discrete size and composition; without the peptides the calcium phosphate structures would grow randomly and precipitate. This discovery led de Kruif and Holt to propose that such nanoclusters are the centerpiece of casein micelle structure (de Kruif *et al.*, 2002).

The formation of nanoclusters with a radius of 2.3 nm would drive micelle formation by randomly binding phosphoproteins causing an inverted micelle, and then more proteins could coat this new hydrophobic surface and in turn, bind more calcium phosphate until a size limited colloid is formed. There are about 800 of these amorphous calcium phosphate nanoclusters in an average sized casein micelle (~100 nm in diameter), (**Fig. 2.12**).

This nanocluster model is supported by the earlier rheomorphic theory of casein structure (Holt *et al.*, 2003) and the recent small-angle X-ray scattering (SAXS) data by Pignon *et al*. (2004).

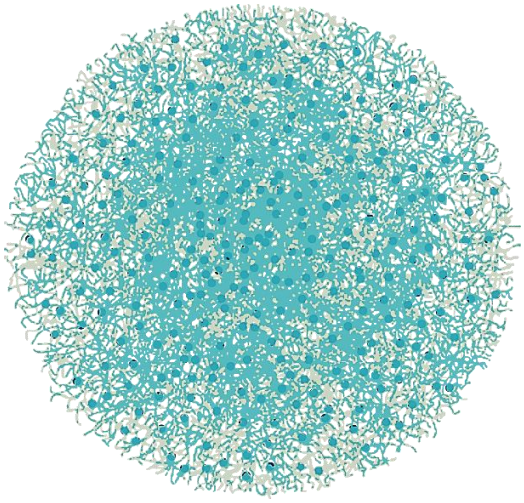


Fig. 2.12. The nanocluster model for casein micelles. Casein monomers are thread-like, while the dark circles represent calcium phosphate nanoclusters. Reproduced from Phoebe, (2007).

The casein micelle model proposed by Horne (1998) (**Fig. 2.13**) considered the surface chemistry of the individual caseins and concluded that protein-protein interactions were indeed important, but in essence the model retains the rheomorphic concept.

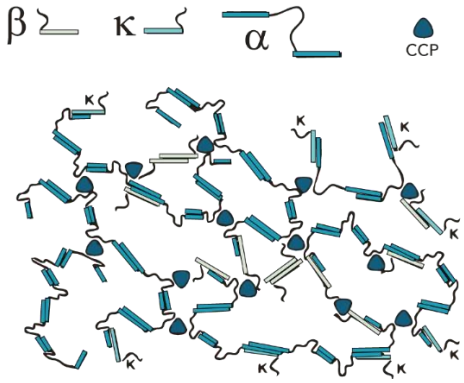


Fig. 2.13 The model of Horne – dual bonding model of casein micelles using casein monomers as indicated. Protein-protein interactions occur between hydrophobic regions (rectangular bars) while the protein hydrophilic regions (loops) bind to calcium phosphate clusters (triangles). *κ*-Casein is monomeric and on the surface. Reproduced from Phoebe, (2007).

Taken from this point of view, the micelles would have a substructure, rather than a distribution of calcium phosphate within a rather uniform protein matrix, but it would not be the same as that envisaged in the original sub-micellar hypothesis (Dalgleish, 2011). In effect, this embodiment of the nanocluster model can be taken to invert the sub-micellar model; instead of casein particles linked by calcium phosphate, there are calcium phosphate/casein particles linked by noncovalent bonds. What is important about the nanocluster model is that it provides a distinct mechanism for the formation of the casein micelles via the interaction between the serine phosphate groups of the caseins and calcium phosphate (Holt *et al.*, 2003).

Dalgleish and Corredig (2012) showed that the main details of micellar substructure come from physical measurements from two sources, electron microscopy and small angle neutron scattering (SANS) or X-rays (SAXS). Electron microscopy, both transmission electronic microscope (TEM) and scanning electronic microscope (SEM), has been used for many years to try to elucidate micellar structure. In particular, the earlier work by TEM on gold replicas of freeze-fractured micelles showed a granular appearance that was taken to indicate the presence of submicelles (Schmidt, 1982, Karlsson *et al.*, 2007) (**Fig. 2.14**).

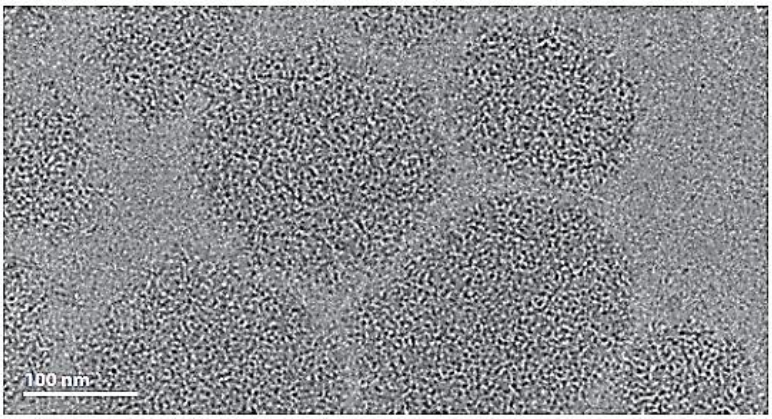


Fig. 2.14 Cryo-transmission electron microscopy image of casein micelles, showing the relatively even distribution of calcium phosphate clusters (dark points) within a distributed matrix of protein. Samples were prepared by vitrification in liquid ethane, and the measurement was made at a temperature below 97 K. Reproduced from Dalgleish and Corredig (2012).

However, as pointed out above, the nanocluster model also provides in-homogeneities within the micelle. A relatively recent study of the TEM of micelles has severely criticized much of the earlier results from microscopy, suggesting that many observed structures could be artifacts arising from the fixation and staining procedures that are essential in TEM (McMahon and Oomen 2008). This study gave evidence that the structure was more of an extended web rather than one consisting of subunits and suggested that water channels could exist throughout the micelle. It provided some support for the nanocluster model, where the nanoclusters surround regions of low density associated with hydration. Similar results have been proposed by Harte (2011), where an open structure of the micelle with water channels is proposed. Other studies, using cryo-TEM, where staining is not used, again give images that do not appear to be consistent with the sub-micellar model, but rather show a semiregular dispersion of calcium phosphate clusters within a reticulated protein matrix (Marchin *et al.*, 2007, Knudsen and Skibsted, 2010). Studies using SEM (scanning electronic microscope) also depend on the methods of sample preparation and whether or not the samples are metal coated. The most detailed micrographs of uncoated specimens using field emission SEM do not show submicelles; rather, they show an irregular structure (**Fig. 2.15**) with apparently tubular features and with deep clefts that seem to be leading to the interiors of the micelles (Dalgleish *et al*., 2004). Coating of these structures with gold for standard TEM does give a more sub-micellar appearance (McMahon and McManus, 1998), but this may be an artifact

arising from the gold coating. Thus, more recent methodologies in electron microscopy result in images that do not appear to support a classical sub-micellar structure (de Kruif *et al.*, 2012).

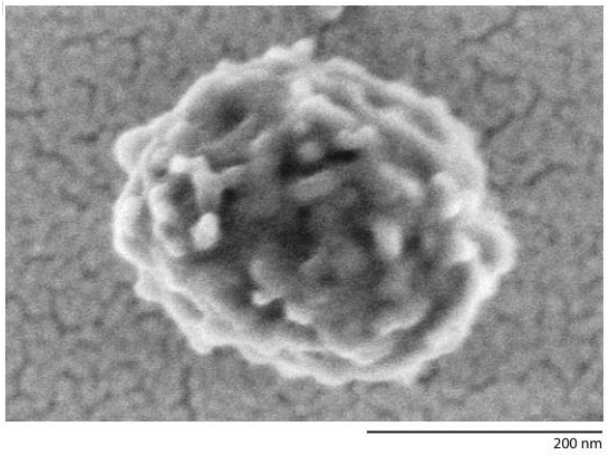


Fig. 2.15 Field-emission scanning electron microscopy image of a casein micelle, chemisorbed to a carbon film and fixed but not stained or metal coated. Reproduced from Dalgleish and Corredig (2012).

According to Dalgleish and Corredig (2012), SAXS studies have generally been interpreted on the basis of a nanocluster model (Marchin *et al.*, 2007), although detailed interpretations of the scattering profiles differ substantially. Shukla *et al.* (2009) proposed an uneven distribution of ellipsoidal calcium phosphate nanoclusters within the micelles. On the other hand, using a variant of SANS on thin films of micelles, Metwalli *et al.* (2009) postulated the existence of mini-micelles, which are present in micellar suspensions prepared from powders derived from the ultrafiltration and diafiltration of milk. A most recent interpretation of the SAXS profile has been given by Bouchoux *et al.* (2010) on the basis of studies of the scattering of micelles that are progressively concentrated and dehydrated by osmotic stress, where it is claimed that the micelle contains hard regions of protein/calcium phosphate within a highly hydrated sponge-like structure. This last interpretation is interesting because it explicitly takes into account the large hydration of the micelle and how the loss of this water during osmotic stressing affects the micellar structure. The proposed structure is similar to that suggested by Dalgleish (2011) in a recent review (**Fig. 2.16**).

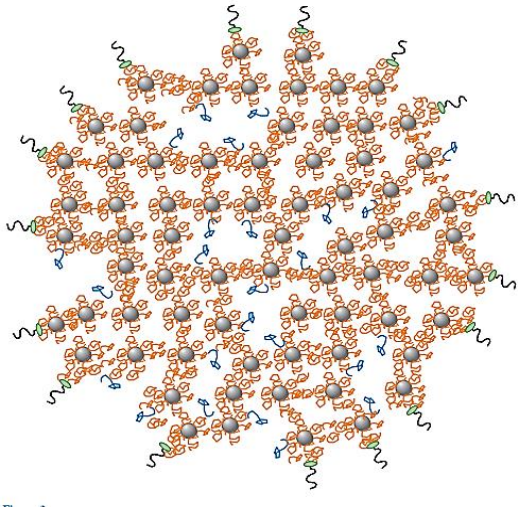


Fig. 2.16. Schematic section through a micelle, showing the regions of water within the structure. The α_s- and β-caseins (orange) are attached to and link the calcium phosphate nanoclusters (grey spheres). Some β-casein (blue) hydrophobically binds to other caseins and can be removed by cooling. The para-κ-casein (green) and the caseinomacropeptide chains (black) are on the outermost parts of the surface. Not drawn to scale, and the sizes of the water channels are exaggerated for clarity. Reproduced from Dalgleish and Corredig (2012).

This proposed structure is important because it is known that the micelle is to some extent porous given that β-casein can be removed from the micelle by cooling (Creamer *et al.*, 1977) and large molecules can penetrate the micelle (Colsenet *et al.*, 2005, Le Feunteun and Mariette, 2007). The dual binding model (Horne, 2008) invokes hydrophobic interactions as being important in linking the caseins or nanoclusters together, but such interactions would appear to preclude the presence of water in the micelle interior. The suggestion has been made that water channels within the micelle can be stabilized by β-CN interacting with the hydrophobic portions of the nanoclusters, to give a structure such as is shown in **Fig. 2.16** (Dalgleish, 2011). Such a model shows a relatively even distribution of the nanoclusters but also contains pores stabilized by β-casein; this accounts for the hydration of the particles, the presence of β-CN in the micelle interior, and the observation of more rigid regions formed from linked nanoclusters.

E. Colloidal stability of casein micelles

Like all other particles in solution, casein micelles are in constant Brownian motion and frequently collide. Collision can lead to either aggregation caused by overall attractive forces, or separation caused by overall repulsive forces. The stability of intact micelles against aggregation

shows that repulsive forces are dominating and this is caused by two mechanisms: (a) electrostatic repulsion and (b) steric repulsion.

One of the phenomena in milk keeping casein micelles from spontaneous aggregation is *steric repulsion* between micelles (Hansen, 2010). Repulsion can occur when two micelles come close enough for their κ-CN hairy layers to interact. The reason for this repulsion is that the local concentration of polymers is increased, causing an increase in osmotic pressure. To counteract this process, solvent is sucked into the gap between the micelles, which drives them apart. Steric repulsive forces can be quite strong if the polymer chain density is high (Walstra *et al.*, 2006). Another closely related phenomenon is *electrostatic repulsion*. Particles in aqueous solutions most often bear an electric charge. In the micelle, the surface potential is generally around -25 mV. The caseins generally have negative overall charge at pH values relevant to cheese making, and especially the CMP part of κ -CN carries high-negative charge. However, electrostatic repulsion cannot alone account for the stability of casein micelles. Calculations based on the Derjaguin, Landau, Verwey and Overbeek theory (DLVO), which considers the effects of electrostatic repulsion and van der Waals attraction, show that casein micelles would not be stable towards aggregation if electrostatic repulsion were the only repulsive effect (Payens, 1979). At physiological pH the potential is, however, negative, which causes the particles to repel each other, when they approach. As the pH decreases the surface potential approaches zero, which is one of the main reasons why pH is a factor affecting coagulation rate (Dalgleish (2011). There are not only repulsive forces in the colloidal system of milk. In fact, Van der Waals attraction forces along with calcium binding complex formation are believed to be the dominating reasons for micelle aggregation (Lucey, 2003). According to Dalgleish and Corredig (2012), most, if not all, of the κ-CN is present on the surfaces of the particles (Dalgleish *et al.*, 1989). This κ-CN causes the micelle to be stable against aggregation, because part of the molecule, the macropeptide (residues 106–169 of the protein), appears to be extended from the micellar surface to create a layer, estimated to be 5–10 nm thick, around the particles (Horne, 1986; de Kruif and Zhulina, 1996). This hairy layer provides steric and electrostatic stabilization to the micelles so that they cannot approach each other closely (**Fig. 2.17**).

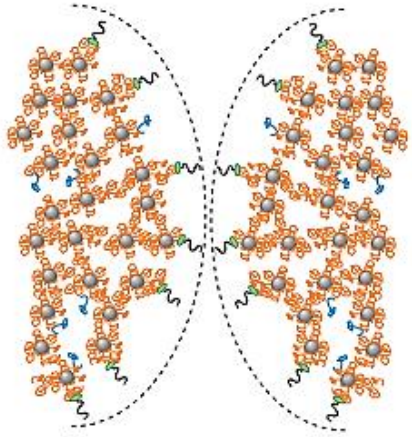


Fig. 2.17. Diagrams of interacting micelles (only the interacting parts are shown, not the whole particles). (a) Native micelles are sterically stabilized by macropeptide hairs. The zone of action of the steric effect is indicated by dashed lines. Reproduced from Dalgleish and Corredig (2012).

F. Micelle Aggregation

In rennet cheeses, the process of cheese making is based on a simple, specific, proteolytic reaction that causes the destabilization of protein particles in milk, which aggregate and form a gel network. Thus, cheese curd is a protein-based gel in which the hydrolyzed casein micelles form a continuous network containing fat globules. The final structure of the gel will be a function of other factors, including, but not limited to, temperature, pH, calcium concentration and milk processing history. By modulating the early stages of structure formation or manipulating the cheese curd it is possible to obtain the large variety of cheese products available today. The reaction is initiated by the enzyme chymosin (in most rennets), which hydrolyzes specifically *κ*-CN followed by calcium- induced aggregation of the rennet-altered micelles. Upon hydrolysis of this protein, the overall free energy of association between the micelles decreases and the casein particles stick to one another forming a three-dimensional gel (**Fig.2.18**). Removal of the macro-peptides from the surface of the casein micelles reduces their zeta potential from −20 mV to −10 mV and removes the steric stabilizing layer (Castillo, 2001). The hydrophilic macro-peptides diffuse into the surrounding medium while the para-*κ*-casein remains attached to the micelle core (the macro-peptides represent ~30 % of *κ*-casein, i.e., 4–5 % of total casein; this unavoidable loss must be considered when calculating the yield of cheese).

The apparent importance of micellar charge in the coagulation of rennet-altered micelles suggests that pH should have a major influence on the secondary phase of coagulation. However, Pyne (1955) claimed that pH has essentially no effect on the coagulation process although the rate of firming of the resultant gel is significantly increased on reducing the pH (Kowalchyk and Olson, 1977).

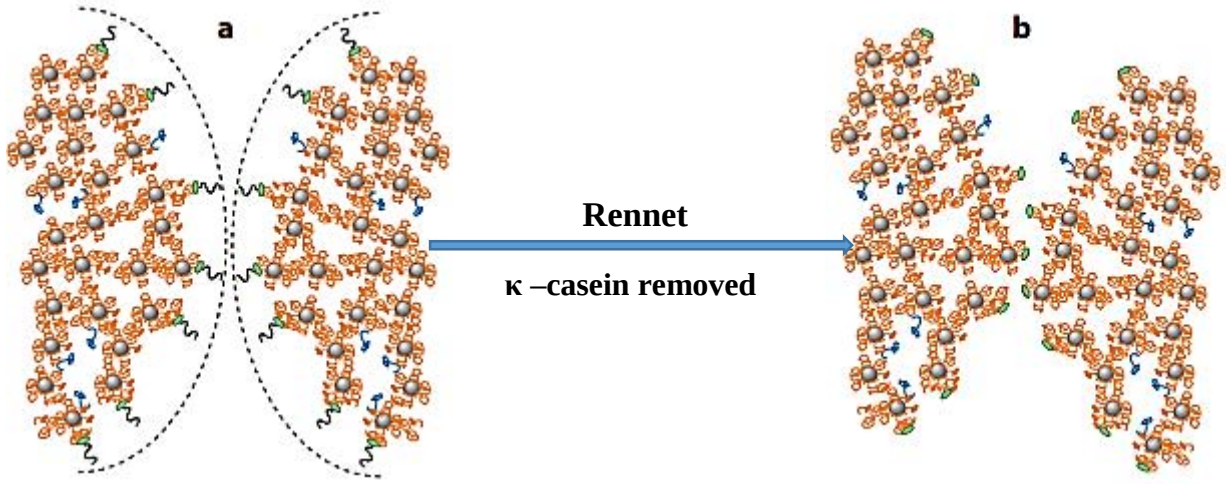


Fig.2.18. a) Native micelles are sterically stabilized by macropeptide hairs. The zone of action of the steric effect is indicated by dashed lines. **b)** Renneted micelles where the hairs have been removed by chymosin, allowing close approach of the micellar surfaces. Reproduced from Dalgleish and Corredig (2012).

Hansen (2010) stated that primary (enzymatic hydrolysis) and secondary (casein micelle aggregation) stages of rennet coagulation overlap to some extent during cheese making. Micelles start to aggregate when the greater part (60 -80%) of the *κ*-CN hairs have been cleaved so that the steric (and electrostatic) repulsive forces have been diminished sufficiently.

The more *κ*-CN chymosin has removed the greater the rate of flocculation, because a greater number of free non-hairy sites are available; *κ*-CN denuded micelles are referred to as para-*κ*-CN, depicted in **Fig.2.19**.

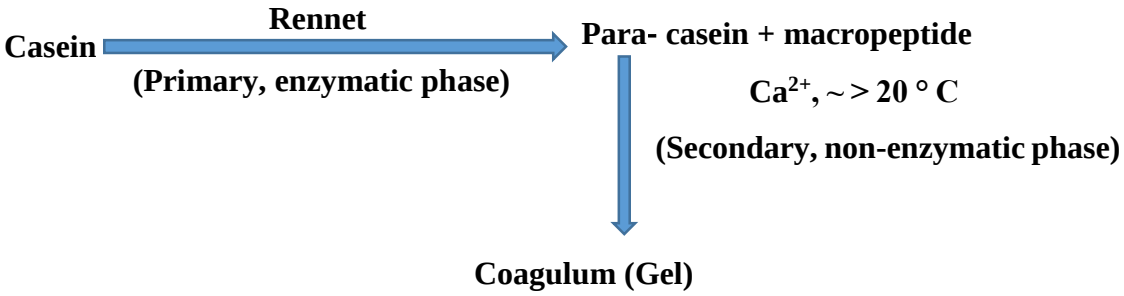


Fig.2.19.Mechanism of the rennet coagulation of milk. The primary phase involves enzymatic hydrolysis of *κ*-CN, while the secondary stage involves aggregation of the rennet-altered (para-*κ*-CN) micelle into a three- dimensional gel network or coagulum. Reproduced from Fox *et al.* (2000).

For aggregation to occur, a sufficient Ca^{2+} concentration is also required because Ca^{2+} diminishes the electrostatic repulsion between micelles and Ca^{2+} ions and can make bridges between negative sites in the para-CN micelles. In this process, pH also plays an important role since lowering the pH will increase the Ca^{2+} activity (Walstra *et al*., 2006). Dalgleish and Corredig (2012), informed that during the first stage of the enzymatic reaction, there is an increase in the diffusion coefficient of the micelles because of the gradual removal of their polyelectrolyte hairy layer (de Kruif, 1992). The milk viscosity decreases until more than 85% of the *κ*-CN is hydrolyzed (Tunier and de Kruif 2002). At this point, the steric stabilization generated by the few remaining *κ*-CN hairs is insufficient to keep the micelles apart, and they begin to aggregate and eventually to gel (Dalgleish, 1979, Sandra *et al.*, 2006, Kethiredipalli *et al.*, 2010).

The clotting time (t_{clot}) is defined as the time taken from the addition of the coagulant until the first visible flocks are formed in a milk film. In an undisturbed milk sample, flocks will continue to grow, and eventually will extend to the entire volume of milk.

The firmness of the gel will continue to grow, and the time to cut (t_{cut}) is then defined as the time from coagulant addition until the gel has the firmness needed to start cutting. It is worthwhile to note that while the strength of coagulants is defined by methods based on clotting time, the time to cutting is of much more practical importance, since it marks the appropriate time for initiation of the next step in the cheese making process. The assembly of renneted micelles into a gel has been studied using various forms of viscometry, electron microscopy and light scattering.

2.2.3.1.2 Kinetics of enzymatic milk coagulation

Van Hooydonk *et al*. (1984) and Dalgleish (1993) informed that since the breakdown of the *κ*-CN substrate is essentially a single-step enzyme catalyzed reaction, it seems reasonable to suppose that the kinetics of the proteolysis should obey the standard Michaelis-Menten formulation. In this, the instantaneous rate of the reaction (i.e. the rate at which substrate *S* is converted into product), *v*, is given by the relation:

$$\mathbf{Eqn\ (2.1)} \qquad v = -\frac{d[S]}{dt} = \frac{V_{max} \cdot [S]}{(K_m + [S])}$$

where [*S*] is substrate (*κ*-CN) concentration, V_{max} is the maximum rate of proteolysis at infinite substrate concentration and K_m represents the dissociation constant of the enzyme-substrate complex. The renneting reaction has been analyzed in this way in a number of studies (Dalgleish, 1979; Chaplin and Green, 1980). The reaction of synthetic peptides with chymosin has also been shown to obey the Michaelis- Menten behavior (Visser *et al.*, 1980). However, it should be noted that this behavior is not always observed, because of the relation between the concentration of substrate and the constant K_m. It can be seen from equation (2.1) that if [*S*] is much larger than K_m, a reaction which is zero-order with respect to the concentration of substrate will be observed, at least in the early part of the reaction. Conversely, if K_m » [*S*] the reaction becomes apparently first order:

$$\mathbf{Eqn\ (2.2)} \qquad v = -\frac{d[S]}{dt} = \frac{V_{max} \cdot [S]}{K_m}$$

The rate of firming of renneted milk gels is influenced by the type of rennet, especially under unfavorable conditions, e.g. high pH, low (Ca^{2+}) (Richardson *et al*., 1971; Kowalchyk and Olson, 1979; Ustumol and Hicks, 1990).

2.2.3.1.3 Factors that influence rennet-induced coagulation of milk

Rennet-induced coagulation of milk is influenced by a number of factors, such as pH, calcium concentration, protein content, enzyme concentration and processing treatments (e.g. cold storage, heat treatment, high-pressure treatment, and ultrafiltration…. etc.) (Gunasekaran and Ay, 1996; Daviau *et al.*, 2000). Due to the differences in the compositions and constituents of milk

from different species, rennet-induced coagulation properties of these milks vary; furthermore, caution should be exercised because of the large variation in composition and properties of milk between breeds of any particular species, and conflicting results reported in the literature. Comparative studies of rennet coagulation properties of milks of different breeds within species were reported by Storry *et al*. (1983) and Bencini (2002).

A. Milk Protein

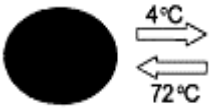
According to Fox (2003a), the coagulation time of milk decreases markedly with protein (and thus casein) content. However, further increases in milk protein level might result in a slight increase in gelation time, an effect attributable to the decreasing rennet to casein ratio, which necessitates an increase in the time required to generate sufficient hydrolysis of κ-CN to induce aggregation. As a result, although many authors observed a decrease in gelation time with increasing protein concentration (Reuter *et al.,* 1981; Lucisano *et al.,* 1985; Mehaia and El-khadragy, 1998; O'Callaghan *et al.,* 1999a) some authors found no decrease or even an increase in clotting time, when protein concentration increased. For instance, Dalgleish (1980) claimed that increasing the casein concentration by ultra filtration or addition of a milk ultrafiltrate brought about an increase in final curd firming while clotting time was not affected or just slightly increased. Similarly, Culioli and Sherman (1978), Schmutz and Puhan (1978), Garnot *et al.*, (1982) and Castillo *et al.* (2003b) found that clotting time increased with increasing protein content. In theory, if the enzyme is not saturated by the substrate, at a constant rennet/casein ratio, the rennet clotting time decreases with increasing casein concentration and vice versa. The maximum curd-firming rate and curd firmness increase more than proportionally with protein level (Guinee *et al.,* 1996). Hence, small variations in the protein content of milk, as can occur throughout the cheese making season, exert a relatively large effect on the coagulation properties of rennet. The positive effects of the higher milk protein content on the rennet coagulation properties probably ensue from the higher level of gel-forming protein, which increases the proximity of casein micelles and thus augments the rate of casein aggregation. One of the economic attractions in using ultrafiltration-concentrated milk in cheese making is the savings from using less rennet. Cheese made from ultrafiltration-concentrated milk ripens more slowly than normal, due partly to slower proteolysis, for which there may be a number of causes, including the lower ratio of rennet to casein.

B. Milk Fat

Increasing fat content in the range 0.1-10% (*w/w*) while maintaining the protein level constant (e.g., at 3.3%, *w/w*) enhances the rennet coagulation properties, as reflected by decreases in coagulation time and set-to-cut time and higher values for curd-firming rate and curd firmness (Mateo *et al*., 2009). Indeed, in a milk in which the level of fat plus protein is maintained constant, increasing the fat level results in significant decreases in curd firming rate and curd firmness (Broome *et al.*, 1998). In commercial cheese manufacture, where standardization of milk protein to a fixed level (e.g., by ultrafiltration of skim milk) is not normally practiced, curd-firming rate and curd firmness increase progressively upon adding cream to a fat level of about 4% (*w/w*) and decrease rapidly thereafter (Broome *et al.*, 1998). The decrease is due to the dilution effect on the protein, which eventually offsets the benefits of increasing the fat content. From physical and structural considerations, the effect of increasing the fat level in a milk where the absolute level of gel-forming protein is constant is probably twofold: 1) the concomitant increase in viscosity with fat content probably restricts the movement of gel strands and thereby contributes to a higher gel rigidity; 2) simultaneously, the increasing number of fat globules causes the gel strands to become more elongated to surround and occlude the obstructing fat globules; this results in thinner and weaker gel strands (Mateo *et al*., 2009)..

C. Cold Storage

Cold storage of milk on farms is a common practice to reduce the collection costs, however is not considered to be beneficial for the cheese making properties of milk. Basically, it is the equilibrium of the milk salts that is disturbed during cold storage and this may create a problem for further processing into cheese. However, such storage results in dissociation of caseins, in particular *β*-casein, from the micelle and solubilization of CCP, which can affect rennet-induced coagulation of milk (Walstra *et al.*, 2006). Cold storage of milk at low temperatures increases coagulation time and may cause weaker curd and a higher fat, protein and fines losses in the whey. This is mainly due to solubilization of calcium phosphate (**Fig 2.20**) and *β*-CN from the micelles and to progressive degradation of casein by plasmin and proteolytic enzymes from psychotropic microorganisms. Casein micelle structure, and coagulation and curd-forming properties can be partially re-established by a normal pasteurization, e.g. 72 °C for 15s (Qvist, 1979), or by holding the milk after cold storage for 30–60 min at 60–65 °C (Reimerdes *et al.*, 1977).



$$ca^{2+} + H(Po_4)^{2-} \qquad H(Po_4)^{2-} + H^{+} \Longleftrightarrow H(Po_4)^{-}$$

(a) Casein micelle and milk serum (b) milk serum

Fig 2.20. Balance of colloidal calcium phosphate (CCP) and the milk serum phosphates **(a)** between the casein micelle and the milk serum, and **(b)** in the milk serum. Reproduced from Walstra *et al.* (2006).

Storage of bovine milk at a low temperature increases RCT and reduces the rate of development of coagulum firmness (Raynal and Remeuf, 2000). Conflicting results have been reported on the effect of cold storage on rennet coagulation properties of ovine and caprine milk; Raynal and Remeuf (2000) reported that such storage has little effect on rennet coagulation properties of caprine or ovine milk, whereas De la Fuente *et al.* (1998) observed increases in the RCT of caprine or ovine milk on cold storage. Less pronounced cold-induced solubilization of calcium and caseins in ovine or caprine milk upon cold storage may explain the smaller effect of cold storage on renneting properties of such milks compared to bovine milk.

D. Milk pH

The pH of the milk strongly influences the rennet-induced coagulation of milk. The effect of pH is mainly on the first (enzymatic) stage of rennet coagulation. As the pH of the milk decreases, the enzyme moves closer to its optimum pH, speeding up the reaction. Optimum pH for the action of chymosin in milk is 6.0, but the optimum pH is lower for isolated caseins or synthetic peptides. A reduction in milk pH to a value in the range 6.6–6.0 results in a reduction in the rennet coagulation time (RCT) of bovine (Shalabi and Fox, 1982), ovine (Pellegrini *et al.*, 1997), caprine (Castillo *et al.*, 2000), and buffalo milk (Fakhr El-Dien, 1994) (due to reduced electrostatic repulsion) and a faster rate of increase in gel firmness. According to Law and Tamime (2010) the pH has a large effect on coagulation and the properties of the curd, as a reduction in pH will speed up the rate of κ-CN hydrolysis and the subsequent aggregation of casein micelles. Lowering the pH and increasing the temperature of the milk from normal values (~pH 6.6 and 31 °C) allow the coagulation to occur at a lower degree of κ-CN hydrolysis (Guinee and Wilkinson, 1992). A moderate decrease in milk pH (e.g., to pH 6.4) results in modest solubilization of the calcium from the casein micelles, which leads to a faster gel formation and a firmer curd. However, a higher degree of calcium solubilization leads to extensive demineralization of casein micelles,

which results in weaker and more flexible curd gels (Choi *et al*., 2007). For some soft cheeses, a step of extensive demineralization is required before coagulant addition to obtain the desired structure and body of the mature cheese (Choi *et al*., 2007).

E. Heat treatment

Heat treatment of bovine milk at a temperature >70 °C increases its RCT, compared with unheated milk, with both the primary and secondary phase being hindered (Dalgleish, 1990; Vasbinder *et al*., 2003) although it is thought that the effect on the secondary stage is far larger than that on the primary stage (Van Hooijdonk *et al*., 1987; Vasbinder *et al*., 2003). Heat-induced impairment of rennet coagulation of bovine milk is probably due to the association of denatured *β*-lactoglobulin with the casein micelle. The negative effects of heat treatment on RCT can be at least partially reversed, if the heat treatment is not too severe, either by the addition of calcium or by reducing pH. In contrast to bovine milk, heat treatment of caprine milk at up to 90 °C has little effect on its RCT (Calvo, 2002; Montilla *et al*., 1995; Raynal and Remeuf, 2000), whereas in milk of certain goat breeds, heat treatment may even lead to reduction in RCT (Alloggio *et al*., 2000). The effects of heat treatment on the primary phase of rennet coagulation also differ significantly between species; heat treatment reduced the rate of CMP release in bovine milk, but had no effect on the rate of CMP release in caprine or ovine milk (Calvo and Leaver, 2000).

F. Milk acidification

Many cheese making processes involve an acidification step, usually occurring simultaneously or previously to chymosin activity. A mild acidification can improve rennet coagulation properties, by shortening the coagulation time and strengthening the gel (Dalgleish and Law, 1988). As the pH decreases, the surface of the casein micelles changes and micellar calcium is released in the serum phase (Dalgleish and Law, 1988). At a more acidic pH there is also a faster release of CMP. With decreasing pH, the steric repulsion between casein micelles decreases and the attractive forces, as described quantitatively using an adhesive hard sphere model, increase (de Kruif, 1997). At about pH 5.6, there is a change in the apparent diameter of the micelles (Alexander and Dalgleish, 2005). Hence, the extent of CMP release necessary to cause the micelles to aggregate becomes smaller as the pH is decreased (van Hooydonk *et al*., 1986).

G. Enzyme concentration

The effect of enzyme concentration on the coagulation is directly related to time of reaction and gel firmness. There are many equations to describe the effect of enzyme concentration on clotting time, which are valid within certain limits of enzyme concentration, temperature, and pH. The earliest attempt to describe the kinetics of the clotting process was made in the 1870s by Storch and Segelcke. They stated that the clotting time was inversely related to the concentration of rennet used to clot the milk, i.e.

$$CT = \frac{K}{[E]} \qquad \textbf{Eqn } (\mathbf{2.3})$$

where ***CT*** is the clotting time, ***K*** is a constant , **[*E*]** is the enzyme concentration

To a first approximation, this equation is valid but a further refinement was postulated by Holter (1932) and further rearranged by Foltmann (1959) to give the familiar equation:

$$CT = \left(\frac{K}{[E]}\right) + A \qquad \textbf{Eqn } (\mathbf{2.4})$$

where **[*E*]** is the enzyme concentration and ***A*** is a constant. Clotting time is affected by both enzymatic and aggregation phases of coagulation, so ***A*** in this equation refers to the time needed for the second phase, which is not enzyme dependent (Lucey, 2002). According to Eqn. 4 when the enzyme concentration **[*E*]** is large, clotting time tends to ***A*** and clotting time depends mostly on the aggregation. This preserves the observed linear dependence of the clotting time on the inverse of the enzyme concentration. In such a form, the equation is acceptable for use with a wide range of enzyme concentrations (McMahon and Brown, 1983). It may be taken as a test of any more advanced model of the reaction that it reduces to the Holter formulation, which is amply supported by empirical experimental evidence. However, the relationship given in Eqn. 4 is not descriptive, in the sense that the particular mechanistic significance of the two constants, ***A*** and ***k***, is not established, and indeed they depend on the composition of the milk (Lucey, 2002).

H. Coagulation temperature

The coagulation of renneted micelles is very temperature-dependent. Temperature seems to have a larger effect on the aggregation phase than on the enzymatic phase. This results because

the temperature coefficient (Q_{10}) of the primary reaction is of the order of 2 while that of secondary reaction is about 11-16 (Brulé and Lenoir, 1987; Lucey, 2002). As a result, bovine milk does not coagulate at temperatures below 18-20 °C unless (Ca^{2+}) is increased. The marked difference between the temperature dependence of the enzymatic and non-enzymatic phases of rennet coagulation has been exploited in studies on the effects of various factors on the rennet coagulation of milk, in attempts to develop a system for the continuous coagulation of milk for cheese or casein manufacture and in the application of immobilized rennets (Lucey, 2002). The very high temperature dependence of rennet coagulation suggests that hydrophobic interactions play a major role. Calcium ions are essential for the coagulation of rennet-altered micelles (although the binding of Ca^{2+} by casein is not affected by renneting). Above 20 °C, the coagulation time decreases with increasing temperature to a broad minimum at 40-45 °C and then increases again as the enzyme becomes denatured (Singh and Waungana, 2001). In cheese making, rennet coagulation normally occurs at an average temperature of ~31 °C, well below the optimum temperature. The lower temperature is necessary to optimize the growth of mesophilic starter bacteria, which have an optimum growth temperature of about 27-28 °C and will not grow, nor perhaps even survive, above 40 °C (Swaisgood, 2003). In addition, the structure of the coagulum is improved at the lower temperature, which is therefore used even for cheeses made using thermophilic cultures (Fox *et al.*, 2000). The optimum temperature for curd formation at pH 6.5 is in the range of 34–38 °C for most commercial coagulants. In practice, coagulation is usually done at temperatures from 30 to 35 °C to have adequate control over curd firmness at cutting, and to give the starter culture suitable conditions to start fermenting the milk.

I. Calcium concentration

It is well recognized that the addition of calcium chloride to milk has positive effects on texture and cheese curd yield (Udabage *et al.*, 2001). Calcium ions are essential for aggregation and gelation of casein micelles, and the reason behind this mechanism has yet to be fully established. Para-κ-CN binds to calcium ions more strongly than κ-CN (Bringe and Kinsella, 1986). Indeed, although free calcium ions shield negatively-charged amino acid residues, reducing the overall charge of the casein micelles, this cannot be the only reason behind its essential role in rennet- induced aggregation of casein micelles. In milk, addition of 1 mM calcium chloride does not increase the enzymatic activity, as shown by kinetics of CMP release (Sandra *et al.*, 2012), but it shortens coagulation time and forms stiffer gels (van Hooydonk *et al.*, 1986; Sandra *et al.*, 2012).

The aggregation of the casein micelles occurs at a lower degree of κ-casein hydrolysis, by decreasing the extent of solvation of the polyelectrolyte brush around the micelles (vanHooydonk *et al*., 1986; de Kruif, 1999). The mode of addition of calcium (fast addition as $CaCl_2$ or slowly by dialysis) to the milk does not seem to have an impact on the aggregation behavior (Sandra *et al.*, 2012). A recent study monitored milk coagulation after addition of rennet using diffusing wave spectroscopy and showed no difference in behavior as a function of CMP release with 1 mM calcium added, in spite of the earlier onset of coagulation of the micelles (Sandra *et al*., 2012). These results may suggest that calcium ions play a major role in strengthening short range interactions. Evidence of calcium binding to the micelles after rennet coagulation was also shown in a study on mixed acid and rennet coagulation, whereby soluble calcium was shown to be reduced during early acidification after complete CMP release (Salvatore *et al*., 2011). It is also important to note that although additional calcium increases stiffness of the gels, too much calcium added to milk can form weaker gels (Fox *et al*., 2000; Udabage *et al*., 2001). While the amount of soluble calcium is of great significance in the early stages of rennet gelation, differences in the concentration of colloidal calcium affect the structural rearrangements of the gel network (Choi *et al*., 2007). Loss of colloidal calcium phosphate will weaken the internal structure of the micelles once the gel has formed, and will affect the forces stabilizing the gel, namely, hydrogen bonds, van der Waals interactions and calcium bridges. Hence, the presence of colloidal calcium is an important factor in determining the rheological properties of the gel network (Choi *et al*., 2007).

2.2.3.2 Cutting the coagulum

The cutting of the curd is normally done either at a predefined time after rennet addition or when the cheese maker empirically determines that the curd has the right properties for cutting; often by cutting the curd with a knife and visually evaluating the surfaces and the splitting of the milk gel.

In enzymatic cheese, the process of "cutting the curd" allows the whey to drain. Depending on what cheese is being made, the gel is cut down to curd grains of different size, allowing them to retain a certain amount of moisture (Bennett and Johnston, 2004). The actual cutting can be done in a few ways. Some cheese makers prefer to cut the curd by hand with a long-handled contraption that has thin cutting edges, resembling a rake with several small blades (Kamin and McElroy, 2015). It can also be cut by machine. For a drier end result, the curds are cut smaller,

thus expelling more whey. For a softer and creamier cheese, the curds are left slightly larger, with more whey being retained. At this step, a lot of variations can take place, depending on the type of cheese being made. The renneting or coagulation time is typically ~30 min (Fox *et al.*, 2000). Before the coagulum is cut, a simple test is normally carried out to establish its whey eliminating quality (Fox *et al.*, 2000). Typically, a knife is stuck into the clotted milk surface and then drawn slowly upwards until proper breaking occurs (**Fig 2.21**).

There are many signs observed by cheese makers which show that the curd may be considered ready for cutting: **1)** a glass-like splitting can be observed, **2**) if it breaks cleanly when the flat blade of a cutting knife is inserted at 45° angle to the surface and then raised slowly and finally a clean whey is observed in the opening that is neither to milky (cut too soon) or too clear (cut too late), **3**) taking a sample using a "sample straw". The test involves inserting a long sanitized straw into the curd, plugging the top of the straw with the finger, and removing the loaded straw. The straw should be placed on a flat surface and slowly drag backwards as the finger is removed from the straw opening at the top. The curd is ready when it snakes across the surface, holds structure, and yields only clear whey.

Fig 2.21 Visual signs of completing the coagulation and starting the curd cutting process. Reproduced from Greenlivingaustralia.com

Cutting gently breaks the curd up into grains with a size of 3 – 15 mm depending on the type of cheese being manufactured. The finer the cut, the lower the moisture content in the resulting

cheese. The cutting tools can be designed in different ways. Stainless-steel knives (vertical and horizontal) are widely used for cutting the coagulum.

In large modern vats, cutting knives are fixed in the vats and serve to cut the coagulum and stir the curds/ whey (Kosikowski and Mistry, 1997). The designed open and closed cheese vats employ only vertical knives (**Fig 2.22**). The knives are also used as an agitation mechanism, so that the curd is cut with the sharp side of the blades, the stirring of the curd-whey mixture is carried out with the blunt side facing forward with respect to rotation.

The duration of cutting the coagulum is around 20 - 30 min (Robinson, 1993), depending on the size of the curd particle required, type of cheese manufactured and/or the size of the vat. For example, 'fine' or 'coarse' size curd particles are used for the manufacture of hard and semi-hard cheese varieties, respectively.

Fig 2.22 Commercial cheese vat.

The coagulum is cut at a very low speed, and the speed is progressively increased to avoid fat and casein losses in the whey which could influence the yield of cheese. For most varieties, the gel is cut/broken using implements which in some cases are traditional and characteristic of the variety. The optimum modulus for the gel at cutting is about 40 Pa, but varies from 20 to 60 Pa (Fox *et al*., 2000), depending on the protein content of the milk; losses of fat and protein in the whey increase with weaker or stronger gels. Rennet- or acid- coagulated milk gels are quite stable under quiescent conditions but if cut or broken, they synerese extensively, expelling whey (Bennett and Johnston, 2004). It thus seems that controlling firmness at cutting time can be important for controlling cheese yield and moisture content. According to van Hooydonk and van den Berg

(1988), the construction of the cheese vat, including its cutting equipment has an important impact on the optimum gel firmness at cutting.

2.2.3.3 Syneresis

As it was mentioned above, the rennet coagulation process is essentially similar for all cheese varieties and the structure of the coagulum is also similar. The gel is subjected to a series of treatments, the principal object of which is to remove whey from the gel and effectively concentrate the casein and fat to the degree characteristic of the cheese variety (O'Connell and Fox, 2000). Rennet- or acid-coagulated milk gels are quite stable if left undisturbed, but when they are cut or broken or subjected to external pressure, the para-CN matrix contracts (more intensity in rennet gels), expressing the aqueous phase of the gel (known as whey) (Dejmek and Walstra, 2004). This process, known as *syneresis*, enables the cheese maker to control the moisture content of the cheese; hence, not only the activity of microorganisms and enzymes in the cheese, but also the biochemistry of ripening and the stability and quality of the finished cheese. Syneresis is defined as shrinkage of a gel and this occurs concomitantly with expulsion of whey (Horne and Banks, 2004). Syneresis also could be defined as a process whereby whey is separated from curd particles and, as a result of the expulsion of whey the curd particles shrink in volume. It is probably more correct to describe gel shrinkage as a process that forces or 'squeezes' whey out of the matrix through the gel pores. The aqueous phase in rennet gels is mostly physically trapped and not chemically bound. It is useful to define spontaneous syneresis as the contraction of a gel without the application of any external force (e.g., centrifugation) and this is related to instability of the gel network (i.e., due to large scale rearrangements) (walstra, 1993). The higher the moisture content of cheese, the faster it will mature but the less stable ripening will be. High-moisture cheeses have a much greater propensity to develop off-flavors than low-moisture varieties (walstra *et al.*, 1985). The tendency to exhibit syneresis in gels can be viewed as the reverse of the swelling behavior (Lucey, 2002). Cheese making can be viewed as a dehydration process and syneresis is the crucial method by which most of the moisture is lost from curd particles. Since syneresis is the main method available to cheese makers for controlling cheese moisture content, it is also the process that is mostly manipulated during cheese making and various dehydration approaches help to facilitate differentiation between cheese varieties.

2.2.3.3.1 Mechanism of Syneresis

The initial rennet-induced gel should be viewed as a weakly stabilized, transient (dynamic) network. The interactions between rennet-altered micelles are weak (ionic bridges, hydrophobic interactions) and the resultant matrix has high bond mobility (or bond relaxation, as indicated by the high values for the loss tangent parameter from rheological measurements). If bonds between aggregating particles are reversible (at least for a short period after gelation), rearrangements may occur in the aggregates/clusters formed as well as in the gel network. In the initial rennet gel network, bonds are breaking and reforming, which increases the possibility of rearrangements.

After gelation, there is ongoing particle fusion and the formation of additional cross-links between the caseins. With increasing time after renneting, rennet gels increase in stiffness and in resistance to deformation, which act to reduce the ability of the network to rearrange its microstructure. Thus, waiting for the gel to become firmer before cutting makes it harder for that gel to undergo extensive syneresis and therefore the cheese has higher moisture content. There is some tendency or driving force promoting increased casein interactions (van Vliet and Walstra 1994). It is possible that the renneted micelles have surfaces that are only partly attractive (due to the high pH) and this promotes shuffling of particles to reduce repulsion (increase attraction) (Walstra *et al.*, 1985). It could also be that the completion of the hydrolysis of all the κ-CN hairs by rennet, after the formation of a weak network, alters the attractive/repulsive balance in the system. The incorporation of additional particles in the network (i.e., micelles where hydrolysis of κ-casein was completed only after network formation) results in the formation of new physical crosslinks between protein strands, which may promote tensile stresses in the system resulting in strand breakage. Micelles that are only partly attached to the network (dangling ends) at the point of gelation could become 'fully' attached to the matrix with aging (Green and Grandison, 1993).

An important aspect of the syneresis mechanism in cheese curd is the ability of the initial gel (coagulum) to retain its shape after cutting. Cheese makers wait until they can subjectively determine that the gel can withstand the cutting process. As described previously, often they evaluate this by cutting the gel with a spatula/knife and they observe if the cut gel surface does not rapidly collapse. The retention of structure in the curd pieces is critical in the creation of a large amount of exposed surfaces through which whey can easily be expelled (Walstra *et al.*, 2006). The weight of the curd particles and gravity as well as collisions between curd particles (e.g., as a result

of stirring) enhance the compression/deformation of curd particles, which promotes squeezing out of whey. In order for rennet gels to undergo syneresis, the network must be flexible enough to be able to rearrange itself into a smaller and more compact matrix. Syneresis of gels can occur either spontaneously or more commonly as a result of some physical stresses applied during cheese making (Lucey, 2002).

Syneresis can also occur in gels due to environmental changes, for example, decrease in pH or increase in temperature. It has been suggested that there is in rennet gels an 'endogenous syneresis pressure', that is, a pressure within the gel that is causing spontaneous syneresis or the syneresis of wetted gels (Horne and Banks, 2004).

It has not been possible to measure experimentally this endogenous pressure since the predicted values are very low. The rate of syneresis increases initially as a function of time after cutting but decreases at longer times, presumably due to fusion of *para*-CN micelles and a reduction in the permeability of the contracting network. The rearrangement process is accelerated and is more extensive at high temperatures (Horne and Banks, 2004). Aging of rennet-induced gels results in a coarsening (sometimes called 'microsyneresis') of the gel (i.e., rearrangements) and an increase in the fractal dimensionality.

In rennet-induced milk gels, low gel stiffness (elastic modulus) and high values of the loss tangent ($tan\delta$ at low frequencies) are important rheological conditions that facilitate rearrangements of bonds (when these rheological measurements are made at approximately the same timescale over which rearrangement processes related to syneresis in these gels are estimated to occur).

Rearrangements of casein particles into a more compact structure would increase the number of bonds and hence decrease the total free energy of the system. However, the particles are part of the gel network, which must be deformed or broken locally to form new junctions. In cheese making, conditions such as cutting, stirring, acid production, and the increase in temperature that occurs during cooking all encourage syneresis and the rearrangement processes that facilitate syneresis of the gel network (Lucey, 2011a) (**Fig 2.23**).

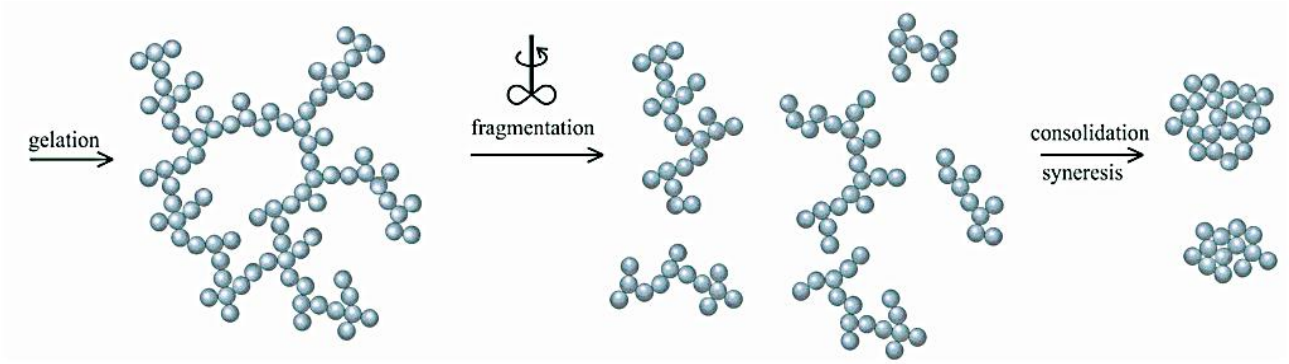


Fig. 2.23 Illustration of strands of the para-CN micelles forming new links, causing breaking of a strand elsewhere in the gel network. Reproduced from Walstra *et al.* (2006).

Syneresis is augmented by increasing temperature, pH and applied pressure, e.g. stirring. According to Weber (1989) and Castillo (2001), the classification of the factors affecting the syneresis process are shown in (**Fig. 2.24**).

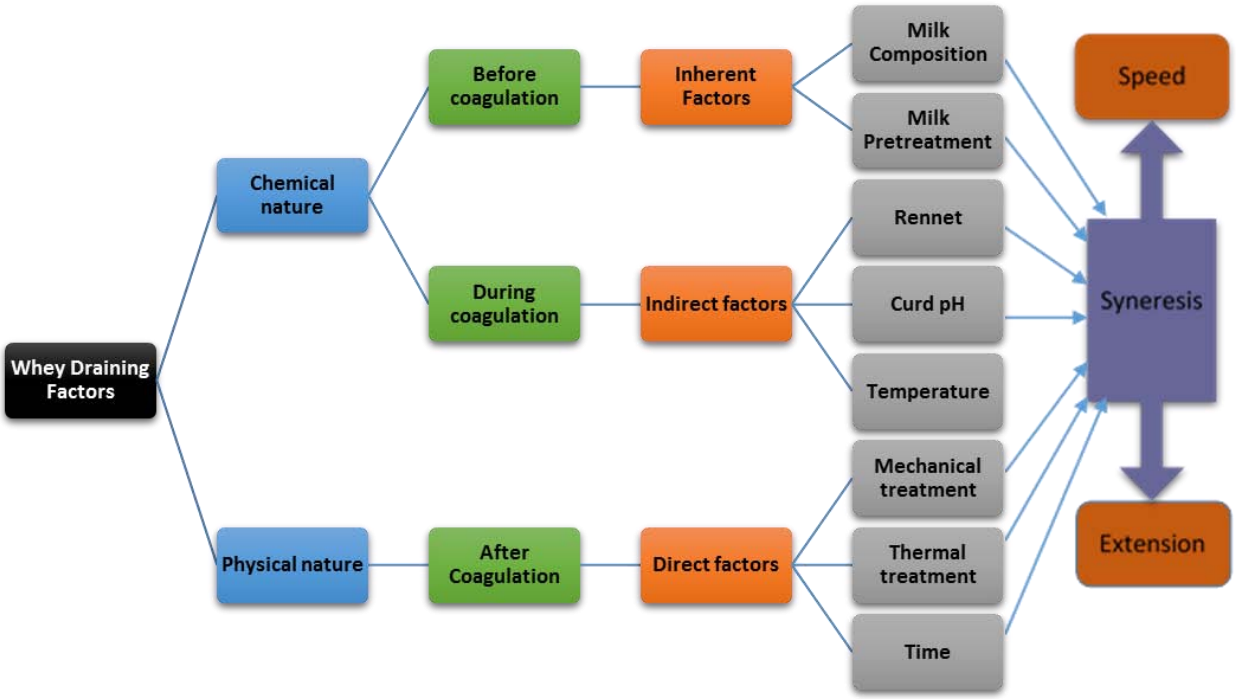


Fig. 2.24 Factors affecting syneresis. Adapted from Weber (1989) and Castillo (2001).

2.2.3.3 Curd molding and pressing

Curd is typically transferred to molds of the cheese characteristic shape and size (**Fig. 2.25**). The principal purpose of molding is to allow the curd to form a continuous mass; matting of high-moisture curds occurs readily under their own weight but pressing is required for low-moisture cheese (Fox *et al*., 2000).

Pressed cheeses are submitted to a pressing system after have been molded with the purpose of assist final whey expulsion, provide texture, shape the cheese, and provide a rind on cheeses with long ripening periods.

Fig. 2.25. Vertical pressing unit with pneumatically operated pressing plates. Reproduced from Bylund (2003).

Pressing should be gradual at first, because initial high pressure compresses the surface layer and can lock moisture into pockets in the body of the cheese (Bylund, 2003). The intensity of pressure and the length of the pressing process vary with the type of cheese; it typically ranges between 0.1 and 1.5 kg/cm^2 (Everard *et al*., 2011) where the smallest pressure is usually applied to fresh cheese.

2.2.3.4 Salting

In cheese, as in many foods, salt normally functions as a condiment. But salt has other important effects, such as retarding starter activity and bacterial processes associated with cheese ripening (Kosikowski *et al.*, 1997). Application of salt to the curd causes more moisture to be expelled, both through an osmotic effect and a salting effect on the proteins. The osmotic pressure

can create suction on the surface of the curd, causing moisture to be drawn out. With few exceptions, the salt content of cheese is 0.5 – 2%. The exchange of calcium for sodium in para-caseinate that results from salting also has a favorable influence on the consistency of the cheese, which becomes smoother (Bylund, 2003).

In general, the curd is exposed to salt at a pH of 5.3 – 5.6 for ~5 – 6 hours (Bylund, 2003). Brine salting is the most commonly used system for salting the curd, which consists in placing the cheese in a container with brine. The containers should be placed in a cool room at about 12 – 14 °C, as it shown in **Fig. 2.26.**

Fig. 2.26. Cheese salting. Reproduced from http://www.bloomberg.com

2.2.3.5 Ripening

Ripening refers to the biochemical, microbiological, structural, physical and sensory changes that occur during storage post manufacture and transform the fresh curd to a cheese with the desired characteristics. It has a major effect on the quality of most cheese varieties, apart from those belonging to the category of un-ripened cheese including fresh rennet and acid cheeses and some ingredient cheeses. However, even here storage can influence quality depending on temperature, humidity and packaging. For most rennet curd cheeses, ripening is a critical process and varies from ~4 weeks for Camembert to 2 years in the case of mature Parmesan cheese. During this period, the curd or cheese undergoes a number of changes, which facilitate the transformation (Fox *et al.*, 1996; McSweeney, 2004): (a) glycolysis (sugar metabolism), (b) proteolysis (hydrolysis of protein and peptides), (c) lipolysis (hydrolysis of triacylglycerols), and (d) mineral

equilibrium. These changes are in turn associated with related changes in pH, protein hydration, fat coalescence, and swelling of the casein matrix, to an extent depending on the cheese variety (Everett and Auty, 2008). The changes that occur during ripening are significantly influenced by storage conditions (time, temperature, packaging), with the magnitude of the effects depending on the manufacturing process used (e.g. salt distribution, level of rennet retention), composition (e.g., pH, levels of Ca^{2+}) and microbiology. The humidity of the environment must be controlled for the ripening of many varieties, mainly those with a surface microflora, such as smear and mould ripened cheeses. Its control, which involves cycling at different stages, is essential for controlling: (a) extent of surface drying, (b) moisture loss, (c) growth of the surface flora, (d) development of correct surface skin and (e) the levels of textural/flavor changes (Spinnler and Gripon, 2004; Hélias *et al*., 2007). Some brine-salted cheeses are stored at lower relative humidity to encourage development of a rind, which protects the cheese against undesirable surface growth and the loss of moisture (weight) (Fox and Cogan, 2004).

2.3 Factors affecting cheese yield

Cheese yield is affected by many factors including milk composition, amount and genetic variants of casein, milk quality, somatic cell count (SCC) in milk, milk pasteurization, coagulant type, vat design, curd firmness at cutting, and manufacturing parameters (Banks *et al.*, 1981; Lawrence, 1993b; Lucey and Kelly, 1994; Walsh *et al*., 1998; Fenelon and Guinee, 1999).

2.3.1 Lactation

The concentrations of the various milk constituents vary during lactation (Lucey and Kelly, 1994), and the content of fat and protein is much higher in colostrum milk than in normal milk. From around lactation week 5, it has been shown that the fat and protein content of the milk is at its minimum with a steady increase during further lactation, while the concentration of lactose decreases slowly during lactation (Fox *et al.*, 2000). Guinee *et al.* (2007) showed that the protein content of milk increased during lactation with a similar increase of cheese yield. At very late lactation the protein content of the milk dropped and thereby also the cheese yield. As very late lactation milk normally contains more serum proteins, it exhibits more hydrophilic properties and a higher milk pH; in addition, when the content of SCC is high and the content of casein low, the coagulation properties of the milk are reduced. Late lactation milk has previously been considered

inferior for cheese making, since SCC increases and the content of whey protein increases at the expense of casein; however, Kefford *et al.* (1995) showed that if the cows were offered a high quality diet these changes did not occur in late lactation milk.

2.3.2 Seasonal variation

Seasonal variations of the composition of milk, in particular those regarding the protein or casein content, markedly affect the cheese yield of most cheese productions (Barbano and Sherbon, 1984; Paolo *et al.*, 2008). Barbano and Sherbon (1984) and Ozimek and Kennelly (1993) have underlined an analogous trend for the yield in Cheddar cheese, with minimum values in the months of June, July and August and the maximum ones in correspondence with the autumnal months. Banks and Tamime (1987) indicated that the climatic conditions and the physiological state of the cows exercise a determining role on the contents of fat and casein and on the coagulation properties, factors that, as a result, markedly affect the cheese yield.

2.3.3 Microbial quality of milk

Milk from healthy animals is practically sterile when it is drawn from the udder; however, as soon as it leaves the udder it is contaminated by various micro- organisms (Auldist *et al.*, 1996). Milk used for cheese making must be of good microbiological quality, with a low total count of bacteria, absence of pathogenic and detrimental bacteria, and a low count of psychrotrophic bacteria, which produce heat-resistant proteases and lipases that may reduce yield and cause undesirable flavors in the ripened cheese.

2.3.4 Somatic cell count

Milk with a high SCC (>500,000 cells/mL milk) reduces cheese yield (Auldist *et al.*, 1996), and such milk is associated with higher proteolytic activity, lower concentration of fat and casein and a higher content of whey proteins, especially serum albumin and immunoglobulin. The SCC increases as a result of mastitis infection (Sharma *et al.*, 2011). The somatic cells contain a plasmin activator that converts plasminogen to plasmin in the mammary gland (Lucey and Kelly, 1994). According to de Rham and Andrews (1982), plasmin represents one-third of the total protease activity in milk with high SCC. Plasmin degrades mainly β- and α_{s2}-CN into peptides, and might be slightly active at 5 °C (van den Berg *et al.*, 1996). In addition to decreased cheese yield, the increased proteolytic activity in milk caused by increased levels of SCC has been shown to

influence the cheese composition decreasing the protein content, and increasing proteolysis (Cooney *et al*., 2000; Grandison and Ford, 1986). Cheese made from milk with high numbers of SCC exhibited a decreased firmness and elasticity and an increased stickiness and off-flavor (Grandison and Ford, 1986). Auldist *et al.* (1996) found the same effects of high SCC on cheese characteristics, though the effect of high SCC was more detrimental for cheese quality in late lactation milk than in early lactation milk.

2.3.5 Effect of subclinical mastitis

As indicated above, the health condition of the mammary gland dictates the rate of milk secretion and has a multitude of effects on the quantity, quality, and processing properties of the produced milk (Martinez *et al*., 2011). Lawrence (1993a) characterized the factors that can affect milk composition and cheese yield and quality identifying among those mastitis. Intramammary infection (**IMI**) or mastitis, an inflammation response, is considered to exert one of the most negative impacts on dairy ruminant's health (including small ruminants), farmer's income, and public health (Leitner *et al*., 2004; Leitner *et al*., 2011a). Mastitis causes economic losses to dairy farmers due to reduced milk yield (Heringstad *et al*., 2003b). Mastitis can appear both clinical and subclinical, being the latest form the most prevalent one (Maréchal *et al.*, 2011). Subclinical mastitis (SCM**)** is the presence of infection in the mammary gland without apparent signs of local inflammation or systemic affection and requires a diagnostic test for detection (Harmon, 1994b). The main pathogenic bacteria causing SCM in sheep is coagulase-negative *Staphylococci* **(*CNS*)**. A more detailed description of causes, pathophysiology, and diagnosis of SCM in sheep can be found in Hogeveen (2005). Absence of clinical signs in sheep with SCM in combination with the nonexistence of relevant changes in the milk appearance prevents the detection of the infection by the farmer. The prevalence of subclinical mastitis in dairy sheep flocks may occur up to 15 - 40% and if all animals are milked into the milk tank, 15-30% of the milk would be of infected glands (Leitner *et al*., 2004, 2006, 2008, and 2011). Thus, as a result of the high incidence and prevalence, SCM can be considered as one of the main sources of economic losses in milk production worldwide (Gonzalo *et al*., 2002; Leitner *et al*., 2007, 2008). Moreover, as in dairy sheep the entire milk is used for chesses production, reduction of milk quality along with the loss of quantity can be devastating to the milk producers.

It is well documented that mastitis, even in its predominant subclinical form, impairs milk quality through the activation of the immune system and leads to changes in milk synthesis in the alveolus. As a result of the infection, there is increase in the release of deteriorative enzymes into the milk from the bacteria and the host cells, reduced lactose and casein concentrations, increased pH and higher level of whey protein and mineral content (Merin *et al.*, 2008). Proteolysis of casein leads to increased levels of γ-caseins and proteose-peptones **(p-p)** (Le Roux *et al*., 1995). During SCM, milk SCC increases, and milk composition and functional composition change (Caraviello *et al.,* 2003). The types of somatic cells during SCM change to mostly white blood cells, which add many proteolytic and lipolytic enzymes to milk and in addition, more blood serum leaks into the milk (Le Roux *et al.,* 2003). Protein breakdown in milk produced by sheep with SCM is caused primarily by an enzyme called plasmin, which is found commonly in both milk and in blood plasma and can cause extensive damage to milk protein specially casein in the udder prior to milking. Moreover, when milk is cooled, plasmin continues to break casein down but at a slower pace. Plasmin is extremely heat stable, therefore, pasteurization cannot inactivate it and will continue to damage casein during dairy product manufacture and storage (Abdelgawad *et al.,* 2016). As a result, the milk casein does not aggregate properly resulting in longer rennet coagulation time and a weak coagulum, which in turn leads to increased whey fat and protein losses and larger curd moisture content, all of which derives into lower cheese yield (Barbano *et al.*, 1991; Auldist *et al*., 1995; Auldist and Hubble, 1998; O'Brien, *et al.*, 2001). In summary, SCM can affect cheese yield by: 1) higher content of plasmin and other proteolytic and lipolytic enzymes, 2) damaged casein, 3) poor curd formation (longer flocculation time, slower rate of curd firming, and reduced maximum firmness), and 4) increased pH and altered calcium-phosphate-caseinate balance (Banks *et al*., 1981; Lawrence, 1993b). All these can lead to soft, less elastic, sticky and grainy cheese texture and reduction in cheese yield as well as increasing flavor intensity, usually with off flavors (Barbano *et al*., 1991).

2.3.6 Effect of mixing different milks from different species

The popularity of dairy products made from milk of small ruminants is increasing among researchers and the dairy industry, due to their peculiar taste and nutritional properties. Compositional differences between ewe milk and cow milk, mainly in proteins and fats, account for the different technological and sensorial characteristics of cheeses. Ewe milk contains higher

protein and fat levels than cow milk (Kindstedt *et al.*, 2004). Moreover, it is characteristic for the presence of small fat globules with an easily oxidizable weaker membrane. The lipids contain a higher percentage of short-chain fatty acids (FA), such as caproic, caprylic, and capric acids, which give a typical flavor characteristic (Park, 2001). Lipolysis in ewe cheeses is faster than in cow cheeses, contributing to an important flavor development (Park *et al.*, 2007). Renneting parameters in cheese making from ewe milk are affected by its physicochemical properties, including pH, larger casein micelles, more calcium per casein weight, and mineral contents in milk, which cause differences in coagulation time, coagulation rate, curd firmness, and amount of rennet needed (Park *et al.*, 2007).

Goats produce only approximately 2% of the world total annual milk supply (Park, 1990). Goat milk differs from cow milk from its higher digestibility, alkalinity, buffering capacity, and certain nutritional and therapeutic properties (Park, 2000). The composition of goat milk is similar to cow milk, although it has a smaller fat globular size and a whiter color than cow milk, as goats convert all β-carotene into vitamin A. Goat milk fat contains more than 20 volatile branched-chain FA, including 4-methiloctanoic and 4-ethyloctanoic acid, which contribute to mutton-type and goat-type flavor, respectively (Sheehan *et al.*, 2007). It is poor in casein; casein micelles contain more calcium, inorganic phosphorus, and non-centrifugal caseins. They are less solvated, less heat stable, and lose β-CN more quickly than cow milk casein micelles (Park *et al.*, 2007). Renneting time for goat milk is shorter than for cow milk, and the weak consistency of the gel explains the low cheese yield (Park *et al.*, 2007). Almost half of the cheese produced in Spain is made with different mixtures of milk from cow, sheep and goat (ICEX, 2004). The type of milk and its proportions determine the texture, smoothness and flavor of these cheeses. Bovine milk provides consistency and acidity, while sheep milk incorporates creamy and spicy flavors and goat milk enhances white color.

The quality and organoleptic characteristics of these varieties are positively influenced by the presence of milk from small ruminants (Ha and Lindsay, 1991; Molina *et al.*, 2000). However, because seasonal production of milk by these species has large fluctuations between summer and winter, it is difficult to maintain a standardized composition in cheese manufacture throughout the year (Barron *et al.*, 2001). From the economic point of view, cow milk tends to be the basic ingredient as it reduces the production cost, while increasing the proportion of goat and sheep milk

contributes to increase the product quality. The production of cheese using mixtures of milk (hereafter *"mixed milk cheese* –**MMC**–*"*) has achieved widespread acceptance in Spain. A large variety of MMCs are typically manufactured in Spain such as *"Cabrales"*, *"Picón"*, *"Gamonedo"*, *"Iberico"*, etc. Proportions of the different milk types were established by cheese producers and the Ministry of Agriculture in 1987 (*"*Orden de 9 de julio de 1987*"*). This regulation, which applied to MMCs except for those subjected to specific quality standards, officially recognized three types of MMCs: *"Hispánico"*, *"Ibérico"* and *"Mesta"*. According to this regulation, *"Ibérico"* cheese requires at least 50, 30 and 10% of cow, goat and sheep milk, respectively. *"Hispanico"* cheese requires a minimum of 50% cow and 30% sheep milk, while *"Mesta"* cheese requires sheep and cow milk proportions of no less than 75 and 15%, respectively. Mentioned legislation has been recently repealed by the *"Real Decreto 262/2011, de 28 de febrero"* that establishes for *"Ibérico"* cheese a maximum of 50% cow milk and a minimum of 15% for both goat and sheep milks. MMCs are very important for the Spanish cheese sector, not only for the proportion of sales it represents, but also because of technology differences required for appropriate processing of mixtures of different types of milk. Sheep, goat and cow milk show marked differences in their colloidal structure and chemical composition, which introduces additional difficulties, compared with cheeses made with one type of milk, as regards the control of coagulation and the selection of cutting time.

According to Evtodienco *et al.*, (2015) cheeses made from a mixture of milk of goats and sheep are more required and have higher taste qualities than those of pure milk of goat and sheep. Regarding to the color of cheese in samples of sheep cheese and mixture of up to 75%, the sheep milk contributes the color from the white to yellowish-white, that is influenced by the increased amount of fat, but not decreases its quality. Evtodienco *et al.* (2015) concluded that the goats milk in the manufacture of cheese leads to its bleaching. Referring to the taste of cheese, it can be said that the mixture of 25% of sheep milk to goat milk practically doesn't influence the cheese taste (Evtodienco *et al.*, 2015). The addition of up to 75% of goat milk to sheep milk does not influence the taste either, being specific the sheep cheese taste. It can be concluded that the goats milk in proportions of up to 75% does not influence the quality of sheep cheese, but improves its sensory qualities (Evtodienco *et al.*, 2015).

2.4 Inline monitoring of milk coagulation and curd syneresis and prediction of cutting time using optical sensors

Holm (2003) defines sensors as tools that respond to one or more properties of food and transform the response into a signal, usually electrical in nature. Sensors are powerful tools for real-time measurement of the physical-chemical changes of raw materials, finished products and by-products during food processing (Castillo, 2016). The measurements obtained by sensors installed in line, in combination with appropriate controllers and actuators, allow activation/deactivation of automatic mechanisms, implementation of alarm systems and corrective measures, improving the efficiency of industrial processes and quality of the final product.
Castillo, (2016) mentioned that according to the signal processing the sensors can be classified into two groups: ***primary sensors***, that determine a specific property directly (e.g., temperature), and ***analytical or intelligent sensors***, which detect variations of a parameter as function of time and use a prediction equations to estimate control technological parameters such as the cutting time in making cheese.

According to Kress-Roger (1993) sensors are classified according to their use into four groups: ***"inline sensors"*** are installed directly on the walls of a main pipe. Some inline sensors are installed on the inner face of the pipeline and contact with food, while others are installed on the outer wall, either directly or through a window, which depends on the type of sensor. The ***"online sensors"*** are installed on the wall of a duct derived from the main line to accommodate the sensor. This type of sensors requires therefore the provisional diversion of an aliquot of food through a *bypass,* which allows the return of the sample to the main flow after the measure, or the complete extraction of the sample through a line bleeding. Finally, ***measures "at-line"*** and ***"off-line"*** are made using laboratory instruments located in the production area (at-line) or in a chemical laboratory (off-line).

According to Castillo (2016) the European Concerted Action" (ASTEQ, *artificial sensing techniques for the evaluation of quality)* classifies sensors according to the nature of the signal in the following groups: ***a) biosensors*** employing enzymes or antibodies; ***b) selective agent sensors,*** employing certain compounds or complex materials; ***c) ultrasound***; ***d) frequency variation***; ***e) electrical***; and ***f) electromagnetic***, which includes, among others, optical sensors. ***Electromagnetic*** sensors are classified depending on the wavelength used. For example, electromagnetic sensors

employing infrared light are subdivided in near infrared (700-2,500 nm), mid-infrared (from 2,500 to 30,000 nm), far infrared (1,000,000 nm) and thermography sensors (1-15 microns). We can also classify the optical sensors in absorption-transmission and scattering sensors, depending on the type of interaction light/matter used in the measurement.

Currently, a large number of optical sensors are available commercially. Sensors measuring visible light to determine color (colorimeters), infrared sensors for the determination of chemical components, quality control, and temperature measurement in food, etc. Scientific and technological efforts in this area have focused in recent decades in the development of robust, simple and inexpensive sensors (Castillo, 2016). A new generation of sensors emerges strongly thanks to the reduced capacity of light penetration in food, availability of small optical fiber spectrometers, the robustness of the new systems of data acquisition and the use of optical fibers and electronic micro-components (Castillo, 2016). These advantages joins the great versatility of optical sensors in relation to the measurement configuration.

Optical sensors not only allow the quantitative determination of physicochemical properties as the concentration of certain food components, but in most cases facilitates performing measurements of qualitative nature that are not proportional to physical properties or chemical-specific as in the previous case, but which are proportional to important process control parameters. The development of an optical control technology requires not only the design of the sensor itself, but also to establish algorithms that allow the estimation of the technological parameters necessary for optimizing the efficiency of food processing.

During the last few decades several optical sensors of interest to the food industry have been developed. Payne *et al.* (1993b) proposed a NIR light scattering optical sensor employing two optical fibers to monitor milk coagulation and estimate cutting time. The NIR light scattering sensor also allows predicting Berridge clotting time (Castillo, 2001) and the gelation time determined by rheometry (Castillo *et al.*, 2006b). Payne (2000) introduces light scattering sensors that monitor the concentration of solids in cheese making effluents. Gillette *et al.* (2002) designed another light scattering sensor using three optical fibers to determine the attenuation of light within the milk. Based on this technology, these authors developed a sensor that measures the concentration of fat in milk and cream. Danao and Payne (2003) developed an optical transmission

sensor, which detected the interface between two different transition liquids flowing through the pipes system. Castillo *et al*. (2004) found a significant correlation between the milk coagulation kinetics measured by a NIR light scattering sensor and the kinetic parameters of the curd syneresis process. Based on this interaction, these authors (Castillo *et al.*, 2005b) developed a whey separation sensor (LFV -*"Large Field of View"* - sensor) that estimates the gelation and cutting times in cheese making, and also allows to estimate the moisture content of the curd in the cheese vat.

2.3.1 Inline and off-line monitoring milk coagulation and cutting time

The milk coagulation process, in particular, during cheese production has received a great deal of attention. Real-time estimation of curd firming and prediction of cutting time are essential for milk coagulation control during cheese making. Changes in the composition of milk and coagulation conditions exert a significant impact on the hardness of the curd and therefore cutting time. The optical selection of both clotting and cutting times is more important in small and some automated plants in which the variations, both in composition of different batches of milk and coagulation procedures, are more pronounced. According to Castillo (2016) the smaller scale factories also tend to have more flexible production schemes that help increase the variability of the duration of the coagulation process. Conversely, the larger factories are strongly automated and production schemes are perfectly programmed which often prevents for cutting time modification. For this reason, usually they resort to milk standardization for control. But unfortunately, there is always some risk of unforeseen changes in processing conditions and even human errors, which could result in significant economic losses as a result of large-scale production sequence (Castillo, 2016).

In these cases, an inline sensor objectively monitoring the evolution of coagulation and hardness of the curd could provide real-time information that would allow appropriate measures to minimize the loss of fat and fines. It is well known that cutting time selection depends on rheological and microstructural properties of gels, such as coagulum firmness and rearrangement capability that, in turn, depend on coagulation factors, milk composition, and milk pretreatment. In addition, it is well known that the anticipated cut of the gel translated into a loss of yield due to the weakness of the protein structure, while its delay increases the performance but also the water content of the curd, which decreases the quality of the cheese by altering the tuned up. It

therefore seems logical to assume that there must be an optimum time for cutting the gel (Castillo, 2001). For this reason, cutting time selection greatly affects moisture, yield, and quality of cheese and whey fat losses. As Johnston *et al*. (1998) pointed out, cutting and stirring speeds can also exert a marked impact on curd particle size and/or fat losses to the whey at draining. At constant cutting and stirring speeds.

The economical impact in terms of yield and quality of a defective cutting time selection has not been rigorously reported (Castillo, 2006b). In practice, gel is usually cut after a predetermined reaction time or upon the operator's judgment based on subjective evaluation of textural and visual gel properties. Cutting the curd after a predetermined time is a very common practice, but it is questionable because the factors that affect curd firmness and gel microstructure could vary the optimum cutting time. Cutting the curd by relying on empirical inspection is accurate and acceptable if the evaluation is made properly (Castillo, 2006b). A plethora of devices have been developed for that purpose over the past eight decades for monitoring of coagulation and/or cutting time estimation (gelograph, formoghraph, viscometer, rheometer, etc.). In general, these systems are studying destructive rheological properties and not practical for inline use. The alternative is nondestructive measurement equipment based on thermal, electrical and light waves, vibrations and ultrasonic conductivity. Vibrational, thermal and optical techniques are arousing great expectations, but the great development of fiber optic systems and automatic data processing is giving advantage to optical methods. At the moment, a fully effective and adequate system to determine objectively ideal cutting time is not available, although some existing methods can faithfully reproduce the cutting time set by the cheese maker (Castillo, 2016).

A comprehensive classification of milk coagulation and monitoring techniques was published by Castillo (2006b). Among the various techniques described, the group that stands out is based on optical parameters from light backscatter (LB) measurement. Optical backscatter measurement techniques using optical fibers have several inherent advantages, including the measurement of light through the equivalent of small path lengths and the miniature size of optoelectronic components. Optical techniques are also very suitable for inline measurement using optical fibers and can be continuous and nondestructive (Castillo, 2006b).

A fiber optic sensor technology used to measure LB has been demonstrated to be one of the most promising inline, nondestructive methods for monitoring milk coagulation such as those performed by the inline sensor *CoAguLite* developed by Payne *et al.* (1993a). This technique has become commercially available for inline monitoring of cheese production.

The LB sensor measures changes in backscatter of infrared light at 880 nm (**Fig. 2.27**). The use of two optical fibers spaced ~0.7 mm apart (**Fig. 2.27a**) to transport the light is a unique optical configuration that yields a strong signal proportional to the changes that occur in the protein structure during coagulation. Light from a light-emitting diode (LED) is transferred to the milk through a fiber, and the light backscattered from the milk is transmitted through an adjacent fiber to an optical detector. **Fig. 2.27b** shows a picture of the LB fiber optic sensor (Model 5 CoAguLite, Reflectronics Inc., Lexington, Kentucky). The LB signal contains information about the physical changes, the size and/or structure of casein micelles during enzymatic hydrolysis and gelation. The LB profile increases sigmoidally as milk coagulation proceeds for acid-, rennet-, or mixed-induced coagulation. Increased signal depends on the type of milk (e.g., 5 to 21% in goat milk and 50% skimmed cow milk). The LB ratio is generated by dividing the voltage (V) from the sensor by the voltage V_0 obtained by averaging the voltages obtained during the first minute after enzyme addition. The LB ratio begins with a value of land represents the increase in signal during coagulation (**Fig. 2.27c**).

The LB ratio profile has a latent period (**Fig. 2.27c, I**) during which enzymatic reactions predominate with no detectable change in LB response. The LB ratio increases with particle size increase during network formation due to casein micelle cross-linking. As a result, a sigmoidal period is obtained (**Fig. 2.27c,II**) during which aggregation reactions are predominant, as well as an asymptotic period (**Fig. 2.27c,III**) during which cross-linking proceeds at an ever-decreasing rate while curd firming is progressing. The LB ratio typically increases 20, 50, and 260% during coagulation for goat milk, skim cow milk, and yogurt or cottage cheese, respectively (Payne and Castillo, 2007). From a technological point of view, an interesting parameter, t_{max}, (time from enzyme addition to the inflection point of the LB ratio profile) was obtained from the LB profile (**Fig. 2.27c**). This parameter is strongly correlated with the enzymatic hydrolysis constant (Saputra, 1992), allowing the optical sensor indirectly determine the rate of the hydrolysis reaction. When the temperature, pH or calcium levels or enzyme changed, t_{max} varies accordingly.

Castillo (2001) observed that: a) t_{max} varied inversely with the enzyme concentration according to the Foltmann equation, as Berridge clotting time, and b) the slope at t_{max} followed an Arrhenius-type equation as a function of temperature (in the absence of significant rennet heat inactivation). As indicated above and as it is shown in **Fig. 2.27c,** the LB profile not only contains information about the hydrolysis rate constant, but also contains information about the rates of micelle aggregation and gel firming. Castillo (2001) obtained the values of micelle aggregation and gel firming rates constants from LB profile (k_1 and k_2 respectively) using three levels of protein and five levels of coagulation temperatures.

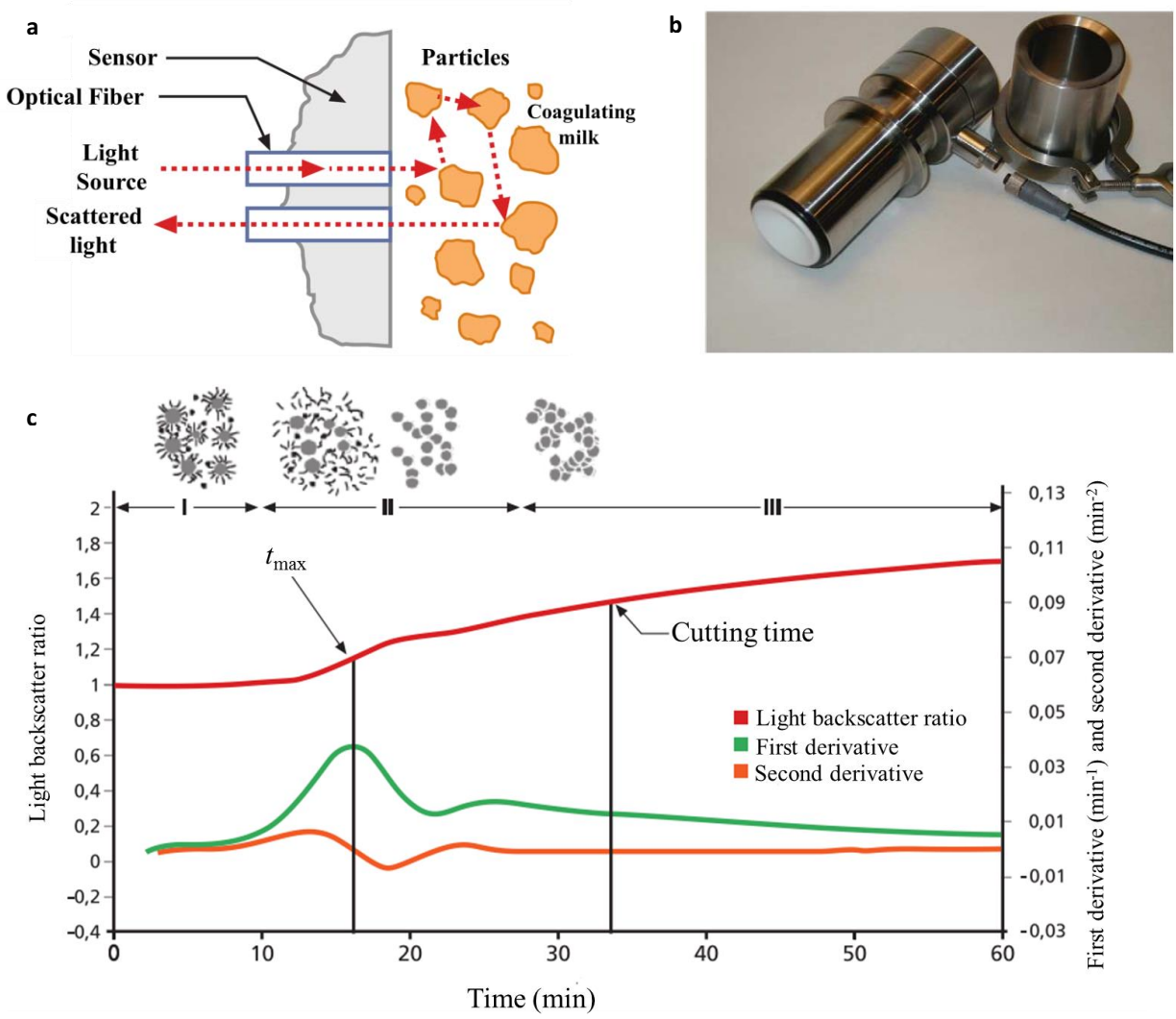


Fig. 2.27 (a) Schematic of LB sensor used for milk coagulation monitoring. (b) Picture of the light backscatter sensor. (c) LB profile and its first and second derivatives as a function of time. (I) Latent period; (II) sigmoidal period; (III) asymptotic period. (Reproduced from Payne and Castillo, 2007).

Thus, Castillo (2001) indicated that it was not surprising that the optical t_{max} parameter is highly correlated with the cutting time. Payne *et al.* (1993a) developed a prediction equation for predicting cutting time (t_{cut}).

$$\textbf{Eqn (2.5)} \qquad t_{cut} = \beta t_{max}$$

The regression coefficient, β, typically varies between 1.15 and 2.4, depending on the type of enzyme used and the type of milk as well as the type of the product manufactured. The coefficient β is selected in the plant to replicate the cheese maker's judgment of cutting time. The value of β calibrates the system and is the only variable that the cheese maker has to determine. Equation 6 is suitable only when the protein concentration is constant. Castillo (2001) observed that β decreases significantly with increasing the protein concentration and for that he developed an algorithm that corrects prediction cutting time depending on the protein content:

$$\textbf{Eqn (2.6)} \qquad t_{cut} = \beta t_{max} \cdot (1 + \gamma\ \%\ \text{protein})$$

The constant γ corrects the value of β according to the concentration of protein. The coagulation sensor is also useful for prediction of other characteristic parameters of coagulation. Thus, t_{max} allows prediction of Berridge clotting time (Castillo, 2001) and gelation time determined by the rheometer (Castillo *et al*., 2006b). Coagulant activity of commercial "rennet" is usually determined by reference methods from the IDF (International Dairy Federation) based on the use of Berridge clotting time (t_{bc}) as traditional method of clotting time determination. This method is based on the observation of the initial casein flocs on the glass wall of a test tube, so its reproducibility and repeatability depend greatly on the operator.

A recent study by Tabayejnehad *et al.* (2009) compares the coagulant activity obtained by the Berridge method and an LB infrared method. This instrument was designed (Department of Biosystems and Agricultural Engineering, University of Kentucky) to determine the coagulant activity. This instrument employs the same optical technology used for cutting time prediction (described above) but adapted for specific laboratory uses (**2.28 a,b**). This method is consistent and accurate for studying the kinetics of the coagulating enzymes. The instrument is conveniently

equipped with two measurement vats for determination in duplicate and includes a comprehensive temperature control that allows the exact determination of the clotting activity of rennet, according to the IDF and ISO standards. The system monitors the temperature of the milk and also measures NIR light backscatter at 880 nm and pH in both vats. During coagulation, a computerized data acquisition system written in Microsoft Visual Basic.net was used to collect, store and analyze the data for determination of optical time parameters. Computerized data acquisition system calculates in real time the first and second derivatives of the LB profile with respect to time to obtain five optical parameters (t_{2max}, t_{max}, t_{2min}, t_{2max2} and t_{2min2}) as described by Castillo (2001). Therefore the proposed method represents an optical objective, accurate and simple alternative to Berridge method for determining the milk-clotting activity of rennet.

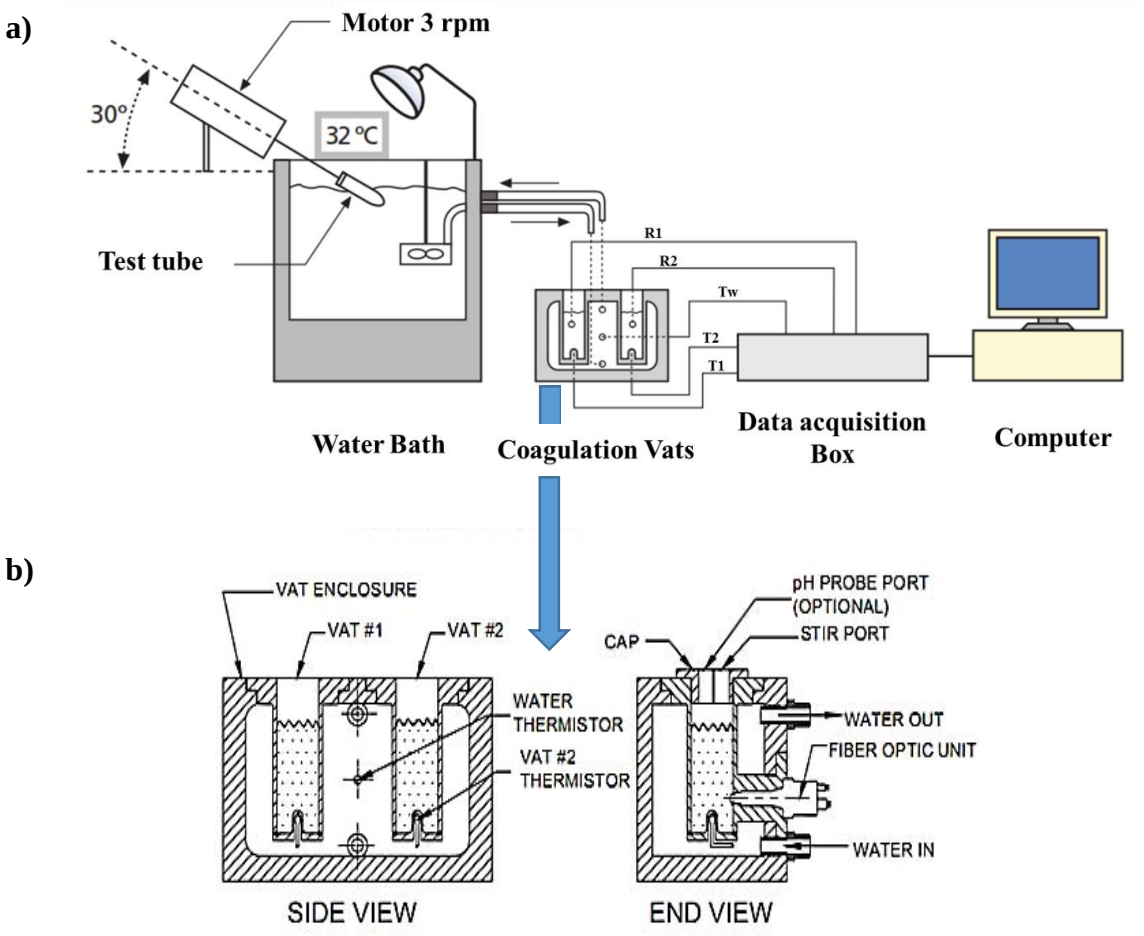


Fig. 2.28 Schematic of the laboratory instrument for measure near infrared light backscatter (880nm) during milk coagulation. Connections: R1 = light backscatter sensor for Vat 1; R2 = light backscatter sensor for Vat 2; T1 = thermistor for Vat 1; T2 = thermistor for Vat 2; and Tw = thermistor for circulating water inside the coagulation vat enclosure. Reproduced from Tabayejnehad *et al.* (2009).

According to Hori (1985), a thermal conductivity sensor detects changes in convective heat transfer from a ‘‘hot wire’’ to the surrounding milk that are caused by the variation in viscosity during coagulation. Hot wire sensor has been demonstrated to measure very accurately the gelation point that, in turn, can be used to predict cutting time. However, because viscosity increases exponentially between the beginning of aggregation and the onset of visual coagulation, the hot wire is not well suited for measuring gel stiffness. Some rheological nondestructive methods have been proposed for gelation and firming measurement.

With small deformation rheometry (Bohlin *et al.*, 1984), the gelation process is not affected by the measurement because the renneted milk is subject to a harmonic low-amplitude shear strain (or stress depending on the rheometer) at a certain angular frequency within the linear viscoelastic limits of the sample. Under these conditions, the gel network recovers instantaneously when the stress is removed. This method allows continuous measurement of well-defined rheological parameters such as elastic and viscous moduli, G' and G'', complex rigidity modulus, G^*, and the loss factor or $tan\delta = G''/G'$. The elastic modulus, G' is direct measurement of gel firmness, while $tan\delta$ is related to the viscoelastic properties and rearrangement capability of the gel (Castillo, 2006a).

2.3.2 Inline monitoring curd syneresis

One of the most important and critical steps in cheese making is syneresis, to regulate moisture content, minerals and lactose from the curd, which in turn influences directly the maturation phase. Many common defects in cheese are due to the high water content of the curd at the beginning of ripening (Castillo, 2016). During maturation, the lactic acid bacteria produce enzymes that modify the sensory attributes of the curd by transforming it into cheese (McSweeney and Fox, 1993). Improving syneresis control would result in more adjusted values of lactose, minerals, pH and moisture from the curd at the beginning of ripening, increasing control over the process and reducing the proportion of cheese that violate quality standards. Walstra *et al.* (1985) and Walstra (1993) have reviewed the various techniques that have been developed to measure syneresis: **a)** measuring the curd shrinkage determined by changes in mass, height, area or volume; **b)** measuring the amount of whey expelled or the degree of dilution of an added tracer; **c)** determining dry matter content in the curd grains; **d)** determining the curd grains density.

Despite the numerous methods proposed for assessment of syneresis, at industrially level this process is still controlled empirically worldwide by mechanical treatment and control of parameters such as time and processing temperature.

Although many authors claim that the kinetics of syneresis can be described by an equation of first order (Marshall, 1982; Castillo *et al.*, 2005a), few authors have directly studied the contraction of the curd by the difficulty of measurement (Castillo, 2016). Castillo *et al*. (2006b) have determined the curd contraction by a laser displacement sensor, confirming that it fits a first order kinetics. The dilution of fat globules in serum during syneresis has been also studied by Castillo *et al*. (2005a), and follows a first order kinetics, suggesting that the fat concentration could be used as *"inner tracer"* for monitoring syneresis. Based on this hypothesis, the authors have developed a NIR diffuse reflection sensor (980 nm) called large field of view (LFV sensor; **Fig. 2.29**) that allows monitoring both the milk coagulation and whey draining from the curd (Castillo *et al*., 2005b), and predicts curd moisture content as a function of stirring time.

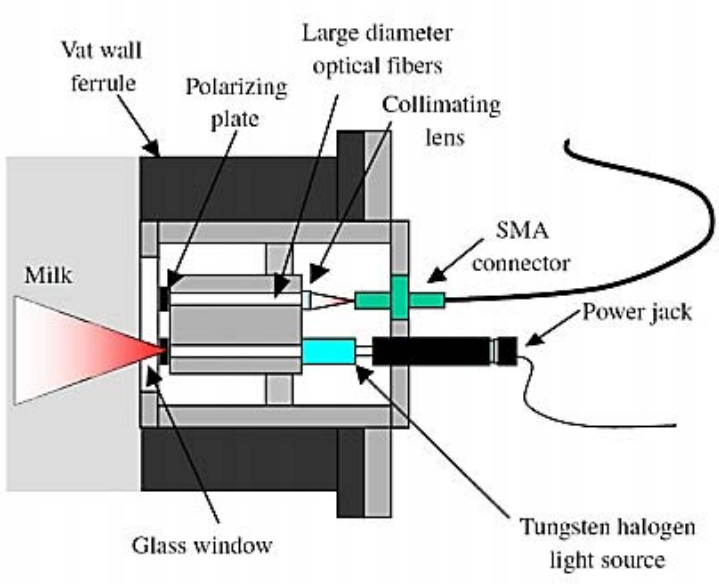


Fig. 2.29. Large Field of View (LFV) fiber Optical sensor for monitoring milk coagulation and curd syneresis in cheese vat. Reproduced from Castillo *et al.* (2005b) and Castillo *et al*. (2007).

The LFV sensor allows continuous stirring by averaging the light intensity scattered by the mixture of curds and whey, despite the heterogeneity of those two phases. Incident light radiation transmits through an optical fiber of 0.5 cm in diameter, a vertical polarizer, and an large diameter optical glass window until it reaches the sample, (*ie*.., the only incident radiation reaching the

sample is vertically polarized). The light reflected/scattered by the sample passes through the optical window and a horizontal polarizer that blocks vertically polarized radiation and reaches the detector through another 0.5 cm diameter optical fiber.

Since only the light, which has rotated 90° after interacting with the sample reaches the detector, the sensor is considerably more sensitive to scattered light that changes orientation when passing through the sample than to specular radiation caused on the surface of the optical window or reflected on the surface of the curd grains without rotating. Removing specular reflection aims to increase the sensitivity of the sensor to some of the phenomena occurring in the serum sample and curd during the draining process. The intensity ratio derived from the sensor sigmoidally grows (~23%) during milk clotting and decreases logarithmically during the draining process (**Fig. 2.30**).

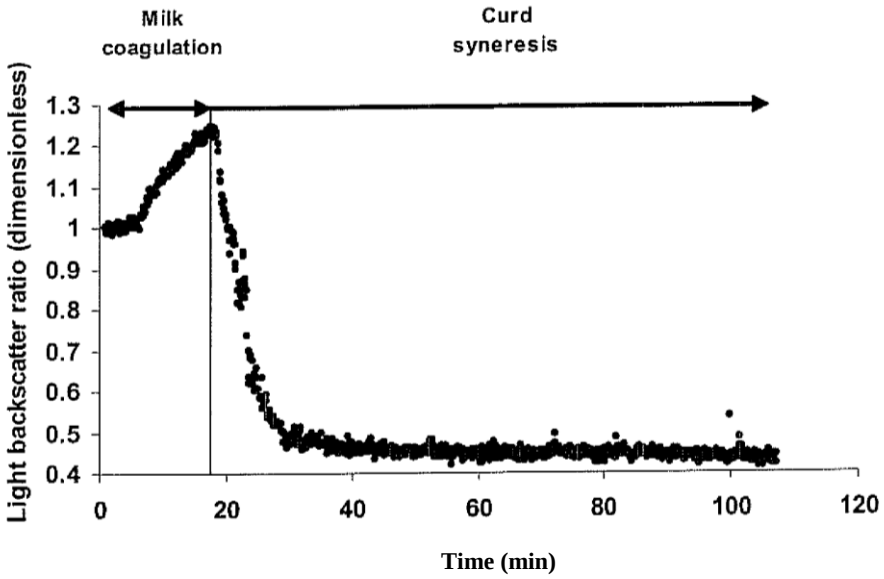


Fig. 2.30 Typical LFV response from the syneresis sensor during both milk coagulation and curd syneresis. Reproduced from Castillo *et al.* (2007)

The decrease of the signal during the whey separation, which varies between 25 and 61% according to the experimental conditions, increases with temperature, suggesting that the sensor is sensitive to changes in the kinetics of syneresis caused by this factor.

The LFV optical technology has the additional advantage of responding to the aggregation of the casein micelles and hardening of the curd during coagulation of milk. Therefore, the optical sensor monitors not only syneresis but prior process of milk clotting, allowing prediction of the

cutting time and humidity changes in the curd during draining by using a single sensor. In other words, the LFV sensor has the potential to provide useful information of most chemical and/or physical phenomena of interest for process control occurring in the cheese vat such as enzymatic hydrolysis, micelle aggregation, gel firming and curd syneresis.

Currently, the LFV syneresis sensor is being scaled up. Data obtained so far are certainly promising, but the industrial implementation still requires an effort. If this implementation is successful, this optical technology would have a great impact on quality and homogeneity of the cheese worldwide.

Chapter Three

Objectives

Chapter Three: Objectives

3.1 General objective

The general objective of this dissertation was to evaluate the impact of milk mixture (i.e., different proportions of goat, sheep and cow milk) and low quality milk (i.e., milk from animals with subclinical mammary infections) in the prediction of clotting time, cutting time, syneresis rate and several other cheese making indexes based on monitoring milk coagulation and syneresis using NIR light backscatter sensor technologies.

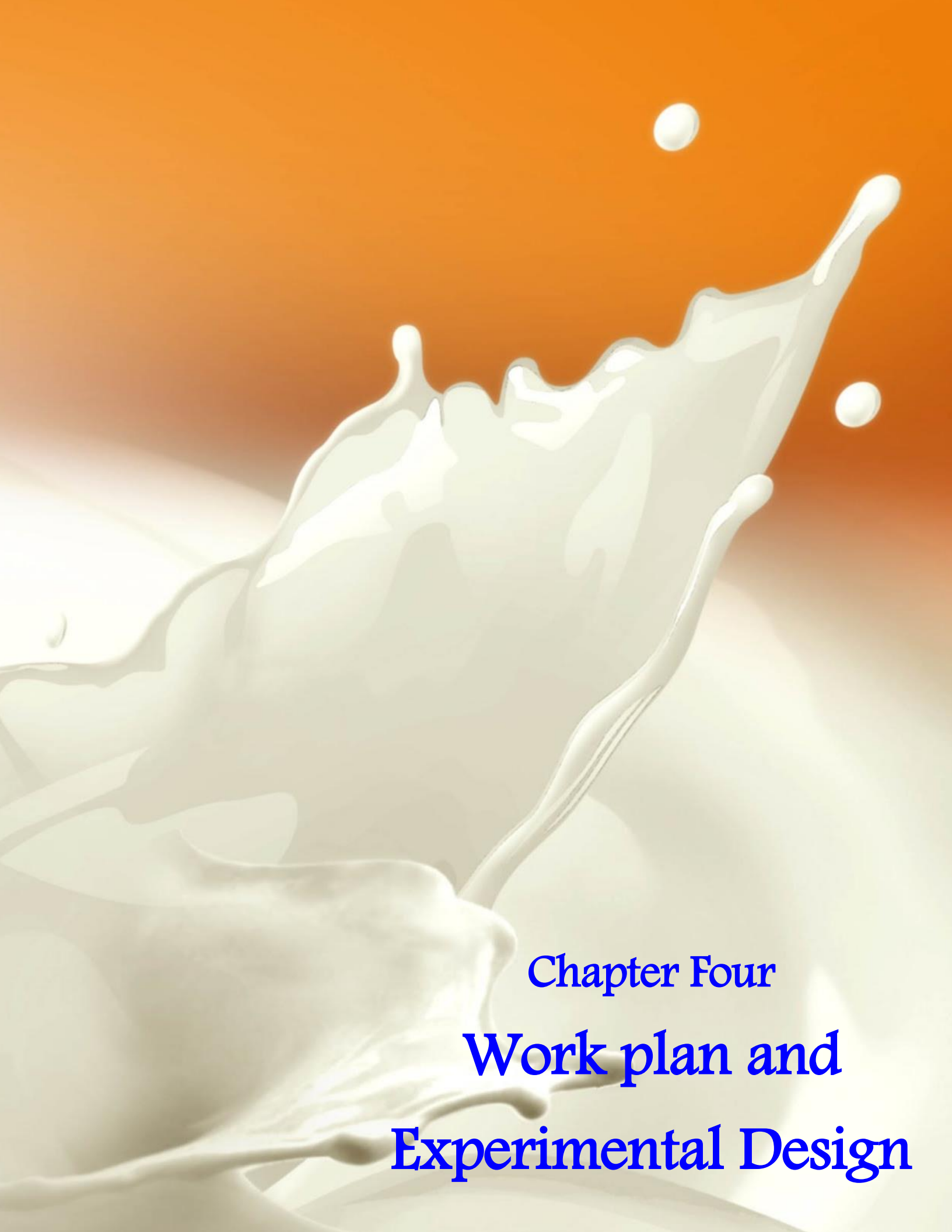
To achieve the objective of this PhD study, the general objective was divided into the following specific objectives:

3.2 Specific objectives

1- Studying the effect of mixing milk from different species (goat, sheep and cow) on the near infrared light backscatter profile and optically-derived parameters during enzymatic coagulation of milk induced at different temperatures and enzyme concentrations.

2- Evaluating the effect of subclinical mammary infection and breed on the near infrared light backscatter profile and optically-derived parameters during mixed coagulation and curd syneresis of sheep milk.

3- Developing prediction models for clotting and cutting times and other relevant cheese making indexes (i.e., rheological parameters, curd moisture content, cheese yield, yield of whey and fat losses to whey) suitable for milk mixtures and milk from animals suffering subclinical mastitis using optically-generated predictors.



Chapter Four

Work plan and Experimental Design

Chapter Four: Work Plan and Experimental Design

To achieve the proposed objectives of this doctoral dissertation, three experiments were carried out.

The results obtained from the three experiments have led to three articles. Two of them are published at high standard peer-reviewed scientific journals and the remaining one is under review.

In the first experiment (**Fig. 1**), a completely randomized factorial design with three factors and three replications (n=3) was used to study the effect of different milk coagulation temperatures (*27, 32* and *37° C*), various enzyme concentration levels (*200* and *400 mg·L*$^{-1}$) and several milk mixtures proportions (*Mix.1* and *Mix.2*) on the light backscatter profile through monitoring the milk coagulation and cutting time using a NIR light backscatter sensor lab-scale tester (CoAguLab, Department of Biosystems and Agricultural Engineering, University of Kentucky), a small amplitude oscillatory dynamic rheometer (Thermo Haake GmbH, Karlsruhe, Germany) and visual determination of both clotting and cutting time. Treatments were assigned to experimental units in random order to eliminate bias from unknown sources of variation. The replicas are needed to estimate the residual variation, to improve the estimation of the effect of treatments. Also, this type of experimental design allows to study the possible interactions between factors (enzyme concentrations, coagulation temperatures and milk mixtures), while saving time and effort. During the coagulation and cutting time monitoring process, many optical parameters were obtained from the light backscatter and the rheometer profiles. According to the data obtained from this experiment and the maximum R^2 procedure from SAS, the best one-, two- and three-variable models for predicting several cheese making indexes such as visual and rheological clotting and cutting times and *tan* δ were obtained. Details regarding the materials and methodology used and the results and discussion are developed widely in chapter 6.

The second experiment (**Fig. 2**) was undertaken to investigate the effect of subclinical mastitis in the coagulation properties of ewe milk using a light backscatter sensor as well as to evaluate its influence in the use of light backscatter for lab-scale prediction of both rheological and visual clotting and cutting times. An experimental design with two different dairy sheep

breeds, Lacaune (***LC***) and Manchega (***MN***), was used to establish base for milk of uninfected animals. The influence of subclinical mastitis was studied using *LC* sheep with one gland uninfected and the contralateral infected with CNS (*ILC*). Each milk type was tested twice. A total of 32 lactating dairy sheeps of the two breeds (MN; n = 14; *LC*, n = 18) at mid to end lactation (80–150 days in milk) were used. The animals were selected for the study according to bacterial udder infection as described by Rovai *et al.* (2014) using a half-udder model to assess the uninfected or infected glands IMI status with various CNS species. This model has been extensively used (Gonzalez-Rodriguez *et al.*, 1995; Leitner *et al.*, 2004, 2006, 2008, 2011a; Martí De Olives *et al.*, 2013) with excellent results and it enables to study the negative effect of SCM on milk yield and quality with high statistical reliability. The model eliminates the significant individual variations between individual animals such as genetic, lactation and stage of lactation, nutrition, farm and environment. All these animal variations are completely neutralized when the unit of comparison corresponds to the two glands of the same animal. Two replications (n = 2) of the experiment were carried out. A NIR light backscatter sensor, a rheometer and visual determinations (discussed above) were used to monitor milk coagulation. The best prediction models for predicting the visual and rheological clotting and cutting times and *tan δ* were obtained. Details regarding the material and method used and the results and discussion are presented in chapter 7.

The third experiment was quite similar to the second experiment regarding the experimental design, as it is observed in **Fig. 3**. This experiment was performed to validate the visual cutting time prediction models obtained in the second experiment at a pilot plant scale using a ten-liter cheese vat as well as to study the effect of the same factors evaluated in the mentioned experiment on syneresis and whey separation and develop prediction models for complementary cheese making indices such as moisture content, cheese yield, fat losses, etc., that were not evaluated in the lab-scale previous experiment. Two replications (n = 2) of the experiment were conducted at pilot plant scale using a double-jacket cheese vat (Type CAL 10L, Pierre Guerin Technologies, Mauze, France). The cheese vat contained two sensors. The first one was a NIR light backscatter sensor (CoAguLite model 5, Reflectronics Inc., Lexington, KY) for monitoring milk coagulation and cutting time and the second sensor was a LFV (Large field of view sensor, designed at the University of Kentucky) for monitoring curd syneresis and whey separation connected to a miniature fiber optic spectrometer (model HR2000CG-UVNIR, Ocean

Optics B.V., Duiven, Netherlands). The best prediction models for predicting the visual clotting and cutting times at pilot plant scale as well as several cheese making indices i.e., curd moisture content, yield of cheese, whey yield and fat losses to whey, were obtained among other parameters. Details regarding the materials and methods used, the results obtained and the corresponding discussion are presented widely in chapter 8.

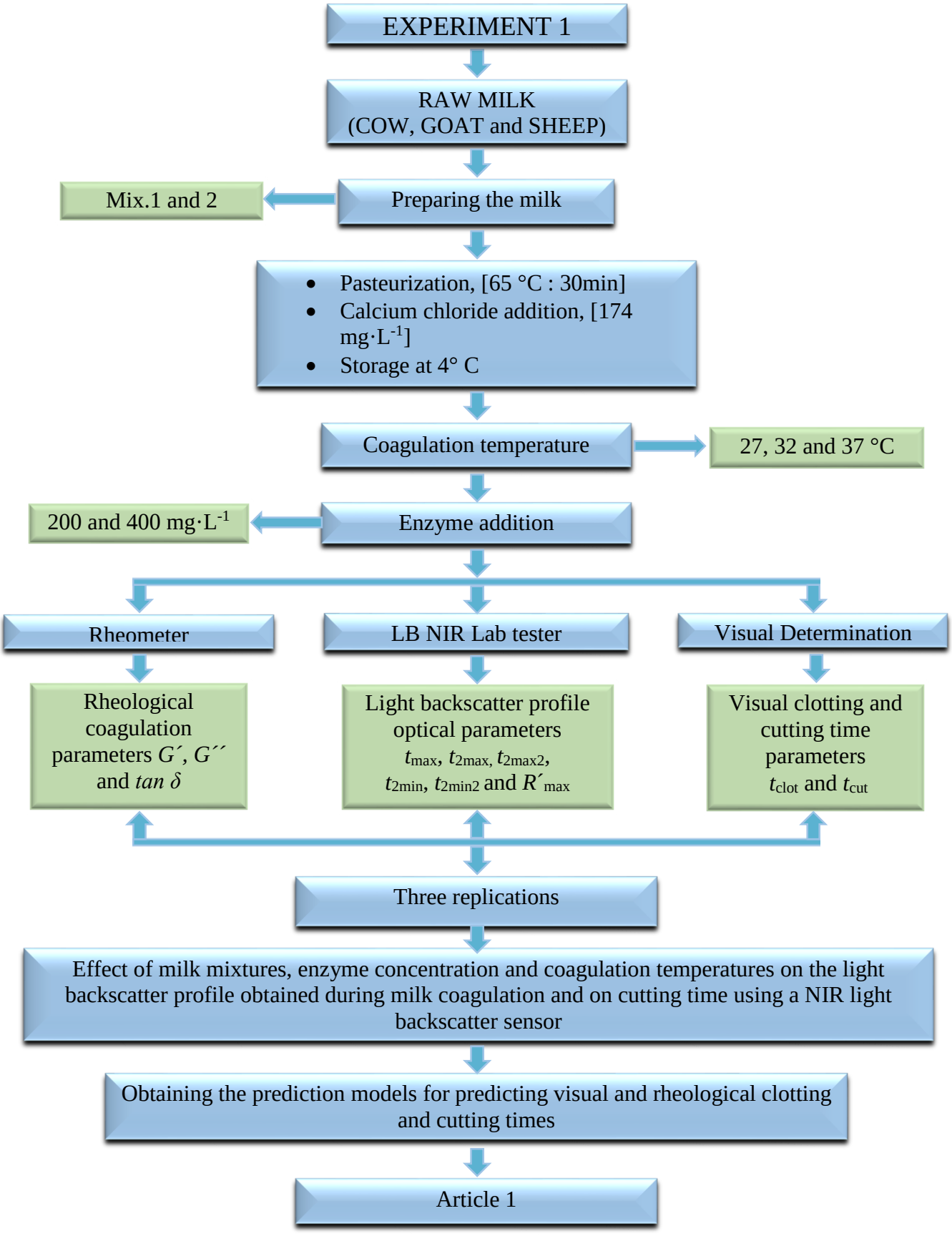


Fig. 1 Experimental design of the first experiment.

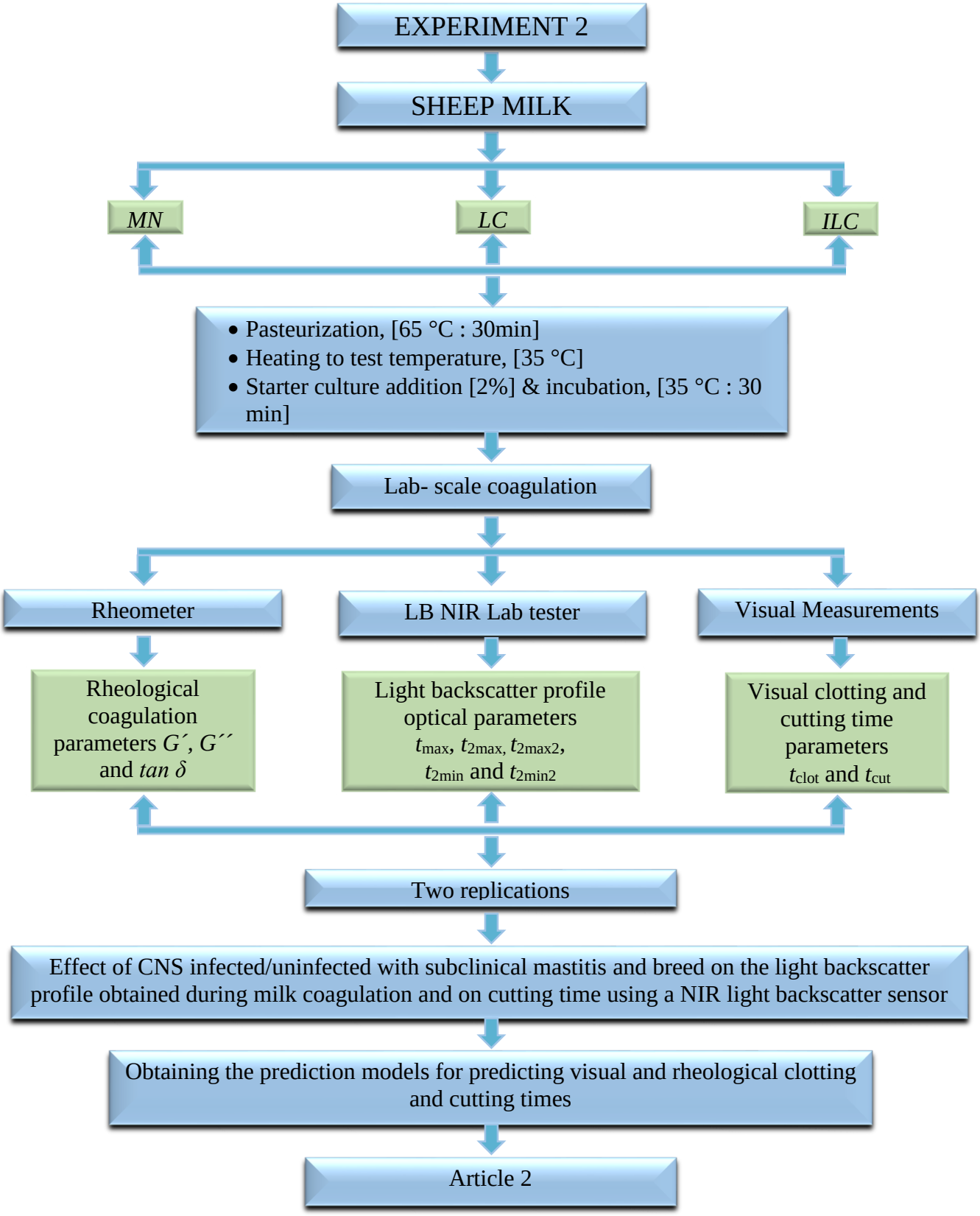


Fig. 2 Experimental design of the second experiment.

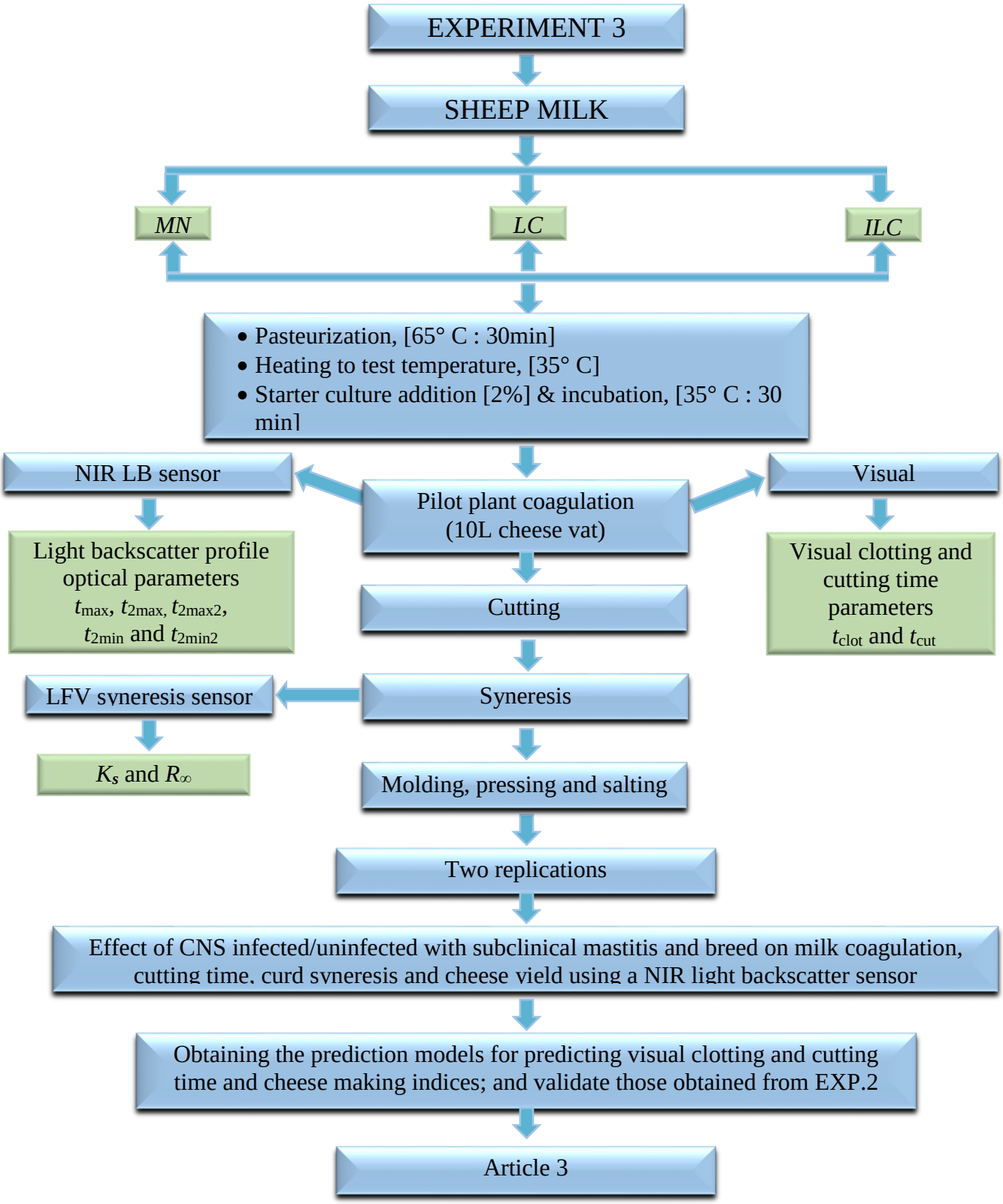


Fig. 3 Experimental design of the third experiment.


GRÀCIES
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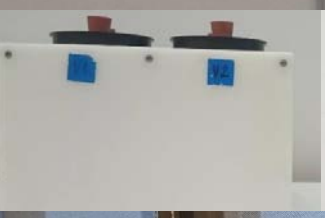

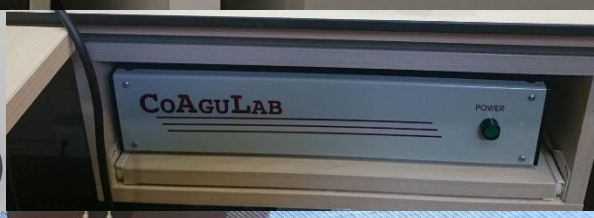
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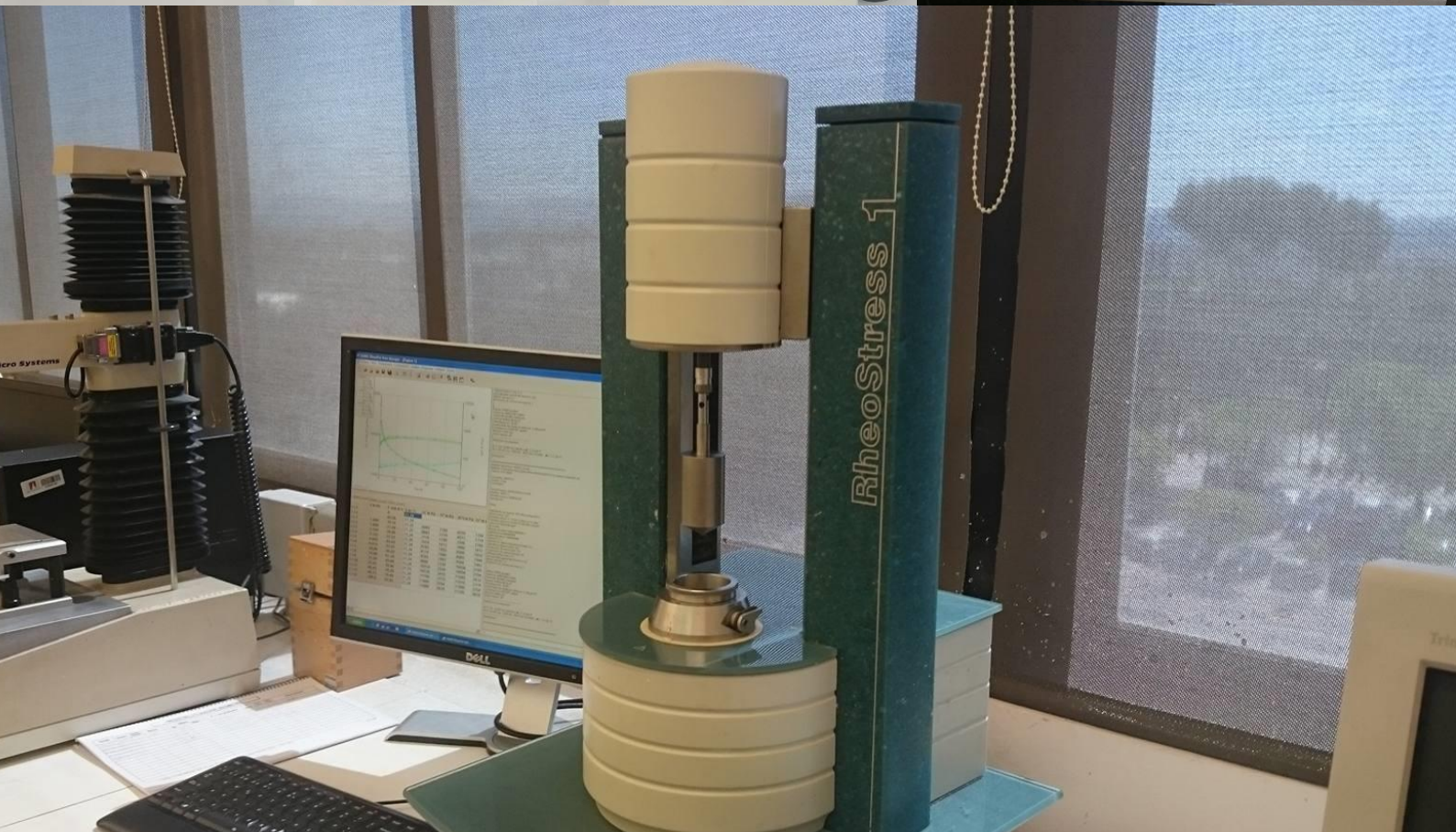
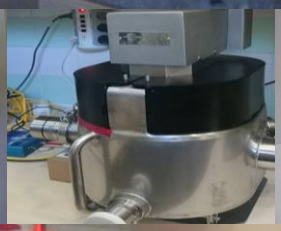

RheoStress 1
DELL



Chapter Five

Material and Methods

Chapter Five: Material and Methods

5.1 Composition of the milk.

Animal care conditions and management practices agreed with the procedures stated by the Ethical Committee of Animal and Human Experimentation of the Universitat Autònoma de Barcelona (Bellaterra, Spain; CEEAH reference 09/771) and the codes of recommendations for the welfare of livestock of the Ministry of Agriculture, Food and Environment of Spain.

Unpasteurized and un-homogenized goat and sheep milk were obtained from the Experimental Farm of the Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain), while the cow milk was obtained from Can Badó farm (S.A.T. Can Badó, La Roca del Valles, Spain). Immediately after milk was received in the Centre Especial de Recerca Planta de Tecnologia dels Aliments (**CERPTA**) at UAB, (Bellaterra, Spain), milk samples (100 mL) were taken for the chemical analysis, then milk was stored at ~4 °C until it was used (typically within the first 24 h from milk reception) to prepare the corresponding milk batches, .

5.2 Testing procedure

The different batches of milk were vat pasteurized at 65 °C for 30 min and immediately cooled and stored at 4 °C until used in the same day of milk collection.

In the test day, milk was heated to the target coagulation temperature using a water bath. Depending on the experiment, calcium chloride was added (exp.1) or not (exp. 2 and 3) to the milk after heating. In exp.1 a constant amount of 0.74 mL of the prepared calcium chloride solution was added per liter of milk to each milk sample. This calcium solution was calculated to deliver 174 mg of anhydrous calcium chloride ($CaCl_2$) per liter of milk. Animal rennet was added to the milk at the target concentration and then milk was quickly stirred for 30 s.

For the lab-scale experiment, a 500 mL milk sample was split immediately after enzyme addition (Calf rennet) into four aliquots. Two 80 mL aliquots were placed in the two measuring vats of the NIR coagulation measurement apparatus to obtain the light backscatter parameters, a 40 mL aliquot was placed in the rheometer to determine the rheological parameters and an 80 mL aliquot was placed in a water bath to determine the visual coagulation parameters. The lag

time between the enzyme addition and the activation of the respective data acquisition systems was measured with a laboratory chronometer and was adjusted to be less than 1.5 min.

For pilot plant scale, 11 L milk were place in cheese vat for cheese manufacturing, and left until thermal equilibrium was achieved. Calf rennet was added at a target amount and the mixture was rapidly stirred during 30 s. Once enzyme was added to the milk, milk coagulation monitoring was initiated with the NIR sensors (CoAguLab and CoAguLite for coagulation and LFV for syneresis).

5.3 Cutting the coagulum, syneresis process, pressing and cheese salting

Once the gel reached the adequate firming for cutting, it was cut. The cutting operation consisted of 3 cutting/resting cycles of one-min duration for a total of 3 min. Cutting was performed at 16 rpm during the first cycle and 24 rpm during the second and third cycles. The cutting/resting duration within the cycles was 40/20 s. During the third stopping phase the *LFV* sensor window was cleaned with a small brush to remove any remaining gel adhered to the surface of the sensor. After cutting, the cutters were substituted by the stirrers and the syneresis procedure started at t = 4 min at a temperature of 35 ºC and a stirring speed of 20 rpm. After syneresis, curd and whey drained from the vat were weighted and aliquots of curd (300 g) and whey (100 mL) were taken for the chemical analysis. Then, the rest of the curd was placed into two-three cylindrical mould (150 ×130 mm) and pressed (0.5 kg/cm^2 for 60 min). The curd placed in each mould was weighted. After 60 min, the pressed cheeses were extracted from the moulds, reversed, and the pressure was increased to 1 kg/cm^2 for 3 h and later on the pressure was increased again to 1.5 kg/cm^2 for 12 h. After pressing, the cheeses were salted for 4 h at 14 ºC by immersion in brine [19% NaCL solution; density, 1.143 g mL^{-1}, adjusted to pH ~5.2 using lactic acid (PURAC FCC 88, corbion purac inc., Barcelona, Spain)]. Once the cheeses were salted, they were weighted and one aliquot of fresh cheese (300 g) was taken for chemical analysis. The cheese was stored at the ripening chamber at a temperature of 14 ºC and 85% relative humidity. Whey and cheese samples were taken to the laboratory in a portable refrigerator. Chemical analysis of cheese and whey was carried out in triplicate.

5.4 Light Backscatter Monitoring

5.4.1 Lab-scale milk coagulation monitoring technology

A lab-scale milk coagulation tester designed at the University of Kentucky (Tabayejnehad, *et al.*, 2009) which is commercialized by Reflectronics, Inc., Lexington, KY, CoAguLab (**Fig. 5.1**) was used to monitor milk coagulation in exps.1 and 2. The sensor uses changes in light backscatter (LB) of infrared light to monitor milk coagulation. As the enzymatic cleavage of the micelles proceeds, the LB ratio (R) increases while the micelle network forms a gel (Payne and Castillo, 2007).

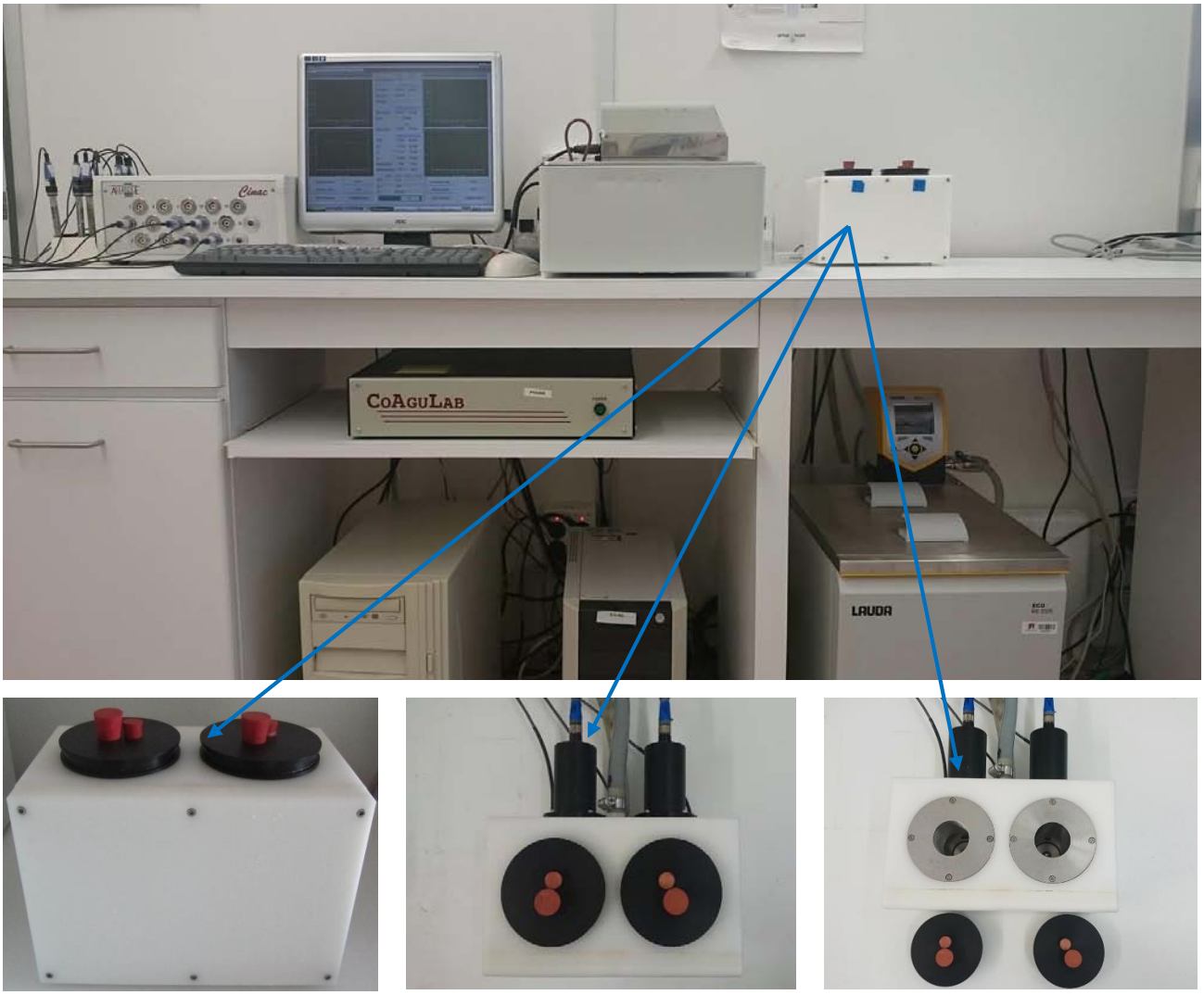


Fig. 5.1 Showing the CoAguLab tester from different views**.**

5.4.1.1 The coagulation measurement apparatus and the measurement set up

Two vats were designed into the apparatus to make precise comparisons feasible. Each vat was instrumented to measure near infrared light backscatter, temperature and pH (not used in this study) during coagulation. The sample vats had an inside diameter of 36 mm, height of 115 mm and contained a maximum sample volume of 98 mL. The sample vats were fabricated of stainless steel with the interior surfaces polished for easy cleaning. A plastic cap was used to cover each vat during testing to prevent evaporative cooling. The cap provided access to the vats for mixing the enzyme and inspecting the sample visually. The coagulation measurement apparatus had an inlet/outlet connection to a water bath as shown in **Fig. 5.2a**.

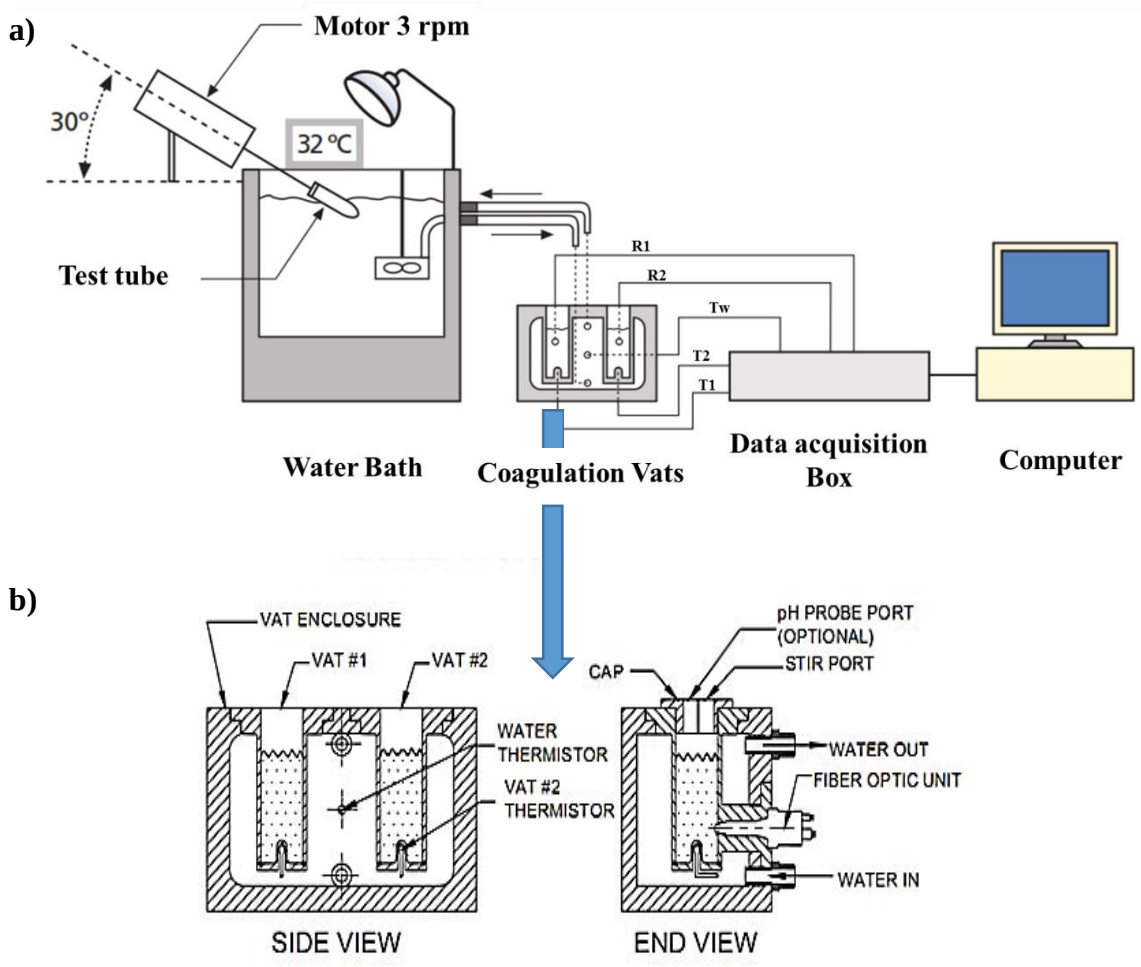


Fig. 5.2 Schematic of the laboratory instrument for measure near infrared light backscatter (880nm) during milk coagulation. Connections: R1 = light backscatter sensor for Vat 1; R2 = light backscatter sensor for Vat 2; T1 = thermistor for Vat 1; T2 = thermistor for Vat 2; and Tw = thermistor for circulating water inside the coagulation vat enclosure. (Reproduced from Tabayejnehad, *et al.* (2009*)*

Linear thermistors (Model OL-710-PP, Omega Engineering, Stamford, CT) were used to measure the temperature of the milk samples (vats #1 and #2) and circulating water bath. A fiber optic unit (Model 5, Reflectronics, Inc., Lexington, KY) was used to measure near infrared light backscatter at 880 nm. The fiber optic unit was installed horizontally in each vat as shown in **Fig 5.2b**. The fiber optic units directed near infrared light from a light-emitting diode (**LED**, Model L2791, Hamamatsu Corp., Bridgewater, NJ) into the milk sample and returned the backscattered light to a detector (Model TSL250, TAOS, Plano, TX) using two optical fibers of 600 μm diameter spaced 0.7 mm apart. The use of two optical fibers spaced 0.7 mm apart to transport the light is a unique optical configuration that yields a strong signal proportional to the changes that occur in the protein structure during coagulation (Castillo *et al*., 2000).

A computerized data acquisition system written in Microsoft Visual Basic.net was used to collect, store and analyze the data for determination of optical time parameters during milk coagulation. The analog signals for temperature and light backscatter were digitized using a 16-bit analog to digital board (Model USB-1608FS, Measurement Computing Corp., Norton MA).

The initial voltage response ($\boldsymbol{V_0}$) was calculated by averaging the first ten data points after correction for the 1V zero offset. A light backscatter ratio ($\boldsymbol{R}$) was calculated by dividing the sensor output voltage (less the 1V zero output) by V_0. The first derivative ($\boldsymbol{R'}$) of the light backscatter ratio profile was calculated by conducting linear least-squares regression on the most recently collected 4 min of data. The calculated slope was assigned to the midpoint of the data subset used. The second derivative ($\boldsymbol{R''}$) was calculated in a similar manner. Thirteen optical time parameters were defined by the different maxima and minima of the light backscatter profile derivatives as a function of time. Those optical parameters are defined in **Table 5.1** and were classified as suggested by Castillo *et al*. (2000).

Table 5.1. Definition of main optical parameters derived from the light backscatter ratio profile.[a]

Parameter	Units	Definition
t_{max}	min	The elapsed time from enzyme addition to the first maximum of R'
t_{max2}	min	The elapsed time from enzyme addition to the second maximum of R'
t_{2max}	min	The elapsed time from enzyme addition to the first maximum of R''
t_{2min}	min	The elapsed time from enzyme addition to the first minimum of R''
t_{2max2}	min	The elapsed time from enzyme addition to the second maximum of R''
t_{2min2}	min	The elapsed time from enzyme addition to the first minimum of R''
R_{max}	Dim[y]	The value of R at t_{max}
R_{2max}	Dim[y]	The value of R at t_{2max}
R_{2min}	Dim[y]	The value of R at t_{2min}
R_{2max2}	Dim[y]	The value of R at t_{2max2}
R_{2min2}	Dim[y]	The value of R at t_{2min2}
R'_{max}	min^{-1}	The value of the first derivative of R at t_{max}
R''_{max}	min^{-1}	Value of R'' at t_{max}

[a] R, light backscatter ratio; R', first derivative of R as a function of time; R'', second derivative of R as a function of time.
[y] Dim: Dimensionless.

5.4.1.2 Temperature control

Precise temperature control is essential for accurate comparison of simultaneous enzymatic reactions. The coagulation measurement apparatus was designed to provide identical temperatures in the two milk sample vats by circulating one stream of water from a water bath around both vats.

The temperature difference between the water bath and the milk in the vats was minimized by using a plastic vat enclosure **(Fig. 5.2b)** and other plastic parts to minimize heat transfer losses. Plastic hoses connected the vat enclosure to the circulating water bath. A circulating water bath (Lauda ECO Gold RE 2025, Brinkman Instruments, Inc., Westbury, NY) having a reported temperature control of ± 0.04 °C was used to circulate water through the vat enclosure at a flow setting of ~8 L min^{-1}. Since the internal water volume of the vat enclosure was measured to be 1.6 L, the design provided for a water volume exchange rate of 5 times per min. Precise temperature control was confirmed for the coagulation measurement apparatus at 32° C.

The temperature difference between the milk sample in a vat and circulating water in the water bath was accurately measured using a precision thermistor (model 5831 A, Omega Engineering, Stanford, CT, resolution ± 0.01°C). The mean of the temperature measurements

was 32.04 and 32.02 °C for the water bath and sample, respectively. The average temperature difference between the sample and water bath was thus 0.02 °C. Therefore the equilibrium sample temperature in the vat is approximately equal to the water bath temperature, and as a result, the temperature control of the vat and the circulating water bath is the same.

5.4.2 In-line light backscatter monitoring instruments during cheese manufacture

In-line monitoring of milk coagulation and curd syneresis in an 11L double-jacket cheese vat (Type CAL 10L, Pierre Guerin Technologies, Mauze, France) (**Fig. 5.3a**) was performed using two different fiber optic sensor technologies, the CoAguLite (**CL**) (**Fig. 5.3b**) and the Large Field of View (**LFV**) sensors (**Fig. 5.3c**), which were placed in the wall of the cheese vat.

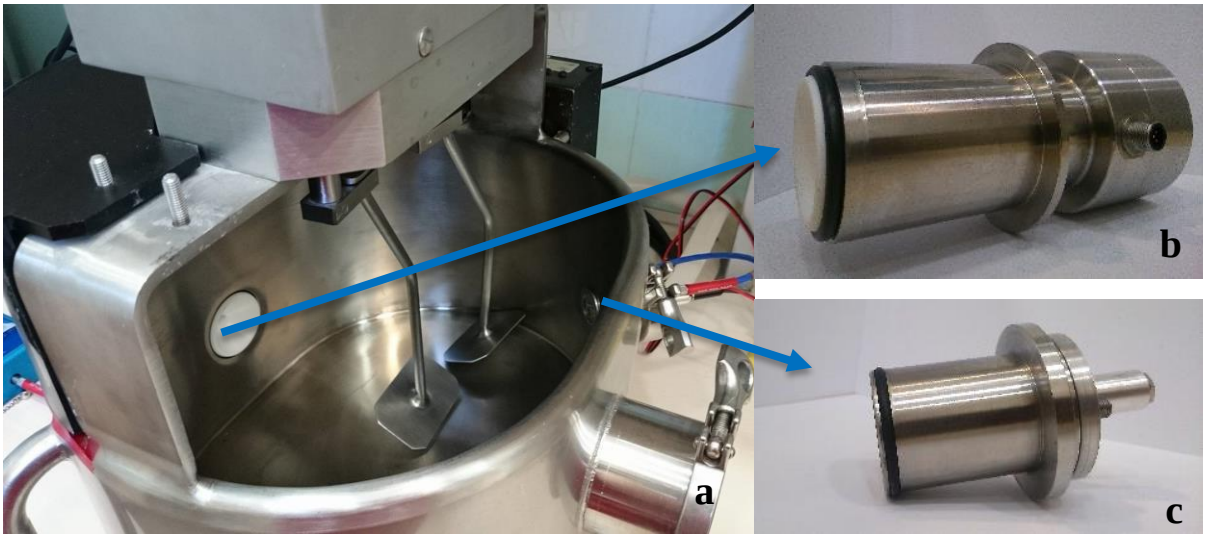


Fig. 5.3. (a) Cheese Vat showing the twin stirring blades used to stir the milk and the whey and curd mixture.
(b) The CoAgulite sensor used for monitoring milk clotting and cutting time.
(c) LFV sensor used for monitoring curd syneresis during cheese manufacture

Optical response from both sensors was continuously monitored from the time of rennet addition to the end of syneresis. Coagulation temperature was controlled (35 ± 0.1 °C) using the double-jacket cheese vat supplied with temperature controlled water through a couple of plastic coils connected to the water bath digital refrigerated immersion thermostat (OvanTherm C-TC00E, Suministros Grupo Esper, S.L. ES). Milk temperature was measured with a precision thermistor (model 5831 A, Omega Engineering, Stamford, CT; resolution ± 0.1 °C; accuracy ± 0.2 °C).

5.4.2.1 CoAguLite light backscatter monitoring technology

The CoAguLite sensor (model 5, Reflectronics Inc., Lexington, KY, USA) (**Fig. 5.3b**) is an optical fiber light backscatter sensor that has been used to monitor milk coagulation and predict both clotting and cutting times (Payne and Castillo, 2007).

This sensor used near infrared light at 880 nm and consisted of two 600 µm diameter fibers. The light from a light emitting diode (**LED**) is transferred to milk sample by an optical fiber, while the other fiber transmitted the radiation scattered by the milk particles present in the milk to a silicon photo-detector (TSL 245, TAOS, Dallas, TX , USA) **Fig. 5.4**. Response data were collected every 6 s.

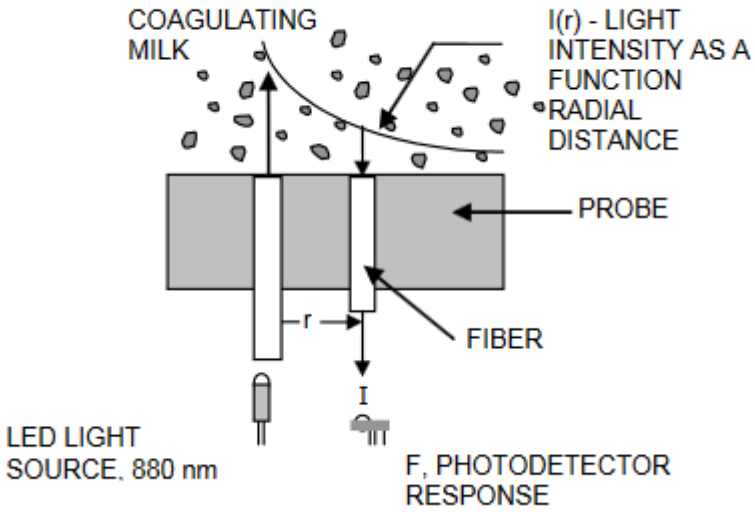


Fig. 5.4. Origin of the sensor signal dispersion in milk during coagulation. According to Payne and Castillo (2007).

This optical configuration provides a signal proportional to the changes occurring in the protein structure of milk during coagulation. The signal contains information about physical changes, the size and/or structure of the casein micelles during enzymatic hydrolysis and gel formation. The light backscatter profile was obtained using the method described by Castillo *et al.* (2000). Output voltage was zeroed to 1 V. Response data were collected every 6 s. The initial voltage response ($\boldsymbol{V_0}$) was calculated by averaging the first ten data points after correction for the 1V zero offset. A light backscatter ratio (***R***) was calculated by dividing the sensor output voltage (less the 1V zero output) by V_0.

The light backscatter profile yields parameters of technological interest. The optical time parameters were defined as for the CoAguLab by the maxima and minima of the light backscatter profile derivatives as a function of time. Those optical parameters are defined in **Table 5.1** and were classified as suggested by Castillo *et al.* (2000). The coagulation sensor is also useful for the prediction of important coagulation indicators. Several linear models have been developed for predicting clotting and cutting time optically in two different types of milk (i.e., cow and goat milk) but those models have never been tested in mixtures of cow, goat and sheep milk or in milk from animals or having mammary infections.

The CoAguLite sensor was tested by Fagan *et al.* (2007a) for monitoring coagulation and syneresis, but the sensor output during syneresis included a high degree of scatter due to the two-phase mixture of curd pieces and whey. This problem was attributed to the optical fiber employed (0.6 mm diameter) had a small field of view in relation to a typical curd piece (5 – 10 mm diameter).

5.4.3 Optical monitoring of curd syneresis using the Large Field of View sensor

The LFV sensor was a syneresis sensor prototype designed and built at the University of Kentucky (Castillo *et al.*, 2005b; Castillo *et al.*, 2007). A schematic for the sensor design as published by Fagan *et al.* (2007a) is shown in **Fig. 5.5.**

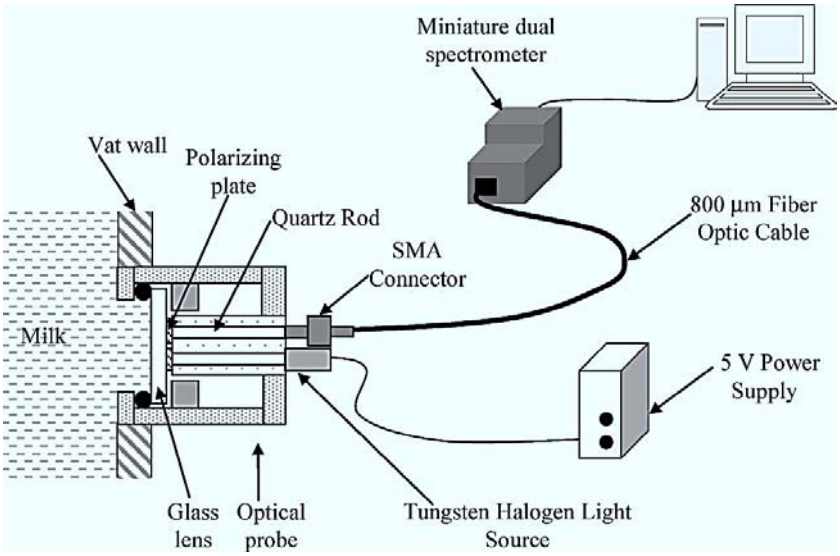


Fig. 5.5 Schematic of the LFV sensor and optical configuration used for monitoring milk coagulation and curd syneresis (Prototype I). According to Payne and Castillo (2007).

The LFV prototype I evolved into prototype II after a number of optical improvements (Castillo *et al.*, 2007) as it is shown in **Fig. 5.6.** In the LFV prototype II, a light from a 6 W tungsten halogen source (model LS1B, Ocean optics, Inc., Dunedin, FL, USA; spectral range of 360–2000 nm) was transmitted through a large diameter (0.5 cm) optical fiber (model FTICR19733, Fiberoptics Technology, Inc., Pomfret, CT, USA), a vertical polarizer (model 43-782, Edmund Optics, Inc., Barrington, NJ, USA), and a glass window (model 02 WBK 224, 27 Melles Griot Inc., Rochester, NY, USA) to the sample as it shown in **Fig. 5.6**.

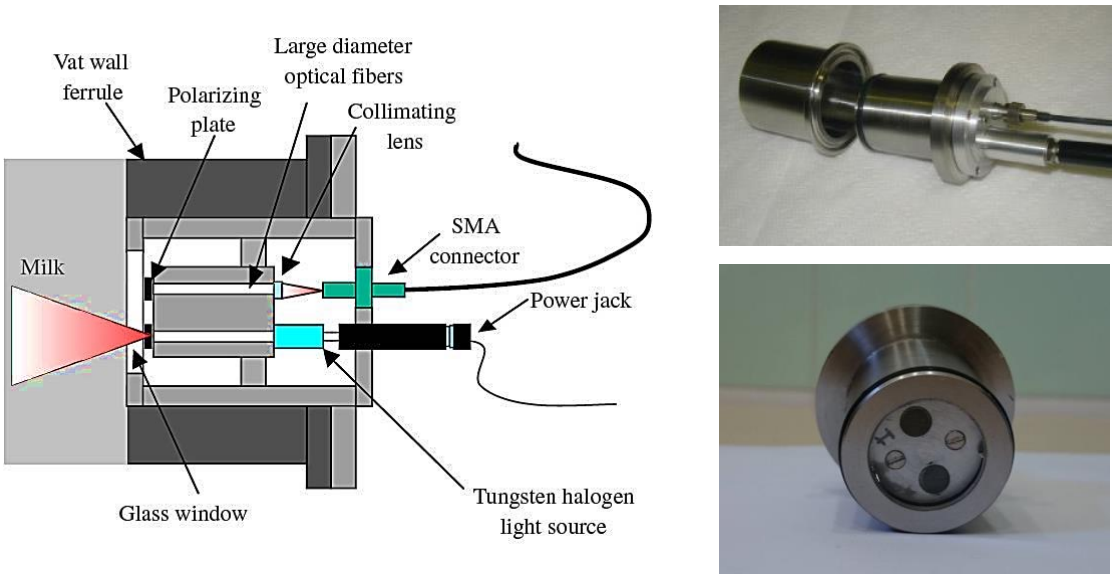


Fig. 5.6 Schematic of the large field of view sensor configuration (Prototype II).

The large-diameter (2 cm) glass window allows scattered light to be collected from a large area. Another polarizing plate allows for the selective detection of horizontally polarized light to ensure that any light reflected by the window was eliminated. Scattered light is transmitted through another optical fiber and a collimating lens (Edmund Optics Inc.) that focuses the scattered light onto a 800 µm diameter fiber optic cable (Spectran Specialty Optics, Avon, CT, USA) to a miniature fiber optic spectrometer (model HR2000CG-UVNIR, Ocean Optics B.V., Duiven, Netherlands). Spectra were collected over the range of 300–1100 nm with a resolution of 0.7 µm.

The integration time was set to 6s by the computer software (SpectraSuite software v. 5.1, Ocean Optics Inc.). Each spectral scan was automatically processed by subtracting the dark background spectral scan. Each spectral scan was reduced to 41 averages by dividing them into 20 nm wavebands with mid-wavelengths of $280 + 20n$ ($1 \leq n \leq 41$), giving 41 wavebands in the

range (300–1100 nm) and averaging the optical response for the wavelengths constituting each waveband.

The voltage readings (sensor output) for the first minute of data were averaged within each waveband to calculate the initial voltage response, V_0. The voltage intensity at every waveband, $V(w)$, was divided by its corresponding $V_0(w)$ to obtain the light backscatter ratio, $R(w)$. The kinetic rate constant (min^{-1}) for the syneresis process, K_S, was estimated from the LFV light backscatter ratio at 980 nm, $R(980)$ (on ahead just R^*). To that end, the LFV sensor response during syneresis was fitted using the least squares method and the Solver utility tool from Microsoft Excel software (version 15.0.4979, Microsoft) to a first order equation as follows:

$$R_t^* = R_\infty^* + (R_0^* - R_\infty^*)e^{-K_{LFV}t} \qquad \textbf{Equ. (5.1)}$$

where R^*_t was the light backscatter ratio at time t (min), R_∞ was the light backscatter ratio at an infinite time, R^*_0 was the light backscatter ratio at cutting time (i.e., at time zero for the syneresis process), and k_{LFV} was the kinetic rate constant (min^{-1}) for the *LFV* sensor response during syneresis.

According to Castillo *et al.* (2005b, 2007) and Fagan *et al.* (2008), the kinetic rate constant for the *LFV* sensor response (k_{LFV}) is highly related with the constant of syneresis of the cheese (k_S). **Fig. 5.7** shows typical LFV sensor profile obtained from syneresis sensor during milk coagulation and curd syneresis

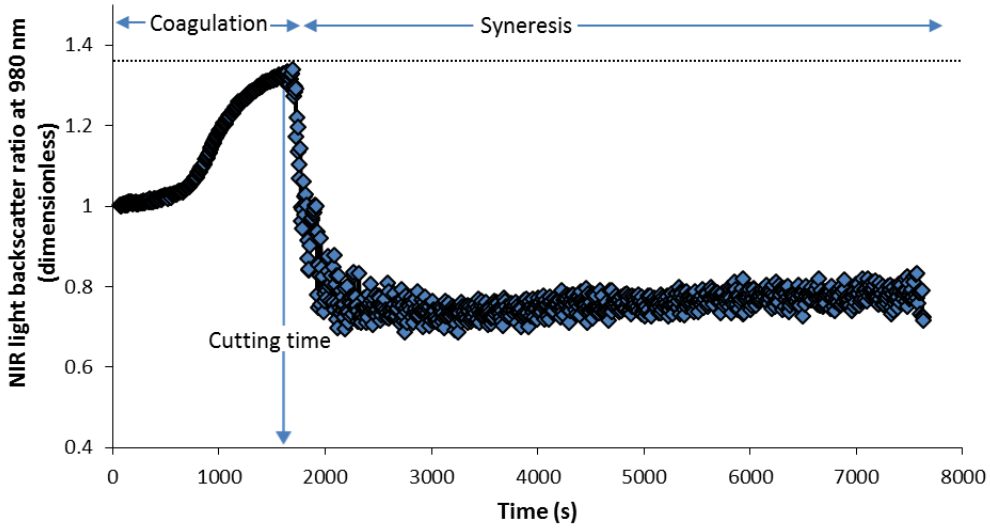


Fig. 5.7 Typical LFV sensor response from syneresis LFV sensor during milk coagulation and curd syneresis.

5.5 Rheological determination of gelation and cutting time

Monitoring the milk coagulation process was also performed using small amplitude oscillatory rheometry (SAOR) as it is shown in **Fig.5.8**, which was performed using a Universal Dynamic rheometer Thermo-Haake Rheo Stress (RS1) (Thermo Haake GmbH, Karlsruhe, Germany) connected with a compressor CARAT 106/E (FIAC S.P.A. Bologna, Italia) and coupled to a Haake water bath for the precise control of temperature using a circulating water bath (Thermo Scientific Hake A10, SC100 –Thermo scientific fisher Inc., USA) having a control accuracy of ±0.01 °C. A concentric cylinder sensor (Z34) with diameters of 34 and 36.88 mm for the inner and outer cylinders, respectively, was used in this experiment.

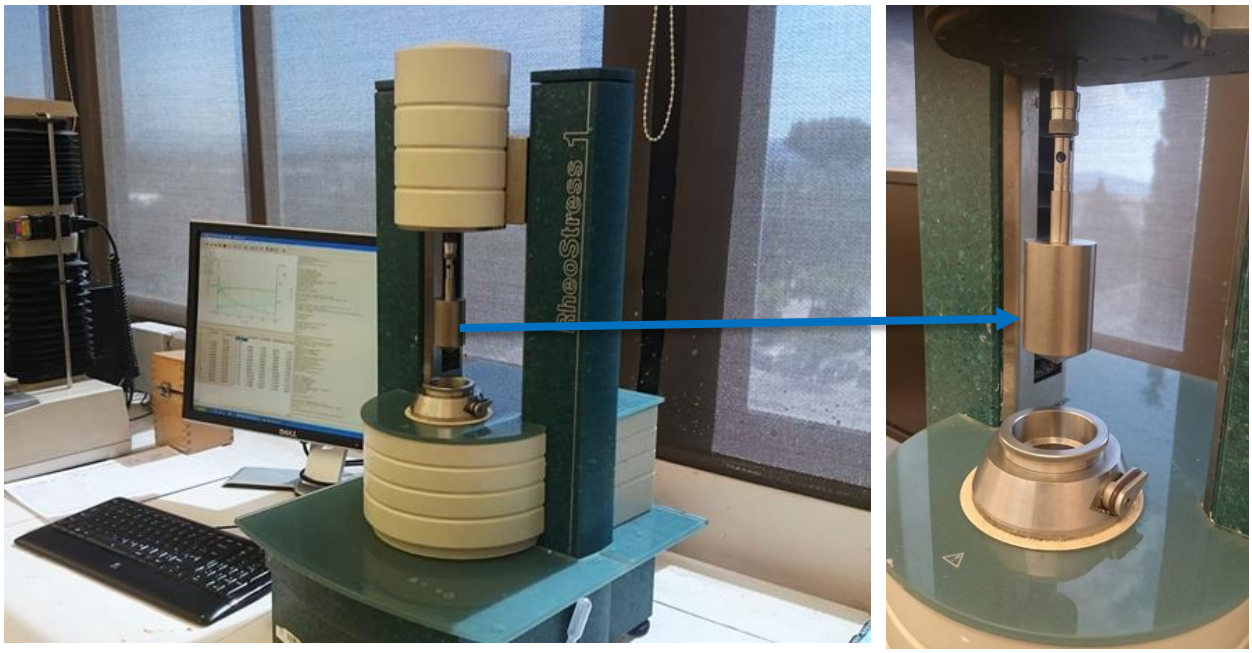


Fig. 5.8. Small amplitude oscillatory Rheometer (SAOR) with a concentric cylinder sensor (Z34).

The tests were performed by applying a deformation of 3%, which is within the linear viscoelastic region for rennet-induced gels (Zoon *et al.*, 1988), and a frequency of 1 Hz. An aliquot of 40 mL of milk with enzyme was transferred to the measuring system (cylinder of the rheometer) that was pre-warmed to the assay temperature. A thin layer of vegetable oil was added on the exposed milk surface to prevent evaporative cooling. The rheological data were collected every 25 s by 3 Rheowin Job Manager Software. Rheological parameters determined were the elastic or storage modulus (***G´***), the viscous or loss modulus (***G´´***) and *tan* δ or loss tangent (***tan* δ** = *G´´/ G´*). Gelation time (t_{gel}) was defined as the time when the gels reached a $G' \geq 1$ Pa, while two cutting times ($t_{G'30}$ and $t_{G'60}$) were defined as the time when the gels

reached a G' of 30 and 60 Pa, respectively. Gel assembly times **(t_{GE30} and t_{GE60})** were estimated as the lag time elapsed between t_{gel} and $t_{G'30}$ or $t_{G'60}$, respectively. Normalized gel assembly times **(t_{NGE30} and t_{NGE60})** were normalized against its respective t_{max} values by dividing t_{GE30} and t_{GE60} by t_{max}. The rheological parameter, ***tan* δ,** was measured when G' = 30 Pa. (i.e., *tan* δ at cutting).

5.6 Visual clotting and cutting time determination

The 80 mL milk aliquot that was placed in a beaker and maintained in a water bath (Clifton, -Nickel electro LTD, western-S- Mare Somerset Inc.), having a control accuracy of ±0.01 °C) at the target experimental temperature was used to determine both visual clotting and cutting times. Clotting time **(t_{clot})** was determined visually by dipping a spatula in the gel for clear evidence of small flocks of casein on the blade surface. The detection of curd cutting time **(t_{cut})** was determined introducing the spatula and pulling it out, and observing that the spatula surface was completely free of coagulum clots or residues, as proposed by Nicolau *et al*. (2010).

5.7 Compositional Analysis, of milk, curd, cheese and whey

Determination of the fat (F), total protein (N × 6.38) (P), casein (CN), lactose (L) and total milk solids (TS) was performed at **Laboratori Interprofessional Lleter de Catalunya** (ALLIC, Cabrils, Barcelona, Spain) using **Milkoscan 5000** (Foss Electric, Hillerød, Denmark). SCC was also measured at ALLIC using a **Fossomatic 5000** (Foss Electric). Milk, curd and cheese protein content (M_P, C_P and Ch_P), was determined also using Dumas methods (Dumas, JAOAC 59, 141, 1976). Gerber (IDF Standard 83, 1987 and IDF, 152A, 1997) were used to determine the fat content of milk, curd, cheese and whey. Milk total solids were determined using a convention oven (IDF Standard 86, 1981). Curd, whey and cheese total solids were determined using a convention oven as well (IDF Standard 4A, 1982). The, **SCC** of milk was determined according to Rovai *et al* (2015).

5.8 Materials

Calcium Chloride: A calcium chloride solution 40% *w/v* was prepared using dihydrate calcium chloride ($CaCl_2 \cdot 2H_2O$; Panreac Química S.A., Montcada i Reixac, Barcelona, Spain). A constant amount of 0.74 mL of the prepared calcium solution was added per liter of milk to

each milk mixture after pasteurization. This calcium solution aliquot was calculated to deliver 174 mg of anhydrous calcium chloride ($CaCl_2$) per liter of milk.

Enzyme: Calf rennet (~70% chymosin, ~30% pepsine) with strength of 1:10000 was obtained from Laboratories' Arroyo (Santander, Spain) and used to induce milk coagulation.

Bacterial culture: Lyophilized mixed starter cultures containing *Lactococcus lactis* subs *lactis* and *L. lactis* subs *cremoris* (Danisco A/S, Copenhagen, Denmark) were used.

Chemical reagents:

Fat concentration was determined by Gerber method using sulfuric acid (H_2SO_4) solution (90-91% *w/w*; M = 98.08, δ = 1.82 g cm^{-3}) from Scharlab (Mas d'en Cisa, Spain) and using 3-methyl-1 butanol ($C_5H_{11}OH$, M = 88.15) from Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain). For pH measurements, standard buffer solutions were used (pH buffers 7.00 and 4.01; Crison Instruments, S.A, Alella, Barcelona, Spain). For pH adjustment, sodium hydroxide solution 1M (Scharlab) and hydrochloric acid 1M (Panreac Química) was used.

5.9 Statistical Analysis

Regression analysis of the data collected was performed using the Statistical Analysis System package SAS® 9.3 (Statistical Analyses System Institute, Inc., Cary, NC 27513, 2011). The analysis of variance (**ANOVA**) was performed using the general linear model (**GLM**) procedure of the Statistical Analysis System (SAS® 9.3, 2012). The linear model used was $Y_i = \mu + MT_i + \varepsilon_i$ where Y was the dependent variable studied, μ was the overall mean, MT_i was the fixed effect of the factors studied, and ε_i was the error term. Pearson correlation coefficients, **r**, were calculated using the correlation "**CORR**" procedure of SAS®. Least-square means (**LSMEANS**) and significance of each treatment were computed using type IV sum of squares (**Type IV SS**). Least-square means predicted differences were used to determine differences between all treatments. Treatment means were considered to be statistically different at $P < 0.05$.

The best one-, two-, and three-parameter prediction models for predicting several dependent variables including visual clotting and cutting times and rheological gelation and cutting times, as well as some other cheese making indices were obtained using the

"**MAXIMUM R^2**", including all independent variables, parameters derived from the LB and several calculated parameters obtained by multiplying *LB* time-based parameters (eg. t_{max}, t_{2min} or t_{2max}) by either milk protein concentration (M_P), milk fat concentration (M_F), milk total solids concentration (M_{TS}), milk fat to protein ratio (***FP***) or temperature (***T***). Complementary models were developed using the "**GLM**" (linear regression), and "**NLIN**" (non-linear regression) procedures of **SAS®,** as described on Abdelgawad *et al*. (2011; 2014; 2016).

Chapter Six

Using a Fiber Optic Sensor for Cutting Time Prediction in Cheese Manufacture from a Mixture of Cow, Sheep and Goat Milk

Chapter Six: Using a Fiber Optic Sensor for Cutting Time Prediction in Cheese Manufacture from a Mixture of Cow, Sheep and Goat Milk

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6.1 ABSTRACT

NIR light backscatter technology has been proven successful for monitoring cow milk coagulation and predicting cutting time but has never been tested with milk mixtures. In Spain ~40% of the cheese produced is made from cow, sheep and goat milk mixtures. The aim of this study was to evaluate if the proposed optical technology could be used to monitor milk coagulation and predict cutting time in milk mixtures. A randomized factorial design with three factors and three replicates was employed. Cow, goat and sheep milk was mixed in two different proportions. Milk mixtures were coagulated at constant calcium chloride addition level, pH and fat concentrations using two different enzyme concentrations and three coagulation temperatures (N=36 tests). Milk coagulation was monitored using small amplitude oscillatory rheometry and a NIR fiber optic light backscatter sensor. Simultaneously, clotting time was visually evaluated. Optical parameter t_{max} was highly correlated ($0.80<r<0.99$, $P<0.0001$) with the rheological and visual parameters studied. Enzyme concentration and temperature had a significant effect ($P<0.05$) on optically-, rheologically-, and visually-derived parameters. Milk mixture proportion was not significant for optical parameters related to clotting time but was significant for the aggregation rate and rheological parameters related to curd firming and syneresis. Models for predicting cutting time were developed successfully with $R^2 = 0.93$. Results strongly suggest that milk mixture proportion exerts an effect on gel assembly (i.e., on both aggregation and curd firming) and syneresis. This finding has important implications for inline process control when goat and sheep milk are used.

Keywords: Milk, Coagulation, Predicting, Light backscatter, clotting time, Cutting time.

6.2 INTRODUCTION

Milk coagulation is typically induced by acidification or enzymatic action to form a continuous, solid curd that entraps fat globules and some water (Castillo *et al.*, 2006a). The most common method of milk coagulation is enzymatic coagulation. Once a sufficiently firm gel has been obtained, the gel needs to be cut into small pieces (curd grains) to induce syneresis (i.e., the expulsion of whey as a result of curd grains shrinkage). This operation increases the gel surface/volume ratio allowing the whey to escape while the gel network is rearranging and contracting (Castillo, 2006b). Traditionally, the curd is cut after a predetermined time from the enzyme addition or upon the operator's judgment based on empirical evaluation of firmness and visual appearance of the gel properties. Cutting the coagulum after a pre-fixed time is questionable, since variations in milk properties and processing conditions affect curd firmness and gel microstructure, modifying the optimum cutting time. A number of authors have noted the disadvantages of an inappropriate cutting time selection (Hori, 1985; Payne *et al*., 1993a; Passos *et al*., 1999). Real-time estimation of curd firming and cutting time is essential for cheese making as those two factors exert a substantial impact in both cheese yield and quality (Bakkali *et al.*, 2001).

A plethora of devices have been developed for milk coagulation and gel firming monitoring over the past seven decades. A comprehensive classification of those devices was published by (Castillo, 2006a). In general, those systems studying rheological properties are destructive and not practical for inline application.

To date, an objective and effective method to determine optimum cutting time is not available, although some existing methods can consistently reproduce the cutting time subjectively selected by the cheese maker. An inline optical sensor, which was designed to measure changes in light backscatter of infrared light at 880 nm, was proposed by Payne *et al*. (1993b) to predict cutting time. This technology has been specifically developed for cow milk, and adapted successfully to goat milk (Castillo 2001). However, it has never been tested on milk mixtures having different proportions of cow, goat and sheep milk. Previous studies have shown that light backscatter does not only depend on milk composition (fat and protein percentages) but also on casein micelle and fat globule sizes. Milk from different animal species has both different milk composition and particle size distribution (Payne *et al*., 1993b; Castillo

2001). Almost half of the cheese produced in Spain is made with different mixtures of milk from cow, sheep and goat (ICEX, 2004).

The production of cheese using mixtures of milk (hereafter *"mixed milk cheese* –**MMC**– *"*) has achieved widespread acceptance in Spain. A large variety of MMCs are typically manufactured in Spain such as *"Cabrales"*, *"Picón"*, *"Gamonedo"*, *"Ibérico"*, etc. Proportions of the different milk types were established by the Ministry of Agriculture in 1987, in compliance with cheese producers (*"*Orden de 9 de julio de 1987*"*), but this law has been recently repealed (*"*Real Decreto 262/2011, de 28 de febrero*"*).

MMCs are very important for the Spanish cheese sector, not only for the proportion of sales it represents, but also because of technological differences required for appropriate processing of the different milk mixtures encountered. Sheep, goat and cow milk show marked differences in their colloidal structure and chemical composition, which introduces additional difficulties, compared with cheeses made with one type of milk, as regards the control of coagulation and the selection of cutting time.

The general objective of this paper was to evaluate if near infrared light backscatter could be used to monitor milk coagulation and predict cutting time in cheese made from different proportions of cow, goat and sheep milk. This general objective was divided into two specific objectives: **a)** evaluate the effect of milk mixture proportions, enzyme concentration, and coagulation temperature on the light backscatter profile, and **b)** obtain the best prediction models for several milk coagulation indicators and cutting time.

6.3 MATERIAL AND METHODS

6.3.1 Experimental design

A randomized factorial design with three factors (a, b and c) and three replications (n = 3) was used to determine the effect of different milk coagulation temperatures, enzyme concentration levels and milk mixture proportions on the light backscatter profile during milk coagulation and on the prediction of clotting and cutting time during cheese manufacturing. Different levels of the experimental factors were selected to obtain light backscatter profiles under a wide range of milk coagulation conditions. Two levels of "*milk mixture"* were prepared using cow, goat and sheep milk as detailed in **Table 6.1**.

Table 6.1. Types of milk mixtures used in the experiment.

	Cow (%)	Goat (%)	Sheep (%)
Mixture 1	60.0	30.0	10.0
Mixture 2	75.0	12.5	12.5

Those two levels of milk mixture were established according to typical industrial practice for MMCs typically manufactured in Spain in compliance with current Spanish regulations (Real Decreto 262/2011, de 28 de febrero). Milk mixtures were coagulated using two *"enzyme concentrations"* (200 and 400 $mg \cdot L^{-1}$) at three *"temperatures"* (27, 32 and 37 °C) at constant fat concentration, calcium chloride ($CaCl_2$) addition level and pH (4.5%, 174 $mg \cdot L^{-1}$, and 6.5, respectively). A total of 36 tests (N = nabc = 3·2·2·3 = 36) were conducted under this design. A fresh batch of each type of milk was obtain for each replication to reconstitute mixtures 1 and 2 (i.e., mixtures for each replication were made using a new batch of the three milk types).

The different treatments of each replication were conducted in random order. **Fig. 6.1** shows the flow chart of the experimental design.

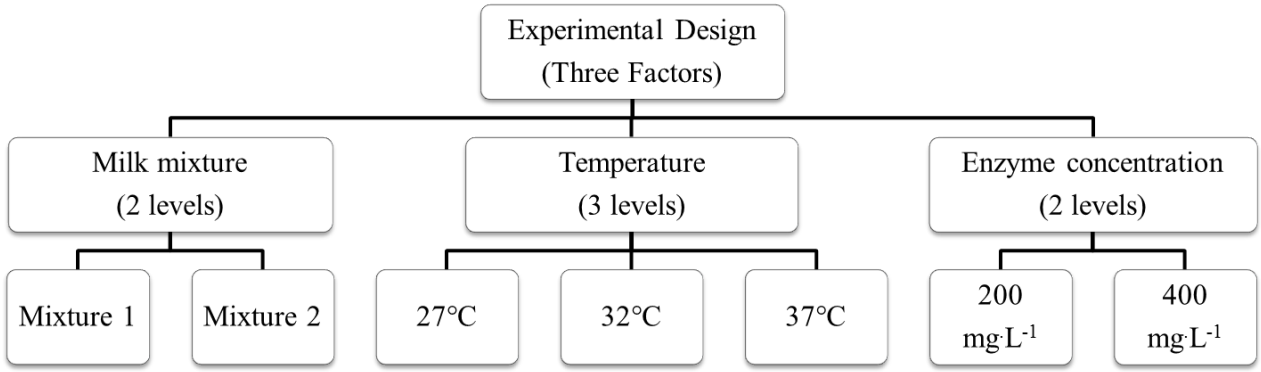


Fig. 6.1. Flow diagram of the experimental design.

Milk coagulation was monitored using small amplitude oscillatory rheometry (**SAOR**) and near infrared (**NIR**) light backscatter. Simultaneously, clotting time was visually determined.

6.3.2 Materials

Milk: Goat and sheep milk were obtained from the Universitat Autònoma de Barcelona (**UAB**) farm while the cow milk was obtained from Can Badó farm (S.A.T. Can Badó, La Roca del Vallès, Spain). Immediately after milk was received in the Centre Especial de Recerca Planta de Tecnologia dels Aliments (**CERPTA**) at UAB, milk was stored at ~4 °C until it was used (typically within the first 24 h from milk reception) to prepare the corresponding milk mixtures.

Calcium Chloride: A calcium chloride solution 40% *w/v* was prepared using dehydrate calcium chloride ($CaCl_2 \cdot 2H_2O$; Panreac Química S.A., Montcada i Reixac, Barcelona, Spain). A constant amount of 0.74 mL of the prepared calcium solution was added per liter of milk to each milk mixture after pasteurization. This calcium solution aliquot was calculated to deliver 174 mg of anhydrous calcium chloride ($CaCl_2$) per liter of milk. ***Enzyme***: Calf rennet (~70% chymosin, ~30% pepsine) with strength of 1:10000 was obtained from Laboratories' Arroyo (Santander, Spain) and used to induce milk coagulation. ***Chemical reagents***: Fat concentration was determined by Gerber method using sulfuric acid (H_2SO_4) solution (90-91% *w/w*; M = 98.08, δ = 1.82 g cm^{-3}) from Scharlab (Mas d'en Cisa, Spain) and using 3-methyl-1 butanol ($C_5H_{11}OH$, M = 88.15) from Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain).

For pH measurements, standard buffer solutions were used (pH buffers 7.00 and 4.01; Crison Instruments, S.A, Alella, Barcelona, Spain). For pH adjustment, sodium hydroxide solution 1M (Scharlab) and hydrochloric acid 1M (Panreac Química) was used.

6.3.3 Milk mixtures preparation

Unpasteurized and unhomogenized cow, goat and sheep milk was stored at 4 °C right after collection and until it was used to prepare the two milk mixture types established by the experimental design.

Gerber (IDF Standard 83, 1987) and Dumas methods (Dumas, JAOAC 59 and 141; 1976) were used to determine the fat and protein content of milk, respectively. Milk total solids were determined using a convention oven (IDF Standard 86, 1981). Ash content of milk was

determined by dry ashing (AOAC 954.46, 2000) using a high temperature muffle furnace. Lactose was calculated by difference.

Table 6.2 shows the average composition of the milk used in this study.

Table 6.2. Means and standard deviation for fat, protein, moisture content, minerals and lactose of cow, goat and sheep milk and milk mixtures 1 and 2.

Milk Type		Fat (%)		Protein (%)		Moisture (%)		Minerals (%)		Lactose (%)	
	N	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Cow	4	3.86	0.25	3.29	0.09	86.0	0.50	0.70	0.03	4.38	0.57
Goat	4	5.00	0.41	3.81	0.05	86.0	0.00	0.70	0.12	4.48	0.46
Sheep	4	7.08	1.06	6.06	0.34	81.0	0.00	0.74	0.10	5.13	1.43
Mixture 1[1]	4	4.83	0.24	3.75	0.09	86.0	0.00	0.75	0.10	4.67	0.34
Mixture 2[2]	4	4.58	0.10	3.72	0.09	86.1	0.25	0.72	0.03	4.86	0.36

[1]**Mixture 1**, cow (60.0%), goat (30.0%) and sheep (10.0%)**;** [2]**Mixture 2**, cow (75.0%), goat (12.5%) and sheep (12.5%)**; N,** number of milk batches**;** $\bar{x}$ means**; SD,** standard deviation.

On the day after collection and for each replication, a batch of each one of the two milk mixtures was prepared in compliance with the experimental design (**Fig. 6.1**), by mixing the corresponding proportions of cow, goat and sheep milk. Milk mixtures were skimmed at ~45 °C using a small cream separator (Elecrem1-125I/h, Elecream, Vanves, France). Fat content of resulting skimmed milk mixtures and corresponding cream batches were determined using the Gerber method to calculate the adequate proportions of skimmed milk mixture and cream for adjustment of the fat concentration of the final milk mixtures to 4.5%. Mixtures proportions were calculated based on mass balance. The final milk mixtures were vat pasteurized at 65 °C for 30 min. Then a constant amount of calcium chloride solution (0.74 mL per liter of milk) was added to each milk mixture. The milk was stirred for 3 min and stored in a cooler until the temperature reached 23 °C. A linear regression between mL of 1 M HCl dilution and pH was conducted at 23 °C and used to predict the amount of acid needed to adjust the pH of milk to 6.5. The day before the experiment, milk samples were tested for pH and the amount of acid required for pH adjustment was calculated. Milk samples were placed into glass containers and the acid was added slowly and with continuous stirring. A constant sample dilution rate was maintained by using de-ionized water as needed to bring the total volume added (HCL and de-ionized water) to the milk to a volume of 5.5 mL. Note that milk pH adjustment was done after calcium chloride addition to account for the decrease of pH induced by the calcium chloride addition. After pH adjustment, milk sample aliquots of 500 mL were placed into glass bottles. The milk samples were then stored in a 4 °C cooler until used. A final pH adjustment was made

before coagulation at the target temperature by adding either 1.0 M HCL or 1.0 M NaOH. The average adjusted pH values for milk mixtures 1 and 2 were 6.54 ± 0.04 and 6.56 ± 0.02, respectively.

6.3.4 Testing procedure

Fig. 6.2 shows the flow chart of the testing procedure. Before every experiment, the corresponding 500 mL milk mixture sample was heated to the target coagulation temperature using a water bath. Then, the sample pH was determined and a final pH adjustment was made when necessary. Animal rennet was added to the milk at the target concentration and the milk was quickly stirred for 30 s.

Immediately, the milk was split into four aliquots. Two 80 mL aliquots were placed in the two measuring vats of the NIR coagulation measurement apparatus to obtain the light backscatter parameters, a 40 mL aliquot was placed in the rheometer to determine the rheological parameters and an 80 mL aliquot was placed in a water bath to determine the visual coagulation parameters.

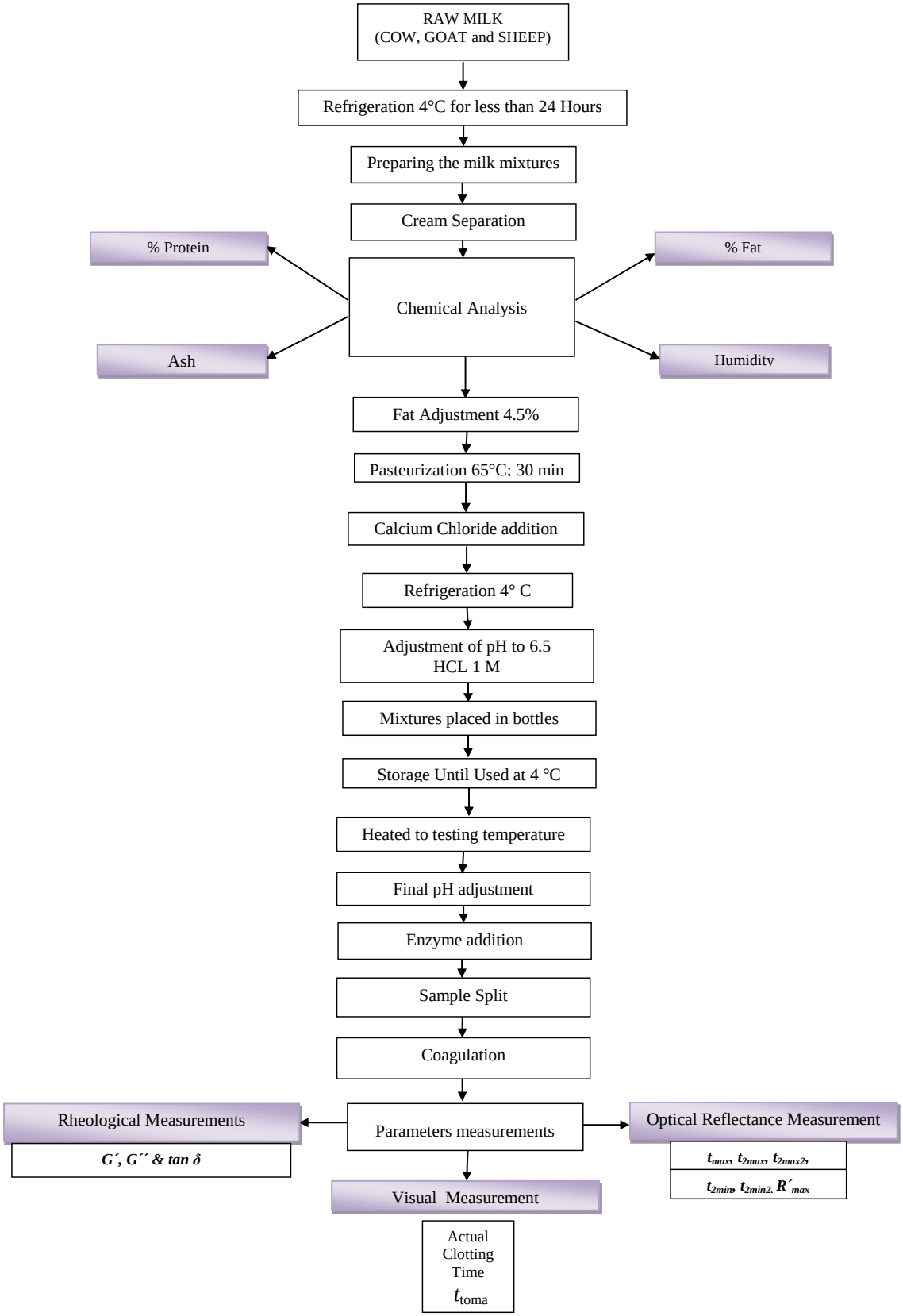


Fig. 6.2. Flow diagram of procedure for testing coagulation of milk mixtures.

6.3.5 Light Backscatter Monitoring

A coagulation measurement apparatus was designed and developed to measure near infrared light backscatter during milk coagulation (Department of Biosystems and Agricultural Engineering, University of Kentucky). For more detailed description of the coagulation measurement apparatus see chapter five: material and methods, section: 5.4.1. Thirteen optical time parameters were defined by the maxima and minima of the light backscatter profile derivatives as a function of time. Those optical parameters are defined in detail in **Table 5.1**

6.3.6 Rheological determination of gelation and cutting time

Small amplitude oscillatory rheometry (SAOR) was used for determination of rheological milk coagulation and cutting time parameters. More details about the equipment used as well as the rheological parameters obtained during the coagulation process are presented at chapter five: material and methods, section 5.5.

6.3.7 Visual clotting and cutting times determination

Determination of visual clotting and cutting time procedure are described in chapter five: material and methods, section: 5.6.

6.3.8 Statistical Analysis

The analysis of variance (**ANOVA**) was performed using the general linear model (**GLM**) procedure of the Statistical Analysis System (**SAS 9.2.3., 2009**). Pearson correlation coefficients, **r**, were determined by the correlation (**CORR**) procedure of SAS. Least-square means (**LSMEANS**) and significance of each treatment were computed using type IV sum of squares (**Type IV SS**). Linear regressions were tested on collected data for predicting several dependent variables using the **maximum R^2**, GLM, and nonlinear (**NLIN)** procedures of **SAS.** Further details about the statistical analysis are found in in chapter five: material and methods, section: 5.9.

6.4 RESULTS AND DISCUSSION

6.4.1 Analysis of variance

The experimental design used in this study allowed the evaluation of the effect of milk mixture, enzyme concentration and coagulation temperature on the optical and rheological time parameters characterizing coagulation as well as on the prediction of cutting time and some other clotting indicators. As detailed in the material and methods section, for each replication two different milk mixtures were obtained by mixing different proportion of cow, goat and sheep milk. However, compositional analysis of the milk mixtures showed that there were minimal differences between batches. The average composition for protein and moisture content was 3.75 and 86.0% for mixture 1 and 3.72 and 86.1% for mixture 2, respectively (for more details see **Table 6.2**).

An analysis of variance (ANOVA) was conducted to determine the main sources of variation in the dependent variables studied. Enzyme concentration "$\boldsymbol{E_0}$", temperature "***T***", type of milk mixture "***Mix***" and the qualitative variable "***Rep***" (to quantify the effect of replication –which also included the effect of the milk batch–) were selected as main effects in the preliminary ANOVA model. The main interactions "***Mix* x $\boldsymbol{E_0}$**", "***Mix* x *T***" and "***T* x $\boldsymbol{E_0}$**" were also included. **Table 6.3** shows the ANOVA for the dependent variables studied, including the R^2 values and *F* statistics for most important dependent variables. The ANOVA model was highly significant for all optical, visual and rheological parameters evaluated (P<0.001). Temperature was found to have a highly statistically significant effect on all dependent variables tested (P<0.001). Enzyme concentration had also statistically significant (P<0.001) effect on all dependent variables tested except for the parameter *tan* δ. Milk mixture was found to have an statistically significant (P<0.001) effect on the parameters $t_{G'30}$, $t_{G'60}$, t_{firm} and *tan* δ but was not significant for the parameters t_{max}, t_{clot}, and t_{gel}. Replication was found to be not statistically significant for dependent parameters except for $t_{G'60}$ and *tan* δ (P<0.05). The interaction "*T x* E_0" had a statistically significant effect on the parameters t_{max}, t_{clot}, t_{gel} and $t_{G'30}$. The interactions "*Mix* x E_0" and "*Mix* x *T*" did not show a statistically significant effect on any dependent parameter.

Table 6.3. Analysis of variance and **F** statistic for optical, visual and rheological dependent variables[1, 2].

Model			Main Factors				Interactions			Co-Variables	
			Rep. (DF=2)	***Mix.*** (DF=1)	***T*** (DF=2)	***E_0*** (DF=1)	***Mix x E_0*** (DF=1)	***Mix x T*** (DF=2)	***T x E_0*** (DF=2)	***FP*** (DF=1)	***pH*** (DF=1)
Parameter	R^2	F	F	F	F	F	F	F	F	F	F
t_{max}	0.948	25.4^{c}	2.39^{ns}	0.020^{ns}	45.9^{c}	202^{c}	0.59^{ns}	0.84^{ns}	12.5^{c}	3.52^{ns}	0.82^{ns}
R'_{max}	0.911	14.1^{c}	5.11^{a}	5.32^{a}	37.0^{c}	61.1^{c}	0.84^{ns}	1.46^{ns}	2.82^{ns}	0.05^{ns}	0.11^{ns}
t_{clot}	0.953	27.8^{c}	0.540^{ns}	0.020^{ns}	48.9^{c}	232^{c}	0.37^{ns}	1.23^{ns}	3.92^{a}	0.71^{ns}	7.17^{a}
t_{gel}	0.949	25.6^{c}	3.28^{ns}	0.230^{ns}	45.9^{c}	221^{c}	0.00^{ns}	0.50^{ns}	7.43^{b}	0.51^{ns}	1.29^{ns}
$t_{G'30}$	0.943	22.8^{c}	2.85^{ns}	7.59^{a}	60.2^{c}	116^{c}	0.07^{ns}	0.70^{ns}	4.19^{a}	0.86^{ns}	3.32^{ns}
$t_{G'60}$	0.943	22.7^{c}	6.62^{b}	30.4^{c}	47.6^{c}	69.1^{c}	2.61^{ns}	0.70^{ns}	2.04^{ns}	0.02^{ns}	10.6^{b}
t_{firm}	0.927	17.6^{c}	2.63^{ns}	17.3^{c}	51.2^{c}	36.5^{c}	0.46^{ns}	0.93^{ns}	1.32^{ns}	0.77^{ns}	4.29^{ns}
$tan\ \delta$	0.989	133^{c}	5.11^{a}	39.9^{c}	$708.^{c}$	0.340^{ns}	1.01^{ns}	1.44^{ns}	0.18^{ns}	0.23^{ns}	1.40^{ns}

[1]Number of observations, N=32; *Rep*., replication; *Mix*., Milk Mixture; *T*, temperature; E_0, enzyme concentration; *Mix x* E_0, interaction between *Mix* and E_0; *Mix* x *T*, interaction between *Mix* and *T*; *T* x E_0, interaction between *T* and E_0; *FP*, fat to protein ratio.
[2]R^2, determination coefficient; F, ANOVA F-statistic; DF, degree of freedom; $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$; ^{ns}not significant; dependent variables explained in the text.

Finally, the co-variable *pH* was not statistically significant for the dependent parameters except for $t_{G'60}$ and *tan* δ, while the co-variable *FP* was found to be not statistically significant for the parameters evaluated.

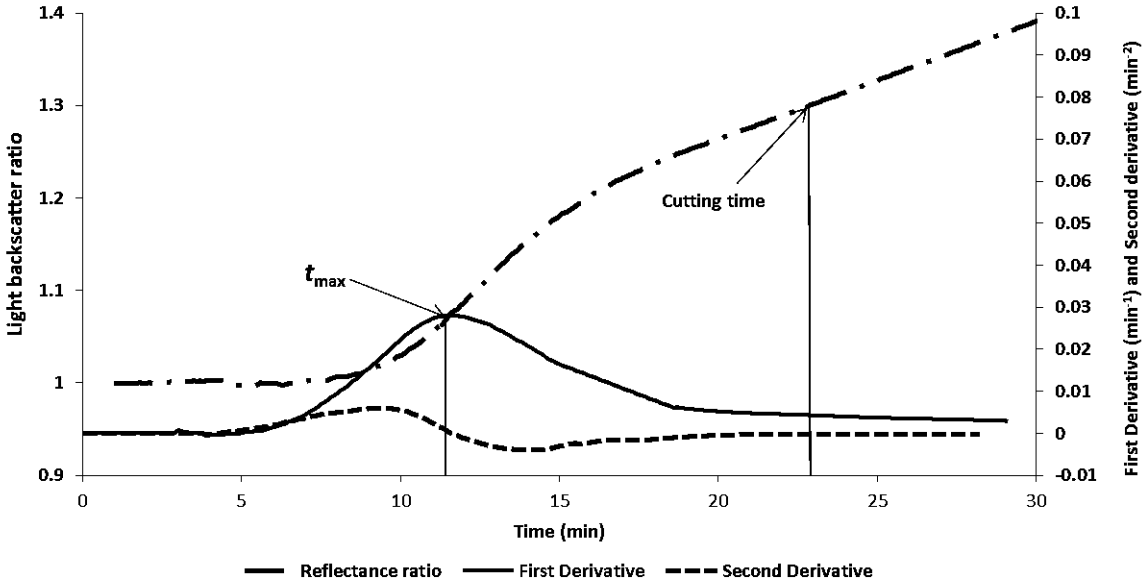


Fig. 6.3. Typical LB profile and its first and second derivatives as a function of time. t_{max}, inflection point of the light backscatter ratio; Cutting time, here defined as the time when the gel reached *G*'= 30 Pa.

6.4.2 Typical light backscatter profile during milk coagulation

As previously observed by Payne and Castillo (2007) in cow and goat milk, the light backscatter (**LB**) ratio profile obtained when coagulating milk mixtures containing different proportions of cow, goat and sheep milk typically began with a value of one and represented the increase in signal during coagulation **(Fig. 6.3).**

As described in previous studies using only cow or goat milk, the LB ratio profile had a latent period during which enzymatic reactions predominate with no detectable change in backscatter intensity. The LB ratio increased as particle size increases during network formation due to casein micelle cross-linking. As a result, a sigmoidal period can be distinguished during which aggregation reactions predominate, as well as an asymptotic period during which cross-linking proceeds at an ever-decreasing rate while curd firming is developing.

As a consequence of the sigmoidal shape of the LB ratio profile, a unique parameter is obtained: the time from enzyme addition to the inflection point of the LB ratio profile, t_{max}**.** According to Payne and Castillo (2007), the extent of hydrolysis at t_{max} is near completion and the onset of aggregation is located between the induction period (the time from enzyme addition to a point at which the magnitude of the first derivative of the normalized LB ratio is equal to 0.025) and t_{max}. From t_{max}, gel assembly proceeds until it is firm enough for cutting.

6.4.3 Effect of temperature on milk coagulation

It is well known that temperature affects both the primary (enzymatic hydrolysis) and secondary (aggregation reaction) phases of milk coagulation (McMahon and Brown, 1984; van Hooydonk and van den Berg, 1988). These authors observed that the aggregation phase is more sensitive to temperature, which has far less effect on the enzymatic hydrolysis than on the aggregation reaction. In fact, the temperature coefficient (Q_{10}) for the hydrolysis of κ-casein reported by several authors varies between 1.3 and 2, while the aggregation Q_{10} ranges between 11 and 30 (McMahon and Brown, 1984; van Hooydonk and van den Berg, 1988). LSMs of optical time parameters $\boldsymbol{t_{max}}$, R'_{max} and $\boldsymbol{t_{clot}}$ at 27, 32 and 37 °C are presented in **Table 6.4**.

Table 6.4. Influence of main effects on the optical, visual and rheological dependent variables[1].

Model	***Mix.***		***E*₀**		***T***		
	LSM		LSM		LSM		
Parameter	[2]Mix.1	[3]Mix.2	200 mg L^{-1}	400 mg L^{-1}	27 °C	32 °C	37 °C
$\boldsymbol{t_{max}}$	22.89[a]	23.05[a]	29.98[a]	15.97[b]	30.04[a]	20.97[b]	17.91[c]
$\boldsymbol{R'_{max}}$	0.013[a]	0.017[b]	0.001[a]	0.019[b]	0.001[a]	0.016[b]	0.022[c]
$\boldsymbol{t_{clot}}$	28.03[a]	28.20[a]	37.43[a]	18.79[b]	37.42[a]	24.94[b]	21.99[c]
$\boldsymbol{t_{gel}}$	34.69[a]	33.95[a]	45.04[a]	23.61[b]	44.94[a]	30.71[b]	27.32[bc]
$\boldsymbol{t_{G'30}}$	65.59[a]	56.27[b]	77.75[a]	44.11[b]	87.49[a]	51.40[b]	43.89[c]
$\boldsymbol{t_{G'60}}$	90.02[a]	64.24[b]	95.22[a]	59.05[b]	110.6[a]	63.18[b]	57.65[bc]
$\boldsymbol{t_{firm}}$	32.77[a]	23.26[b]	34.39[a]	21.64[b]	44.71[a]	21.58[b]	17.76[bc]
$\boldsymbol{tan\ \delta}$	0.275[a]	0.284[b]	0.279[a]	0.279[a]	0.251[a]	0.275[b]	0.312[c]

[1]Number of observation, N=32; *Mix.*, Milk Mixture; [2]Mixture 1, cow (60.0%), goat (30.0%) and sheep (10.0%); [3]Mixture 2, cow (75.0%), goat (12.5%) and sheep (12.5%); *T*, temperature; E_0, enzyme concentration;
[a]Least squares means (LSM) with the same letters are not significantly different P<0.05.
[b]Dependent variables explained in the text.

Both t_{max}, and t_{clot} parameters decreased significantly (P<0.0001) as temperature increased. The optical parameter R'_{max} increased significantly (P<0.0001) as temperature increased.

Rheological properties of milk gels are crucial for cheese manufacture. As discussed in the introduction, it is well known that there is an optimum gel firmness at which the gel should be cut to achieve maximum retention of fat an optimum curd moisture content that will, in turn, maximize product yield and quality. It is known that the storage modulus of the gel will increase with time after gelation until (if left for a long enough period) it reaches a plateau; that is, ultimate firmness (Fagan *et al*., 2007c). However, under certain circumstances, if the gel is not cut, G' may then subsequently decrease with time (Zoon *et al*., 1988) due to microsyneresis, which occurs when regions of the gel become dense and whey is forced into pores that grow in diameter. LSMs of rheological time parameters t_{gel}, $t_{G'30}$, $t_{G'60}$, t_{firm} and *tan* δ at 27, 32 and 37 °C are presented in **Table 6.4**. It is generally accepted that increasing the coagulation temperature decreases t_{gel} (Castillo *et al*., 2000). The effect of temperature on t_{gel}, $t_{G'30}$, $t_{G'60}$, t_{firm} and *tan* δ was highly significant (P<0.0001). Gels Made at 27 °C had larger t_{gel}, $t_{G'30}$, $t_{G'60}$ and t_{firm}. By contrast gels made at 37 °C had shorter values for these four time parameters. Interestingly, there was a significant (P<0.0001) increase in *tan* δ with increasing gelation temperature, which shows a larger tendency for network rearrangement in gels made at higher temperature. The interaction "T x E_0" was significant (P<0.05) for the parameters t_{gel} and $t_{G'30}$ and not significant for the parameters $t_{G'60}$, t_{firm} and *tan*δ.

However, the interaction "*Mix* x *T*" was found to be not significant (P<0.05). As per optically-generated time parameters, the effect of temperature on rheologically-derived time parameters was not linear.

6.4.4 Effect of enzyme concentration on milk coagulation

LSM values of optical, visual and rheological time parameters t_{max}, R'_{max}, t_{clot}, and t_{gel} for the two different enzyme concentrations (200, 400 mg L^{-1}) evaluated in this study are presented in **Table 6.4.** It was found that enzyme concentration had highly significant effect (*P<0.0001*) on all those time-based parameters. Specifically, it was observed that increasing the enzyme concentration resulted in a decrease in t_{max}, t_{clot} and t_{gel} and increase the optical parameter R'_{max}. These results are in agreement with previous studies. Storch and Segelcke

(1874) observed that visual rennet clotting time was inversely proportional to the enzyme concentration and this was described by the equation:

$$t_{clot}E_0 = K \qquad \textbf{Eqn (6.1)}$$

where t_{clot} was clotting time, E_0 was enzyme concentration and K was a constant. This rule is only valid in a narrow pH, enzyme concentration and temperature range. For this reason, Holter (1932) proposed an equation that was modified later by Foltmann (1959) as follows:

$$t_{clot} = \frac{a}{[E_0]} + b \qquad \textbf{Eqn (6.2)}$$

where a and b are constants and $[E_0]$ the enzyme concentration. In fact, the plot of clotting time versus the inverse of the enzyme concentration is linear, and the constants a and b depend not only on the method of determining the clotting time but also on the conditions, such as pH, temperature, temperature history of the milk, calcium and protein (van Hooydonk and van den Berg, 1988). The Foltmann equation was used to model the change in t_{max} with enzyme concentration by Castillo *et al.* (2000). The regression equation obtained was t_{max}=0.444/ $[E_0]$ + 0.0659 (R^2 =0.9998). Similar equations were obtained in our study for t_{max}, t_{clot} and t_{gel}. This confirmed that the three time parameters decreased inversely with enzyme concentration ($R^2 \geq$ 0.958).

As mentioned above, the interaction "*T* x E_0" was significant (*P<0.05*) for the parameters t_{max} and t_{clot}, while the interaction "*Mix* x E_0" was not significant for any of the parameters evaluated (*P<0.05*). The Interaction "*T* x E_0" for t_{max} is shown in **Fig.6.4**.

As it could be observed, the effect of enzyme concentration was more obvious as temperature decreased. LSMs of rheological time parameters, $t_{G'30}$, $t_{G'60}$, t_{firm}, and *tan* δ for the two different enzyme concentrations (200, 400 mg L^{-1}) are presented in **Table 6.5**4 It was found that the effect of enzyme concentration on rheological time parameters t_{gel}, $t_{G'30}$, $t_{G'60}$ and t_{firm} was highly significant (*P<0.0001*).

An increase in the enzyme concentration resulted in a consistent decrease in all the rheological time parameters evaluated. However, no significant effect of the enzyme concentration on the rheological parameter *tan* δ (*P<0.05*) was observed.

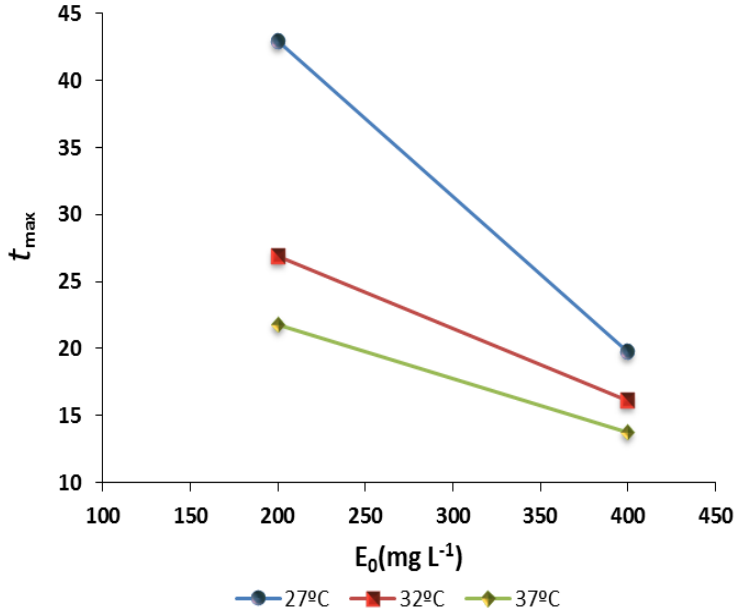


Fig. 6.4. Effect of enzyme concentration on the optical time parameters, t_{max} at different coagulation temperatures.

This strongly suggests that the enzyme concentration has a strong and significant effect on the gel assembly process but has no effect on the gel tendency to shrink and expel the whey (i.e., rearrangement capability of the gel is not affected by E_0; *tan* δ is constant –Table 5–). The interaction "*T* x *E0*" was statistically significant for the rheological parameters t_{gel} and $t_{G'30}$ (*P<0.05*) but not significant for $t_{G'60}$, t_{firm} and *tan* δ. As mentioned above, the interaction "*Mix.* x *T*" was found to be not statistically significant for rheologically-generated parameters.

6.4.5 Effect of milk mixture on milk coagulation

LSM values of optical parameters $t_{max,}$ t_{clot}, R'_{max}, and t_{gel} for the two different milk mixtures (Mix.1, Mix.2) are presented in **Table 6.4**. It was observed that the milk mixture proportion does not have a significant effect (P<0.05) on optical, visual or rheological indicators of gelation, which suggest that mixing milk from different species might not exert an effect on the enzymatic phase of milk coagulation. In addition, and as it could be observed, the milk mixture proportion have a significant effect (P<0.05) on the optical parameter R'_{max}. It's also observed that the interactions "*Mix* x *T*" and "*Mix* x E_0" were found to be not significant for any of those two parameters (P<0.05). LSM values of rheologically-derived time parameters related to firming development and gel microstructure, $t_{G'30}$, $t_{G'60}$, t_{firm} and *tan*δ, for Mix.1 and

2 are presented in **Table 6.4**. Milk mixture had a highly significant (*P<0.0001*) effect on the mentioned rheological parameters as shown in **Fig. 6.5.**

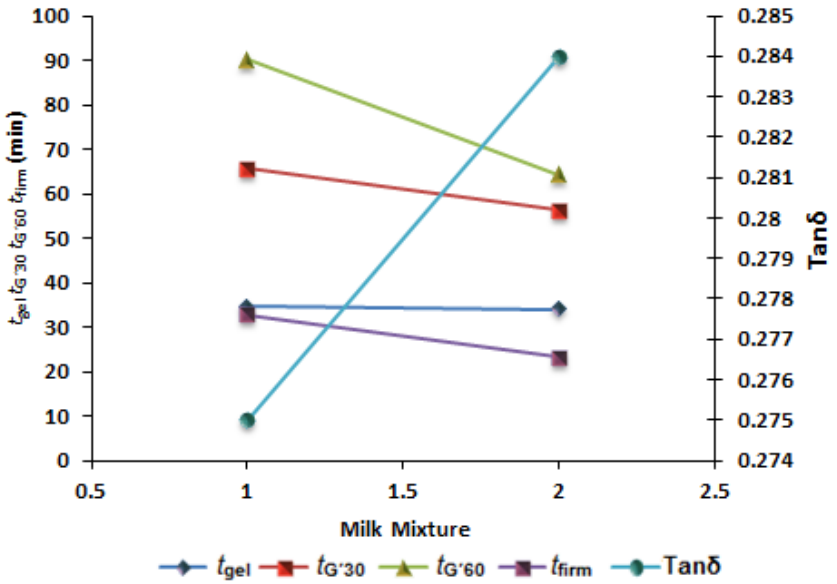


Fig. 6.5. Effect of milk mixture on the rheological time parameters t_{gel}, $t_{G'30}$, $t_{G'60}$, t_{firm} and *tan* δ.

Gels made using Mix.1 had larger firming times as compared to gels made from Mix. 2. In other words, decreasing the proportion of cow milk resulted in larger $t_{G'30}$, $t_{G'60}$ and t_{firm} values.

A highly significant (*P<0.0001*) effect of the milk mixture type on the rheological parameter *tan* δ was also observed. Mix. 2 increased the value of the rheological parameter *tan* δ. This result indicates that, even when the milk mixture proportion might not exert an effect on the enzymatic phase of milk coagulation, increasing the cow milk proportion results on faster gel firming rate, which contributes to originate a weaker gel with more tendency for rearrangement (i.e., with increased syneresis capabilities). This conclusion is reinforced with the observed effect of the mixture proportion on light backscatter parameter R'_{max}. This optical parameter is the maximum rate of increase in the light backscatter profile during coagulation, which, according to Castillo *et al.* (2006b), is correlated to the rate of casein micelle aggregation during the primary network formation. It was observed that increasing the proportion of cow milk in the mixture (Mix. 2) increased R'_{max} by 25% (P<0.0134), probably as a result of an increase in the casein micelle aggregation reaction rate

6.4.6 The relationship between time-based milk coagulation parameters

Our results show a close relationship between the LB derived parameters and both rheological and visual parameters obtained during milk coagulation. All the optically-generated time-based parameters (e.g., t_{max}) were significantly correlated with the visually-generated parameter, t_{clot}, and the rheologically-generated parameters, t_{gel}, $t_{G'30}$, $t_{G'60}$, t_{firm} and *tan* δ, with Pearson correlation values (r) ranging between 0.76 and 0.97 ($P<0.0001$). As observed in **Fig. 6.6(a)**, the correlation encountered between t_{max} and t_{clot} was strong and significant (r = 0.973, $P<0.0001$). These results confirm that irrespective of temperature, enzyme concentration and milk mixture, t_{max} is useful for indirectly measuring the enzymatic activity of enzymes for milk coagulation.

The correlation observed between t_{max} and t_{gel} was also very significant (r = 0.974, $P<0.0001$) as showed in **Fig.6.6 (b).** Note that the observed time difference between those two parameters is larger than that existing between t_{max} and t_{clot}, which, in a practical sense, makes it easier to use t_{max} as a potential indicator for the rheological gelation time. The optical parameter t_{max} and the rheological cutting time indicators, $t_{G'30}$ and $t_{G'60}$, were also significantly correlated (r = 0.904 and 0.790, respectively, $P<0.0001$) **Figs. 6.6(c)** and **6.6(d).** Note that the R^2 for the regression between t_{max} and $t_{G'60}$ is substantially smaller than the one encountered between t_{max} and $t_{G'30}$. This unexpected fact has been attributed to the differences induced by the two milk mixture proportions used in this study on the rate of gel assembly (as discussed before), which resulted on higher and higher scatter on the regressions plots as the gel formation proceeded.

6.4.7 Prediction of visual clotting time

The maximum R^2 procedure of SAS® was utilized to obtain the best one-, two-, and three-parameter models for predicting t_{clot}, t_{gel}, $t_{G'60}$ and *tan* δ as indicated in chapter five: material and methods, section 5.9.

In accordance with McMahon *et al.* (1984b) the lag time between the addition of the enzyme and the appearance of visible clots, is believed to be a consequence of the time needed for the enzyme to produce sufficient amount of aggregated *nuclei* material and the time needed for this material to aggregate. According to these authors, visual clotting time is an estimate of actual clotting time defined as the instant when an extended space network of casein particles

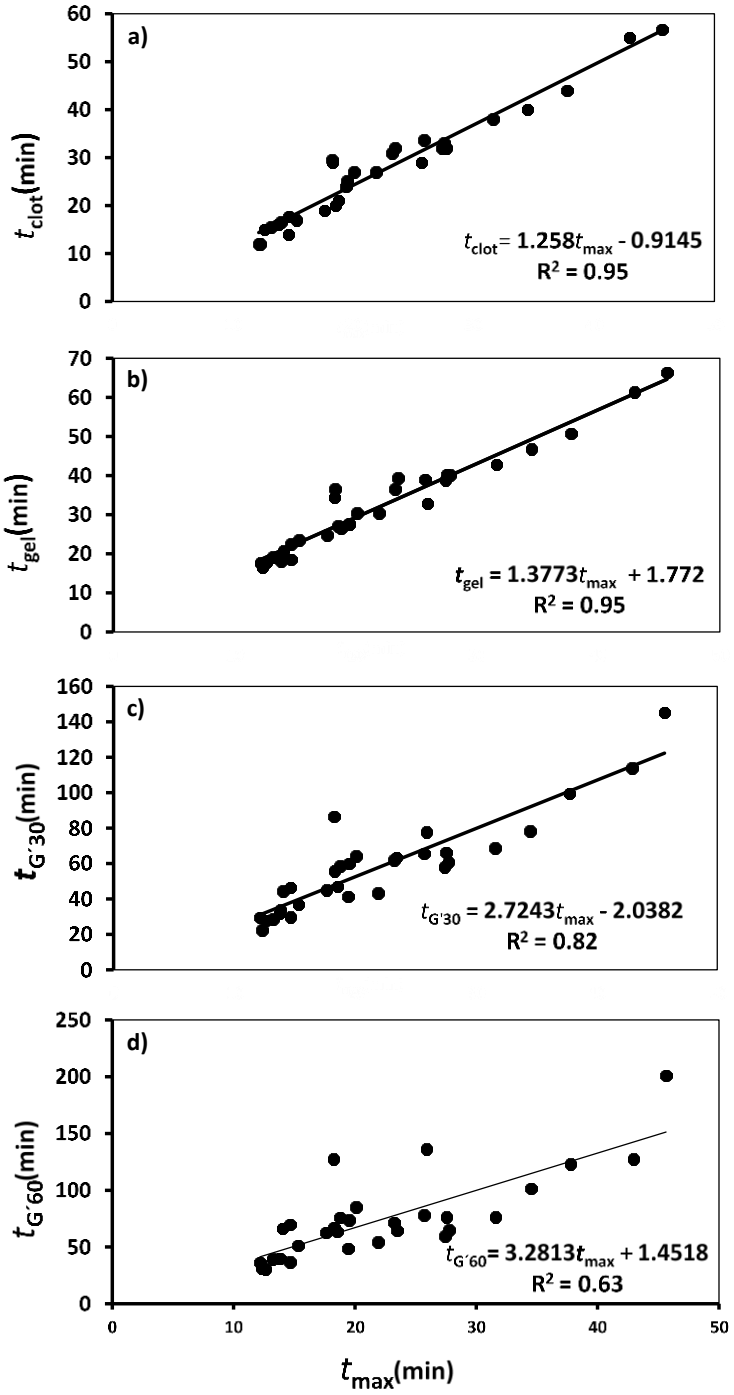


Fig. 6.6. The linear correlation between the optical predictor, t_{max}, and the cheese making indices, t_{gel}, t_{clot}, $t_{G'30}$ and $t_{G'60}$.

is formed. Actual clotting time can be estimated by non-visual parameters such as rheological or optical parameters. The difference between the observed lag time and the actual lag time is a function of the precision of the equipment used, as well as the physical parameters chosen for measuring it (McMahon et al., 1984b; Eck, 1989).

The factors t_{max} · protein (t_{max}p), fat to protein ratio (*FP*) and enzyme concentration (E_0), were consistently found to be the best descriptors of t_{clot} throughout the various linear regression models tested as shown in **Table 6.5**.

Table 6.5. Models for the prediction of the visually-defined gelation time (t_{clot}).[a]

Model		β_0	β_1	β_2	β_3	R^2	SEP (min)
I* **	$t_{clot}=\beta_0+\beta_1 t_{max}p$	-0.747^{ns}	0.339***	-	-	0.952	2.57
II* **	$t_{clot}=\beta_0+\beta_1 t_{max}p+\beta_2 FP$	19.57^{ns}	0.335***	-15.8*	-	0.958	2.43
III* **	$t_{clot}=\beta_0+\beta_1 t_{max}p+\beta_2 E_0+\beta_3 FP$	27.6*	0.307***	-0.012^{ns}	-17.4*	0.963	2.32

[a]N = 36. Predictors are defined in the text; β_0, β_1, β_2, β_3, regression coefficients; R2, determination coefficient (corrected for the means); SEP, standard error of predictions; [ns]Not significant; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

According to the observed SEP and R^2, values, the best one-parameter algorithm was Model I, which contained the parameter t_{max}p and the intercept. In this model, only the regression coefficient assigned to the term t_{max}p was significantly different from zero while the intercept was found to be not significant. The best two-parameter prediction algorithm was Model II. This model included, in addition to t_{max}p, *FP* and the intercept. As in Model I, in Model II, the intercept was also found to be not significant. In Model II, the SEP was only reduced by 0.14 min as compared to Model I. Finally, the best model having three-parameters was Model III, which contained t_{max}p, E_0, *FP* and the intercept. The regression coefficients for t_{max}p and *FP* as well as the intercept were found to be significant while the coefficient for E_0 was not significant. Model III had a slightly higher R^2 (R^2 = 0.963) as compared with Models I and II. Even though, Model III had the smallest SEP and highest R^2, it was not considered practical for in-line prediction of clotting time due to the large number of significant coefficients needed to implement it and to the slight decrease in SEP that was observed compared with Model I. Thus, Model I was preferred for in-line application, because it was based on an easily measurable time-parameter and only needed one significant regression coefficient to predict t_{clot}. This results show the potential of the parameter t_{max}p for in-line prediction of clotting time in manufacture of MMCs.

6.4.8 Prediction of rheologically-generated gelation time

The maximum R^2 procedure of SAS® was utilized to obtain the best one-, two-, and three-parameter models for predicting the gelation time measured by the rheometer as shown in **Table 6.6**. The parameters t_{max}, t_{max} · fat (t_{max}f) and enzyme concentration (E_0) were

consistently found to be the best descriptors of the rheological gelation time t_{gel} throughout the various linear regression models tested.

Table 6.6. Models for the prediction of the rheological gelation time (t_{gel}).[a]

Model		β_0	β_1	β_2	β_3	R^2	SEP (min)
I* **	$t_{gel}=\beta_0+\beta_1 t_{max}$	1.426 ^{ns}	1.432***	-	-	0.948	3.015
II* **	$t_{gel}=\beta_0+\beta_1 t_{max}+\beta_2 t_{max}f$	1.7284 ^{ns}	2.408***	-0.211 ^{ns}	-	0.953	2.917
III* **	$t_{gel}=\beta_0+\beta_1 t_{max}+\beta_2 t_{max}f+\beta_3 E_0$	8.616*	2.418***	-0.239 ^{ns}	-0.014 ^{ns}	0.958	2.803

[a]N = 36. Predictors are briefly defined in the text; β_0, β_1, β_2, β_3 regression coefficients; R^2, determination coefficient (corrected for the means); SEP, standard error of predictions; ^{ns}Not significant; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Model I was the simplest model to predict t_{gel}. It had a high determination coefficient ($R^2 = 0.948$) using just t_{max} as predictor. Only the regression coefficient assigned to the term t_{max} was significantly different from zero while the intercept was found to be insignificant. Model II was the best model having two parameters and contained, in addition to t_{max}, the parameter $t_{max}f$ and the intercept. However, only the regression coefficient term for t_{max} was found to be significant. In Model II, the SEP was only reduced by 0.1 min, as compare to Model I. Finally the best model having three parameters was Model III. This model included not only t_{max}, and $t_{max}f$ but also E_0, and the intercept. However, the SEP for Model III was only slightly smaller than that for Model II. In addition, only the regression coefficient term for t_{max} and the intercept were found to be significant. Model II and Model III were considered to be less desirable as in-line prediction algorithms due to the small improvement in SEP, the large number of predictive terms and because the magnitude of the predictions in both cases was affected by milk composition (i.e., fat concentration). Model I was preferred for inline prediction as it was based on an easily measurable time-parameter t_{max} and only required one significative regression coefficients to predict t_{gel}.

6.4.9 Prediction of the rheologically–determined cutting time

Cutting time (t_{cut1}) was defined as the time when gels reached a $G' \geq 30$ Pa ($t_{G'30}$). The maximum R^2 procedure was used to select the best one-, two- and three-parameter models for predicting rheological cutting time. The parameter, $t_{max} \cdot$ protein ($t_{max}p$), $t_{max} \cdot$ fat to protein ratio ($t_{max}FP$), $t_{max} \cdot$ fat concentration ($t_{max}f$), and coagulation temperature (T) were found to be the best descriptors of t_{cut}, as shown in **Table 6.7.**

Model I, the best one-parameter algorithm, included the parameter t_{max}p and the intercept. Model I had the highest value of SEP and the lowest determination coefficient (R^2 =0.8). In this model, only the regression coefficient assigned to the term t_{max}p was significant.

Table 6.7. Models for the prediction of the rheological cutting time ($t_{cut1} = t_{G30'}$)[a]

Model		Coefficient	Estimate	R^2	SEP (min)
I* **	$t_{cut1}=\beta_0+\beta_1 t_{max}p$	β_0	-2.034 [ns]	0.832	11.17
		β_1	0.739***		
II* **	$t_{cut1}=\beta_0+\beta_1 t_{max}p+\beta_2 t_{max}FP$	β_0	1.491 [ns]	0.887	9.297
		β_1	1.977***		
		β_2	-3.748***		
III* **	$t_{cut1}=\beta_0+\beta_1 t_{max}p+\beta_2 t_{max}f+\beta_3 T$	β_0	58.134***	0.926	7.830
		β_1	2.304***		
		β_2	-1.339***		
		β_3	-1.524***		
IV* **	$t_{cut1}=\beta_0 t_{max}(1+\Upsilon_1 FP+\Upsilon_2 Mix.+\Upsilon_3 T)$	β_0	11.166***	0.930	7.804
		Υ_1	-0.489***		
		Υ_2	-0.029***		
		Υ_3	-0.003 [ns]		

[a]N = 36. Predictors are defined in the text; β_0, β_1, β_2, β_3, Υ_1, Υ_2, Υ_3 regression coefficients; R^2, determination coefficient (corrected for the means); SEP, standard error of predictions; [ns]Not significant**;** **P<0.05, **P<0.01, ***P<0.001*.

Model II could be obtained by adding t_{max}FP into Model I, while Model III substituted the term t_{max}FP by t_{max}f and incorporated the term coagulation temperature. In Model II, the intercept was found to be insignificant. Comparing Model I with Model II, the SEP was reduced in 1.9 min and the determination coefficient reached a value of R^2 = 0.887. Comparing Model III with Model I and II, Model III had the smallest SEP and the largest R^2 (SEP = 7.83 min; R^2 = 0.926). Even though, Model III was not considered practical for in-line prediction due to the large number of significant coefficient needed to implement it and to the slight decrease in SEP that was observed compared with Model I.

Cutting time prediction was alternatively evaluated by defining cutting time at a higher level of firming. Thus, t_{cut2} was defined as the time when gels reached a $G' \geq 60$ Pa ($t_{G'60}$). Models for prediction of t_{cut2} were not discussed in detail to avoid redundancy as the terms selected by the Maximum R^2 procedure of SAS were very similar to those for prediction of $t_{G'30}$. Terms for prediction of t_{cut2} were introduced by the Maximum R^2 procedure of SAS in the Models in the following order: t_{max}p, t_{max}FP, and t_{max}Mix (i.e., *Mix.* · t_{max}). Note that the mixture

proportion (*Mix.*) was not included in the models for prediction of t_{cut1}. Inclusion of the term ¨*Mix*¨ in the cutting time models suggested the importance of this factor, which is affecting the aggregation and firming rate, on selecting an adequate cutting time when milk mixtures are coagulated, especially when firming levels larger than 30 Pa are selected for cutting.

On the other hand, it needs to be highlighted that a practical prediction model for industrial application would preferably contain only one parameter. A one-parameter model could be adjusted in the plant for calibrating the predicted cutting time with the operator observed cutting time by adjusting the one variable. Castillo *et al*. (2003a) found that the algorithm $t_{cut} = \beta_0\, t_{2min}\, (1 + \gamma\, \text{protein})$ adequately predicted t_{cut} for a wide range of protein concentrations (3% to 7%), where γ was considered constant (in goat milk γ = -0.067). This model contained a LB parameter, t_{2min}, and a protein term that corrected the gradient β_0 depending on protein content by using the constant. Thus, theoretically, only the gradient β_0 would require in plant calibration. These authors claimed that the proposed prediction algorithm is based on the fact that gradient β_0 decreases significantly as protein concentration increases. It was expected that the effect of protein concentration on this gradient is constant if estimated within a wide range of protein concentrations. Thus, based on the described model by Castillo *et al*. (2003a), an alternative nonlinear Model was tested using NLIN procedure of SAS that would be more practical for in-line prediction of cutting time when using milk mixtures (Model IV, **Table 6.7**).

Model IV was developed taking also into account that in most of the models obtained for prediction of $t_{G'30}$ and $t_{G'60}$ the intercept was not significant, and that the optical parameter t_{max} was selected by the Maximum R^2 procedure of SAS in most of the prediction terms. Consequently, Model IV had no intercept, and contained only one optical prediction parameter, t_{max}, and a complex term that corrected the gradient β_0 depending on the fat to protein ratio, the mixture proportion and the coagulation temperature. This model had R^2 and SEP values of 0.930 and 7.804 min, respectively. This model assumed that the effect of *FP*, *Mix*. and *T* on the gradient β_0 is constant. Thus, industrial implementation of this model would require accurate estimation of the γ values in the laboratory within wide ranges of the three factors. Once the three factors would have been accurately estimated, only the gradient β_0 would require in-plant calibration. **Fig.6.7** shows the relationship between the actual and the predicted cutting time using Model IV.

Another aspect of importance with regard to the proposed prediction models is the large

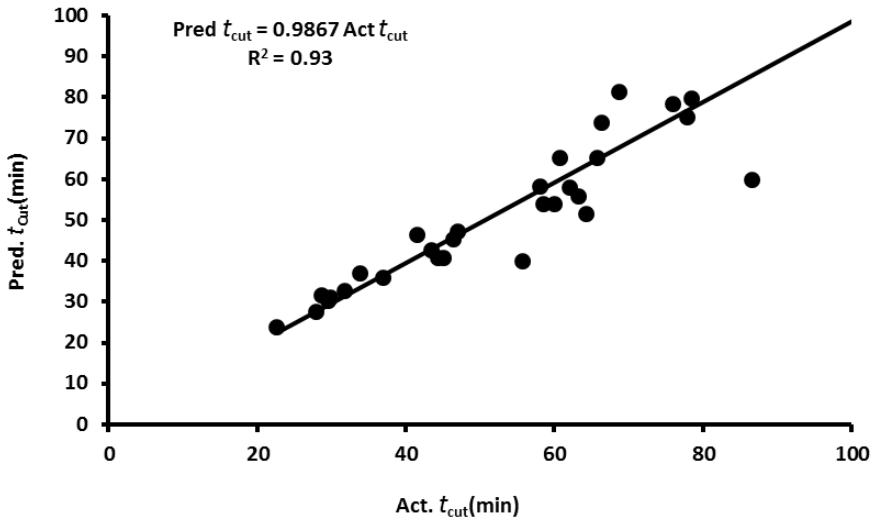


Fig. 6.7. The correlation between actual and predicted cutting time using model IV. See Model IV in Table 6.7.

SEP observed. Models obtained for prediction of $t_{G'30}$ and $t_{G'60}$ required four regression coefficients to reduce the SEP to a value of ~7 min. This error was considered too large (CV = 13.4%) for acceptable prediction of cutting time. This was partially attributed to the reduced light backscatter response originated by increasing proportions of goat and sheep milk in the milk mixtures, which reduced the precision of the software for generating the light backscatter parameters. This precision lost tended to increase the scatter of the predictors obtained by the system, which very likely, resulted in an increase of the SEP value. The large CV observed was also originated by the small number of milk mixture proportion levels evaluated in the experimental design used. For this reason, a reduction of SEP is warranted before implementing the proposed technology in the industry. Reduction of SEP is probably twofold: a) using a more suitable experimental design to gather more information about the effect of the milk mixture proportions on the formation of the gel (i.e. a better understanding of the milk mixture proportions on the rate of casein micelle aggregation and gel firming will help to identify more adequate predictors for cutting time), and b) modifying the optical and/or electronic configuration of the sensor to increase its response during coagulation when milk mixtures are used for cheese making.

6.4.10 Prediction of the tan δ value at cutting time

The loss tangent (*tan* δ) is the ratio between the viscous or loss modulus (G″) and elastic or storage modulus (G′), i.e., *tan* δ = G″/G′; where δ is the phase difference between stress and strain (Marchesseau *et al*., 1997). The value of this rheological parameter is related to the rearrangement capability of the gel (Castillo *et al*., 2006b, d). Thus, an early estimation of the *tan* δ value that is expected to be achieved at cutting time would be very useful to foresee the tendency of the gel to undergo syneresis at cutting. According to the maximum R^2 procedure of SAS, the best descriptors for *tan* δ at cutting (i.e., *tan* δ when *G´* reaches a value of 30 Pa) were coagulation temperature, milk mixture and fat/protein ratio. **Table 6.8** shows the best one, two- and three- parameters models for predicting *tan* δ at cutting.

Table 6.8. Models for the prediction of the rheological cutting time (tan δ) using light backscatter[a]

Model		β_0	β_1	β_2	β_3	R^2	SEP
I* **	$tan\ \delta=\beta_0+\beta_1 T$	0.079***	0.006***	-	-	0.927	0.007
II* **	$tan\ \delta=\beta_0+\beta_1 Mix+\beta_2 T$	0.073***	0.006*	0.006***	-	0.941	0.006
III* **	$tan\ \delta=\beta_0+\beta_1 Mix+\beta_2 T+\beta_3 FP$	-0.009 [ns]	0.009***	0.006***	0.062**	0.959	0.005

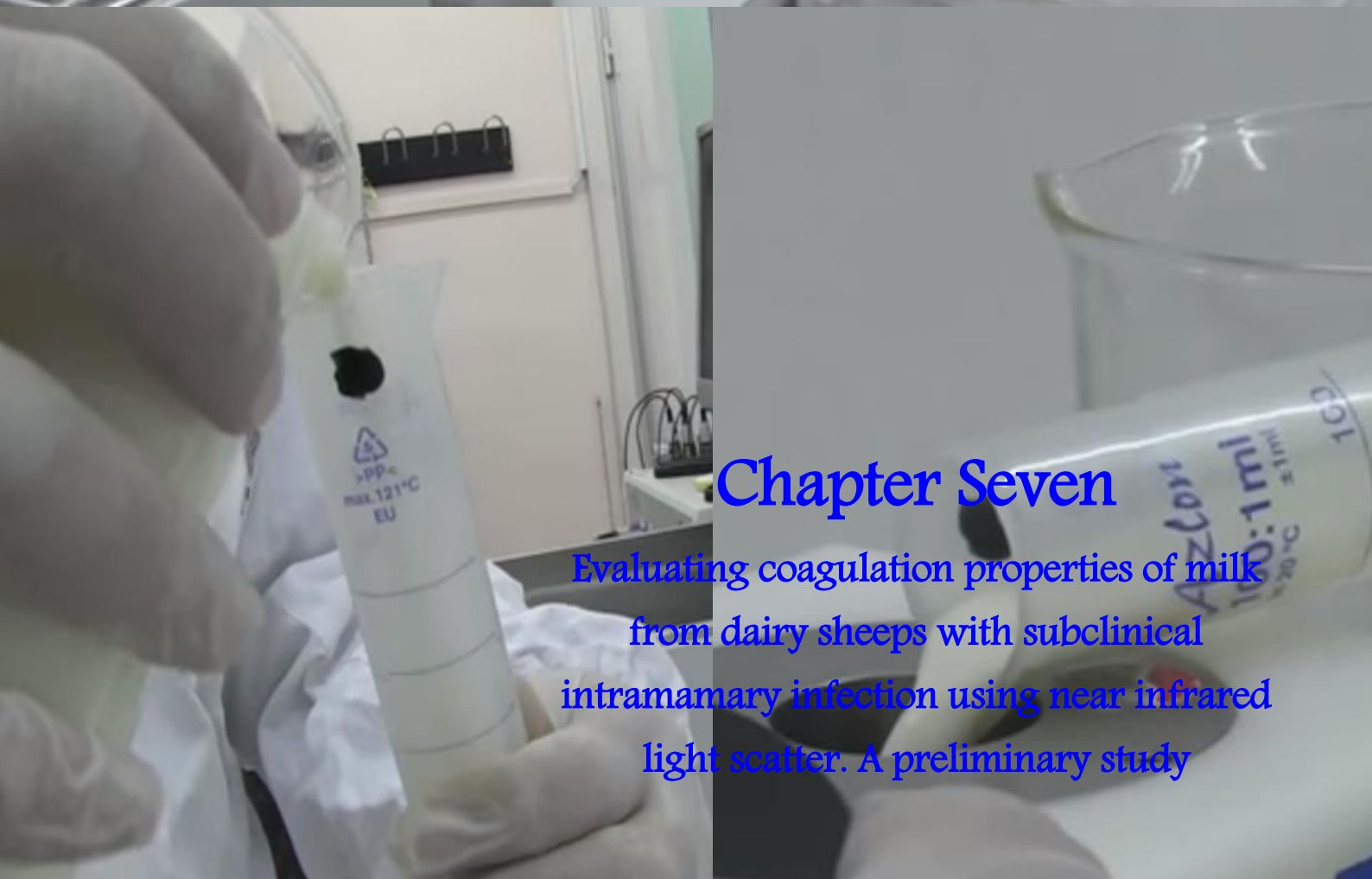
[a]N = 36. Parameters are defined in the text; β_0, β_1, β_2, β_3 regression coefficients; R^2, determination coefficient (corrected for the means); SEP, standard error of predictions; [ns]Not significant**;** *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Model I was the best model containing the predictor, coagulation temperature, and an intercept. Both regression coefficients in this model were significant. The best two-parameter model was Model II. It included, in addition to coagulation temperature, the milk mixture proportion and the intercept. Again, all the regression coefficients in this model were significant. Finally, the best model having three parameters was Model III, which contained in addition to the previous predictors, the parameter fat to protein ratio.

6.5 CONCLUSIONS

Mixing different proportion from cow, goat and sheep milk and coagulating the mixtures with different enzyme concentrations and at different coagulation temperatures resulted in a significant change in visual, optical and rheological parameters, which indicated that the change in milk mixture type affected the reactions of casein aggregation and gel firming. Time based parameters generated from the light backscatter profile were a function of the hydrolysis rate, which varied with temperature and enzyme concentration but not varied with the milk mixture. Enzyme concentration and temperature had a significant effect ($P<0.05$) on optically- (t_{max}, t_{2max}, t_{2min}), rheologically- (t_{gel}, $t_{G´30}$, $t_{G´60}$, $tan\delta$ and t_{firm}) and visually-derived parameters (clotting time). A highly significant effect of the milk mixture type on optical parameter $R´_{max}$ (optical estimate of aggregation rate) and the rheological parameter $tan\ \delta$ was also observed. This results indicate that, even when the milk mixture proportion might not exert an effect on the enzymatic phase of milk coagulation, increasing the cow milk proportion resulted on faster gel firming rate, which contributed to originate a weaker gel with more tendency for rearrangement (i.e., with increased syneresis capabilities).

The maximum R^2 procedure was used to select the best one-variable, two-variable and three-variable models for predicting Berridge clotting, gelation and cutting times. Models obtained for prediction of $t_{G´30}$ and $t_{G´60}$ required four regression terms to reduce the SEP to a value of ~7 min. This error was considered too large (CV = 13.4%) for acceptable prediction of cutting time. This was partially attributed to the reduced light backscatter response originated by increasing proportions of goat and sheep milk in the milk mixtures, which reduced the precession of the software for generating the light backscatter parameters. This precision lost tended to increase the scatter of the predictors obtained by the system, which very likely, resulted in an increase of the SEP value. For this reason, a reduction of SEP is warranted before implementing the proposed technology in the industry. Reduction of SEP is probably twofold: a) using a more suitable experimental design to gather more information about the effect of the milk mixture proportions on the formation of the gel (i.e. a better understanding of the milk mixture proportions on the rate of casein micelle aggregation and gel firming will help to identify more adequate predictors for cutting time), and b) modify the optical and/or electronic configuration of the sensor to increase its response during coagulation when milk mixtures are used for cheese making.



Chapter Seven

Evaluating coagulation properties of milk from dairy sheeps with subclinical intramamary infection using near infrared light scatter. A preliminary study

Chapter Seven: Evaluating coagulation properties of milk from dairy sheeps with subclinical intramamary infection using near infrared light scatter. A preliminary study

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7.1 ABSTRACT

Loss of milk quality caused by subclinical infection in dairy sheep has a negative effect on cheese manufacture. As milk from each single animal is not systematically evaluated for somatic cell count, milk from animals with undetected subclinical mastitis often reaches the refrigeration tanks, mixing with normal milk and reducing its technological suitability for cheese manufacture. This study was undertaken to investigate the effect of subclinical mastitis in the coagulation properties of ewe milk using a light backscatter fiber optic sensor**.** Manchego-type cheese was manufactured using milk from Lacaune and Manchega sheep. Milk from infected and non-infected udders was coagulated and monitored at laboratory scale using both a NIR fiber optic light backscatter sensor and a rheometer. Simultaneously, clotting and cutting times were visually evaluated by an experienced cheese maker. Optical parameters t_{max}, t_{2max}, and t_{2min} were highly correlated ($0.914 < r < 0.999$, $P < 0.001$) to the visually and rheologically derived clotting and cutting times and with somatic cell count. It was observed that milk from animals with no udder bacterial infection, irrespectively of the breed, had quite similar clotting and cutting times. On the other hand, milk from animals having subclinical infection caused by coagulase-negative *Staphylococcus* had longer coagulation and cutting time. Prediction models using light backscatter parameters alone or in combination with protein/solids concentration were successfully obtained for visually determined clotting and cutting times, rheologically derived gelation and cutting times and for $\tan\delta$ at cutting with R^2 values ranging from 0.799 to 0.999. Our results suggest that early detection of subclinical mastitis and milk coagulation monitoring using light scatter can diminish the negative impact of mixing milk of infected animals, when milk is used for cheese manufacture.

Keywords: Sheep; subclinical mastitis; clotting time; cutting time; cheese; rheology; light backscatter; optical sensor; prediction.

7.2 INTRODUCTION

Poor clotting properties represent cheese yield losses and poor cheese quality, requiring the adoption of technological modifications to minimize these negative impacts (Martins *et al.*, 2000). For this reason, a large number of devices have been developed to monitor milk coagulation and gel firming over the past seventy years. A comprehensive classification of those devices was published by Castillo (2001) and Castillo *et al.* (2006c). Rheological systems in general, are destructive and not practical for inline application. As yet, there is not an ¨optimum¨, efficient, non-destructive, and 100% reliable cutting time determination method available, although some existing methods can consistently reproduce the cutting time selected by subjective evaluation of the cheese maker. An inline optical sensor designed to measure changes in light backscatter of infrared light at 880 nm was proposed by Payne *et al*. (1993a) to predict cutting time. This technology has been tested as a useful tool to monitor milk coagulation. In particular, the fiber optic, light backscatter, near-infrared (NIR) sensor CoAguLite (Reflectronics Inc., Lexington, Kentucky, USA) is an inline sensor that has been well documented to monitor milk coagulation and predict clotting, gelation and cutting times (Castillo, 2001). This technology has been specifically developed for cow milk, and adapted successfully to goat milk (Castillo, 2001). However, it has never been used to evaluate the effect of milk from animals with subclinical mastitis on milk coagulation. Previous studies (Castillo *et al*, 2001, 2005c, 2006a; Abdelgawad *et al*. (2014) have shown that light backscatter and milk coagulation do not only depend on milk fat and protein percentages but also on casein micelle and fat globule structure and size distribution. Milk from infected and uninfected animals has both different milk composition and particle structure/size distribution.

The health condition of the mammary gland dictates the rate of milk secretion and has a multitude of effects on the quantity, quality, and processing properties of the produced milk. Intramammary infection (**IMI**) or mastitis, an inflammation response, is considered to exert one of the most negative impacts on dairy ruminant's health, including small ruminants, farmer's income and public health (Leitner *et al*., 2004; Leitner *et al*., 2011b). The major pathogenic agents causing subclinical mastitis **(SCM)** in sheep are coagulase-negative *Staphylococci* **(CNS)** as indicated by (Leitner *et al*., 2004; Leitner *et al*., 2011b). Absence of clinical signs in sheep with SCM in combination with the nonexistence of relevant changes in the milk appearance prevents the detection of the infection by the farmer. The prevalence of subclinical

mastitis in dairy sheep flocks may occur up to 15 - 40% and if all animals are milked into the milk tank, 15-30% of the milk would be of infected glands (Leitner *et al.*, 2004, 2006, 2008, and 2011a). Thus, as a result of the high incidence and prevalence, **SCM** can be considered as one of the main sources of economic losses in milk production worldwide (Gonzalo *et al*., 2002; Leitner *et al*., 2007, 2008). Moreover, as in dairy sheep the entire milk is used for cheeses production, reduction of milk quality along with the loss of quantity can be devastating to the producers.

It is well documented that mastitis, even in its predominant subclinical form, impairs milk quality through the activation of the immune system and leads to changes in milk synthesis in the alveolus. As a result of the infection, there is increase in the release of deteriorative enzymes into the milk from the bacteria and the host cells, reduced lactose and casein concentrations, increased pH and higher level of whey protein and mineral content (Merin *et al* 2008). Proteolysis of casein leads to increased levels of γ-caseins and proteose peptones **(p-p)** (Le Roux *et al*., 1995). Studies reported that different bacteria may cause different types of physico-chemical damage to the milk (Leigh *et al.*, 1997; Silanikove *et al*., 2014). By activation of the host's innate immune system, milk from udders infected with different types of bacteria but with similar somatic cell count **(**SCC) may present different milk composition alterations related to a bacteria specific mixture of leukocyte populations and leukocyte-associated proteases (Coulon *et al*., 2002; Merin *et al.* 2008; Leitner *et al*., 2006; Silanikove *et al*., 2014). As a result, high SCC has been associated with prolonged rennet clotting times and a weak coagulum, which, in turn, lead to increased increased cheese yield (Leitner *et al.*, 2006).

All sheep milk is destined for manufacturing of dairy products, mainly cheese. Milk quality is critical for high quality sheep milk cheeses and dairy products. Thus milk from SCM animals compromises its functional properties and hence its technological suitability (e.g., rennet coagulation time and curd firmness), which in turn impacts negatively on quality of dairy products. Likely, the three main reference quality parameters used to evaluate milk in the cheese manufacturing facilities are milk fat and protein as well as SCC. Milk fat and protein contents are related directly to the cheese yield, and as a result, for the most part, milk is paid according to the useful material content (fat + protein content) (Ramón *et al.*, 2006). The SCC is also an important index for milk quality and in many (bovine milk) and some (ovine and goat milk) countries it is used as a criterion for milk payment to producers as well.

The objective of this study was to investigate if near infrared light backscatter could be used to monitor milk coagulation and predict cutting time in Manchego-type cheese made from CNS infected/uninfected type of milk and predict several relevant milk coagulation indicators, including clotting and cutting times.

7.3 MATERIALS and METHODS

7.3.1 Experimental Design

The study was carried out at the Experimental Farm of the SGCE (Servei de Granges i Camps Experimentals) of the Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain). A total of 32 lactating dairy sheeps of two breeds (Manchega, MN; n = 14; Lacaune; *LC*, n = 18) at mid to end lactation (80 – 150 days in milk) were used. The animals were selected for the study according to bacterial udder infection as described by Rovai *et al.* (2014) using a half-udder model to assess the uninfected or infected glands IMI status with various CNS species. Teats were disinfected by dipping in an iodine solution (P3-ioshield) and dried with disposable paper towels. The first 3 milk squirts were discarded. Following cleaning, the teats were disinfected with 70% ethanol and left for approximately 1 min to evaporate any remaining alcohol. Milk of the first squirts were again discarded and 3- to 4-mL samples were collected from each udder half into sterile tubes (Eurotubo Deltalab, code 429946; Deltalab SL, Rubí, Spain) for bacteriological testing done on the same day. A loopful (0.01 mL) was streaked onto blood-agar plates (Bacto-Agar; Difco Laboratory, Le Pont-de-Claix, France) containing 5% washed sheep red blood cells. Plates were incubated at 37 °C and examined for bacterial growth after 18 and 42 h. An infection was assumed to have occurred if five or more similar colony-forming units were present in the incubated sample of milk.

The half-udder model has been extensively used (González-Rodriguez *et al*., 1995; Leitner *et al*., 2004, 2006, 2008, 2011a; Martí-De Olives *et al*., 2013) and it enables to study the negative effect of SCM on milk yield and quality with high statistical reliability. The model eliminates the significant individual variations between individual animals such as genetic, lactation and stage of lactation, nutrition, farm and environment. All these animal variations are completely neutralized when the unit of comparison corresponds to the two glands of the same animal. Two different dairy sheep breeds ***LC*** and ***MN*** were used to establish base for milk of uninfected animals.

The influence of IMI was studied using *LC* sheep with one gland uninfected and the contralateral infected with CNS **(*ILC*)**. Each milk type was tested twice. Before and during the study, bacterial infection and individual glandular milk yield, as well as composition and SCC were tested as previously described (Rovai *et al.*, 2014). Dairy animals were sampled during the morning milking. The individual gland was milked by machine and the entire milk was collected from previously chosen group of ewes (2-4 milking´s) producing more than 1 L day^{-1} to obtain enough amount of milk for cheese production. Ewe milk samples were coagulated using a Manchego-type cheese procedure**.**

Milk coagulation was monitored by rheological and optical methods. Small Amplitude Oscillatory Rheology assays **(SAOR)** were performed using a rheometer while light backscatter **(LB)** of coagulating milk was measured using a near infrared **(NIR)** light backscatter laboratory scale tester (CoAguLab). Simultaneously, both clotting time and cutting times were visually determined by an experienced cheese maker.

Rheological results were used as an objective reference to which visually determined clotting and cutting times could be compared.

The Ethical Committee on Animal and Human Experimentation (CEEAH) of the UAB approved all the experimental and animal care procedures.

7.3.2 Materials

Milk: Immediately after milk was received in the Centre Especial de Recerca Planta de Tecnologia dels Aliments (**CERPTA**) at UAB, (Barcelona, Spain), milk was stored at ~4 °C until it was used (typically within the same day of milk reception).

Bacterial culture: Lyophilized mixed starter cultures containing *Lactococcus lactis* subs *lactis* and *L. lactis* subs *cremoris* (Danisco A/S, Copenhagen, Denmark) were used.

Enzyme: Calf rennet (~70% chymosin, ~30% pepsine) with strength of 1:10000 was obtained from Laboratories' Arroyo (Santander, Spain) and used to induce milk coagulation at a dose of 350 $mg \cdot L^{-1}$.

7.3.3 Milk batches preparation

Unpasteurized and unhomogenized sheep milk (*LC* and MN) was stored at 4 °C right after collection and until it was used. Determination of fat (F), total protein (N × 6.38) (P), casein (CN), lactose and total milk solids (S) was performed at Laboratori Interprofessional Lleter de

Catalunya (ALLIC, Cabrils, Barcelona, Spain) using Milkoscan 5000 (Foss Electric, Hillerød, Denmark). SCC was also measured at ALLIC using a Fossomatic 5000 (Foss Electric). The different batches of milk were vat pasteurized at 65 °C for 30 min and immediately cooled and stored at 4 °C until used in the same day of milk collection. The milk was heated to the test target temperature prior to coagulation.

7.3.4 Testing procedure

Fig. 7.1 shows the flow chart of the testing procedure.

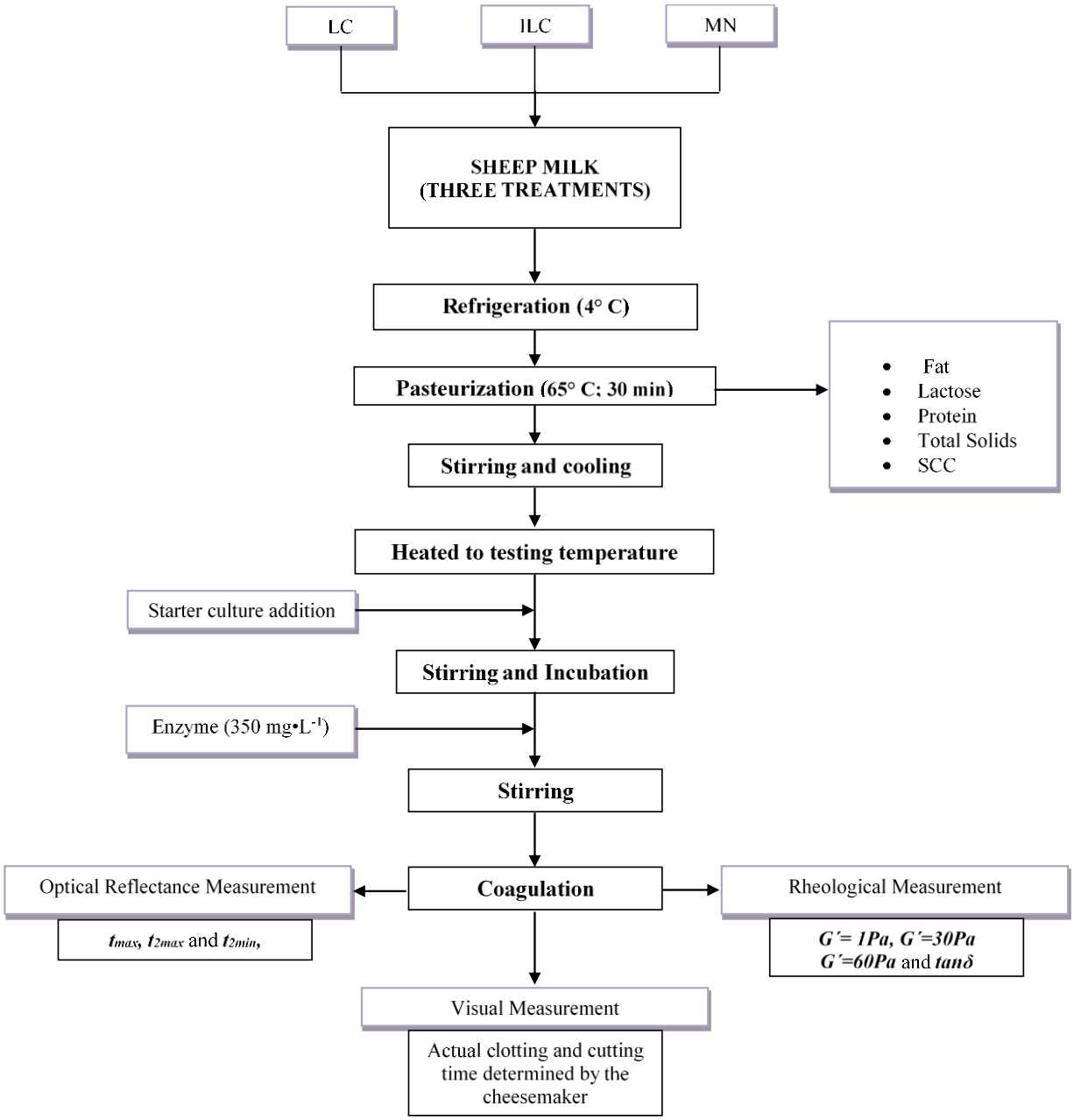


Fig.7.1. Flow chart of testing procedure. *MN*, Manchega; *LC*, Lacaune; *ILC*, Lacaune CNS-infected milk; *SCC*, somatic cell count. The rest of parameters are explained in the text.

Before each experiment, a 500 mL ewe milk sample was placed on a beaker and heated to the target coagulation temperature (35 °C) using a water bath. When thermal equilibrium was achieved, the starter culture was added to the milk (2%), and the mixture was stirred and incubated at the target temperature for 30 min. After the incubation, the rennet was added at a concentration, E_0 = 350 mg·L^{-1} milk, and the sheep milk was quickly stirred during 30 s and immediately, split into four aliquots. Milk samples were split between the CoAguLab, Rheometer and the water bath according to the method described in chapter five: material and methods, section 5.2.

7.3.5 Milk coagulation monitoring

Detailed description of the monitoring milk coagulation procees using CoAguLab, rheomoter and visual determination as well as the obtained parameters using these pieces of equipment, can be found in chapter five: material and methods, sections 5.4.1, 5.5. and 5.6.

7.3.6 Statistical Analysis

The data were analyzed using the Statistical Analysis System (SAS® 9.3 Statistical Analyses System Institute, Inc., Cary, NC, USA, 2011). Pearson correlation coefficients, **r**, were determined by the correlation (**CORR**) procedure of SAS. The analysis of variance (**ANOVA**) was performed using the general linear model (**GLM**) procedure of SAS.

The linear model used was $Y_i = \mu + MT_i + \varepsilon_i$ where Y was the dependent variable studied such as t_{max} , t_{2max} , t_{2min}, t_{clot}, t_{gel}, t_{cut}, $t_{G'30}$, $t_{G'60}$, t_{GE30}, t_{GE60} and *tan* δ, μ was the overall mean, MT_i was the fixed effect of milk type (*MN*, *LC*, *ILC*), and ε_i was the error term.

Detailed description of statistical methods used for analysing the data obtained from this experiment can be found in chapter chapter five: material and methods, section: 5.9.

7.4 RESULTS and DISCUSSION

7.4.1 Chemical composition of milk and analysis of variance.

Table 7.1 shows the average chemical composition and SCC for the three milk types evaluated in this study.

Table 7.1. Average milk composition and somatic cell count for each milk type evaluated in this study[1]

	Group		
Parameter	***MN***	***LC***	***ILC***
Fat (%)	7.10±0.14	6.79±0.04	7.74±1.09
Protein (%)	6.69±0.01	5.49±0.35	5.65±0.31
Casein (%)	5.65±0.19	4.58±0.35	4.61±0.02
Lactose (%)	4.62±0.04	4.62±0.04	4.37±0.09
Total Solids (%)	12.54±0.22	11.09±0.40	11.07±0.33
Log SCC	5.12±0.15	5.32±0.04	6.27±0.09

[1]Number of observations, N=6; *MN*, Manchega; *LC*, Lacaune; *ILC*, Infected Lacaune; *SCC*, somatic cell count.

An ANOVA analysis was carried out to determine whether the type of milk (*MN*, *LC*, *ILC*) significantly affected the dependent variables studied.

ANOVA results are presented in **Table 7.2**, including the $\boldsymbol{R^2}$ values and ***F*** statistics for most important dependent variables.

The analysis of variance (ANOVA) model was significant for all optical (t_{max}, t_{2max}, and t_{2min}), visual (t_{clot} and t_{cut}) and rheological ($t_{G'30}$, $t_{G'60}$ and $tan\delta$) parameters evaluated (P<0.05). Significant differences were found between milk from healthy Lacaune and CNS-Infected Lacaune, while the differences between milks from healthy animals of both breeds, Lacaune and Manchega, were not significant.

7.4.2 Effect of breed in milk composition and coagulation properties

Many factors are affecting the milk composition and yield such as genetic, physiological, environmental, and nutritional factors as well as the milking system. This is highly important in the small ruminants because many different breeds and management procedures exist. As it is described by (Moussaoui, 2009), milk yield is greater in *LC* than in MN ewes.

However, our results showed that MN milk contained greater fat and protein but similar lactose concentrations than *LC* milk (**Table 7.1**).

According to (Moussaoui, 2009) breed had no significant effect on milk non-protein nitrogen (**NPN**) or whey protein percentages. Since milk fat and protein contents are directly related to cheese yield, typically, milk is paid according to the useful material content (**UMC**; fat + protein content) (Ramón *et al*., 2006).

Our study confirms that the UMC was greater in MN than in *LC* ewes, which may reflect a greater economic value for the milk of MN ewes.

The SCC is an important index for milk quality and in many countries it is used as a criterion for milk payment to producers. In the current study, the low SCC in both milk categories, MN and *LC*, corresponded to high quality milk (**Table 7.2**).

Microbiological and chemical composition of milk exert and effect on milk clotting properties, which are critical from a technological point of view, as they influence milk gel microstructure and, as a result, subsequent cheese making operations such as whey separation, and curd ripening (Abdelgawad *et al*., 2011). It must be emphasized that poor clotting properties lead to cheese yield losses and reduced cheese quality, requiring the adoption of technological corrective actions (Martins *et al*., 2000).

According to Tyrisevä *et al*. (2004), the breed differences in milk coagulation aptitude and cheese quality are mainly based on the differences in chemical composition of milk and milk protein genotypes. As referred by Alichanidis and Polychroniadou (1997), sheep milk coagulation is quicker than that usually found for cow milk, leading to faster micelar aggregation and curd firming.

In our study, it was observed that milk of *LC* ewes seemed to start aggregating earlier and yielded a firm curd quicker as compared to milk of MN ewes (i.e., time at which a gel firmness of 30 and 60 Pa was attained was shorter in *LC* milk). LSMs of optical time parameters t_{max}, t_{2max}, and t_{2min}, visual clotting and cutting times (t_{clot} and t_{cut}), and rheological cutting times ($t_{G'30}$, $t_{G'60}$) were consistently smaller for *LC* milk than for MN milk (as an average, for *LC* milk the times were 1.24 min shorter for the seven time variables considered). However, gel assembly times, t_{GE30} and t_{GE60}, were as an average, 0.79 min longer for *LC* than for MN (**Table 7.2**).

Table 7.2. Analysis of variance and influence of main effects on the optical, visual and rheological dependent variables[1]

Analysis of Variance			Influence of main effects on dependent variables				
Model					Main effect (DF=2)		
	R^2	F	Parameter		***MN*** (LSM)	***LC*** (LSM)	***ILC*** (LSM)
V_0	0.953	13.6*	V_0	volts	1.53[a]	1.46[a]	1.36[b]
t_{max}	0.813	8.67*	t_{max}	(min)	10.6[a]	8.98[a]	23.7[b]
t_{2max}	0.778	7.02*	t_{2max}	(min)	8.99[a]	7.40[a]	20.6[b]
t_{2min}	0.824	9.35*	t_{2min}	(min)	12.1[a]	10.4[a]	25.6[b]
t_{clot}	0.818	9.02*	t_{clot}	(min)	10.8[a]	9.90[a]	25.2[b]
t_{gel}	0.793	7.69*	t_{gel}	(min)	11.6[a]	10.1[a]	27.8[b]
t_{cut}	0.711	4.94*	t_{cut}	(min)	16.0[a]	14.8[a]	40.7[b]
$t_{G'30}$	0.792	7.62*	$t_{G'30}$	(min)	13.9[a]	12.9[a]	36.8[b]
$t_{G'60}$	0.787	7.43*	$t_{G'60}$	(min)	15.1[a]	14.6[a]	40.9[b]
t_{GE30}	0.747	1.97*	t_{GE30}	(min)	2.34[a]	2.89[a]	8.19[b]
t_{GE60}	0.736	1.86*	t_{GE60}	(min)	3.52[a]	4.54[a]	11.9[b]
t_{NGE30}	0.750	4.51*	t_{NGE30}	(dim^2)	0.22[a]	0.32[a]	0.36[b]
t_{NGE60}	0.751	4.52*	t_{NGE60}	(dim^2)	0.34[a]	0.50[a]	0.52[b]
$tan\delta$	0.728	1.66***	$tan\delta$	(dim^2)	0.32[a]	0.32[a]	0.34[b]
SCC	0.959	35.9**	***SCC***		5.12[a]	5.32[a]	6.27[b]

[1]Number of observations, N=6; *MN*, Manchega; *LC*, Lacaune; *ILC*, CNS-Infected Lacaune. Least squares means (LSM) with the same letters (in the same row) are not significantly different P<0.05. Dependent variables explained in the text. R^2, determination coefficient; F, ANOVA F-statistic; DF, degree of freedom; **P<0.05, **P<0.01, ***P<0.001.*
[2]Dimensionless

Even when these trends were not significant, they were found to be clearly consistent, and in agreement with previous work (Castillo *et al*., 2006a, 2006b; Leitner *et al*. 2004, 2008). These results were attributed to the observed differences in protein and casein concentration between the two sheep breeds. As shown in Table 1, MN milk had higher protein and casein concentration than *LC* milk. Castillo (2001) and Castillo *et al*. (2003b) observed that, at coagulation conditions where the coagulating enzyme might be saturated by the substrate, hydrolysis of κ-casein is the limiting reaction (Carlson *et al*., 1987), yielding longer clotting and cutting times, even when the aggregation and firming rate is increased by increased protein concentration (*MN vs. LC*).

In order to further evaluate the mentioned hypothesis, the gel assembly times were normalized against its respective t_{max} values. To that end, two parameters, t_{NGE30} and t_{NGE60} were calculated by dividing t_{GE30} and t_{GE60} by t_{max}. In accordance with (Castillo *et al*., 2003a), who claims that the aggregation of casein micelles start around t_{max}, a significant level of curd gel assembly is likely to begin a small and unknown period of time after the onset of aggregation.

The calculated normalized firming times, t_{NGE30} and t_{NGE60}, were 46% and 52% larger, respectively, in *LC* milk as compared with MN milk **(Table 7.2)**. These results clearly show that a smaller percentage of protein in *LC* milk reduced the time required for κ-casein hydrolysis reaction, inducing a decrease in cutting time, but at the same time originated a decrease of the micelle aggregation/firming rate in this type of milk with regard to MN milk. Finally, no significant differences (P>0.05) were encountered in the gel tendency for curd rearrangement and whey separation as ***tanδ*** value (dimensionless) when the gel reached 30 Pa was 0.32 in both MN and *LC* gels.

7.4.3 Effect of subclinical mastitis infection in milk composition and coagulation properties

Mastitis, an inflammatory reaction of the mammary gland to an infection, is also known to have a multitude of effects on the quantity, quality, and processing properties of the produced milk (Waston and Buswell, 1984). Leitner *et al*. (2004) clarified that the milk yield of the uninfected udder halves was significantly higher than that of the infected halves. In the current

study, milk from *ILC* had higher protein, casein and low fat concentrations, but the diffrences were not significant (P>0.05) while lactose concentration was significantly lower than *LC* (P<0.05) (**Table 7.1**).

A lower concentration of lactose has been observed by other authors (Batavani *et al*., 2007) in milk from infected animals. In infected animals' milk, decreased lactose content balances the osmotic effect of increased mineral content.

Many reports have described the changes occurred in milk yield and/or composition associated with SCM. Regarding quality control, the increase of milk SCC is the main marker for the detection and diagnosis of mastitis (Viguier *et al*. 2009). In our study, milk from *LC* glans contained ($\log_{10}$ SCC mL^{-1} = 5.32), while milk from infected, *ILC*, glands contained much higher SSC ($\log_{10}$ SCC mL^{-1} = 6.27) (**Table 7.2**) and the difference was highly significant (P<0.001). Leitner *et al*. (2008) classified bulk milk quality in three categories according to its SCC: high quality (SCC $mL^{-1} < 800{,}000$), medium quality ($800{,}000 \leq$ SCC $mL^{-1} < 1.5 \cdot 10^6$), and low quality (SCC $mL^{-1} \geq 1.5 \cdot 10^6$)**.** According to this classification and as expected, milk quality was high and low for *LC* and *ILC*, respectively.

Since changes in chemical composition of milk from animals with SCM are small and SCC is not measured in every animal a wide proportion of milk from animals having SCM is transformed into cheese.

Even when the composition of raw milk is hardly affected by subclinical mastitis, the quality of dairy products can deteriorate (Merin *et al*. 2008). Besides the risks of bacterial contamination accompanying products based on raw milk, changes in the composition of mastitic milk can impair the cheese manufacture processes.

LSMs of $\boldsymbol{V_0}$, optical time parameters $\boldsymbol{t_{max}}$, $\boldsymbol{t_{2max}}$, and $\boldsymbol{t_{2min}}$, visual time parameters, $\boldsymbol{t_{clot}}$, and $\boldsymbol{t_{cut}}$, rheologically-derived parameters related to firming development and gel microstructure, $\boldsymbol{t_{gel}}$, $\boldsymbol{t_{G'30}}$ $\boldsymbol{t_{G'60}}$, $\boldsymbol{t_{GE30}}$, $\boldsymbol{t_{GE60}}$ and $\boldsymbol{tan\delta}$, for both *LC* and *ILC* milk are presented in **Table 7.2**. It was observed that milk from animals having SCM had a highly significant effect (P<0.05) on all the parameters studied as compared to milk from healthy animals.

Curiously, the initial voltage, V_0, was significantly smaller in *ILC* milk than in *LC* milk, even when the percentages of milk fat, protein and casein were slightly (but not significantly)

higher. This is unexpected. According to previous studies (Castillo 2001; Castillo *et al*., 2005a; Castillo *et al*., 2003a) increased fat and protein concentration is known to increase V_0. This might suggest severe demineralization of the casein micelles in *ILC* milk. This might be expectable, as proteolytic enzymes in *ILC* milk have been reported to cause loss of colloidal calcium phosphate (**CCP**) (Caldwell, 2012) and demineralization of casein micelles has been related to a decrease in light backscatter (Castillo *et al*., 2006a, 2006b).

On the other hand, all optical, visual and rheological time-based parameters related to milk coagulation were substantially larger on *ILC* milk as compared to *LC* milk **(Table 7.2)**. As an average, infection increased all time-based coagulation parameters by 2.5-2.8 times depending on the parameter considered. Unexpectedly, normalized firming times, t_{NGE30} and t_{NGE60} did change significantly with the subclinical infection but in a much smaller proportion than previously mentioned time parameters, suggesting that observed coagulation delay in *ILC* was due to delay in both enzymatic step of coagulation and gel assembly.

These results are in agreement with Srinivasan and Lucey (2002), Moussaoui *et al*. (2004) and Leitner *et al*. (2004). These authors stated that the effect of rennet and, therefore, the coagulation process may be impeded by only partial hydrolysis of κ-CN and more pronounced hydrolysis of the other caseins by enzymes such as plasmin and cathepsin.

A slight but significant ($P<0.05$) increase of the loss tangent, ***tanδ***, was also observed in milk from *ILC* when compared with *LC*, which indicates a weaker casein network with increased rearrangement capability in gels obtained from *ILC* milk. A higher loss tangent represents more mobile bonds and strands that are more susceptible to rearrangement, which in turn is typically associated with increased syneresis tendency (Castillo *et al*., 2006a, 2006b). However, in the case of mastitic milk, the integrity of the casein micelle is compromised and a higher loss tangent might not be directly related to enhanced rearrangement and syneresis capabilities. The loss of integrity of casein micelles in *ILC* milk (i.e., hydrolyzed caseins) might lead to poor contractibility of the gel as compared to intact rennet gels (Lucey., 2011b) (i.e., gel with decreased syneresis capability). However, as it will be discussed later (chapter 8), our results obtained in a ten-liter cheese vat pointed our to an increased syneresis tendency in ILC curd.

Although Maréchal and Thiéry (2011) and Abdelgawad and Ahmed (2011) indicated that some studies have reported no effect of high SCC (>500,000 cells·mL^{-1}) on rennet coagulation properties, in most studies, high SCC has been clearly associated with a significant increase in rennet clotting time, slower rate of curd firming and lower curd firmness, which lead to low quality of the final cheese. Batavani *et al*. (2007) suggested that late coagulation observed in milk obtained from infected animals was due to diminished concentration of the calcium, phosphorous, and potassium in milk from animals with SCM. As it is known, the lactose is the key factor balancing the osmotic changes between blood and the udder milk. As it was discussed previously, SCM leads to increased sodium and chloride and decreased calcium and potassium in the infected milk while reducing the lactose percentage. Batavani *et al*. (2007) and Tsioulpas *et al.* (2006) clarified that the content of minerals has a pronounced effect on the technological properties of milk, as it affects its susceptibility to renneting, fouling of heat exchangers, gelation and sedimentation. Changes in the mineral equilibrium in the infected milk lead to longer coagulation compared to the normal milk.

7.4.4 Correlations between optical, rheological and visual milk coagulation parameters

Our results show a close relationship between light backscatter (LB) parameters and both visually and rheologically-derived parameters obtained through milk coagulation as it is shown in **Table 7.3**.

The optically-generated time-based parameters (t_{max}, t_{2max} and t_{2min}) were significantly correlated with (a) visually-obtained parameter (t_{clot} and t_{cut}); (b) rheological parameters (t_{gel}, $t_{G'30}$, $t_{G'60}$, t_{GE30} and t_{GE60}); and (c) SCC, with Pearson correlation values (r) ranging between 0.914 and 0.999 (P<0.01). A slight correlation (but not significant) was observed between $tan\delta$ and LB parameters (0.570 < r < 0.670). Since the correlations between the three optical parameters evaluated and the rest of indicators behaved similarly, only the discussion of the optical parameter t_{max} is included in the text to avoid redundancy.

Table 7.3. Pearson correlation between parameters[1].

Parameter	t_{max}	t_{2max}	t_{2min}
t_{clot}	0.996^{c}	0.991^{c}	0.998^{c}
t_{gel}	0.999^{c}	0.999^{c}	0.996^{c}
t_{cut}	0974^{c}	0.996^{c}	0.994^{c}
$t_{G'30}$	0.987^{c}	0.995^{c}	0.989^{c}
$t_{G'60}$	0.973^{c}	0.993^{c}	0.986^{c}
t_{GE30}	0.961^{b}	0.963^{b}	0.949^{b}
t_{GE60}	0.957^{b}	0.960^{b}	0.947^{b}
$tan\delta$	0.619^{ns}	0.570^{ns}	0.670^{ns}
SCC	0.933^{b}	0.914^{b}	0.935^{b}

[1]Number of observations, N=6; Dependent variables explained in the text.
^{ns}Not significant; $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$; ^{ns}not significant; ^{ns}not significant.

The strong and significant correlation encountered between t_{max} and both gelation indicators, t_{clot} and t_{gel}, showed that, t_{max} is useful for detection of gelation time even when milk from animals having SCM is present. It was also detected that the differences in time between t_{max} and t_{clot} were slighter than those existing between t_{max} and t_{gel} (**Table 7.2**), which, in practice, simplifies the implementation of t_{max} as a potential indicator for rheological-determined gelation time.

Similarly, and surprisingly, the linear correlation observed between the optical parameter t_{max} and each visual and rheological cutting time indicator was not affected by the presence/absence of SCM, suggesting that even when all time-based- parameters were significantly delayed in milk from infected animals as compared to healthy animals, the relation between t_{max} and cutting time is stable, which in practice should allow for adequate cutting time predictions when milk from infected animals is mixed in the refrigeration tank with milk from normal animals.

7.4.5 Prediction algorithms of gel assembly indicators

Models for predicting visual (t_{clot}, t_{cut}) and rheological (t_{gel}, $t_{G'30}$, $t_{G'60}$, $tan\delta$) indicators of gel assembly were obtained using the maximum R^2 procedure of SAS®. Best predictor

selected by maximum R^2 procedure for predicting visual clotting time, t_{clot}, was the optical parameter t_{2min} while for the rheological gelation time t_{gel} was t_{2max}. According to the observed SEP and R^2 values, the best one-parameter algorithm models for predicting t_{clot} and t_{gel} were Model I and Model II, as shown in **Table 7.4,** which contained the parameters t_{2min} and $t_{2max,}$ respectively in addition to the intercept.

In these models, the regression coefficients assigned to the terms t_{2min} and t_{2max} were significantly different from zero while in both cases the intercept was found to be not significant. It was observed that the time parameters t_{2min} and t_{2max}, comprising for the most part information about the rate of casein micelle aggregation (Castillo *et al*., 2006d), contained the required information for predicting visual and rheological gelation times accurately, and irrespectively of the sheep breed and infection status, with a SEP of ~0.6 and ~0.4 min, respectively. Model I and II were very suitable for inline application because they both were based on an easily measurable time-parameter and only needed one significant regression coefficient to predict t_{clot} and t_{gel} **(Fig. 7.2a and Fig. 7.2b)**. As a result, these models were simplified without giving up any significance prediction capability to equation **7.1 and 7.2**, respectively (model III and IV**, table 4**) (SEP < 0.77 min).

$$\textbf{Eqn } (\mathbf{7.1}) \qquad t_{clot} = \beta_1 t_{2min}$$

$$\textbf{Eqn } (\mathbf{7.2}) \qquad t_{gel} = \beta_1 t_{2max}$$

The maximum R^2 procedure suggested that the best predictors for visual cutting time t_{cut} were t_{max} and t_{2max} while for the rheological cutting times $t_{G'30}$ and $t_{G'60}$ was t_{2max}. The best one-parameter algorithm for prediction of visual cutting time, t_{cut}, was Model V, as shown in **Table 7.4 (Fig. 7.2c),** which contained the parameter t_{max} and the intercept. In this model, only the regression coefficient assigned to the term t_{max} was significantly different from zero with SEP of ~ 1.2 min; thus, removing the intercept would only increase the SEP from 1.2 to 1.6 (Model VI). The best two-parameter prediction algorithm was Model VII; this model was generated by adding the intercept into Model V and replacing t_{max} with t_{2max}. In this Model, the three regression coefficients were significant and the SEP was reduced by 0.65 min when incorporating the DM (Dry Matter) term into Model V.

Table 7.4. Models for the prediction of gelation, cutting time parameters and tan δ value at cutting time.[1]

Predicted Model	Model ID	Model Equation	Coefficient β_0	β_1	B_2	B_3	R^2	SEP (min)
t_{clot}	I***	$t_{clot}=\beta_0+\beta_1 t_{2min}$	-1.3^{ns}	1.03***	-	-	0.996	0.58
t_{gel}	II***	$t_{gel}=\beta_0+\beta_1 t_{2max}$	-0.094^{ns}	1.3***	-	-	0.995	0.42
t_{clot}	III***	$t_{clot}=\beta_1 t_{2min}$	-	0.967***	-	-	0.976	0.77
t_{gel}	IV***	$t_{gel}=\beta_1 t_{2max}$	-	1.3***	-	-	0.995	0.37
t_{cut}	V***	$t_{cut}=\beta_0+\beta_1 t_{max}$	-2.3^{ns}	1.8***	-	-	0.917	1.2
t_{cut}	VI***	$t_{cut}=\beta_1 t_{max}$	-	1.7***	-	-	0.860	1.55
t_{cut}	VII***	$t_{cut}=\beta_0+\beta_1 t_{2max}+\beta_2 DM$	16.07*	-1.1398*	1.94***	-	0.998	0.55
$t_{G'30}$	VIII***	$t_{G'30}=\beta_0+\beta_1 t_{2max}$	-1.4^{ns}	1.8***	-	-	0.973	1.33
$t_{G'30}$	IX***	$t_{G'30}=\beta_0+\beta_1 t_{2max}+\beta_2 t_{2max}P$	-0.376^{ns}	2.675***	-0.159***	-	0.997	1.12
$t_{G'30}$	X***	$t_{G'30}=\beta_1 t_{2max}$	-	1.7***	-	-	0.997	1.38
$t_{G'30}$	XI***	$t_{cut}=\beta_0 t_{2min}(1+\Upsilon P)$	2.19	-0.0674	-	-	0.983	2.42[a]
$t_{G'30}$	XII***	$t_{cut}=\beta_0 t_{2max}(1+\Upsilon P)$	2.7***	-0.062***	-	-	0.999	0.35
$t_{G'60}$	XIII***	$t_{G'60}=\beta_0+\beta_1 t_{2max}$	-1.7^{ns}	2.0***	-	-	0.986	1.81
$t_{G'60}$	XIV***	$t_{G'60}=\beta_1 t_{2max}$	-	1.9***	-	-	0.978	1.84
tanδ	XV***	$tan\delta=\beta_0+\beta_1 V0$	0.607***	-0.063*	-	-	0.799	0.005[2]
tanδ	XVI***	$tan\delta=\beta_0+\beta_1 V0+\beta_2 t_{2max}F$	0.456***	-0.098**	0.0002**	-	0.965	0.002[2]
tanδ	XVII***	$tan\delta=\beta_0+\beta_1 V0+\beta_2 t_{2max}F+\beta_3 t_{2max}FP$	0.275***	-0.112**	0.0003**	-0.001*	0.998	0.001[2]

[1]N = 6. Predictors are defined in the text; β_0, β_1, β_2, β_3 regression coefficients; R^2, determination coefficient (corrected for the means); SEP, standard error of predictions; [ns]Not significant; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

[2]Dimensionless.

[a] Regression coefficients taken from Castillo *et al*. (2003b).

Although incorporating milk composition related information reduced the SEP to about a half, model V was still preferable for inline application due to practical reasons.

Model VIII, was the best one-parameter algorithm for prediction of rheological cutting time $t_{G'30}$. It had an $R^2 = 0.973$ and contained the optical parameter t_{2max} and the intercept, as shown in **Table 7.4**.

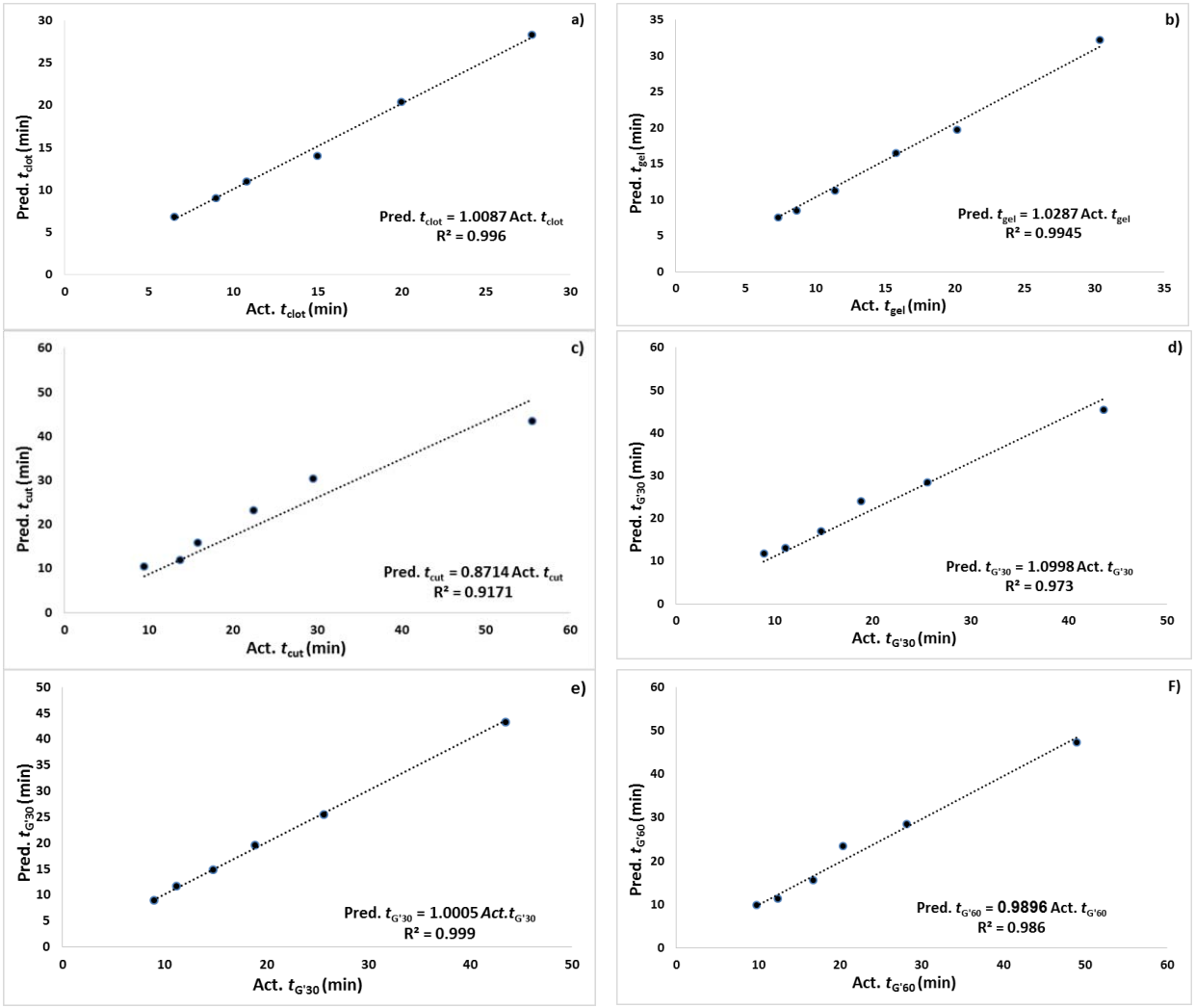


Fig. 7.2. The correlation between actual and predicted some cheese making indices. **a)** Correlation between actual and predicted visual clotting time using model I (Table 7.4). **b)** Correlation between actual and predicted rheological gelation time using model II (Table 7.4). **c)** Correlation between actual and predicted visual cutting time using model V (Table 7.4). **d)** Correlation between actual and predicted rheological cutting time ($t_{G'30}$) using model VIII (Table 7.4). **e)** Correlation between actual and predicted rheological cutting time ($t_{G'30}$) using model XII (Table 7.4). **f)** Correlation between actual and predicted cutting time ($t_{G'60}$) using model XIII (Table 7.4).

In this model, as in previous ones, only the regression coefficient assigned to the term t_{2max} was significant. Model IX (**Table 7.4**) was obtained by adding the optical parameter $t_{2max}P$

into Model VIII, which increased R^2 from 0.973 into 0.997. Even when Model VIII had larger SEP as compared to Model IX (1.33 *vs*. 1.12) the previous one was preferred for inline applications (**Fig. 7.2d)**, and was simplified to equation 7.3, (model X, **Table 7.4**) (SEP =1.38 min) as the intercept was not significant.

$$\textbf{Eqn (7.3)} \qquad t_{G'30} = \beta_1 t_{2\max}$$

Castillo *et al*. (2003b) found that the algorithm (equation 7.4) adequately predicted t_{cut} for a wide range of protein concentrations (3% - 7%), where γ was considered constant (in goat milk γ = -0.067) and (R^2=0.983) (**Table 7.4** (Model XI)).

$$\textbf{Eqn (7.4)} \qquad t_{\text{cut}} = \beta_0 t_{2\min}\,(1 + \gamma\, protein)$$

Such equation is simple but at the same time contains a time-based predictor of cutting time, t_{2min}, and a protein term that corrected the gradient β_0 depending on protein content by using the constant. Thus, theoretically, only the gradient β_0 would require in plant calibration. These authors claimed that the proposed prediction algorithm is based on the fact that gradient β_0 increases significantly as protein concentration increases. It was expected that the effect of protein concentration on this gradient is constant if estimated within a wide range of protein concentrations. Thus, based on the described model by Castillo *et al*. (2003a), an alternative nonlinear Model was tested using NLIN procedure of SAS that would be more practical for inline prediction of cutting time when using milk from CNS-infected glands. Model XI have high SEP (2.42 min) with R^2 0.983. However, taking also into account that in most of the models obtained for prediction of $t_{G'30}$ and $t_{G'60}$ the intercept was not significant, and that the optical parameter t_{2max} was selected by the Maximum R^2 procedure of SAS in most of the prediction terms, a modified version of model XI was tested (Model XII). Model XII had R^2 and SEP values of 0.999 and 0.35 min (reduced by a factor of 7 as compared to model XI), respectively. This model assumed that the effect of *P*, on the gradient β_0 is constant (-0.06; models XI and XII). Thus, for industrial implementation of this model only the gradient β_0 would require in-plant calibration. **Fig. 7.2(e)** shows the relationship between the actual and the predicted cutting time using Model XII.

From our results, we observed that the prediction models for predicting rheological cutting time are working successfully with all the breeds and infection status tested.

Cutting time prediction was alternatively evaluated by defining cutting time at a higher level of firming, $t_{G'60}$ .Models developed were similar to those for $t_{G'30}$ **Table 7.4** (Models XIII and XIV) **(Fig. 7.2f)**.

7.4.6 Prediction of the tan δ value at cutting time

The ratio between the viscous modulus (G″) and the storage modulus is defined as *tan*δ, i.e., *tan*δ = G″/G′; where δ is the phase difference between stress and strain (Marchesseau *et al*., 1997). The value of this rheological parameter is relevant to the rearrangement capability of the gel (Castillo *et al*., 2006b,). Thus, an early estimation of the *tan* δ value that is expected to be achieved at cutting time would be very useful to foresee the tendency of the gel to undergo syneresis at cutting. A high *tan*δ shows increased rearrangement capability and syneresis tendency in the gel as a result of augmented proneness of gel bonds and strands for breaking/relaxing (Vliet *et al*., 1991).

The maximum R^2 procedure of SAS suggested that, the best descriptors for *tan* δ at cutting (i.e., *tan* δ when *G´* reaches a value of 30 Pa) were V_0, t_{2max}F and t_{2max}FP. **Table 7.4** shows the best one-, two- and three- parameters models for predicting *tan* δ at cutting time.

Fig. 7.3 shows the correlation between actual and predicted (tan δ) at cutting time ($t_{G'30}$) using model XVII in **Table 7.4**.

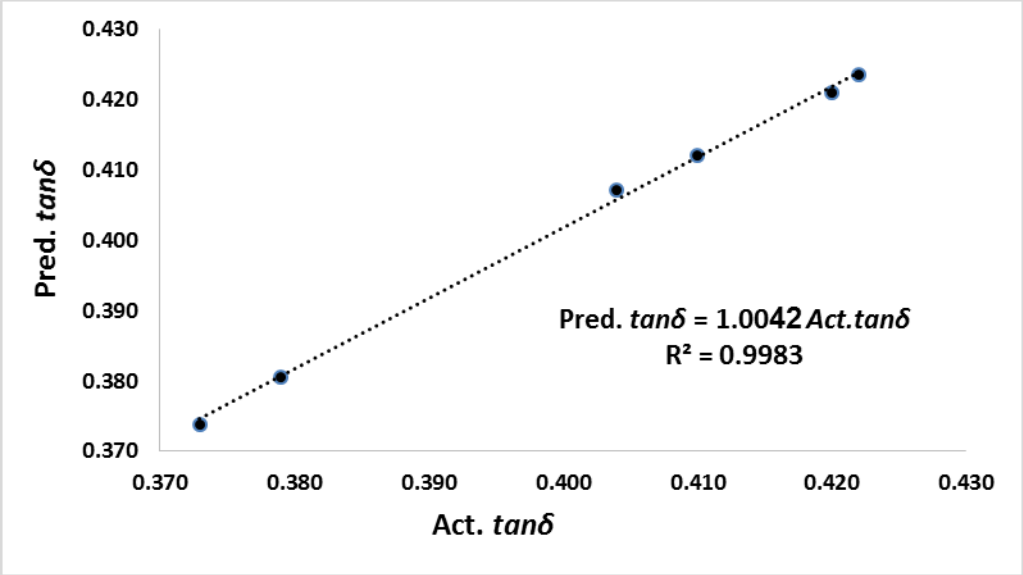


Fig. 7.3. The correlation between actual and predicted (tan δ) value at cutting time ($t_{G'30}$) using Model XVII. See Model XVII in Table 7.4.

From our results, we conclude that V_0 and t_{2max} combined with chemical composition (F and FP) contain all information required for accurate prediction of tan δ. Selection of the response-based optical parameter V_0 *by* the maximum R^2 procedure of SAS was attributed to the existing relationship between the V_0 and both fat and protein concentration. In addition, it is well know that syneresis depends mainly on the permeability and porosity of the curd as well as the endogenous pressure all of which is correlated with both fat and protein percentages in milk. (Castillo *et al*., 2006d) Chemical structure of the casein matrix is directly related to the endogenous pressure, and fat plays no direct role in the formation of the coagulum, but it plays an indirect role by plugging the network pores and vacuoles. A high percentage of fat could also obstruct the neo-formation of links between the caseins during syneresis (Castillo *et al*., 2000). According to our finding, tan δ value at cutting can be predicted as soon as t_{2max} appears. Note that these parameter typical occur before rheological gelation time. No reference was found in the literature by our group regarding early inline prediction of tan δ value at cutting.

7.5 CONCLUSIONS

Our study confirms the effect of subclinical mammary gland infection in sheep with coagulase-negative *Staphylococci* on both milk composition and coagulation properties.

Milk from animals with subclinical mastitis had higher protein, casein, fat concentration and SCC as well as lower lactose concentration than the healthy animals. The observed decrease of lactose concentration balanced out the osmotic effect of increased mineral content due to the infection.

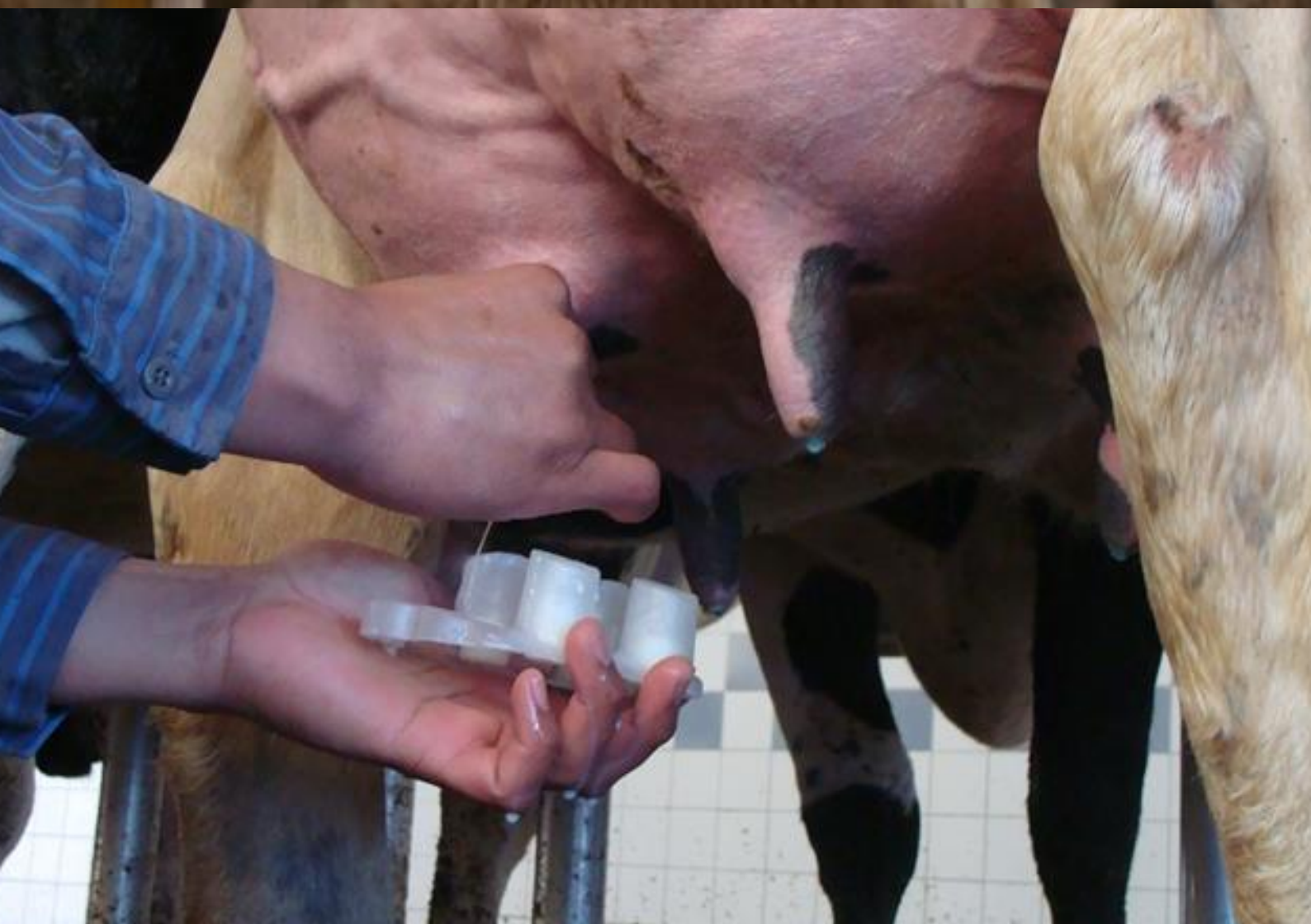
It was observed that the MN milk contained larger fat and protein concentration than *LC* milk while both milk types had similar lactose concentration. Results suggest that the smaller percentage of protein in *LC* as compared to MN milk originated a decrease of the micelle aggregation/firming rate in *LC* milk but, at the same time, it also enhanced the rate of κ-casein hydrolysis reaction, which as a whole resulted in a slight (consistent but not significant) decrease of cutting time. However, no significant differences ($P \geq 0.05$) in the gel tendency for curd rearrangement and whey separation between MN and *LC* ewe milk (as shown by similar *tan* δ values) were observed.

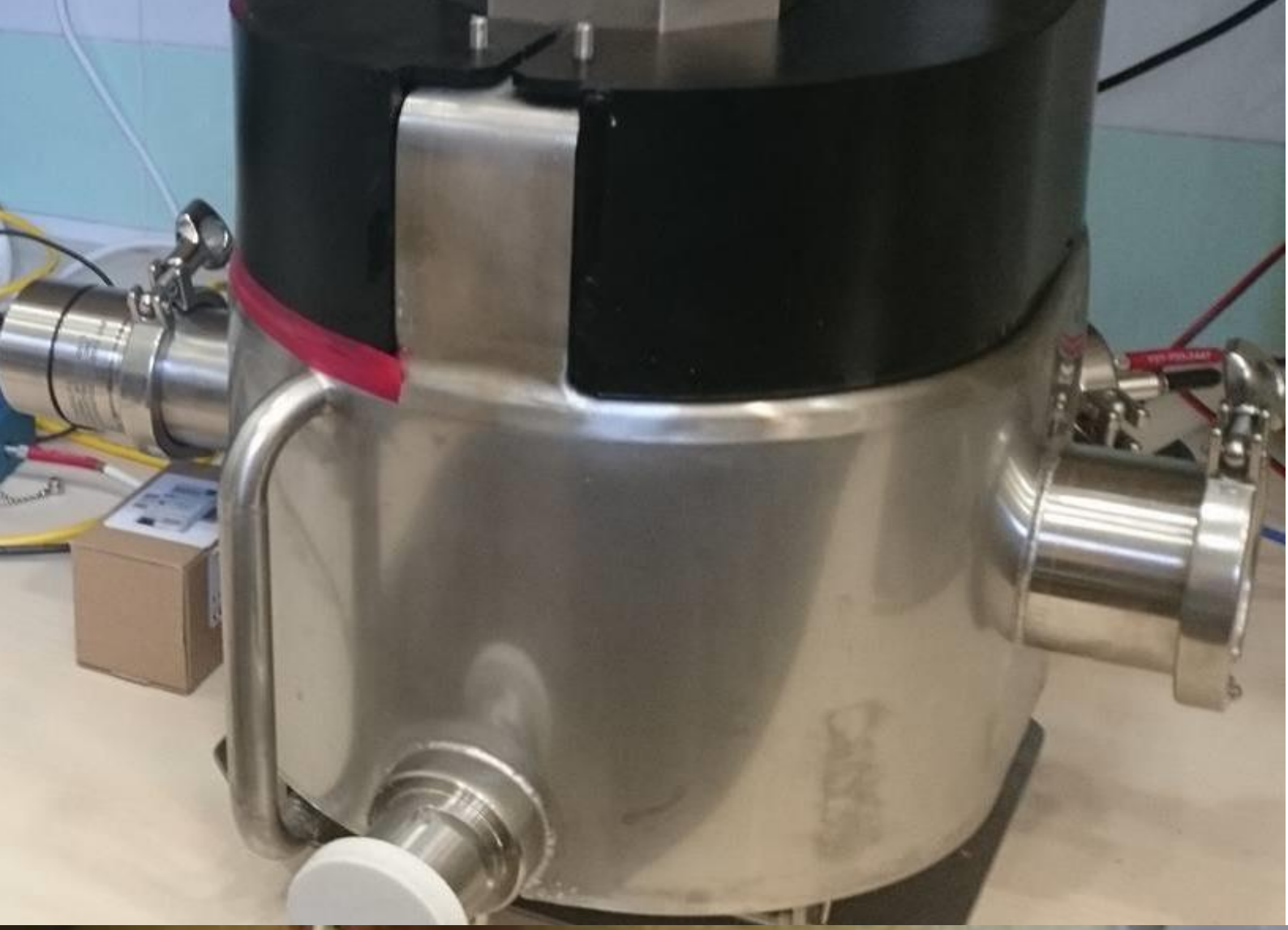
Time based parameters generated from the LB profile varied significantly with SCM infection. Milk of MN ewes seemed to start aggregating and firming later than *LC* ewe's milk. However, gel assembly times, t_{GE30} and t_{GE60}, were as an average, 0.79 min longer for *LC* than for MN. This trend was quite consistent but not significant. These results clearly suggest that a smaller percentage of protein in *LC* milk decreased the time required for κ-casein hydrolysis reaction, inducing a decrease in cutting time, even when the reduced casein concentration also originated a decrease of the micelle aggregation/firming rate in this type of milk with regard to MN milk.

SCM increased significantly ($P<0.05$) optical, rheological and visual parameters in *ILC* as compared with *LC*. These results are in agreement with previous studies reporting that SCM resulted in partial hydrolysis of κ-CN and more pronounced hydrolysis of the other caseins, which affect the coagulation process.

It was observed that milk initial voltage V_0 from LB (affected by fat and protein percentage in the milk) was smaller in milk from animals infected with SCM than in healthy animals even when both milk types have almost the same percentage of fat and protein. This is surprising as according to previous studies increased fat and protein concentration is known to increase V_0. This might suggests severe demineralization of the casein micelles in *ILC* milk. This is expectable as proteolytic enzymes in *ILC* milk have been reported to cause loss of colloidal calcium and demineralization of casein micelles has been related to a decrease in light backscatter. These results suggest that inline LB parameter, V_0, could be used to discriminate milk from infected animals inline.

Irrespectively of the health status or the breed, prediction models using light backscatter parameters alone or in combination with protein/solids concentration were successfully obtained for visually determined clotting and cutting time, rheologically derived gelation and cutting times and for $\tan\delta$ at cutting with R^2 values ranging from 0.799 to 0.999.





Chapter Eight

NIR inline cheese-vat monitoring of milk coagulation and curd syneresis in sheeps with subclinical mastitis

Chapter Eight: NIR inline cheese-vat monitoring of milk coagulation and curd syneresis in sheeps with subclinical mastitis

(Submitted to *Food Control Journal*)

8.1 ABSTRACT

Subclinical mastitis (**SCM**) is one of the most prevalent diseases in dairy cattle, which causes changes in milk. The objective of this study was to evaluate the effect of **SCM** on cheese manufacture from ewe milk using a *NIR* optical sensor technology. The milk used in this study was collected from two different ewe breeds and from glands with no infection and glands infected with various coagulase negative staphylococci (**CNS**). Milk was quickly pasteurized, and coagulated at constant temperature and enzyme concentration. Coagulation was monitored using a *NIR* light backscatter sensor, while whey separation was monitored using a large field of view (*LFV*) syneresis sensor. Optical parameter t_{max} was highly correlated ($r \geq 0.969$) to both visual clotting and cutting times and prediction models were successfully obtained for the two parameters. Results suggested that subclinical infection with **CNS** have an impact on cheese manufacture.

Keywords: Cheese; clotting time; cutting time; yield, light backscatter; prediction; inline; subclinical mastitis

8.2 INTRODUCTION

In some European Mediterranean countries, such as Spain, France Italy, Bulgaria and Rumania, cheese industry plays an important role supporting their large sheep milk production (Martinez *et al*., 2011). Lawrence (1993a) characterized the factors that can affect milk composition and cheese yield identifying mastitis among those. Mastitis causes economic losses to dairy farmers due to reduced milk yields (Heringstad *et al*., 2003). Mastitis can appear both clinical and subclinical, being the latest form the most prevalent one (Maréchal *et al.*, 2011). Subclinical mastitis (SCM**)** is the presence of infection in the mammary gland without apparent signs of local inflammation or systemic affection and requires a diagnostic test for detection (Harmon, 1994a; Wilson *et al*., 1995). The main pathogenic bacteria causing SCM in sheep is coagulase-negative *Staphylococci* **(CNS)**. A more detailed description of causes, pathophysiology, and diagnosis of SCM in sheep can be found in Hogeveen (2005).

On many farms, SCM is economically important because of the long-term effect of chronic infections on total milk yield. Persistent long-term infections with SCM damage milk secretory cells and result in reduced milk production, affecting the yield and quality of milk and, consequently, cheese production and quality. During SCM, the somatic cell count (SCC) increases, and both milk composition and functionality change (Caraviello *et al.*, 2003). During the infection, changes occur in the distribution of the cells by increase of leucocytes (Leitner *et al*., 2012), which add many proteolytic and lipoytic enzymes to milk and in addition, more blood serum leaks into the milk (Le Roux Y. *et al.*, 2003). Protein breakdown in milk produced by sheep with SCM is caused primarily by plasmin, which is found commonly in both milk and blood plasma and can cause extensive damage to milk protein, specially casein, in the udder prior to milking (Leitner *et al*., 2006). Moreover, when milk is cooled, plasmin continues to break casein down but at a slower pace. Plasmin is extremely heat stable, therefore, pasteurization cannot inactivate it and will continue to damage casein during dairy product manufacture and storage (Abdelgawad *et al.*, 2016). As a result, the milk casein does not aggregate properly resulting in longer rennet coagulation time and a weak coagulum, which in turn leads to increased whey fat and protein losses, which derives as a whole into lower cheese yield (Barbano *et al.*, 1991; Auldist *et al.*, 1995; Auldist & Hubble, 1998; O'Brien *et al.*, 2001).

SCM can affect cheese yield by: 1) higher content of plasmin and other proteolytic and lipoytic enzymes, 2) damaged casein, 3) poor curd formation (longer flocculation time, slower

rate of curd firming, and reduced maximum firmness), and 4) increased pH and altered calcium-phosphate-caseinate balance (Banks *et al*., 1981; Lawrence, 1993b). Albenzio *et al*. (2002) and Maréchal *et al.* (2011) clarified that cheese made from the milk of infected animals negatively affects the chemical processes that occur during cheese maturation; despite the fact that the curd mass of cheese made from milk taken from uninfected glands was equal to the curd mass of cheese made from milk taken from infected glands, the final product from milk of the infected glands had lower yield and quality (Merin *et al*., 2004).

Optical sensor technologies based on either light backscatter or transmission have been proven as a successful tool for monitoring milk coagulation (Payne *et al.,* 1993; O'Callaghan *et al*., 1999; Castillo *et al.,* 2000). In particular, the optical fiber light backscatter (880 nm) sensor, CoAguLite^{TM} is a well-documented inline sensor technology to monitor milk coagulation and predict both clotting and cutting times in various types of milk (Payne *et al.,* 1993; Castillo, 2001; Castillo *et al.,* 2005a; Abdelgawad, 2011; Abdelgawad *et al.,* 2014, 2016). In previous studies, a large field of view sensor (*LFV*) using 980 nm light for monitoring both milk coagulation and curd syneresis was designed by Castillo *et al.* (2005a) and evaluated over a range of cutting times, temperatures and calcium chloride levels (Fagan *et al*., 2007a).

Both fiber optic sensor technologies mentioned above, CoAguLite^{TM} and *LFV*, have been specifically developed for cow milk. The CoAguLite^{TM} sensor was tested successfully with goat milk (Castillo, 2001), sheep milk (Nicolau *et al*., 2010) and mixtures of milk from different animals (Abdelgawad *et al.,* 2014). However, no information is available regarding the effect of mixing in the refrigeration tank normal milk with milk from animals with SCM. As discussed by Abdelgawad *et al.* (2016), failure to detect subclinical infection by the farmer and high prevalence (up to 15 to 40%) result in milk from uninfected and subclinically infected glands in the tank. If all animals in a dairy sheep flock having the mentioned prevalence are milked into the milk tank, about 15 to 30% of the milk would be of infected glands (Leitner *et al*., 2004, 2006, 2008, and 2011a).

The objective of this study was to evaluate if light backscatter could be used to monitor coagulation and whey separation processes during Manchego-type cheese manufacture at pilot plant level (10L vat) using milk from subclinically infected or uninfected ewe. This main objective was divided into three specific objectives: **(a)** studying coagulation, cutting and syneresis properties during cheese manufacture from different ewe breeds (Lacaune (***LC***) and

Manchega (***MN***) using a pilot plant scale *NIR* light backscatter sensor, **(b)** investigating the effect of SCM by CNS on the light backscatter profile of coagulation and whey separation during the cheese making process, and **(c)** validate visual cutting time prediction models from our previous studies; and obtaining complementary prediction models for several cheese making indices i.e., curd moisture content, cheese yield, yield of whey and fat losses to whey.

8.3 MATERIALS and METHODS

Milk was obtained from the Experimental Farm of the Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain). A total of 32 lactating dairy sheep of two breeds (*MN*; n=14, *LC*; n=18) at mid to end lactation (80-150 days in milk) were used. These animals housing, management, bacterial infection, milking and sampling for bacteriological testing was handled using a half-udder model to assess the uninfected or infected glands intramammary status with various CNS species as described by Rovai *et al.* (2015) and Abdelgawad *et al.*, (2016). The half-udder model has been widely used (Gonzalez-Rodriguez *et al*., 1995; Leitner *et al*., 2004, 2006, 2011a; Martí-De Olives *et al*., 2013) with excellent results in order to study the passive effect of the SCM on milk yield and quality with high statistical accuracy. The experimental model removes the significant variations between individual animals (e.g., genetic, stage of lactation, nutrition, etc.) as the unit of comparison corresponds to the two glands of the same animal. The two different dairy sheep breeds: *LC* and *MN* were used to establish base for milk of uninfected animals. The influence of intramammary infection was studied using *LC* sheep with one gland uninfected and the contralateral infected with CNS (***ILC***). Each milk type was tested twice.

The milk coagulation experiments were conducted at Centre Especial de Recerca Planta Tecnologia dels Aliments (CERPTA) (UAB). Unpasteurized and unhomogenized ewe milk (*LC*, *ILC*, *MN*) was stored at 4 °C right after collection and until it was pasteurized and coagulated (**Fig. 8.1**). Before pasteurization, samples of milk were taken for chemical analysis and determination of the chemical composition was performed as described by Abdelgawad *et al.* (2016). On the test day the different batches of milk were pasteurized at 65 °C for 30 min and then immediately cooled to the target milk coagulation temperature (35 °C). The corresponding 10L ewe milk was added to a 10L cheese vat, and left until thermal equilibrium was achieved.

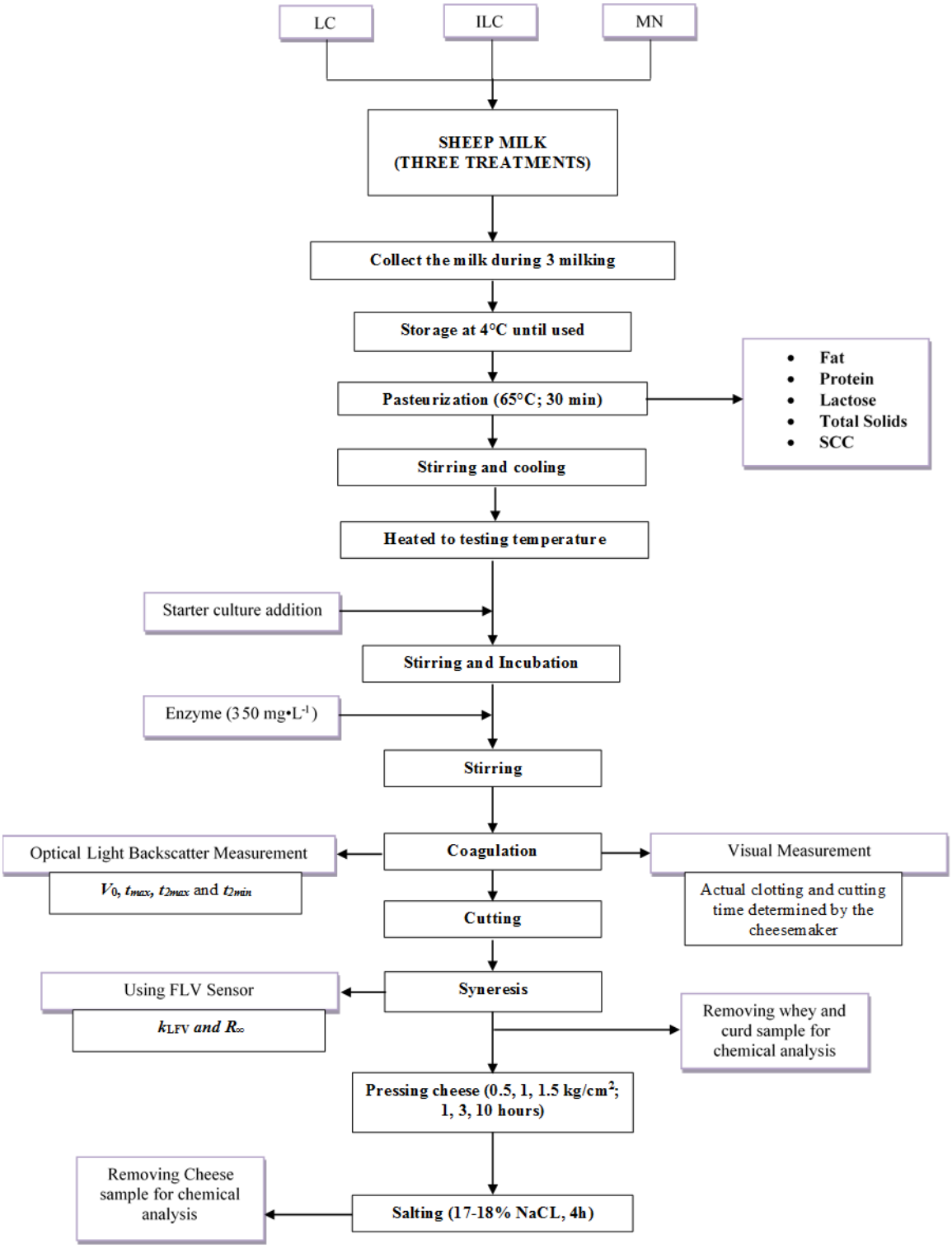


Fig.8.1. Flow chart of testing procedure. *MN*, Manchega; *LC*, Lacaune; *ILC*, Lacaune CNS-infected milk; *SCC*, somatic cell count. The rest of parameters are explained in the text.

Then a Manchego-type cheese starter (Poveda *et al.*, 2004), which was previously prepared as described by (Abdelgawad *et al.*, 2016), was added to the milk (2% *w/w*), and rapidly stirred with the milk and incubated at the target temperature for 30 min. After the incubation, bovine rennet (Abdelgawad*et al.*, 2016) was added at a concentration (E_0= 350 mg·L^{-1} milk) and the mixture was rapidly stirred during 30 s. Once enzyme was added to the milk, milk coagulation monitoring was initiated.

8.3.1 In-line light backscatter monitoring instruments

Pilot plant scale, in-line monitoring of milk coagulation and curd syneresis in a 10L double-jacket cheese vat (CAL 10L, Pierre Guerin Technologies, Mauze, France) (**Fig. 8.2a**) was performed using two different fiber optic sensor technologies, the CoAguLite™ (***CL***) (**Fig. 8.2b**) and the Large Field of View (***LFV***) sensors (**Fig. 8.2c**), which were placed in the wall of the cheese vat.

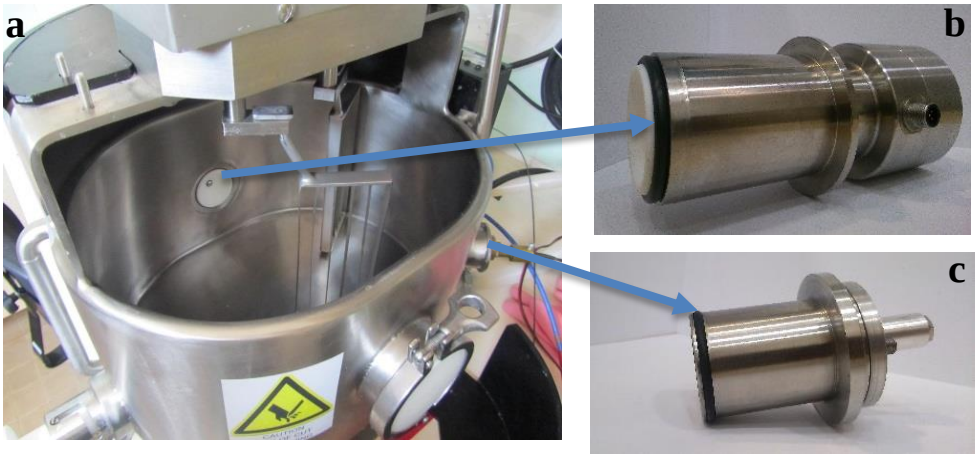


Fig. 8.2. (a) Cheese Vat showing the twin cutting blades used to cut the milk gel. (b) The CoAguLite™ sensor used for monitoring milk clotting and cutting time. (c) LFV sensor used for monitoring milk coagulation and curd syneresis during cheese manufacture.

Optical response from both sensors was continuously monitored from the time of rennet addition to the end of syneresis. Clotting time (t_{clot}) and cutting time (t_{cut}) were visually determined by an experienced cheese maker as described by Abdelgawad *et al.* (2014). Coagulation temperature was controlled (35 ± 0.1 °C) using the double-jacket cheese vat supplied with temperature controlled water through a couple of plastic coils connected to the water bath digital refrigerated immersion thermostat (OvanTherm C-TC00E, Suministros Grupo Esper, S.L. ES). Milk temperature was measured with a precision thermistor (model

5831 A, Omega Engineering, Stamford, CT; resolution ± 0.1 °C; accuracy ± 0.2 °C). Flow chart of the testing procedure is summarized in **Fig. 8.1.**

A *CL Sensor* (**Fig. 8.2b**) (model 5, Reflectronics Inc., Lexington, KY) transmitted near-infrared light at (880 nm) through two 600 μm diameter fibers. One fiber transmitted infrared radiation into the milk sample while the other fiber transmitted the radiation scattered by the milk particles to a silicon photodetector. Response data were collected every 6 s. Output voltage was zeroed to 1 V. Response data were collected every 6 s. The initial voltage response ($\boldsymbol{V_0}$) was calculated by averaging the first ten data points after correction for the 1V zero offset. A light backscatter ratio ($\boldsymbol{R}$) was calculated by dividing the sensor output voltage (less the 1V zero output) by V_0. The first ($\boldsymbol{R'}$) and second ($\boldsymbol{R''}$) derivatives of the light backscatter ratio profile as a function of time were calculated by as described by Castillo (2001). Three coagulation optical parameters: t_{max}, t_{2max}, and t_{2min}, were obtained from the light backscatter profile. t_{max} was the elapsed time from enzyme addition to the first maximum of $\boldsymbol{R'}$, t_{2max} was the elapsed time from enzyme addition to the first maximum of $\boldsymbol{R''}$and t_{2min} was the elapsed time from enzyme addition to the first minimum of $\boldsymbol{R''}$.

Further details on the *CL* sensor and data acquisition system as well as on the light backscatter profile regeneration and parameters definition were presented by Castillo *et al*, (2000; 2006b), Payne and Castillo (2007) and Abdelgawad *et al*. (2014).

It was necessary to calibrate the CoAgulite^{TM} before using the device in the coagulation monitoring process since the type of milk, fat globule seize, and fat/protein ratio are mainly factors affecting the initial voltage detected by the sensor. Calibration process described in details as it was described by Castillo (2002) and Nicolau *et al.* (2015).

The *LFV* sensor was a syneresis sensor prototype designed at the University of Kentucky (Castillo *et al.*, 2005b; Castillo *et al.*, 2007) **Fig. 8.2c**. A schematic for the sensor design as published by Fagan *et al*. (2007a) is shown in **Fig. 8.3.** Light from a 6 W tungsten halogen source (model LS1B, Ocean optics, Inc., Dunedin, FL, USA; spectral range of 360–2000 nm) was transmitted through a large diameter (0.5 cm) optical fiber (model FTICR19733, Fiberoptics Technology, Inc., Pomfret, CT, USA), a vertical polarizer (model 43-782, Edmund Optics, Inc., Barrington, NJ, USA), and a glass window (model 02 WBK 224, 27 Melles Griot Inc., Rochester, NY, USA) to the sample. The large-diameter (2 cm) glass window allows

scattered light to be collected from a large area. Another polarizing plate allows for the selective detection of horizontally polarized light to ensure that any light reflected by the window was eliminated. Reflected light is transmitted through another optical fiber and a collimating lens (Edmund Optics Inc.) that focuses the scattered light onto a 800 µm diameter fiber optic cable (Spectran Specialty Optics, Avon, CT, USA) to a miniature fiber optic spectrometer (model HR2000CG-UVNIR, Ocean Optics B.V., Duiven, Netherlands). Spectra were collected over the range of 300–1100 nm with a resolution of 0.7 µm. The integration time was set to 6s by the computer software (SpectraSuite software v. 5.1, Ocean Optics Inc.). Each spectral scan was automatically processed by subtracting the dark background spectral scan. Each spectral scan was reduced to 41 averages by dividing them into 20 nm wavebands with mid-wavelengths of $280 + 20n$ ($1 \leq n \leq 41$), giving 41 wavebands in the range (300–1100 nm) and averaging the optical response for the wavelengths constituting each waveband. The voltage readings (sensor output) for the first minute of data were averaged within each waveband to calculate the initial voltage response, V_0. The voltage intensity at every waveband, $V(w)$, was divided by its corresponding $V_0(w)$ to obtain the light backscatter ratio, $R(w)$. The kinetic rate constant (min^{-1}) for the syneresis process, k_{LFV}, was estimated from the *LFV* light backscatter ratio at 980 nm, $R(980)$ (on ahead just R^*). To that end, the *LFV* sensor response during syneresis was fitted using a least squares method and the Solver utility tool from Microsoft Excel software (version 15.0.4979, Microsoft) to a first order equation as follows:

$$\textbf{Eqn (8.1)} \qquad R_t^* = R_\infty^* + (R_0^* - R_\infty^*)e^{-K_{LFV}t}$$

where R^*_t was the light backscatter ratio at time t (min), R_∞ was the light backscatter ratio at an infinite time, R^*_0 was the light backscatter ratio at cutting time (i.e., at time zero for the syneresis process), and k_{LFV} was the kinetic rate constant (min^{-1}) for the *LFV* sensor response during syneresis.

According to Castillo *et al.* (2005b, 2007) and Fagan *et al.* (2007b), the kinetic rate constant for the *LFV* sensor response (k_{LFV}) is highly related with the constant of syneresis of the cheese (k_S). Costa et al. (2012) used k_{LFV} as an estimate of kinetic rate constant. Another syneresis parameter, ΔR, was obtained from the *LFV* sensor response by subtracting R_∞ from R_0.

8.3.2 Sampling, compositional analysis, and yield

Once the gel reached the adequate firming for cutting, it was cut. The cutting procedure, sampling of curd, whey and cheese and further information about the compositional analysis of curd, whey and cheese are found in chapter five: material and methods, section 5.3 and 5.7.

During the cheese processing, the weight of milk transformed into cheese, ***M***; as well as the generated weight of curd, ***C***; salted cheese, ***Ch***; and whey, ***W*** were recorded. A number of samples were collected to determine the percentage of milk components such as: lactose, $\boldsymbol{M_L}$; fat, $\boldsymbol{M_F}$; protein $\boldsymbol{M_P}$; casein, $\boldsymbol{M_{CS}}$; total solids, $\boldsymbol{M_{TS}}$ as well as the percentage of whey components such as: fat, $\boldsymbol{W_F}$; protein, $\boldsymbol{W_P}$ and the percentage of cheese components such as: moisture content, $\boldsymbol{Ch_M}$; fat, $\boldsymbol{Ch_F}$; protein $\boldsymbol{Ch_P}$; total solids, $\boldsymbol{Ch_{TS}}$.

Wet basis cheese yields, $\boldsymbol{ChY_W}$, and dry basis cheese yields, $\mathbf{ChY_D}$, were calculated from mass balance, while theoretical Van Slike yield**,** $\boldsymbol{ChY_T}$, was calculated using Van Slyke equation (Van Slyke, 1894).

8.3.3 Statistical Analysis

Detailed description of statistical methods used for analysing the data obtained from this experiment can be found in chapter chapter five: material and methods, section: 5.9, and chapter seven section 7.3.6.

8.4 RESULTS and DISCUSSION

8.4.1 Analysis of variance

An (ANOVA) analysis was conducted to determine the major sources of variation in the studied dependent variables as a function of the main treatments (***MN***, ***LC*** and ***ILC*****)**. **Table 8.1** presented the results for the most important dependent variables studied, including the $\boldsymbol{R^2}$ values and ***F*** statistics. Models were found to be statistically significant for all dependent variables tested ($P<0.05$). Most of the significant differences observed were found between milk from *LC* and *ILC* except for chemical composition parameters excluding M_L (i.e., M_F, M_P, M_{CS}, M_{TS}). Significant differences were also observed for M_F, M_P, M_{CS}, M_{TS}, ChY_w, ChY_D, ChY_T, ΔR, and k_{LFV} between healthy animals of both breeds, *LC* and *MN*.

8.4.2 Typical light backscatter profile during milk coagulation

The typical light backscatter (***LB***) ratio profile during coagulation has been widely shown by several authors (Payne and Castillo, 2007; Abdelgawad, 2011; Abdelgawad *et al.*, 2014, 2016; Nicolau *et al.*, 2015). It has been stated that the general shape of the profile observed in this study (*MN*, *LC* and *ILC*) is similar in cow, goat, sheep milk and mixtures of milk from different ruminants (cow, goat and sheep). The *LB* ratio profile obtained during coagulation, which usually begins with a value of one and represents the increase in signal during coagulation, includes latent, sigmoidal, and asymptotic periods, irrespective of the type of milk being coagulated. For more details regarding the typical *LB* profiles during coagulation, see Abdelgawad *et al.* (2014). In this study, it was confirmed that milk form *LC* breed suffering subclinical mastitis (i.e., *CNS-ILC*) presented typical *LB* coagulation profiles as compared with healthy *LC* and *MN* animals. The major difference observed was not related to the shape of the profile but to the curve displacement to the right as a result of a decreased coagulation velocity.

8.4.3 LFV sensor spectral response during coagulation and whey separation

Previous work (Castillo *et al.*, 2005; Fagan, 2007a) showed that light backscatter at 980 nm was potential for monitoring milk coagulation as well as the status of syneresis in the cheese vat using just a single sensor. Using the *LFV* sensor, Castillo *et al.* (2005a) and Fagan *et al.* (2007a) observed a sigmoidal increase of the *LB* ratio during coagulation followed by an exponential signal decrease after gel cutting (i.e., curd syneresis stage) at different wavelengths.

For both stages: coagulation and syneresis, the *LFV* sensor response was greatest at 980 nm as compared to other wavelengths. In addition, at this wavelength the *LFV* sensor also incorporated less noise than at other wavelengths. These results were widely confirmed in the preliminary stages of our study and, as a result, 980 nm was selected as the optimum monitoring wavelength in this research.

A typical light backscatter profile derived from the *LFV* sensor during coagulation and syneresis at a wavelength of 980 nm is shown in **Fig. 8.3**.

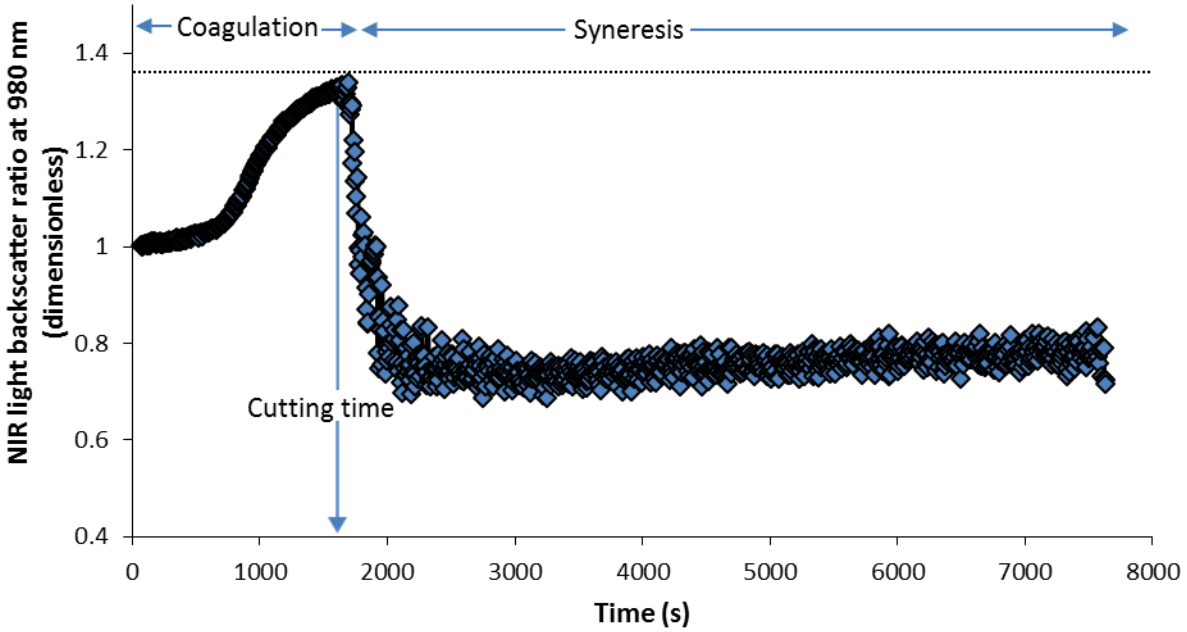


Fig. 8.3. Typical LFV sensor response (average of two replicates) during coagulation and syneresis. Time is measured from starter addition (t = 0 min) for the syneresis process.

8.4.4 Effect of breed in the composition and coagulation of the milk, curd syneresis and cheese yield

The effect of breed on LSMs of milk composition parameters (M_L, M_F, M_P, M_{CS}, and M_{TS}) is shown in **Table 8.1**.

MN milk had higher protein, fat, casein and total solids concentration than *LC* milk ($P < 0.05$) in agreement with Abdelgawad *et al.* (2016) and Moussaoui (2009). As expected, no significant difference with regard to SCC was encountered between *LC* and *MN* milks, as observed by Abdelgawad *et al.* (2016). The level of *SCC* (log SCC) in both *LC* and *MN* milks corresponded to high quality milk according to classification of sheep milk suggested by Leitner (2008). No significant difference was observed for lactose concentration. In agreement with this, Moussaoui (2009) claimed that breeding had no effect on milk lactose concentration.

It is well-known that cheese yield is affected mainly by milk composition and technological pretreatments applied to milk (Lawrence, 1993a). A plethora of factors affect milk composition (e.g., breed, stage of lactation, seasonal changes, etc.) while large variations are encountered regarding cheese making milk pretreatments (e.g., milk standardization, heat treatment, homogenization of milk, etc.). Also Abdelgawad *et al.* (2016) stated that poor milk

clotting typically leads to losses in cheese yield and low cheese quality, which might require an adaptation in the technological process. A detail list of factors affecting cheese yield can be found at Lawrence (1993b) and Abdelgawad and Ahmed (2011).

Table 8.1. Analysis of variance and influence of main effects on the dependent variables studied[1]

	Analysis of Variance		Influence of main effects on dependent variables				
					Main effect (DF=2)		
Model	R^2	F	Parameter		***MN*** (LSM)	***LC*** (LSM)	***ILC*** (LSM)
$\mathbf{M_L}$	0.895	11.6*	$\mathbf{M_L}$	(%)	4.620[a]	4.620[a]	4.370[b]
$\mathbf{M_F}$	0.116	0.190*	$\mathbf{M_F}$	(%)	7.100[a]	6.785[b]	6.735[b]
$\mathbf{M_P}$	0.887	11.8*	$\mathbf{M_P}$	(%)	6.690[a]	5.485[b]	5.560[b]
$\mathbf{M_{CS}}$	0.903	14.0*	$\mathbf{M_{CS}}$	(%)	5.655[a]	4.580[b]	4.605[b]
$\mathbf{M_{TS}}$	0.898	13.3*	$\mathbf{M_{TS}}$	(%)	12.54[a]	11.09[b]	11.07[b]
$\boldsymbol{V_0}$	0.384	0.930*	$\boldsymbol{V_0}$	(volts)	2.605[a]	2.515[b]	2.480[c]
$\boldsymbol{t_{max}}$	0.989	132**	$\boldsymbol{t_{max}}$	(min)	5.950[a]	5.650[a]	14.75[b]
$\boldsymbol{t_{2max}}$	0.978	66.2**	$\boldsymbol{t_{2max}}$	(min)	4.600[a]	4.200[a]	12.25[b]
$\boldsymbol{t_{2min}}$	0.869	9.95*	$\boldsymbol{t_{2min}}$	(min)	7.200[a]	6.650[a]	16.70[b]
$\boldsymbol{t_{clot}}$	0.935	21.4*	$\boldsymbol{t_{clot}}$	(min)	6.500[a]	6.450[a]	15.40[b]
$\boldsymbol{t_{cut}}$	0.932	20.4*	$\boldsymbol{t_{cut}}$	(min)	11.55[a]	10.75[a]	26.10[b]
$\mathbf{ChY_W}$	0.771	5.04*	$\mathbf{ChY_W}$	(%)	21.94[a]	20.18[b]	18.77[c]
$\mathbf{ChY_D}$	0.164	0.290*	$\mathbf{ChY_D}$	(%)	78.76[a]	74.88[b]	72.16[c]
$\mathbf{ChY_T}$	0.870	3.06*	$\mathbf{ChY_T}$	(%)	31.76[a]	28.96[b]	25.46[c]
Wh	0.833	7.49*	**W**	(kg)	7.830[a]	7.975[a]	8.200[b]
ΔR	0.248	0.490*	**ΔR**	(Dim^y)	0.489[a]	0.569[b]	0.728[c]
$\boldsymbol{k_{LFV}}$	0.297	0.640*	$\boldsymbol{k_{LFV}}$	(min^{-1})	0.336[a]	0.466[b]	0.563[c]
***log* SCC**	0.959	35.9**	***log SCC***		5.170[a]	5.320[a]	6.270[b]

[1]Number of observations, N=6; *MN*, Manchega; *LC*, Lacaune; *ILC*, Infected Lacaune with subclinical mastitis; R^2, determination coefficient; F, ANOVA F-statistic; M_L, percentage of lactose in the milk; M_P, percentage of protein in the milk; M_F, percentage of the fat in the milk; M_{CS}, percentage of the casein in the milk; M_{TS}, percentage of the total solids in the milk; V_0, milk initial voltage; t_{max}, elapsed time from enzyme addition to the first maximum of the first derivative; t_{2max}, elapsed time from enzyme addition to the first maximum of the second derivative; t_{2min}, time to the first minimum of the second derivative; t_{clot}, clotting time monitored visually; t_{cut}, cutting time monitored visually ; ChY_W, cheese yield wet basis; ChY_D , cheese yield dry basis; ChY_T, cheese yield using van slyke equation; *Wh* , the weight of whey; ΔR, was obtained from the *LFV* sensor response by subtracting R_∞ from R_0. k_{LFV}, estimated apparent kinetic rate constant (min^{-1}) for the syneresis process; *SCC*, log somatic cell count

[a]Least squares means (LSM) with the same letters are not significantly different P<0.05.

*Analysis of variance.

[2]R^2, determination coefficient; F, ANOVA F-statistic; DF, degree of freedom;

Dim^y, Dimensionless

**P<0.05, **P<0.01, ***P<0.001*; [ns]not significant;

The effect of the breed on the optical parameter V_0 is shown in **Table 8.1**. V_0 was higher in *MN* as compared with *LC*. It is well documented that V_0 increases with increasing protein and fat contents in milk (Castillo *et al.*, 2003b), in agreement with Abdelgawad *et al.* (2016). The observed positive correlation between V_0 and both M_F and M_P in **Table 8.2** supports the mentioned effect of fat and protein content in milk on V_0.

Table 8.2. Pearson correlation between parameters[1].

Parameter	M_L	M_P	M_F	M_{CS}	t_{max}	t_{2max}	t_{2min}
V_0	0.286^{ns}	0.798^{b}	0.892^{b}	0.611^{ns}	-0.422^{ns}	-0.465^{ns}	-0.331^{ns}
t_{max}	-0.915^{b}	-0.386^{ns}	-0.256^{ns}	-0.459^{ns}	-	0.997^{c}	0.919^{b}
t_{2max}	-0.887^{b}	-0.411^{ns}	-0.320^{ns}	-0.464^{ns}	0.997^{c}	-	0.912^{a}
t_{2min}	-0.882^{b}	-0.490^{ns}	-0.308^{ns}	-0.653^{ns}	0.919^{b}	0.912^{a}	-
t_{clot}	-0.884^{b}	-0.281^{ns}	-0.314^{ns}	-0.384^{ns}	0.979^{c}	0.978^{c}	0.929^{b}
t_{cut}	-0.932^{b}	-0.276^{ns}	-0.223^{ns}	-0.426^{ns}	0.969^{c}	0.959^{b}	0.949^{b}
ChY_W	0.306^{ns}	0.762^{a}	0.921^{b}	0.834^{a}	-0.864^{c}	-0.827^{a}	-0.866^{a}
W_Y	-0.885^{b}	-0.666^{ns}	-0.379^{ns}	-0.807^{a}	0.816^{a}	0.807^{a}	0.882^{a}
Log SCC	-0.869^{b}	-0.428^{ns}	-0.458^{ns}	-0.473^{ns}	0.971^{c}	0.993^{c}	0.966^{b}

[1]Number of observations, N=6; M_L, percentage of lactose in the milk; M_P, percentage of protein in the milk; M_F, percentage of the fat in the milk; M_{CS}, percentage of the casein in the milk; V_0, milk voltage; t_{max}, elapsed time since enzyme addition to the first maximum of the first derivative; t_{2max}, elapsed time since enzyme addition to the first maximum of the second derivative; t_{2min}, time to the first minimum of the second derivative; t_{clot}, clotting time monitored visually; t_{cut}, cutting time monitored visually; ChY_W, cheese yield wet basis; W_Y, weight of whey; *SCC*, log somatic cell count
$^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$; ^{ns}not significant;

The effect of breed in milk clotting properties is also shown in (**Table 8.1**). According to Abdelgawad *et al.* (2016), there was no significant breed effect on milk coagulation ability when comparing *MN* and *LC* milks. However, these authors found a consistent decrease of milk coagulation time parameters in *LC* milk as compared to *MN* milk. As it can be observed in **Table 8.1**, our results clearly confirm the results obtained by Abdelgawad *et al.* (2016). LSMs of optical time parameters, t_{max}, t_{2max}, and t_{2min}, and visual time parameters, t_{clot}, and t_{cut}, were consistently larger in *MN* milk than in *LC* milk, although the tendencies were found to be not significant (P<0.05). These trend was attributed to the differences in protein and casein concentration between the two sheep breeds.

As it is indicated in **Table 8.1**, *LC* milk had lower protein and casein concentration than *MN* milk. Castillo (2001) and Castillo *et al.* (2003a) determined that at clotting conditions where the coagulation enzyme could be saturated by the substrate, hydrolysis of κ-casein

becomes the limiting reaction (Carlson *et al.*, 1987) and, as a result, it might yield longer clotting and cutting times, even if the aggregation and firming rates are increased by increased protein concentration (*MN vs. LC*).

The effect of breed in the syneresis kinetics is shown in **Table 8.1**. It is well-known that during syneresis, the curd matrix shrinks due to rearrangement of casein micelles, resulting in expulsion of whey from the curd grains (Castillo *et al*., 2006b).

It is also well documented that casein is the main milk component affecting curd firmness, syneresis rate, and moisture retention and, as a result, affecting cheese quality and yield (Lawrence, 1993c). Syneresis influences protein and fat losses in whey, which in turn affects cheese yield. LSMs of optical parameter $\boldsymbol{k}_{\mathbf{LFV}}$ (apparent syneresis kinetic rate constant, min^{-1}, estimated using light backscatter changes during whey separation in the cheese vat), whey yield and ΔR for both *MN* and *LC* milk are presented in **Table 8.1**. **Fig. 8.4a,b** showed the typical *LFV* light backscatter ratio as a function of time during milk coagulation and curd syneresis for *MN* and *LC* cheese manufacture.

Fig. 8.5a,b. shows that the experimental LB data after gel cutting, *R*, fitted consistently a first order-based kinetic model (Eqn. 8.1) in both types of milk: *MN* and *LC* (R^2 = 0.94 and 0.91).

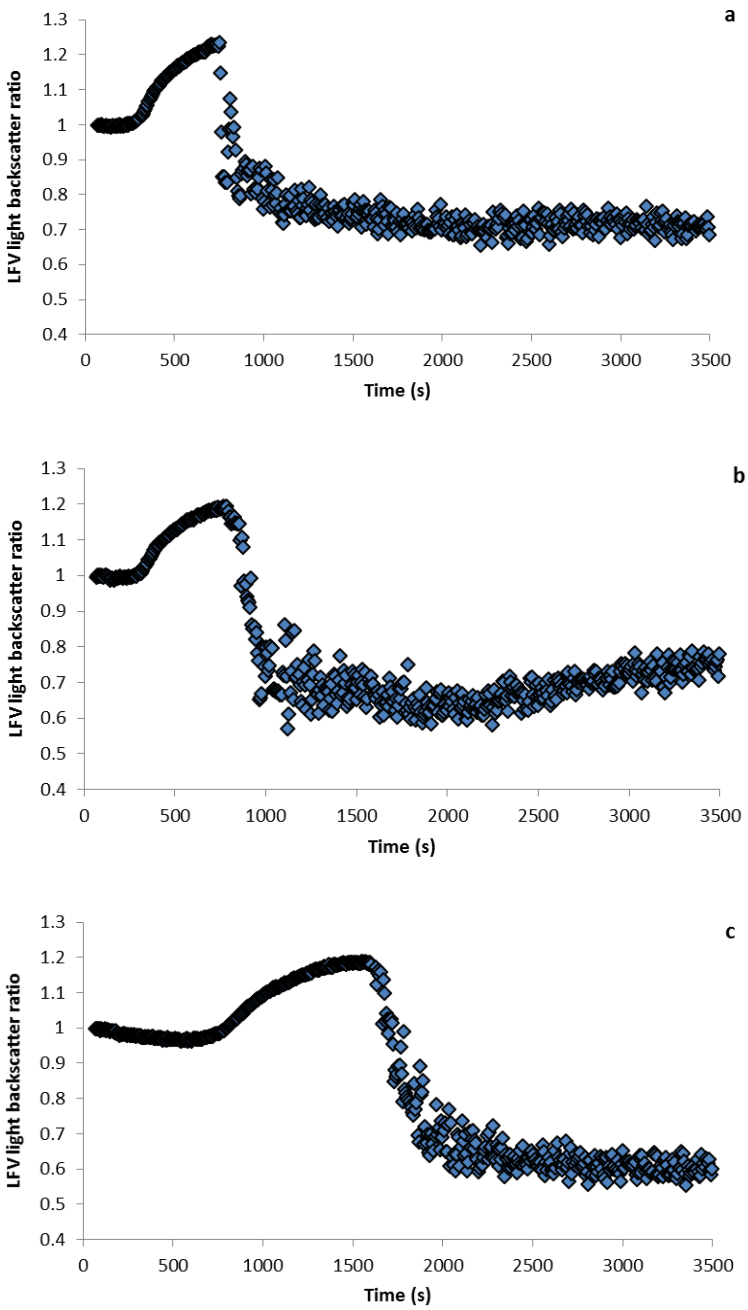


Fig. 8.4. Average of two LFV sensor profile replicates for treatments (a: *MN*, Manchega), (b: *LC*, Lacaune), (c:*ILC*, Lacaune CNS-infected milk) under temperature 35 °C.

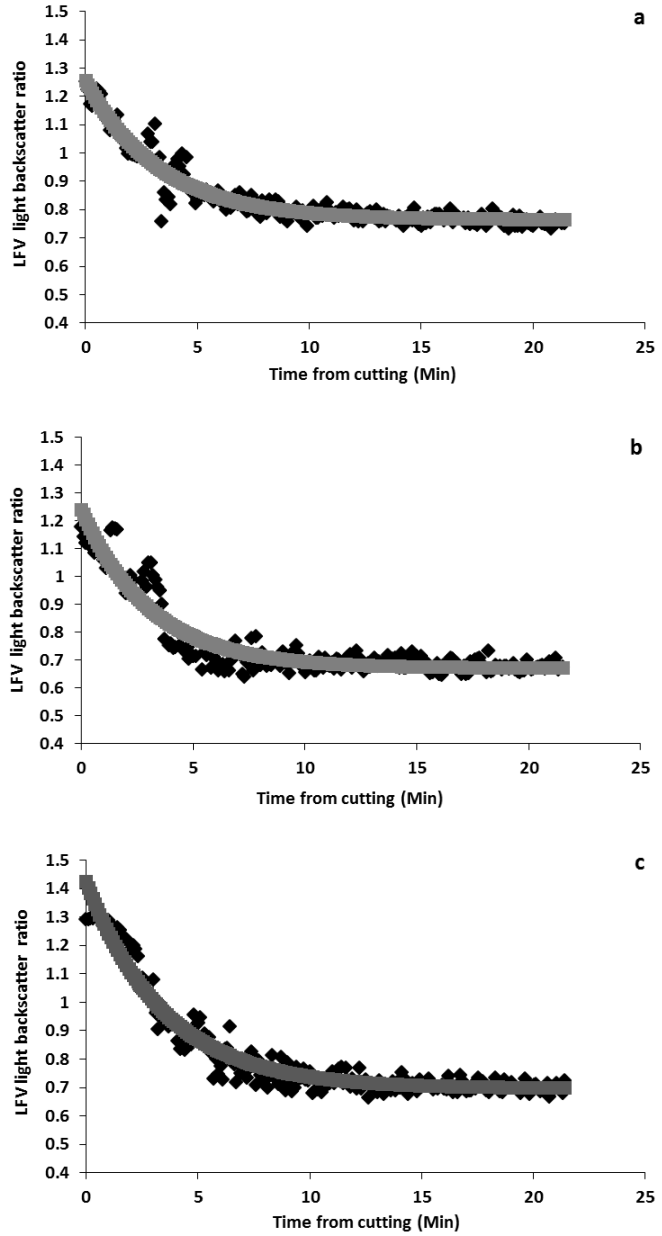


Fig. 8.5. Kinetics of the LFV light backscatter ratio as a function of time during syneresis at 35 °C of the average of two replicates for the three milk types. (a: *MN*, Manchega), (b: *LC*, Lacaune), (c:*ILC*, Lacaune CNS-infected milk) at 35 °C. The average value of the experimental data (♦) at the test conditions is shown (n = 2). The solid line is the theoretical (fitted or predicted) curve assuming first order kinetics (eqn. 1).

As it was stated in the material and methods section, the *LFV* response after cutting is highly correlated with syneresis rate constant of the cheese. As a result, data from LFV sensor after cutting were adjusted to a first order equation (Equ.8.1) to obtain k_{LFV}.

As it is stated in **Table 8.1** and graphically shown in **Fig 8.6a**, the breed has a significant effect on $\boldsymbol{k_{LFV}}$. Since the LSMs of k_{LFV} was significantly ($P < 0.05$) higher in *LC* curd as compared with *MN* curd, higher whey separation was expected in that type of milk (*LC*) during syneresis.

However, this tendency was observed but not significant ($P > 0.05$) as it is shown in **Table 8.1**, and **Fig. 8.6b**, maybe as a result of variation between replications of the treatments. Note that whey is likely one of the cheese making components more directly associated to mass balance losses during cheese making. However, a significant and positive correlation (r =0.998; $P < 0.05$) between whey yield (W_Y) and ΔR (**Fig 8.6c)** was observed, which suggest that the more curd whey release occurring in the cheese vat during the stirring period the larger the *LFV* sensor response (i.e., larger ΔR value). Taking the correlation between ΔR and W_Y into consideration, thus, the significantly larger k_{LFV} and ΔR values observed in LC milk (**Table 8.1** and **Fig 8.6a)** is consistent with the significantly decreased cheese yield encountered in this type of milk as compared to *MN* one **(Table 8.1** and **Fig 8.6b)**. Previous studies showed that the variation of the milk composition due to the animal breeding has an effect on both curd and cheese yield (Lawrence, 1993a). This results can be graphically observed in **Fig. 8.6d**, which shows the normalized average of the *LFV* sensor response for *LC* and *MN* curd after cutting; *MN* milk shows a smaller whey separation rate and yield (as whey yield seems to be directly proportional to ΔR) when it is compared with LC milk.

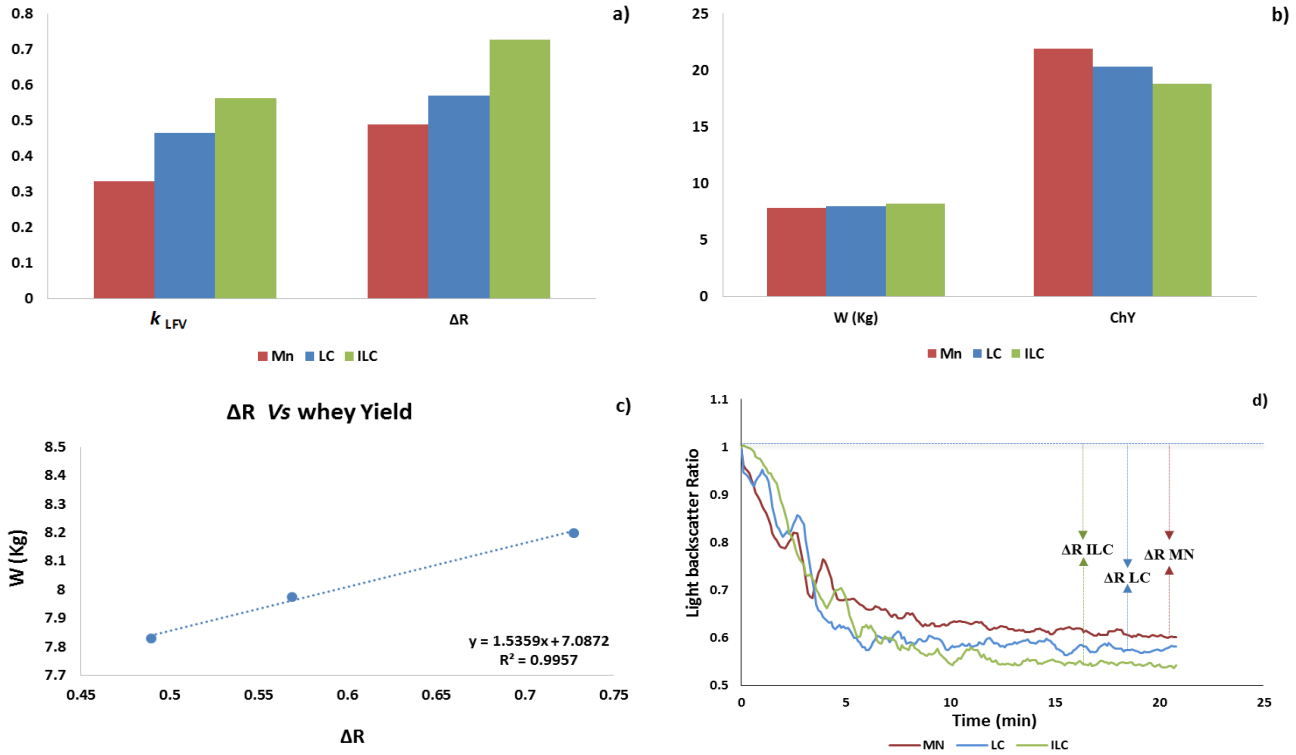


Fig. 8.6. **a)** The effect of animal breed and subclinical mastitis infection on apparent syneresis kinetic constant (k_{LFV}) and **ΔR**. **b)** The effect of animal breed and subclinical mastitis infection on both cheese yield wet basis and whey yield. **c)** Correlation between the whey yield and **ΔR.** **d)** Normalized LFV light backscatter ratio profiles as a function of time during syneresis at 35 °C (each profiles is the average of two replicates).

8.4.5 Effect of subclinical mastitis in the composition and coagulation of milk, curd syneresis and cheese yield

It is well documented that subclinical mastitis affects milk quality, quantity and processing properties increasing SCC, changing both milk composition and functional (i.e., technological) properties (Dragana, *et al.*, 2012). McCarthy *et al*. (1988), Fthenakis *et al*. (1991), and Leitner *et al*. (2003) reported that the intramammary infection, even if restricted to subclinical levels, affect the milk yield of sheep negatively (Watson and Buswell, 1984). However, quantifying this effect is difficult because 1) in most cases, only one gland is infected; therefore, the effect is diluted when measurements are made on a whole-animal basis; and 2) individual animal variability, breed (in the case of mixed breeds in the same flock), age, and stage of lactation introduce considerable variability.

The effect of subclinical mastitis on LSMs of milk composition parameters is presented in **Table 8.1.** It was observed no significant ($P > 0.05$) difference in milk composition parameters between *LC* and *ILC*, except for lactose content. M_L was significantly lower ($P<0.05$) in *ILC* as compare with *LC*. Batavani *et al*. (2007) and many other authors (Auldist and Hubble, 1998; Carolina *et al.*, 2013, Leitner *et al*., 2011 and Abdelgawad *et al.*, 2016) observed that animals infected with subclinical mastitis produced milk with lower concentration of lactose as compare with healthy animals. As it is shown in **Table 8.2,** a negative and significant correlation between the log of SCC and M_L was observed, indicating that infection with SCM led to both a decrease in milk lactose concentration and an increase in SCC (Villalobos *et al*., 2015).

As it is known, the lactose is the key factor balancing the osmotic changes between blood and the udder milk (i.e., increase of minerals content in milk). The more probable reason for depressed lactose concentrations in the infected animals is the leakage of lactose out of milk via the para-cellular pathways that proliferate during mastitis (Auldist and Hubble, 1998). Therefore, the low lactose concentrations are dependent on the severity of damage to the tight junctions (Bruckmaier *et al*., 2004). Evidence for this exists in the elevated concentrations of lactose in the blood and urine of mastitic cows. Since lactose is synthesized only in the mammary gland and is not secreted through the baso-lateral surface of the mammary epithelium in significant quantities and is not metabolized elsewhere in the body, the plasma level of

lactose provides a measure of the leakage rate of material from the lumen of the mammary gland into the blood stream (Nguyen *et al*., 1998).

The reduction in lactose content is depressed as the mechanism regulating the concentration of the major ions, potassium, sodium, chloride and bicarbonate is impeded due to subclinical mastitis (Harmon, 1994b). This will increase the concentration of ions and by necessity to maintain osmotic equilibrium decrease lactose concentrations.

Furthermore, according to Auldist *et al*. (1995), many of the common mastitis-causing organisms are capable of fermenting lactose. The lower concentrations of lactose in mastitic milk may be partly due to the activities of these organisms.

Also as expected, a significant (P<0.05) SCC increase was encountered in *ILC* milks as compare with *LC* (**Table 8.1**), as observed by Abdelgawad *et al.* (2016). The log SCC in *ILC* milks corresponded to low quality milk according to classification of sheep milk suggested by Leitner (2008).

The effect of subclinical mastitis on the optical parameter $\boldsymbol{V_0}$ is shown in (**Table 8.1**). Since, it is well known that fat and protein are the most important scatterers present in milk (Castillo, 2001), and there was no significant difference in milk fat and protein concentrations between *LC* and *ILC*, the observed significant difference in V_0 was not expected. However, a decrease in V_0 induced by subclinical mastitis was also observed by Abdelgawad *et el.* (2016).

This might suggest severe demineralization of the casein micelles in *ILC* milk as it was suggested by Abdelgawad *et al*., (2016). Caldwell (2012), reported that proteolytic enzymes in *ILC* milk causes loss of colloidal calcium phosphate (**CCP)** and Castillo *et al*., (2006a, b) related demineralization of casein micelles with a decrease in light backscatter. Walstra and Jenness (1984), claimed that a decrease CCP reduces the light scattering capability of the micelles. This might explain the change of the V_0 value between *ILC* and *LC* milks.

The effect of the subclinical mastitis in milk clotting properties, is shown in **Table 8.1**. It was observed that subclinical mastitis had a significant effect (P<0.05) on all milk coagulation parameters, in agreement with Abdelgawad *et al.* (2016). LSMs of the optical and visual milk clotting parameters (t_{max}, $t_{2max,}$ t_{2min}, and t_{clot},) and visual cutting time (t_{cut}) were significantly *($P<0.05$)* larger in *ILC* milk as compared with *LC* milk. Coagulation time-based

parameters increased by a range of 2.4-2.9 times, depending on the parameter being investigated when compare with *LC* milks. Many previous studies have related mastitis with poor milk coagulation. For instance, high *SCC* has been significantly correlated with an increase in rennet clotting time, with slower rate of curd firming, and reduced curd firmness, (Maréchal *et al.*, 2011, Villalobos *et al*., 2015), which in turn, led to poor yield and low cheese quality. This trend was in agreement with our results which, in addition, were supported by the observed positive and significant correlation between log SCC and both types of coagulation indicators evaluated in this study (optical -t_{max}, t_{2max}, t_{2min}- as it is shown in **Table 8.2** and visual -t_{clot} and t_{cut}- time based parameters).

Thus, although subclinical mastitis does not affect total protein and fat concentrations in milk, it seems to have effect on the casein fraction of milk proteins (Maréchal *et al.*, 2011). Thus, it seems that mastitis affects the casein composition. Concentration of soluble casein has been shown to be higher, while concentration of micellar casein becomes lower in mastitic milk than in milk from healthy animals (Sharma and Randolph, 1974). Many authors, Srinivasan and Lucey (2002), Moussaoui *et al*. (2004) and Leitner *et al*. (2004), stated that the effect of rennet and, therefore, the coagulation process may be impeded by only partial hydrolysis of κ-CN and more pronounced hydrolysis of the other caseins by enzymes such as plasmin and cathepsin, as which consistent with our results.

The effect of subclinical mastitis on both syneresis kinetics and cheese yield is shown in **Table 8.1.** LSMs of optical parameter $\boldsymbol{k_{LFV}}$, whey yield and ΔR for both *LC* and *ILC* milk are presented in **Table 8.1**. Typical *LFV* light backscatter ratio as a function of time during milk coagulation and curd syneresis for *LC* and *ILC* cheese manufacture is shown in **Fig. 8.4b,c**. **Fig. 8.5b,c** shows that the experimental *LB* data after gel cutting (t = 0), R, fitted consistently a first order syneresis kinetic model (Eqn. 1) in both types of milk: *LC* and *ILC* (R^2 = 0.91 and 0.95).

It was observed that the subclinical mastitis increased significantly (P<0.05; **Table 8.1)** $\boldsymbol{k_{LFV}}$ and ΔR in *ILC* curd as compared with LC curd, as it is graphically shown in **Fig. 6a. A**lso, both higher whey yield and lower cheese yield were observed in *ILC* as compare with LC (**Fig. 8.6b** and **Table 8.1).** Thus, as it was explained in section 3.4 (effect of the breed), likely, subclinical mastitis originated an increase in the extent and rate of syneresis, (**Figs 8.6a, c, d)**,

causing the observed changes in whey and cheese yield in *ILC*. These results were also supported by the existing correlation of the optical time parameter, t_{max}, with whey and cheese yields (**Table 8.2**). As it can be observed, a positive correlation ($P < 0.001$) between t_{max} and whey yield, while cheese yield was negatively correlated with t_{max} ($P < 0.01$).

The observed increase on the extent and rate of syneresis in *ILC* is also supported from rheological data previously observed by our research group. It is well documented that higher ***tanδ*** indicate more mobile bonds and strands that are more susceptible to rearrangement, which in turn is typically associated with increased syneresis tendency (Castillo *et al*., 2006a, 2006b). According to Abdelgawad *et al.* (2016), ***tanδ*** at cutting time was significantly higher in *ILC*, compared to healthy animals (*LC*), in a lab-scale experiment, suggesting increased syneresis capability in *ILC* gels.

8.4.6 Algorithms for prediction of cheese making parameters

8.4.6.1 Validation of previous cutting time prediction algorithms

Abdelgawad *et al.* (2016) predicted visual cutting time using a NIR light backscatter lab-scale tester and several prediction models (models I, II and III, **Table 8.3)** in milk from *MN*, *LC* and *ILC*. To validate those three models, they were fitted to our current data.

It was observed that these models worked successfully with a complete independent data set for predicting cutting time with R^2 ranging from 0.94 to 0.96 **(Fig. 8.7),** and irrespectively of the health status of the animals.

Table 8.3. Models for prediction of cutting time according to Abdelgawad, *et. al.* (2016): validation.[a]

Model		R^2	
ID	**Equation**	**Abdelgawad *et al.* (2016)**	**Current study**
I* **	$t_{cut} = 1.70 \cdot t_{max}$	0.863	0.944
II* **	$t_{cut} = 2.19 \cdot t_{2min}(1+0.0674 \cdot P)$[b]	0.983	0.977
III* **	$t_{cut} = 2.70 \cdot t_{2max}(1+0.0620 \cdot P)$	0.999	0.959

[a]N = 6. Predictors are defined in the text**;** R^2, determination coefficient (corrected for the means)**;**
**P<0.05, **P<0.01, ***P<0.001*; [ns]not significant;
[b] Regression coefficients taken from Castillo *et al*. (2003).

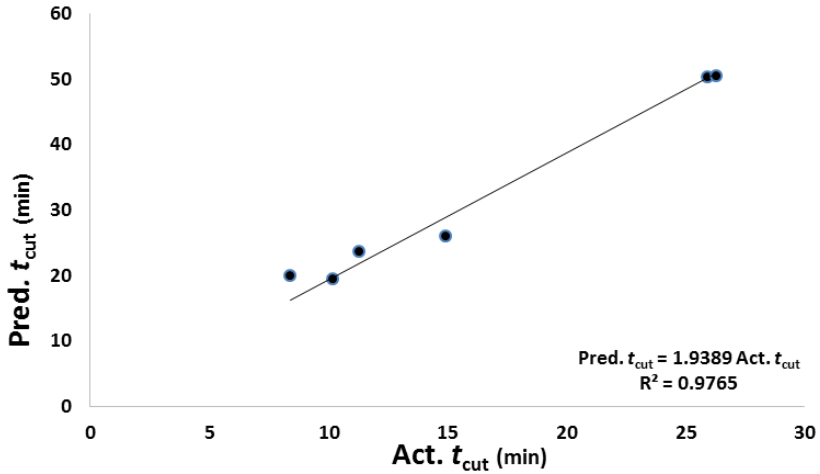


Fig. 8.7. Correlation between actual and predicted visual cutting time (t_{cut}) using model II (Table 8.3).

The maximum R^2 procedure of SAS® was used to obtain the best one-, and two-parameter prediction models for predicting visual clotting (t_{clot}) time as well as to evaluate alternative, improved prediction equations for cutting time.

As expected, a strong linear correlation (r = 0.93; P< 0.001) between the visual clotting time (t_{clot}) and optical time parameters t_{max}, t_{2max} and t_{2min} was obtained (**Table 8.2**). Thus, as it is shown in **Table 4,** Models I and II were selected (maximum R^2 procedure of SAS) for predicting t_{clot}, which contained the parameters t_{2max}**P* (both models) and *FP (model II)*, in addition to the intercept.

In these two models, all the regression coefficients estimated were significantly different from zero. Model I had smaller R^2 and higher SEP than Model II (R^2 of 0.975 and SEP of 0.76 min versus R^2 of 0.997 and SEP of 0.28 min, respectively). Model I (**Fig. 8.8a**) used an easily measurable and reproducible time-parameter in addition to the intercept to predict $t_{clot.}$

It was observed that simplifying this model using only the optical parameter t_{2max}P (ie., without the intercept) increasing SEP substantially (0.47 min) (model III, **Table 8.4; Fig. 8.8b**).

The best t_{cut} predictor was t_{max}P (models IV and V) while t_{2min}P was the second best t_{cut} predictor (model V) as it is shown in **Table 8.4.** Even though model IV had smaller R^2 and higher SEP as compare with model V, Model IV **(fig. 8.8c)** was preferred for inline application due to its simplicity (note that it uses only one significant predictor). Since the intercept in this model was not significant, it was simplified further to yield model VI (SEP 1.89 min) **(fig. 8.8d).**

These results agreed with Abdelgawad *et al.* (2016) in that the prediction models for predicting visual clotting and cutting times are working successfully with all the breeds and infection status tested.

Table 8.4. Models for the prediction of gelation and cutting time parameters.[a]

Predicted Model	Model		Coefficient				R^2	SEP (min)
	ID	Equation	β_0	β_1	B_2	$B3$		
t_{clot}	**I*** **	$t_{clot} = \beta_0+\beta_1 t_{2max}P$	2.13**	0.19***	-	-	0.975	0.76
t_{clot}	**II*** **	$t_{clot} = \beta_0+\beta_1 t_{2max}P+\beta_2 FP$	9.10***	0.19***	-6.08**	-	0.997	0.28
t_{clot}	**III*** **	$t_{clot} = \beta_1 t_{2max}P$	-	0.24***	-	-	0.986	1.23
t_{cut}	**IV*** **	$t_{cut} = \beta_0+\beta_1 t_{max}P$	0.33^{ns}	0.31***	-	-	0.944	2.11
t_{cut}	**V*** **	$t_{cut} = \beta_0+\beta_1 t_{max}P+\beta_2 t_{2min}P$	1.54^{ns}	0.19**	0.09**	-	0.984	1.30
t_{cut}	**VI*** **	$t_{cut} = \beta_1 t_{max}P$	-	0.31***	-	-	0.991	1.89

[a]N = 6. Predictors are defined in the text**;** β_0, β_1, β_2, regression coefficients; R^2, determination coefficient (corrected for the means)**;** SEP, standard error of predictions**;**
**P<0.05, **P<0.01, ***P<0.001*; [ns]not significant;[b]

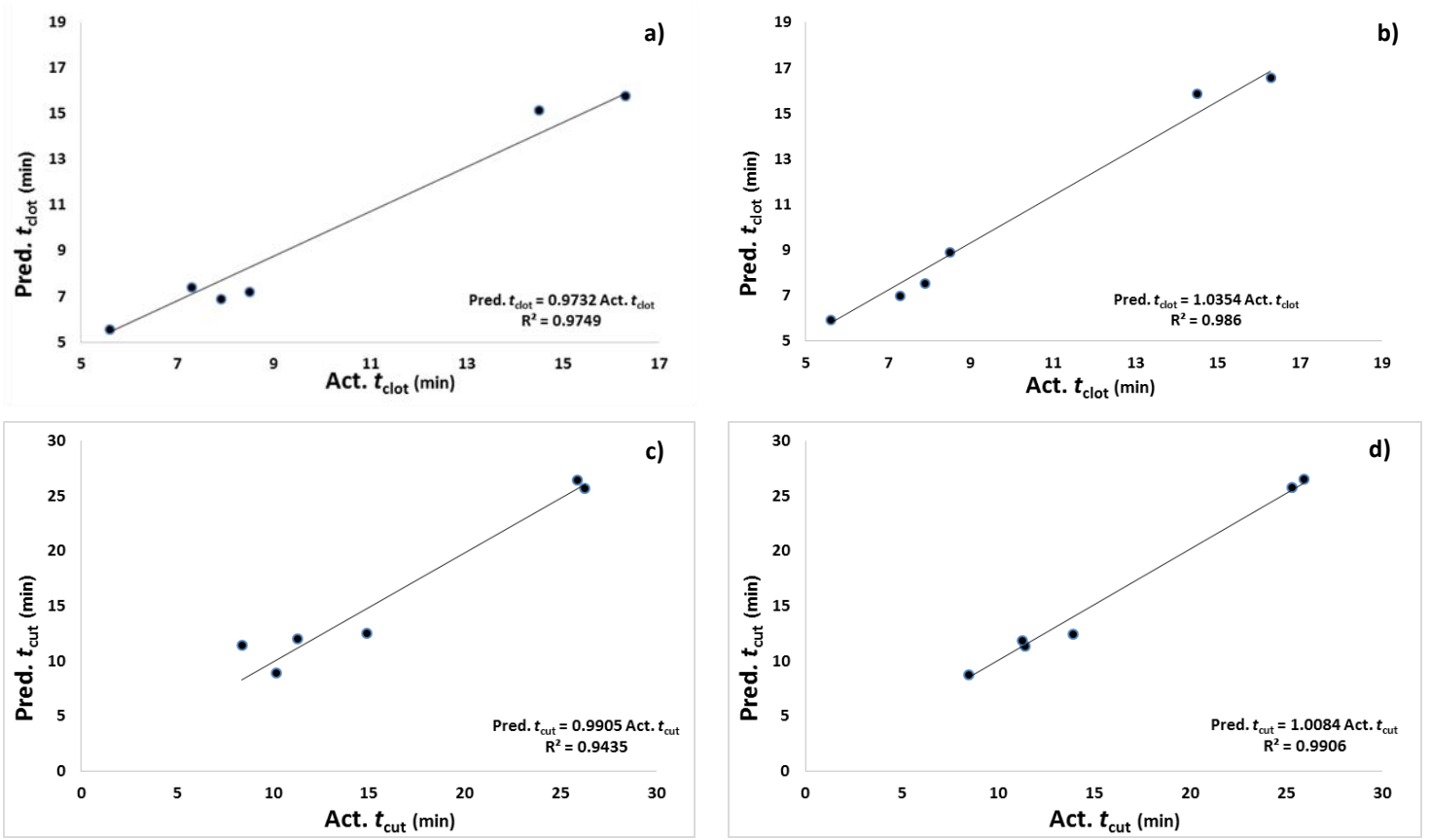


Fig. 8.8. a**)** Correlation between actual and predicted visual clotting time (t_{clot}) using model I (Table 8.4). **b)** Correlation between actual and predicted visual cutting time (t_{clot}) using model III (Table 8.4). **c)** Correlation between actual and predicted visual cutting time (t_{cut}) using model IV (Table8.4). **d)** Correlation between actual and predicted visual cutting time (t_{cut}) using model VI (Table 8.4).

8.4.6.2 Prediction of cheese yield and curd syneresis parameters

The parameters $FP_{,}$ and $t_{2min}P$ were found to be the best descriptors of fat content in cheese Ch_F throughout the various linear regression models tested. Model I was the simplest model to predict Ch_F. It had a determination coefficient of 0.578 using just *FP* as predictor as it is shown in **Table 8.5**. Model II was the best model having two parameters and included, in addition to *FP*, the optical parameter $t_{2min}P_{,}$ and the intercept with higher determination coefficient and smaller SEP as compare with model I. Note that incorporation of an optical parameter into the prediction model I almost increased R^2 by a factor of ~2, reducing the SEP ~14 times. All predictors used in models I and II were statistically significant.

Model III, and IV were the best one-, and two-parameter models for predicting Ch_{TS} as it is shown in **Table 8.5**. Using only R_{max} as a predictor for Ch_{TS} did not allow to reduce the SEP below 2.15%, while adding as predictor $t_{2max}P$ to model III reduced the SEP to 0.79%, which represented a 2.7 times prediction error decrease.

Regarding ChY_W prediction, a three-parameter model (Model V; **Figs. 8.9a**) was found to predict this cheese making index using as predictors the optical parameters R_{max}, t_{2max} and t_{2min}. All the regression coefficient were significant.

The best algorithms for predicting W_Y, $W_{P,}$ and W_F, were models VI, VII, and VIII, respectively as it is shown in **Table 8.5**.

Model VI contained the optical parameter $t_{2min}P$, FP and R_{max} in addition to the intercept with R^2 =0.989 and SEP=0.03. While model VII contained the V_0, *L* and *TS* in addition to the intercept, with R^2 =0.998 and SEP=0.2. Finally, model VIII contained the optical parameters t_{2min} and V_0 in addition to *TS* as predictors for W_F, in this model the regression coefficient was 0.930 and SEP was 0.030. The regression coefficient terms corresponding to all the predictors and the intercept for the three discussed models were significant as it shown in **Table 8.5**.

The parameters *L*, *TS*, and R_{max} were consistently found to be the best descriptors of the kinetic rate constant ($\boldsymbol{k}_{\mathbf{LFV}}$) throughout the various linear regression models tested as it is shown in **Table 8.5**. Model IX was the best three-parameter model to predict $\boldsymbol{k}_{\mathbf{LFV}}$ with R^2 = 0.994 and SEP = 0.03 min (**Figs. 8.9b**)

Table 8.5. Models for the prediction of cheese yield and curd syneresis indicators.[a]

Predicted Model	Model		Coefficient				R^2	SEP
	ID	Equation	β_0	β_1	B_2	B_3		
Ch_F	**I*** **	$Ch_F=\beta_0+\beta_1 FP$	14.8***	12.5***	-	-	0.578	1.26%
Ch_F	**II*** **	$Ch_F=\beta_0+\beta_1 FP+\beta_2 t_{2min} P$	13.2***	15.3***	-0.03***	-	0.980	0.09%
Ch_{TS}	**III*** **	$Ch_{TS}=\beta_0+\beta_1 R_{max}$	382**	-319**	-	-	0.728	2.15%
Ch_{TS}	**IV*** **	$Ch_{TS}=\beta_0+\beta_1 R_{max}+\beta_2 t_{2max} P$	307**	-253***	0.09***	-	0.972	0.79%
ChY_W	**V*** **	$ChY_W=\beta_0+\beta_1 t_{2max}+\beta_2 t_{2min}+\beta_3 R_{max}$	145***	0.32***	-0.48***	-116***	0.992	0.21%
W_Y	**VI*** **	$W_Y=\beta_0+\beta_1 R_{max}+\beta_2 FP+\beta_3 t_{2min} P$	-2.19*	8.87**	0.46*	0.01***	0.989	0.03kg
W_P	**VII*** **	$W_P=\beta_0+\beta_1 L+\beta_2 TS+\beta_3 V_0$	3.00***	-1.30***	0.30***	0.40***	0.998	0.20%
W_F	**VIII*** **	$W_F=\beta_0+\beta_1 TS+\beta_2 V_0+\beta_3 t_{2min}$	2.50***	0.15***	-1.30***	0.01***	0.930	0.03%
k_{LFV}	**IX*** **	$k_{LFV}=\beta_0+\beta_1 L+\beta_2 TS+\beta_3 R_{max}$	11.1***	1.30***	-0.08***	-14.7***	0.994	0.03 min^{-1}

[a]N = 6. Predictors are defined in the text**;** β_0, β_1, β_2, regression coefficients; R^2, determination coefficient (corrected for the means)**;** SEP, standard error of predictions**;** *$P<0.05$, **$P<0.01$, ***$P<0.001$; [ns]not significant;[b]

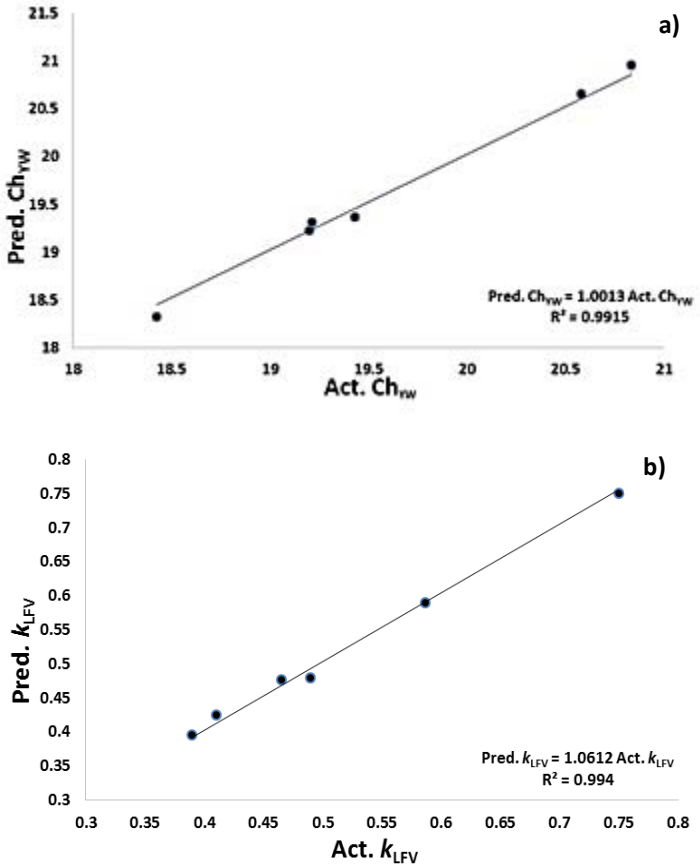


Fig. 8.9. **a)** Correlation between actual and predicted cheese yield wet bases (**Ch**Y_W) using model V (Table 8.5). **b)** The correlation between actual and predicted k_{LFV} using Model IX (Table 8.5).

8.5 CONCLUSIONS

NIR infrared light backscatter and *LFV* syneresis sensors were used to monitor milk coagulation, cutting time, and whey separation processes during Manchego-type cheese manufacture using sheep milk from healthy animals or individuals infected with subclinical mastitis. The effect of infection and sheep breed (Lacaune and Manchega) on the milk coagulation and curd syneresis monitoring procedure was also investigated.

Results shows significant differences ($P<0.05$) in milk composition between different ewes breeds, *MN* milk contained higher concentrations of protein, fat, and total solids as compare with *LC*, while no significant differences in milk composition were encountered between milk from *LC* and *ILC*, except for lactose concentration and SCC, which were lower and higher, respectively, in *ILC* due to the SCM infection.

Significant differences were observed for V_0 (the light backscatter initial average voltage) during the coagulation of the three types of milk (*MN*, *LC* and ILC). The differences observed between *MN* and *LC* were attributed to the different milk fat and protein concentration between the two types of milk, while between *LC* and *ILC*, those difference were more likely due to the severe demineralization of the casein micelles and corresponding decrease of CCP in *ILC*. Those phenomena in *ILC* were probably originated by the increased activity of proteolytic enzymes in *ILC* milk.

Using milk from different sheep breeds resulted in no significant effect on milk coagulation ability when comparing *MN* and *LC* milks, even when the coagulation time indicators in *MN* were consistent but not significantly larger than in *LC* milk, likely due to the higher content of protein in *MN* milk as compare with *LC*.

Subclinical mastitis increased significantly all optical and visual coagulation and cutting time parameters. As a result, lower aggregation and curd firming rates were observed in *ILC* as compare with *LC*. This trend was attributed to the partial hydrolysis of κ-CN and more pronounced hydrolysis of the other caseins by enzymes such as plasmin and cathepsin, which apparently impaired the coagulation process.

Breed and SCM had a significant effect on both curd syneresis process and cheese yield. A slight but significant increase in whey release rate resulted in lower cheese yield in *LC* as

compared with *MN* animals. Additionally, a highly significant increase in syneresis rate and extent was originated in *LC* animals suffering subclinical mastitis, which turned into reduced cheese yield.

Apparently, the combination of light backscatter parameters and protein concentration are required for generate the prediction model for predicting both visual clotting and cutting time while the prediction models for predicting some cheese yield indicators require the light backscatter parameters in combination with the chemical composition of the milk with R^2 values ranging from 0.578 to 0.998 irrespectively of the health status or the breed.

Visual cutting time prediction models from our previous studies were successfully validated using an independent data set obtained using a ten-liter cheese vat.

Chapter Nine

General conclusions

Chapter Nine: General conclusions

1. Near infrared light scattering sensor technology in combination with small amplitude oscillatory rheology allow monitoring, evaluating and studying the impact of rennet- or mixed-induced coagulation of milk mixtures (i.e., different proportions of goat, sheep and cow milk at different coagulation temperatures and enzyme concentrations) and low quality milk (i.e., milk from sheep infected with subclinical mastitis) in order to evaluate the effect of those factors in the prediction of clotting time, cutting time, curd syneresis and some other cheese making indexes during Manchego-type cheese manufacture.
2. The effect of different enzyme concentrations and coagulation temperatures on coagulation of milk mixtures resulted in a significant change in visual, optical and rheological parameters related to casein hydrolysis/aggregation and gel firming. To this regard:
    a. Enzyme concentration and coagulation temperature had a significant effect on visual, optical, and rheological indicators of clotting time suggesting that both factors influenced casein hydrolysis. However, no significant effect of milk mixtures on the enzymatic phase of coagulation was observed as revealed by the lack of significant differences on milk clotting indicators with regard to this factor.
    b. The significant effect of milk mixtures on the optical parameter R'_{max}, and rheological parameters $t_{G'30}$, $t_{G'60}$ and $tan\delta$ indicated that increasing the cow milk proportion resulted in faster micelle aggregation and gel firming rate, which contributed to originate a weaker gel with an opener network and more tendency for rearrangement (i.e., with increased syneresis capability).
3. Sheep breed and subclinical mammary gland infection with coagulase-negative staphylococci exerted an effect on both milk composition and coagulation properties. Specific conclusions in that regard were as follows:
    a. Lacaune milk contained smaller fat and protein concentration than manchega milk while both milk types had similar lactose concentration. These differences were consistent with the higher light backscatter intensity observed, at enzyme addition time, in manchega milk as compared with lacaune milk.
    b. Milk of manchega ewes seemed to start aggregating and firming later than lacaune ewe milk. However, gel assembly times were longer for lacaune than for manchega.

This trend, which was quite consistent but not significant, suggested that a smaller percentage of protein in lacaune milk decreased the time required for *κ*-casein hydrolysis reaction, inducing a decrease in cutting time, even when the reduced casein concentration also originated a decrease of the micelle aggregation/firming rates in this type of milk with regard to manchega milk.

c. Milk from healthy animals had significantly lower somatic cell count and higher lactose concentration than milk from animals infected with subclinical mastitis. The reduction on lactose concentration in milk from animals with subclinical mastitis balanced out the osmotic effect of increased mineral content due to the infection.

d. Subclinical mastitis increased significantly all optical, rheological and visual clotting and cutting time indicators showing the clear negative impact of subclinical mastitis on the milk coagulation process. As a result, lower aggregation and curd firming rates were observed in infected animals. These detrimental milk coagulation effects of subclinical mastitis was attributed to partial hydrolysis of *κ*-CN and more pronounced hydrolysis of the other caseins by enzymes such as plasmin and cathepsin, which apparently impaired coagulation.

e. Taking in account that totals solids of lacaune and infected lacaune were not significantly different, the significant changes in intensity of light backscatter encountered at enzyme addition time between healthy and infected animals suggested severe demineralization and/or size reduction of the casein micelles occurred as a results of subclinical mastitis, which would require further confirmation using complementary instrumental techniques.

4. Breed and subclinical inframammary infection had a significant effect on both curd syneresis process and cheese yield. A slight but significant increase in whey release rate resulted in lower cheese yield in lacaune as compared with manchega animals. Additionally, a highly significant increase in syneresis rate and extent was observed in lacaune animals suffering subclinical mastitis, which turned into reduced cheese yield.
5. As compared with previous results obtained using just milk from one species, mixture of milk from three species (goat, sheep and cow –two levels of mixture-) did not largely affected prediction of gelation parameters such as visual clotting time and rheological gelation time although prediction algorithms benefited from incorporating a compositional factor (e.g., protein or fat concentrations). Nonetheless, the standard error of prediction

was about double (2 min) than when only milk from one type is coagulated. However, prediction of cutting time parameters (visually- and rheologically-determined) was more challenging, requiring as predictors t_{max}, protein concentration and mixture type to reduce the standard error of prediction to a value of 7-8 min. These results, clearly show that a reduction in the standard error of prediction (i.e., an improved prediction algorithm) is warranted before implementing the proposed technology in the industry, if mixtures with different proportions of milk from several species are to be coagulated.

6. The loss tangent at cutting time was successfully estimated as a function of the fat/protein ratio, mixture level and temperature, which is expected to contribute to foresee the tendency of the gel to undergo syneresis.
7. Irrespectively of the health status or the breed, prediction models using light backscatter parameters alone or in combination with protein/solids concentration were successfully obtained for visually determined clotting and cutting times, rheologically derived gelation and cutting times, $\tan\delta$ at cutting, several cheese yield indicators and the syneresis rate constant.
8. Visual cutting time prediction models from our previous studies were successfully validated using an independent data set obtained using a ten-liter cheese vat.

Chapter Ten

Refrences

Chapter Ten: References

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