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Milk and dairy products: evaluation of bioactive components by analytical techniques

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Summary

Milk and dairy products are important source of bioactive compounds useful to satisfy the nutritional and physiological needs of any newborns of mammalian species and useful to guarantee adequate growth and development of infants as well as provide a complete nourishment of adults. Physico-chemical, nutritional and organoleptic properties of the main constituents (i.e. lipid and protein fractions) and the "minor" components have a crucial role in the quality of milk and milk products. Although in the past decades dietary milk fat was often regarded as harmful for the human health, recent researches suggest that milk contains specific fatty acids with nutritional, metabolic and physiological health benefits. For these reasons, a major attention is given to the quantity and quality of total fat intake. In the recent years, as a result of the new concept of multifunctional agriculture and the changing behaviours about diet, consumer demands in favor of high-quality, security and safety dairy products are increased. Moreover, milk proteins and milk-derived bioactive peptides are recognized to have a high nutritive value, several health-promoting functional activities and excellent technological properties. Accordingly, growing interest in the development of functional dairy products and preparation of infant formulae for babies who cannot be breast-fed, has been give in order to meet the specific consumer's requests.

This manuscript presents the main results obtained during my PhD research aimed to evaluate the main bioactive lipids and proteins in milk and dairy products using innovative analytical techniques. The first part of this work is an overview about lipid and protein compositions of bovine and human milks. A particular emphasis is given to the main biologically active properties of lipids, proteins and peptides and a briefly description of the analytical techniques applied for their evaluation is reported.

The experimental section of this manuscript is divided in two sections where are reported the main results obtained during my research activities on dairy products (***Section I***) and human milks (***Section II***) in order to characterize their bioactive compounds for functional food applications. Particularly, in the first section, the bioactive lipid fractions of dairy products (milk cream and butter) from bovine milk were characterized by chromatographic and spectroscopic techniques. In the second section, lipidomic and proteomic profiling approaches were performed on human milks (colostrum, pre-term and term samples) collected from a milk bank in Northern Ireland.

Introduction

1. Milk composition and structure

Milk is defined as the fluid secretion of mammary glands of all mammalian species. The primary natural function of milk is to completely accomplish the nutritional and physiological requirements of the neonate species, as well as to promote the growth and development of children. Moreover, milk may be considered like as a source of nourishment for the adult humans.

Because the nutritional and physiological requirements are specific-specific and change as the neonate matures, the composition of milk shows very marked inter-species differences. Inter-species differences in the concentration of many of the minor constituents are even greater than those of the macro-constituents[1]. Inter-species differences in the quantitative composition of milk probably reflect differences in the metabolic processes of the stage of lactation and the nutrients requirements of the suckling neonate[2].

This section will describe the main chemical, physico-chemical properties of the major components of bovine and human milk including their quantitative variations. In addition, structural elements will be considered in order to point out *how* the main constituents are present the milk's system.

1.1 The major components of bovine and human milks

Although milk is a liquid food (87% water), it contains an average of 13% total solids and 9% solids-not-fat, an amount comparable to the solids content of many other foods (***Figure 1***). It may contain more than 100 different kinds of molecules[3].

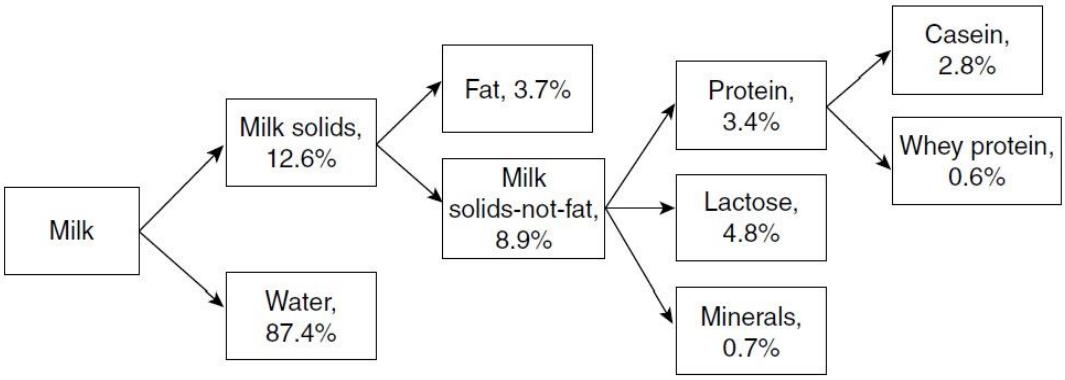

Figure 1. Major constituents of milk[4]

[1] Fox P.F. and McSweeney P.L.H. (1998) Production and utilization of milk. Part I. In: *Dairy Chemistry and Biochemistry*, Blackie Academic & Professional Ed, an imprint of Thomson Science, 2-6 Boundary Row, London SE1 SHN, UK.

[2] Jenness R. and Sloan R.E. (1970) The composition of milks of various species. A review. *Dairy Science*. Abstract. 32, 599–612.

[3] Miller G.D., Jarvis J.K. and McBean L.D. (2006) The importance of milk and milk products in the diet. In: *Handbook of Dairy Foods and Nutrition*, Third Edition. CRC Press Taylor & Francis Group, 6000 Broken Sound Parkway NW, Suite 300.

Overall milk contains specific protein, fats designed to be easy digested, most have lactose, minerals, vitamins, and other components which may have important role[5].

The constituents of the milk of the other main dairy species are generally similar to those of bovine milk but they differ in varying amounts (***Table 1***). In particular, the concentration of milk's constituents and their properties are influenced by several factors, including genetic factors (individually of the species and breed), stage of lactation, health and nutritional status of dairy animals or humans. Therefore, the flexibility of milk as raw material, the processability and functionality of milk and dairy products, resides in the chemical and physico-chemical properties of its constituents, many of which are unique and easily isolated from milk permitting the production of valuable food ingredients[1].

Component	Bovine	Human	Goat	Sheep
Protein	3.4	1.0	2.9	5.5
Casein	2.8	0.4	2.5	4.6
Fat	3.7	3.8	4.5	7.4
Lactose	4.6	7.0	4.1	4.8
Ash	0.7	0.2	0.8	1.0

Table 1. Proximate composition (WT%) of bovine, human, goat and sheep milks[6].

1.1.1 Composition of milk lipids

The term "lipids" is defined as those organic compounds in food and tissues that are insoluble in water, soluble in organic solvent (e.g. chloroform, ether), contain hydrocarbon groups as primary parts of the molecule. Compound classes covered in this definition include fatty acids (FAs), acylglycerols, FA esters and isoprenoid hydrocarbons. Other compounds also included are often considered as belonging to different classes, such as carotenoids, sterols, and the vitamins A, D, E and K.

Lipids tend to classified as "simple" or "complex", referring to the size or structural details of the molecules. Simple lipids include FAs, hydrocarbons, and alcohols, all of which are relatively

[4] Chandan R. (1997) *Dairy-Based Ingredients*, Eagan Press, St. Paul, MN.
[5] Jensen R.G. (1995) *Handbook of milk composition*. Academic Press, San Diego, California.
[6] Jenness R. (1974) The composition of milk. In: *Lactation.* B:L: Larson and V.R. Smith, Ed, Volume II, pp 3-107. Academic Press, New York.

"neutral" in term of charge. Complex lipids, such as phospholipids and glycolipids, are relatively more charge and are also refereed to as "polar"[7].

Milk lipids, referred to as "milk fat", is an important component of many dairy products, various food products (e.g. bakery products, chocolate), and also has non-traditional application (e.g. in cosmetics)[8].

The milk fat content can vary from about 3.0 to 6.0%, but typically is in the range 3.5 to 4.7% [9]. Bovine milk lipids are similar in structure to the milk lipids of other species, but differences in individual composition and their proportion may occur. Generally, milk lipids are largely composed of triacylglycerols (TGs), small amounts of diacylglycerols (DGs), monoacylglycerols (MGs), phospholipids (PLs), free fatty acids (FFA), cholesterol and cholesteryl esters (***Table 2).*** Trace amounts of fat-soluble vitamins, β-carotene and fat-soluble flavouring compounds are also present in the bovine milk lipids.

Lipid Class	**Weight percentage**	
	Bovine milk	***Human milk***
Triglycerides	97.5	98.2
Diglycerides	0.3	0.7
Monoglycerides	0.027	T
Free fatty acids	0.027	0.4
Phospholipids	0.6	0.26
Cholesterols	0.31	0.25
Cholesteryl ester	T	T

Table 2. Composition of individual lipids classes in bovine and human milks (weight, % of the total lipids)[10]. T, Trace.

The lipids are secreted in milk in the form of colloidal assemblies called milk fat globules (0.1-20 μm in diameter) emulsified in the aqueous phase (87%) and surrounded by the milk fat globule membrane (MFGM), which serve as an emulsion stabilizer.

[7] Nicholas D.S. and Sanderson K. (2002).The nomenclature, structure, and properties of food lipids. In: *Chemical and Functional Properties of Food Lipids.* Zdzislaw Z. E. Sikorski and Anna Kolakowska, Eds. CRC Press, Boca Raton, USA.

[8] Zegarska Z.A. (2003) Milk lipids. In: *Chemical and Functional Properties of Food Lipids*. Sikorski Z. E. and Kolakowska A., Eds. CRC Press, Boca Raton, USA.

[9] MacGibbon A.K.H. and Taylor M.W. (2006) Composition and structure of bovine milk lipids. In: *Advanced Dairy Chemistry. Volume 2 Lipids*. Third Edition. Fox P.F. and McSweeney P.L.H., Eds. Springer, USA.

[10] Christie W.W. (1995) Composition and structure of milk lipids. In: *Advanced Dairy Products* – 2 (Second Edition). P.F. Fox, Eds. Chapman & Hall, London.

Many factors can affect the milk lipid concentration, particularly, species, breed, climate, genetics, stage of lactation, health (mastitic infection or other diseases), plane of nutrition, interval between milking, and the point during milking when the sample is taken[11]. Moreover, milk lipids show variability in FA composition and in the size and stability of the globules. These variations are responsible for the rheological properties which in turn can have a marked effect on the functional properties of a number of dairy products, for the chemical stability and nutritional properties of fat-containing milk and dairy products, such as milk creams, butter and cheese. Regarding the nutritional properties, milk lipids contribute unique characteristics to the appearance, texture, flavour, and satiability of dairy foods, and are a source of energy, essential fatty acids (EFA), fat-soluble vitamins, and several other potential health-promoting components[12,13].

1.1.2.1. Fatty acid profile of milk lipids

Fatty acids (FAs) are mainly bound in acylglycerol (tri-, di-, and mono-), PLs, glycolipids, cholesteryl esters that altogether make up the greatest part of milk lipids, and only in a small amounts of 0.1 – 0.4 wt % they occur in free form [14].

Milk lipids, especially ruminant lipids, contain a very wide range of FAs than any other lipid system – up to 400 and 200 distinct acids have been detected in bovine and human milk lipids, respectively. However, the vast majority of FAs are present in trace quantities[15].

In ruminant species, a large portion of these FAs are produced as intermediates during the lipid metabolism in the rumen linked to microbial activity. Milk FAs of ruminants are either take up from plasma lipoproteins (60% of the total FA in milk) or they are synthesised in the mammary gland from acetate and 3-hydroxybutyrate (3-HB) derived from rumen fiber digestion. The main metabolic pathway involves two key enzyme: acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS) enzyme complex[16].

As such, the FAs in human milk have three origin: diet, biosynthesis in the mammary gland and adipose, liver, and other tissue mobilization. In man glucose-derived acetyl-CoA is the major source of C_2 units for C8:0 to C14:0 FAs. The magnitude of the contribution of each source to the total content depends on the amount of carbohydrates and FA composition of the diet, time elapsed since

[11] Fox P.F. (2003) Dairy product safety and quality. Part I. The major constituents of milk. In: *Dairy processing: Improving quality.* Smit G., Ed. CRC Press, Boca Raton, USA.
[12] German J.B. and Dillard C. J. (1998) Fractionated milk fat: composition, structure, and functional properties. *Food Technology*, 52, 33.
[13] Parodi P.W. (2004) Milk fat in human nutrition. *Australian Journal of Dairy Technology*, 59, 3.
[14] Molkentin J. (1999) Bioactive lipids naturally occurring in bovine milk. *Nahrung* 43, 185-189.
[15] Jensen R.G. (2002) The composition of bovine milk lipids: January 1995 to December 2000. *Journal of Dairy Science* 85, 295-350.
[16] Innis S.M. (2007) Human milk: maternal dietary lipids and infant development. *Proceedings of the Nutritional Society*, 66, 397-404.

the last meal and factors affecting the FA mobilization in the adipose tissue. The change occur rapidly, depending on the energy condition of the locating mother[17].

The milk FA composition is the most variable among the natural edible fats. Whereas, milk FA composition is linked to intrinsic (animal breed, genotype, lactation and pregnancy stage) and extrinsic (environmental, diet) factors[18]; human milk FAs are also influenced by cultural tradition, social and economic status, and the lactating mother's metabolism (individually)[19]. Although maternal diet appears to be the most important variable determining both bovine and human milk's FA composition[20], several important differences in the effect of dietary lipids, as well as in the mammary gland FA synthesis, occur between ruminants and non-ruminants species[16].

FAs can be divided into several groups with respect to their structure, physiological role and biological effects[21]. On the basis of chain length, ranging from butyric acid – with four carbon atoms – to FAs with 26 carbon atoms, and the number of double bonds (0 to 6), FAs are classified in:

- *Saturated fatty acids* ($C_nH_{2n}O_2$).

Saturated FAs (SFA) do not contain any doubles bonds and can be divided into subgroups according to their chain length in short-chain SFA (C4-C10, SC-SFA), medium-chain SFA (C12-C16, MC-SFA) and long-chain SFA (C > 17, LC-SFA)[14].

Most of the FA (C4:0 to C16:0) arising from the de novo synthesis are saturated, because the delta-9 desaturase has very low activity with FAs shorter than 18 carbon chain length, although a small portion of C14:0 and C16:0 is desaturated to C14:1 and C16:1[18]. The *de novo* FA content found in human milk for word population ranging from 12.7% to 42.5% of the total FA. Usually, a diet high in carbohydrates and low in fat content stimulates glucose synthesis in human milk of medium-chain FAs (MC-FA) or *de novo* FAs (C6-C14:0) which occurs in the cytoplasm of the mammary gland[22]. SFA in whole bovine milk accounts approximately 57% of the total milk lipids while in mature human milk[23] from 39.0-51.3%.

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[17] Chilliard Y. and Ferlay A. (2004) Dietary lipids and fresh forage interactions on cow and goat milk fatty acid composition and sensory properties. *Reproduction Nutritional Development* 44, 467-492.

[18] Chilliard Y., Ferlay A., Mansbridge R.M and Doreau M. (2000). Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trams and conjugated fatty acids. *Annales de Zootechnie* 49, 181-205.

[19] Finlay D.A., Lonnerdal B., Dewey K.G. and Grivetti L.E. (1985) Breast milk composition: fat content and fatty acid composition in vegetarian and non-vegetarian. *American Journal of Clinic Nutrition* 41, 787-800.

[20] Lock A.L. and Bauman D.E. (2004) Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids* 39, 1197-1206.

[21] Tvrzicka E., Kremmyda L.-S., Stankova B. and Zak A. (2011) Fatty acids as biocompounds: their role in human metabolism, health and disease – A review. Part 1: Classification, dietary sources and biological functions. *Biomedical Papers of the Faculty of Medicine and Dentistry of Palacky University*, Olomouc Czech Republic, 155(2), 117-130.

[22] Silva M.H.L., Silva M.T.C., Brandao S.C.C., Gomes J.C., Peternelli L.A. and Franceschini S.C.C. (2005) Fatty acid composition of mature breast milk Brazilian women. *Food Chemistry* 93, 297-303.

[23] Koletzko B and Thiel&Abiodun P.O. (1992) The fatty acids composition of human milk in Europe and Africa. *Journal of Pediatrics*, 120, 62-70.

Milk lipids is characterize by the presence of relative high concentration (10.6-12.8 wt % bovine milk) of SC-SFA. In contrast, human milk has very little SC-FA (< 1%) content[24]. They are particularly well digestible, as they hydrolysed preferentially from TGs and they transferred directly from the intestine to the portal circulation without re-synthesis of TGs. Thus, there is only a low tendency of adipose formation. Additionally, it has long been known that not all SFA have the same effect on blood cholesterol levels. Specifically, SC-SFA generally contribute either a neutral or reducing effects in blood cholesterol levels as well as triglycerides levels[25].

Beneficial effects of human health have been associated with the butyric acid (C4:0) content in bovine milk lipids[26]. There is no obvious relation between C4:0 contents in bovine milk fats and feeding condition of cows. In fact, C4:0 and other SC-SFA concentrations are classically either unchanged or only slightly reduced by increased lipid supplementation in the diet or body lipid mobilization[27]. Since butyric acid is specific for milk fat of ruminants, low concentration or absent of C4:0 was noticed in milk fats of non-ruminant species[8].

MC-SFA, such as lauric (C12:0) and myristic (C14:0) FAs, are produced by mammary gland synthesis with an average of about 12% in human milk and 18% in cows' milk, while palmitic acid (C16:0) represents about 20-25% of human and cows' milk total fatty acids[28]. These FAs have been related with an increase the level of total and low-density lipoprotein (LDL) cholesterol concentration in plasma leading to a high risk of cardiovascular disease, whereas LC-SFA is reported to be neutral concerning its effects on lipoprotein cholesterol levels[29].

In addition, LC-SFA express several metabolic peculiarities to decrease the melting point of milk lipids. These FAs (C18 and longer) are derived from blood FAs originating from the diet or from mobilization of body fat store.

- *Monounsaturated fatty acids* ($C_nH_{(2n-2)}O_2$).

Monounsaturated fatty acids (MUFA) are defined as components containing one double bond between adjacent carbon atoms. The presence of even this sample functionality introduce two important variables into MUFA structure and nomenclature. First, the double bond may be present

[24] Garcia C. and Innis S. (2013) Structure of the human milk fat globule. *Lipid Technology* 25(10), 223-226.
[25] Miller G.D., Jarvis J.K. and McBean L.D. (2007) The importance of milk and milk products in the diet. In: *Handbook of Dairy Foods and Nutrition.* Third Edition. National Dairy Council. CRC Press. Boca Raton, USA
[26] Parodi P.W. (1999). Conjugated linolenic acid and other anticarcinogenic agents of bovine milk fat. *Journal of Dairy Sciences*, 82, 1339-1349.
[27] McNamara D.J. (1992) Dietary fatty acids, lipoproteins, and cardiovascular disease. *Advance in Food Nutrition Research*, 36, 253.
[28] Lopez-Lopez A., Lopez-Sabater M.C., Campoy-Folgoso C., Rivero-Urgell M. and Cstellote-Bargallò A.I. (2002). Fatty acid and sn-2 fatty acid composition in human milk Granada (Spain) and in infant formulas. *European Journal of Clinical Nutrition*, 56, 1242-1254.
[29] Elgersma A., Tammingab S. and Ellen G. (2006) Modifying milk composition through forage. *Animal Feed Science and Technology*, 131, 207-225.

in a number of possible location along the carbon chain, giving rise to different MUFA isomers. Second, the double bond may be in either *cis* or *trans* geometry ***(Figure 2).***

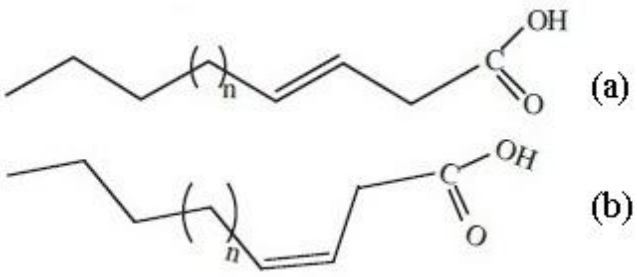


Figure 2. Generic structure of various fatty acids type: (a) trans-monounsaturated, (b) cis-monounsaturated.

In the nature, the vast majority of all double bonds in FAs are in *cis* geometry.

The average MUFA content in bovine milk fat is around 30% and in mature human milk fat is around 40%. The geometric and positional isomers are primarily the result of biohydrogenation activity of polyunsaturated fatty acids in the rumen.

Oleic acids (*cis*-9 C18:1) is the predominant MUFA in bovine and human milk fat and represents the major FA positional *cis* isomers of octadecenoic acid.

Milk fat also contains *trans* MUFA isomers, mainly *trans*-C18:1 and *trans*-C16:1, in amounts of approximately 3.7% and 0.13%, respectively[14]. Levels of *trans*-C18:1 from 2-5% were detected in human milk.

- *Polyunsaturated fatty acids*.

Polyunsaturated fatty acids (PUFA) are long-chain fatty acids that contain 18 or more carbons with two or more double bonds. PUFA are described using a structural designation that identifies the FAs by the number of carbon atoms, and the number and position of the unsaturated double bonds (***Figure 3***).

Depending upon the position of the first double bond proximate to the methyl end of the FAs, these FAs are categorized as *n*-9, *n*-6, or *n*-3 (also called ω-9, ω-6, ω-3) series, with the first double bonds being between carbons 9 and 10, carbons 6 and 7, and carbons 3 and 4, respectively. The two predominant PUFA series are the *n*-6 and *n*-3 FA. Within each PUFA class, there are many different FAs. The major dietary *n*-6 PUFA is linoleic acid (C18:2 *n*-6, LA) and the primary *n*-3 PUFA is α-linolenic acid (C18:3 *n*-3, ALA), whereas eicosapentaenoic acid (C20:5 *n*-3; EPA) and docosahexaenoic acid (C22:6 *n*-3; DHA), are a quantitatively minor component of total PUFA[30].

[30] Gebauer S., Harris W.S., Kris-Etherton P.M. and Etherton T.D. (2005) Dietary n-6:n-3 fatty acid ration and health. Chapter 11. In: *Healthful Lipids.* Akon C.C. and Lai O-M Eds, AOCS Press, Champaign, Illinois.

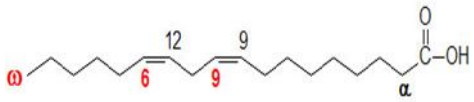
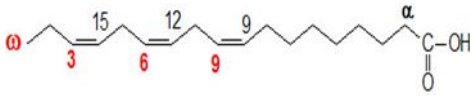
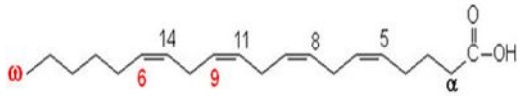
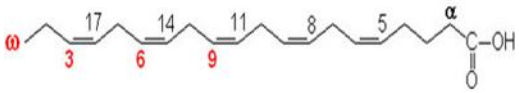
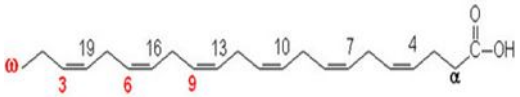
Numerical Symbol	*Common Name and Structure*
$18:2^{\Delta 9,12}$	Linoleic acid (LA) 
$18:3^{\Delta 9,12,15}$	α-Linolenic acid (ALA) 
$20:4^{\Delta 5,8,11,14}$	Arachidonic acid (AA) 
$20:5^{\Delta 5,8,11,14,17}$	Eicosapentaenoic acid (EPA) 
$22:6^{\Delta 4,7,10,13,16,19}$	Docosahexaenoic acid (DHA) 

Figure 3. Representative structure of the common polyunsaturated fatty acids.

The three classes of PUFA are metabolized by a common series of enzyme system. Humans are capable of desaturating stearic acid (C16:0) to form oleic acid (C18:1*n*-9) by means of a Δ^9 desaturase enzyme. However, humans lack the ability to insert a double bond at the *n*-6 or *n*-3 position of a FA carbon chain because of the absence of the Δ^{12} and Δ^{15} desaturases. The presence of these two enzymes is required for the production of LA and ALA, which is why these FA are essential and must be derived from the diet[30].

C18:0 (stearic acid) $\xrightarrow{\Delta 9\text{-desaturase}}$ C18:1 *n*-9 (oleic acid) $\xrightarrow{\Delta 12\text{-desaturase}}$ C18:2 *n*-6 (linoleic acid)

LA and ALA are metabolized by the same enzyme system, by alternating desaturation and elongation steps, generating two different pathways of subsequent metabolic products of up to 22 or more carbon atoms, called *n*-6 and *n*-3 long-chain PUFA (***Figure 4***). LA is the precursor of the

bioactive *n*-6 PUFA such as arachidonic acid (C20:4 *n*-6, AA); ALA is the precursor of the bioactive *n*-3 PUFA series, including EPA and DHA.

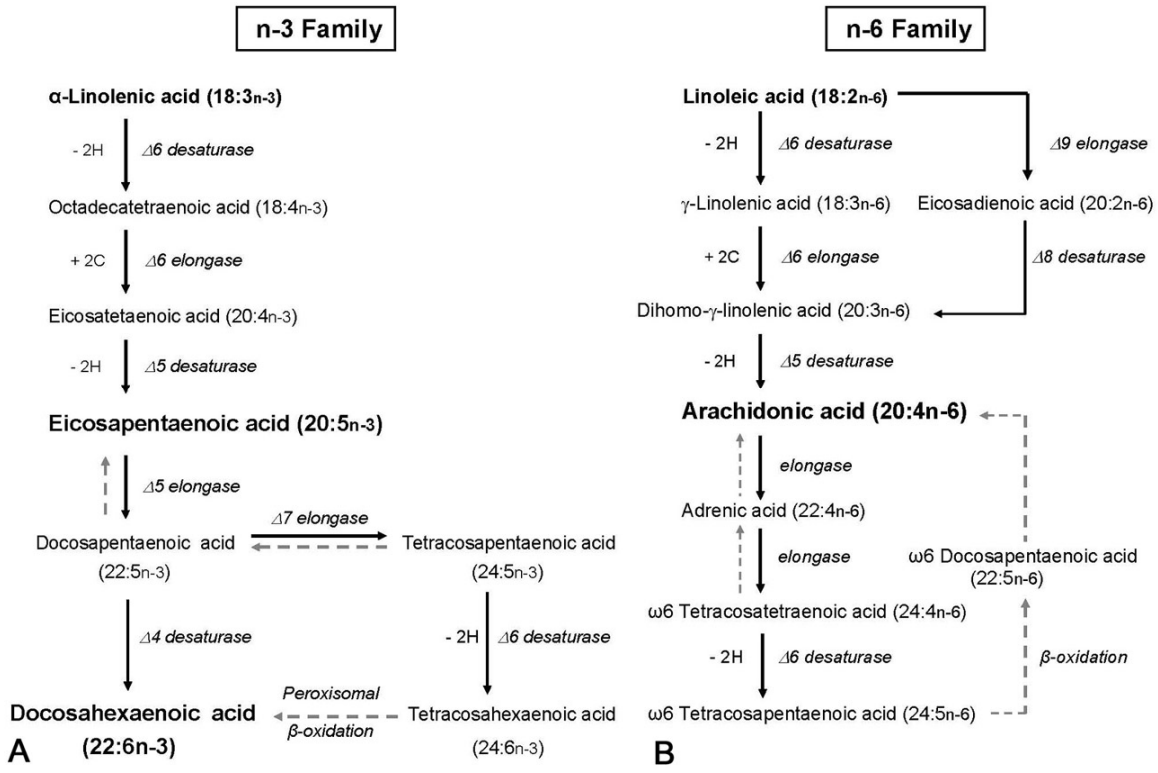


Figure 4. Metabolic pathways for *n*-3 (A) and *n*-6 fatty acids (B).

Both metabolic pathway of *n*-3 and *n*-6 FAs result in the synthesis of related but unique bioactive compounds that regulate many important biological processes. The absolute amounts and relative proportions of LA and ALA affect the respective metabolism of each FA, as well as the metabolism of other FAs. Consequently, in order to establish the main effects on metabolisms of PUFA, more attention is given to the changes in the quantity and ratio of *n*-6 and *n*-3 FAs. These changes are very important because the greatest health benefits are associated to adequate intake of LA and ALA[30].

In human milk, over of 200 FAs of different chain length and unsaturation have been identified, including LA, ALA and their longer-chain metabolites AA and EPA and DHA, respectively. The levels of *n*-6 and *n*-3 FAs in human milk vary widely and among different population, and they are readily changed by the maternal dietary intake of the respectively FAs. In fact, LA and ALA cannot be formed by mammalian cells, and as the result all *n*-3 PUFA and *n*-6 PUFA secreted in human milk must be derived from the maternal diet, either directly or after storage or further metabolism in maternal tissue[16].

In several recent studies analyzing FA composition of mature milk from women living in industrialized countries, the percentage contribution of total *n*-6 LC-PUFA ranged from 0.83% to 1.40%, whereas total *n*-3 LC-PUFA ranged from 0.27% to 0.48% of total FA[31].

Although the levels of AA is relatively stable in breast milk, the levels of DHA vary widely and among different populations[32]. Moreover, the contents of EFA vary with the duration of lactation, particularly the levels of LA and ALA increase with milk maturation, whereas the percentage of LC-PUFA of both *n*-3 and *n*-6 series decrease markedly during the first month after child birth[31].

Human milk contains measurable quantities of total conjugated linoleic acid (CLA) and this concentration vary significantly all over the world and can be strongly influenced by the diet and CLA intake[33].

In bovine milk, PUFA are not synthesis by ruminants tissue, therefore their concentration in milk is closely related to the quantities absorbed in the intestine, hence the quantities leaving the rumen. Those quantities may be increased by dietary PUFA intake and by factors which decrease rumen biohydrogenation[17].

Bovine milk PUFA (~ 5% of total FA) mainly comprise LA (1.5-2%) and ALA (0.3-0.9%) and its positional and geometric isomers[14.] Milk fat also contains *trans*-C18:2 isomers in average amounts of approximately 0.5%. The content of CLA isomers ranges from 0.2 to 2%, and *cis*-9, *trans*-11 C18:2 (rumenic acid) acid dominates[8].

In recent years there has been considerable interest in the beneficial effects of PUFA due to their involvement in several biochemical and physiological metabolic process and very intensive research has been conducted on *trans*-FA (TFA) and CLA. The reason are, firstly, the recently acquired knowledge about the beneficial effects of CLA on human health[26,34,35]. Secondly, the controversy effects of the specific *trans* isomers on serum cholesterol levels in the different lipoprotein classes[36,37] and on fat synthesis/secretion.

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[31] Koletzko B., Rodriguez-Pamero M., Demmelmair H., Fidler N., Jensen R. And Sauerwald T. (2001) Physiological aspects of human milk lipids. *Early Human Development* 65, S3-S18.

[32] Croizer G. (2005) Long.chain polyunsaturated fatty acid-supplemented formula and antioxidant balance in preterm infants. Chapter 9. In: *Healthful Lipids.* Akon C.C. and Lai O-M Eds, AOCS Press, Champaign, Illinois.

[33] Luna P., Juárez M. and Angel de la Fuente M. (2007) Fatty acid and conjugated linoleic acid isomer profile in human milk fat. *European Journal of Lipid Science and Technology* 109, 1160-1166.

[34] Griinari J.M. and Bauman D.E. (1999) Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In: *Advances in Conjugated Linoleic Acid Research*, Vol. 1, pp. 180-200.Yurawecz M.P., Mossoba M.M., Kramer J.K.G., Pariza M.W., Nelson G.J. Eds Champaign, AOCS Press, Illinois.

[35] Stanton C., Lawless F., Kjellmer G., Harrington D., Devery R., Connolly J.F. and Murphy J. (1997) Dietary influences on bovine milk cis-9, trans-11- conjugated linoleic acid content. *Journal of Food Sciences.* 62, 1083-1086.

[36] Willett W.C., Stampfer M.J., Manson J.E., Colditz G.A., Speizer F.E., Rosner B.A., Sampson L.A. and Hennekens C.H. (1993) Intake of trans fatty acids and risk of coronary heart disease among women. *Lancet* 341, 581-585.

[37] Wolff R.L. (1995) Content and distribution of trans- 18:1 acids in ruminant milk and meat fats. Their importance in European diets and their effect on human milk. *JAOCS* 72, 259-272.

The potential health benefits of *n*-3 PUFA, *n*-6 PUFA and CLA will be dealt with in the next chapter (*Chapter 2*), as well as the effects of TFA on fat quality and human health.

1.1.2.2. Triglyceride composition and structure

Triglycerides (TGs) make up about 98% of the fat in human and cow' milk and are comprised of glycerol to which 3 FAs are esterified. The composition of TGs is usually defined in terms of kinds and amounts of FA present. However, structure also includes the distribution of FA within TG molecule and among the TG molecules, as well as the identification of the individual molecular species of TG.

TG of milk fat are made up of a wide variety of FAs and consequently the complex mixture of TGs differs considerably in molecular weight and degree of unsaturation. Because the number of different FAs is great, the number of different TGs is much greater. According to the total number of carbon atoms, milk fat TG extend from C24 to C54 and their can be distinguished in short-chain TGs (C24-C34), medium-chain TGs (C36-C44) and long-chain TGs (C46-C54)[38].

Diet, season, stage of lactation, and individual factors of mammalian species, influence the proportion of individual TG types. The FA composition and the positional distribution of the three FA on glycerol backbone of the TGs (***Figure 5)*** reflect the mechanism of milk fat biosynthesis.

The FA in TG structure are important in determining the relative digestion of TG and the nutritional effects of TG and individual FAs. In addition, physical properties such as crystallization and the melting point temperature, which have an important role on the texture and taste of milk fat products, cannot be explained completely in terms of the properties of individual FAs, but rather depend on the properties of entire TG[39].

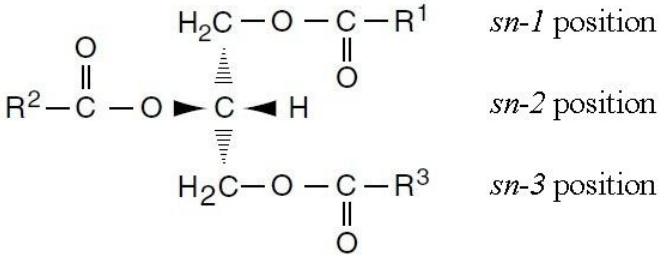

Figure 5. Triglycerides structure. R denotes the aliphatic chain. R^1, R^2 and R^3 denotes the stereo-specific number (*sn*) of the three acyl position.

[38] Mele M., Contarini G., Cercaci L., Serra A., Buccioni A., Povolo M., Conte G., Funaro A., Banni S., Lercker G. and Secchiari P. (2011) Enrichment of Pecorino cheese with conjugated linoleic acid by feeding dairy ewes with extruded linseed: effect on fatty acid and triglycerides composition and on oxidative stability. *International Dairy Journal* 21, 365-372.

[39] Creamer L.K. and MacGibbon A. (1996) Some recent advances in the basic chemistry of milk proteins and lipids. *International Dairy Journal* 6, 539-568.

It is know that the distribution of FAs among the three stereo-specific numbering (*sn*) position in milk TG is not random, but are found selectively placed.

For example, human milk has a high percentage of total palmitic acid (C16:0) at the *sn*-2 position (over 60%)[16], while bovine milk fat C16:0 is predominantly esterified in the *sn*-1, 3 position of the TG. It has been shown that C16:0 is absorbed most readily as its 2-monoacylglycerol than free C16:0 which formed insoluble soaps with calcium and magnesium[40].

The most abundant TGs in human milk, as *sn*-1/3-*sn*-2-*sn*-1/3, are C12:0-C16:0-C16:0, C16:0-C16:0-C18:1 and C18:2 *n*-6-C16:0-C12:0 which represent over 30% of all human milk TG. Human milk also show specific positioning of MC-FA with most esterified to *sn*-3 position[41], while C18:0 is preferentially esterified at *sn*-1, C18:1 and C18:2 at *sn*-1 and *sn*-3 position[42].

Several studies on position distribution of FAs in TG from bovine milk showed that the C4:0, C6:0 and C8:0 FA are almost entirely esterified at the *sn*-3 position. The C10:0, C12:0 and C14:0 FAs are preferentially at the *sn*-1 and *sn*-2 position. C18:0 is esterified preferentially at position *sn*-1 whereas C18:1 showed a preference for *sn*-1 and *sn*-3 position. The *trans*-MUFA are incorporated in the TG in the same manner as their *cis* isomers[43].

In human milk the distribution of FAs in TG is unique with most of the C16:0 at *sn*-2, C12:0 at *sn*-3, C18:0 at *sn*-1, and C18:1 and C18:2 at *sn*-1 and *sn*-3[42].

1.1.2.3. Compound lipids

Milk fat is composed of 97-98% of TGs and 2-3% of minor compound lipids. Compound lipids are also called polar lipid, because they contain charge, that is, acidic and/or basic groups. They are strongly amphipolar properties and are virtually insoluble in water as well as in oil. They are highly surface active and form the typical bilayers that are the basic structure of cellular membrane. In milk they are mainly present in the fat globule membrane and in the poorly defined lipoproteins particles.

The most important polar lipids are phospholipids (PLs). PLs are amphiphilic molecules with a hydrophobic tail and a hydrophilic head group and they are dived in two main groups: glycerolphospholipids and sphingolipids. In the ***Figure 6*** is reported the chemical structure of phospholipid (a) and simplified way to draw a phospholipid (b).

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[40] Yang T., Xu X., He C. and Li L. (2003) Lipase-catalyzed modification of lard to produce human milk fat substitutes. *Food Chemistry* 80, 473-481.

[41] Garcia C. and Innis S. (2013) Structure of the human milk fat globule. *Lipid Technology* 25 (10), 223-226.

[42] Jensen G., Bitman J., Carlson S.E., Couch S.H. Hamosh M. and Newburg D.S. (1995) Milk lipids. A. Human Milk Lipids. In: *Handbook of Milk Composition.* Academic Press, San Diego, California.

[43] Parodi P.W. (1992) Positional distribution of fatty acids in the triglycerides classes of milk fat. *Journal of Dairy Sciences* 49, 73-90.

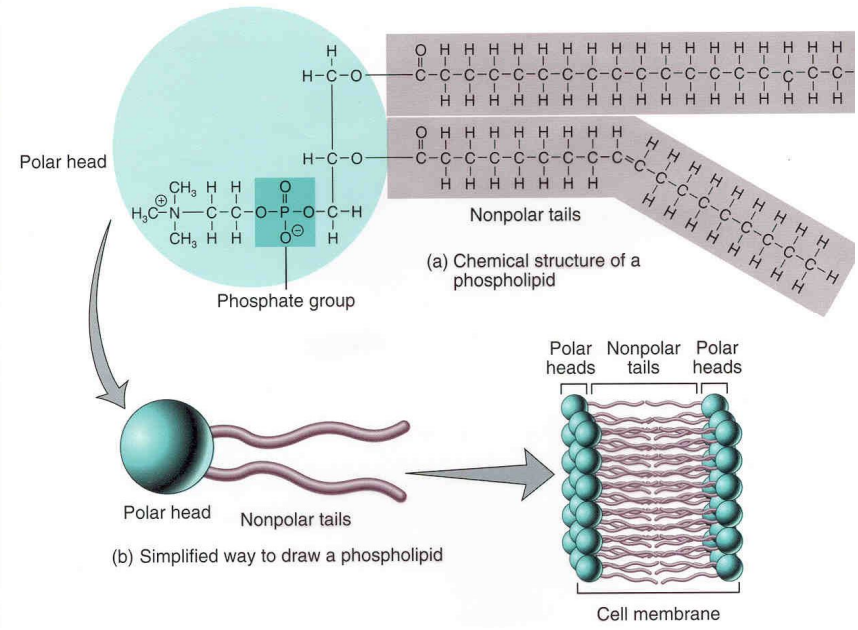

Figure 6. Chemical structure of phospholipid.

The glycerolphospholipids ***(Figure 7)*** consist of glycerol, backbone on which two FAs are esterified. A phosphate residue with different organic groups (e.g. choline, ethanolamine, serine, inositol) may be linked on the third hydroxyl group. The two FA, mainly represented by UFA, are esterified at *sn*-1 and *sn*-2 position of the glycerol backbone. Generally, these FAs are more unsaturated than the TG fraction of milk.

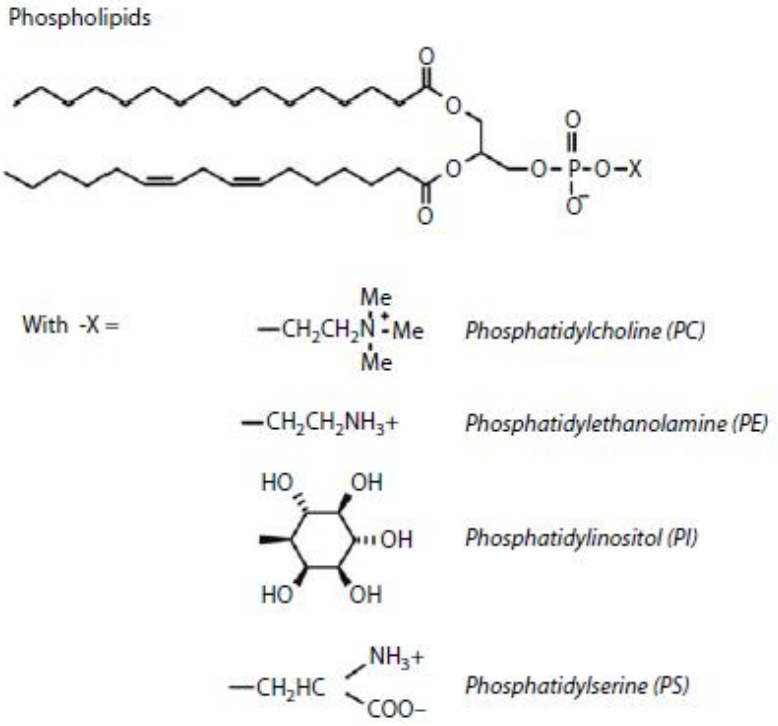

Figure 7. Structure of the principal glycerolphospholipids in milk fat.

Sphingolipids are derived from sphingosine; sphingomyelin (SM) is the dominant species and it is composed of a phosphorylcholine head group and a FA linked to the amine nitrogen of sphingoid long chain base (***Figure 8***). A ceramide is formed when the amino group of this sphingoid long chain base is linked with a FA. On this ceramide unit, an organophosphate group can be bound to form a sphingophospholipid (e.g. phosphocholine in the case of sphingomyelin) or a saccharide to form sphingoglycolipids (glycosilceramides).

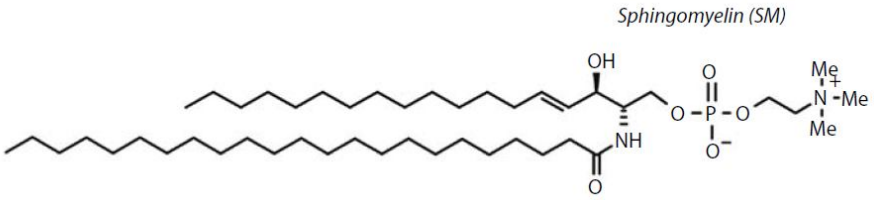

Figure 8. Structure of the sphingomyelin (SM).

Glycerolphospholipids and sphingolipids are quantitatively the most important polar lipids in milk. They represent about 0.5-1% of milk fat and about 60-70% of the polar lipids in milk are in the MFGM, placed mainly in the external bilayer membrane. Among minor PLs, even though lyso-derivatives of PE and PC such as lysophosphotidylethanolammine (LPE), lysophophatidylcholine (LPC) have been reported in milk[44,45], several authors consider that they are probably artifacts caused by careless sample preparation, or that they could be due to lipolytic enzyme activity[46]. Ceramide monohexoside (glucosylceramide, GluCer) and ceramide-dihexexoside (lactosylceramide, LaCer) and plasmogens have often been included by several authors as PLs[47].

1.1.2.4. Unsaponifiable lipids

The unsaponifiable fraction of milk lipids includes sterols, vitamins (mainly A, E and D), hydrocarbons, and alcohols. Sterols are the major fraction of unsaponifiable lipids[8].

Human milk has a high cholesterol content (10–20 mg/dl or 250–500 mg/100 g fat). Cholesterol is the major milk sterol contributing 90.1% to the total sterol content, followed by desmosterol (8.6% of total sterols)[31]. Most of the cholesterol is located in milk fat globule membrane and the amount is

[44] Sanchez-Juanes F., Alonso J.M., Zancada L. and Hueso P. (2008) Glycosphingolipids from bovine milk and milk fat globule membrane: a comparative study. Adhesion to enterotoxigenic Escherichia coli strains. *Biological Chemistry* 390(1), 31-40.

[45] Keen T.W. and Patton S. (1995). The structure of milk: implication for sampling and storage. The milk lipid globule membrane. In: *Handbook of Milk Composition*. Jensen R.G Ed, Academic Press, San Diego, California.

[46] Rombaut R., Camp J.V. and Dewettinck K. (2005) Analysis of phospholipids and sphingolipids in dairy products by a new HPLC method. *Journal of Dairy Sciences* 88, 482-488.

[47] Fong B.Y, Norris C.S. and MacGibbon A:K.H. (2007) Protein and lipid composition of bovine milk-fat-globule-membrane. *International Dairy Journal* 17, 275-288.

not affected by diet or by maternal plasma levels, but is correlated with fat content. About 15% occurs as the ester[42]. The quantities of phytosterols are negligible[31].

Bovine milk contains 10 to 20 mg/dl of cholesterol or 308 to 606 mg/100 mg fat in whole milk containing 3.3% fat. The amount is positively correlated with fat content on the dairy products. Cholesterol, which accounts for 95% of total sterols, is associated with PLs; accordingly it is located mostly in the fat globule membrane, the remainder being dissolved in the fat. About 10% of the cholesterol is esterified. Small amounts of 7-dehydrocholesterol (from 0.7 to 4% of total sterols), and phytosterols (less than 1%) are present[48].

1.1.2 Nitrogen composition of milk

In milk, the three nitrogen (N)-containing fraction are caseins (CNs, insoluble at pH 4.6 at 20°C in bovine milk and insoluble at pH 4.3 plus $CaCl_2$ in human milk) , whey protein (soluble at pH 4.6 at 20°C in bovine milk and soluble at pH 4.3 plus $CaCl_2$ in human milk) and nonprotein nitrogen (NPN)[49].

These proteins are present in aqueous phase (whey proteins), casein micelles consisting of predominantly of casein proteins and only 1-2 % and 4-5% in the MFGM of bovine and human milk, respectively[50,51].

The protein composition of milk (***Table 3***) has been widely studied because of its relationships with composition, nutritional and technological properties of milk and dairy products containing them[11].

In this section will be described the nitrogenous components of bovine and human milks, because it is known that the type of major milk proteins, their amino acid sequence, their relative abundance and the content of NPN fractions varies considerably between species[52].

Moreover, the nitrogenous composition of milk is affected by most of the same factors that affect the concentration of fat, i.e. breed, individuality, season, nutritional status, health, stage of lactation and gestational age[49,53].

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[48] Jensen G. and Newburg D (1995) Milk lipids. B. Bovine milk lipids. In: *Handbook of Milk Composition*. Jensen R.G Ed, Academic Press, San Diego, California.

[49] Alston-Mills B. (1995). Nitrogenous components of milk. G. Non protein nitrogen composition in bovine milk. In: *Handbook of Milk Composition*. Jensen R.G Ed, Academic Press, San Diego, California.

[50] Riccio P. (2004) The proteins of the milk fat globule membrane in the balance. *Trends Food Science and Technology* 14, 458-461.

[51] Liao Y., Alvarado R., Phinney B. and Lönnerdal B. (2011) Proteomic characterization of specific proteins in the human milk casein fraction. *Journal of Proteome Research* 10, 5409-5415.

[52] Smolenski G., Haines S., Kwan F. Y.-S., Bond J., Farr V., Davis S.R., Stelwagen K. and Wheeler T.T. (2007) Characterisation of host defence proteins in milk using a proteomic approach. *Journal of Proteome Research* 6, 207-215.

[53] Bauer J. and Gerss J. (2011) Longitudinal analysis of macronutrients and minerals in human milk produced by mothers of preterm infants. *Clinical Nutrition* 30, 215-220.

Protein	Concentration (g/l)	
	Bovine milk	*Human milk*
Total protein	36.0	9.0
Total Casein	29.5	2.7
α_{s1}-casein	11.9	Not present
α_{s2}-casein	3.1	Not present
β-casein	9.8	2.3[c]
γ-casein[d]	-	1.2
κ-casein	3.5	0.4
Total whey protein	6.3	67.3
β-Lactoglobulin	3.2	
α-Lactalbumin	1.2	1.9
Immunoglobulins (A, M and G)	0.7	1.3
Serum albumin	0.4	0.4
Lactoferrin	0.1	1.5
Lactoperoxidase	0.03	
Lysozyme	0.0004	0.1
Miscellaneous	0.8	1.1
Proteose-peptone	1.2	-
Glycomacropeptide	1.2	-

Adapted from Séverin S. and Wenshui X. (2005)[54], Lönnerdal B. and Atkinson S. (1995)[55].

Table 3. Amounts of protein in bovine and human milks. [c] Non precisely determined. Calculated based on κ-casein being 15% or less of total casein. [d] γ-casein is a product of the proteolysis of the C-terminal of β-casein.

1.1.2.1 Caseins

Caseins is not a globular proteins; it associates extensively and is present in milk in large aggregates, the casein micelles, which also contain the colloidal calcium phosphate (CCP).

Milk caseins (CNs) are the major class of proteins of the most mammalian species (e.g. bovine milk) but this is not the case for human milk[55]. In fact, caseins in bovine milk constitute about 80% of total proteins, whereas in human milk comprise 10-50% of total proteins[56,57].

[54] Séverin S. and Wenshui X. (2005) Milk biologically active components as nutraceuticals: review. *Food Science and Nutrition* 45, 645-656.

[55] Lönnerdal B. and Atkinson S. (1995) Nitrogenous components of milk. A. Human milk proteins. In: *Handbook of Milk Composition*. Jensen R.G Ed, Academic Press, San Diego, California.

[56] Armaforte E., Curran E., Huppertz T., Ryan C.A., Caboni M.F., O'Connor P.M., Ross R.P., Hirtz C., Sommerer N., Chevalier F. and Kelly A.L. (2010) Proteins and proteolysis in pre-term and term human milk and possible implications for infant formulae. *International Dairy Journal* 20, 715-723.

Particularly, natural bovine milk contains about 3 - 3.5% protein by weight and human milk contains about 1% protein by weight. According to the genetically determined primary structures, the casein (CN) group can be sub-divided into α_{s1}-CN, α_{s2}-CN, β- (+ γ)-CN and κ-CN occurring in bovine milk in the approximate proportion of 4:1:4:1, whereas human milk casein consists of primarily β-CN and κ-CN[58] and does not contain α_{s2}-CN[56]. κ-CN is heavily glycosylated, with the carbohydrate content of human milk κ-CN being higher than that of bovine κ-CN.

Milk protein system is very heterogeneous due to post-translation modifications and the presence of genetic polymorphism. As the results of post-translational proteolysis by plasmin and plasminogen, a number of small (proteose - peptone) and large peptides (γ-CN) are present in varying concentration in milk[59].

The polymorphism seems to be correlated to milk composition and to influence some processing properties of bovine milk[58].

1.1.2.2 Whey proteins

Whey proteins or serum proteins is the term to describe the milk proteins remaining in the serum after precipitation of casein or after casein is removed. Whey proteins are globular molecules with a substantial content of α-helix motifs, in which the acidic/basic and hydrophobic/hydrophilic amino acids are distributed in a fairly balanced way along their polypeptide chains[60]. It is possible to separate them into various groups depending on origin (milk proteins, whey proteins) or function (enzyme, binding proteins, immunoglobulins); however, there are no stringent borderline between these categories and proteins may belong to several groups (e.g. alkaline phosphate – a serum protein, most likely also a mammary-derived protein – an enzyme , and a zinc-binding protein)[55].

In bovine milk, whey protein fraction is approximately 16.5% of total nitrogen, whereas in human milk whey protein is the major part of proteins (60-80%) and the casein is a smaller fraction (10-50%)[49,56,61].

Two whey proteins (~ 16.5 % of total nitrogen) β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) in a ratio 3:1 were detected in bovine milk[58]. Human milk does not contain β-Lg and the most abundant whey protein is α-La[56].

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[57] Liao Y., Alvarado R., Phinney and Lönnerdal B. (2011) Proteomic characterization of specific minor proteins in the human casein fraction. *Journal of Proteome Research* 10, 5409-5415.

[58] Bordin G., Cordeiro Raposo F., de la Calle B. and Rodrigues A.R. (2001) Identification and quantification of major bovine milk proteins by liquid chromatography. *Journal of Chromatography A* 928, 63-76.

[59] Swaisgood H.S. (1995) Nitrogenous components of milk. F. Protein and amino acid composition of bovine milk. In: *Handbook of Milk Composition*. Jensen R.G Ed, Academic Press, San Diego, California.

[60] Madureira A.R., Pereira C.I., Gomes A.M.P., Pintado M.E., Malcata F.X. (2007) Bovine whey proteins – Overview on their main biological properties. *Food Research International* 40, 1197-1211.

[61] Liao Y., Alvarado R., Phinney B. and Lönnerdal B. (2011) Proteomic characterization of human milk whey proteins during a twelve-months lactation period. *Journal of Proteome* 10, 1746-1754.

Additionally, bovine milk, like the milk of other species, contains low levels (10% of the whey proteins) of serum-derived protein such as serum albumin (BSA), numerous enzyme such as plasmin and lactoperoxidase, immunoglobulins, and complement proteins, hormones/growth factors such as the IGF family and lactoferrin[52].

Lactoferrin (LF), the first iron-binding protein described in milk, consists of 703 amino acids with a molecular weight of 80 kDa. Bovine milk contains between 0.02 and 0.35 mg/ml of LF, depending on the period of lactation – whereas human milk contains ca. 4.00 mg/ml and therefore LF represent an important fraction of the total human milk protein content[56,60].

The main proteinase in human milk and bovine milk as well, is plasmin, a heat-stable alkaline serine proteinase with a relatively broad specificity on the caseins, occurs in milk like as a complex system including plasminogen (PG), plasminogen activators (PA), inhibitors[62]. The plasmin system components interact together and with other components of milk to promote or inhibit proteolysis in milk and dairy products[63]. Thus, final plasmin activity in milk depends not only on the amount of PG, PA and inhibitors but also on the processing and storage conditions of milk[64].

Proteolysis may have beneficial effects on the texture and flavour of dairy products, particularly during the manufacture or ripening of cheese. However, uncontrolled proteolysis or unwanted proteolysis can detrimentally effect the quality of milk and dairy foods[62,63,65]. For example in bovine milk, inflammation of mammary gland can result in high hydrolysis rate and concentration of undesirable products that are indicators of low quality of milk and dairy products[56].

Conversely, in human milk and especially in pre-term human milk, the higher activity of plasmin on α-CN and β- CN, may have an important nutritional role for low-birth weight infants[56].

1.1.2.3 Nonprotein nitrogen (NPN) fractions

The nonprotein nitrogen (NPN) fractions of milk consist of free amino acids, urea, uric acid, nucleotides, creatine, creatinine and small peptides[49,56].

The NPN concentration in bovine milk ranging from 5 to 6%. Urea N contributes as much as 35-48% of the total NPN fractions, and its level is dependent strictly on dietary changes[49].

Compared to bovine milk, the NPN fractions of human milk (20 to 25% of total nitrogen) is higher and is generally considered to provide adequate protein and nitrogen for the term infants. Moreover,

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[62] Kelly A.L., O'Flaherty F. and Fox P.F. (2006) Indigenous proteolytic enzyme in milk. A brief overview of the present stage of knowledge. *International Dairy Journal* 16(6), 563-572.

[63] Ismail B. and Nielsen S.S. (2010) Invited review: plasmin protease in milk: current knowledge and relevance to dairy industry. *Journal of Dairy Science* 93(11), 4999-5009.

[64] Schroeder D.L., Nielsen S.S. and Hayes K.D. (2008) The effect of raw milk storage temperature on plasmin activity and plasminogen activation in pasteurized milk. *International Dairy Journal* 18, 114-119.

[65] Nielsen S.S. (2002) Plasmin system and microbial proteases in milk: characteristics, roles, and relationship. *Journal of Agricultural and Food Chemistry* 50, 6628-6634.

the absolute amount of NPN is significantly greater in pre-term milk than in term-milk. This is generally related to the growth requirements of the neonate because free amino acid and peptides in NPN should be more readily available than intact proteins[56].

1.1.2.4 Variability of nitrogen components

The nitrogen fraction of milk is composed of a heterogeneous mixture of N-containing substance and their composition/structure and concentration is influenced by genetic make up of the mammalian species, its physiological condition (especially stage of lactation and gestational interval)[55], and environmental factors (especially seasonal differences while the dietary protein has little effect on milk protein content)[49].

Particularly in bovine milk, the protein concentration changes significantly during the first days post-partum and the greatest change occurs in the whey protein fraction. In fact, compared to mature milk, the milk secreted immediately after birth (colostrum) contains higher levels of lactalbumins, lactoglobulins, and especially immunoglobulins (IgG_1, IgG_2, IgA, IgM), peptides (lactoferrin, tranferrin), hormones (insulin, prolactin, thyroid hormones, cortisol), growth factors, prostaglandins, enzyme, cytokines, acute-phase proteins, nucleuotides, polyamines, minerals (iron, magnesium and sodium salts), (pro)vitamins: especially D-carotene, vitamins A, E, D, B, cell elements − lymphocytes, monocytes, epithelial cells etc. Therefore, the intake of colostrum in the first hours after birth confer passive immunity to the newborn and is extremely important for the regulation of growth and functional maturation of gastrointestinal tract of the organism[66,67].

In the case of human milk, during the first day of lactation (*colostrum period*) and in milk from mothers delivering prematurely (pre-term milk), the concentration of whey proteins is very high, whereas casein is virtually undetectable; then whey proteins gradually declines to a relatively low level of 0.8-10% in mature milk and casein concentration increases from 20% of total protein content of human milk during early lactation to ~ 45% during late lactation[51]. These compositional differences can only partly be explained by an increased volume of milk being produced and by an adaptation to the higher protein requirements of premature infants. In the other hands, the milk volume produced during early lactation is very low as well as the milk volume produced by women giving birth prematurely[53,56]. As result, casein concentration increased gradually during the postpartum weeks of lactation and there is no “fixed” ratio whey to casein in human milk. The larger proportion of whey proteins in colostrum than mature milk is due to very high concentration

[66] Gauthier S.F., Pouliot Y. and Maubois J-L. (2006) Growth factors from bovine milk and colostrum: composition, extraction and biological activities. *Lait* 86, 99-125.

[67] Georgiev I.P. (2008) Differences in chemical composition between cow colostrum and milk. *Bulgarian Journal of Veterinary Medicine* 11(1), 3-12.

of secretory IgA and LF. In contrast, some milk proteins, like β-CN, are absent or present in very low concentration during early lactation[56]. These differences in protein levels between colostrum, pre-term and term human milk are related to the rapidly growing of premature infant, whose protein requirement has been estimated to be greater than that of the more mature infant[53,56].

1.2. Structural elements of bovine and human milks

Structure can be defined as the geometrical arrangement of the (chemical) components in a system. In milk, the chemical components (mainly lipids and casein) are located in separate compartments or phases and their structure affect some properties of the system, like viscosity, optical appearance and physical stability. ***Figure 9*** shows a schematic representation of the main structural elements of milk.

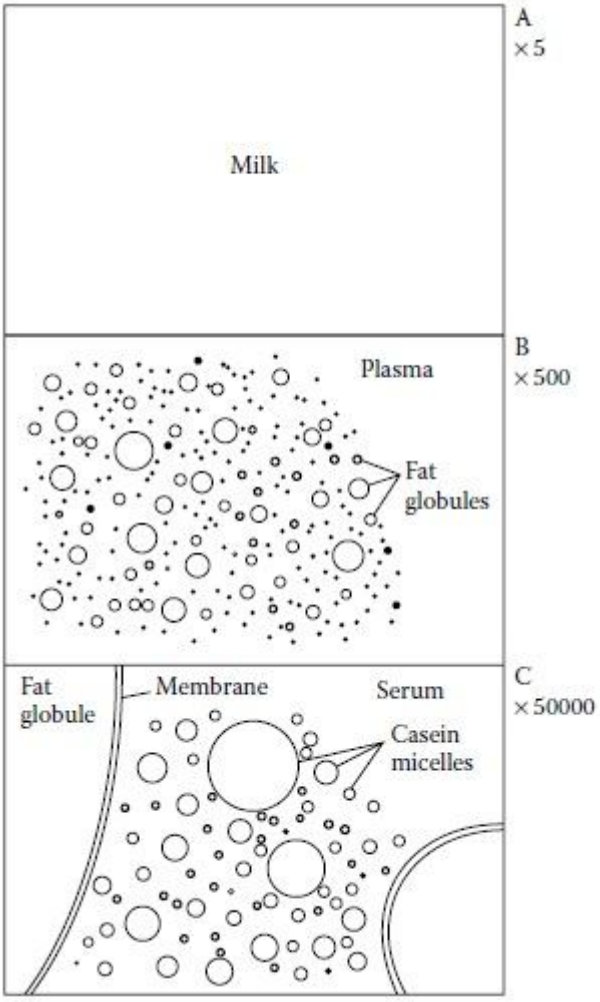

Figure 9. Milk viewed at different magnifications, showing the relative size of structural elements. (**A**) Uniform liquid - the liquid is turbid and thus cannot be homogeneous; (**B**) Spherical droplets, consisting of fat, in a liquid (plasma), which is still turbid; (**C**) The plasma contains casein micelles. The remaining liquid (serum) is still opalescent, so it must contain other particles. The fat globules have a thin membrane of different constitution[68].

[68] Mulder H. and Walstra P. (1974) *The Milk Fat Globule Emulsion Science as Applied to Milk Products and Comparable Foods.* Pudoc, Wageningen, The Netherlands.

The knowledge of molecular structure and properties of milk elements is essential for understanding the many changes that occur in it and the natural function of milk's biomolecules to develop novel food or ingredients with valuable technological and nutritional characteristics. The processability and functionality of milk and dairy products are determined by the properties and concentrations of its principal structural elements (***Table 4***): proteins, lipids, lactose and salts.

	Milk			
		Plasma		
			Serum	
	Fat Globule	**Casein Micelles**	**Globular Proteins**	**Lipoprotein Particle**
Main components	Fat	Casein, water, salt	Serum protein	Lipids, proteins
To be consider as	Emulsion	Fine dispersion	Colloidal solution	Colloidal dispersion
Content (% dry matter)	4	2.8	0.6	0.01
Volume Fraction	0.05	0.1	0.006	10^{-4}
Particle Diameter[a]	0.1-10 µm	20-400 nm	3-6 nm	10 nm
Number per ml	10^{10}	10^{14}	10^{17}	10^{14}
Surface area (cm^2/ml milk)	700	40000	50000	100
Density (20°C; Kg · m^{-3})	920	1100	1300	1100
Visible with	Microscope	Ultramicroscope		Electron Microscope
Separable with	Milk separator	High-speed centrifuge	Ultrafiltration	Ultrafiltration
Diffusion rate (mm in 1 h)[a]	0.0	0.1-0.3	0.6	0.4
Isoelectric pH	~ 3.8	~ 4.6	4-5	~ 4

Note: numerical value are approximate average

[a] For comparison, most molecules in solution are 0.4 to 1 nm diameter, and diffuse, say, 5 mm in 1 h. (1 mm = 10^3µm = 10^6 nm = 10^7 Å.

Table 4. Properties of the main structural elements of milk[69].

[69] Walstra P., Geurts T.J., Noomen A., Jellena A. and van Boekel M.A.J.S. (1999) Part I: Milk – Composition, Structure and Properties. In: *Dairy Technology – Principles of Milk Properties and Processes*, Marcel Dekker, INC. New York, USA.

Milk contains many particles of colloidal especially fat globules and casein micelles; together they make up 12 to 15% of the milk by volume.

Fat globule are a major structural elements of cells. All species, including humans, secrete milk lipids in similar, highly specialized fat globules that are unique to milk. Each fat globule of milk comprised of a central core containing mostly TG is enveloped in a layer of surface-material know as the milk fat globule membrane (MFGM). A small part of the lipids of milk is found outside the fat globule.

Casein micelles occur in the form of colloidally dispersed particles consisting of proteins along with colloidal calcium phosphate (CCP) which is comprised of calcium, magnesium, phosphate, and citrate. They contain also small quantities of some other proteins, such as part of the proteose peptone and certain enzymes. The micelles are voluminous, holding more water than dry matter. They have a negative charge[70].

Moreover, *serum proteins* in molecular form or as very small aggregates, *lipoproteins particles* and *cells* are present in milk[69].

1.2.1 Milk fat globule membrane (MFGM)

Lipids, composed mainly of TGs, are secreted in milk as colloidal globules of variable size (0.1 – 10 μm). The size distribution of milk fat globules results from the well-regulated biological mechanism of assembly and extrusion of intracellular lipid micro-droplets through the apical plasma membrane of the epithelial cells of the mammary gland[71].

The structure and composition of the MFGM depend on the mechanism of secretion. From a structural point of view, the MFGM is organized as a trilayer (thickness = 10-50 nm), with an electron dense material on the inner membrane face composed by polar lipids and protein derived from the endoplasmic reticulum, and a bilayer membrane derived from apical plasma membrane of the mammary epithelial cells that surrounds fat globules when they are secreted (***Figure 10***).

Lipids and proteins are asymmetrically arranged. In particular, the choline-containing PLs, PC and SM, and the glycosphingolipids are largely located on the outside of the membrane, while PE, PI and PS are mainly concentrated on the inner surface of the membrane. Carbohydrate moieties appear to be uniformly distributed over the external membrane surface[72].

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[70] Phadungath C. (2005) Casein micelle structure: a concise review. *Songklanakarin Journal of Science and Technology* 27(1), 201-2012.

[71] Heid H.W. and Keenan T.W. (2005) Intracellular origin and secretion of milk fat globules. *European Journal of Cell Biology* 84, 245-258.

[72] Lopez C., Briard-Bion V., Menard O., Rousseau F., Pradel P. and Besle J-M. (2008) Phospholipids, sphingolipid, and fatty acid composition of the milk fat globule membrane are modified by the diet. *Journal of Agriculture and Food Chemistry* 56, 5226-5236.

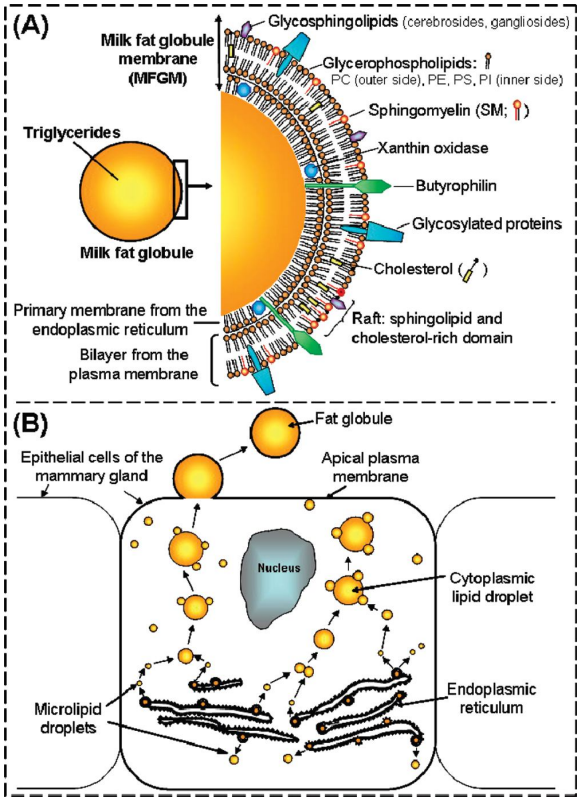

Figure 10. Schematic representation of (**A**) the structure of milk fat globule membrane and (**B**) the pathway for the intracellular origin, growth, and secretion of milk fat globules[72].

The gross composition of MFGM is given in ***Table 5***.

Component	mg/100 g fat globules	mg/100 g MFGM dry matter
Protein	1800	70
Phospholipids	650	25
Cerebrosides	80	3
Cholesterol	40	2
Monoglycerides	+[a]	-
Water	+	-
Carotenoids + Vit.A	0.04	0.0
Fe	0.3	0.0
Cu	0.01	0.0
Total	***> 2570***	***100***

Table 5. Estimated average composition of the milk fat globule membrane[73]; +[a] present, but quantity unknown.

[73] Walstra P., Wouters J.T.M. and Geurts T.J. (2006) *Dairy Science and Technology*. CRC Press, Boca Raton, FL, USA. pp 197-512.

As viewed, MFGM is highly structured and the majority of the MFGM comprises membrane-specific protein, mainly glycoprotein (20 to 60%), TGs, gycerophospholipids (33% of the MGFM), sphingolipids (mainly SM), glycolipids, cholesterol, enzyme and other minor compounds.

The MFGM is subject to changes in composition and structure from the moment the fat globule leaves the mammary secretory cell as well as upon the milk harvesting and milk handling. Moreover, physiological (animal) factors, physical/mechanical and environmental factors affect the stability of MFGM[74,75].

Due to their origin, unique composition and structure, MFGM polar lipids and proteins play an important technological and nutritional role. The MFGM shows good emulsifying properties and provides a preventive action against enzymatic degradation by lipases. Additionally, some MFGM components and metabolites, formed during digestion, provide benefits for human and this is related to the suggestion that MFGM may have interesting nutraceutical properties[76].

1.2.1.1. Lipids of the milk fat globule membrane

The lipids of the MFGM are primarily polar lipids, although neutral lipids can also occur. The latter are TGs, DGs, MGs, cholesterol and its ester. It was often mentioned that the MFGM contains a significant amount of high-melting TGs[77], even if this must be attributed to the isolation methods of the MFGM-prepare[78,79], as during isolation from milk, these MFGM-fragments can easily become contaminated by TG crystals. Several studies have been achieved on the polar lipid content of milk and dairy products. The polar lipid content is positively related to the surface area of MFGM, and negatively correlated with the diameter of the globules in human and bovine milk.

The total amount of polar lipid content in milk ranges from 0.25 to 0.96 g/100g of fat with some differences from human to bovine milk in individual proportion of polar lipid.

The major species of polar lipids of bovine milk fat, expressed as percentage of total polar lipids, are PE (26.4-72.3), PC (8.0-45.5), SM (4.1-29.2%) followed by PI (1.4-14.1%) and PS (2.0-16.1%)[80]. Glucosylceramide (GluCer), lactosylceramide (LacCer) and gangliosides (Gang) were

[74] Evers J.M (2004) The milkfat globule membrane – compositional and structural changes post secretion by the mammary secretory cell. *International Dairy Journal* 14, 661-664.

[75] El-Loly M.M. (2011) Composition, properties and nutritional aspects of milk fat globule membrane – a Review. *Polish Journal of Food and Nutrition Science* 61(1), 7-32.

[76] Hintze K.J., Dallin S., Burtenshaw I and Ward R.E. (2011) Nutraceutical properties of milk fat globular membrane. Chapter 16. In: *Biotechnology and Biopolymers*. Elnashar M. Ed, 2004–2013 InTech - Open Access Company.

[77] Wooding F.B.P. and Kemp P. (1975) High melting point triglycerides and milk fat globule membrane. *Journal of Dairy Research* 49, 419-426.

[78] Walstra P. (1974) High melting triglycerides in fat globule membrane – artifact. *Netherlands Milk and Dairy Journal* 28, 3-9.

[79] Walstra P. (1985) Some components on the isolation of fat globule membrane. *Journal of Dairy Research*, 52, 309-312.

[80] Contarini G. and Povolo M. (2013) Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies. *International Journal of Molecular Sciences* 14, 2808-2831.

also found in trace amounts in the MFGM[81,82]. Less numerous are the studies aiming to determine the polar lipid composition of milk of different mammalian species. Specifically, human milk is characterize by a high content of SM, followed by PC, PE, PI and PS[8384]. In comparison to the quantitative distribution of PLs, human MFGM has relatively less PC but relatively more PE and SM than does bovine MFGM, whereas PS and PI account for a higher proportion of PLs in bovine MFGM than in human MFGM[45]. Furthermore, human milk PE contains about 40% of plasmogens and 60% of diacyl species, compared to 10% of plasmogens and 90% of diacyl-PE in mature bovine milk[84].

The mean values of main polar lipids in milk fat from bovine and human milk are reported in the ***Table 6***.

Polar Lipids	*Bovine milk*	*Human milk*
PE	33	28
PI	4	4.5
PS	10	9
PC	29	24
SM	22	31

Table 6. Average distribution of the major PLs in milk fat from bovine and human species. Value are expressed as percentage (%) of total PLs[24] .

The variability of polar lipid composition of the MFGM material may be associated by differences in isolation, purification and analytical techniques, as well as by other factors such as processing (mainly homogenization), environmental factors, animal factor, period of lactation and diet.

Focusing of the FA composition, the SC- and MC-FA (C4-C14) typically for milk fat, are virtually absent in the PLs fraction. In particular, PE is highly unsaturated, followed by PI and PS, whereas PC contains more saturated FA in comparison with the other PLs. Fong *et al*. (2007)[47] detected only a small amount of very long chain FAs (carbon atoms > 20) esterified in the glycerolphospholipids PE, PC, PI and PS. Completely different is the FA composition of dairy SM. Although LC-FA

[81] Lopez C., Briard-Bion V., Menard O., Beaucher E., Rousseau F., Fauquant J., Leconte N. and Robert B. (2011) Fat globule membrane from whole milk according to their size: Different composition and structure of the biomembrane, revealing sphingomyelin-rich domains. *Food Chemistry* 125, 355-368.

[82] Dewettinck K., Rombaut R., Thienpont N., Le T.T., Messens K. and Camp J.V. (2008) Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal* 18, 436-457.

[83] Benoit B., Fauquant C., Daira P., Peretti N., Guichardant M. and Michalski M.C. (2010) Phospholipids species and minor sterols in French human milk. *Food Chemistry* 120, 684-691.

[84] Garcia C., Lutz N.W., Confort-Gouny S., Cozzone P.J., Armand M. and Bernard M. (2012) Phospholipids fingerprints of milk from different mammalian species determined by ^{13}P NMR: towards specific interest in human health. *Food Chemistry* 135, 1777-1778.

occur, nearly all of them are saturated[80]. This uncommon high degree of saturation gives SM the ability to form with, cholesterol, the so-called "lipid rafts", rigid domains involved in different important cellular process like signal transduction, cell sort, endocytocis and cholesterol trafficking. Similarly, the dietary intake of SM lowers cholesterols adsorption in the intestines by lowering the membrane fluidity of the liposome[82].

1.2.1.2. Proteins of the milk fat globule membrane

Extensive proteomic studies of the MFGM have demonstrated that MFGM has a large number of polypeptides, at least 50, ranging from 10 to 300 kDa. A similar number of protein polypeptides can be see in human milk HMGM[85].

The protein components of bovine MFGM (***Table 7***) mainly consist of xanthine oxidase (155 kDa), butyrophilin (67 kDa), glycoprotein B (48 kDa), mucin MUC1 (200 kDa), the redox enxyme, CD36 (77 kDa), the adhesive glycoprotein, MFG-F8 (PAS-6/7), adipocyte differentiation-related protein and fatty acid-binding protein (13 kDa). Furthermore, new minor proteins including polymeric Ig receptor protein, apolipoproteins E and A1, 71-kDa heat-shock cognate protein, clusterin, lactoperoxidase, Ig heavy chain and peptidylprolyl isomerase A, were found in bovine MFGM[86].

Protein	*Molecular Weight (kDa)*
Mucin I (MUC1)	160-200
Butyrophilin(BTN)	66-67
Adipophilin (ADPH)	52
Xanthine oxidase (XO)	150
Cluster of differentiation (CD36)	76-78
Fatty acid binding protein (FABP)	13
Periodic acid shift 6/7 (PAS 6/7)	48-54
Periodic acid shift III (PAS III)	95-100
Breast cancer type 1 (BRCA1)	210

Table 7. Some proteins of bovine milk fat globule membrane (MFGM)[75,85,86]

[85] Spitsberg V.L. (2005) Invited review: bovine milk fat globule membrane as a potential nutraceutical. *Journal of Dairy Science* 88, 2289-2294.

[86] Kuchta A.M., Kelly P.M., Stanton C. and Devery R.A. (2012) Milk fat globule membrane – a source of polar lipids for colon health? A review. *International Journal of Dairy Technology* 65, 1-19.

Quantitatively, the major MFGM protein is glycoprotein butyrophilin (about 40% of total MFGM protein) and the second representative protein of the MFGM is xanthine oxidase (12-13% of the total MFGM protein). Other proteins are present in MFGM each at 5% or less[85].

1.2.2. Casein micelles

Casein micelles are supramolecules consisting of multiple molecular entities organized by hydrophobic interactions and electrostatic binding. The latter type of binding occurs via bridges formed between charged parts of the casein subunits, calcium and phosphate. In fact, the casein micelles does not only consist of casein subunits, but calcium, phosphate, and to some extent other ions like magnesium and citrate form an insoluble aggregate namely colloidal calcium phosphate (CCP). The casein micelles contain also small quantities of some other protein, such as part of the proteose-peptone and certain enzyme. These aggregates give milk its characteristic white appearance and these noncovalent intermolecular bonds are responsible of the integrity of the casein micelles. They have a negative charge[55,57,87].

Since the casein micelles has important for the nutritional, physical stability and rheological properties of milk and dairy products, their structure have been extensively reviewed. In the past 50 years, numerous models for the structure of casein micelles regarding coat-core structure, sub-micelles, and internal structure, have been proposed[88].

Essentially, the most widely accepted model for the micelle is build up from sub-micelles held together by hydrophobic interaction between proteins and by CCP linkages (***Figure 11***)[88, 89].

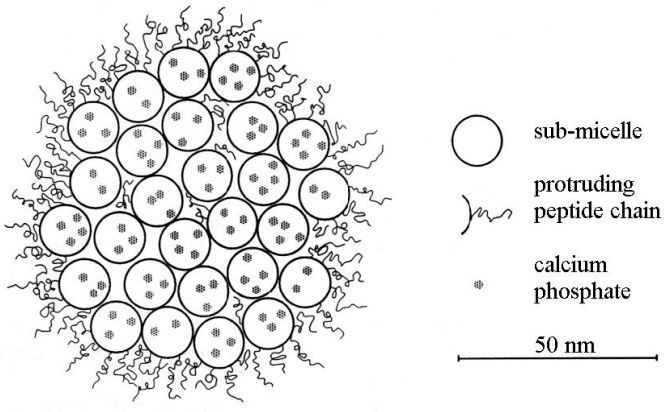

Figure 11. Schematic model of a cross-section through a casein micelle.
According to this model the κ-CN are located near the outside of the micelle with hydrophilic part of the C-terminal end protruding from the micelle surface to form a "hairy" layer that will avoid further aggregation of sub-micelles by steric and electrostatic repulsion[88].

[87] Jensen R.G., Blanc B. and Patton S. (1995) The structure of milk. B. Particulate constituents in human and bovine milks. In: *Handbook of Milk Composition.* Jensen R.G Ed, Academic Press, San Diego, California.
[88] Phadungath C. (2005) Casein micelle structure: a concise review. *Songklanakarin Journal of Science and Technology* 27(1) 201-212.
[89] Walstra P. (1999) Casein sub-micelles: do they exist? *International Dairy Journal* 9, 189-192.

However, several alternative models have been proposed. Particularly, two new internal structure models have been proposed by Holt C.1992[90], later in 1996[91] and Horne D. 2003[92] in which the caseins act as an inhibitors of the growth of calcium phosphate precipitates (***Figure 12***) and where the state of association of casein proteins is governed by a balance of attractive hydrophobic interactions and electrostatic repulsion (***Figure 13***). Consequently, micelles are stable, and they do not usually flocculate[88].

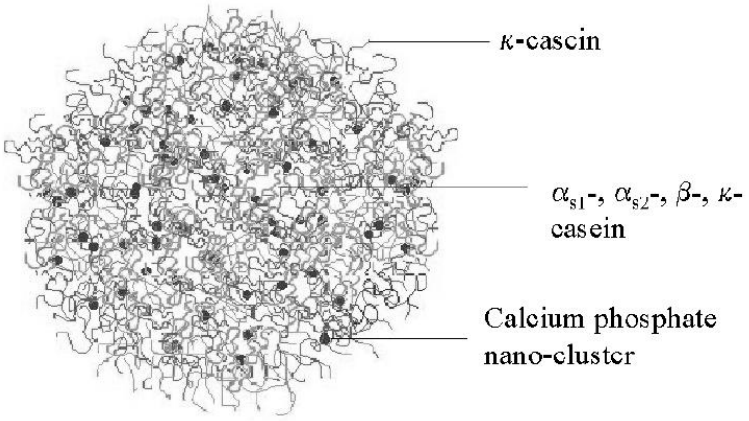

Figure 12. Casein micelle model where the casein micelles as a tangled web of flexible casein networks forming a gel-like structure with micro-granules of colloidal calcium phosphate through the casein phosphate centre, and the C-terminal region of κ-CN extends to for a hairy layer[90,91].

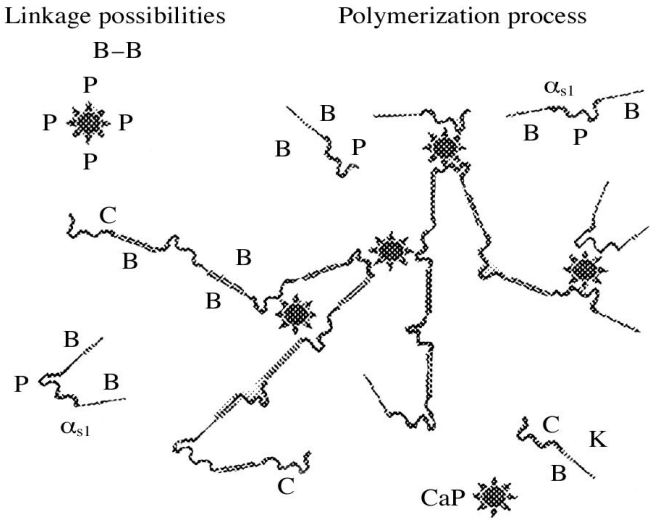

Figure 13. Dual-binding model show interaction between α_{s1}- β and κ-CN[92].

[90] Holt, C. (1992) Structure and properties of bovine casein micelles. *Advances in Protein Chemistry* 43, 63–151.
[91] Holt C. and Horne D.S. (1996) The hairy casein micelle: evolution of the concept and its implications for dairy technology. *Netherlands Milk and Dairy Journal* 50, 85-111.
[92] Horne, D.S. (2003) Caseins – micellar structure. In: *Encyclopedia of Dairy Sciences*, pp 1902–1909. Roginski H., Fuquay J. and Fox P.F., Eds, Academic Press, London, UK.

Therefore, the two main feature of these models are the integrating role of CCP and the surface layer consisting predominantly of κ-CN. Furthermore, hydrophobic interaction are the driving force for the formation of casein micelles, while the electrostatic repulsion are limiting the growth of polymers or in other words defining the degree of polymerization[88].

Milk casein micelles exist as relatively large spherical particles with an average diameter of ~ 120 nm (range 50-600 nm), suspended in a soluble salt solution[93].

The casein micellar system in the milk differ considerably. The casein subunit of bovine milk consist of α_{s1}- ,α_{s2}-, β-, and κ-CNs; while human milk contains only β- and κ-CN. Moreover, the level of phosphorylation between human and bovine milk β-CN is different. Human β-CN has six possible phosphorylation levels (i.e. 0, 1, 2, 3, 4, 5 phosphate groups) compare to the fully phosphorylated bovine β-CN. This differences in the level of phosphorylation play an important role to the capacity of these different form to assemble micelles and may be significant during the digestion of β-CN by infants[55,94]. Moreover, human κ-CN contains 40-60% of carbohydrates with a numerous possibilities of structural variants, whereas bovine κ-CN contains only 10% of carbohydrates. The highly glycosylated κ-CN has a structure-stabilization role[55].

Considering these differences in phosphorylation and glycosylation levels, as well as the tendency of proteolysis, human and bovine milks are characterized by a additional heterogeneity.

In human milk only 15% of the calcium is linked to casein and in bovine milk the amount is about 65%. The casein in human milk are more difficult to isolate requiring acidification to pH 4.3 and addition of $CaCl_2$ compared to the isolation of bovine milk caseins by acidification to pH 4.6 at 20°C[87].

Although the concentration of casein is lower in human milk (0.2-0.5 g/dl of milk) than in bovine milk (2.2-2.8 g/dl of milk), the numbers of micelles (7×10^{15}) are about the same. The shape of bovine milk casein micelles ranging in size from 50 - 500 nm in diameter (average about 120 nm), whereas the micelles in human milk are considerably smaller (30-75 nm in diameter)[55,95].

The integrity of casein micelles is affected by thermodynamic equilibrium between the micelles and their surroundings. The main change probably is proteolysis of β-CN into γ-CN and proteose peptone by plasmin. Moreover, the stability and behaviour of structure of casein micelles is strongly dependent on external factor, especially pH, temperature and milk salts. Some of these changes are reversible, whereas others are not or partly so[73].

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[93] O'Kennedy B.T., Mounsey J.S., Murphy F., Duggan E. and Kelly P.M. (2006) Factors affecting the acid gelation of sodium caseinate. *International Dairy Journal* 16, 1132-1141.

[94] McCarthy N.A., Kelly A.L., O'Mahony J.A. and Fenelon M.A. (2013) The physical characteristics and emulsification properties of partially dephosphorylated bovine β-casein. *Food Chemistry* 138, 1304-1311.

[95] Fox P.F. and McSweeney P.L.H. (1998) Milk proteins.. In: *Dairy Chemistry and Biochemistry*, Blackie Academic & Professional Ed, an imprint of Thomson Science, 2-6 Boundary Row, London SE1 SHN, UK.

2. Bioactive components in milk and dairy products

The term *bioactive components* refers to compounds either naturally existing in food or ones formed and/or formulated during food processing that may have physiological and biochemical functions when consummated by humans. Several studies have been applied on bioactive components of milk and dairy products for application in functional foods and for potential pharmaceutical use. A food can be said to be functional if it contains a component (whether or not a nutrient) that benefits one or a limited number of functions in the body in a targeted way that is relevant to either the state of well-being and health or the reduction of the risk of a disease, or if it has physiologic or psychologic effect beyond the traditional nutritional effect[96].

Milk is often considered as a functional food since it contains an array of different bioactive components, such as protein, antibacterial peptides, immunoglobulins, various important vitamins and minerals in substantial quantities, carbohydrate in the form of lactose (***Figure 14).*** Also, approximately one third of the fat in whole milk is monounsaturated and small amounts of EFA as well as other components at low concentrations (so-called "minor" components) are provided. Milk is one of the major sources of CLA in the diet, although it is a minor component of milk fat.

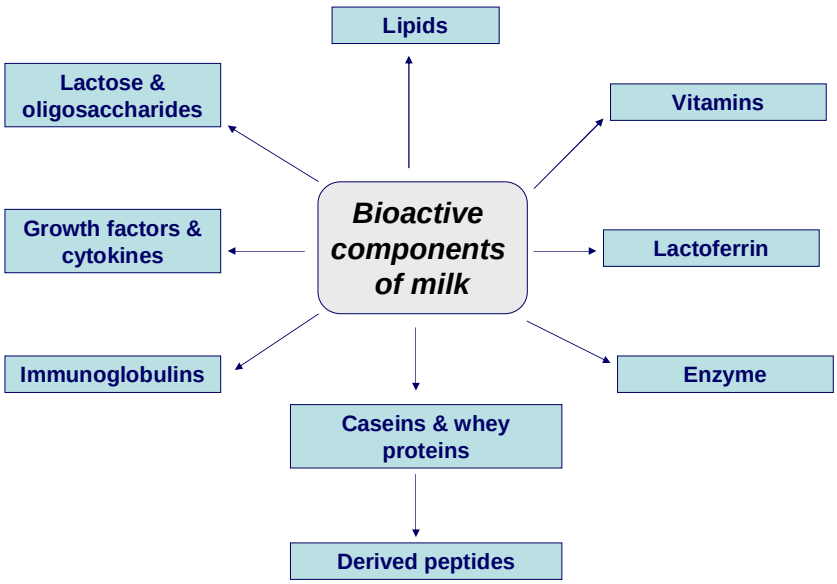

Figure 14. Schematic representation of major bioactive functional compounds derived from milk[97].

Due to the presence of a large number of bioactive compounds, milk and dairy products provide chemical, physiological, and nutritional functionalities and characteristic that have strong potential

[96] Roberfroid M.C. (2000) Concepts and strategy of functional food science: the European perspective. *American Society for Clinical Nutrition* 71, 1660-1664.
[97] Park Y.W. (2009) Overview of bioactive components in milk and dairy products. In: *Bioactive Components in Milk and Dairy Products.* Park Y.W. Ed, Wiley – Blackwell, Iowa, USA.

for beneficial effects on human health. Four major areas of bioactivity of milk components have been categorized: 1) gastrointestinal development, activity and function; 2) infant development; 3) immunological development and function; and 4) microbial activity, including antibiotic and probiotic action[97].

2.1 Bioactive lipids

Milk fat represents a rich source of biologically active molecules, many of which offer potential benefits on human health. Particularly, nutritional aspects in milk has perceived increasing importance and interest of food scientists, nutritionists, health professionals, and general consumers. Therefore, a general overview of the nutritional aspects of the lipid fractions is given, because of a several health-promoting effects have been attributed to individual lipid components of milk and dairy products. It is well known that lipids are the vital components of cell membrane with multiple forms and functions because they are involved in many inter- and intra-cellular metabolic process[98]. In this section, special attention is given to short-, medium-chain SFA, stearic acid (C18:0), MUFA, long-chain PUFA (LC-PUFA), *cis*, *trans* and conjugated FA, which are involved as positive or negative predisposing factors for human health[99].

- *Short-, medium-chain SFA (SC-SFA, MC-SFA) and stearic acid (C18:0)*

The dietary intake of SFA increase the concentration of serum low-density lipoprotein (LDL) cholesterol, which is a risk factor for cardiovascular disease (CVD) and in particular for coronary heart disease (CHD). Whilst in general SFA raise total cholesterol and LDL-cholesterol, early studies have identified that individual FAs have markedly different effects[100].

Particularly, milk fat is now recognized among few food sources of butyric acid (C4:0), a short-chain SFA (SC-SFA), produced by gastrointestinal microflora during the fermentation of dietary fibre. The C4:0 produced by this formation is utilized both as an energy source and as a source of carbon for colonic epithelial cells. C4:0 also serves as essential role in the normal development of colonic cell proliferation, induces differentiation in a wide spectrum of cancer cell lines including those of the breast and colon. Moreover, C4:0 is capable of inducing apoptosis in several hepatic cells lines with a possible to provide anti-cancer benefits to the liver[101,13].

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[98] Parodi P.W. (2006) Nutritional significance of milk lipids. In: *Advance Dairy Chemistry*. Volume 2. Lipids. Fox P.F. and McSweeney P.H.L., Eds, Third Edition. Springer, New York, pp 601-636.

[99] Talpur E.N., Bhanger M.J., Khooharo A:A. and Zuhra Memon G. (2008) Seasonal variation in fatty acid composition of milk from ruminants reared under the traditional feeding system of Sindh, Pakistan. *Livestock Science* 118, 166-172.

[100] Givens D.I (2008) Symposium on "The challenge of translating nutrition research into public health nutrition". Session 4: challenges facing the food industry in innovative for health impact on CVD risk of modifying milk fat to decrease intake of SFA and increase intake of cis-MUFA. *Proceeding of the Nutritional Sciences* 67, 419-427

[101] Watkins S.M., Carter L.C., Mak K., Tsau J., Yamamoto S. and German J.B. (1999) Butyric acid and tributyrin induce apoptosis in human hepatic tumour cells. *Journal of Dairy Research* 66, 559-567.

A summary of these related cell-growth-inhibiting effects induced by C4:0 is outline by Parodi *et al*. (2006)[98].

Regarding MC-SFA, lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids have been implicated in increasing total and serum LDL-cholesterol concentrations. In contrast, stearic acid (C18:0), that account for the majority of SFA in milk, is considered to be neutral in this regard[51,17] SFA are also associated to reduce insulin sensitivity, which is a key factor in the development of the metabolic syndrome[13,100].

These aspects underline the possibility to reduce levels of consumption of SFA by the use of low-fat/high-carbohydrate diet, or by displacing SFA with both *cis*-MUFA and PUFA to obtain not only favourable changes in plasma cholesterol pools but other health related outcomes[100,102].

- *Cis-monounsaturated fatty acids (cis-MUFA)*

Oleic acid (*cis*-9 C18:1) is the most prominent MUFA. Although the *cis*-9 C18:1 is not an essential FA, it is very important because, in addition to the usual functions of FAs (source of energy and structural components), it reduces the melting point of TGs, thus provide the liquidity required for the formation, transport and metabolism of milk fat globules[103].

Moreover, as reported by several authors[104,105,106], human diet high in *cis*-9 C18:1 reduces total and LDL cholesterol levels when substituted for C12:0, C14:0, or C16:0 and when the basic diet containing less *cis*-9 C18:1. Although, high-MUFA/low-SFA diet has been shown to result in a more favourable metabolic profile with respect to total cholesterol, high density lipoprotein (HDL) cholesterol, and triglyceride concentrations than a conventional diet or a low-fat/high-carbohydrate diet, some evidence indicates that a high intake of MUFA may increase the risk of cardiovascular disease[107].

- *Trans fatty acids (TFA)*

TFA are naturally present in ruminant milk and meat; they are formed by bacterial biohydrogenation in the rumen of cows, sheep and goats. Mainly, TFA occurring in cow milk fat are *trans*-C18:1 followed by a small amounts of *trans*-C18:2, *trans*-C16:1 and *trans*-C14:1. Their

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[102] Williams C.M., Francis-Knapper J.A., Webb D., Brookes C.A., Zampelas A., Tredger J.A., Wright J., Calder P.C., Yaqoob P., Roche H. and Gibney M.J. (1999) Cholesterol reduction using manufactured foods high in monounsaturated fatty acids, a randomised cross-over study. *British Journal of Nutrition* 81,439-446.

[103] Jensen R.G. (1999). Lipids in human milk. *Lipids* 34, 1243-1271.

[104] Mattson F.H. and Groundy S.M. (1985) Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids lipoprotein in man. *Lipid Research,* 26, 194-202.

[105] Zock P.L. (1995) Dietary fatty acids and risk factors for coronary heart disease: Controlled studies in healthy volunteers. *Thesis*, Wageningen.

[106] Berry E.M., Eisenberg S., Haratz D.. Friedlander Y., Norman Y., Kaufmann N.A. and Stein Y. (1991) Effects of diet rich in monounsaturated fatty acids on plasma lipoproteins – the Jerusalem Nutrition Study: high MUFAs vs high PUFAs. *American Journal of Clinical Nutrition*, 53, 899-907.

[107] Kris-Etherton, P. et al. (2001) AHA scientific statement: summary of the scientific conference on dietary fatty acids and cardiovascular health, *Journal of Nutrition* 131, 1322.

levels in cow milk (generally ranges from 1.8 to 7.6%) can vary considerably depending on the season and animal's diet[108,109].

Vaccenic acid (*trans*-11 C18:1, VA) is the major components of ruminant fat while elaidic acid (*trans*-9 C18:1) is generally regarded as the isomer typical for the industrial hydrogenation[110]. VA is also an intermediate in the biohydrogenation of PUFA 18-carbon FA to C18:0 (stearic acid) in the rumen, and is the major precursor of CLA in milk fat. The importance of VA lies in its role as a precursor of the main isomer of CLA, rumenic acid (*cis*-9, *trans*-11 C18:2, RA), physiologically the most relevant bioactive compound present in milk. This synthesis is catalyzed by Δ9-desaturase enzyme and not only occurs in the bovine mammary gland, but also in the human tissue[111,112]. Therefore, TFA are also present in human milk, the level being highly correlated to the maternal diet. Their level (from 2% to 18% of total fatty acids) in human milk influence the plasma TG level of breast-fed infants[113].

In order to evaluate the potential effects of TFA it is important to know how much and what kind of TFA are present in milk lipids because individual TFA isomers could have differing physiological effects. Metabolic and epidemiological studies have revealed the raising effects of *trans*-C18:1 FA and *trans*-C16:1 FA, especially when the latter is associated with *trans*-C20:1, on plasma total and LDL-cholesterol concentration with a simultaneous reduction of HDL-cholesterol concentration[114] and with an unfavourable increase of the potentially atherogenic lipoprotein (a) level[115,116]. These changes may cause premature atherosclerosis and CHD. Thus, compared with SFA and *cis*-MUFA, the overall effect of increased intakes of *trans* FAs is a less favourable LDL/HDL ratio, which is an additional increase in the risk of CVD.

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[108] Precht D. and Molkentin J. (1997). Effect of feeding on conjugated cis delta 9, trans delta 11-octadecadienoic acid and other isomers of linoleic acid in bovine milk fats. *Nahrung* 41, 330-335.

[109] Wolff, R.L., C.C. Bayard and Fabien R.J. (1995) Evaluation of Sequential Methods for the Determination of Butterfat Fatty Acid Composition with Emphasis on trans-18:1 Acids, Application to the Study of Seasonal Variations in French Butters. *Journal of the American Oil Chemists' Society* 7, 1471-1483.

[110] Weggemans R.M., Rudrum M. and Trautwein E.A. (2004) Intake of ruminant versus industrial trans fatty acids and risk of coronary heart disease – what is the evidence? *European Journal of Lipid Sciences and Technology* 106, 390-397.

[111] Turpeinen A., Mutanen M., Aro A., Sakminen I., Basu S., Plamquist D.L. and Griinari J.M. (2002). Bioconversion of vaccenic acid to conjugated linoleic acid in humans. *American Journal of Clinical Nutrition* 76, 504-510.

[112] Recio I., De la Funete M.A., Juàrez M. and Ramos M. (2009) Bioactive compounds in sheep milk. In: *Bioactive Compounds in Milk and Dairy Products.* pp 83-104. Young W. Park Ed, Wiley-Blackwell, Iowa, USA.

[113] Dionisi F., Golay P.A. and Fay L.B. (2002) Influence of milk fat presence on the determination of trans fatty acids in fats used for infant formulae. *Analytica Chimica Acta* 465, 395-407.

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[115] Mensink R.P., Zock P.L., Katan M.B. and Hornstra G. (1992) Effect of dietary cis and trans fatty acids on serum lipoprotein [a] levels in humans. *Journal of Lipid Research* 33, 1493-1501.

[116] Nestel P., Noakes M. and Belling B. (1992) Plasma lipoprotein lipid and Lp[a] changes with substitution of elaidic acid for oleic acid in the diet. *Journal of Lipid Research* 33, 1029-1036.

Moreover, *trans*-isomer of C18:1 and specifically, *trans*-10 C18:1 is considered to have a major role in the mechanism for the reduction in milk fat[117].

Further studies have demonstrated that *trans*-9 C18:1 and *trans*-9, *trans*-12 C18:2 are responsible to inhibit prostaglandin synthesis, and the isomer *trans*-9, *trans*-12 C18:2 is associated also with a variety of toxicological and physiological aberrations[118].

In contrast to *trans*-9, *trans*-12 C18:2, the rumenic acid (*cis*-9, *trans*-11 C18:2, RA) exclusively exhibits positive biological effects on human health. For these reasons, the potential cancerogenic properties of TFA are rather controversial discussed[119,120].

Besides, several of the large studies, which established that intake of TFA increases CHD risk, showed a significant inverse association with intake of animal or dietary TFA, a non-significant inverse trend or at least no change with increasing intake of TFA from such source[121].

Since the melting point of TFA is very different from that of the corresponding *cis* isomers, and because lipid fluidity in cell structure could limit fat globule packaging and/or secretion, it is likely that the percentage of FAs, as well as their position in the glycerol molecule, contribute together via the fluidity of milk fat to an overall regulatory mechanism[17].

- *Long chain polyunsaturated fatty acid (LC-PUFA)*

In recent years there has been considerable interest in the beneficial physiological effects of the long-chain (LC) *n*-3 polyunsaturated FAs (PUFA) such as DHA and EPA and *n*-6 LC-PUFA such as AA. These fatty acids derived from the diet, or from endogenous synthesis from their respectively parental EFA. In particular, LA is the precursor of the bioactive *n*-6 PUFA, such as AA; while ALA is the precursor of the bioactive *n*-3 PUFA, EPA and DHA (***Figure 4***).

Optimal balance of *n*-6/*n*-3 PUFA ratio contribute to a lower risk of CHD by a reduction of pro-thrombotic and pro-inflammatory state. These FAs are important since they are structural components of cellular membrane, performing the function of growth and development of tissue and organs which occurs intensely during the first months of life[122].

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[117] Griinari J.M., Chouinard P.Y. and Bauman D.E. (1997) Trans fatty acids hypothesis of milk fat depression revised. In: *Proceeding of the Cornell Nutrition for Feed Manufactures*, pp 208-216. Ithaca, NY, Cornell University Press.

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[121] Pfeuffer M. and Schrezenmeir J. (2006) Impact of trans fatty acids of ruminants origin compared with those from partially hydrogenated vegetable oils on CHD risk. *International Dairy Journal* 16, 1383-1388.

[122] Uauy R., Mena P. and Rojas C. (2000) Essential fatty acids in early life: structural and functional role. *Proceedings of the Nutritional Society* 59(1), 3-15.

Particularly, *n*-3 PUFA and *n*-6 PUFA are precursors of the bioactive prostaglandins, thromboxanes, and leukotrienes. These compounds have a regulatory function in various biochemical processes including immune response[123].

Furthermore, DHA is the major structural components of phospholipids in the retina, brain and central nervous system[124,125] and AA, the most important *n*-6 LC-PUFA, is an abundant constituent of the central nervous system[123] and is involved in growth and cell signaling, in part through its precursorship of eicosanoids and lipoxins[126].

Evidence for anti-atherogenic, anti-thrombotic, anti-inflammatory and immuno-suppressive action of DHA and EPA are confirmed from epidemiological and experimental studies[30].

- *Conjugated linoleic acid (CLA)*

Conjugated linoleic acid (CLA) is a collective term to describe one or more positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12 C18:2) with a conjugated double bond system (***Figure 15***) that are formed by biohydrogenation and oxidation processes in nature. Conjugated double bonds are usually at position 7-9, 8-10, 9-11, 10-12, 11-13; each double bond can be in either *cis* or *trans* configuration[98].

Ruminant-derived food products (dairy and meat) are the major dietary sources of CLA, and *cis*-9,*trans*-11 C18:2 also called "rumenic acid" (RA), is the predominant CLA isomer (about 75 to 90 % of total CLA isomers) in natural lipids[127].

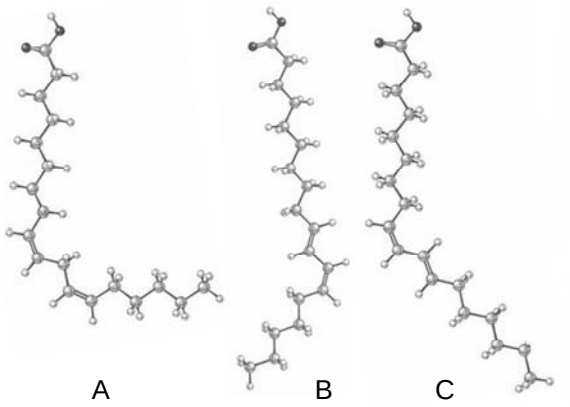

Figure 15. Chemical structure of linoleic acid (A), *trans*-10, *cis*-12 conjugated linolenic acid (B) and rumenic acid (C).

[123] Weber N. and Mukherjee K.D. (2005) Lipids in infant formulas and human milk fat substitutes. Chapter 25. In: *Healthful Lipids.* Akon C.C. and Lai O-M Eds, AOCS Press, Champaign, Illinois.

[124] Anderson R.E., O'Brian P.J., Wiegand R.D., Koutz C.A. and Stinson A.M. (1999) Conservation of docoesahexaenoic acid in the retina. *Advance in Experimental Medicine and Biology* 318, 285-294.

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[126] Innis S.M. (2003) Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. *Journal of Pediatrics* 143, S1-S8.

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The second most common isomer is *trans*-7, *cis*-9 CLA isomers, representing about 10% of total CLA. Each of other CLA isomers is at a low concentration (less than 0.5% of the total CLA isomers) when present[20,98].

CLA in ruminant milk arises both directly and indirectly from incomplete microbial hydrogenation of PUFA in the rumen (***Figure 16***), by anaerobic bacteria, e.g. *Butyrovibrio fibrisolvents* and primarily from endogenous synthesis by Δ9-desaturase of vaccenic acid *(trans*-11 C18:1, VA) in the mammalian tissue[128] (***Figure 17)***.

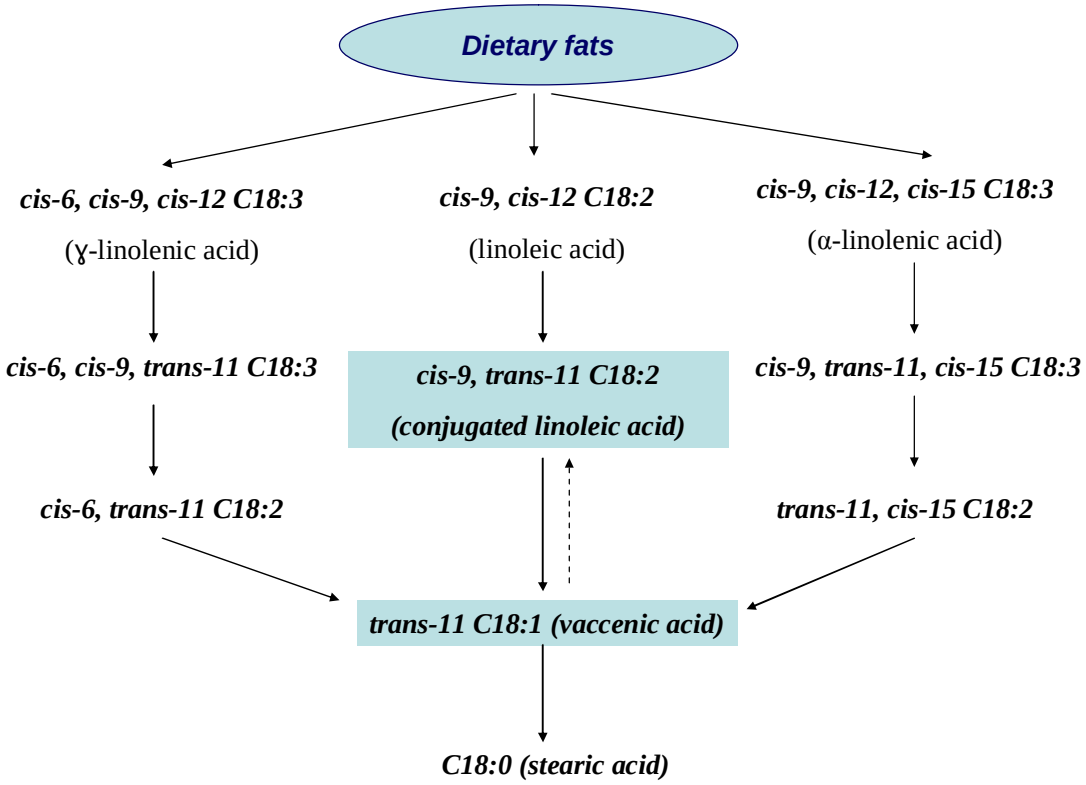

Figure 16. Predominant pathway of ruminal biohydrogenation on unsaturated C_{18} fatty acids involving *cis*-9, *trans*-11 C18:2.

As reported in the ***Figure 16***, RA in not an intermediate in the biohydrogenation of linolenic acid, but the biohydrogenation of both linoleic and linolenic acid produces VA as an intermediate. Therefore, a close relationship was observed between the levels of VA and RA in milk fat[34,129-131].

[128] Griinari J.M., Corl B.A., Lacy S.H., Chouinard P.Y., Nurmela K.V.V. and Bauman D.E. (2000) Conjugated linoleic acid is synthesized endogenously in lactating dairy cow by Δ9-desaturase. *Journal of Nutrition* 130, 2285-2291.
[129] Jiang J., Björerck L., Fondén R. and Emanuelson M. (1996) Occurrence of conjugated cis-9,trans-11-octadecadienoic acid in bovine milk: effects of feed and dietary regimen. *Journal of Dairy Science* 79, 438-445.
[130] Jahreis G., Fritsche J. and Steinhard H. (1997) Conjugated linoleic acid in milk fat: high variation depending on production system. *Nutrition Research* 17, 1479-1484.
[131] Lawless F., Murphy J.J., Harrington D., Devery R., and Stanton C. (1998) Elevation of conjugated cis-9,trans-11-octadecadienoic acid in bovine milk because of dietary supplementation. *International Dairy Science* 81, 3259-3267.

The final step in the rumen biohydrogenation of linoleic and linolenic acids is a further reduction of the *trans*-monoenes, producing stearic acid (C18:0). Since this reduction is generally the rate-limiting step, there is often an accumulation of TFA in the rumen[132].

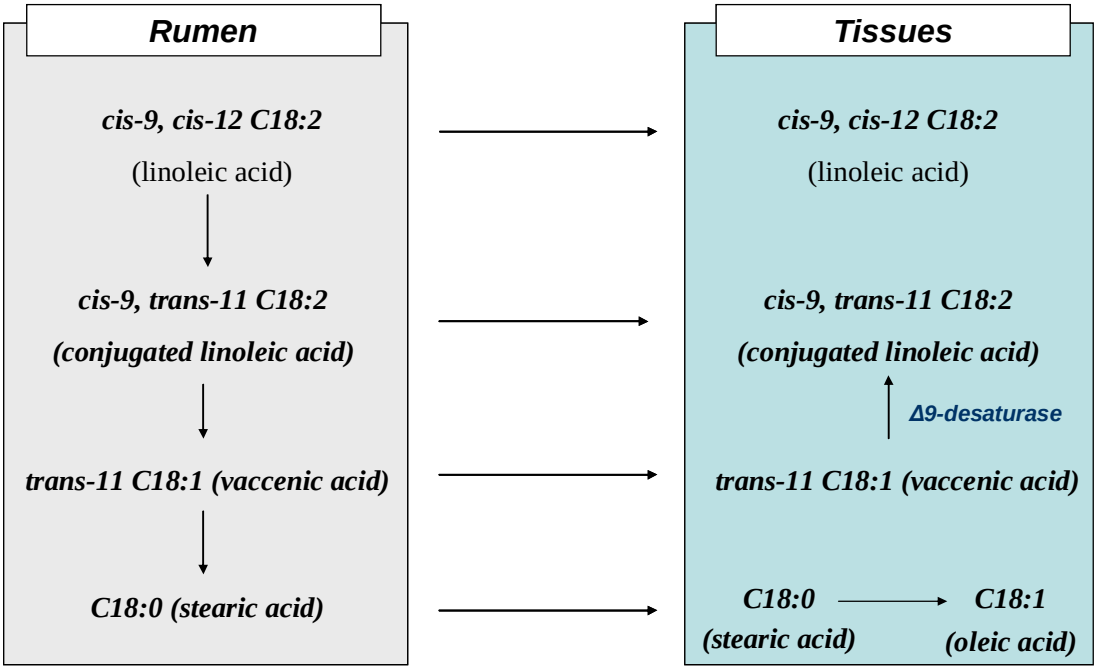

Figure 17. Pathway of conjugated linoleic acid (CLA) biosynthesis.

Typical concentration of CLA in milk fat are 3-6 mg/g of fat, but the levels of CLA vary markedly from 2 to 53.7 mg/g fat. Variation in CLA concentration in milk and dairy products is influenced by many factors, such as geographical regions, season, stage of lactation, parity and breed[133,134,135]. However, the feeding strategies (type of diet and level of intake) is the most significant factors affecting the CLA content of milk fat[136].

Especially, it is know that the major effective dietary treatments for increasing milk CLA are those that both increase the dietary intake of unsaturated fat source, especially 18-carbon PUFA (amount limited at 7% of total lipids), and modify the rumen environment with a consequent shift of the

[132] Bauman D.E and Lock A.L. (2006) Conjugated linoleic acid: biosynthesis and nutritional significance. In: *Advance Dairy Chemistry*, Volume 2: Lipids, Third Edition, Fox P.F and McSweeney P.H.L., Springer, New York, USA.
[133] Kelly M.L., Berry J.R., Dwyer D.A., Griinari J.M, Chouinard P.Y., Van Amburgh M.E. and Bauman D.E. (1998a) Dietary fatty acid spurce affect conjugated linoleic acid concentrations in milk from lactating dairy cows. *Journal of Nutrition* 128, 881-885.
[134] Kelsey J.A., Corl B.A., Colller R.J. and Bauman D.E (2003) The effect of breed parity, and stage of lactation on conjugated linoleic acid (CLA) in milk fat from dairy cows. *Journal of Dairy Science* 86, 2588-2597.
[135] Collomb M., Schmid A., Siebre R., Wechsler D. and Ryhänen (2006) Conjugated linoleic acids in milk: variation and physiological effects. Review. *International Dairy Journal* 16, 1347-1361.
[136] Stanton C., Murphy J., McGrath E. and Devery R. (2003) Animal feeding strategies for conjugated linoleic acid enrichment of milk. In*: Advances in Conjugated Linoleic Acid Research*, Volume 2, Sébédio J-L and Christie W.W, Adlof R.O. Eds, AOCS Press, Champaign, IL.

biohydrogenation pathways. The result is an accumulation of VA, thereby increasing the rumen outflow of this precursor for the endogenous synthesis of CLA[132].

Thus, the highest CLA levels in milk fat were obtained from fresh pastures or organic diet and diets supplemented with vegetable oils, oilseeds and marine oils[17,137-140].

Moreover, the effects of manufacturing conditions, storage and ripening on the CLA levels in milk and dairy products have been studied by many authors[141-143].

Although the structural complexity of CLA means that it is very difficult to identify the isomers with biological activity, interest in the levels of CLA into the human diet has increased in the last 20 years, because of a large number of biomedical studies across a variety of animal models have demonstrated health benefits of CLA isomers[144].

The many physiological effect of biologically-active CLA isomers appear to be the results of the interaction of these FA with numerous metabolic signaling pathways[145].

The majority of studies on the potential positive effects on human health, including anti-carcinogenic, anti-atherogenic, anti-diabetic, anti-obesity, and enhancement of immune system, have been conducted on RA and *trans*-10, *cis*-12 CLA, the two main CLA isomers. These major isomers have shown additive, independent, or antagonistic effects[132,146,147].

Limited researches have been focused on other CLA isomers. This is not due to the other isomers having no effects, but rather that their effects are currently unknown[145].

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[137] Dhiman T.R., Anad G.R., Satter L.D. and Pariza M.W. (1999) Conjugated linoleic acid content of milk from cows fed different diet. *Journal of Dairy Science* 82, 2146-2156.

[138] Bauman D.E., Baumgard L.H., Corl B.A. and Griinari J.K (2001) Conjugated linoleic acid (CLA) and the dairy cow. In: *Recent Advances in Animal Nutrition 2001*, Garsworthy P.C and Wiseman J., Eds, Nottingham University Press, Nottingham.

[139] Chilliard Y., Ferlay A. and Doreau M. (2001) Effect of different types of forage, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids *Livestock Production Science* 70, 31-48.

[140] Prandini A., Sigolo S. and Piva G. (2009) Conjugated linoleic acid (CLA) and fatty acid composition of milk, curd and Grana Padano cheese in conventional and organic farming system. *Journal of Dairy Research* 76, 278-282.

[141] Chamba J.F., Chardigny J.M., Perreard E., Chappaz S., Richert R., Steinhart H. and Sèèbèdio J-L. (2006) Conjugated linoleic acid (CLA) content of French Emmental cheese: effect of the season, region of production, processing and culinary parameters. *Lait* 86, 407-480.

[142] Bisig W., Eberhard P., Collomb M. and Rehberger B (2007) Influence of processing on fatty acid composition and the content of conjugated linoleic acid in organic and conventional dairy products – a review. *Lait* 87, 1-19.

[143] Prandini A., Sigolo S., Tansini G., Brogna N. and Piva G. (2007) Different level of conjugated linoleic acid (CLA) in dairy products from Italy. *Journal of Food Composition and Analysis* 20, 472-479.

[144] Korhonen H.J. (2009) Bioactive components in bovine milk. In: *Bioactive Components in Milk and Dairy Products.* Chapter 2. Park Y.W. Ed, Wiley-Blackwell, Iowa, USA.

[145] Park Y. and Pariza M.W. (2007) Mechanism of body fat modulation by conjugated linoleic acid (CLA). *Food Research International* 40, 311-323.

[146] Pariza M.W., Park Y. and Cook M.E. (2001) The biological active isomers of conjugated linoleic acid. *Progress in Lipid Research* 40, 283-289.

[147] Lobos-Ortega I., Revilla I., Gonàlez-Martìn M.I., Hernàndez-Hierro J.M., Vivar-Quintana A. and Gonzalez-Pèrez C. (2012) Conjugated linoleic acid content in cheeses of different composition during six months of ripening. *Czech Journal of Food Sciences* 30(3), 220-226.

Particularly, several studies have been confirmed that *trans*-10, *cis*-12 CLA is responsible for the effects on body composition i.e. lowering of body weight and fat mass, whereas RA is neural. Moreover, animal and human trials have found controversial results on anti-diabetic effect of *trans*-10, *cis*-12 CLA. Therefore, further studies are necessary to clarify this issue and define the mechanism of action of *trans*-10, *cis*-10 CLA on glucose levels and insulin sensitivity[135,148].

Results from animal experiments on anti-atherogenic affects of dietary CLA mix or individual isomers supplementation have been shown to reduce of serum cholesterol and TG concentrations with a positive effects of blood-vascular system that can influence vascular homeostasis[148]. These positive effects of dietary CLA intake, especially of RA, may be beneficial in preventing heart diseases in human. To date, it is not know what effect long-term ingestion of different concentration of CLA (mix or individual isomers) will have on incidence of atherosclerosis in man[148]. Therefore, will be required new human and animal investigations before to assess the benefits of specific CLA isomers in the prevention of vascular diseases.

The anti-carcinogenesis activity of CLA has been clearly established with *in vitro* cell culture systems and *in vivo* animal models for a wide range of cancer type, such as skin papillomas, fore-stomach neoplasia, and preneoplastic lesions and tumors in the colon and mammary gland. The anti-carcinogenic activities of CLA involve by different mechanisms: a) modulation of cell proliferation and apoptosis, b) regulation of gene expression, c) influence on eicosanoid synthesis and metabolism, and d) anti-oxidative mechanisms. However, potentially anti-carcinogenic effects differ according to CLA isomers, type and site of the cell/organ and stage of carcinogenesis (initiation, promotion, progression)[132,149.]

It is clear that the two major CLA isomers influence the eicosanoid synthesis and therefore they can enhance some specific immune functions in animals and man although published results differ for both within and between species studies[148].

The estimated average daily intake of CLA from ruminant-derived products ranges from 0.19 to 1.0 g CLA and varies for different countries. The estimated average total CLA intake ranges from 95 to 440 mg and varies for different countries, consumption habits and differing CLA values in food. Optimal dietary intake remains to be established, nevertheless hypotheses based on epidemiological animal studies range between 95 mg and 3.5 g/d. However, all recommendations not based on experimental human data should be taken with caution[135].

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[148] Wahle K.W., Heys S.D. and Rotondo D. (2004) Conjugated linoleic acids: are they beneficial or detrimental to health? *Progress in Lipid Research* 43, 553-587.

[149] Banni S., Heyes S.D. and Wahle K.W.J. (2003) Conjugated linoleic acids as anticancer nutrients: studies *in vivo* and cellular mechanisms. In: *Advances in Conjugated Linoleic Acid Research*, Volume 2, Sébédio J-L and Christie W.W, Adlof R.O. Eds, AOCS Press, Champaign, IL.

- *Lipid fraction of MFGM*

The lipid fraction of MFGM have been received increasing interest due to their nutritional and technological properties.

Polar lipids of MFGM have been related with many health benefits. Particularly, sphingolipids are considered functional ingredients, because they are involved in structural and regulatory functions such as the regulation, growth, proliferation, differentiation, and apoptosis of cells. Sphingolipids also affect the age-related diseases, blood coagulation, immunity and inflammatory responses In particular SM and its metabolites (ceramide, sphingosine and sphingosine 1-phosphate) are linked to numerous biological effects on human health concerning their anticarcinogenic properties, cholesterolemia-lowering effects and anti-bacterial activities[80,85,150].

Functions on neuronal system relating the attenuation of ageing-cell process and relating the restoring of normal memory on a variety of task have been shown in animal study by PS intake. Moreover, positive results by clinical studies were obtained on Alzheimer's disease at elevated doses of PS. The contribution of PS on human health of dairy products is not significant because PS is only present in a small amounts in milk-derived. Protective effects of PC on liver and gastrointestinal mucosa against toxic chemical attack and acute or chronic viral damage have been reported in several studies[75,150].

Is well known that SM and PC provide a source of choline, which was officially recognized as an essential nutrient by the Institute of Medicine (IOM) in 1998. It is thought to have an impact on diseases such as liver disease, atherosclerosis, and, possibly, neurological disorders because implicated in a wide-ranging roles in human metabolism[151].

Cholesterol, located mainly in MFGM, is important for its physiological functions. The high levels of cholesterol plasma are associated with an increase of risk of cardiovascular disease (CVD); nevertheless, cholesterol play an essential role in the body as a structural component of cellular and sub-cellular membrane, plasma lipoproteins and nerve cells. Therefore, the functionality of body cells depends on compensatory regulation system (endogenous synthesis in the liver, excretion in the intestine and feedback reactions) in blood cholesterol levels. In addition, cholesterol is a metabolic precursor of bile acids and steroid hormones including vitamin D. These metabolic functions are essential to life[152,153].

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[150] Rombaut R. and Dewettinck K. (2006) Properties, analysis and purification of milk polar lipids. *International Dairy Journal* 16, 1362-1373.

[151] Zeisel H.S. and Da Costa K-A. (2009) Choline: an essential nutrient for public health. *Nutritional Reviews* 67(11), 615-623.

[152] Park Y.W (2009) Bioactive components in goat milk. In: *Bioactive Components in Milk and Dairy Products*. Chapter 3, Park Y.W. Ed, Wiley – Blackwell, Iowa, USA.

[153] Cichon R.M. (2003) Lipids in human nutrition. In: *Chemical and Functional Properties of Food Lipids*. Sikorski Z. E. and Kolakowska A., Eds. CRC Press, Boca Raton, USA.

2.2 Bioactive proteins and peptides

Milk proteins are known to provide a source of energy and the amino acids essential to growth and maintenance of various body functions. In addition, they also posses biological, physicochemical and sensory properties of protein-rich foods. In recent years, the multiple functional properties of major milk proteins are largely characterized due to the rapidly expanding knowledge about physiologically active peptides that are encrypted within intact proteins[154].

The primary and secondary structures of major human and bovine milk proteins are well characterized and their molecular weight and potential bioactivities are shown in ***Table*** 7.

Protein	**Molecular weight (Da)**	**Biological Activities**
Total Casein (α_{s1}, α_{s2}, β, and κ)	14.000-22.000	Ion carrier (Ca, PO_4, Fe, Zn, Cu), precursor for bioactive peptides
β-Lactoglobulin	18.400	Vitamin carrier, potential antioxidant, precursor for bioactive peptides, fatty acids binding
α-Lactalbumin	14.200	Lactose synthesis in mammary gland, Ca carrier, immunomodulatory, precursor for bioactive peptides, potentially anticarcinogenic
Immunoglobulins	150.000-1000.000	Specific immune protection, potential precursor for bioactive peptides
Serum albumin	66.300	Precursor for bioactive peptides
Lactoferrin	80.000	Antimicrobial, antioxidative, anticarcinogenic, anti-inflammatory, iron transport, cell growth regulation, precursor for bioactive peptides, immunomodulatory
Lactoperoxidase	78.000	Antimicrobial, synergic effects with immunoglobulins, lactoferrin, and lysozyme
Lysozyme	14.000	Antimicrobial, synergic effects with immunoglobulins, lactoferrin, and lactoperoxidase
Glycomacropeptide	8.000	Antimicrobial, antithrombotic, probiotic, gastric hormone regulator
Growth Factors	6.400-30.000	Stimulation of cell growth, intestinal cell protection and repair, regulation of immune system

Adopted from Séverin S. and Wenshui X. (2005)[54], Korhonen H.J. (2009)[144].

Table 7. Potential biological functions of major human and bovine milk proteins.

[154] Korhonen H. and Pihlanto A. (2006) Bioactive peptides: production and functionality. *International Dairy Journal* 16, 945-960.

Milk proteins are considered the most important source of bioactive peptides. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health by reducing the risk of chronic diseases or boosting natural immune protection (***Figure 18***)[144,154-156].

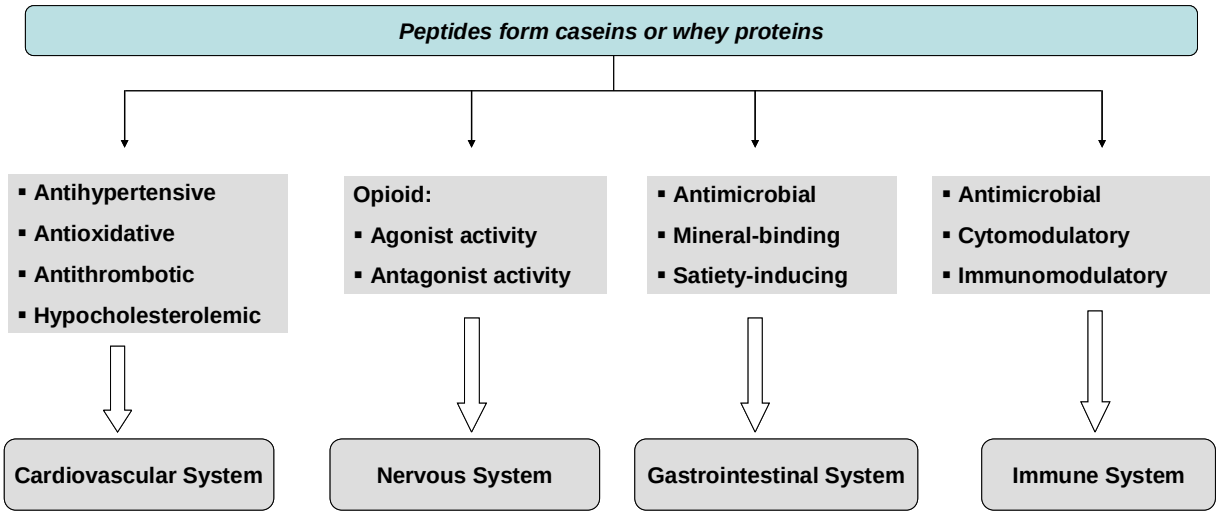

Figure 18. Physiological functionality of milk-derived bioactive peptides.

Peptides are inactive within the sequence of the parent protein and can be released in three ways: a) through hydrolysis by digestive enzyme, b) through hydrolysis by proteolytic microorganism and c) through the action of proteolytic enzyme derived from microorganisms or plants. Therefore, these active peptides have been found in enzymatic protein hydrolysis and fermented dairy products, but they can also be released during gastrointestinal digestion of proteins[154,156].

Milk-derived bioactive peptides may exert a number of specific activities, such as antihypertensive, antioxidative, antimicrobial, immunomodulatory, opioid or mineral-binding activities[144,154].

The activity is based on the inherent amino acid composition and sequence. Bioactive peptides usually contain 3-20 amino acid residues per molecule and specific peptide sequences are known to have two or more different biological activities[157].

Bioactive peptides represent potential health enhancing components for novel food, dietary supplements and pharmaceutical applications[158]. Due to their physiological and physiochemical versatility and bio-specific health benefits, a novel concept of "personalized nutrition" can be

[155] Kitts D.D. and Weiler K. (2003) Bioactive proteins and peptides from food source. Application of bioprocess used isolation and recovery. *Current Pharmaceutical Design* 9, 1309-1323.
[156] Clare D.A. and Swaisgood H.E. (2000) Bioactive milk peptides: A prospectus. Invited Review. *Journal of Dairy Science* 83, 1187-1195.
[157] Korhonen H. and Pihlanto-Leppälä A. (2004) Milk-derived bioactive peptides: formation and prospects for health promotion. In: *Handbook of Functional Dairy Products. Functional Foods and Nutraceuticals Series 6.0.* Chapter 6. Shortt C. and o'Brien J., Eds, CRC Press, Florida, USA.
[158] Meisel H. (1998) Overview on milk protein-derived peptides. *International Dairy Journal* 8, 363-373.

development. For these reasons, milk protein-derived peptides as highly prominent ingredients for health-promoting functional foods provide interesting opportunities to the dairy industry for expansion of its field of operation[154].

3. Evaluation of bioactive lipid fractions, bioactive proteins and peptides: a brief overview of analytical techniques

Milk is a very flexible and basic raw material for the dairy products industry. Particularly, dairy products can be considered as the most diverse of all food groups characterized by unique properties. For these reasons, a very wide range of analytical techniques have been used and specific preparative and separate determination can be applied to identify and quantify macro- and micro-constituents, including bioactive components[159].

In the case of lipid fraction, gas chromatography (GC) coupled with flame ionization detector (FID) is the most commonly used analytical method to assess the FA profile including minor bioactive FAs and TG composition of bovine and human milks and dairy products as well as to evaluate eventually adulteration of milk fat. In the recent years, rapid GC analysis (fast, very fast, ultra fast) have been defined with a possibility to minimise analysis times and increase the number of samples for analysis without impairing the quality of results and consequently reduce cost of analysis and increase laboratory productivity[160-162].

Also, gas-chromatography mass-spectrometry (GC-MS) has been gaining importance for the detection and identification of the molecular structure of lipid components[163,164].

In the chromatography field, comprehensive two dimensional gas-chromatographic methods (GC x GC) have been recently applied for the milk FA analysis. This multidimensional techniques offers a significant increase in the separation power of components in complex lipid samples (e.g. milk or dairy product samples) in comparison with one-dimensional GC. However, the application of GC x GC is not an easy task and several parameters (amount of injection, columns, temperature programs, gas flows in both dimensions, and modulation period) need to be checked[165,166].

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Currently, infrared (IR) spectroscopy is being used in addition to chromatographic methods or to replace traditional methods of analysis for the characterization of lipid fraction and also for the evaluation of moisture, sugars, casein, cholesterol, and contaminants in milk and dairy products[167,168]. This technique is simple, rapid, inexpensive, non-destructive and sensitive[169].

The combination of IR and chemometric tools, has increased popularity in food science, and has been widely using for dairy product analysis[170,171]. Near infrared (NIR) spectroscopy has been widely studied for the rapid in-line screening determination of major food components[167,169]. The mid-IR (MID) region (from 400 to 4000 cm^{-1}) is an extremely useful area of study especially when spectroscopy procedures based on attenuated total reflectance (ATR) Fourier transform infrared (FTIR) are applied. FT-MIR analysis provide qualitative information of functional groups and offer the possibility for quantitative analysis of organic constituents, such as total *trans* FA content in milk fats (infrared spectrum from 956 to 976 cm^{-1})[172-174].

Several methods have been employed for extraction, separation and detection of PLs in milk and dairy products[175,176]. Concerning the isolation of phospholipid fraction from other lipid classes, several techniques such as thin layer chromatographic (TLC), column chromatography and solid phase extraction (SPE) have been applied. Separation and quantification of different phospholipid classes is generally achieved by high performance liquid chromatography (HPLC) combined with a UV or evaporative light scattering (ELS) detectors[177,178]. Moreover, HPLC in combination with

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mass spectrometry (MS) detector offers structural information of phospholipid molecular species within milk and dairy products because of its high sensitivity, specificity and simplicity in the analytical procedures[175]. Nowadays, spectroscopic methods e.g. UV-VIS spectrophotometry, FT-MIR, NIR and ^{31}P-NMR has been applied for PLs analysis[176].

As regards the identification and quantification of bioactive proteins and milk-peptides, numerous analytical techniques have been developed. These techniques comprise gel-electrophoresis, liquid chromatography (LC) and immunochemical methods. Compared with conventional liquid chromatography, the new analytical applications such as high performance liquid chromatography (HPLC) as well as ultra performance liquid chromatography (UPLC) allow for higher resolution, better peak shape, reproducibility responses and the speed of analysis. On the basis of the greater resolving power of the chromatographic columns, reverse phase (RP) - HPLC is the most accurate and efficient techniques to separate, evaluate and monitor casein fractions, whey proteins and minor small peptides[179,180].

Structural information on proteins is provided using spectroscopy methods, such as FT-IR spectroscopy that is the main methods used to obtain information on the secondary structure of proteins, and NMR spectroscopy used for the determination of the three-dimensional structure of the proteins[180].

Recently, to acquire more information about peptide sequencing from enzymatic mapping for structural characterization of proteins (proteomics), detection of low abundance milk proteins and MFGM proteins, identification and quantification of biologically active peptides and evaluation of proteolysis process, two complementary analytical strategies (gel-based and gel-free approaches) in combination with mass spectrometry analysis are used[51,57,179,181].

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Experimental Section

Section I

Dairy products from bovine milk - Bioactive lipids analysis by chromatographic and spectroscopic techniques

This section describes the main results obtained during my PhD activities performed on dairy products (milk creams and butters) from bovine milk in order to evaluate their bioactive lipid composition by using chromatographic and spectroscopic techniques.

The first chapter (***Chapter I***) deals with the evaluation of effects of dairy cow's dietary supplementation with extruded linseed on bioactive compounds in outcrop milk creams, destined to butter production, collected from local dairy farm located in the production area of the Parmigiano-Reggiano Cheese. Fatty acid composition and triglyceride profile of milk fat were analyzed by traditional gas-chromatography combined with flame ionization detector (GC-FID). Moreover, the combination of infrared spectroscopy and chemometric method was applied to determine their ability to discriminate between milk cream samples in response to differences in dairy cow's diet and the use of supplemented of extruded linseed.

The second chapter (***Chapter II***) is a preliminary study concerning the application of fast gas-chromatography coupled with flame ionization detector (fast GC-FID) to determine the fatty acid composition in dairy fat. A chromatographic performances comparison between two short highly polar capillary columns is presented. Having regard to all results obtained until now, the use of cyanopropyl siloxane capillary column (BPX-70, 10m) was chosen for fast and routinely analysis of fatty acids on dairy samples in the subsequent two chapters (***Chapter III and Chapter IV***).

Further studies are being done on the development and optimization of the fast-chromatographic method described in this research manuscript in order to ensure the practical *applicability* of fast-fatty acid analysis for several foodstuffs.

Chapter I

Lipid fraction of outcrop milk creams collected in the Parmigiano Reggiano cheese area in response to extruded linseed supplementation: GC-FID and FT-MIR evaluation

(Submitted to an International Journal)

Lipid fraction of outcrop milk creams collected in the Parmigiano Reggiano cheese area in response to extruded linseed supplementation: GC-FID and FT-MIR evaluation

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Abbreviated running headline: **value-added by-product of dairy industry**

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Abstract

This study evaluated the effects of cow's dietary supplementation with extruded linseeds on the fatty acid and triglyceride composition of creams obtained by natural creaming of cows' milk and collected in the "Parmigiano-Reggiano cheese" protected designation of origin area. Moreover, the spectroscopic profiles of each cream were recorded.

The inclusion of linseed, in the diet of experimental dairy cows' resulted in creams with increased unsaturated fat levels and healthy fatty acids such as omega-3 and conjugated linoleic acid isomers, as well as higher levels of long-chain triglycerides. Moreover, the combination of infrared spectroscopy and chemometric methods, was able to differentiate outcrop milk creams based upon differences in cows' diets and supplemented extruded linseed.

Key words: milk creams, extruded linseed, GC-FID, FT-MIR.

1. Introduction

Bovine milk fat is an important part of the human diet in many countries, which represents up to 75% of the total consumption of fat from ruminant animals (Chilliard et al., 2000b). Although dairy products provide only 15-25% of the total fat in the human diet, there is an increasing awareness amongst consumers regarding the consumption of dairy products and the associated risk of obesity, coronary heart disease and tumours (Williams 2000), mostly ascribed to the saturated fatty acids (SFA) content. Specifically, the medium-chain SFA (MC-SFA) that account for the majority of fatty acids (FAs) in bovine milk, have been associated with an increase of low-density lipoprotein (LDL) cholesterol concentration, leading to a high risk of cardiovascular diseases. The negative effects on human health may be reduced by lowering the intake of undesirable SFA and/or by making alterations in the FA profile of milk (Elgersma et al., 2006). However, recent studies have suggested that milk fat contains specific FAs, that are often related to health enhancing properties for humans (Secchiari et al., 2003; Williams 2000; Bauman et al., 2006; Park and Pariza 2007). These FAs, that pertain to the group of polyunsaturated fatty acids (PUFA), include the conjugated linoleic acid isomers (CLA) and omega-3 fatty acids (*n*3-PUFA), dominated by cis-9, trans-11C18:2 and C18:3*n*3 respectively. It is know that the FA composition of milk is influenced by several factors (Griinari and Bauman 1999), and the cows' diet is considered to be amongst the leading factors (Buccioni et al., 2012). Many studies have focused on the development of feeding strategies that foster the seasonal availability of fresh forage and the supplementation of unsaturated fat sources, such as extruded linseed, to enhance the content of CLA and *n*3-PUFA in dairy product and lower the amounts of SFA (Renna et al., 2010; Jones et al., 2005; Ryhanen et al., 2005).

Milk FAs are mostly esterified with 3-carbon glycerol to form triglycerides (TGs), in which more than 400 FAs have been identified (Jensen and Newburg 1995). Studies have highlighted the biological importance to humans of the stereospecific distribution of FAs in TGs (Innis 2011; Jensen 1995) but, the effect of the increase in unsaturated fatty acids (UFA) content of TGs in fat-rich dairy products, has received less attention than total FA composition. The mechanism behind the stereospecific positioning of FAs into TGs indicates that the esterification is directed to produce the required TGs regardless of changes in dietary FAs, suggesting that quantities of TGs may vary but not their structure (Jensen et al., 2002). Researchers have studied UFA-enriched cows' milk TGs considering their physicochemical properties (Smet et al., 2010; Bugeat et al., 2011) or structural characteristics (De Peters et al., 2001) Mele et al. (Mele et al., 2011) reported the TG profile of UFA-enriched ewe cheese, but data is not available regarding cows' milk TG profile in response to supplemental extruded linseed. According to the available literature, modifications in milk FA composition and TG species, play a wide number of roles in milk quality, including

physical and processing characteristics, as well as nutritional and organoleptic properties of milk and dairy products (Williams 2000). In fact, dietary manipulations will undoubtedly affect not only the lipid fraction, but also the interaction and the variation of specific constituents, in addition to the characteristic molecular structure of dairy products. It was recently reported that sheep's and cow's milk samples produced under different feeding systems, were correctly discriminated by mid- and near-infrared spectroscopy (MIR and NIR) combined to chemometric tools (Coppa 2012; Karoui et al., 2011). Yet to date, no published data have considered the effect of supplemental extruded linseed on the spectroscopic properties of dairy products.

The aim of the current study was to characterize the lipid composition of outcrop creams, obtained by supplementing the traditional diet of dairy cows with extruded linseed, produced by natural creaming during the Parmigiano-Reggiano cheese-making process. The research objective is that of improving outcrop creams destined to butter production, with a stable level of enrichment in bioactive compounds. Therefore, the FA composition, including minor components such as CLA, trans-11C18:1 and *n*3-PUFA, and the TG profile, were analyzed by gas chromatography combined with a flame ionization detector (GC-FID). The relationship between FAs and TGs of outcrop milk creams were studied. Finally, the ability of Fourier Transform Mid infrared spectroscopy (FT-MIR) in combination with artificial neural networks (ANN), have been evaluated for their ability to discriminate between outcrop milk cream samples in response to the differences in dairy cow's diet and the use of supplemented extruded linseed.

2. Materials and Methods

The study was conducted with a total of 34 outcrop cream samples. Each sample was obtained by natural creaming of the evening milk for 12 hours, previously the addition of morning whole milk, during the Parmigiano-Reggiano cheese-making process.

The samples were collected weekly from January to May 2011, in a dairy farm in the Reggio Emilia province, located in the production area of the Parmigiano-Reggiano Cheese. Before and during the investigation, two groups of 300 multiparous Holstein milking cows were fed with a traditional diet, based on the use of concentrate and hay (*dry feeding period*) and fresh forage *(fresh forage feeding period)*, permitted by the Parmigiano-Reggiano cheese consorce (CFPR).

In particular, milk samples were taken from week 1 to week 8, during the "*dry feeding period*", consisting of alfalfa hay (11 kg), mixed hay (4 kg) and a concentrate supplement (13 kg) consisting of a combination of maize seeds (48%), sugar beet pulp (2%), wheat bran (10%), wheat meal (5%), sunflower meal (9%), barley meal (8%), soybean meal (18%), minerals and vitamins; and from

week 9 to week 17, during the "*fresh forage feeding period*", consisting of fresh forage (25 kg) obtained from natural meadows and mixed permanent meadows.

Traditional diet was offered at variable levels dependent on milk yield but focused on maintaining a maximum energy intake. While the control group was fed only with the traditional diet, the linseed group was fed with 0.4 kg/d of extruded linseeds. Throughout the entire experimental period the two groups of milk cows were fed and housed separately. Milk yield of individual cows were recorded during the entire experiment. Compared to the traditional diet, feeding the traditional diet with extruded linseeds was associated with an increase in cow's milk yield of 0.8 kg/d and reduction in milk fat content of 0.07%. Although, the addition of extruded linseeds to the traditional diet did not disturb the cow reproductive performances or well being. Moreover, the linseed group showed a increased of ovulatory activity in postpartum interval.

Cream samples of each experimental groups were obtained during the early phases of the Parmigiano-Reggiano cheese making process, carried out in the same dairy farm by natural surface skimming in open steel tubs. Sample (250 mL) was collected and stored at our laboratories at -20°C until fat extraction.

2.1 Fatty acid composition of milk cream

Lipid extraction was performed in duplicate on cream samples according to Hara – Radin (Hara and Radin 1978) and transesterification to the corresponding fatty acid methyl esters (FAME) following the method of Christie (Christie 1982).

Methyl undecanoate (C11:0; 1 mg/mL) was used as internal standard (IS). The FAME were analyzed in duplicate with a Clarus 500 gas chromatograph (Perkin Elemer, Shelton, CT, USA), equipped with a flame-ionization detector (FID), automatic injector, and split injector port. The separation of the FAME was performed with a BPX70 fused silica capillary column (50 m, 0.22 mm i.d., 0.25μm f.t.), coated with 70% cyanopropil polysilphenylenesiloxane film from SGE (VWR International, Fontenay-sous-Bois, France).

The GC-FID conditions were used according to Gori et al. (Gori et al. 2011). The injector and detector temperatures were set at 240°C, and the helium (carrier gas) was maintained at a flow rate of 0.75 mL/min. The oven temperature was programmed for 100 to 115°C at 1.5°C/min, then to 180°C at 58°C/min for 10 min, and finally to 240°C at 3°C/min for 10 min. The split ratio was set at 1:60. Peaks were routinely identified by comparison of the retention times with fatty acid methyl ester GLC 463, CLA standard mixtures UC-59M from Nu-Check (Elysian, MN, USA), and published isomeric data (Kramer et al., 2004). FAs were quantified by comparing the peak area of

each compound with that of IS, and the composition was expressed as weight percentage of total FAME (mg/100mg of FAME).

2.2 Validation of the analytical method

The analytical method used for FAME analysis was subjected to validation, and the instrumental limits such as coefficient of variation (CV%), linearity, limit of detection (LOD) and limit of quantification (LOQ) were calculated.

To verify the repeatability of the method used for the fat extraction and FAME derivatization, the GC analysis was repeated five times on a single milk cream sample, and the coefficient of variation was calculated for all FAs quantified. The instrumental limits were determined using the coefficient of variation of the IS peak migration time by injecting five times a blank sample added with 0.5 mg of methyl C11:0.

The linearity of the method used was verified in range of 25-100% of the injection volume prescribed by the method. A blank sample added with different amounts of methyl C11:0 (0.25 µL, 0.4 µL, 0.5 µL, 0.8 µL and 1µL) was analyzed in duplicated. LOD and LOQ of the method were calculated according to literature (European Pharmacopeia 2005). LOD was determined as the signal to noise ratio of 3:1 and LOQ was determined as a signal to noise ratio of 10:1 of averaged value of C11:0 obtained during linearity determination experiments.

2.3 Triglyceride analysis of milk cream fat

TG analysis was conducted on the fat phase previously extracted for FA analysis. Briefly, from a concentration of 10 mg/mL of milk cream fat in hexane, 1.0 µL of solution were injected in duplicate into a GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector (FID) and an AOC-20s auto sampler (Shimadzu Corporation, Kyoto, Japan). Tricaprylin (0.25mg/mL) was used as internal standard. TG separation was performed with a Rtx-65 TG fused silica capillary column (30 m, 0.25 mm i.d., 0.10 µm f.t.) with 35% dimethyl, 65% diphenyl polysiloxane from Restek (Chromatography Products, Superchrom Milano). Experimental chromatography condition were as follows: the initial oven temperature of 240°C was raised to 370°C at a rate of 2.5 °C/min. The injector and detector temperatures were set at 360°C. The air and helium flow rates were of 400 and 40 mL/min, respectively. Hydrogen was used as a carrier gas at a flow rate of 1.75 mL/min. The split ratio was set at 1:30. The identification of TGs was carried out by comparison with the retention time of pure standard (trilinolein, triolein, tristearin, tripalmitin, trimyristin, trilaurin, tricaprin and tricaprylin). The method applied for the analysis is based on the separation of classes of milk fat TGs according to their total number of

carbon atoms (CN; sum of the three FA) (Povolo et al., 2008; Fontecha et al., 2005). On the basis of chain length, sixteen group of peaks, that corresponded to TGs from 24 to 54 CN, and, distinguished in short-chain TGs (C26-C34, SC-TG), medium-chain TGs (C36-C44, MC-TG) and long-chain TGs (C46-C54, LC-TG) were identified and quantified.

2.4 FT-MIR analysis

Frozen outcrop milk cream samples were thawed slowly at 4 °C and about 40 mL of samples were placed in a 70 mL screw-cap plastic tube and centrifuged at 5000 rpm at 4 °C to remove excess of water. Each sample was melted in an oven at 35 °C oven and stored for 30 minutes, Analysis was carried out using a Tensor 27TM FTIR spectrometer system (Bruker Optics, Milan, Italy) equipped with a RocksolidTM interferometer and a DigiTectTM detector coupled to an attenuated total reflectance (ATR) accessory. Approximately, 1 mL of the upper layer was deposited directly on the ZnSe 11 reflection crystal ATR accessory (Specac Inc., Woodstock, GA, USA) equipped with a temperature controller set at 35 ± 2 °C. All the spectra were acquired (32 scans/sample or background) in the range of 4000–700 cm^{-1} at a Fourier transform (FT) resolution of 4 cm^{-1}.

2.5 Data analysis

FA and TG composition data were analyzed with one-way analysis of variance (ANOVA) using the STATISTICA 6 software package (StatSoft Inc., Tulsa, OK, USA). Differences between the treatment means were compared at the 1% and the 5% level of significance ($p < 0.01$ and $p < 0.05$) using Fisher's least significance difference (LSD) test. The index of stearoyl-CoA desaturase (Δ9-desaturase) activity was calculated according to Malau-Aduli et al. (Malau-Aduli et al., 1997).

The FT-IR spectra were analysed by artificial neural network (ANN) using STATISTICA Neural Networks 4.0 (StatSoft Inc., Tulsa, OK, USA). The regions between 4000 and 3050 cm^{-1}, and between 2410 and 2260 cm^{-1}, were deleted before processing data: the former region because it contained no useful chemical information and contributed to instrumental noise, and the latter because of its low signal to noise ratio and the presence of fluctuations that are independent of sample composition (Maggio et al., 2009). Two Multi-Layer Perceptron (MLP) with Back-propagation learning algorithm were performed to set up networks capable of classifying samples with different cow diet (dry or fresh feeding) or with the presence of extruded linseeds in the feeding. For both MPL, the input values were represented by spectrum data (490 points).

One nominal output variable, with the possibility to assume the X_1 or X_2 character, was used to perform classification tasks where the objective is to determine to which of a number of classes the input case belongs. For the ANN built to classify the samples as a function of cow diet, X_1 represent

dry feeding while X_2 fresh feeding. For the ANN built to classify the samples as a function of presence of linseed, X_1 symbolizes linseeds while X_2 not linseeds.

The ANN performs classification by checking output unit activation levels against two thresholds: the accept threshold and the reject threshold. Values above the accept threshold are considered to be on, and values below the reject threshold are considered to be off. The output values are interpreted as confidence levels. The accept threshold was set at 0.9, while the "reject thereshold" was set at 0.1. These values dictated that the confidence level cannot be less than 90%.

The original datasets were randomly divided into a training set (60%), a verification set (20%) and a test set (20%) for all ANNs tested.

For the input layer, a linear function was used, while for the hidden and the output layers a logistic activation function was used In order to build a good ANN, it is important to determine a suitable number of neurons in the hidden layer. Generally the number of neurons in the hidden layer is empirically determined depending on data quantity and behaviour; too many neurons can show a small training error but high generalization error due to overfitting (Romani et al., 2012). Therefore different node numbers in the hidden layer momentum were tested, looking for the best classification ability. An early stopping technique was used to select the number of training cycles to avoid overfitting, using the verification set to monitor the prediction error. Training was repeated 5 times and the network's predictions were averaged, since with ANNs convergence is influenced by the initial weight value. The learning rate and momentum parameters were used to control the size of weight adjustment along the descending direction and for dampening oscillations of the iterations.

3. Results and discussion

3.1 Fatty acid composition

Forty-one FAs were identified and quantified in the 34 milk cream samples analyzed: 15 were SFA, 15 monounsaturated fatty acids (MUFA), and 11 PUFA. The coefficient of variation for each FA, calculated over five analytical milk cream replicates, ranged from 0.01 to 2.24. The coefficient of variation of the IS peak migration times was 3×10^{-4}, while the LOD and LOQ were 0.15 and 0.2 mg/100mg of FAME, respectively. The obtained results for LOD and LOQ suggested that the method is sensitive enough for this purpose. The value of the determination coefficient ($R^2 = 0.99$) showed excellent linearity of the calibration curve for the method.

The results of individual FA composition of milk creams and FA classes for both groups of dairy cows are summarized in ***table 1*** and ***table 2***, respectively.

Fatty acids	*Milk cream*						
	Control group			**Linseed group**			*p value*
	mean		CV	mean		CV	
C4:0	2.22	±	0.50	2.03	±	0.45	NS
C6:0	1.45	±	0.34	1.42	±	0.30	NS
C8:0	0.84	±	0.20	0.89	±	0.19	NS
C10:0	2.05	±	0.37	2.27	±	0.40	*
C12:0	2.71	±	0.50	2.91	±	0.32	NS
C13:0	0.08	±	0.01	0.12	±	0.02	**
C14:0	10.21	±	0.57	10.22	±	0.53	NS
C15:0	1.15	±	0.07	1.13	±	0.07	NS
C16:0	30.54	±	1.13	27.11	±	0.74	**
C17:0	0.72	±	0.05	0.62	±	0.03	**
C18:0	12.38	±	0.64	11.96	±	0.65	*
C20:0	0.21	±	0.03	0.17	±	0.01	**
C22:0	0.09	±	0.01	0.07	±	0.01	**
C24:0	0.04	±	0.03	0.03	±	0.02	**
C12:1 c	0.05	±	0.01	0.07	±	0.01	**
C13:1 t	0.13	±	0.01	0.10	±	0.01	**
C14:1	1.10	±	0.06	1.10	±	0.10	NS
C16:1 c	1.43	±	0.26	1.34	±	0.08	**
C16:1 t	0.11	±	0.11	0.12	±	0.10	NS
C18:1 t9	0.57	±	0.06	0.74	±	0.10	**
C18:1 t10	0.43	±	0.59	0.77	±	0.64	*
C18:1 t11	1.71	±	0.62	2.12	±	0.64	*
C18:1 t12	0.26	±	0.06	0.39	±	0.11	**
C18:1 c 9	22.99	±	1.30	23.48	±	1.01	NS
C18:1 t15	0.45	±	0.20	0.57	±	0.25	*
C18:1 c 11	0.37	±	0.17	0.53	±	0.17	**
C18:1 c 12	0.17	±	0.10	0.25	±	0.22	**
C18:1 c 13	0.28	±	0.05	0.42	±	0.03	**
C18:1 c 15	0.04	±	0.02	0.05	±	0.02	**
C18:2 9t12t	0.35	±	0.04	0.45	±	0.03	**
C18:2 *n*6	2.59	±	0.28	3.87	±	0.17	**
C18:2 c9, t11	0.80	±	0.07	1.03	±	0.05	**
C18:2 c,c	0.19	±	0.05	0.18	±	0.03	NS
C18:3 *n*6	0.10	±	0.01	0.08	±	0.01	**
C18:3 *n*3	0.71	±	0.06	0.89	±	0.07	**
C20:2 c11 c14	0.03	±	0.01	0.03	±	0.01	NS
C20:3 *n*6	0.12	±	0.01	0.14	±	0.01	**
C20:4 *n*6	0.16	±	0.02	0.20	±	0.01	**
C20:5 *n*3	0.07	±	0.01	0.06	±	0.01	**
C22:5 *n*3	0.11	±	0.01	0.11	±	0.01	NS

Table 1. Individual fatty acid composition (expressed in mg/100mg of total FAME) in outcrops milk creams from cows fed control and linseed diet. Significant differences, * : $p<0.05$; **: $p<0.01$; NS, not significant.

Classes of Fatty acids	*Milk cream*						
	Control group			**Linseed group**			*p value*
	mean		CV	mean		CV	
SFA	64.69	±	1.89	60.92	±	1.38	**
SC-SFA (C4-C10)	6.57	±	1.36	6.61	±	1.24	NS
MC-SFA (C12-C16)	44.68	±	1.49	41.48	±	1.24	**
LC-SFA (>C17)	13.44	±	0.68	12.83	±	0.68	**
MUFA	30.09	±	1.54	32.04	±	1.18	**
PUFA	5.22	±	0.48	7.04	±	0.28	**
UFA	35.31	±	1.89	39.08	±	1.38	**
TFA	4.02	±	0.30	5.26	±	0.32	**
CLA	0.99	±	0.10	1.21	±	0.07	**
***n*3-PUFA**	0.89	±	0.08	1.06	±	0.07	**
***n*6-PUFA**	2.96	±	0.31	4.29	±	0.18	**
SFA/UFA	1.84	±	0.16	1.56	±	0.09	**
index of Δ9 desaturase	32.62	±	1.55	34.38	±	1.03	**
***n*6-PUFA/*n*3-PUFA**	3.34	±	0.20	4.07	±	0.24	**
SC-FA (C4-C10)	6.57	±	1.36	6.61	±	1.24	NS
MC-FA (C12-C16)	47.51	±	1.55	44.19	±	1.32	**
LC-FA (C17-C22)	48.75	±	2.21	51.91	±	1.82	**

Table 2. Classes of fatty acid (expressed in mg/100mg of total FAME) in outcrops milk creams from cows fed control and linseed diet. Significant differences, * : $p<0.05$; **: $p<0.01$; NS, not significant.

The inclusion of extruded linseeds in the diet of dairy cows resulted in a significant increase ($p < 0.01$) of biological compounds in milk cream fat, such as MUFA, PUFA, essential fatty acids (EFA including *n*3-PUFA and *n*6-PUFA), trans-fatty acids (TFA), in particular trans-11C18:1, and total CLA content (cis-9,trans-11 C18:2 plus other unseparated isomers). Conversely, significantly lower ($p < 0.01$) content of SFA was measured in milk cream samples from the linseed group than that observed in the control group. Moreover, milk creams from the linseed group showed the lowest SFA/UFA ratio (1.56) than those of the control group (1.84). These results are in agreement with other investigations about the effect of plant lipid supplementation in the diet of dairy cows (Ryhanen et al., 2005; Chilliard et al., 2007; Hurtaud et al., 2010).

All groups of FA were significantly affected by extruded linseed integration except short chain FAs (SC-FA). This result may be correlated with the presentation of the dietary supplementation (protected fat), as noted in earlier studies where the proportion of C6:0 and C8:0 decreased only when unprotected unsaturated fat sources were administered (Ashes et al., 1997; Chilliard et al., 2003).

The content of medium chain FAs (MC-FA), especially MC-SFA, was lower ($p < 0.01$) in milk cream of the linseed group than that from the control group. In fact, levels of C12:0 and C14:0 did

not differ significantly between the two groups, whilst the content of C16:0 was lower ($p < 0.01$) in milk creams from dairy cows fed with the extruded linseeds diet, according to Hurtaud et al. (Hurtaud et al., 2010).

Decrease in C16:0 yield or content may be attributed to the inhibitory effects of long chain FAs (LC-FA) on mammary FA synthesis, through a direct inhibitory effect on acetyl CoA carboxylase activity and can be leaded to the reduced availability of acetate and 3-hydroxybutyrate (3-HB) for mammary FA synthesis (Chilliard et al., 2000a).

In addition, significant increases ($p < 0.01$) of LC-FA were observed in milk creams from the linseed group with a significant decrease ($p < 0.05$) of C18:0. As reported by other studies, the increase in the proportion of most of the C18:1 and C18:2 isomers may be associated with the biohydrogenation activity of the rumen, with is stimulated by supplementing an unsaturated fat source such as oil or oilseeds (Chilliard et al., 2000a; Chilliard et al., 2000b; Luna et al., 2008; Kim et al., 2009). Moreover, the spreadability index, cis-9C18:1/C16:0, was higher ($p < 0.01$) in milk cream from the linseed group than in the control group. These modifications in FA composition, and in particular in the spreadability index were responsible for linear decreases in the final melting temperature and solid fat content (Couvreur et al., 2006).

Results in this study also show that the concentration of C18:3*n*3 in milk creams from the linseed group increased significantly ($p < 0.01$) and this may be attributed to the FA composition of linseed because a rich source of C18:3*n*3. Previous studies about the effect of fat-supplemented rations on milk FA profile, found that linseeds increased the proportion of C18:3*n*3 up to 1.5% of total FA (Kennelly 1996; Mansbridge et al., 1999) and the C18:3*n*3 content was maximized when cows were fed with full fat linseeds (Secchiari et al., 2003). Other authors found that the incorporation of linseed oil or seeds in the diet resulted in no increase (Kelly et al., 1998) or significant increase of C18:3*n*3 content with a stronger response to linseed oil with a high-concentrate diet than with a high-hay diet (Chilliard et al., 2000a; Loor et al., 2002; Collomb et al., 2004). Contradictory results have been obtained with calcium salts of linseed oil (Chouinard et al., 1998; Brzoska et al., 1999).

The trend to a higher proportion of C18:3 in milk when linseeds are used could be due to a partial protection by whole linseed against biohydrogenation due to the location of oil in the seed or meal (Chilliard et al., 2000). According to our experiment, the most recent studies regarding the effects of linseed based supplements on milk FA profile, suggested that the C18:3*n*3 content in milk fat was dietary linseed dose dependent, in particular to dietary C18:3*n*3 intake (Schori et al., 2006; Chilliard et al., 2009). Moreover, as reported by other investigations, a fraction of these PUFA can bypass the rumen biohydrogenation and directly incorporated in the TGs of milk fat in the mammary gland (Luna et al., 2008).

The trans-11C18:1 was the major isomer among trans octadecanoic acids (trans C18:1) in milk creams fat, and the highest level was found in the linseed group. This result may be explained by a change of microbial activity associated with PUFA biohydrogenation in the rumen, in particular by a sharp increase in rumen transformation of cis-9, cis-12C18:2 or C18:3*n*3 to trans-11C18:1, which would be later taken up by the udder and then desaturated by Δ9-desaturase to yield cis-9, trans-11C18:2 (Chilliard and Ferlay 2004). The total CLA content and in particular cis-9, trans-11C18:2 concentration increased significantly ($p < 0.01$) in dairy cows fed the enriched ration (1.21 mg/100mg FAME) compared to the control group (0.99 mg/100mg FAME). The total CLA concentration varied between 0.80 - 1.15 mg/100mg FAME and 1.09 - 1.31 mg/100mg FAME, during the control and experimental feeding period, respectively (***Figure 1***).

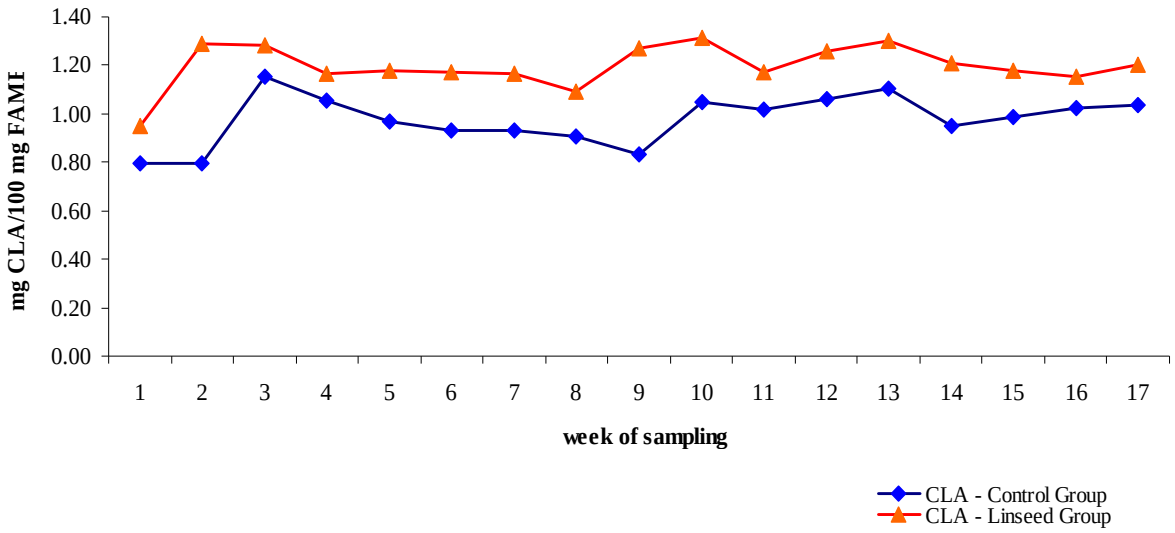

Figure 1. Distribution of total CLA (mg/100mg FAME) of milk cream samples during the linseed supplementing period.

The increase of CLA levels in response to dietary factors is consistent with values reported in the literature (Dhiman et al., 1997; Chilliard et al., 2000a; Hurtaud et al., 2010).

Results from the current investigation show that it is possible to obtain high concentration of CLA in milk fat during the extruded linseed treatment and that the linseed group maintained a relatively constant CLA content across the time period. Increases in the concentration of CLA and TFA in milk fat can be explained by the production of intermediates formed during ruminal biohydrogenation of linseeds and since C18:3 is not a precursor of CLA in the rumen, this suggests that feeding linseed results in a large increase in the production of ruminal trans-11C18:1, which can be used by the mammary gland for CLA synthesis (Chilliard et al. 2000a). In fact, in the current study, the Δ9-desaturase index calculated according to Malau-Aduli et al. (Malau-Aduli et al., 1997)

was significantly higher in linseed group than in the control group, and the highest value of index probably corresponded to the highest content of cis-9, trans-11C18:2 converted in the mammary gland from trans-11C18:1. In cows milk, about 70% of cis-9, trans-11C18:2 originates from conversion of trans-11C18:1 by Δ9-desaturase in the mammary gland and other tissues (Griinari et al,. 2000; Bauman et al., 2001).

3.2 Triglyceride composition

Figure 2 shows the chromatograms of TG profile of milk cream samples.

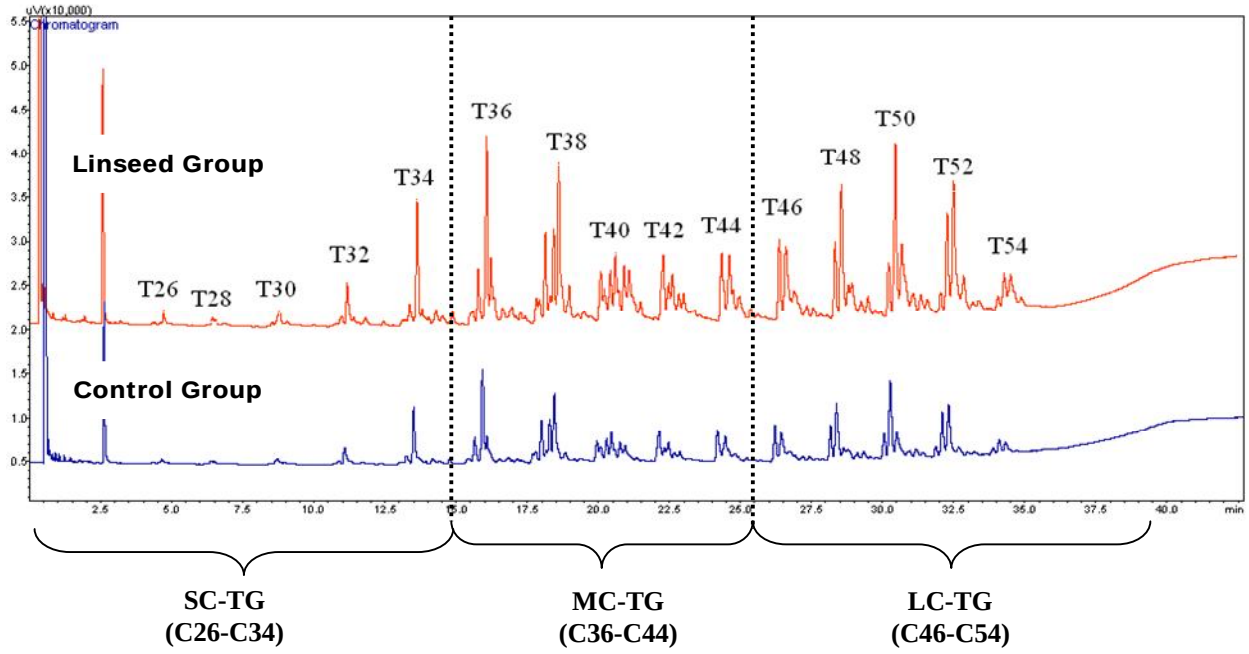

Figure 2. Gas chromatograms profile of triglycerides of milk cream samples. Separation of classes of TGs according to their total number of carbon atoms.

The differences observed in the FA composition between control and linseed groups were also detected in the TG composition (***Table 3***).

The mean content of SC-TG in milk creams from the linseed group slightly increased, without differing significantly to those from the control group. The explanation of the observed variations in the SC-TG of linseed group may be related to SC-FA composition of milk cream fat that did not showed significant differences between two groups. The mean content of MC-TG was lower in milk creams from the linseed group. This variations can be due to *de novo* synthesis occurring in the mammary gland, which is limited to SC- and MC-FA (from C4 to C16) that are non-randomly esterified into *sn*-1, *sn*-2 and *sn*-3 positions of the glycerol backbone, with the exception of C4 and C6 that are esterified to the *sn*-3 position (Jensen et al., 2002).

Triglyceride	*Milk cream*						
	Control group			**Linseed group**			*p value*
	mean		CV	mean		CV	
T26	0.47	±	0.06	0.52	±	0.05	**
T28	0.52	±	0.09	0.58	±	0.09	*
T30	1.05	±	0.14	1.20	±	0.11	**
T32	2.53	±	0.26	2.67	±	0.18	NS
T34	6.27	±	0.30	5.94	±	0.28	**
T36	11.50	±	0.33	10.47	±	0.32	**
T38	13.24	±	0.36	12.45	±	0.33	**
T40	9.73	±	0.29	10.19	±	0.30	**
T42	6.70	±	0.21	6.99	±	0.21	**
T44	6.80	±	0.26	6.95	±	0.19	*
T46	7.74	±	0.26	7.95	±	0.39	*
T48	9.61	±	0.18	9.46	±	0.23	**
T50	11.41	±	0.39	10.98	±	0.25	**
T52	9.38	±	0.67	9.48	±	0.54	NS
T54	3.04	±	0.50	4.15	±	0.56	**
SC-TG (T26-T34)	10.84	±	0.56	10.92	±	0.43	NS
MC-TG (T36-T44)	47.97	±	0.86	47.06	±	0.94	**
LC-TG (T46-T54)	41.19	±	1.20	42.02	±	1.22	**

Table 3. Triglyceride composition (expressed in mg/100mg of total fat) of outcrops milk creams from cows fed control and linseed diet. Significant differences, * : p<0.05; **: p<0.01; NS, not significant.

The rate of FA synthesis in the mammary gland decrease by supplemental LC-FA, and the administration of protected fat sources prevent ruminal biohydrogenation of unsaturated fatty acids, providing UFA post-ruminally for milk fat synthesis (Palmquist et al., 1993; Demeyer and Doreau 1999). In fact, LC-TG (in particular the C50 to C54 TG) predominantly contain plasma-derived LC-FA (mostly C18 FA) that are replaced principally into *sn*-1 and *sn*-3 positions (DePeters et al., 2001; Jensen et al., 2002).

This finding was also observed in the current investigation, and most of the increase was noted with TG52 and TG54 contents from milk creams of the linseed group, in particular with TG54 that showed significant increase ($p < 0.01$) when compared to than from the control group. Finally, as reported by earlier investigations, a high content in TG54 was coincident with high levels of C18:1 and C18:2 FAs that resulted in a highly improved spreadability of the butter (Precht 2001).

3.3 FT-MIR

The characteristic infrared spectra of milk cream and the peak assignments are shown in ***figure 3***. As reported in our previous investigations (Gori et al., 2012), these regions contains absorbance from fundamental valence vibration of functional groups of molecules such as the TGs ester linkage

(C–O and C=O stretching and bending) and acyl chain (C–H stretching and bending), *cis* and *trans* insaturation of FAs (–C=C–H stretching and –HC=CH bending), amide I and II of proteins (N–H bending) and carboxylate group of side chain of several aminoacids (O–C–O stretching) (Karoui et al., 2003).

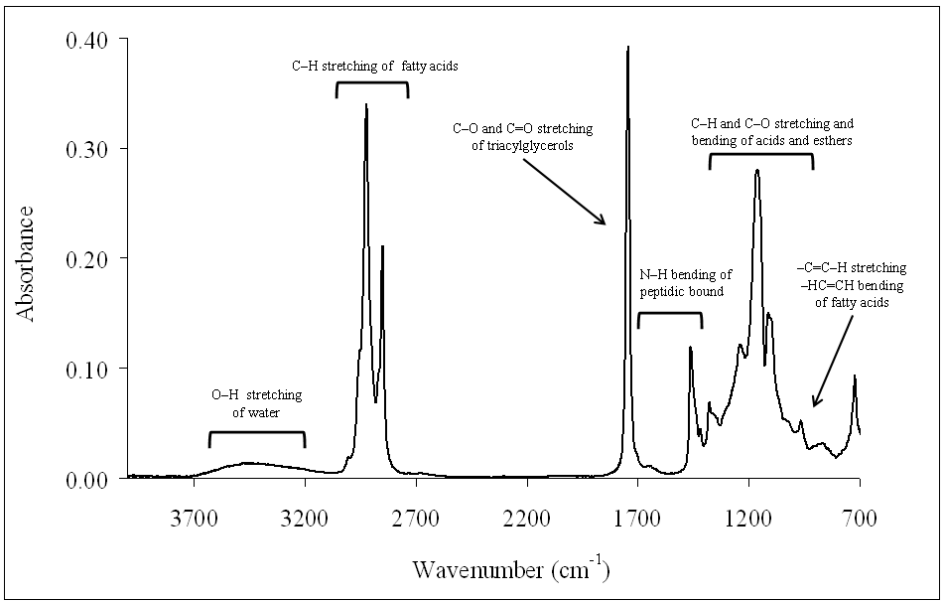

Figure 3. ATR-MIR spectra of an outcrop milk cream sample in the 4000–650 cm^{-1} region. The bands and vibrational modes of functional groups were assigned tentatively.

The success of the classification model was evaluated in different ways: (a) recognition ability as percentage of the samples in the training and verification set correctly classified; (b) predictive ability as percentage of the samples in the test set correctly classified by using the developed model during the training step; and (c) classification ability as percentage of samples in both training, verification and test sets correctly classified by the model (Cajka et al., 2009).

Results of training, verification and test of the best MLP neural networks are summarized in ***table 4***.

The best structure of the best MLP neural networks tested to classify samples with different cow's diet (dry or fresh feeding) or with the presence of extruded linseeds in the feeding was characterized by 5 neurons in the hidden layer. A large number of nodes did not increase the network performance. All the ANNs were obtained with iterations number of about 2000, a momentum value of 0.3 and a learning rate of 0.1.

The prediction abilities, were 93% and 86%, for the different cow's diet and for the presence of extruded linseeds, respectively.

	Correct classified samples (%)			*Nodes*	*Iterations*
	Training set	**Verification set**	**Test set**		
Diet	100	100	93	5	2010
Presence of linseed	100	93	86	5	2025

Table 4. Overall summary of MPL neural networks models to predict cow's diet (*dry feeding period* or *fresh forage feeding period*) and the presence of extruded linseed.

Similar results were obtained by Gori et al (Gori et al., 2012) to discriminate feeding regimen (traditional or unifeed) of butters by using infrared spectroscopy and artificial neural networks. In fact the samples were classified with a predictive ability of 90.0% (confidence levels of 95%) for samples collected in spring. Concerning other diary product, by using the near infrared spectroscopy combined with partial least squares analysis, Coppa et al. (Coppa et al., 2012) have correctly classified (classification performance major of 91% in cross validation) milks from different type of cows feeding, while Andueza et al. (Andueza et al., 2013) have discriminated between cheeses from pasture-fed and preserved forage-fed cows with a classification performance of 96% (cross-validation).

4. Conclusion

In this investigation, the effect of the dietary supplementation with extruded linseeds on outcrop milk cream samples produced in the Parmigiano Reggiano cheese area were studied.

This study confirmed that the extruded linseed supplementation in dairy cow diet strongly affected milk FA and TG composition. Compared with the control group, linseed group showed an improved FA profile, exhibiting a better SFA/UFA ratio, a lower content of the total amount of SFA, in particular of MC-SFA, and a higher content in unsaturated fraction. Specifically, biologically active FA such as C18:3*n*3 and total CLA increased significantly, as well as the trans-11C18:1 content. The increased concentration of cis-9, trans-11C18:2 (+ 30%) and the precursor trans-11C18:1 (+ 24%) in outcrop milk creams of the linseed group, appears to confirm that supplementing extruded linseeds into cow's diet stimulates cis-9, trans-11C18:2 production from dietary cis-9, cis-12C18:2 and C18:3*n*3.

As a consequence of the different FA profile, differences in quantitative composition of TGs were detected by the comparison of milk creams from the linseed and control group. Extruded linseed supplementation in the ration of dairy cows did not significantly affect the SC-TG composition, whilst reduced the MC-TG and increased the LC-TG of milk cream fat. Among the latter group a

remarkable increment of TG54 was observed in milk creams from the linseed group. This result, together with the high cis9-C18:1/C16:0 ratio found in the linseed group, was associated with the ameliorating effect on rheological properties such as butter spredability. Thus, the higher proportion of LC-FA and lower proportion of MC-FA may explain a portion of the variations of TG quantities in the linseed group. Moreover, the possibility to classify outcrop milk creams on the basis of the different cow's diet and the presence of extruded linseeds with an accuracy higher than 93% and 86% respectively, was obtained by the combination of FT-MIR and ANN. The spectral regions between 3050 - 2410 cm^{-1} and 2260 - 700 cm^{-1} appeared to contains specific vibrational frequencies of functional groups that may describe the effects of the cow's dietary manipulation The spectroscopic approach allowed for a minimal sample preparation without producing environmentally unacceptable waste, high-throughput and ease of operation, and may offer specific advantages to dairy manufacturers in the online monitoring of the multiple organoleptic and nutritional characteristics of the resulting product.

The high quality of milk that characterize the dairy production in this defined area can transmit high collective performance throughout the supply chain, generating a beneficial multiplier effects that will be transferred finally to the adjacent by products. In fact the feeding strategy used in this study was adopted to improve the level of bioactive compounds when the fresh forage was not available for dairy cows, leading to the production of dairy products that would be much more beneficial to consumers, and with the advantage for producers to obtain value-added by products with a higher quality than the industrial standard.

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Chapter II

Determination of bioactive fatty acids in dairy products by fast gas chromatography (fast GC) coupled with flame ionization detector (FID): performance comparison of two short highly polar capillary columns. *A preliminary study*.

Determination of bioactive fatty acids in dairy products by fast gas chromatography (fast GC) coupled with flame ionization detector (FID): performance comparison of two short highly polar capillary columns. *A preliminary study*.

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Abstract

Separation of fatty acid methyl esters (FAME) in butter samples has been applied to evaluate the analytical performance of two short highly polar capillary column, cyanopropyl siloxane (BPX-70, 10 meters) and ionic liquid (SLB-IL111, 15 meters) columns, for fast gas-chromatography coupled to flame ionization detector (FID). The research purpose was to optimize the fast gas-chromatography method for routinely analysis of bioactive fatty acids in butter and dairy fats.

During this investigation, about 50 fatty acids were separated in less than 8 minutes by both columns. The best results in terms of analytical performances assessed during the optimization and validation procedures were achieved by using BPX-70 column.

For these reasons, the use of BPX-70 column was chosen for fast and routinely analysis of fatty acids on butter fat.

Key words: bovine milk, fast gas-chromatography, ionic liquid stationary phase, cyanopropyl siloxane stationary phase.

1. Introduction

The fatty acid (FA) composition of dairy fat has been widely studied due to its importance in human nutrition and health (Molketin et al., 1999). To date more than 400 different FAs have been identified in dairy products: from 4 to 26 carbons, branched or not, saturated or unsaturated (up to six double-bonds) and conjugated, and with an extensive number of positional and geometric isomerical possibilities (Jensen 2002).

Gas chromatography (GC) combined to long highly polar capillary columns, have represented by far the most suitable analytical technique to analyze this complex FA composition. In particular, cyanopropyl siloxane and ionic liquid stationary phases have been reported to be the most effective capillary columns for the resolution of critical separation of *cis* and *trans* as well as conjugated isomers (Delmonte et al., 2011).

The ideal GC method for the analysis of FAs in milk would be in a single run, but this is not yet possible, especially for the resolution of all C18:1 isomers and conjugated linoleic acid (CLA) isomers; to avoid isomers overlap, a prior separation followed by fractionation steps by Ag^+-TLC and Ag^+-HPLC are needed (Kramer 2008). This well established analytical approach have the disadvantage to be time consuming, requiring more than 60 minutes of analysis time for each samples, and this factor becomes particularly important when routinely analysis have to be carried out, and a fast response is requested.

The technological innovation over the past years to speed up the analysis time in GC, has led to the evolution of analytical instruments that support fast flow rates, high inlet pressure, rapid temperature programming rates and sensitive detection system (Korytàr 2002). Furthermore, the introduction of new generation narrow bore capillary columns have given the advantage to preserve the efficiency in peaks separation, increasing the interest to investigate on FA separations by fast GC in complex biological matrices such as human plasma, fish oil and milk, and on their analytical performances during method optimization with the respect to the conventional GC columns (Mondello et al., 2004; Bondia-Pons et al., 2004; Cruz Hernandez 2007). Since the primary goal of fast GC is to achieve the desired resolution of compounds or the critical pair of compounds in the shortest possible time, a particular attention should be paid towards optimization of the experimental conditions because a fully optimized chromatographic method is an ad-hoc compromise between sample nature, column and carrier gas, optimal temperature programme, sample capacity (Matisová and Dömötörová 2003).

Therefore the aim of the present study was to set up a specific, accurate, time-saving and reliable fast GC method for the determination of fatty acid methyl esters (FAME) in butter samples, which are considered the most complex edible fats due to the wide diversity in nature of FAs. Two short

highly polar capillary columns were tested, namely the 10m BPX-70 (70% Cyanopropyl Polysilphenylene-siloxane stationary phase) and the 15m SLB-IL111 [1,5-Di(2,3-dimethylimidazolium)pentane bis(trifluoromethylsulfonyl)imide phase] in order to determine which of the two columns lead to the highest chromatographic performances for the separation of butter FAME. During the initial stage of method validation the effect of fast temperature programming rate on peak retention times, areas and power of resolution was evaluated, then the optimized methods for the analysis of FAME in butter were subjected to validation on both the selected GC columns considering specific parameters, such as precision, accuracy, linearity, limit of quantification (LOQ) and limit of detection (LOD).

2. Materials and Methods

2.1 Sampling and sample preparation

The 52 FAME GLC 463 and UC-59M CLA mixture were supplied from Nu-Check (Elysian, MN, USA) and the alkane standard solution C8-C20 was acquired from Sigma-Aldrich (Saint Louis, MO, USA).

The butter sample was kindly donated by a local dairy farm in Reggio Emilia (Italy), and the FA composition was determined after extraction of total lipids in accordance with Hara and Radin (Hara and Radin 1978). About 20 mg of fat was methylated with ethereal diazomethane (Fisier and Fiser 1967) and then the FAs were transesterified according to Christie (Christie1982). For each preparation 1 mg of methyl undecanoate (C11:0) from Sigma-Aldrich (Saint Louis, MO, USA) was added as internal standard.

2.2 GC-FID analyses: operative conditions

FAME were analyzed on a GC 2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a split–splitless injector, an AOC-20i autoinjector and a flame ionization detector (FID). All data were collected by GC Solution software (Shimadzu, Kyoto, Japan).

The BPX-70 capillary column (10 m, ID 0.10 mm, 0.10 µm f.t.; SGE, Melbourne, Australia) was initially operated at a oven temperature programme set at 50 °C for 0.2 min, then increased at a rate of 120 °C/min to 175 °C, where it remained for 2 min, then increased at a rate of 20 °C/min to 220 °C, and then increased at a rate of 50 °C/min to 250 °C where it remained for the last 0.3 min. Hydrogen was used as a carrier gas at a flow rate of 0.8 mL/min, and the split ratio was set at 1:100.

The SLB-IL111 capillary column (15 m, ID 0.10 mm, 0.08 µm f.t.; Sigma-Aldrich, Saint Louis, MO, USA) was initially operated at an oven temperature programme set at 50 °C for 0.3 min, then increased at a rate of 40 °C/min to 165 °C, where it remained for 1.2 min, then increased at a rate of

10 °C/min to 180 °C, where it remained for 0.5 min and then increased at a rate of 70 °C/min to 250 °C. Hydrogen was used as a carrier gas at a flow rate of 0.6 mL/min, and the split ratio was set at 1:100. The analyses for the evaluation of fast temperature programming rates on BPX-70 and SLB-IL111 , were carried out from 50 °C to 200 °C , with constant oven temperature programme set at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 120 °C/min. In all the configurations the typical injection volume was 0.3 μL, and FID parameters as follows: make-up gas was He at a flow rate of 30 mL/min; H_2 flow rate was 30mL/min; the air flow rate was 400 mL/mi and FID sampling rate was 12 ms.

FAME in butter samples were routinely identified by comparison of the retention times of standard mixtures and published isomeric data (Kramer et al., 2004). Quantification of individual FAs was based on the obtained peak area and the quantity was expressed as weight percentage of total FAME.

2.3 Validation of the analytical method

The resolution between peaks was estimated on 18 butter replicates as measure of how completely two adjacent peaks were separated from each other. The values were automatically calculated by GC Solution software (Shimadzu, Kyoto, Japan) during peak integration. According to the European Pharmacopoeia criteria (European Pharmacopoeia 2005), a resolution lower than 1.5 was considered to correspond to peaks not baseline separated. The accuracy of the assay is the closeness of the test value obtained to the nominal value. It is calculated by determining trueness and precision (%Recovery, %RSD). It was determined as the percentual recovery of C11:0 standard spiked at a different concentrations (0.5, 0.75, 1, 1.25, 1.50 mg/mL) in 5 butter replicates, and then analyzed individually in triplicate. The analyte concentrations were calculated from daily calibration curve and the accuracy was calculated by the ratio of this calculated concentration versus the theoretical (spiked) concentration. Intraday and interday precisions were developed to evaluate the repeatability of the GC method. A butter extract was injected (n = 6) on the same day (intraday precision) for 3 consecutive days (interday precision, n = 18). The relative standard deviations (RSDs) of analysis time and peak area were determined. Linearity was expressed as the linear regression of instrument response for 9 increasing concentration of C11:0 (IS) (4.85, 9.7, 24.25, 48.5, 97, 242.5, 485, 970, 1940 mg/mL) injected in triplicate. The detection and quantification limits (LOD and LOQ), expressed in ppm, were calculated following the International Conference on Harmonization guidance (ICH, 1996) from 3 S_a/b and 10 S_a/b respectively, where S_a is the standard deviation of blank response injected in triplicate, and b is the slope of the linear calibration curve.

3. Results and discussion

3.1 Study of the influence of temperature on the polarity of the stationary phases

Since increasing the temperature programming rates is a good practise to increase the speed of analysis (McNair and Reed 2000), the effects of 12 linear monogradients (temperature rates from 10° to 120°C/min) at a constant flow rate (0.8 and 0.6 mL/min for the BPX-70 and SLB-ILL111 columns, respectively) were evaluated on column related parameters such as retention times, peak areas and resolution with the purpose to guarantee an adequate separation of a mixture contained analyte with different boiling points. To do so, the alkane standard solution C8-C20 was used.

As reported in ***Figures 1*** and ***2***, the retention time as well as the resolution of the hydrocarbons solution decreased with the progressive temperature rates increment. Comparing the two columns, the cyanopropyl siloxane column (BPX-70) showed a slightly higher peak retention time variation with the increase of temperature rate than the ionic liquid column (SLB-IL111) (***Figure 1A-B***). Particularly, ***Figure 1*** shows the variability of alkanes ranging from C9 to C20, while C8 was not reported due to the solvent fronting effect.

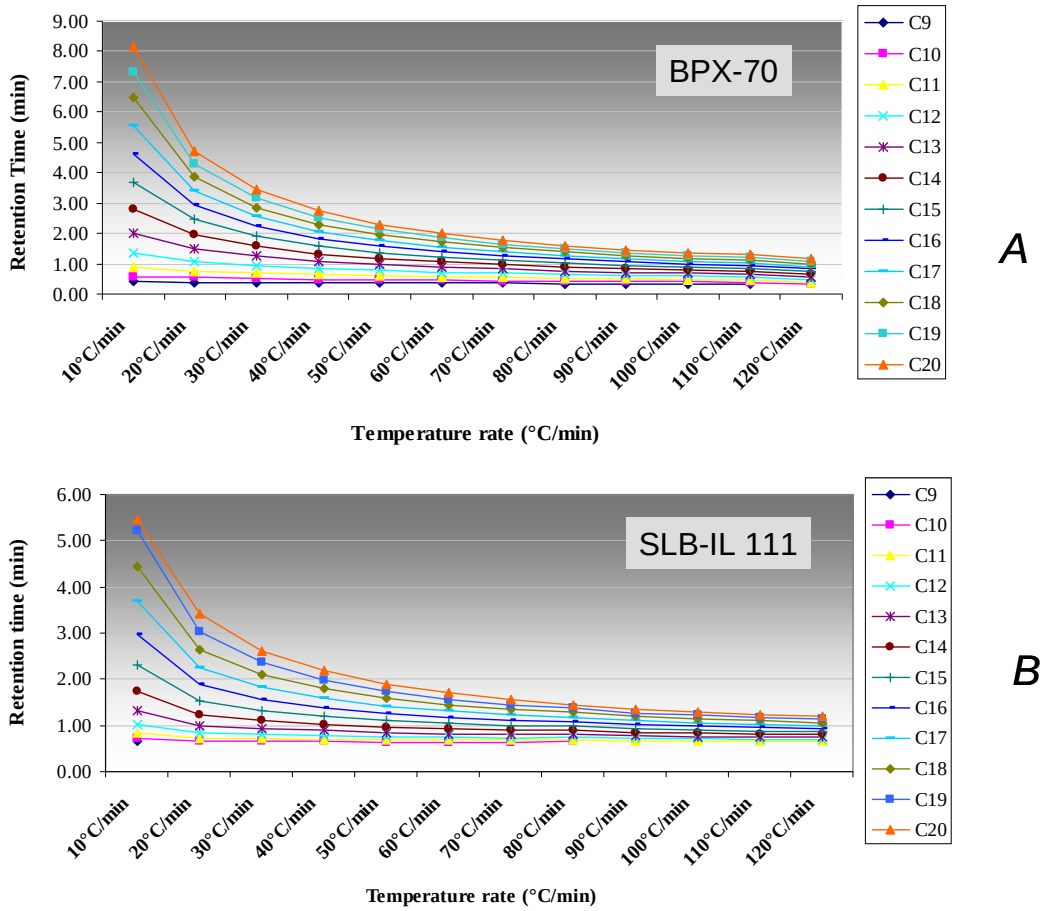

Figure 1. Effect of temperature rate (°C/min) on the retention rime (min) of alkane standard solution C8-C20 by fast gas-chromatography analysis using a 10m BPX-70 (A) and 15m SLB-IL111 (B) columns.

Furthermore, it was observed (***Figure 2A-B***) that the C10 was the first alkane well resolved using BPX-70 column in the temperature rate interval from 10° to 110°C/min, while on SLB-IL111, the C10 alkane was only resolved only at 10°C/min temperature rate and the subsequent C11 alkane in the temperature rate interval from 10° to 80°C/min. The other hydrocarbons were well separated in all temperature rates checked. As a consequence, peak resolution was affected by the specific columns retention characteristic at a different temperature rates, especially on low chain alkanes. This performance can be ascribed to the higher polarity of the ionic liquid coating SLB-IL111 column than the homologous cyanopropyl siloxane BPX-70 columns (Sigma-Aldrich 2011; Anderson et al., 2002).

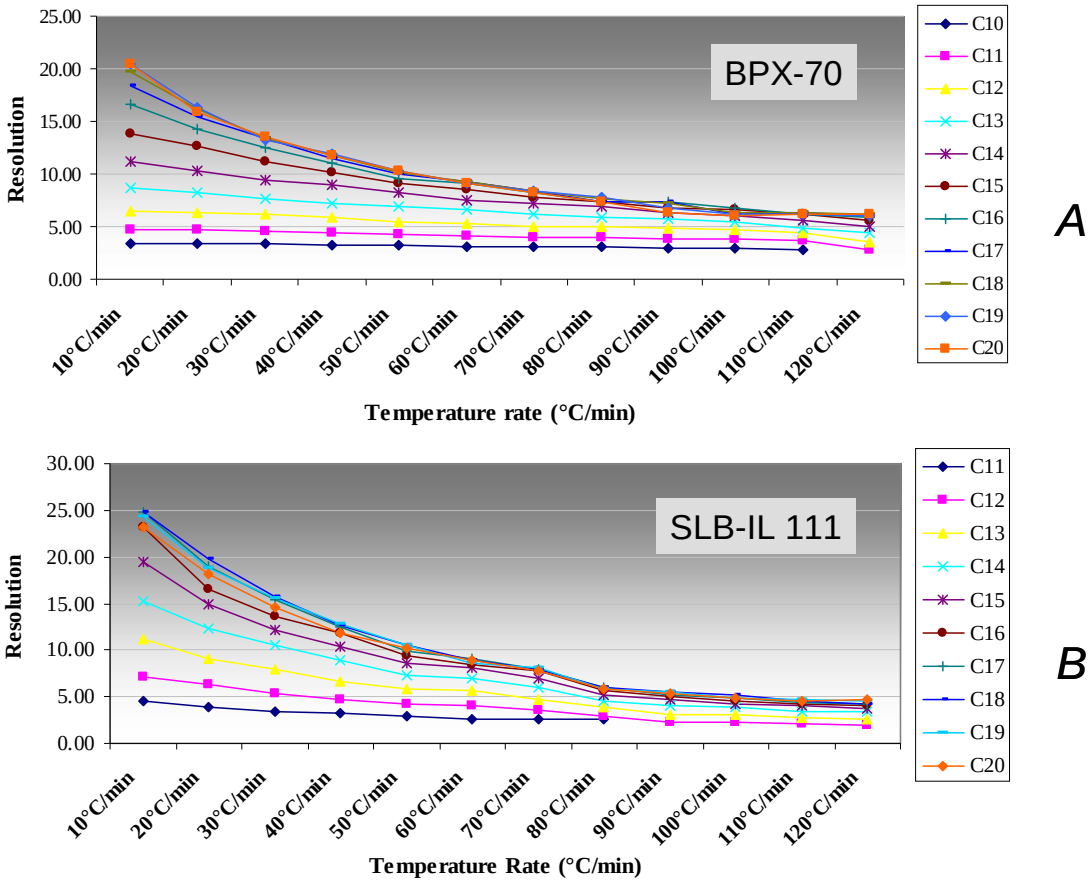

Figure 2. Effect of temperature rate (°C/min) on the resolution of alkane standard solution C8-C20 by fast gas-chromatography analysis using a 10m BPX-70 (A) and 15m SLB-IL111 (B) columns.

As reported in ***Figure 3,*** peak areas remained constant at temperature rates from 10°C/min to 70°C/min, and then they increased from 80°C/min to 120°C/min on both the columns. This tendency might be attributed to the polarity of the capillary columns stationary phases investigated, which is known to be temperature sensitive (Huang et al., 2007; Castello et al., 1997) The only

exception is represented by the trend of C9 alkane on BPX-70 (***Figure 3A***) and the C9 and C10 alkenes (***Figure 3B***), whose peak areas seemed to be influenced by the solvent fronting as the temperature rate increased.

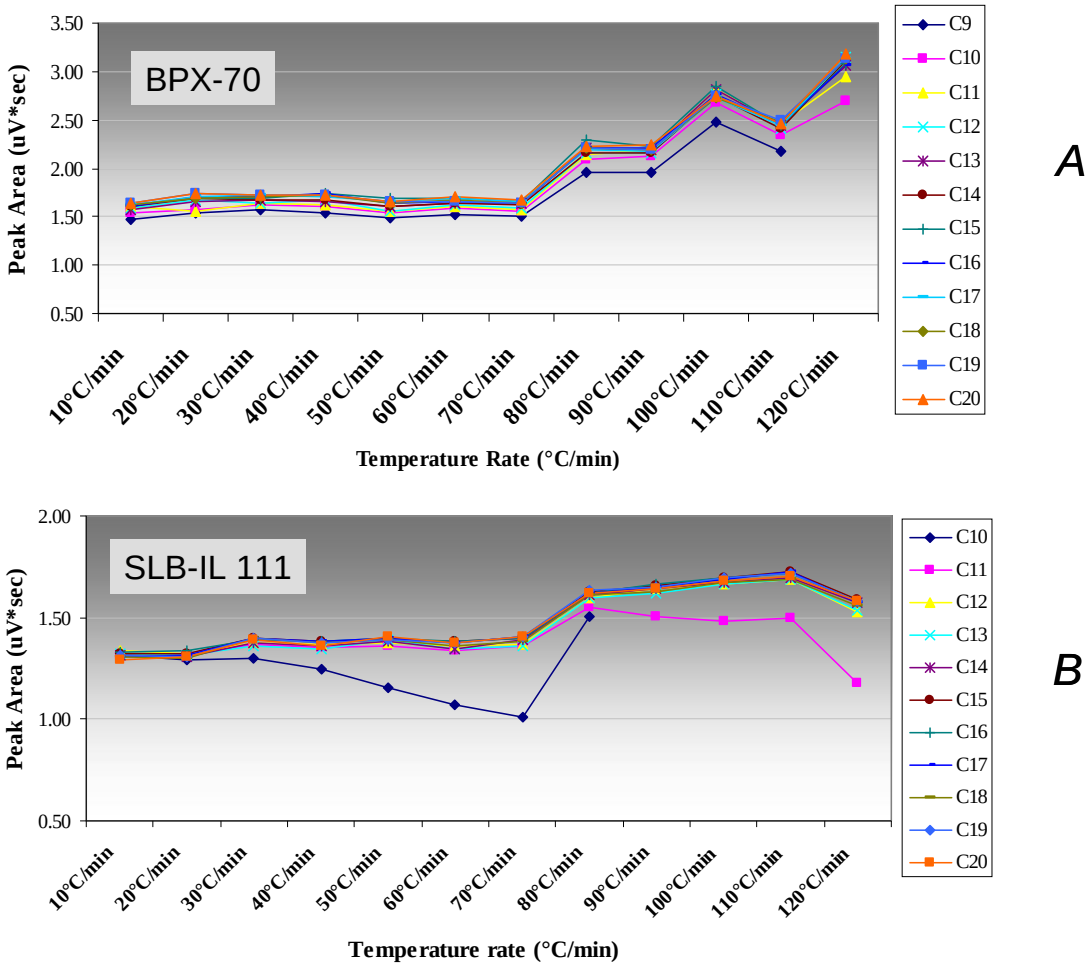

Figure 3. Effect of temperature rate (°C/min) on the peak areas (uV*sec) of alkane standard solution C8-C20 by fast gas-chromatography analysis using a 10m BPX-70 (A) and 15m SLB-IL111 (B) columns.

3.2 Fast GC method optimization

To optimize the separation of FA in the butter extract different separation factors were considered. To this end, two commercial standard mixtures (52 FAME GLC 463 and UC-59M) were used to establish the best resolution of FAs, from C4:0 to long-chain polyunsaturated FAs (LC-PUFA), with special emphasis on the separation of *cis* and *trans* C18:1 isomers, *n*-3 and *n*-6 FA, and CLA. The first fast temperature programs at 120 °C/min from 50 °C to 175 °C and 40 °C/min from 50 °C to 165 °C adopted for BPX-70 and SLB-IL111 respectively, were selected because they provided the best compromise for the resolution from C4:0 to C18:0 FAs (***Table 1, Table 2, Figure 4A-B***).

FAME	Retention time		Resolution	Intra-day repeatability		Inter-day repeatability	
	minutes	RSD(%)		mg/100mg FAME	RSD(%)	mg/100mg FAME	RSD(%)
C4:0	0.51	0.09	--	2.43	1.66	2.32	4.04
C6:0	0.75	0.11	11.66	1.64	0.66	1.62	1.93
C8:0	0.97	0.07	12.38	1.05	1.43	1.04	1.70
C10:0	1.17	0.10	6.56	2.44	1.45	2.56	4.07
C12:0	1.36	0.16	--	2.88	1.43	2.90	1.20
C12:1 c	1.46	0.16	--	0.07	4.84	0.09	19.45
C13:0	1.52	0.16	*	0.21	6.41	0.27	19.91
C14:0	1.58	0.16	4.00	10.02	1.05	10.06	0.89
C14:1	1.65	0.15	--	1.07	1.66	1.06	1.63
C15:0	1.72	0.15	2.77	1.12	1.81	1.12	1.31
C15:1c	1.81	0.16	3.29	0.36	6.61	0.41	12.57
C16:0	1.91	0.17	5.63	27.19	0.95	27.27	0.73
C16:1 t	1.97	0.15	--	0.20	9.61	0.21	14.74
C16:1 c	1.99	0.16	--	1.17	2.27	1.15	2.62
C17:0	2.14	0.15	*	0.67	1.92	0.68	2.65
C17:1 c	2.25	0.15	4.81	0.30	11.36	0.28	9.96
C18:0	2.47	0.16	5.28	10.62	1.36	11.21	5.41
C18:1 t9	2.55	0.15	--	0.50	13.02	0.58	17.73
C18:1 t10-t11	2.57	0.15	--	2.07	3.19	2.11	3.08
C18:1 c9-t12-t15	2.61	0.15	--	21.33	3.82	20.45	4.51
C18:1 c 11	2.63	0.14	--	0.54	12.25	0.53	11.64
C18:1 c 12	2.66	0.13	--	0.32	6.63	0.33	5.43
C18:1 c 13-c14	2.69	0.14	*	0.10	13.50	0.10	19.27
C18:1 c15	2.72	0.14	*	0.32	6.53	0.32	8.71
C18:1 c16	2.75	0.13	*	0.25	7.32	0.26	10.41
C18:2 n6	2.87	0.13	2.17	2.42	2.22	2.35	4.15
C18:2 c9, t11	3.43	0.11	4.58	0.69	2.93	0.66	7.09
C18:3 n3	3.26	0.13	4.71	0.58	3.15	0.56	17.25
C18:3 n6	3.06	0.14	3.91	0.12	14.42	0.12	4.86
C19:0	2.91	0.14	*	0.30	9.09	0.30	9.76
C20:0	3.48	0.10	*	0.25	5.92	0.25	6.69
C20:1 c5	*nd*	*nd*		*nd*		*nd*	
C20:1c8-c11/CLA§	3.63	0.08	1.84	0.23	13.16	0.23	12.63
C20:2	nd	nd		nd		nd	
C20:3 n6	4.12	0.06	3.58	0.10	10.92	0.09	20.06
C20:4 n6	4.22	0.06	3.19	0.13	12.24	0.11	17.07
C20:5 n3 (EPA)	4.54	0.04	3.21	0.19	14.45	0.24	21.91
C22:0	4.42	0.04	6.92	0.18	8.45	0.18	11.24
C22:2	*nd*	*nd*		*nd*		*nd*	
C22:4	*nd*	*nd*		*nd*		*nd*	
C22:5 n3	5.3	0.03	5.65	0.09	11.05	0.09	16.62
C24:0	5.15	0.03	3.86	0.07	11.89	0.07	14.77

-- : not resoluted

*: resolution <1.5

nd: not detected

CLA§ unidentified CLA isomers

Table 1. Resolution, intra-day and inter-day repeatability values of butter FAME obtained on 10m BPX-70 column.

FAME	Retention time		Resolution	Intra-day repeatability		Inter-day repeatability	
	minutes	RSD(%)		mg/100mgFAME	RSD(%)	mg/100mgFAME	RSD(%)
C4:0	0.95	0.10	--	2.61	0.97	2.63	1.38
C6:0	1.35	0.10	19.06	1.74	0.64	1.76	1.46
C8:0	1.80	0.11	22.84	1.09	2.07	1.11	2.12
C10:0	2.23	0.08	23.82	2.51	0.68	2.52	1.22
C12:0	2.63	0.07	10.73	2.93	0.91	2.94	1.35
C12:1 c	*nd*	*nd*		*nd*		*nd*	
C13:0	2.82	0.08	8.67	0.12	6.84	0.13	18.79
C14:0	2.99	0.05	8.08	10.21	0.22	10.23	0.59
C14:1	3.14	0.04	*	0.75	0.78	0.73	6.11
C15:0	3.16	0.05	*	1.05	2.15	1.13	7.60
C15:1c	*nd*	*nd*		*nd*		*nd*	
C16:0	3.34	0.05	9.69	27.60	0.03	27.66	0.47
C16:1 t	3.43	0.06	3.48	0.10	9.43	0.09	15.40
C16:1 c	3.47	0.35	*	1.61	0.67	1.36	35.36
C17:0	3.52	0.04	*	0.61	1.38	0.63	2.15
C17:1 c	*nd*	*nd*		*nd*		*nd*	
c18:0	3.74	0.04	7.48	11.22	1.90	11.30	1.54
c18:1 t8-t9-t10	3.84	0.05	--	0.59	4.05	0.71	17.44
C18:1 t11-t12	3.87	0.05	--	1.84	1.87	1.74	7.25
C18:1 c9-t15	3.89	0.03	--	20.80	1.51	20.82	1.09
C18:1 c 11	3.91	0.05	--	0.54	26.65	0.51	15.26
C18:1 c 12	3.94	0.04	--	0.29	4.79	0.30	8.69
C18:1 c 13	4.05	0.04	--	0.06	25.92	0.06	21.48
C18:1 c 14	4.07	0.05	--	0.07	13.29	0.06	16.39
C18:1 c 15	4.10	0.04	--	0.14	4.16	0.14	4.91
C18:1 c 16	4.14	0.06	--	0.20	2.37	0.22	7.00
C18:2 n6	4.17	0.04	*	2.26	1.19	2.21	2.37
C18:2 c9, t11 (CLA)[§]	4.77	0.03	8.08	0.61	2.38	0.58	4.36
C18:3 n6	4.41	0.04	4.30	0.03	96.32	0.03	67.81
C18:3 n3	4.59	0.03	5.88	0.56	3.81	0.55	3.29
C19:0	3.99	0.05	--	0.59	3.05	0.56	9.41
C20:0	4.28	0.07	--	0.23	8.32	0.22	7.22
C20:1 c8	4.46	0.06	2.09	0.11	12.71	0.12	11.21
C20:1 c11	4.49	0.07	*	0.06	24.65	0.07	16.39
C20:2	*nd*	*nd*		*nd*		*nd*	
C20:3 n6	5.15	0.04	4.55	0.07	28.55	0.07	15.17
C20:5 n3 (EPA)	5.85	0.04	16.09	0.05	13.58	0.05	43.68
C22:0/CLA**	5.02	0.07	6.22	0.18	1.39	0.18	10.01
C22:2/c20:3 n3	5.33	0.04	7.16	0.14	7.03	0.12	14.73
C22:5 n3	6.71	0.01	30.00	0.07	37.40	0.09	25.11
C24:0	5.87	0.08	*	0.08	27.25	0.09	25.19

-- : not resoluted

*: resolution <1.5

nd: not detected

CLA[§]: unidentified CLA isomers

Table 3. Resolution, intra-day and inter-day repeatability values of butter FAME obtained on 15m SLB-IL111 column.

The significant presence of short chain FAME in butter fat imposed the choice of an initial column temperature of 50 °C. In particular, the cyanopropyl siloxane column was capable to resolve the medium chain saturated FAME (C12:0 to C18:0) from the *cis* MUFA isomers in the 52 FAME mix and butter sample (***Figure 4A***) whereas, on the ionic liquid column, peculiar overlaps occurred the 52 FAME mix between the saturated FAME and the *cis* MUFA isomers with one less carbon atom, such as C12:0 with the *cis* C11:1, C13:0 with the *cis* C12:1, C14:0 with the *cis* C13:1, C15:0 with the *cis* C14:1 and C16:0 with *cis* C15:1 (***Figure 4B***). Accordingly with earlier investigation, this behaviour can be ascribed to the lower polarity of BPX-70 with the respect of SLB-IL111 column (Delmonte et al. 2011).

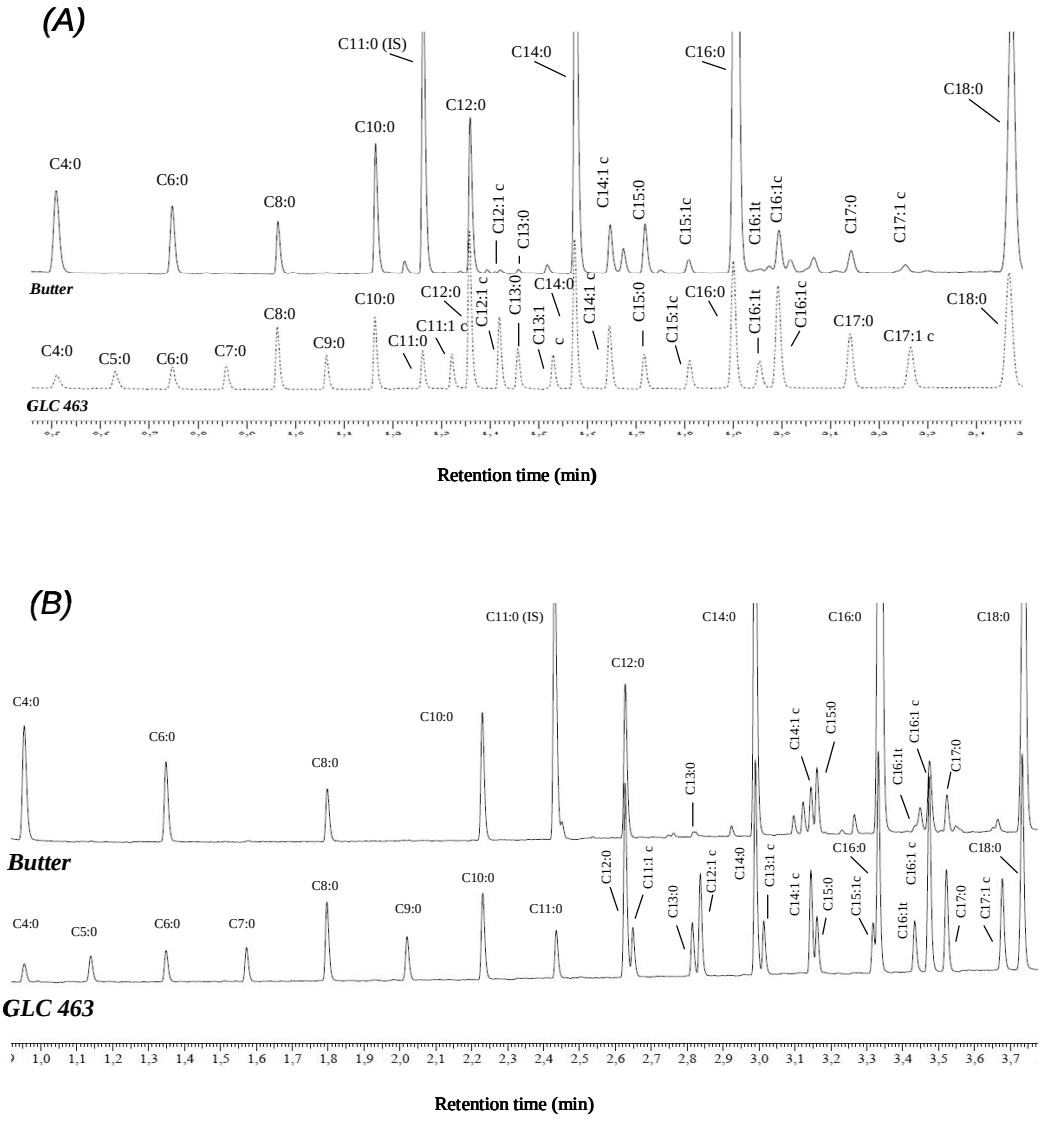

Figure 4. Chromatographic region from C4:0 to C18:0 fatty acids. Separation obtained by fast gas-chromatographic using 10m BPX-70 (A) and SLB-IL111 (B) columns.

The reason of the choice of the first temperature increments was to improved the separation of *cis* and *trans* C18:1 isomers, minimizing the isomer overlaps in the in the subsequent isothermal steps. Although *cis* and *trans* C18:1 isomers were partially resolved on both BPX-70 and SLB-IL111 columns, it was enough to integrate the area peaks and quantify their sum in butter (***Figure 5 A-B***).

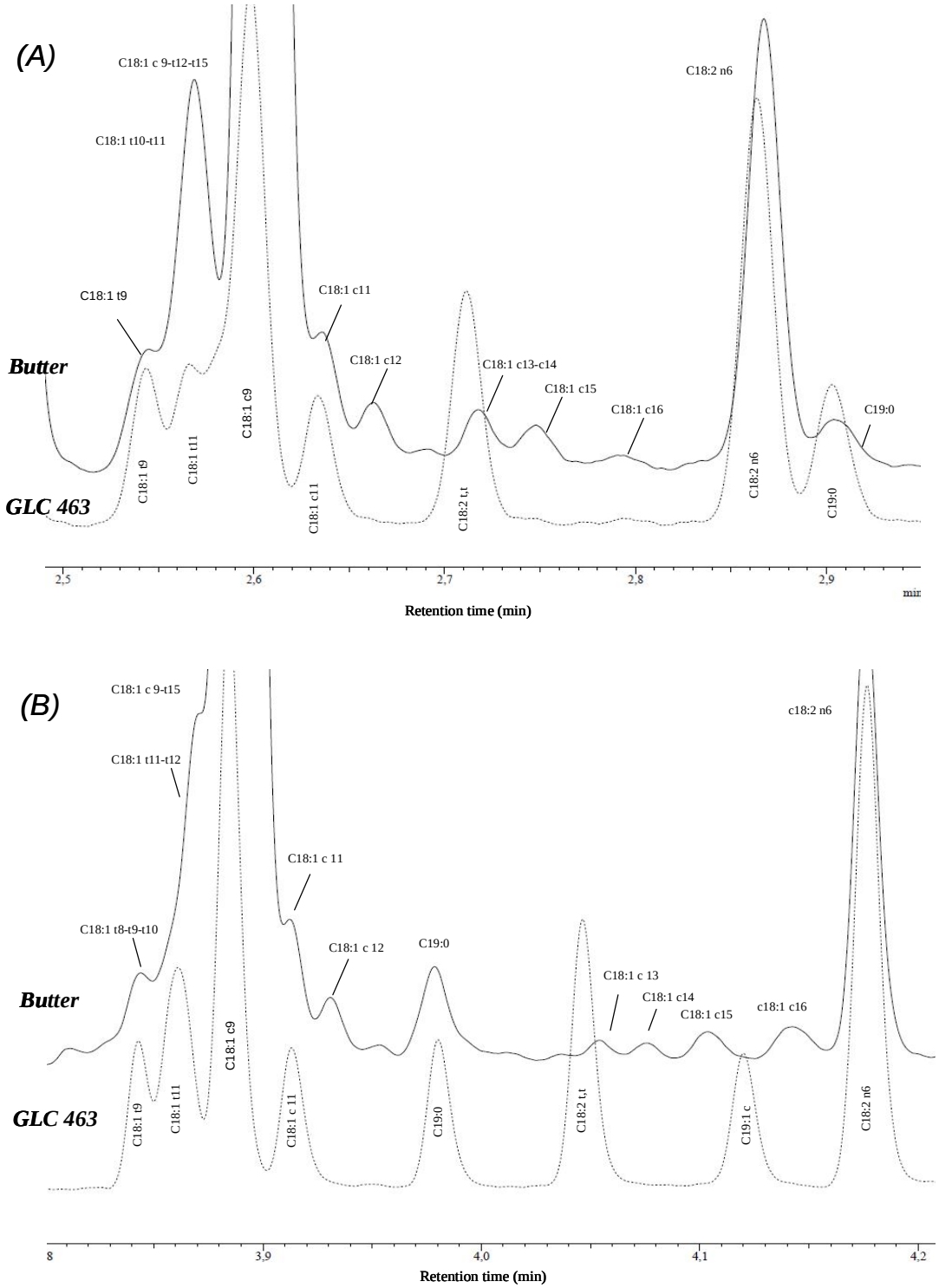

Figure 5. Chromatographic region of C18:1 isomers. Separation obtained by fast gas-chromatographic using 10m BPX-70 (A) and SLB-IL111 (B) columns.

The second temperature programs were selected on BPX-70 and SLB-IL111 for the elution of PUFA on 52 FAME mixture and butter sample with satisfactory resolution, although some co-elution occurred over the *n*3- and *n*6-PUFA (***Table 1, Table 2*** and ***Figure 6A-B***).

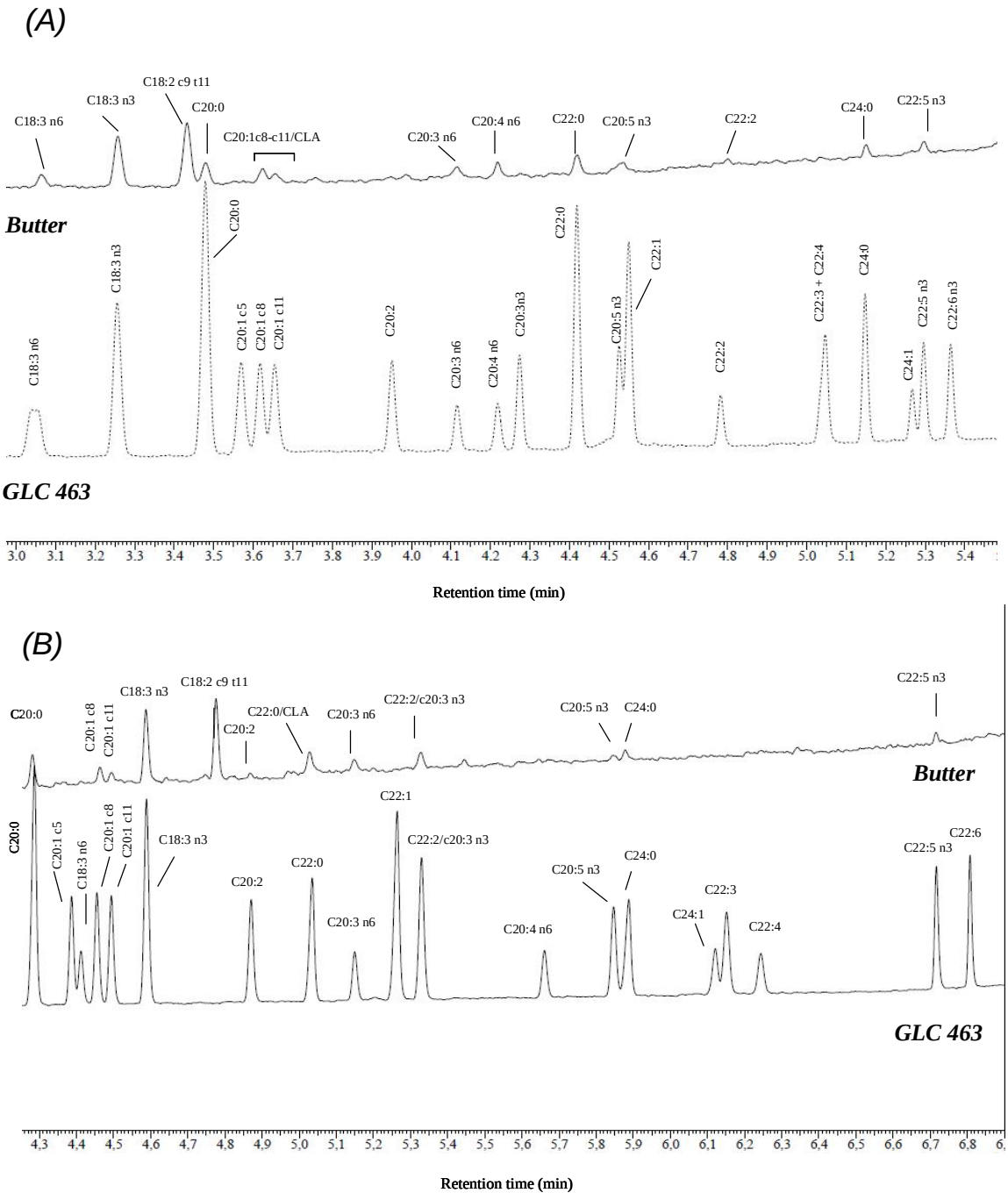

Figure 6. Chromatographic region of *n*3- and *n*6-PUFA. Separation obtained by fast gas-chromatographic using 10m BPX-70 (A) and SLB-IL111 (B) columns.

In fact, on the BPX-70 the C18:2*n*6 showed a near to baseline resolution from C19:0, and 18:3*n*6 coeluted with C19:1, while on SLB-IL111 were perfectly separated. The C18:3*n*3 was fully

resolved but in a different elution order: on BPX-70 the C18:3*n*3 eluted before the C20:0 while on SLB-IL111 it eluted after the C20:0, between C20:1 and C20:2 isomers. With BPX-70 column, the C20:5*n*3 was not completely resolved from C22:1 and the C22:3 co-eluted with C22:4, and with SLB-IL111 the C20:3*n*6 and C20:5*n*3 co-eluted with C22:2 and C24:0, respectively. Furthermore, a special attention was paid on CLA isomers (***Figure 7A-B***).

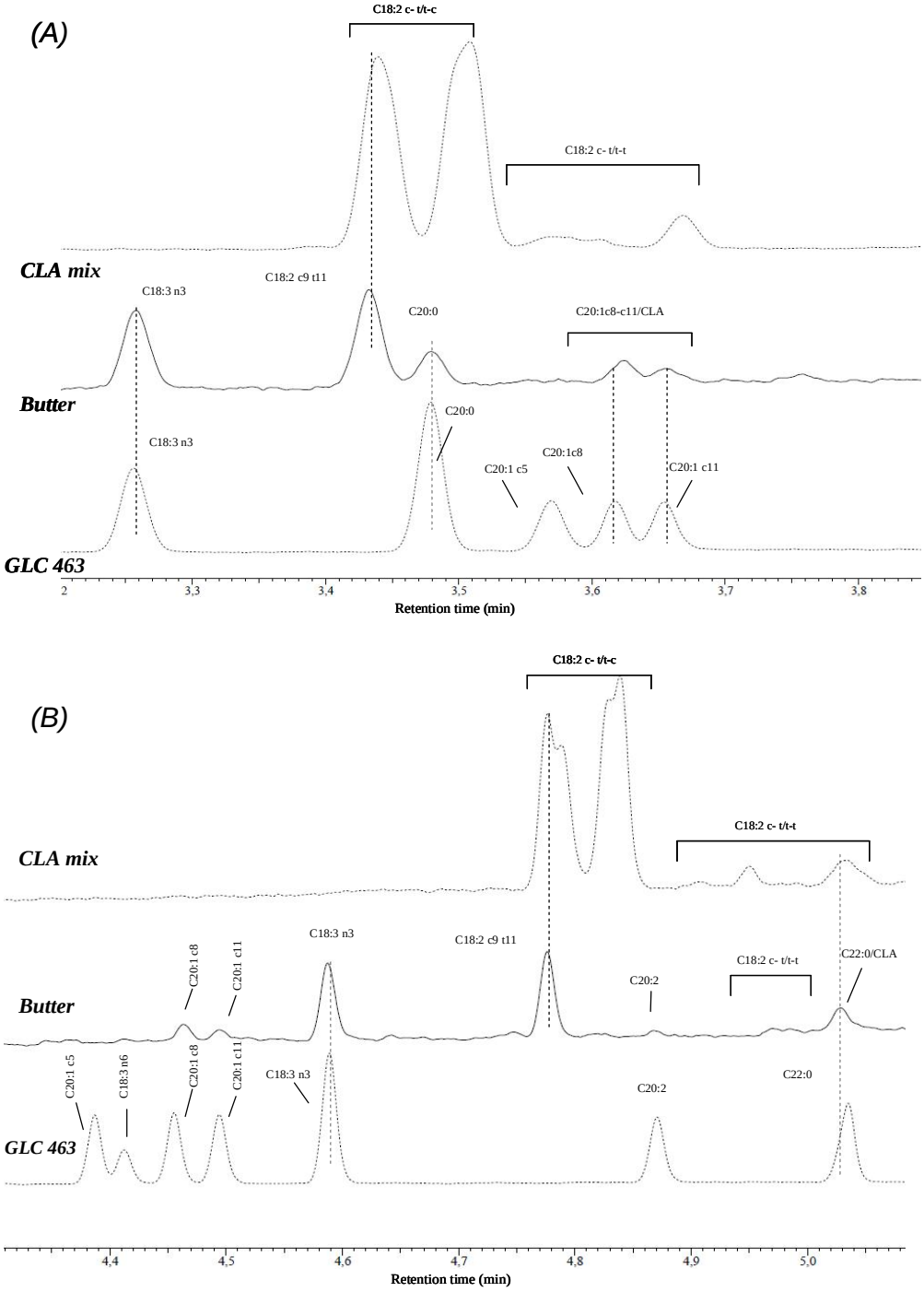

Figure 7. Chromatographic region of CLA isomers. Separation obtained by fast gas-chromatographic using 10m BPX-70 (A) and SLB-IL111 (B) columns.

The *cis*-9, *trans*-11 C18:2 CLA was baseline resolved in butter sample by both the columns: on BPX-70 slightly before the C20:0 (***Figure 7A***), and on SLB-IL111 between C18:3*n*3 and C20:2 isomers (***Figure 7B***). However, it raised the peaks tentatively identified as minor CLA isomers showed substantial overlap with *cis*-C20:1 isomers by using the BPX-70, while on SLB-IL111 co-eluted with C22:0.

As reported in ***Figure 7 A-B*** clearly appeared the impossibility to separate all the geometric CLA isomers on UC-59M mixture under the experimental chromatographic conditions. In fact, it is well established that for a better resolution of all the possible combinations of double bond position and *cis/trans* geometric isomers in commercial CLA preparations, the complementary use of Ag^{+}-HPLC is currently the most effective way for their separation and quantification (Kramer 2008). In contrast, dairy fat such as butter fat contains primarily *cis*-9, *trans*-11 C18:2 (90% of total CLA), followed by small amount of *trans*-7, *cis*-9 C18:2 and *trans*-11, *cis*-13 C18:2 isomers (Kramer et al., 2004), and several investigation reported the unfeasibility to resolve the coelution of the two most abundant CLA isomers by GC under any temperature programme conditions (Luna et al. 2005; Delmonte et al. 2011). Notwithstanding, in the current investigation a significant result were obtained: the analysis of complex FA composition of dairy fat and the separation of the most abundant CLA isomers in dairy fat were achieved in less than 6 minutes in both experimental chromatographic conditions by using short length polar capillary columns, resulting a good alternative for rapid and efficient routine analysis of dairy fat than traditional analysis with middle and long length polar capillary columns.

3.3 Fast GC method validation

Validation experiments were carried out to obtain the analytical parameters of the methods. Therefore, precision of the analytical method was verified through the intra-day and inter-day repeatability of the butter sample analyzed on BPX-70 and SLB-IL111 under the selected temperature configurations. As it can be observed in ***Table 1*** and ***Table 2*** the average RSD% of retention time were significantly low with both the column and agreed with earlier investigation about fast GC (Bondia-Pons et al., 2006). Furthermore, the RSD% of FAME content on the column tested, highlighted a higher precision of BPX-70 with lower intra- and inter-day repeatability values than SLB-IL111, 6.38% and 9.20% versus 10.30% and 12.19%, respectively.

The nearness to the true value of the quantitative data obtained by the two chromatographic approaches, was evaluated through the recovery of different amount of C11:0 in spiked butter sample. The percentage of recovery reported in ***Table 3***, suggested better accuracy performances of

BPX-70 than SLB-IL111. In fact, the percentage of average recovery on BPX-70 was of 100.07% with a RSD% of 1.89%, while on SLB-IL111 was of 69.26% with a RSD% of 18.14%.

Spiked Concentration (mg/mL)	10m BPX-70		15m SLB-IL111	
	Measured Concentration (mg/mL)	Recovery (%)	Measured Concentration (mg/mL)	Recovery (%)
0.50	0.51	102.21	0.33	65.3
0.75	0.73	97.09	0.41	54.0
1.00	1.01	100.93	0.64	64.2
1.25	1.25	100.20	0.95	75.7
1.50	1.50	99.92	1.31	87.1
Average recovery (%)	**100.07**		**69.26**	
RSD%	**1.89**		**18.14**	

Table 3. Accuracy - Recovery (%) of methyl undecanoate (methyl C11:0) in spiked butter samples (n = 5) on 10m BPX-70 and 15m SLB-IL111 columns.

With regards to the linearity of the detector, calculated over 9 increasing concentration of C11:0 (IS), the BPX-70 showed a R^2 of 0.9998 while the SLB-IL111 a R^2 of 0.9874.

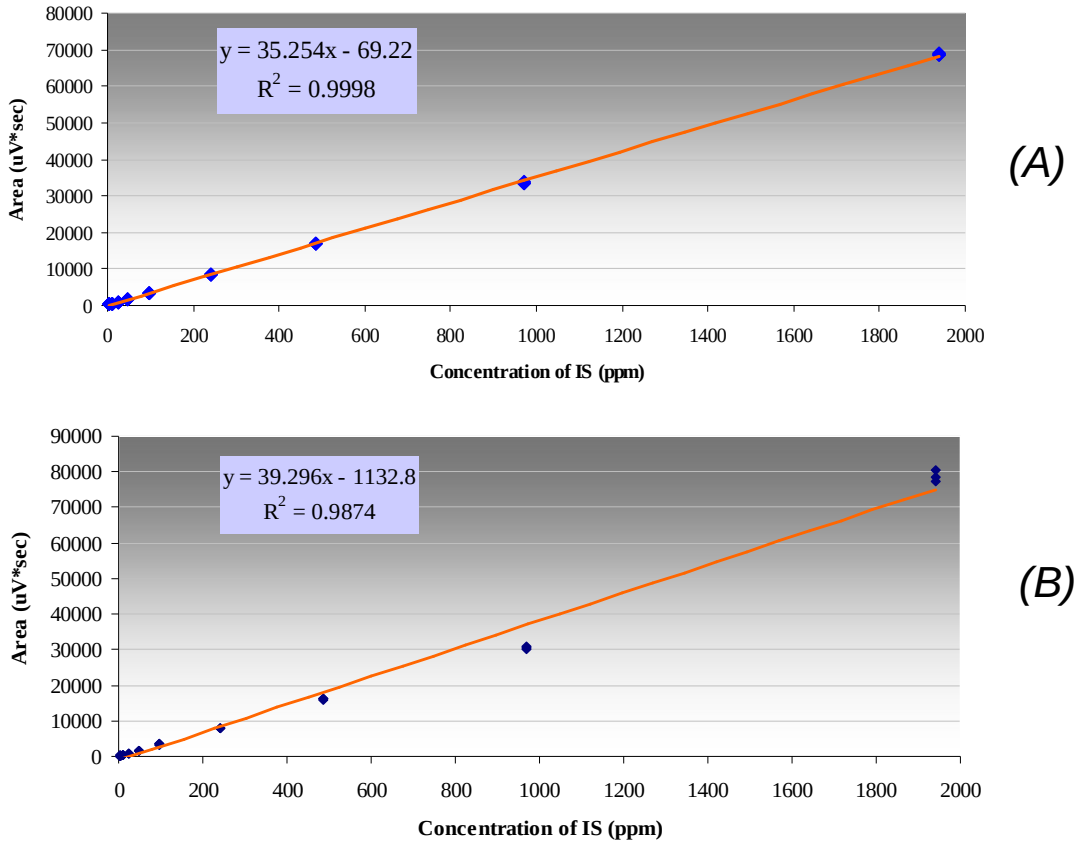

Figure 8. Linearity obtained by fast gas-chromatographic using 10m BPX-70 (A) and SLB-IL111 (B) columns.

Detection and quantification limits values indicated similar performances of BPX-70 and SLB-IL111. The cyanopropyl siloxane column showed a LOD and LOQ of 0.19 and 0.63 ppm, while the ionic liquid column of 0.18 and 0.60 ppm.

4. Conclusion

In the current investigation two short highly polar capillary columns were tested for routine FA analysis in butter by fast GC: the 10m BPX-70 cyanopropyl siloxane phase coated column, and the 15m SLB-IL111 ionic liquid phase coated column. For each column, the effect of fast temperature programming on analysis performances as well as qualitative and quantitative evaluation of the analytical method developed was studied.

High temperature rates seemed to affect particularly the ionic liquid column than the cyanopropyl siloxane column, probably due to the higher polarity of the SLB-IL111 than the BPX-70. This result suggested to adopt lower temperature rates on SLB-IL111 than BPX-70 during fast GC method optimization, to increase peaks resolution and reduce the column bleeding. The elution order of FA resulted to be characteristic of the tested capillary columns, and this may be due to the to peculiar stationary phases, polarities, and finally the different temperature programs.

The analysis time of complex FA composition of butter fat on BPX-70 and SLB-IL111 required 6.39 min and 7.38 min, respectively. Both the columns showed acceptable resolution except for *cis* and *trans* C18:1 isomers that were partially resolved, while the *cis*-9, *trans*-11 C18:2 CLA and C18:3*n*3 were fully separated.

Overall the BPX70 capillary column showed the best analytical performances during the fast-GC method optimization and validation, and resulted more efficient than SLB-IL 111 for fast and routinely analysis of FA on butter fat. Finally, the experimental chromatographic conditions adapted for the analysis of butter fat, might be extended for the FA analyses on dairy fat.

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Chapter III

Application of fast gas-chromatography for a rapid screening of fatty acid composition in butter samples: a comparative study between experimental PUFA-enriched butters from the Parmigiano-Reggiano cheese area and some European commercial brands.

Application of fast gas-chromatography for a rapid screening of fatty acid composition in butter samples: a comparative study between experimental PUFA-enriched butters from the Parmigiano-Reggiano cheese area and some European commercial brands.

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Abstract

In this research study, fast gas-chromatography technique was used to investigate the fatty profile of butter samples. Particularly, the fatty acid composition of experimental butters produced in the Parmigiano-Reggiano cheese area from milking cows fed with the traditional diet supplemented with 300 g/d of extruded linseed were compared with some European commercial butters.

Compared to European commercial butters, experimental samples showed a lower value of saturated fraction replaced by a higher level of bioactive fatty acids (*n*3-PUFA, *n*6-PUFA, VA, RA). These FA contents probably may be related to the unsaturated-lipid supplementation to the diet of dairy cows. Therefore, this industrial research support the possibility for the Parmigiano-Reggiano cheese industry to obtain by-products that may be more beneficial to consumers and with greater added value respect to the industrial standards.

Key words: bioactive fatty acids, PUFA, butter, fast gas-chromatography

1. Introduction

Butter is the one of the most complex of all edible fats with a wide range of fatty acids (FAs) identified to date. The saturated fraction (SFA) account for the majority of total FAs, followed by mono- and poly-unsaturated fractions (MUFA and PUFA, respectively) (Elgersma et al., 2006). Milk FA composition has a number of effects on fat quality, including several aspects, such as physicochemical, organoleptic and nutritional properties of milk and dairy products (Ashes et al., 1997). In recent years, nutritional aspect of milk fat has received increasing importance both from the consumer and dairy-industry point of views, because bioactive FAs offer an potential commercial advantage to produce health-promoting functional dairy products (Gill 2002; Akalin et al., 2006; Talpur et al., 2008). Although there is evidence that dietary SFA contribute to heart disease, early studies suggest that individual FAs have markedly different effects (Givens 2008). Particularly, lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids have been associated with increased total and low-density lipoprotein (LDL) cholesterol levels in human plasma, whereas stearic acid (C18:0) has been shown to be have a neutral or beneficial effects on human health (Grummer 1991; Givens 2008; Cocchi et al., 2009; Chen et al., 2013).

The potentially adverse effects of individual trans-fatty acids (TFA) isomers on risk of atherosclerosis and coronary heart disease (CHD) have been also reported by several authors (Mensink and Katan 1993; Nestel et al., 1992; Williams 2000; Mensink et al., 2003). Nevertheless, vaccenic acid (*trans*-11 C18:1, VA) has an important role as a precursor of the rumenic acid (*cis*-9, *trans*-11 C18:2, RA) that is the most relevant isomer of conjugated linoleic acid (CLA) which exhibits positive biological effects on human health. Therefore, the deleterious effects on cardiovascular diseases and cancerogenic properties of TFA are controversial discussed (Chilliard and Ferlay 2004; Hodge et al., 2004; King at al., 2005).

In contrast, milk fat provide biologically active FAs, such as butyric acid (C4:0), CLA, *cis*-MUFA, *n*-3 PUFA, *n*-6 PUFA, with multiple vital functions because they are involved in several inter- and intra-cellular metabolic process (Parodi 2006). Numerous studies confirm the beneficial effects on human health of specific FAs related to prevention of many chronic diseases (Zock 1995; Watkins et al., 1999; Uauy et al., 2000; Williams 2000; Bising et al., 2007).

These findings underline the interest of modulating the milk FA composition. In fact, one of the objective of the milk industry is to improve the nutritional quality of milk fat by reducing SFA, and in particular the MC-SFA (C12 – C16) level, in favour to both *cis*-MUFA and PUFA (Contarini et al., 1996; Williams 2000; Elgersma et al., 2006; Givens 2008). These changes can be occur mostly by feeding strategies contemplating the supplementation of unsaturated fat source (Chilliard and Ferlay 2004; Egger et al., 2007; Talpur et al., 2008; Puppel et al., 2012).

This industrial investigation aimed to determine by fast gas-chromatography the FA profile of experimental butters produced in the Parmigiano-Reggiano cheese area, in response to extruded linseed supplementation into the traditional diet of milking cows, based on the use of concentrate, hay and fresh forage, permitted by the Parmigiano-Reggiano cheese Consorce (CFPR), and to compare the ameliorating effect with some European commercial butters.

2. Materials and Methods

2.1 Samples

A total of 54 butter samples purchase at local markets in Europe were analyzed in this study. The set of samples include 12 experimental butters produced at a local dairy farm in the Parmigiano-Reggiano cheese Consorce (CFPR) from milk creams obtained by natural creaming of milk from dairy cows fed a traditional diet supplemented with 300 g/d of extruded linseed, 21 Italian and 21 non-Italian butters. About 50 g of each butter was placed in 100 mL plastic screw cap containers, and stored at - 40° C until fatty acid analysis.

2.2 Sample preparation and fast gas-chromatography analysis

The FA composition of butter samples was determined after extraction of total lipids in duplicate in accordance with Hara and Radin (Hara and Radin 1978). About 20 mg of fat was methylated with ethereal diazomethane (Fisier 1967) and then the FA transesterified according to Christie (Christie 1982). The methyl undecanoate (C11:0; 1 mg/mL) was used as internal standard (IS).

The fatty acid methyl esters (FAME) were analyzed on a GC 2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a split–splitless injector, an AOC-20i autoinjector and a flame ionization detector (FID). All data were collected by GC Solution software (Shimadzu, Kyoto, Japan). The separation of the FAME was performed with a BPX70 fused silica capillary column (10m, ID 0.10 mm, 0.10 µm f.t.), coated with 70% cyanopropil polysilphenylenesiloxane film from SGE (VWR International, Fontenay-sous-Bois, France). The injector and detector ports were set at 250 °C. The oven temperature programme was initially set at 50 °C for 0,2 min, then increased at a rate of 120 °C min^{-1} to 175 °C, where it remained for 2 min, then increased at a rate of 20 °C min^{-1} to 220 °C, and then increased at a rate of 50 °C min^{-1} to 250 °C where it remained for the last 0,3 min. Hydrogen was used as a carrier gas at a flow rate of 0.8 mL/min, and the split ratio was set at 1:100.

The analytical method was subjected to validation and specific chromatographic parameters, such as accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ) and precision, were calculated.

Peaks were routinely identified by comparison of the retention times with fatty acid methyl ester GLC 463, CLA standard mixtures UC-59M from Nu-Check (Elysian, MN, USA), and published isomeric data (Kramer et al. 2004). FA were quantified by comparing the peak area of each compound with that of IS, and the composition was expressed as weight percentage of total FAME (mg/100mg of FAME).

2.3 Statistical analysis

Statistical analysis was performed using the STATISTICA 6 software package (StatSoft Inc., Tulsa, OK, USA). Significant differences (at *p*-level < 0.05) between means of individual FA and principal groups of FA data of experimental, Italian and non Italian butters were explored using an one-way analysis of variance (ANOVA) followed by Fisher's least significance difference (LSD) post-hoc test.

3. Results and discussion

Fast gas-chromatography combined with flame ionization detector (fast GC-FID) was applied to characterize the FA profile of butter samples. The analysis of each sample was conducted in less than 7 min. A short highly polar capillary column (10m BPX-70, 70% Cyanopropyl Polysilphenylene-siloxane stationary phase) was selected due to the high-performances for the separation of butter FAME, exhibited during the stage of optimization and validation of analytical method (*Experimental Section, Chapter II*). A summary results are shown in the ***Table 1.***

Analytical Parameters	**10m BPX-70**	
Accuracy	Average recovery (%)	100.07
	Standard deviation	1.89
	RSD	1.89
Linearity	$R^2 = 1$	
LOD	0.19 ppm	
LOQ	0.63 ppm	
Precision	Intra-day repeatability (RSD%)	6.24
	Inter-day repeatability (RSD%)	8.99

Table 1. Chromatographic performances of 10m BPX70 column**.**

The FA composition of experimental, Italian and foreign butters was reported in ***Table 2*** and ***Table 3***. A total of 46 FAs were identified and quantified: 18 SFA, 14 MUFA and 14 PUFA. Significant differences ($p < 0.05$) in saturated and unsaturated fractions among the experimental and commercial butter samples were found.

Individual fatty acids	Experimental			Italian			Foreign		
	mean		CV	mean		CV	mean		CV
C4:0	2.49	±	0.12[a]	2.51	±	0.09[a]	2.69	±	0.19[b]
C6:0	1.73	±	0.10[a]	1.74	±	0.07[a,b]	1.80	±	0.11[a]
C7:0	*nd*	±	*nd*	*nd*	±	*nd*	0.04	±	0.02
C8:0	1.09	±	0.10[a]	1.13	±	0.05[a]	1.14	±	0.12[a]
C9:0	0.04	±	0.03[a]	*nd*	±	*nd*	0.03	±	0.01[a]
C10:0	2.61	±	0.24[a]	2.74	±	0.13[a]	2.70	±	0.36[a]
C12:0	2.86	±	0.55[a]	3.30	±	0.22[b]	3.28	±	0.47[b]
C13:0	0.11	±	0.04[a]	0.09	±	0.01[b]	0.08	±	0.02[b]
C14:0	10.90	±	0.30[a]	11.34	±	0.40[b]	11.10	±	0.83[a,b]
C15:0	1.14	±	0.05[a]	1.15	±	0.06[a]	1.13	±	0.09[a]
C16:0	29.02	±	0.79[a]	31.93	±	1.02[b]	32.02	±	2.32[b]
C17:0	0.58	±	0.04[a]	0.59	±	0.05[a]	0.58	±	0.07[a]
C18:0	11.07	±	0.83[a]	10.84	±	0.58[a]	10.43	±	1.31[a]
C19:0	0.24	±	0.08[a]	0.25	±	0.02[a]	0.24	±	0.03[a]
C20:0	0.16	±	0.01[a]	0.19	±	0.08[a]	0.18	±	0.05[a]
C22:0	0.08	±	0.02[a]	0.08	±	0.02[a]	0.10	±	0.04[b]
C24:0	0.04	±	0.04[a]	0.04	±	0.01[a]	0.05	±	0.02[a]
C12:1 c	0.06	±	0.01[a]	0.09	±	0.01[b]	0.07	±	0.02[a]
C14:1	1.14	±	0.19[a]	1.15	±	0.09[a]	1.14	±	0.13[a]
C15:1c	0.25	±	0.01[a]	0.26	±	0.03[b]	0.25	±	0.04[a]
C16:1 c	1.36	±	0.39[a]	0.86	±	0.52[b]	1.24	±	0.52[a]
C16:1 t	0.42	±	0.23[a]	0.14	±	0.02[b]	0.19	±	0.06[b]
C17:1 c	0.24	±	0.01[a]	0.24	±	0.03[a]	0.27	±	0.05[b]
C18:1 t10	0.69	±	0.17[a]	0.53	±	0.08[b]	0.49	±	0.09[b]
C18:1 t11	2.01	±	0.53[a]	1.74	±	0.23[a]	1.85	±	0.77[a]
C18:1 c9-t12	22.43	±	0.80[a]	21.20	±	0.78[b]	21.14	±	1.26[b]
C18:1 c11	0.73	±	0.16[a]	0.56	±	0.06[b]	0.58	±	0.11[b]
C18:1 c12	0.48	±	0.13[a]	0.35	±	0.08[b]	0.30	±	0.12[b]
C18:2 t9t12	0.36	±	0.03[a]	0.09	±	0.01[b]	0.11	±	0.02[b]
C18:1 c13-c14	0.33	±	0.19[a]	0.27	±	0.03[a]	0.31	±	0.06 [a]
C18:1 c14	0.23	±	0.11[a]	0.10	±	0.02 [a]	0.13	±	0.05 [a]
C18:1 c15-c16	0.15	±	0.09[a]	0.32	±	0.03[b]	0.37	±	0.07[c]
C18:2 *n*6	2.82	±	0.37[a]	2.28	±	0.34[b]	1.97	±	0.50[c]
C18:2 c9, t11 (RA)	0.78	±	0.10[a]	0.53	±	0.15[b]	0.61	±	0.21[b]
C20:1 c5,8,11/C18:2 c,c (CLA isomers)	0.20	±	0.02[a]	0.21	±	0.03[a]	0.25	±	0.14[a]
C18:3 *n*6	0.17	±	0.19[a]	0.18	±	0.04[a]	0.18	±	0.12[a]
C18:3 *n*3	0.76	±	0.10[a]	0.52	±	0.11[b]	0.52	±	0.23[b]
C20:2 c11 c14	0.03	±	0.04[a]	*nd*	±	*nd*	0.02	±	0.01[a]
C20:3 *n*6	0.16	±	0.25[a]	0.12	±	0.02[a]	0.09	±	0.03[a]
C20:4 *n*6 (AA)	0.12	±	0.03[a]	0.15	±	0.02[b]	0.11	±	0.03[a]
C20:5 *n*3 (EPA)	0.07	±	0.03[a]	0.09	±	0.02[a]	0.08	±	0.04[a]
C22:2	0.05	±	0.02[a]	0.02	±	0.02[b]	0.07	±	0.02[a]
C22:3/22:4	0.02	±	0.02[a]	0.04	±	0.01[a]	0.03	±	0.01[a]
C22:5 *n*3 (DPA)	0.05	±	0.04	*nd*	±	*nd*	*nd*	±	*nd*
C24:1	0.05	±	0.04[a]	0.06	±	0.02[b]	0.07	±	0.02[b]
C22:6 *n*3 (DHA)	0.01	±	0.02	*nd*	±	*nd*	*nd*	±	*nd*

Table 2. Individual FA composition (expressed in mg/100mg FAME) of experimental, Italian and foreign butter samples. Mean ± CV with the same letter are not significantly different at the 0.05 probability level.

Classes of fatty acids	Experimental			Italian			Foreign		
	mean		CV	mean		CV	mean		CV
SFA	64.13	±	1.32[a]	67.92	±	1.21[b]	67.57	±	2.43[b]
SC-SFA (C4-C10)	7.94	±	0.53[a]	8.12	±	0.30[a,b]	8.37	±	0.56[b]
MC-SFA (C12-C16)	44.04	±	0.96[a]	47.80	±	1.48[b]	47.62	±	3.15[b]
LC-SFA (C>17)	12.15	±	0.92[a]	11.99	±	0.62[a]	11.58	±	1.41[a]
MUFA	30.32	±	1.92[a]	27.59	±	0.89[b]	28.08	±	1.75[b]
PUFA	5.42	±	0.81[a]	4.50	±	0.49[b]	4.35	±	0.76[b]
TFA	3.33	±	0.43[a]	2.77	±	0.28[b]	2.98	±	0.87[a,b]
Total CLA isomers	0.95	±	0.17[a]	0.74	±	0.15[b]	0.86	±	0.24[a,b]
C18:2 c9, t11 (RA)	0.78	±	0.11[a]	0.53	±	0.15[b]	0.61	±	0.21[b]
***n*3-PUFA**	0.87	±	0.14[a]	0.61	±	0.12[b]	0.60	±	0.23[b]
***n*6-PUFA**	3.28	±	0.55[a]	2.73	±	0.38[b]	2.36	±	0.58[c]
***n*3-PUFA/*n*6-PUFA**	3.77	±	0.41[a]	4.65	±	1.21[a]	4.44	±	1.93[a]
SFA/UFA	1.80	±	0.11[a]	2.12	±	0.12[b]	2.10	±	0.22[b]

Table 3. Classes of FAs (expressed in mg/100mg FAME) of experimental, Italian and foreign butter samples. Mean ± CV with the same letter are not significantly different at the 0.05 probability level.

Compared with Italian and foreign butters, experimental sample showed the lowest content of total SFA fraction. Specifically the MC-SFA (C12-C16) level, that are associated with an increase of LDL-cholesterol concentration and therefore leading to a high risk of cardiovascular disease (CVD), was significantly lower ($p < 0.05$) in experimental butters than in commercial butters (-7.9 % and -7.5% respect to Italian and foreign butters, respectively), whereas no significant differences were found between Italian and Foreign butter samples.

The unsaturated fatty acids (UFA) fraction, including MUFA and PUFA, was significantly higher in experimental butter than in commercial butters. The major differences were detected for the PUFA content between experimental vs. commercial butters (an increase of 20.5% and 24.5% compared to Italian and foreign butter, respectively). In turn, *n*3-PUFA and *n*6-PUFA were significantly higher in experimental butter than in commercial samples. In particular, the content of C18:2*n*6 (linoleic acid, LA) and C18:3*n*3 (alpha-linolenic acid, ALA), the main precursors of bioactive *n*6- and *n*3-long chain PUFA (LC-PUFA), were significantly higher in experimental butters than in commercial samples. Particularly, C22:5*n*3 (DPA) and C22:6*n*3 (DHA) were detected only in experimental samples (***Table 2***).

The experimental butter was characterized by a higher levels of VA, the predominant isomer of TFA in milk fat. This value probably may be related to the highest level of RA observed in experimental samples (+ 46.7% compared to Italian butters and + 28.4% compared to foreign butters). In fact, VA is produced naturally during the partial biohydrogenation of LA and ALA in the rumen and it is converted to RA in the mammary gland by Δ^9-desaturase enzyme (Griinari et al., 2000; Chilliard and Ferlay 2004).

The reduced MC-SFA content and the consequent enrichment of bioactive MUFA, PUFA and total CLA contents in experimental butters could be attributed to the inclusion of extruded linseed in the traditional diet of dairy cows. These changes in the FA composition affect not only the nutritional properties but also the texture characteristics of butter. Therefore, the spreadability index (*cis*-9 C18:1/C16:0) that is responsible for the linear decreases in the final melting temperature and solid fat content (Couvreur et al., 2006) was calculated for all butter samples as a measure of rheological quality. As expected, experimental butters showed a higher value of spreadability index respect to commercial butter samples. This outcome has also been observed by Hurtaud (Hurtaud et al., 2010) who reported that the FA profile of milk fat is strongly affected when the diet of dairy cows was added with extruded linseed.

4. Conclusion

In the present study, the FA composition of butter fat was obtained by fast GC-FID analysis with a reduction of GC run time and with a major chromatography efficiency to the traditional GC-FID application. From a point of view of the bioactive FA composition of butter samples, a lower value of MC-SFA with a simultaneous higher levels of MUFA, PUFA and total CLA were observed in experimental butters respect to those detected in European commercial butters. These findings seem to be related with the feeding strategy used in this industrial investigation, in order to improve the levels of bioactive compounds in experimental butter samples, leading to the production of dairy products that may be more beneficial to consumers. Although the changes in the FA composition of milk fat can be attributed to a number of factors (i.e. genetic factors, health status of mammary gland, stage of lactation, environment and season), the feeding manipulation is a more practical way to modulate the FA profile of milk and dairy products, especially when unsaturated fat source is added to the diet. Therefore, this study support the possibility for a sustainable production of value-added by-products from the Parmigiano-Reggiano cheese Consorce with a higher nutritional and rheological qualities than the industrial standards.

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Chapter IV

Influence of the cow's diet on bioactive lipid composition of milk creams obtained by natural creaming: application of HPLC-ELSD, HPLC-ESI-MS and GC-FID techniques.

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Influence of the cow's diet on bioactive lipid composition of milk creams obtained by natural creaming: application of HPLC-ELSD, HPLC-ESI-MS and GC-FID techniques.

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Abstract

Bioactive lipid components (*n*3-PUFA and *n*6-PUFA, CLA and phospholipids) were determined in cream samples obtained by natural creaming of milk that are by-products of some industrial cheese-making process. Milk was collected after the supplementation of dairy cows' diet with an unsaturated-fat source (extruded linseed and fresh forage).

The addition of an unsaturated fat source into the traditional diet of milking cows significantly ($p < 0.01$) decreased the mean SFA content and significantly ($p < 0.01$) increased the mean UFA content. In milk creams from the linseed group the MUFA and PUFA content significantly increased (+ 7.3% and + 36% respectively). The total phospholipid content of milk cream from cows fed with linseed diet was significantly ($p < 0.01$) higher than that of cream samples from the control group, with an increase of 11%.

Because of that, the inclusion of extruded linseed into to the traditional diet of dairy cows may be adopted to enhance the bioactive fatty acid and phospholipid composition of dairy products.

1. Introduction

Milk is a highly variable biological fluid that contains many nutrients useful for the nutritional and physiological needs of the young of the milk producing mammal. The chemical components of milk are located in several compartments or phases. In particular, lipids (3 - 5%) are secreted in the form of colloidal assemblies, called milk fat globules, suspended in the aqueous phase. Milk fat globules contain a non-polar core consisting of triglycerides (TGs, accounting for ~ 98% of total lipid content) coated with bipolar materials such as polar lipids, proteins, cholesterol, enzymes and other minor components surrounded by a bilayer membrane that is referred to as the milk fat globule membrane (MFGM). The structure and composition of MFGM is dependent on the mechanism of secretion (Lopez et al., 2011) from specialized regions of apical plasma membrane of mammary epithelial cells, and from endoplasmic reticulum (ER) and perhaps from other intracellular compartments (Jensen and Newburg 1995). Various factors, such as genetic factor, environmental condition, animal factor and diet, number and stage of lactation, processing (mainly homogenisation) can alter the chemical composition of MFGM (Britten et al., 2008; Graves, et al., 2007; Lopez et al., 2008). The ruminant's diet has a crucial role in milk lipid composition with important consequences on technological proprieties, chemical – physical, and organoleptic characteristics of milk and dairy products. As a result many of the milk lipid components, including fatty acids (FAs), TGs and phospholipids (PLs), that have functional properties for humans may be manipulated by feeding strategies. Several studies have reported that the inclusion of an unsaturated fat source into the diet of dairy cows affects the profile and percentage of bioactive polyunsaturated fatty acids (PUFA) e.g. conjugated linoleic acid isomers (CLA) and essential FAs (*n*3-PUFA and *n*6-PUFA) (Mele et al., 2011; Secchiari et al., 2003) with a simultaneous decrease of medium-chain saturated fatty acids (MC-SFA), which have previously been associated with an increase of low-density lipoprotein (LDL) cholesterol resulting in a higher risk of cardiovascular diseases (Elgersma et al., 2006).

Since the structure and composition of TGs is usually defined in terms of the kinds and amounts of FA present in the milk lipid fraction (more than 400 FAs) and since the distribution of FAs in milk TG is not random (Bugeat et al., 2011), the identification of TG species is a difficult although it is well-known that the structure of TGs influences the action of lipolytic enzymes and consequently their absorption (Jensen 2002), the melting and crystallization behaviour and the rheological properties of milk fat, less data is available regarding the effects of the increase in unsaturated fatty acids (UFA) on the TG content in enriched-fat dairy products than those reported for the total FA composition.

Recent studies have highlighted that polar lipids and single PLs exert positive biological activities on human health (Contarini and Povolo 2013; Rombaut et al., 2005; Sanchez-Jaunes et al., 2009) and emulsification role of the membrane in several food system by virtue of both lipophilic and hydrophilic properties (Donato et al., 2011; Thompson and Singh 2013). However, little information is available in the literature regarding the FA composition and specific structure of PLs classes. The PLs of the MFGM consists mainly of glycerolphospholipid and sphingolipids. Glycerolphospholipids (approximately 1% of total lipids) including phosphatidylethanolamine (PE, 19.8 - 42%), phosphatidylinositol (PI, 0.6 - 13.6%), phosphatidylserine (PS, 1.9 - 19%) phosphatidylcholine (PC, 19.2 - 37.3%) and sphingolipids are dominated by sphingomyelin (SM) (Lopez et al., 2011). These are amphiphilic molecules with a hydrophobic tail and a hydrophilic head group (Dewettinck et al., 2008). The glycerolphospholipids contain a phosphate residue onto which different organic groups may be linked. Sphingolipids can contain a similar organophosphate group or a mono- or disaccharide (glycosphingolipids) (Rombaut et al., 2005). Considering the fatty acids (FAs) distribution of glycerolphospholipids, recent investigations reported that milk glycerophospholipids are rich in unsaturated FAs (C18:1, C18:2, C18:3) which have low melting temperatures and play an essential role in the fluidity of the MFGM. Conversely, sphingolipids (mainly SM) contains long and very long saturated FA chain (C16:0, C18:0, C22:0, C23:0, C24:0) with high melting temperatures (Bitman and Wood 1990; Fong et al., 2007).

The aim of this study was to expand the current knowledge on the bioactive components of total lipid and MFGM of milk creams obtained by the supplementation of dairy cows' diet with an unsaturated-fat source (extruded linseed and fresh forage). The growing interest in bioactive lipids, as food ingredients and in their healthy properties makes it important to study milk's constituents in milk creams destined to industrial butter production.

The PLs profile was determined using high performance liquid chromatography coupled with evaporative light scattering detector (HPLC-ELSD). The specific FA composition of PLs was determined using gas chromatography combined with flame ionization detector (GC-FID) analysis with a previous preparative thin layer chromatography (TLC) and the molecular species of each individual PL class by HPLC combined with mass spectrometry (HPLC-MS). This work also investigates the total FA and TG composition of milk creams by GC-FID analysis.

2. Materials and Methods

2.1 Samples

A total of 24 cow's milk cream samples were analysed in this study. The milk samples were collected from January to May 2013, on a dairy farm in Italy. Before and during the study, two

groups of 300 multiparous Holstein cows were fed based on the traditional diet, based on the use of fresh forage, hay and pellets. Milk samples were collected from 1 to 5 week during the *dry feeding period* consisting of hay, concentrate and from 6 to 12 week during the *fresh forage period* consisting of hay, concentrate and fresh forage. While the control group was fed only with the traditional diet, 400 g/d of extruded linseed was integrated to the traditional diet of the linseed group. Throughout the entire experimental period the two groups of milking cows were fed and housed separately.

Milk cream samples from each group were obtained by natural creaming of milk for 12 hours in open steel tubs during the early phase of the cheese-making process and destined to butter production. Finally, milk cream samples were collected and stored at our laboratories at - 20°C until fat extraction.

2.2 Lipid Extraction

A modification of the Folch method described by Boselli et al. (Boselli et al., 2001) was used to extract total lipids of the milk cream samples. Firstly, 200 mL of chloroform/methanol 1/1 (v/v) was added in milk cream sample (5 g) and each sample was homogenized for 3 min using an ultrasound bath at room temperature and all samples were maintained at 60°C for 20 min. Secondly, 150 mL of chloroform was added and the mixture was homogenized again for 2 min. The homogenate was centrifuged at 2000 rpm for 3 min. Successively, in order to obtain a better separation between organic matter and the water phase, 75 ml of potassium chloride (c = 1 mol/L) was added, shaken for about 1 min, and left overnight at 4 °C. The organic phase was separated from the water phase by a separatory funnel and successively, filtered over anhydrous sodium sulphate. Finally, the organic solvent of lipid extract was evaporated under using a vacuum evaporator (bath temperature: 40 °C) and the lipid fraction was dried under nitrogen. The final dry residue was weighed and stored in 5 ml of *n*-hexane/*i*-propanol 4/1 (v/v) at -18 °C until further analyses. Each extraction was performed two times.

2.3 Phospholipids Determination by HPC-ELSD

Prior to analysis of the PL profile of milk cream samples, lipid extracts (400 mg) were purified using small solid-phase extraction (SPE) cartridges according to Avalli and Contarini (Avalli and Contarini 2005). The identification and quantification of the PLs classes was performed using high-performance liquid chromatography (HPLC; HP 1200 Series, Agilent Technologies, Palo Alto, California, USA) combined with a evaporative light scattering detector (ELSD; PL-ELS1000, Polymer laboratories, Church Stretton, Shropshire, UK). The dried and filtered compressed nitrogen

was used as the nebulizing gas at a flow rate of 1.0 l/min and the temperature was set at 50 °C. The evaporating temperature was 85 °C. A silica column, 150 x 3 mm with 3µm particle diameter (Phenomenex, Torrance, CA, USA) was used. The column was maintained at room temperature (25 °C). The method used for separation of the PLs was the same used by Verardo et al. (Verardo et al., 2013a) with some modifications. The HPLC system was software controlled by Agilent ChemStation (Agilent Technologies, Santa Clara, CA, USA) whilst chromatogram registration and data processing were assessed by ClarityLite (ver. 2.4.0.190, DataApex, Praha, The Czech Republic). Identification of PLs was carried out by comparison of retention time of pure standards. To obtain the quantitative evaluation an external calibration was used. A serial diluted solution (from 1 to 500 µg ml^{-1}) of PE, PI, PS, PC and SM was injected in triplicate, and the average data were subjected to linear regression analysis (y = ax+b). The sum of glycerolphospholipids (PE, PI, PS and PC) and sphingomyelin (SM) concentration was regarded as total PL concentration. The results were expressed in mg/g fat.

2.4 Determination of phospholipids molecular species by HPLC-MS

HPLC-MS analyses were performed according to Montealegre et al. (Monteleagre at al., 2012). In briefly, purified lipid extract were analysed using an HP 1100 Series from Agilent Technologies (Palo Alto, California, USA) including a degasser, a binary pump delivery system and an automatic liquid sampler coupled to a mass spectrometer (mod. G1946A) from Agilent Technologies (Palo Alto, California, USA) that operated in negative and positive atmospheric pressure ionisation-electrospray source (API-ES) mode. The chromatographic conditions employed for the HPLC-ELSD protocol were also used for the HPLC-MS analysis. MS analysis of PLs was performed using ESI in negative and positive ion mode. The instrumental settings were as follows: drying gas flow (nitrogen): 9 L min^{-1}; nebulizer pressure: 50 psi; gas drying temperature: 350 °C; capillary voltage: 5000 V; fragmentor voltage: 100 mV; mass scan range: 500 – 1000 m/z.

2.5 Isolation of phospholipids by thin layer chromatography (TLC) and fast gas chromatographic determination of phospholipids fatty acids and total fatty acid profile of milk creams.

The total FAs of milk cream samples and the FA of PL analysis were determined by Verardo et al. (Verardo et al., 2013a). The fatty acid methyl esters (FAME) were measure on a GC-2010 Plus gas chromatograph from Shimadzu (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector (FID), a programmed temperature injector and a fused silica capillary column BPX70 (10 m x 0.1 mm i.d., 0.2 µm f.t.) from SGE Analytical Science (Melbourne, Australia),

according to the method of Verardo et al. (Verardo et al., 2013a). Peak identification was accomplished by comparing peak retention times with GLC-463 from Nu-Check (Elysian, MN, USA) and FAME 189-19 standard mixtures from Sigma-Aldrich Chemicals (St. Louis, MO, USA). FA were quantified by comparing the peak area of each compound with that of methyl undecanoate (C11:0; 1 mg/mL) that was used as an internal standard (IS), and the composition was expressed as weight percentage of total FAME (mg/100 mg of FAME).

2.6 Determination triglyceride composition of milk creams by capillary gas-chromatography analysis.

TG analysis was conducted on the lipid phase previously extracted for the PLs and FA characterization. Briefly, 1.0 µL of organic solution (10 mg/mL of milk cream lipid in hexane) was injected in duplicate into a GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector (FID) and an AOC-20s auto sampler (Shimadzu Corporation, Kyoto, Japan). TGs separation was performed with a Rtx-65 TG fused silica capillary column (30 m, 0.25 mm i.d., 0.10 µm f.t.) with 35 % dimethyl, 65 % diphenyl polysiloxane from Restek (Chromatography Products, Superchrom Milano, Italy). The initial oven temperature of 240 °C was raised to 370 °C at a rate of 2.5 °C/min. The injector and detector temperatures were set at 360 °C. The air and helium flow rates were of 400 and 40 mL/min, respectively. Hydrogen was used as a carrier gas at a flow rate of 1.75 mL/min. The split ratio was set at 1:30. The identification of TG was carried out by comparison with the retention time of a pure standard (trilinolein, triolein, tristearin, tripalmitin, trimyristin, trilaurin, tricaprin and tricaprylin) and quantification was achieved by use of an internal standard (Tricaprylin, 0.25 mg/mL). The methodology for the analysis was based on the separation of classes of milk fat TGs according to the total number of carbon atoms (CN; sum of the three FA) (Fontecha et al., 2005; Povolo et al., 2008).

2.7 Statistical Analysis

Resulting data from PL composition and their fatty acid profiles, FA and TG composition of milk creams were analyzed with one-way analysis of variance (ANOVA) using the STATISTICA 6 software package (StatSoft Inc., Tulsa, OK, USA). Differences between the treatment means were compared at the 1% and the 5% level of significance ($p < 0.01$ and $p < 0.05$) using Fisher's least significance difference (LSD) test. Factorial ANOVA univariate analysis was employed to evaluate the effects of feeding (dry feeding period, fresh forage feeding period) and linseed supplementation (control group, linseed group).

3. Results and discussion

3.1 Phospholipids content of milk creams

In order to determine the PLs in the cream samples by HPLC-MS, the method used in a previous research Verardo et al. (Verardo et al., 2013a) was modified to avoid the persistent memory effect of triethylamine (TEA) (Rutters et al., 2000). Because of that, another chromatographic method that was developed for the determination of PLs in olive oil Verardo et al. (Verardo et al., 2013b) that use formic acid buffer with ammonium instead TEA was considered. The first step was to evaluate the possibility of analysing the cream PL extracts without previous purification. Due to the matrix effect, the results confirmed that a purification by SPE was necessary when TEA was not used (data not shown). In accordance with Avalli and Contarini (Avalli and Contarini 2005), the silica SPE columns were used for the PL purification.

Milk cream samples from control and linseed group were analyzed on PL content and the distribution of the individual PL species (***Table 1***).

Concentration of phospholipids (mg/g fat)								**% of phospholipids**	
Phospholipids	**Milk cream**							**Milk cream**	
	Control group			**Linseed group**			***p value***	**Control group**	**Linseed group**
	mean		**CV**	**mean**		**CV**			
PE	1.00	±	0.17	1.14	±	0.17	**	28.7	29.7
PI	0.14	±	0.02	0.15	±	0.02	NS	4.1	3.9
PS	0.13	±	0.02	0.13	±	0.02	NS	3.6	3.4
PC	1.44	±	0.18	1.63	±	0.23	**	41.4	42.5
SM	0.77	±	0.10	0.82	±	0.10	**	22.2	21.4
sum PLs	3.48	±	0.41	3.84	±	0.40	**		

Table 1. Concentration of phospholipids (mean ± CV) in milk creams obtained from cows fed control and linseed diet and relative proportion of each classes of phospholipids. Results of the analysis of variance by Fisher's least significance difference (LSD) test. (**) $p < 0.01$; (NS) not significant.

In agreement with other author's observation (Lamothe et al., 2008; Lopez et al., 2008; Rombaut, et al., 2005; Rombaut et al., 2006) the total amount of PL (glycerolphospholips + SM) content ranged from 0.23 to 0.45 % (w/w). According to the literature (Contarini and Povolo, 2013; El Loly, 2011) five PLs were detected in the analyzed samples. In this study, the total PL content of milk cream from cows fed with linseed diet was significantly ($p < 0.01$) higher than that of cream samples from the control group, with an increase of 11%. In fact, total PL content in cream from the linseed group was 3.8 mg/g of fat in contrast to the control group sample that have a total PL content of 3.5 mg/g. This increase agrees with the results of Lopez et al. (Lopez et al., 2008) that suggested that linseed

supplementation caused a reduction of milk fat globule size. This reduction in milk fat globule size significantly increased the surface area and, consequently, the PL-containing membrane per unit of milk fat. As reported for the total PLs, the amount of the major phospholipid PC was significantly ($p < 0.01$) affected by linseed supplemented diet; its concentration was significantly increased by 13.4%. The concentration of the other major PLs significantly increased in cream samples from linseed cow-fed, with the following percentages observed: PE + 14.2% ($p < 0.01$) and SM + 6.6% ($p < 0.01$). No statistical differences ($p < 0.05$) were reported for PE and PI that were determined in lower quantities (their sum was < 8% of total PLs). The content of choline-containing PLs (PC + SM) increased 10.8% and represented approximately 64 % of total PLs.

A univariate analysis of variance was used to assess the effects of the subject and between-subjects (***Table 2***). The p-level shows substantial differences among the PL fraction.

Phospholipids	**Feeding**	**Linseed**	**Feeding x linseed**
PE	0.000041 (**)	0.006374 (*)	0.490617 (NS)
PI	0.805285 (NS)	0.075559 (NS)	0.116243 (NS)
PS	0.805050 (NS)	0.295700 (NS)	0.281687 (NS)
PC	0.069970 (NS)	0.001962 (*)	0.433510 (NS)
SM	0.276759 (NS)	0.001976 (*)	0.240105 (NS)
sum PLs	0.001747 (*)	0.005687 (*)	0.423944 (NS)

Table 2. Effects of feeding and linseed-supplemented on phospholipids concentration of outcrop milk creams. (*) $p < 0.01$; (**) $p < 0.0001$; (NS) not significant.

In general, PE, PC, SM and total PLs were higher when linseed supplementation was applied. PE and total PL composition were also, influenced by the single effect of feeding e.g. higher levels of PE ($p < 0.0001$) and total PLs ($p < 0.01$) were observed when fresh forage was used. Any PL change were associated with the combined effects of feeding and linseed supplementation.

Figure 1 shows the trends of total and single PL during the study. ***Figure 1A*** reports the trends of total PLs during the twelve weeks of the study. Briefly, total PLs were statistically ($P < 0.05$) higher (excluding the fifth week) in the linseed supplemented group than in the control group. The lowest PL content were observed in the sixth week in the control and the linseed group; corresponding to the change of feeding from dry feed to fresh forage.

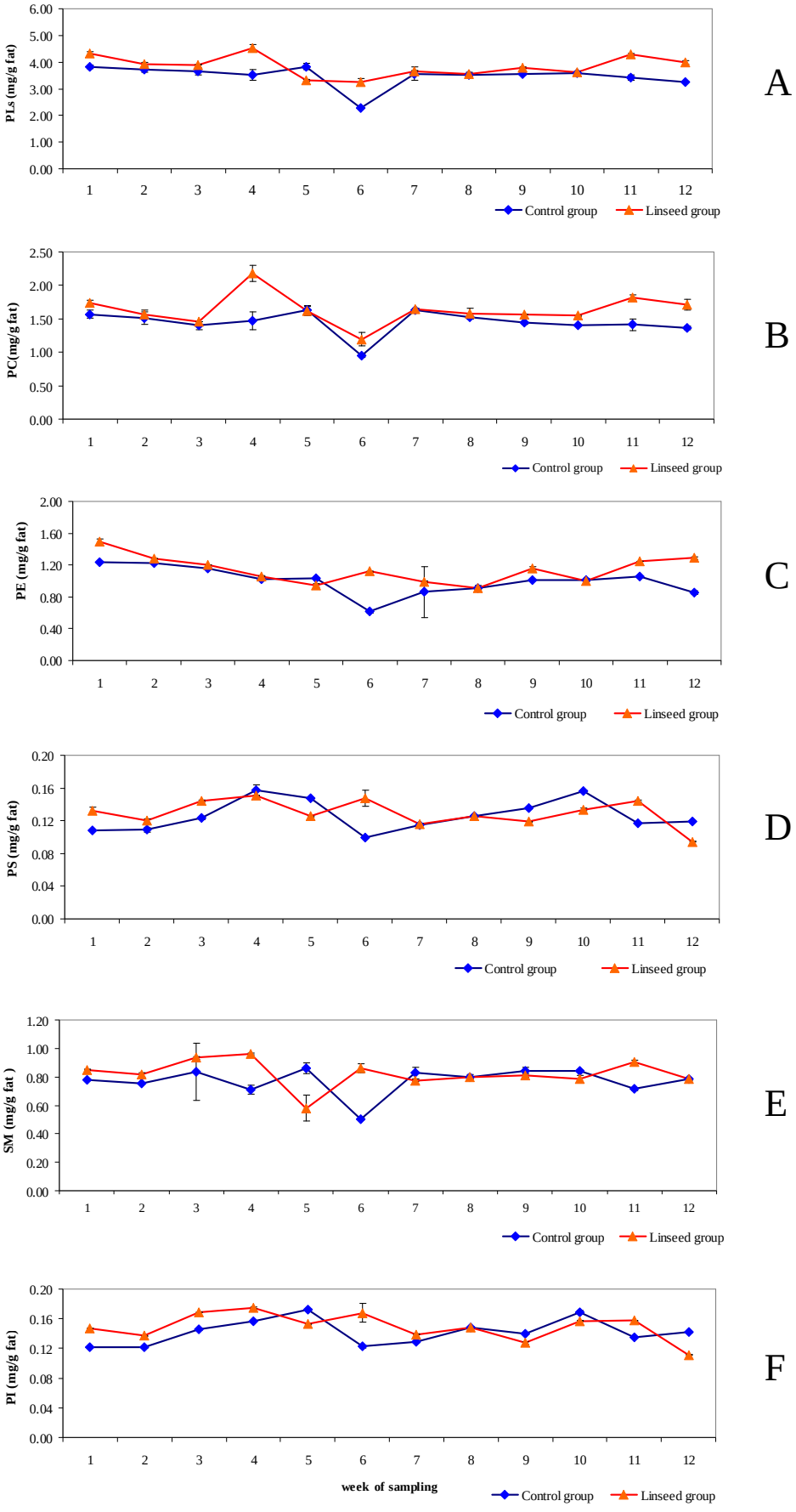

Figure 1. Distribution of phospholipids (expressed in mg/g fat) of milk creams during the experimental period

Figures 1B, 1C, 1D, 1E and ***1F*** show the trend of PC, PE, PS, SM and PI, respectively, during the study period. As reported for total PLs, PC trend (***Figure 1B***) confirmed that this phospholipid was contained in higher quantities in the linseed group with the highest concentration observed in the fourth week (during dry feeding); whilst the lowest concentration was observed for both experimental group during the sixth week coinciding with the start of the fresh forage period. PE, PS and PI behaved similarly (***Figures 1C, 1D and 1F***); with opposite effects observed between the control and the linseed group during the sixth week when cows changed their feeding from dry to fresh forage. Effectively in this week, PE, PS and PI content was lower for the control group and higher in the linseed group. ***Figure 1E*** shows the SM amounts; linseed group synthesized the highest quantities of SM at 4, 6 and 11 week while the control group reported the lowest concentration of SM during the same weeks.

3.2 Molecular species characterization and fatty acids distribution in phospholipids

The distribution of fatty acids of the individual PL species in milk cream samples determines the physical and rheological characteristics of the dairy products and in particular, the MFGM structure. Consequently, HPLC coupled with electrospray ionisation - mass spectrometry (HPLC-ESI-MS) confirmed the presence of the five principal PLs. Analysis in negative-ion mode of the PE, PI and PS and in positive-ion mode of PC and SM were used according to Verardo et al. (Verardo et al., 2013a) (***Table 3***).

Compared to the results of Verardo et al. (Verardo et al., 2013a) other molecular species were identified. The ESI-MS spectrum of PI in negative ion mode revealed an additional molecular species at m/z 833 corresponding to PI with C16:0/C18:2 or C16:1/C18:1. The ESI-MS spectrum of PS in negative ion mode revealed two additional molecular ion clusters, compared to Verardo et al. (Verardo et al., 2013a) at m/z 790 and 804. These molecular species correspond to C18:0/C18:0 or C14:0/C22:0 or C16:0/C20:0 and C14:0/C24:0 or C18:2/C20:5, respectively. Moreover, the ESI-MS spectrum of SM acquired in positive ion mode showed an additional peak at m/z 817 corresponding to C18:0/C24:0 or C20:0/C22:0 molecular species.

The main molecular species identified for PE was highly unsaturated and according to Sanchez-Jaunes et al. (Sanchez-Juanes et al., 2009) it was composed of molecular species with high levels of C18:1 and C18:2. PS and PI showed a lower degree of unsaturation compared to PE, and the principal FAs in their molecular structures were C18:0, C18:1 and C18:2. The high degree of unsaturation of these glycerophospholipids, that were localized in the inner layer of MFGM, may play an essential role in the fluidity of the membranes (Gallier et al., 2010; Lopez et al., 2011; Sanchez-Jaunes et al., 2009).

By contrast, according to Gallier et al. (Gallier et al., 2010) PC and SM, located in the outer layer of MFGM, are the highly saturated PLs present in cream samples. PC was more saturated compared to the other glycerolphospholipids and its content was lower in the sixth week of the study, when the feeding of the cows changed from dry to fresh forage. This suggests that, the supplementation of fresh forage caused a higher contribution to UFA and resulted in a lower synthesis of PC. As reported by several authors (Fong et al., 2007; Lopez et al., 2008), long (C > 17 carbon atoms) and very long saturated FA chains (C > 20 carbon atoms) were observed in the FA composition of SM. The presences of long and very long chain FAs and the high degree of saturation of SM fatty acids contribute to the structure of the MFGM by maintaining its rigidity (Gallier et al., 2010). In greater detail, this high degree of saturation gives SM the ability to form with cholesterol rigid domains in the cellular membrane, with higher melting points and a higher degree of packing in comparison to the glycerophospholipids (Dewettinck et al., 2008). Similarly to PC, the lowest amount of SM was reported in the control samples when the of fresh forage commenced.

Selected ion	m/z	Tentatively identification	Control group		Linseed group	
			min	max	min	max
PE						
[M-H]$^-$	688.5	C14:0/C18:1	3.99	4.16	3.53	4.18
	690.5	C16:0/C16:0; C14:0/C18:0	2.15	2.73	1.68	6.13
	714.5	C14:0/C20:0; C16:0/C18:2	8.74	9.92	9.56	10.23
	716.5	C16:0/C18:1; C14:0/C20:1	13.55	13.55	11.38	12.19
	738.5	C18:2/C18:2; C18:3/C18:1	3.07	4.14	3.23	4.52
	740.3	C18:2/C18:1; C18:0/C18:3	13.70	17.00	16.57	18.63
	742.7	C18:1/C18:1; C18:0/C18:2; C20:2/C16:0	26.84	31.68	27.57	29.32
	744.6	C18:0/C18:1	11.26	11.32	9.66	10.56
	764.5	C16:0/C22:5; C18:3/C20:2; C18:0/C20:5	4.01	5.44	4.55	4.82
	766.6	C18:2/C20:2	5.85	6.10	4.51	7.20
PI						
[M-H]$^-$	807.6	C14:0/C18:1	1.64	4.67	3.43	5.25
	809.3	C14:0/C18:0; C16:0/C16:0	0.71	0.97	1.97	2.55
	833.0	C16:0/C18:2	8.56	13.38	10.50	13.21
	835.3	C16:0/C18:1	5.06	7.20	4.36	5.12
	837.5	C16:0/C18:0; C14:0/C20:0	3.66	5.79	3.77	6.28
	861.6	C18:0/C18:2; C18:1/C18:1	25.37	29.81	25.07	26.28
	863.5	C18:0/C18:1	22.06	25.33	19.69	21.58
	885.6	C18:2/C20:2; C20:1/C18:3;	12.19	13.78	12.44	16.94
	887.7	C20:0/C18:3; C20:1/C18:2; C22:3/C16:0	8.63	11.20	8.04	13.51
PS						
[M-H]$^-$	760.5	C18:1/C16:0; C14:0/C20:1	1.32	3.12	5.03	5.55
	784.5	C18:1/C18:2; C14:0/C22:3; C18:0/C18:3	2.46	6.67	1.38	5.89
	786.5	C18:1/C18:1; C14:0/C22:2; C16:0/C20:2; C18:0/C18:2	21.11	28.90	13.58	34.03
	788.5	C18:1/C18:0	28.14	29.63	18.38	33.01
	790.0	C18:0/C18:0; C16:0/C20:0	10.28	12.82	3.10	32.59
	804.5	C14:0/C24:0; C18:2/C20:5	18.65	26.91	13.69	24.72
	810.5	C20:1/C18:3; C18:2/C20:2	1.11	2.56	0.84	1.27

	812.6	C20:0/C18:3; C18:1/C20:2; C20:1/C18:2	2.39	3.92	3.04	3.90
PC						
	678.5	C14:0/C14:0	7.83	9.70	7.57	8.79
	692.5	C14:0/C15:0	1.99	2.16	1.97	2.08
	706.5	C16:0/C14:0	20.94	22.21	19.85	20.75
	720.6	C16:0/C15:0; C14:0/C17:0	4.29	4.39	4.07	4.27
	730.5	C18:2/C14:0	1.00	1.18	1.23	1.39
	732.5	C18:1/C14:0; C16:0/C18:1	5.07	5.86	5.79	6.14
	734.6	C16:0/C16:0; C14:0/C18:0; C15:0/C17:0	14.63	16.29	15.27	15.41
$[M-H]^+$	746.6	C15:0/C18:0; C20:0/C14:1	1.72	1.87	1.83	1.89
	758.7	C16:0/C18:2; C14:0/C20:2	7.40	7.79	8.60	9.10
	774.7	C17:0/C18:1; C15:0/C20:1; C:20:0/C15:1	1.17	1.23	1.11	1.28
	782.2	C18:2/C18:2	3.04	3.08	3.14	3.39
	784.6	C18:1/C18:2	7.38	7.44	7.65	8.54
	786.7	C18:1/C18:1; C16:0/C20:2	12.29	12.54	11.98	12.54
	788.7	C18:1/C18:0; C16:0/C20:1	6.71	6.79	6.29	6.41
	806.7	C20:5/C18:1; C16:0/C18:1; C16:0/C22:6	0.98	1.03	0.81	0.95
SM						
	677.8	C14:0/C18:0; C16:0/C16:0; C15:0/C17:0	3.93	3.96	3.56	4.03
	689.6	C15:0/C18:1; C15:1/C18:0; C20:0/C14:1	3.90	4.66	3.74	4.15
	691.7	C17:0/C16:0; C15:0/C18:0	0.96	1.02	0.78	0.95
	703.6	C18:1/C16:0; C14:0/C20:1	19.70	21.86	18.58	20.26
	705.8	C17:0/C17:0; C18:0/C16:0; C14:0/C20:0; C22:6/C14:1	3.99	4.34	3.73	4.41
	717.7	C18:1/C17:0; C15:0/C20:1; C20:0/C15:1; C18:0/C17:0	1.69	1.73	1.61	1.70
	759.6	C14:0/C24:1; C18:0/C20:1; C20:0/C18:1	5.90	6.40	6.41	7.32
	761.8	C18:0/C20:0; C14:0/C24:0	4.39	4.41	4.52	5.10
$[M-H]^+$	773.8	C15:0/C24:1; C24:0/C15:1; C18:2/C22:6	7.98	8.57	7.62	8.33
	775.8	C15:0/C24:0; C22:6/C18:1	6.38	6.45	6.13	6.23
	787.7	C16:0/C24:1; C20:0/C20:1	9.75	10.14	9.37	11.14
	789.6	C16:0/C24:0	5.89	6.04	6.36	6.43
	801.7	C17:0/C24:1	8.40	8.99	5.84	7.86
	803.6	C17:0/C24:0; C20:1/C22:6	3.48	3.66	3.53	3.68
	813.8	C18:1/C24:1; C24:0/C18:2	1.62	1.64	1.71	1.89
	815.3	C18:1/C24:0; C18:0/C24:1	6.32	7.17	6.01	6.64
	817.7	C18:0/C24:0	2.22	2.42	2.14	8.24

Table 3. Distribution of molecular species of phospholipids in outcrop milk cream samples by HPLC-ESI-MS analysis (% area of total methyl esters) from cows fed control and linseed diet.

The total FAs content of PLs in the milk creams was analysed by fast-GC-FID and data were compared to elucidate the principal differences between milk creams from cows fed with the traditional diet (control group) and the extruded linseed-supplemented traditional diet (linseed group). Particularly, in the ***Table 4*** are reported the mean contents, maximum and minimum levels of FAs for both control and linseed groups, expressed in mg/100mg FAME. In this study, the short-chain fatty acid (C4-C10) were not detected in the composition of PLs in the milk cream samples. This is in agreement with those reported by other authors (Dewettinck et al., 2008; Sanchez-Jaunes et al., 2009) for the composition of PL fraction of milk.

Fatty Acids	Milk creams										
	Control group					Linseed group					
	min	max	mean		CV	min	max	mean		CV	*p value*
C14:0	5.78	6.91	6.41	±	0.45	4.65	5.97	5.31	±	0.54	**
C15:0	2.86	4.14	3.31	±	0.46	1.54	3.16	2.23	±	0.68	**
C16:0	20.96	24.71	22.76	±	1.60	18.48	20.68	19.63	±	0.83	**
C17:0	1.50	1.93	1.71	±	0.16	0.85	1.86	1.27	±	0.43	*
C18:0	9.24	11.14	10.16	±	0.78	7.65	16.23	11.60	±	3.75	NS
C20:0	0.45	0.70	0.57	±	0.10	0.23	0.74	0.41	±	0.20	NS
C24:0	0.77	1.08	0.95	±	0.12	0.50	1.07	0.79	±	0.25	NS
C14:1	0.26	0.63	0.43	±	0.12	0.24	0.47	0.34	±	0.09	NS
C15:1 c	0.39	1.36	0.88	±	0.33	0.21	1.66	0.98	±	0.51	NS
C18:1 t9	0.22	0.72	0.44	±	0.19	0.19	1.07	0.57	±	0.36	NS
C18:1 t10, t11, c9, t12, t15	19.25	22.32	20.70	±	1.13	20.25	25.27	22.59	±	2.48	NS
C18:1 c11	0.50	0.68	0.58	±	0.08	0.71	1.15	0.85	±	0.17	**
C18:1 c12	0.12	0.65	0.42	±	0.20	0.29	0.48	0.39	±	0.08	NS
C18:1 c13	0.26	0.52	0.42	±	0.10	0.35	0.55	0.45	±	0.07	NS
C18:1 c15, c16	0.15	0.56	0.43	±	0.16	0.20	0.36	0.28	±	0.08	*
C20:1	2.07	3.01	2.60	±	0.41	2.41	2.78	2.63	±	0.19	*
C24:1	*nd*	*nd*	*nd*			0.79	1.73	1.26	±	0.47	*
C18:2 t9,t12	0.71	1.09	0.87	±	0.14	0.37	0.95	0.67	±	0.24	NS
C18:2 *n*6	2.06	2.48	2.29	±	0.18	4.21	5.02	4.56	±	0.35	**
C18:2 (CLA) c9,t11	0.24	0.41	0.30	±	0.06	0.18	2.63	1.24	±	0.97	*
C18:3 *n*6	2.17	3.95	2.62	±	0.70	1.92	3.19	2.39	±	0.47	NS
C20:2 c11, c14	1.84	2.41	2.14	±	0.23	2.12	4.75	3.35	±	1.31	NS
C20:5 *n*3 (EPA)	0.98	1.60	1.29	±	0.28	0.00	2.29	1.27	±	0.88	NS
C22:2	13.73	15.69	15.12	±	0.77	6.50	17.77	12.02	±	5.60	NS
C22:3	1.42	1.96	1.60	±	0.24	1.10	5.10	3.16	±	1.89	NS
C22:6 *n*3 (DHA)	0.87	1.08	0.97	±	0.07	0.86	2.76	1.85	±	0.97	*
SFA	42.63	49.13	45.88	±	2.70	37.64	45.30	41.24	±	3.03	*
UFA	50.87	57.37	54.12	±	2.70	54.70	62.36	58.76	±	3.03	*
MUFA	24.94	29.25	26.92	±	1.57	26.50	29.84	28.25	±	1.09	NS
PUFA	25.77	29.27	27.20	±	1.37	28.20	33.96	30.51	±	2.21	*
TFA	0.22	0.72	0.44	±	0.19	0.19	1.07	0.57	±	0.36	NS
CLA	0.24	0.41	0.30	±	0.06	0.18	2.63	1.24	±	0.97	*
***n*3-PUFA**	1.91	2.68	2.26	±	0.34	2.01	3.85	3.13	±	0.69	*
***n*6-PUFA**	4.23	6.40	4.91	±	0.80	6.23	7.95	6.95	±	0.57	**
***n*6 PUFA /*n*3 PUFA**	1.76	2.67	2.19	±	0.31	1.81	3.31	2.32	±	0.55	NS
SFA/UFA	1.46	1.89	1.69	±	0.18	1.11	1.61	1.36	±	0.19	*
SC-SFA (C4-C10)	*nd*										
MC-SFA (C12-C16)	31.19	35.97	33.80	±	1.9	27.52	29.99	28.49	±	0.94	**
LC-SFA (>C17)	64.03	68.81	66.20	±	1.9	70.01	72.48	71.51	±	0.94	**
SFA	42.63	49.13	45.88	±	2.70	37.64	45.30	41.24	±	3.03	*

Table 4. Fatty acid composition of phospholipids isolated by milk creams (expressed in mg FA/100mg FAME). Results of the analysis of variance by Fisher's least significance difference (LSD) test.

(*) $p<0.05$; (**) $p<0.01$; (NS) not significant.

PL composition of milk creams from the linseed group showed a lower ($p < 0.01$) content of MC-FA (C12-C16) and a higher ($p < 0.01$) content of long and very long chain FAs (C > 17 carbon

atoms) than those reported for the PL composition of milk creams from the control group. Moreover, we found that the content of MUFA, PUFA, and minor bioactive lipids, such as CLA, *n*3-PUFA and *n*6-PUFA were greater in the PL composition of milk cream from the linseed group. Saturated C14, C15, C16, C17 FAs and C18:1 c15, c16 FA were detected in significantly ($p < 0.05$) lower content in PLs of creams from the control group. Contrary, C18:1 c11, C18:2 *n*6 and C18:2 c9, t11 (CLA) were significantly ($p < 0.05$) higher in samples from the linseed group. Moreover, C24:1 was detected only in PLs of the linseed group. The differences observed in the FA composition of the PLs in the milk from cows fed with two diets, shows that the diet rich in polyunsaturated FA affected the lipid composition of MFGM. Therefore, MFGM consists of a high content of long and very long chain FAs with one or more unsaturated bond that determine its rigidity (Gallier et al.,2010).

3.3 Fatty acid and triglyceride composition of milk creams.

The determination of the FA composition of milk cream samples was undertaken using fast GC-FID analysis. Results of the FA composition are presented in the ***Table 5***.

Classes of fatty acids	**Milk cream**						
	Control group			**Linseed group**			***P value***
	mean		**CV**	**mean**		**CV**	
SFA	64.90	±	2.00	60.86	±	1.55	**
SC-SFA (C4-C10)	6.66	±	1.10	6.57	±	1.35	NS
MC-SFA (C12-C16)	44.80	±	1.66	41.38	±	1.30	**
LC-SFA (>C17)	13.45	±	0.68	12.91	±	0.57	**
UFA	35.10	±	2.00	39.14	±	1.55	**
MUFA	29.91	±	1.64	32.08	±	1.32	**
PUFA	5.19	±	0.52	7.05	±	0.31	**
TFA	4.06	±	0.32	5.30	±	0.34	**
CLA	0.98	±	0.10	1.22	±	0.06	**
***n*3-PUFA**	0.88	±	0.08	1.05	±	0.07	**
***n*6-PUFA**	2.95	±	0.33	4.31	±	0.19	**
***n*6-PUFA/*n*3-PUFA**	3.34	±	0.17	4.12	±	0.25	**
SFA/UFA	1.86	±	0.17	1.56	±	0.10	**
SC-FA (C4-C10)	6.66	±	1.10	6.57	±	1.35	NS
MC-FA (C12-C16)	47.65	±	1.70	44.07	±	1.35	**
LC-FA (>C17)	48.55	±	2.28	52.05	±	1.90	**

Table 5. Fatty acid composition (expressed in mg/100mg of total FAME) in milk creams from cows fed control and linseed diet. Results of the analysis of variance by Fisher's least significance difference (LSD) test.
(*) $p<0.05$; (**) $p<0.01$; (NS) not significant

The addition of an unsaturated fat source into the traditional diet of milking cows significantly ($P < 0.01$) decreased the mean SFA content from 64.9 ± 2.0 mg/100mg of FAME to 60.9 ± 1.5 mg/100mg of FAME and significantly ($p < 0.01$) increased the mean UFA content from 35.1 ± 2.0 mg/100mg of FAME to 39.1 ± 1.5 mg/100mg of FAME. In milk creams from the linseed group the MUFA and PUFA content significantly ($p < 0.01$) increased (+ 7.3% and + 36% respectively). When compared to the control group, the linseed group showed a higher concentration of bioactive UFA, including TFA (+ 30.7%), total CLA (+ 24.8%), *n*3-PUFA (+ 18.7%) and *n*6-PUFA (+ 46.1%). Since PUFA are not synthesized by tissues in ruminants, their concentration in milk is dependent on the amount absorbed in the intestine and therefore on the amount which leave the rumen. Consequently, these amounts may be increased by the use of feed rich in PUFA and by factors which decrease their rumen biohydrogenation. Moreover, the unsaturated fat content in milk depends on the action of intestinal and mammary desaturase activity that functions to convert SFA to MUFA. This helps offset the extensive hydrogenation that occurs in the rumen and probably ensures sufficient fluidity of milk fat for efficient secretion from the mammary cell (Grummer, 1991). Milk FAs were divided according to Secchiari et al. (Secchiari et al., 2003) into short-chain (C4-C10, SC-FA), medium-chain (C12-C16, MC-FA) and long-chain (> C17, LC-FA). The average content of SC-FA did not differ significantly between two groups of milk cream samples, whilst the content of MC-FA significantly ($p < 0.01$) decreased by 7.5% and the content of LC-FA significantly increased by 7.2% in milk cream samples from the linseed group. The major intake of LC-FA arising from the diet, or from body fat mobilization results in a decrease in the percentage of MC-FA in milk fat. This may be due to both a higher secretion of LC-FA from the blood (dilution effect) and a lower de novo synthesis of FAs that are mainly saturated (from C4:0 to C16:0) (Chilliard et al., 2000).

Special attention is given to the trend of SFA, UFA, MUFA and PUFA content of milk creams samples across the all sampling weeks. The mean SFA content was lower ($p < 0.01$) in milk creams from the linseed group than in milk creams from the control group, however during the dry feeding period (from 1 to 5 week of sampling) the value of SFA of the linseed group increases while there was an decrease during the transition from the dry feeding period to fresh forage feeding period (from 5 to 6 week of sampling) and finally, during the fresh forage feeding period (from 6 to 12 week of sampling) the SFA content increases again. The different general trend was observed for the control group until the eighth week of sampling showing a decrease of SFA content, even though after this week this content increases slightly as well as the linseed group. Comparing the two groups of milk creams, after the first dry feeding period where the linseed group showed a decrease of in the concentration of UFA, the inclusion of fresh forage (from 5 to 6 week of

sampling) into the dry diet resulted in an increase of unsaturated fat (+ 3.4% for UFA; + 2.1% for MUFA and + 9.5% for PUFA). After this time these increment decreased to values close to those recorded for the dry feeding period.

As result of the high unsaturated fat content, especially linolenic acids (C18:3), of extruded linseed and fresh forage offered into the traditional diet of the linseed group, more pronounced decreases in SFA and increases in UFA and MUFA were observed than in the control group. These changes in FA levels in milk cream samples suggest that the extent of various metabolic pathway, such as adipose tissue metabolism, mammary lipogenesis and ruminal biohydrogenation, mainly depend on the lipid profile of the diet. For reason not yet elucidated, from 5 to 6 week of sampling, PUFA content of the linseed group increased less (+ 9.5%) compared to that of the control group (+ 16.9%).

As FA synthesis, uptake and desaturation all contribute to a pool of FAs available for esterification of glycerol to primarily form TGs (comprising 97 – 98% of the total milk lipids) this study also focused on the TG composition of samples using GC-FID analysis (***Table 6*).**

Classes of Triglyceride	**Milk cream**						
	Control group			**Linseed group**			***p value***
	mean		**CV**	**mean**		**CV**	
T26	0.48	±	0.06	0.53	±	0.05	**
T28	0.51	±	0.10	0.57	±	0.09	*
T30	1.05	±	0.16	1.20	±	0.10	**
T32	2.55	±	0.28	2.69	±	0.18	*
T34	6.26	±	0.32	5.93	±	0.31	**
T36	11.44	±	0.35	10.46	±	0.36	**
T38	13.14	±	0.35	12.40	±	0.34	**
T40	9.63	±	0.25	10.14	±	0.31	**
T42	6.67	±	0.23	6.95	±	0.17	**
T44	6.82	±	0.29	6.94	±	0.19	NS
T46	7.78	±	0.28	7.97	±	0.44	NS
T48	9.66	±	0.17	9.47	±	0.24	**
T50	11.46	±	0.43	11.01	±	0.22	**
T52	9.43	±	0.76	9.51	±	0.54	NS
T54	3.11	±	0.56	4.23	±	0.57	**
SC-TG (T26-T34)	10.85	±	0.64	10.92	±	0.42	NS
MC-TG (T36-T44)	47.71	±	0.82	46.88	±	0.89	**
LC-TG (T46-T54)	41.44	±	1.26	42.20	±	1.14	*

Table 6. Triglyceride composition (expressed in mg/100mg of total fat) of outcrop milk creams from cows fed control and linseed diet. Results of the analysis of variance by Fisher's least significance difference (LSD) test. (*) $p<0.05$; (**) $p<0.01$; (NS) not significant

Fifteen peaks, corresponding to TGs with 26 – 54 carbon atoms, were identified and quantified. The TG classes identified were classified according to their carbon atoms chain length into short-chain (C26-C34, SC-TG), medium-chain (C36-C44, MC-TG) and long-chain (C46-C54, LC-TG). As consequent results of the differences observed in the FA composition between control and linseed group, differences in the TG composition were detected. As previously observed for SC-FA, the mean content of SC-TG did not show significant differences between the two groups of milk creams. Additionally, the mean content of MC-TG was lower ($p < 0.01$) and LC-TG was higher ($p < 0.05$) in milk fat from cows fed the enriched diet in comparison to the control diet. Differences in TG composition were observed between the control group and the linseed group in agreement with previous studies on the composition of these molecules (DePeters et al., 2001; Mele et al., 2011) in response to lipid supplementation of feeding dairy cows.

Taken as a whole, these results confirm that the modification of the diet of milking cows constitutes a natural way to sharply and rapidly modulate milk FA composition (Chilliard and Ferlay, 2004) especially when the dietary intake of lipid supplements are comprised predominantly (90 - 95%) of UFA with a chain length greater than 14 carbon atoms (typically C18 FA accounting for more than 75% of total UFA).

Therefore, an increase in long-chain UFA (LC-UFA), especially bioactive FA, and LC-TG can influence the chemical, physical, nutritional and organoleptic properties of fat-rich dairy products (Mele et al., 2011; Oriz-Gonzales et al., 2007).

3.4 Comparison of fatty acid composition of total lipid (fat globules) and phospholipids fatty acid composition.

The main differences in the FA composition of the total lipid (fat globules) and PLs were compared (***Table 7***). The SC-FA (C4-C10) were not detected in PL fraction in agreement with previous studies (Fauquant et al., 2007; Fong et al., 2007) who identified and quantified FAs in the MFGM starting from C10:0. Aside from the traditional diet of the milking cows, the composition of PLs was characterized by a lower ($p < 0.01$) content of MC-FA and a higher ($p < 0.01$) content of LC-FA than total lipid in agreement with other author observations (Jensen and Nielsen 1996; Lopez et al., 2008). As reported by Lopez et al. (Lopez et al., 2008) the current study confirmed that the content of UFA was significantly ($p < 0.01$) higher in the PLs than in the total lipid of cream, while the content of SFA was lower ($p < 0.01$). In our study the content of MUFA was significantly lower ($p < 0.01$) in the PLs than in the total lipids while conversely the content of PUFA was higher ($p < 0.01$).

Classes of fatty acids	Linseed Group (traditional diet + linseed)							Control Group (traditional diet)						
	Phospholipids			Total Lipids			*p value*	Phospholipids			Total Lipids			*p value*
	mean		CV	mean		CV		mean		CV	mean		CV	
SFA	43.38	±	1.54	60.86	±	1.55	**	45.88	±	2.70	64.90	±	2.00	**
UFA	58.76	±	3.03	39.14	±	1.55	**	54.12	±	2.70	35.10	±	2.00	**
MUFA	28.25	±	1.09	32.08	±	1.32	**	26.92	±	1.57	29.91	±	1.64	**
PUFA	30.51	±	2.21	7.05	±	0.31	**	27.20	±	1.37	5.19	±	0.52	**
TFA	0.57	±	0.36	5.30	±	0.34	**	0.44	±	0.19	4.06	±	0.32	**
CLA	1.2	±	1.0	1.2	±	0.1	NS	0.3	±	0.1	1.0	±	0.1	**
***n*3-PUFA**	3.13	±	0.69	1.05	±	0.07	**	2.26	±	0.34	0.88	±	0.08	**
***n*6-PUFA**	6.95	±	0.57	4.31	±	0.19	**	4.91	±	0.80	2.95	±	0.33	**
SC-FA (C4-C10)	*nd*			6.66	±	1.1		*nd*			6.57	±	1.35	
MC-FA (C12-C16)	28.49	±	0.94	44.07	±	1.35	**	33.80	±	1.90	47.65	±	1.70	**
LC-FA (>C17)	71.51	±	0.94	52.05	±	1.90	**	66.20	±	1.90	48.55	±	2.28	**

Table 7. Fatty acid composition (expressed in mg FA/100mg FAME) of phospholipids and total lipid from cows fed control and linseed diet. Results of the analysis of variance by Fisher's least significance difference (LSD) test. (*) $p<0.05$; (**) $p<0.01$; (NS) not significant

Particularly, the total amounts of *n*3-PUFA and *n*6-PUFA, was 2.6 - 3-fold and 1.6 - 1.7-fold higher respectively, in the PLs compared to the total lipids. These results are in agreement with previous studies which reported that the principal precursor of the *n*3-PUFA and *n*6-PUFA were 2.3 - 2.7-fold and 1.2 - 1.7-fold higher in the PLs than in the corresponding cream (Jensen and Nielsen 1996). The content of TFA was significantly ($p < 0.01$) lower in the PLs than in the corresponding total fat of milk creams. Additionally, different FA composition in the PLs and total lipid was observed between the linseed and the control group for the total CLA concentration. Even though the total CLA concentration in the total lipid and in the PLs was higher ($p < 0.01$ and $p < 0.05$, respectively) in milk cream from the linseed group than in the milk cream from the control group, no significant difference in the total CLA composition of the PLs and total lipid was detected when the unsaturated-enriched-diet was offered to milking cows. On the contrary, the control group showed a significantly ($p < 0.01$) higher content of total CLA in the total lipids than in the PLs.

For these reasons it is possible to conclude that the inclusion of extruded linseed into the diet of dairy cows results in a significant increase of total CLA concentration both in the PLs (from 0.3 ± 0.1 to 1.2 ± 0.1 mg FA/100mg FAME) and in the total lipid (from 1.0 ± 0.1 to 1.2 ± 0.1 mg FA/100mg FAME).

4. Conclusion

These results presented here suggest that the inclusion of extruded linseed into to the traditional diet of dairy cows may be adopted to enhance the bioactive FA and PL composition of dairy products.

Although milk FA is linked to intrinsic (animal breed, genotype, lactation and pregnancy stage) and extrinsic (environmental) factors, the feeding may be considered a strategic factor to modify the bioactive lipid profile of milk especially when the feed is rich in an unsaturated fat source and when correlated to the availability of fresh forage rather than dry diet. Therefore, the research applied in this study provides several advantage for the dairy industry to obtain products and by-products with added-value both technologically and nutritionally.

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Section II

Human milk: proteomic and lipidomic characterizations

The research work described in this section has been undertaken in parallel to a major current project on human milk biochemistry during my training period at School of Food and Nutritional Science, University College Cork, Ireland from 4 January to 3 July, 2013, under the supervision of Professor Alan Kelly. The objectives of this study was to compare the protein and lipid profiles of human milk at different stage of lactation and gestational age. The major constituents of human milk (proteins and lipids) that are essential to neonate growth and nutrition will be characterized to provide a comprehensive profiling of the differences between pre-term human milk against colostrum and mature human milk.

The first part of the research work (***Chapter V***) which looked at protein differences occurring in colostrum, pre-term and term human milk was realized using proteomic methodologies and techniques, including 1-dimensional electrophoresis (1DE), 2-dimensional electrophoresis (2DE) and enzyme assays, in order to better understand the changes in human milk proteome as result of premature delivery compared to colostrum and milk from mothers that delivered at full term.

The second part of the research work (***Chapter VI***) is focused on lipid profile in order to evaluate the principal differences in lipid-based compounds, with particular attention to bioactive fatty acids, between colostrum, pre-term and term human milk. The study on fatty acid composition of human milk was carried out by fast gas-chromatography (fast GC) combined with flame ionization detector (FID).

To date, further researches within this project will being done on a greater human milk sample numbers, especially on longitudinal milk samples from mothers of pre-term infants, in order to confirm the preliminary results and to establish how the extent of prematurity and early lactation influences the protein, metabolite and fatty acid profiles of milk.

This research will be useful to improve the knowledge about pre-term infant growth and development and will help in the optimization of neonatal nutrition requirements.

Chapter V

Proteomic characterization of colostrum, pre-term and term human milks

Proteomic characterization of colostrum, pre-term and term human milks

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Abstract

Human milk proteins and protein-derived bioactive peptides provide nutritional benefits, regulatory functions, neurodevelopment, immunological protection and antimicrobial effects, ensuring the growth and development of newborn in early life, especially for pre-mature infants. In fact, the protein composition of milk from pre-term mothers is known to differ from that of term mothers. The objective of this study was to compare the protein content, individual protein profile, plasmin activity and peptides in colostrum, pre-term and term human milks, and study how they differ with gestational age. For this purpose, proteomic methodologies and techniques were applied, including 1-dimensional electrophoresis, 2-dimensional electrophoresis and enzyme assays. Compared to term milk (T), pre-term (PT) milk was found to contain higher total nitrogen level, plasmin activity, lactoferrin (LF), serum albumin and α-lactalbumin (α-lac) levels, in contrast to the lower content of β-casein (β-CN). These finding supports the greater bioavailability of bioactive protein and protein-derived bioactive peptides that arise from plasmin activity, for the adequate growth, development and protection of premature breastfed infants. This research will contribute to extend the knowledge about the differences of the many abundance of proteins in colostrum, pre-term and term milks and, therefore, to meet neonatal nutritional and physiological requirements.

Key words: human milk, casein, protein, plasmin activity

1. Introduction

Human milk meets all of the nutritional needs of healthy infants and provides not only the necessary nutrients required for growth, but also a large numbers of bioactive compounds that are involved in milk synthesis and that likely contribute to the neonatal maturation, protection and subsequent development of the infant (L'Abbé and Friel 1998; Bauer and Gerss 2011).

Human milk contains a wide array of proteins and peptides that contribute to its unique qualities and provide biological activities, ranging from antimicrobial effects to immuno-stimulatory functions (Armaforte et al., 2010), especially for pre-mature babies (Molinari et al., 2012).

The protein composition of human milk is affected by two main factors: gestational age at birth of the mothers and stage of lactation. It is well known that during the early stage of lactation (colostrum period) and in breast milk from mothers delivering prematurely (pre-term milk) the total protein content is higher compared to milk from mothers delivering at term (term-milk) (Atkinson 1995). The casein and whey protein concentrations of both pre-term and term human milks rapidly declines during the first month of lactation and declines more slowly with progressive weeks of lactation (Lönnerdal 2003). In particular, while the whey protein fraction is very high during early lactation and in pre-term milk, the casein is not detectable or is very low. Afterwards, the casein concentrations increase and the total whey proteins decreases, partially due to an increased volume of milk being produced (Lönnerdal 2003; Armaforte 2013). These changes are related to a not "fixed" ratio of whey protein to casein in human milk. Generally, the ratio 60:40 (whey to casein) is typical during the normal course of lactation (Kunz and Lönnerdal 1990, Lönnerdal 2003).

The non-protein nitrogen (NPN) concentration in human milk represents ~20-25% of total nitrogen and shows the same trend as the protein contents. In fact, the NPN content is significantly higher in early lactation and in pre-term milk than in term milk as a consequence of a greater nutritional and physiological needs of neonate and premature babies to amino acids and peptides that are readily available than intact proteins (Atkinson et al., 1980).

Colostrum and milk also contains numerous active enzymes with multifunctional roles and their levels in milk are related to the lactation period (L'Abbé and Friel 1998). Active enzymes are involved in nutrient digestion, gastrointestinal function, nutrient delivery to the infant (Hamosh 1989), anti-microbial and antiviral functions (Isaacs and Thomar 1991; Hamosh 1991). Plasmin is the most predominant proteolytic enzyme in human milk and higher levels were detected in pre-term milk that in term milk, leading to high concentration of hydrolysis products of α_{s1}-casein and β-caseins (Ferranti et al., 2004; Armaforte et al., 2010), which are be useful for the adequate growth and development of premature newborns.

Several comparative studies concerning the protein composition of colostrum, pre-term and term human milk have been applied in the proteomic field. However, some variables such as collection methodology, gestational age, lactation period, inter-individual variability affect the total protein content of human milk (Atkinson 1995, Bauer and Gerss 2011) and they also influence the levels of many individual proteins.

The main objective of this study was to investigate the differences in total protein content, protein profile, plasmin activity, and peptides on colostrum, pre-term and term human milks and how they differ during with gestational age. Therefore, this proteomic study will not only give a indication of protein content in colostrum, pre-term and term human milks but will also characterize the changes in the main abundance of many individual proteins in addition to quantifying the plasmin activity in milk samples. Identifying and quantifying the main individual proteins is important, firstly, because specific proteins could play a significant roles in infant growth and development, and, secondly, because the differences in the protein fractions of milk are related to the changes occurring in the mammary gland during pregnancy and lactation.

The amount of total nitrogen (% N) and non-protein nitrogen (% NPN) was determined by the Kjeldahl method. In order to monitor the changes occurring in the protein profiles of human milk and, in particular, to describe how the stage of lactation affects the relative abundance of many high abundance and nutritionally important proteins, 1-dimensional electrophoresis (1DE), and 2-dimensional electrophoresis (2DE) was used. Furthermore, the activity of plasmin was determined using a standard spectrophotometric method.

2. Materials and Methods

2.1 Collection of human milk bank samples

Human milk (HM) samples were collected from a milk bank in Northern Ireland. Full ethical approval has been granted and four colostrum (C) samples, two pre-term (PT), and five term (T) human milks and one undetermined (CT) milk, were kindly donated by milk bank for this preliminary study. Pre-term milks were collected from mothers who delivered between 30 and 37 completed weeks of gestation and term milks from mothers with infants born after 38 weeks of gestation.

2.2 Nitrogen determination

The amount of total nitrogen (% N) and non-protein nitrogen (% NPN) in each of the human milk samples were determined by the Kjeldahl method (IDF 1986, International Dairy Federation, Brussels, Belgium).

2.3 Plasmin Activity

Sample of HM were prepared according to Aslam and Hurley (Aslam and Hurley, 1996) with some modification. Milks were centrifuged at 10,000 g for 25 min at 4°C and the aqueous phase was collected. Centrifugation was repeated until no fat was visible on the top. The supernatant was passed through a 45 µm filter and stored at -80 °C until plasmin assay.

The activity of plasmin were determined in duplicate on the defatted, filtered aqueous phases of human milk according to a spectrophotometric assay described by Fantuz et al. (Fantuz et al., 2001). Briefly, 250 µL of reaction mixture was obtained by the addition of 125 µL of 0.1 M Tris-HCl buffer (pH 7.4), 70 µL of double distilled water (ddH_2O), 25 µL of 0.6 mM of D-Val-Leu-Lys p-nitroanilide dihydrochloride (V7127; Sigma-Aldrich Chemical Co.) and 30 µL of samples. An analysis in which samples was replaced by buffer was used as control to detect spontaneous breakdown of the substrates. The reaction mixture was incubated at 37 °C for 3 h and A_{405} nm was measured at 30-min intervals with a 96-well microtiter plate reader.

The rate of p-nitroanilide formation was calculated from the linear portion of the absorbance versus time curve. Plasmin activity was expressed as units/mL of milk, where one unit was defined as the amount of enzyme that produced a change in absorbance of 0.001 at 405 nm in 1 min.

2.4 Protein identification (One- and two-dimensional electrophoresis)

Analytical SDS-PAGE electrophoresis (1DE) was performed on human milk (1.5 µL) using 4-20% polyacrylamide gels (4-20% Precise™, Tris-Glycine Gels, 12 well, Thermo Pierce) under reducing conditions. Gels were stained with coomassie (Bio-Rad, Bio-Safe™ Coomassie, G-250 Stain). Images were scanned (HP Scanjet G4010) and were analysed using TotalLab™ Quant (v12) Software (TotalLab, UK). All human milk samples were analyzed in triplicate and the replicates were used to provide average values for the gels.

For analytical 2D gel electrophoresis (2DE), human milk (5 µL) proteins were separated in the first dimension on 7 cm, pH 4-7 strips (Ready Strip™ IPG Strips, Bio-Rad). Strips were equilibrated and separated in the second dimension on pre-cast 4-20 % gels (Mini-Protean® TGX™ Gels, Bio-Rad). 2D gels were scanned (HP Scanjet G4010) and the main differences between samples were evaluated.

3. Results and Discussion

3.1 Nitrogen Determination

The mean total nitrogen (% N) content of milk from colostrum, pre-term and term human milks was 0.31 %, 0.23 % and 0.17 %, respectively (***Figure 1***). Thus, colostrum samples had 34.8 % more

nitrogen than pre-term human milk and 82.4 % more than term human milk samples. In particular, the levels of nitrogen in pre-term human milk samples were higher (+35.8%) than that of the five term human milk samples. This can be associated with a rapid growth and development of premature infants that have high protein requirements.

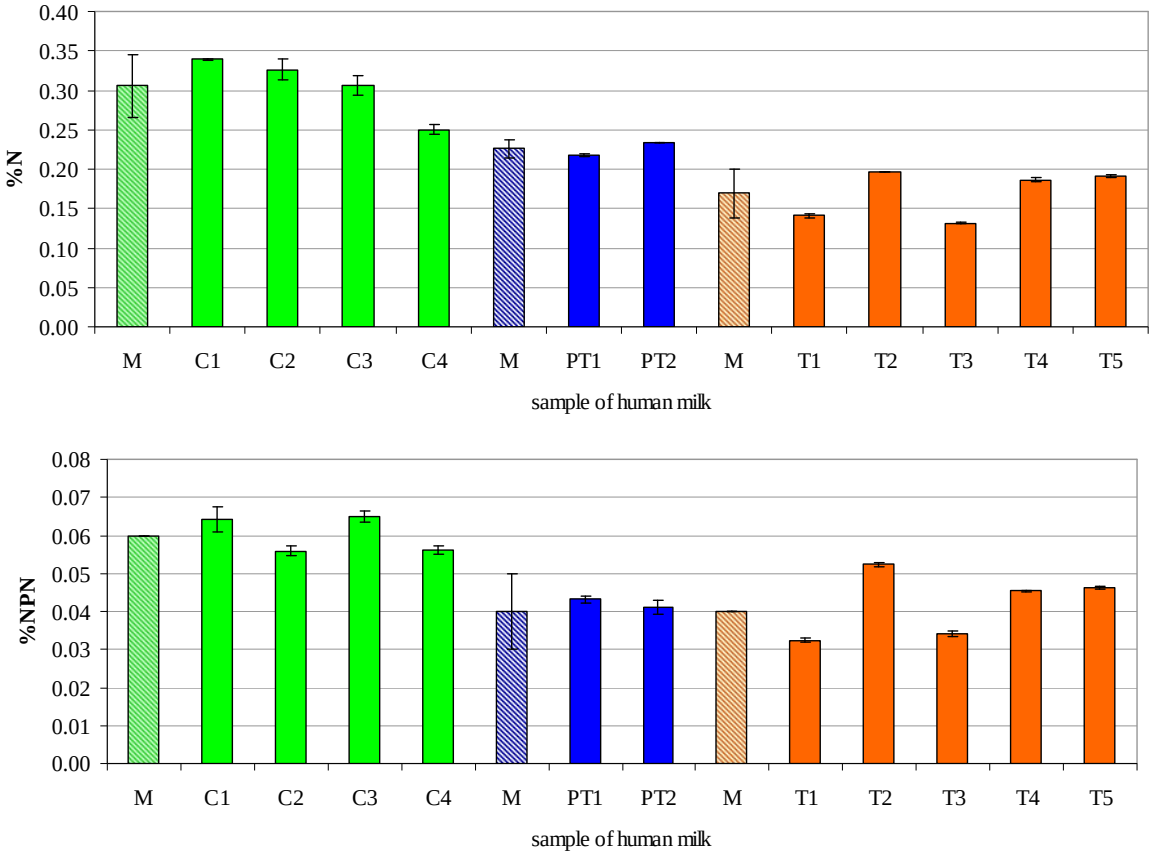

Figure 1. Total nitrogen (%N) and non-protein nitrogen (%NPN) contents of the colostrum, pre-term and term human milks; M: average; C: colostrum; PT: pre-tern and T: term.

The non-protein nitrogen (%NPN) contents of each samples, consisting of creatine, creatinine, urea, free amino acids and small peptides, were quantified. In this investigation, the mean content of NPN (%) of colostrum samples was higher (+50 %) compared to pre-term and term human milk samples (***Figure 1***), whereas no differences were observed between pre-term and term human milks.

Finally, the true milk protein (TP) content was estimated by subtracting the NPN value from total nitrogen, and then by multiplying the value for the conventional human milk protein conversion factor of 6.25 (Lönnerdal 2003). The mean TP content (%) of colostrum samples (1.53 ± 0.23 %) and pre-term milks (1.15 ± 0.08 %) was greater than the mean value calculated for term milk samples (0.79 ± 0.14 %).

3.2 Plasmin activity

The results of plasmin activity of human milk samples, expressed in Units/mL of milk, are reported in the ***Figure 2***.

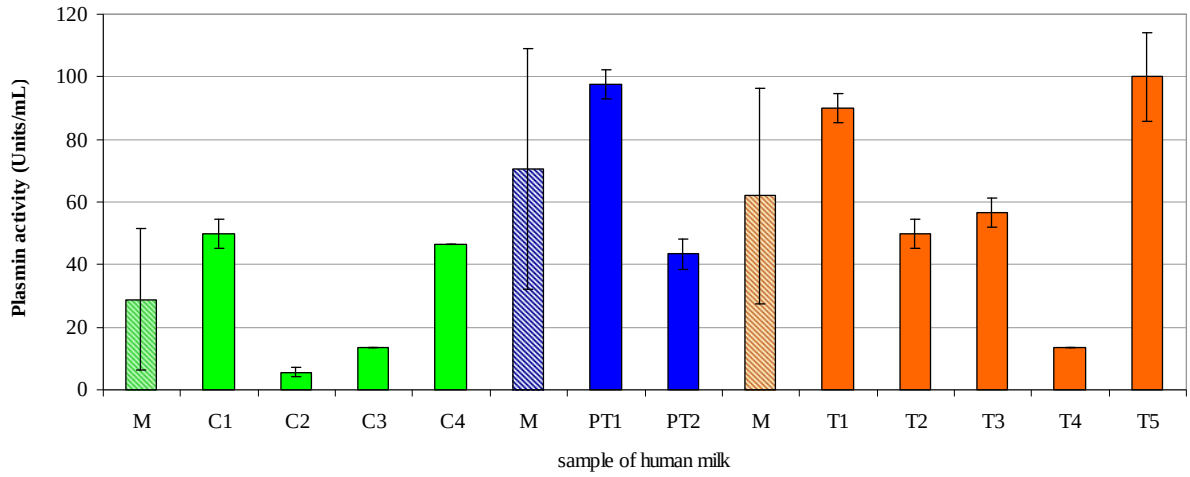

Figure 2. Plasmin activity (Units/mL of milk) of the colostrum, pre-term and term human milks; M: average; C: colostrum; PT: pre-tern and T: term.

The plasmin activities in colostrum samples were lower than those in pre-term and term human milks, possibly due to the presence of elevated level of inhibitors in milk produced during the first days after calving. Furthermore, in accordance with the findings of Armaforte (Armaforte et al., 2010) plasmin activity of pre-term milks (although only a small number of samples was determined) was higher than the plasmin activity of term milk samples.

3.3 Protein identification by one-electrophoresis (SDS-PAGE) analysis and two-dimensional (2DE) electrophoresis.

SDS-PAGE was carried out on a total of 12 human milk samples: 4 colostrum (C), 2 pre-term (PT), 5 term (T) and 1 undetermined (CT) samples The principal bands in all samples corresponded to lactoferrin (LF), serum albumin, β-casein (β-CN) and α-lactalbumin (α-lac). As expected, β-lactoglobulin, an important protein in bovine milk, was not present in human milk samples.

The intensity of the bands, corresponding to individual proteins, varied greatly between groups of colostrum, pre-term and term milks (intra-variability) rather than the samples belonging the same group (inter-variability). In particular, the intensity of the bands was compared to highlight the main differences in individual protein profile of samples collected at different gestational ages.

For these reasons, all human milk samples were analyzed in triplicate and the replicates were used to provide an average value for the gels. The same bands of the gels were selected and the their intensity as expressed as percentage of total protein (%).

Therefore, qualitative and quantitative analysis were performed and the result are reported in ***Figure 3*** and ***Figure 4***, respectively.

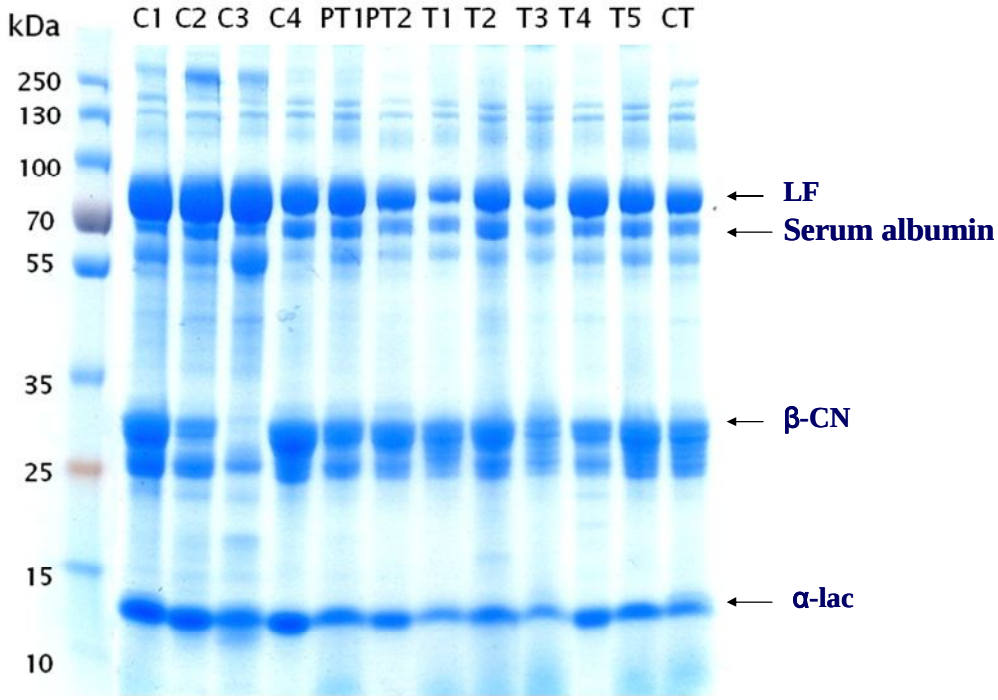

Figure 3. SDS-PAGE electrophoretograms of colostrum (C1, C2, C3, C4), pre-term (PT1, PT2), term (T1, T2, T3, T4, T5) and undetermined (CT) milk samples. LF: lactoferrin, β-CN: β-casein, α-lac: α-lactalbumin.

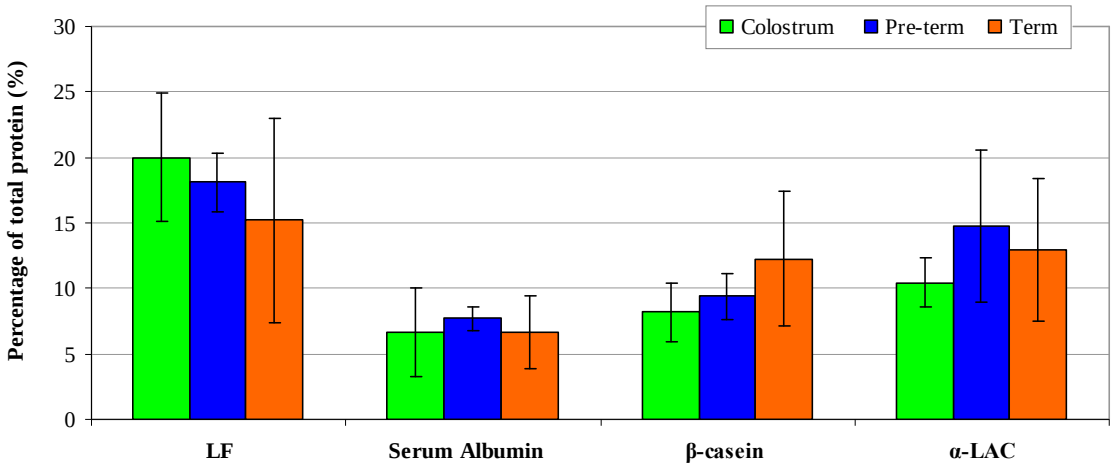

Figure 4. SDS-PAGE analysis of different protein composition of colostrum, pre-term and term human milk samples. LF: lactoferrin, β-CN: β-casein, α-lac: α-lactalbumin, expressed as percentage of total protein (%).

In human milk samples, the most abundant protein was LF, followed by α-lac, β-CN and serum albumin. In particular, colostrum samples showed the highest content of LF and lowest content of β-CN and α-lac. Comparing the 1DE data between pre-term and term samples, the mean value (%

of total protein) of LF, serum albumin and α-lac were determined in pre-term milks. In contrast, pre-term human milks showed a lower content of β-CN than term human milk samples and this was confirmed by 2DE (***Figure 5***). This may be as a result of the higher hydrolysis by plasmin activity of β-CN in pre-term milk samples. In fact, it is well known that, in human milk, plasmin is responsible for proteolytic cleavage of β-CN and also α_{s1}-CN, with a consequent formation of lower-molecular mass polypeptides and peptides which have essential physiological body functions and immune protective role for breastfed infants, especially for pre-mature babies (Armaforte et al., 2010; Clare and Swaisgood 2000).

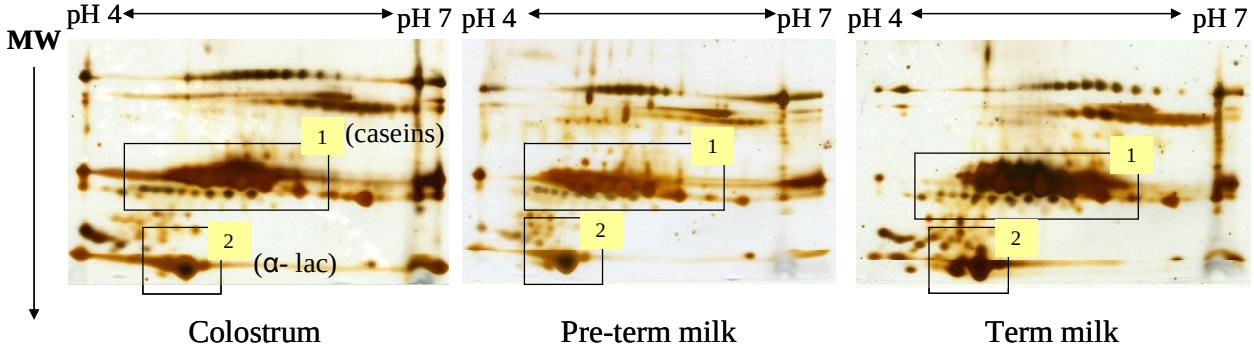

Figure 5. 2DE analytical gels of a representative colostrum, pre-term and term human milk samples. Identification of principal spots of casein (1) and α-lac: α-lactalbumin (2).

The casein spots (corresponding to intact proteins) appear to be in higher abundances in the term milks than in the colostrum and pre-term milks. Similarly, the abundance of α-lac appears to be higher in term human milk and lowest in pre-term milk samples. The latter evidence seems to contradict the result of 1DE (SDS-PAGE) and, therefore, further analysis will be carry out to define these differences.

4. Conclusion

The present study highlight the differences in protein content, the changes in abundance of many individual proteins, and the enzyme activity between breast milk from women delivering prematurely and breast milk from women delivering at term.

Results obtained showed that the higher nitrogen content in pre-term milks may be associated with the rapid growth and development of premature infants that have higher protein requirements and a higher proteolytic activity of plasmin, especially on β-CN, leading to the production of β-CN breakdown products (including bioactive peptides) could contribute to rapid development, immunological protection and regulatory functions of premature babies. These results can be

confirmed by 1D and 2D electrophoresis which showed that casein bands, and spots, were higher abundance in term milk samples than in colostrum and pre-term milks. In conclusion, proteomic for colostrum, pre-term and term human milks suggest a differences in physiological response of enzyme and/or protein expression by mammary gland to improve milk digestibility and to ensure immunological protection for breastfed prematurely newborn babies, a finding that may have relevance for further investigations of human milk samples from different groups of neonates.

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Chapter VI

Fatty acid composition of colostrum, pre-term and term human milks in Irish mothers: a preliminary study

Fatty acid composition of colostrum, pre-term and term human milks in Irish mothers: a preliminary study

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Abstract

Fatty acid composition, in particular bioactive fatty acids such as essential *n*3- and *n*6-polyunsaturated fatty acids (*n*3-PUFA and *n*6-PUFA) and their longer-chain polyunsaturated fatty acid, including arachidonic acid (C20:4 n6) and docosahexaenoic acid (DHA), were identified and quantified by fast gas-chromatography analysis in full term colostrum, pre-term and term milks sourced from a milk bank in Northern Ireland. While the saturated, monounsaturated and polyunsaturated fatty acids fractions did not differ between full term colostrum and term milks, level of long-chain polyunsaturated fatty acids was significantly higher in colostrum samples. Regarding variation among the gestational age groups, pre-term milks showed the significantly highest content of medium-chain and long-chain saturated fractions compared to term milks. Conversely, essential fatty acids and long-chain polyunsaturated fatty acids were significantly lower in the milk from mothers who delivered infants before term that the concentration present in term milks. These findings observed among the maternal groups at different stage of lactation were probably related to the variation in functional maturity of the mammary gland and/or related to the diet of Irish mothers.

Key words: human milk, bioactive fatty acids, colostrum, pre-term milk, term milk.

1. Introduction

Human milk contains all the exacting amounts of required nutrients, including bioactive fatty acids (FAs), to satisfy the nutritional and physiological needs of full-term infants in early life and to prevent the incidences of obesity, diabetes and cardiovascular disease in later life (ESPGAN, Committee in Nutrition 1982, 1991; Department of Health and Society Security 1998; American Academy of Pediatrics 1982; Jensen 1995; López-López et al., 2002).

The suitability of breast milk as a source of proteins, carbohydrates, lipids and other nutrients for pre-term infants, in order to fulfil their requirements and consequently reduce infant morbidity, is widely debated (Hintz et al., 2011).

Human milk FAs may be originate from the diet, mammary gland synthesis and adipose, liver and other tissue mobilization (Jensen 1996). Maternal diet during pregnancy, and in particular, the amount of carbohydrates and the type of FAs intake, besides the time elapsed since the last meal, are the predominant factors which affect the adipose tissue mobilization (Jensen 1989). Therefore, the differences in FA composition of breast milk from mothers mainly arise from the maternal diet as well as geographic origin and socio-cultural conditions of lactating mothers (Finley et al., 1985; Koletzko et al., 1988; Hayat et al., 1999a; Del Prado et al., 2001; Silva et al., 2005; Lauritzen et al., 2006; Innis 2007; Martin et al., 2012). Furthermore, duration of pregnancy (Bitman et al., 1983; Elias and Innis 2001; Moltó-Puigmartí et al., 2011) stage of lactation (Gibson et al., 1981; Szabó et al., 2010) and individual metabolism of lactating mothers are relevant factors.

Numerous studies concerning the FA composition of breast milk, have confirmed the important role in the neonatal growth and development of essential *n*3 and *n*6 polyunsaturated fatty acids (*n*6-PUFA and *n*3-PUFA) and their derivatives, the long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (AA) and docosahexaenoic acid (DHA) (Carlson et al., 1996; SanGiovanni et al., 2000; Elias and Innis 2001; Innis 2007; van Goor et al., 2009).

The aim of this study was to compare the FA profile, with particular attention to bioactive FAs, of human milk samples in order to determine the main differences between milks from mothers that deliver prematurely (pre-term milks) and those that deliver at full-term (term milks). In addition, full-term colostrum and mature milks were compared to assess the main changes occurring between samples collected at different stage of lactation. The FAs present in human milks were identified and quantified by fast gas-chromatography combined with flame ionization detector (fast GC-FID). The results of this research will be useful to better understand the variability of FA composition between milk secreted by Irish mothers delivering at different gestational age and during the progression of lactation. Moreover, this study contributes to defining the bioactive FA requirements

of pre-mature infants and consequently could be helpful in designing, optimizing and/or fortifying functional infant formulae.

2. Materials and Methods

2.1 Milk samples collection

Breast milk samples were obtained from mothers of term and pre-term infants. Five samples of full term colostrum (C), five samples of pre-term (PT) milk and five samples of term (T) milk were sourced from a milk bank in Northern Ireland. Pre-term milks were collected from mothers who have delivered at pre-term between 30 and 37 completed weeks of gestation and term milks from mothers with infants born after 38 weeks of gestation. Ethical protocol and prior informed consent for the use of these human milk samples for research purpose was approved by the milk bank. All milk samples were stored in sterile tubes at – 80°C until fat extraction.

2.2 Milk fractionation and lipid extraction

Milk samples (5.25 mL) were thawed at room temperature, 52.5 µL of protease inhibitor (PI) and $CaCl_2$ (final concentration of 60 mM) were added, pH was adjusted to 4.3 and each sample was centrifuged at 30000 rpm at 4°C for 1 h. The milk cream layer was removed with a metal spatula, and whey and casein fractions were collected and stored at -80°C.

Total lipid was extracted in duplicate from milk cream layer according to Hara and Radin (Hara and Radin, 1978). Briefly, to ~ 100 mg of milk cream was added 1.8 mL of *n*-hexane: isopropanol (3:2, v/v) and the mixture was homogenized with vortex for 30 seconds, then 1.2 mL of aqueous sodium sulphate (prepared from 1 g of the anhydrous salt and 15 mL of water) was added and mixed for 1 minute. The mixture was centrifuge at 2500 g for 3 minutes to allow the separation between non-lipids layer (aqueous phase) and upper lipid-rich layer (organic phase). The organic phase was transferred into pre-weighted tubes and dried under nitrogen (N_2). The lower phase was again extracted and the organic phase was recovered.

The total lipid fraction was stored at – 18°C in the presence of a solution of *n*-hexane: isopropanol (4:1, v/v) until fatty acid methyl esters (FAME) preparation.

2.3 Fast gas chromatography analysis

Methyl esters of free fatty acids (FFA) were prepared by reaction with ethereal diazomethane (Fisier and Fisier 1967) and the fatty acid methyl esters (FAME) was obtained followed the base-catalyzed transesterification method described by Christie (Christie 1982). After the addition of ethereal diazomethane (0.1 mL), each aliquots of extracted lipid (~ 20 mg) was added to 0.5 mL of

internal standard (IS) solution (C11:0; 2 mg/mL of *n*-hexane) and dissolved in 0.5 mL of *n*-hexane, 40 µL of methyl acetate and 40 µL of sodium methoxide in methanol (1M). The solution was mixed for 30 seconds and after 15 minutes at room temperature the reaction was stopped by adding 30 µL of a saturated solution of oxalic acid in diethyl ether. After a brief agitation, the mixture was centrifuged at 1500 g for 2 minutes and the supernatant, containing FAME, was collected into 2 mL auto-sampler vials fitted with a reductor.

FAME in human milk was determined in duplicate using a GC 2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a split–splitless injector, an AOC-20i autoinjector and a flame ionization detector (FID). All data were collected by GC Solution software (Shimadzu, Milan, Italy). The separation of the FAME was performed with a BPX70 fused silica capillary column (10m, ID 0.10 mm, 0.10 µm f.t.), coated with 70% cyanopropil polysilphenylenesiloxane film from SGE (VWR International, Fontenay-sous-Bois, France). The injector and detector ports were set at 250 °C. The oven temperature programme was initially set at 50 °C for 0.2 min, then increased at a rate of 120 °C/min to 175 °C, where it remained for 2 min, then increased at a rate of 20 °C/min to 220 °C, and then increased at a rate of 50 °C/min to 250 °C, where it remained for the last 0.3 min. Hydrogen was used as a carrier gas at a flow rate of 0.8 mL/min, and the split ratio was set at 1:100.

Peaks were routinely identified by comparison of the retention times with fatty acid methyl ester GLC 463, CLA standard mixtures UC-59M from Nu-Check (Elysian, MN, USA), and published isomeric data (Kramer et al. 2004). FA were quantified by comparing the peak area of each compound with that of IS, and the composition was expressed as relative amounts (% wt/wt of total FAME).

2.4 Statistical analysis

Fatty acid results of colostrum, pre-term and term milks were evaluated with the STATISTICA 6 software package (StatSoft Inc., Tulsa, OK, USA). Statistical analysis as performed using one-way analysis of variance (ANOVA) and the differences between the means of data for human milk groups were compared at the 5% level of significance ($p < 0.05$) using Tukey HSD test.

3. Results and discussion

In the present study, a total of thirty-three FA were identified and quantified in human milks by fast GC-FID analysis using a fused silica short capillary column with a run time less of 7 minutes.

In order to evaluate the main differences of lipid composition between full term colostrum, pre-term and term milks, individual FA (***Table 1***) and classes of FA (***Table 2***), expressed as mean ± standard deviation (SD), were compared.

Individual fatty acids	**Colostrum**			**Pre-Term**			**Term**		
	mean		**SD**	**mean**		**SD**	**mean**		**SD**
C8:0	0.24	±	0.07 [a]	0.32	±	0.06 [a]	0.24	±	0.11 [a]
C10:0	1.13	±	0.58 [a]	1.75	±	0.17 [b]	1.28	±	0.27 [a]
C12:0	5.05	±	2.31 [a]	6.60	±	0.64 [a]	4.97	±	1.32 [a]
C14:0	6.20	±	1.67 [a]	7.76	±	0.71 [b]	5.94	±	1.42 [a]
C14:1	0.27	±	0.05 [a]	0.43	±	0.11 [b]	0.33	±	0.05 [a]
C15:0	0.37	±	0.07 [a]	0.49	±	0.12 [b]	0.41	±	0.05 [a,b]
C16:0	23.49	±	2.51 [a]	26.14	±	0.64 [b]	22.32	±	2.3 [a]
C16:1 t	0.53	±	0.17 [b]	0.39	±	0.05 [a]	0.46	±	0.08 [a,b]
C16:1 c	2.62	±	0.79 [a]	2.21	±	0.44 [a]	2.93	±	0.89 [a]
C17:0	0.32	±	0.07 [a]	0.36	±	0.05 [a]	0.34	±	0.05 [a]
C17:1 c	0.24	±	0.03 [a]	0.23	±	0.05 [a]	0.24	±	0.03 [a]
C18:0	7.25	±	0.92 [a,b]	7.80	±	0.93 [a,b]	6.70	±	0.98 [a]
C18:1 t*9*	0.56	±	0.08 [b]	0.42	±	0.07 [a]	0.39	±	0.1 [a]
C18:1 t*10*-t*11*	0.65	±	0.15 [a]	0.89	±	0.29 [b]	0.70	±	0.08 [a,b]
C18:1 c*9*-t*12*-t*15*	32.53	±	1.8 [a]	29.01	±	1.18 [b]	33.67	±	3.08 [a]
C18:1 c*11*	3.12	±	0.32 [a]	2.11	±	0.05 [b]	3.26	±	1.21 [a]
C18:1 c*12*-c*13*	0.25	±	0.04 [a]	0.27	±	0.03 [a]	0.38	±	0.22 [a]
C18:1 c*15*-c*16*	0.31	±	0.06 [a]	0.28	±	0.02 [a]	0.34	±	0.12 [a]
C18:2 *n6*	9.74	±	1.87 [a,b]	8.86	±	0.59 [a]	10.78	±	0.98 [b]
C18:3 *n6*	0.19	±	0.04 [a]	0.19	±	0.04 [a]	0.26	±	0.07 [a]
C19:1	0.10	±	0.01 [a]	0.08	±	0.01 [a]	0.13	±	0.03 [b]
C18:3 *n3*	1.09	±	0.55 [a,b]	0.84	±	0.13 [a]	1.35	±	0.42 [b]
C18:2 c9, t11 (CLA)	0.29	±	0.07 [a]	0.38	±	0.15 [a]	0.35	±	0.09 [a]
C20:0	0.34	±	0.06 [b]	0.26	±	0.05 [a]	0.22	±	0.04 [a]
C20:1	0.61	±	0.11 [b]	0.48	±	0.06 [a]	0.45	±	0.12 [a]
C20:2 *n*6	0.38	±	0.14 [b]	0.24	±	0.01 [a]	0.19	±	0.04 [a]
C20:3 *n*6	0.53	±	0.18 [b]	0.32	±	0.05 [a]	0.25	±	0.05 [a]
C20:4 *n*6 (AA)	0.49	±	0.07 [b]	0.28	±	0.06 [a]	0.36	±	0.14 [a]
C22:0	0.17	±	0.09 [a]	0.14	±	0.05 [a]	0.11	±	0.02 [a]
C20:5 *n*3 (EPA)	0.20	±	0.03 [b]	0.13	±	0.02 [a]	0.15	±	0.02 [a]
C22:4 *n*6	0.15	±	0.08 [b]	0.06	±	0.01 [a]	0.08	±	0.04 [a]
C22:5 *n*3 (DPA)	0.20	±	0.06 [a]	0.14	±	0.05 [a]	0.19	±	0.07 [a]
C22:6 *n*3 (DHA)	0.37	±	0.09 [b]	0.14	±	0.05 [a]	0.23	±	0.1 [a]

Table 1. Individual fatty acid composition (expressed in % wt/wt of total FAME) in colostrum, pre-term and term human milk samples. Mean ±SD with the same superscript letter are not significantly different at the 0.05 probability level.

Classes of fatty acids	Colostrum			Pre-Term			Term		
	mean		**SD**	**mean**		**SD**	**mean**		**SD**
SFA	44.57	±	3.22 [a]	51.62	±	0.42 [b]	42.52	±	2.49 [a]
MC-SFA (C12-C16)	35.15	±	1.89 [a]	43.06	±	1.12 [b]	36.48	±	3.10 [a]
LC-SFA (>C17)	8.09	±	1.11 [a,b]	8.57	±	1.00 [b]	7.37	±	1.01 [a]
MUFA	41.78	±	2.75 [a]	36.79	±	0.56 [b]	43.29	±	1.4 [a]
PUFA	13.65	±	2.25 [a]	11.59	±	0.63 [b]	14.19	±	1.19 [a]
UFA	55.43	±	3.22 [a]	48.38	±	0.42 [b]	57.48	±	2.49 [a]
TFA	1.74	±	0.26 [a]	1.70	±	0.3 [a]	1.55	±	0.18 [a]
***n*3-PUFA**	1.87	±	0.67 [a]	1.25	±	0.11 [b]	1.91	±	0.49 [a]
***n*6-PUFA**	11.50	±	1.69 [a]	9.96	±	0.64 [b]	11.93	±	1.17 [a]
***LC-PUFA (C>20-24*)**	2.34	±	0.4 [b]	1.32	±	0.14 [a]	1.45	±	0.4 [a]
AA/DHA	1.68	±	0.26 [a]	2.31	±	1.64 [a]	1.38	±	0.39 [a]
SFA/UFA	0.81	±	0.11 [a]	1.07	±	0.02 [b]	0.74	±	0.08 [a]

Table 2. Classes of fatty acid (expressed in % wt/wt of total FAME) in colostrum, pre-term and term human milk samples. Mean ±SD with the same superscript letter are not significantly different at the 0.05 probability level.

Saturated fatty acid (SFA) fraction

Saturated fatty acids (SFA) constitute 44.57 %, 51.62 % and 42.52 % of total FAs in colostrum, pre-term and term milks, respectively. These values fall within a European Range and, according to previously studies, the average value of SFA content of term milks ranges from 39.0 % to 51.3 % of total FAs, whereas the SFA content of colostrum ranges from 37.24 % to 46.88 % of total FAs (Gibson and Kneebone 1981; Koletzko 1992; Hayat et al., 1999a and 1999b; Fidler and Koletzko 2000; López-López et al., 2002; Silva et al., 2005).

Pre-term milks showed a significantly higher content of total SFA compared to term milk samples, while no significant differences were observed in SFA, including MC-SFA and LC-SFA contents, between colostrum and term milks. As regards the individual SFA, the highest contents of lauric (C12:0), myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids were detected in pre-term milk. Therefore, the concentration of MC-SFA and LC-SFA was significantly affected by gestational age. Although no significant differences were highlighted between full-term colostrum and term milks, the mean value of MC-SFA (C12:0, C14:0 and C16:0) showed a reduction as lactation progressed. Overall, C16:0 was the major SFA quantified in all human milk samples. Generally, the concentration of C16:0 represents 20-25 % of human milk FAs (Innis et al., 1994). It is well established that the high percentage of C16:0 in human milk is a potential source of energy (Koletzko et al., 1992; Hayat et al., 1999b), especially for pre-mature breastfed newborns.

The differences found in the MC-SFA content and composition of colostrum, pre-term and term human milks can either be related to the variations in mammary *de novo* synthesis capability, or

associated with the diet consumed by the lactating women (Gibson and Kneebone 1981; Bitman et al., 1983; Silva et al., 2005; Innis 2007). In particular, there is some evidence that the high intakes of carbohydrates by lactating women results in an increase in these FAs (Silva et al., 2005; Innis 2007). Moreover, FA with a short chain length are easily and more readily absorbed than longer-chain FAs by newborns and pre-mature babies and, therefore, differences in the MC-SFA detected among the maternal groups may be also related to digestive system capacity of the infants who are born at different gestational age (Bitman et al., 1983).

Long-chain saturated fatty acids (LC-SFA, C > 17), arising from diet or adipose tissue, were significantly higher in pre-term milks sample than in term milks. Moreover, the sum of FA with 17 or more carbon atoms did not significantly vary when comparing colostrum to pre- or term human milks. Human milk samples collected at different gestational age during this investigation showed a low value of LC-SFA (8.09 % colostrum, 8.57 % pre-term, 7.37 % term) compared to previous studies, where their mean concentration ranged from 23.8 % to 30 % (Kneebone et al., 1985; Jensen 1996; Silva et al., 2005).

Unsaturated fatty acid (UFA) fraction

The unsaturated fraction account for 55.43 %, 48.38 % and 57.48% of the total FAs in colostrum, pre-term and term milks, respectively. The major unsaturated fraction of all samples was represented by monounsaturated fatty acids (MUFA), where oleic acid (C18:1c9), considered to be an important source of energy and a structural component, was the predominant FA. Term milks showed the highest content of UFA. In agreement with a previously study (Mólto-Puigmartí et al., 2011), no significant difference as observed between full term colostrum and term milks for the MUFA level. The lowest content of MUFA was determined for pre-term milks. Thus, in this study, the content of MUFA was not affected by stage of lactation, but significant differences were observed according to the duration of pregnancy between pre-term and term mothers.

Polyunsaturated fatty acids (PUFA) were 13.65 %, 11.59 % and 14.19 % in colostrum, pre-term and term milks, respectively. In all human milk samples, linoleic acid (C18:2*n*6) and α-linolenic acid (C18:3*n*3), which are considered essential because cannot synthesized by mammary cells, constituted the major components of the PUFA fraction. Therefore, the levels of C18:2*n*6 and C18:3*n*3 secreted in milk are probably related to maternal dietary fat composition.

In particular, the C18:2*n*6/C18:3*n*3 ratio in colostrum (10.00 ± 2.75 %), pre-term (10.95 ± 2.58 %) and term (8.78 ± 3.03) milks was in the desirable range (5:1 to 15:1) established by ESPGAN Committee in Nutrition 1991, and no significant differences ($p > 0.05$) were observed between colostrum, pre-term and term milks.

Just as for the MUFA content, pre-term milks showed a significantly lower concentration of PUFA compared to colostrum and term milks, whereas no significant differences were found between full-term colostrum and term milks. The same significant differences between human milk samples were also found for *n*3-PUFA and *n*6-PUFA levels.

Compared to human milks used in this study, colostrum showed a significantly higher content of long-chain polyunsaturated fatty acids (LC-PUFA, C > 20-24) than term milks, while no significant differences were detected between term and pre-term milks. The levels found for bioactive FAs, including C20:4*n*6 (AA), C20:5*n*3 (EPA), C22:6*n*3 (DHA), were significantly higher in colostrum samples than in pre-term and term milks. However, the ratio of AA/DHA did not change between samples secreted at different gestational age. These findings have confirmed earlier studies in which colostrum samples showed a high concentration of AA and DHA (Bitman et al., 1983; Xiang et al., 2000; Mólto - Puigmartí et al., 2011) and pre-term milks the lowest contents (Jensen 1999). Since pre-mature birth is an abnormal event, the lowest proportion of bioactive FAs, can be due to immaturity of mammary gland (Jensen 1999). Full-term colostrum samples showed a higher concentration of LC-PUFA and a lower concentration of *n*-3 and *n*-6 essential fatty acids (EFA) than term milks. These changes can probably be due to the biological variability of lactating women at different gestational age and dietary FA intake. It is assumed that the breastfed newborns during the first week after birth have a high requirements for LC-PUFA, because AA and DHA are important structural components of cell membranes and are involved in numerous basic life process affecting the infant growth and nervous system development (Elias and Innis 2001; Xiang et al., 2000; van Goor et al., 2009; Bortolozo et al.,2013).

The total concentration of total *trans* FAs (TFA) were found to be less than 2 % of total FAs in colostrum, term and pre-term milk samples. This value was lower compared to the range values from 2 to 18 % of TFA reported in several studies (Chappell et al., 1985; Innis and King 1999, Hayat et al., 1999a; Friesen and Innis 2006). Moreover, although the TFA concentrations decrease from colostrum to mature milks, no significant differences were reported between different groups of mother's milk. Statistical analysis ($p < 0.05$) of cis-9, trans-11 C18:2 (rumenic acid, CLA) did not show differences between milk samples. Therefore, it is possible to consider that the CLA and TFA concentrations were not related with length of gestation or stage of lactation but probably that their concentrations are correlated with intake of dietary bioactive FAs by lactating mothers.

4. Conclusion

The outcome of the current study showed how the bioactive FAs composition of human milks varies with gestational age and throughout lactation. Interestingly, a significantly higher content of

SFA (MC-SFA and LC-SFA) and significantly lower content of *n*-3 and *n*-6 PUFA as detected in pre-term than in term milks and colostrum samples. Moreover, comparing the LC-PUFA of full term colostrum and breast milk from mothers of term infants, AA and DHA significantly decreased throughout lactation. These differences probably reflect the nutritional needs of the newborns, especially for the adequate energy supply and bioactive FAs intake.

Milk from mothers of pre-term infants provide a high saturated fraction to meet the energy request and the functional development of digestive system of infants, but it is likely that the low-LC-PUFA concentration does not satisfy the bioactive FAs requirement of pre-mature babies.

The FA composition of full term colostrum probably reflects the adaptation of mammary gland to secreting required quantities of LC-PUFA for the growth and neural development of the newborns, especially during the first week after birth. During the lactation period, the LC-PUFA concentration decrease and *n*3- and *n*6-PUFA levels increased, but not significantly.

Presumably, as the consequent of the differences in functional maturity of the mammary gland and/or dietary fat and carbohydrates intakes by Irish mothers, a quantitative variability of bioactive FA composition was observed in the current human milk study.

This preliminary investigation was conducted to improve the current knowledge regarding the bioactive FA profile of human milk and to better understand the differences between colostrum, pre-term and term milks. These results could be useful to design a functional infant formula in order to ensure the required bioactive FA intake of infants during early life, particularly in supporting the growth and development of pre-mature babies.

In order to extend the information regarding lipid fraction of human milk collected at different gestational age and lactating period, further studies will be done on phospholipids and cholesterol contents. Moreover, new human milk samples should be collect at different stages of lactation from mothers delivered at term, pre-term and very-preterm. In this way, it will be possible to have a high representativeness of a larger population and the study will be able to better satisfy the neonatal lipid requirements.

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Final Conclusions

Milk and dairy products are recognized to have an important role in human nutrition due to the content of numerous “bioactive” components with potential health benefits. Bioactive components comprise specific proteins, protein-derived peptides, lipids and carbohydrates. Moreover, physico-chemical, technological and organoleptic properties of milk proteins and lipids influence the processability and functionality of milk as a valuable raw material of dairy products and as a nourishment for the newly-born babies, children and adults.

For these reasons, the evaluation of bioactive lipids were performed in PUFA-enriched dairy products from bovine milk and human milk samples by traditional and innovative analytical techniques (gas-chromatography, fast gas-chromatography, liquid chromatography and spectroscopy analysis). Additionally, proteomic methodologies and techniques (including 1-dimensional electrophoresis, 2-dimensional electrophoresis and enzyme assays) were used to characterize the differences occurring in colostrum, pre-term and term milks to better understand the changes occurring in the protein profile of human milk during different gestational age and stage of lactation.

The results achieved during the research activity on lipid profile of dairy products have shown the possibility to modulate the bioactive lipids by diet integration of dairy cows with unsaturated fat source (extruded linseed) in order to obtain functional dairy products and value-added by-products.

The proteomic and lipidomic outcomes related to the human milk project work may contribute to improve the knowledge about pre-term milk composition with the aim to develop fortified infant formulas and therefore, satisfy the requirements of premature babies and guarantee optimal growth conditions of infants during early life.

In conclusion, qualitative and quantitative analysis of bioactive compounds using fast and innovative analytical techniques could be useful for the milk- and dairy-industry to design functional ingredients and foods with new technological properties and/or specific biochemical, physiological and nutritional characteristics.