PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
ESCUELA DE INGENIERÍA

BIOAVAILABILITY OF INTERESTERIFIED LIPIDS IN FOOD EMULSIONS

MARIEL IRMA FARFÁN MARTÍNEZ

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences.

Advisor:

PEDRO BOUCHON AGUIRRE

Santiago de Chile, July, 2014

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To my family

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ABSTRACT

The importance of lipids in food goes beyond their role as a source of energy and nutrients. They are also partially responsible for key organoleptic properties, such as texture or flavor release, and satiety, which are difficult to duplicate in fat-free formulations. In addition, lipids have been associated to the development of numerous diseases, but also, to the prevention of several ones, mainly due to the unique effect of essential fatty acids. Unfortunately, physicochemical and nutritional properties of lipids are not frequently assembled. There are fats whose physical properties are suitable for specific applications, but are not nutritionally recommended. On the other hand, some lipids may have an excellent fatty acids profile, but their distribution in the triacylglycerol molecule may not allow an optimal absorption. In addition, the food matrix where lipids are located may be also a key factor to determine the extent to which lipids are absorbed.

The hypothesis of this study was that through enzymatic interesterification it is possible to synthesize a structured fat with nutritional advantages –high in n-3 and zero *trans* fatty

acids- and a melting profile similar to a commercial fat which bioavailability may be modulated through the new TAG structure, and by emulsion design.

Accordingly, the main objective of this thesis was to get a better understanding of the effect of enzymatic interesterification, in the synthesis of plastic fats, as well as the effect of emulsification, in the *in vivo* postprandial bioavailability of fatty acids.

First, the effect of interesterification on lipids structuring was analyzed. To do so, chemical and enzymatic interesterifications of binary blends were compared, in order to obtain structured lipids with zero *trans* fatty acids and high linolenic acid content, and adequate functional properties. Fully hydrogenated soybean oil (FHSBO) and walnut oil (WO) blends were used as raw materials. Sodium methoxide and Lipozyme TL IM were used as chemical and enzymatic catalysers, respectively. FHSBO/WO mass-ratios of 20/80, 40/60 and 60/40 were evaluated, and total interesterification was determined by stabilization of the solid fat content (SFC). Chemical interesterifications were stabilized at 10 min for the 20/80 blend and at 15 min for the 40/60 and 60/40 blends. Whereas, all enzymatically interesterified blends were completed at 120 min. A significant reduction in the SFC for all the mass ratios was produced after completing the chemical or the enzymatic interesterification. Chemically and enzymatically interesterified FHSBO/WO mass ratio 40/60 showed the plastic curve of an all-purpose-type shortening rich in polyunsaturated fatty acids, especially high in linolenic acid C18:3n3 and with zero *trans*. Enzimatic interesterification was chosen for additional studies, due to its stereospecificity.

Since, the fatty acids profile is a critical factor in the nutritional properties of fats, but also stereochemistry may play a fundamental role, the bioavailability of fatty acids in the interesterified mix was analyzed. To do so, linseed-oil and palm-stearin blends were compared to their interesterified mix, when using a sn-1,3 stereospecific lipase, to determine if there was any difference in terms of faty acids availability when using this technology. Both blends had the same fatty acids profile, but a different SFC, due to the change in TAGs stereochemistry. Sprague-Dawley male rats were fed with either IE or nIE blends through an intragastric feeding tube after 18 h fasting, and physiological serum was

used as control. Blood samples were collected after test meals administration and postprandial plasmatic fatty acids profiles were determined. Results showed that modification of the melting profile through interesterification, without altering the bioavailability determined by sn-2 stereochemistry, could delay lipid absorption at the beginning, with peaks of absorption of C18:3n3 at 1.5 and 4.5 h for IE and nIE test meals, respectively, but it had no effect on total lipid absorption.

Food structure is also responsible of lipids bioavailability. An appropriate design of the food matrix could either, restrict or facilitate the release of fatty acids to let them available for absorption, such as in emulsions. To get a better understanding of this phenomenon, we evaluated the effect of emulsification, as well as the use of different surface-active agents, on the postprandial bioavailability of interesterified-lipids in O/W emulsions after oral gastric feeding Sprague-Dawley rats. Stable O/W emulsions, prepared with sodium caseinate (Sc) or chitosan (Ch) were fed as test meals. A non-emulsified blend was used as control (C), and physiological serum was the blank (B). Sprague-Dawley rats were intragastrically tube-fed with any Sc, Ch, C or B test meals and postprandial blood samples were collected. Results showed that emulsification may increase lipid absorption, as determined after feeding Sc emulsions, which was 20% higher than C in total FAs. However, this result could not be generalized. Interesterified-lipids that were emulsified with Ch were equally absorbed as those contained in non-emulsified interesterified-lipids/distilled-water blends. It was suggested that this could be due to a limited access of pancreatic lipases to lipids due to the pH increase in the duodenum (pH>pKa), which may reduce Ch solubility and may counterbalance the positive effect of size reduction. This suggests that emulsification, as a food matrix design tool, is not the only responsible for a higher bioavailability, but the interactions that occur during digestion between the stabilizer and surroundings, are also crucial.

Overall, this study confirms that interesterification seems to be an appropriate tool to synthesize a structured fat with a good nutritional profile and adequate functional properties. Certainly, food matrix design is also of great concern, as is reflected in this study, which set-ups the basis for future research with respect to the need to get a better

understanding of the interactions between nutrients and its environment. Different approaches can be used to design an appropriate food structure, which may maximize or reduce lipids and/or specific fatty acids bioavailability, as needed. As such, this study contributes to the growing field of food structure design and highlights how structure can be tailored to meet new consumers' demands.

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PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
ESCUELA DE INGENIERÍA

BIODISPONIBILIDAD DE LIPIDOS INTERESTERIFICADOS EN EMULSIONES ALIMENTARIAS

Tesis enviada a la Dirección de Investigación y Postgrado en cumplimiento parcial de los requisitos para el grado de Doctor en Ciencias de la Ingeniería.

MARIEL IRMA FARFÁN MARTÍNEZ

RESUMEN

La importancia de los lípidos en los alimentos va mas allá de su rol como fuente de energía y nutrientes. También son parcialmente responsables de propiedades organolépticas clave, tales como textura, liberación del sabor, y saciedad, la cual es difícil de duplicar en formulaciones libres de grasa. Además, los lípidos han sido asociados al desarrollo de numerosas enfermedades, pero también a la prevención de varias otras, principalmente debido al efecto de ácidos grasos esenciales. Desafortunadamente, las propiedades fisicoquímicas y nutricionales de los lípidos no se encuentran juntos con frecuencia. Existen grasas cuyas propiedades físicas son aptas para aplicaciones específicas, pero no son nutricionalmente recomendables. Otros, en cambio, pueden tener un excelente perfil de ácidos grasos, pero su distribución en la molécula de triacilglicerol podría no permitir una absorción óptima. Además, la matriz alimentaria donde los lípidos están ubicados, puede ser un factor clave para determinar el grado en el cual los lípidos son absorbidos.

La hipótesis de este estudio fue que a través de interesterificación enzimática es posible sintetizar lípidos estructurados con un buen perfil de ácidos grasos –alto en acidos grasos n-3 y cero *trans*- y un perfil de fusión similar al de una grasa comercial, cuya

biodisponibilidad puede ser modulada, además de la nueva estructura de TAG, a través del diseño de emulsiones.

De acuerdo a esto, el objetivo principal de esta tesis fue conseguir un mejor entendimiento del efecto de la interesterificación enzimática, en la síntesis de grasas plásticas, así como de la emulsificación, en la biodisponibilidad postprandial de lípidos *in vivo*.

Primero se analizó el efecto de la interesterificación en la estructuración de lípidos. Para ello, se compararon las interesterificaciones química y enzimática de mezclas binarias en la obtención de lípidos estucturados con cero *trans* y un alto contenido de ácido linolénico (C18:3n3), además de adecuadas propiedades funcionales. Aceite de soya full hidrogenado (fully hydrogenated soybean oil, FHSBO) y aceite de nuez (walnut oil, WO) fueron usados como materias primas. Metóxido de sodio y Lipozyme TL IM fueron usados como catalizadores químico y enzimático, respectivamente. Mezclas FHSBO/WO en relaciones de masa de 20/80, 40/60 y 60/40 fueron evauladas, y la interesterificaión total se determinó por la estabilización en su contenido de grasa sólida (solid fat content, SFC). La mezclas interesterificadas químicamente fueron estabilizadas a los 10 min para al mezcla 20/80 y a los 15 min para las mezclas 40/60 y 60/40. Mientras, todas las mezclas enzimáticamente interesterificadas se completaron a los 120 min. Una reducción significativa en el SFC para todas las composiciones se produjo después de completar las interesterificaciones química o enzimática. Las mezclas 60/40 interesterificadas química y enzimáticamente mostraron la curva plástica de un *shortening* multipropósito, rico en ácidos grasos poliinsaturados, especialmente en C18:3n3 y cero *trans*. La interesterificación enzimática fue escogida para estudios adicionales debido a su estereoespecificidad.

Dado que, el perfil de ácidos grasos es un factor crítico en las propiedades nutricionales de las grasas, pero también la estereoquímica juega un rol fundamental, la biodisponibilidad de ácidos grasos en las mezclas interesterificadas fue analizada. Para ello, mezclas de aceite de linaza (linseed oil, LO) y esterarina de palma (palm stearin, PS) fueron comparadas con sus mezclas interesterificadas usando una lipasa estereoespecífica en las posiciones sn 1 y 3, para determinar si había alguna diferencia en términos de biodisponibilidad cuando se usaba esta tecnología. Las dos mezclas tenían el mismo perfil

de ácidos grasos, pero distinto SFC, debido al cambio en la estereoquímica de los triacilgliceroles. Ratas Sprague-Dawley macho fueron alimentadas con las mezclas interesterificada (IE) o no interesterificada (nIE) a través de un tubo de alimentación bucogástrico luego de 18 h de ayuno. Se utilizó suero fisiológico como control. Se tomaron muestras de sangre luego de la administración de los *test meals* y se determinó el perfil de ácidos grasos plasmáticos en el periodo postprandial. Los resultados mostraron que la modificación en los perfiles de fusión de las mezclas a través de la interesterificación, sin alterar la biodisponibilidad determinada por la posición sn-2, puede retrasar la absorción de los lípidos al principio de la digestión, con peaks de absorción de C18:3n3 a 1,5 y 4,5 h postadministración, para IE y nIE, respectivamente, pero esto no tiene un efecto en la absorción total de los lípidos.

La estructura alimentaria es también responsable de la biodisponibilidad de lípidos. Un diseño apropiado de la matriz alimentaria podría restringir o facilitar la liberación de ácidos grasos y dejarlos disponibles para la absorción, tal como en la emulsificación. Para comprender mejor éste fenómeno, evaluamos el efecto de la emulsificación, así como el uso de diferentes agentes emulsificantes, en la biodisponibilidad postprandial de lípidos estructurados en emulsiones O/W en ratas. Emulsiones O/W estables, preparadas con caseinato de sodio (Sc) o quitosán (Ch) fueron dosificadas como *test meals*. Una mezcla no emulsificada fue utilizada como control (C), y suero fisiológico fue usado como blanco (B). Ratas Sprague-Dawley fueron intragástricamente alimentadas con Sc, Ch, C o B, y se tomaron muestras de sangre en el periodo postprandial. Los resultados mostraron que la emulsificación puede aumentar la absorción de lípidos, como se determinó luego de la administración de emulsiones Sc, cuya absorción de AG totales es 20% mayor que el control. Sin embargo, éste resultado no puede ser generalizado. Los lípidos interesterificados que fueron emulsificados con quitosán, fueron igualmente absorbidos que aquellos en la mezcla lípidos interesterificados/agua destilada, no emulsificados. Se sugirió que esto puede ser debido a un acceso limitado de la lipasa pancreática a los lípidos. El mayor pH que se encuentra en el duodeno (pH>pKa quitosan) puede reducir la solibilidad de Ch, y puede contrapesar el efecto positivo de la pre-emulsificación. Esto sugiere que la emulsificación, como herramienta para el diseño de matrices alimentarias,

no es la única responsable de una mayor biodisponibilidad, sino que las interacciones que ocurren entre el estabilizante y los alrededores durante la digestión, es también crucial.

En general, este estudio confirma que la interesterificación parece ser una herramienta apropiada para la sintesis de grasas estructuradas con un buen perfil nutricional y propiedades funcionales adecuadas. Sin duda, el diseño de matrices alimentarias es también un asunto de interés, como se refleja en este estudio, el cual establece las bases para una futura investigación con respecto a la necesidad de conseguir un mejor entendimiento de las interacciones entre los nutrientes y su entorno. Distintos enfoques pueden ser utilizados para el diseño de una estructura alimentaria apropiada, la cual puede maximizar o reducir la biodisponibilidad de lípidos y/o ácidos grasos específicos, según necesidad. Como tal, este estudio contribuye al creciente campo del diseño de estrucuras alimentarias y resalta como la estructura puede ser personalizada para satisfacer las demandas de los consumidores.

Miembros de la Comisión de Tesis Doctoral
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LIST OF PAPERS

This thesis is based on the following papers, referred in the text by respective chapters:

Chapter 2. Farfán, M., Bouchon, P. (2014). Comparison of chemical and enzymatic interesterification of fully hydrogenated soybean oil and walnut oil to produce a fat base with adequate nutritional and physical characteristics. *Food Technology and Biotechnology*, Submitted.

Chapter 3. Farfán, M., Villalón, M.J., Ortíz, M.E., Nieto, S., and Bouchon, P. (2013). The effect of interesterification on the bioavailability of fatty acids in structured lipids. *Food Chemistry*, 139: 571-577.

Chapter 4. Farfán, M., Villalón, M.J., Ortíz, M.E., Nieto, S., and Bouchon, P. (2015). *In vivo* postprandial bioavailability of interesterified-lipids in sodium-caseinate or 2 chitosan based O/W emulsions. *Food Chemistry*,171: 266-271.

PROCEEDINGS

Some of this work has been presented at international congresses as follows:

Farfán, M., Bouchon, P., Álvarez, A., y Gárate, A. (2009). Comparación de los métodos de interesterificación química y enzimática en la obtención de lípidos estructurados. *VII Congreso Iberoamericano de Ingeniería de Alimentos*, Bogotá, Colombia. Poster.

Farfán, M., Villalón, M.J., Ortíz, M.E., Nieto, S. and Bouchon, P. (2013). Effect of Enzymatic Interesterification on Fatty Acids Bioavailability in Structured Lipids. *9th World Congress of Chemical Engineering (WCCE9)*, Seoul, Korea. 18-23 Agosto. Oral.

Farfán, M., Villalón, M.J., Ortíz, M.E., Nieto, S. and Bouchon, P. (2013). Effect of enzymatic interesterification on fatty acids bioavailability. *5th International Symposium on Delivery of Functionality in Complex Food Systems- Physically-Inspired Approaches from the Nanoscale to the Microscale*, Haifa, Israel. 30 Septiembre - 4 Octubre. Oral.

1 INTRODUCTION

The importance of lipids in food goes beyond their role as a source of energy and nutrients (Watkins and German, 2002). They also add flavor, texture, and satiety to foods, properties that are difficult to duplicate in fat-free formulations (Akoh, 2002). As such, lipids cannot be eradicated from food products even though intake of fats has been shown to be related to the development of numerous diseases. However, fatty acids (FAs) such as n-9, n-6, and n-3 families, play an important role in the prevention of diseases and, more importantly, some of them perform essential functions in the development of organs and as a precursor for mediating biochemical and physiological responses (Narayan *et al*., 2006).

Essential fatty acids

We cannot cut lipids out of our diet entirely, and this would not be advisable in any case. FAs perform many functions that are necessary for normal physiological function and good health (Spector, 1999). The human body can produce most of them using other nutrients. However, there are essential FAs such as n-3 and n-6 that are not synthesized in the body and must therefore be obtained from food products. Essential FAs have membrane function. C18:2n-6, C20:4n-6 (arachidonic acid, AA), and C22:6n-3 (docosahexaenoic acid, DHA) take some of the lipids that form the permeable barrier of the epidermis and influence plasticity and membrane fluidity in the brain or retina, becoming essential for brain function and vision. Also, AA and C20:5n-3 (eicosapentaenoic acid, EPA) are substrate for eicosanoids synthesis (Spector, 1999; Whelan, 2008). Those derived from AA are mostly pro-active, whereas EPA-derived ones are inhibitory (Schmitz and Ecker, 2008). Given that these regulatory molecules are produced through the same metabolic pathway, the amount of essential FA ingested is not trivial. Excessive amounts of n-6 and a very high n-6/n-3 ratio such as those found in a Western diet of 15-16.7/1 promote the pathogenesis of many diseases (Simopoulos, 2004). Thus, an optimal ratio of n-6/n-3 varies from 1-4/1, but absolute amounts of n-3 and n-6 and the individual essential FAs must be considered (Simopoulos, 2004; Stanley, 2007; Riediger *et al*., 2008).

Table 1.1: Melting temperatures of fatty acids (O'Brien, 2004).

Fatty acid	Abbreviation	Carbon atoms	Double bonds	Melting point (°C)
Butyric	C4:0	4	0	-8.0
Caproic	C6:0	6	0	-3.4
Caprylic	C8:0	8	0	16.7
Capric	C10:0	10	0	31.6
Lauric	C12:0	12	0	44.2
Myristic	C14:0	14	0	54.4
Myristoleic	C14:1n-5	14	1 *cis*	18.5
Palmitic	C16:0	16	0	62.9
Palmitoleic	C16:1n-7	16	1 *cis*	45.0
Margaric	C17:0	17	0	61.3
Margaroleic	C17:1n-9	17	1 *cis*	57.5
Stearic	C18:0	18	0	69.6
Oleic	C18:1n-9	18	1 *cis*	16.0
Elaidic	C18:1n-9	18	1 *trans*	43.7
Linoleic	C18:2n-6	18	2 *cis*, *cis*	-7.0
Linolelaidic	C18:2n-6	18	2 *trans*, *trans*	56.0
Linolenic	C18:3n-3	18	3 *cis*, *cis*, *cis*	-13
Arachidonic	C20:0	20	0	75.3
Behenic	C22:0	22	0	79.9
Erucic	C22:1n-9	22	1 *cis*	33.5
Lignoceric	C24:0	24	0	84.2

Lipids intake: risks and benefits

The positive effects mentioned above are related to the intake of essential FAs, and include the most intensively studied cardioprotective and anti-inflammatory effects as well as the neuroprotective and anticancer effects of n-3 essential FAs, which can mainly be explained by changes in eicosanoids metabolism (Uauy and Valenzuela, 2000; Kang, 2005; Narayan *et al*., 2006). Monounsaturated FA C18:1n-9 and C18:2n-6 also have a protective effect against cardiovascular diseases, mainly through reduction of total and LDL-cholesterol and increased HDL-cholesterol (Kris-Etherton *et al*., 2004; Thijssen and Mensink, 2005). Despite this beneficial effect, eicosanoids derived from n-6 FAs have been associated with many chronic conditions such as cardiovascular, autoimmune, and inflammatory diseases (Simopoulos, 2004; Schmitz and Ecker, 2008). Finally, adverse effects have been attributed to the consumption of saturated and *trans* FAs. C12:0, C14:0, and C16:0 are the most potent total and LDL-cholesterol-raising FAs, while *trans* FAs also lower HDL-

cholesterol and increase triglycerides concentrations. In contrast, C18:0 and other saturated FAs (C4:0-C10:0) lower total and LDL-cholesterol concentrations, with a similar effect on serum lipoproteins to that of C18:1n-9. However, C18:0 also reduces HDL cholesterol concentration (Wahrburg, 2004; Thijssen and Mensink, 2005).

Table 1.2: Triglyceride liquidity and functionality (O'Brien, 2004).

TAG/FA pattern	Melting point (°C)	Double bonds	Functionality
Liquidity zone 1: chilled temperature			
Linoleic-linoleic-linoleic	-13.3	6	Nutrition
Oleic-linoleic-linoleic	-6.7	5	Clarity
Palmitic-linoleic-linoleic	-5.6	4	Lubricity
Palmitic-linoleic-oleic	-2.8	3	
Oleic-oleic-linoleic	-1.1	4	
Stearic-linoleic-linoleic	1.1	4	
Liquidity zone 1: room temperature			
Oleic-oleic-oleic	5.6	3	Lubricity
Stearic-linoleic-oleic	6.1	3	Clarity
Palmitic-oleic-oleic	15.6	2	
Stearic-oleic-oleic	22.8	2	
Palmitic-linoleic-palmitic	27.2	2	
Liquidity zone 1: body temperature			
Stearic-linoleic-palmitic	30.0	2	Structure
Stearic-linoleic-stearic	32.8	2	Aeration
Palmitic-oleic-palmitic	35.0	1	Lubricity
Stearic-oleic-palmitic	37.7	1	Moisture
Stearic-oleic-stearic	41.7	1	barrier
Liquidity zone 1: heated temperature			
Palmitic-palmitic-palmitic	56.1	0	Structure
Stearic-palmitic-palmitic	60.0	0	Lubricity
Stearic-stearic-palmitic	61.1	0	Moisture
Stearic-stearic-stearic	65.0	0	barrier

Physicochemical characteristics of lipids

The physical properties of fats are defined by the chemistry of triacylglycerols (TAGs) and FAs. Chain length, unsaturation degree, and configuration of FAs as well as the position in which each FA is attached determine how they will be absorbed and their physical and

functional properties. The most important physical properties of lipids are crystallization and melting (Gunstone, 2006).

Short chain FAs (4 to 10 carbon atoms) are liquid at room temperature, but the melting point increases with the chain length (Table 1.1). Longer saturated FAs are solid. However, unsaturated FAs with the same chain length are liquid at room temperature by effect of unsaturation. Finally, *trans* FAs have similar physical properties to SFAs but generate nutritional concerns. As edible lipids are mainly TAGs, stereochemistry determines the melting profile and crystalline habit, defining the functionality of fats (Table 1.2) (O'Brien, 2004). Palmitic FA C16:0 can provide the desired plasticity, smooth texture, aeration, and creamy properties in food products. However, these properties are present when C16:0 crystallizes in β' form, which is also related to its TAG position and to the FAs composition of fats (Gunstone, 2006; O'Brien, 2004).

Lipids metabolism

The nature of each FA and its position on TAG will determine how easily it will be absorbed. Lipids' metabolism is basically the hydrolysis of TAGs and the resulting release of free FAs and sn-2 MAG, which will eventually be absorbed (Mu and Høy, 2004; Ramírez *et al*., 2001; Phan and Tso, 2001). TAGs are first hydrolyzed by lingual and gastric lipases into the stomach. FAs arranged mainly on sn-3 are released. These enzymes show a higher efficiency in short and medium chain FAs. Between 70 and 90% of total lipids consumed reach the proximal duodenum as TAGs, while the remainder is a mixture of DAGs and FAs. These partially hydrolyzed products, together with chewing process in the mouth, shear forces by gastric motility, and amphipathic qualities of bile salts increase the contact surface between lipids and the aqueous phase, which allows pancreatic lipase action (Jackson and McLaughlin, 2006; Mu and Høy, 2004; Phan and Tso, 2001). Pancreatic lipase must be attached to co-lipase in order to act. Together they preferentially hydrolyze FAs in the sn-1 and sn-3 position of TAG. However, at most 25% of dietary TAGs can be completely hydrolyzed to glycerol and three free FAs (Kris-Etherton *et al*., 2005; Ramírez *et al*., 2001).

Once the FAs have been hydrolyzed, their fate depends on their hydrophobicity, which is determined by their chain length and degree of saturation. Short and medium chain saturated free FAs (C4:0 - C10:0) are absorbed by passive diffusion, are then bounded to albumin to be transported via portal way to the liver. This mode of absorption may occur in the stomach and duodenum (Osborn and Akoh, 2002; Marten *et al*., 2006; Carlier *et al*., 1991). When the chain length increases, the ability of the intestinal content to pass through the aqueous phase diminishes, due to the increased hydrophobicity (Tso *et al*., 2004; Ramírez *et al*., 2001). Longer saturated and unsaturated FAs may be emulsified with bile salts and sn-2 MAG in mixed micelles, which help lipids cross the unstirred water layer and reach the microvilli membrane, where they are absorbed by the jejunal enterocyte (Carlier *et al*., 1991; Ramírez *et al*., 2001). Absorption may occur by passive diffusion at high concentration of any chain length FA, or through a carrier-dependent process where a membrane FA binding protein binds the apolar FA to transfer it inside of the enterocyte, preferably at a lower substrate concentration (Carlier *et al*., 1991; Stremmel, 1988). In the enterocyte, TAGs are resynthesized, packaged into macromolecules -chylomicrons-, and transported by lymphatic mode to peripheral tissues and the liver (Decker, 1996; Ramírez *et al*., 2001).

With an increased melting point resulting from a longer chain length and a lower degree of unsaturation, the solubilities of FAs in the aqueous medium are restricted. As such, FAs with higher melting points are incorporated in the mixed micelle less efficiently and are not absorbed properly (Ramírez *et al*., 2001; Tso *et al*., 2004), while the FA attached to the sn-2 position is always absorbed as sn-2 MAG. Thus, saturated free FAs remain in the gut for longer periods of time and are exposed to interaction with excess luminal calcium to form insoluble calcium-fatty soaps, which reduce saturated FA absorption and increase saturated FA fecal content (Denke *et al*., 1993). Figure 1.1 presents a general outline of the fate of FAs depending on their nature and position in the TAG molecule. Several studies have reported higher excretion of fat when saturated FAs C16:0 and C18:0 are mainly attached to sn-1 and sn-3. These significantly lower absorption efficiencies suggest that the position of FAs in TAGs influences lipids absorption and metabolism.

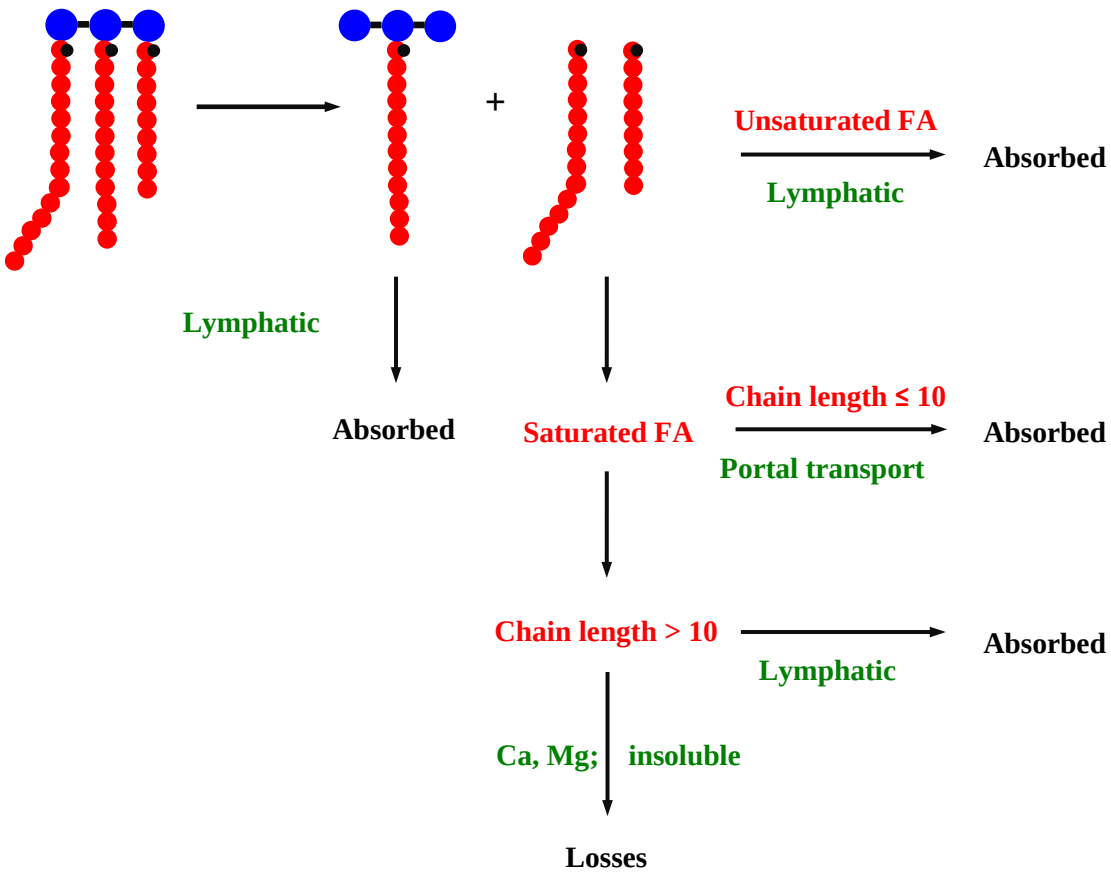


Figure 1.1: Schematic representation of hydrolysis and absorption of TAGs.

This also suggests that highly hydrophobic FAs that melt above body temperature, such as C18:0 (Tm=70°C), have a lower absorption as free FA (Brink *et al*., 1995; Small, 1991). Also, whole TAGs could be absorbed less when they are solid at body temperature. *In vitro* studies (Bonnaire *et al*., 2008) reported slower digestion of solid particles compared to liquid ones when the FAs composition of TAGs is unchanged. Asselin *et al*. (2004) studied the postprandial lipid response of fats with different melting points *in vivo*. They report delayed gastric emptying, lower intestinal fat absorption, and higher excretion of fats in the group that was fed fat with high melting points. This supports the results of studies carried out on humans (Yli-Jokipii *et al*., 2003) where fats studied have 27.6 and 18.3% of solid fat content (SFC) at 30°C. The liquid fat was easily emulsified and therefore more rapidly absorbed than the solid fat. As the position of FAs in the sn-1 and 3 positions or in the sn-2

position partially determines the extent of absorption, the relocation of FAs through lipid modification appears to be the way to control lipid absorption.

Modification of lipids

The usual incompatibility of the technological and nutritional properties of most natural oils and fats may be improved using technological or biological methods in order to expand their uses. Biological methods include genetic engineering and crop or animal control (Gunstone, 2006). Conventional seed breeding and genetic engineering have been used to nutritionally or technologically enhance oils. This makes it possible to reduce levels of saturated FAs and increase levels of C18:1n-9 for nutritional reasons, reduce levels of C18:3n3 that improve oil stability, or increse levels of saturated FAs to add plasticity to fats. Linola™, NuSun™ and Canola oils are commercial oils with improved stabilities and FA profiles obtained through crop modification (Murphy, 2006). In addition, animal diets may be changed to modify the FAs profile of the fat in meat, milk, or eggs, mainly for non-ruminants. Modification of ruminant fats deserves special attention due to the fact that these animals bio-hydrogenate fats during metabolism (Scheeder, 2006).

Blend, fractionation, and hydrogenation

Blend, fractionation, and hydrogenation are technological methods that modify the structure and composition of lipids with the aim of improving their physical, chemical, and nutritional properties. Blend and fractionation are physical processes that produce value-added fats and oils but may produce minor components which could affect their oxidative stability (Gunstone, 2006; O'Brien, 2004). Hydrogenation is a chemical process by which hydrogens are added to the double bonds of unsaturated FAs. It may be carried out completely to obtain fully hydrogenated hardfats with a zero IV (iodine value), a FAs profile of mainly C16:0 and C18:0, and melting points of 56-65°C (List, 2006). If the reaction is only partially completed, an extended plastic range and a good oxidative stability may be reached, but *trans* FAs may be also produced (O'Brien, 2004; Idris and Mat Dian, 2005). *Trans* FAs are largely responsible for these desired physical properties

due to the fact that *trans* FAs are straight chains that behave like saturated FAs. However, *trans* FAs are also responsible for the aforementioned adverse health effects (Hunter, 2006).

Interesterification

Interesterification is a reaction that exchanges FAs within and between TAG molecules and maintains the FA profile and saturation degree of the reagents (Rodrigues and Gioelli, 2003; Karabulut *et al*., 2004). Interesterification products present a different TAGs stereochemistry, and may have melting and crystallization characteristics which differ from the original oil or fat (O'Brien, 2004). Also, nutritional properties may be improved through this process (Klinkesorn *et al*., 2004). Interesterification usually refers to three reactions: acidolysis, alcoholysis, and ester–ester exchange. There are two ways to carry them out: chemical and enzymatically (Xu *et al*., 2006). Interesterification reagents and products are outlined in Figure 1.2.

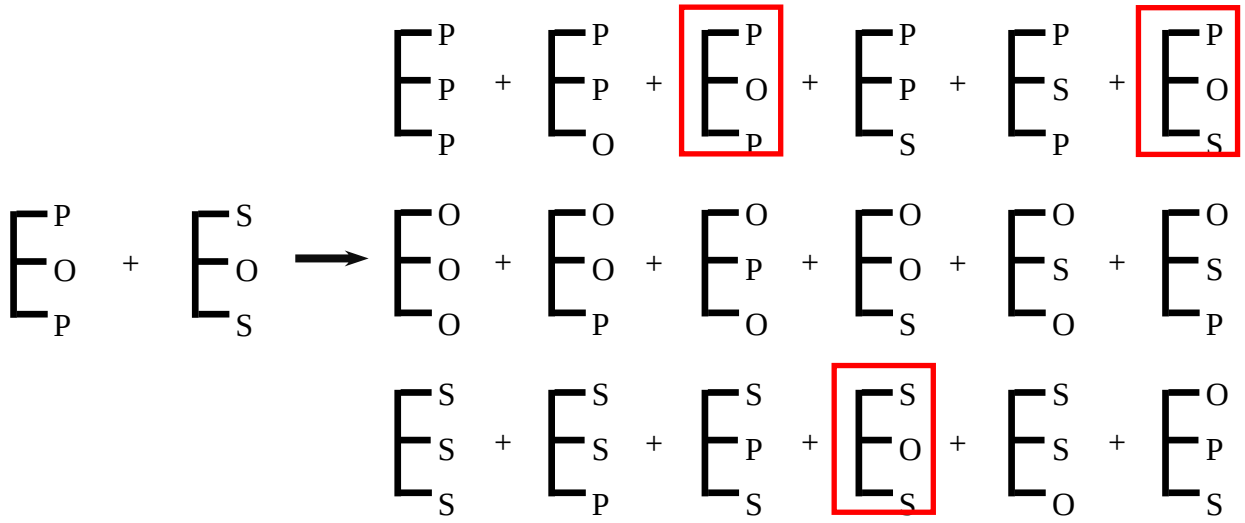


Figure 1.2: Schematic illustration of interesterification. By randomized chemical interesterification, all 18 isomers are theorically produced, while only the boxed isomers may be produced by 1,3-specific enzymatic interesterification.

Chemical interesterification

Chemical interesterification has been used to replace hydrogenation since the 1970s (Xu *et al*., 2006). Chemical interesterification may spontaneously occur at 250°C or higher. However, most processors use metal salts, alkali hydroxide, and mainly alkoxide and alkali metals as initiators of the process, which is now carried out at temperatures of 60-80°C (Liu, 2004; O'Brien, 2004). A color change to brown signals the start of the reaction, which can be completed in 30 min. The reaction is considered complete when it reaches an equilibrium that is based on the composition of the starting material and is predictable based on the laws of probability. Chemical interesterification is stopped by adding water or acid, which must be removed later (Xu *et al*., 2006; O'Brien, 2004). Chemical interesterification is easy to use and scale up, relatively inexpensive, and readily available. However, this process lacks positional specificity, and considerable amounts of side products may be formed, which results in product losses. Furthermore, the use of high temperatures can lead to deterioration of the finished product (Mangos *et al*., 1999; Osborn and Akoh, 2002; Yang *et al*., 2003).

Enzymatic interesterification

It is also possible to increase control over the reaction using commercial lipases from plant, animal, and microbial sources. This is called enzymatic interesterification, a process that was patented in the 1970s (Xu *et al*., 2006). It produces fats with a more defined structure due to the stereospecificity of catalyst. For instance, it is possible to hydrolyze the 1,3-positions, incorporating FA at these sites without changing the FA in the sn-2-position (Wang *et al*., 2006; Hamam *et al*., 2005). As we also see in chemical interesterification, the reaction is complete when it reaches the equilibrium, which is verified by solid fat content control (O'Brien, 2004). The enzymatic process offers milder reaction conditions and a lower degradation of unstable FA and produces fewer side products than the chemical process (Osório *et al*., 2001; Rønne *et al*., 2005). Its greatest advantage is its selectivity. Although lipases may still hydrolyze the 1,3-positions, the process has changed since the 1970s. New cost-effective immobilized lipases offer more opportunities to produce an

inexpensive product with improved properties. Furthermore, the product is easily recovered, and there is minimal waste (Yang *et al*., 2003; Xu *et al*., 2006).

Improvement of lipids through interesterification

Lipid modification changes the nutritional and physical properties of fats and oils. The plastic behavior of fat, which depends on the temperature at which the fat crystallizes and melts, and the ratio and type of solid and liquid fat (Gunstone, 2006) may be altered by interesterification. As this process changes the TAGs profile, which affects the crystalline structure of fats and modifies the SFC, a new and different plastic behavior is obtained. Interesterification can thus modify plasticization, hardness, texture, solubility and other characteristics of lipids that affect organoleptic properties such as spreadability, thickness, and flavor release (O'Brien, 2004; Karabulut *et al*., 2004; Zhang *et al*., 2004). Figure 1.3 shows the SFC of fats blends before and after chemical interesterification. In this case, the randomization of FAs significantly reduces the SFC of blends, which extends the application of these specific fat blends (Petrauskaite *et al*., 1998). Interesterification also generates nutritional improvements. Enzymatic interesterification is a stereospecific process. This makes it possible for instance to introduce FAs of interest while the original FA on the sn-2 position of the TAG remains unchanged. Several studies have reported the production of fats enriched in n-3 polyunsaturated FAs with nutraceutical purposes (Osório *et al*., 2001; Ramírez *et al*., 2001) or in medium chain FAs (Jennings and Akoh, 2001; López-Hernández *et al*., 2005) with a therapeutic aim. Those TAGs have a strategic configuration that allows for improved absorption of n-3 and medium chain FAs, which is especially useful in situations like poor lipid absorption, which is seen in patients with cystic fibrosis (López-López *et al*., 2001; Innis *et al*., 1995). It is also possible to improve both the physical and nutritional properties of fat. Shortenings and margarine fat bases have the required physical properties and are *trans* FA-free. They are produced by interesterification as well (Lee et al., 2008; Gosh and Bhattacharyya, 1997).

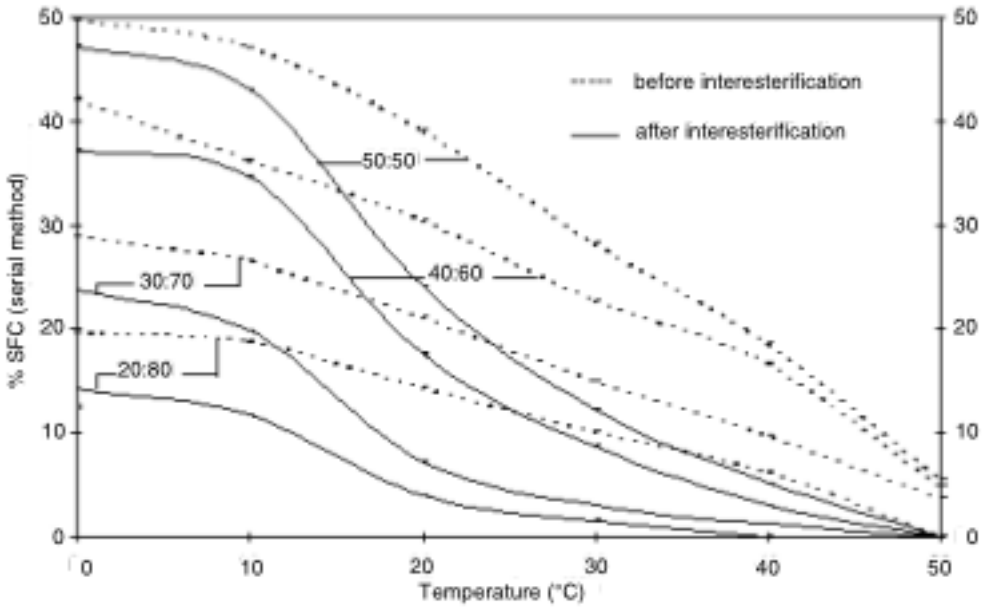


Figure 1.3: Solid fat content (SFC) profiles of blends of palm stearin and soybean oil before and after chemical interesterification (Petrauskaite *et al*., 1998).

Structured lipids

More complete knowledge of lipid metabolism and the powerful tool of interesterification have given researchers the ability to synthesize lipids that meet a specific need and improve their nutritional or functional properties (Osborn and Akoh, 2002). Structured lipids (SLs) are lipids obtained by changing fatty acid composition and/or positional distribution in the glycerol backbone. Commercial SLs like Betapol™ (Lipid Nutrition) and Salatrim (short and long acyltriglyceride molecule) have been produced. Betapol™ is synthesized by enzymatic interesterification to obtain the distinctive fat structure of human breast milk, with about 70% C18:0 on the sn-2 position of TAG. This structure improves fat absorption and reduces C18:0 losses by formation of calcium soaps, which also diminishes calcium availability (Kennedy *et al*., 1999; López-López *et al*., 2001). Salatrim is a family of lipids structured by chemical interesterification of highly hydrogenated vegetable oils with triglycerides of short-chain FAs (C2:0, C3:0 and/or C4:0), with a lower energetic value (5kcal/g) (Softly *et al*., 1994; Smith *et al*., 1994). The ratio of short chain FAs to long chain FAs is varied in order to obtain fats with different physical and functional properties, with the secondary purpose of mimicking conventional fats such as

cocoa butter (Akoh and Min, 2002). Other SLs are synthesized for nutraceutical purposes. Medium chain FAs attached at sn-1 and sn-3 positions of TAG are quickly hydrolyzed and absorbed, while an unsaturated long chain FA is attached to the sn-2 position. In this way, quick energy and essential FA may be provided to patients under enteral nutrition (Lee and Akoh, 1998; Osborn and Akoh, 2002).

Lipids bioavailability

The design and synthesis of fats that meet specific needs is a step forward in the attempt to control the absorption of lipids. However, there are other issues involved in the amount of lipids, or any other nutrient, that is ultimately used by the body. Providing definitions of two concepts will help us arrive at a better understanding of this issue (Parada and Aguilera, 2007; McClements *et al*., 2009).

Bioaccessibility: is the amount of an ingested nutrient that is released from a food matrix and becomes available to be absorbed.

Bioavailability: is the amount of an ingested component that is actually absorbed and becomes available for a metabolic or structural function, or as a source of energy.

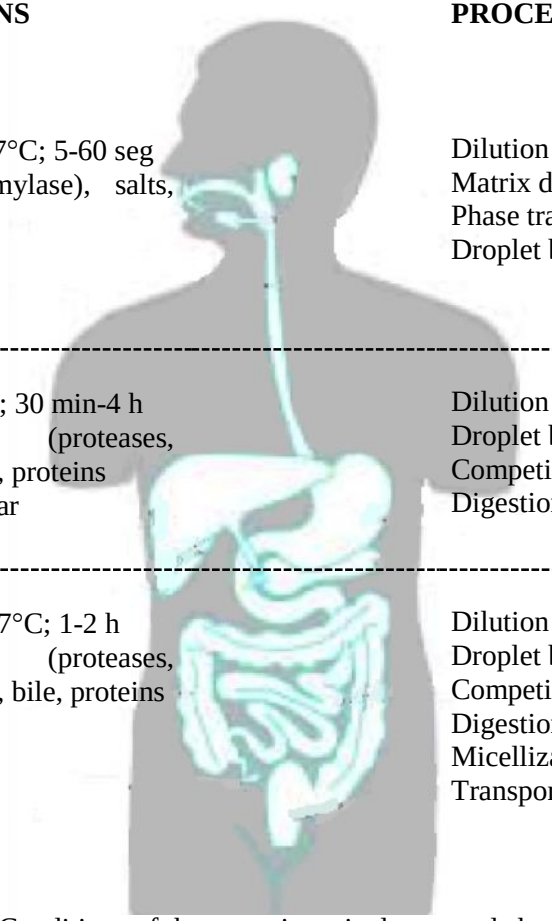
Understanding these concepts is key for a hierarchical design of structures of functional foods.

Lipid bioavailability depends on the bioaccessibility and physiological processes -transport and metabolism- that are affected by the stereochemistry of TAGs and the nature of FAs (McClements *et al*., 2009). As such, lipid modification with the resulting changes in the state of aggregation, or physical properties, alters its bioavailability (Yli-Jokipii *et al*., 2003; Asselin at al., 2004). In fact, randomization of FAs through interesterification may reduce saturated FAs

excretion (Lien *et al*., 1993), or generate a differentiated postprandial lipaemia (Christensen and Høy, 1996; Hodge *et al*., 1999). Enzymatic interesterification may increase the bioavailability of C18:0 and calcium in human milk fat substitutes, as is mentioned above. These changes at the molecular level have shown the effect on lipid bioavailability, which can also be modified through control of the bioaccessibility.

Food structure

The natural organization of a product involves molecules and organelles that are compartmentalized into cells and tissues. The complex systems displayed by processed foods as a result of interactions among proteins, carbohydrates, and lipids correspond to food structures. Food structure has been studied because it is responsible for sensory properties such as texture perception, flavor release, and appearance, which impact product acceptability. Also, food structure affects the shelf-life and physical and rheological properties of food prior to ingestion (Singh *et al*., 2009). However, the physicochemical and structural changes that occur in foods after ingestion are just as important as those that happen before, as they are partially responsible of their digestibility and subsequent use as a source of nutrients (McClements *et al*., 2009; Singh *et al*., 2009). A better understanding of food properties, digestion, and absorption may allow us to design food matrices that release or trap certain nutrients, determining their bioaccessibility. Figure 1.6 summarizes the conditions offered by the gastrointestinal tract and changes in lipids that may occur during digestion. It also illustrates the changes that an emulsion may undergo given different pHs, shear forces, or enzymes. An ideal food matrix may be able to encapsulate and protect bioactive components during storage, transport, and utilization and then release them at specific sites within the gastrointestinal tract, or increase or decrease the digestibility and absorption of specific nutrients (McClements *et al*., 2009).



CONDITIONS	PROCESSES ON LIPIDS	CHANGES IN EMULSION
Mouth pH 5-7; 35-37°C; 5-60 seg enzymes (amylase), salts, mucin high shear	Dilution & mixing Matrix disruption Phase transition Droplet breakdown/coalescence	bridging flocculation depletion flocculation
Stomach pH 1-3; 37°C; 30 min-4 h enzymes (proteases, lipases), salts, proteins moderate shear	Dilution & mixing Droplet breakdown/coalescence Competitive adsorption Digestion	coalescence disruption
Intestine pH 6.8-7.5; 37°C; 1-2 h enzymes (proteases, lipases), salts, bile, proteins low shear	Dilution & mixing Droplet breakdown/coalescence Competitive adsorption Digestion Micellization Transport & absorption	competitive adsorption enzyme adsoption interfacial layer hydrolysis

Figure 1.4: Conditions of the gastrointestinal tract and changes that may occur during lipid digestion (adapted from Singh *et al*., 2009 and McClements *et al*., 2009).

Food matrix design: emulsions design

The lipids in most processed foods are present as emulsions, which can be end products or part of a more complex food system (Singh *et al*., 2009). An emulsion is formed during lipid digestion through chewing, shear forces by gastric motility, and amphipathic qualities of bile salts (Mu and Høy, 2004). An emulsion is a dispersion of droplets of one liquid into another immiscible liquid, with an interfacial layer between the two. Figure 1.5 shows an emulsion at different scales and surface-active agents: surfactant, extended and aggregated biopolymers, solid particles or multilayers stabilizer (Singh *et al*., 2009). As emulsions are thermodynamically unstable, changes in the surroundings such as different pH and temperature added to active enzymes, proteins and salts at different ionic strengths may produce flocculation or coalescence of a stabilized emulsion (see Figure 1.4). Lipids in food emulsions may be hydrolyzed as well. We do not have a great deal of information about the digestion of emulsions, but there is evidence that interfacial properties, size of droplets, SFC, and surrounding matrices may affect the rates of gastric emptying and lipolysis (Lundin and Golding, 2009; Borel *et al*., 1994; Armand *et al*., 1999). The composition of emulsions can be manipulated to control the stability of emulsion before consumption. However, changes after emulsion intake are still unclear. More research is needed to design food structures and strategies for controlling lipid bioavailability (Singh *et al*., 2009).

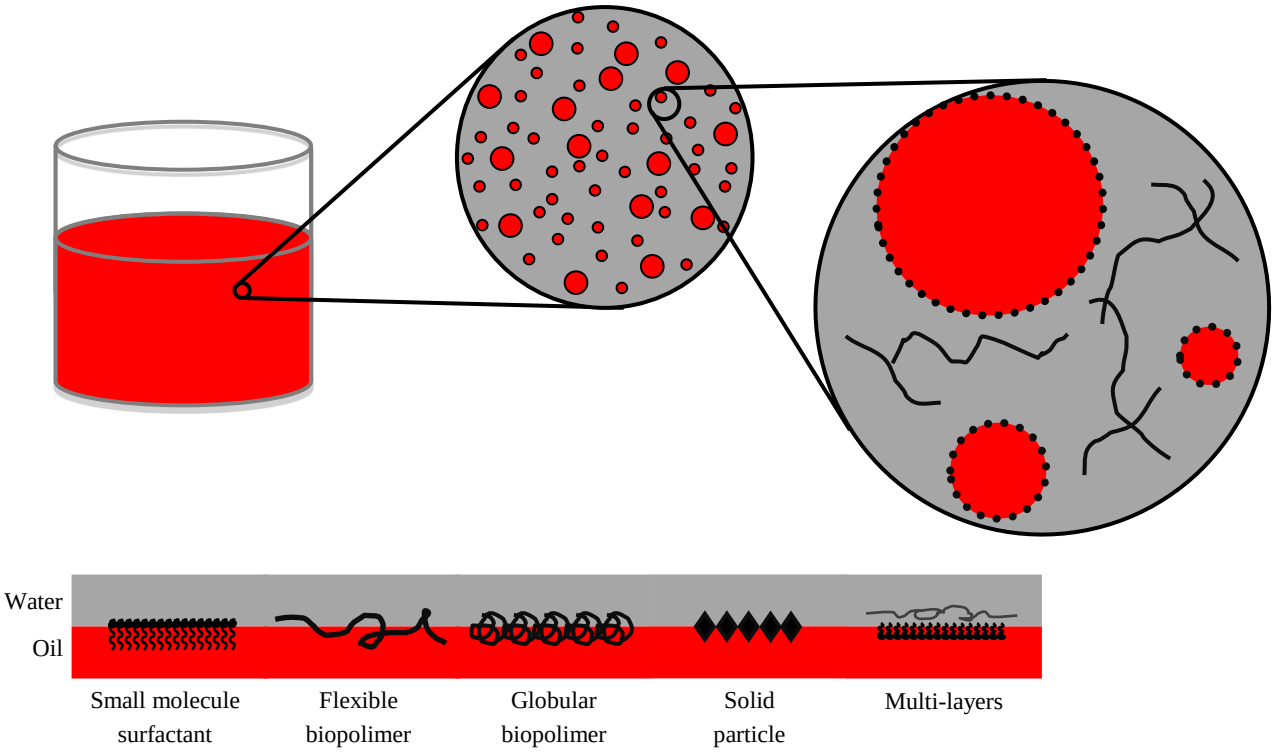


Figure 1.5: O/W emulsion at different scales and surface-active agents that stabilize emulsions.

Our problem

Some fats have physical properties that are suitable for specific applications and others which are not nutritionally recommended. Some lipids have an excellent profile of FAs, but their distribution in TAGs molecules does not allow for optimal absorption. A fat-free diet is not a viable option due to the essential nature of some FAs. Furthermore, lipids affect the physicochemical properties of food. So, how do we maintain the benefits and remove the adverse effects of fats? Lipids modification may be the answer. However, synthesizing perfect TAGs and lipids is not the solution. Due to metabolic and food structure issues, the amount of lipids taken in and the amount of lipids absorbed are not the same.

1.1 Hypothesis and objectives

The hypothesis of this study was that through enzymatic interesterification it is possible to synthesize a structured fat with nutritional advantages –high in n-3 and zero *trans* fatty acids- and a melting profile similar to a commercial fat which bioavailability may be modulated through the new TAG structure, and by emulsion design.

Accordingly, the main objective of this thesis was to get a better understanding of the effect of enzymatic interesterification, in the synthesis of plastic fats, as well as the effect of emulsification, in the *in vivo* postprandial bioavailability of fatty acids.

The specific objectives are:

- To compare chemical and enzymatic interesterification of binary blends to obtain structured lipids with zero *trans* FAs and high linolenic acid content, and adequate functional properties.
- To evaluate the effect of enzymatic interesterification on FAs bioavailability, when using a sn-1,3 stereospecific lipase, that is, without modifying the bioavailability determined by sn-2 stereochemistry, to understand if there could be any drawback in the *in vivo* postprandial bioavailability when using this technology.
- To study the effect of emulsification, as well as the use of different surface-active agents, on the *in vivo* postprandial bioavailability of interesterified-lipids in O/W emulsions.

Accordingly, a general overview of the outline of the thesis is summarized Figure 1.6.

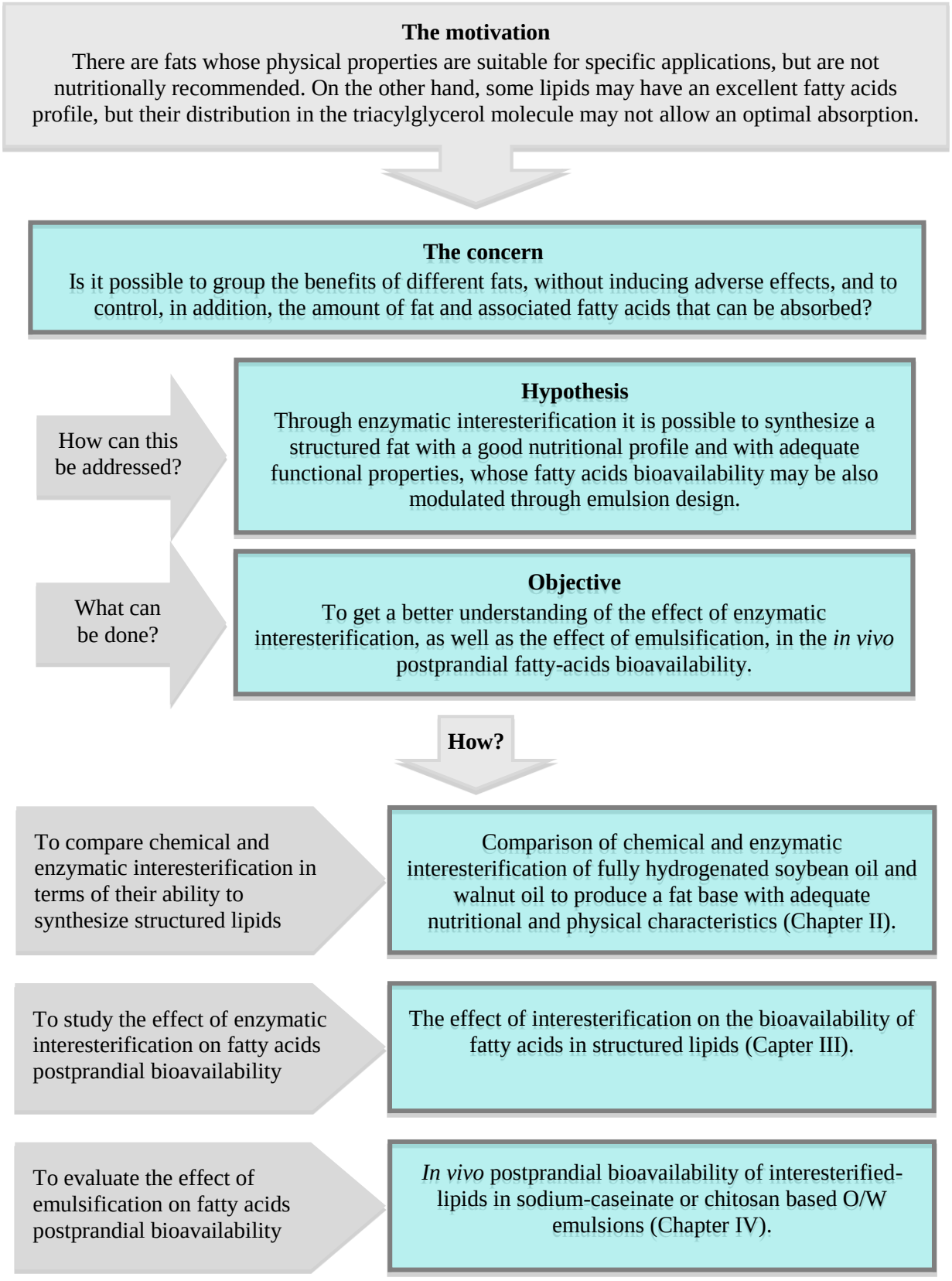


Figure 1.6: Thesis overview.

2 COMPARISON OF CHEMICAL AND ENZYMATIC INTERESTERIFICATION OF FULLY HYDROGENATED SOYBEAN OIL AND WALNUT OIL TO PRODUCE A FAT BASE WITH ADEQUATE NUTRITIONAL AND PHYSICAL CHARACTERISTICS

Abstract

Physical, chemical, and nutritional properties of natural lipids depend on the structure and composition of triacylglycerols (TAGs). Unfortunately, physicochemical and nutritional properties of lipids are not frequently assembled. Interesterification is a possible technology that may be used to synthesize a structured fat with a good nutritional profile and adequate functional properties. In accordance, the aim of this study was to compare chemical and enzymatic interesterification of binary blends of fully hydrogenated soybean oil (FHSBO) and walnut oil (WO), to synthetize a fat base with adequate nutritional and physical characteristics. FHSBO/WO mass-ratios of 20/80, 40/60 and 60/40 were evaluated, and total interesterification was determined by stabilization of the solid fat content (SFC). Chemical reactions were completed at 10 min for the 20/80 blend and at 15 min for the 40/60 and 60/40 blends. Enzymatically interesterified blends were stabilized at 120 min for all of the mass ratios. Complete interesterification significantly reduced the SFC of blends at any composition. Chemically and enzymatically interesterified FHSBO/WO mass-ratio 40/60 showed the plastic curve of an all-purpose-type shortening rich in polyunsaturated FAs, especially linolenic acid, with zero *trans* FAs.

2.1 Introduction

Lipids are the highest energy source of the three macronutrients (carbohydrates, proteins, and lipids). They also add flavor, texture and satiety to foods. Slight differences in the fat produce significant changes in the food. For instance, a fat that performs well in baked products will not work well enough in ice cream, producing a pasty and waxy feeling instead of a pleasant cooling effect (O'Brien, 2004). On the other hand, some fats may have excellent physical properties for specific applications but are not recommended due to nutritional concerns. Those lipids may not be absorbed as expected or may have deleterious effects on health due to the presence of certain FAs (FAs). Each of the three FA bonded to sn-1, sn-2 or sn-3 positions of the glycerol backbone can vary in regard to chain length, number and position of double bonds, and geometrical configuration. These characteristics give lipids their physical, chemical, and nutritional properties (Babayan, 1987; Garaiova *et al*., 2007).

The optimal physical, chemical and nutritional properties of an oil or fat are not always mutually compatible (Gunstone, 2006). As such, lipid modification is a good way to give them specific functionalities, increase their oxidative stability, or improve their nutrition value. The temperature at which fats crystallize and melt or the ratio and kind of solid and liquid fats that produce a specific plastic behavior are physical properties that are specific for each application (Gunstone, 2006). Concerns about *trans* configuration or actions to reverse the obesity epidemic in which the aim is to reduce oil absorption stand in contrast to the need to increase absorption in situations like the poor lipid absorption in an immature digestive system or in patients with cystic fibrosis (López-López *et al*., 2001; Innis *et al*., 1995). Our knowledge of lipid metabolism has played a part in the development of structured lipids (SLs), which were reviewed previously, and may be obtained by technological or biological methods, expanding their uses. The biological methods include genetic engineering and crop or animal control (Gunstone, 2006). The technological methods include blending and fractionation, which are physical processes that produce value-added fats and oils (O'Brien, 2004). However, it is not always easy to predict what will happen to minor components which could affect the products' oxidative

stability (Gunstone, 2006). Hydrogenation solved this problem, as mentioned before, allowing for the production of fats with creaming properties, frying stability, sharp melting properties, or other functional characteristics for specific applications while enhancing oxidative stability (O'Brien, 2004). However, hydrogenated fats contain the two least desirable FAs: saturated and *trans* (Idris and Mat Dian, 2005; List, 2006). Full hydrogenation is an alternative that produces hardfats which may be used to prepare low to zero-*trans* commercial fats through interesterification (Petrauskaite *et al*., 1998; Ribeiro *et al*., 2009). An interesting raw material which can be used in this process is fully hydrogenated soybean oil (FHSBO). This is a relatively low-cost product with high C18:0 content and saturated FA of ~85%. It is not atherogenic and has none of the adverse effects on cardiovascular diseases reported with shorter FAs (C12:0, C14:0 and C16:0) (Ribeiro *et al*., 2009).

Interesterification is a reaction through which it is possible to rearrange the FAs in the triacylglycerol (TAG) molecule so that the TAG composition is changed but the FAs profile is preserved (Rodrigues and Gioielli, 2003; Klinkesorn *et al*., 2004; Liu *et al*., 2006). There are two ways to carry out interesterification: chemical and enzymatic which were explained previously. However, it should be pointed out that enzymatic process offers a better control of the reaction, and produces a more defined structure and a lower degradation of long-chain polyunsaturated FAs (Fomuso and Akoh, 1997; Osório *et al*., 2001; Rønne *et al*., 2005). In this respect, raw materials such as walnut oil (WO) are of great interest. Walnuts are unique within the nut family due to their high poly-unsaturated fatty acid content, specifically C18:3n3, and ratio of C18:2n6/C18:3n3 of 4/1, which has shown to decrease the risk for heart disease (Davis *et al*., 2007)

In accordance, the aim of this study was to compare chemical and enzymatic interesterification of binary blends of FHSBO and WO and evaluate them in the synthesis of a zero *trans* and high C18:3n3 fat with a plastic behavior similar to that of a commercial one.

2.2 Materials and methods

2.2.1 Materials

Raw materials for enzymatic and chemical interesterification were fully hydrogenated soybean oil (FHSBO) supplied by Watt's S.A. (Santiago, Chile) and walnuts donated by Valbifrut S.A. (Santiago, Chile). Walnut oil (WO) was obtained by cold-pressing. The product was also neutralized and bleached (Bailey, 1961). FHSBO and WO were stored at 4°C in a nitrogen atmosphere until the experiments were conducted. Raw materials were chosen because of the the saturated FAs content of FHSBO and associated high melting point (68-75°C), and the high C18:3n3 content of walnut oil (10%), as shown in Table 2.1. For chemical interesterification, analytical grade sodium methoxide (95%, Sigal, Chile) and citric acid monohydrate (Merck, Chile) were used. For enzymatic interesterification, Granotec Chile S.A donated Lipozyme TL IM, an immobilized and sn-1 and sn-3 stereospecific enzyme obtained from *Thermomicea lanuginosus*.

Table 2.1: Fatty acids composition of raw materials, and FHSBO/WO in different mass ratios blends.

Fatty acid	FHSBO (%)	WO (%)	80/20 (%)	60/40 (%)	40/60 (%)
nid	0.38	0.59	0.422	0.464	0.506
C12:0	0.98	-	0.784	0.588	0.392
C14:0	0.44	0.03	0.358	0.276	0.194
C15:0	0.05	-	0.04	0.03	0.02
C16:0	10.06	7.65	9.578	9.096	8.614
C16:1/17:0	0.20	0.16	0.192	0.184	0.176
C18:0	87.18	2.4	70.224	53.268	36.312
C18:1isom	-	0.31	0.062	0.124	0.186
C18:1tw9	0.03	-	0.024	0.018	0.012
C18:1cw9	0.17	16.36	3.408	6.646	9.884
C18:2isom	0.05	1.86	0.412	0.774	1.136
C18:2w6	0.06	56.52	11.352	22.644	33.936
C18:3isom	-	0.32	0.064	0.128	0.192
C18:3w3	-	13.78	2.756	5.512	8.268
C22:0	0.37	0.02	0.3	0.23	0.16
C24:0	0.04	-	0.032	0.024	0.016

2.2.2 Chemical interesterification

Three blends of FHSBO/WO mass ratios (20/80, 40/60 and 60/40) were chemically interesterified following the procedure described by Rodríguez *et al*. (2001). Briefly, each blend was dried under vacuum conditions (100 mm Hg) and heated with constant stirring (150 rpm) in a thermoregulated bath until it reached 90±2°C. Next, 0.5% w/w sodium methoxide was added and the reaction was carried out for 10, 15 or 60 min. Citric acid monohydrate was added to stop the reaction (1.78 g/g sodium methoxide) and kept for 5 min. The interesterified blend was then washed three times with distilled water to remove the soap produced and any residue of sodium methoxide or citric acid monohydrate.

2.2.3 Enzymatic interesterification

Three blends of FHSBO/WO mass ratios (20/80, 40/60 and 60/40) were enzymatically interesterified following the method reported by Abigor *et al*. (2003). Each blend was dried under vacuum conditions (100 mmHg) and heated with constant stirring (150 rpm) in a thermoregulated bath until it reached 70±2°C. Next, 5% (w/w) Lipozyme TL IM was added. The reaction was carried out for 30, 120 or 240 min. In order to stop the reaction, the enzyme was removed by filtration. All of the chemical and enzymatically interesterified blends were stored at 4 °C in a nitrogen atmosphere.

2.2.4 Solid fat content (SFC)

Because interesterification modifies the melting profile of lipids, which become constant when equilibrium is reached (Idris and Mat Dian, 2005), the SFC was measured and used as an indicator of this. The SFC of interesterified blends was measured using pulsed Nuclear Magnetic Resonance (p-NMR) according to the AOCS Official Method Cd 16-81 (1993). Briefly, dry and filtered samples were placed in glass tubes and completely melted (10 min, 60 °C) and then solidified (0 °C, 30 min). Samples were then allowed to melt in water at 10.0, 21.1, 26.7, 33.3 and 40.0 °C for 15 min. Finally, the SFC was measured in a Bruker Minispec PC120s p- NMR analyzer (Bruker Analytische Mestechnik, Rheinstetten, Germany).

2.2.5 Fatty acids profile

Methylated FAs of the TAGs were analyzed in an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA). A fused silica capillary column BPX70 (50 m, 0.25 lm film; SGE, Incorporated, Austin, TX, USA) was used. Samples were run with hydrogen as the carrier gas between 160 and 230°C at a rate of 2°C/min. Standard fatty acid methyl esters (FAME) from Merck (Merck, Darmstadt, Germany) were used for identification purposes.

2.2.6 Statistical analysis

All of the analyses were carried out in triplicate and the results are expressed as mean ± standard error (SEM). Statistical differences between the times and mass ratios were determined using one-way analysis of variance and Fisher's test. Differences were considered significant at $P<0.05$. Statistical analysis was performed using Statgraphics 4.0 (StatPoint, Inc., Virginia, USA).

The methodology is summarized on the Figure 2.1.

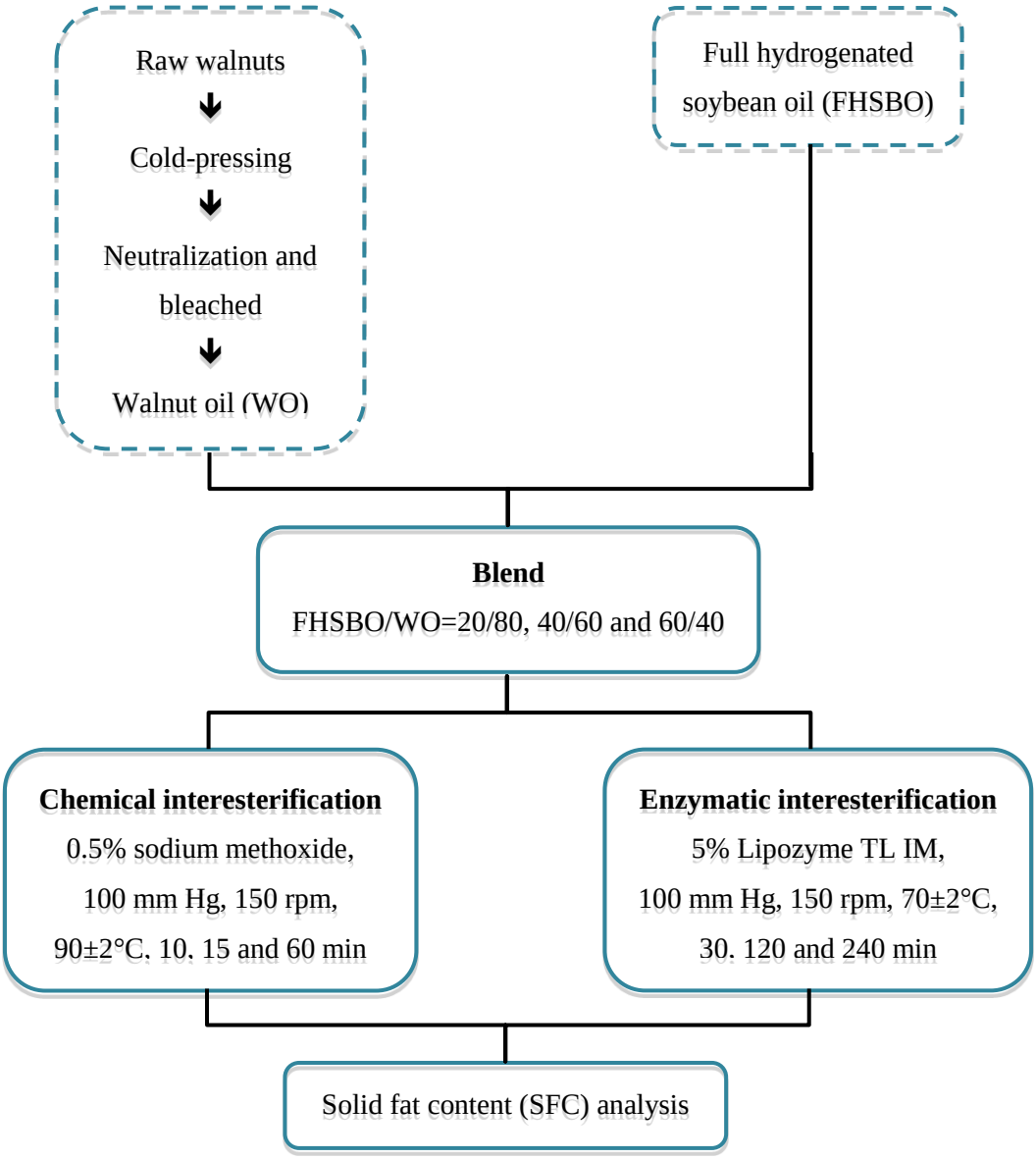


Figure 2.1: Overview of methods for chemical and enzymatic interesterification.

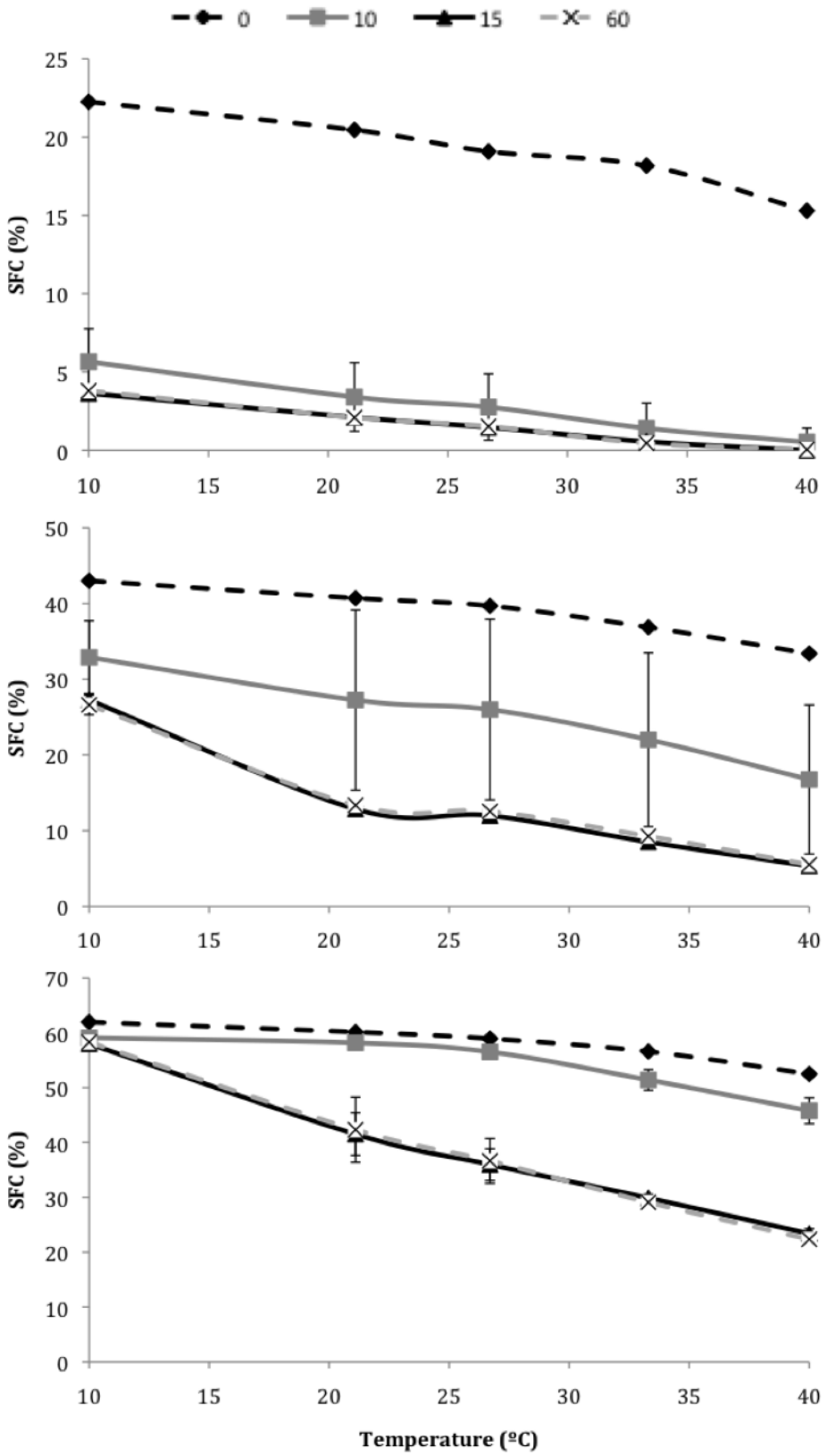


Figure 2.2: Solid fat content (SFC) of Chemically interesterified blends mass ratios 20/80 (A), 40/60 (B), and 60/40 (C) FHSO/WO.

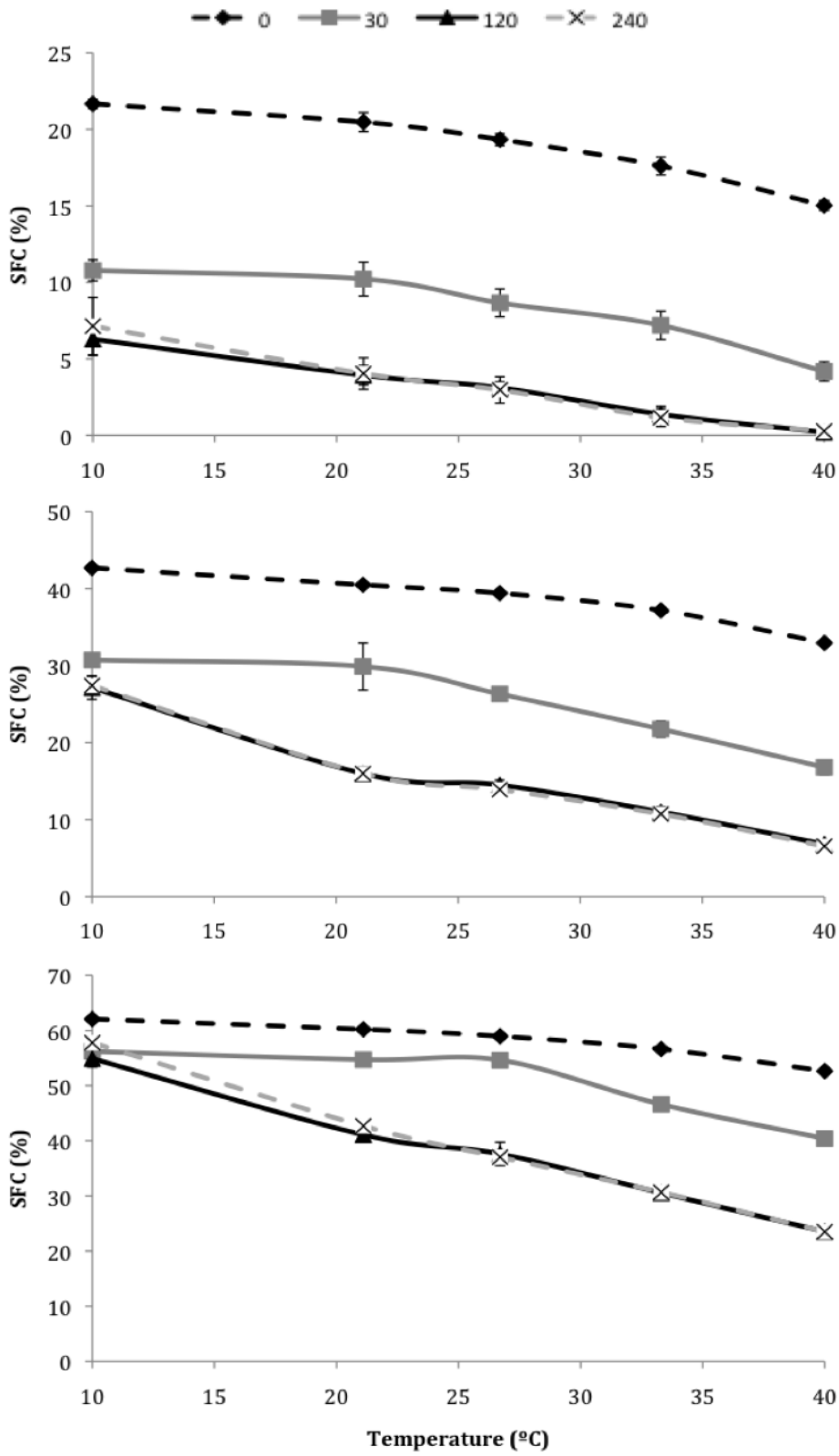


Figure 2.3: Solid fat content (SFC) of enzymatically interesterified blends mass ratios 20/80 (A), 40/60 (B), and 60/40 (C).

2.3 Results and discussion

Chemical and enzymatic interesterifications were carried out to obtain a fat base with a high content of C18:3n3 and zero-*trans*, as shown in Table 2.1.

2.3.1 Chemical interesterification:

Figure 2.2 shows the SFC of blends interesterified for 0 (non interesterified), 10, 15 and 60 min with FHSBO/WO mass ratio of 20/80, 40/60 and 60/40. As we expected, blends with higher FHSBO content had the highest SFC values. FHSBO is composed exclusively of saturated FA and may contain traces of unsaturated FA (Table 2.1). As the content of WO in the blend increased, the SFC values decreased due to the high content of unsaturated FA. The chemical interesterification produced the same effect for all of the compositions: this reaction reduces SFC at all temperatures. Our results echo those reported in other studies (Rodríguez *et al*., 2001; Norizzah *et al*., 2004; Klinkesorn *et al*., 2004) that produced melting profiles at lower temperatures after interesterification; however, the contrary effect -an increase on SFC produced by interesterification- may also happen (Kellens, 2000; Rodrigues and Gioelli, 2003). The conditions, temperature and catalyst concentration chosen allowed thermodynamic equilibrium to be reached after a few minutes. This effect was reflected in the stabilization of melting profiles. No significant differences in SFC with subsequent measurements were found at 10 min for the 20/80 blend and 15 min for blends 40/60 and 60/40. Furthermore, the beginning of the reaction was retarded with increasing content of WO. While the 20/80 blend had interesterified at 10 min, the melting profile of the 40/60 blend after 10 min of interesterification was found at significantly lower temperatures than at the beginning of the reaction, though it still had not stabilized. Interestingly, this particular blend (Fig. 2.2B) shows evident variability after 10 min (high SEM), probably denoting a transition state, which reflects how unstable the SFC of a blend may be during the interesterification. It can be also noticed that after 10 min of interesterification, the melting profile of the 60/40 blend had not changed significantly from the profile observed in the non-interesterified blend. These results suggest that the beginning of the reaction is delayed with an increase in the content of FHSBO. Comparing our results to those reported in relation to the reaction time may not

be relevant because it might vary widely (5 min to 6 h, or even longer) depending on conditions such as the catalyst concentration, temperature and solubility of catalysts on the reactants (Klinkesorn *et al*., 2004). In general, the reaction is accelerated with the increase in catalyst concentration and temperature, but the effect of solubility of catalyst depends on the raw materials, mass ratios, and catalyst itself.

2.3.2 Enzymatic interesterification

Figure 2.3 shows the melting profiles of FHSBO/WO blends considering 20/80, 40/60 and 60/40 mass ratios interesterified for 0 (non interesterified), 120 and 240 min. As we observed with chemical interesterification, blends with higher FHSBO content present melting profiles at the highest temperatures, which are reduced with the addition of WO. Also, the SFC of all compositions decreases as the reaction progresses. Díaz, Gamboa and Gioelli (2003) synthesized functional TAGs using chemical and enzymatic interesterification. They reported that the addition of a solid fat to a liquid oil increases the SFC of the blend, which decreases after interesterification, as we found in our experiments. They also reported SFC at lower temperatures after interesterification of pure palm kernel fat. For all of the FHSBO/WO blends, the thermodynamic equilibrium was achieved at 120 min when the SFC was stabilized. This coincides with the results of the study by Undurraga *et al*. (2001) which reported total interesterification after 80-120 min under similar conditions (65°C, Lypozime TL IM). However, as is the case in chemical interesterification, the rate of the enzymatic reaction is affected by temperature and catalyst concentration. Interesterification speeds up when temperature increases until it reaches a maximum level. At that point, it starts to slow down as the temperature increases due to the loss of enzyme activity (Namal Senanayake and Shahidi, 2002). The catalyst concentration generally has the same effect. As the enzyme load increases, the reaction rate is accelerated, but above a certain amount, there is no effect (Wang *et al*., 2006).

2.3.3 Interesterified blends as a fat base for margarine

In order to obtain an interesterified fat similar to a fat base for margarine, the melting profiles of a commercial fat base and interesterified blends were compared. Figure 2.4

shows the melting profiles of the interesterified mixtures and the commercial base. Although differences between the curves of the commercial base and the mixtures studied are observed, the 40/60 blend presents physical behavior similar to that of the commercial base. Chemical and enzymatically interesterified blends exhibited a spreadability as good as commercial fat with 27% of solid fat at 10°C; a SFC not greater than 32% is essential for good spreadability at refrigeration temperature. Interesterified blends also showed good stability and resistance to oil exudation at room temperature with a SFC over 10% at 21.1°C (13 and 16% for chemical and enzymatically interesterified blends, respectively). The poorest behavior of interesterified blends was at body temperature. Under these conditions, the contents of 9 and 11% of solids at 33°C for chemical and enzymatically obtained products produced a waxier sensation in the mouth. Ideally, SFC should be less than 3.5% at 33.3°C (Criado *et al*., 2007; Karabulut *et al*., 2004; Seriburi and Akoh, 1998).

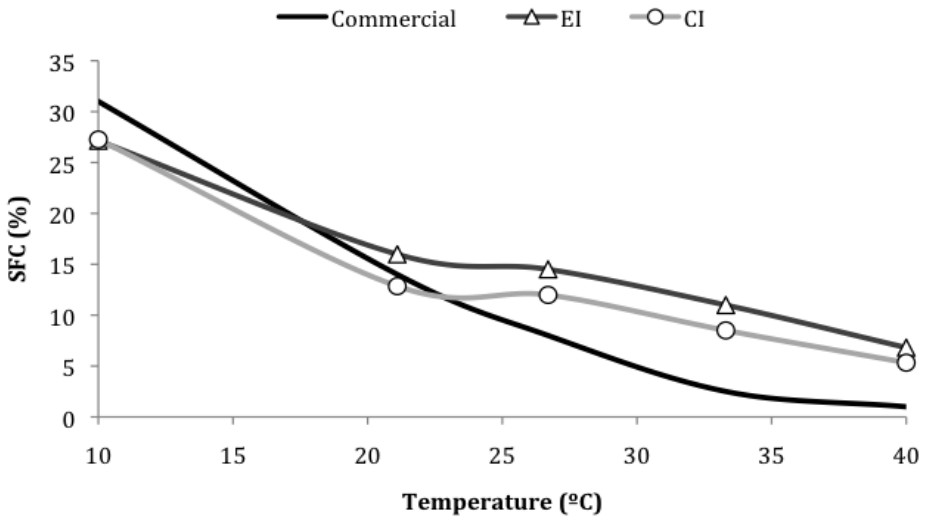


Figure 2.4: Comparision of chemical and enzymatically interesterified blends mass ratios 40/60 FHSO/WO, and a commercial fat base.

However, the SFCs of both interesterified blends had plasticity curves that fall within the range of all-purpose-type shortening fats. According to List *et al*. (1995), the SFC required at 10, 21.1, 26.6, 33.3 and 40°C are 18–23, 14–19, 13–14, 12–13 and 7–11%, respectively. Interesterified blends achieved these requirements, but a 27% SFC at 10°C it was slightly higher than shortening fats.

There are no physical differences between chemical and enzymatically interesterified blends. Other differences between chemical and enzymatically interesterified fats may be produced by the process itself. The main advantage of the enzymatic process over the chemical one is that the latter produces complete randomization of FA, while enzymatic interesterification may be either substrate specific -differentiated by chain length- or stereospecific. The stereospecificity of commercial enzymes allows them to produce structured lipids with mainly nutritional advantages. Essential FA are usually bonded at the sn-2 position of TAG; if these oils are enzymatically interesterified, the essential FA remains at the sn-2 position, where they are more easily absorbed as sn-2 monoacylglycerol than free FA (Willis *et al*., 1998). This improved absorption characteristic is not maximized in chemical interesterification. Enzymatic interesterification is mainly used when a very specific structure is required, as in Betapol.

2.4 Conclusions

Through this study it was possible to demonstrate the suitability of chemical and enzymatic interesterification to produce a fat-base rich in polyunsaturated FAs, especially high in linolenic acid, and with zero *trans*. Fully hydrogenated soybean oil seems to be an adequate raw material to increase the solids fat content, since it is high in palmitic acid and does not have as many detrimental effects as shorter saturated FAs. On the other hand, walnut oil was a good source of linolenic acid and contributed to the plastic behavior of the interesterified mix. No differences in the products obtained using chemical and enzymatic processes were found, thus, both approaches could be used to meet technological requirements. However, further studies should be carried out to determine if there is any difference in terms of the nutritional attributes of the different interesterified mixes.

A**cknowledgements**

The authors would like to acknowledge the Pontificia Universidad Católica de Chile's Central Nursery and Department of Physiological Sciences (Faculty of Biological Sciences), the financial support provided by the institution's VRI and the financial support received from Conicyt through Anillo Project ACT1105/2012: Healthy Food Matrix Design.

3 THE EFFECT OF INTERESTERIFICATION ON THE BIOAVAILABILITY OF FATTY ACIDS IN STRUCTURED LIPIDS

Abstract

Fatty acid (FA) profile is a critical factor in the nutritional properties of fats, but, stereochemistry may also play a fundamental role in the rate and extent to which FAs are absorbed and become available. To understand better this phenomenon, we evaluated the bioavailability of FAs in linseed-oil and palm-stearin blends compared to their interesterified mix, when using a sn-1,3 stereospecific lipase, to determine if there was any difference in terms of FA availability when using this technology. Test meals were fed through an intragastric feeding tube on Sprague-Dawley male rats after 18 h fasting. Postprandial blood samples were collected after meal or physiological serum (control) administration and the FA profile of plasma lipids was determined. Results showed that modification of the melting profile through interesterification, without altering the bioavailability determined by sn-2 stereochemistry, could delay lipid absorption at the beginning, but it had no effect on total lipid absorption.

3.1 Introduction

The technological applications of fat depend on its physical and chemical properties. These and nutritional properties are limited by the composition of fatty acids (FAs) and the stereochemistry of triacylglycerols (TAGs) of fats (Gunstone, 2006). In order to improve technological applications and preserve the sensorial attributes of fats, scientists have modified them using fractionation and blending. However, the preferred way to achieve the desired semi-solid consistency is hydrogenation (Ribeiro *et al*., 2009). In cases where hydrogenation is partial, this may lead to the production of *trans* FAs, which are known to have detrimental health effects (Hunter, 2006).

Although fractionation and blending are still used, interesterification is an alternative process that also alters the physical properties of fats without producing the undesirable *trans* FA (Rodrigues and Gioelli, 2003). Interesterification, as we mentioned before, is a reaction that involves FA redistribution between and within TAG molecules, until a thermodynamic equilibrium is reached (Karabulut *et al*., 2004; Idris and Mat Dian, 2005). The resulting products maintain the FA profile and saturation degree of the starting blends (Rodrigues and Gioelli, 2003; Karabulut *et al*., 2004), but present a different TAGs stereochemistry, which results in new physical-chemical characteristics and nutritional properties (Klinkesorn *et al*., 2004). Those two kinds of reactions are chemical interesterification (CI) and enzymatic interesterification (EI), with the most important advantage of EI is regiospecificity, which is produced as lipases may hydrolize the sn-1 and sn-3 positions, incorporating FA at these sites without changing the FA in the sn-2 position (Wang *et al*., 2006). EI is thus most suitable for interesterifying fats and oils that are more sensitive to high temperatures and for producing technological and nutritionally superior fats (Yankah and Akoh, 2000; Chu *et al*., 2001; Zhang *et al*., 2004). These new fats, that were previously defined, are structured lipids (SL), and are synthesized to improve the physicochemical and nutritional characteristics of natural fats (Lopez-Hernandez *et al*., 2005). SLs are used to achieve more efficient delivery of specific FA for nutritional and medical purposes. The synthesis of SL allows for the supply of the desired FA and TAGs profiles (Hamam *et al*., 2005). Furthermore, interesterification allows the

introduction of essential FA into oils, which may be useful in the treatment of certain clinical disorders (Hamam *et al*., 2005).

However, the FA profile is not the only factor that determines the nutritional properties of fats. Stereochemistry plays a fundamental role in lipid bioavailability (Singh and Singh, 2011; Parada and Aguilera, 2007). The nature of each FA and its position on TAG will determine whether or not it will be absorbed, as we explained previously. As summary, sn-2 monoacylglycerols are absorbed by lymphatic way, regardless of the FA's nature; while free FAs may be either: (a) absorbed by portal way; (b) absorbed by lymphatic way; or (c) lost as Ca or Mg salts depending on the nature of the FA (Mu and Høy, 2004). In accordance lipids bioavailability could be modified to meet specific needs, which in turn would change the triacylglycerol structure, state of aggregation, and physical properties of the food matrix (Yli-Jokipii *et al*., 2003; Asselin *at al*., 2004).

In fact, randomization of FAs on TAG molecules may determine the bioavailability of specific FA. *In vivo* studies (Lien *et al*., 1993) have demonstrated significant differences in total lipid fecal excretion after randomization. Similarly, plasma or chylomicron TAGs have been evaluated after the intake of a randomized fat. Some researchers (Christensen and Høy, 1996; Hodge *et al*., 1999) have concluded that there are differences between randomized and non-randomized groups absorption rate during the first few hours. However, the nature of FA has taken precedence over stereochemistry (Christensen *et al*., 1995). Other studies have evaluated the bioavailability determined by the physical state. Bonnaire *et al*. (2008) reported that fats with different melting profiles and equal FA composition presented slower digestion of solids than liquid particles in *in vitro* assays. Asselin *et al*. (2004) reported different absorption rates between high melting profile fats and those with medium-low melting profiles within the first 3 hours in *in vivo* tests. Moreover, the high melting profile group excreted a larger amount of fat ($P<0.05$) than other groups. However, it is important to consider that these fats had different FA profiles since they were obtained from milk fat fractionation, thus, it was not possible to isolate the effect of the nature of the FA over the melting point of the fat. Yli-Yokipii *et al*. (2003) evaluated the effects of different melting profiles with an equal FA profile and found easier emulsification and faster absorption at 30ºC in liquid fat as compared to solid fat (SFC of

18.3 and 27.6%, respectively). This agrees with the results presented by Lien *et al*. (1993), who reported that tri-saturated TAGs (higher melting point) are not as easily digested and absorbed as TAGs with a lower melting point (containing unsaturated FAs or short-chain saturated FAs). In accordance, the aim of this study was to evaluate the bioavailability of FA in linseed-oil and palm-stearin blends compared to their interesterified mix, when using a sn-1,3 stereospecific lipase, that is, without modifying the bioavailability determined by sn-2 stereochemistry, to understand if there could be any drawback in terms of FA availability when using this technology.

3.2 Materials and methods

3.2.1 Dietary fats

In order to obtain dietary fats, we developed binary blends of linseed oil (LO) and palm stearin (PS) which were either interesterified (IE) or non-interesterified (nIE). LO and PS were chosen because of the high melting point (49ºC) and saturated FA content (>60%) of PS and the high ω-3 content (~50%) of LO. Three blends of LO/PS mass ratios (70/30, 60/40 and 50/50) were evaluated in order to have a wide range of solid fat content (SFC). LO was purchased from Fontevita (Nutra Andes Ltd., Santiago, Chile), and PS was supplied by Cox & Co. (Santiago, Chile) as Danfat MIX-4547. Most important FAs of raw materials and mixes are shown in Table 3.1.

In order to interesterify, we used Lipozyme TL IM as a catalyst (Blumos S.A., Santiago, Chile), which only hydrolyses the sn-1 and sn-3 positions of TAGs. Briefly, each blend was melted and dried under vacuum conditions. The catalyst was added at 5% (w/w) and the reaction was performed under vacuum conditions (100 Torr) and agitation (150 rpm) at 70ºC for 120, 180, 240, and 300 min (Zhang *et al*., 2004; López-Hernández *et al*., 2005). The catalyst was separated by filtration and interesterified blends were stored at 4ºC under a nitrogen atmosphere.

3.2.2 Animals and test meals

Forty-eight male eight week-old Sprague-Dawley rats weighing ~300 g were given free access to a 4% fat commercial diet and water. They were divided into three groups. One was given a test meal with interesterified fat (IE), one had non-interesterified fat (nIE), and one was used as a control (C) group. After 18 h of fasting with free access to water, the rats were anesthetized in an induction chamber with isofluorane/oxygen, and intubated with an oral gastric feeding tube. 1 ml of test meal at 36ºC or 1 ml physiological serum for C group was administrated via the feeding tube and recovery was allowed. The rats were returned to their cages until blood sample extraction at 0 (before administration), 1.5, 3, 4.5, 8, and 12 h after the test meal or physiological serum administration.

In order to obtain the blood sample, the rats were anesthetized in the induction chamber and then arranged in surgery position with anesthesia flow through a nosecone. The abdominal cavity was opened and blood was collected from the abdominal aorta using a K_2EDTA vacutainer tube. After blood extraction, rats were sacrificed by exsanguination, which was verified by perforating the diaphragm.

All procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of Pontificia Universidad Católica de Chile.

Table 3.1: Main fatty acids composition of raw materials as well as linseed oil (LO) and palm stearin (PS) mixes used in this study.

	Fatty acids (%)					
	C14:0	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n3
LO	-	5.60±0.006	4.44±0.010	23.97±0.010	16.68±0.006	49.31±0.006
PS	1.14±0.006	57.52±0.006	6.87±0.010	27.66±0.012	6.81±0.010	-
70/30	0.34±0.006	21,18±0.010	5,17±0.010	25.08±0.006	13.72±0.006	34.52±0.012
60/40	0.46±0.006	26.37±0.006	5.41±0.010	25.45±0.006	12.73±0.006	29.59±0.010
50/50	0.57±0.006	31.56±0.010	5.66±0.006	25.82±0.006	11.75±0.010	24.66±0.010

LO: linseed oil; PS: palm stearin; 70/30, 60/40, and 50/50 are mass ratios of LO/PS blends.

3.2.3 Analytical procedures

3.2.3.1 Determination of interesterification kinetics through solid fat content measurements

Since interesterification modifies the melting profile of lipids, which is unstable until thermodynamic equilibrium is reached (Idris and Mat Dian, 2005), the solid fat content (SFC) was used as indicator of this equilibrium. The SFC of enzymatically interesterified blends was measured by pulsed Nuclear Magnetic Resonance (p-NMR) according to AOCS Official Method Cd 16-81 (1993). Briefly, dry and filtered samples were put into glass tubes and completely melted (10 min, 60ºC) and then solidified (0ºC, 30 min). Thereafter, samples were allowed to melt into a thermostated bathwater at 10.0, 21.1, 26.7, 33.3, and 40.0ºC for 15 min. Finally, the SFC was measured in a Bruker Minispec PC120s p-NMR analyzer (Bruker Analytische Mestechnik, Rheinstetten, Germany).

3.2.3.2 FA profile of dietary fats

The fatty acid methyl esters concentration of raw materials and interesterified blends was determined by conversion into corresponding methyl esters of FAs residues followed by GC (gas chromatograph HP 5890 and a capillar column BPX-70, 50 m, 0.25 μm). This was carried out according to AOCS Official Method Ce 1-62 (1997).

3.2.3.3 FA profile of plasma lipids

Blood samples were centrifuged at 3000 rpm, 4ºC for 10 min. Plasma was separated and stored at -18ºC until the lipids were isolated. Lipids were extracted using the method described by Bligh and Dyer (1959), converted into methyl esters using the method described by Morrison and Smith (1964), and analyzed by GC using a GC Hewlett Packard 7890 (column J and W DB-FFAP, 30 m, 0.25 mm ID, 0.25 um, FID detector).

3.2.4 Statistical analysis

The absorption studies were performed in parallel to reduce differences caused by external factors. The results are expressed as the mean ± standard error (SEM) of 3 rats in each group. Statistical significance of differences between groups was determined using one-way ANOVA followed by the Tukey HSD test. Differences were considered significant at

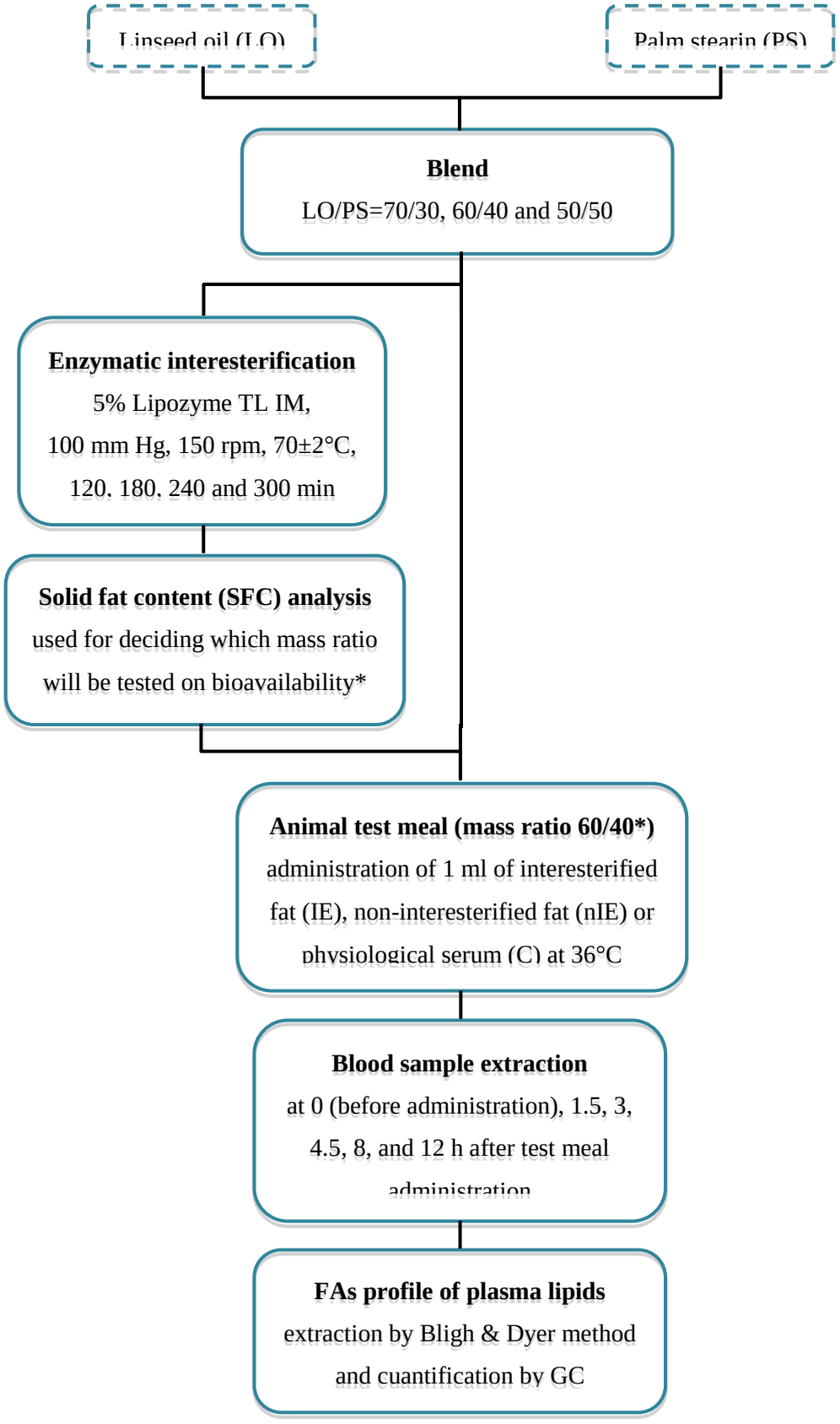


Figure 3.1: Overview of methods for bioavailability of structured lipids.

$P<0.05$. Statistical analysis was performed using Statgraphics 5.0 (Manugistic Inc., Oackland, Va., USA). Areas under the curve were determined using GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA).

An overview of this methodology is shown in Figure 3.1.

3.3 Results and discussion

3.3.1 Dietary fats

Interesterification modifies the melting profile of lipids, which varies until thermodynamic equilibrium is reached. The SFC was used as an indicator of this equilibrium. Figure 3.2 shows the melting profiles of the nIE blends (0 min) and blends interesterified during 120, 180, 240, and 300 min of 70/30, 60/40 and 50/50 weight ratios mixes of LO/PS. There are significant differences between nIE blends and the interesterified blends in all mixes. Further, for all compositions there are no differences between melting profiles at 120, 180, 240 and 300 min of interesterification. This suggests that thermodynamic equilibrium is reached at 120 min of process, which is enough to modify the melting profile of lipids. Prolonged exposure of the blend to high temperatures could lead to the thermo oxidative degradation of the finished product. Other authors have reported shorter and longer periods of interesterification (Yankah and Akoh, 2000; Ibrahim *et al*., 2008), but this depends on enzyme load and activity, the variety of TAGs in the raw material, FA-enzyme affinity, and temperature (Wang *et al*., 2006).

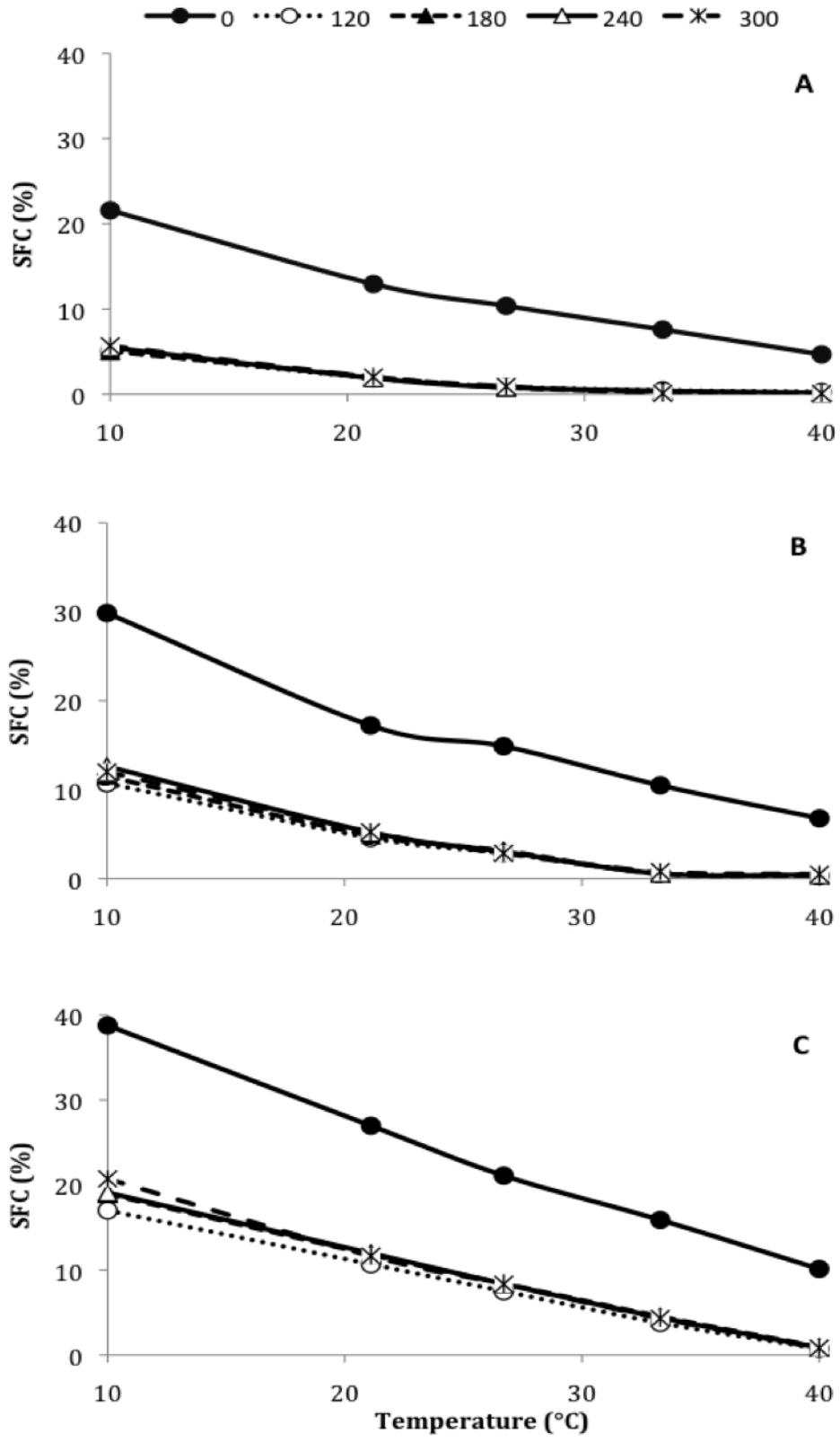


Figure 3.2: Melting profiles of non-interesterified blends (0 min) and interesterified blends during 120, 180, 240, and 300 min of 70/30 (A), 60/40 (B), and 50/50 (C) weight ratio mixes of LO/PS.

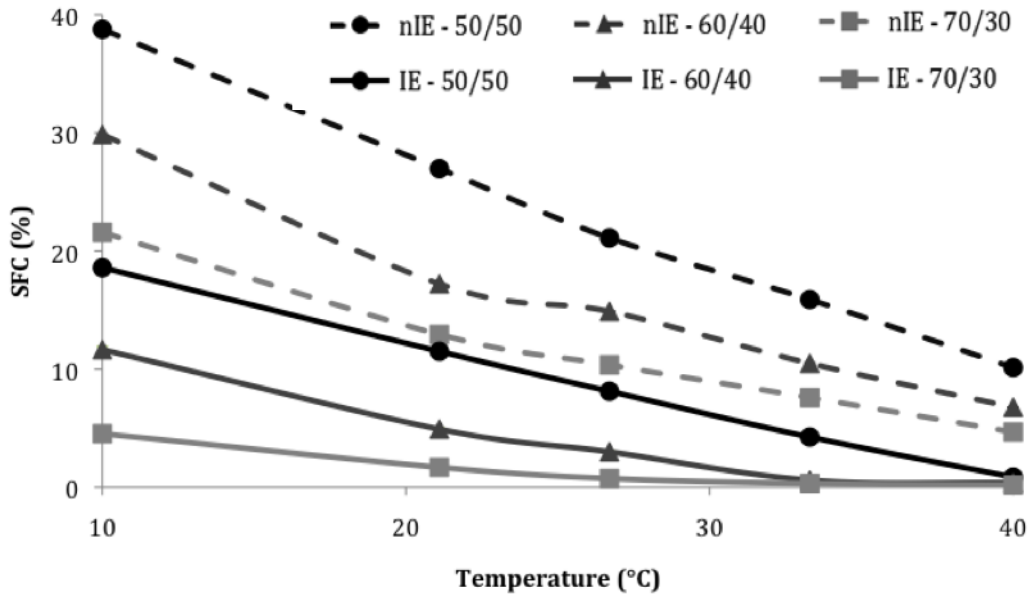


Figure 3.3: Melting profiles of interesterified (IE, at equilibrium) and non-interesterified (nIE) blends of 70/30, 60/40, and 50/50 weight ratio mixes of LO/PS.

For all nIE blends, the interesterification process reduced the SFC at every temperature measured, as reported in other studies (Chu *et al*., 2001; Criado *et al*., 2007). As reflected in this work, the effect of interesterification is notorious in the presence of high-melting TAG species, reducing significantly the SFC of the mix. Figure 3.3 shows the melting profiles of 70/30, 60/40 and 50/50 IE and nIE LO/PS blends. As was expected, when a higher PS content is used higher melting profile is observed, since PS is a natural hard component. Thus, 50/50 has higher SFC than 60/40 and this has higher SFC than 70/30. In accordance, 50/50 IE and nIE blends curves have a stronger slope and may be considered plastic for a wider temperature range but are waxier than 60/40 and 70/30 blends. These results are in agreement with those reported by Chu *et al*. (2001) and Criado *et al*. (2007).

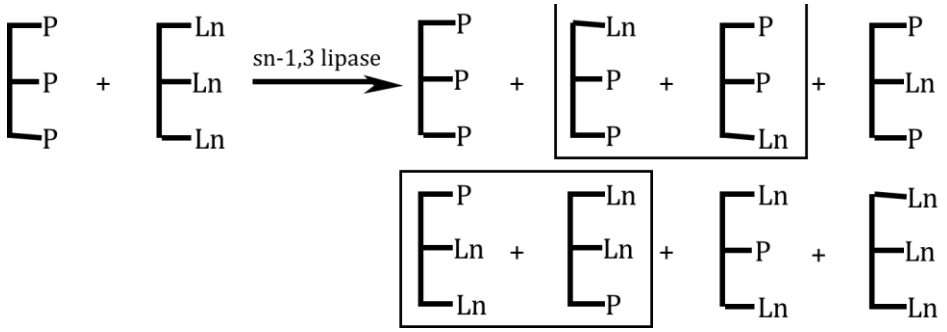


Figure 3.4: Reaction of two characteristic TAGs of PS (tripalmitin: PPP) and LO (trilinolein: LnLnLn), when using sn-1,3 stereospecific lipase. TAGs in each rectangle are considered to be equal.

3.3.2 Test meals

We decided to use 60/40 blends to evaluate the bioavailability of lipids, since at administration temperature (36ºC), the IE blend was completely liquid and had a higher difference in SFC respect to the nIE mix, compared to the 50/50 blend. This allowed us to evaluate whether or not the interesterification process together with the physical state of the matrix had an influence on lipid absorption. PS is rich in palmitic acid (P) and tripalmitin (PPP), while LO is rich in linolenic acid (Ln) and trilinolein (LnLnLn) (Xu, 2000). As an example, Figure 3.4 shows the reaction scheme of these two characteristic substrates (PPP) and (LnLnLn), when using sn-1,3 stereospecific lipase, to illustrate the different combinations that may result. As PS and LO contain other TAGs as substrates in addition to PPP and LnLnLn, all previous configurations might be found in both nIE and IE blends but at different proportions: nIE is richer in trisaturated and disaturated TAGs than the IE blend. As a result, different SFCs are obtained at the same temperature.

The fatty acid profiles of test meals and the concentration of major FA in plasma lipids before and after test meal administration are shown in Table 3.2. No statistical differences were observed in absorption of total FA and individual FA between IE and nIE test meals under the conditions used for the study. However, it is important to know that the effect of the test meal intake was quickly reflected in lipemia. For all FA ingested, the individual concentrations increased significantly after test meal dosification. Increases were even observed in those administered at a lower amount or not at all as C18:0 and C20:4n6 (5.4 and 0% of dietary fat), though to a lesser extent.

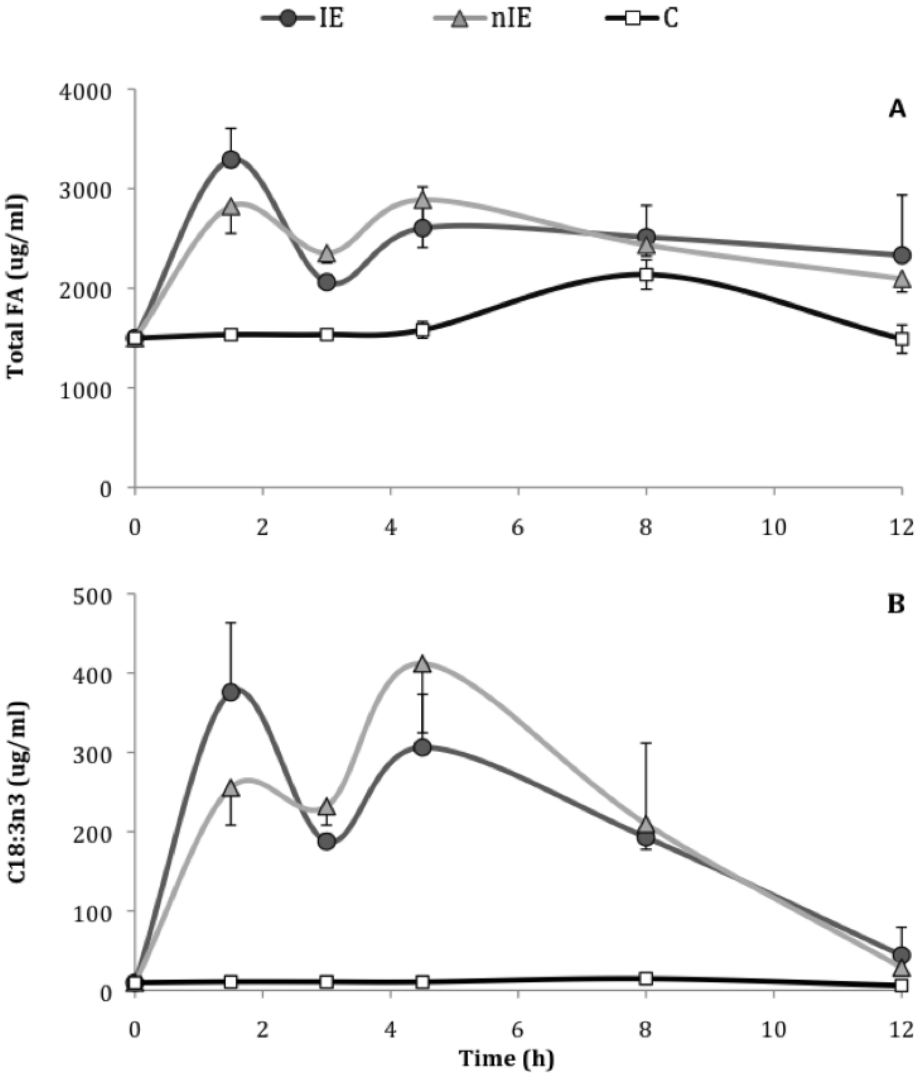


Figure 3.5: Absorption curve of Total FA (A) and C18:3n3 (B) after interesterified (IE), non-interesterified (nIE) and control sample administration.

Before any test meal administration, the content of 18:3n3 was lower than 10 μg/ml. After the administration, the 18:3n3 content increased to 375.84 and 255.31 μg/ml in the first measurement for IE and nIE test meals, respectively. Similar results were observed in the studies using rats published by Christensen and Høy (1996) and Christensen *et al*. (1995). They reported null content of EPA or DHA (essential FAs) before test meal administration and significant increases after administration. This is possible because an essential FA cannot be synthesized *de novo* by humans and other mammalians. As such, C18:3n3 was defined as our biomarker. But how could plasma in fasting state be as rich in other essential FA as C18:2n6 and C20:4n6? Although plasma lipids reflect the dietary fat ingested over a period of a few hours (mainly transported by chylomicrons), other lipoproteins synthesized in enterocyte in interprandial and fasting states transport important amount of lipids as well. The lipoproteins responsible for TAG transport at these moments are very low-density lipoproteins (VLDLs), which may be synthesized in both the liver and enterocyte. The VLDLs of enterocyte transport remained and have sloughed enterocyte lipids. Therefore, FAs that are plentifully ingested may appear in interprandial and fasting states including essential FAs as C18:2n6 and its desaturated and elongated product C20:4n6.

A similar effect is observed for total FA, as shown in Figure 3.5A, where an unexpected increase is observed at t=8 h in the control group. The variability in serum triglycerides may be due to the prolonged fasting and carbohydrate starvation with the resulting need for sources of energy (Man and Albrink, 1956).

It is also important to note the rates at which lipids are absorbed. Figure 3.5B shows that the absorption kinetics of 18:3n3 in the nIE test meal appeared to be slightly retarded than the one present in the IE test meal, showing absorption peaks after 4.5 and 1.5 h, respectively . This may be attributed to the higher melting point of nIE test meal that is richer in trisaturated and disaturated TAGs, which may retard the absorption of the meal. This coincides with the findings of Asselin *et al*. (2004), who reported lower absorption and higher lipid fecal losses for high melting profiles as compared to low and medium

Table 3.2: Main fatty acids composition of test meals and concentration in plasma lipids after interesterified (IE) and non-interesterified (nIE) test meal administration (μg/ml).

Fatty acid	Percentage in test meal (%)	Concentration of fatty acids in plasma lipids before (0 h) and after 1.5, 3, 4.5, 8 and 12 h test meal administration (μg/ml)										
		IE test meal						nIE test meal				
		0	1.5	3.0	4.5	8.0	12	1.5	3.0	4.5	8.0	12
14:0	0.46	7.08 ±0.87	17.18 ±1.93	9.68 ±0.13	11.38 ±2.16	21.59 ±3.75	13.82 ±1.64	12.99 ±1.99	11.42 ±0.74	14.39 ±3.42	17.66 ±2.79	12.91 ±0.90
16:0	26.37	320.59 ±23.22	764.69 ±82.06	425.13 ±15.47	595.08 ±92.36	551.56 ±69.86	517.23 ±46.43	618.08 ±68.49	496.55 ±18.27	631.24 ±110.00	782.75 ±154.16	492.38 ±23.87
18:0	5.41	169.66 ±19.18	256.48 ±13.23	207.91 ±7.04	243.44 ±28.26	231.76 ±11.75	255.39 ±22.70	239.85 ±19.48	220.90 ±4.19	240.89 ±29.39	282.38 ±36.63	239.17 ±5.81
18:1n9	25.45	241.10 ±15.11	623.48 ±64.25	343.01 ±9.59	471.78 ±84.97	440.33 ±72.85	365.52 ±41.85	484.71 ±52.17	403.77 ±25.52	553.86 ±98.21	583.79 ±116.30	300.58 ±15.30
18:2n6	12.73	307.84 ±17.05	604.02 ±48.70	353.98 ±15.30	472.40 ±73.33	446.81 ±81.35	454.34 ±38.27	518.20 ±44.09	438.53 ±19.52	530.11 ±87.03	598.94 ±107.73	419.41 ±23.30
18:3n3	29.59	9.56 ±0.30	375.84 ±87.27	187.68 ±9.15	306.26 ±66.84	192.86 ±39.62	44.10 ±11.83	255.31 ±47.04	231.98 ±23.71	411.90 ±87.27	400.08 ±129.60	28.56 ±1.62
20:4n6	-	268.20 ±19.94	342.02 ±21.69	323.31 ±8.77	288.82 ±33.71	343.53 ±19.44	414.49 ±43.54	401.12 ±20.71	321.60 ±5.73	283.33 ±28.98	323.70 ±26.24	381.46 ±15.32
Total	100	1498.54 ±55.76	3290.21 ±316.32	2060.22 ±72.97	2603.70 ±413.53	2512.60 ±317.79	2330.22 ±201.76	2823.47 ±273.41	2351.21 ±96.97	2886.86 ±478.02	2433.97 ±112.21	2094.46 ±44.44

melting profiles. However, this study compared fats with different FA profiles and thus enhanced the differences between the test meals. It is important to note that the control group's C18:3n3 levels were constant and almost zero during the experiment. This confirms the usefulness of this FA as biomarker. Finally, plasma C18:3n3 has practically disappeared at t=12 h.

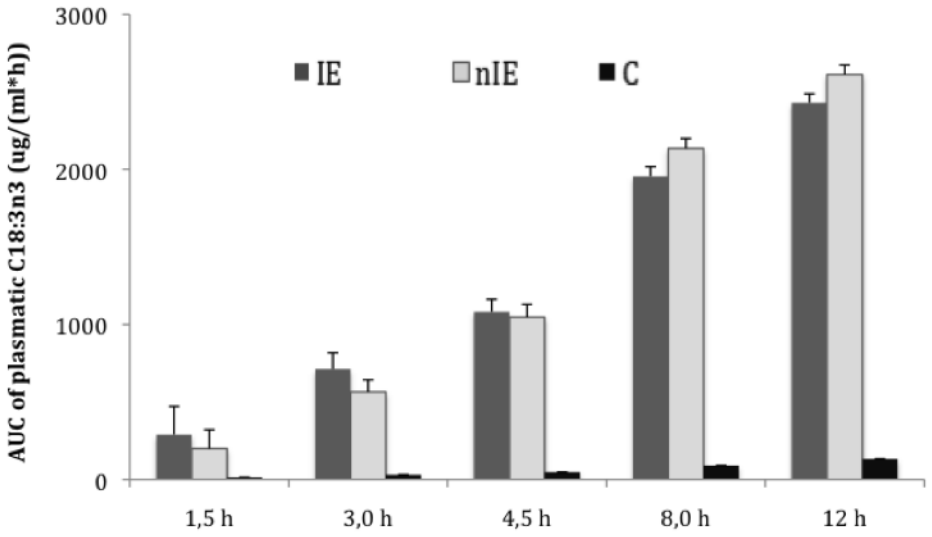


Figure 3.6: Areas under the C18:3n3 plasma concentration time curve (AUC) after interesterified (IE), non-interesterified (nIE) and control sample administration.

Figure 3.6 shows the area under the C18:3n3 plasma concentration time curve (AUC) after nIE and IE test meals as well as the control, which reflects the absorbed FA at all measurements. Due to the retarded maximum concentration of individual FA of nIE compared to IE, there are differences in the means, but they are not significant at any time. These findings suggest that different melting profiles do not interfere with absorption of C18:3n3 when the FA profile is preserved. In this way, C18:3n3 is equally bioavailable in both semisolid and liquid fats.

3.4 Conclusions

This study was designed to evaluate enzymatic interesterification as a tool for modifying fats in order to improve their physical properties and observe these changes on lipid absorption.

Our results indicate that the methodology and conditions used are good tools for evaluating the bioavailability of lipids elaborated with modified fats. Furthermore, it proves the suitability of C18:3n3 as a biomarker. Our results show that a higher melting point could delay lipid absorption at the beginning, but that it has no effect on total lipid absorbed. Finally, the results of this study suggest that modifying the melting profiles of fat blends through interesterification, without altering the bioavailability determined by sn-2 stereochemistry, while maintaining the FA profile, does not alter the bioavailability of such fats. Enzymatic interesterification has proved to be instrumental to achieve this modification in fats and, thus, as an effective tool to introduce essential FA that could be absorbed in the same way. We believe that the interesterified blend might be used in food emulsions, which is the focus of our ongoing research.

A**cknowledgements**

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4 *IN VIVO* POSTPRANDIAL BIOAVAILABILITY OF SODIUM CASEINATE OR CHITOSAN EMULSIFIED STRUCTURED-LIPIDS

Abstract

Recent studies have shown that it should be possible to control lipids bioavailability through food structure. But, to be successful, the gastrointestinal-tract physiological conditions must be considered. To get a better understanding of this phenomenon, we evaluated the effect of emulsification, as well as the use of sodium caseinate or chitosan, on the postprandial bioavailability of emulsified interesterified-lipids after oral gastric feeding Sprague-Dawley rats. We verified that emulsification may increase lipid absorption, as determined after feeding sodium-caseinate emulsions. Though, this result could not be generalized. Interesterified-lipids that were emulsified with chitosan were equally absorbed as those contained in non-emulsified interesterified-lipids/distilled-water blends. This may be due to a limited access of pancreatic lipases to lipids due to the pH increase in the duodenum (pH>pKa), which reduces chitosan solubility and may counterbalance the positive effect of size reduction. Further studies are needed to confirm this hypothesis.

4.1 Introduction

The importance of lipids in human diet goes beyond their energetic, structural and regulatory functions in the body; they confer specific sensory attributes to foods, which are critical for consumers' acceptance (O'Brien, 2004). Their intake may be either related to the development or to the prevention of several diseases (Wahrburg, 2004). Thus, it is of interest to understand and control the amount of lipids that is released from a food matrix and becomes available to be absorbed, the previously explained concept of bioaccessibility, as well as their bioavailability (McClements *et al*., 2009).

The FAs profile is certainly a critical factor in fat absorption, but the stereochemistry of TAGs also plays a fundamental role. As we explained before, free FAs and sn2-monoacylglycerols are absorbed in different modes. Monoacylglycerols are readily absorbed and transported through lymphatic way, whereas free FAs absorption and transport depends on their chain length and degree of unsaturation. Shorter FAs are absorbed faster than longer ones, whereas unsaturated FAs are absorbed faster than saturated ones (when comparing the same chain length). Thus, fats with a higher melting point are poorly absorbed, as in long-chain saturated free FAs absorption (Ramírez *et al*., 2001; McClements *et al*., 2009). Changes of the solid fat content, without altering the free FAs profile of fats, may also affect lipids absorption. In fact, Farfán *et al*. (2013) showed that modification of the melting profile through interesterification may delay lipids absorption kinetics, compared to a non-interesterified mix, without altering total lipids absorption.

Lipids bioavailability may be also affected by the diet. Nutrients can interact with partially-digested lipids or with any other molecule involved in lipid digestion, reducing its bioavailability. For instance, divalent cations, as calcium and magnesium, may form insoluble soaps with long-chain saturated free FAs, increasing their excretion (Ramírez *et al*., 2001; Mu and Høy, 2004). On the other hand, polyphenols from berries or tea may inhibit lipase activity (Juhel *et al*., 2000; Nakai *et al*., 2005; McDougall *et al*., 2009).

Certainly, it should be possible to reduce or maximize lipids bioavailability through food structuring. But, to be successful, the effect of the gastrointestinal-tract physiological conditions must be considered (Parada and Aguilera, 2007; Lundin and Golding, 2009). Accordingly, emulsification has shown to be an appropriate technique to control lipids bioavailability. *In vitro* studies have reported that emulsions produced with different interfacial compositions have large differences in lipids digestion (Lundin and Golding, 2009) and that the rate of lipids hydrolysis increases when decreasing droplet size (Borel *et al*., 1994). This has been confirmed in *in vivo* studies, which in turn have also reported that apparently fat assimilation would not be affected by differences in initial droplet size (Armand *et al*., 1999).

Proteins, carbohydrates, and other natural or manufactured substances may be used to stabilize emulsions (McClements *et al*., 2008). Sodium caseinate is a widely used emulsifier (Dickinson and Golding, 1997), which can increase pancreatic-lipase accessibility to lipids, as shown in *in vitro* studies (Mun *et al*., 2006). On the other hand, the intake of carbohydrates, specifically dietary fibers, may interfere with the digestive process by either changing the viscosity of the bolus, binding to bile salts or by forming a protective coating over emulsified lipids (Beysseriat *et al*., 2006). Chitosan, the deacetylated form of chitin, is an abundant and widespread polycationic biopolymer, which has shown good emulsifying properties (Moschakis *et al*., 2010) due to its structural heterogeneity, which includes a strongly hydrophilic component (D-glucosamine) and some less deacetylated hydrophobic (N-acetylated) residues (Klinkesorn, 2013). In addition, *in vivo* studies have reported that chitosan intake can reduce plasma cholesterol, total plasmatic lipids, and total liver lipids, and at the same time, it may increase lipids excretion (Han *et al*., 1999; Chiang *et al*., 2000). Also, *in vitro* studies have reported that lipids hydrolysis may be reduced in chitosan stabilized emulsions when compared to other stabilizers (Beysseriat *et al*., 2006; McClements *et al*., 2008; Helgason *et al*., 2009).

In accordance, the aim of this paper was to study the effect of emulsification, as well as the use of sodium caseinate or chitosan, on the *in vivo* postprandial bioavailability of emulsified structured-lipids.

4.1 Materials and methods

4.1.1 Synthesis of dietary fat and test meals

Two oil-in-water emulsions (test meals) were developed to evaluate the effect of emulsification as well as the effect of sodium caseinate or chitosan on lipids bioavailability. To do so, test meals were prepared by mixing an interesterified fat with an emulsifier solution, as explained below.

Dietary fats were synthesized by enzymatic interesterification of linseed oil (LO) and palm stearin (PS), as explained in Farfán *et al*. (2013). LO was purchased from Fontevita (Nutra Andes Ltd., Santiago, Chile) and PS was supplied by Cox & Co. (Santiago, Chile) as Danfat MIX-4547. Briefly, blends of LO/PS (60/40 mass ratio) were interesterified using 5% (w/w) of Lipozyme TL IM (Blumos S.A., Santiago, Chile), a stereospecific catalyst that only hydrolyses the sn-1 and sn-3 positions of TAGs. The reaction was carried out under vacuum (100 Torr) and agitation (150 rpm) at 70°C for 120 min. After this time, the blend reached thermodynamic equilibrium, as previously reported. The interesterified fat was kept at 4°C under a nitrogen atmosphere. Most important FAs of the raw materials and the interesterified mix are shown in Table 4.1.

A sodium caseinate solution was prepared by dispersing 10% (w/w) of sodium caseinate (Protevit-HV Ultra Top 2, supplied by Blumos S.A., Chile) in distilled water after stirring for 2 h at 40°C. On the other hand, a chitosan solution was prepared by dispersing 3% (w/w) of chitosan powder (medium molecular weight with 75-85% deacetylation degree, Sigma-Aldrich, USA) in a 0.15 M acetic acid solution. As reported by Mun *et al*. (2006), at a relatively low pH (<6.5), chitosan is positively charged and tends to be soluble.

Each emulsion was prepared by homogenizing 40% (weight) interesterified fat with 60% (weight) of either the sodium caseinate or the chitosan solution with a high-speed blender (Ultra Turrax®T25 digital, Ika®-Werke, Germany) for 2 min at 20,000 rpm. The emulsions were then stored at 4°C for 15 h before use.

4.1.2 Characterization of sodium caseinate or chitosan emulsions test-meals

Emulsions were characterized in terms of stability, globule size distribution and rheological properties.

4.1.2.1 Stability

The creaming stability was determined in triplicate by transferring 10 ml of fresh emulsion into a test tube, sealed and stored at 4°C. The phase separation of a creamy layer at the top and a serum layer at the bottom was monitored daily for 7 days (Surh *et al*., 2006; Khalloufi *et al*., 2008).

4.1.2.2 Oil droplet-size determination

Since droplets were larger than 1 μm, they were directly measured using optical microscopy (Boom, 2008). Emulsions were observed using an Olympus BX-61 TRF (Tokyo, Japan) microscope equipped with a high-resolution CCD color camera (CoolSnap Pro Color, Photometrics Roper Division Inc., Tucson, USA), which was used for digital image acquisition. Images were then analyzed using Image ProPlus 4.5 (Media Cybernetics, Silver Spring, USA). A circle was fit on each globule and the corresponding diameter was obtained. Thereafter, the Sauter mean diameter (d_{32}), which corresponds to an area-volume mean diameter, was determined according to the following equation (McClements, 2005):

$$d_{32} = \frac{\Sigma_{i=1} n_i d_i^3}{\Sigma_{i=1} n_1 d_i^2} \qquad \text{Equation (1)}$$

where n_i is the number of droplets with a diameter d_i. Determinations were carried out at 20°C in triplicate.

4.1.2.3 Rheological properties

A stress-controlled rheometer (Physica MCR 301, Anton Paar GMbH, Graz, Austria) with a 25-mm diameter parallel plate geometry, separated by a 1-mm gap, was used to carry out all the measurements. Dynamic viscoelastic properties were determined by frequency

swept tests in a frequency range of 0.628-628 rad/s. The linear viscoelastic regions of both emulsions were previously determined by amplitude swept tests γ=0,01-100% at a fixed frequency (f=6.28 rad/s). Rheological tests were carried out at 20°C in duplicate, after averaging 3 measurements per replicate.

Table 4.1: Fatty acid composition of raw materials, interesterified blends and dosis administrated though sodium caseinate and chitosan test meals.

Fatty acid	Linseed oil (%)	Palm stearin (%)	Interesterified blend (%)	Administrated dosis (mg)
C14:0	-	1.14±0.006	0.46±0.006	1.84±0.004
C16:0	5.60±0.006	57.52±0.006	26.37±0.006	105.48±0.004
C18:0	4.44±0.010	6.87±0.010	5.41±0.010	21.64±0.006
SFA	10.04±0.012	65.53±0.013	32.23±0.013	128.96±0.008
C18:1n-9	23.97±0.010	27.66±0.012	25.45±0.006	101.80±0.004
C18:2n-6	16.68±0.006	6.81±0.010	12.73±0.006	50.92±0.004
C18:3n-3	49.31±0.006	-	29.59±0.010	118.36±0.006
TFA	100±0.018	100±0.020	100±0.019	400.04±0.012

4.1.3 Animal tests

Sixty male eight-week old Sprague-Dawley rats weighing ~300 g were given free access to a 4% fat commercial diet and water. They were divided into four groups. One group was given the sodium-caseinate emulsion (Sc), another one the chitosan emulsion (Ch) and a third group was fed with a control test meal (C), which consisted of a non-emulsified interesterified-fat/distilled-water blend using the same mass ratio (40/60). In addition, a blank group (B) was fed with physiological serum.

Overall, after 18 h of fasting with free access to tap water, the rats were anesthetized in an induction chamber with isofluorane/oxygen, and intubated with an oral gastric feeding tube. 1 g of test meal at 36°C or 1 g of physiological serum was administrated via the feeding tube, and recovery was allowed (Table 4.1 shows most important FAs that were fed in each 1-g test meal). The rats were returned to their cages until blood sample extraction at 0 (before administration), 1.5, 3, 4.5, and 8 h after test meal or serum administration.

Afterwards, the rats were anesthetized in the induction chamber and arranged in surgery position with an anesthesia flow through a nosecone. The abdominal cavity was opened and rats were exsanguinated by abdominal aortic blood puncture using a K_2EDTA vacutainer tube. Rats were sacrificed by exsanguination, which was verified by perforating the diaphragm. All procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of Pontificia Universidad Católica de Chile.

4.1.4 Postprandial fat absorption

Fat absorption was determined as total plasmatic lipids during the postprandial period as explained as follows. The collected blood samples were centrifuged at 3,000 rpm during 10 min at 4°C. Plasma was separated and stored at -18°C until lipid extraction and analysis. The extraction of lipids was carried out using the method described by Bligh and Dyer (1959). Lipids were then methylated (Morrison and Smith, 1964) and analyzed by gas chromatography using a GC Hewlett Packard 7890 (column J and W DB-FFAP, 30 m, 0.25 mm ID, 0.25 um, FID detector).

4.1.5 Statistical analysis

Animal tests were performed in parallel to reduce differences of external factors. The results are expressed as the mean ± standard error (SEM) of 3 rats in each group for each individual data point. Statistical significance of differences between groups was determined using two-way ANOVA followed by the Tukey HSD test. Differences were considered significant at $P<0.05$. Areas under the curve and statistical analysis were determined using GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA).

The methodology described above is overviewed in Figure 4.1.

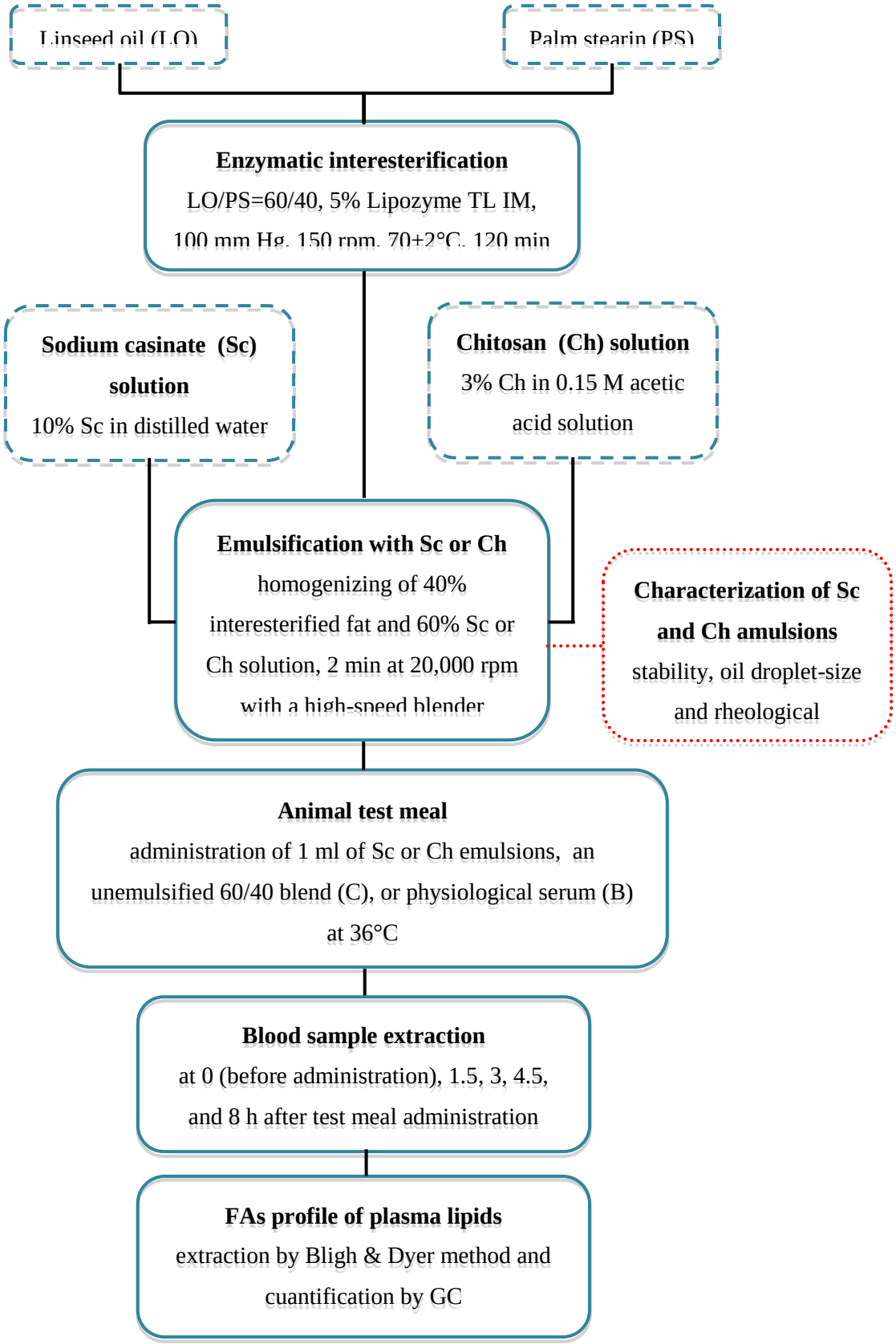

Figure 4.1: Overview of methods for bioavailability of emulsified structured lipids.

4.2 Results and discussion

4.2.1 Characterization of sodium caseinate or chitosan emulsions test-meals

Emulsions were characterized in terms of stability, globule size distribution and rheological properties to understand a possible link between these parameters and fat absorption.

4.2.1.1 Stability

No creaming or phase separation was determined in any emulsion. This was probably due to the high concentrations of sodium caseinate or chitosan that were used, as reported by other authors (Jafari et at., 2008; Liang *et al*., 2014). As we used a high concentration of sodium caseinate, a firm, percolating droplet network must have been formed, which fixed the droplets by a strong force (Dickinson and Golding, 1997; Liang *et al*., 2014). Similarly, the concentration of medium molecular weight chitosan (with 75-85% deacetylation degree) that was used ensured a good emulsifying capacity (Klinkesorn, 2013).

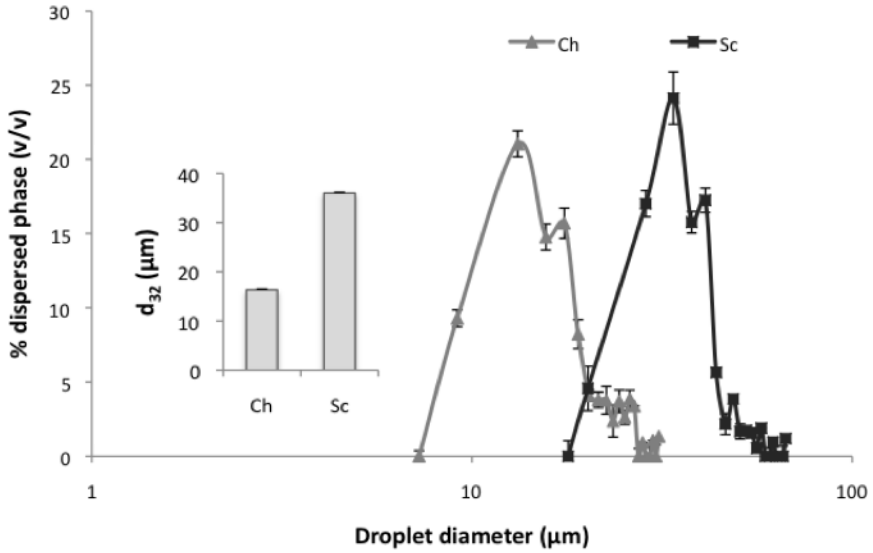

Figure 4.2: Droplet size distribution and respective Sautier mean diameter (d_{32}) of O/W emulsion stabilized by sodium caseinate (Sc) or chitosan (Ch).

4.2.1.2 Oil droplet size

Sodium caseinate and chitosan emulsions had a mono-modal particle size distribution with mean particle diameters of d_{32}=36 μm and d_{32}=16 μm, respectively, as shown in Figure 4.2. This indicates that, under these experimental conditions, chitosan was able to form and stabilize smaller droplets than sodium caseinate. This information could indicate that lipids in the chitosan emulsion could be better absorbed. But, other factors than droplet size may play an important role, as will be explained in the next section.

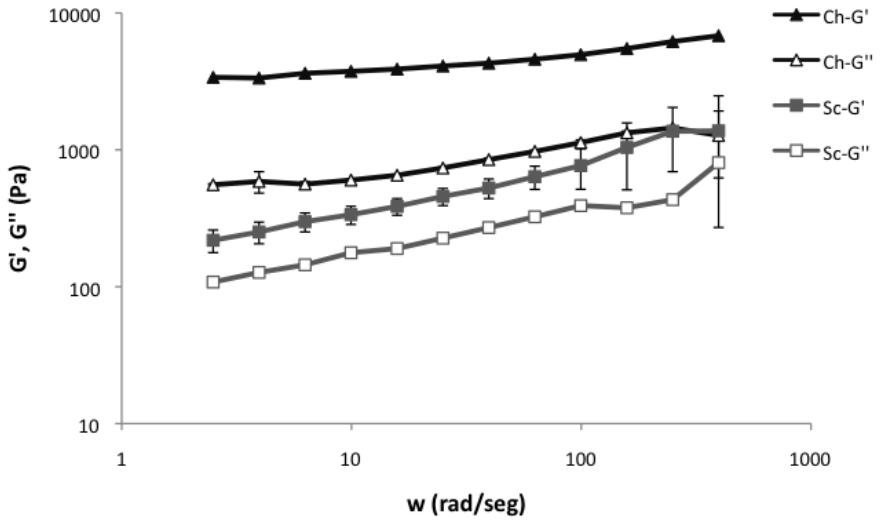

Figure 4.3: Evolution of linear viscoelastic properties for emulsion containing 40% interesterified fat and stabilized by protein (P) or carbohydrate (Q).

4.2.1.3 Rheological properties

Figure 4.3 shows the evolution of the shear storage and loss moduli, G' and G'', of both emulsions during the rheological measurements, inside the linear viscoelastic region. G' was always much higher than G'' in both cases, denoting a predominantly gel-like behavior (Batista *et al*., 2006; Romero *et al*., 2008). In addition, the higher values of G' and G'' found in the chitosan emulsion indicate a better developed three-dimensional

structure. Overall, in concentrated emulsions as we used, strong inter-particle interactions are usually observed. Particles are almost touching each other, conferring a high viscoelasticity (Rahalkar, 1992).

4.2.2 Postprandial lipids absorption after sodium caseinate or chitosan emulsions feeding

The postprandial absorption of selected lipid fractions was analyzed over time to understand its relationship to test-meal composition.

4.2.2.1 Postprandial lipids absorption over time

The absorption curves of linolenic acid (C18:3n3), palmitic acid (C16:0), saturated FAs (SFA) and total FAs (TFA) in plasma lipids, before and after test meal administration, are shown in Figure 4.4. We focused on linolenic acid absorption since this essential fatty acid is present in high amounts in linseed oil and is practically not found on the bloodstream during the fasting state, thus, it is a suitable biomarker. On the other hand, palmitic acid is present in high amounts in palm stearin and was the most abundant saturated fatty acid in the interesterified mix.

As we expected, the test meal intake was quickly reflected in lipemia. All curves showed a similar pattern, that is, an increase after 1.5 or 3 h, which slightly decreased for increasing times. Overall, sodium-caseinate test meals showed a better absorption than chitosan test meals at the beginning (t=1.5 h). These differences were statistically significant ($p<0.05$) when comparing C16:0, SFA, and TFA concentrations, however, no statistical differences were found in C18:3n3 absorption. In addition, no significant differences were found in postprandial lipid absorption between the chitosan based emulsion and the control mix at any time. Also, the blank group remained at the basal level.

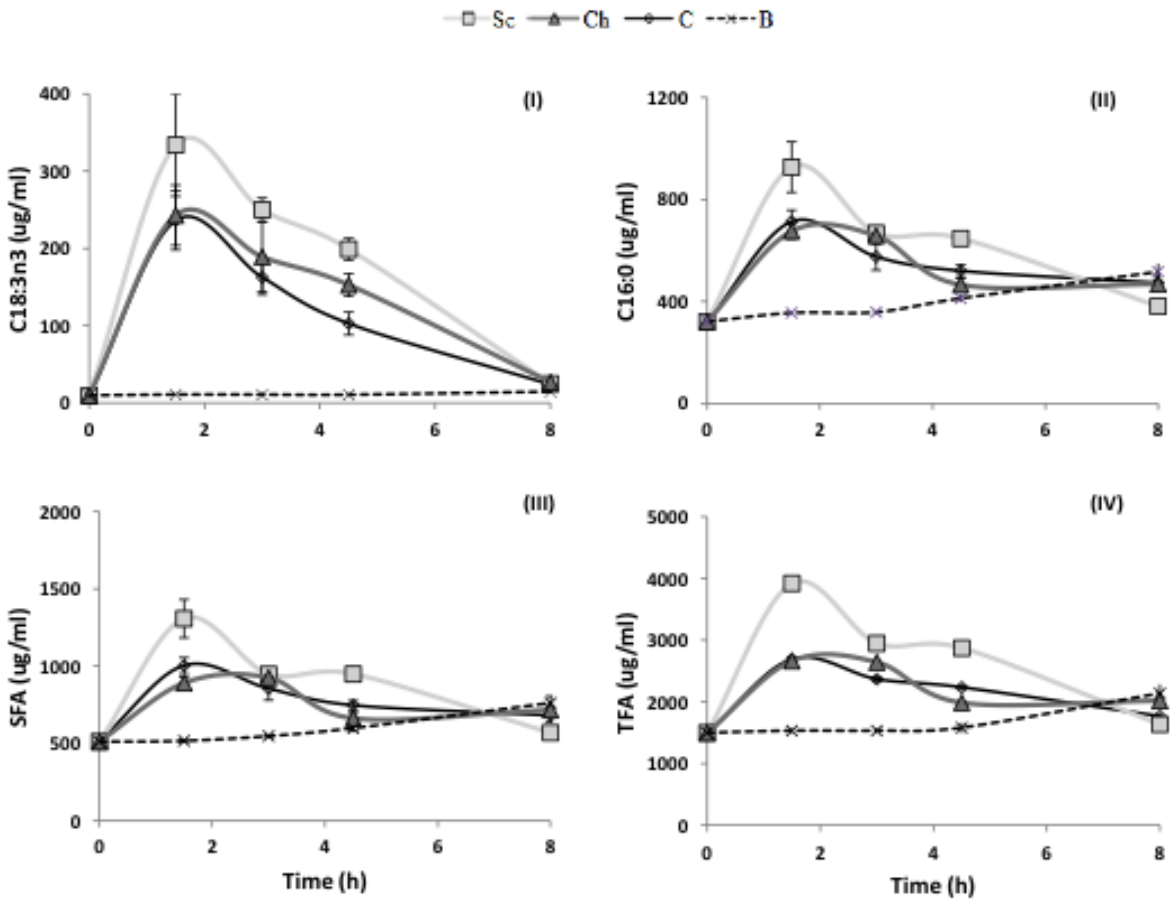

Figure 4.4: Absorption of C18:3n3 (I), C16:0 (II), SFA (III), and TFA (IV), after the administration of chitin (Ch) and sodium caseinate (Sc) test meals, control (C), or blank (B).

Figure 4.4 also shows important amounts of C16:0, SFA, and TFA, before test meal administration. These blood lipids correspond to those released from the enterocyte or the liver during the fasting state. At the end of the experiment (t=8 h), the amounts of C16:0, SFA, and TFA decreased once again, up to their basal level (no statistical differences were found, $p>0.05$). Interestingly, an increase in their content was observed in the blank group (only fed with physiological serum) at t=8 h, which was only significant in SFA. This agrees with results obtained in a previous work (Farfán *et al*., 2013), and is attributed to the prolonged fasting and carbohydrate starvation, and the consequent fatty acid mobilization (Owen *et al*., 1969).

On the other hand, C18:3n3 concentration was approximately 10 μg/ml, before oral gastric feeding (Figure 4.4). This low concentration proved that C18:3n3 was practically not found in the bloodstream during the fasting state, and thus, was an appropriate biomarker. After feeding any test meal (Sc, Ch or C), the amount of C18:3n3 was quickly reflected. The peaks were obtained in the first measurement (t=1.5 h). They were statistically higher than the ones found in the blank group, which remained at the basal level. All concentrations diminished gradually, up to the basal level at t=8 h.

4.2.2.2 Total postprandial lipids absorption over time: area under the curve

Figure 4.5 shows the areas under the C18:3n3, the C16:0, the SFA and the TFA plasma concentration time curves (AUC), which reflect the total absorbed amounts at all measurements after oral gastric feeding. As expected, lipids absorption after test-meal feeding was significantly higher at all times compared to lipids absorption after physiological-serum feeding (blank, B). Results were certainly consistent we those reported in Figure 4.4. Sodium-caseinate emulsions feeding resulted in a significantly higher absorption of C16:0 (after 4.5 h), SFA (after 4.5 h) and TFA (after 3 h), compared to those obtained after chitosan-emulsion or control feedings. No significant differences were obtained in C18:3n3 total absorption when comparing both emulsions, however, mean values were always higher in sodium-caseinate based emulsions. Also, a significant difference between the sodium-caseinate emulsion and the control was observed after 8 h. Overall, no significant differences were found in the total postprandial absorption of any lipid fraction, between the chitosan-based emulsion and the control mix at any time.

The higher absorption observed after sodium-caseinate emulsion feeding, confirms the effectiveness of emulsification on lipid absorption. Results agree with those reported by Garaiova *et al*. (2007) and Vors *et al*. (2013) who reported an enhanced absorption of emulsified fat compared to non-emulsified fat, during postprandial absorption in humans.

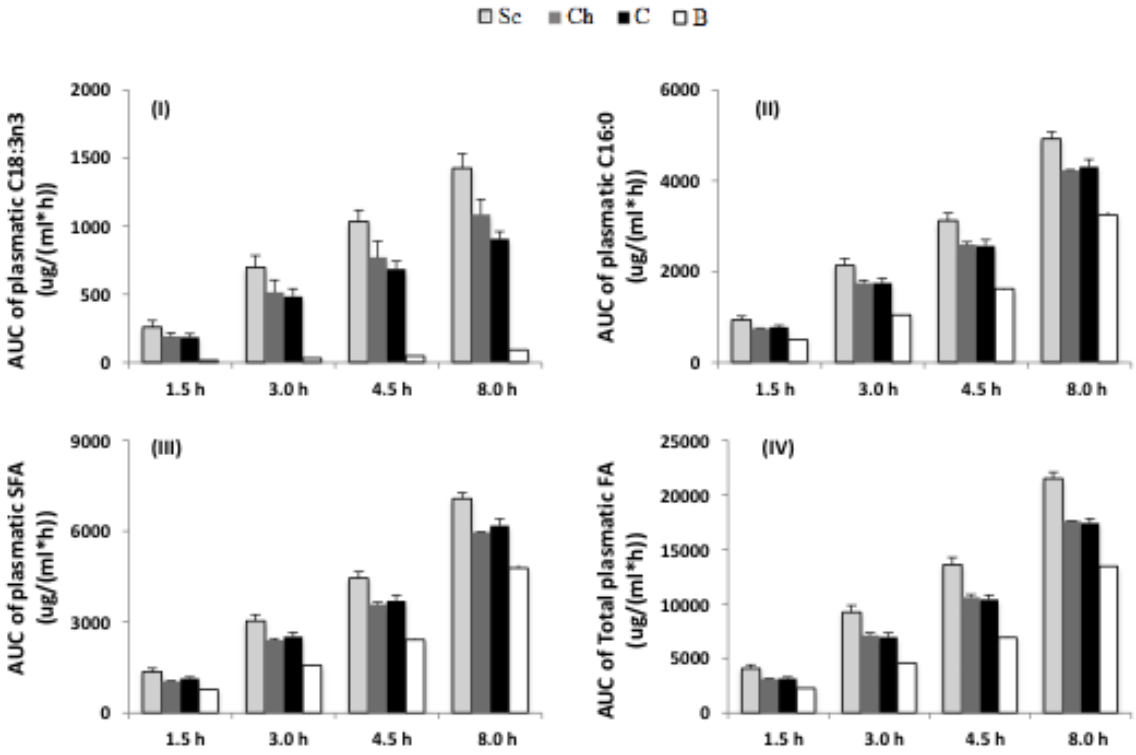

Figure 4.5: Area under 18:3n3 (I), 16:0 (II), saturated (SFA, III), and total (TFA, IV) plasma concentration time curve (AUC) after sodium caseinate (Sc) and chitosan (Ch) test meals, control (C), and blank (B) sample administration.

But, why postprandial lipid absorption after oral gastric feeding a chitosan-based emulsion was not improved? This behavior could be a consequence of pH variation through the gastro-intestinal track. In fact, chitosan has shown to have gelling properties when the pH is greater than its pKa (>6.3), due to the lower solubility of its deprotonated form (Klinkesorn, 2013). Thus, when the pH rises (as in the duodenum), chitosan-stabilized droplets become more rigid, entrapping the emulsion (Beysseriat *et al*., 2006). This may restrict the access of pancreatic lipase to lipids within the droplets, as explained by Agulló *et al*. (2003) and Mun *et al*. (2006). This behavior could be intensified by a direct interaction between chitosan and the active site of the pancreatic lipase, reducing its enzymatic activity, as explained by Mun et al. (2006). If this happened, the limited access of pancreatic lipases to lipids could counterbalance the positive effect of size reduction,

resulting in a null net effect. Conversely, the lower postprandial lipid absorption found in chitosan-based emulsions could be due to the breakdown of the emulsion in the small intestine, into oil and water phases.

4.3 Conclusions

Throughout this study, we were able to successfully evaluate the effect of emulsification, as well as the use of sodium caseinate or chitosan, on the postprandial bioavailability of emulsified interesterified-lipids after oral gastric feeding Sprague-Dawley rats. In addition, we confirmed the suitability of linolenic acid as plasma biomarker.

Overall, we confirmed that through emulsification it is possible to increase lipid absorption, as determined after feeding sodium-caseinate emulsions. However, this result could not be generalized. Interesterified-lipids emulsified with chitosan were not better absorbed than those contained in non-emulsified interesterified-lipids/distilled-water blends. This may be due to a limited access of pancreatic lipases to lipids due to the pH increase in the duodenum, which may counterbalance the positive effect of size reduction, or to the breakdown of the emulsion in the small intestine. Future studies may consider a deep analysis of the physical stability of the chitosan-based emulsions at different pH values, as well as additional in vitro studies under simulated gastrointestinal conditions to analyse lipase digestibility

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5 CONCLUSIONS

Throughout this thesis it was possible to conclude that chemical and enzymatic interesterification are suitable processes to produce structured lipids that introduce essential fatty acids, with zero *trans*, which may have the plastic behaviour of a commercial fat. Specifically, through this technology, it appears that it is possible to meet the nutritional benefits desired by consumers, with the technological properties required by producers. Certainly, an appropriate choice of raw materials and adequate process conditions are essential. Structured lipids could also mimic specific solid content profiles and associated melting kinetics, to produce plastic fats, which in turn could allow defining some of the organoleptic properties of food.

In relation to the inclusion of desired fatty acids, such as polyunsaturated ones, to make the most of the new structure, they must be absorbed. As shown in this study, a suitable way to evaluate fatty acids bioavailability, may be through postprandial plasmatic lipids analysis after animal feeding. Specifically, it was found that the measurement of plasmatic lipids, using linolenic acid as biomarker -when present in the raw material- was an effective way to determine lipids bioavailability. As linolenic acid cannot be synthesized *de novo*, it is a natural biomarker, easy to use, that allows avoiding additional costs.

In relation to the comparison between an interesterified mix and a direct blend, it was possible to conclude that fats with a higher melting point, produced by a different stereochemistry of triacylglycerols, did not show a reduced bioavailability, but a delayed absorption. Specifically, it was possible to conclude that modification of the melting profile of fat blends through interesterification, without altering the bioavailability determined by sn-2 stereochemistry, while maintaining the fatty acids profile, does not alter the bioavailability of such fats. Accordingly, enzymatic interesterification was proved to be instrumental to produce fats with desired physical properties, and to introduce fatty acids of interest while keeping them bioavailable.

Certainly, the food matrix is also of great concern, as reflected in this study. Different approaches can be used to design an appropriate food structure, which may maximize or reduce lipids and/or specific fatty acids bioavailability, as needed. Nutrients are compartmentalized into cell and tissues, and also in multicomponent structured matrices in the case of processed foods. Consequently, nutrients need to be released from these structures to become available for absorption. In the case of lipids, food emulsions are of particular interest, since they are widely used in the food industry. This study showed that it is possible to increase lipids bioavailability through O/W emulsification, probably due to the larger area exposed for hydrolysis. However, this depended on the interactions between the surface-active agent that was used and its surroundings, to a large extent. Sodium caseinate allowed increasing fatty acids bioavailability, whereas lipids that were emulsified with chitosan were equally absorbed as those contained in non-emulsified blends. This was thought to occur due to a limited access of pancreatic lipases to lipids due to the pH increase in the duodenum (pH>pKa), which may reduce chitosan solubility and thus, could counterbalance the positive effect of size reduction.

Certainly, further studies are needed to confirm the previous hypothesis and overall, a better understanding of the interactions between nutrients and its surroundings should be taken into consideration when designing an appropriate food structure to maximize or reduce the bioavailability of any compound. In the case of lipids, specific targets could be either to increase or to decrease the absorption of a specific fatty acid, depending on its nutritional value.

Nutrient bioavailability is a burning topic in food technology. The ability of a food matrix to effectively deliver specific nutrients, or conversely, trap them, is under development. Even though *in vitro* studies, which are cheaper and faster, may be used, *in vivo* studies consider all the complex interactions between nutrients and the several components of the diet, as well as real body conditions. On the other hand, it is difficult to isolated particular effects, and effort should be made to analyze the individual factors that may affect compounds bioavailability. In this respect, chitosan seems to have a good potential to reduce lipids bioavailability, but certainly additional studies should be made to reveal the

real mechanisms. This could well be achieved through the combination of *in vitro* and *in vivo* studies, to further contribute to the growing field of food structure design.

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