IMPACT OF LIPID OXIDATION ON DIGESTIBILITY OF DAIRY PROTEINS IN O/W EMULSIONS WITH AUTOXIDIZING LIPIDS

ERIKA LORENA SOTO CHAVARRO
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Director
MÓNICA OBANDO CHAVES
M.Sc Science and technology of Milk

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Director: MONICA OBANDO CHAVEZ y la codirección de ANGELICA SANDOVAL ALDANA

Jurado No.1: GUILLERMO ARRAZOLA
Jurado No.2: JONH JAIRO MENDEZ ARTEAGA

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2.2.1 Stripping of soybean and fish oil.................................................................25
2.2.2 Oxidation....................................................................................................25
2.2.3 Determination Of Primary Oxidation Products: Peroxide Value (POV) IDF Method. ..................................................................................................................26
2.2.3.1 Principle. ..................................................................................................26
2.2.3.2 Preparation of reagents. .........................................................................26
2.2.3.3 Calibration curve for peroxide value (POV) determination. .................27
2.2.3.4 Analysis of sample. .................................................................................27
2.2.3.5 Data calculations.....................................................................................27
2.2.4 Determination of secondary oxidation products: p-Anisidine value (P-AV) ....28
2.2.4.1 Principle..................................................................................................28
2.2.4.2 Procedure................................................................................................28
2.2.4.3 Data calculations.....................................................................................29
2.2.5 Experimental setup: interaction between autoxidized lipids and dairy proteins in o/w emulsion. .................................................................................................29
2.2.5.1 Preparation of the oil-in-water (O/W) emulsion........................................29
2.2.6 Determination of secondary lipid oxidation products ....................................30
2.2.6.1 Malondialdehyde (MDA) .......................................................................30
2.2.6.2 Hexanal. ..................................................................................................30
2.2.7 In-Vitro Digestion of emulsions: static model.............................................31
2.2.7.1 Procedure................................................................................................31
2.2.7.2 Static gastric phase.................................................................................31
2.2.7.3 Gastrointestinal digestion (Duodenal phase)..........................................31
2.2.8 Extraction of protein after in vitro digestion and determination of protein content by kjeldahl.................................................................32
2.2.8.1 Protocol for extraction of protein in digested and non-digested samples. ......32
2.2.8.2 Determination of nitrogen content by Kjeldahl......................... 32

2.2.8.2 Data calculations. The amount of nitrogen was calculated according to the
following equation:......................................................................................................... 33

2.2.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.. ................. 33

2.2.10 Statistical treatment of data.............................................................................. 34

3 RESULTS ................................................................................................................ 35

3.1 OIL OXIDATION STATUS OF THE STRIPPED FRESH AND OXIDIZED OILS .... 35

3.2 SECONDARY LIPID OXIDATION PRODUCTS IN EMULSIONS ......................... 36

3.3 DIGESTIBILITY IN EMULSIONS .......................................................................... 38

3.4 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS
(SDS-PAGE) ................................................................................................................. 38

4 DISCUSSION ............................................................................................................. 42

5 CONCLUSION ........................................................................................................... 50

RECOMMENDATIONS ............................................................................................... 51

REFERENCES .............................................................................................................. 52

APPENDIX ................................................................................................................. 63
LIST OF TABLES

Table 1 Characterization of oils at three oxidation levels .......................................................... 35
Table 2 Experimental and theoretical MDA content in O/W emulsions stabilized with casein and whey proteins .......................................................................................................................... 36
Table 3 Experimental and theoretical hexanal content in O/W emulsions stabilized with casein and whey proteins .......................................................................................................................... 37
Table 4 Digestibility in O/W emulsions .......................................................................................... 38
LIST OF FIGURES

Figure 1 Schematic representation of simple emulsions, oil in water emulsion (O/W) and water in oil emulsion (W/O). .................................................................................................................. 13

Figure 2 Theoretical development of primary and secondary oxidation products as a function of time in lipid oxidation .................................................................................................................. 18

Figure 3 Malondialdehyde (MDA) structure. ................................................................................. 20

Figure 4 Hexanal structure .................................................................................................................. 20

Figure 5 Electrophoretic pattern of emulsions containing casein (A) or whey protein (B) with fish oil after 24 h incubation at 4°C. ......................................................................................... 39

Figure 6 Electrophoretic pattern of emulsions containing casein (A) or whey protein (B) with soybean oil after 24 h incubation at 4°C. ......................................................................................... 40
The impact of lipid oxidation in the digestibility of milk proteins (casein and whey protein) in O/W emulsions from soybean and fish oil with different levels of oxidation (fresh, intermediate and high) were studied after incubation (24 hours, at 4ºC). Malondialdehyde (MDA) and hexanal were determined as markers of secondary lipid oxidation products. Emulsions were digested using an in-vitro gastrointestinal digestion (static model). After digestion, the remaining amount of nitrogen was determined in order to calculate the protein digestibility. SDS gel electrophoresis was used to visualize the aggregation or fragmentation of proteins. The results showed that casein based emulsions presented the higher concentrations of secondary products bound to protein in soybean and fish oil, hexanal 0.84 mg/mL and MDA 0.70 mg/mL at high oxidation level, respectively. Also the higher losses in protein digestibility occurred in this emulsions, soybean oil (57.3%) and fish oil (64.6%). It was observed that digestibility decreased gradually with increasing the oil oxidation levels. The electrophoretic analyses for digested samples of emulsions containing fish oil, confirm the presence of protein aggregates which were resistant toward in vitro gastrointestinal digestion process. The impact of autoxidized lipids on the protein digestibility loss in O/W emulsions, particularly for casein with soybean and fish oils at high oxidation level was evidenced. The aggregation occurs and this protein becomes more resistant to digestion as a result of the chemical interaction with the autoxidized lipid. This effect was greater for casein probably due to its chemical structure related to a possible hydrophobic interaction in the emulsion interface.

**Keywords:** lipid oxidation, dairy proteins, O/W emulsions, digestibility, soybean oil, fish oil.
RESUMEN

El impacto de la oxidación lipídica en la digestibilidad de proteínas lácteas (caseína y proteína del suero) en emulsiones O/W con aceites de soya y pescado, con diferentes niveles de oxidación fueron estudiados después de ser sometidos a incubación (24 horas, 4ºC). Malondialdehido (MDA) y hexanal fueron determinados como marcadores de productos secundarios de oxidación lipídica. Las emulsiones fueron digeridas usando un modelo estático in vitro de digestión gastrointestinal. Después de la digestión la cantidad de nitrógeno remanente fue determinada con el fin de calcular la digestibilidad proteica. La técnica de electroforesis (SDS-PAGE) fue usada para visualizar la agregación o fragmentación de proteínas. Los resultados mostraron que las emulsiones estabilizadas con caseína presentaron altas concentraciones de productos secundarios de oxidación lipídica enlazados a la proteína tanto en el aceite de soya, con hexanal (0.84 mg/mL), como en el de pescado, con MDA (0.70 mg/mL), en altos niveles de oxidación. Asimismo las altas pérdidas en la digestibilidad proteica se produce en estas mismas emulsiones con caseína en altos niveles de oxidación con aceite de soya (57.3%) y con aceite de pescado (64.6%). Se observó una disminución en la digestibilidad proporcional al incremento del nivel de oxidación de los aceites. Además, se confirmó la presencia de agregados de proteína en las muestras digeridas de las emulsiones con aceite de pescado los cuales resistieron a la digestión gastrointestinal. Se evidenció el impacto de los lípidos auto-oxidados sobre la pérdida de digestibilidad proteica en las emulsiones O/W, especialmente para caseína con aceite de soya y pescado en altos niveles de oxidación. Como resultado de la interacción proteínas con lípidos auto-oxidados se producen agregados de proteínas que se tornan más resistentes a la digestión gastrointestinal. Este efecto fue observado en gran medida en caseína probablemente debido a su estructura química relacionada a una posible interacción hidrofóbica en la interfase de la emulsión.

Palabras clave: oxidación lipídica, proteínas lácteas, emulsiones O/W, digestibilidad, soybean oil, fish oil.
INTRODUCTION

There is currently a growing demand for food products which prevent nutrition-related diseases and improve the physical and mental state of people, in this regard, milk products have been conducting for new market needs. This trend has created a need in the market to create and design new dairy products with extra properties which go further than just nutrition, such as those who contribute to prevent chronic diseases and cardiovascular problems (Dawczynski, Martin, Wagner and Jahreis, 2010). The intrinsic quality of dairy foods with added functional fatty acids is influenced by the possible occurrence of chemical processes such as oxidation of lipids and the consequent formation of new chemical substances that alter the organoleptic properties of the food, along with its nutritional value and functional value which may even be harmful to the body. However, enrichment of foods with omega-3 polyunsaturated fatty acid (PUFA) should be evaluated carefully, since they are highly susceptible to oxidation (Cucu, Devreese, Mestdagh, Kerkaert and De Meulenaer, 2011).

This susceptibility claims to have precautions in processing and storage to ensure product quality and safety (Mestdagh, Cucu and De Meulenaer, 2011). Thus, recent studies have focused on investigating the effect of lipid oxidation on the nutritional quality of dairy products (Kolanowski and Weissrodt, 2007; Livney, 2010). Since it is known that products of lipid oxidation not only interfere with the functionality of proteins by decreasing their nutritional value, but also the oxidation products are potentially toxic and lead to a risk for human health (Papastergiadis, 2014; Cucu et al, 2011; Mestdagh et al, 2011).

However, understanding the interaction between secondary lipid oxidation products and dairy proteins that leads to the formation of complexes and their impact on digestibility of proteins is still poorly understood. For these reason, the purpose of this study was to study the impact of the secondary lipid oxidation on the digestibility of dairy proteins, casein and whey protein, in O/W emulsions with the addition of autoxidized lipids, as
well as need to contribute to the understanding of the factors involving in these phenomena which it can be controlled to ensure the nutritional quality of these foods.
1. LITERATURE REVIEW

1.1 EMULSIONS

An emulsion is known as a disperse system in which two liquids media are one and in which one liquid is dispersed in the other as droplets (McClements, 2004a). Generally, these two liquids are water and oil. Although these dispersions are thermodynamically unstable with tendency to coalescence, flocculation or creaming (Tcholakova, Denkov, Ivanov and Campbell, 2006), the emulsions can be stabilized by adding substances before homogenization. These substances can be emulsifiers, surfactants or surface active polymers (McClements and Decker, 2000).

Emulsions can be typically classified as oil in water (O/W) and water in oil (W/O) systems. In O/W emulsions the oil droplet forming the dispersed phase and water is the continuous phase. On the contrary, in W/O emulsions the water is forming the dispersed phase and the oil is the continuous phase (Chung and McClements, 2014). In figure 1 a representation of the two types of simple emulsions is shown.

**Figure 1** Schematic representation of simple emulsions, oil in water emulsion (O/W) and water in oil emulsion (W/O).

<table>
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<th>Water continuous phase</th>
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Adapted from Chung and McClements, 2014.
The emulsifiers have a polar part that dissolves well in the aqueous phase and a nonpolar part that is soluble in the oil phase. Consequently, those molecules are absorbed in the interphase between water and oil (Hoffmann and Reger, 2014), forming a protective membrane against the aggregation of the droplets (Dickinson, 1992; McClements, 2004a), by generating repulsive forces between them. Emulsifiers commonly used in the food industry are amphiphilic proteins, phospholipids, protein–carbohydrate conjugates and small molecule surfactants. (McClements and Decker, 2000).

Some of the proteins widely used as emulsifiers in the food industry are whey protein, casein, soy and egg protein (McClements, 2004b). Proteins are considered as surface active polymers due to presence of hydrophobic groups and also because multipolar compounds that confer high chemical reactivity (Hoffmann and Reger, 2014).

The forces involve in the stabilization and destabilization of emulsions are mainly van der Waals, attractive forces, electrostatic interactions and steric factors. At pH values far from the isoelectric point of proteins, electrostatic repulsion occurs, preventing the dispersed droplets in the emulsion to come close together. Furthermore the interactions between protein-stabilized droplets can be influenced for the presence of certain ions, especially calcium (Singh and Ye, 2009).

1.2 DAIRY PROTEINS

In food systems, dairy proteins have found many applications due to their functional properties, including their ability to form stable emulsions (Singh and Ye, 2009). The main proteins in milk are casein and whey protein. Caseins are phosphoproteins with a random coil structure and precipitate at their isoelectric point (P.I 4.6). Whey proteins do not contain phosphorus, are globulins and remain in solution at P.I 4.6. In cow milk, caseins correspond to 82% of the milk protein approximately, whereas the remaining 18% are whey proteins. (Singh and Ye, 2009).
1.2.1 Casein. Casein is produced from skimmed milk by addition of hydrochloric or lactic acid precipitating caseins near their isoelectric point of pH 4.6. At this pH caseins are at their lowest point of solubility due to the decrease of intermolecular repulsion. Caseins have excellent holding water capacity and fat emulsification properties (Singh and Ye, 2009). They have an open tertiary structure due to its high content of proline, a feature that allows easy access for proteolytic cleavage by gastric proteases (Livney, 2010).

The amphipathic nature of the caseins is defined by both hydrophobic and hydrophilic residues and also due to positive and negative charges of aminoacid side chain groups distributed in different areas of the protein. This amphipatic nature contributes to their ability to stabilize oil in water emulsions (Horne, 2009).

1.2.2 Whey proteins. Represent 18-20% of the total nitrogen content in milk approximately. β-lactoglobuline is the main whey protein, followed by the α-lactalbumin that represents 20% of the whey protein fraction. Other fractions are bovine serum albumin and immunoglobulins. Whey proteins are highly susceptible to heat induced denaturation leading to coagulation (Jovanović, Barać and Maćej, 2005).

Whey proteins have secondary, tertiary and quaternary structure and in many cases most of them are globular proteins (Singh and Flanagan, 2006). In emulsions, whey proteins are rapidly absorbed, unfolding and reorienting at the oil-water interfase and (Singh and Flanagan, 2006).

1.3 POLYUNSATURATED FATTY ACIDS (PUFA’s)

In recent decades, many nutritional and epidemiological studies have linked the consumption of polyunsaturated fatty acids (PUFAs) omega-3 (i.e, α-linolenic acid, eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) to a significant decrease in some risk factors of cardiovascular disease, diabetes type 2, rheumatoid arthritis, Crohn disease, and chronic obstructive pulmonary disease (Simopoulos, 1999). Thus, the potential benefits of omega 3 fatty acids has stimulated a growing
interest in food fortification, particularly with oils rich in these fatty acids (Betti et al., 2009; Iafelice et al., 2008; Ye, Cui, Taneja, Zhu and Singh, 2009).

The high susceptibility of polyunsaturated lipids to lipid oxidation has restricted their incorporation into many food products, however which is unfortunate because greater consumption of polyunsaturated lipids is beneficial to health (Watkins and German, 1998) and is recommended in dietary guidelines (Kritchevsky, 1998). Progress in the development of PUFA-enriched foods with desirable nutritional and physical attributes depends on the availability of improved methods of controlling their oxidative stability, which in turn relies on a deep understanding of the mechanisms of lipid oxidation (McClements and Decker, 2000).

PUFA’s are found mainly in fish oil a source of omega-3 fatty acids, especially long-chain docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3). Fish oil is characterized by its high degree of unsaturation, the high content of the long-chain omega-3 type PUFAs (Tehrany et al., 2012). While the major source of fatty acid in soybean oil are PUFAs corresponding to alpha-linolenic acid (C-18: 3), 7-10% and linoleic acid(C-18: 2), 51% (Dhiman et al., 2000).

1.4 LIPID OXIDATION

The lipid oxidation process consists of a complex sequence of chemical changes that result from the interaction of lipids with reactive oxygen species (ROS). The mechanism of lipid oxidation in food depends on the nature of the reactive species present and their physicochemical environment (McClements and Decker, 2000).

1.4.1 Mechanisms of lipid oxidation. The oxidation process can be initiated by exposure to light (photo-oxidation); the presence and the activity of lipooxygenases (enzymatic oxidation) and by the direct reaction with oxygen (auto-oxidation), in this section only autoxidation process will be addressed.
1.4.1.1 Auto-oxidation. Oxidation of unsaturated lipids mainly by autoxidation proceeds through free radical chain reactions involving four steps: initiation, propagation, branching, and termination.

- **Initiation**: occurs by the loss of a hydrogen radical from an allylic position of an unsaturated fatty acid, and hence an alkyl radical is formed (Reaction 1). Abstraction of hydrogen occurs in the presence of initiators. Hydroperoxides present as impurities and redox metals produce radicals that act as initiators (Márquez, Holgao and Velasco, 2014). Initiation is more effective at high temperature and induced by U.V light.

  \[ \text{Initiation: } RH \rightarrow R\cdot + H\cdot \]  
  
  **Reaction 1**

- **Propagation**: the alkyl radical reacts with oxygen at rates controlled by diffusion to form a peroxyl radical (Reaction 2), which triggers the chain reaction by abstracting a hydrogen atom from another unsaturated lipid molecule and giving rise to a hydroperoxide as the primary oxidation product and a new alkyl radical that propagates the reaction chain (Reaction 3) (Márquez et al., 2014).

  \[ \text{Propagation: } R\cdot + O_2 \rightarrow ROO\cdot \] 
  \[ \text{ROO}\cdot + RH \rightarrow ROOH + R\cdot \]  
  
  **Reaction 2**
  **Reaction 3**

- **Branching**: consists of hydroperoxide decomposition and hence leads to the increase in the concentration of the free radicals. Decomposition of hydroperoxides is first monomolecular (Reaction 4) and becomes bimolecular (Reaction 5) when the hydroperoxide concentration is high enough. Latter reactions require lower activation energy (Márquez et al., 2014).

  \[ \text{Monomolecular} \quad \text{Bimolecular} \]  
  \[ \text{ROOH} \rightarrow RO\cdot + HO\cdot \]  
  \[ 2 \text{ROOH} \rightarrow \text{ROO}\cdot + \text{RO}\cdot + \text{H}_2\text{O} \]  
  
  **Reaction 4**
  **Reaction 5**
• **Termination**: radicals react between each other to yield relatively stable nonradical species (some reactions are 6 and 7). All stages of oxidation occur simultaneously in a complex series of sequential and overlapping reactions (Márquez et al., 2014).

\[
\text{ROO}^\bullet + \text{ROO}^\bullet \rightarrow \text{ROOR} + \text{O}_2 \quad \text{Reaction 6}
\]
\[
\text{R}^\bullet + \text{ROO}^\bullet \rightarrow \text{ROOR} \quad \text{Reaction 7}
\]

### 1.4.2 Primary lipid oxidation products: Hydroperoxides

Hydroperoxides are unstable compounds and decompose into radicals (mainly alkoxy and hydroxyl radicals) that follow different pathways to produce a great variety of secondary oxidation products, (i.e. ketones, aldehydes, epoxides, alcohols, hydrocarbons, acids). Hydroperoxides form and decompose simultaneously and constitute the most abundant compounds under low and moderate temperature conditions. When hydroperoxides are accumulated at relatively high levels, their decomposition becomes faster than their formation, and the overall oxidation rate increases exponentially (Márquez et al., 2014).

The decrease in the concentration of hydroperoxides occurs when the decomposition rate towards secondary lipid oxidation products exceeds the hydroperoxide formation rate (Frankel, 2005; Shahidi and Wanasundara, 2002). In the figure 2, it is observed that during the auto-oxidation process, the primary oxidation products are predominant in the early stages, whereas secondary oxidation products dominate at later stages.

**Figure 2.** Theoretical development of primary and secondary oxidation products as a function of time in lipid oxidation

Adapted from Frankel, 2005.
1.4.3 Secondary lipid oxidation products. Secondary lipid oxidation products are low molecular weight and typically volatile compounds that cause rancidity. Combinations of different decomposition compounds give different sensory properties (Fennema, Parkin and Damodaran, 2007; Frankel, 1985).

From the decomposition of hydroperoxides from ω-3 fatty acids a large variety of secondary oxidation products such as epoxides, ketones, aldehydes, alkanes etc. are produced. Particularly, the formation of aldehydes is considered to be relevant because of their low sensorial threshold and because of their reactivity towards biopolymers such as protein, RNA, DNA. Thus they are considered as toxic. (Esterbauer, Schaur and Zollner, 1991).

1.4.3.1 Malondialdehyde (MDA). One of the most studied secondary lipid oxidation products is malondialdehyde (MDA), which is widely used as a marker of oxidative stress (Onyango and Baba, 2010). This aldehyde is produced by multiple cleavages of cyclic hydroperoxides which are formed from fatty acids containing three or more double bonds (linolenic acid and higher) (Schaich, Shahidi, Zhong and Eskin, 2013). Hydroperoxy epidioxides of ω-3 and ω-6 PUFAs and bicycloendoperoxides have been identified as the main precursors of MDA (Papastergiadis, 2014).

MDA is considered as a highly toxic and mutagenic compound able to interact with macromolecules by binding with proteins, DNA and RNA forming a molecule known adduct. It has been demonstrated that MDA reacts readily with DNA to form adducts of deoxyguanosine and deoxyadenosine which can be mutagenic. Adducts have already been detected in human tissues (Lykkesfeldt, 2007).
1.4.3.2 Hexanal. Hexanal is an aldehyde formed by breakdown of hydroperoxides of omega 3 and 6 fatty acids (Zhou and Decker, 1999). According to Sanchez, Rodriguez, López and Paseiro, 2004, one of the best known synthetic pathways for hexanal occurs during oxidation of linoleic acid (linoleic acid) through 13-hydroperoxide.

The hexanal content is related directly with undesirable flavors and compound is readily detected due to its low odor threshold, below 5 ng g⁻¹ (Ha, Seo, Chen, Hwang and Shim, 2011). Hexanal has been identified as the main volatile aldehydes generated from lipid peroxidation in human milk (Elisia and Kitts, 2011). This compound has become a marker of lipid peroxidation, and is considered one of the main products of lipid oxidation due to the fact that it increases consistently during storage (Panseri, Soncin; Chiesa and Biondi, 2011).

Figure 4 Hexanal structure

Adapted from Holmback et al (2008)
1.5 INTERACTION BETWEEN PROTEINS AND SECONDARY LIPID OXIDATION PRODUCTS

In biological tissues and food systems, proteins are prone to degradation due to exposure to lipid oxidation products and their decomposition by-products, leading to the formation of protein-lipid complexes (Gardner, 1979). The strong interaction between proteins and lipids allow moreover that oxidation reactions can transferred easily from lipids to proteins (Wu, Zhang and Hua, 2009; Viljanen, 2005).

Moreover proteins can be subjected to fragmentation, aggregation and changes in hydrophobicity. All this modification leads to a loss of their nutritional value (Wu et al., 2009; Headlam and Davis, 2004; Tironi, Tomás and Añón, 2007).

Secondary lipid oxidation products, such as aldehydes, can both physically bind or react covalently to proteins (Gardner, 1979). There are two major pathways for reaction between unsaturated aldehydes and amino acids from proteins: Michael addition and Schiff’s base reactions. The formation of a Schiff’s base occurs when electrophilic carbon atoms of aldehydes and ketones are targets of nucleophilic attack by amines, as a result of this reaction the oxygen of the carbonyl group is replaced with nitrogen of the amine, resulting in the formation of a Schiff base. In the Michael addition the reaction occurs between carbon atom of the aldehyde and the nucleophilic amino acid residues (the e-amino group of lysine, the imidazole fraction of histidine or the sulfhydryls group of cysteine) (Viljanen, 2005).

Malondialdehyde, a bifunctional aldehyde (containing two functional groups) is a very reactive compound which reacts with nucleophilic amine groups to form covalent adducts with lysine residues of proteins. Other amino acids in which MDA may also react are histidine, tyrosine, arginine and methionine. This reaction lead to a loss of specific amino acid residues, protein cross-linking and fluorescence formation. Functional properties of the protein are reduced (Wu et al., 2009).
Hexanal, a saturated aldehyde, could potentially react with proteins, peptides, amino acids, polyamines, and sulfhydryls through Schiff base reactions. The study carried out by Zhou and Decker, 1999, suggests that the interaction between the carbonyl group of hexanal and Leu-His occurs with both the free amino acid group and the imidazole nitrogens.

Meynier, Rapon, Dalgalarondo and Genot, 2004, carried out a research about the ability of hexanal and t-2-hexenal to form covalent bonds with whey protein and sodium caseinate in aqueous solution. The results of the study suggest that covalent binding, leading to aggregation of proteins, losses in Histidine and Lysine, and the formation of fluorescent compounds, in the presence of t-2-hexenal and hexanal binding of aldehyde to proteins and their aggregation was also observed. In both cases, protein modifications were accompanied by a decrease in Triptophane fluorescence due to changes in protein conformation and in the environment of the amino acid.

1.6 DIGESTION

Digestion is the process in which the food is transformed into small components which can be more easily assimilated and absorbed by the body. The human gastrointestinal tract consist in a set of organs (mouth, stomach, small and large intestine) that interact and cooperate with each other, playing an important role in digestion and absorption of food.

Several researchers have studied the digestion using an in vitro static gastrointestinal digestion model with food matrices like emulsions (Picarello, Mamone, Chiara, Addeo and Ferranti, 2013; Malinauskyte, 2014; Stuknytė, Cattaneo, Masotti and De Noni, 2015). These in vitro tests exhibited several advantages such as versatility, time efficiency and costs. Moreover they are more practical compared to bioassays using experimental animal models (in vivo) (Picarello et al., 2013). The in vitro gastrointestinal digestion model involves mainly two phases: the gastric and intestinal phase.
In the gastric phase that begins in the stomach, the proteins are metabolized through proteolytic enzymes and due to the high acidity of the environment. Pepsine, the enzyme included in this phase, has an affinity for cleaved peptide bonds involving aromatic amino acids such as phenylalanine, tyrosine and tryptophan and hydrophobic residues (leucine). Furthermore it has been demonstrated that pepsine can breakdown bonds of acidic amino acids such as glutamic acid (Nik, Wright and Corredig, 2010). Proteolytic ability of pepsine depends on the tertiary structure of the protein and its conformation under hydrophobic or hydrophilic environments. Consequently the casein disordered structure is readily digested by pepsin; in contrast the β-lactoglobulin shows greater resistance to proteolysis in its native state, which is related to its highly folded conformation in solution (Nik et al., 2010).

In the intestinal phase, the partially digested emulsion, that is part of the chyme formed in the stomach phase is mixed with substances including bile salts, phospholipids, salts, bicarbonate, proteases (trypsin and chymotrypsine) and lipases (pancreatic lipase and phospholipase). After entering the duodenum, chyme is mixed with sodium bicarbonate, causing an increase in pH (1-3 in the stomach to the duodenum 5.8-6.5) where enzymes act more efficiently (McClements and Li, 2010).

The protease trypsin catalyzes the peptide chain at the terminal carbon (C-terminal) of the aliphatic amino acids, especially lysine and arginine; while chymotrypsine acts on aromatic residues such as phenylalanine, tyrosine and tryptophan. The peptides/protein hydrolysis does not occur solely because lipase are present (Singh and Ye, 2013). Phospholipids and bile salts from the liver (via the gall bladder), facilitate the emulsification of fats.

Lipase and bile salts inducing lipid hydrolysis in the duodenum, generating lipid digestion products as free fatty acids, cholesterol, monoacylglycerol, etc., that are solubilized within micelles and then are transported to epithelial cells for absorption. The function of bile salts is to displace any protein or peptide remaining in the surface of the oil droplet after protease activity. (McClements and Li, 2010).
2 MATERIALS AND METHODS

2.1 MATERIALS

Soybean oil and fish oil were obtained from Carrefour (Belgium N.V.) and Smit Zoon (The Netherlands) respectively; p-anisidine reagent (99% purity) was purchased from Acros Organics. Silica gel 60 was obtained from Merck (Germany), aluminum oxide (Al₂O₃) was obtained from Sigma-Aldrich (Switzerland), ferrous chloride, barium chloride (BaCl₂.2H₂O), ammonium thiocyanate (NH₄CNS, 99%), hydrochloric acid (37%) and iso-octane (99.5%) were purchased from Chem-Lab (Zedelgem, Belgium). Glacial acetic acid (99.7%), petroleum ether, methanol and hexane (99%) were from Fisher Scientific (UK). Potassium dihydrogen phosphate a.r. (KH₂PO₄) from Chem-Lab (Zedelgem, Belgium) and potassium phosphate dibasic (HK₂P O₄, ≥ 99.9% pure) from Sigma-Aldrich (St. Louis, MO, USA), hydrochloric acid (HCl 25%), sulfuric acid (H₂SO₄ 98%) and NaOH (99.8%) were obtained from Chem-Lab (Zedelgem, Belgium). Trichloroacetic acid (CCl₃COOH, TCA) (99+ %) was obtained from Acros organics (New Jersey, USA). Kjeltab CX tablets made up of 5 g potassium sulphate (K₂SO₄) and 0.5 g copper (II) sulphate (CuSO₄.5H₂O) were obtained from Thompson & Capper Ltd. (Cheshire, UK). Boric acid (H₃BO₃) was purchased from VWR (Leuven, Belgium). Mixed indicator was obtained from Merck (Darmstadt, Germany). Auto distillator Kjeltec 2200 (Foss Tecator). All reagents were analytical grade.

Sodium caseinate (Miprodan 30) and whey protein isolate (Lacprodan DI 9224) were purchased from Acatris food (Belgium).

Pepsin from porcine pancreas (P6867), lipase from porcine pancreas (Type II, 100 – 400 units/mg protein), bile extract porcine (B8631), trypsin from porcine pancreas (T0303), α-chymotrypsin from bovine pancreas (type lyophilized powder, ≥ 40 units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
2.2 METHODS

2.2.1 Stripping of soybean and fish oils. Soybean and fish oils were purified by stripping minor components such as pigments, tocopherols and sterols in a two-step procedure. In the first step, 50 g of oil were mixed with 50 mL of hexane. The solvent/oil mixture was then passed through a 40 cm (height) x 4 cm (diameter) glass column previously filled with SiO$_2$ activated was wrapped with aluminum foil to avoid light-induced oxidations during the purification process. The column was rinsed three times with 50 mL of hexane. The oil/solvent mixture was collected in an aluminum-covered round bottom flask. The solvent was evaporated using a rotavapor, then 40 g of each oil was mixed with 56 mL of petroleum ether and passed through a glass column (38 cm Height x 4 cm diameter) packed with petroleum ether and activated aluminum oxide (achived at 200 °C overnight in muffle furnace). The glass column was rinsed with 100 mL of hexane and the stripped oil was collected, rotavaporated (40°C), poured into a brown bottle and flushed with nitrogen to remove solvent traces and prevent oxidation during storage at –20 °C until use.

2.2.2 Oxidation. The stripped oils were oxidized under accelerated temperature conditions; the aging was carried out in an oven at 60 °C from 0-24 hours in the dark with air in the headspace of the container. The oxidation status for fresh and aged stripped oils was determined by the peroxide value (POV) and p-anisidine (p-AV) value.

Fresh stripped oil and oils with two different levels of oxidation were selected for the experiment. Based on studies with oils during frying process by Aladedunye and Przybylski, 2009, the p-AV values of the oils were used to classify oils oxidation levels as follows: fresh status is the stripped oil without heat treatment that contain the lowest concentration of lipid oxidation products, intermediate and high level were obtained heating the oils at 60°C according to oxidation kinetics on stripped fish and soybean oil performed on a previously study (Quintero, 2014).
2.2.3 Determination Of Primary Oxidation Products: Peroxide Value (POV) IDF Method.

2.2.3.1 Principle. The POV value was determined using the International Dairy Federation, 1991, spectrophotometric method with modifications according to Shantha and Decker, 1994, based on the ability of hydroperoxides to oxidize Fe$^{2+}$ (ferrous) to Fe$^{3+}$ (ferric) ions that once formed, react with ammonium thiocyanate in an acidic medium to form ferric-thiocyanate (chromophores), a red-violet complex with absorption spectra at 500-510 nm. It comprises a weighted amount of sample in a mixture of dichloromethane/methanol and addition of iron (II) chloride and ammonium thiocyanate. After a fixed reaction time, the optical density is measured.

2.2.3.2 Preparation of reagents. The iron (II) chloride stock solution was prepared under indirect, dimmed light as follows: 0.4 g of barium chloride (BaCl$_2$.2H$_2$O) dissolved in 50 mL of distilled water was gently mixed with a solution of 0.5 g of iron sulphate (FeSO$_4$.7H$_2$O). Then 2 mL of concentrated hydrochloric acid solution (10 N) was added to the resulting solution which was precipitated and filtrated until a clear liquid was obtained. The iron (II) chloride solution was poured in a brown bottle and stored until further use. The solution of ammonium thiocyanate (NH$_4$SCN) was prepared by weighting 30 g of NH$_4$SCN in a volumetric flask and diluting until 100 mL with distilled water.

Iron (III) stock solution was prepared as follows: 0.5 g of iron sulphate (FeSO$_4$.7H$_2$O) was dissolved in about 50 mL of 10 N hydrochloric acid then 2 mL of 30 % (w/w) hydrogen peroxide solution were added, the excess of hydrogen peroxide was removed by boiling for 5 minutes. Afterwards the solution was cooled down at room temperature (20 °C) and diluted with distilled water until 500 mL. From the previous solution 1 mL was transferred to a 100 mL volumetric flask and the volume completed with a mixture of dichloromethane: methanol (70:30) (Fe (III) concentration 10 mg/mL).
2.2.3.3 Calibration curve for peroxide value (POV) determination. The Fe (III) standard was used to prepare a calibration curve with concentrations ranging from 1 – 40 µg. In order to generate the chromophores, the final step comprised of the addition of 50 µL of ammonium thiocyanate to each sample. Then the samples were vortexed (2 – 3 s) and after 5 minutes the optical density was measured in a spectrophotometer at 500 nm. The solvent blank was made up by the mixture of dichloromethane: methanol (70:30).

The calibration curve was obtained in triplicate, the absorbance of samples were plotted versus Fe (III) concentrations expressed as µg Fe (III). The best fitting straight line through the points was used to calculate the slope.

2.2.3.4 Analysis of sample. A reagent blank was prepared (E_{reagent blank}) as follows: 10 mL of dichloromethane: methanol (70:30) mixture were mixed with 50 µL of ammonium thiocyanate, then 50 µL of Fe (II) solution was added. This blend was mixed for 2 – 3 s and the absorbance was recorded after 5 min at 500 nm.

For the determination of POV in either fresh or aged stripped oil samples 20 – 30 mg of oil depending on oxidation rate was weighted in a 10 mL volumetric flask wrapped with aluminum foil, then the sample was diluted until mark with dichloromethane: methanol (70:30). From the diluted solution 100 µL was taken and added to a test tube containing 10 mL of dichloromethane: methanol (70:30). Then 50 µL of ammonium thiocyanate were added, the mix was vortexed and 50 µL of Fe (II) were added to trigger the reaction. Finally the absorbance was measured spectrophotometrically after 5 min at 500 nm. All the data were recorded using a 1 cm path length quartz cell.

2.2.3.5 Data calculations. The peroxide value of oils, expressed as milliequivalents/Kg was calculated according to:

\[ \text{POV} = \frac{\text{Corrected absorbance} \times m}{55.48 \times W \times 2} \]
Where:
Corrected absorbance = $E_{\text{sample}} - E_{\text{reagent}}$

$m$ = slope of calibration curve
$W$ = mass in grams of the sample
$55.84$ = atomic weight of Iron

2.2.4 Determination of secondary oxidation products: p-Anisidine value (P-AV)

2.2.4.1 Principle. The p-anisidine value is a measurement of secondary oxidation products which resulted from decomposition of fatty acid hydroperoxides. The method is based on the reaction of p-anisidine and aldehydes, principally 2,4-dienals and 2-alkenals. As a consequence of the aforementioned reaction a yellow-colored compound is formed and which is detectable spectrophotometrically at 350 nm.

2.2.4.2 Procedure. The determination of secondary oxidation products in oxidized oils was based on American Oil Chemists Society, 1993, Official Method Cd 18-90. 0.25 g of p-anisidine reagent was dissolved in 100 mL of glacial acetic acid. The solution was stored at 4 °C and protected from light.

The sample size was taken depending on the oil oxidation rate as follows: 0.5 g for fresh and 0.2 g for oxidized oil were weighted in a 25 mL volumetric flask previously wrapped with aluminum foil to avoid direct exposition of sample to light. Then the sample was dissolved in isooctane and diluted to the mark.

For the spectrophotometric measurements isooctane was used as a blank was measured at 350 nm. Blank reagents was prepared by mixing 5 mL of isooctane and 1 mL of p-anisidine in a test tube. The mixture was vortexed and the optical density was recorded after 10 min.
A similar procedure was followed for test solution. 5 ml of dissolved oil was mixed with 1 mL of \( p \)-anisidine reagent, and the absorbance was recorded exactly after 10 min of reaction.

### 2.2.4.3 Data calculations.
All determinations including blanks readings were carried out in triplicate. \( p \)-anisidine value (P-AV) was calculated from the expression:

\[
P - AV = \frac{25 \times (1.2A_s - A_b)}{W}
\]

Where:
\( A_b \) = Absorbance of the solution before addition of \( p \)-anisidine reagent at 350 nm.
\( A_s \) = Absorbance of the solution after addition of \( p \)-anisidine reagent at 350 nm.
\( W \) = mass of oil examined in test solution in grams (g).

### 2.2.5 Experimental setup: interaction between autoxidized lipids and dairy proteins in o/w emulsion.

#### 2.2.5.1 Preparation of the oil-in-water (O/W) emulsion.
The oil in water pre-emulsions were prepared in 0.1M potassium phosphate buffer (pH 7.4) (continuous phase) mixing 6 mg/mL of protein (sodium caseinate or whey protein) and 3% of stripped soybean or fish oil (fresh, medium or high oxidation level) (disperse phase) with an Ultra-Turrax equipped with a S 25N–18G dispersion device during 3 min at 9000 rpm. This coarse emulsion was then homogenized for 1 pass (250 bar) at 50°C using a microfluidizer.

After homogenization, 100 mL of the emulsion was poured into graduated (250 mL) DURAN\textsuperscript{®} laboratory glass bottles, which were tightly closed making use of screw caps. Solutions of sodium caseinate and whey protein were prepared as controls. Samples were prepared in triplicate for each treatment including also the controls and then incubated at 4 °C during 24 hours under dark.
2.2.6 Determination of secondary lipid oxidation products

2.2.6.1 Malondialdehyde (MDA). The method of Papastergiadis, Mubiru, Van Langenhove and De Meulenaer, 2012, was followed to determine MDA. Trichloroacetic acid was added until final concentration of 15% to precipitate the protein and samples were kept on ice for 10 min, after that centrifugation at 13000 g per 30 min was done to get a clear supernatant. The top layer was discarded and 1 mL of supernatant was mixed with 3 mL of TBA reagent (40 mM dissolved in 2 M acetate buffer at pH 2.0) in a closed test tube and heated in a boiling water bath for 40 min. The reaction mixture was cooled prior to the addition of 1 mL of methanol. After filtration 20 µL of the sample was injected into a Varian C18 HPLC column (5 µm, 150 × 4.6 mm), held at 30 °C. The mobile phase consisting of 50 mM KH2PO4 buffer solution, methanol, and acetonitrile (72:17:11, v/v/v, pH 5.3) was pumped isocratically at 1 mL/min. Fluorometric detector excitation and emission wavelengths were set at 525 and 560 nm, respectively. For quantification, standard solutions of MDA in 7.5% TCA were prepared from 1,1,3,3-tetraethoxypropane (TEP) and calibration curves were prepared at a concentration ranging from 0.6 to 10 µM.

2.2.6.2 Hexanal. Hexanal formation in emulsions and in fish or soybean oil was evaluated using headspace solid-phase microextraction (HS-SPME) combined with gas chromatography–mass spectrometry (GC–MS). A total of 1 mL of emulsion was placed in a glass vial (size 10 mL, H 22.5 mm × 46 mm) and mixed with 2 mL of 0.02 M Na2HPO4, 0.02 M KH2PO4 pH 2 buffer. Butylated hydroxyanisol (BHA) dissolved in methanol was added in the vial at a final concentration of 2.8 M and 15 µL of internal standard of Hexanal–d12 was incorporated in the sample.

Then, the vial was sealed with a PTFE septum cup and was subjected to HS-SPME extraction. The SPME fiber (75 µm Carboxen/PDMS, Supelco, Bellefonte, PA,USA) was inserted into the headspace of the vial and left there for 30 min at 70 °C. Volatile compounds were desorbed by inserting the fiber into the injection port of an Agilent 7890A chromatograph (Agilent Technologies, Palo Alto, CA) operated in splitless mode.
for 10 min at 240 °C. Helium was used as carrier gas with a constant flow rate of 1.3 mL/min. The compounds were separated on a DB-624 column (60 × 0.25 mm x 1.4 μm). The oven temperature program began with 5 min at 50 °C for 5 min, increase to 4 °C/min to 140°C, then 30 °C/min increase to 240 °C for 10 minutes. An Agilent 5975C inert XL mass spectrometry detector was used and detection was carried out on the total ion current obtained by electron impact at 70 eV. The selected ion was 56 for hexanal. External calibration curves were prepared using a hexanal standard (Sigma–Aldrich).

2.2.7 In-Vitro Digestion of emulsions: static model

2.2.7.1 Procedure. For digestion of emulsion samples, the oral phase was considered negligible due to the fast swallowing thus, static gastric phase and gastrointestinal digestion (duodenal phase) were carried out as follows, according to the method described by Wickham, Faulks and Mills, 2009, with some modifications.

2.2.7.2 Static gastric phase. After incubation of the samples (24 hours, 4°C) glass laboratory bottles were taken out from cold room and immediately was initiated sampling. For this purpose 6 falcon tubes per treatment (including solutions) were filled with 5 mL of sample: 3 tubes were disposed for digestion and the rest were identified as non-digested samples.

First of all, the pH of arranged samples for digestion was adjusted at 2.0 by adding 8 M HCl, then 10 μL of dissolved enzyme (10 mg pepsine/1 mL of water) was incorporated, vortexed and incubated at 37 °C during 2 hours with constant shaking.

2.2.7.3 Gastrointestinal digestion (Duodenal phase). Initially the pH was adjusted to 6.5 by adding NaOH 8 M. Then, a mix containing 1 g of lipase (100-400 units/mg L3126) and 2.5 g of bile salts in 50 mL of buffer phosphate 5mM were prepared and 400 μL was added to each falcon tube. In addition, 15 μL of CaCl₂ was incorporated as well.
Afterwards, the trypsin and chymotrypsin were prepared (10 mg/ mL of 0.1 M HCL) and 10 µL of each one was incorporated to the emulsions. Then, samples were incubated at 37 °C for 2.5 hours with constant shaking. After incubation the pH of the samples was adjusted to 5.0 by adding 8 M HCL.

2.2.8 Extraction of protein after in vitro digestion and determination of protein content by kjeldahl.

2.2.8.1 Protocol for extraction of protein in digested and non-digested samples. The protein pellet was obtained according to Cucu et al., 2011, using TCA (trichloroacetic acid) as follows:

After the in vitro digestion, 5 mL of TCA (30% w/v) were added to each falcon tube containing 5 mL of emulsion (TCA 15% final concentration). Then, samples were incubated on ice for 10 min. The tubes were subsequently centrifuged (13000 g x 10 min at 4 °C). The supernatant was discarded and the remaining pellet was dissolved by adding 2 mL NaOH, 10% w/v. The dissolved pellets were poured in digestion tubes to determine percentage of nitrogen in samples by Kjeldahl method. The same process was undertaken for non-digested samples (in triplicate).

2.2.8.2 Determination of nitrogen content by Kjeldahl. The nitrogen content was determined according with the Kjeldahl method (Association Of Analytical Communities, 1981), that consists in a procedure of catalytically supported mineralization of organic material in a boiling mixture of sulfuric acid and sulfate salts at temperatures between 340–370 °C. In the digestion process the organically bonded nitrogen is converted into ammonium sulfate. The digest having been made alkaline with 50% NaOH is steam distilled with 2% boric acid to release the ammonia which is trapped and titrated with standard hydrochloric acid.
2.2.8.2 Data calculations. The amount of nitrogen was calculated according to the following equation:

\[ N \ (g) = \frac{14 \ast N \ast V1}{1000} \]

Where:
N = Nitrogen content expressed as mg/g
N = Normality of HCl
V1= Volume of consumed HCl

The digestibility percentage was calculated according to the following equation:

\[ \%D = 1 - \left( \frac{\text{mg nitrogen digested samples}}{\text{mg nitrogen non digested samples}} \right) \times 100 \]

2.2.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was carried out according to Laemmli, 1970. Possible protein crosslinking or fragmentation was studied using a 4% acrylamide stacking gel and a 15% acrylamide resolving gel (for protein separation). An aliquot of 20 μL (previously diluted until 1 mg protein/mL) of digested and non-digested samples was added to 20 μL Laemmli buffer containing mercaptoethanol. Then samples were heated at 95 °C during 5 min and centrifuge at 10000 rpm. Subsequently, 20 μL of samples and 10 μL of molecular weight marker (precision plus standard) were loaded on the gel. Electrophoresis was performed at constant voltage of 150 V. The gels were stained with Coomasie blue® for 1 hour afterwards were incubated with water for 1 hour. The gels were further read using the scan Gel Doc TM EZ Imager with Image Lab TM Software (Bio-Rad) the molecular masses of the proteins were recorded.
2.2.10 Statistical treatment of data. The data concerning digestibility, MDA and hexanal were determined in triplicates. One way two factors Analysis of Variance (ANOVA) with significance levels of $P<0.05$, was applied to detect differences between samples and were compared by LSD test. All statistical analyses were performed using SPSS 18 statistics package.
3 RESULTS

3.1 OIL OXIDATION STATUS OF THE STRIPPED FRESH AND OXIDIZED OILS

The evaluation of the oxidation status of the stripped fish and soybean oils was performed by using classical and generic parameters such as peroxide value (POV) and p-anisidine value (p-AV) in order to determine the level of primary and secondary lipid oxidation products respectively. In addition the hexanal and MDA of the oils has averaged as were (Table 1).

**Table 1 Characterization of oils at three oxidation levels**

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>Oxidation levels</th>
<th>POV [meq O₂/ kg Oil]</th>
<th>P-AV [-]</th>
<th>MDA [µg/g]</th>
<th>Hexanal [µg/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean</td>
<td>Fresh</td>
<td>0.49 ± 0.02</td>
<td>0.15 ± 0.04</td>
<td>5.42 ± 1.30</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>21.31 ± 1.30</td>
<td>50.29 ± 1.28</td>
<td>16.54 ± 0.07</td>
<td>134.10 ± 9.05</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>25.87 ± 0.80</td>
<td>97.48 ± 2.37</td>
<td>19.47 ± 1.40</td>
<td>231.56 ± 5.88</td>
</tr>
<tr>
<td>Fish</td>
<td>Fresh</td>
<td>1.73 ± 0.03</td>
<td>1.36 ± 0.55</td>
<td>13.83 ± 4.43</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>24.97 ± 1.00</td>
<td>52.41 ± 1.90</td>
<td>68.97 ± 7.06</td>
<td>3.72 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>28.83 ± 1.88</td>
<td>104.44 ± 2.65</td>
<td>85.31 ± 11.33</td>
<td>5.07 ± 0.46</td>
</tr>
</tbody>
</table>

From: the author

Notes: Data are presented as mean (n=3) ± Standard deviations. All three oxidation levels of the fish oil showed higher MDA values than soybean oil. While the soybean oil showed higher hexanal concentration for all oxidation levels compared to the fish oil.
3.2 SECONDARY LIPID OXIDATION PRODUCTS IN EMULSIONS

In Tables 2 and 3 the MDA and hexanal content measured in the emulsion are shown. On basis of the original MDA and hexanal content of the respective oil (theoretical) and the experimentally obtained aldehyde content, the amount of aldehyde bound to the protein could be estimated by the difference between them.

Experimental MDA concentrations in emulsions containing fresh soybean oil were not detected. In emulsions containing fresh fish oil, theoretical MDA concentrations (0.414 µg/mL) were lower than the experimental for whey protein (0.86 µg/mL) and casein (0.56 µg/mL) respectively and hence it was not possible to calculate the bound MDA. The whey protein and casein emulsions at intermediate and high oxidation with stripped fish oil showed higher concentrations than the emulsions with soybean oil.

Table 2 Experimental and theoretical MDA content in O/W emulsions stabilized with casein and whey proteins

<table>
<thead>
<tr>
<th>EMULSIONS</th>
<th>Experimental MDA in emulsions [µg/mL]</th>
<th>Theoretical MDA in emulsions [µg/mL]</th>
<th>Bound MDA in emulsions [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey intermediate soybean</td>
<td>0.32 ± 0.1 a</td>
<td>0.49</td>
<td>0.17</td>
</tr>
<tr>
<td>Casein intermediate soybean</td>
<td>0.46 ± 0.1 a</td>
<td>0.49</td>
<td>0.03</td>
</tr>
<tr>
<td>Whey high soybean</td>
<td>0.46 ± 0.1 a</td>
<td>0.59</td>
<td>0.12</td>
</tr>
<tr>
<td>Casein high soybean</td>
<td>0.60 ± 0.1 a</td>
<td>0.60</td>
<td>0.00</td>
</tr>
<tr>
<td>Whey intermediate fish</td>
<td>1.90 ± 0.1 b</td>
<td>2.07</td>
<td>0.17</td>
</tr>
<tr>
<td>Casein intermediate fish</td>
<td>1.88 ± 0.3 b</td>
<td>2.07</td>
<td>0.19</td>
</tr>
<tr>
<td>Whey high fish</td>
<td>2.16 ± 0.1 c</td>
<td>2.56</td>
<td>0.40</td>
</tr>
<tr>
<td>Casein high fish</td>
<td>1.86 ± 0.1 b</td>
<td>2.56</td>
<td>0.70</td>
</tr>
</tbody>
</table>

From: the author

Notes: Experimental data are presented as mean (n=3) ± Standard deviations. Different letters between oxidation levels: intermediate and high (in rows) are considered
statistically different at the 95% confidence level. MDA detection limit: 0.3 µg/mL. The theoretical MDA content was based on the amount of MDA originally present in each oil (Table 1). The amount of bound MDA was calculated by subtracting the experimental MDA content in the emulsion from the theoretically expected value.

Hexanal was not detected in emulsions containing fresh oils. Emulsions containing soybean oil at an intermediate and high oxidation level, showed higher hexanal values than the emulsions with fish oil. Furthermore, analysis of the experimental hexanal concentration (Table 3) showed statistically significant differences between emulsions containing soybean oil at different oxidation levels. This behavior was different for emulsions containing fish oil which showed a low hexanal content.

**Table 3** Experimental and theoretical hexanal content in O/W emulsions stabilized with casein and whey proteins

<table>
<thead>
<tr>
<th>EMULSIONS</th>
<th>Experimental hexanal in emulsions [µg/mL]</th>
<th>Theoretical hexanal in emulsions [µg/mL]</th>
<th>Bound hexanal in emulsions [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey intermediate soybean</td>
<td>4.60 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.02</td>
<td>-0.57</td>
</tr>
<tr>
<td>Casein intermediate soybean</td>
<td>4.22 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.02</td>
<td>-0.19</td>
</tr>
<tr>
<td>Whey high soybean</td>
<td>6.36 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.96</td>
<td>0.60</td>
</tr>
<tr>
<td>Casein high soybean</td>
<td>6.12 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.96</td>
<td>0.84</td>
</tr>
<tr>
<td>Whey intermediate fish</td>
<td>0.09 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td>Casein intermediate fish</td>
<td>0.07 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11</td>
<td>0.04</td>
</tr>
<tr>
<td>Whey high fish</td>
<td>0.11 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td>Casein high fish</td>
<td>0.09 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Notes: Experimental data are presented as mean (n=3) ± Standard deviations. Different letters superscripts between oxidation levels: intermediate and high (in rows) are considered statistically different at the 95% confidence level. Hexanal detection limit: 0.038 µg/mL. The theoretical hexanal content was based on the amount of hexanal...
originally present in the each oil (Table 1). The amount of bound hexanal was calculated by subtracting the experimental hexanal content in the emulsion from the theoretically expected value.

### 3.3 DIGESTIBILITY IN EMULSIONS

The protein digestibility percentage of the emulsions is shown in Table 4. A decrease was observed in digestibility mainly in emulsions containing casein with soybean or fish oil. Emulsion containing high oxidized soybean oil showed the lowest digestibility percentage.

**Table 4** Digestibility in O/W emulsions

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>Protein digestibility %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh oil</td>
</tr>
<tr>
<td>Casein Fish oil</td>
<td>71.43 ± 2.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Whey Fish oil</td>
<td>74.32 ± 0.94&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Casein Soybean oil</td>
<td>75.84 ± 3.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Whey Soybean oil</td>
<td>74.53 ± 1.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

From: the author

Notes: Data are presented as mean (n=3) ± Standard deviation. Different letter superscripts between oxidation levels: fresh, intermediate and high (in rows) are considered statistically different at the 95% confidence level.
3.4 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The electrophoretic patterns of the emulsions containing fish or soybean oils are shown in Figures 4 and 5 respectively. In Figure 4A (casein emulsions) and 4B (whey protein emulsions) containing fish oil, the formation of aggregates with high molecular weight was observed. A fraction of the aggregates could even not enter to the gel (lanes 5, 7 and 9). In the same lanes intensive bands with a molecular mass below 10 kDa were observed. On the contrary in the electrophoretic pattern for casein and whey containing soybean oil, shown in Figures 5A and 5B respectively, neither aggregates in the at the top of gel nor intensive bands below 10 kDa were observed in digested lanes.

**Figure 5.** Electrophoretic pattern of emulsions containing casein (A) or whey protein (B) with fish oil after 24 h incubation at 4°C.

Figure 6  Electrophoretic pattern of emulsions containing casein (A) or whey protein (B) with soybean oil after 24 h incubation at 4°C.

(A): lane 1: standard, lane 2: casein with fresh oil non digested, lane 3: casein with fresh oil digested, lane 4: casein with medium oxidized oil non digested, lane 5: casein with medium oxidized oil digested, lane 6: casein with high oxidized oil non digested, lane 7: casein with high oxidized oil digested. (B): lane 1: standard, lane 2: whey protein with fresh oil non digested, lane 3: whey protein with fresh oil digested, lane 4: whey protein with medium oxidized oil non digested, lane 5: whey protein with medium oxidized oil digested, lane 6: whey protein with high oxidized oil non digested, lane 7: whey protein with high oxidized oil digested
4 DISCUSSION

Fresh soybean and fresh fish oil presented the lowest peroxide value (POV) as shown in Table 1. However between the oil at intermediate and high oxidation level a notable difference was not observed for hydroperoxides. The p-anisidine value (p-AV) for both oils at the high oxidation level were twice the intermediate level value. These results suggested the possibility that the speed of decomposition of the hydroperoxides into secondary products exceeds the formation rate as indicated by the theory (Shahidi and Wanasundara, 2002).

Malondialdehyde and hexanal were determined in fish and soybean oils as secondary lipid oxidation products markers. In Table 1, MDA was found at higher concentrations at the three oxidation levels of fish oil with respect to soybean oil. The major source for the production of MDA are fatty acids highly unsaturated, with more than three double bonds in their structure, such as alpha linolenic acid [C 18:3 (n-3)], eicosapentaenoic acid [EPA 20: 5 (n-3)] and docosahexaenoic acid [DHA 22: 6 (n-3)] (Egert et al, 2012), EPA and DHA are present in fish oil in an amount of 18.4 g and 7.9 g/100 g fatty acids, respectively (see Appendix 1).

Analysis of variance (ANOVA) of the experimental concentrations of MDA (Table 2) showed that emulsions containing soybean oil were not statistically significant different compared with the intermediate and high oxidation levels, probably because of low content of MDA precursors in soybean oil. Emulsions containing fish oil had higher MDA concentration than emulsions containing soybean oil. The highest MDA content could be related to the high content of this aldehyde in the highly oxidized fish oil.

The experimental and theoretical MDA content presented in Table 2 showed that in the casein and whey protein based emulsions containing fish oil, the theoretical values expected for MDA were higher than the experimental ones. This may suggest that part of the MDA presented in the emulsion was bound to the protein. The casein based
emulsion containing fish oil (at high oxidation level) was the system with more bound MDA (0.70 mg/mL). The ability of MDA to form adducts and crosslinking with biomolecules such as proteins, DNA and RNA has been extensively reported (Esterbauer et al., 1991; Del Rio, Stewart and Pellegrini, 2005; Lykkesfeldt, 2007). This molecule is very prone to reaction with the nucleophilic side chain of cysteine, histidine and lysine residues due to its chemical structure (a three carbon aldehyde) (Esterbauer et al., 1991).

In addition, it has been reported that MDA can react with the free amino groups (especially the ε-amino group of lysine group) of proteins and therefore introduce carbonyl groups to this biomolecule to form adducts (Wu et al., 2009). Several authors suggest that the formation of crosslinks between MDA and the amino acids of the proteins is performed by Schiff base and Michael addition reactions (Refsgaard, Tsai and Stadtman, 2000; Wu et al., 2009; Gardner, 1979).

While the MDA was detected mainly in fish oil, the hexanal showed high concentrations in soybean oil, 134 µg/g and 231 µg/g at intermediate and high oxidation level, respectively (Table 1). Hexanal is a decomposition product of linoleic acid which is presented mainly in soybean oil (50.55 g/100g fatty acids, Appendix 1).

In emulsions containing soybean oil (at high oxidation level) the theoretical values expected for hexanal were higher than the experimental ones as a shown in Table 3, indicating that the system with more bound hexanal was casein based emulsion with soybean oil at high oxidation level (0.84 mg/mL). Other studies have also demonstrated that hexanal interact with amino acids, peptides and proteins (Refsgaard et al., 2000; Meynier et al., 2004; Zhou and Decker, 1999). The ability of hexanal to bind covalently to dairy proteins leading to protein aggregation and modification was reported by Meynier et al., 2004. Besides the interaction of proteins that contain sulfhydryl and amino groups with saturated aldehydes as hexanal has been studied by Zhou and Decker, 1999, showing that as a result of the interaction of histidine-hexanal, the hexanal carbonyl group reacts with the free α-amino group and imidazole nitrogen.
Moreover, it was observed that in emulsions containing soybean oil the experimental concentration of this aldehyde at intermediate oxidation level exceeded the expected concentration (theoretical). Probably due to the favored hexanal production even after the incubation period of these emulsions (Table 3). The hexanal formation can be performed through multiple pathways in chain reactions (alkoxyl, peroxyl radicals and hydroperoxides decomposition) (Shaich, 2013) and therefore with the accumulation of hexanal it was not possible to calculate the aldehyde bound to protein.

Another possible explanation is that other secondary lipid oxidation products apart from hexanal such as epoxy, ketones, dimers, alcohols and other aldehydes (not measured in this study), which could interfere with the hexanal binding sites with protein. Thus, this compound remained free in the continuous phase of the emulsion and available for its detection due to its high stability as a saturated aldehyde (Shahidi and Zhong, 2005; Zhou and Decker, 1999).

In the electrophoretic patterns for non-digested samples in casein and whey protein based emulsions containing fish and soybean oil (Figures 4 and 5), the formation of protein aggregates with high molecular weight (> 250 kDa) were observed. These aggregates were formed probably as a consequence of the interaction protein-protein and also protein with secondary lipid oxidation products (Cucu et al., 2011). The major targets for the oxidation are proteins and these reactions can produce modifications in this biomolecule that include oxidation of the side chain groups, backbone fragmentation, aggregation and consequently loss of functional properties of the proteins (Zhu et al., 2009). It has been reported that lipid oxidation-derived aldehyde is involved in cross-links between protein chains leading to the formation of high mass (250 kDa) protein aggregates (Sayre, Lin, Yuan, Zhu and Tang, 2006). Also Cucu et al., 2011, reported the strong influence of the kind of oil and its oxidation status in the protein oxidation. This study showed that fish oil and the highly oxidized soybean oil induced oxidation in whey protein, observed mainly in the increase of carbonyl content, loss of lysine, changes in amino acid composition and protein aggregates formation.
According to Schaich, 2013, all lipid oxidation products react in some way with proteins in parallel oxidation reactions. In this progressive damage known as co-oxidation, secondary lipid oxidation products, particularly aldehydes, acted in the later stages of lipid oxidation introducing different types of protein oxidation products. Lipid-protein complexes (aggregates, adducts and crosslinking formation) in addition to generate conformational and functional changes, can also interfere with the bioavailability of proteins and amino acids. As a consequence it may decrease the bioavailability of amino acid residues and modify the digestibility, which negatively affects the nutritional values of proteins (Lund, Heinonen, Baron and Estévez, 2010).

Secondary lipid oxidation products are capable to covalently bind to amino acids (Gardner, 1979) and thus block the action sites of the enzymes involved in the process of in vitro gastrointestinal digestion. The enzymes are unable to cleavage bonds involving specific amino acids and lead to a potential reduction in the protein digestibility.

As reported in the protein digestibility percentage (Table 4), in emulsions containing fish oil a decrease in the protein digestibility percentage at higher oxidation levels was observed, particularly in casein based emulsions. It is well known that the high oxidation susceptibility of fish oil (rich in PUFAs EPA and DHA) leads to the formation of the hydroperoxides and secondary oxidation products that are able to interact with the side chain of amino groups and therefore, inducing generation of protein modifications and/or degradation (Mestdagh et al., 2011). This degradation was even more pronounced when the lipids in the emulsion were more unsaturated and thus more prone to oxidation (Frankel, 2005).

Protein digestibility loss with increasing oxidation level in whey protein based emulsions with soybean oil was not observed (Table 4). These results could be due to a poor interaction between the hydrophobic amino acid of whey protein with the oxidation products of soybean oil in the emulsion interface. It has been reported that the polarity of the oil phase influence strongly on the degree of protein conformational re-
arrangement and the way in which amino acid are physically oriented in the O/W emulsion interface, due to the oil acts as a solvent of hydrophobic amino acids (Zhai, Day, Aguilar and Wooster, 2013). Also Maldonado, Wilde, Mulholland and Morris, 2012, reported that in emulsions the lower polarity oil (tetradecane) induced a greater attraction of the β-lactoglobulin hydrophobic residues towards the oil phase and therefore their strong interaction compared to olive oil. Consequently, polarity of the oil phase is one of the factors that might determine the adsorption of the protein at the interface leading to lipid-protein interaction.

On the contrary, in casein based emulsions containing soybean oil there was a difference in the digestibility percentage at intermediate and high oxidation levels with respect to fresh oil. Surprisingly emulsions with soybean oil presented a dramatic decrease in the protein digestibility loss. This result could be related to soybean oil generating sufficient secondary oxidation products that lead to the formation of aggregates. Also its chemical structure consists mainly of triacylglycerols that contain multiple oxidized fatty acids residues that could interact with nucleophilic amino acid residues in proteins which induce to protein aggregation (Cucu et al., 2011; Schaich, et al., 2013).

The protein digestibility loss results (Table 4) showed that the higher losses occurred in casein based emulsions at high oxidation level for soybean oil (57.3%) and fish oil (64.6%). It was observed that digestibility decreased gradually with increasing the oil oxidation levels. This is probably due to the open and unfolded structure of casein in the O/W emulsion, in which the protein exposes its hydrophobic groups in order to be absorbed in the emulsion interface covering completely the oil droplet (Aynié, Le Meste, Colas and Lorient, 1992). This structural conformation facilitates the interaction of casein with the secondary oxidation products from soybean and fish oils. Fomuso, Corredig and Akoh, 2002 and Hu, McClements and Decker, 2003, suggested that due to the flexible structure of caseins, these tend to form crosslinking in the presence of oxidized lipids. Also Chao and Standman, 1997, established that oxidative modification
could simultaneously result in exposure of hydrophobic groups due to protein partial unfolding and cross-linking hydrophobic interactions, resulting in aggregation.

On the contrary in whey protein based emulsions, significant losses of protein digestibility percentage were not observed; this can be due to its structure (rigid and compact) and thermal stability. In this study, temperature below 60°C were used to prepare emulsions and incubation at -4°C were not enough to unfold the protein completely, exposing its hydrophobic groups and covering fully the oil droplet. Therefore the interaction of whey protein with the secondary lipid oxidation products of the oxidized oils in the emulsion interface was partial and incomplete. Whey protein digestibility loss percentages were low or imperceptible particularly in emulsions containing soybean oil with the increase in oxidation level (Table 4). The interaction of whey protein with secondary lipid oxidation products at 70°C has been reported by Cucu et al., 2011 and Mestdagh et al., 2011, evidencing the formation of aggregates, fragmentations, among other protein modifications.

Also, Nicolai, Britten and Schmitt, 2011, suggest that when the β-lactoglobulin is heated, higher than 60°C, its structure is modified and becomes more mobile. Buried hydrophobic groups and the thiol group in the native state became exposed and interact with other molecules.

Several authors have been reviewed other factors involved in the interaction of proteins and oil droplet in the O/W emulsion interface and the differences between casein and whey protein behavior as emulsifiers (Hu et al., 2003; McClements, 2004a; Zhai et al., 2013., Horn et al., 2011; Singh and Ye, 2009). One factor is the unfolding and rearrangement of protein native structure, which is related with temperature, as previously discussed. Proteins as emulsifiers reduced the system free energy and therefore the interfacial tension due to their adsorption at the emulsion interface. In consequence caseins (flexible proteins) are more effective at reducing the interfacial tension due to its higher proportion of non-polar groups in contrast to whey protein (rigid protein) that have fewer non-polar groups (Dickinson and McClements, 1995).
Hu et al., 2003, showed that the amino acid composition and its physical orientation (e.g., toward the lipid or water face) for each type of protein might influence their interaction with the oil droplet in the O/W emulsion interface and oxidative stability. However more detailed studies about the role of amino acids in the oxidative stability in protein-stabilized emulsions are needed.

Another factor that might influence the protein interaction is the droplet size. The smaller droplet could be easier to oxidize because it has larger contact area on the surface and consequently it presented greater interaction with oxidized lipids and its products (McClements and Decker 2000). Hu et al., 2003, and Horn et al., 2011, showed that smaller droplets were formed in casein than in whey based emulsions. However it was not reported a direct correlation between the tendency for decreasing droplet size and increased oxidation. This suggests that other factors besides droplet size may influence lipid-protein interactions in O/W emulsions.

The SDS-PAGE analysis for digested samples of emulsions containing fish oil, display the smearing towards the top of gel with molecular mass higher than 250 kDa (Figure 4 A and B) corresponding to protein aggregates. These molecules showed a resistance towards proteolysis, despite of the enzymatic treatment with proteases in gastric and duodenal phases in the in-vitro gastrointestinal digestion of emulsions. Zamora and Hidalgo, 2000, observed in electrophoretic gels that protein aggregates with high molecular weight were more resistant to digestion.

According to Zhang, Xiao and Ahn, 2013, in general heavily oxidized proteins could present less digestibility due to protein aggregation, whereas slightly oxidized proteins are easily digested by proteases. This phenomenon occurs probably due to the conformational changes that expose some buried peptide bonds for enzyme hydrolysis and increase degradation susceptibility. Otherwise, strong oxidation environment can result in the formation of protein cross-linkings, which further leads to protein
aggregation. These cross-linkings and aggregates are more resistant to enzymatic degradation compared to native protein forms (Davies, Lin and Pacifici, 1987).

Figure 4 also showed that the gels of casein (A) and whey protein (B) based emulsions containing fish oil, had intense bands below the 10 kDa in the digested samples at the three oxidation levels (fresh, intermediate and high). This small protein fragments could indicate a backbone fragmentation. Similar result has been reported previously in whey protein emulsion with fish oil upon photo-oxidation (Mestdagh et al., 2011). Oxidants such as reactive aldehydes can directly attack the backbone of a protein to cause fragmentation and conformational changes in the secondary and tertiary structure of the protein (Zhang et al., 2013).

In contrast, in the electrophoretic patterns of the protein emulsions containing soybean oil showed in Figure 5 A and B, it was no observed smearing neither towards the top of the gel (up to 250 kDa), nor below 10 kDa. This may possibly indicate that the protein binding sites for dye Coomassie blue® (basic and aromatic amino acids) were occupied by secondary oxidation compounds. In fact, it has been reported that the amino acids lysine, arginine, histidine and tyrosine (which are the Coomassie blue® binding sites) are also the main targets for attack by lipid radicals and secondary lipid oxidation products as hexanal and MDA (Schaich, 2008).

Even though in this study the possible effect of the interaction of MDA and hexanal with milk proteins (casein and whey) in emulsion were observed, it is not appropriate to attribute the decrease in protein digestibility only to these two secondary oxidation products. Lipid oxidation is a dynamic process in which is produced, transform and react continuously several oxidation compounds via multiple pathways. Thus, it is known that epoxides are up to 1000 times more reactive with proteins than aldehydes, generating unidentified products that have no organoleptic changes (Schaich, 2008). Other lipid oxidation products as dimers and polymers are capable to reduce the digestibility and can damage the intestinal mucosa (Schaich, 2013).
5. CONCLUSION

To conclude, the impact of autoxidized lipids on the protein digestibility loss in O/W emulsions particularly for casein with soybean and fish oils at high oxidation level was evidenced. The casein emulsion with soybean oil, at high oxidation level, was the system with the most hexanal bound to protein and also the greatest protein digestibility loss. As a result of the chemical interaction of the autoxidized lipid with dairy proteins, the aggregation occurs and the protein becomes more resistant to digestion. This effect was greater for casein probably due to its chemical structure related to a possible hydrophobic interaction at emulsion interface.
RECOMMENDATIONS

The results of this study provide important evidence of the impact of lipid oxidation on protein digestibility in emulsions, which affects the nutritional value of dairy foods enriched with polyunsaturated fatty acids, generating implications for production, shelf life and consumer welfare. The consumption increase of functional foods where the matrices are dairy emulsions (e.g. yoghurt, flavored milk, salad dressing, nutritional beverages, infant formula, etc) should promote studies in order to understand the physical and chemical processes occurring in this type of emulsions to ensure quality.
REFERENCES


Papastergiadis, A. (2014). A *contribution to the risk assessment in relation to the formation of toxic aldehydes in foods as a result of lipid oxidation*. (PhD dissertation). Faculty of Bioscience Engineering, Ghent University, Gent.


### APPENDIX A FAT'TY ACID PROFILE OF THE FISH AND SOYBEAN OILS.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Fish oil</th>
<th>Soybean oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g Fatty acid/100g fatty acids</td>
<td>g Fatty acid/100 g fatty acids</td>
</tr>
<tr>
<td>C14:0</td>
<td>7,10</td>
<td>0,08</td>
</tr>
<tr>
<td>C16:0</td>
<td>15,02</td>
<td>10,57</td>
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<tr>
<td>C16:1</td>
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</tr>
<tr>
<td>C18:0</td>
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<td>3,95</td>
</tr>
<tr>
<td>C18:1c9</td>
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</tr>
<tr>
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</tr>
<tr>
<td>C18:2</td>
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</tr>
<tr>
<td>C18:3n-3</td>
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</tr>
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</tr>
<tr>
<td>C20:2</td>
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<td>--</td>
</tr>
<tr>
<td>C20:4</td>
<td>1,00</td>
<td>--</td>
</tr>
<tr>
<td>C20:5 EPA</td>
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<td>--</td>
</tr>
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</tr>
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<td>C22:6 DHA</td>
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<td>--</td>
</tr>
<tr>
<td>Others fatty acids</td>
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<td>0,77</td>
</tr>
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</table>

*From Quintero 2014*