THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Science des aliments et Nutrition

École doctorale GAIA

Unités de recherche UMR QUALISUD (Montpellier) & UMR IATE (Montpellier)

Étude des mécanismes de l'oxydation lipidique et des antioxydants dans des matrices alimentaires complexes en vue d'améliorer leur stabilité et la bioaccessibilité des lipides : Exemple des préparations pour nourrissons et farines infantiles enrichies en acides gras polyinsaturés à longues chaînes

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Le 14 décembre 2023

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UNIVERSITÉ DE MONTPELLIER

A ma famille, et mes amis.

« Je pense que tout est possible à qui rêve, ose, travaille et n'abandonne jamais. »

Xavier Dolan

Pour commencer ce manuscrit, je souhaite exprimer ma reconnaissance envers toutes les personnes qui ont contribuées de près ou de loin à la réalisation de ce travail de thèse. A mon sens la réussite de ce projet est indissociable du soutien de ces personnes, dont l'impact va bien au-delà de la simple rédaction du texte final.

Je souhaite tout d'abord vivement remercier l'équipe projet en commençant par mes directeurs de thèse : Pierre Villeneuve et Claire Bourlieu-Lacanal. Merci de m'avoir choisie et d'avoir rendu possible la concrétisation de cette thèse. Ce fût trois années riches et intenses parfois difficiles mais votre soutien m'a permis d'aller au-delà de ce que je pensais être capable de réaliser. Merci infiniment pour votre confiance du début à la fin du projet, pour votre bienveillance et pour tous ce que vous m'avez appris. Vous avez eu un impact autant sur le plan professionnel en contribuant très largement à ma construction entant que chercheuse, que sur le plan personnel en m'aidant à m'affirmer et à prendre confiance en moi.

Claire, tu as été, tout au long de ma thèse, un réel modèle et je te remercie pour ton soutien jusqu'à la dernière minute de la rédaction et bien au-delà ! Merci de m'avoir formée et motivée comme tu l'as fait, et surtout d'avoir supportée mes boulettes (fréquentes) pendant ces trois ans (style renverser les 15 L d'eau MQ que tu venais juste d'aller chercher à l'autre bout de Montpellier). Je te suis infiniment reconnaissante pour ta patience et ton dévouement ; avoir eu la chance de t'avoir comme directrice de thèse est un véritable privilège, pour lequel je te dois mille merci.

Pendant ce projet, j'ai eu la chance d'être suivie et encadrée par Youna Hemery et Erwann Durand. Merci à vous pour votre implication dans mon projet de thèse, pour vos précieux conseils et pour tous les moments que nous avons partagés. Un merci particulier à toi Youna pour ton accueil dans les laboratoires de l'IRD, ce fût un vrai refuge dans les moments difficiles.

Je souhaite également remercier chaleureusement Nathalie Barouh et Bruno Barèa pour votre implication dans mon encadrement de thèse et dans ma formation en tant que chercheuse. Vous m'avez enseigné la rigueur scientifique et avez cultivé ma curiosité et mon envie de réussir. Merci pour votre soutien sans faille, votre présence et de manière générale pour tout le temps que vous m'avez accordé. Vous avez été, et comme je vous appelle souvent, mes « Papa & Maman labo », bien plus que des encadrants vous m'avez permis de grandir à travers vos conseils. Merci pour toutes les discussions scientifiques ou non, les rires et les centaines d'heures de labo que nous avons partagé. Et surtout merci pour les tonnes de bonbons (parce que je reste malgré tout une grande enfant) !

Un grand merci à mes deux stagiaires, Marine Tournaux et Noémie Dormoy pour votre efficacité et votre implication dans le projet. Je n'aurais pas pu espérer mieux pour ma première expérience d'encadrement. Vous avez été formidables, de vraies Power Rangers !

Je tiens à exprimer ma reconnaissance à l'ensemble des membres de mon jury de thèse composé de Fernando Leal-Calderon, Marie-Caroline Michalski, Amélie Deglaire, Thierry Durand, Charlotte Jacobsen, Jean-Charles Martin, Benjamin Choque et Frédéric Carrière. Je vous remercie pour l'attention que vous avez porté à mon travail et pour notre échange scientifique à l'occasion de ma soutenance.

Je souhaite également remercier l'ensemble des personnes avec qui j'ai eu la chance collaborer et/ou interagir en commençant par les membres de mon comité de thèse et notamment Claire Berton-Carabin, Frédéric Carrière et Véronique Vié pour vos précieux conseils et nos discussions scientifiques autour de mon projet au cours des différents CSI. Un grand merci à Simon Bugeat, pour tes conseils au début de ma thèse et à Philippe Guesnet pour la co-écriture notre revue CND. Merci pour votre gentillesse et accessibilité, ce fut un réel plaisir d'avoir eu l'occasion de collaborer avec vous.

Un grand merci à l'INRAE et au CIRAD d'avoir généreusement financé ce projet, et sans qui sa concrétisation aurait été impossible.
Le succès de cette thèse repose également en grande partie sur le soutien et l'aide précieuse de tous les membres des équipes dans lesquelles j'ai été chaleureusement accueillie à commencer par l'équipe Graines de l'INRAE avec notamment Valérie Micard, Cécile Baron ou encore Valérie Lullien-Pellerin. Un merci particulier à Reine Barbar et Carole Antoine-Assor pour votre aide et implication dans mon projet sur des parties pour lesquelles mon niveau était proche de zéro quand je suis arrivée. La pluralité de vos expertises et de celles des personnes de l'équipe a été un réel atout qui a fortement contribué à l'enrichissement de mes connaissances. Je souhaite également remercier infiniment le quatuor Mélina, Pauline, Laetitia, Jeremy. Merci pour votre aide, votre écoute, votre soutien et pour tous nos rires partagés. Ce fut un réel bonheur de travailler en votre compagnie !
Je remercie aussi vivement la plateforme PLANET, et notamment Adrien Reau, Leslie Lhomond, George Marval et Charlène Fabre pour votre expertise, votre aide et vos conseils pour le broyage de mes matières premières, qui nous ont parfois fait des nœuds au cerveau.

Du côté CIRAD, mon deuxième organisme d'accueil et certainement celui où j'ai passé le plus de temps, je voudrais chaleureusement remercier toutes les personnes de l'équipe lipide et plus largement de l'équipe 1 avec qui j'ai pu travailler et eu la chance d'interagir au cours de ces 3 ans. Merci notamment

à Marc Lebrun de m'avoir formée à l'utilisation de la DHS et accompagnée dans l'analyse de ces résultats. Je remercie également les personnes de la Hall Techno pour le temps que vous m'avez accordé et l'ensemble des ressources que vous avez mis à disposition du projet.
A mes collègues de bureau/vie pendant ces 3 ans passés au CIRAD ou encore présents, un grand merci pour notre entraide, votre bonne humeur et pour tous les moments partagés (surtout pour manger des gâteaux !) à commencer par Marie-Amélie et Charles avec qui tout a débuté dans des circonstances assez particulières à moitié au labo à moitié confinés. Une pensée également pour Mignon, Celia, Millena, Imane, Camille, Bee, Marianne, Baptiste et bien d'autres …
Un grand merci également à Jeanne (Janey pour les intimes), rencontré dans un congrès et finalement devenue une très bonne amie et un important pilier de vie. Pour nos vacances sur un autre continent, nos chaï à répétition au Starbucks et notre entraide infaillible, merci infiniment !

J'ai eu la chance et l'opportunité de réaliser une partie de mes expérimentations au sein des laboratoires de l'IRD. Merci à tous les membres de l'équipe pour votre accueille, votre aide et implication dans ces manips et notamment à Morgane Chapron, Isabelle Rochette, Christèle Vernière et Claire Moquet-Rivier. Une mention spéciale pour Nelly, merci pour ta présence, ton soutien et ton réconfort dans les bas (assez fréquents). Je suis très heureuse d'avoir pu partager ça avec toi (même si parfois on se paniquée l'une l'autre) !

Ces remerciements n'en seraient pas si j'omettais de remercier mon acolyte de bureau et bien plus encore, Juliette. Merci infiniment d'avoir été là, de m'avoir soutenue et aidé, sans toi cette thèse n'aurait pas été la même. Merci pour tous les fous rires (ça se compte en milliers !), les voyages et aussi les bêtises que nous avons partagées (RIP mon vélo et mon arcade sourcilière). Merci d'avoir ris à toutes mes blagues sans exception et d'avoir supporté mon humour parfois « lourdingue ». Tu as été comme un rayon de soleil dans mes journées et la meilleure voisine de bureau que j'aurai pu espérer, même si on était parfois « un peu » turbulentes (en même temps qui a la chance d'avoir une de ses meilleures amies à moins de 2 mètres pendant 3 ans ?). Aristote a dit « *Il n'y a point de génie sans un grain de folie* » alors merci d'avoir cultivé ma folie peut être que comme ça, un jour, j'atteindrais le génie.

Dans mes autres acolytes il y a, bien évidemment, Gaëlle. Je pense que je pourrais écrire une deuxième thèse sur toutes les raisons pour lesquelles je souhaite te remercier (je suis cap !). Tu as été un vrai pilier autant sur le plan professionnel, en m'encourageant chaque jour (et surtout en passant une semaine à relire ma thèse et à me nourrir pendant la rédaction !), que sur le plan personnel par ta présence et ton soutien sans faille. Merci pour toutes nos expériences culinaires (c'était délicieux), nos

blabla run, nos rando/treks (et toutes les galères qui vont avec), nos week-ends à faire mille activités, nos voyages, les dizaines de concerts/festivals, nos sessions escalade, padel, yoga et même boxe, et surtout d'avoir été la best partenaire pour faire tout ça. Je te remercie du fond du cœur d'avoir pris soin de moi pendant ces 3 années !

Ma dernière acolyte (et pas des moindre), Apo *alias* mon Tamtam, même avec 700 km de distance tu as été présente chaque jour pour me soutenir et me rappeler qu'il fallait être fière de ce que je réalisais. Tu as toujours su trouver les mots justes dans les moments difficiles et être heureuse avec moi dans les moments de bonheur. Merci pour nos centaines de visio à dire n'importe quoi, dans n'importe quelle circonstance, à n'importe quelle heure (du jour ou de la nuit). Tu as très largement contribué à mon équilibre pendant ces 3 ans, et pour ça, merci infiniment ! Je te remercie aussi pour tous les moments que nous avons partagés, avec beaucoup beaucoup de rires notamment grâce au 1308 photos partagées sur WhatsApp, et je sais que ces moments seront encore nombreux !

J'ai la chance immense d'avoir été entourée par des personnes merveilleuses tous au long de ma thèse et notamment mes amis, sans qui ces trois années n'auraient pas été les mêmes. Merci à ceux qui font partie de ma vie depuis déjà longtemps, tout comme ceux rencontrés tout au long de ma thèse. Je vous remercie du fond du cœur pour votre présence, votre écoute, pour m'avoir fait décompresser, voyager, rire, sortir, et surtout d'avoir maintenue ma santé mentale à flot ! Merci pour tous les moments que l'on a partagés et ceux à venir. Je pense notamment à Pp, Léa, Ana, Flo, Mathou, Hugo, Auré, Juliette, Ophélie, Zoé, Marion, Léna et bien d'autres…
Une mention spéciale pour Thomas, mon coloc (le préféré de tous). J'appréhendais énormément d'arriver dans cette ville qui m'était, jusqu'alors, inconnue, mais tout a changé quand je t'ai rencontré. Je suis très heureuse d'avoir pu vivre ma première année de thèse avec toi. Merci pour ta bonne humeur constante, pour toutes ces soirées à regarder Top Chef, faire des dégustations à l'aveugle ou juste à être affalé dans le canapé en disant n'importe quoi, et surtout merci d'avoir partagé tous ces moments avec moi !

Pour terminer je voudrais remercier ma famille pour votre présence, votre soutien sans faille, votre écoute (même quand vous n'y comprenez rien à ce que je raconte) et surtout votre amour immense. Merci d'avoir été là aussi bien dans les moments difficiles que dans les moments de joies et d'avoir sans cesse cru en moi. Je suis très heureuse d'avoir eu la chance de partager ça avec vous et d'avoir pu vous rendre fière. Cette fois promis, Papa, Maman j'arrête les études !

Merci à tous pour ces trois merveilleuses années !

Mathilde

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ALA: α-Linolenic Acid
ARA: Arachidonic Acid
Aw: Water Activity
BHA: Butylated Hydroxyanisole
BHT: Butyl Hydroxy Toluene
BSSL: Bile Salt-Stimulated Lipase
CCL: Critical Chain Length
CD: Conjugated Dienes
CLSM: Confocal Laser Scanning Microscopy
CMC: Critical Micelle Concentration
DAG: Diacylglycerol
DHA: Docosahexaenoic Acid
DPL: Dairy Phospholipids
DSC: Differential Scanning Calorimetry
EFSA: European Food Safety Authority
EPA: Eicosapentaenoic Acid
EU: European Union
FA: Fatty Acid
FAME: Fatty Acids Methyl Esters
FFA: Free Fatty Acid
GC: Gas Chromatography
HIV: Human Immunodeficiency Virus
HMP: High Melting Point
HPH: High Pressure Homogenization
HPLC: High Performance Liquid Chromatography
IDA: Iron Deficiency Anemia
IF: Infant Flours
IFF: Infant Follow-on Formulas
IMP: Intermediate Melting Point
LA: Linoleic Acid
LC-PUFA: Long Chain Polyunsaturated Fatty Acid
LOX: Lipoxygenase
MDA: Malondialdehyde
MFG: Milk Fat Globule
MFGM: Milk Fat Globule Membrane
MUFA: Monounsaturated Fatty Acid
OA: Oleic Acid
PC: Phosphatidylcholine
PCA: Principal Component Analysis
PE: Phosphatidylethanolamine
PEM: Protein-Energy Malnutrition
PI: Phosphatidylinositol
PL: Phospholipid
PLA2: Phospholipase A2
PLRP1: Pancreatic Lipase Related Protein 1
PS: Phosphatidylserine
PUFA: Polyunsaturated Fatty Acid
PV: Peroxide Value
RA: Retinyl acetate
RE: Retinol Equivalent
RP: Retinyl Palmitate
SFA: Saturated Fatty Acid
SM: Sphingomyelin
TAG: Triacylglycerol
TBA: Thiobarbituric Acid
TBARS: Thiobarbituric Acid Reactive Substances
TCA: Trichloroacetic Acid
TCEP: Tris(2-carboxyethyl)phosphine
TE: Total Energy
TLC: Thin-Layer Chromatography
TMCA: 3-4-5-trimethoxy-trans-cinnamic acid
UNICEF: United Nations International Children's Emergency Fund
UPLC: Ultra Performance Liquid Chromatography
VAD: Vitamin A Deficiency
VMC: Vitamin and Mineral Complex
WHO: World Health Organization
WPI: Whey Protein Isolate

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GENERAL INTRODUCTION

"Let food be thy medicine and medicine be thy food" - Hippocrate, 400 BC

This ancient quote underlines the importance of nutrition and its effect on overall health. It encourages to consider dietary practices as a key component of both short- and long-term health.

This principle is even more applicable during infancy, a key window of development. The "1,000-days" concept, referring to the critical period of infant development from conception to the age of two, emphasizes the importance of providing adequate nutrition at this stage of life (Martorell 2017). Indeed, nutritional intake is essential to support the rapid growth and development characteristic of this period, and is also involved in programming effects (Koletzko, Brands *et al.* 2017, Van Der Beek and Oosting 2020). During the first two years of life, development is usually divided into three stages (Lönnerdal and Hernell 2016). During the first stage, from 0 to 6 months, exclusive breastfeeding is strongly recommended by the WHO (WHO 2011). However, for various reasons, breastfeeding is not always possible and/or desired, and it is estimated that only 48% of infant under 6 months are exclusively breastfed (WHO 2022). Infant formulas can then be chosen as a breast milk substitute. The second stage of development, from 6 to 12 months, is a pivotal period during which breast milk and/or infant follow-on formulas (IFF) are no longer able to cover all the energy, macro- and micronutrient requirements of infants. In order to supplement the milk intake (breast milk or IFF), complementary foods such as infant flours (IF) are gradually introduced into the infant diets (WHO 2003, WHO 2009, WHO 2021). Finally, in the third age, from 12 to 24 months, dietary diversification continues until it becomes exclusive.

Among macronutrients, lipids are the main energy suppliers during infancy, providing 40-55% of calorie intake (FAO 2001, FAO and WHO 2004, Delplanque, Gibson *et al.* 2015, EFSA 2017). In addition to serving as an energy storage form, lipids are involved in membrane structure and the generation and propagation of various physiological signals. Consequently, lipids play an essential role during infancy, particularly on the cognitive, visual, immune, and digestive development (Koletzko and Rodriguez-Palmero 1999, Guesnet, Ailhaud *et al.* 2013, Cusick and Georgieff 2016, Miles, Childs *et al.* 2021). Dietary lipids are mainly found in their neutral form, namely triacylglycerol (TAG), composed of three fatty acids (FA) esterified to a glycerol backbone. However, other forms are also found such as phospholipids, which include two FA esterified on a glycerol with a polar compound, or sphingolipids, composed of a single FA, a sphingosine and a polar residue. These differences in structure confer different physico-chemical and nutritional properties to lipids, and determine their biological role. Among FA, some are classified as essential due to the human organism inability to synthesize them (Delplanque, Gibson *et al.* 2015). This is the case for the metabolic precursors and leaders of the non-interconvertible omega 6 (ω6) and omega 3 (ω3) groups. These are linoleic acid (LA, carbon chain of

18 with 2 double bonds, C18:2) and α-linolenic acid (ALA, carbon chain of 18 with 3 double bonds, C18:3). These FA, through a specific pathway of successive enzymatic desaturations-elongations, lead to the biosynthesis of other long-chain polyunsaturated FA (LC-PUFA, carbon chain equal to or greater than 20) such as docosahexaenoic acid (DHA, carbon chain of 22 with 6 double bonds, C22:6 ω3) and arachidonic acid (ARA, carbon chain of 20 with 4 double bonds, C20:4 ω6), which are essential for the cognitive development and visual acuity of infants (Carlson, O'Loughlin *et al.* 2019, Koletzko, Bergmann *et al.* 2020). A deficiency in these FA can have significant long-term consequences, increasing the risk of developing certain mental and/or metabolic diseases in adulthood. It is therefore recognized that lipid intake is both quantitatively and qualitatively important during infancy.

To ensure an adequate nutritional intake, particular attention must be given to the composition of infant foods. PUFA-source raw materials or product fortification are therefore commonly used. However, due to their multiple double bonds, these FA are prone to lipid oxidation, which is one of the main alteration phenomena in food products. This auto-catalytic free-radical reaction is initiated by external factors such as exposure to light, elevated temperature or the presence of oxygen, radical species and transition pro-oxidant metals (Stephens 1928, Frankel 1980, Kamal-Eldin 2003, Schaich 2005, Berton-Carabin, Ropers *et al.* 2014, Laguerre, Bily *et al.* 2017, Villeneuve, Bourlieu-Lacanal *et al.* 2021). It is generally admitted that lipid oxidation can occur *via* three chemical pathways: auto-oxidation, enzymatic oxidation or photo-oxidation. Autoxidation, induced by triplet oxygen, is the major oxidation pathway in processed products. The reactions involved in oxidation mechanisms are usually presented according to a simplified three-step scheme including initiation, propagation and termination steps leading to the formation of primary oxidation products (hydroperoxides), which then react to form volatile compounds belonging mainly to the aldehyde and ketone family (Schaich 2020). Lipid oxidation results in a decrease in nutritional properties (loss of unsaturated FA and fat-soluble vitamins) and organoleptic properties (formation of volatile compounds), but also exposes to potentially toxic oxidation products.

In this context, and as detailed in the section “Aim and strategy”, this PhD project aims at understanding the physico-chemical parameters that are at play in IFF and IF oxidative stability and bioacessibility.

The present manuscript is divided into seven chapters. The first chapter reviews the current state of knowledge about the issues related to infant nutrition, with a particular focus on the lipid fraction of the diet. This chapter also presents the challenges associated with the oxidative sensitivity of infant foods and the various strategies currently in place to control this phenomenon.

The second chapter presents the formulation of the systems studied and describes all the experimental techniques used to characterize these systems and assess oxidation and digestion kinetics.

The third chapter is devoted to the study of the oxidative stability of IFF according to lipid formulation and structural parameters.

The fourth chapter pursues the same objective but is applied to the dry granular systems represented by IF.

The fifth chapter presents the results obtained from the study of nutrient bioaccessibility in a limited number of systems with optimized oxidative stability and nutritional properties.

The sixth chapter is devoted to a general discussion of all the results, organized around a comparison of liquid emulsified systems with dry granular systems.

Finally, the seventh chapter concludes this manuscript and provides possible perspectives for completing this work.



CHAPTER 1

Bibliographic synthesis

This chapter is organized into three main sections. The first one provides a review of current knowledge about infant nutrition, their nutritional needs in macro and micronutrients, their metabolism, and their role in development. A particular focus is given to lipid compounds (FA and fat-soluble vitamins), which are the objects of study in this thesis. In this first part, the worldwide nutritional status of infant under the age of five will be presented, along with the strategies implemented against nutritional deficiencies. Finally, this section describes the different sources of nutrients available to infant of different ages. A second section is dedicated to understanding lipid oxidation and its impact on the nutritional properties of infant foods. Other compounds may also be affected by these mechanisms and are also described in this section. Finally, the third part presents the various strategies currently in place to optimize the lipid profile for the infant needs and to improve their oxidative stability.

This chapter is based in part on the review "Supplementation of infant formula with docosahexaenoic and arachidonic acids: Effects on child development and difficulties associated with their introduction" published in the journal Cahiers de Nutrition et Diététique in 2022 (Cancalon, Barouh et al. 2022).



Cahiers de nutrition et de diététique 57 (2022) 370–383

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ALIMENTS

Supplémentation des formules infantiles en acides docosahexaénoïque et arachidonique : effets sur le développement de l'enfant et difficultés associées à leur introduction

Supplementation of infant formula with docosahexaenoic and arachidonic acids: Effects on child development and difficulties associated with their introduction

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1. Infant nutrition

1.1. Nutrients requirements and metabolic role

Infancy is a period of very rapid growth and development, resulting in specific nutritional needs. The 1,000-days concept, which covers the period from conception until two years of age, underlines the importance of this vulnerable stage of life for the proper infant development and long-term health (Cusick and Georgieff 2016, Martorell 2017, Schwarzenberg, Georgieff *et al.* 2018, Darmaun 2020). The emergence of this concept has led to the development of tools for assessing the nutritional status of infants under five worldwide, and encourages the promotion of appropriate nutritional practices during this key window of development (WHO 2021). Nutritional requirements during infancy vary according to age. Specific recommendations for each age group have been established as shown in **Table 1** for energy and nutrient intake (FAO 2001, FAO and WHO 2004, EFSA 2017).

Macronutrients, including lipids, proteins and carbohydrates, are energy providers (energy density of 9, 4 and 4 Kcal/g, respectively) as well as carriers of bioactive compounds. Micronutrients, on the other hand, have no energy value, but are involved in numerous physiological mechanisms. Carbohydrates, which correspond to organic polymers composed of carbon chains with alcohol and aldehyde or ketone functions, have mainly an energetic function and will not be detailed in this manuscript.

Table 1. Nutritional intake recommendations for infant under 3 years of age (FAO 2001, FAO and WHO 2004, EFSA 2017)

	0 - 6 months	6 - 12 months	1 - 3 years
Energy (Kcal/d)	515	765	1,165
Proteins (g/d)	12.5	14	15
Lipids (% of TE)	50 - 55	40	35 - 40
LA (% of TE)	4	4	4
ALA (% of TE)	0.5	0.5	0.5
DHA (mg/d)	100	100	100 (<24 months)
DHA + EPA (mg/d)	-	-	250 mg (>24 months)
ARA (mg/d)	140	-	-
Vitamin A (µg RE/d)	375	400	400
Vitamin D (µg/d)	10	10	10
Vitamin E (mg/d)	3	5	6
Vitamin C (mg/d)	25	30	30
Iron (mg/d)	-	8	5

TE: total energy; RE: retinol equivalent

1.1.1. Lipids

Lipids have several definitions but the most general one is based on their physical property: they are organic compounds characterized by their limited solubility in water and their solubility in organic solvents. Lipids include a wide variety of compounds which can be categorized according to their biological functions (storage, structural or signaling lipids), or their composition as presented in **Figure 1** (Fahy, Subramaniam *et al.* 2005). Dietary lipids are mainly in their neutral form, *i.e.* TAG composed of three FA esterified on a glycerol backbone (esterification positions *sn-1*, *-2* or *-3*). FA are classified according to their carbon chain length (short <8 carbons, medium 8-12 carbons, long > 12 carbons, very long > 20 carbons), their saturation degree (saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA) FA) and their unsaturation position (omega 3, 6, 9 *i.e.* ω3, ω6 and ω9, respectively). These different characteristics determine the properties of FA, notably their melting point, energy content, solubility, digestibility and metabolic function.

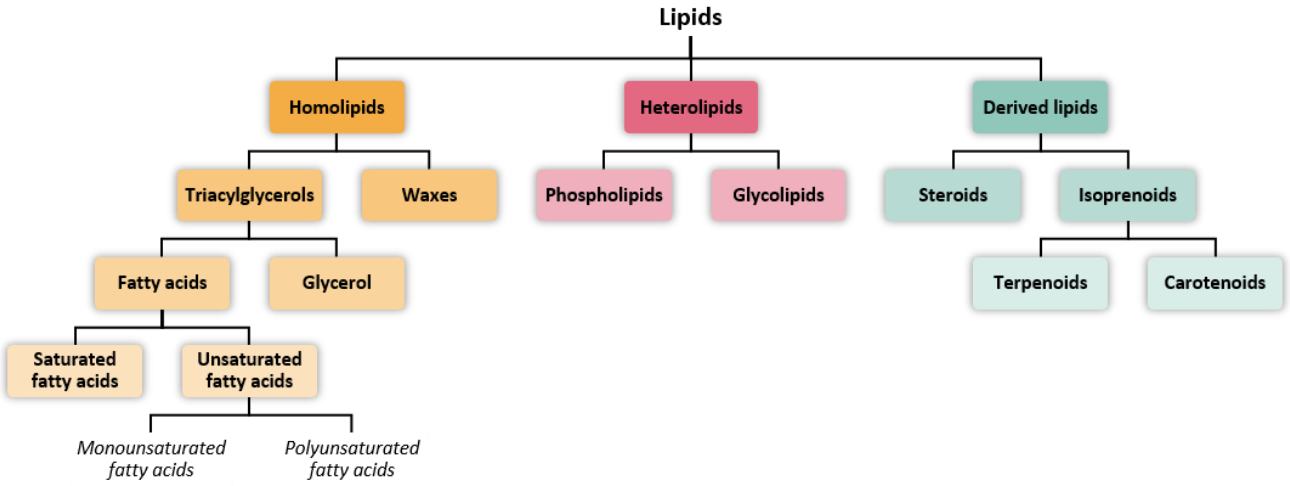


Figure 1. Lipids classification

During the first year of life, lipids are the main energy suppliers, providing 40-55% of total calories *via* their β-oxidation (**Table 1**). Infants lipid requirements are more than four times higher than those of an adult per unit of body weight (**Figure 2**).

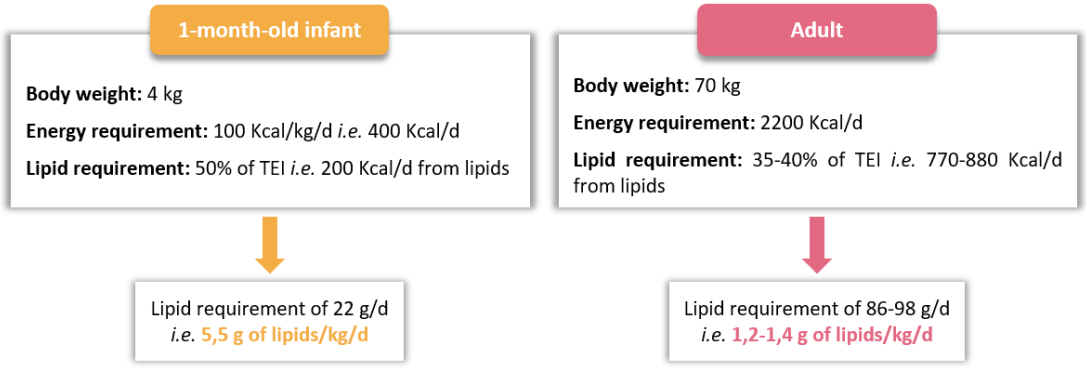


Figure 2. Lipid requirements for infants and adults (Guesnet, Ailhaud *et al.* 2013, EFSA 2014)

The quality of lipid intake is also essential due to their significant involvement in supporting rapid growth and proper development. Recommendations regarding lipid profile during infancy have therefore been established (**Table 1**).

SFA are mainly mobilized as energy providers. They also have a structural and stability function and are involved in lipid digestion (see section *1.2.1*). The potential functionalities of MUFA, mainly in the form of oleic acid (OA, C18:1 ω9) in the infant diet, have not been extensively studied, and their nutritional importance is not yet fully known. On the other hand, PUFA are extensively studied, and their importance in infant nutrition is thoroughly reviewed. PUFA are involved in numerous vital functions as fundamental components and regulators of cell membrane structure and function, precursors of endogenous regulators of cell signaling and gene expression (Calder 2015, Harayama and Shimizu 2020). Through enzymatic conversion *via* cyclooxygenase and lipoxygenase pathways, they are also precursors to a series of oxygenated metabolites called eicosanoids. These compounds, including prostaglandins, prostacyclins, thromboxanes and leukotrienes, modulate cardiovascular, immune and pulmonary functions (Dyall, Balas *et al.* 2022).

The human enzymatic system lacks the ability to endogenously insert a double bond into the FA structure at positions ω6 and ω3. Consequently, the metabolic precursors of the non-interconvertible families namely LA for ω6 and ALA for ω3 are classified as essential FA and must be provided by the diet. To cover essential FA requirements, it is recommended that 4 and 0.5% of total energy be supplied daily by LA and ALA, respectively (**Table 1**). Both LA and ALA lead though the same specific pathway of successive desaturation-elongation to the biosynthesis of other long-chain polyunsaturated FA (LC-PUFA, carbon chain than more 20 carbons) such as DHA (carbon chain of 22 with 6 unsaturations, C22:6 ω3) and ARA (carbon chain of 20 with 4 unsaturations, C20:4 ω6), which are essential for cognitive development and visual acuity in infants (**Figure 3**). Recommendations have also been established concerning the ω6/ω3 ratio, which should be balanced and as close as possible to 5 (below 15 during infancy) (Clark, Makrides *et al.* 1992, EFSA 2013). Indeed, due to the mobilization of the same biochemical pathways, an imbalance in this ratio can lead to a reduction in the conversion of ALA into ω3 LC-PUFA and DHA, thus favoring the ω6 pathway, such imbalance could result in pro-inflammatory effects, for example (Simopoulos 2010, Miles, Childs *et al.* 2021).

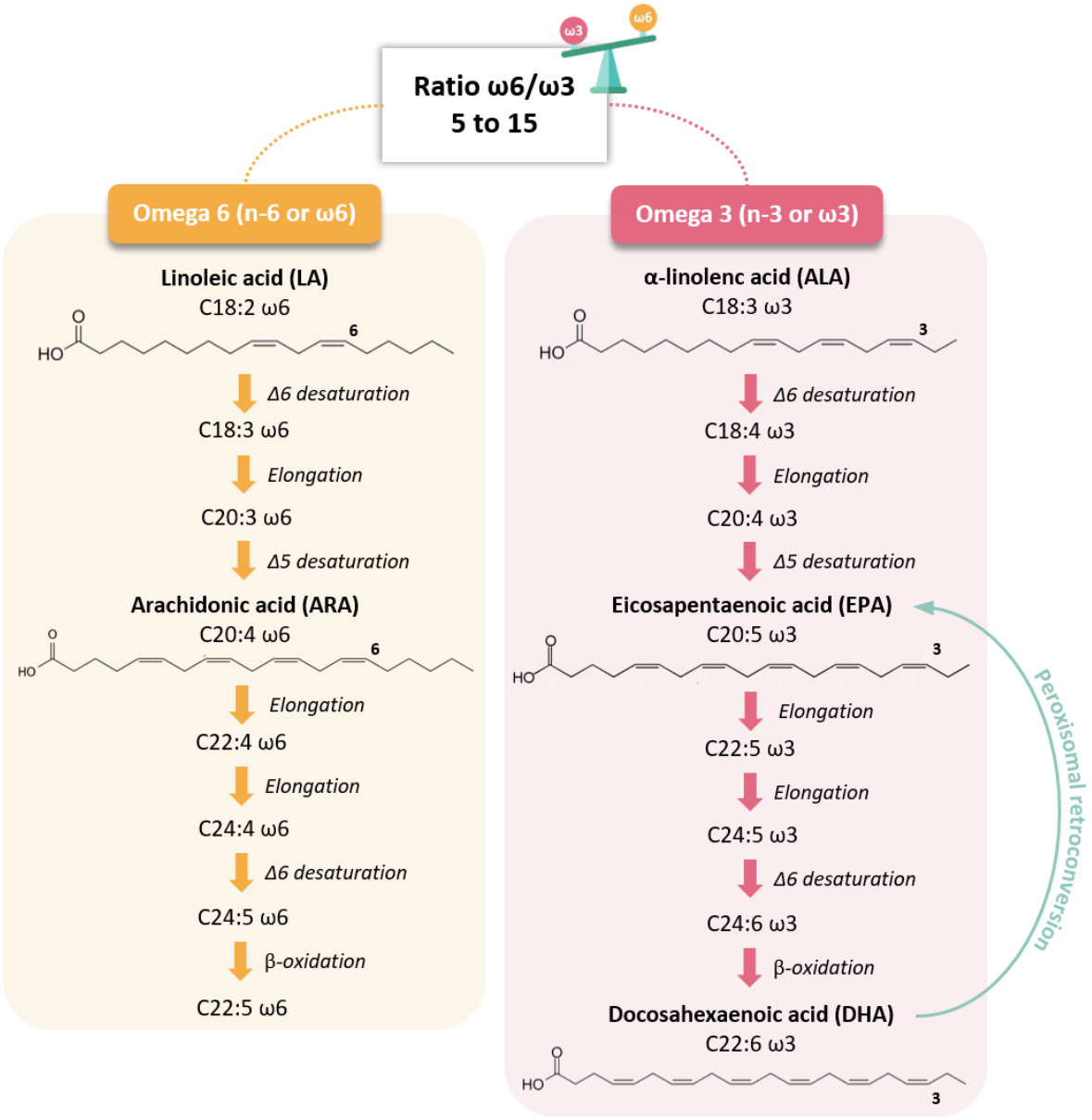


Figure 3. Biochemical pathways for the elongation of LA and ALA into LC- PUFA

The essential nature of LA was demonstrated in the 1960s in infants who showed dryness, thickening and peeling of the skin after several months of feeding with infant formulas lacking dietary fats. The addition of LA to the diet, at 1% of energy, rapidly reduced and then eliminated these skin symptoms (ANSES 2011). ARA is a crucial FA to infant growth and brain development, as it is involved in membrane structure, cell signaling and gene regulation. It is also the precursor of multiple cellular mediators (*e.g.* prostanoids, endocannabinoids, epoxides, isoprostanes, resolvins) after metabolization by several enzymatic and non-enzymatic oxidation pathways. Some of these mediators, such as prostacyclin I2, activate the development of white adipose tissue (Ailhaud, Massiera

et al. 2006). It has been reported that ARA deficiency by invalidation of the LC-PUFA biosynthesis pathway in mice led to alterations in their motor neurodevelopment (Harauma, Yasuda *et al.* 2017).

ALA has no physiological role, but its longer-chain derivative DHA is particularly abundant in the phospholipids of the nervous system (brain, retina) (Alessandri, Guesnet *et al.* 2004). In the brain, DHA accretion occurs during the period of rapid growth, synaptogenesis and myelination, *i.e.* in humans from the last trimester of pregnancy to up 2 years of age (1,000-days concept). The essential nature of ω3 PUFA has mainly been established in pre-clinical models for infants. It has been widely demonstrated that the significant reduction in cerebral membrane DHA content in rodents and monkeys during the perinatal development period (gestation-lactation), generally induced nutritionally by a maternal diet lacking ω3 PUFA and/or imbalanced in the ω6/ω3 balance, was accompanied by a reduction in learning and visual discrimination capacities (visual acuity, electroretinogram b-wave amplitude) (Alessandri, Guesnet *et al.* 2004). The DHA accumulation period represents a window of vulnerability, as it appears difficult to fully correct the membrane deficit and functional disorders in adults due to DHA deficiency during the 1,000-days period, despite the early reintroduction of ω3 PUFA into the diet (Anderson, Neuringer *et al.* 2005). On the basis of these pre-clinical data, the indispensability of ω3 PUFA, and ALA in particular, was established for infants, with a recommended intake of between 0.45 and 0.6% of total energy (Simopoulos, Leaf *et al.* 2000).

In addition to the importance of esterified FA on TAG, an increasing amount of experimental and clinical data also highlights the nutritional significance of minor lipids such as phospholipids (PL), sphingolipids, gangliosides and cholesterol (Bourlieu, Barouh *et al.* 2021). PL are the main membrane constituents and are notably involved in signal transmission and recognition (Bourlieu-Lacanal, Cheillan *et al.* 2018). Sphingolipids are involved in the regulation of cardiometabolic homeostasis, intestinal health, and inflammatory signaling pathways (Zheng, Fleith *et al.* 2019). Gangliosides account for around 10% of the brain total lipid mass. Cholesterol is used as a substrate for the synthesis of bile acids, lipoproteins, vitamin D and hormones. It has been reported that the high concentration of cholesterol in breast milk favors its homeostasis in adolescence and adulthood, with a lower concentration of total serum cholesterol in adults who were breastfed during infancy compare to those who were formulas fed (Owen, Whincup *et al.* 2002, Bourlieu-Lacanal, Bouzerzour *et al.* 2015).

Whether essential or not, all FA contribute to the rapid growth of the infant during the first year of life. Furthermore, a significant proportion of the acetyl-CoA resulting from β-oxidative lipid degradation is actively recycled in the *de novo* synthesis of lipids (such as FA, cholesterol), as well as in the ketone body pathway (Cunnane and Crawford 2014). Ketone are the primary energy substrates for the brain during this stage of life. These recycling and ketogenesis pathways involve SFA and MUFA but also essential PUFA. This is the case with ALA, which is reported to be recycled approximately 200 times more actively than metabolized into ω3 LC-PUFA such as DHA (Cunnane, Menard *et al.* 1999).

1.1.2. Proteins

Proteins are organic polymers composed of chains of more than 30 amino acids (< 30 amino acids are peptides) linked by peptide bonds (primary structure). There are 20 different amino acids, 9 of which are essential during infancy, as they cannot be synthesized by the human body and must be supplied *via* the diet. Essential amino acids in infant include: leucine, isoleucine, valine, threonine, methionine, phenylalanine, tryptophan, lysine and histidine (not essential in adulthood) (WHO 2002). The composition and sequence of peptide chains, as well as the three-dimensional structure adopted, determine the properties and functions of different proteins. Secondary structure refers to the way peptide chains are stretched, coiled or folded in space. The tertiary structure corresponds to the overall three-dimensional conformation of proteins resulting from the interaction of the various amino acids of the primary structure. Some proteins, known as quaternary proteins, are composed of several protein subunits. The quaternary structure describes the spatial arrangement of these subunits and the interactions between them. Proteins are essential for healthy growth and development during infancy. They are also involved in the metabolic programming effect, which has an impact on long-term well-being. Proteins, in addition to being energy carriers, have first and foremost a structural role as they are the building blocks for the elaboration and replacement of all cells and tissues. They play a signaling role under the form of hormones and neurotransmitters, controlling the regulation of secretory functions, for example. Proteins are involved in transport and immune response functions, and are also used as reaction catalysts (enzymes). Therefore, protein intake has a major impact on development, and both qualitative (total protein intake) and qualitative (individual amino acid intake) recommendations have been established. During the first month of life, the protein requirements of infants are more than three times higher than those of an adult male, based on body weight (**Figure 4**). From 6 to 12 months of age, infants still have a high protein requirement, which is 40% higher than in adulthood (Nutten 2016).

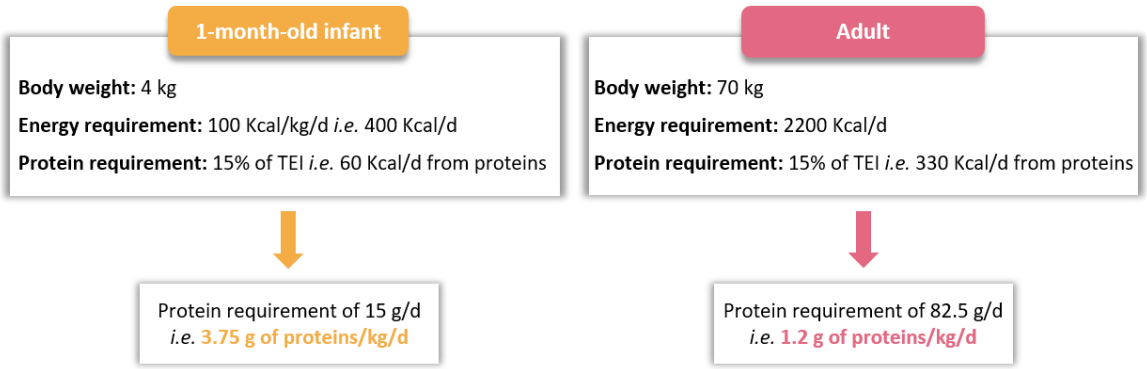


Figure 4. Protein requirements for infants and adults (Nutten 2016)

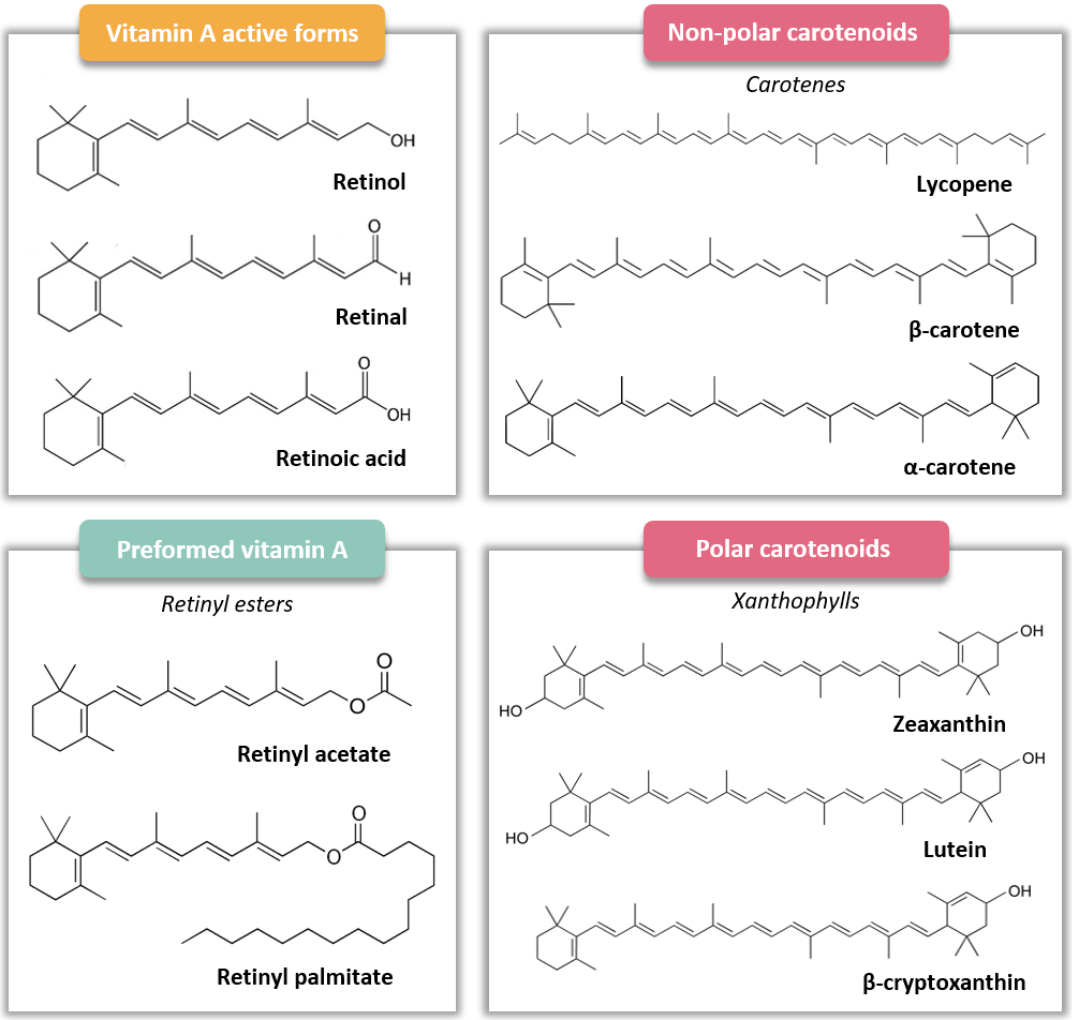
However, a high-protein intake during infancy is associated with a rapid increase in body mass index, resulting in an increase in adipogenesis and therefore an increased risk of developing overweight and obesity later in infancy and adulthood (Koletzko, von Kries *et al.* 2009, Luque, Closa-Monasterolo *et al.* 2015, Koletzko, Demmelmair *et al.* 2019).

1.1.3. Vitamins and minerals

1.1.3.1. Vitamin A

Vitamin A refers to life essential group of unsaturated fat-soluble organic compounds with similar structures and related metabolically to all-*trans*-retinol (EFSA 2006). As the body is unable to produce vitamin A, it must be supplied by the diet. Depending on the origin of the food, vitamin A can be supplied under various chemical forms. Animal products provide mainly retinol and preformed vitamin A (retinyl esters as retinyl acetate and palmitate) but also some carotenoids, while plant products provide only carotenoids (**Figure 5**). Carotenoids can be classified into two main groups according to their polarity: non-polar carotenoids called carotene, which include for example lycopene, β-carotene and α-carotene, and polar carotenoids called xantophylls, including zeaxanthin, lutein and β-cryptoxanthin. Only carotenoids with at least one unsubstituted β-ionone ring can be converted in the body through enzymatic process into the active form of vitamin A and are therefore provitamin A (*i.e.* around 50% of carotenoids can be converted). The most abundant provitamin A are β-carotene (representing up to 75% of the provitamin A carotenoid intake), α-carotene and β-cryptoxanthin (Conaway, Henning *et al.* 2013).

Vitamin A is mainly stored as long-chain retinyl esters and carotenoids in the liver, kidneys and adipose tissue. In target cells, retinol is converted to all-*trans*-retinal, which in turn is oxidized to all-trans-retinoic acid, the main biologically active metabolite (Li, Wongsiriroj *et al.* 2014). Vitamin A is involved in numerous physiological functions, such as regulating the immune system (limiting the occurrence of serious infection) or the development of the visual system as a precursor of 11-*cis*-retinal, the chromophore of rhodopsin (light-sensitive pigment in rods of eye) (Carazo, Macáková *et al.* 2021). Vitamin A plays a role in intracellular communication and is also involved, in its retinoic acid form, in the regulation of stem cell differentiation. Vitamin A, and carotenoids in particular, have antioxidant activity due to their hydrophobic chain of isoprenoid subunits. This structure enables them to quench singlet oxygen and scavenge free radicals (Palace, Khaper *et al.* 1999).



Conversion factors: 1 µg RE = 3.3 UI = 1.15 µg RA = 1.83 µg RP = 6 µg β-carotene = 12 µg other carotenes

Figure 5. Chemical forms of vitamin A (EFSA 2006, Maoka 2020)

1.1.3.2. Vitamin E

Vitamin E refers to fat-soluble organic compounds including two homologous series of tocochromanols synthesized by plants, namely tocopherols and tocotrienols (EFSA 2006). Each series includes four isomers *i.e.* α, β, γ and δ (**Figure 6**). α-tocopherol exhibits the highest biological activity (β-tocopherol = 0.4 TE/mg, γ-tocopherol = 0.2 TE/mg, and for δ-tocopherol = 0.1 TE/mg) (Shils and Shike 2006). Vitamin E is characterized by its antioxidant properties (Barouh, Bourlieu *et al.* 2021). It contributes to lipid and body membrane protection by limiting oxidative stress due to its ability to neutralize free radicals (Burton and Ingold 1981). Vitamin E plays a role in preventing the development of certain chronic diseases with its anti-inflammatory properties (Jiang 2014).

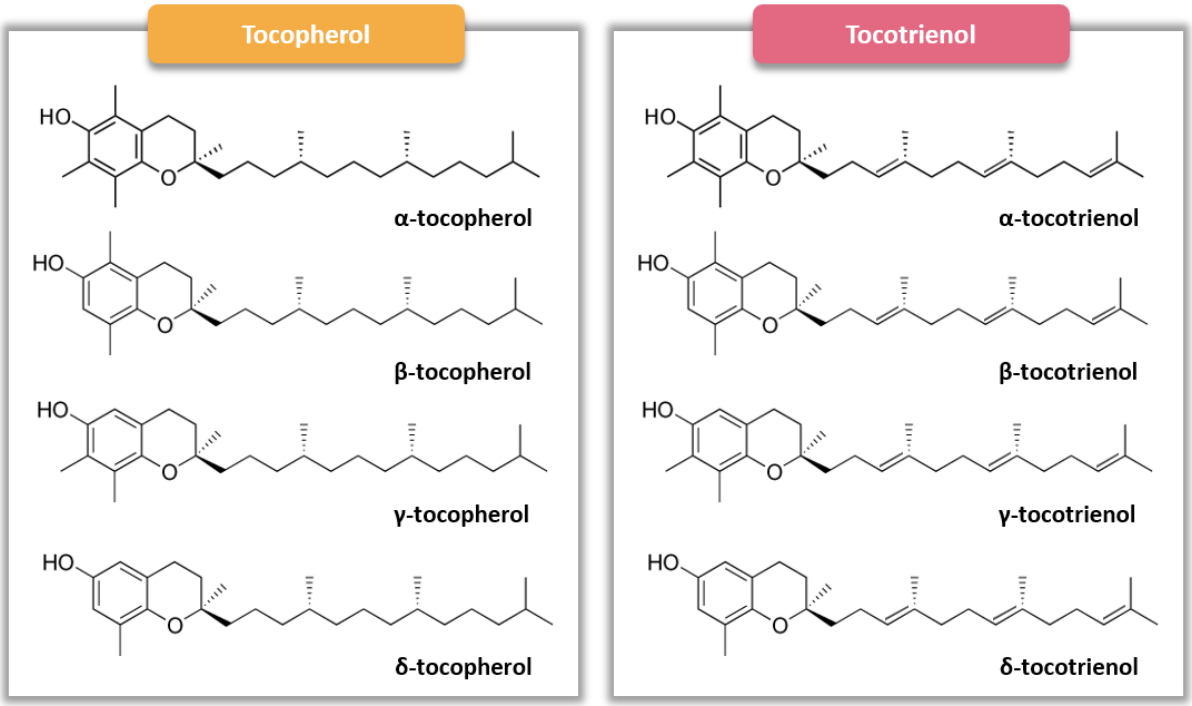


Figure 6. Chemical forms of tocopherols and tocotrienols (Reboul 2017)

1.1.3.3. Vitamin C

Ascorbic acid is the active form of vitamin C. It is involved in the proper functioning of the immune system by supporting the epithelial barrier function against pathogens for example (Carr and Maggini 2017). Ascorbic acid is characterized by its antioxidant properties, which limit the formation of free radicals and acts in synergy with tocopherols by promoting the regeneration of tocopherol radicals formed during the neutralization of free radicals back to their active form (Barouh, Bourlieu *et al.* 2021). Ascorbic acid is also involved in the process of iron absorption by chelation or simply by maintaining the iron in the reduced (ferrous, Fe^{2+}) form (Fairweather-Tait, Fox *et al.* 1995, World Health, Food *et al.* 2004).

1.1.3.4. Iron

Iron exists in two main forms: heme iron provided by animal products and non-heme iron provided by plants. Iron is characterized by low bioacccessibility, ranging from 5 to 35% (Abbaspour, Hurrell *et al.* 2014). It plays an essential role in oxygen transport by forming hemoglobin (an iron molecule bound to each of the four protein subunits), enabling it to bind with oxygen. Iron is also involved in the development of the central nervous system, participating in the formation and functioning of neurons and in myelogenesis (McCann, Perapoch Amadó *et al.* 2020).

1.2. Nutrients digestion and metabolism

The digestive system, like many other physiological processes, undergoes significant development during uterine life and continues during the post-natal period up to the age of two (Abrahamse, Minekus *et al.* 2012). Consequently, the infancy period is marked by immaturity of the enzymatic and secretory functions involved in the nutrient digestion process. The variations in digestive conditions between adults and infants at different stages of development resulting from this immaturity are difficult to define precisely, as they would involve invasive *in vivo* clinical studies which are ethically prohibited. However, the review by Bourlieu, Ménard *et al.* (2014) offers a broad overview of knowledge concerning the specificities of digestive conditions in infants aged 0 to 6 months.

In adults, the digestive process is usually segmented into three main phases: the oral phase in the oral cavity, the gastric phase in the stomach and intestinal phase concentrating for lipid digestion in the duodenal part where most lipolysis occurs (Minekus, Alminger *et al.* 2014). In infants under 12 months of age, food is mostly liquid or semi-solid. As the residence time of food in the oral cavity is limited, the oral phase is generally omitted (Ménard, Bourlieu *et al.* 2018).

1.2.1. Lipids

Lipid digestion begins in the stomach under the action of gastric juice secreted by the parietal and non-parietal cells of the gastric mucosa (**Figure 7**) (Iqbal and Hussain 2009). Lipid hydrolysis is initiated by gastric lipase (pH optimum from 3 to 5), whose enzymatic activity reaches adult maturity at birth (Bourlieu, Ménard *et al.* 2014). However, gastric lipase has a limited action and is responsible for only 10 to 30% of total lipid hydrolysis, although this rate depends on the type of meal (breast milk has a significantly higher rate of gastric lipolysis than infant formulas) (Pafumi, Lairon *et al.* 2002, Bourlieu, Ménard *et al.* 2015, Liu, Lin *et al.* 2022). The regioselectivity of gastric lipase to *sn-3* leads to the release of free FA (FFA) and 1,2-diacylglycerol (DAG). In addition, during the gastric phase, lipids are emulsified (segmented into fine droplets) by the mechanical action of the stomach, leading to an increase in specific surface area, which favors contact with the various digestive enzymes. The chyme, consisting of the bolus and gastric secretions, is then emptied into the intestine *via* the duodenum. The speed of chyme transit from the gastric to the intestinal compartment is linked to the type of meal, with gastric half-emptying times of 36 and 72 minutes for breast milk and infant formula respectively in pre-term infants (Ewer, Durbin *et al.* 1994). The presence of intrinsic BSSL (bile salt-stimulated lipase) in breast milk, at 0.5 to 1% of total protein, promotes TAG hydrolysis and accelerates transit (Baba, Downs *et al.* 1991). The FA present in the chyme and the lipolysis products then stimulate the release of cholecystokinin, favoring the exocrine pancreas to secrete pancreatic enzymes and bile salts. Pancreatic enzymes involved in lipolytic reactions include pancreatic lipase, PLRP1 (Pancreatic Lipase Related Protein 1) and 2, PLA2 (Phospholipase A2) and BSSL. Pancreatic lipase, although immature

during infancy, is the main intestinal enzyme involved in TAG digestion and, in the presence of colipase, is responsible for 56% of total TAG hydrolysis in adults (Carriere, Barrowman *et al.* 1993). It has a *sn-1,3* regiospecificity and typoselectivity for short- and medium-chain FA. The lipolysis products are therefore mainly FFA and *sn-2* MAG. BSSL is a non-specific lipolytic enzyme activated by bile salts, capable of hydrolyzing all positions. Under the action of bile salts, the hydrolysis products are solubilized into micelles and released at the brush border of enterocytes, where they are absorbed by two mechanisms: simple diffusion or active transport. Lipolysis products are re-esterified in enterocytes as TAG and associated with proteins in the Golgi apparatus to form chylomicrons, the body lipid transporter. Chylomicrons are transported from the basal pole of enterocytes *via* vesicles into the bloodstream to peripheral tissues, including liver, muscle and adipose tissue, where they are metabolized (Marcil, Peretti *et al.* 2004).

Vitamins E and A have similar absorption pathways to FA (Debier and Larondelle 2005, Conaway, Henning *et al.* 2013, Reboul 2017). Retinyl esters are hydrolyzed by pancreatic and intestinal enzymes to form retinol. Retinol is absorbed into enterocytes by active transport or by passive diffusion (Reboul 2013). Carotenoids, on the other hand, are absorbed as such in enterocytes. In enterocytes, retinol is esterified with long-chain FA and incorporated into chylomicrons. For carotenoids, two pathways are possible: i) 50% are converted into retinal and then reduced into retinol, which is esterified with long-chain FA and incorporated into chylomicrons, ii) the remaining 50% are directly incorporated into chylomicrons as an unaltered form (Carazo, Macáková *et al.* 2021). Depending on the chemical form considered, retinol conversion factors differ, ranging from 1.15 for 1 µg retinol equivalent from retinyl acetate to 6 for β-carotene and 12 for other provitamin A carotenoids (EFSA 2015). Carotenoids have limited bioaccessibility ranging from 23.5 to 47.5% and require a minimum of 1.55% fat to be micellarized (Conboy Stephenson, Ross *et al.* 2021). The presence of long-chain FA also enhances their bioaccessibility.

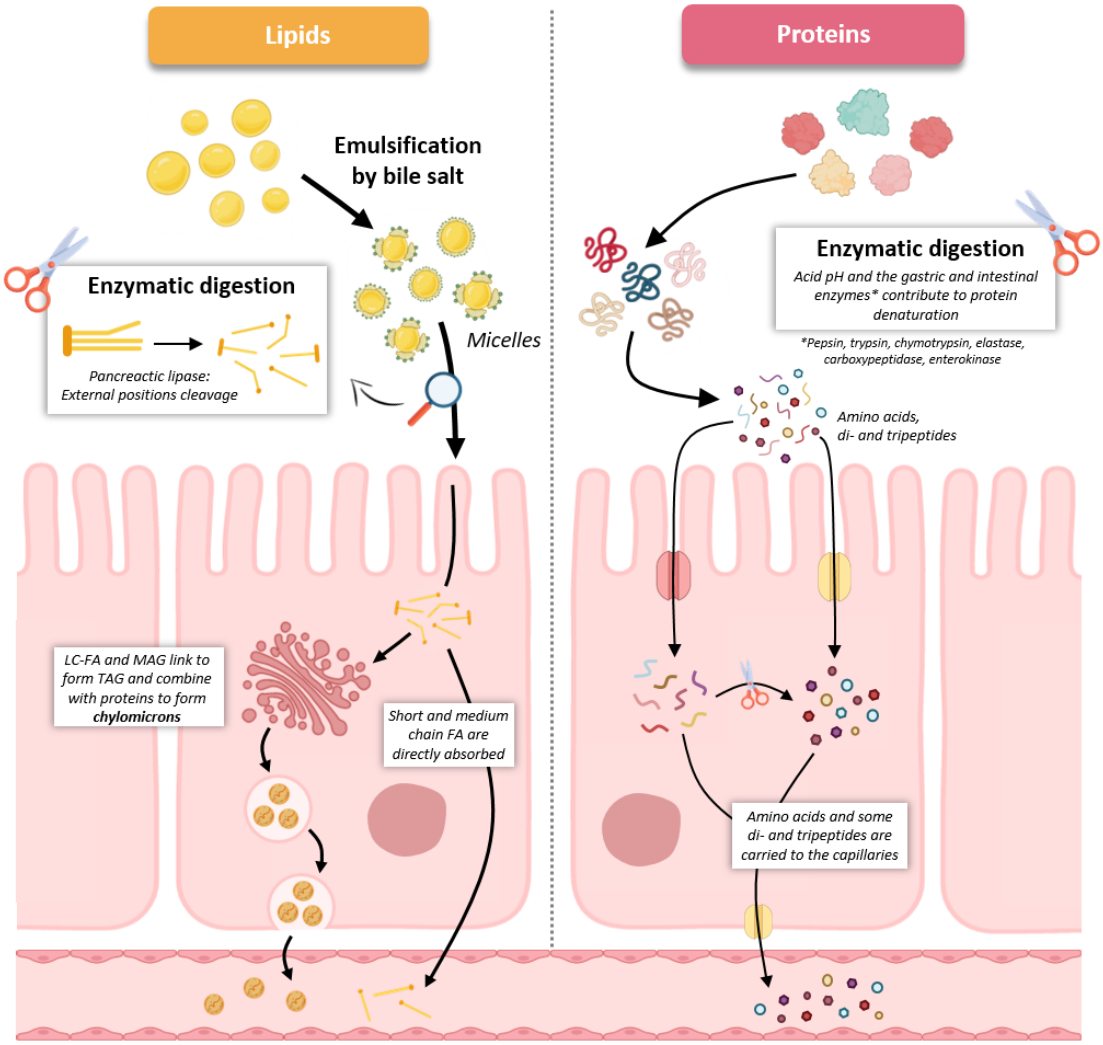


Figure 7. Lipids and proteins digestion processes (Lindquist and Hernell 2010, Lee, Jo *et al.* 2023)

1.2.2. Proteins

Proteolysis begins during the gastric phase, under the action of pepsin, secreted in the form of pepsinogen (inactive form) whose auto-catalytic activation into pepsin takes place at pH below 4 (Blanco and Blanco 2017). Although pepsinogen is mature from birth, at 1-month pepsin reaches only 18% of its adult activity, due to the relatively low conversion rate of pepsinogen to pepsin at gastric pH above 4 (around 5-6) at this stage of development (Bourlieu, Ménard *et al.* 2014). From 6 months onwards, the gastric pH allows full activity of the enzyme, reaching maturity (pH = 3) at two years of age. Pepsin specifically hydrolyzes peptide bonds with aromatic amino acids. The proteolysis products therefore include polypeptides with leucine, tyrosine and tryptophan in the N-terminal position (**Figure 7**). Pepsin has a limited action and is responsible for only 15% of total proteolysis (Bourlieu, Ménard *et al.* 2014). As a result, most proteins reach the intestinal compartment partially or not at all hydrolyzed. As with lipids, during gastric emptying, chyme is progressively emptied into the intestine at the level of the duodenum, the major site of proteolysis (Lee, Jo *et al.* 2023). The speed of transit is associated

with the nature of the chyme proteins, with caseins, for example, emptying significantly more slowly than serum proteins. In the duodenum, protein hydrolysis occurs under the action of enzymes of the intestinal juice. All the enzymes involved in proteolysis are secreted in zymogenic form and are activated by a cascade reaction leading to the release of trypsinogen activated in trypsin by brush border enterokinases. Enterokinas activity in infants is only 20% of its activity at 4 years of age (Bourlieu, Ménard *et al.* 2014). Trypsin, whose activity at birth is 90% that of an adult, is the main intestinal enzyme involved in protein digestion. It hydrolyzes proteins into amino acids and di- and tripeptides. Peptidases from the brush border of enterocytes also contribute to protein hydrolysis, and their action increases the total proteolysis rate from 54% to 74% (Picariello, Miralles *et al.* 2015). Over 90% of the proteolysis products are then absorbed into the enterocytes of the intestine by active transport or simple diffusion (Blanco and Blanco 2017). Post-absorption in the enterocytes, intracellular proteolysis of di- and tripeptide occurs under the action of endoproteases. Amino acids are diffused or transported from the basal pole of enterocytes into the bloodstream, where they are directed towards various metabolic pathways.

1.4. Infant diet

1.4.1. Breast milk

Breast milk is considered as the optimal food for infant nutrition, and is often referred to as the “gold standard” (Jensen 1999, Innis 2007, Andreas, Kampmann *et al.* 2015, Bourlieu-Lacanal, Bouzerzour *et al.* 2015, Dror and Allen 2018, Perrella, Gridneva *et al.* 2021). The WHO recommends exclusive breastfeeding for the first six months of life, followed by complementary breastfeeding up to the age of two (WHO 2021). Breastfeeding is associated with numerous health benefits, including a reduced risk of developing cardiovascular disease, overweight, obesity, type 2 diabetes or immune system disorders, and improved cognitive development (Binns, Lee *et al.* 2016). Breast milk is a complex and dynamic biofluid, adapted to the evolving and age-specific nutritional needs of infants (Andreas, Kampmann *et al.* 2015). Thus, three main phases are distinguished according to the evolving composition of breast milk: colostrum during the first 3 days of lactation, transitional milk secreted up to two weeks of lactation, followed by mature milk from around three weeks onwards (Ballard and Morrow 2013). The composition of breast milk also evolves over the course of a single lactation, with nutrient density decreasing with lactation time. The composition of breast milk, and particularly its lipid composition, is influenced by numerous external factors, the most influential being the mother's diet (Bravi, Wiens *et al.* 2016). It has been shown that a vegan diet leads to high levels of the precursor PUFA *i.e.* LA and ALA, while a diet rich in fish-rich diet promotes significant concentrations of LC-PUFA, notably DHA (Innis 2014). Although variable, the average lipid composition of breast milk has been extensively reviewed by (Jensen 1999) and other authors (Ballard and Morrow 2013, Andreas,

Kampmann *et al.* 2015, Guo 2021). The lipid fraction of breast milk is unique in terms of its composition, with a complex lipid profile, and its structure, with a particular FA regiodistribution on TAG and the organization of lipids in the form of a natural colloidal structure, *i.e.* the milk fat globule (MFG) (Bourlieu and Michalski 2015). Breast milk lipids have an average total content of 3.8% (3 to 5%) and are predominantly (around 98%) in the form of TAG. SFA accounts for 34-47% of total lipids, mainly C16:0 (17-25%), 70% esterified in the *sn-2* position (Innis 2011). The preferential esterification of palmitic acid in the internal position of TAG favors the absorption of lipids and calcium by limiting the formation of insoluble calcium soaps that are indigestible (López-López, Castellote-Bargalló *et al.* 2001, Bar-Yoseph, Lifshitz *et al.* 2013). The higher the rate of esterification of palmitic acid to *sn-2*, the greater the absorption of lipids and the less calcium is excreted in the feces. Short- and medium-chain FA (C < 12) esterified mainly to *sn-3* are also present in breast milk. They are therefore released by gastric lipase and have the advantage of not inhibiting enzymatic activity, unlike longer-chain FA which tend to trap the enzyme. MUFA are the second most abundant FA, accounting for 31% to 43% of total FA (Delplanque, Gibson *et al.* 2015). OA is the most abundant MUFA, mainly esterified in the *sn-1* and *-3* positions. Breast milk is also rich in PUFA, which account for 12 to 30% of total FA. Breast milk is a carrier of both precursor FA, with 0.1% to 24.3% LA and 0.7% to 1.9% ALA, and their longer-chain derivatives, with 0.45% to 0.86% ARA and 1.09% to 1.03% DHA. Breast milk corresponds to a natural oil-in-water emulsion. All TAG are located in the MFG core, dispersed in an aqueous phase containing the protein fraction, carbohydrates, minerals and water-soluble vitamins. The lipid fraction of breast milk also includes minor compounds such as PL (over 50% represented by sphingomyelin - SM) and cholesterol (the main form of sterol), which account for 0.26 to 0.80 and 0.25 to 0.34% of total lipids respectively (Benoit, Fauquant *et al.*, Keating, Curtis *et al.* 2013). These compounds are localized in the MFG membrane (MFGM). The MFGM consists of a PL trilayer, with the first membrane deriving from the endoplasmic reticulum and the outer bilayer deriving from apical membrane of lactocytes, and a wide variety of compounds (Bourlieu and Michalski 2015). The presence of SM and cholesterol in the MFGM as lipid ordered phase leads to the formation of lipid rafts, conferring local high degree of rigidity to the membrane (Bourlieu, Mahdoueni *et al.* 2020). Several authors have reported the involvement of the MFGM in infant development, notably due to its polar lipid and protein composition (Brink and Lönnerdal 2020, Silva, Colleran *et al.* 2021, Chai, Oh *et al.* 2022). For example, it has been shown to stimulate the development of the cerebral and immune systems. Breast milk is also a carrier of fat-soluble vitamins, such as vitamins E at 2.95 to 3.64 mg α-tocopherol equivalent/L. Vitamine A is also present in various chemical forms with a retinoid content of 300 to 800 µg/L. The retinol to ester ratio is variable but around 10:90 w/w with predominant retinyl palmitate. Breast milk provides carotenoids at 50 to 200 µg/L including β-caroten, lutein and lycopene (Jensen, Ferris *et al.* 1992, Dror and Allen 2018, Xi, Wang *et al.* 2023).

Breast milk has a specific protein profile, with an average total content of 1% and a casein/whey ratio of 30:70 that evolves with lactation. Enzymes such as BSSL are also present in the protein fraction of breast milk. It is directly involved in the auto-lipolysis of breast milk and might support digestive enzymes in its lipid digestion process.

1.4.2. Infant formulas

Despite recommendations related to breastfeeding, only 48% of infants under 6 months are exclusively breastfed worldwide, and 70% are still breastfed up to 12 months (Coulm 2021, WHO 2022). When breastfeeding is not possible and/or not desired, various alternatives are available. Human donor milk from a lactarium, for example, can be used for the nutrition of preterm for instance, but more commonly infant formulas are used as a substitute for breast milk. To meet the evolving nutritional needs of infants, infant formulas are designed for each different stages of development. IFF are specifically designed for the 6 to 12 months period. The composition of infant formulas is governed at international level by the *Codex Alimentarius* CX 156/1987, and at European level by CE Directive 2016/127 (**Table 2**). These regulations impose a list of authorized ingredients for the formulation of IFF, as well as minimum and maximum threshold values for energy density and macro- and micronutrients content.

Table 2. Threshold values for IFF composition imposed by European and international regulations and the typical composition of mature breast milk for 100 mL (Guo 2021)

	EU regulation CE 2016/127		***CODEX Alimentarius*** CX 156/1987		**Breast milk**
	Min	Max	Min	Max	
Energy (kcal)	60	70	60	85	67
Lipids (g)	2.64	4.2	1.8	5.1	3.8
SFA (g)	-	-	-	-	1.8
MUFA (g)	-	-	-	-	1.5
PUFA (g)	-	-	-	-	0.4
LA (mg)	300	840	180	-	338.2
ALA (mg)	30	70	-	-	45.6
ARA (mg)	-	42	-	-	27.4
DHA (mg)	12	35	-	-	13.8
Carbohydrates (g)	5.4	9.8	-	-	7
Proteins (g)	1.08	1.75	1.8	4.7	1
Vitamin A (µg)	42	79.8	45	191.3	53
Vitamin D (µg)	1.2	2.1	0.6	2.55	0.1
Vitamin E (mg)	0.36	3.5	0.28	-	0.5
Vitamin C (mg)	2.4	21	4.8	-	4.7
Iron (mg)	0.36	1.4	0.6	1.7	0.04 - 0.08

Since the first European decree on the composition of condensed and powdered milk for human consumption in 1961, the regulations have been regularly updated to align with nutritional recommendations based on the composition of breast milk (the golden standard) and scientific knowledge (**Figure 8** ; Bourlieu, Deglaire *et al.* 2017). Since February 2020, the EU regulation CE 2016/127 has, for example, imposed a reduction in the upper threshold value for protein content in order to limit the risk of developing overweight in adulthood (Koletzko, Demmelmair *et al.* 2019). In this new European directive, the limit contents for certain essential FA have also been revised, with an increase in the lower limit for LA and the upper limit for ALA, and an obligation to supplement formulas with DHA at 20 to 50 mg per 100 kcal (*i.e.* 0.5 to 1% of total FA), which was previously optional. However, there is still a regulatory gap concerning the addition of ARA, which is not mandatory despite its proven importance for infant development (Koletzko, Bergmann *et al.* 2020, Tounian 2020, Tounian, Bellaïche *et al.* 2021).

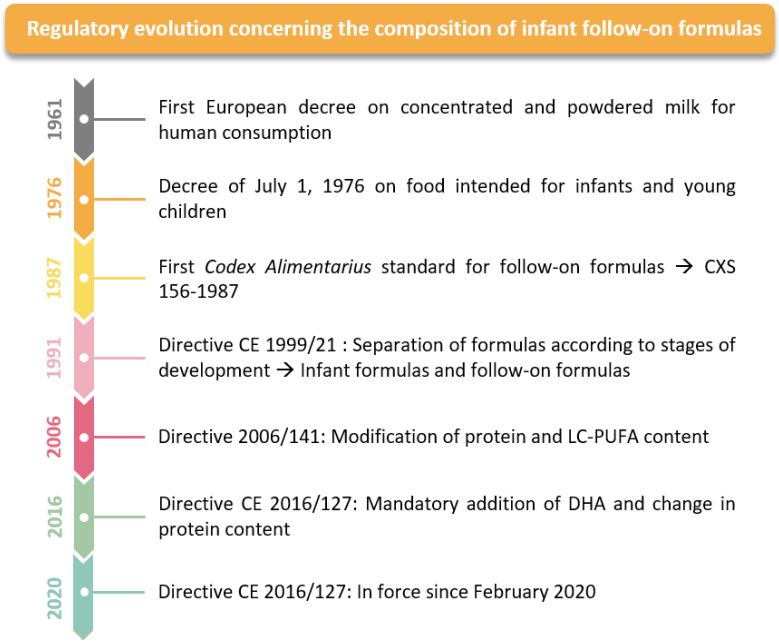


Figure 8. Regulatory changes concerning the composition of infant formulas in Europe (adapted from Bourlieu, Deglaire *et al.* 2017)

The lipid fraction in breast milk is under the form of fat globules around 4 µm in diameter, consisting of a TAG core surrounded by a phospholipid trilayer with a wide variety of compounds at the interface (Michalski, Briard *et al.* 2005). In infant formulas, lipids are in form of dispersed lipid droplets around 0.4 µm in diameter, with a TAG core surrounded by a protein-stabilized monolayer. These structural differences have a strong influence on nutrient digestibility by modifying proteolysis and lipolysis kinetics (Bourlieu, Ménard *et al.* 2015). FA profiles and their regiodistribution on TAG also have an impact on lipid absorption. In infant formulas, the lipid phase results from a mixture of oils, mainly of

vegetable origin. For example, palm, coconut, sunflower and rapeseed oils are commonly used. Depending on the vegetable oil source used, the FA profile varies greatly, particularly in terms of SFA content and the abundance of short (C4-C8) and medium (C10-C12) chains. As for short and medium chains FA, with the exception of coconut oil, whose main FA is lauric acid (C12:0), vegetable oils contain little or none (Jensen, Ferris *et al.* 1992, Delplanque, Du *et al.* 2018). As a result, this category of lipids, which can be metabolized very rapidly *via* the portal vein, is poorly represented in infant formulas based on vegetable oils, compared with breast milk (Dubois, Breton *et al.* 2008). A notable difference between infant formulas and breast milk is also observed in the regiodistribution of FA on TAG. This is the result of the difference between lipids of animal origin (saturated in the internal position) and those of vegetable origin (saturated in the external position) (Innis 2011). In breast milk, around 70% of palmitic acid (C16:0), the predominant SFA, is esterified in the internal position (*sn-2*) of the TAG. On the other hand, in the vegetable oils that composed infant formulas, this FA is mainly located in the outer positions (*sn-1* and *-3*), which modifies digestion kinetics and the absorption rates of lipids as well as certain minerals such as calcium and magnesium (Bourlieu, Ménard *et al.* 2015). It is accepted that the TAG organization of breast milk, with esterified palmitic acid preferentially in the internal position (*sn-2*), enables better lipid absorption by avoiding the formation of insoluble soaps (calcium palmitate) which are directly excreted. The distribution of FA positions in breast milk with a majority of PUFA are esterified in the outer positions (*sn-1* and *-3*), while LC-PUFA, such as DHA and ARA, are mainly located in the inner position (*sn-2*), improves their bioavailability (Damerau, Ahonen *et al.* 2023). The minor lipid profile also varies between infant formula and breast milk. A majority of the PL species in breast milk are represented by SM, whereas in the vegetable oils included in IFF this compound is absent. Similarly, cholesterol is present in breast milk and is not represented in vegetable oil-based IFF. In this context, the reintroduction of bovine dairy fat in the oil fractions of IFF, which was progressively replaced by vegetable oils from the 1970s onwards particularly for economic reasons, is strongly encouraged by the scientific community (Delplanque and Baudry 2015, Bourlieu, Deglaire *et al.* 2017, Huërou-Luron, Lemaire *et al.* 2018). Indeed, bovine milk has similarities with breast milk in terms of lipid structure (in the form of fat globules with a similar milk fat membrane), FA composition (presence of short-, medium- and very long-chain SFA, SM and cholesterol) and esterification on the TAG backbone with the palmitic acid mainly in the internal position (**Figure 9**). On the other hand, bovine milk is deficient in DHA and ARA. Hokkanen, Frey *et al.* (2022) have calculated a similarity index based on various lipid elements (including the distribution of FA contents and their regiodistibution, droplet size, minor lipid contents and different types of PL) between breast milk and infant formulas based only on vegetable oils or including bovine dairy fat. Infant formulas based only on vegetable oils had an index of 0.56 *versus* 0.68 when bovine dairy fat was included, demonstrating the potential for lipid profile optimization (biomimicry enhancement) *via* the reintroduction of this raw material.

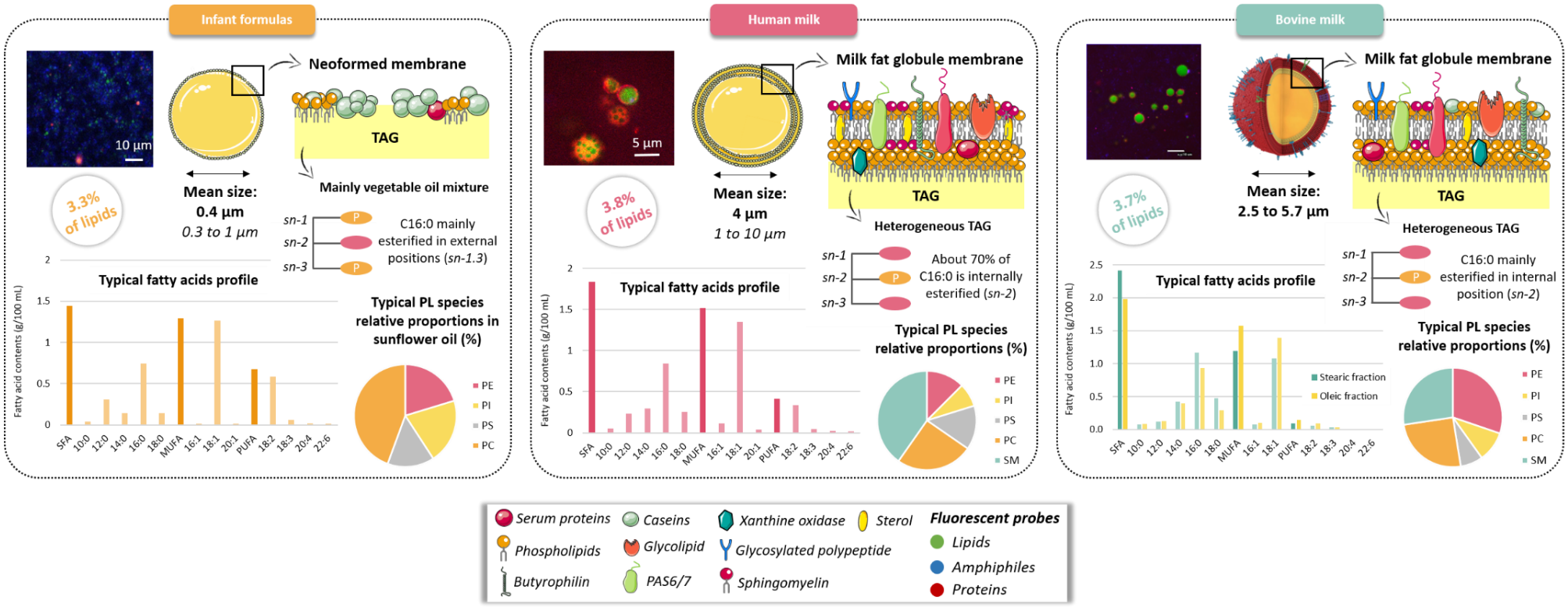


Figure 9. Structure and lipid composition of infant formula, mature breast milk and bovine milk (adapted from Bourlieu, Deglaire *et al.* 2017)

The differences between breast milk and infant formulas are reflected in variations in circulating lipid levels. A large number of clinical studies carried out since the 1980s have systematically reported that circulating ARA and especially DHA levels during the first year of life remain systematically lower in infant formula infants-fed, compared with breast-fed infants (low-weight premature infants and those born at term) (Guesnet and Alessandri 1995). Data from infants dead from sudden death syndrome who ingested this type of infant formula confirmed these observations, at least for DHA. The overall body DHA balance was negative in these infants born at term during the first six months of life (- 0.9 g), showing a strong mobilization of body stocks present at birth for DHA accumulation in the brain. However, this accumulation remains insufficient (0.4 g *versus* 1 g in breast-fed infants) (Cunnane, Francescutti *et al.* 2000). These differences in body status can be explained by the composition of human breast milk, which, unlike the majority of infant formulas marketed until the 2010s, contained appreciable quantities of long-chain derivatives, notably preformed DHA (Ailhaud, Massiera *et al.* 2006). In this way, artificially-fed infants cover their long-chain DHA needs exclusively through endogenous biosynthesis and mobilization of their body reserves, a biosynthesis whose capacity is ultimately insufficient to meet physiological requirements. In low-birth-weight premature infants, who have an immature bioconversion system and no body stores of PUFA (precursors and long-chain), it appears more convincing that supplementing the diet with DHA can correct the lower level of visual acuity (ANSES 2011). For infants born at term, the clinical functional data are controversial, due in particular to the great heterogeneity of the populations studied (variability in levels of desaturation and body reserves of PUFA at birth). These results have led various government agencies to recommend a balanced ω6/ω3 ratio and the addition of DHA and ARA to infant formulas, based on the levels found in breast milk. It has been shown in human newborns (premature and full-term) that consumption of infant formulas low in ω3 PUFA and high in ω6 PUFA, compared with breast-fed infants, leads to reduced DHA concentration in erythrocyte and brain membranes, and delayed maturation of visual functions.

Differences in the composition of the protein fraction between breast milk and IFF have been observed. In breast milk, the total protein content ranges from 0.8 to 1.2 g/100 mL in average which is around three times lower than in bovine milk (3.4 g/100 mL), one of the major sources of protein in IFF (Jenness 1986, Lönnerdal and Atkinson 1995). Moreover, the protein profile is also different with mainly whey (casein/whey ratio of 30:70) in breast milk, whereas in IFF, caseins predominate (casein/whey ratio of 80:20).

1.4.3. Diet diversification

Around 6 months of age, breast milk and/or IFF are no longer sufficient to cover all energy, macro- and micronutrient requirements of infant (**Figure 10**; (WHO 2009)). Moreover, at this stage of development, infant acquired a certain digestive and neuromuscular maturity, giving them the ability to consume and assimilate complex foods. In order to cover the nutritional gap between needs and the intake provided by breast milk and/or IFF, the official guidelines recommend a progressive introduction of complementary foods from 4/6 months of age onwards (WHO 2000, WHO 2003, WHO and UNICEF 2003). This specific period marks the transition from an exclusively milky diet to a diversified diet. Nevertheless, the intake provided by breast milk and/or IFF remains the majority until the age of 12 months. Breastfeeding and providing appropriate complementary foods have been recognized by the WHO and UNICEF as the most significant practices for enhancing infant health. An appropriate complementary food must meet four criteria (WHO 2000):

- Timely: introduce at the right time when infant's requirements exceed the intake provide by breast milk and/or IFF (not too early or late).
- Adequate: provide sufficient energy, macro and micronutrients to meet the nutritional needs of a growing infants.
- Safe: ensure hygienic storage and preparation.
- Properly fed: Adequate frequency to ensure sufficient consumption.

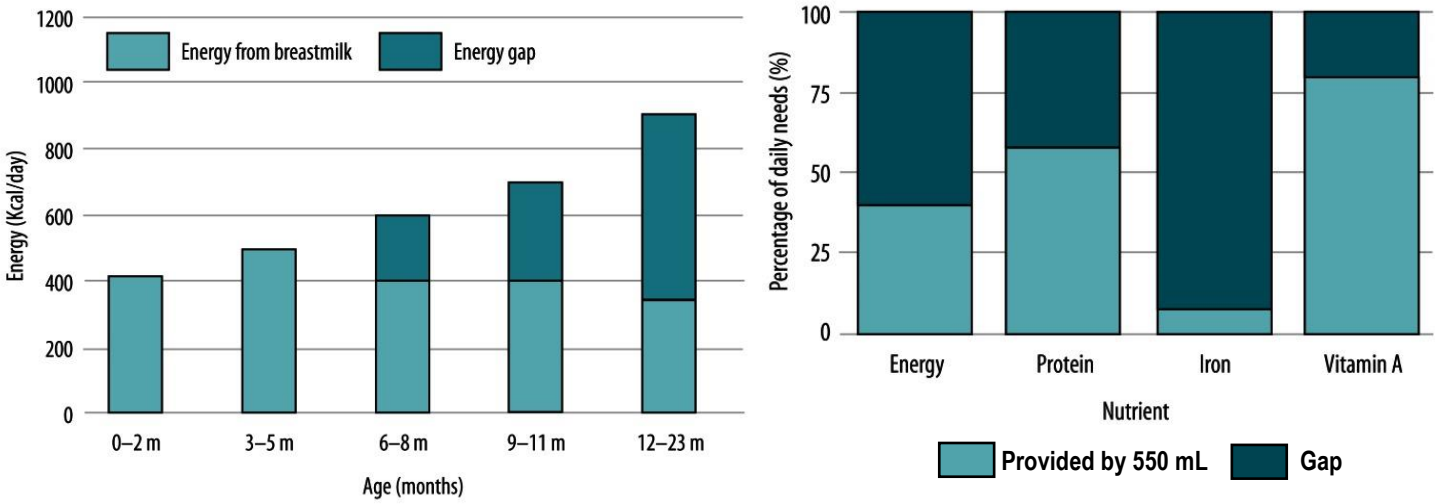


Figure 10. Age-dependent coverage of energy requirements by breast milk and nutritional gaps to be filled by complementary foods in breast-fed infants aged 12 to 23 months (WHO 2009)

Infant cereals (hereafter named IF) are among the most popular complementary foods, with over 50% of infant consuming them around 6 months of age. (National Institute of Population, Training *et al.* 2005, Roess, Jacquier *et al.* 2018). IF are globally consumed, but particularly in the southern regions.

IF are consumed after reconstitution with water or milk and cooking until it become a thick porridge namely gruel. IF can be processed cereal-based foods, which are, according to European regulation 2006/125/EC, composed of at least 25% cereals and/or starchy roots in the final mixture on a dry weight basis. They can be divided into four categories including for example:

- "Simple cereals which are or have to be reconstituted with milk or other appropriate nutritious liquids."
- "Cereals with an added high protein food which are or have to be reconstituted with water or other protein-free liquid."

However, industrial IF are often sold at high prices. IF can also be home-made prepared flours or blends of local flours from cereal and tubers (Bamba, Gbogouri *et al.* 2018). Despite their great potential as energy and nutrients carriers, inappropriate nutritional composition and practices associated with their use are widespread. Dimaria, Schwartz *et al.* (2018) showed that among 32 IF marketed in Benin, Burkina Faso, Ghana and Senegal only 7 had a satisfactory nutritional composition based on 10 nutritional specifications including, for example, lipid (8 IF adequate) and iron (24 IF adequate) contents. The prevalence of poor nutritional quality in FI has also been reported by Oladiran and Emmambux 2022 who associate this issue with gruel high viscosity combined with low energy and protein density. Anti-nutritional factors such as the presence of trypsin inhibitors (phytic acid for example) in crudes raw materials composing IF contribute to poor nutritional quality by limiting nutrient bioaccessibility (Park, Ahn *et al.* 2006). Inappropriate complementary feeding practices with for example early introduction are commonly observed. The prevalence of very early introduction (< 4 months) ranged from 0 to 34% in Europe and was around 30% in the United States (Nantel and Gingras 2023).

1.3. Nutritional status of infant and foods fortification strategies

1.3.1. Global nutritional status of infant under five of age

Feeding practices including breastfeeding and providing appropriate complementary foods have a pivotal role in determining the optimal development of infant and their nutritional status. Although nutritional status is influenced by many factors such as social, economic and environmental factors, inadequate diet intake and inappropriate feeding practices among other factors are associated with an increased risk of developing malnutrition problems. Malnutrition includes undernutrition and macro- and micronutrient deficiencies, as well as excessive intakes and problems of overweight and obesity. Nutritional status is described by standard indicators of physical growth (National Institute of Population, Training *et al.* 2005):

- Height-for-age: used to assess stunting, which represents a long-term effect. Stunting reflected cumulative effect of malnutrition as inadequate nutritional intake over a long period of time. It may also be the consequence of recurrent and chronic diseases.
- Weight-for-height: used to asses wasting, which represents a short-term effect. Wasting reflected recent inadequate nutritional intake. It may also be the consequence of recent diseases.
- Weight-for-age: used to evaluate under or overweight. This indicator is closely linked to the other indices (it is possible to be underweight because of stunting or wasting). It is a clinical tool for continuous assessment of nutritional progress and growth.

The global prevalence of these three indices was updated in 2022 by WHO (**Figure 11**). The prevalence of stunting, wasting and overweight was globally 22.3, 6.8 and 5.6% of infants under 5, respectively. Despite a decrease in prevalence of 10.7% between 2000 and 2022, stunting remains the most prevalent malnutrition disorder, affecting more than 148 million infants under 5. The most affected regions of the world are Africa and South Asia, where over 30% of infants under 5 are stunted. Wasting is the second most prevalent malnutrition disorder, affecting nearly 45 million infants under 5, particularly in South Asia (15% of infants under 5 are wasted). The global prevalence of overweight remained relatively stable between 2000 and 2022 (+0.3%), affecting 37 million infants under 5. The most affected regions are the Middle East and North Africa (10.3% of infants under 5 are overweight). These data show that malnutrition is predominantly in terms of numbers associated with problems of undernutrition.

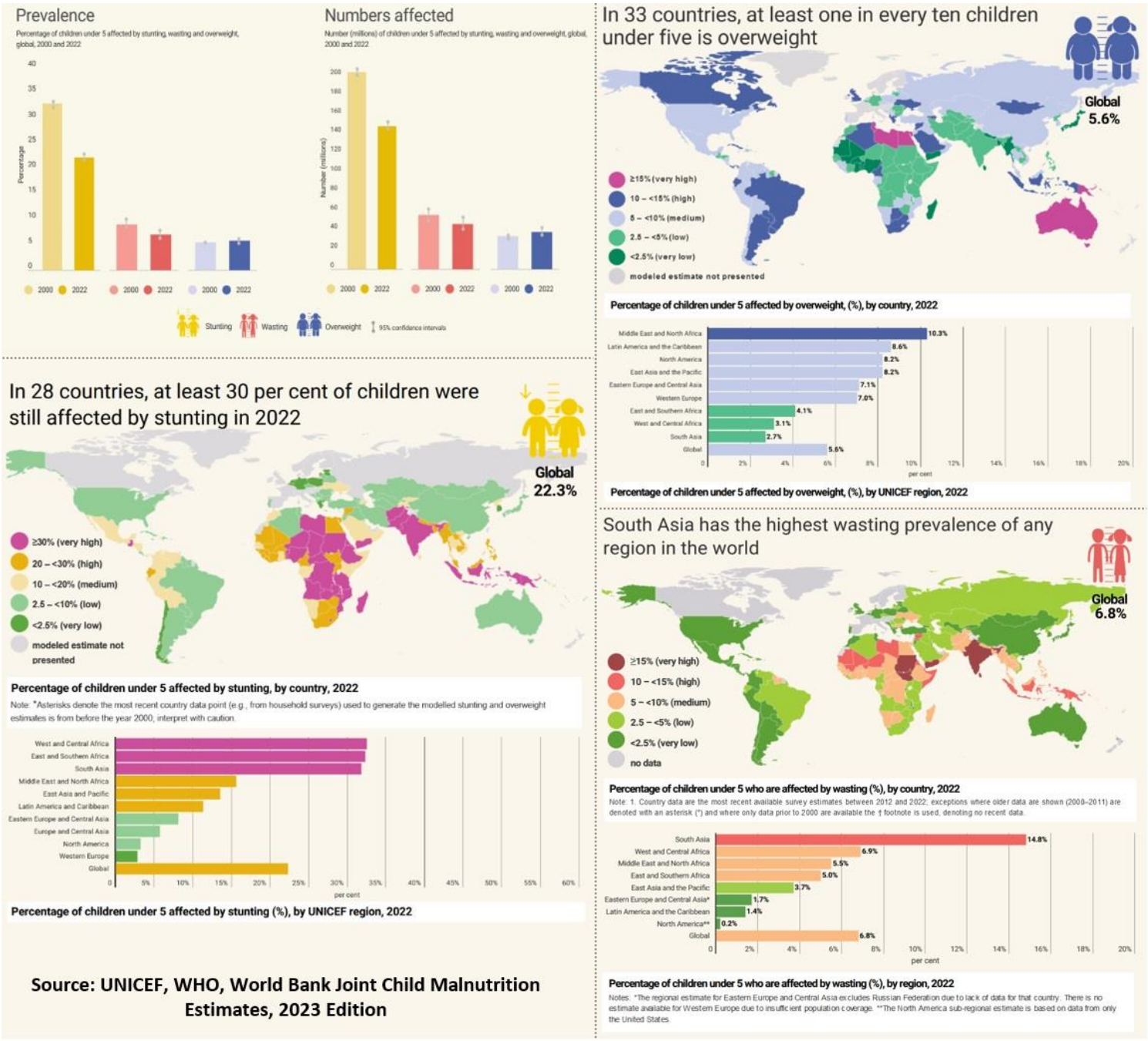


Figure 11. Global prevalence of stunting, wasting and overweight in infant under 5 of age (WHO 2023)

1.3.2. Imbalanced lipid intake

Protein-energy malnutrition (PEM), whose main clinical syndromes are marasmus and kwashiorkor, is due to severe energy and/or protein deficiencies which coincides with the diet diversification period. PEM is associated with increased mortality and numerous co-morbidities, including malaria, chronic diarrhea and favor various infections spreading such as HIV. The poor nutritional quality of complementary foods and feeding practices are among the main contributors to this malnutrition

disorder from 6 months onwards (Oladiran and Emmambux 2022). The overall protein content of complementary foods has been reported to be adapted to infant's nutritional needs (Dimaria, Schwartz *et al.* 2018). However, the quality of the protein profile and the bioaccessibility of nutrients must also be considered (Lutter and Rivera 2003). With regard to lipids, the main energy carriers, it has been reported that their quantitative intake during dietary diversification period was lower than recommended. (Lutter and Rivera 2003) reported from unpublished 24-h recall data collected by the Pan American Health Organization from Bolivia (in 2001), Ecuador (in 2002) and Peru (in 1999) that only 9 to 13% of the total energy intake of breastfed infants aged 6 to 24 months was derived from lipids in complementary foods. A low lipid content leads concomitantly to a low supply of essential FA and LC-PUFA. No-compliance with instructions for use, such as over-dilution of IF, is also commonly observed, leading to a reduction in nutritional density. Going deeper into qualitative analysis, it has been reported that in developing countries the percentage of stunting was inversely related to DHA and ARA intake (Forsyth, Gautier *et al.* 2016). The National Individual Study of Food Consumption 3 (INCA3) conducted in France from 2014 to 2015 showed that the lipid profile of the dietary intake of infants aged from 0 to 11 months was imbalanced, with, for example, DHA consumption 14 times below the recommendation and overconsumption of LA (**Table 3**). DHA and ARA intake values are dependent on many factors such as the geographical, economic, social, and cultural situation of the country, but the value reported by INCA3 seems particularly low for DHA. Schwartz, Dube *et al.* (2010) reported, for example, that in Germany daily intakes were 47 and 72 mg/day of DHA and ARA at 6 months of age, respectively. Intakes dropped to 28 and 24 mg/day of DHA and ARA at 9 months of age, respectively, as a result of reduced consumption of breast milk and/or IFF and the gradual introduction of complementary food. This decrease was even more marked in low-income countries with poor access to marine sources, such as Gambia, where DHA intake fell from 21 to 1 mg/kg body weight between 6 and 24 months (Prentice and Paul 2000). Complementary foods such as IF are often plant-based and therefore contribute very little to the intake of preformed LC-PUFA *i.e.* DHA and ARA. (Michaelsen, Dewey *et al.* 2011, Forsyth, Gautier *et al.* 2016).

Table 3. Adequacy between nutritional intake recommendations and average PUFA consumption in infants under 12 months of age (FAO 2001, FAO and WHO 2004, ANSES 2017, EFSA 2017)

	Recommendations		**Average consumption by infants aged from 0 to 11 months**
	0 - 6 months	**6 - 12 months**	
LA (g/d)	2.3	3.4	4
ALA (g/d)	0.3	0.4	0.5
DHA (mg/d)	100	100	7.1
ARA (mg/d)	140	-	22.4

Over the last few decades, changes in dietary habits have led to an increase in LA consumption (mainly due to the use of vegetable oils such as soybean and sunflower oil) (Blasbalg, Hibbeln *et al.* 2011, Simopoulos 2011). The direct result of these dietary changes was an increase in LA content in breast milk and IFF, for which the use of vegetable oils became widespread from the 1970s onwards (thus replacing animal fats as dairy fat). Despite its essential character, an excess of LA marked by a high LA/ALA ratio (recommended ratio for infant between 5 and 15) reduces the efficiency of the ALA bioconversion into DHA through competition with the metabolic pathways of synthesis (Carlson, Schipper *et al.* 2021). In addition, some authors have reported that the capacity to synthesize LC-PUFA from precursors is limited during infancy. The use of stable isotopes, for example, has shown that endogenous synthesis rates throughout the body remain inadequate to guarantee stable LC-PUFA levels in plasma and erythrocytes (Hadley, Ryan *et al.* 2016). The pre-clinical study by Makrides, Neumann *et al.* (2000) showed that reducing the LA/ALA ratio from 10:1 to 5:1 in infant formula led to an improvement in ω3 status, with a 30% increase in DHA concentration in plasma and erythrocyte PL. These levels nevertheless remained lower than those of breast-fed infants. Similar results were reported by Clark, Makrides *et al.* (1992), with an increase in circulating DHA content from 53 to 73%, while decreasing LA/ALA from 19 to 3, or in the study by Jensen, Prager *et al.* (1997), in which the authors studied ratios ranging from 44 to 4.8. However, these studies did not show any beneficial effect of lowering the LA/ALA ratio on visual performance. The relationship between precursor intake and the mobilization of LC-PUFA for infant development is therefore not solely a result of the LA/ALA ratio, but also depends on the presence of preformed LC-PUFA. Makrides, Neumann *et al.* (1995) reported that infant breast-fed or fed with DHA-enriched infant formula had greater visual evoked potential than infants who did not receive supplemented infant formula. In addition, supplementation of infant formula led to a maintain of erythrocyte DHA birth level until the end of the trial. Erythrocyte ARA content, on the other hand, was lower than in breast fed or non-supplemented infants, but was not associated with adverse effects. More recently, the pre-clinical study by Carlson, O'Loughlin *et al.* (2019) showed in a mouse model that a diet containing only DHA and ARA as sources of ω3 and ω6, respectively (no precursors present) was capable of ensuring normal growth and development. The current body of knowledge on the need to limit the prevalence of imbalanced lipid profiles has led government agencies to review regulations concerning the composition of IFF in Europe, for example. However, the addition of ARA is still facultative in IFF.

Precursor FA ratio balancing and LC-PUFA fortification of both IFF and complementary foods as IF appears to be reliable strategies for improving the lipid profile of the intakes of infants under 12 months, and in particular their ω3 status (Patel, Desai *et al.* 2022, Gsoellpointner, Thanhaeuser *et al.* 2023).

1.3.3. Micronutrients deficiency

Malnutrition disorders are often associated with multi-micronutrient deficiencies. The most globally prevalent micronutrient deficiencies are vitamin A, iron, iodine and zinc.

Vitamin A deficiency (VAD), defined as a serum retinol concentration below 0.70 µmol/L, affects around a third of infants aged between 6 and 59 months with the highest rate recorded in sub-Saharan Africa where 48% of children are concerned (**Figure 12.a**) (Stevens, Bennett *et al.* 2015). In 2013, WHO classified VAD as a public health problem. Low vitamin A stores at birth and poor dietary intake are often at the root of VAD development in infants. VAD is associated with adverse health effects, such as blindness, stunted growth and an increased risk of diseases development and morbidity from severe infections (WHO 2009). VAD can also cause impaired iron metabolism, and thus be associated with increased susceptibility to the development of anemia (Kraemer and Zimmermann 2007).

Iron deficiency, defined as a hemoglobin concentration below 110 g/L in children, results in most cases in anemia, which in 2019 affected 39.7% of infants under 5 years of age according to (Gardner and Kassebaum 2020) (42.6% in 2011; (WHO 2015)). Iron deficiency anemia (IDA), also classified as a major global health problem because of its high prevalence (**Figure 12.b**), is often due to low iron stores at birth and inadequate nutritional intake. It is associated with an increased risk of mortality and a reduced immune response. IDA also has long-term effects of brain with cognitive, behavioral, and neurodevelopment deterioration.

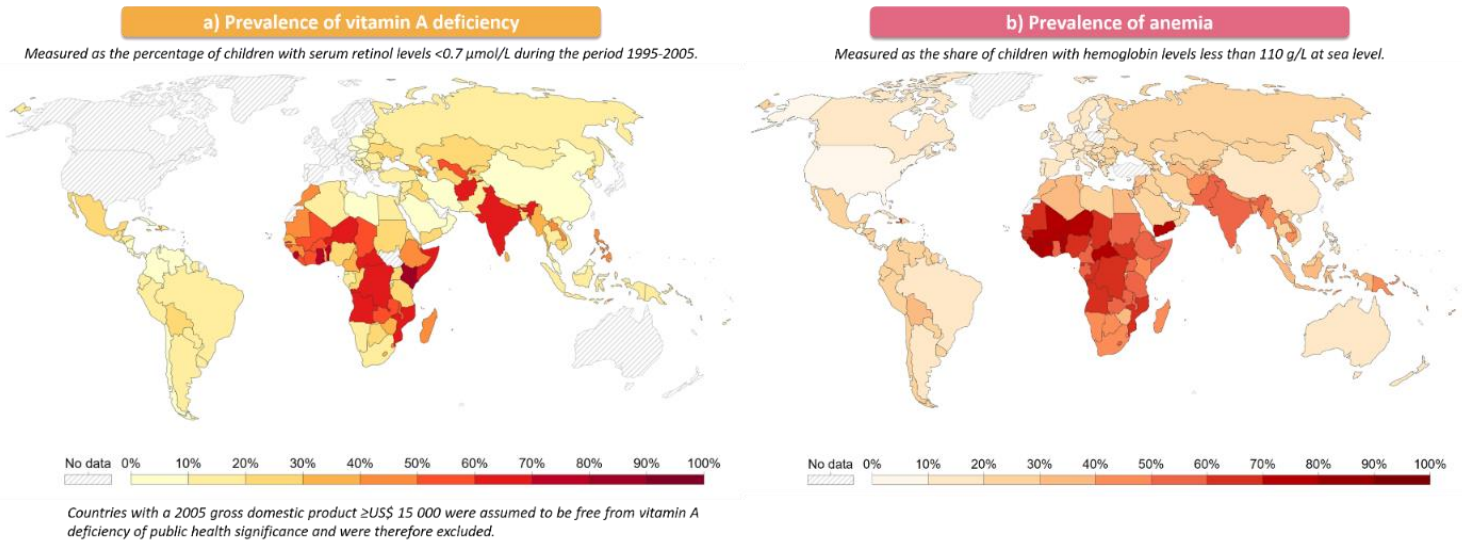


Figure 12. Prevalence of a) vitamin A deficiency during the period 1995-2005 and b) anemia in 2019 in in pre-school children (aged under-5) (Ritchie and Roser 2017)

1.3.4. Infant food fortification

Poor nutritional intake, particularly from complementary foods, is a major cause of undernutrition. In addition, limited digestive capacity up to the age of two (enzyme immaturity) and low food intake (25%

of foods offer not consumed even among stunting infants) further exacerbate the difficulties of meeting infants requirements. Nutritional quality rather than quantity is key aspect of infant diet. To compensate for micronutrient deficiencies, various strategies have been put in place by government health organizations. Supplementation, which refers to the periodic administration of pharmacological preparations of nutrients in capsule or injection form, is used when immediate benefits are necessary (FAO 2001). For vitamin A, the global guidelines recommend periodic supplementation with a high dose of vitamin A every 4-6 months to all infants aged 6 to 59 months living in high prevalence VAD areas. This strategy has proven its efficiency since it lowered all-cause mortality from 12 to 24%. However, WHO estimates that only 59% of targeted infants were reached in 2022.

Fortification of infant foods, which refers to the addition of one or more nutrients, is also a common strategy that is cost effective on a large scale (FAO 2001). Fortification is often achieved by adding Vitamin and mineral complex (VMC). (Lutter and Rivera 2003) have proposed proportions of nutrients that complementary foods must provide, calculated on the basis of age-specific recommendations and the nutritional contribution of breast milk. For example, the authors recommend that all iron should be provided by complementary foods. For vitamin A, on the other hand, the authors propose a contribution of 5% of total intake from complementary foods, which seems particularly low in view of the prevalence of VAD, especially in southern regions. This low fortification rate may be linked to various factors. The authors did not consider the variability of breast milk composition. However, lipid content, and in particular vitamin A content, is strongly influenced by the mother diet. Bioaccessibility rates and fortification needs in at-risk populations were also not taken into account.

The fortification of infant foods with vitamin A is therefore recommended and mandated for IFF. The guidelines on food fortification with micronutrients (WHO 2006) have recognized IFF and IF as suitable vehicles for vitamin A diet supplementation. However, due to its significant sensitivity to oxidative degradation, its supplementation must be accompanied by the addition of antioxidants. Furthermore, since vitamin A is fat-soluble, it is transported by lipids, and these compounds must therefore be co-ingested. For example, the bioaccessibility of carotenoids requires a minimum of 1.55% fat to be micellarized. The chemical forms of vitamin A commonly used for fortifying infant foods are retinyl esters, including retinyl acetate and retinyl palmitate, as well as provitamin A compounds such as β-carotene. Various studies have reported that fortifying infant foods with these chemical forms of vitamin A had a significant effect on serum retinol concentration, thereby reducing the prevalence of VAD. For instance, Lopez-Teros, Quihui-Cota *et al.* (2013) reported this effect in their randomized, controlled clinical trial. The authors used fortified milk (196 µg RE/d in 250 mL servings) in children aged 3 to 6 years administered for 3 months. A 40% increase in total body vitamin A was observed in children who received the fortified milk. Mackey, Albrecht *et al.* (2013) demonstrated that enriching infant formulas with carotenoids (lutein, lycopene, and β-carotene) significantly improved plasma

carotenoid content (with a dose-response for lutein) in infants aged 15 to 35 days compared to a control group that did not receive enriched infant formulas. However, this plasma level remained lower than for exclusively breastfed infants. Carotenoids therefore seemed to be more bioaccessible when delivered through breast milk.

Studies have also been conducted on dry systems, such as Rahman, Ahmed *et al.* (2015) study on fortifying wheat flour with retinyl palmitate (3030 µg RE/kg of flour). This study included 352 children aged 6 to 15 years. The authors showed a significant increase in serum retinol (+0.12 µmol/L) for the group that received fortified flour for 6 months (no significant difference in serum retinol concentration between the groups at the baseline of the trial). A decrease in the prevalence of VAD from 67% to 48% was observed compared to a decrease from 63.6% to 60.3% in the control group that did not receive fortified flour.

Walter, Dallman *et al.* (1993) studied the impact of supplementing IFF with ferrous sulfate (12 mg/100 g IFF powder) and IF with iron electrolytic (55 mg/100 g IF) on the prevention of IDA in 515 infants aged 4 to 15 months. The authors reported that both IFF and IF were effective vehicles for iron supplementation (no significant difference between matrix types), with a significantly lower percentage of anemic children (hemoglobin levels below 105 g/L) at 8 months in the groups that received fortified foods. The authors also emphasized the importance of ascorbic acid in the iron absorption process, which remains relatively low due to its low bioavailability. This effect was also highlighted by Fairweather-Tait, Fox *et al.* (1995) who reported no significant difference in iron bioavailability for 9-month-old infants according to the four iron-enriched weaning foods tested (using ^{54}Fe, ^{57}Fe and ^{58}Fe stable istopes), but demonstrated a significant improvement in iron bioavailability in the presence of ascorbic acid (2 times greater than without ascorbic acid). More recently, Harrison, Hays *et al.* (2022) study on 208 infants aged 6 to 18 months concerning the iron fortification of IF in the form of ferrous fumarate (7.5 mg/100 g IF) showed similar results, with a reduction of over 40% in the prevalence of anemia in the group receiving fortified IF. Different chemical forms of iron can therefore be used to fortify infant foods. Studies have focused on the impact of the chemical form of iron on its bioavailability. For example, Pérez-Expósito, Villalpando *et al.* (2005) reported that the addition of iron in the form of ferrous sulfate significantly improved its bioavailability compared to ferrous fumarate or the reduced ion form Na_2EDTA. Davidsson, Kastenmayer *et al.* (2000) compared ferric fumarate and pyrophosphate and found the fumarate form to be more bioavailable. Finally, Ziegler, Fomon *et al.* (2011) reported equivalent bioavailability for ferric fumarate and iron electrolytic forms. The choice of iron supplementation form for infant foods therefore appears to be of prime importance in limiting the prevalence of IDA.

2. Lipid oxidation

2.1. Mechanism of fatty acids oxidation

2.1.1. General mechanism

Lipid oxidation refers to the chemical mechanisms by which unsaturated lipids react with oxygen, leading to their degradation. This complex phenomenon has been studied for over a century and is one of the main causes of food spoilage (Stephens 1928, Frankel 1980, Kamal-Eldin 2003, Schaich 2005, Berton-Carabin, Ropers *et al.* 2014, Laguerre, Bily *et al.* 2017, Villeneuve, Bourlieu-Lacanal *et al.* 2021). It leads to both a loss of organoleptic properties, with the appearance of off-flavors, and nutritional properties, with the alteration of unsaturated lipids. In addition, the reaction leads to the formation of potentially toxic oxidation products. According to the reaction initiation factor, lipid oxidation can occur *via* three pathways: autoxidation, enzymatic oxidation or photo-oxidation. Autoxidation, induced by triplet oxygen ($^3O_2$), is the predominant lipid oxidation pathway in processed food products. It is a self-catalytic reaction, historically described as a free radical chain reaction with three main stages: initiation, propagation and termination. First of all, it leads to the formation of primary oxidation compounds, *i.e.* hydroperoxides, which then react to form secondary oxidation compounds corresponding to volatile substances (very low sensory detection threshold of the order of parts per million - ppm).

Initiation

The high activation energy of the initiation step prevents it from occurring spontaneously. Thus, it occurs under the influence of a physical (temperature, light) or chemical (transition metal ions, free radicals) initiator. It consists of a homolytic breakdown of a hydrogen in the allenic position relative to the double bond of an unsaturated FA (LH), leading to the formation of a $L^\bullet$ radicals (reaction 1). Among the initiators, hydroxyl radicals ($HO^\bullet$) have the highest reaction rate (reaction 2).

(1) $$LH \rightarrow H^+ + L^\bullet$$

(2) $$LH + HO^\bullet \rightarrow H_2O + L^\bullet$$

The formation of the $L^\bullet$ radical can also occur *via* a number of different reaction pathways, including the action of transition metal ions (M^{n+}) on unsaturated FA (reaction 3) or on hydroperoxides (LOOH) already present in the system (reactions 4 and 5). The radicals may also be the product of the thermal degradation of hydroperoxides (reaction 6 and 7).

(3) $$LH + M^{3+} \rightarrow H^+ + L^\bullet + M^{2+}$$

(4) $LOOH + M^{2+} \rightarrow OH^{-} + LO^{\bullet} + M^{3+}$

(5) $LOOH + M^{3+} \rightarrow H^{+} + LOO^{\bullet} + M^{2+}$

(6) $LOOH \rightarrow HO^{\bullet} + LO^{\bullet}$

(7) $2\ LOOH \rightarrow LO^{\bullet} + LOO^{\bullet} + H_2O$

Propagation

The reaction rate during the initiation phase is relatively slow, but the $L^{\bullet}$ radicals formed are characterized by significant instability. Oxidation then accelerates with the rapid reaction of $L^{\bullet}$ radicals with triplet oxygen, leading to the formation of peroxide radicals ($LOO^{\bullet}$) as described in reaction 8. Peroxide radicals being unstable, they react with other unsaturated FA by abstracting off a hydrogen, leading to the formation of the primary oxidation products *i.e.* hydroperoxide and a new $L^{\bullet}$ radical (reaction 9).

(8) $L^{\bullet} + {}^{3}O_2 \rightarrow LOO^{\bullet}$

(9) $LOO^{\bullet} + L'H \rightarrow LOOH + L'^{\bullet}$

The newly formed $L^{\bullet}$ radical can also react with the oxygen triplet and another unsaturated FA and so on. At this stage, the reaction occurs at a high rate, leading to the exponential formation of hydroperoxides, usually quantified as peroxide values (PV). Stabilization of the radical state by rearrangement of the double bonds (electron delocalization) conducts to the formation of conjugated dienes and/or trienes.

Termination

The reaction is stopped by the decomposition of the hydroperoxide and the reaction of the radicals formed between them. This step leads to the formation of non-radical species including a wide variety of compounds, the most common being carbonyls, alcohols, aldehydes, ketones, epoxides and hydrocarbons (more exhaustively described in section *2.2*). The non-radical compounds produced depend not only on the nature of the original unsaturated FA, but also on the hydroperoxide decomposition pathways, of which there are many. This stage is therefore complex, as one hydroperoxide can generate different secondary compounds.

(10) $LOO^{\bullet} + LOO^{\bullet} \rightarrow$ Secondary oxidation products (non-radicals compounds)

The lipid oxidation reaction described as three main stages is a simplified global vision of the process. Indeed, in view of the diversity and reactivity of the compounds produced and the complexity of the dispersed systems composition, lipid oxidation involves a series of competing and interconnected reaction pathways. (Schaich 2005) has proposed a more exhaustive description of the underlying mechanisms, questioning the chain-reaction character of the process (**Figure 13**).

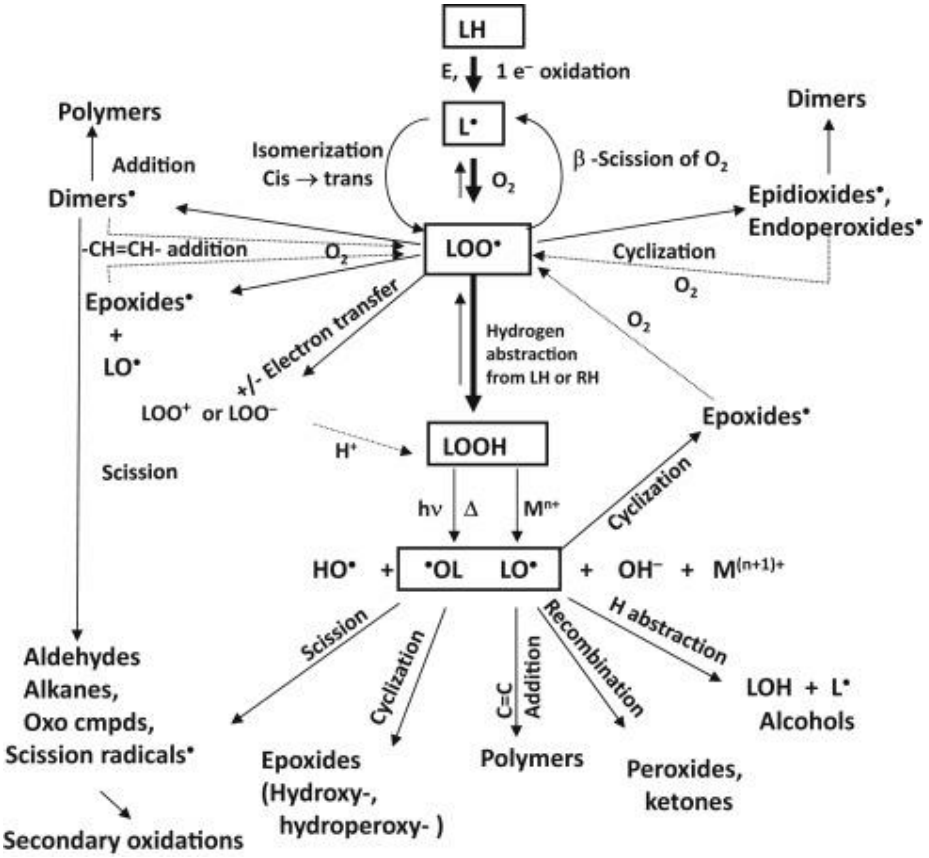


Figure 13. Comprehensive scheme of lipid oxidation (Schaich 2005)

The constituents of the dispersed systems play a crucial role in determining which oxidation pathway will occur. Some raw materials, for example, have high levels of endogenous oxidative enzymes such as certain cereals, legumes or brans (*e.g.* soybean, cowpea). Enzymatic oxidation is not usually a dominant reaction pathway in dispersed systems (granular or emulsions) and is initiated by the presence of enzymes such as lipoxygenase (LOX), polyphenol oxidase, and peroxidase. After initiation, this oxidation pathway follows the same reaction pattern as auto-oxidation, with the formation of free radicals leading to the loss of unsaturated FA and the formation of oxidation products. Enzymatic oxidation can be limited by inhibiting the enzymatic activity in the raw materials. Enzyme inactivation by heat treatments or pH modifications (deviate from the enzymes optimum pH) can be used.

Photo-oxidation is also not usually the dominant reaction pathway in processed products, but is influenced by the presence of photo-sensitive pigments such as chlorophyll. Following photo-initiation, the reaction steps follow the same pattern as for auto-oxidation.

2.1.2. Lipid oxidation substrate

Lipid oxidation concerns all compounds belonging to the lipid class that possess one or more unsaturations, including FA and fat-soluble vitamins. Sensitivity to oxidation depends, among other factors, on the number of unsaturations. ALA has been reported to be more than twice as reactive as LA, which is up to 40 times more reactive than OA (Holman and Elmer 1947, Mao, Chen *et al.* 2020). LC-PUFA are therefore considered highly susceptible to lipid oxidation. The FA profile of food products is one of the determining factors in the oxidative sensitivity. However, other factors also come into play, such as the chemical structure of lipids in free form as FFA, or esterified as PL or TAG. In FFA form, FA are more exposed and therefore more sensitive to oxidation. It has also been reported that DHA is less oxidizable in PL form than in TAG form (Song and Miyazawa 1997, Lyberg, Fasoli *et al.* 2005). Nevertheless, the majority of lipids in food products are in TAG form. TAG oxidation sensitivity seems to be associated with the position of the FA on the glycerol backbone, but there is no clear consensus on this effect in the literature. Indeed, (Neff and List 1999) concluded that esterification of PUFA at the *sn-1* and *3* positions in high stearic soybean oil improved resistance to oxidation. However, other authors have reported opposite results, with a decrease in oxidative stability for PUFA in the external positions rather than at the *sn-2* position (Wada and Koizumi 1986, Neff and El-Agaimy 1996, Ogasawara, Ogawa *et al.* 2020). Park, Terao *et al.* (1983) reported that FA position had no significant effect on oxidative stability. It was hypothesized that this was linked to the randomization process, which led to lower levels of endogenous antioxidants (notably tocopherols) and TAG composed of three PUFA, such as tri-eicosapentaenoyl glycerol and tri-docosahexaenoyl glycerol composed of three Eicosapentaenoic Acids (EPA) and DHA, respectively (Park, Terao *et al.* 1983, Kimoto, Endo *et al.* 1994). (Wijesundera, Ceccato *et al.* 2012) showed that oxidative stability was improved when the DHA was in the internal position on purified TAG. The author used purified TAG composed of a DHA with two palmitic acids arranged as DHA-PA-PA or PA-DHA-PA or with two OA as DHA-OA-OA or OA-DHA-OA. Similar results were observed for TAG composed of two ALA and one LA, with improved oxidative resistance with the ALA in *sn-1* and 3 (ALA-LA-ALA) rather than *sn-1* and *2* or *sn-2* and *3*. For a TAG composed of two LA and one ALA, the greater oxidation resistance was observed for the arrangement with the ALA in the *sn-3* position (Miyashita, Frankel *et al.* 1990). This effect is attributed to interactions between the external and internal positions. Thus, when a FA is oxidized by interaction with the adjacent position, oxidation is propagated. These various studies showed that interactions were favored between ALA or ALA with LA rather than between LA.

Fat-soluble vitamins are also affected by lipid oxidation, which like FA, leads to a loss of biological activity and the formation of oxidation products. Tocopherols exhibit antioxidant activity attributed to their ability to neutralize free radicals (Barouh, Bourlieu *et al.* 2021). They act as a sacrificial agent during lipid oxidation, protecting FA. Tocopherols, by releasing an electron to neutralize the free radical, lead to the formation of tocopherol radicals, which possess a resonance structure making them unreactive and therefore unable to initiate lipid peroxidation. Moreover, tocopherol radicals are capable of reacting with each other to form tocopherol dimers, or with lipid peroxyl radicals to form tocopherol-lipid peroxide complexes (Ma, Wang *et al.* 2023).

Vitamin A is characterized by its important reactivity, which translates into a high sensitivity to oxidation (Palace, Khaper *et al.* 1999, Ross 2016, Nieva-Echevarría, Goicoechea *et al.* 2017). Retinol is considered as the most sensitive chemical form. Retinyl esters, including retinyl palmitate and acetate, have shown improved resistance to oxidation, mainly due to their decreased polarity, which impacts their localization in dispersed systems (less close to interfaces) (EFSA 2016, Ross 2016).

2.1.3. Lipid oxidation in dispersed systems

2.1.3.1. Oil-in-water emulsion

An oil-in-water emulsion is a multiphase system composed of lipid droplets dispersed in a continuous water phase. The immiscibility of the dispersed and continuous phases results in thermodynamic instability of the system. To avoid spontaneous separation of the two phases, surfactants such as emulsifiers adsorb to the oil/water interface, reducing surface tension and stabilizing the system (**Figure 14**). The presence of emulsifiers at the interface induces electrostatic and/or steric repulsion, depending on the nature of the adsorbed molecules, thus maintaining the physical stability of emulsions. Emulsifiers are typically categorized into three groups (Berton-Carabin, Ropers *et al.* 2014, Ghelichi, Hajfathalian *et al.* 2023):

- Low-molecular-weight emulsifiers: these are synthetic surfactants or natural polar lipids, either neutral (zwiterrionic form) or charged (cationic or anionic form), and are mainly represented by lecithins.
- High-molecular-weight emulsifiers: these are amphiphilic biopolymers and are mainly represented by proteins.
- Solid particles: these are nano or micro solid particles capable of adsorbing at interfaces. These emulsifiers are used in Pickering emulsions (Berton-Carabin, Schröder *et al.* 2021).

Interface properties are linked to the nature and concentration of the emulsifiers used, as well as to the chemical environment (droplet size, pH, ionic strength) (McClements and Decker 2000, Berton-Carabin, Sagis *et al.* 2018). The choice of emulsifier determines the thickness of the interface *i.e.* from

0.5 to 1 nm and from 1 to 15 nm for a surfactant and protein monolayer, respectively. It also affects the interface coverage, going from 30 to 40% for proteins, to around 100% for surfactants (Kroll 1992, Berton-Carabin and Villeneuve 2023).

In an emulsion, the interface is the location of contact between the unsaturated lipids present in the droplets and the pro-oxidant compounds (metals transition ions) present in the aqueous phase. Thus, the interfacial area is the site of lipid oxidation initiation (**Figure 14**). The properties of the interface not only play a role in maintaining the physical stability of the emulsion, but are also strongly implicated in the oxidative stability of the overall system (Berton-Carabin, Ropers *et al.* 2014).

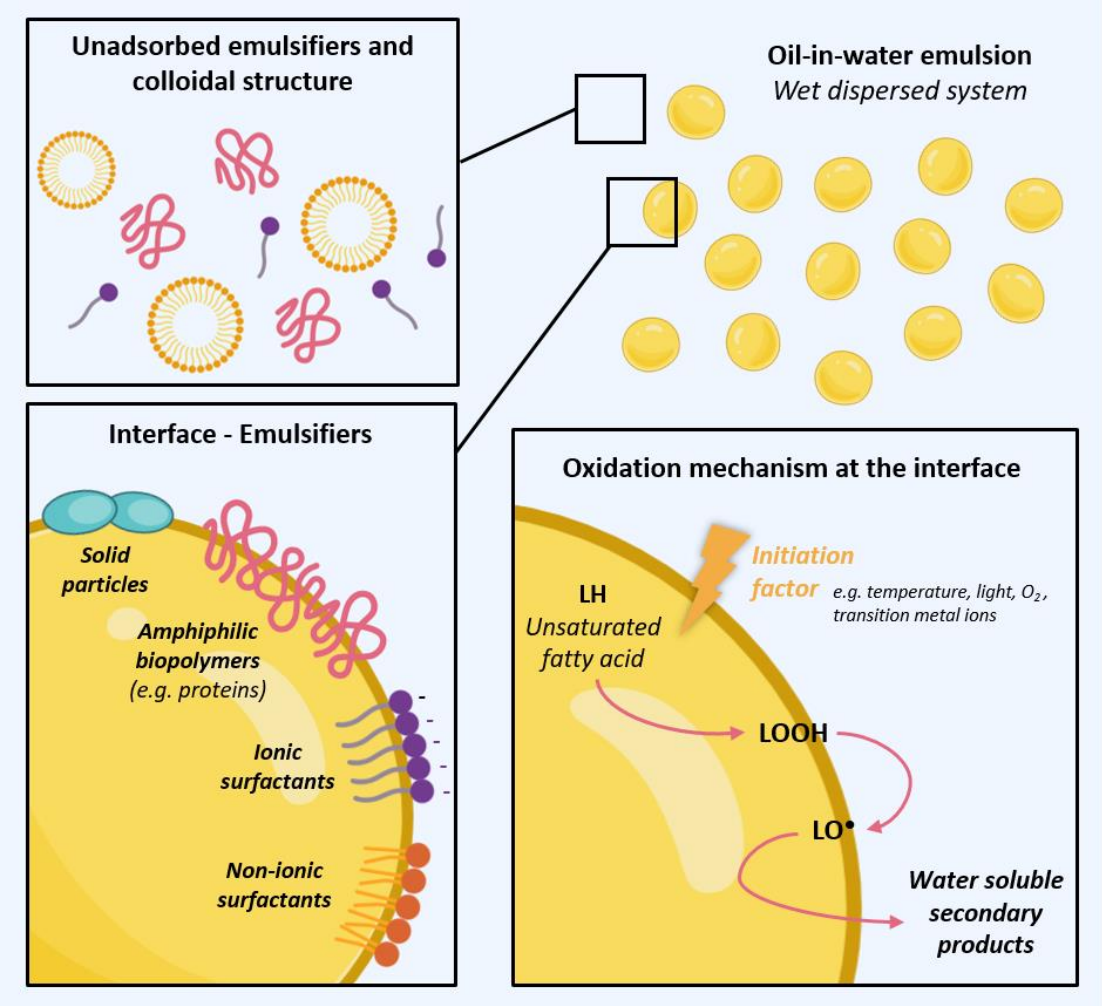


Figure 14. Compounds organization in oil-in-water emulsion and oxidation mechanism at the interface (adapted from (Berton-Carabin, Ropers *et al.* 2014)

Using a fluorescent probe (BODIPY665/676) and Confocal Laser Scanning Microscopy (CLSM), (Raudsepp, Brüggemann *et al.* 2014) demonstrated that radical transfer between lipid droplets can occur in oil-in-water emulsions. This result suggests that lipid oxidation is not an isolated event occurring at the level of each individual droplet, but a dynamic phenomenon that can propagate to the

whole system from a single initiation. The mechanisms involved in the propagation of oxidation in oil-in-water emulsions have not yet been elucidated, but several hypotheses are proposed to explain the spreading of oxidation to the entire system (Laguerre, Bily *et al.* 2017, Laguerre, Tenon *et al.* 2020, Villeneuve, Bourlieu-Lacanal *et al.* 2021). Two limiting factors in the diffusion of oxidation need to be considered: the high reactivity of radicals (short life span) decreases the propagation distance and the polarity of compounds limits the propagation mode.

The first hypothesis involves water transfer. This pathway only concerns water-soluble compounds, which would be diffused between droplets *via* the water phase. However, this hypothesis was quickly dismissed, as its contribution seems limited due to the hydrophobic nature of the lipid radicals. The second hypothesis suggests radical transfer by collision-exchange-separation transfer. This process involves contact between two adjacent droplets, leading to an exchange of compounds including radicals. However, this mode of propagation is relatively slow. The third hypothesis corresponds to micelle-assisted transfer. The amount of emulsifier used to ensure the stability of food emulsions often exceeds the amount needed to coat the droplet surfaces, resulting in a substantial portion of emulsifiers remaining unabsorbed at the interface. Unabsorbed emulsifiers can associate in the water phase to form colloidal structures such as micelles. Micelles could then act as radical carriers, allowing radicals to propagate between droplets. LOOH formed during lipid oxidation have a more important half span life compared to radicals (more stable compounds) and are surface-active. These compounds could aggregate at the surface and self-assemble to form micelles (or co-micelles with emulsifiers already present in the system). The colloidal structures thus formed can then diffuse *via* the water phase to other droplets, contributing to the propagation of oxidation. The presence of micelles would therefore be associated with a pro-oxidant effect. However, it has also been suggested that micelles may act as antioxidant reservoirs, leading to a better diffusion of these molecules at the interface area and thus limiting oxidation (Ferreira da Silveira, Laguerre *et al.* 2021).

The presence of unadsorbed compounds in the aqueous phase, due to the use of a concentration above the critical micelle concentration (CMC), may also have an antioxidant effect for example with the metals chelation activity of proteins (Berton-Carabin, Ropers *et al.* 2014). However, more investigations are needed to elucidate the involvement of colloidal structures in lipid oxidation.

2.1.3.2. Granular system

Lipid oxidation has been extensively investigated in bulk and emulsified systems, but few studies have focused on low-moisture systems including granular systems such as flours. A search on Web of Science in 2023 with the keywords (lipid oxidation AND emulsion) yields 2573 results, whereas only 428 results are obtained for (lipid oxidation AND flour). The oxidative stability of IF and cracker has been investigated (Hemery, Laillou *et al.* 2018, Moustiés, Bourlieu *et al.* 2019, Gumus and Decker

2021). However, emulsions are characterized by oil/water interfaces and water saturation. Low-moisture systems are characterized by multiple interfaces such as air/lipid, starch/lipid, and starch/protein/lipid interfaces and low water activity (Aw < 0.5). Thus, oxidation propagation mechanisms are assumed to be different from emulsions (Barden and Decker 2016). Aw has a significant effect on the lipid oxidation rate. Water being the vehicle for hydrophilic compounds, low Aw results in limited diffusion of compounds including pro-oxidizing agents such as metals transition ions. Accordingly, the rate of lipid oxidation is the lowest at Aw around 0.2 to 0.4 (Nelson and Labuza 1992). Sensitivity to oxidation increases with increasing Aw due to the greater mobility of pro-oxidant compounds favoring contact with unsaturated lipids at interfaces. An Aw of less than 0.2 also leads to greater sensitivity to lipid oxidation. In fact, water can have both a protective and a pro-oxidant effect. The protective effect observed at Aw levels of 0.2 to 0.4 is attributed to the formation of a bound water monolayer (water saturation of polar groups lipids). This bound water is non-reactive (limited mobility) and prevents direct exposure of lipids to oxygen (oxygen solubility up to 5 times lower in the aqueous compartment than in oily fractions) (Cuvelier, Soto *et al.* 2017). At very low Aw, the protective barrier formed by the bound water monolayer is no longer present, leading to direct lipid contact with oxygen. Initiation of oxidation *via* the action of transition metals in granular systems is therefore not the predominant pathway. This is one of the reasons why wheat flour is considered a good iron carrier.

Initiation of lipid oxidation in flour occurs firstly during milling. Cells are disrupted, promoting contact between unsaturated lipids, enzymes including LOX, which are responsible for enzymatic oxidation, and minerals (pro-oxidant metal ions) and oxygen, which are responsible for the autoxidation pathway. Propagation then appears to take place from proximal to proximal between adjacent lipids. However, the oxidation kinetics of granular systems are slower than those of emulsified systems, due to the limited mobility of the compounds. Moreover, as suggested by Wei, Zhu *et al.* (2021), peroxyl radicals are formed more rapidly than hydroperoxides. In the presence of endogenous antioxidants in flours such as tocopherols (contact between oxidizable lipids and endogenous antioxidants favored during milling), peroxyl radicals can be neutralized and form more stable antioxidant radicals rather than lipid hydroperoxides, resulting in a delay on the conversion into secondary product.

2.2. Lipid oxidation products

The decomposition of hydroperoxides leads to the formation of a wide variety of non-radical compounds belonging mainly to the aldehyde, ketone and alcohol families. The non-radical compounds produced depend both on the nature of the original unsaturated FA and on the decomposition pathways of the hydroperoxides, which are multiple. This stage is therefore complex, as one hydroperoxide can generate different secondary compounds. The most commonly formed

secondary compounds include nonanal for ω9 FA, propanal, 1-penten-3-one or 4-hydroxy-2-hexenal (4-HHE) for the ω3 FA and hexanal, (E)-2-heptanal, pentanal or 4-hydroxy-2-nonenal (4-HNE) for the ω6 FA (Frankel 1987, Schaich 2005, Nogueira, Scolaro *et al.* 2019). These compounds lead to the formation of off-flavors and a reduction in organoleptic properties. In addition, these secondary lipid oxidation products are well absorbed and pass into the plasma and blood in various preclinical and clinical studies (Grootveld, Atherton *et al.* 1998, Jenkinson, Collins *et al.* 1999, Kim, Gallaher *et al.* 1999, Awada, Soulage *et al.* 2012). If these oxidized products pass the intestinal barrier more works is needed to trace their tissue accumulation. Oxidized lipids could also promote the development of certain pathologies, for example by forming adducts with proteins and thus blocking their function, by affecting vital cell functions through membrane damage and enzymes inactivation or by acting as tumor promoters *via* chromosomal damage (Guéraud, Atalay *et al.* 2010). Oxidized lipids can affect the gut microbiota, as highlighted by Lei, Yang *et al.* (2021) in a preclinical model of mice fed oxidized lipids who developed colonic inflammation and dysbiosis. However, the toxicity of oxidized lipids in the diet is controversial and it is difficult to assess the deleterious effects directly associated with their consumption. A clear distinction needs to be made between oxidation compounds produced *in situ* by oxidative stress and those produced *in vitro* by auto-oxidation in an emulsion, for example. Oxidized lipids generated *in situ* in tissues are highly cytotoxic and contribute to a wide range of pathologies, including ageing, cardiovascular issues and certain cancers. Oxidized lipids in the diet, on the other hand, must undergo digestion and pass through the intestinal barrier before being transported to target tissues, which can considerably reduce their damage potential. Indeed, the endogenous defense mechanisms are able to restrict the levels and types of oxidized lipids that are absorbed and can decompose, detoxifie, and eliminate the absorbed ones (Schaich 2020). It is important to note that most toxicological studies on the effect of consuming oxidized lipids have been carried out on animals with doses or levels of oxidation that are unrealistic for human consumption. However, a randomized, double-blind, placebo-controlled clinical trial in humans has shown that the consumption of 8 g/d of oxidized fish oil for 7 weeks had no effect on a variety of *in vivo* markers of oxidative stress, lipid peroxidation or inflammation (Ottestad, Vogt *et al.* 2012).

Oxidation of LC-PUFA can also lead to the formation of oxygenated metabolites known as oxylipins. These compounds can be generated enzymatically, such as prostaglandins, resolvins, protectins or thromboxanes, or non-enzymatically, such as isoprostanes and neuroprostanes. Oxilipins are biomarkers of oxidative stress and have numerous biological activities, including anti-inflammatory properties (Bosviel, Joumard-Cubizolles *et al.* 2017, Bilodeau, Gevariya *et al.* 2021, Durand, Bultel-Poncé *et al.* 2023).

2.3. Oxidation of others compounds such as proteins and phenolic compounds

In this manuscript the focus is on lipid oxidation, but this phenomenon is not isolated and may also involve other non-lipid compounds. Oxidation and lipidation (reaction of lipid oxidation products with proteins) of proteins are among the main chemical reactions leading to their degradation in foods (Hellwig 2020). As with lipids, proteins oxidation is induced by environmental factors (light, temperature, oxygen, pro-oxidant metals, presence of radicals) and leads firstly to the formation of reactive protein radicals and then to oxidation compounds belonging to the aldehyde and ketone families. The sensitivity of proteins to oxidation depends on their amino acid composition, those with aromatic rings, such as tryptophan or tyrosine, are easily oxidized. Oxidation alters the structure of proteins and consequently modifies their inherent functionality (Xiong and Guo 2021, Geng, Liu *et al.* 2023). Covalent bonds between lipid oxidation products and proteins, as well as lipid-induced protein aggregation, can also occur.

Phenolic compounds are mainly degraded by oxidation and isomerization reactions. Phenolic oxidation or browning leads to the production of brown compounds and a reduction in their antioxidant capacity. It can occur enzymatically, in the presence of the polyphenol oxidase and peroxidase enzymes, or non-enzymatically (Igor Otavio, Cristine Vanz *et al.* 2017). For the non-enzymatic pathway, phenolic compounds are oxidized to quinones catalyzed by oxygen and pro-oxidant metals, which self-polymerize or combine with amino acids and proteins to produce brown compounds (Su, Geng *et al.* 2022).

3. Strategies to improve lipid profile and oxidative stability

Simultaneously optimizing the lipid profile and oxidative stability of IFF and IF is therefore very challenging. To meet the nutritional needs of infants, fortification of products with LC-PUFA and micronutrients (notably vitamin A and iron) is necessary, but leads to a reduction in oxidative stability. Various strategies have been reported in the literature to overcome this actual concern, along two main axes: modifying formulation or structural lipid parameters (**Figure 15**).

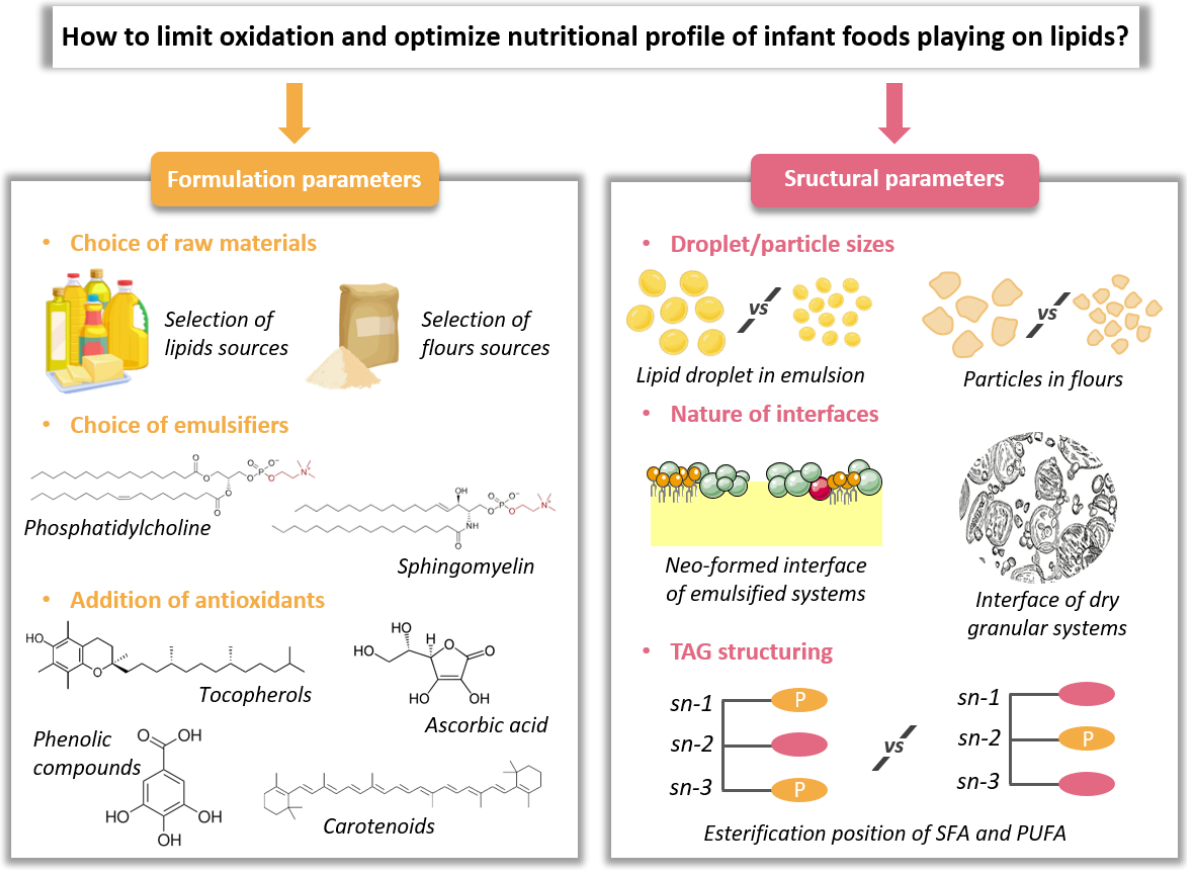


Figure 15. Strategies to optimize both lipid profile and oxidative stability in infant food products

3.1. Formulation parameters

3.1.1. Choice of raw materials

The choice of raw materials for IFF and IF plays a crucial role in optimizing their lipid profiles and oxidative stability. The initial level of oxidation of the raw materials determines, in part, the oxidative stability of the final product during storage. Indeed, once the oxidation initiation stage has been completed, the phenomenon spreads rapidly throughout the product. Strict control of the oxidation level when receiving raw materials, and the establishment of specifications with the various suppliers, therefore contribute to improved stability. Furthermore, the choice of raw materials determines the lipid composition of the final product, and therefore its sensitivity to oxidation. Fats and flours containing high levels of unsaturated lipids are instinctively more sensitive than more saturated ones although differences in PUFA susceptibility to oxidation is observed and well described in literature, and should be used with care (in dark, under modified atmospheres, at low temperatures). The fats and flours constituting IFF and IF are also carriers of antioxidant compounds that improve resistance to oxidation (see section *3.1.2*). A good knowledge of the raw materials composition therefore contributes to the better control of oxidation mechanisms. The lipid content of raw materials and,

concomitantly, of the final product, has an impact on oxidation resistance. In oil-in-water emulsions such as IFF, it has been reported that an increase in lipid content favors resistance to oxidation (Kargar, Spyropoulos *et al.* 2011). Conversely, in low-moisture foods, an increase in lipid content has been shown to increase sensitivity to oxidation (Gumus and Decker 2021). The physical state of lipids also has an impact on oxidation sensitivity. In breast milk, LC-PUFA are mainly localized in the larger MFG surrounded by long-chain SFA, with high melting point (HMP) leading to a partial surface crystallization (Michalski, Ollivon *et al.* 2004). This specific organization could have an impact on oxidative stability through the formation of a lipid shell protecting LC-PUFA (steric barrier). Okubanjo, Loveday *et al.* (2019) and Salminen, Helgason *et al.* (2017) have reported, for example, that lipid shell formation at interfaces in emulsified systems results in improved oxidative stability. These results were attributed to reduced contact between pro-oxidant compounds and unsaturated lipids, and limited oxygen diffusion at the interface (Tikekar and Nitin 2011). However, the exudation of unsaturated lipids (migration towards the surface) induced by crystallization can, on the contrary, promote lipid oxidation (Okuda, McClements *et al.* 2005). Finally, processing of raw materials may improve oxidative stability. Heat treatment, for example, results in enzymatic inactivation, inhibiting the enzymatic oxidation pathway (LOX) or the production of FFA (lipase), which are more sensitive to oxidation (Van Den and Mendoza 1982, Zheng, Wang *et al.* 2023).

3.1.2. Addition of antioxidants

One of the most commonly studied strategies for limiting lipid oxidation is the addition of antioxidants or antioxidant mix. Antioxidants are defined as compounds capable of delaying and/or decreasing the rate of oxidation mechanism. Regulations concerning their use in IFF and IF establish maximum levels for certain antioxidants of, for example, 3.5 and 21 mg/100 mL in IFF and 3 and 12.5 mg/100 Kcal in IF (25 mg/100 Kcal of vitamin C in the case of iron-fortified IF) for vitamins E and C, respectively. Antioxidants can be classified according to their action mode (Ghelichi, Hajfathalian *et al.* 2023) :

- Chain breaker antioxidants: these are antioxidants capable of reacting directly with lipid radicals to inhibit oxidation. This category includes free radical scavengers such as tocopherols, phenolic compounds, ascorbic acid and certain carotenoids.
- Other antioxidants: these are antioxidants that inhibit oxidation indirectly. This category includes, for example, metals chelators (EDTA, certain phenolic compounds), oxygen scavengers (ascorbic acid) or singlet oxygen quenchers (carotenoids) and compounds capable of regenerating the active form of other antioxidants (PL, ascorbic acid, carotenoids).

Some antioxidants have multi-functions and can therefore act *via* several mechanisms. Antioxidants can also act in conjugation, offering a synergy effect including mechanisms of regeneration, combination, sacrifice or by dimerization and adduct. Synergies by regeneration have been reported

between tocopherols and PL, or tocopherols and tocotrienols with β-carotene, which favor the conversion of the oxidized form of tocopherols, produced by the neutralization of free radicals, back to the active form (Schroeder, Becker *et al.* 2006, Zou and Akoh 2015, Samdani, McClements *et al.* 2018, Liang, Liu *et al.* 2023). Combination synergy, on the other hand, involves the simultaneous use of different antioxidant functions, such as metal chelation in combination with free radical scavenging (Ma, Wang *et al.* 2023).

The antioxidants efficiency depends on many factors, including their polarity and that of their environment, which determines their localization, as well as their concentration or the presence of other compounds. The antioxidants efficiency according to their localization is described by two theories: the “polar paradox” and the “cut-off effect”. The polar paradox predicts the efficiency of an antioxidant according to its polarity and that of the chemical environment (Frankel, Huang *et al.* 1994, Frankel, Huang *et al.* 1996). Thus, a fat-soluble antioxidant is more effective in a polar environment, whereas a water-soluble antioxidant is assumed to be more effective in a non-polar environment. In fact, these differences in polarities between the environment and the antioxidant enable the antioxidant to locate itself close to the interfaces, the oxidation initiation site. For example, tocopherols (fat-soluble) have been reported to be more effective than ascorbic acid (water-soluble) in oil-in-water emulsions. The cut-off effect theory is a continuation of the polar paradox and predicts the effectiveness of an antioxidant in a dispersed system such as an oil-in-water emulsion according to its fat-solubility. This theory was established with rosmarinic esters with different chain lengths increasing their fat-solubility (Laguerre, López Giraldo *et al.* 2009, Laguerre, López Giraldo *et al.* 2010). The more fat-soluble an antioxidant was, the more effective it was, until the Critical Chain Length (CCL) was reached at which point the antioxidant's effectiveness declined. Chain lengths of 8 to 12 carbons were the most effective. However, this parameter is dependent on the physico-chemical properties of the overall system (Alemán, Bou *et al.* 2015). This theory is based on the partitioning of antioxidants between emulsion compartments. At the CCL, the antioxidant is localized at the interface and is therefore the most effective. These different theories contribute to the selection of antioxidants adapted to the system to be stabilized (**Table 4**).

Regarding dry systems, several authors have suggested that the most effective antioxidants are the most fat-soluble (Barden, Barouh *et al.* 2015, Ten Klooster, Villeneuve *et al.* 2022). However, other authors have reported an important stabilizing effect with the addition of cereal bran, which is rich in phenolic compounds (Van Wayenbergh, Struyf *et al.* 2020, Van Wayenbergh, Coddens *et al.* 2023). The authors suggest that both a physical effect of the bran by trapping the oil in the fibers (protective effect against moisture and contact with pro-oxidants) and a chemical protective effect linked to the diversity of "lipid components" present in the bran acted as stabilizing factors.

More research is therefore needed to better understand the mechanisms involved in dry systems.

Table 4. Effect of antioxidant addition in dispersed oil-in-water emulsions or dry granular systems

Reference	Dispersed systems characteristics	Antioxidant	Results
Oil-in-water emulsions			
Satué-Gracia, Frankel *et al.* (2000)	Infant formulas emulsion containing or not iron (0 to 220 µM) stored 20 days at 50°C	12.5 to 110 µmol/L of lactoferrin	Highest antioxidant capacity at a lactoferrin/iron molar ratio of 2:1, with over 75% inhibition of hydroperoxide and hexanal formation in all samples
Uluata, McClements *et al.* (2015)	1% medium-chain TAG oil-in-water emulsion stored 20 days from 10 to 45°C	0.01 to 200 mmol/L of ascorbic acid	↗ oxidative stability in presence of ascorbic acid up to 20 mmol/L Pro-oxidant effect at higher concentration
Zou and Akoh (2015)	Infant formulas emulsion containing structured TAG stored 28 days at 37°C	50 ppm of α-tocopherol, β-carotene, ascorbyl palmitate, ascorbic acid, citric acid, and their combinations	Ascorbyl palmitate is more effective but less than an N_2-inerted atmosphere Strongest synergy between α-tocopherol and β-carotene
Samdani, McClements *et al.* (2018)	1% stripped soybean oil-in-water emulsion	15 µmol/kg of PS or PE and their combination with 3.0 µmol/kg of δ- or α-tocopherol or tocopherols mix	Antioxidative synergy order: δ-tocopherol and PS ≥ δ-Tocopherol and PE > α-tocopherol and PS > α-tocopherol and PE
Tang, Cao *et al.* (2023)	5% stripped peony seed oil-in-water emulsion stored 18 days at 28°C	Total concentration of 10 to 30 µmol/L of tocopherols (α, β, γ and δ) and PL (PC, PE, PI)	Synergy between tocopherols and PL (PL may improve tocopherols distribution at the interface and its regeneration)
Dry systems			
Baik, Suhendro *et al.* (2004)	Microencapsulated fish oil powder	250 ppm of α-tocopherol or ascorbyl palmitate or 1,000 ppm of α-tocopherol	Surface fat was 10x less stable than encapsulated fat (without antioxidant), α-tocopherol more efficient than ascorbyl palmitate at both concentrations
Wang, Li *et al.* (2020)	Infant formulas powder containing or not DHA and/or ARA with metals	2500 and 125 ppm of vitamins C and E	Considerable variability in results, addition of DHA and ARA ↗TBARS, addition of metals or vitamins did not affect TBARS
Ten Klooster, Villeneuve *et al.* (2022)	Spray-dried stripped sunflower and fish oils-in-water emulsion stored 25 days at 40°C with 50% relative humidity	600 µmol/kg oil of alkyl chain length of gallic acid esters from 0 to 16 carbon atoms	↗ oxidative stability for antioxidant with more than 8 carbon alkyl chain
Tzima, Brunton *et al.* (2021)	Fat-filled milk powders with sunflower oil stored 90 days at 40 °C with 23% relative humidity	200 ppm BHT/BHA (1:1) 77, 200 or 308 ppm mix of carnosol, carnosic acid and rosmarinic acid	↗ oxidative stability ↗ antioxidant concentration, mix of phenolic compounds less effective than BHT:BHA
Van Wayenbergh, Langenaeken *et al.* (2023)	Wheat bran enriched with 20% soybean oil containing 0.8% RP stored up to 8 weeks at 60°C with 70% relative humidity	Bioactive compounds of wheat bran including phenolic compounds tocopherols and tocotrienols	↗ oxidative stability of retinyl palmitate with heat treated wheat bran

PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PI: Phosphatidylinositol, PS: Phosphatidylserine

TBARS: Thiobarbituric Acid Reactive Substances

3.1.3. Interface composition

In oil-in-water emulsions, the initiation of oxidation occurs at the oil/water interfaces, whose composition is modulated by the source of emulsifier. European regulation CE 2016/127 governing the composition of IFF allows the use of lecithins, mono- and diacylglycerols and glycerophospholipids. Certain proteins such as serum proteins (including whey protein isolate (WPI)) or caseins also have emulsifying properties and can be used. Depending on the composition of the interfacial layer, its charge (dependent on the pH of the aqueous phase), its thickness and the emulsifier concentration, the properties of the interface can offer a protective effect against lipid oxidation (Ghelichi, Hajfathalian *et al.* 2023). However, it is important to keep in mind that emulsifier concentration is almost systematically higher than CMC, resulting in the presence of unadsorbed emulsifiers which may have an antioxidant effect and may hide the contribution of the emulsifiers located at the interface (Berton-Carabin, Ropers *et al.* 2014). In general, protein-based emulsifiers are reported to be the most effective at limiting oxidation, independently of pH (**Table 5**). Among proteins, caseins have a superior effect in limiting lipid oxidation compared to WPI. This difference is due to several factors:

- Their amino acid composition: caseins are composed of phosphorylated serines with metal chelation activity, whereas WPI contain more residues with sulfhydryl groups (cysteine, tyrosine) with free radical scavenging activity.
- Their structures: caseins are unstructured proteins, whereas WPI are globular proteins. This difference impacts their flexibility and hence their adsorption onto the interfacial layer.
- Interface-covering capacity: caseins are reported to have greater interface-covering capacity than WPI (this difference is notably linked to their structure) (Hunt and Dalgleish 1994).
- Interface thickness: caseins form thicker interfaces than WPI, around 10 nm *versus* 1 to 2 nm, respectively (Dalgleish, Srinivasan *et al.* 1995). Increased interface thickness helps limit lipid oxidation by forming a steric barrier (antioxidant activity through physical properties).
- Their charges: a high density of cationic charges at the interface results in an effective electrostatic repulsion of pro-oxidant metals. However, at neutral pH these proteins are negatively charged (acid isoelectric point). An anionic charge in the aqueous phase (unadsorbed protein) contributes to metal chelation capacity.

Differences in oxidation-limiting efficacy between dairy phospholipids (DPL) and lecithins are also reported and probably due to their different PL composition and the important residual WPI load remaining in DPL. DPL are mainly composed of SM, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), while vegetable lecithins contain PE, PC and phosphatidylinositol (PI). Their antioxidant activity is attributed to the presence of amino groups. In addition, their FA

composition, particularly unsaturated FA, determines their flexibility and consequently the distance between their active group and the interface (**Table 5**).

Table 5. Effect of emulsifier source and concentration on oxidative stability of oil-in-water emulsion

Reference	Oil-in-water emulsion characteristics	Emulsifier		Results
		Source	Concentration	
Fomuso, Corredig *et al.* (2002)	10% of purified structured oil-in water emulsion (pH = 7.0)	Tween 20 Sucrose ester WPI MAG Lecithin	0.25 or 1%	↗ oxidative stability with ↗ of emulsifier concentration (excepted for WPI) Oxidative stability based on hydroperoxide content: WPI > Lecithin > Tween and MAG > Sucrose esters
Hu, McClements *et al.* (2003)	5% corn-oil-in-water emulsion (pH = 3.0)	Casein SPI Soy protein	0.5 to 1.5%	Oxidative stability based on hydroperoxide and hexanal content: Casein > WPI > Soy protein > Sucrose esters
Horn, Nielsen *et al.* (2011)	70% fish oil-in-water emulsion (pH = 4.5 or 7.0)	Soy lecithin DPL 20 or 75% WPI Sodium caseinate	2.8%	Oxidative stability based on PV and volatiles compounds content: Sodium caseinate > WPI > DPL 20 > Soy lecithin > DPL 75 at pH = 7.0
Nielsen, Horn *et al.* (2013)	5% fish oil-in-water emulsion (pH = 3.0 or 7.0)	WPI Sodium caseinate Soy lecithin DPL 20 or 75%	0.2 or 0.75%	Oxidative stability based on PV and volatiles compounds content: Sodium caseinate > WPI > DPL 20 > Soy lecithin > DPL 75
Drapala, Mulvihill *et al.* (2018)	3.5% soybean oil-in-water emulsion (pH = 6.8)	Lecithin	0 to 5%	↗ oxidative stability with ↗ of lecithin concentration up to 1% w/w No additional effect at 2-5%
Bourgeois, Couëdelo *et al.* (2020)	20% rapeseed oil-in-water emulsion (pH = 5.5)	Lecithin only or co-emulsified with proteins extracted from canola oil bodies	7.4% with ratio lecithin/protein from oil bodies of 1:0, 1:1 or 1:3	Based on hydroperoxide and CD content ↗ Oxidative stability with ↗ of protein from oil bodies content
Wang, Yu *et al.* (2022)	10% flaxseed oil-in-water emulsion (pH = 7.0)	WPI Soy lecithin Tween 20	1.5 or 2%	Oxidative stability based on hydroperoxide and TBARS content: WPI > Soy lecithin > Tween 20

In dry granular systems, initiation occurs at the air/lipid interface. Consequently, surface lipids are more prone to oxidation. Better encapsulation of these lipids can improve oxidative stability, notably by modulating particle size (Schorno, Manthey *et al.* 2010).

3.2. Structural parameters

3.2.1. Droplet and particle sizes

In both emulsified or granular systems, oxidation initiation occurs at interfaces whose nature differs according to the type of dispersed system (oil/water interface in IFF and air/lipids interface in IF).

The decrease in droplet size in an emulsion leads to an increase in specific surface area, which favors contact between unsaturated lipids and pro-oxidants present in the water phase. However, there are many contradictory results in the literature concerning the effect of droplet size on oxidative stability (**Table 6**). Authors who have reported a stabilizing effect through droplet size reduction attribute this result to better adsorption of emulsifiers at the interface, leading to greater interfacial coverage (steric barrier) (Nakaya, Ushio *et al.* 2005, Let, Jacobsen *et al.* 2007, Atarés, Marshall *et al.* 2012). In addition, surfactants such as lecithin or proteins often possess antioxidant capacity, and their adsorption to the droplet surface leads to the formation of a protective antioxidant interface. Authors who have reported a negative effect of droplet size reduction on oxidative stability attribute these results to an increase in specific surface area favoring contact between pro-oxidants and unsaturated lipids (Lee, Choi *et al.* 2011, Kuhn and Cunha 2012, Li, McClements *et al.* 2020). In addition, the processes used to reduce droplet size cause shear stress, local temperature elevation, cavitation and contact with air, which may favor oxidation initiation. Lethuaut, Métro *et al.* (2002) reported differences in stability according to droplet size over short periods, then stability independent of droplet size when oxygen concentration became limited. A compensatory effect of the antioxidant properties of the compounds at the interface and the increase in specific surface area could explain why no significant effect was observed (Osborn and Akoh 2004, Kiokias, Dimakou *et al.* 2007). As suggested by Sørensen, Baron *et al.* (2007), it would appear that oxidative stability is more dependent on the composition of the interface and the chemical environment rather than the droplet size.

Table 6. Effect of lipid droplet size in oil-in-water emulsions on lipid oxidation stability

Reference	Oil-in-water emulsion characteristics	Droplet size	Method	Results
↘ droplet size ↗ oxidative stability				
Nakaya, Ushio *et al.* (2005)	10% of TAG from cod liver and soybean oils in citrate-phosphate-buffer stabilized by sucrose esters	0.8 to 12.8 µm	Hydroperoxide and hexanal formation and O_2 consumption	Significantly lower oxidation product and residual oxygen for smaller droplet size
Let, Jacobsen *et al.* (2007)	Enriched milk with 0.5 or 1% of cod liver oil only or with rapeseed oil	0.5 to 1.4 µm	PV evolution and volatiles compounds formation	Emulsion with smaller droplet size were less oxidized
Atarés, Marshall *et al.* (2012)	20% of sunflower oil (untreated or purified) in phosphate buffer stabilized by WPI	0.3 to 51 µm	PV evolution and TBARS fomation	Significantly lower oxidation product and residual oxygen for smaller droplet size
↘ droplet size ↘ oxidative stability				
Lee, Choi *et al.* (2011)	0.5% of fish oil in phosphate buffer stabilized by WPI	0.07 to 0.33 µm	TBARS formation	Significantly higher TBARS content for smaller droplet size
Kuhn and Cunha (2012)	30% of flaxseed oil in water stabilized by WPI	0.5 to 2.2 µm	PV evolution	Significantly higher PV content for smaller droplet size
Li, McClements *et al.* (2020)	0.2% of soybean oil in SDS solution stabilized by xanthan gum	2 to 40 µm	Hydroperoxide and hexanal formation and flow cytometry	Faster oxidation of small droplet in a polydisperse emulsion
No effect of droplet size on oxidative stability				
Lethuaut, Métro *et al.* (2002)	30% of stripped sunflower oil in water stabilized by BSA	0.4 to 8.1 µm	O_2 consumption and CD formation	Non-limiting O_2 concentration: ↘ droplet size ↘ oxidative stability Limiting O_2 concentration: Droplet size-independent stability
Osborn and Akoh (2004)	10 or 30% of structured lipids from canola and oil and caprylic acid in phosphate buffer stabilized by WPI	0.3 to 2.7 µm	PV, anisidine and TOTOX values evolution	No effect of droplet size on lipid oxidation rate
Kiokias, Dimakou *et al.* (2007)	30% of sunflower oil in distilled water stabilize by WPI	0.5 to 1.9 µm	CD and TBARS formation	No clear effect of droplet size

Contradictory results concerning the effect of particle size on oxidative stability are also reported for dry dispersed systems. Linke, Hinrichs *et al.* (2020) evaluated the effect of a size range from less than 50 µm to over 125 µm in a spray-dried emulsion containing 8% of fish oil. The finest particles (size below 50 µm) were oxidized significantly faster than the larger ones, with a quadratic relationship between total interfacial area and hydroperoxide concentration (if interfacial area doubles, hydroperoxide concentration quadruples). The authors suggested that oxygen diffusion is limited in

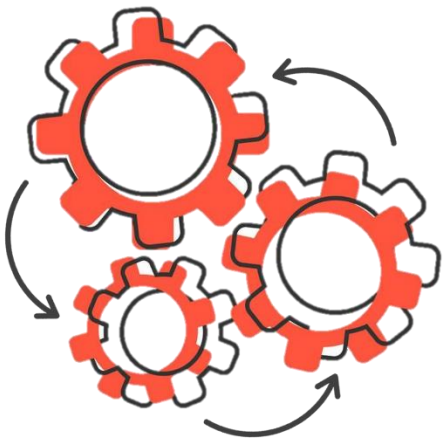
the case of larger particles, with a required time for oxygen to reach the particle center by diffusion increasing proportionally with particle diameter. The structure and interfacial composition of spray-dried emulsions highly differs from natural particles obtained when milling seeds. However, similar results have been reported by Schorno, Manthey *et al.* (2010) on the effect of milled flaxseed particle size. The authors showed that fractions with finer particle sizes (below 600 µm) had a surface lipid content three times higher than the coarser fraction (above 600 µm), which increased sensitivity to oxidation. Indeed, surface lipids in direct contact with oxygen are more prone to oxidation. Lee, Lee *et al.* (2013) also showed that reducing the size of soybean flour from 1.7 to 0.34 mm resulted in an increase in the content of secondary compounds measured by the thiobarbituric acid reactive substances (TBARS) method. However, Rosa, Barron *et al.* (2013) suggested that there is a linear relationship between the specific surface area and the antioxidant capacity of wheat bran, with an antioxidant capacity 1.5 times higher when the average particle size (D50) was reduced from 172 to 30 µm. The increase of specific surface area leads to cell rupture and release of active components. Rohfritsch, Canelli *et al.* (2021) evaluated the antioxidant potential of cereal bran (including wheat and rice) to improve the oxidative stability of fish oil, testing the effect of different milling processes. Particle size reduction from 429 to 117 µm for wheat bran and from 170 to 68 µm for rice bran had no significant effect on stabilization. The authors suggested that the coarse fraction size was already relatively small. Moreover, while increasing the specific surface area leads to better release of antioxidant compounds, it also increases exposure to oxygen and thus promotes oxidation. These two phenomena could therefore occur simultaneously and thus explain the non-significant effect of particle size reduction.

3.2.2. Lipid organization and TAG structuring

Breast milk lipids have a specific lipids organization under the form of MFG as described previously. In addition, the core composition of MFG is heterogeneous, with the smallest globules containing mainly medium-chain SFA (ranging from 8 to 12 carbons), while larger globules contain long-chain SFA forming a lipid shell (partial surface crystallization) (Michalski, Ollivon *et al.* 2004, Fauquant, Briard *et al.* 2005). Thus, the LC-PUFA present in the largest MFG are protected by this lipid shell (steric barrier) improving their oxidative stability. Another structural specificity of breast milk is the preferential esterification of palmitic acid and PUFA in *sn-2* position of TAG. All these parameters contribute to explain the better oxidative stability of breast milk compared to infant formulas (liquid or dispersed powder) reported by Michalski, Calzada *et al.* (2008). From a nutritional point of view, the esterification of palmitic acid and DHA in the internal position improves lipid bioavailability. Palmitic acid in *sn-2* central position limits the formation of insoluble soaps (calcium palmitate) which are directly excreted (Dubois, Breton *et al.* 2008). Christensen, Høy *et al.* (1995) showed on male albino Wistar rats that TAG structured with EPA

and DHA predominantly in *sn-2* favored their absorption and lymphatic transport compared to randomized TAG. More recently, Linderborg, Kulkarni *et al.* (2019) in their study on animal models, highlighted a lower fecal secretion of DHA when it is esterified in *sn-2* compared to external positions. In addition, the sensitivity of FA to oxidation is suspected to depend on their esterification position, with the protective effect attributed to the *sn-2* position. Damerau, Ahonen *et al.* (2023) in agreement with Wijesundera, Ceccato *et al.* (2012) showed improved oxidative stability of DHA in *sn-2* with or without the addition of α-tocopherol in regio- and enantiopure TAG. The authors reported that without the antioxidant addition, the *sn-3* position was slightly more stable than *sn-1*, whereas in the presence of α-tocopherol, DHA in this same position underwent a more rapid oxidation rate than the *sn-1* position, which could be explained by a diastereomeric interaction between α-tocopherol and DHA in *sn-1*. Structuring part of the oil mixtures used in the composition of IFF could therefore enable both an improvement in nutritional profile and oxidative stability by increasing palmitic acid contents in the internal position, while retaining the LC-PUFA localization of vegetable oils (esterification in *sn-2*). The formulation of structured TAG analogous to breast-milk lipids, obtained by enzymatic interesterification or acidolysis reaction, has been the subject of several studies (Maduko, Park *et al.* 2008, Pina-Rodriguez and Akoh 2010, Ilyasoglu 2013, Nagachinta and Akoh 2013, Bryś, Górska *et al.* 2021). Other authors encourage the reintroduction of dairy fats, particularly of bovine origin, which are quite similar to breast milk in both composition and structure (Delplanque and Baudry 2015, Baars, Oosting *et al.* 2016, Bourlieu, Deglaire *et al.* 2017). For instance, the position of FA on bovine milk TAG backbone is more biomimetic of breast milk than that of vegetable oils, with up to 45% of palmitic acid in the *sn-2* position (compared to only 10 to 20% in vegetable oils) (Hageman, Danielsen *et al.* 2019).

Regarding dry granular systems, for spray-dried systems, the organization of lipids in free form (on the surface of the grain) or trapped in the powder grain strongly modifies its susceptibility to oxidation (higher if in free form) (Garcia-Martinez, Rodriguez-Alcala *et al.* 2010). Nevertheless, analysis of this free fat in a limited set of infant formulas over 30 days after pack opening showed good stability over this period, in line with post-opening storage recommendations for powdered infant formulas (Zunin, Boggia *et al.* 2015)



AIM AND STRATEGY

This thesis focused on optimizing both the nutritional properties and the oxidative stability of the lipid fraction of two matrices consumed between 6 and 12 months of age, namely liquid follow-on infant IFF and IF. The aim of this work was to answer the following research question:

What are the key physico-chemical levers to control the oxidative stability of dispersed systems enriched in LC-PUFA while maintaining an acceptable bio-accessibility of lipids?

The overall strategy implemented to address this issue is presented in **Figure 16**. The choice of matrices studied covers two types of dispersed systems: liquid emulsified systems (oil-in-water emulsion) represented by IFF, and dry granular systems represented by IF.

The first part of the strategy was devoted to the study of IFF. For this purpose, an IFF with a simplified aqueous phase named model IFF was formulated according to the composition of commercial IFF and European regulations. In order to improve the lipid profile (composition and structure) and identify the key levers impacting oxidative stability, the model IFF was declined into a panel of model systems with various formulations and structural parameters. All model IFF were subjected to storage tests under accelerated conditions to assess oxidation kinetics. Model IFF that demonstrated improved resistance to oxidation and nutritional properties were then subjected to an *in vitro* digestion process to evaluate the impact of optimization on nutrient bioaccessibility.

The second part of the strategy was devoted to the study of another type of staple food for infant, *i.e.* IF. The same steps as for IFF were applied to IF. Firstly, the lipid profile was optimized based on the composition of globally marketed IF and international regulations. Subsequently, storage tests were conducted to understand the oxidation mechanisms in dry granular systems and to identify key parameters for controlling this phenomenon. Finally, systems with the most significant nutritional and oxidative stability optimization underwent *in vitro* digestion to assess the nutrients bioaccessibility in this type of dry dispersed system.

The third part of the strategy was dedicated to comparing the two types of system in terms of their oxidative stability, diffusion mechanisms involved in oxidation, nutritional properties, and the potential of each system as lipid nutrient carriers.

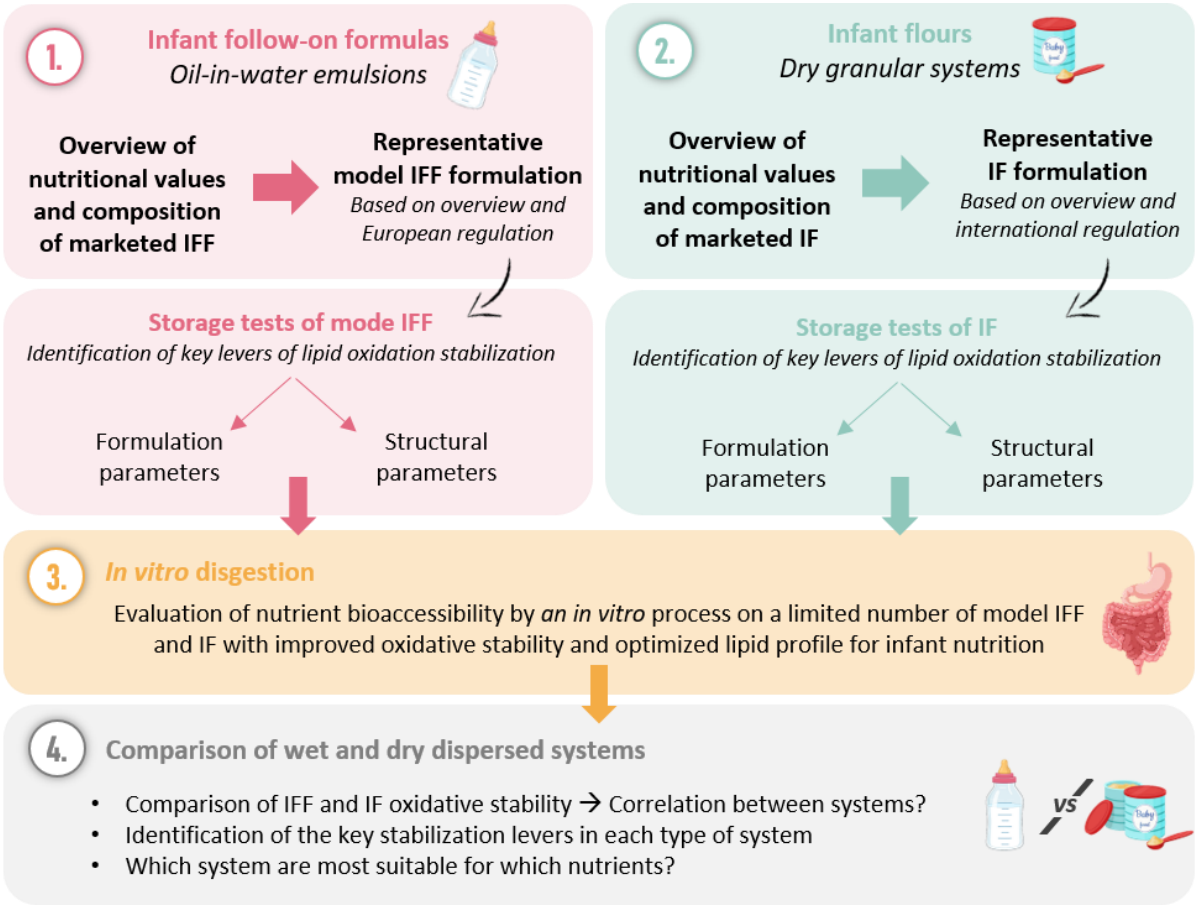
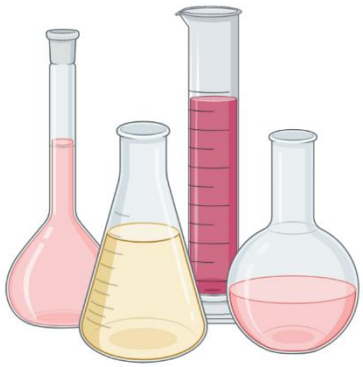


Figure 16. Global strategy



CHAPTER 2

Materials and methods



This chapter is devoted to the description of all the experimental techniques used in this thesis. The techniques used in the research articles presented in Chapters 3, 4 and 5 have been extracted and gathered in this chapter. Firstly, the materials (solvents, reagents, analytical standards and raw materials) used in the experiments are detailed. A second section describes the steps involved in the formulation and preparation of the model IFF and IF studied (a detachable memento contains the definition of all the emulsified and granular systems studied). The third part of this chapter describes the storage tests carried out to evaluate the oxidation kinetics of the different systems, with a description of each physical and chemical characterization techniques used. The fourth section describes the *in vitro* digestion procedures performed on a limited number of systems demonstrating good oxidative stability. In this section, digesta characterization techniques for assessing the bioaccessibility of FA, vitamin E and proteins are also presented.

Infant follow-on formulas
Oil-in-water emulsions

Infant flours
Dry granular systems

Model systems formulation and preparation

Formulation based on marketed infant follow-on and regulations → Preparation and fortification

Formulation based on marketed infant flours and regulations → Preparation and fortification

Oxidation kinetics - Storage test

20 days | 25 or 40°C | 110 rpm or no shaking

Sampling times

days: D0 D2 D6 D9 D15 D20

Structural characterizations:
- Droplet size distribution
- Confocal laser scanning microscopy
- Differential scanning calorimetry

Chemical characterizations:
- Fatty acid profile
- Primary oxidation compounds
  Peroxide value
- Secondary oxidation compounds
  Thiobarbituric acid reactive substances
- Protein oxidation rate
- Vitamins content (A, E and C)

5, 6 or 9 months | 25 or 40°C

Sampling times *(example of the 6-months storage)*

months: M0 M0.5 M1 M2 M3 M6

Structural characterizations:
- Particle size distribution
- Confocal laser scanning microscopy

Chemical characterizations:
- Dry matter and water activity
- Fatty acid profile
- Primary oxidation compounds
  Peroxide value, conjugated dienes
- Secondary oxidation compounds
  Volatiles compounds
- Vitamins content (A, E and C)
- Phenolic compounds
- Lipoxygenase activity

Static *in vitro* digestion

Preparation and digestion:
- Selection and preparation of IFF
- Static *in vitro* digestion procedure

Initial characterizations:
- Droplet size distribution
- Fatty acid profiles
- Initial vitamin E content

Digestas characterizations:
- Free fatty acid released profile
- Lipolysis
- Proteolysis
- Vitamins E bioaccessibility

Preparation and digestion:
- Selection and preparation of IF
- Static *in vitro* digestion procedure

Initial characterizations:
- Texture (flow rate)
- Fatty acid profile
- Initial vitamin E content
- Trypsin inhibitory activity

Digestas characterizations:
- Free fatty acid released profile
- Lipolysis
- Proteolysis
- Vitamin E bioaccessibility

1. Chemicals and raw materials

All the solvents, reagents, analytical standards and raw materials used are listed in **Table 7** and **Table 8**.

Table 7. References of solvents, reagents, analytical standards and other materials used

Solvents	Suppliers	References
1,4-Dioxane	Sigma-Aldrich	296309
1-Butanol	Sigma-Aldrich	33065
2,2,4-Trimethylpentane	Sigma-Aldrich	32291
Acetic acid	Sigma-Aldrich	33209
Acetone	Sigma-Aldrich	179124 and 270725
Chloroform	Sigma-Aldrich	472476
Diethyl ether	Sigma-Aldrich	14775
Ethanol	Sigma-Aldrich	32221-M
Ethyl acetate	Sigma-Aldrich	33211-M
Hexane	Sigma-Aldrich	34859 and 446903
Hydrochloric acid	Sigma-Aldrich	30721
Methanol	Sigma-Aldrich	34860 and 32213
Methyl tert-butyl ether	Sigma-Aldrich	432032
Phosphoric acid	Sigma-Aldrich	30417
Propan-2-ol	Sigma-Aldrich	34863
Pyridine	Sigma-Aldrich	270970
Sulfuric acid	Legallais	131058.1612
Reagents	**Suppliers**	**References**
16:0 Liss Rhod PE	Aventi Polar Lipids	810158P
2,4-dinitrophenylhydrazine	Sigma-Aldrich	D199303
4-bromophenylboronic acid	Sigma-Aldrich	B75956
Acetyl chloride	Sigma-Aldrich	00990
Ammonium carbonate	Sigma-Aldrich	207861
Ammonium thiocyanate	Sigma-Aldrich	221988
Anhydrous sodium sulfate	Sigma-Aldrich	31481
Azide	Sigma-Aldrich	S8032
Barium chloride, dehydrated	Sigma-Aldrich	217565
Calcium chloride	Sigma-Aldrich	499609
Calcium chloride dihydrate	Sigma-Aldrich	C8106
Cumene hydroperoxide	Sigma-Aldrich	247502
Decylamine	Sigma-Aldrich	D2404
Fast green FCF	Sigma-Aldrich	F7258
Ferrous sulfate	Sigma-Aldrich	F8633
Folin-Ciocalteu	Sigma-Aldrich	F9252
Guanidine hydrochloride solution	Sigma-Aldrich	G9284
Leucine	Sigma-Aldrich	61819
Linoleic acid	Sigma-Aldrich	62230
Lipidtox	Invitrogen	H34475
Magnesium chloride hexahydrate	Sigma-Aldrich	M9272
Monobasic potassium phosphate	Sigma-Aldrich	P0662
Ninhydrin	Sigma-Aldrich	N7285

N-Methyl-N-(trimethylsilyl)trifluoroacetamide	Sigma-Aldrich	69479
Nα-Benzoyl-L-arginine hydrochloride 4-nitroanilide	Sigma-Aldrich	B3133
Pefabloc	Sigma-Aldrich	76307
Phenolphthalein	Sigma-Aldrich	343355
Phosphate buffer	Sigma-Aldrich	P2272
Potassium chloride	Sigma-Aldrich	P3911
Sodium acetate	Sigma-Aldrich	S5761
Sodium chloride	Sigma-Aldrich	31434
Sodium hydroxide	Sigma-Aldrich	S5881
Sodium methoxide solution	Sigma-Aldrich	156256
Thiobarbituric acid (TBA)	Sigma-Aldrich	T5500
Trichloroacetic acid (TCA)	Sigma-Aldrich	T6399
Tris(2-carboxyethyl)phosphine (TCEP)	Sigma-Aldrich	C4706
Tween 20	Sigma-Aldrich	P1379
Analytical standards	**Suppliers**	**References**
3-4-5-trimethoxy-trans-cinnamic acid (TMCA)	Sigma Aldrich	T70408
3-heptanol	Sigma Aldrich	109363
Gallic acid	Sigma Aldrich	G7384
Glyceryl triheptadecanoate	Sigma Aldrich	T2151
Hexanal	Sigma Aldrich	18109
Infant/Adult Nutritional Formula II	Sigma Aldrich	NIST1869
Lutein	Sigma Aldrich	7168
Mixture of 37 FAME components	Sigma Aldrich	CRM47885
Retinol	Sigma Aldrich	R7632
Retinyl acetate	Sigma Aldrich	PHR1236
Retinyl palmitate	Sigma Aldrich	PHR1235
Tocopherol complex	Millipore	613424
Tridecanoic acid	Sigma Aldrich	T7517
α-carotene	Sigma Aldrich	40395
α-tocotrienol	Sigma Aldrich	T0452
β-carotene	Sigma Aldrich	C-9750
Other	**Suppliers**	**References**
B. licheniformis – Protease	Megazyme	E-BSPRT
Bicinchoninic acid assay kit	Sigma-Aldrich	BCA1
Bile salt	Sigma-Aldrich	B3883
Gastric lipase	Lipolytech	RGE15
Pancreatic lipase	Sigma-Aldrich	P7545
Trypsin	Sigma-Aldrich	T2600000

Enzymes activity:

B. licheniformis - Protease: 6 U/mg of protein (40°C, pH 8.0 on casein)

Rabbit gastric lipase extract: lipase: >15 U/mg powder; Pepsine ≥ 500 U/mg powder

Pancreatic lipase: 8xUSP

Table 8. References and suppliers of raw materials used for model IFF and IF formulation

Infant follow-on formulas studies	
Raw materials	**Suppliers**
ARA-rich oil	DSM Nutritional, La Garenne Colombes, France
Coconut oil	Huilerie Moog SAS, Bram, France
Dairy fat	Corman, Limburg, Belgium
Dairy phospholipid	Corman, Limburg, Belgium
DHA-rich oil	DSM Nutritional, La Garenne Colombes, France
IFF VMC	DSM Nutritional Products South Africa, Isando, South Africa
Lactose	Ingredia, Arras, France
Commercial IFF (LIFF_A)	Carrefour, Montpellier, France
Commercial IFF (LIFF_C)	Carrefour, Montpellier, France
Commercial IFF (LIFF_G)	Lafayette de l'Arc pharmacy, Montpellier, France
Commercial IFF (LIFF_L)	Lafayette de l'Arc pharmacy, Montpellier, France
Maltodextrine	Sigma Aldrich
Oleic sunflower oil	Cuisinor, Saint-Médard de Guizières, France
Palm oil	Cargill, Paris, France
Rapeseed oil	Casino, Montpellier, France
Red palm oil	La pangée, Montpellier, France
Skim milk	Sodiaal, Paris, France
Soy lecithin	Novastell, Etrepagny, France
Sunflower oil	Casino, Montpellier, France
Whey protein	Ingredia, Arras, France
Infant flours studies	
Raw materials	**Suppliers**
Amaranth leaves	Le panier exotique, Montpellier, France
ARA-rich powder from fungi	DSM Nutritional, La Garenne Colombes, France
Black rice	Bongran, Arles, France
Commercial infant flour (CIF)	Internal network
Cowpea flour	Ô Sondaga, Montpellier, France
DHA-rich powder from microalgae	DSM Nutritional, La Garenne Colombes, France
Flour VMC	DSM Nutritional Products South Africa, Isando, South Africa
Groundnut flour	Internal network
Maize flour	Moulin des Moines, Krautwiller, France
Millet seeds	Internal network
Rice flour	Moulin des Moines, Krautwiller, France
Soy flour	Markal, Saint-Marcel-lès-Valence, France
Teff flour	Moulin des Moines, Krautwiller, France
Wheat flour	Moulin des Moines, Krautwiller, France
Wholemeal oat flour	Moulin des Moines, Krautwiller, France

Cowpea, wholegrain oat and millet flours, black rice bran and amaranth leaves powders were prepared prior to the experiments. Cowpea flour was sieved to 700 µm. The coarsest fraction (>700 µm) was ground using an MM500 ball mill (Retsch, Éragny, France) under cryogenic conditions. Grinding cycles were 30 seconds at 30 Hz. The coarse regrind fraction and the sieved fraction (<700 µm) were mixed, then part of the cowpea flour was heat-treated for 10 minutes at 90°C using a UF450 stove (Memmert,

Schwabach, Germany) to inhibit potential lipoxygenase activity and antinutritional factors. Wholegrain oat flour was sieved to 1 mm. The coarsest fraction (>1 mm) was ground using an MM500 ball mill (Retsch, Éragny, France) under cryogenic conditions. Grinding cycles were 30 seconds at 30 Hz. The coarse regrind fraction and the sieved fraction (<1 mm) were mixed. Millet seeds were roasted at 180°C for 10 minutes to inhibit the expression of anti-nutritional factors using a UF450 stove (Memmert, Schwabach, Germany). Millet flour was obtained by hulling roasted millet seeds with a DMS 500N abrassif huller (Electra, Poudenas, France). Two hulling cycles at a speed of 54 kg/h were applied, at 2,000 rpm for the first and 500 rpm for the second. The hulled grains were then ground with an UPZ 100 shearing mill (Hosokawa-Alpine, Augsburg, Germany) to reach a grain size of 150 µm in mode. Black rice bran was obtained by hulling black rice with a DMS 500N abrassif huller (Electra, Poudenas, France). Two hulling cycles at a speed of 54 kg/h were applied, at 2,000 rpm for the first and 1,000 rpm for the second. The bran obtained was then ground under cryogenic conditions with an MM500 (Retsch, Éragny, France), using 30 second grinding cycles at 30 Hz. A 10-minutes heat treatment at 105°C in a UF55 stove (Memmert, Schwabach, Germany) was performed to inactivate the enzymes. Amaranth leaf powder was obtained by freeze-drying amaranth leaves with a COSMOS (Cryotec, Saint-Gély-du-Fesc, France), followed by cryogenic grinding with an MM500 ball mill (45 seconds, 30 Hz; Retsch, Éragny, France).

2. Model system preparation

2.1. Model infant follow-on formulas

The first part of the thesis (described in Chapter 3) was devoted to studying the oxidation kinetics of model IFF according to different structural and formulation parameters.

2.1.1. Model infant follow-on formulas formulation

The model IFF composition was based on that of the marketed products. For this purpose, the labelled nutritional values of 91 IFF from the global market were collected and statistically analyzed (Cancalon, Hemery *et al.* 2023). The target nutritional values of model IFF were therefore set to the average values obtained in the overview. The oil phases composition of model IFF were determined by linear programming to reach the target nutritional values using Microsoft Excel Solver (Excel 2016, https://www.microsoft.com/fr-fr/microsoft-365/excel). Thus, four oil mixtures were formulated depending of the main source of SFA *i.e.* based on refined palm (PO), red palm (RPO), coconut (CO) oils or dairy fat (DF). Model IFF were declined in a panel of modalities according to different formulation parameters (different sources of saturated fats, emulsifiers or chemical forms of vitamin A) and structure (droplet size, heterogeneity of droplet composition or emulsifiers mode of incorporation).

Two droplet sizes were selected, one representative of commercial liquid IFF with an average diameter of 0.45 µm. The second droplet size was intended to be closer to the micrometer to avoid overprocessing, while ensuring a certain physical stability. Droplets of 0.7 and 0.4 µm were studied. To fortify model IFF, a VMC has been formulated according to the overview of the 91 marketed IFF nutritional value and European regulation. The IFF VMC was made of a mixture of vitamin A as retinyl acetate (3593 µg RA/g), vitamin C as sodium ascorbate (81 µg/g), vitamin D as cholecalciferol (588 mg/g) and iron as dried iron sulfate (49 mg/g). Nineteen model IFF were studied (**Table 9** and memento side A):

- PO_NE_L and PO_NE_S: PO based model IFF non-enriched with VMC with a droplet size of 0.7 or 0.4 µm, respectively (L stands for large and S for large).
- PO_L and PO_S: PO based model IFF enriched with VMC, a vitamin A normalization with retinol and a droplet size of 0.7 or 0.4 µm respectively.
- PO_RA_L: PO based model IFF enriched with VMC, a vitamin A normalization with retinyl acetate and a droplet size of 0.7 µm.
- PO_2RA_L: PO based model IFF enriched with VMC, a vitamin A overage as form of retinyl acetate and a droplet size of 0.7 µm.
- PO_DLDC_L and PO_DLDC_S: PO based model IFF enriched with VMC, a vitamin A normalization with retinol and heterogeneous lipid droplet composition (blend of two pre-emulsions, one with high and low melting point oils, *i.e.* palm with ARA and DHA oils, the other with intermediate melting point oils, *i.e.* sunflower, oleic sunflower and rapeseed oils) with a droplet size of 0.7 or 0.4 µm, respectively.
- PO_LWP_L and PO_LWP_S: PO based model IFF enriched with VMC, a vitamin A normalization with retinol and constituted of assembly of two pre-emulsions, one composed of the oil phase homogenized with the water phase (stabilization with proteins) and the other of soy lecithin homogenized with the water phase (*i.e.* lecithin previously homogenized in water phase) with a droplet size of 0.7 or 0.4 µm, respectively.
- PO_DPL_L and PO_DPL_S: PO based model IFF enriched with VMC, a vitamin A normalization with retinol, DPL as emulsifier and a droplet size of 0.7 or 0.4 µm respectively.
- RPO_L and RPO_S: RPO based model IFF enriched with VMC, a vitamin A normalization with retinol and a droplet size of 0.7 or 0.4 µm respectively.
-CO_L and CO_S: CO based model IFF enriched with VMC, a vitamin A normalization with retinol and a droplet size of 0.7 or 0.4 µm respectively.
- DF_L and DF_S: DF based model IFF enriched with VMC and a droplet size of 0.7 or 0.4 µm respectively.
- DF_DPL_L and DF_DPL_S: DF based model IFF enriched with VMC, DPL as emulsifier and a droplet size of 0.7 or 0.4 µm respectively.

Table 9. Oil phase composition, levels of fortification and normalization in vitamins and minerals of model IFF

	PO_NE	PO	PO_RA	PO_2RA	PO_DLDC	PO_LWP	PO_DPL	RPO	CO	DF	DF_DPL
Oil phase omposition (%w/w)											
Palm oil	67.8	67.8	67.8	67.8	67.8	67.8	67.8	-	-	-	-
Rapeseed oil	18.4	18.4	18.4	18.4	18.4	18.4	18.4	15.4	18.6	12.6	12.6
Sunflower oil	9.1	9.1	9.1	9.1	9.1	9.1	9.1	12.0	14.4	19.4	19.4
High oleic sunflower oil	3.1	3.1	3.1	3.1	3.1	3.1	3.1	-	34.9	11.1	11.1
Coconut oil	-	-	-	-	-	-	-	-	30.6	-	-
Dairy fat	-	-	-	-	-	-	-	-	-	55.3	55.3
Red palm oil	-	-	-	-	-	-	-	71.0	-	-	-
ARA oil	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
DHA oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Soy lecithin	0.2	0.2	0.2	0.2	0.2	0.2	-	0.2	0.2	0.2	-
Dairy phospholipid	-	-	-	-	-	-	0.8	-	-	-	0.8
Vitamins and minerals fortification (mg/L)											
Retinol	-	0.1	-	-	0.1	0.1	0.1	0.1	0.1	-	-
Retinyl acetate	-	-	0.1	0.3	-	-	-	-	-	-	-
Tocopherol	-	9.1	9.1	9.1	9.1	9.1	9.1	9.1	-	6.1	6.1
IFF VMC	-	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0

2.1.2. Model infant follow-on formulas preparation

The water and oil phases were prepared 24 hours prior to model IFF preparation and stored at 4°C according to Bourlieu-Lacanal, Bouzerzour *et al.* (2015) and Drapala, Auty *et al.* (2016) with slight modifications (Memento side A). The water phase was obtained after solubilization at 50°C of 20.5 g/L lactose in ultrapure water and mixing at room temperature with the other compounds, namely 39.0 g/L of maltodextrin, 32.9 g/L of skim milk and 3.5 g/L of serum protein isolate. The oil phases were obtained by blending the fats in the proportions described in **Table 9**. For PO_DLDC_L and PO_DLDC_S, the oil phase has been separated into two pre-mixtures, one with high and low melting point oils, *i.e.* palm with ARA and DHA oils, the other with intermediate melting point oils, *i.e.* sunflower, oleic sunflower and rapeseed oils. Soy lecithin and DPL were used as emulsifiers and added to the oil mixtures. Soy lecithin was added at 2 g/L (40% PL *i.e.* 0.8 g/L of PL) in the oil phase except for PO_LWP_S and PO_LWP_L in which soy lecithin was added in the water phase. DPL was also added in the oil phase at 8 g/L (11% PL *i.e.* 0.88 g/L of PL) and concomitantly increases protein content (+0.4 g/100 mL). For PO_LWP_L and PO_LWP_S, soy lecithin was added to the water phase (instead of oil phase). To be representative of IFF industrial manufacturing processes, vitamin and mineral fortification and standardization was carried out just prior to the model IFF homogenization as described in **Table 9**. 20 mg/100 mL of IFF VMC was added to the oil phase (to be representative of industrial practices). Vitamin E content was normalized by adding α-tocopherol to the oil phase. Normalization of vitamin A content was performed on the total retinoid content (including retinol and retinol ester) by adding retinol, the active form of vitamin A, or retinyl acetate to the oil phase. The oil phase (oil mixture and emulsifier) was added at 3.4% (v/v) to the water phase previously heated to 70°C. Both phases were pre-emulsified by two cycles of 5 minutes at 5,000 rpm using a Silverson L5M (Silverson, Longmeadow,

USA). Model IFF were homogenized with an APV-1000 lab homogenizer (SPXflow, Charlotte, North Carolina, USA) using two processes to obtain emulsions with different droplet sizes. The first homogenization process involved eight cycles at pressures of 100/30 bar and resulted in droplets of 0.7 µm. The second homogenization process involved eight cycles at pressures of 350/40 bar and resulted in a droplet size of 0.4 µm. For PO_DLDC_L and PO_DLDC_S two pre-emulsions were prepared, one with the water phase and a premix of high and low melting point oils, the other with the water phase and premix of intermediate melting point oils. For PO_LWP_L and PO_LWP_S two pre-emulsions were also prepared, one composed of the oil phase homogenized with the water phase (stabilization with proteins) and the other of soy lecithin homogenized with the water phase. For each IFF model, the two pre-emulsions were then assembled after homogenization to have an overall chemical composition equivalent to the reference model IFF PO_L and PO_S.

2.2. Model infant flours

The second part of the thesis (described in Chapter 4) was devoted to studying the oxidation kinetics of IF according to different structural and formulation parameters.

2.2.1. Model infant flours formulation

The composition of IF was determined by linear programming according to a set of constraints using Microsoft Excel Solver and presented in **Table 10**. Target nutritional values were set according to the composition of products on the market and international regulations concerning the composition of infant processed cereal-based foods. Two IF were formulated, one representative of IF produced and marketed in southern regions (SIF) and the other representative of products from northern regions (NIF). The composition of these IF includes the four most commonly used flours depending on the production and marketing region *i.e.* maize, soy, groundnut and millet flours for SIF and rice, wheat, maize and wholegrain oat flours for NIF. SIF and NIF were fortified only in minerals (including calcium at 0.324 mg/100g IF) and subjected to a 5-month storage test with temperature variations as described in section *3.1.2*. and memento B. Their stability was compared with that of commercial IF (CIF).

In order to optimize their lipid profiles, omega-3 source flours, namely teff and cowpea flours, were introduced into the composition to formulate an optimized IF (OIF). Different OIF stabilization strategies were tested. Firstly, based on structural parameters (particle size), omitting vitamin and mineral fortification. Thus, three IF were subjected to an 9-month storage test with temperature variations as described in section *3.1.2*:

- OIF_NE: optimized flour non-enriched with flour VMC
- OIF_BRB_NE: optimized flour non-enriched in flour VMC with black rice bran

- OIF/RM_BRB_NE: optimized regrind flour (finer particle size) non-enriched in flour VMC with black rice bran

Secondly, OIF stabilization strategies based on formulation and process parameters (heat treatment and addition of exogenous antioxidant sources) with VMC fortification were tested. The flour VMC corresponds to a mixture of vitamin A (retinyl acetate, 6601.8 µg RA/g), vitamin E (α-tocopheryl acetate, 69.9 mg/g), vitamin C (sodium ascorbate, 385.4 mg/g), vitamin D (cholecalciferol, 88.6 µg/g) and iron (ferrous sulfate dried, 47.7 mg/g). Thus, five flours were subjected to a 6-month storage test:

- OIF: optimized flour enriched with flour VMC
- OIF_HT: optimized flour with heat-treated cowpea flour (to inactivate potential lipoxygenase activity) enriched with flour VMC
- OIF_HT_PUFA: optimized flour with heat-treated cowpea flour enriched with LC-PUFA (including DHA and ARA powder) and flour VMC
- OIF_HT_PUFA_AL: optimized flour with cowpea flour heat-treated with amaranth leaves powder enriched with LC-PUFA and flour VMC
- OIF_HT_PUFA_BRB: flour optimized with cowpea flour heat-treated with black rice bran enriched with LC-PUFA and flour VMC

Table 10. IF composition and levels of fortification in vitamins and minerals

	CIF	SIF	NIF	OIF_NE	OIF_BRB_NE	OIF/RM_BRB_NE	OIF	OIF_HT	OIF_HT_PUFA	OIF_HT_PUFA_AL	OIF_HT_PUFA_BRB
Composition (%w/w)											
Maize flour	-	29.8	20.5	-	-	-	-	-	-	-	-
Soy flour	20.0	15.3	-	24.7	23.9	23.9	24.7	24.7	23.6	22.9	22.9
Groundnut flour	10.0	5.0	-	-	-	-	-	-	-	-	-
Millet flour	60.0	49.9	-	-	-	-	-	-	-	-	-
Rice flour	-	-	21.7	-	-	-	-	-	-	-	-
Wheat flour	-	-	39.6	-	-	-	-	-	-	-	-
Wholegrain oat flour	-	-	21.6	-	-	-	-	-	-	-	-
Cowpea flour	-	-	-	41.5	40.1	40.1	41.5	-	-	-	-
Heat treated cowpea flour	-	-	-	-	-	-	-	41.5	39.6	38.5	38.5
Teff flour	-	-	-	33.7	32.5	32.5	33.7	33.7	32.1	31.2	31.2
DHA powder	-	-	-	-	-	-	-	-	2.9	2.8	2.8
ARA powder	-	-	-	-	-	-	-	-	1.6	1.6	1.6
Amaranth leaves powder	-	-	-	-	-	-	-	-	-	2.9	-
Black rice bran	-	-	-	-	3.0	3.0	-	-	-	-	2.9
Vitamins and minerals fortification (%w/w)											
Flour VMC	-	-	-	-	-	-	0.2	0.2	0.2	0.2	0.2

2.2.2. Model infant flours preparation

IF were obtained by assembling raw materials in the proportions described in **Table 10**. For enriched IF 0.2 g/100 g of VMC was added. IF were mixed using a Turbula T10B powder mixer (Willy A. Bachofen AG, Muttenz, Switzerland), with a three-dimensional movement of shaking, rotating and turning for 5 minutes.

3. Oxidation kinetics

3.1. Storage test conditions

3.1.1. Infant follow-on formulas

To avoid microbial proliferation 0.02% (w/w) of sodium azide was added to all model IFF. Model IFF were aliquoted in quadruplicate into 40 mL hermetically closed brown tubes with headspace of 3.82 mL containing O_2 at atmospheric pressure. Model IFF were stored for 20 days at either 40°C with 110 rpm orbital stirring with a IKA KS 4000 i control incubator (IKA, Staufen, Germany) or at 25°C without stirring. Samplings were made before high pressure homogenization and after 0, 1, 3, 6, 9, 15 and 20 days of storage. Once collected, samples were flushed under nitrogen and stored at -20°C until further analysis.

3.1.2. Infant flours

IF were aliquoted in quadruplicate into polyethylene terephthalate/aluminum/polyethylene (PET/ALU/PE) trilayer Doypack® bag (20 g of IF in each). For the first storage test (*i.e.* with IF representative of commercial products non-fortified in vitamins and minerals), the IF were stored for 5 months. In order to simulate the temperature variations that occur during distribution circuits, the IF were stored for 5 months including 2 weeks at 25°C, then after 1-month storage at 4°C, the samples were stored again for the remainder of the test at 25°C. Samplings occurred at the initial point (M0), after 2, 3 and 5 months of storage (M2, M3, M5).

For the second storage test (*i.e.* with optimized IF non-enriched in vitamins and minerals) the IF were stored for 9 months including 2 weeks at 25°C, then after 1-month storage at 4°C, the samples were stored again for the remainder of the test at 25°C. Samplings occurred at the initial point (M0), after 2, 3, 5 and 9 months of storage (M2, M3, M5, M9).

For the third storage test (*i.e.* with optimized IF enriched in vitamins and minerals), the IF were stored for 6 months at 25 or 40°C. Samples were taken at the initial point (M0), after two weeks of storage (M0.5), or after 1, 2, 3 and 6 months of storage (M1, M2, M3, M6). Once sampled, IF were stored at - 20°C until further analysis.

3.2. Structural characterizations

3.2.1. Particles size distribution

3.2.1.1. Droplets size in emulsions

The particle size distribution of model IFF was determined using a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). Measurements were performed at refractive indices of 1.458 (milk fat; (Bourlieu, Rousseau *et al.* 2012) and 1.33 (water) with agitation of 1500 rpm. About 500 µL of model IFF were diluted in 100 mL of distilled water in a measurement cell to reach an obscuration rate between 5 and 10%. The surface-weighted mean diameter (D[3,2]) and the distribution mode were measured. Data were collected using Mastersizer 3000 software (Malvern Instruments, Worcestershire, UK).

3.2.1.2. Grain size in granular systems

Particle size distribution of IF was measured by laser light diffraction techniques with an LS 13 320 (Beckman Coulter, California, USA) using air as the carrier fluid. Measurements were taken at refractive indices of 1.54 (cellulose) and 1.00 (air). The volume-weighted mean diameter (D[3,2]) and a three-point specification of D10, D50 and D90 which describes the diameter where ten, fifty and ninety percent of the distribution has a smaller particle size, respectively were measured. Data were collected using Adopt software (Beckman Coulter, California, USA).

3.2.2. Confocal laser scanning microscopy

Samples microstructure was observed by CLSM system (Leica SP8, Heidelberg, Germany) using an inverted microscope. Multiple images were obtained using two He-Ne lasers (excitation at 543 and 633 nm), an argon laser (excitation at 488 nm) and a 40× water-immersion or a 10x dry objective for model IFF and IF, respectively. Three fluorescent dyes were used to localize non-polar lipids with Lipidtox® (0.2:100 v/v, λex 488 nm – λem 590 nm, Invitrogen), polar lipids with Rd-DOPE® (1:100 v/v, 16:0 Liss RhodPE 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), λex 543 nm – λem 590 nm, Aventi polar lipids) and proteins with Fast green FCF (6:100 v/v, Sigma-Aldrich). For model IFF, the three dyes were added to 200 µL of the sample at least 10 minutes before observations. For IF, the dyes and 50 µL of ethanol were added in 100 mg of IF at least 24 hours before observation. Samples were then deposited on a glass slide. Leica LAS X software (Wetzlar, Germany) was used for the images collection and analysis.

3.2.3. Differential scanning calorimetry

The crystallization behavior of model IFF oil phases was characterized using a differential scanning calorimeter (DSC Q200, TA Instruments, New Castle, USA). Cooling was carried out with a refrigerated

cooling system (TA Instruments, New Castle, USA) with Nitrogen as purge gas. The TA Instruments Software was used to record and analyze the thermograms. 10 mg of each oil mixture were loaded into aluminum DSC pans with inverted lids (TA Instruments, New Castle, USA) and sealed. An empty, hermetically sealed, aluminum pan was used as reference. The temperature range was adapted to each oil mixture as follows: isothermal for 10 minutes at 60°C, 40 or 30°C for PO, CO and RPO or DF, respectively followed by cooling and isothermal at -40°C for 5 minutes. The scan rate was 5°C.min^{-1}.

3.3. Chemical characterizations

3.3.1. Dry matter and water activity

Dry matter of IF was determined by measuring the difference in mass after drying 1 g of sample at 104°C for 4 hours. Aw was also determined on 1 g of sample at 25°C using a PRE aw-meter (Aqualab, Hopkins, USA).

3.3.2. Lipids extractions

Total lipids were extracted using the Folch method with slight modifications. For model IFF, 200 µL were mixed with 80 µL of HCL 0.1 N and 1.2 mL of Folch mixture (chloroform/methanol 2:1, v/v). Samples were vortexed, then 50 µL of NaCl (0.73%) and 300 µL of Folch mixture were added. After centrifugation for 5 minutes at 1500 rpm (Rotina 380R, Hettich, Westphalie, Germany), the organic phase was collected and hereafter named model IFF Folch extract. For IF, 600 mg were hydrated with 2.4 mL of ultrapure water for 10 minutes. 360 µL HCL 0.1N and 10 mL of Folch mixture were added. Samples were mixed with an IKA T18 ultra turrax (IKA, Staufen, Germany). Three consecutive washes with 240 µL of NaCl 0.73% and 2.4 mL of Folch mixture were performed, with 10-minutes centrifugation at 1500 rpm (Rotina 380R, Hettich, Westphalie, Germany) between each wash. The organic phase was collected and hereafter named IF Folch extract.

3.3.3. Fatty acid profiles

The FA profiles were determined by gas chromatography (GC) following methylation of Folch extracts according to the NF T30-233 standard method with slight modifications. 500 µL sodium methylate solution with phenolphthalein were added to 6 mg of previously evaporated under nitrogen model IFF or IF Folch extracts. After heating at 65°C for 10 minutes, 500 µL hydrochloric methanol were added. The mixture was again heated at 65°C for 10 minutes, then 1 mL of hexane and 1 mL of water were added. The organic phase was collected and analyzed by GC after a centrifugation at 1500 rpm for 5 minutes with a CR412 centrifuge (Jouan Thermo Electron, Waltham, USA). A Focus GC (Thermo Electron Corporation, Massachusetts, USA) was used and equipped with a split injector (ratio of 1/20), a CP-Cil 88 Varian capillary column (50 m×0.25 mm with 0.2 µm film thickness; Chrompack, Mid-

delburg, Netherlands), and helium (1 mL.min^{-1}) was used as carrier gas. FA methyl esters (FAME) were analyzed by a flame ionization detector and ChromCard software data system (version 2005, Thermo FisherScientific, Massachusetts, USA). The column temperature started from 150°C, reached 225°C with a rise of 5°C.min^{-1} and was maintained for 10 minutes. The injector and detector temperatures were 250 and 270°C, respectively. FAME were identified using the retention time of external standards of methyl esters mixture and quantified with an internal standard.

3.3.4. Lipid oxidation compounds

3.3.4.1. Primary oxidation compounds

3.3.4.1.1. Peroxide values

The amounts of primary oxidation compounds monitored by the measurement of the PV, were determined according to Ferreira da Silveira, Laguerre *et al.* (2021). For model IFF, after an extraction on 350 µL of model IFF using 750 µL of isooctane/isopropanol (3:1 v/v), a dilution of the supernatant with methanol/butanol (3:7 v/v) was performed. For IF, 5 mg of previously evaporated under nitrogen IF Folch extracts were diluted with methanol/butanol (3:7 v/v). Samples were then deposited in a microplate UV star 96 well COC F-bottom (Greiner Bio-One, Courtaboeuf, France). 2.5 µL of aqueous ammonium thiocyanate (300 mg/mL) and ferrous solution (0.144 mol/L) were added in each well. After an incubation of the microplate at 25°C for 10 minutes with a stirring of 1,000 rpm in PHMP-4 microplate thermoshaker a (grant instruments ltd Shepreth, Cambridge, England), absorbances were measured at 500 nm using an Infinite M200 microplate reader (Tecan, Gröedig, Austria). Data collection was made using Magellan software (Tecan, Gröedig, Austria). PV were determined using a standard calibration curve of cumene hydroperoxide and were expressed as meqO_2/kg oil.

3.3.4.1.2. Conjugated dienes

The amount of primary oxidation compounds was also monitored by the measurement of the conjugated dienes in IF according to NF T60-223 (AFNOR, 2011) with slight modifications. 3 mg of previously evaporated under nitrogen IF Folch extracts were diluted in isooctane and 200 µL were deposited in a Quartz 96 well microplate (Hellma Analytics, Paris, France). Absorbance was measured at 233 nm with an Infinite M200 microplate reader (Tecan, Gröedig, Austria). Data were collected with Magellan software and results were expressed in mmol/kg oil.

3.3.4.2. Secondary oxidation compounds

3.3.4.2.1. Thiobarbituric acid reactive substances

For model IFF, secondary oxidation compounds were quantified by measuring the TBARS method according to Yi, Ning *et al.* (2019), with slight modifications. 50 µL of model IFF were mixed with 200

µL of the reagent solution (150 mg/mL of trichloroacetic acid, 3.75 mg/mL of thiobarbituric acid and 0.25 mol/L of HCl) and heated at 95°C for 15 minutes. Samples were then cooled in an ice bath for 5 minutes and centrifuged for 10 minutes at 5,000 rpm using a Pico 21 centrifuge (ThermoFisher Scientific, USA). 200 µL were then deposited in a microplate UV star 96 well COC F-bottom (Greiner Bio-One, Courtaboeuf, France). The absorbance was read using a microplate reader (Infinite M200, Tecan, Gröedig, Austria) at 532 nm with the Magellan software. The calibration curves were set with 1,1,3,3-tetramethoxypropane. Results were expressed as mg malondialdehyde (MDA)/kg oil.

3.3.4.2.2. Volatiles compounds

Secondary oxidation products were determined by measuring volatile compounds, *i.e.* hexanal and pentanal. 2 g of IF were placed in a 10 mL vial. Dynamic HeadSpace (DHS) method was used to extract the volatile compounds using a Gerstel autosampler (Gerstel, Mülheim an der Ruhr, Germany). 1 µL of 3-heptanol (0.821 mg/mL) solution was added as an internal standard. The samples were equilibrated for 10 minutes at 35°C. The headspace was swept using a nitrogen flow at 25 ml/min stirred at 500 rpm and the volatile compounds were collected on a Tenax TA trap at 40°C. Residual water was removed with an additional purge flow at 100 ml/min at 50°C during 1 minute. Desorption and analysis were carried out on a gas chromatography coupled to mass spectrometry (GC-MS) using an Agilent 7890B GC (Agilent Technologies, Santa Clara, USA). Splitless desorption of volatile compounds was performed using an automatic thermal desorption unit maintained at 50°C and then reached 310°C for 3 min with a rise of 120°C/min. The desorbed compounds were cryofocused using a CIS4 cooled injection system in which the temperature started from 2°C reached 300°C with a rise of 12°C/sec and was maintained for 3 minutes. A DB-Wax column (60 m x 250 µm x 0.25 µm) was used. The analyzer and source temperatures were 150°C and 230°C, respectively. Data were analysed using MassHunter software version B.08.00. Volatile compounds were identified by using the NIST08 library (Wiley, New Jersey, USA) and their semi-quantification as assessed using the internal standard peak area.

3.3.5. Protein oxidation rate

Oxidation reactions are also known to affect dairy proteins (Hellwig, 2019), therefore the protein-bound carbonyl content was analyzed in model IFF according to the protocol adapted from Sante-Lhoutellier, Aubry *et al.* (2007). 200 µL of model IFF were mixed with 1.8 mL of isopropanol. The samples were then centrifuged at 14,000 g for 5 minutes (Sorval Lynx 4000, Thermo Fisher Scientific, Massachusetts, USA) and the supernatant was discarded. 500 µL of 2,4-dinitrophenylhydrazine solution in 2 N hydrochloric acid were added to each sample. A blank was made by performing a similar procedure, except that the DNPH solution was replaced by 500 µL of 2 N hydrochloric acid. All samples were incubated for 60 minutes at room temperature in the dark and vortexed every 10 minutes. 500 µL

of a 40 wt.% TCA solution were added and the samples were placed on ice for 10 minutes. The samples were centrifuged at 14,000 g for 5 minutes (Sorval Lynx 4000, Thermo Fisher Scientific, Massachusetts, USA) and the supernatant was removed. The pellet was washed twice with 1 mL ethyl acetate/ethanol mixture (1:1, v/v) and once with 1 mL isopropanol. Between each washing step, the samples were centrifuged again in the aforementioned conditions, and the supernatant was removed. The pellets were finally dissolved in 1 mL of 6 M guanidine hydrochloride solution, at 40°C overnight under moderate agitation. After a final centrifugation at 14,000 g for 5 minutes, the spectrum of the supernatant was recorded between 300 and 700 nm using an UV–visible spectrophotometer (DR-3900, HACH Lange, Germany). The determination of the soluble protein concentration in the supernatant was performed with a bicinchoninic acid assay kit. The carbonyl content was calculated according to equation 1, where 22,000 is the molar extinction coefficient of carbonyls for an optical path of 1 cm, and is expressed as µmol carbonyls/g soluble protein.

$$[Carbonyls] = \frac{(Absorbance\ at\ 370\ nm - Absorbance\ at\ 700\ nm)}{22000} \text{ (eq 1)}$$

3.3.6. Vitamins content

3.3.6.1. Vitamin A

The different chemical forms of vitamin A (retinol, retinyl acetate, retinyl palmitate and carotenoids) were quantified by High Performance Liquid Chromatography (HPLC). For model IFF, extraction method was adapted from Montúfar, Laffargue *et al.* (2010). 1 mL of model IFF was mixed with 2 mL of hexane/isopropanol (3:2 v/v) extraction solvent and centrifuged for 5 minutes at 1500 rpm with a Rotina 380R centrifuge (Hettich, Westphalie, Germany). The hexane phase was collected and evaporated under nitrogen. 3.2 mL of acetone were added and samples were cooled for at least 3h at -20°C, leading to TAG crystallisation. Frozen samples were centrifuged at 900 g at -9°C for 20 min (Eppendorf 5810R, Hamburg, Germany). TAG were removed by rapid sampling of the acetone phase and filtration through a 0.2 µm Minisart SRP4 PTFE filter (Sartorius, Germany). For IF, the extraction was adapted from the method described by Moustiés, Bourlieu *et al.* (2019) with slight modifications. 5 g of IF were hydrated with 100 mL ultrapure water for 10 minutes with magnetic stirring at 300 rpm. 10 mL of the suspension were collected and 20 µL of protease were added. Samples were then placed in a water bath at 37°C for 30 minutes with regular shaking (manual turning). 8 mL of an ethanol/hexane mixture (4:3, v/v) were added and mixed with an IKA T18 ultra turrax (IKA, Staufen, Germany) for 30 seconds at 18,000 rpm. After 45 minutes of centrifugation at 4°C at 8,500 g (Rotina 380R, Hettich, Westphalie, Germany), the hexane phase was collected, evaporated under nitrogen and mixed with 1 mL acetone. The extracts were filtered through a 0.2 µm Minisart SRP4 PTFE filter

(Sartorius, Germany). 50 µL of samples were injected into a Thermo Scientific Ultimate 3000 HPLC system (Thermo electron, Massachusetts, USA) using a C30 column (250 × 4.6 mm, 3.0 µm, YMC Europe GMBH, Dinslaken, Germany) and a photodiode array detector (Vanquish PDA, Thermo Scientific). The column temperature was maintained at 30 °C and elution was performed at a flow rate of 1 mL.min^{-1} using the following method, adapted from Courraud, Berger *et al.* (2013): A = methanol/water (60:40 v/v), B = methanol/methyl tert-butyl ether/water (28.5:67.5:4 v/v/v), (i) 55% A and 45% B for 1 min, then (ii) a linear gradient from 55% A and 45% B to 10% A and 90% B for 2 min, followed by (iii) a linear gradient to 100% B for 5 min, (iv) a step with 100% B for 2 min, (v) a linear gradient to 100% C for 5 min, then (vi) a 8-min rinse step using 100% ethyl acetate, (vii) a 3-min return to initial conditions, followed by (viii) a re-equilibration step for 4 min. The detection wavelength was set at 325 nm. Calibration curves, spectra and retention times of analytical standards were used to identify and quantify the various chemical forms of vitamin A.

3.3.6.2. Vitamin E

Four distinct tocopherols (α, β, γ and δ) and γ-tocotrienol were quantified according to the ISO-FDIS 9936 standard. 4 mg of previously evaporated under nitrogen model IFF or IF Folch extracts were diluted in hexane. For IF, samples were filtered through a 0.45 µm Minisart RC filter. HPLC analysis was performed with an Ultimate 3000 (Thermo electron, Massachusetts, USA) equipped with a silica column (250 mm × 4.6 mm, 5 µm, Delaware USA) and a fluorescence detector FL 3000 (Thermo electron, Massachusetts, USA). The mobile phase consisted of hexane/dioxane (97:3 v/v) in isocratic conditions with a flow rate was 1.3 mL.min^{-1}. The column temperature was maintained at 25°C. Fluorescence detection was set at 290 and 330 nm for excitation and emission, respectively. The injection volume was 100 µL and the calibration curves were realized with standard solutions of each tocopherols isomers.

3.3.6.3. Vitamin C

Vitamin C content was quantified by ascorbic acid determination with an Ultra Performance Liquid Chromatography (UPLC) system using trichloroacetic acid (TCA) in the presence of TCEP as a reducing agent according to method ISO 20635:2018. 2 g of samples were mixed with 16 mL of TCEP 25% and 8 mL of TCA 15%. The samples were homogenized for 30 seconds using a T18 ultra turrax (IKA, Staufen, Germany) then 10 mL of ultra-pure water was added. After centrifugation for 10 minutes at 10,000 g at 20°C (Rotina 380R, Hettich, Westphalie, Germany), 2 mL of supernatant was collected and mixed with 2 mL of sodium acetate solution (0.5 mol/L, pH 5.4) and 6 mL of mobile phase adjusted to pH 5.4 (sodium acetate solution (50 mmol/L, pH 5.4), decylamine (1.6 g/L), acetonitrile (1%), TCEP (40 mg/L)). The samples and mobile phase were filtered through 0.2 µm RC. UPLC analysis was performed with an

Acquity system (Waters, Massachusetts, USA) equipped with a BEH C18 column (2.1 mm X 50 mm, 1.7 µm, Waters, Massachusetts, USA) and a UV-VIS detector. The injection volume was 5 µL. Elution was carried out under isocratic conditions at a flow rate of 0.35 mL/min. Column and injector temperatures were maintained at 25°C at 10°C, respectively. Detection was set at 265 nm. Ascorbic acid was identified and quantified using an analytical standard calibration curve.

3.3.7. Free phenolic compounds

The free phenolic contents of IF were determined by HPLC. 100 mg of IF were incubated with 2 mL of extraction solvent consisting of ethanol/eau (60:40 v/v) and 40 µL of an internal standard (TMCA, 0.42 mmol/L) for 1 hour at 60°C with constant magnetic stirring of 800 rpm. The samples were then centrifuged for 10 minutes at 10,000 rpm (5425 R G, Eppendorf, Montesson, France) and filtered through 0.2 µm Minisart SRP4 PTFE filter (Sartorius, Germany). Samples were injected in HPLC using an Alltima C18 column (250 × 4.6 mm; Alltech associates, Inc., Deerfeld, Illinois) and a photodiode array detector Waters 996 (Millipore Co., Milford, Massachusetts). Detection was performed by UV absorbance at 280 nm. A gradient elution was used using acetonitrile and sodium acetate buffer (0.05 M, pH 4.0) at 1 $mL.min^{-1}$ at 35°C, in linear gradients from: 15/85 to 35/65 in 30 minutes, 35/65 to 60/40 in 0.5 minutes, 60/40 to15/85 in 4.5 minutes, and finally maintained at 15/85 for 5 minutes. External (Gallic acid) and internal standards, spectra and retention times were used to identify and estimate the total free phenolic content.

3.3.8. Lipoxygenase activity

LOX activity was determined using a linoleic acid solution containing Tween 20 and adjusted pH of 9.0 with a NaOH solution as substrate. 100 mg of IF were incubated with 2 mL of phosphate buffer (66.7 mM, pH 5.5) for 3 hours at 4°C with magnetic stirring. The samples were then centrifuged for 15 minutes at 5,000 g (Rotina 380R, Hettich, Westphalie, Germany) and filtered through 7-9 µm fast ashless wattman paper. The samples were diluted several times with the phosphate buffer to determine enzymatic activity at different concentrations. 8 to 15 µL of samples were placed in a Quartz 96 well microplate (Hellma Analytics, Paris, France). 34 µL of linoleic acid solution and 249.7 µL of phosphate buffer were added. Absorbance was read at 234 nm every 30 seconds for 10 minutes using an Infinite M200 microplate reader (Tecan, Gröedig, Austria).

4. Static *in vitro* digestion

The third part of the thesis (described in Chapter 5) was devoted to studying the bioaccessibility of lipid compounds (PUFA, vitamins A and E) and proteins in nutritionally optimized model IFF and IF that have demonstrated good overall oxidative stability.

4.1. Sample preparation

Infant formulas

Among the model IFF, DF_DPL_L and PO_RA_L were subjected to the static *in vitro* digestion procedure. Model IFF were formulated and prepared as described in sections *2.1.1* and *2.1.2*. A commercial liquid IFF (LIFF_L) was also selected as reference. To prevent microbial development, 0.02% sodium azide was added to each IFF.

Infant flours

For the IF, OIF_HT_PUFA_BRB (sections *2.2.1* and *2.2.2*) and a commercial infant flour reference (CIF) were selected to undergo static *in vitro* digestion. The flours were rehydrated to 9% using ultra-pure water. The flour suspensions were then boiled for 5 minutes to obtain infant gruels. To avoid microbial growth, 0.02% sodium azide was added to each gruel.

4.2. Initial characterizations

4.2.1. Droplets size in emulsions

The droplet size distribution of model IFF was measured by laser light diffraction technique as described in section *3.2.1.1*.

4.2.2. Texture (flow rate)

The infant gruels texture was measured after cooking using a Bostwick consistometer (Polyvisc FV, PCE instruments, Soultz-sous-Forêts, France). The gruel was cooled to 50°C at room temperature and then 50 mL were placed in the filling compartment of the Bostwick consistometer. The gruel was gently stirred until it reached the measuring temperature of 45.0 ± 1.0°C. The gate keeper was removed, freeing the gruel. The gravity flow distance of the gruel from the first compartment to the second was measured after 30 seconds.

4.4.3. Initial fatty acid profile

The initial FA profiles of model IFF and IF were determined by GC after a methylation as described in section *3.3.3*.

4.4.4. Initial fat-soluble vitamin contents

The initial contents of vitamins A and E were determined by HPLC as described in section *3.3.6.1.* and *3.3.6.2.*, respectively.

4.4.5. Trypsin inhibitory activity

Trypsin inhibitory activity was quantified in IF. 50 mL of 10 mM NaOH were added to 1 g of IF and pH was adjusted to 9.5 using an HCl solution. Sample suspensions were stored at 4°C overnight. Samples were then diluted with water to 100 mL and kept at 4°C for 15 minutes to sediment. Dilution using cold water was performed to obtain a trypsin inhibition rate between 40 to 60%. 0.5 mL of dilute samples, 2 mL water, and 5 mL of Nα-Benzoyl-L-arginine hydrochloride 4-nitroanilide solution (0.6 mg/mL) were mixed and placed at 37°C for 10 minutes. Then, 0.5 mL of trypsin solution (absorbance of 0.38 +/- 0.05) were added. After 10 minutes at 37°C, 0.5 mL of 30% acetic acid solution was added to stop the reaction and samples were centrifuged for 5 minutes at 5,000 rpm (Heraeus, Megafuge 16R, Thermo, Massachusetts, USA). Absorbance of supernatant was measured at 410 nm (Ultrospec 2000, Pharmacia Biotech, Amersham, United Kingdom).

4.3. Static in vitro digestion procedure

The *in vitro* digestion conditions used were set up to simulate digestion of full-term infants aged 6 months onward (**Figure 17**). As the residence time of infant foods in the mouth is short, the oral phase was omitted. The *in vitro* static digestion process used therefore included two stages, a gastric phase lasting 60 minutes and an intestinal phase lasting 120 minutes. Gastric digesta were sampled after 10 and 60 minutes of digestion, namely G10 and G60, respectively. Intestinal digesta were sampled after 10, 60 and 120 minutes of digestion, namely I10, I60 and I120, respectively. Samples were either used directly for evaluation of proteolysis rate as described in *4.4.3.* section or frozen at -20°C after addition of enzyme inhibitors, *i.e.* 50 µL of 4-bromophenylboronic acid (1 M) per mL of gastric digesta or 50 µL of Pefabloc (0.1 M) per mL of intestinal digesta.

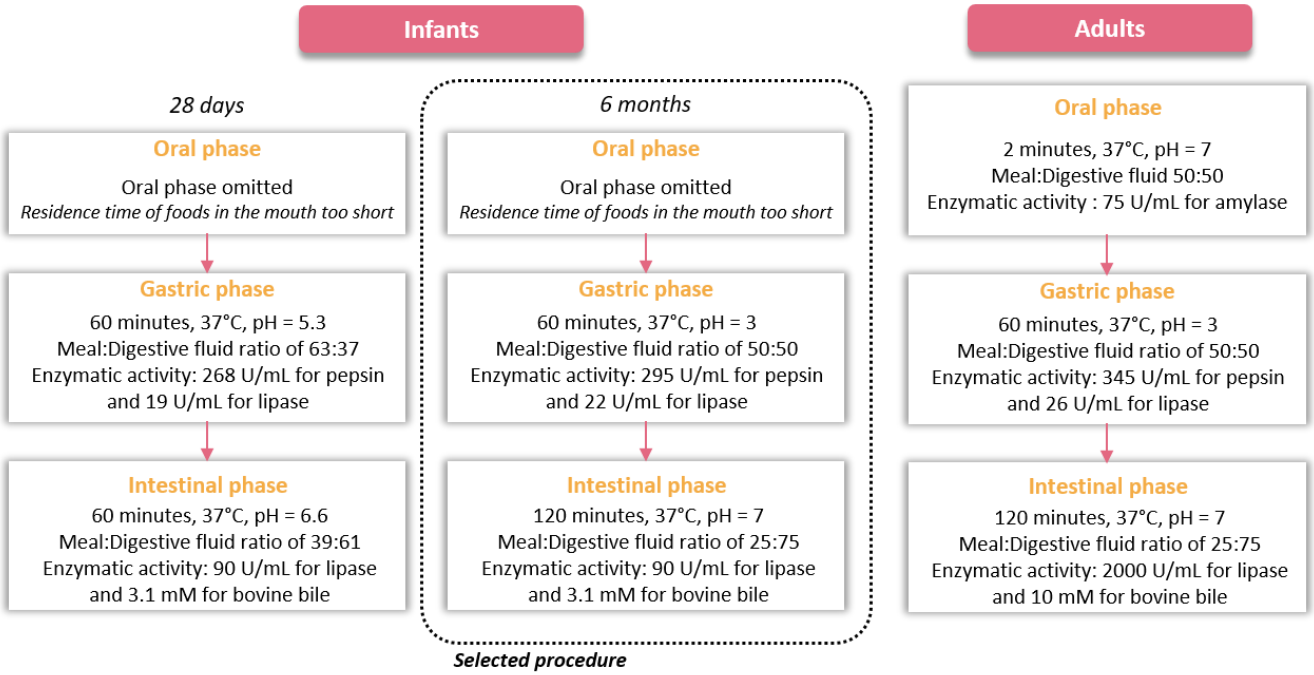


Figure 17. Static *in vitro* digestion model conditions in infants and adults (adapted from Bourlieu, Ménard *et al.* 2014, Minekus, Alminger *et al.* 2014, Ménard, Bourlieu *et al.* 2018)

Gastric phase

Simulation of the gastric digestion was performed at 37°C with rotary stirring for 60 minutes. The meal:gastric secretion ratio was set at 50:50 with an initial pH of 3 adjusted using a 1M HCl solution. The simulated gastric fluid was composed of 6.9 mM potassium chloride, 0.9 mM monobasic potassium phosphate, 25 mM sodium bicarbonate, 47.2 mM sodium chloride, 120 µM magnesium chloride hexahydrate and 500 µM ammonium carbonate. Enzymes activities were set up at 22 and 295 U/mL of total gastric content for lipase and pepsin, respectively. Both enzymes were added using a rabbit gastric extract which covers 100% of enzymatic activity. After 60 minutes, the gastric phase was stopped by raising the pH to 7 using a 1M NaOH solution.

Intestinal phase

Simulation of the intestinal digestion was performed at 37°C with rotary agitation for 120 minutes. The meal:total secretions (including gastric and intestinal fluids) ratio was set at 20:80 with a previously adjusted pH to 7 using a 1M NaOH solution. The simulated intestinal fluid was composed of 6.8 mM potassium chloride, 0.8 mM monobasic potassium phosphate, 42.5 mM sodium bicarbonate, 9.6 mM sodium chloride and 1.1 mM magnesium chloride hexahydrate. Bovine bile salts were added at 3.1 mM and pancreatin from porcine pancreas was added to rise a lipase activity of 90 U/mL of intestinal content.

4.4. Digesta characterization

4.4.1. Free fatty acid profile

FFA profiles were assessed at the end points of the gastric (G60) and intestinal (I120) phases by GC following derivatization by silylation. Firstly, FFA were extracted after an acidification by addition of 75 µL of methanol/sulphuric acid (14:1, v/v) containing 0.7 mg/mL BHT to a volume of digesta equivalent to 3 mg of fat. 500 µL of hexane and glass beads were added and after an incubation at 40°C under stirring at 400 rpm overnight the samples were centrifuged at 16,000 g for 10 minutes at 4°C (Rotina 380R, Hettich, Westphalie, Germany). The hexane phase was collected and evaporated under nitrogen. A second extraction using 500 µL of hexane with stirring at 400 rpm for 2 hours at 40°C was performed on the remaining lower phase. After centrifugation at 16,000 g for 10 minutes at 4°C (Rotina 380R, Hettich, Westphalie, Germany), the hexanic phase was added to the previously collected and evaporated under nitrogen upper phase. 200 µL of extract were then derivatized by silylation by adding 25 µL of (trimethylsilyl)trifluoroacetamide and 25 µL of pyridine. The extracts were incubated for 20 minutes at 50°C and then 0.5 µL were injected into a gas chromatography coupled with a flame ionization detector (GC-FID). A Shimadzu GC2010 Plus (Shimadzu, Noisiel, France) was used and equipped with an automatic sampling system (AOC 20i), a temperature programmable injector with flow splitting (ratio of 5) and a ZebronTM ZB-5HT polyimide-coated fused silica phase capillary column (15 m x 0.25 mm x 0.1 µm, Phenomenex, Le Pecq, France). Helium was used as carrier gas with a flow rate of 1 mL·min^{-1}. The injector temperature started from 290°C, reached 400°C with a rise of 200°C.min^{-1} and was maintained for 36.45 minutes. The oven temperature gradient started from 150 to 230°C with a rise of 5°C·min^{-1}, then from 230 to 250°C (2°C·min^{-1}), and reached 390°C (35°C·min^{-1}). The oven temperature was maintained at 390°C for 5 minutes. The detector temperature was 400°C. FFA were identified and quantified using a calibration curve of analytical standards and internal standards (including C13:0 and C17:0). Signals were collected and analyzed using LabSolutions software (Shimadzu, Noisiel, France).

4.4.2. Lipolysis rate

The lipolysis rate was determined by measuring lipid classes by thin-layer chromatography (TLC). After lipid extraction of the digesta using the Folch method described above (section *3.3.2*), the Folch extracts were spotted on a silica gel HTPLC plate using an ATS4 (Camag, Muttenz, Switzerland). Lipid standards namely TAG, 1,2- and 1,3-DAG, MAG, and FFA were also spotted on each plate. Elution was carried out using an ADC2 automatic developing chamber (Camag, Muttenz, Switzerland) with a solvent consisting of a mixture of hexane/ether/formic acid (70:30:1 v/v/v) up to 80 mm. The plates were then dried at room temperature and immersed in the developer solution (copper/phosphoric acid/ethanol/water 50:40:25:390, v/v/v/v) with a chromatogram immersion device 3 (Camag,

Muttenz, Switzerland). After drying at room temperature, the plates were placed at 180°C for 10 minutes. Spots were read at 500 nm using a TLC Scanner 3 (Camag, Muttenz, Switzerland) and collected using Visioncat software (Camag, Muttenz, Switzerland). The spots were identified according to the migration distance of the analytical standards.

4.4.3. Proteolysis rate

The proteolysis rate was determined by assaying free amino groups using the ninhydrin method. The digesta samples were centrifuged at 4°C for 15 minutes at 10,000 rpm (Avanti JXN-26, Rotor JA-20.1, Beckman Coulter, California, USA). 50 µL of supernatants were collected and diluted with ultra-pure water. 200 µL of samples were mixed with 200 µL of ninhydrin 2%. After 30 minutes at 100°C, the samples were cooled in an ice bath for 5 minutes. 50 µL of 50% ethanol were added and 270 µL were placed in a UV star 96 well COC F-bottom (Greiner Bio-One, Courtaboeuf, France) to read the absorbance at 570 nm using a Spark microplate reader (Tecan, Männedorf, Switzerland). Free amino groups were quantified using a leucine calibration curve.

4.4.4. Vitamin E bioaccessibility

After two successive centrifugations of the digesta from 10 to 10,000 rpm and 30 min at 18,7000 rpm (Avanti JXN-26, Rotor JA-20.1, Beckman Coulter, California, USA), the micellar phase was collected and extracted by Folch method (section *3.3.2.*). The vitamin E content was determined by HPLC as described in section *3.3.6.2*.

5. Statistical analysis

All analyses were performed in quadruplicates with possibly two repetitions of measurement except for the CLSM, which was carried out in duplicate. The results are presented as mean ± SD. Statistical significance was determined by one-way ANOVA using R software (R.2.13.0, http://cran.r-project.org). Differences were considered statistically significant at $P < 0.05$.



CHAPTER 3

Oxidative stability of follow-on infant formulas

This chapter is dedicated to presenting the results related to the study of oxidative stability of IFF and the optimization of their lipid profile for infant nutrition (**Figure 18**). In the first part, the composition and the nutritional values of nearly a hundred IFF available in the global market have been collected. Following a comprehensive statistical analysis of this overview, eighteen model IFF were formulated. These model IFF were studied in three distinct sections. The first section was devoted to identifying key lipid formulation parameters that could enhance both the nutritional profile of IFF and their oxidative stability. The second section focused on identifying lipid structural parameters leading to improved oxidative stability. Lastly, the third section aimed to evaluate the impact of vitamin A chemical forms and the homogenization process on its stability.

In order to complement and validate the results for extrapolation to real systems, the final part of this chapter is dedicated to comparing the oxidative stability of a limited number of IFF models with commercially available follow-up infant formulas having similar chemical compositions. This endeavor offers valuable insights into the practical applicability of the established results.

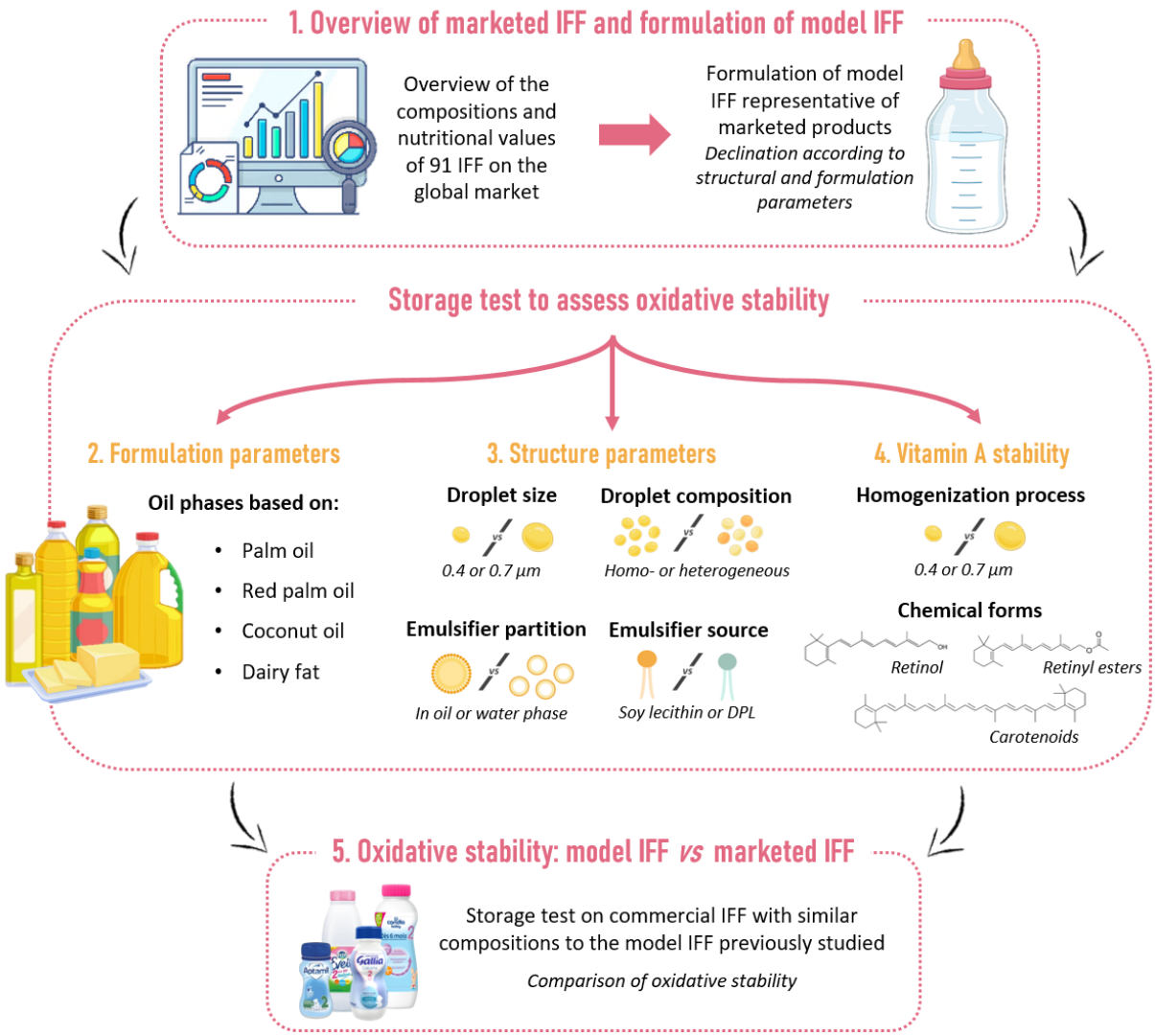


Figure 18. Strategy of chapter 3

1. Panorama of IFF composition and nutritional values

Despite the recommendations regarding breastfeeding, only 48% of infants aged under 6 months are exclusively breastfed, and 33% are still breastfed up to the age of 12 months (WHO 2022). IFF are therefore widely consumed throughout the world. In order to identify a typical IFF composition a market analysis was carried out.

This section is partly based on a dataset entitled: "Panorama of the nutritional composition of IFF commercialized worldwide in 2021" published in the repository Dataverse INRAE (Cancalon, Hemery et al. 2023) and a data paper entitled: "Dataset of the nutritional composition of follow-on infant formulas commercialized worldwide in 2021" published in Data in Brief journal (Cancalon, Hemery et al. 2023).



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Panorama of the nutritional composition of follow-on infant formula commercialized worlwide in 2021

Version 2.0

Cancalon, Mathilde; Hemery, Youna Morgane; Barouh, Nathalie; Thomopoulos, Rallou; Durand, Erwann; Villeneuve, Pierre; Bourlieu-Lacanal, Claire, 2023, "Panorama of the nutritional composition of follow-on infant formula commercialized worlwide in 2021", https://doi.org/10.57745/CLER60, Recherche Data Gouv, V2, UNF:6:+hluX/UnRsD6C9z1D+2ByQ== [fileUNF]

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Data Article

Dataset of the nutritional composition of follow-on infant formulas commercialized worldwide in 2021

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The ingredients list and nutritional values of marketed IFF were collected from December 2020 to April 2021, directly on the product packaging or on the websites of the different brands (**Figure 19**). The dataset exclusively includes only "classic" IFF (no anti-reflux or hypoallergenic formulas...) that are widely consumed and were listed in order to covering more than 60% of the market share by volume. In total, data collection includes 91 IFF. After data collection, each IFF was anonymized by assigning a unique product code. The data were then structured to form the dataset, with standardized lists of ingredients to maintain anonymization and present the information consistently in the same format. The sources of fats, emulsifiers and vitamins A and E were also extracted from the ingredient lists. It is worth noting that data included in the dataset are subject to change, particularly in case of regulatory changes, and therefore needs to be regularly updated.

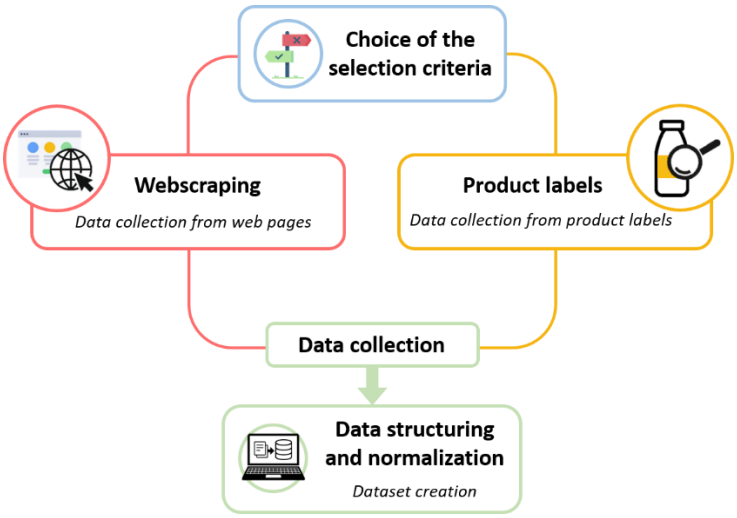


Figure 19. Data collection methodology

Value of the data

- These data are useful to companies or researchers in the field of infant nutrition.
- The dataset is a tool to help in the formulation of infant formulas for companies or representative model systems for laboratory research.
- Companies or intervention programs aimed at improving infant formulas composition can use our data as a benchmark.
- Our data are useful to companies to position their product in relation to the market and regulations.
- The dataset can be used to identify the most used raw materials with a focus on the lipid fraction (oils mixtures, sources of vitamins A and E fortification, type of emulsifiers).
- These data constitute a support for the estimation of the nutritional intake and the contribution of each ingredient. These data are complementary to the CIQUAL, USDA or FCEN databases.

The dataset is provided as a text file (.txt) and three Excel Spreadsheet (.xlsx) files, each including one sheet:

The ***Summary.txt*** file provides information about the aim and the method used to generate this dataset in three points. The "aim of the dataset" section describes the objective, the "Selection criteria " section lists the criteria for data inclusion and the "Data collection method" section defines the data collection method used. This file also gives additional information such as the abbreviations list and the specificities on the list of ingredients (decreasing order of weight of ingredients not necessarily respected) and the sources of vitamins A and E (addition of an asterisk when the source is not specified).

The ***Nutritional values.xlsx*** file provides information about the age group in which the product is consumed, the list of ingredients, the composition of the oil mixture, the source of emulsifier and vitamin A and E fortification, the preparation conditions per 100 mL and the nutritional values per 100 g of powder, 100 mL of preparation or 100 kcal. Thus energy, fats content with details on the content of SFA, MUFA and PUFA and specific FA (LA, ALA, DHA and ARA) are listed. The carbohydrates and proteins contents (with casein and serum protein contents) are also provided. Finally, the contents of vitamins A, E, C, D and iron and copper are presented. When the values were not available the boxes are left empty. This file is organized as described in **Figure 20**.

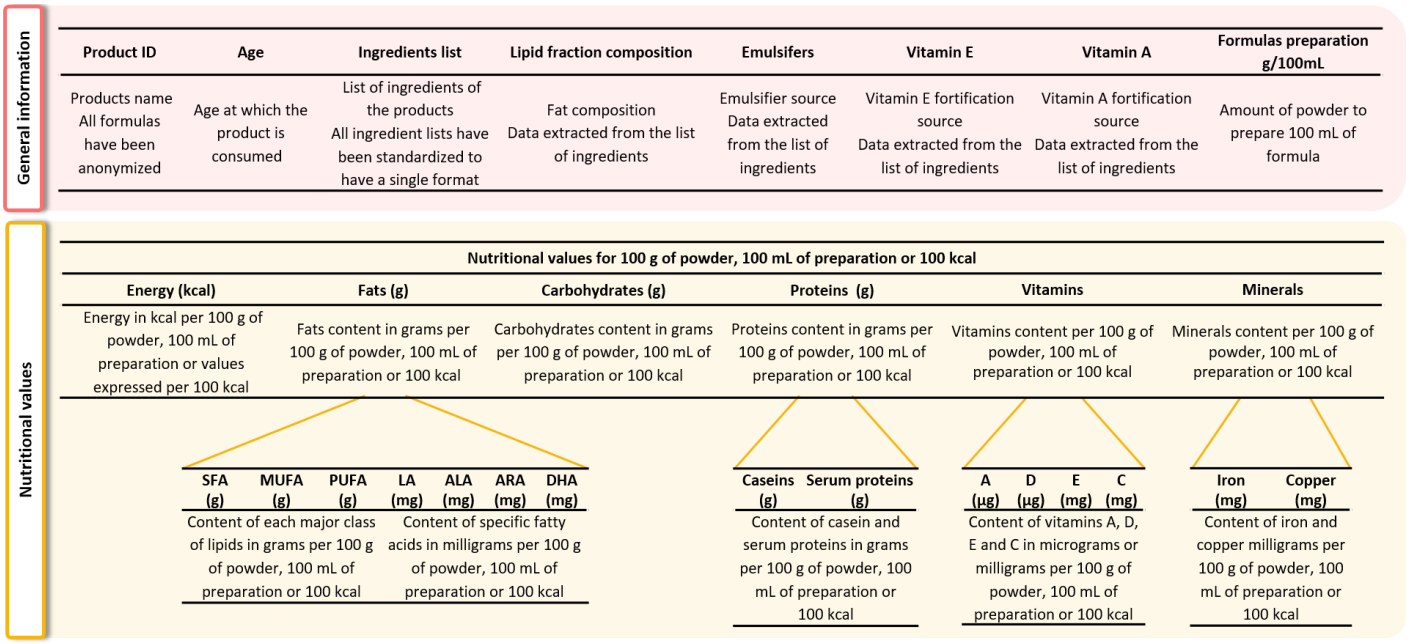


Figure 20. Organization and information provided in the “Nutritional values.xlsx” file

The ***Overview.xlsx*** file provides information about the number of values available on the product labels or on the website, the average (with standard deviations), maximum and minimum nutritional values for 100 g of powder, 100 ml of preparation or 100 kcal.

The ***Examples of regulation.xlsx*** *file* provides information concerning the range of contents in certain macro and micronutrients governed by the international regulation (*Codex Alimentarius* STAN 156/1987) and the European regulation (UE regulation 2016/127).

Analysis of the dataset showed a high degree of homogeneity in the nutritional values of commercial IFF, and good compliance with the threshold values imposed by *CODEX Alimentarius* CX156/1987 at global level and Decree CE2016/127 at European level (**Table 11**). The average DHA content of IFF from the dataset is 22.0 ± 0.3 mg/100 Kcal, which is in line with, but close to, the regulatory minimum limit. The dataset also highlighted that the majority of lipid fractions composed IFF are based exclusively on mixtures of vegetable oils, mainly palm, coconut, rapeseed and sunflower oils. Only 17.6% of IFF whose fat sources were mentioned include lipids of animal origin (mainly bovine sources). 78% of IFF include PUFA-rich oils from marine, algal and fungal sources. Concerning surfactants, soy lecithin is present in 72.5% of the IFF in the dataset. Comparison with the average nutritional values of breast milk shows a similar overall lipid content, but differences are observed between lipid classes for example IFF contain less SFA than breast milk. Regarding PUFA profile, significant differences were observed between IFF and breast milk. The overall PUFA content is higher in IFF, moreover, breast milk contains on average 1.5 times less LA and 3.8 times more ARA than IFF. However, ALA and DHA levels are relatively similar.

Table 11. Overview of the nutritional values of IFF available on the global market for 100 mL and comparison with European regulation effective since February 2020 (EU regulation CE 2016/127) and the typical composition of mature breast milk (Guo 2021, Cancalon, Hemery *et al.* 2023)

	Infant follow-on formulas Mean ± SD	**EU regulation CE 2016/127**		***CODEX Alimentarius* CX 156/1987**		**Breast milk**
		Min	Max	Min	Max	
Energy (kcal)	67.2 ± 1.3	60	70	60	85	67
Lipids (g)	3.3 ± 0.2	2.64	4.2	1.8	5.1	3.8
SFA (g)	1.1 ± 0.4	-	-	-	-	1.8
MUFA (g)	1.5 ± 0.4	-	-	-	-	1.5
PUFA (g)	0.6 ± 0.1	-	-	-	-	0.4
LA (mg)	502.2 ± 0.1	300	840	180	-	338.2
ALA (mg)	53.7 ± 12.6	30	70	-	-	45.6
ARA (mg)	9.4 ± 5.6	-	42	-	-	27.4
DHA (mg)	14.8 ± 4.0	12	35	-	-	13.8
Carbohydrates (g)	7.8 ± 0.4	5.4	9.8	-	-	7
Proteins (g)	1.4 ± 0.2	1.08	1.75	1.8	4.7	1
Caseins (g)	0.8 ± 0.3	-	-	-	-	0.3
Vitamin A (µg)	61.5 ± 6.4	42	79.8	45	191.3	53
Vitamin D (µg)	1.4 ± 0.3	1.2	2.1	0.6	2.55	0.1
Vitamin E (mg)	1.2 ± 0.4	0.36	3.5	0.28	-	0.5
Vitamin C (mg)	9.8 ± 2.3	2.4	21	4.8	-	4.7
Iron (mg)	0.976 ± 0.155	0.36	1.4	0.6	1.7	0.04 - 0.08
Copper (mg)	0.052 ± 0.006	0.036	0.070	-	-	0.02 - 0.04

2. Impact of formulation parameters on oxidative stability and nutritional profile

Firstly, formulation parameters were studied by assessing the impact of different sources of saturated FA on the oxidative stability and nutritional profile of IFF.

This part is based on the research article entitled: "Comparison of the effect of various sources of saturated FA on IFF oxidative stability and nutritional profile" published in the Food Chemistry journal in 2023. To ensure the uniformity of the present manuscript, some parts have been slightly modified.

Food Chemistry 429 (2023) 136854

Contents lists available at ScienceDirect

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Comparison of the effect of various sources of saturated fatty acids on infant follow-on formulas oxidative stability and nutritional profile

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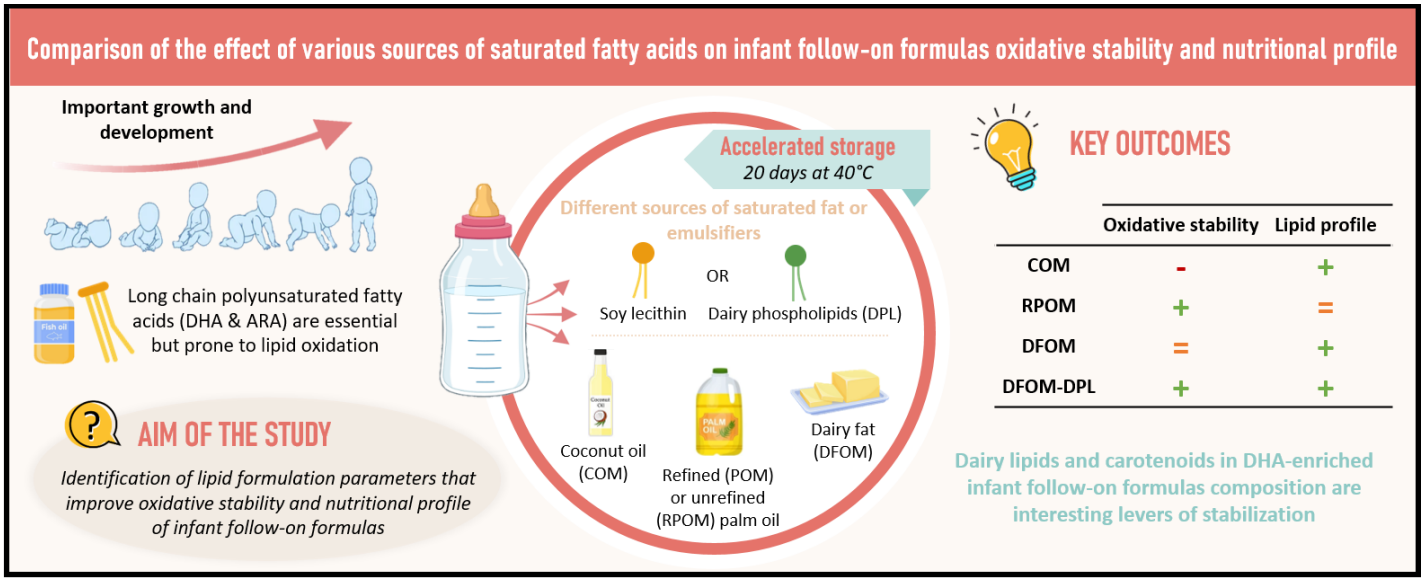
Abstract

Fortification of IFF with DHA, which is prone to lipid oxidation, is required by European regulation. This study aimed to identify lipid formulation parameters that improve the nutritional profile and oxidative stability of IFF. Model IFF were formulated using different lipid and emulsifier sources, including refined (PO) or unrefined red palm oil (RPO), coconut oil (CO), dairy fat (DF), soy lecithin, and DPL. After an accelerated storage, RPO_L and DF_DPL_L had improved oxidative stability compared to other model IFF. Specifically, they had a PV twice lower than PO_L and 20% less loss of tocopherols for DF_DPL_L. This higher stability was mainly explained by the presence of compounds such as carotenoids in RPO_L and sphingomyelin in DFO_DPL_L very likely acting synergistically with tocopherols. Incorporation of dairy lipids and carotenoids into DHA-enriched IFF compositions seems promising to enhance their stability and nutritional quality.

Keywords

Infant formula, nutritional needs, fortification, palm oil, lipid oxidation, docosahexaenoic acid (DHA)

Graphical abstract





2.1. Introduction

The infancy period and especially the first two years of life, which are included in the 1,000-days concept, is a crucial period of human development (Martorell 2017). At this stage of life, nutrition is essential to support the very rapid growth and development and the infant diet is highly involved in the programming effects (Koletzko, Brands *et al.* 2017). Among the nutrients, lipids are the dominant energy provider during infancy, with a supply of 45 to 55% of the calories *via* their β-oxidation. They are also structural membrane components and precursors of several cell mediators involved in the cerebral, visual, intestinal and immune development of infants (Delplanque, Gibson *et al.* 2015).

To determine the optimal lipid supply for neonatal nutrition, breast milk is often considered as the "gold stallion". Although its composition is affected by many factors (one of the most influential being the mother's diet), it remains the most appropriate source of nutrients for infant's needs (Jensen, 1999). In breast milk, lipids are found as the very specific form of MFG, *i.e.* a trilayered membrane of polar lipids and proteins that stabilizes a core of complex TAG. These latter contain a significant amount of SFA, but also essential FA – LA (C18:2 ω6) and ALA (C18:3 ω3) - precursors of LC-PUFA such as ARA (C20:5 ω6) and DHA (C22:6 ω3). When breastfeeding is impossible or fails for various reasons, infant formulas can be administrated and are developed specifically to meet the needs of infants at a given age. During the second stage of development, which extends from 6 to 12 months, the infant's diet is mainly composed of a dairy fraction *via* breast milk and/or IFF. The IFF composition is governed by strict regulations and evolves to be in line with the recommendations, especially concerning the content of LC-PUFA. Since 2020, the addition of DHA to IFF has been mandatory in European countries (EU regulation CE 2016/127). The addition of ARA is not required yet, but it is strongly recommended by the scientific community (Tounian, Bellaïche *et al.* 2021). It is therefore usually added in the composition of IFF.

Because of their multiple unsaturated double bonds, LC-PUFA are very prone to lipid oxidation and this degradation leads to the appearance of a multitude of neo-formed products, some of which are detrimental to human health (Schaich 2020). Because of this susceptibility to oxidation, a higher inclusion level of LC-PUFA in IFF is challenging. Oxidation also affects antioxidants such as fat-soluble vitamins (vitamins A and E) that are first degraded. This oxidation can be limited by several strategies of which the most common is the addition of antioxidants (Cancalon, Barouh *et al.* 2022). Selecting the protein sources is also possible to limit oxidation using, for example, among whey proteins lactoferrin which is able to chelate metal ions and thus to block their pro-oxidant effect (Satué-Gracia, Frankel *et al.* 2000, Elias, Kellerby *et al.* 2008). Recent regulatory evolutions have reinforced the need to identify efficient stabilization levers, including those related to the formulation and particularly to lipid formulation.

In commercial IFF, the oil phase is a mixture of oils mainly of vegetable origin in which palm oil is the most frequent source of SFA. Moreover, IFF are generally enriched in vitamins (especially vitamins A and E) and minerals (Delplanque, Du *et al.* 2018). Depending on the source of oil used, the FA profile varies greatly, especially in terms of the amount of short (C4-C8) and medium (C10-C12) chains. Numerous studies have shown that these FA play an important role during infancy period, particularly because they are more easily lipolyzed and metabolized by the portal vein pathway than long-chain FA (Yuan, Wang *et al.* 2022). In this regard, dairy fats, whose most common commercial source worldwide is bovine source, are the most biomimetic of breast-milk lipids but they need to be supplemented in PUFA for their use in IFF. TAG fraction found in dairy fat can be also supplemented by dairy polar lipids, a concentrated source of SM and LC-PUFA glycerophospholipids that have beneficial nutritional effects and may improve the IFF stability (Bourlieu-Lacanal, Cheillan *et al.* 2018).

The impact of several parameters (addition of antioxidants or proteins sources) on the resulting oxidative stability of IFF is largely documented. However, to the best of our knowledge, the comparison of various saturated fats sources on IFF oxidative stability and nutritional profile has never been undertaken. The presence and type of SFA lowers the melting point of oil mixtures possibly leading to partial crystallization, which in an emulsified system leads to the formation of a physical barrier that could then limit oxidation phenomena.

The present study aims at comparing different lipid sources of SFA in model IFF representative of marketed products (palm, red palm and coconut oils and dairy fat). In all these sources the SFA content account for more than 45% of their FA profiles (**Table 12**). The set hypothesis was therefore that variation in these lipid sources would modulate their oxidative stability.

Model IFF were thus formulated with equivalent chemical composition and structure, *i.e.* with a normalization of vitamins E and A, SFA, MUFA, PUFA, DHA and ARA contents and similar droplet size.

Table 12. Fatty acid profile of oils used for model IFF formulation

	Palm oil	Red palm oil	Coconut oil	Dairy fat olein	Rapeseed oil	Sunflower oil	High oleic sunflower	ARA rich oil	DHA rich oil
SFA	47.2 ± 0.3	44.5 ± 0.1	92.5 ± 0.0	53.5 ± 0.5	6.7 ± 0.4	11.2 ± 0.0	8.6 ± 0.1	21.6 ± 1.1	27.1 ± 0.1
C12:0	0.1 ± 0.0	-	47.1 ± 0.0	3.5 ± 0.1	-	-	-	-	3.7 ± 0.0
C16:0	41.0 ± 0.3	36.9 ± 0.2	9.3 ± 0.1	25.3 ± 0.1	4.1 ± 0.0	6.0 ± 0.0	3.7 ± 0.0	9.6 ± 0.1	11.0 ± 0.1
MUFA	42.9 ± 0.3	46.7 ± 0.2	6.3 ± 0.0	42.6 ± 0.2	65.5 ± 0.3	32.9 ± 0.0	80.5 ± 0.1	18.3 ± 0.1	23.6 ± 0.2
PUFA	9.9 ± 0.1	8.8 ± 0.1	1.1 ± 0.0	3.9 ± 0.7	27.8 ± 0.6	55.9 ± 0.1	10.9 ± 0.1	59.6 ± 0.1	49.2 ± 0.3
C18:2 ω6	9.9 ± 0.1	8.4 ± 0.1	1.1 ± 0.0	2.9 ± 0.7	18.8 ± 0.2	55.8 ± 0.0	10.9 ± 0.1	7.7 ± 0.1	0.7 ± 0.0
C18:3 ω3	-	-	-	-	-	-	-	2.8 ± 0.1	-
C20:4 ω6	-	-	-	-	-	-	-	45.0 ± 0.5	-
C22:6 ω3	-	-	-	-	-	-	-	0.1 ± 0.1	47.9 ± 0.3

2.2. Materials and methods

All the experimental materials and techniques are detailed in Chapter 2. In this study, the following model IFF were investigated (see memento side A):

- PO_NE_L
- PO_L
- RPO_L
- CO_L
- DF_L
- DF_DPL_L

2.3. Results

2.3.1. Model IFF development and standardization validation in term of chemical composition and structure

To develop model IFF abiding by the regulation and representative of marketed IFF, a survey of the nutritional values of 91 IFF was undertaken (**Table 11**) and used to establish target values. The oil phase compositions were then fixed by linear programming with constraints applied the FA profile to standardize the contents of SFA, MUFA and PUFA (total and LA, ALA, ARA and DHA contents) and fit with the target. Thus, as shown in **Table 13**, four oil mixtures were formulated.

Table 13. Oil phase composition, fatty acids profile and initial PV of model IFF

	Target value	PO_NE_L	PO_L	PO_RA_L	CO_L	RPO_L	DF_L	DF_DPL_L
Oil phase composition (% w/w)								
Palm oil	-	67.8	67.8	67.8	-	-	-	-
Rapeseed oil	-	18.4	18.4	18.4	18.6	15.4	12.6	12.6
Sunflower oil	-	9.1	9.1	9.1	14.4	12.0	19.4	19.4
High oleic sunflower oil	-	3.1	3.1	3.1	34.9	-	11.14	11.1
Coconut oil	-	-	-	-	30.6	-	-	-
Dairy fat	-	-	-	-	-	-	55.3	55.3
Red palm oil	-	-	-	-	-	71.0	-	-
ARA oil	-	0.6	0.6	0.6	0.6	0.6	0.6	0.6
DHA oil	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Soy lecithin	-	0.2	0.2	0.2	0.2	0.2	0.2	-
Dairy phospholipid	-	-	-	-	-	-	-	0.8
Fatty acid profile (% w/w)								
SFA	33.3	36.5 ± 0.6	35.9 ± 0.9	34.7 ± 0.1	37.6 ± 3.4	35.3 ± 0.1	35.0 ± 1.3	32.9 ± 0.5
C12:0	-	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	18.4 ± 2.7	0.1 ± 0.0	2.8 ± 0.2	1.9 ± 0.1
C16:0	-	30.9 ± 0.5	30.3 ± 0.9	28.8 ± 0.1	6.7 ± 0.1	28.9 ± 0.1	16.6 ± 0.7	16.4 ± 0.4
MUFA	45.5	44.6 ± 0.4	45.2 ± 0.5	46.0 ± 0.1	42.9 ± 3.0	45.5 ± 0.2	42.9 ± 1.1	46.8 ± 0.5
PUFA	18.2	18.4 ± 0.2	18.4 ± 0.3	19.3 ± 0.2	19.4 ± 0.4	19.3 ± 0.1	21.4 ± 0.1	20.3 ± 0.2
LA C18:2 ω6	15.6	16.3 ± 0.2	16.3 ± 0.2	16.7 ± 0.1	17.3 ± 0.4	17.1 ± 0.2	17.9 ± 0.2	17.7 ± 0.2
ALA C18:3 ω3	1.6	1.5 ± 0.0	1.5 ± 0.0	1.6 ± 0.0	1.5 ± 0.0	1.6 ± 0.2	1.7 ± 0.2	1.5 ± 0.0
ARA C20:4 ω6	0.3	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.0
DHA C22:6 ω3	0.5	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
LA/ALA	9.7	11.0	11.0	10.6	11.3	10.7	10.8	11.5
PV ($meqO_2/kg$)								
Initial PV	-	3.2 ± 0.2	2.5 ± 0.4	2.9 ± 0.4	3.0 ± 0.4	5.6 ± 0.4	3.2 ± 0.3	3.4 ± 0.3

In order to evaluate the fit between the formulated model IFF by linear programming and the target values the percentage of deviation was calculated. It represents the difference between the FA profiles measured and those targeted for the main classes (*i.e.* SFA, MUFA and PUFA contents) and was calculated for each class as follows (eq 2):

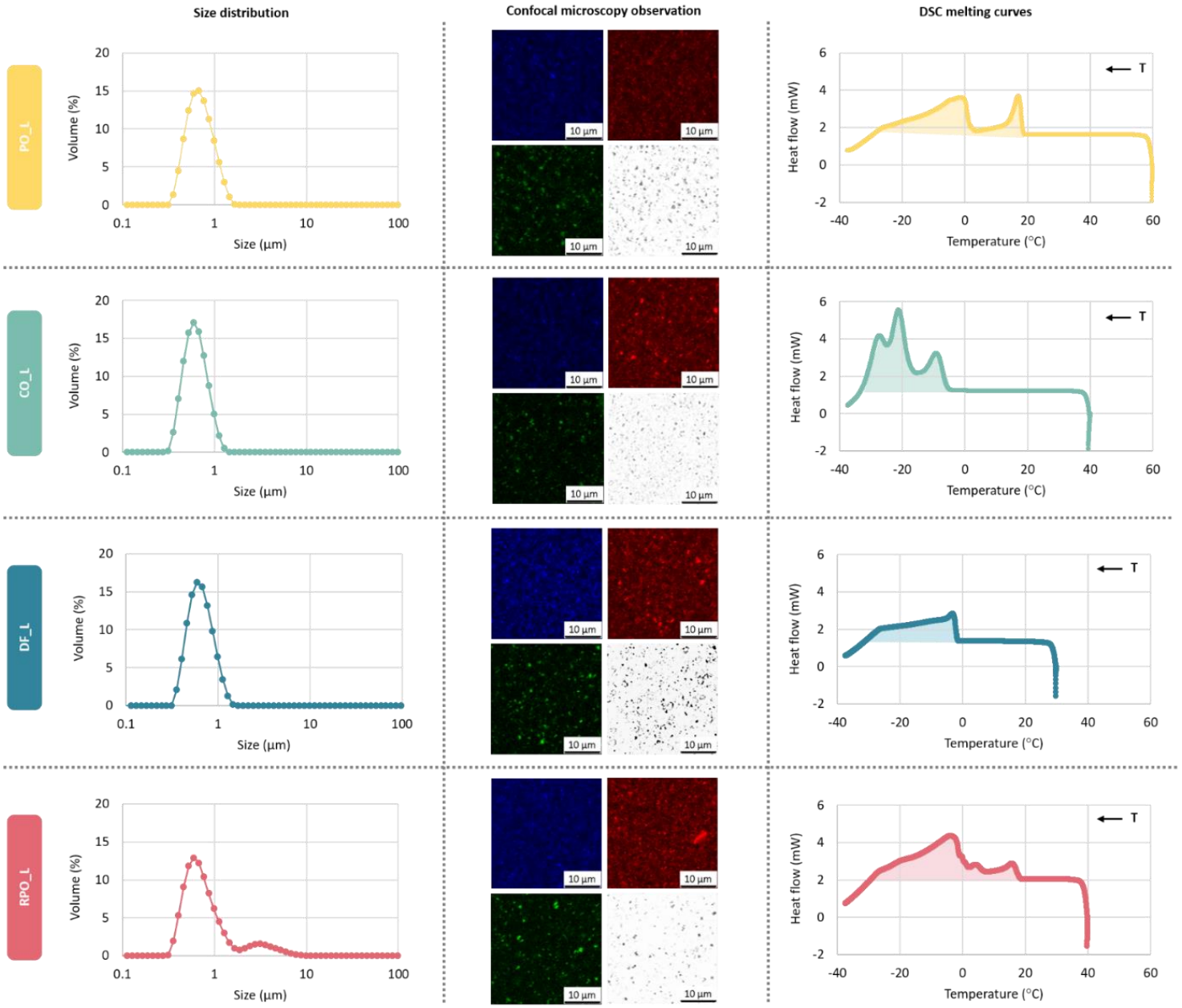
$$\text{Percentage of deviation} = \frac{|(\text{Target value} - \text{Measured value})| \times 100}{\text{Target value}} \quad \text{(eq 2)}$$

The percentage of deviation was on average of 5.2%. Thus, model IFF could be validated and considered as representative of the marketed IFF embraced in the survey (**Table 11**).

The measurement of the initial FA profiles showed that the linear programming resulted in an equivalent SFA content ranging from 32.93% to 37.60% of the FA in all model IFF. The main SFA was palmitic acid for the palm oil-based reference model IFF (PO), the olein dairy fat-based model IFF (DF) and the red palm oil-based model IFF (RPO), in which it represented 30.58%, 16.55% and 28.86% of FA, respectively. In the coconut oil-based model IFF (CO) the main SFA was lauric acid at a level of 18.42%. The contents of MUFA, PUFA, LA, ALA, ARA and DHA were also measured at the initial point and were equivalent in all model IFF. The LA/ALA ratio was on average of 10.97 ± 0.31.

In addition, a structure standardization was also carried and targeted a droplet size distribution centred on 0.7 µm in mode. All model IFF distribution and structures were assessed both by granulometry and CLSM (**Figure 21**). The results showed a similar monomodal droplet size distribution centered on 0.7 µm in all model IFF. Moreover, CLSM confirmed a good structure homogeneity with core of non-polar lipids stabilized by proteins and amphiphile compounds. The distribution size was also followed at days 9 and 20. Creaming occurred especially at 40°C but did not lead to coalescence and was reversible. Thus, before sampling a redispersion was performed by repeated inversion of tubes and was effective. Creaming was similar in all model IFF and can be classically described following Stokes law.

The crystallization behaviour of the four oil mixtures was assessed by DSC (**Figure 21**). The results showed that depending the SFA source the thermogram profiles varied strongly. The thermograms of PO showed an initiation of crystallization from 19.2°C and two major crystallization events with extremes at 17.1 and -1.4°C. CO had a crystallization that started from -4.5°C and was characterized by three major crystallization events with extremes respectively at -9.3, -21.2 and -27.4°C. DF was characterized by a homogeneous and continuous crystallization range starting from -1.5°C with an extremum at -3.2°C. Finally, the crystallization initiation of RPO occurred from 18.5°C and was characterized by two major events at 15.6 and -4.0°C.

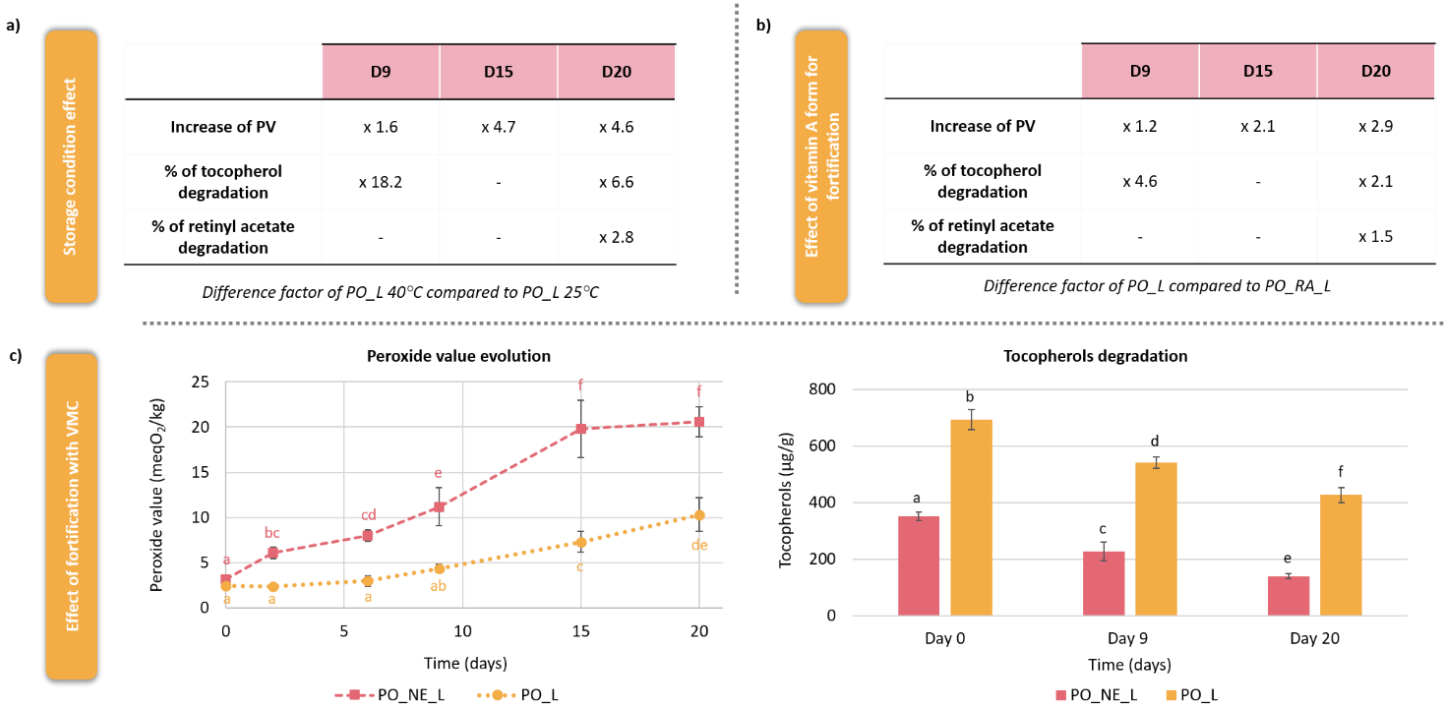


Particles size distribution was assessed by laser light scattering (left panel); confocal laser scanning micrograph were collected using a 40× water-immersion objective (middle panel) with each time four micrographs: top left blue colored micrograph: proteins; top right red colored: amphiphiles compounds; bottom left green colored: lipids; bottom right: transmission light micrograph; melting points were assessed by DSC (left panel).

Figure 21. Characterization of the structure and physical state of model IFF

In order to determine the storage conditions a preliminary test was carried out. PO_NE_L model IFF were stored 20 days either at 40°C under a constant stirring of 110 rpm or at 25°C without stirring (**Figure 22.a**). The results showed that under the least pro-oxidant conditions (25°C without stirring) model IFF remained stable throughout the incubation period. In order to initiate oxidation for the purpose of the study, the temperature was set at 40°C with a constant stirring.

All model IFF, except the negative control (PO_NE_L), were enriched with a VMC. The enrichment in VMC aims at contributing to the nutritional intake of vitamins and minerals necessary for the development of infants. Moreover, addition of VMC is expected to alter the lipid oxidation kinetics. Indeed, VMC are made not only of antioxidant compounds (vitamins E and C) but also of iron sulfate that could have a potential pro-oxidant effect. In order to quantify the antioxidant capacity of VMC and evaluate its protective effect, the reference model IFF were either fortified (PO_L) or not fortified (PO_NE_L). **Figure 22.b** showed that the enrichment of model IFF delays oxidation and leads to a loss of tocopherols approximately 1.5 times less important (38.5% of loss in PO_L against 60.4% of loss in PO_NE_L) and a PV twice lower at day 20. In the non-enriched model IFF, only the endogenous tocopherols of the raw materials acted as a barrier against oxidation which explains its faster degradation.



Different letters indicate a significant difference (P<0.05) between IF at a given time.

Figure 22. Effect of a) storage condition (25 or 40°C), b) vitamin A form (retinol or retinyl acetate) used for fortification and c) fortification in VMC on tocopherols loss and PV evolution in model IFF based on palm oil

2.3.2. Selection of the most discriminating indicators of oxidation among the monitored parameters

To determine which indicators of oxidation level were the most informative (among: the evolution of PV, TBARS, FA profile and degradation of tocopherols and vitamin A), their statistical variations along the accelerated storage test and between model IFF were analysed. A correlation matrix was established (**Table 14**). TBARS did not undergo any significant evolution during the kinetics (maximum

value of 49.3 mg MDA/kg oil after 20 days of storage) although all model IFF contained 1.8% to 2.0% of FA of ω3 series (ALA and DHA). The oxidation level of the IFF model was not advanced enough, consequently, no significant evolution of the FA profiles, especially the DHA content, was observed. At this stage, primary oxidation products are formed, and fat-soluble vitamins are degraded first, acting as sacrificial agents. The correlation matrix indicated that PV and tocopherols content underwent a significant evolution during storage and were strongly correlated throughout the storage.

Table 14. Correlation matrix of all the oxidation indicators monitored in IFF during storage at 40°C

	PV	Tbars	Tocopherols	Vitamin A	LA	ALA	DHA	ARA
PV	1.0000	0.6807	-0.7332	-0.5969	0.0381	0.0696	-0.2141	0.1524
Tbars	0.6807	1.0000	-0.8407	-0.5367	-0.0695	-0.0889	0.1233	-0.1823
Tocopherols	-0.7332	-0.8407	1.0000	0.6825	0.2109	0.1762	-0.0883	0.2473
Vitamin A	-0.5969	-0.5367	0.6825	1.0000	0.3493	0.3406	0.2544	0.2281
LA	0.0381	-0.0695	0.2109	0.3493	1.0000	0.4490	0.2320	0.4734
ALA	0.0696	-0.0889	0.1762	0.3406	0.4490	1.0000	-0.0138	0.4695
DHA	-0.2141	0.1233	-0.0883	0.2544	0.2320	-0.0138	1.0000	-0.4942
ARA	0.1524	-0.1823	0.2473	0.2281	0.4734	0.4695	-0.4942	1.0000

2.3.3. Influence of vitamin A form

A preliminary characterization of the raw materials showed that they contained significant levels of vitamin E and various forms of vitamin A. In order to make all model IFF comparable, α-tocopherol was added to normalize the vitamin E content in all model IFF. For vitamin A, the levels were normalized across all model IFF based on the content of DF_L, as this was the model IFF with the highest intrinsic vitamin A content (**Table 15**). Normalization was performed by adding retinol, the biologically active form of vitamin A, in PO_L, CO_L, DF_L and DF_DPL_L or with retinyl acetate, a retinol ester, in PO_RA_L. The comparison of PO_L and PO_RA_L showed that tocopherol losses and PV were significantly lower for model IFF in which normalization was performed with retinyl acetate (**Figure 22.c**). In addition, a retinyl acetate degradation 1.5 times lower was observed in model IFF normalized with retinyl acetate. PO_RA_L would appear to be more resistant to oxidation than PO_L in which normalization had been performed by addition of retinol.

Table 15. Levels of model IFF fortification with VMC and levels of normalization of tocopherols and vitamin A contents

	Target value	PO_NE_L	PO_L	PO_RA_L	CO_L	RPO_L	DF_L	DF_DPL_L
Supplementation (mg/L)								
Retinol	-	-	0.1	-	0.1	0.1	-	-
Retinyl acetate	-	-	-	0.1	-	-	-	-
Tocopherol	-	-	9.1	9.1	-	9.1	6.1	6.1
VMC	-	-	200.0	200.0	200.0	200.0	200.0	200.0
Tocopherol contents (mg/L)								
Total	23.0	12.0 ± 0.5	23.6 ± 1.2	26.3 ± 1.4	23.3 ± 2.1	18.9 ± 1.8	24.3 ± 2.5	23.7 ± 1.1
α-tocopherol	-	7.6 ± 0.4	10.0 ± 0.5	23.4 ± 2.9	17.7 ± 1.6	3.2 ± 2.1	11.1 ± 1.9	21.4 ± 1.0
Vitamin A content								
Total (µg RE/L)	-	-	493.4 ± 13.0	462.9 ± 29.6	298.8 ± 13.6	2029.9 ± 79.9	658.0 ± 46.4	586.7 ± 62.4
Retinol (µg RE/L)	-	-	-	-	-	-	-	-
Retinyl acetate (µg RA/L)	849.1	-	566.0 ± 15.3	530.9 ± 34.0	342.7 ± 15.6	467.4 ± 19.0	708.1 ± 49.0	599.9 ± 70.2
Retinyl palmitate (µg RP/L)	-	-	-	-	-	-	74.6 ± 6.7	116.8 ± 2.2
Carotenoid (mg/L)	-	-	-	-	-	13.3 ± 0.6	-	-
α-carotene (mg/L)	-	-	-	-	-	7.1 ± 0.3	-	-
β-carotene (mg/L)	-	-	-	-	-	5.1 ± 0.2	-	-
9-cis-β-carotene (mg/L)		-	-	-	-	1.1 ± 0.1	-	-

RE: retinol equivalent; RA: retinyl acetate; RP: retinyl palmitate.

2.3.4. Comparison of palm oil with other SFA sources

2.3.4.1. Comparison of palm oil with olein of dairy fat

PO_L was compared to a model IFF in which the main SFA source was dairy fat olein. As shown in **Figure 23.a**, PO_L and DF_L have equivalent PV at day 20 of 10.31 and 11.48 meqO_2/kg on average, respectively. In addition, tocopherol losses were 38.5% for PO_L *versus* 42.0% for DF_L (**Figure 23.b**). The determination of tocopherol isomers (**Figure 23.c**) showed that γ-tocopherol was the main isomer in PO_L and DF_L and represented 54.0 and 49.3% of the total tocopherol, respectively followed by α-tocopherol (42.5 and 45.8% of total tocopherol, respectively). During the kinetic, the loss of tocopherol isomer followed the same behavior in each of these model IFF. α-tocopherol is mostly degraded and the fraction of γ-tocopherol thus increased at day 20. A higher loss of retinyl acetate was observed in PO_L compared to DF_L after 20 days of storage and was of 75.9 and 29.6%, respectively (**Figure 23.d**).

2.3.4.2. Comparison of palm oil with coconut oil

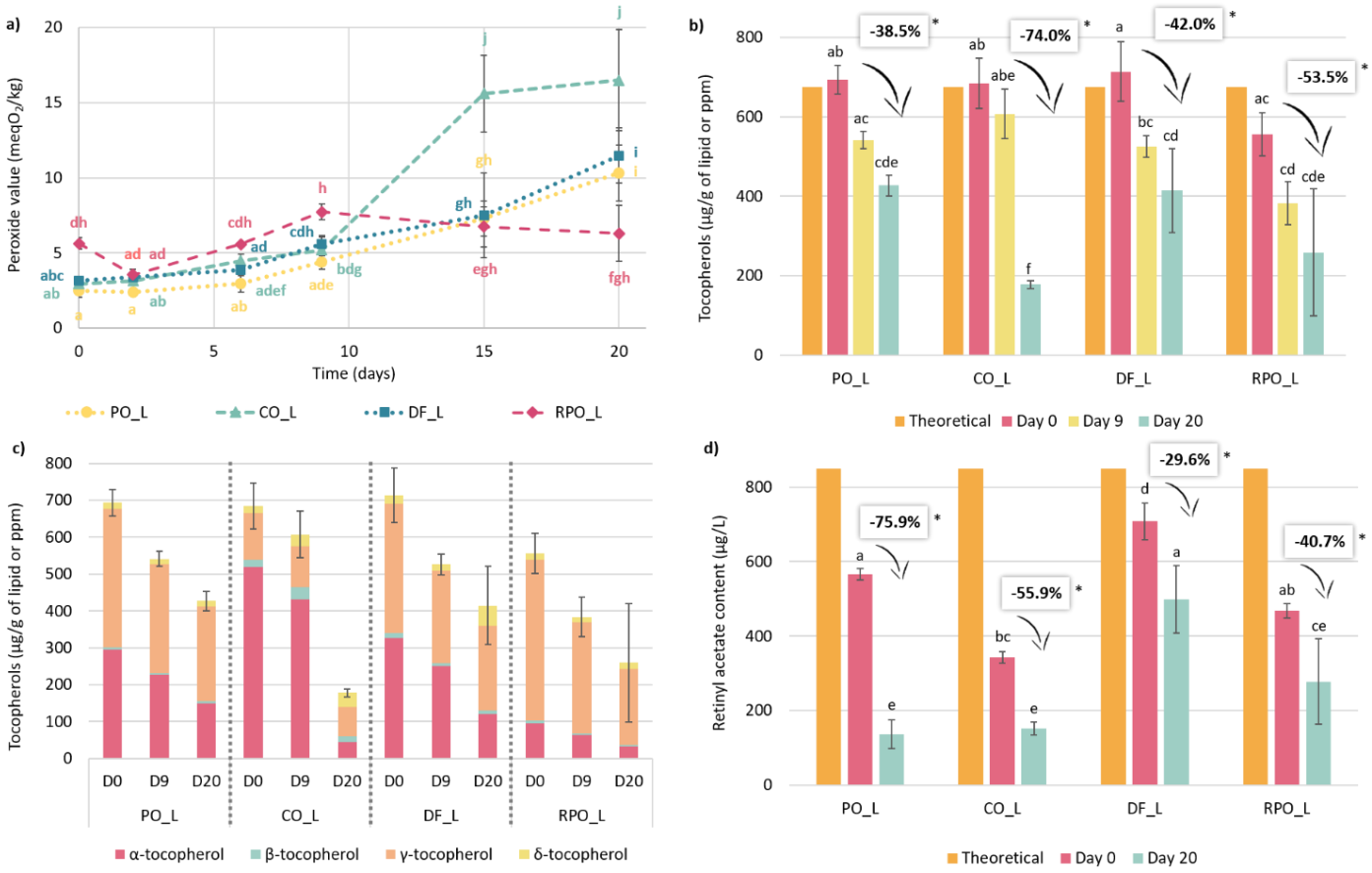
PO_L was compared to a model IFF in which SFA were mainly provided by coconut oil (CO_L) and their oxidative stability was evaluated. As shown in **Figure 23.a**, at day 20 the PV of CO_L was 1.5 times higher than that of PO_L (16.49 *versus* 10.31 meqO_2/kg, respectively). In addition, **Figure 23.b** also indicated that the loss of tocopherols in CO_L was nearly twice more than in PO_L and amounted to a loss of 74% of total tocopherols at day 20. At initial point, 75.8% of the total tocopherols in CO_L corresponded to α-tocopherol and was the predominant isomer (**Figure 23.c**). The γ-tocopherol, which was the second most abundant isomer, represented only 18.5% of total tocopherols. At day 20, the α-

tocopherol content significantly reduced in CO_L, this isomer then represented only 24.4% of total tocopherols. The important degradation of tocopherols in CO_L may relate to the loss of α-isomer.

2.3.4.3. Comparison of palm oil with red palm oil

The impact of the refining process on oxidative stability and nutritional properties was evaluated by comparing PO_L with the red palm oil-based model IFF (RPO_L). The measurement of PV is based on a colorimetric method. The PV results were overestimated due to the coloration of red palm oil and therefore not representative. At the initial point the PV of RPO_L, which amounted to 5.63 meqO_2/kg (**Table 13**), was more than two times higher than the initial value of PO_L. Despite this overestimation, the PV of RPO_L at day 20 was 1.6 times lower than that of PO_L (6.29 *versus*. 10.31 meqO_2/kg, respectively).

Almost no PV evolution was observed during the storage in RPO_L while this value more than quadrupled in PO_L (**Figure 23.a**). A degradation of 53.5% of total tocopherols was observed in RPO_L *versus* 38.5% in PO_L (**Figure 23.b**). At initial point, γ-tocopherol was the main isomer and thus represented 54.0 and 78.2% of total tocopherols in PO_L and RPO_L, respectively (**Figure 23.c**). The second most abundant isomer was α-tocopherol in each model IFF and represented for 42.5 and 16.9% of the total tocopherols in PO_L and RPO_L, respectively. A decrease in α-tocopherol content was observed at day 20 in these two model IFF. The degradation of tocopherols may therefore be attributed to a loss in the α-isomer. Red palm oil is one of the richest vegetable oils in tocotrienols. Therefore, γ-tocotrienol was also one of the major forms of vitamin E found in RPO_L. At initial point an average content of 170.2 ppm of γ-tocotrienols was present in the RPO_L. At day 20, 58.3% of γ-tocotrienols were degraded in these model IFF (average content of 71.0 ppm at day 20). A loss of 75.9% of retinyl acetate was observed in PO_L compared to 40.7% in RPO_L, *i.e.* almost two times less important (**Figure 23.d**).



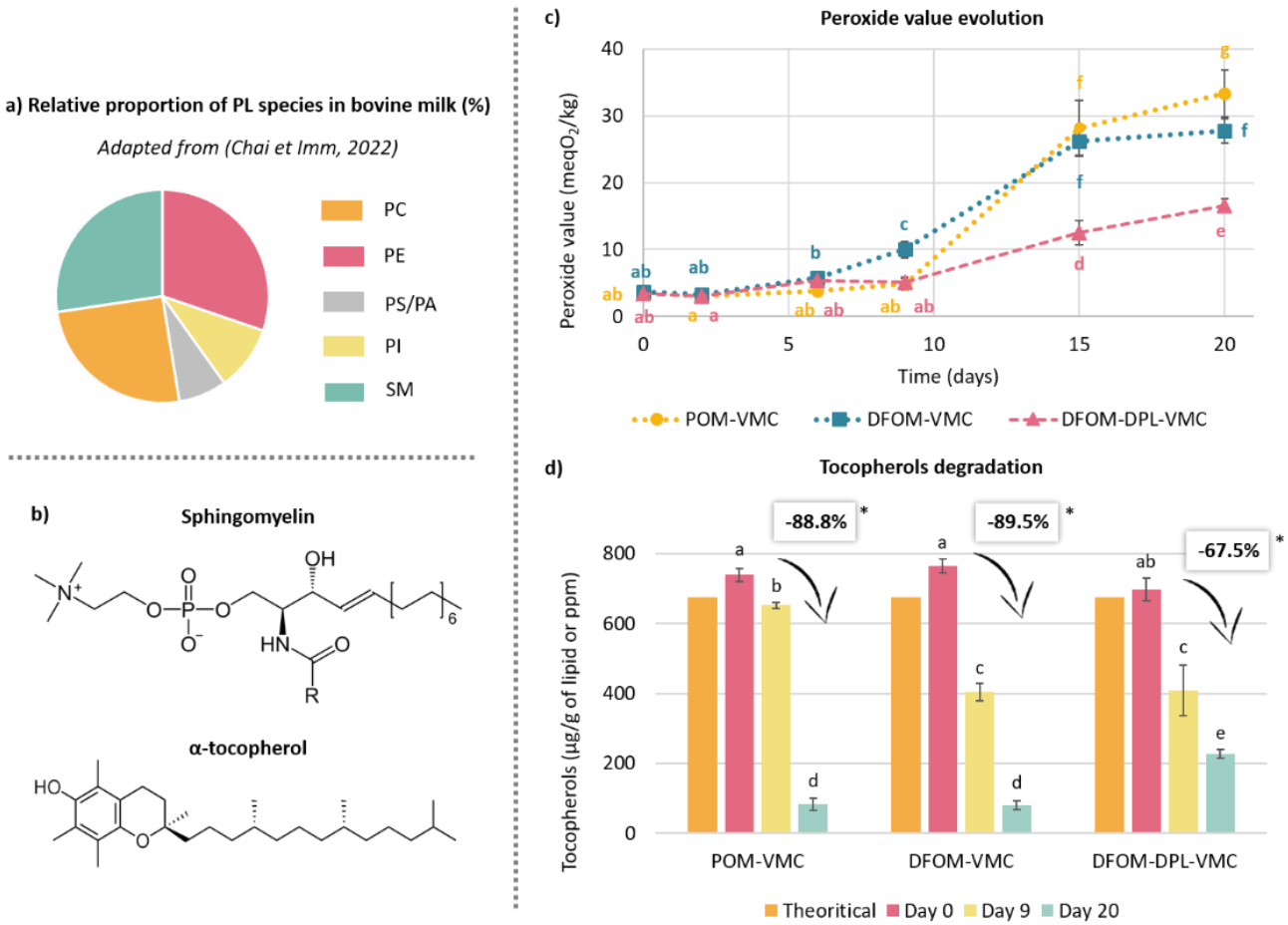
** stands for percentage of tocopherol degradation between initial point and day 20.*

Different letters indicate a significant difference (P<0.05) between IF at a given time.

Figure 23. Effect of refined palm oil substitution by other SFA (coconut oil, dairy fat or non-refined palm oil (red palm oil)) on a) PV evolution, b) tocopherols loss, c) tocopherols isomers partition and d) retinyl acetate content in model IFF over storage at 40°C

2.3.5. Combined use of dairy fat and phospholipids to stabilize IFF

To further investigate the effect related to the introduction of dairy raw materials in model IFF composition, another challenge test was performed. The objective was to substitute soy lecithin by a DPL in DF_DPL_L which leads to a higher content of some PL species like SM (**Figure 24.a**) (Chai, Oh *et al.* 2022). This model IFF was then compared to both PO_L and DF_L which are stabilized with soy lecithin. The results first confirmed the ones obtained concerning the comparison of PO_L and DF_L (**Figure 23.)**. Thus, despite faster oxidation kinetics than for the previous model IFF (resulting in higher PV and tocopherol degradation at day 20), these two model IFF seemed to have equivalent oxidative stabilities. DF_DPL_L had PV twice lower than PO_L (**Figure 24.c**). Moreover, the degradation of tocopherols in DF_DPL_L was less important than in model IFF with soy lecithin with a loss of 67.5% of tocopherols at day 20 against about 89% for PO_L and DF_L (**Figure 24.d**).

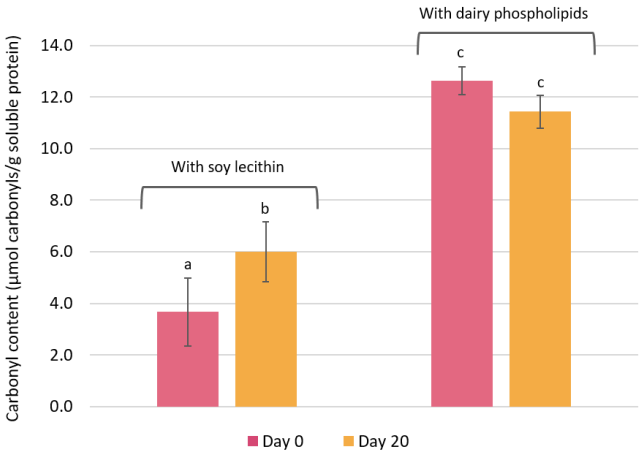


**stands for percentage of tocopherol degradation between initial point and day 20.*

Different letters indicate a significant difference (P<0.05) between IF at a given time.

Figure 24. a) Partition of PL species in bovine milk, b) chemical structure of SM and α-tocopherol and effect of introduction of dairy lipids on c) PV evolution and d) tocopherols loss in model IFF

It is expected that the composition of the oil droplets surface depends on the amount and type of PL used, which will directly alter the amount of dairy proteins adsorbed at this locus. In turn, protein localization in emulsions is known to be related to the propensity of proteins to oxidize (Berton, Ropers *et al.* 2012), which is why protein-bound carbonyls were measured in selected model IFF. This analysis showed that the amount of carbonylated proteins was overall fairly low in all tested model IFF (**Figure 25**), initially and throughout storage (<13 µmol/g soluble protein). Yet, the carbonyl content was higher in the DPL-stabilized IFF model than in those stabilized with soy lecithin, even in freshly prepared IFF.



Different letters indicate a significant difference (P<0.05) at a given time.

Figure 25. Carbonyl content in model IFF depending on the emulsifier source (soy lecithin or DPL)

2.4. Discussion

An important result of our study is that in the presence of antioxidants (added under the form of VMC), model IFF showed an overall good stability despite the addition of LC-PUFA (DHA and ARA in particular) and an acceptable nutritional profile (ω6/ω3 ratio below 15). More specifically, our study indicates that the lipid sources can modulate the oxidative stability and the nutritional profile quality of IFF with an improvement with the introduction of dairy lipids (dairy fat olein and PL). The lipid sources also influence the chemical composition of fat-soluble vitamins. Among them, vitamin A and tocopherol isomers were key factor influencing model IFF oxidation stability and some isomers and chemical forms were more stable than other (interest of retinyl acetate, carotenoids and γ-tocopherol). In addition, among the oxidation descriptive indicators studied, once again and in agreement with literature, the evolution of PV and the tocopherols degradation were reliable descriptors of oxidation kinetics.

2.4.1. Tocopherol content and isomers composition are key element of IFF stabilization

The indicators choice to evaluate the oxidation level of model IFF, *i.e.* the evolution of PV and tocopherol contents, and their strong negative correlation is in agreement with Jiang, Yang *et al.* (2021). These authors also demonstrate that the measurement of volatile compounds and the monitoring of vitamin C contents were reliable indicators for estimating the oxidation rate. In our study, we confirm that tocopherols act as sacrificing agents and are able to limit oxidation by several reaction pathways (Barouh, Bourlieu *et al.* 2021). Tocopherols can react with the radicals formed during the oxidation process by donating their phenolic hydrogen. This ability is related to the low dissociation energy of the phenolic O-H bond. Tocopherols can also react with singlet oxygen to induce a return to the triplet oxygen ground state. In addition, tocopherols are able to develop synergic effects

with several compounds (PL like SM, vitamin C or carotenoids for example) with different physico-chemical interactions resulting in tocopherol regeneration. Finally, they can chelate transition metals by forming a complex that will block the pro-oxidant activity of these compounds.

The results showed that the oxidation kinetics differed according to the tocopherols isomers composition, for instance illustrated on CO_L that has a lower resistance to oxidation than PO_L. These results are consistent with Let, Jacobsen *et al.* (2005) who compared the ability of different antioxidants (α and γ-tocopherols, ascorbyl palmitate and EDTA) to prevent oxidation in fish-oil-enriched milk emulsion. These authors showed that when added individually, γ-tocopherol inhibits oxidation more effectively than α-tocopherol with a concentration dependent efficacy (maximum at 660 ppm). This study also demonstrated that, in these systems, ascorbyl palmitate was very effective in limiting oxidation.

As described by Barouh, Bourlieu *et al.* (2021), in bulk system the regeneration capacity of tocopherols isomers is quite easily predictable on a given range of concentration (<500 ppm) and is mainly based on the dissociation energy of their phenolic O-H bond. The lower the binding energy, the higher the hydrogen donating power and follows the order: α < β < γ < δ. However, in more complex biphasic systems such as ours, the chemical environment influences the antioxidant efficiency of the different isomers. The results suggest that in our model IFF, γ-tocopherol would have a better regenerative capacity than α-tocopherol.

2.4.2. Vitamin A form is a key parameter of formulation for the IFF stabilization

The results showed that the vitamin A form used to fortify model IFF has an impact on the oxidative stability. Model IFF normalized with retinyl acetate has a better oxidation resistance than those with retinol. This result agrees with the literature in which it is demonstrated that retinol esters are less prone to oxidation than unesterified retinol. Esterification increases the lipophilicity of the retinyl esters and thus modulates its location at the oil-water interface where lipid oxidation mainly occurs (Thorsteinn Loftsson *et al.*, 2014).

Our results seem to indicate that RPO_L has a better oxidative stability compared to PO_L. Refining processes have a strong impact on the chemical composition of palm oil and in particular on its composition in vitamins with antioxidant activities and nutritional benefits for infants. The study of (Abdullah 2018) had shown that red palm oil had a highest antioxidant activity due to its higher carotenoids and vitamin E (tocopherols and tocotrienols) contents compared to refined palm oil. These results indicated that refining processes lead to the loss of antioxidant compounds and are in line with our results.

Red palm oil contains high amount of provitamin A carotenoids, the most abundant of which are α- and β-carotenoids (41.3 and 41% of total carotenoids, respectively ; Loganathan, Subramaniam *et al.*

2017). In addition, an important part of the vitamin E in red palm oil is in the form of tocotrienols. It is well documented that these compounds have important antioxidant properties. Moreover, the presence of both carotenoids and vitamin E provides synergistic effects for protection against oxidation. This effect was demonstrated in the study of Schroeder, Becker *et al.* (2006) that aimed to compare the oxidative stability of liposome as a function of antioxidants (α and γ-tocopherols and tocotrienols and β-carotene) added individually or in combination at three different concentrations (100, 500 or 1,000 ppm). It showed that the induction period of oxidation was significantly increased for systems with combined addition of vitamin E (1,000 ppm) and β-carotene (100 ppm). These results indicate that these compounds interact synergistically. To explain this observation, the authors suggest that tocopherols and tocotrienols would promote the regeneration of carotenoids.

Thus, α-tocopherol acts as a sacrificial agent for carotene regeneration which could explains its faster degradation in our study. However, these results disagree with Yi, Andersen *et al.* (2011) who compare the oxidative stability of fish oil mixed with refined or unrefined palm olein at concentrations of 0, 20 or 50%. These authors showed that red palm olein mixture was more susceptible to oxidation than refined palm olein. These unexpected results, which go against the hypothesis that the intrinsic antioxidant composition of red palm oil makes it less sensitive, could be explained by the study of Henry, Catignani *et al.* (1998) who showed that above a certain concentration (> 500 ppm) carotenoids could have a pro-oxidant effect. The study of Yi, Andersen *et al.* (2011) also showed that in their systems α-tocopherol and tocotrienol were depleted antioxidants, while γ-tocotrienol concentrations remained stable.

From a nutritional point of view, the inclusion of red palm oil in IFF conduct to a greater incorporation of provitamin A carotenoids which are more bioaccessible in such lipid environment and quite efficiently converted into the active form of vitamin A, *i.e.* retinol. Several studies have shown that supplementation of the infant and adults diet (especially pregnant and lactating women) with red palm oil leads to an increase in retinol and β-carotene levels in serum but has no effect on α-carotene level (Dong, Xia *et al.* 2017). Red palm oil supplementation is therefore an effective strategy to fight against vitamin A deficiency disease. However, there are different forms of carotenoids that do not all have the same nutritional properties and antioxidant activity. In addition, the presence of tocotrienol has been reported to have health benefits. They may participate in the regulation of the cholesterol synthesis in the liver and could play an important role in the reduction of the progression of certain cancers (Aggarwal, Kashyap *et al.* 2019).

2.4.3. SFA profile modulates the physical state and influences the oxidation kinetics

Despite standardized levels of total SFA in all model IFF, the type of SFA varies depending on the fat source used. Indeed, while in PO_L and RPO_L the main SFA was palmitic acid and represented 30.31

and 28.86% of total FA, respectively. These same FA represented only 16.55% in DF_L (**Table 13**). DF_L also provides capric (C10:0), lauric (C12:0) and myristic (C14:0) acids with average contents of 1.97, 2.80 and 6.79% of the total FA, respectively. The olein dairy fat has the specificity to be rich in short and medium chains SFA. CO_L also provides a medium chain SFA due to its lauric acid content. These FA, which are found in minimal quantities or even absent in PO_L, are more easily digested and metabolized very quickly by the portal vein (Menard, Chever *et al.* 2014). The introduction of dairy fat and coconut oil in IFF lipid fraction have therefore a nutritional interest, especially in the case of infant. The more important sensitivity to oxidation of CO_L compared to PO_L could also be related to the difference in the chain lengths of the most abundant SFA in each model IFF. The melting point of the oil mixture constituting CO_L is lower than that of PO_L which impact the physical state of lipid especially at 40°C. Calligaris, Sovrano *et al.* (2006) reported in their study that lowering the melting point of an oil mixture makes it more susceptible to lipid oxidation phenomena. From a nutritional point of view, a lower melting point improves the lipid absorption rate.

Based on these results, CO_L is more adapted to the infant nutritional needs than PO_L due to the profile and the bioaccessibility of its FA, however this model IFF may be more sensitive to lipid oxidation.

2.4.4. Interest of dairy raw materials to optimize IFF

The results appear to show that the substitution of palm oil by olein dairy fat did not impact the oxidative stability. It also showed that the combined substitution of palm oil and soy lecithin by dairy fat olein and DPL respectively, would lead to a higher oxidative resistance. The oil mixture based on olein dairy fat provides, for equivalent SFA contents, a more diversified FA profile especially of short and medium saturated chains that is more suitable for infant nutrition. Moreover, a notable difference is observed in the organization of FA on the TAG backbone between lipids of animal and vegetable origin (saturated preferentially in internal or external positions). Indeed, in dairy fat the *sn-2* position is mostly occupied by long chain SFA, while short chain and unsaturated FA are mainly esterified in external positions (*sn-1* and *3*). The study of Jensen (2002) on the composition of bovine milk lipids, the most represented TAG in dairy fat was identified as 18:1-16:0-4:0. In vegetable oils that constitute the fat phase of PO_L, SFA are mainly located in the external positions (*sn-1* and *3*). The TAG organization, with palmitic acid preferentially esterified in the internal position (*sn-2*), as found in dairy fat, is similar to that in breast milk. Furthermore, it is admitted that this organization conduct to a better lipidic absorption by avoiding the formation of insoluble soaps (calcium palmitate) which are directly excreted (Dubois, Breton *et al.* 2008). The TAG structure could also have an influence on the sensitivity to oxidation with a protective effect of the PUFA in the central position because less accessible (Wijesundera, Ceccato *et al.* 2012).

The inclusion of DPL provides very long chains SFA that form rigid domains at the interface which is then more biomimetic of breast milk. It also provides different PL species like SM (**Figure 24**.**a**) (Chai, Oh *et al.* 2022). It has been reported in the literature that these compounds can react with tocopherols and promote their regeneration. The study of Shimajiri, Shiota *et al.* (2013) on the synergistic antioxidant activity of milk SM and α-tocopherol on fish oil TAG showed that the higher the number of amino groups, the greater the synergy. Thus, the synergy between tocopherols with SM enhanced their antioxidant activity. In this study, two interaction mechanisms were proposed. The first would involve a transfer of hydrogen or electrons between an amino group and the tocopherols, thus promoting their regeneration (**Figure 24**.**b**). The second mechanism would involve an interaction between oxidized lipids and the amino group of polar lipids. This positive synergy could explain the better oxidative stability of DF_DPL_L than other model IFF.

2.4.5. Optimization of IFF formulation

IFF aim to mimic the composition and structure of breast milk while ensuring the stability of nutrients throughout storage. In order to optimize the lipid fraction of IFF, the substitution of palm oil by other sources of SFA seems to be aviable strategy. The use of red palm oil conduct to an increase of the oxidation initiation phase due to its antioxidant composition while improving the nutritional status of provitamin A compounds. However, it did not increase the intake of short and medium SFA and minor lipids compounds. The inclusion of bovine dairy fat and DPL provides a lipid structure and a global chemical complexity closer to that of breast milk, in particular thanks to its composition of minor lipids such as cholesterol and other complex lipids (glycerophospholipids or sphingolipids). These minor compounds are lacking in commercial IFF which do not include polar lipid extracts from bovine milk (Delplanque, Gibson *et al.* 2015). In this context, the reintroduction of bovine dairy fat in the oil fractions of IFF, which was progressively replaced by vegetable oils from the 1970s onwards particularly for economic reasons - appears to be aviable strategy (Delplanque and Baudry 2015, Viriato, Queirós *et al.* 2022). Thus, the use of vegetable oils - for their unsaturated FA content -, marine, algal or fungal oils - for their LC-PUFA content - and dairy fat - for its structure and composition, especially in terms of SFA - would lead to reach a balance between lipid oxidation stability and improvement of the nutritional profile of IFF lipid fraction.

2.5. Conclusion

This study compared the oxidative stability of model IFF according to the source of saturated fat used. The importance of the balance between stability and nutritional properties to meet the infant nutritional needs was highlighted. The results showed that in all model IFF, the oxidation phenomena affected primarily the fat-soluble vitamins (A and E) which acted as sacrificial agents. It has also shown that the lipid formulation parameters were key levers to optimize IFF from a chemical and nutritional properties point of view. Based on the tocopherol degradation kinetics and PV evolution the use of coconut oil as the main SFA does not seem to give convincing results in terms of stability. The red palm oil would delay oxidation, and olein dairy fat would conduct to a similar stability to that obtained with palm oil. However, the combined use of dairy fat and DPL led to an improvement in both oxidative stability and nutritional profile by providing a wide variety of components playing a role in the proper development of infant.

MAIN RESULTS

- γ-tocopherol was the most stable tocopherol isomer in model infant formula systems
- Carotenoids improved oxidative stability due to synergy with tocopherols
- Dairy fat provided oxidative stability in infant formulae equivalent to palm oil
- Coconut oil made infant formulae more sensitive to lipid oxidation
- Dairy lipids are of interest both for nutritional profile and oxidative stability

3. Impact of structural parameters on oxidative stability and nutritional profile

In a second step, structural parameters were studied by assessing the impact of droplet size, their core composition, the emulsifier source, and their order of addition on the oxidative stability of IFF.

This section is based on a research article entitled "Stabilization of infant formulas against lipid oxidation: what are the key levers?" submitted to the European Journal of Lipid Science and Technology.

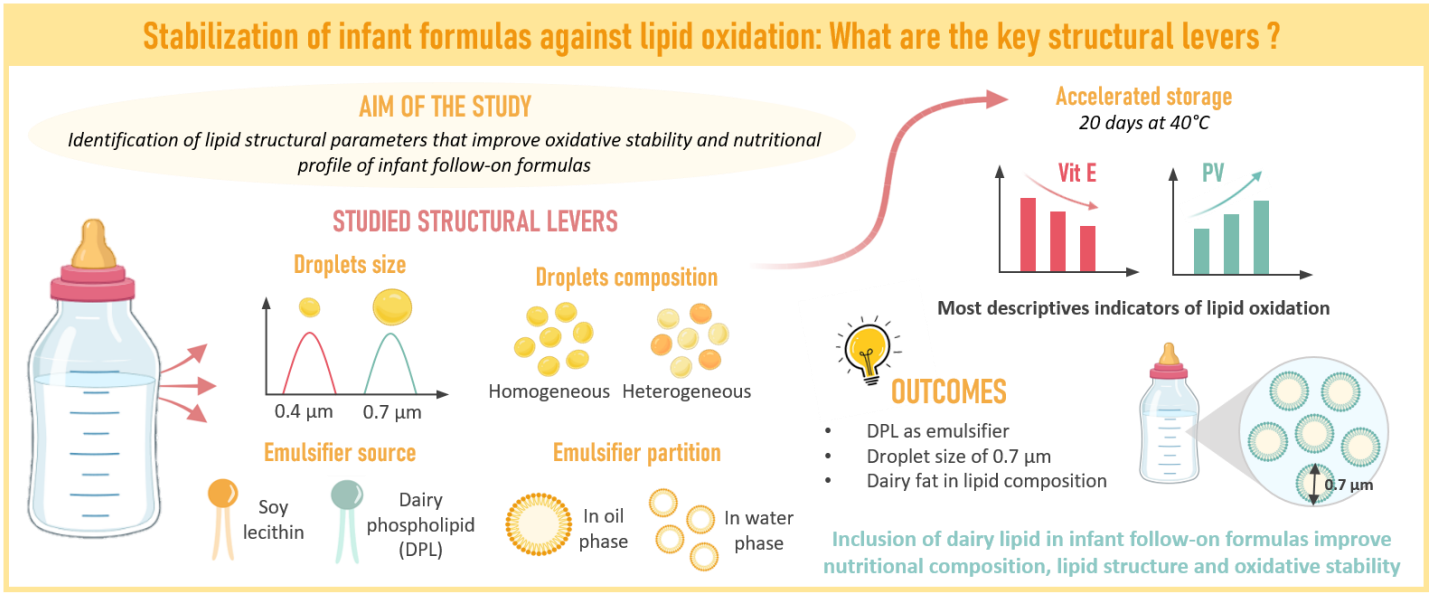
Abstract

IFF mimic human milk. This study aimed to identify the key structural levers that influence oxidative stability of IFF. Representative model IFF of marketed products in term of lipid composition were formulated with varying droplet size, lipid droplet core composition and interfacial composition using different emulsifiers (soy lecithin or DPL). The oxidative stability of model IFF was assessed in accelerated storage conditions. No significant stabilization effect based on the lipid droplet core composition was observed. However, the nature of the interface, influenced by the emulsifier type, had an impact. Model IFF with DPL showed no loss of tocopherols and PV was up to twice lower than those with soy lecithin after 20 days. This effect was particularly pronounced for the 0.4 µm droplets. These results suggest that model IFF with DPL had a greater oxidative stability, likely due to the presence of sphingomyelin and the formation of a rigidified domain at the droplet surface. Model IFF with 0.4 µm droplets stabilized with soy lecithin, especially when added to the water phase, showed a tocopherols loss twice as high as that of IFF with DPL. These results indicate that oxidative stability of IFF is more influenced by the chemical environment rather than droplet size.

Practical applications

IFF aim to ensure an adequate nutritional intake to support the proper development and infant growth. Therefore, IFF must be stable against degradation phenomena such as lipid oxidation and have a composition and structure that are as biomimetic as possible to mature breast milk. This study provides key information for the development of IFF with a lipid composition and structure that are suitable for the infant nutritional needs and have an acceptable resistance to lipid oxidation. More generally, these results can be applied to all dispersed systems in the form of oil-in-water emulsions with a similar composition.

Graphical abstract



3.1. Introduction

Breast milk is a complex and dynamic biofluid recognized as the reference for infant nutrition even if its composition is strongly impacted by many external factors, with one of the most influential being the mother's diet (Innis 2014, Perrella, Gridneva *et al.* 2021). Its specific composition is adapted to the evolving nutritional needs of infants, particularly with regard to its lipid content (Innis 2007). Breast milk provides a complex lipid profile with a majority of SFA (mainly palmitic acid C16:0) that account for more than half of the total FA. Breast milk is also rich in PUFA including essential FA, such as LA and ALA acids and also provides their longer chain derivatives, namely ARA (C20:5 ω6) and DHA (C22:6 ω3) acids, which are notably involved in the infant brain development. Breast milk is also a source of various bioactive compounds that promote optimal development and provide long-term benefits, such as a reduced risk of developing type II diabetes or obesity in adulthood (Andreas, Kampmann *et al.* 2015). The World Health Organization (WHO) recommends exclusive breastfeeding for the first 6 months, followed by mixed breastfeeding until the age of 2 years. However, 48% of infants aged under 6 months are exclusively breastfed, and only 70% of them are still breastfed up to the age of 12 months (WHO, 2022). IFF, which are designed for the period ranging from 6 to 12 months, are therefore widely consumed throughout the world. At the industrial scale, the lipid composition of IFF is submitted to very specific regulations that are regularly updated to align with the nutritional recommendations. For instance, the addition of DHA to IFF is mandated by the EU regulation CE 2016/127 since 2020. These regulatory changes aim to improve the composition of IFF to meet infant nutritional needs.

However, differences in composition and structure between mature breast milk and IFF remain (Deglaire, Lee *et al.* 2023). The lipids in breast milk are mainly found as TAG (98%) and PL (1%) and have a particular structural organization in the form of MFG, ranging from 0.1 to 10 µm droplet size (4 µm on average) (Koletzko, Rodriguez-Palmero *et al.* 2001, Bourlieu and Michalski 2015). MFG are composed of a core of TAG stabilized by a trilayered biological membrane composed mainly of polar lipids and proteins. However, IFF are composed of submicron droplets (0.3 to 0.8 µm) stabilized by milk proteins and vegetable emulsifiers (mainly lecithin) (Delplanque, Gibson *et al.* 2015). The regiodistribution of FA on the triacylglycerol backbone also differs widely due to the use of a mixture of fats almost exclusively of vegetable origin. It has been reported that these differences in structure and composition lead to differences in the efficiency of digestion and assimilation of nutrients, especially of lipids (Bourlieu, Deglaire *et al.* 2017). Some studies also showed that these differences could impact the oxidative stability, with a greater resistance of breast milk compared to IF (Michalski, Calzada *et al.* 2008). Various studies have focused on structural parameters that modulate oxidative stability at different levels. Firstly, at lipid droplet core level with the FA regiodistribution by formulating breast milk fat analogues (Zou, Pande *et al.* 2016). But also depending of their esterification as PL or TAG with an increased stability attributed to PL due to the presence of the

phosphate group and the polar head group (Song and Miyazawa 1997, Lyberg, Fasoli *et al.* 2005). In emulsions, it is now well known that the interface is the lipid oxidation initiation site. Therefore, many studies have focused on the interface properties such as the size and composition to modulate the oxidative stability.

However, to the best of our knowledge, no studies have yet proposed a multi-parameter approach to screen the most efficient structural levers ranging from droplet size, core lipid composition, emulsifier source and its mode of incorporation in model IFF having similar chemical composition.

Therefore, the present study aims at identifying structural parameters that influence oxidative stability of model IFF representative of marketed products. Additionally, particular attention is given to the biomimetic aspect related to these parameters, which may improve the nutritional profile. Model IFF were formulated with equivalent chemical composition with a normalization of their lipid profile (similar contents in SFA, MUFA, PUFA, DHA, ARA, vitamins E and A). The influence of the lipid structure was evaluated by adjusting the droplet size, the core lipid composition the emulsifier source and its mode of incorporation.

3.2. Materials and methods

All the experimental materials and techniques are detailed in Chapter 2. In this study, the following model IFF were investigated (see memento side A):

- PO_L and PO_S
- PO_DLDC_L and PO_DLDC_S
- PO_LWP_L and PO_LWP_S
- PO_DPL_L and PO_DPL_S
- DF_DPL_L and DF_DPL_S

3.3. Results

The aim of this study was to compare the impact of different chemical and physico-chemical parameters on oxidative stability of model IFF. In that context, various model IFF were designed with similar FA composition and vitamin content but varying in their structure (droplet size, core lipid composition, emulsifier source and its mode of incorporation). These model IFF were formulated in compliance with the regulations and were intended to be representative of the marketed IFF. The oxidation rates were monitored during an accelerated storage test of 20 days at 40°C through the evaluation of several indicators of lipid oxidation (PV, TBARS, FA profile evolution and vitamin A and E contents).

3.3.1. Standardization of model IFF and selection of descriptive indicators of oxidation

Model IFF size droplet distribution and structure were assessed both by granulometry and CLSM (**Figure 26** and **Supplementary figure 1**). All model IFF show a similar monomodal droplet size distribution centered on 0.4 or 0.7 µm depending on the homogenization pressures applied. CLSM observations confirmed a good structure homogeneity with a core of non-polar lipids stabilized by proteins and amphiphilic compounds. The size droplet distribution and the CLSM observations showed that creaming occurred but did not lead to coalescence and was reversible. Therefore, before sampling an effective redispersion was achieved by repeated inversion of tubes. Creaming was similar in all model IFF and can be classically described following Stokes law.

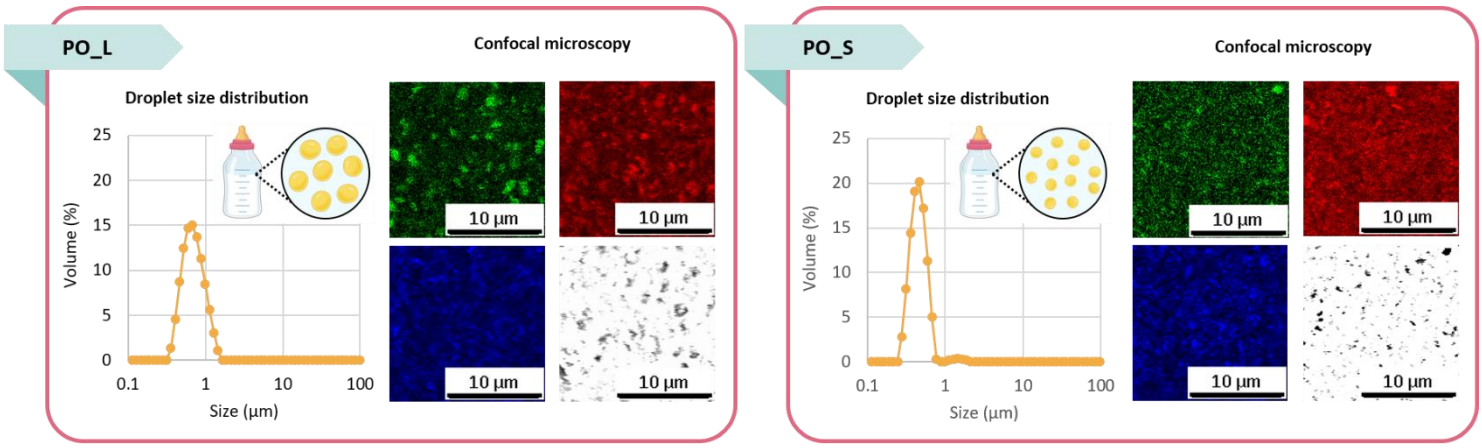


Figure 26. Typical droplet size distribution of model IFF and physical organization in PO_L and PO_S

The FA compositions of model IFF were designed to be representative of marketed products (Cancalon, Hemery *et al.* 2023). As shown in **Figure 27**, the linear programming used to design the various model IFF the resulted in similar initial FA profiles with equivalent SFA (ranging from 32.9 to 38.8% of total FA), MUFA (ranging from 43.3 to 46.9% of total FA) and PUFA (ranging from 17.0 to 20.3% of total FA) contents in all model IFF. The contents of LA, ALA, ARA and DHA were also relatively equivalent and the LA/ALA ratio was 10.7 ± 0.6 on average. Total DHA and ARA content in DF_DPL_S and DF_DPL_L is slightly higher (+0.3%), making them potentially more sensitive to oxidation.



Figure 27. Fatty acid profiles of model IFF at the initial point (D0)

Normalization of initial vitamin A and E contents was performed by supplementation with VMC and the addition of α-tocopherol and retinol (**Table 16** and **Table 17**). The normalization in vitamin A (retinyl acetate with the VMC and retinol) was made prior to emulsification to be representative of industrial-scale practices. All IFF models had an equivalent initial total tocopherol content (653.0 ± 67.6 ppm) (**Table 16** and **Table 17**). However, depending on the emulsification process especially the pressure applied, significant variations of the initial vitamin A content were observed. Indeed, the more drastic homogenization process (to obtain small droplets), the more important loss in vitamin A contents during the process (**Figure 28** and **Figure 29**).

Table 16. Model IFF fortification, normalization of tocopherols and vitamin A contents and PV

	Target value	PO_L	PO_S	PO_DLDC_L	PO_DLDC_S	PO_LWP_L	PO_LWP_S	PO_DPL_L	PO_DPL_S
Supplementation (mg/L)									
Retinol	-	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
Retinyl acetate	-	-	-	-	-	-	-	-	-
Tocopherol	-	9.12	9.12	9.12	9.12	9.12	9.12	9.12	9.12
VMC	-	200	200	200	200	200	200	200	200
Tocopherols contents (ppm)									
Total	676.2	694.2 ± 36.1	688.9 ± 55.6	672.0 ± 3.6	615.5 ± 11.9	651.3 ± 80.4	556.1 ± 15.4	570.8 ± 79.9	535.1 ± 58.6
α-tocopherol	-	294.8 ± 15.5	293.4 ± 24.1	265.3 ± 2.0	224.1 ± 6.1	229.6 ± 36.6	140.8 ± 7.7	161.1 ± 35.2	122.0 ± 24.8
Vitamin A content									
Total (µg RE/L)	-	493.4 ± 13.8	126.9 ± 28.7	383.3 ± 27.1	179.3 ± 31.1	440.6 ± 68.4	189.1 ± 27.4	651.2 ± 66.1	351.8 ± 85.1
Retinyl acetate (µg RA/L)	849.1	566.0 ± 15.8	145.5 ± 33.0	439.6 ± 31.1	205.7 ± 35.6	505.4 ± 78.5	216.9 ± 31.4	746.9 ± 75.8	403.5 ± 97.6
PV (meqO_2/kg)									
Initial PV (day 0)	-	2.5 ± 0.4	1.9 ± 0.4	2.5 ± 0.3	2.2 ± 0.3	1.3 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	0.6 ± 0.1
Final PV (day 20)	-	10.3 ± 1.8	9.4 ± 1.2	10.9 ± 1.9	11.5 ± 1.3	11.8 ± 0.4	16.4 ± 1.9	7.1 ± 0.5	4.4 ± 0.3

Table 17. Model IFF fortification, normalization of tocopherols and vitamin A contents and PV for the additional storage test

	Target value	PO_L	PO_S	DF_DPL_L	DF_DPL_S
Supplementation (mg/L)					
Retinol	-	0.11	0.11	-	-
Retinyl acetate	-	-	-	-	-
Tocopherol	-	9.12	9.12	6.06	6.06
VMC	-	200	200	200	200
Tocopherols contents (ppm)					
Total	676.2	740.0 ± 18.6	710.5 ± 17.7	698.1 ± 33.1	703.1 ± 42.9
α-tocopherol	-	592.3 ± 15.2	567.3 ± 13.0	628.8 ± 29.6	634.2 ± 36.1
Vitamin A content					
Total (µg RE/L)	-	456.3 ± 31.8	422.3 ± 21.3	586.7 ± 62.4	491.5 ± 63.8
Retinyl acetate (µg RA/L)	849.1	450.1 ± 28.1	419.8 ± 21.2	599.9 ± 70.2	537.7 ± 69.0
Retinyl palmitate (µg RP/L)	-	-	-	116.8 ± 2.2	41.7 ± 6.7
PV(meqO_2/kg)					
Initial PV (day 0)	-	3.6 ± 0.5	2.8 ± 0.3	3.40 ± 0.3	3.11 ± 0.3
Final PV (day 20)	-	33.4 ± 3.6	21.8 ± 2.3	16.6 ± 1.0	4.8 ± 0.6

Concerning the evaluation of lipid oxidation rates in model IFF in accelerated storage conditions, five different measurements were performed: evolution of PV, TBARS, FA profile and degradation of tocopherols and vitamin A. The measurement of primary oxidation compounds (here, PV value) is generally considered to be relevant to evaluate lipid oxidation rates at early stage of oxidation, where the formation of secondary oxidation compounds is not yet important. In our case, lipid oxidation was not advanced enough for any model IFF studied in our chosen accelerated conditions. Therefore, TBARS which is a measurement of some secondary oxidation compounds (Laguerre, Lecomte *et al.* 2007) did not significantly increase after 20 days at 40°C. Moreover, TBARS assay is designed to measure MDA which preferentially forms from FA containing at least three double bonds (Dahle, Hill *et al.* 1962). In our case, the amount of such FA especially ALA and DHA ranged from 1.7 to 2.0% of the total FA content. This amount could be too low to guarantee a relevant TBARS measurement. Similarly, the FA profile did not show any significant evolution during storage in all model IFF. This can be attributed to the fact that advanced oxidation stages were not reached during our chosen storage conditions. Finally, a correlation matrix was established to determine the most relevant indicators of the oxidation rate in our model IFF. It is also worth noting that the tocopherols presence in all model IFF induced a lag phase (up to 9 days) and that peroxidation increased when tocopherols were partially or totally consumed.

3.3.2. Impact of model IFF droplets size on oxidation

In order to evaluate the impact of droplet size on model IFF oxidative stability, they were systematically homogenized at two pressures to obtain emulsions with either 0.4 or 0.7 µm droplet sizes. As

presented previously, depending on the chosen emulsification process, significant variations of the initial retinyl acetate content were observed (**Figure 28** and **Figure 29**). In average the loss of retinyl acetate loss was twice as important for the more drastic homogenization process compared to the homogenization process that was applied to obtain larger droplets. Interestingly, this loss seems to be even more pronounced for model IFF designed with soy lecithin as emulsifier, with for instance an initial retinyl acetate content up to four times lower for PO_S compared to PO_L *versus* twice lower for PO_DPL_S compared to PO_DPL_L (**Table 16**). Moreover, in the case of model IFF stabilized with soy lecithin, a higher loss of tocopherols was observed after 20 days of storage for the smaller droplet sizes with for instance 62.8% loss for PO_S against only 38.5% for PO_L. The substitution of soy lecithin with DPL in model IFF resulted in a very limited tocopherols loss after 20 days whatever the droplet size. These results suggest that the coverage of interfacial area by DPL is efficient, offering a good protection of tocopherols *versus* oxidation whatever the droplet size. On the contrary, with soy lecithin, the larger interfacial area obtained with smaller droplet may have been imperfectly covered by this emulsifier, resulting in a weaker protection of tocopherols. This result may also be related to the slightly higher protein content of model IFF with DPL. Concomitantly, no significant differences in the PV evolution were observed as a function of droplet size in the case of model IFF stabilized with soy lecithin (PO_S and PO_L *versus* PO_DLDC_S and PO_DLDC_L). However, for model IFF with a droplet size of 0.4 µm, the addition of soy lecithin to the water phase conduct to a 1.7-fold increase in PV. In the case of DPL-stabilized model IFF, the evolution of PV was significantly less important especially for the smaller droplets (4.4 ± 0.3 meqO_2/kg for PO_DPL_S) than for the larger ones (9.4 ± 1.2 meqO_2/kg for PO_S) after 20 days. These results suggest that the droplet size cannot be considered individually to anticipate the resulting model IFF oxidative stability. Indeed, the chemical environment at the interface probably has a more significant influence than the droplet size alone.

3.3.3. Impact of homogenous or heterogeneous droplet core composition

The impact of the droplet core composition (homogeneous or heterogeneous) on the oxidative stability was evaluated by comparing reference model IFF, *i.e.* PO_S and PO_L with model IFF in which the individual core composition of the droplets was heterogeneous (PUFA and HMP oils droplets or IMP oils droplets) *i.e.* PO_DLDC_S and PO_DLDC_L (comparison of **Figure 28.a** and **Figure 28.b**). The results show no significant differences in the PV evolution after 20 days and reaches on average 10.5 ± 0.9 meqO_2/kg. The tocopherols loss was similar but higher for model IFF with a droplet size of 0.4 µm (62.8% and 65.3% of total tocopherol loss for PO_S and PO_DLDC_S, respectively) compared to the one with a size of 0.7 µm (38.5% and 42.6% of total tocopherol loss for PO_L and PO_DLDC_L, respectively). According to the tocopherols isomers composition, the tocopherols loss was mainly attributed to a loss of α-tocopherol which was the second most abundant isomer after the γ-

tocopherol in these model IFF. Significant differences in retinyl acetate content were measured after processing, but levels after storage were equivalent.

3.3.4. Impact of the interfacial composition

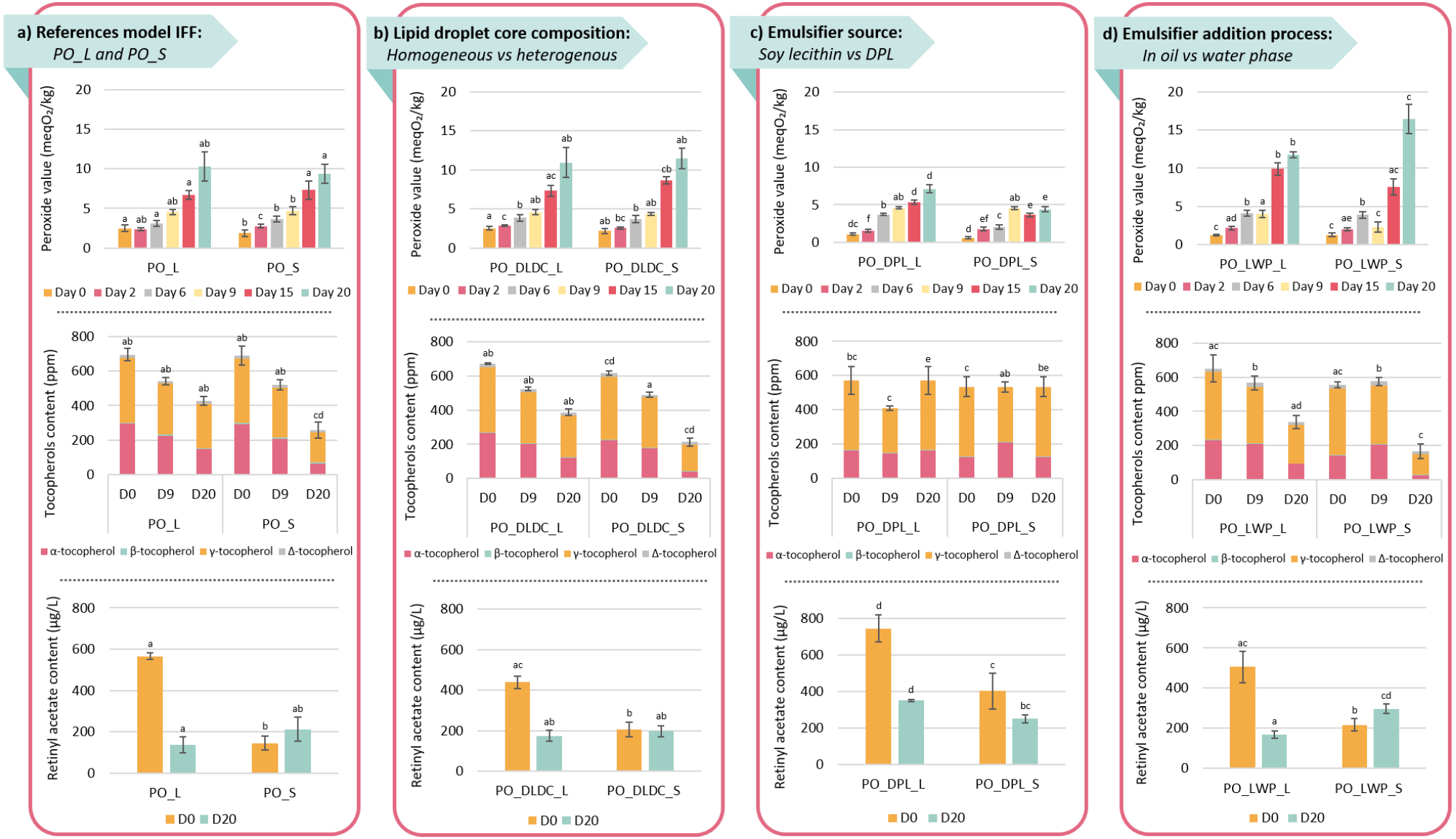
3.3.4.1. Impact of emulsifier type

Lipid oxidation is well known to occur at the lipid/water interface in emulsified system (Berton-Carabin, Ropers *et al.* 2014). Therefore, the interface composition and physico-chemical properties are crucial regarding oxidation rates and are directly correlated with the type of the emulsifier used. Therefore, model IFF that prepared either with soy lecithin *i.e.* PO_S and PO_L or DPL *i.e.* PO_DPL_S and PO_DPL_L were compared to evaluate the impact of the emulsifier source on the oxidative stability. According to PV evolution, model IFF stabilized with a DPL were significantly more stable than the ones stabilized with soy lecithin (comparison of **Figure 28.a** and **Figure 28.b**). This tendency was even more pronounced for the small droplets size with PV of 4.4 ± 0. 3 meqO_2/kg for PO_DPL_S after 20 days *versus* 9.4 ± 1.2 meqO_2/kg for PO_S. In parallel, the total tocopherols content remains constant after 20 days for model IFF with DPL as emulsifier whereas losses of 62.8% for PO_S and 38.5% for PO_L were observed. As expected, these results show that the type of emulsifier modulates the oxidative stability. In our case, DPL demonstrates superior protection against lipid oxidation compared to soy lecithin. Morevover, retinyl acetate losses were significantly lower when using DPL.

3.3.4.2. Impact of the emulsifier partition between the lipid and the water phases

The oxidation rate in emulsions can be also related to the processing conditions (McClements and Decker 2018). Thus, the way the emulsifier is added during emulsification process plays an important role as it will determine the potential presence of emulsifier micellar or colloidal structures. These particular structures are known to play an important role on mass transport phenomena in lipid oxidation (Laguerre, Bily *et al.* 2017, Villeneuve, Bourlieu-Lacanal *et al.* 2021). They could hypothetically be carriers of antioxidants, pro-oxidant or oxidation compounds in emulsion. The oxidative stability of PO_S and PO_L in which soy lecithin was added to the oil phase prior to emulsification processing was compared to PO_LWP_S and PO_LWP_L in which the same emulsifier was added to the water phase also prior to emulsification processing. Depending on the addition process, it is expected that the emulsifiers is more prone to be localized at the interface or to form colloidal structures in the emulsions. The comparison of **Figure 28.a** and **Figure 28.b** show that the incorporation of soy lecithin in the water phase (PO_LWP_S and PO_LWP_L) leads to a higher PV evolution after 20 days whatever the droplet size. Similarly, the tocopherols loss was significantly higher when the emulsifier was added in the water phase. This loss was even more important for small droplets (70.2% loss for PO_LWP_S *versus* 48.2% for PO_LWP_L).

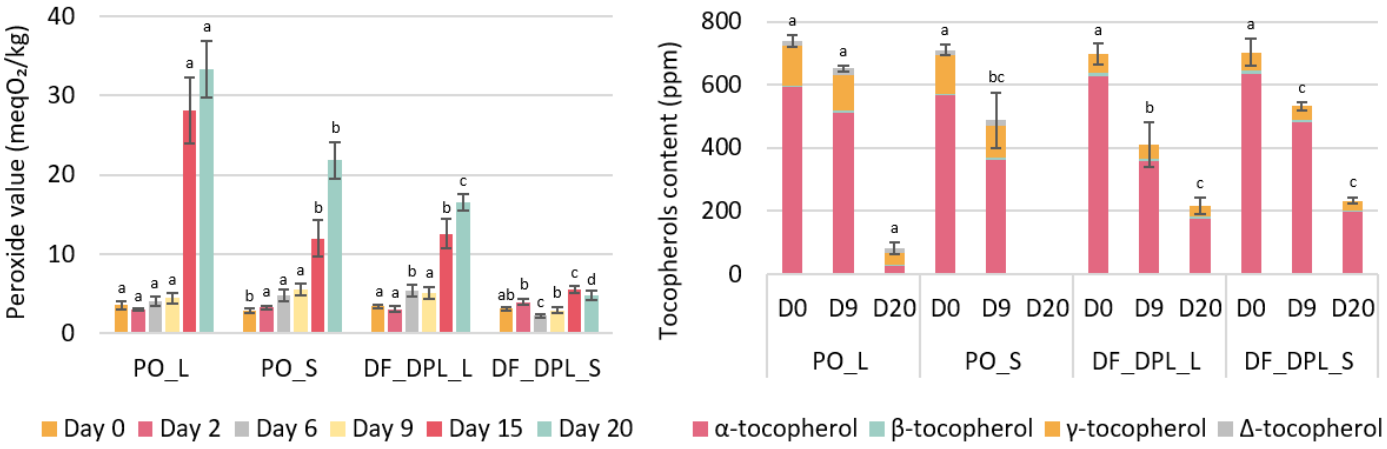
Figure 28. PV, tocopherols and retinyl acetate contents evolution a) in the reference model IFF and depending on a) the lipid droplet core composition, b) the source of emulsifier and c) the emulsifier addition process during accelerated storage



Different letters indicate a significant difference (P<0.05) between model IFF at a given time.

3.3.5. Effect of dairy lipids introduction in model IFF

An additional storage test was performed to further investigate the impact on oxidative stability of the substitution of palm oil by dairy lipids (including DPL and dairy fat olein) (**Figure 29**). Despite a faster oxidation kinetics than for the previous model IFF (resulting in higher PV and tocopherol degradation after 20 days of storage for PO_S and PO_L, model IFF based on dairy lipids were more stable than the ones with palm oil. PV were indeed significantly lower, especially for the smaller droplets for which PV were 9.5 times lower in comparison with model IFF made from palm oil (4.8 ± 0.6 and 21.8 ± 2.3 meqO_2/kg for DF_DPL_S and PO_S, respectively). Moreover, the tocopherols loss in DF_DPL_S and DF_DPL_L was less important than PO_S and PO_L with a loss up to 1.5 times lower. The difference in oxidative stabilities with the prior model IFF studied could be attributed on the fact that in these additional model IFF α-tocopherol was the most abundant tocopherol isomers (85% of the total tocopherols). However, in the prior model IFF the most abundant isomer was the γ- tocopherol and represented at least 54% of the total tocopherols. The post-processing retinyl acetate content was significantly higher in DF_S and DF_L and a lower loss after storage was measured in DF_DPL_S.



Different letters indicate a significant difference (P<0.05) between model IFF at a given time.

Figure 29. Impact of introduction of dairy lipids (dairy fat olein and DPL) in model IFF composition on PV and tocopherols

3.4. Discussion

Results presented in the current study showed that the studied model IFF have an overall good oxidative stability despite the addition of LC-PUFA (in particular DHA and ARA). Among the structural levers that we evaluated regarding their impact on oxidative stability, the most influential being the interface nature that can be modulated depending on the type of the used emulsifier (in our case soy

lecithin *versus* DPL). On the opposite, the droplet size and their core composition homogeneity have a limited impact which is mostly related to the chemical environment, especially to the presence of colloidal vesicles.

3.4.1. Droplet size alone has no strong impact on oxidative stability over the range of IFF model sizes tested

Our results show that the droplets size (0.4 or 0.7 µm) impacts mainly the initial vitamin A content with a more important loss when model IFF were designed with smaller droplets. This observation is most likely explained by higher shearing forces that lead to higher local heating of vitamin A molecules favoring their oxidative degradation during process.

Over the droplet sizes range tested no strong effect on oxidative stability was observed considering this parameter alone illustrated for instance by PO_S and PO_L. The hypothesis most often encountered in literature is that smaller droplets oxidized faster due to their larger specific surface area in comparison with larger droplets. However, it is difficult to distinguish between the effect of droplet size and that of the chemical environment. In fact, homogenization conditions not only modulate droplet size, but also have an impact on other crucial parameters involved in the oxidation reaction rates, such as the emulsifier partitioning with more or less adsorption at the interface (Sørensen, Baron *et al.* 2007). The present study shows that the type of emulsifier and the chemical environment determine stability rather than the droplet size. There are many contradictory results in the literature about the effect of droplet size. Some studies demonstrate that decreasing droplet sizes resulted in increased oxidation (Li, McClements *et al.* 2020), while others show that there was no effect of droplet size on lipid oxidation initiation or propagation (Erdmann, Lautenschlaeger *et al.* 2017), or the opposite, an increased stability with a decreasing of the droplets size (Let, Jacobsen *et al.* 2007). This dissensus supports our hypothesis that other factors such as the interface composition is more important than the total surface area itself.

From a nutritional point of view, droplet size has a very strong impact on the bioaccessibility of lipid compounds with accelerated lipolysis kinetics for smaller droplet sizes and a structure less close to that of breast milk (Bourlieu, Ménard *et al.* 2015, Bourlieu, Deglaire *et al.* 2017). Moreover, processing conditions to obtain smaller droplet may result in a loss of added Vitamin A in model IFF has seen in the present study.

3.4.2. Substitution of soy lecithin by DPL improves oxidative stability of model IFF

In emulsified systems, the interface area is known to be one of the main site of lipid oxidation phenomena (Waraho, McClements *et al.* 2011, Berton-Carabin, Ropers *et al.* 2014, Villeneuve, Bourlieu-Lacanal *et al.* 2021). Our study shows that, for an equivalent lipid composition, the

substitution of soy lecithin by DPL in model IFF improves their oxidative stability (illustrated by the comparison of PO_DPL_S and PO_DPL_L *versus* PO_S and PO_L). A first hypothesis to explain this result is that the thickness and density of the interface is more important for model IFF formulated with DPL. Indeed, DPL provides very long chains SFA (beyond C20:0) that create rigid domains at the interface which may form a steric protective barrier at the interface limiting the pro-oxidant effects of water-dissolved molecules such as metals. DPL also provides different PL species such as sphingomyelin (SM) which are known to have an antioxidant effect themselves or have synergistic effect with tocopherols (Shimajiri, Shiota *et al.* 2013). The efficiency of the synergy could be related to the number of amino groups, which may regenerate tocopherols after a series of rearrangement reactions (Arora, Culler *et al.* 2022). Another hypothesis that could explain these results is that the normalization of PL content has concomitantly slightly increased the protein content for model IFF with DPL compare to soy lecithin that can exhibit antioxidant properties (Nielsen, Horn *et al.* 2013).

The results obtained in our study show that in presence of DPL the smaller the droplet size, the greater the stability. Moreover, the addition of DPL concomitantly increases protein content. Similar results were obtained in the study of Let, Jacobsen *et al.* (2007) in which fish oil and/or rapeseed oil were homogenized with milk at different temperatures and pressures to obtain droplets from 0.5 to 1.36 µm (D[3,2]). The authors show that oxidative stability was improved for smaller droplet sizes and hypothesized that the adsorption of proteins and their rearrangement at the interface was more important in the case of smaller droplets, enabling an interface more fully covered and facilitating the contact with amino groups.

The results showed that stability was even enhanced when DPL was used in combination with dairy fat. Dairy fat provides short and medium saturated chains and minor lipids such as cholesterol and other complex lipids (glycerophospholipids or sphingolipids) that are suitable for infant nutrition. In addition, the organization of its FA on the TAG backbone, with long-chain SFA and LC-PUFA mainly esterified in *sn-2* position, leads to better lipid absorption by avoiding the formation of insoluble soaps (calcium palmitate) which are directly excreted (Dubois, Breton *et al.* 2008).). This organization is also suspected of influencing oxidative stability, with a protective effect for PUFA in the central position, as they are less accessible (Wijesundera, Ceccato *et al.* 2012). Complexifying the chemical composition by introducing dairy lipids is more relevant to mimic HM, and therefore more adapted to the needs of infants (Deglaire, Lee *et al.* 2023). Moreover, despite a slightly lower oxidative stability than PO_DPL_S, droplets of 0.7 µm would be closer to the average size of MFG and avoid over-processing leading to significant losses of vitamin A in particular.

3.4.3. The addition of lecithin to the water phase in model IFF may favor lipid oxidation

Our results show that the presence of colloidal structures in the water phase illustrated by PO_LWP_S and PO_LWP_L were not inert regarding lipid oxidation and result in a decrease of the stability compared to PO_S and PO_L. Lipid oxidation depends not only on the amount of PUFA, but also on the overall reactivity and interactions of the many molecules involved in lipid oxidation, and their location and mobility in the emulsion. More and more evidences show that colloidal structures such as the one studied in PO_LWP_S and PO_LWP_L play a crucial role in mass transport phenomena in lipid oxidation pathway (Villeneuve, Bourlieu-Lacanal *et al.* 2021, Klooster, Schroën *et al.* 2023). According to literature, micelles and colloidal structures may have two antagonistic effects regarding lipid oxidation in emulsified systems. They could act as antioxidant reservoirs, leading to a better diffusion of these molecules at the interface area and thus limiting oxidation. On the contrary, colloidal structure may serve as carriers of oxidation products, leading to their transfer from one droplet to another and promoting the oxidation. The results obtained in the present study seem to agree with the second pathway, as we observed that the addition of lecithin to the water phase diminish oxidative stability of model IFF. Moreover, this decrease in stability could also be attributed to the fact that the addition of lecithin in the water phase concomitantly affects the interfacial composition, by reducing its concentration at the oil-water interface. Consequently, interface coverage and the steric barrier would be lower. This would thus, favor contacts between oxidizing compounds (transition metals or oxidation products such as hydroperoxides) and consumption of lipid-soluble antioxidants such as tocopherols leading to their fast degradation. Moreover, phospholipids and tocopherols are known to have synergistic effect that favor the regeneration of tocopherols (Judde, Villeneuve *et al.* 2003, Osborn and Akoh 2004, Doert, Jaworska *et al.* 2012). The lack of lecithin at the interface due to colloidal structure formation may also limit this synergy effect and could also explain the more important sensitivity to oxidation of PO_LWP_S and PO_LWP_L compared to PO_S and PO_L. Finally, due to functional groups such as phosphates and amines, lecithin can also act as metal chelators that contribute to limit the pro-oxidant effects of iron or copper ions. The lower concentration of lecithin at the interface in PO_LWP_S and PO_LWP_L may also limit this effect at the oxidation initiation site, which could explain their higher susceptibility to oxidation. However, the transition metals in their free form are located in the water phase in direct contact with the colloidal structures, this hypothesis seems limited.

3.4.4. The individual core lipid droplets composition in a population is difficult to control and stabilize

MFG core composition of breast milk is heterogeneous, with the smallest globules containing a higher content of medium-chain SFA (C8-C12) and lower amount of long-chain SFA than the largest globules (Fauquant, Briard *et al.* 2005). LC-PUFA are therefore "encapsulated" in large globules surrounded by

long-chain SFA, with high melting point leading to a partial surface crystallization (Michalski, Ollivon *et al.* 2004). Instinctively, these parameters could impact the oxidative stability due to the formation of a lipid shell protecting LC-PUFA (steric barrier). In our study, the individual composition of the lipid core droplet in PO_DLDC_S and PO_DLDC_L was expected to be heterogeneous to mimic the breast milk as closely as possible. Our results show no significant effect on oxidative stability. However, Okubanjo, Loveday *et al.* (2019) studied the oxidative stability of oil-in-water emulsions by forming a lipid shell, and demonstrated that structuring the interface with solid lipids improved oxidative stability. The authors suggest that this kind of system leads to limited contact between pro-oxidant and oxidizable compounds. Similar results were obtained by Salminen, Helgason *et al.* (2017) who found that the thicker the crystallized shell, the better the oxidative stability. These findings were attributed to a reduced diffusion and mobility of pro-oxidants compounds and oxygen at the interface. Contrary to the previous studies, our results show no significant effect of the lipid core heterogeneity. One hypothesis to explain this contradictory result would be that a homogenization of the composition of the lipid droplet cores occurred during storage. Physical instability, possibly due to the stirring and reduction in viscosity caused by storage at 40°C, could favor collisions between droplets and concomitantly the transfer of lipids between droplet cores. Further investigations will be necessary to determine whether the individual lipid core droplet can be controlled in a such complex food matrix and used to delayed lipid oxidation.

3.5. Conclusion

This study showed that the oxidative stability of model IFF depends on their chemical composition and can be modulated according to different structural levers. Based on the tocopherol degradation kinetics and PV evolution, the most representative indicators of oxidation in our systems, the results showed that in a such complex food matrix it is difficult to manage the individual lipid core droplet composition. The impact of droplet size on lipid oxidation in IFF is limited and highly dependent on other factors such as the chemical environment, and in particular the nature of the interface. The interface quality could be modulated according to the source of emulsifier with a more important oxidative stability when soy lecithin is substituted by DPL. Moreover, the presence of colloidal structures in IFF has an impact on stability, probably by promoting the diffusion of oxidized compounds or limiting the synergy with tocopherols. Thus, considering both issues related to lipid oxidation and those related to nutrition, IFF enriched in LC-PUFA and incorporating dairy lipids may be a reliable strategy to guarantee oxidative stability and an adequate nutritional profile.



MAIN RESULTS

- The influence of the droplet size on the oxidation stability was limited and depended on the chemical environment
- Homogenization pressures impacted the initial vitamin A content
- Biomimetic IFF having large droplet size (0.7 µm) and based on dairy lipids had interesting oxidative stability
- Colloidal structures promoted the diffusion of pro-oxidant compounds and speed up lipid oxidation
- DPL as emulsifier slowed down lipid oxidation

4. Focus on vitamin A oxidative stability

The third step of this chapter was devoted to a focus on the oxidative stability of vitamin A depending on the homogenization process conditions and the chemical form.

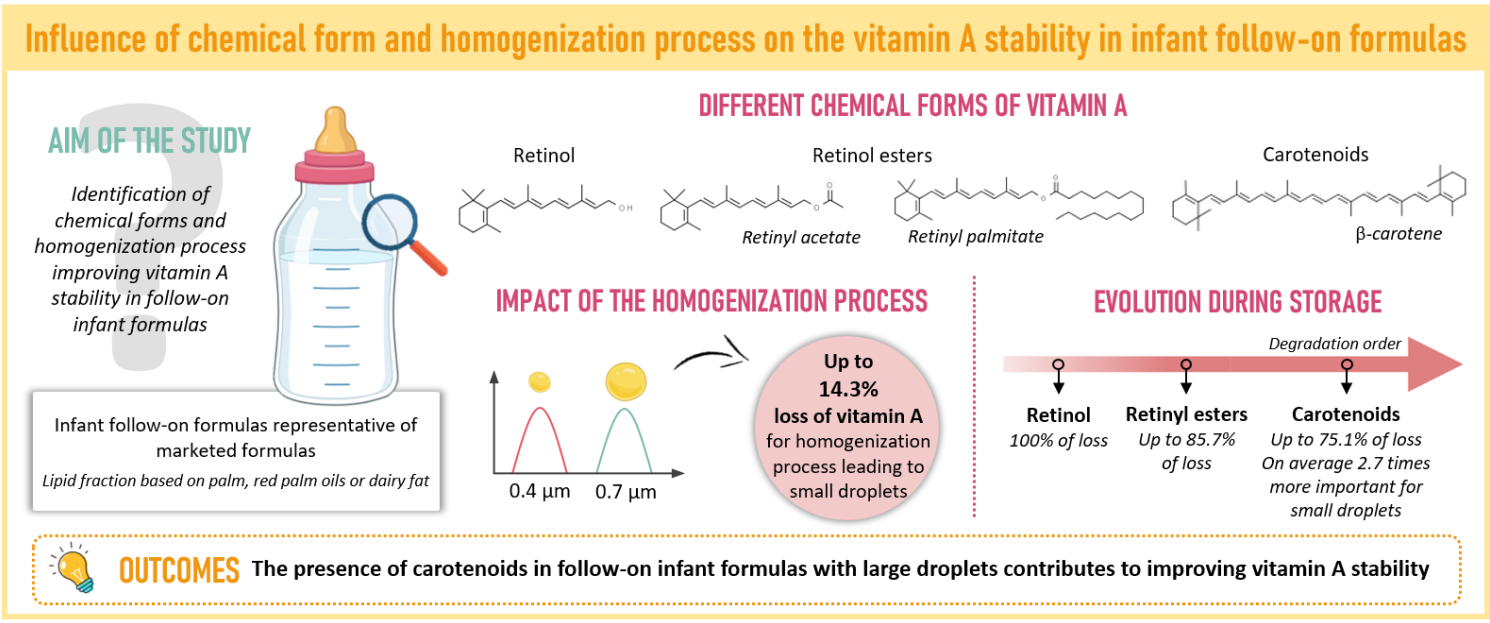
This section is based on a research article entitled "Influence of chemical forms and homogenization process on the stability of vitamin A in infant follow-on formulas" in preparation for submission.

Abstract

Vitamin A is an essential micronutrient for infant development, and its stability is crucial to ensure adequate intake. This study aimed to investigate the vitamin A stability in IFF depending on its chemical forms and on the homogenization process. For this propose, model IFF representatives of commercialized formulas were formulated with varying vitamin A forms (retinol, retinyl esters or carotenoids) and homogenization process applied. Losses of vitamin A were assessed either before or after the homogenization process, or after a storage of 20 days at 40°C. The homogenization process led to a vitamin A loss up to 14.3% at high pressure (350/40 bars, 8 passes) explained by important shearing forces leading to local heating of molecules. The presence of carotenoids and especially β-carotene led to a stabilization effect with a loss of only 40.7% of vitamin A during storage (up to 84.9% without carotenoids). Carotenoids provides a better resistance to lipid oxidation due to their synergetic effect with vitamin E. This study highlights that a good knowledge of vitamin A reactivity during processing and selection of chemical form with potential synergy with other compounds can improve its stability in IFF.

Keywords: infant formulas, vitamin A, retinoids, carotenoids, processing, stability

Graphical abstract



4.1. Introduction

Vitamin A is a crucial micronutrient that plays an essential role in the growth, development, and overall health of infants. (O'Byrne and Blaner 2013). Vitamin A refers to life essential group of unsaturated fat-soluble organic compounds with similar structures and related biological activities to all-*trans*-retinol (EFSA 2006). Under its active form, *i.e.*, retinol (286.46 g/mol), it plays key functions in various biological processes, including in the development of the visual system (retinal is essential in the function of rhodopsin), cell differentiation (retinoid acid in epithelial cells and tissues) and regulation of the immune response. Vitamin A deficiency, defined as a serum retinol concentration below 0.70 µmol/L (0,2 mg/L) is widespread throughout the world, particularly in Africa and Southeast Asia. The World Health Organization has classified vitamin A deficiency as a public health problem. It was estimated that, in 2013, this deficiency affected around a third of children aged between 6 and 59 months (with the highest rate recorded in sub-Saharan Africa, with 48% of children concerned) (Li, Wongsiriroj *et al.* 2014, WHO 2023). Vitamin A deficiency is associated with adverse health effects, such as blindness, stunted growth and an increased risk of diseases development and morbidity from severe infections (WHO 2009). Insufficient vitamin A intake can also cause impaired iron metabolism, and thus be associated with increased susceptibility to the development of anemia (Kraemer and Zimmermann 2007). In order to prevent vitamin A deficiency, various approaches aimed at increasing its intake have been implemented, such as periodic supplementation with a high dose of vitamin A, or fortification of food products. These strategies have shown beneficial effects on vulnerable populations (pregnant women and children) and have led to a reduction in the prevalence of vitamin A deficiency. In the literature, numerous examples of the impact of fortifying food products with vitamin A are available, such as the study of Lopez-Teros, Quihui-Cota *et al.* (2013) on powdered milk, or the study of Sandjaja, Jus'at *et al.* (2015) on oil, and which have been shown to be effective in increasing serum retinol concentration. An exclusive breastfeeding for the first 6 months of life, followed by mixed breastfeeding until the age of 2 years is strongly recommended (WHO 2011). Thus, breast milk is considered as the "golden standard" for infant nutrition despite its composition being impacted by many external factors, with one of the most influential being the mother's diet (Jensen 1999). According to a meta-analysis by Zhang, Ren *et al.* (2022) the vitamin A content of breast milk decreased significantly as lactation progressed, and is estimated at between 339.4 and 431.3 µg/L for mature breast milk. Jensen, Ferris *et al.* (1992) reported retinoid levels of 300 to 800 µg/L and 50 to 200 µg/L of β-carotene, lutein and lycopene in mature breast milk. Breast milk contain vitamin A under the predominant form of esters (palmitate mainly), the retinol to ester ratio is variable but around 10:90 w/w. The composition of IFF, which are consumed from 6 to 12 months of age when breastfeeding is either not desired or not possible, is governed by precise regulations which, in Europe, require the addition of between 420 and 798 µg/L of vitamin A (EU regulation CE 2016/127). In a recent

benchmark analysis of IFF (Cancalon, Hemery *et al.* 2023), the main chemical form of ester detailed was retinyl acetate and second most used form was retinyl palmitate.

However, due to its high reactivity, vitamin A is particularly susceptible to oxidation and isomerization (Palace, Khaper *et al.* 1999, Ross 2016, Nieva-Echevarría, Goicoechea *et al.* 2017). Degradation of vitamin A can be induced by various factors, such as the presence of oxygen, radical compounds or transition metals. Vitamin A is also thermo- and photosensitive. All these factors impact the stability of vitamin A, leading to a significant reduction in its biological activity. The fortification of food products with vitamin A is therefore very challenging. Different chemical forms of vitamin A are used in the food industry, exhibiting different physicochemical properties and stabilities (retinyl esters are more stable than retinol) (EFSA 2016, Ross 2016). Several strategies for improving vitamin A stability are described in the literature, such as its encapsulation, notably for cosmetology applications (Shields, White *et al.* 2018). However, to the best of our knowledge, no studies have focused on the combined impact of the homogenization process and the chemical forms of vitamin A on its stability in systems representative of foods actually consumed such as IFF. Moreover, the strategies proposed are often costly, which limits their application. A better understanding of the reactivity of vitamin A in emulsion such as IFF is therefore necessary to better control its stability.

The aim of the present study is to investigate the impact of homogenization process and different chemical forms of vitamin A on its stability in model IFF representative of marketed formulas. Therefore, model IFF with normalized lipid profile (equivalent FA profile and vitamin E content) but with various droplet sizes and chemical forms of vitamin A (retinol or retinol esters or carotenoids) were formulated. The vitamin A stability was assessed by evaluating the content of each forms before and after homogenization process and during storage under accelerated conditions.

4.2. Materials and methods

All the experimental materials and techniques are detailed in Chapter 2. In this study, the following model IFF were investigated (see memento side A):

- PO_L and PO_S
- PO_RA_L
- PO_3RA_L
- RPO _L and RPO _S
- DF _L and DF_S

4.3. Results

The aim of this study was to compare the stability of different chemical forms of vitamin A (retinol, retinol esters or carotenoids) in model IFF according to the homogenization process and during storage. For this purpose, model IFF representative of formulas on the world market with similar FA profiles and standardized vitamin E and A contents were formulated. Vitamin A standardization was carried out on total retinoids content. Model IFF presented different chemical forms of vitamin A by varying the fat sources which composed the lipid fraction (palm, red palm oils and dairy fat) or by using different chemical forms for normalization (retinol or retinyl acetate). Vitamin A stability was assessed by measuring the levels of the different chemical forms before and after the homogenization process, and after storage with constant orbital stirring at 110 rpm at 40°C in amber tubes.

4.3.1. Structural and composition normalization of model IFF

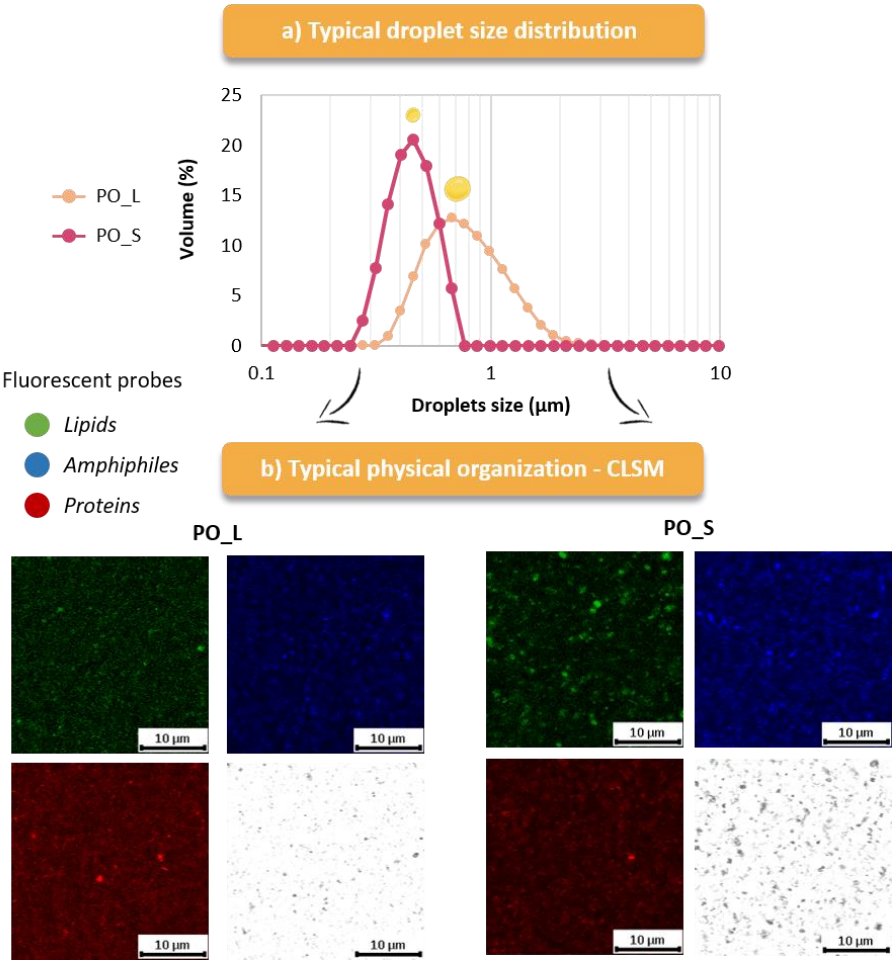
Model IFF were designed to be representative of commercial formulas. Their FA profiles were normalized and characterized in a previous study (Cancalon, Hemery *et al.* 2023). All model IFF had average SFA, MUFA and PUFA contents of 34.0 ± 1.5 %, 45.9 ± 0.4 % and 20.1 ± 1.1 % respectively, including 0.3 ± 0.0 % of DHA and 0.5 ± 0.1 % of ARA. Vitamin E content was also normalized and averaged 751.2 ± 41.7 ppm for all model IFF. Although RPO_ L and RPO_ S contained also in this total γ-tocotrienol representing 161.3 ± 19.6 ppm. In this study, the stability of different chemical forms of vitamin A was studied, and **Table 18** shows the vitamin A profiles of the model IFF. Normalization of vitamin A content was carried out before emulsification on the total content of retinoids (including retinol and retinol esters). Carotenes (α-carotene, β-carotene and 9-cis-β-carotene) were excluded from the standardization, as these provitamins A require bioconversion to retinol in order to be assimilated, which limits their efficacy compared with retinoids (Haskell 2012, Li, Wongsiriroj *et al.* 2014). As result, model IFF had total retinoids contents on average of 12.7 ± 1.4 ppm RE after homogenization, except for PO_3RA_L which there is a retinyl acetate overage leading to an average content of 36.0 ± 2.4 ppm RE. All model IFF contained retinyl acetate, which was provided by VMC and by normalization *via* the addition of retinyl acetate for PO_RA and PO_3RA. Retinyl acetate content was 11.0 ± 0.8 to 14.5 ± 0.9 ppm RE for all model IFF without overages, and 36.0 ± 2.4 ppm RE for PO_3RA in which the impact of vitamin A overage is studied. Retinol was added in PO and RP, 1.9 ± 0.2 ppm RE on average was measured in PO_ L and PO_ S and absent in RPO_L and RPO_S. Depending on the source of fat constituting the lipid fraction, other chemical forms of vitamin A were present. Dairy-based model IFF (DF_L and DF _S) contained on average 3.0 ± 0.6 ppm RE of retinyl palmitate. Red palm oil contains high levels of endogenous carotenoids but no retinoids. RPO therefore contained three forms of carotenoids: β-carotene, 9-cis-β-carotene and α-carotene, with average contents of 25.7 ± 1.2, 17.9 ± 0.9 and 5.3 ± 0.3, respectively.

Table 18. Oil phase composition, levels of vitamins (vitamin A and tocopherols) fortification and normalization and initial contents of the different chemical forms of vitamin A

	PO_L	PO_S	PO_RA_L	PO_3RA_L	RPO_L	RPO_S	DF_L	DF_S
Oil phase omposition (%w/w)								
Palm oil	67.8	67.8	67.8	67.8	-	-	-	-
Red palm oil	-	-	-	-	71.0	71.0	-	-
Dairy fat	-	-	-	-	-	-	55.3	55.3
Rapeseed oil	18.4	18.4	18.4	18.4	15.2	15.4	12.6	12.6
Sunflower oil	9.1	9.1	9.1	9.1	12.0	12.0	12.6	12.6
High oleic sunflower	3.1	3.1	3.1	3.1	-	-	11.1	11.1
ARA oil	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
DHA oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Soy lecithin	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Vitamin fortification (mg/L)								
VMC	200	200	200	200	200	200	200	200
Retinol	0.11	0.11	-	-	0.11	0.11	-	-
Retinyl acetate	-	-	0.14	0.28	-	-	-	-
Tocopherol	9.12	9.12	9.12	9.12	9.10	9.10	6.06	6.06
Initial vitamin E content (ppm of lipids)								
Total vitamin E	740.0 ± 18.6	710.5 ± 17.7	828.3 ± 112.5	803.1 ± 34.1	726.7 ± 51.3	707.6 ± 25.4	764.9 ± 19.6	728.8 ± 54.4
γ-tocotrienol	-	-	-	-	170.2 ± 31.8	152.4 ± 7.3	-	-
Initial vitamin A content (ppm RE of lipids)								
Total vitamin A	14.3 ± 1.0	13.2 ± 0.7	14.5 ± 0.9	36.0 ± 2.4	63.4 ± 2.4	58.9 ± 0.8	12.8 ± 1.8	12.7 ± 0.9
Retinol	2.0 ± 0.20	1.8 ± 0.1	-	-	0.0 ± 0.0	0.0 ± 0.0	-	-
Retinyl acetate	12.3 ± 0.8	11.4 ± 0.6	14 .5 ± 0.9	36.0 ± 2.4	12.7 ± 0.5	11.8 ± 0.4	11.1 ± 1.3	11.0 ± 0.8
Retinyl palmitate	-	-	-	-	-	-	1.6 ± 0.5	1.7 ± 0.1
Carotenoids	-	-	-	-	50.7 ± 1.9	47.1 ± 0.5	-	-
α-carotene	-	-	-	-	18.5 ± 0.8	17.3 ± 0.1	-	-
β-carotene	-	-	-	-	26.7 ± 0.8	24.7 ± 0.2	-	-
9-cis-β-carotene	-	-	-	-	5.5 ± 0.3	5.1 ± 0.2	-	-

ppm RE correspond to µg retinol equivalent/g of lipids.

The structure of the model IFF was also standardized. The typical droplet size distribution and structure of PO_ L and PO_ S at the initial point of storage (D0) assessed by granulometry and CLSM, respectively (**Figure 30**). All model IFF had a similar monomodal droplet size distribution centered on 0.4 or 0.7 µm, depending on the homogenization process applied. CLSM observations showed a similar structural organization in all model IFF, with a core of non-polar lipids stabilized by proteins and amphiphilic compounds.



Particles size distribution was assessed by laser light scattering.

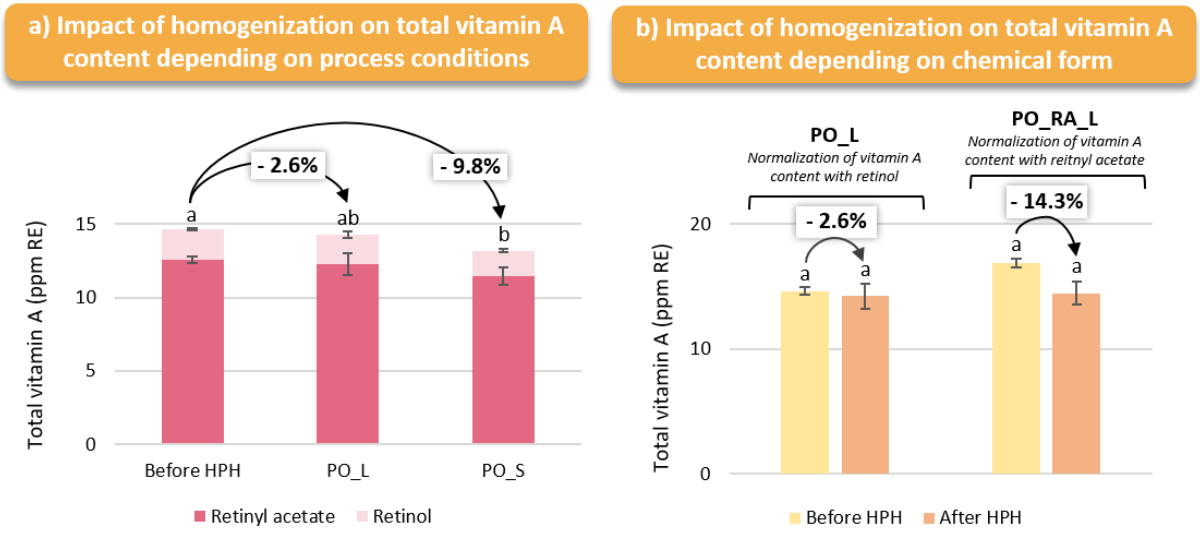
Confocal laser scanning micrograph were collected using a 40× water-immersion objective with each time four micrographs: blue colored marks proteins; red colored marks amphiphiles compounds, green colored marks lipids and white micrograph corresponds to transmission light.

Figure 30. Structural characterization by a) droplet size distribution and b) CLSM observations

4.3.2. Impact of homogenization process conditions on vitamin A stability and its chemical forms

In order to evaluate the impact of the homogenization process on vitamin A stability, total retinoids levels in PO were determined before and after two different homogenization processes leading to droplet sizes of 0.4 or 0.7 µm (**Figure 31.a**). The results showed a non-significant loss of 2.6% in total retinoids for the homogenization process applied to obtain large droplets. On the other hand, for the homogenization process leading to small droplet size, this loss was significant and represented 9.8% of retinoids loss.

The impact of the chemical form of vitamin A was assessed during the homogenization process leading to large droplets and was not significant (**Figure 31.b**).



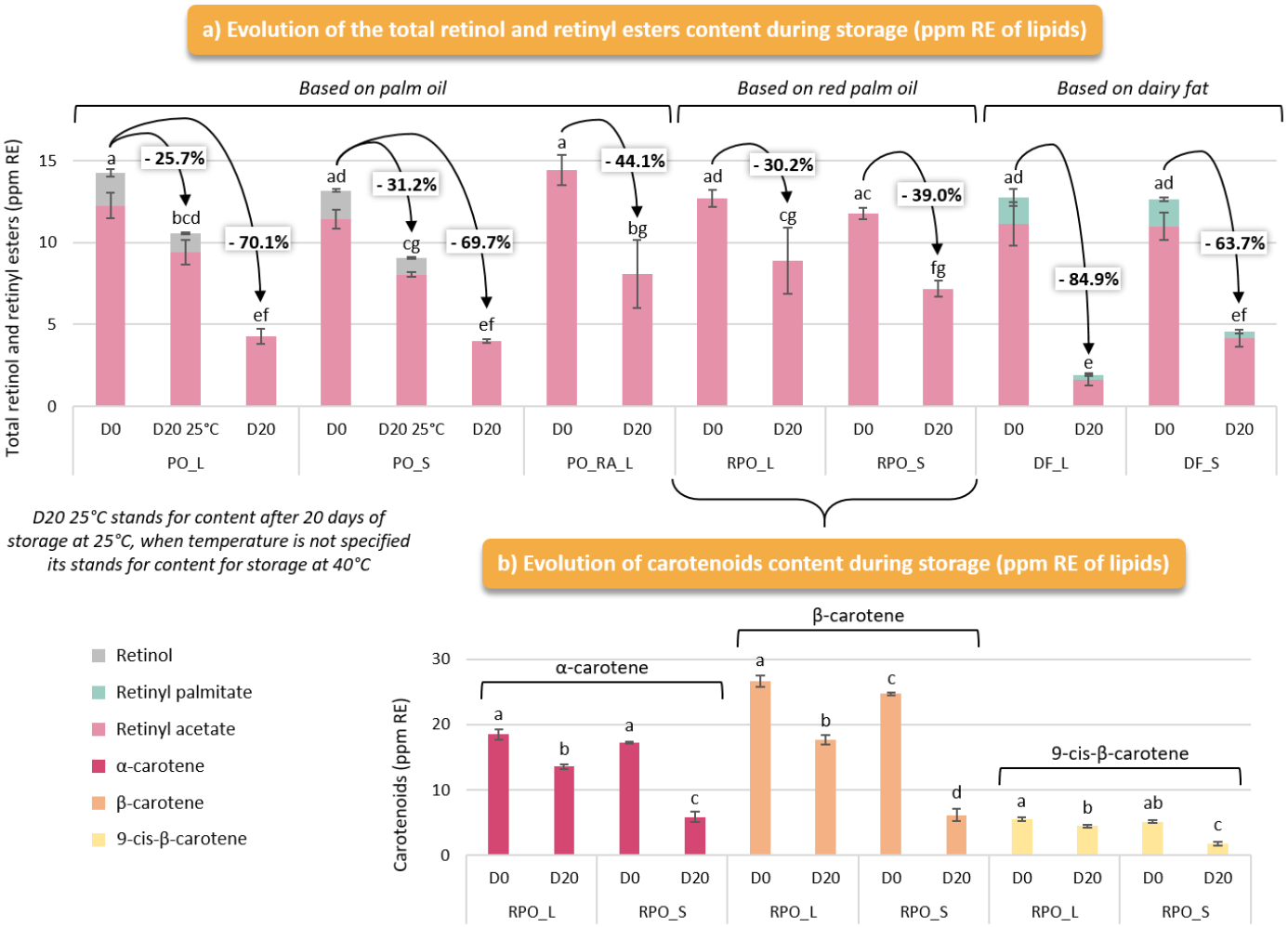
The percentages in the boxes correspond to the loss percentage of vitamin A between before and after the homogenization process (eight cycles at 100/30 bars leading to a droplet size of 0.7 µm). Different letters indicate a significant difference (P<0.05) between model IFF at a given time. ppm RE correspond to µg retinol equivalent/g of lipids.

Figure 31. Impact of the homogenization on total vitamin A content depending on a) process conditions and b) vitamin A chemical form

4.3.3. Impact of chemical form on vitamin A stability during storage

Depending on the fat constituting the lipid fraction, the chemical forms of vitamin A may vary. The impact of the vitamin A profile during storage was assessed by comparing initial vitamin A contents after 20 days in model IFF with different fat compositions. Palm-oil-based model IFF with total retinoids content normalized by the addition of retinol were stored at 25 and 40°C (**Figure 32.a**). Vitamin A losses were more than twice as high, whatever the droplet size, for the model IFF stored at 40°C. These losses were 70.1 and 69.7% in PO_ L and PO_ S, respectively. After 20 days of storage, all retinol was lost in both model IFF. For an equivalent droplet size, normalization of the palm oil-based model IFF with retinyl acetate led to significantly lower losses than with normalization using retinol (44.1 *versus* 70.1% of loss for PO_RA_L and PO_ L, respectively). The introduction of red palm oil into the lipid fraction led after 20 to losses of 30.2 and 39.0% of retinyl acetate (retinoic fraction composed only of retinyl acetate). These losses were significantly lower than in the case of palm-oil-based model IFF using retinol normalization, but equivalent when retinyl acetate normalization was used. Total retinoids contents in RPO_ L and RPO_ S were normalized prior to emulsification by the addition of retinol, however, at the initial point this chemical form of vitamin A was absent whatever the droplet size considered. This suggests that retinol was lost during the homogenization process in in RPO_ L and RPO_ S. Red palm oil is an important source of carotenoids. Thus, three compounds belonging to the

carotenoid class were present in RPO_ L and RPO_ S (**Figure 32.b**). β-carotene was the most abundant, accounting on average for 53.5% of total carotenoids. α-carotene and 9-cis-β-carotene were also present, averaging 38.5 and 8.0% of total carotenoids respectively. The results show significant losses in all three carotenoids during storage. These losses were on average 2.7 times higher for model IFF with a droplet size of 0.4 µm compared to 0.7 µm droplets. Losses in β-carotene were highest, 33.8% for RPO_ L *versus* 75.1% for RPO_ S. The use of dairy fat led to the presence of both retinyl acetate (86.7% of total retinoids) and retinyl palmitate (12.9% of total retinoids) in DF_L and DF_ S. The results show that losses of 84.9 and 63.7% of total retinoids occurred during storage for DF_ L and DF_ S, respectively. These losses were not significantly different according to droplet size.

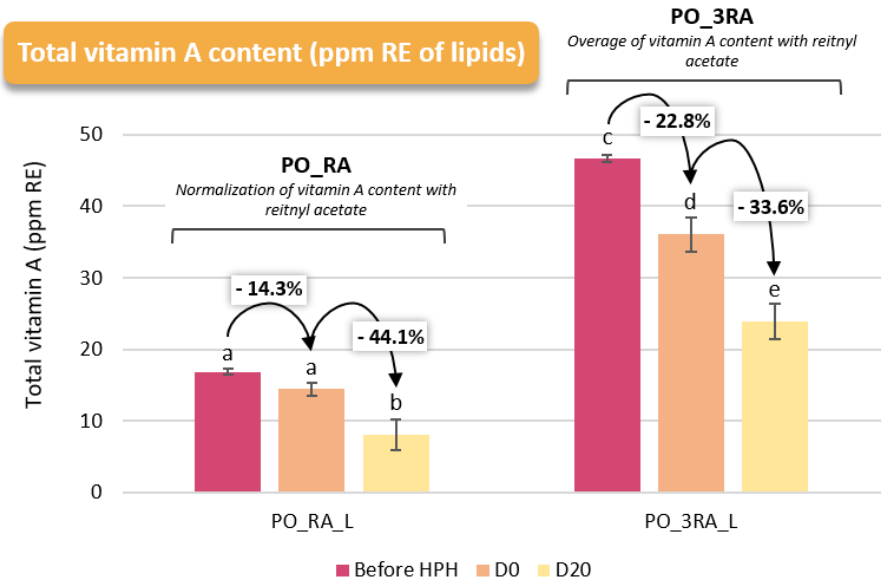


The percentages in the boxes correspond to the loss percentage of vitamin A during the storage at 40°C.
Different letters indicate a significant difference (P<0.05) between model IFF at a given time.
ppm RE correspond to µg retinol equivalent/g of lipids.

Figure 32. Impact chemical forms of vitamin A on the evolution of a) the total retinoids content and b) carotenoids content during 20 days of storage

4.3.4. Impact of overage of vitamin A during process and storage

The impact of vitamin A acetate overage on its stability during the homogenization process leading to small droplets and storage was assessed (**Figure 33**). Results showed a significant vitamin A loss of 22.8% during the homogenization process for PO_3RA_L, which had three times the total vitamin A content (exclusively as retinyl acetate) compared to PO_RA_L. Without overage not significant loss of total vitamin A was observed during the homogenization process. After 20 days of storage, an important loss of vitamin A was observed in both model IFF (44.1% for PO_RA_L and 33.6% for PO_3RA_L) but these losses were not significantly different.



The percentages in the boxes correspond to the loss percentage of vitamin A between before (before HPH) and after (D0) the homogenization process (eight cycles at 100/30 bars leading to a droplet size of 0.7 µm) and during the storage at 40°C (D20).

Different letters indicate a significant difference (P<0.05) between model IFF at a given time.

ppm RE correspond to µg retinol equivalent/g of lipids.

Figure 33. Impact of overage of vitamin A as form of retinyl acetate on the total vitamin A content stability during homogenization process and storage in accelerated conditions

4.4. Discussion

Results presented in the current study showed significant vitamin A losses in the model IFF studied. The homogenization process leading to 0.7 µm droplets had a limited effect on stability. Stability during storage was improved for model IFF normalized with retinyl acetate and in the presence of carotenoids. Carotenoid stability was strongly dependent on droplet size. In contrast, an overage of vitamin A had a limited effect on stability both during processing and storage.

4.4.1. Homogenization process can lead to vitamin A loss

Several studies have shown that encapsulating vitamin A in emulsion systems such as the model IFF in our study leads to improved stability. For example, Tanglao, Kumar *et al.* (2019) showed that the stability of retinyl acetate increased when it was incorporated into a coconut oil-in-water emulsion stabilized by whey protein. Our results showed that for model IFF containing both retinol and retinyl acetate, the homogenization process using high pressures (resulting in small droplet size of 0.4 µm) led to a significant vitamin A loss. A gentler homogenization process (droplet sizes 0.7 µm) showed no effect on post-process vitamin A content. These results suggest that the higher the homogenizing force and concomitantly the smaller the droplet size, the higher the percentage loss of vitamin A during the process. This phenomenon would very likely be due to more important shearing forces leading to local heating of molecules. As described by Sachdeva, Kaushik *et al.* (2021) in their study focusing on the impact of processing conditions on the stability of native vitamin A and retinyl acetate in fortified milk, vitamin A is thermolabile. Indeed, by comparing the effects of three thermal treatments - pasteurization, sterilization and boiling - the authors showed that exposure to temperatures above 63°C (the temperature used for pasteurization) resulted in significant vitamin A losses. However, our results partially disagree with Banasaz, Morozova *et al.* (2021 and 2022) who observed that vitamin A stability increased with homogenization pressure (up to 2,000 bars) and α- tocopherol concentration (maximum effect for the highest of the concentrations tested, *i.e.* 1,000 ppm oil). The authors hypothesized that increasing pressure and concomitantly decreasing droplet size led to more effective interface whey protein coverage, creating a steric barrier that would limit contact between vitamin A, and pro-oxidant or oxidized compounds. At 200 MPa and above, on the other hand, the authors showed a decrease in vitamin A stability, explained by a rise in temperature and more difficult adsorption of whey protein at the newly formed interface. Other studies agree with our results and suggest that stronger homogenization processes lead to chemical instability of the systems. For example, it was observed that an increase in specific surface area during homogenization processes leads to greater exposure to pro-oxidant compounds and can accelerate oxygen uptake during cavitation and local heating due to shear forces (Atarés, Marshall *et al.* 2012, Valencia-Flores, Hernández-Herrero *et al.* 2013). As described in a previous work (Cancalon *et al.* [Under review]), the impact of droplet size alone is difficult to predict in such complex systems and is also linked to the chemical environment (Lethuaut, Métro *et al.* 2002).

4.4.2. Chemical forms of retinyl esters is a key lever of vitamin A stability

Our results showed that vitamin A loss kinetics were dependent on the chemical forms of retinoids present in model IFF. Retinol was totally degraded after the homogenization process in RPO _L and RPO_ S and after 20 days of storage at 40°C in PO_ L and PO_ S, while retinyl acetate alone or in

combination with carotenoids showed improved resistance. These results suggest that the ester form of retinol improves its stability toward oxidation (Ihara, Hashizume *et al.* 1999, Ross 2016). Indeed, esterification increases the hydrophobic character of vitamin A and determine its localization (droplet core or interface) in the emulsion (Laguerre, Bayrasy *et al.* 2015). Retinyl acetate and palmitate, being more hydrophobic (logP = 6.3 and 13.6, respectively), may be localized at the core of the droplet, while retinol, less hydrophobic (log kow = 5.7), is located closer to the interface, where its contact with oxidizing species (mainly pro-oxidant metals) is maximized. The addition of acetate in combination with carotenoids led to an improvement in stability attributed to the antioxidant capacity of carotenoids as discussed below. On the other hand, the combined addition of retinyl acetate and palmitate led to significant losses, but in this case palmitate form represented only 12.9% of total retinoids. Complementary tests using palmitate form only in these systems would be interesting but were not prioritized as the main target was to complement endogenous forms in the oil mixture with the most frequently form of retinyl esters used to fortify IFF on the market, *i.e.* retinyl acetate. Data on breast-milk in which palmitate form is predominant gives good indication of the fair stability of this form in such systems (Dror and Allen 2018).

4.4.3. The presence of carotenoids improves total vitamin A stability

Our results showed that in the presence of carotenoids, the stability of retinyl acetate is improved (losses below 40%), suggesting that these compounds exert antioxidant activity. Similar results were shown by Heinonen, Haila *et al.* (1997) concerning the effect of β-carotene individually or in combination with tocopherols (α and γ isomers) on autoxidation in a 10% oil-in-water emulsion. The authors demonstrated an antioxidant effect of β-carotene in the presence of α-tocopherol. On the other hand, when added individually to the emulsion, β-carotene had a pro-oxidant effect (at 0.45, 2 and 20 ppm), characterized by a faster increase in the PV and higher levels of volatile compounds (hexanal and 2-heptenal in particular). The addition of α-tocopherol inhibited the antagonistic effect of β-carotene, developing an antioxidant synergy between the two compounds. Schroeder, Becker *et al.* (2006) also demonstrated an increase in the oxidation induction phase in the presence of 1,000 and 100 ppm of α-tocopherol and β-carotene, respectively. Various hypotheses have been put forwards in the literature to explain these results, such as a singlet oxygen quenching or free radical scavenging effect of β-carotene or promoting regeneration of radicals back to their active form in the presence of tocopherol has also been proposed. (Dimakou and Oreopoulou 2012) showed that depending on the polarity of the carotenoids (xantophylls *versus* carotenes) and the type of emulsifier, antioxidant activity could be inhibited. Thus, in protein-based emulsions, only polar carotenoids exerted antioxidant activity, limiting the formation of oxidation products. Several hypotheses could explain these observations, such as the distribution of carotenoids between the core of the droplets or at the

interface, the site of oxidation where molecules are in direct contact with the pro-oxidant compounds and would therefore be more effective. This hypothesis would also explain the higher consumption of polar carotenoids in their systems. The hindering of the radical scavenging activity of β-carotene by the properties of the protein based interface could also explain this finding. The antioxidant activity of carotenoids therefore depends on numerous parameters, and explains some of the discrepancies in the literature. It has been reported, for example, that droplet size also has an effect. As observed in the present study, whatever the carotenoid considered, losses were on average 2.7 times more important for 0.4 µm droplets than for 0.7 µm droplets. These results indicate that the smaller the droplet size, the higher the consumption of carotenoids to limit retinoids losses (no significant differences in retinyl acetate losses between RPO _L and RPO_S). Similar results are described by (Zhang and Li 2021) which showed that the loss of β-carotene during storage at 37°C was more important for smaller droplet size. These observations could be due in particular to an increased specific surface area for a decreased size, thus increasing contact with pro-oxidant compounds. In addition to modulating its stability, chemical environment impacts the bioaccessibility of carotenoids. (Meroni and Raikos 2018) showed through an *in vitro* digestion model that an emulsion rich in long-chain FA improved the solubilization of β-carotene in mixed micelles thus increasing its bioaccessibility (by up to 30%). The bioaccessibility of carotenoids, ranging from 23.5 to 47.5%, remains limited compared to other forms of vitamin A, and requires a minimum of 1.55% fat to be micellarized. IFF such as the one studied in the present study are suitable for providing carotenoids. Indeed, despite the need for carotenoids with provitamin A activity to be bioconverted into retinoic form, supplementation with these compounds has proved effective against vitamin A deficiency by helping to raise serum retinol and β-carotene levels (Dong, Xia *et al.* 2017).

4.4.4. Vitamin A overage has a limited effect on stability

Due to the high sensitivity of vitamin A to oxidative degradation, overages leading to a content above the upper recommendation limits are common, and are intended to compensate for possible storage losses (Moustiés, Bourlieu *et al.* 2019). Regulations therefore impose content ranges, from 42 to 68 µg/100 mL in European countries. The results of the present study showed that retinyl acetate overaging of 115.2 µg RE/100 mL in PO_3RA led to a more important loss during processing and had no additional beneficial effect during storage, compared to model IFF without overaging (equivalent losses after 20 days in PO_RA_L and PO_3RA_L). Similar results have been shown in cosmetic products, where higher concentrations of retinoids had no stabilizing effect and could even lead to an increase in sensitivity to degradation (Temova Rakuša, Škufca *et al.* 2021). Overages expose infants to the risk of upper intake leading to hypervitaminosis A. Many adverse effects are associated with vitamin A overconsumption including tetragenecy. The tolerable upper intake level of preformed vitamin A has

been set by EFSA (European Food Safety Authority) at 800 µg RE/day for infants aged 1-3 years (EFSA 2006). A two-fold overage as studied in PO_3RA_L, exposes infant to a daily retinyl acetate dose of 576 to 806 µg RE, considering only infant formulas intake (volume between 6 and 12 months of age of 500 to 700 mL/day). However, the second age period is characterized by the beginning of dietary diversification, with the gradual introduction of complementary foods that can also be carriers of vitamin A. As a result, the daily intake of vitamin A is further increased, concomitantly increasing the risk of overaging. Moreover, the oxidation products of vitamin A are not yet fully understood, but could have possible adverse effects. Better control of stability in such a food matrix seems to be more favorable and should be favored over the practice of overaging.

4.5. Conclusion

The results of this study showed that the chemical forms of vitamin A in model IFF had an impact on its stability during both processing and storage. An increase in homogenization pressure resulted in a significant loss of vitamin A, particularly retinyl acetate. Analyses performed after the storage test showed that the presence of carotenoids improved overall vitamin A stability. The antioxidant activity of provitamin A such as β-carotene can be associated with its anti-free radical activity and its synergy with tocopherols. The physico-chemical properties of the different forms of vitamin A also impacted its stability, with retinyl acetate showing greater resistance during storage than retinol. This study highlights that despite the important sensitivity of vitamin A, a good knowledge of its reactivity during processing and selection of chemical form and potential synergy with other compounds can improve its stability in IFF. In this way, vitamin A overage, which have shown no significant additional beneficial effects, can be avoided.

MAIN RESULTS

- A gentle homogenization process leading to droplet size in the micrometer range has a limited effect on vitamin A loss
- Retinyl acetate has superior stability during the homogenization process and storage than retinol in infant formulas
- The presence of carotenoids in infant formulas improves vitamin A stability
- Carotenoid stability depends on lipid droplet size (0.4 or 0.7 µm)
- Vitamin A overage has no additional beneficial effect and may have adverse effects

5. Comparison of model IFF oxidative stability with commercial IFF

The results obtained in this chapter showed relatively good overall stability of model IFF, with the possibility of optimization by modulating the formulation parameters. However, the model IFF studied are representative in terms of macronutrient composition of the commercial products but have a simplified aqueous phase in which the oxidation-initiating compounds are located. In order to validate the results, four commercial IFF were selected from the panorama and subjected to a storage test under the same conditions as the model IFF, *i.e.* 20 days at 40°C with constant agitation at 110 rpm. The aim was to compare the oxidative stability of the model and commercial IFF. The commercial IFF were selected according to the composition of their lipid phase. Thus, LIFF_A includes palm oil which corresponds to PO-based model IFF, LIFF_G includes coconut oil which corresponds to CO-based model IFF and LIFF_C and LIFF_L include dairy ingredients (whole milk or cream, respectively) which is representative of DF-based model IFF. To our knowledge, no commercial IFF include red palm oil in their composition. To assess the oxidative stability of commercial IFF, the same markers indicating the level of oxidation were monitored used for model IFF were evaluated (including PV, TBARS and FA profile evolution, and vitamins A and E loss).

Initial characterization of the commercial IFF showed total tocopherol contents ranging from 655.2 ± 10.1 to 846.3 ± 26.9 ppm with α-tocopherol as the main isomer, representing more than 50% (**Table 19**). FA profiles were relatively similar across these IFF, with DHA representing 0.75% of total FA on average. Initial PV were low averaging 2.5 meqO_2/kg.

Table 19. Initial characterization (FA profiles, vitamins A and E content and PV) of the commercial IFF

	LIFF_A	LIFF_G	LIFF_C	LIFF_L
Vitamin E content (ppm or µg/g of lipids)				
Total	660.0 ± 14.5	668.2 ± 113.0	846.3 ± 26.9	655.2 ± 10.1
α-tocopherol	459.9 ± 6.8	473.4 ± 79.2	497.4 ± 11.9	524.4 ± 8.2
Vitamin A content (mg RA/L)				
Retinyl acetate	17.6 ± 0.6	20.2 ± 7.1	15.2 ± 1.6	13.3 ± 0.6
Fatty acid (% w/w)				
Total lipids content	3.2	3.2	3.3	3.1
SFA	25.7 ± 0.3	27.6 ± 1.3	23.6 ± 0.4	38.3 ± 0.5
MUFA	52.9 ± 0.4	51.1 ± 1.5	56.6 ± 0.4	42.6 ± 0.4
PUFA	21.4 ± 0.1	21.4 ± 0.2	19.8 ± 0.0	19.1 ± 0.1
LA C18:2 ω6	18.2 ± 0.1	18.3 ± 0.2	16.8 ± 0.0	15.9 ± 0.1
ALA C18:3 ω3	1.9 ± 0.0	1.8 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ARA C20:4 ω6	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DHA C22:6 ω3	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
ω6/ω3	6.6	6.8	5.7	5.5
Initial characterization				
Peroxide value (meqO_2/kg of lipids)	2.5 ± 0.2	2.7 ± 0.4	2.3 ± 0.3	2.4 ± 0.3

The results showed stable PV of less than 3 meqO_2/kg up to the sixth day of storage for all the commercial IFF studied (**Figure 34.a**). An increase in PV was observed after nine days of storage but was nevertheless relatively low, indicating that oxidation levels in commercial IFF were not very advanced. After 20 days of storage, LIFF_G exhibited the highest PV value of all IFF and reached 10 meqO_2/kg. These results are similar to those obtained for model IFF. However, the TBARS measurement showed an increase in secondary oxidation compounds after 20 days of storage in the commercial IFF that had not been observed in the model IFF (**Figure 34.b**). The greater complexity of the mineral and protein phases in commercial IFF could explain this discrepancy in results. Changes in fat-soluble vitamins content were also assessed. Losses ranging from 32 and 71% of total tocopherols were observed depending on the IFF (**Figure 34.c**). LIFF_C, which includes dairy lipids in its composition, had the lowest tocopherol loss rate of all the commercial IFF studied. The tocopherol isomer distribution showed that the α-isomer was in the majority, followed by the γ-isomer. Results on model IFF showed that the higher the γ-tocopherol content, the better the overall stability of the system. Finally, the evolution of retinyl acetate content showed losses of 59 to 73% depending on the IFF after 20 days of storage (**Figure 34.d)**. LIFF_C and LIFF_L which include dairy lipids in their composition, had the lowest loss rates, which had also been demonstrated in the case of model IFF.



Figure 34. Evolution of a) PV, b) TBARS, c) tocopherols loss and isomer distribution and d) retinyl acetate content in commercial IFF over storage at 40°C

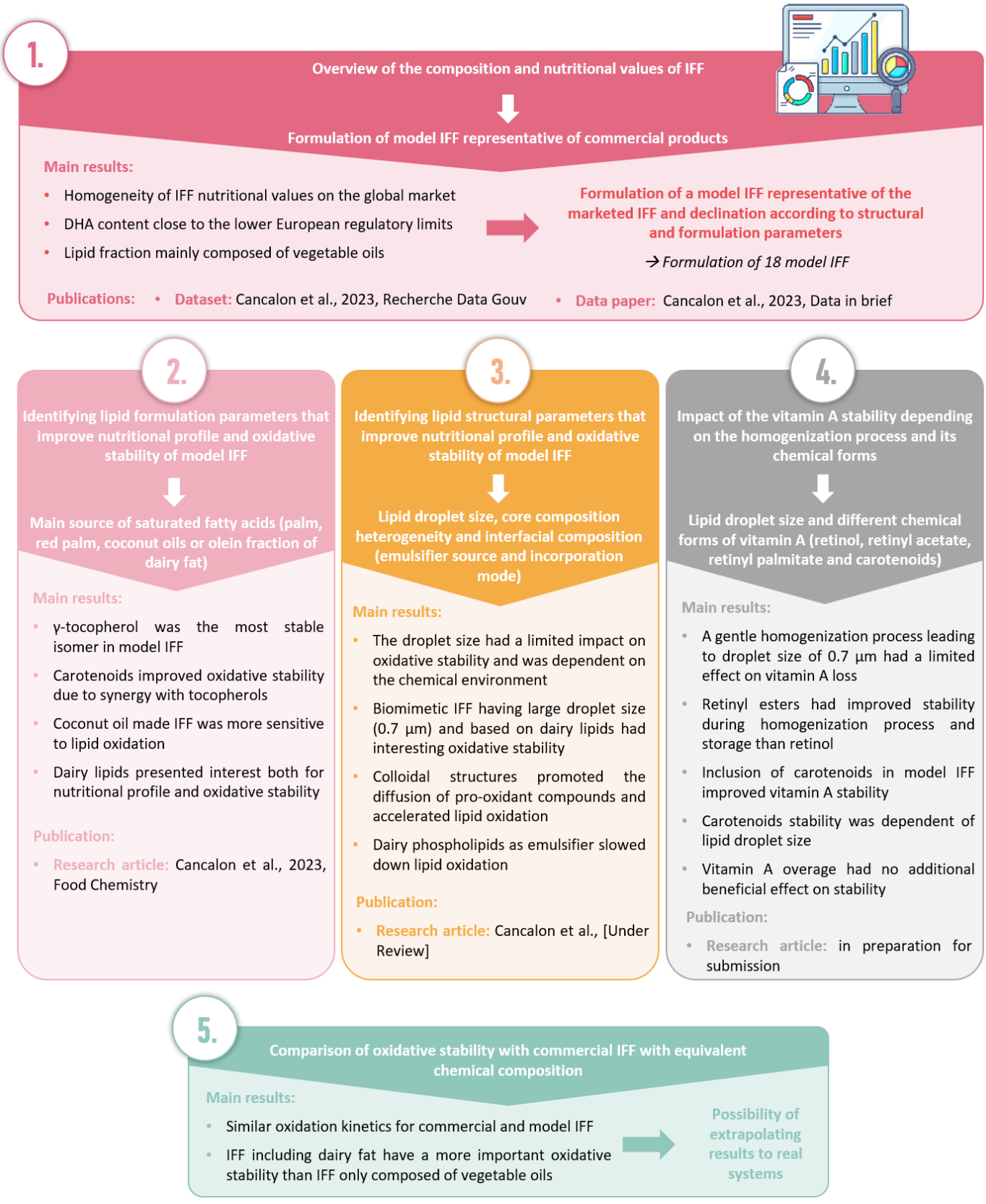


Figure 35. Main results from chapter 3



CHAPTER 4

Oxidative stability of infant flours

In chapter 3, we have demonstrated that the composition and organization of lipids in IFF can be modulated to achieve improved oxidative stability. These complex degradation mechanisms are dependent on the considered matrix. To further understand these mechanisms in other common staple food used for infant nutrition, the stability of IF was characterized thus providing a focus on dry granular systems. In this context, the present chapter is dedicated to presenting the results related to the study of the oxidative stability of dry dispersed systems representatives of IF (**Figure 36**). Similarly to the first chapter on IFF, the first step involved compiling ingredients lists and nutritional values of marketed IF which confirmed a major issue already observed in previous work (Moustiés, Bourlieu *et al.* 2019): an imbalance in the lipid profile and overages in micronutrients. The second step aimed to formulate representative IF based on those present on the market and to perform a physical and biochemical characterization of the most commonly used flours in IF formulation. These IF were then submitted to a storage test and their lipid profile were optimized by introducing sources of ω3 FA. The optimized IF was declined into several systems according to structural (particles size) and formulation (addition of LC-PUFA and antioxidant fractions) parameters to screen different strategies that would conduct to maintain an acceptable oxidative stability with a balanced lipid profile.

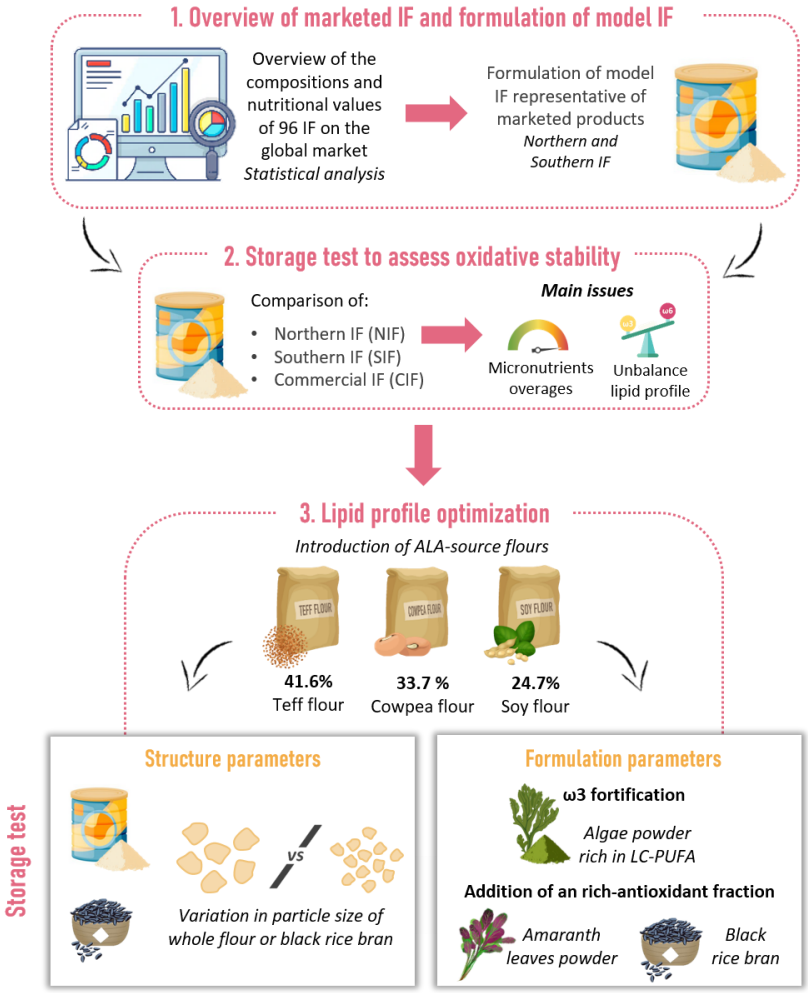


Figure 36. Strategy of chapter 4

1. State of the art of marketed infant flours

1.1. Panorama of infant flours composition and nutritional values

Dietary diversification, which starts between 4 and 6 months, is a pivotal period in infant development. During this period, complementary foods such as IF are progressively introduced. IF are therefore widely consumed throughout the world, particularly in southern countries. In order to identify a typical IF composition a market analysis was carried out.

This section is partly based on a dataset entitled: "Overview of the nutritional composition of infant flours commercialized worldwide in 2021" published in the repository Dataverse INRAE and a data paper entitled: "Dataset of the nutritional composition of infant flours commercialized worldwide in 2021" submitted for publication in Data in Brief journal (Cancalon, Hemery et al. 2023).

The ingredients list and nutritional values of marketed IF were collected from December 2020 to April 2021, with direct collection on the product packaging or by extracting the information from brands website or research articles. The dataset exclusively includes processed cereal-based foods, either single or mixed cereals that require reconstitution with milk or other appropriate liquids, as well as cereals with added protein-rich food that require reconstitution with water or protein-free liquids. In total, data collection includes 96 IF. After data collection, each IF was anonymized by assigning a unique product code. The data were then structured to form the dataset, with standardized lists of ingredients to maintain anonymization and present the information consistently in the same format. The raw material sources were also extracted from the ingredient lists. As the data included in the dataset are subject to change, particularly in case of regulatory changes, they must therefore be regularly updated.

Value of the data

- These data hold value for companies and researchers specializing in infant nutrition.
- The dataset serves as a valuable tool for the formulation of IF.
- Our data can be used as a reference point for companies or intervention programs aiming to enhance the composition of IF.
- Companies can leverage our data to assess their products positioning in the market and compliance with regulations.
- The dataset facilitates the identification of commonly used raw materials involved in the IF formulation and potential micronutrient overages.
- These data can be used to estimate the contribution of each ingredient to the daily nutritional intake.

The dataset is provided as a text file (.txt) and four Excel Spreadsheet (.xlsx) files, each including one sheet:

The ***Summary.txt*** file provides general information. The first point outlines the dataset objective, as detailed in the "Aim of the dataset" section. The second point covers the criteria used to include data, which are listed in the "Selection criteria" section. Lastly, the "Data collection method" section expounds on the approach used to collect the data. Additionally, the sheet contains supplementary information, including an abbreviations list and specificities regarding the list of ingredients (may not necessarily follow a decreasing order based on weight).

The ***Nutritional values.xlsx*** file provides comprehensive data about ingredient lists and their corresponding nutritional values per 100 g of powder or 100 kcal. This includes details on energy, lipids with details on their content in SFA, carbohydrates, and proteins contents. Additionally, the sheet presents information on the contents in vitamins A, C, D, E and minerals such as iron and zinc. Empty boxes indicate unavailable values. The sheet organization follows the structure outlined in **Figure 37**.

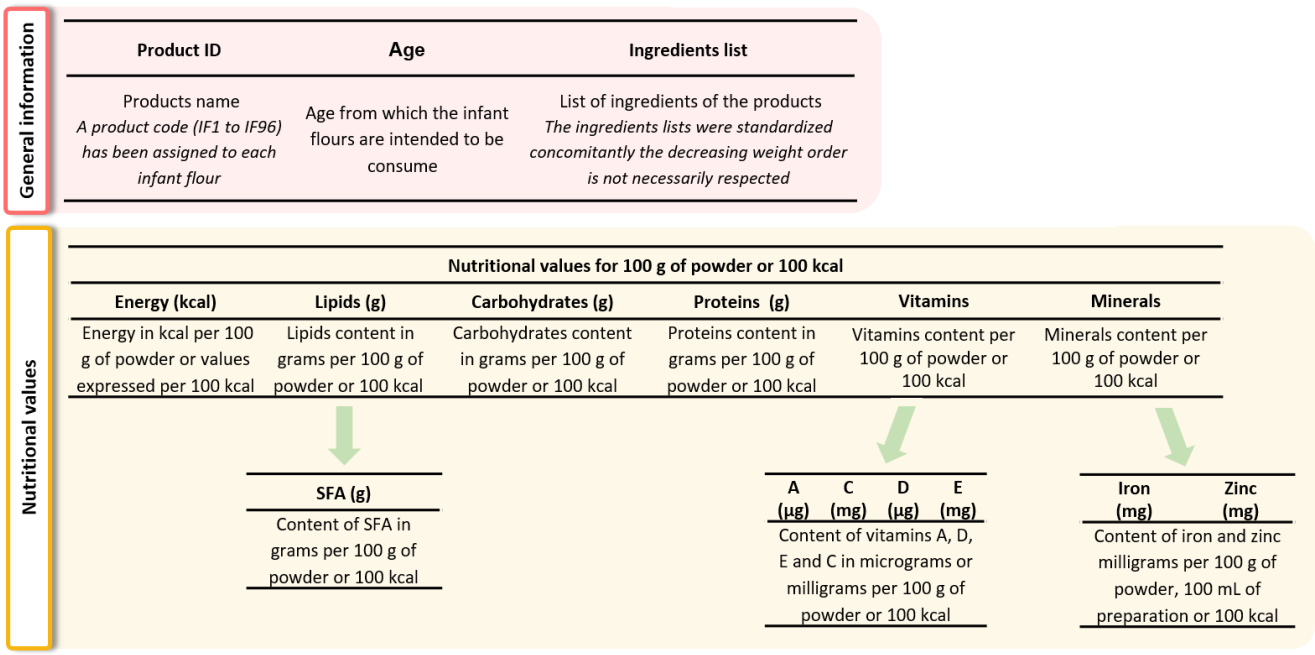


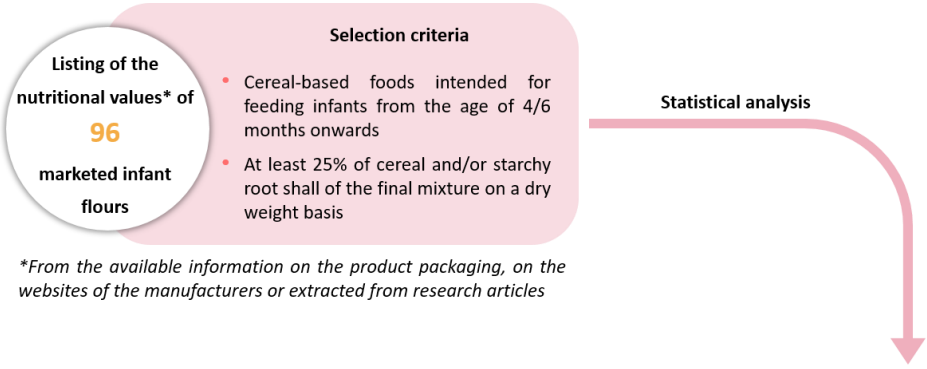
Figure 37. Organization and information provided in the “Nutritional values.xlsx” file

The ***Overview.xlsx*** file provides a summary of the data, including the number of available values on product labels, websites or extracted from research articles or reports. It also includes the average values and their standard deviations, as well as the maximum and minimum nutritional values per 100 g of powder or 100 kcal.

The ***Raw materials.xlsx*** file provides a list of ingredients used in the collected IF composition, along with the corresponding percentage of flours that incorporate each of these ingredients in their formulation.

The ***Example of regulation.xlsx*** file provides non-exhaustive information concerning the specificities about processed cereal-based foods and the range of macro and micronutrients governed by the European regulation (EU regulation 2006/125/CE).

Figure 38 presents a summary of the nutritional values of the 96 IF included in the dataset.



Energy (kcal)	Fat (g)	SFA (g)	Proteins (g)	Carbohydrates (g)	Vitamins				Iron (mg)
					A (µg)	D (µg)	E (mg)	C (mg)	
Number of available value									
91	91	51	91	91	26	25	26	37	59
Mean values ± SD									
400.4 ± 23.6	5.6 ± 3.4	1.3 ± 1.2	11.8 ± 3.2	74.6 ± 7.2	450.2 ± 141.8	7.5 ± 1.4	5.1 ± 1.2	52.2 ± 13.3	16.9 ± 11.1
Minimal values									
94.0	0.2	0.2	2.6	13.0	97.0	1.5	2.6	9.2	2.1
Maximal values									
478.1	18.8	7.1	22.2	90.4	1039.0	11.0	11.0	90.0	83.3

Figure 38. Overview of the nutritional values of IF available on the global market

1.2. Infant flours nutritional values variability

The amount of information available for this dataset was much less compared to IFF. Statistical analysis of the nutritional values of the IF showed considerable variability in macro- and micronutrient content, characterized by large standard deviations and wide gaps between minimum and maximum values. For example, lipid content varied from 0.2 to 18.8 g/100 g IF. The high variability was also highlighted by a principal component analysis (PCA). This multivariate statistical method aimed to visualize the relationships between variables and similarities between individuals. 74.6% of the variance was explained by the first two principal components, however, no IF group could be distinguished (**Figure 39**).

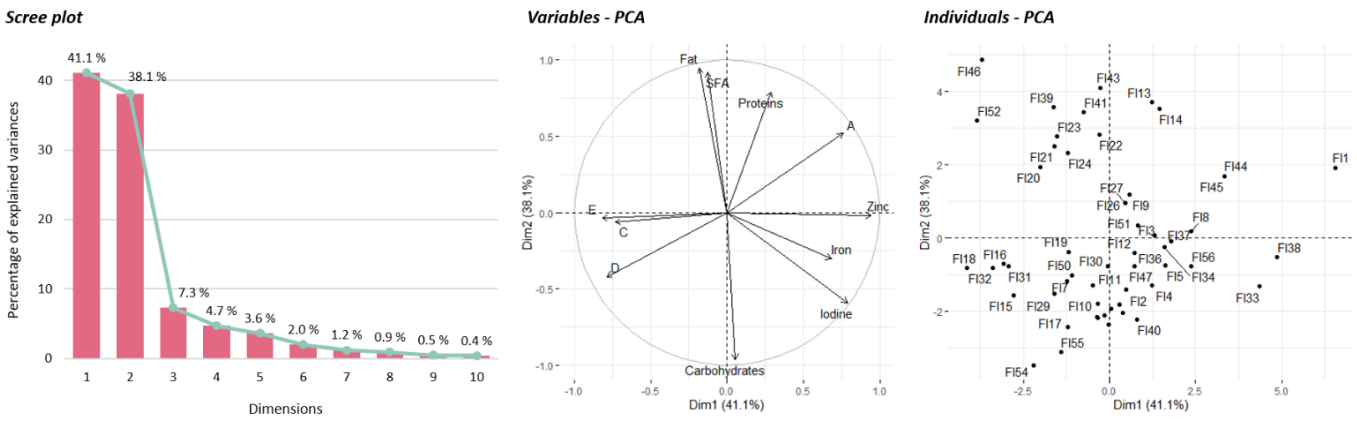


Figure 39. Principal component analysis of IF nutritional values from the dataset

In order to explain this variability, IF were separated into two groups according to their geographical area of production and consumption: southern and northern IF, which therefore refer to IF produced and marketed in the southern or northern regions, respectively. However, the variability in nutritional value was not explained by a geographical factor and remained important. The segregation of IF into two groups has highlighted a significant difference in energy density between southern and northern IF with an average of 421.0 nd 387.9 Kcal/100 g for Southern and Northern IF, respectively.

Table 20. Comparison of the average nutritional values of IF with the threshold values imposed by European and international regulations

	Infant flours Mean ± SD	**Northern infant flours**[1] Mean ± SD	**Southern infant flours**[2] Mean ± SD	**EU Regulation**	
				Min	Max
Energy (kcal)	100	100	100	-	-
Fat (g)	1.4 ± 0.8	1.2 ± 0.8	1.7 ± 0.7	-	3.3
SFA (g)	0.3 ± 0.3	0.4 ± 0.3	0.2 ± 0.1	-	-
Proteins (g)	3.0 ± 0.7	2.8 ± 0.7	3.3 ± 0.8	-	5.5
Carbohydrates (g)	18.5 ± 2.2	18.9 ± 2.2	18.0 ± 2.0	-	-
Vitamin A (µg)	115.4 ± 33.4	95.3 ± 13.7	143.2 ± 45.9	-	180
Vitamin C (mg)	13.5 ± 2.6	13.4 ± 2.8	13.7 ± 2.2	-	12.5/25*
Vitamin D (µg)	2.1 ± 0.4	1.9 ± 0.3	2.5 ± 0.5	-	3
Vitamin E (mg)	1.6 ± 0.6	1.2 ± 0.3	2.1 ± 0.9	-	3
Iron (mg)	4.2 ± 2.6	4.8 ± 3.5	3.9 ± 2.0	-	3
Zinc (mg)	1.5 ± 0.9	1.7 ± 1.5	1.4 ± 0.7	-	2
Iodine (µg)	17.1 ± 5.8	15.2 ± 4.5	21.2 ± 4.6	-	35

[1] *Northern IF refers to IF produced and marketed in northern regions (n = 56)*

[2] *Southern IF refers to IF produced and marketed in southern regions (n = 40)*

**25 mg/100 Kcal of vitamin C in the case of iron-fortified IF*

The comparison of IF nutritional values with the regulatory thresholds imposed by *CODEX CXS 74-1981* showed overages for certain micronutrients, notably iron and vitamins A, E and C (**Figure 40**). These vitamin and mineral overages have already been reported in the literature (Moustiés, Bourlieu *et al.* 2019) and are intended to compensate for any losses during storage.

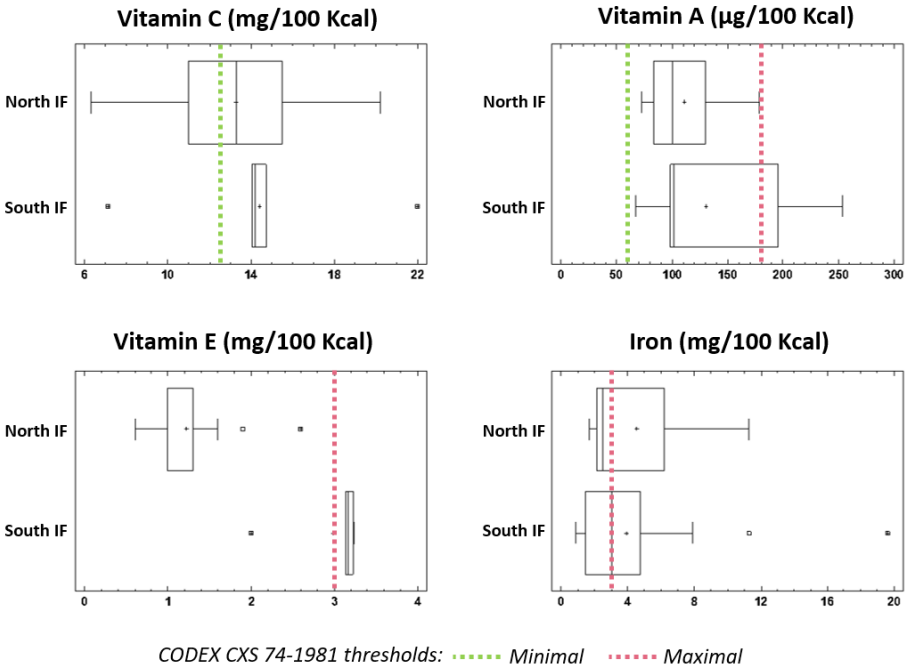


Figure 40. Micronutrients overages in marketed IF compare to the regulatory thresholds of the *CODEX CXS 74-1981*

An analysis of the raw materials in the ingredient lists of the 96 IF was carried out to explain the nutritional values variability reported in the dataset. This analysis showed that the raw materials most commonly used in the North and South IF were different (**Table 21**). Northern IF included a majority of cereal, with 51.8, 32.1, 30.4 and 23.2% of North IF including rice, wholegrain oat, wheat and maize flours, respectively. For Southern IF, the raw materials used are mainly legumes, with 57.5% composed of soy flour and 45% of groundnuts flour, but also cereal, with 30 and 55% of southern IF composed of millet and maize flours, respectively.

Table 21. Percentage of IF included in the dataset containing the ingredients

	Northern IF *N = 56*	**Southern IF** *N = 40*
Milk-base raw material	23.2 %	27.5 %
Added fats	26.8 %	17.5 %
Barley flour	10.7 %	2.5 %
Cassava flour	-	2.5 %
Cowpea flour	-	12.5 %
Fonio four	-	2.5 %
Groundnut flour	-	**45 %**
Maize flour	**23.2 %**	**55 %**
Mil flour	-	7.5 %
Millet flour	1.8 %	**30 %**
Oat flour	14.3 %	10 %
Quinoa flour	5.4 %	-
Rice flour	**51.8 %**	25 %
Rye flour	10.7 %	-
Sorghum flour	-	20 %
Soy flour	1.8 %	**57.5 %**
Spelt flour	1.8 %	-
Tapioca four	7.1 %	-
Tubers flour	-	5 %
Wheat flour	**30.4 %**	25 %
Wholegrain barley flour	5.4 %	-
Wholegrain millet flour	14.3 %	-
Wholegrain oat flour	**32.1 %**	-
Wholegrain quinoa flour	1.8 %	-
Wholegrain rice flour	3.6 %	-
Wholegrain rye flour	8.9 %	-
Wholegrain spelt flour	10.7 %	-
Wholegrain wheat flour	19.6 %	-

In red: most frequently used flours.

The variability in IF nutritional values reported in the dataset was associated to the diversity of raw materials included in their composition. Cereals, which are predominantly used in northern IF, have a lower nutrient density than legumes due to lower lipid (22.5% for soy *versus* 1.7% for wheat) and protein (38.7% for soy *versus* 13.1% for wheat) contents (Stone, Nosworthy *et al.* 2019). The FA profile and concomitantly the LA/ALA ratio also vary according to the raw material used, ranging from 1.9 for cowpea flour to 49.8 for peanut flour (**Figure 41**). Moustiés, Bourlieu *et al.* (2019) also reported these observations in their study focusing on 13 IF mainly marketed in southern countries (Vietnam, Burkina Faso, Madagascar and Benin). The used raw materials were similar to those identified in the dataset, and significant heterogeneity in nutritional values was observed.

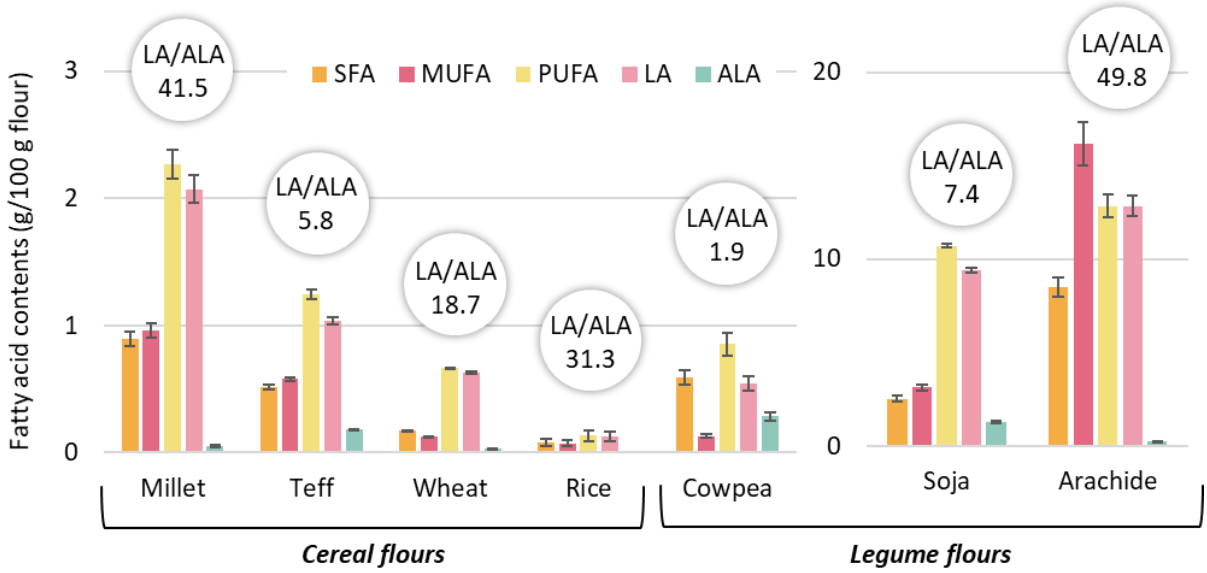


Figure 41. Typical fatty acid profiles of raw materials used in IF composition

2. Oxidative stability of IF representatives of marketed products

Analysis of the panorama of the composition and nutritional values IF on global market has revealed imbalances in their lipid profiles (insufficient total lipid content and/or unbalanced ω6/ω3 ratios), as well as micronutrient overages. But what about their oxidative stability?

To assess the oxidative stability of IF, two distinct formulations were prepared. One formulation, labeled as SIF, was designed to represent IF typically produced and marketed in southern regions. The other, labeled as NIF, was designed to represent IF typically produced and marketed in northern regions. The composition of these IF includes the four most commonly used flours depending on the production and marketing region *i.e.* maize, soy, groundnut and millet flours for SIF and rice, wheat, maize and wholegrain oat flours for NIF. Both SIF and NIF were fortified with minerals, including calcium at a concentration of 0.324 mg per 100 g of IF. Subsequently, they underwent a 5-month storage test, with temperature variations as described in memento B. The stability of these formulations was then compared to that of CIF marketed mainly in southern regions.

The initial characterization of these IF showed variable total lipid contents ranging from 8.8 ± 0.6 to 3.4 ± 0.3 g/100 g IF (dry basis) for CIF and NIF. SIF had an intermediate content of 6.0 ± 0.2 g/100 g of IF. This result is in line with the panorama, which showed significant variability in lipid content in IF on the global market, with a significantly lower content for northern IF. Despite an LA content around twice as high in CIF and SIF compared with NIF, the ω6/ω3 ratios of all these IF are in line with recommendations. Tocopherol levels were also variable (ranging from 1127.2 ± 97.9 to 500.3 ± 203.9 ppm). Both parameters depend on the raw materials used, and may vary according to cultivar (Shammugasamy, Ramakrishnan *et al.* 2015, Tremmel-Bede, Szentmiklóssy *et al.* 2022).

Table 22. CIF, SIF and NIF composition and initial characterization (tocopherol content, lipid profile, PV, moisture and Aw)

	CIF	SIF	NIF
Composition (%w/w)			
Groundnut flour	10.0	5.0	-
Maize flour	-	29.8	20.5
Millet flour	60.0	49.9	-
Rice flour	-	-	21.7
Soy flour	20.0	15.3	-
Wheat flour	-	-	39.6
Wholegrain oat flour	-	-	21.6
Fatty acid (g/100 g of IF dry base)			
Total lipids content	8.8 ± 0.6	6.0 ± 0.2	3.4 ± 0.3
SFA	2.1 ± 0.2	1.5 ± 0.0	0.9 ± 0.1
MUFA	2.7 ± 0.2	2.5 ± 4.8	0.8 ± 0.1
PUFA	4.0 ± 0.3	4.8 ± 0.1	2.2 ± 0.3
LA C18:2 ω6	3.7 ± 0.3	4.3 ± 0.1	1.9 ± 0.2
ALA C18:3 ω3	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0
ω6/ω3	9.3	10.6	9.5
Vitamin E content (ppm or µg/g of lipids)			
Total	1164.3 ± 31.7	679.0 ± 133.1	1527.9 ± 276.9
α-tocopherol	76.9 ± 20.1	84.4 ± 12.9	168.3 ± 29.8
δ-tocopherol	380.9 ± 17.1	188.9 ± 29.6	511.7 ± 85.6
γ-tocopherol	683.8 ± 17.4	396.0 ± 95.1	772.9 ± 163.1
Initial characterization			
Peroxide value (meqO_2/kg of lipids)	2.9 ± 0.6	3.6 ± 0.5	3.3 ± 0.3
Moisture (%)	2.9 ± 0.4	6.6 ± 0.2	8.8 ± 0.1
Aw	0.274 ± 0.029	0.444 ± 0.003	0.589 ± 0.001

In order to assess oxidative stability, the evolution of primary oxidation compounds (by measuring PV) and total tocopherol content was monitored during the 5-month of storage. Following the classic pattern of lipid oxidation, an increase in PV is expected first, followed by a decrease characteristic of the conversion of hydroperoxide to secondary compounds. Surprisingly, the results showed a decrease in PV in both CIF and SIF over the 5-month storage (**Figure 42.a**). Similar results have been reported by Wei, Zhu *et al.* (2021) in their study of the oxidative stability of wholegrain flours from 65 different wheat varieties (stored at 40°C for 20 weeks). The authors observed, in agreement with (Nielsen and Hansen 2008) that oxidation initiation occurs mainly during the process by promoting the production of peroxide radicals and hydroperoxide through contact with oxygen and other inducing factors (cell destruction). The authors suggested that low lipid content (total lipid content of 1.5% in wheat flour) reduces the accumulation capacity of hydroperoxides, and that their rate of formation being

significantly slower than that of peroxyl radicals, these compounds would be rapidly neutralized by nearby antioxidants such as tocopherols, limiting their conversion to hydroperoxide. Another hypothesis that could explain the observed drop in hydroperoxide levels is that they are rapidly converted into secondary compounds, preventing their accumulation from being observed. Furthermore, the results show that tocopherol levels were relatively stable during the first 3 months of storage. However, after 5 months, a significant loss was observed in both CIF and SIF. This loss could be associated to the mobilization of tocopherols to neutralize the hydroperoxide (**Figure 42.b**). In the case of NIF, the results show an increase in PV, which then decreased significantly after 5 months of storage, suggesting that they have either been converted to secondary compounds or neutralized by antioxidants. A significant loss of tocopherols was also observed. Comparison of these results suggests that NIF has the highest sensitivity to oxidation.

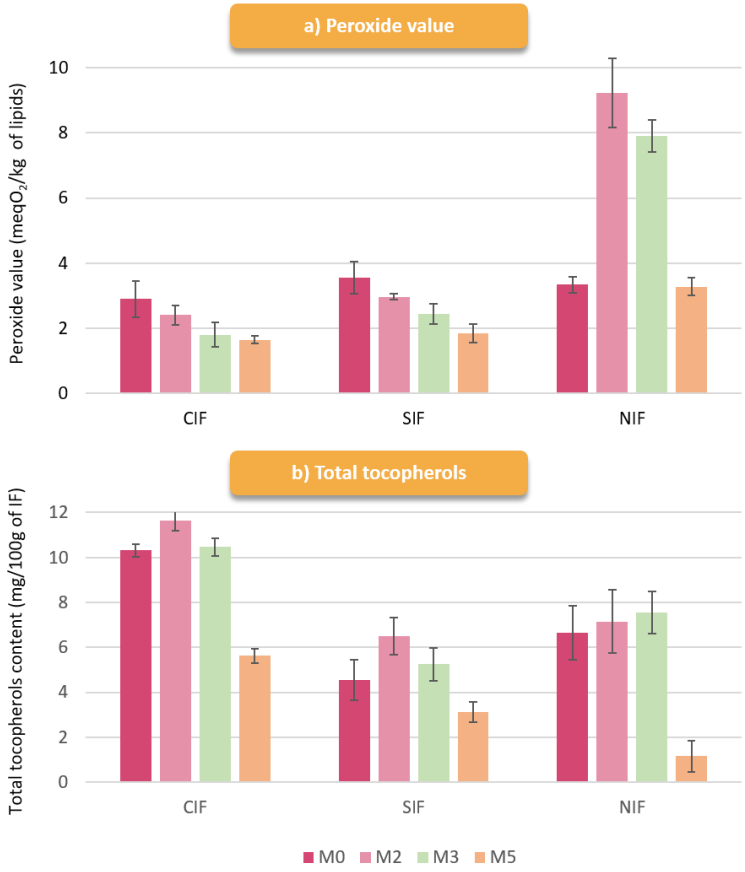


Figure 42. Evolution of a) PV and b) total tocopherols content in CIF, SIF and NIF over the 5-month storage

The results of this first storage test show relatively good stability, particularly for IF from southern regions (CIF and SIF). However, these IF have not been fortified with vitamin A, which is particularly sensitive to oxidation, especially in granular systems (Hemery, Laillou *et al.* 2018). In addition, their lipid profile could be further optimized for the needs of infant.

3. Optimization of IF lipid profile and oxidative stability

3.1. Impact of structural parameters on oxidative stability and nutritional profile

In order to optimize IF lipid profiles, an optimized IF (OIF) was formulated by introducing ω3 source flours, *i.e.* teff and cowpea flours, in combination with the most used raw material in southern IF, soy flour. Southern ingredients were selected as IF are predominantly consumed in these regions of the world. Moreover, in order to identify levers of controlling oxidative stability in dry systems, OIF was enriched with black rice bran (BRB), whose phenolic compound content has been reported as high in the literature (Santos, Barouh *et al.* 2021). The impact of structure was also investigated by varying the particle size of OIF. Three IF, OIF_NE, OIF_BRB_NE and OIF/RM_BRB_NE, fortified with minerals, including calcium at a concentration of 0.324 mg per 100 g of IF were subjected to a 9-month storage test with temperature variations as described in memento B. Their oxidative stability was investigated by monitoring changes in PV and total tocopherol content.

The initial characterization of these IF showed total lipid contents of 4.7 ± 0.1 g/100 g IF (dry basis) on average with a lower ω6/ω3 ratio than commercial IF (CIF, SIF, NIF) due to their lower LA content, *i.e.* 9.3 to 10.6 *versus* 5.6 to 6 (**Table 23**). Tocopherol content was on average of 1351.8 ± 62.7 g/100 g IF.

Table 23. Optimized IF composition and initial characterization (tocopherol content, lipid profile, PV, moisture and Aw)

	OIF_NE	OIF_BRB_NE	OIF/RM_BRB_NE
Composition (%w/w)			
Cowpea flour	41.5	40.4	40.4
Soy flour	24.7	24.0	24.0
Teff flour	33.7	32.7	32.7
Blalck rice bran	-	2.9	2.9
Fatty acid (g/100 g of IF dry base)			
Total lipids content	4.6 ± 0.2	4.8 ± 0.4	4.6 ± 0.6
SFA	0.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.0
MUFA	0.9 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
PUFA	2.9 ± 0.0	2.9 ± 0.0	2.8 ± 0.0
LA C18:2 ω6	2.4 ± 0.0	2.4 ±0.0	2.3 ± 0.0
ALA C18:3 ω3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
ω6/ω3	6	6	5.6
Vitamin E content (ppm or µg/g of lipids)			
Total	1309.5 ± 130.5	1322.1 ± 116.9	1423.8 ± 51.9
α-tocopherol	121.0 ± 16.2	135.1 ± 30.1	153.2 ± 10.0
δ-tocopherol	571.8 ± 39.3	531.3 ± 30.5	536.7 ± 11.1
γ-tocopherol	616.7 ± 78.4	623.2 ± 131.3	672.2 ± 51.4
Initial characterization			
Peroxide value ($meqO_2$/kg of lipids)	4.0 ± 0.9	3.7 ± 1.6	5.2 ± 2.0
Moisture (%)	7.8 ± 0.2	7.3 ± 0.1	7.7 ± 0.3
Aw	0.554 ± 0.002	0.555 ± 0.005	0.559 ± 0.002

In order to evaluate the impact of particle size on oxidative stability OIF/RM_BRB_NE was re-milled. **Figure 43** shows that particle size was consistently reduced from 184.5 ± 7.7 to 102.2 ± 7.7 µm (D50). The size distribution across all IF was multimodal and re-milling of OIF increased the specific surface area from 1006.6 ± 21.5 to 1306.3 ± 79.4 cm^2/mL.

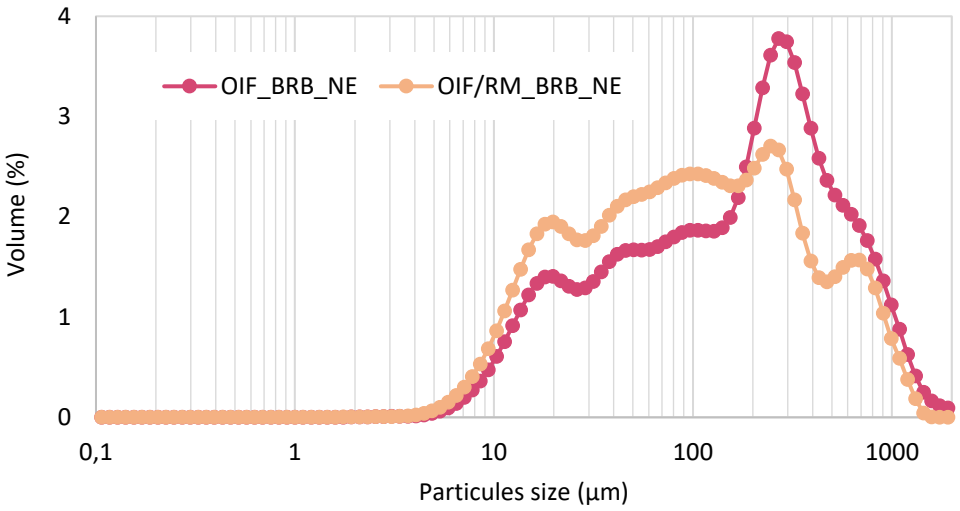


Figure 43. Distribution of particle size of OIF_BRB_NE and OIF/RM_BRB_NE

The results show that the IF are relatively stable to oxidation up to 5 months of storage, after which a significant increase in PV is observed in all the IF, particularly in OIF_BRB_NE and OIF/RM_BRB_NE, which reach an average of 35.2 ± 1.1 $meqO_2/kg$ after 9 months of storage (**Figure 44.a**). In addition, total tocopherols content was relatively stable in OIF_NE, whereas a significant loss was observed after 2 months of storage in OIF_BRBR_NE and OIF/RM_BRB_NE which then remains relatively stable until the end of the storage test (**Figure 44.b**). These results suggest that the presence of BRB results in destabilization of IF, equivalent whatever the particle size. BRB are carriers of phenolic compounds which have an important antioxidant capacity (Santos, Barouh *et al.* 2021), however, they can also be degraded by oxidation reactions (browning) through enzymatic or non-enzymatic pathways and could explain the greater increase in PV in IF including a BRB fraction (Igor Otavio, Cristine Vanz *et al.* 2017, Su, Geng *et al.* 2022). Several authors have nevertheless demonstrated the high antioxidant capacity of cereal bran and its potential as a vitamin A stabilizing agent (Van Wayenbergh, Struyf *et al.* 2020). It should be remembered that the IF studied in this section were only enriched with minerals, and therefore not supplemented with vitamins with antioxidant capacity such as vitamins E or C. It would therefore be interesting to investigate these results further in the presence of a more complex vitamin composition (see section *3.2*).

As with PV evolution, tocopherol loss was independent of particle size of OIF. Furthermore, an additional IF was studied including a fraction of BRB re-milled with a particle size around 50 µm to be close to cell size (data not show). Our results suggest that reducing the particle size of OIF or BRB no effect on oxidative stability (over the size range tested). As reported by Rosa, Barron *et al.* (2013) reduction of particles size may increases the antioxidant capacity of cereal brans by increasing the liberation of bioactive compounds and their contact with unsaturated lipids. On the other hand, such a process also promotes contact with oxygen but also with cellular compounds having a pro-oxidant effect (enzymes and metals) and may therefore promote the oxidation of lipids (Rohfritsch, Canelli *et al.* 2021). This effect may therefore counterbalance the increasing antioxidant capacity and could explain that the not significant effect of size in our study.

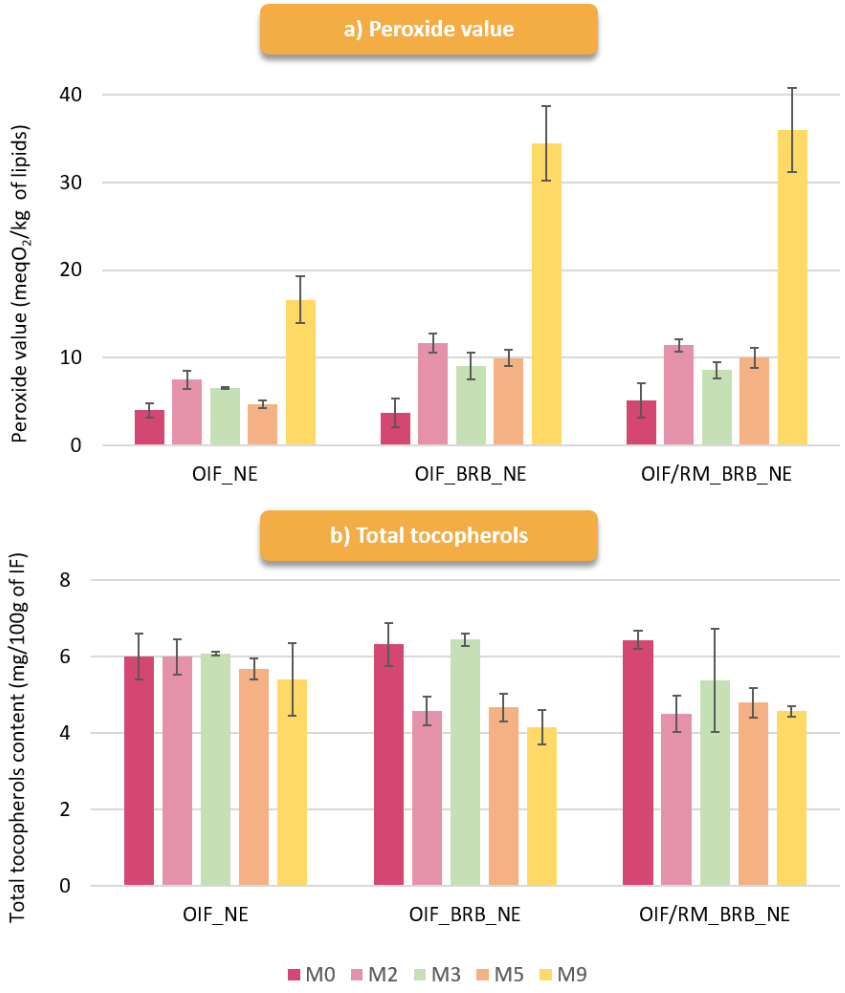


Figure 44. Evolution a) PV and b) total tocopherols content in optimized IF over the 9-month storage

3.2. Impact of formulation parameters on oxidative stability and nutritional profile

Particle size (over the size range tested) has no impact on the oxidative stability of OIF. Moreover, the previous storage test showed a greater sensitivity to oxidation of this IF compared with commercial IF (CIF, SIF and NIF). OIF was also not fortified with vitamins notably A, which is particularly sensitive to oxidation). In this section the impact of formulation parameters on oxidative stability of IF enriched with a VMC and with an optimized lipid profile was studied.

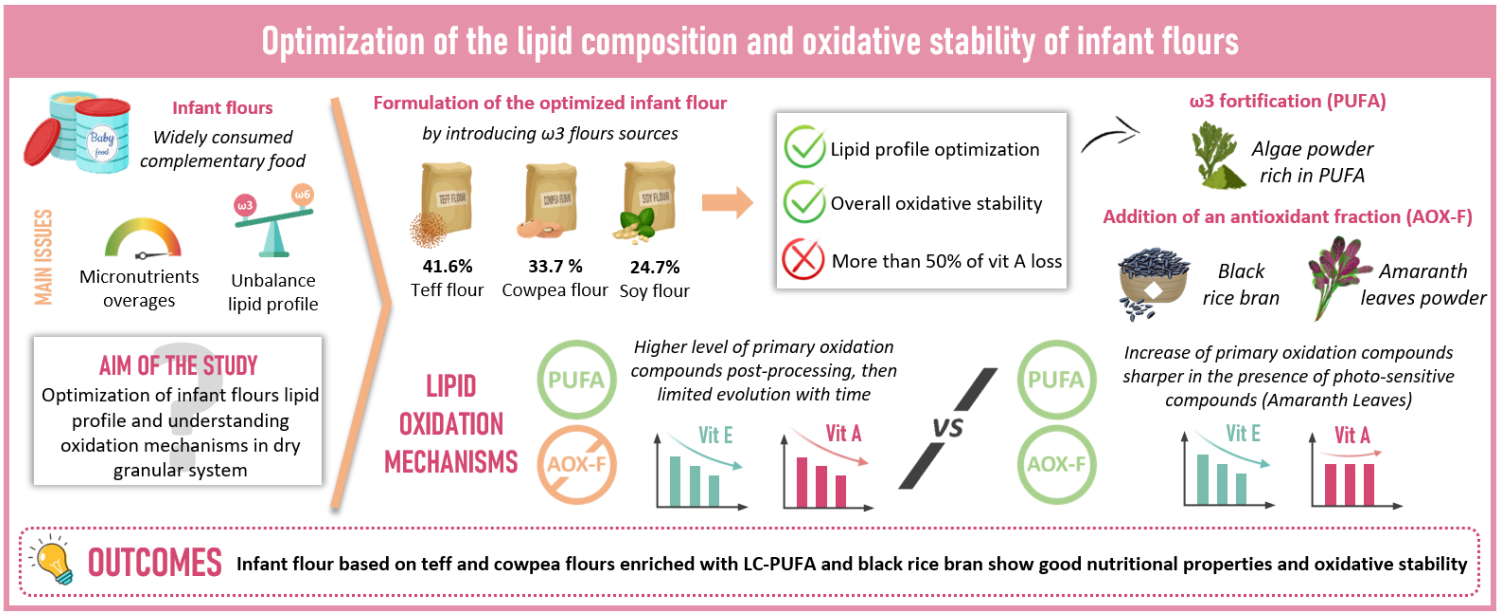
This section is based on a research article entitled "Optimization of the lipid composition and oxidative stability of infant flours" in preparation for submission.

Abstract

From 4/6 months of age, complementary foods such as IF are introduced into the infant diet to supplement the nutritional intake of breast milk and/or infant formula, which are no longer sufficient. However, IF exhibit an unbalanced lipid profile and micronutrient overages. The aim of this study was to optimize both lipid profile and oxidative stability of IF and providing a better understanding of oxidation mechanisms in low-moisture foods. An IF was formulated using essential omega-3 fatty acids-carrier flours (cowpea and teff) and underwent heat treatment (enzymatic inactivation and inhibition of anti-nutritional factors), LC-PUFA and exogenous antioxidant fraction fortification (amaranth leaves and black rice bran). The oxidative stability of IF was monitored during a 6-month storage test at 25°C. The results demonstrate that the inclusion of teff and cowpea flours into the IF formulation leads to a balanced lipid profile (omega-6/omega-3 ratio close to 5) and ensures relatively good overall stability. However, 50% of the vitamin A was lost after 2 weeks of storage. Black rice bran and amaranth leaves acted as vitamin A stabilizer, delaying losses. Initiation of oxidation occurred mainly during processing and propagated at a relatively slow rate, depending on antioxidant composition (faster in the presence of photosensitive compounds).

Keywords: infant flours, lipid oxidation, vitamins, antioxidants, carotenoids, phenolic compounds

Graphical abstract



1. Introduction

Proper nutrition from birth to two years of age is essential to support comprehensive growth and development (Martorell 2017). Around 6 months of age, breast milk is no longer sufficient to cover all energy, macro- and micronutrient requirements of infant (WHO 2021). Moreover, from this age the acquisition of a certain digestive and neuromuscular maturity allows for the consumption and assimilation of complex food. This specific period of development is characterized by the gradual introduction of complementary foods to supplement the nutritional intake of the milky part of the diet, which nevertheless remains the main source of calories until the age of 12 months (WHO 2003). To promote adequate complementary feeding practices, official guidelines have been established by WHO and UNICEF (WHO 2003, UNICEF 2021). However, inappropriate complementary feeding practices are widely reported in the literature, including a high prevalence of early introduction and consumption of nutritionally poor complementary foods (Nantel and Gingras 2023). These inappropriate practices are associated with an increased risk of developing multiple nutritional deficiencies, notably in micronutrients (vitamin A and iron), which can lead to irreversible physical and cognitive consequences (including stunted growth, increased morbidity and mortality). These factors are therefore among the main contributors to malnutrition problems, especially in developing countries.

In many countries, especially in southern regions, IF are one of the first complementary foods introduced into the infant diet, with over 50% of infant consuming them around 6 months of age (National Institute of Population, Training *et al.* 2005, Lange, Visalli *et al.* 2013, Roess, Jacquier *et al.* 2018, Duffy, Kay *et al.* 2019). In a previous study by our group, the nutritional values and ingredient lists of 96 IF marketed worldwide were collected (Cancalon, Hemery *et al.* 2023). Statistical analysis of this dataset revealed considerable variability in nutritional values due to the diversity of raw materials used, notably between northern countries, which mainly used cereals (wheat, oats, maize, rice), and southern countries, which included legumes (soy, groundnut). Lipid content was particularly affected by this heterogeneity, with levels ranging from 0.2 to 18.8 g lipid/100 g IF. These results were in line with the analyses proposed by Moustiés, Bourlieu *et al.* (2019) on several southern IF. Dimaria, Schwartz *et al.* (2018) also showed that among 32 IF marketed in Benin, Burkina Faso, Ghana and Senegal only 8 had an adequate lipid content. Moreover, some commercial IF showed ω6/ω3 ratios of over 30, exceeding international recommendations which indicate an optimal ratio range between 5 and 15 (Moustiés, Bourlieu *et al.* 2019). An imbalance in this ratio, particularly as a result of an excessive LA or deficient ALA content, the essential fa, can reduce the efficiency of the bioconversion of ALA into its longer-chain derivative, *i.e.* DHA, through competition with the metabolic pathways of synthesis. An inadequate lipid profile can affect the proper development of infant as lipids are the main energy suppliers covering up to 55% of caloric intake during infancy and are involved in structural

membrane and in the cerebral, visual, intestinal and immune systems development. Our dataset, in correlation with the Moustiés, Bourlieu *et al.* (2019) study, highlighted the high prevalence of micronutrient overages, particularly of vitamins A and E. The aim of these voluntary overages is to compensate for any losses during IF storage, and thus guarantee a certain content until the expiry date despite the reactivity of these compounds and their sensitivity to oxidation phenomena. Lipid oxidation is a major concern in food products as it is one of the primary causes of alteration. While the mechanisms of lipid oxidation have been extensively studied in emulsified and bulk systems, few studies have focused on low-moisture systems such as IF (Gumus and Decker 2021). Thus there is a gap in current knowledge concerning the mode of propagation of oxidation, which is assumed to be different as a function of the low water activity that limits diffusion of the compounds (Nelson and Labuza 1992). Lipid oxidation can occur according to different chemical pathways mainly auto-oxidation in processed products. However, the presence of intrinsic lipolytic enzymes in some flours and of photo-sensitive compounds in plants could favor other oxidation pathways, namely enzymatic oxidation and photo-oxidation. Plant-based ingredients are also sources of various antioxidant compounds that could provide a protective effect against lipid oxidation. For instance, leafy vegetables such as amaranth and cereal bran are recognized for their antioxidant properties, attributed in particular to the presence of carotenoids and phenolic compounds, respectively (Huang and Lai 2016, Sarker and Oba 2019, Sarker, Hossain *et al.* 2020, Santos, Barouh *et al.* 2021, Fioroni, Mouquet-Rivier *et al.* 2023). Antioxidants can act through a number of mechanisms, including scavenging of free radicals, quenching of singlet oxygen or metal chelation (Ma, Wang *et al.* 2023). However, currently, there is a lack of knowledge regarding the impact of these actions in dry systems.

Optimizing the composition of IF is therefore a real challenge. Several studies have proposed different improvement strategies, mainly based on formulation. However, to the best of our knowledge, no studies have focused on the optimization of the lipid profile with a focus on the quid of lipid oxidation, one of the main causes of food deterioration.

The present study had a double objective: i) to optimize the lipid profile of IF by introducing raw materials that are sources of essential lipids *i.e.* LA and ALA with a balanced ratio, and ii) to provide a better understanding of lipid oxidation mechanisms in dry systems as a function of the addition of exogenous antioxidant fractions. Thus, an IF composed of a mixture of soy, teff and cowpea flours was formulated to obtain a lipid profile adapted to infant needs. This IF underwent various treatments/fortification including heat treatment (enzymatic inactivation and inhibition of anti-nutritional factors), LC-PUFA including ARA and DHA and exogenous antioxidant fraction enrichments (black rice bran and amaranth leaves powder) to improve both its nutritional profile and oxidative stability. Oxidation mechanisms were then evaluated during a 6-month storage test at 25°C.

2. Materials and methods

All the experimental materials and techniques are detailed in Chapter 2. In this study, the following IF were investigated (see memento side B):

- OIF
- OIF_HT
- OIF_HT_PUFA
- OIF_HT_PUFA_BRB
- OIF_HT_PUFA_AL

3. Results and discussion

3.1. Infant flours formulation and standardization

IF were designed to have an optimized lipid profile. Teff, cowpea and soy flours were combined to obtain a balanced FA composition. The choice of raw materials was based on the composition of the IF produced and consumed in southern regions (**Table 24**). LC-PUFA fortification of the IF was performed to further improve the lipid profile. However, such modifications can lead to a more important sensitivity to lipid oxidation. Different strategies were investigated to stabilize the IF formulated. The effect of heat treatment of cowpea flour and the addition of exogenous antioxidant fractions were studied.

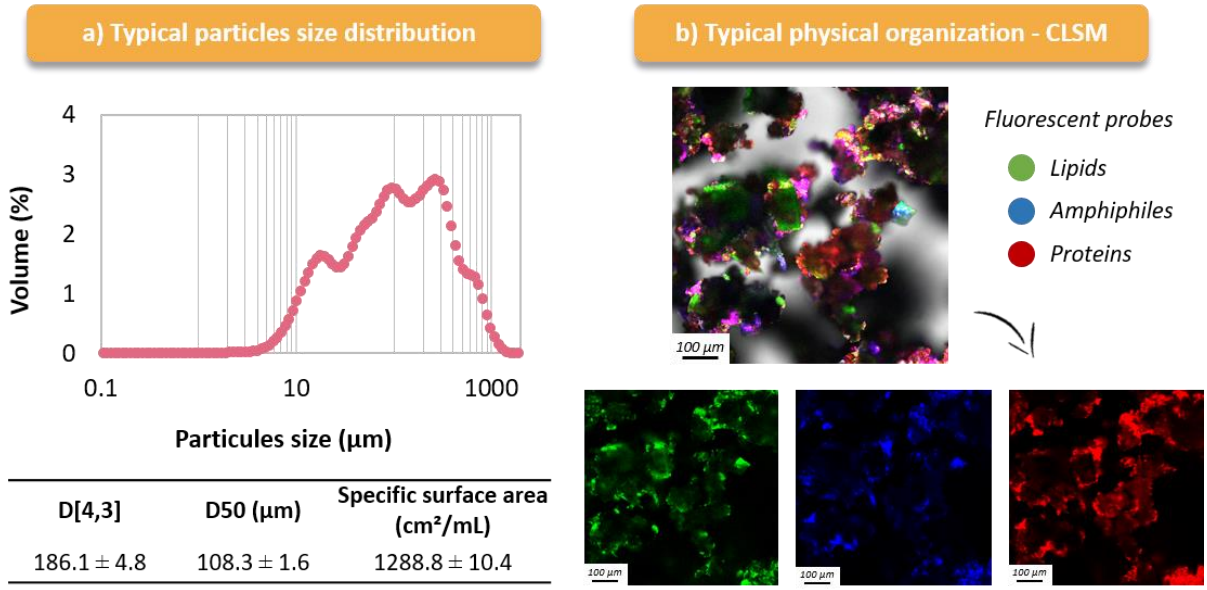
All IF had equivalent vitamin E contents averaging 1773.4 ± 132.4 ppm, with γ-tocopherol as the main isomer averaging 1069.7 ± 85.8 ppm (**Table 24**). The IF were fortified with vitamin A in the form of retinyl acetate, whose content was equivalent in all IF (average 5.8 ± 0.7 µg RA/g IF). OIF_HT_PUFA_AL also contained carotenoids (α and β-carotene and lutein), provided by the addition of amaranth leaves. Vitamin C, in the form of ascorbic acid, was provided either by the addition of VMC or by the addition of ARA and DHA powders (containing ascorbic acid as an antioxidant). Non-enriched IF with LC-PUFA (OIF and OIF_HT) contained 0.6 ± 0.0 mg ascorbic acid/g IF. However, enriched IF with LC-PUFA, contained almost twice as much, averaging 1.1 ± 0.1 mg ascorbic acid/g flour. Heat treatment of cowpea flour led to a lowering of moisture and Aw, which averaged 6.3 ± 0.3% and 0.436 ± 0.015 respectively, compared with 7.3 ± 0.1% and 0.523 ± 0.003 in the case of OIF, for which the cowpea flour had not been heat-treated. All IF had standardized FA profiles with equivalent SFA, MUFA and PUFA contents averaging 1.0 ± 0.1, 1.2 ± 0.2 and 3.4 ± 0.2 g/100 g IF, respectively. The ω6/ω3 ratios of 6.6 were reduced to an average of 3.9 with the addition of LC-PUFA. These ratios are in line with international recommendations which indicate an optimal ratio range between 5 and 15 for infants and improvement compared to commercial IF whose ratios have been reported to reach 30 (Moustiés, Bourlieu *et al.* 2019). These imbalances are linked to an excess of LA and low ALA content in certain raw materials (*e.g.* millet flour ω6/ω3 ratio is over 40). Protein and carbohydrate contents were estimated according to raw material labels at 23.0 and 49.3%, respectively. Macro and micronutrient contents are strongly dependent on the raw materials used, the nutritional composition of flours varies

from one study to another. Araro, Gemechu *et al.* (2020) reported that the formulation of a complementary foods based on sweet potato, brown teff and dark red kidney beans, showed protein and carbohydrate contents ranging from 3.65 to 14.25% and 67.10 to 83.96%, respectively. However, the lipid contents did not meet recommendations. The authors concluded that it was necessary to optimize the lipid fraction of IF.

Table 24. IF composition and initial characterization of their vitamins contents (E, A and C) and their physico-chemical properties (PV, moisture and Aw)

	OIF	OIF_HT	OIF_HT_PUFA	OIF_HT_PUFA_BRB	OIF_HT_PUFA_AL
Composition (%w/w)					
Soy flour	24.7	24.7	23.6	22.9	22.9
Teff flour	33.7	33.7	32.1	31.2	31.2
Cowpea flour	41.5	41.5	39.6	38.5	38.5
ARA rich powder	-	-	1.6	1.6	1.6
DHA rich powder	-	-	2.9	2.8	2.8
Amaranth leave powder	-	-	-	-	2.9
Black rice bran	-	-	-	2.9	-
VMC	0.2	0.2	0.2	0.2	0.2
Vitamin E content (ppm or µg/g of lipids)					
Total	1909.9 ± 65.1	1885.0 ± 169.2	1764.0 ± 112.4	1726.2 ± 59.9	1581.9 ± 144.9
α-tocopherol	139.0 ± 8.5	151.4 ± 27.7	126.8 ± 8.6	115.4 ± 10.2	103.7 ± 14.4
δ-tocopherol	573.1 ± 19.9	568.1 ± 51.2	533.2 ± 31.5	501.3 ± 40.7	544.3 ± 26.0
γ-tocopherol	1163.7 ± 47.1	1135.1 ± 89.3	1073.2 ± 73.0	1027.4 ± 38.7	949.2 ± 89.5
Vitamin A content (dry base)					
Retinyl acetate (µg RA/g)	4.6 ± 0.1	6.3 ± 0.5	6.4 ± 1.1	4.8 ± 0.5	5.8 ± 1.2
Carotenoid (µg/g)	-	-	-	-	13.2 ± 0.9
α-carotene (µg/g)	-	-	-	-	0.9 ± 0.0
β-*carotene (µg/g)*	-	-	-	-	5.7 ± 0.3
Lutein (µg/g)	-	-	-	-	6.6 ± 0.4
Vitamin C content (mg/g of IF dry base)					
Ascorbic acid	0.6 ± 0.0	0.6 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0
Fatty acid (g/100 g of IF dry base)					
Total lipids content	5.9 ± 0.2	5.8 ± 0.3	6.6 ± 0.2	6.4 ± 0.3	6.5 ± 0.1
SFA	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.0	1.0 ± 0.1	1.1 ± 0.0
MUFA	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.0	1.2 ± 0.1	1.2 ± 0.0
PUFA	3.3 ± 0.2	3.0 ± 0.2	3.6 ± 0.0	3.3 ± 0.1	3.4 ± 0.1
LA C18:2 ω6	2.8 ± 0.1	2.6 ± 0.2	2.7 ± 0.0	2.4 ± 0.1	2.5 ± 0.1
ALA C18:3 ω3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
ARA C20:4 ω6	-	-	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
DHA C22:6 ω3	-	-	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
ω6/ω3	6.7	6.6	3.9	4.0	3.7
Initial characterization					
Peroxide value (meqO_2/kg of lipids)	5.3 ± 1.1	3.9 ± 1.8	12.7 ± 1.9	19.8 ± 1.5	23.8 ± 1.1
Moisture (%)	7.3 ± 0.1	6.1 ± 0.1	5.9 ± 0.1	6.7 ± 0.2	6.3 ± 0.1
Aw	0.523 ± 0.003	0.421 ± 0.008	0.427 ± 0.004	0.453 ± 0.001	0.444 ± 0.002
LOX activity (U/mg of IF)	202.9 ± 2.0	60.1 ± 23.5	-	-	-

A standardization of the IF structure was performed and was similar in all systems. **Figure 45.a** shows the typical particle size distribution of OIF. This was a multimodal distribution with 3 extremes centered on 18, 96 and 269 µm, respectively. A similar particle size distribution was measured in all IF with a specific surface area average of 1294 ± 23 cm²/mL. Compound organization was assessed by CLSM as shown in **Figure 45.b** for OIF. The results showed a homogeneous distribution of proteins and lipids in the grains, with a higher concentration on the surface, characterized by greater fluorescent intensity. Amphiphilic compounds, on the other hand, were mainly located at the periphery of the grains.



D[4,3]	D50 (µm)	Specific surface area (cm²/mL)
186.1 ± 4.8	108.3 ± 1.6	1288.8 ± 10.4

Figure 45. a) Typical droplet size distribution b) and physical compounds organization of IF with the example of OIF

3.2. Infant flours optimization by introducing sources of essential fatty acids

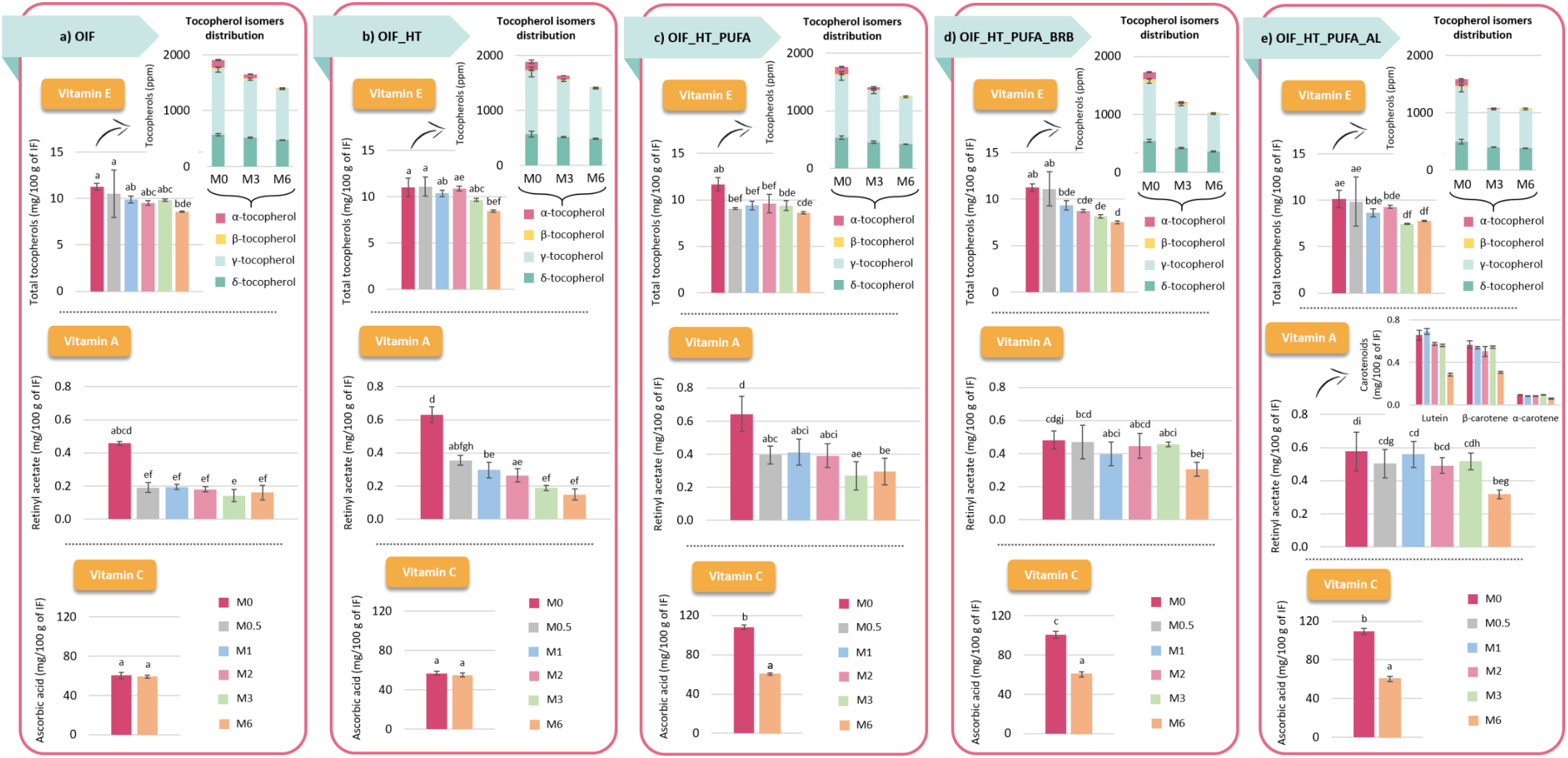
The impact of optimizing the lipid profile of IF by introducing ω3 essential FA-carrier flours on oxidative stability was assessed using an OIF and OIF_HT storage test. Cowpea, which represent 41.5% of IF and contain high amount of ALA (15.3 ± 1.6%), has however the drawback of having an important LOX activity. (Chang and McCurdy 1985) in their study comparing the LOX activity of fourteen legumes classified cowpea in category of legumes with the highest LOX activity (>2,000 U/mg flour). LOX ubiquitous in plant and animal kingdom, expressed at particularly high levels in legumes. They are responsible of the enzymatic oxidation pathway of PUFA, LA being generally the main substrate. The LOX activity promotes formation of hydroxyperoxide that are further degraded into metabolites such as jasmonic acid contributing to plant defense against biotic or abiotic stresses. Several strategies are employed to inactivate LOX, including heat treatment (Shi, Mandal *et al.* 2020). Cowpea flour in OIF_HT were heat-treated to limit this oxidation pathway. This treatment reduced by than three times the LOX

activity in comparison to non-heat-treated IF. More precisely, OIF had an activity of 202.9 ± 2.0 U/mg of flour compared with 60.1 ± 23.5 U/mg of flour for OIF_HT (**Table 24**). The LOX activity of soy, which accounts for 24.7% of IF in our study, was also investigated by other authors that reported 2370 U/mg of flour at pH 6.9 (Chang and McCurdy 1985). However, the soy flour used to formulate the IF in our study was roasted, leading to an inhibition of the enzyme in this raw material.

OIF and OIF_HT both exhibited relatively good overall oxidative stability, except for vitamin A, which underwent significant degradation over short times. **Figure 46.a** and **Figure 46.b** show that heat treatment had no significant effect on tocopherol loss kinetics. Tocopherols content remained stable, however, significant losses were observed after a 6-month storage in the two IF and reached on average 23.6% in OIF and OIF_HT. When looking more specifically to isomers reactivity, α-tocopherol was totally degraded after 6 months of storage in both IF (**Figure 46.a** and **Figure 46.b)**. Tocopherols losses were mainly associated with a loss of γ-tocopherol (the main isomer). The δ-tocopherol remained stable throughout storage. In addition, tocopherols are also fat-soluble, and therefore certainly localized close to vitamin A, and possess free radical scavenging capacity (Ma, Wang *et al.* 2023). Barden, Barouh *et al.* (2015) reported that the lipid oxidation lag phase can be extended by using hydrophobic antioxidants in low-moisture crackers. Ascorbic acid also has a free radical scavenging capacity but its content remained stable over the storage in both IF, probably due to its polarity which limits its mobilization.

Monitoring of retinyl acetate content revealed significant losses of 58.5 and 43.7% in OIF and OIF_HT, respectively within 15 days of storage (**Figure 46.a** and **Figure 46.b**). Retinyl acetate content then remained relatively stable in OIF up to 6 months, with a total loss of 65.0% at the end of storage. In OIF_HT, a more progressive loss of retinyl acetate was observed up to the 6-month storage, reaching 76.5%. This result suggests that the initiation of vitamin A oxidation probably occurs during processing, although it is added after heat treatment in our systems. Lampi, Damerau *et al.* (2015) showed that in non-heat-treated oat grain, lipid hydrolysis occurred immediately after milling, which favored the propagation of lipid oxidation during storage. Mixing process applied our study using a three-dimensional movement to ensure homogeneous raw materials promotes contact between oxidizable lipids including vitamin A and enzymes, minerals and air. As vitamin A is particularly sensitive to heat, light and contact with oxygen (higher reactivity than vitamin E), this compound is degraded primarily (Palace, Khaper *et al.* 1999). Similar losses of vitamin A were reported by Hemery, Laillou *et al.* (2018) in their study on the stability of fortified wheat flour. The authors highlighted vitamin A losses of up to 85% after 3 months of storage under various conditions in PET/ALU bags (25 or 40°C with 65 or 85% relative humidity).

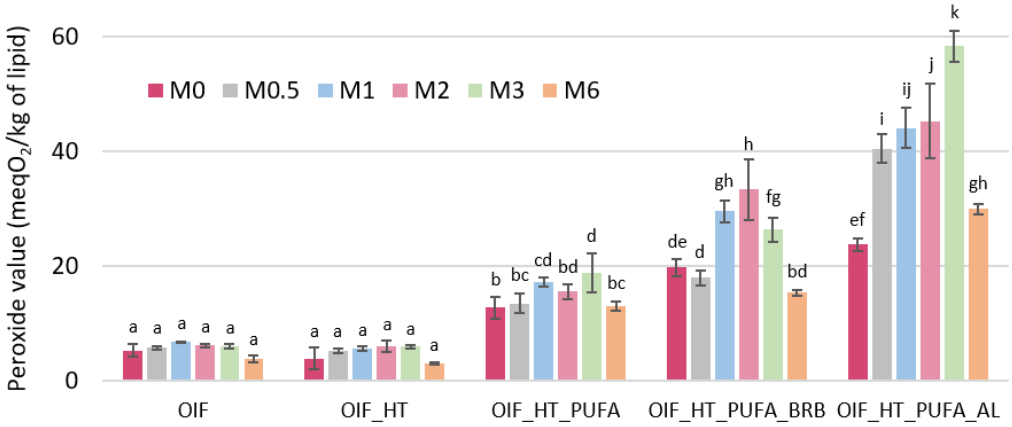
Figure 46. Evolution of the vitamins E, A and C contents over storage



Different letters indicate a significant difference (P<0.05) between IF and time.

Figure 47.a and **Figure 47.b** show the evolution of primary and secondary oxidation products assessed by measuring PV and volatile compounds (hexanal and pentanal), respectively. Primary oxidation compounds remained stable over the 6-month storage period in OIF and OIF_HT, with PV below the regulatory limit in oils of 10 meqO_2/kg. Concerning volatile compounds, only hexanal was detected in OIF after 6 months of storage, at a relatively low level. Consequently, despite the significant degradation of vitamin A, PV and volatile compounds remained stable, suggesting that the peroxyl radicals formed were neutralized by antioxidants to form antioxidant radicals before being converted to hydroperoxide and volatiles compounds as suggested by Wei, Zhu *et al.* (2021). In our work, the radicals could have reacted with tocopherols, as a slow loss of total tocopherols content is measured.

a) Peroxide value



b) Volatiles compounds

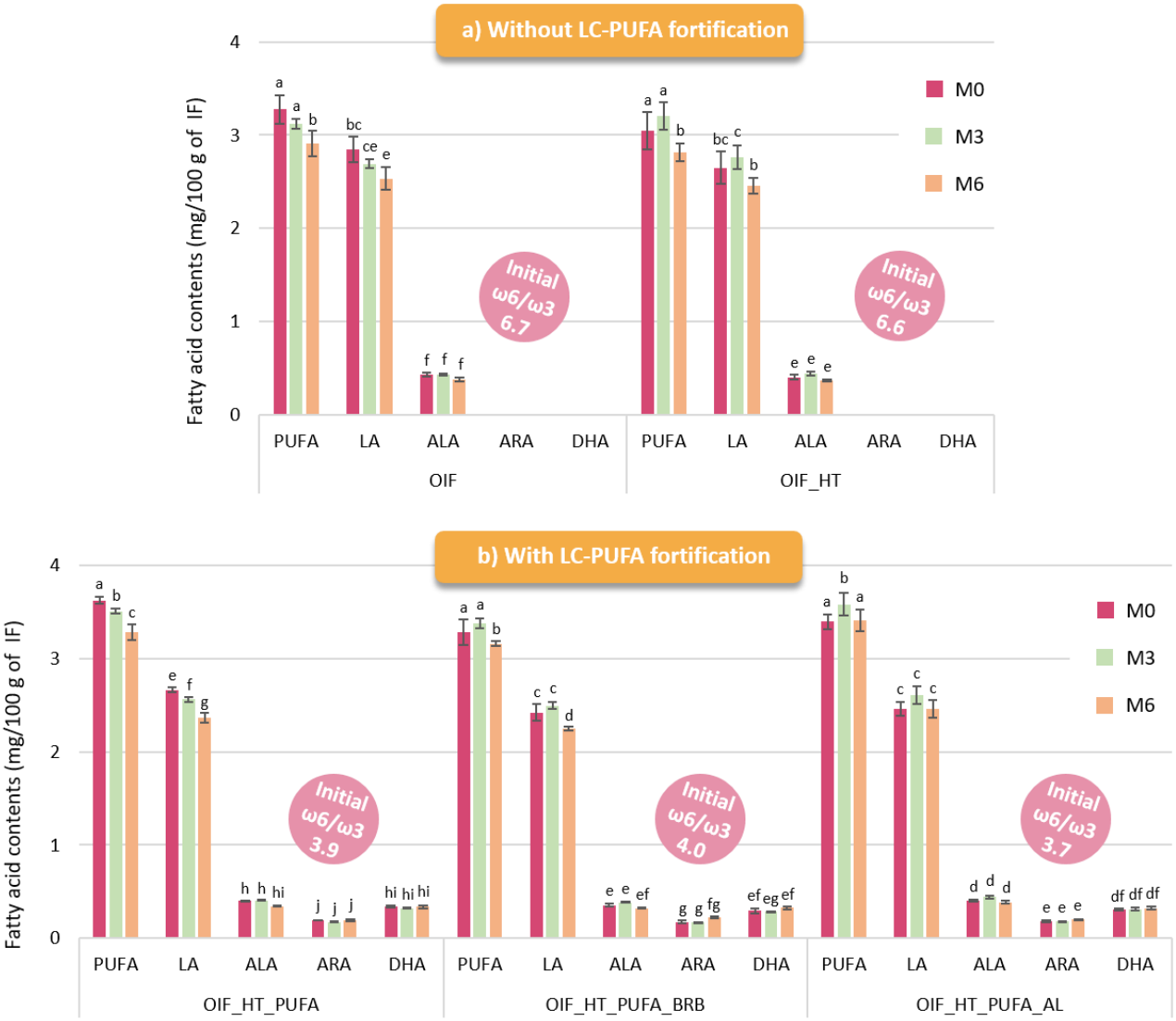
ng eq 3-heptanol/g of IF

	Hexanal		Pentanal	
	M3	M6	M3	M6
OIF	*N.D.*	6.3 ± 1.2^{ac}	*N.D.*	*N.D.*
OIF_HT	*N.D.*	*N.D.*	*N.D.*	*N.D.*
OIF_HT_PUFA	*N.D.*	4.6 ± 0.7^{ad}	*N.D.*	*N.D.*
OIF_HT_PUFA_BRB	4.1 ± 0.7^{a}	11.1 ± 0.9^{cb}	*N.D.*	*N.D.*
OIF_HT_PUFA_AL	5.9 ± 1.3^{ab}	12.8 ± 2.0^{b}	38.3 ± 3.7^{a}	47.8 ± 4.4^{b}

Different letters indicate a significant difference (P<0.05) between IF and time.

Figure 47. Evolution of the oxidation compounds a) PV and b) volatile compounds over the 6-month storage

Finally, monitoring the evolution of the FA profile showed in both IF a non-significant loss of PUFA up to 3-month and a limited but significant loss of on average 9.2% after 6 months of storage (**Figure 48.a**). This loss was attributed to a loss of LA in OIF. However, no significant loss LA occurred in OIF_HT. As previously mentioned, LA is the main substrate for LOX, it loss in OIF could therefore possibly occurred due to enzymatic oxidation pathway.



Different letters indicate a significant difference (P<0.05) between time in an IF.

Figure 48. Evolution of the IF PUFA profiles

3.3. Impact of infant flours fortification with LC-PUFA on oxidative stability

DHA and ARA play an essential role in cognitive and retinal development of infant (Makrides, Neumann *et al.* 1995, Gil, Ramirez *et al.* 2003, Agostoni 2008, Carlson, O'Loughlin *et al.* 2019). These LC-PUFA are derived by endogenous biosynthesis from precursor FA *i.e.* LA for the ω6 and ALA for the ω3. In our

study, OIF_HT was fortified with DHA and ARA (from algal and fungal sources, respectively) to further optimize the lipid profile for infant needs. LC-PUFA fortification as expected modified the FA profile, with a lowering of the ω6/ω3 ratio (**Figure 48.b**). LA content decreased with storage time in a limited extend and at a pace similar to OIF (11.4% of loss after 6 months of storage). In addition, this fortification was expected to reduce oxidative stability due to the high sensitivity of LC-PUFA to lipid oxidation, but the results showed no reduction of ARA and DHA contents in OIF_HT_PUFA.

A loss of 22.6% of total tocopherols after 15 days of storage was measured, mainly associated with a loss of γ-tocopherol (**Figure 46.c**). Tocopherols content then remained stable throughout storage, with a loss of 26.4% after 6 months. The kinetics of retinyl acetate losses in OIF_HT_PUFA were similar to those observed for OIF_HT, with significant losses of 38.5% over the short time (**Figure 46.c**). The content then remained stable until the third month of storage, and reached a total loss of 54.2% after 6 months of storage.

However, OIF_HT_PUFA showed a higher level of PV post-processing compared to OIF_HT (**Figure 47.a)**. PV then had a limited evolution with time up to 6 months of storage. Concerning secondary oxidation compounds, only hexanal was detected after 6 months of storage at relatively low levels (**Figure 47.b**). These results reinforce the hypothesis that oxidation initiation occurs mainly during the process, favoring contact of unsaturated lipids with oxidation-initiating factors (enzyme, minerals and oxygen), and that the propagation rate is then slow due to the limited diffusion of compounds at an Aw around 0.5. An Aw ranging around 0.2 to 0.4 leads to the formation of a monolayer of bound-water providing a protective effect by limiting direct contact with oxygen due to its lower solubility in water than to oil fractions (Cuvelier, Soto *et al.* 2017). Ascorbic acid which is hydrophilic is probably localized at the level of the water monolayer. Monitoring of its content showed a loss of 44.3% after storage (**Figure 46.c**). Thus, a two-fold increase in its content leads to greater losses. This compound is more potentially mobilized for oxygen quenching than for its metal chelation properties. Indeed, (Barden and Decker 2016) highlighted that the effect of metal chelation activity was limited in low-moisture foods due to the low mobility of transition metals, which consequently does not contribute to lipid oxidation in this type of system. The free radical scavenging properties of ascorbic acid may also seem limited by its polarity (no proximity to the radicals formed).

3.4. Introduction of exogenous antioxidant fractions impact oxidation mechanisms

To improve the stability of OIF_HT_PUFA, antioxidant fractions were added *i.e.* black rice bran for its phenolic compounds contents and amaranth leaves powder for its carotenoids content in OIF_HT_PUFA_BRB and OIF_HT_PUFA_AL, respectively.

The addition of an exogenous antioxidant fraction delayed retinyl acetate losses until 3 months of storage. Retinyl acetate loss of only 36.8% after 6 months of storage was measured for

OIF_HT_PUFA_BRB suggesting a vitamin A stabilizing effect of black rice bran (**Figure 46.d**). Several authors have previously reported the retinyl palmitate-stabilizing agent properties of cereal bran including wheat, oat and rice. Van Wayenbergh, Struyf *et al.* (2020) have shown that rice bran effectively improves the stability of vitamin A in the form of retinyl palmitate during long-term storage. A 35% retention of vitamin A was observed after 8 weeks at 60°C with 68% relative humidity in the presence of rice bran, whereas it was totally degraded after less than one week in the absence of bran. The same authors showed that the efficacy of cereal bran was notably dependent on its enzymatic activity, with an improvement in vitamin A stability with heat-treated wheat bran leading to an enzyme inactivation (Van Wayenbergh, Langenaeken *et al.* 2023). Their efficacy was not associated with a single compound, but with a wide range of antioxidant compounds that could act synergistically, such as tocopherols and phenolic compounds, which have high levels in cereal bran (Van Wayenbergh, Coddens *et al.* 2023). The authors also highlighted that wheat bran was more effective than rice bran, due to its higher phenolic compound content, despite the significant ester-linked with polysaccharides or conjugated with mono- or oligosaccharides fraction. In our study, we used black rice bran, which is richer in antioxidant compounds with globally 89 phenolic compounds belonging to flavonoids (52%), phenolic acids (33%), other polyphenols (8%), lignans (6%) and stilbenes (1%) (Santos, Barouh *et al.* 2021).

Similar vitamin A stabilization results were demonstrated in OIF_HT_PUFA_AL, with a retinyl acetate loss of only 44.9% after 6 months of storage (**Figure 46.e**). The addition of amaranth leaves also provided carotenoids, including two carotenes (α and β-carotene) and a xanthophyll (lutein). These compounds are recognized for their antioxidant capacity particularly in emulsified systems (Dimakou and Oreopoulou 2012, Cancalon, Hemery *et al.* 2023), and are probably responsible for the improved vitamin A stability in OIF_HT_PUFA_AL. The proximity of the compounds in the system due to their equivalent polarity enables carotenoids to have a protective effect. The antioxidant capacity of β-carotene is associated with its singlet oxygen quenching or free radical scavenging effect. Studies in emulsified systems have also shown that carotenoids, and in particular β-carotene, act synergistically with tocopherols to promote regenerative effects (Heinonen, Haila *et al.* 1997, Barouh, Bourlieu *et al.* 2021). Lutein, with its system of eleven conjugated double bonds, also possesses important antioxidant properties, contributing to improved vitamin A stability. The evolution of carotenoid contents according to storage time showed that carotenes were degraded only from the sixth month of storage, reaching losses of 37.6 and 46.0% for α and β-carotene, respectively. In the case of lutein, losses were observed in three stages: a stable content up to one month, then a stable loss averaging 13.5% up to the third month of storage, and finally a significant loss of 56.4% after 6 months.

As with OIF_HT_PUFA, the results showed higher post-processing PV than for non-enriched with LC-PUFA IF (**Figure 47.a**). For OIF_HT_PUFA_BRB, the evolution was not as clear-cut and remained

relatively stable during the 6 months of storage, despite a slight increase. A clear increase in PV was measured in the presence of photosensitive compounds such as chlorophyll from amaranth leaves in OIF_HT_PUFA_AL. Furthermore, monitoring of volatile compounds showed the presence of relatively low levels of hexanal after 3 months of storage in OIF_HT_PUFA_BRB and both hexanal and pentanal in OIF_HT_PUFA_AL. A slight increase was measured after 6 months of storage, with higher overall volatile compound contents in OIF_HT_PUFA_AL. These results suggest that the presence of photo-sensitizing compounds particularly from amaranth leaves, destabilized the system, accelerating the production of primary and secondary oxidation compounds. A negative correlation between chlorophyll content and oxidative stability has already been demonstrated in rapeseed and coconut oils, for example (Rukmini and Raharjo 2010, Li, Yang *et al.* 2019). Chlorophyll is capable of initiating photo-oxidation even at very low levels (below 0.1 ppm). Moreover, the rate of photo-oxidation is up to 1,000 to 1,500 times faster than autoxidation (Ghanbari, Anwar *et al.* 2012). Thus, initiation of this oxidation pathway has probably occurred during processing following exposure to light (IF stored in light-barrier bags), leading to electronic excitation of chlorophyll. The excitation energy favors the conversion of ground state triplet oxygen ($^3O_2$) to highly reactive excited state singlet oxygen ($^1O_2$), which promotes lipid oxidation and consequently favors the formation of oxidation compounds.

The results show similar tocopherols loss kinetics which reached a rate of 33.2 and 23.2% after 6 months of storage in OIF_HT_PUFA_BRB and OIF_HT_PUFA_AL, respectively (**Figure 46.d**.and **Figure 46.e**). Tocopherol losses were associated, as in other IF, with a loss of the γ-tocopherol isomer and of the totality of α-tocopherol isomer. Tocopherols are probably mobilized for their free radical scavenging and oxygen quenching properties. In addition, they are able to act synergistically with other compounds such as phenolics and carotenoids by combining their properties or regenerating effect (Ma, Wang *et al.* 2023). The results also showed ascorbic acid losses of 39.9 and 45.1% for OIF_HT_PUFA_BRB and OIF_HT_PUFA_AL, respectively, which were similar to OIF_HT_PUFA suggesting that the addition of antioxidant fraction has no influence on this compound.

Finally, the results show a loss of LA after 6 months for OIF_HT_PUFA_BRB whereas the FA profile did not change during storage for OIF_HT_PUFA_AL (**Figure 48.b**).

Overall, our results show that phenolic compounds appeared to be particularly effective in limiting oxidation in granular systems which is in contrast to Barden, Barouh *et al.* (2015) who reported that the lipid oxidation was more effectively delayed by more hydrophobic antioxidants in low-moisture systems.

Taken together, these results suggest that oxidation initiation occurs mainly in the short times and is dependent on processing conditions and IF composition. IF could be good carriers of both essential FA and LC-PUFA in the presence of endogenous fiber-associated antioxidant fractions and adequate, controlled processing conditions.

4. Conclusion

The formulation of an IF from cowpea and teff flours results in an optimized lipid profile characterized by a balanced ω6/ω3 ratio in line with recommendations. In addition, micronutrient levels have been adjusted in line with international regulations to avoid overages. Despite these formulation changes, IF has relatively good oxidative stability, except for vitamin A, which is lost by more than 50% within the first two weeks of storage. While heat treatment of cowpea had a fairly limited effect, the addition of LC-PUFA impacted the initial oxidation level of IF, but remained relatively stable during storage. Initiation of oxidation occurs mainly during processing emphasizing the importance of controlling the quality of raw materials, as well as milling and mixing conditions. The propagation rate of oxidation is relatively slow in dry systems, due to the limited diffusion of the compounds. However, the kinetics of vitamin loss and oxidation compound formation are linked to the addition of antioxidant fractions. In the absence of antioxidant fraction, vitamin A was rapidly lost. The stability of vitamin A was improved in the presence of black rice bran or amaranth leaves, which provide phenolic and carotenoid compounds, respectively. Oxidation pathways also appear to be impacted by the addition of antioxidants, particularly in the presence of photosensitive compounds such as chlorophyll provided by amaranth leaves. These vitamin loss and oxidation pathway switches need further investigations to understand the interaction between antioxidants, unsaturated lipids and oxidation initiation factors in granular systems.



MAIN RESULTS

- Introducing teff and cowpea flours into infant flours optimizes lipid profile with a ω6/ω3 ratio close to 5
- Oxidation diffusion kinetics were relatively slow in granular systems, except for vitamin A
- Fat-soluble vitamin loss kinetics depended on chemical composition (presence or absence of antioxidants)
- Carotenoids and black rice bran were vitamin A-stabilizing agent

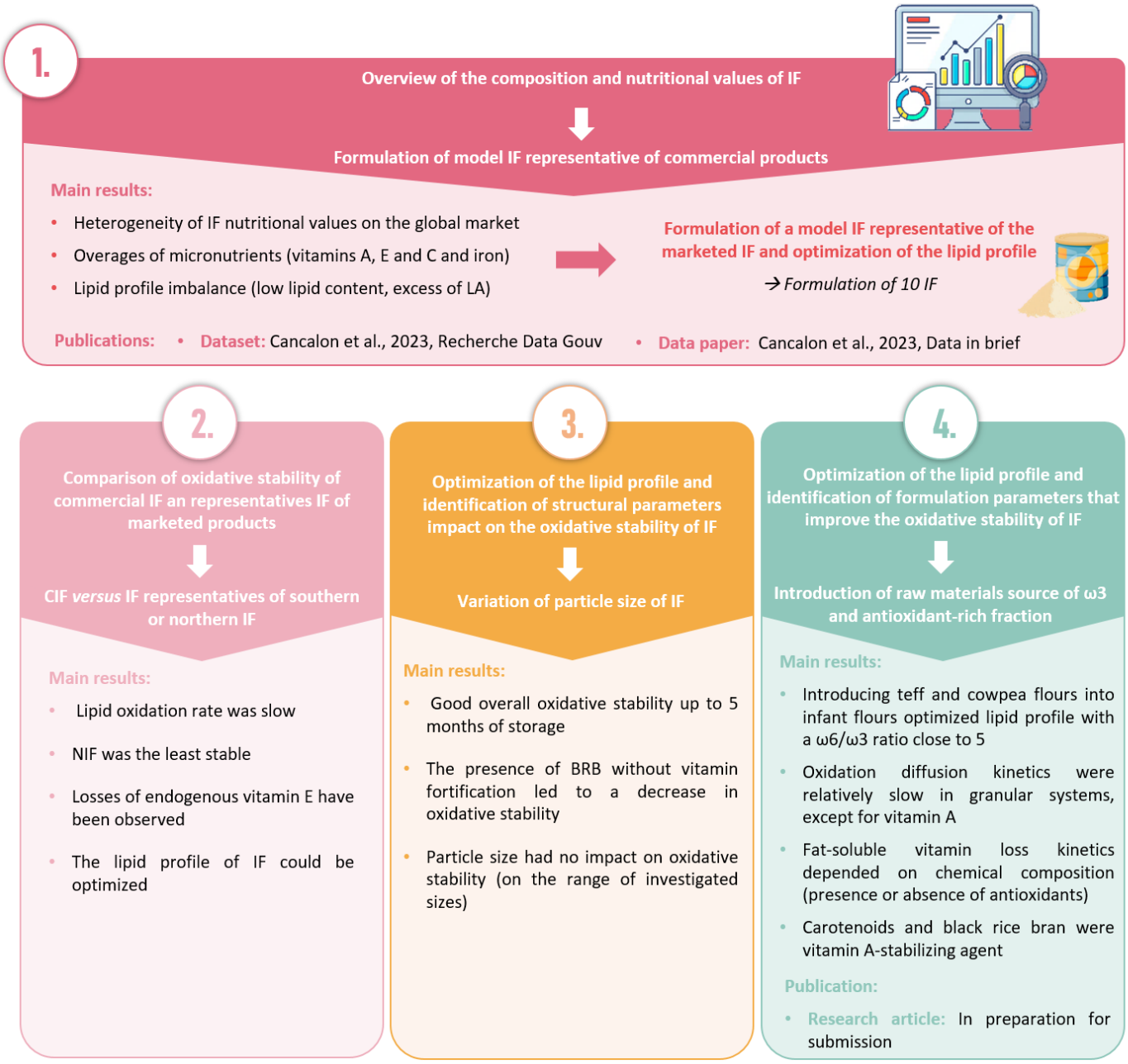
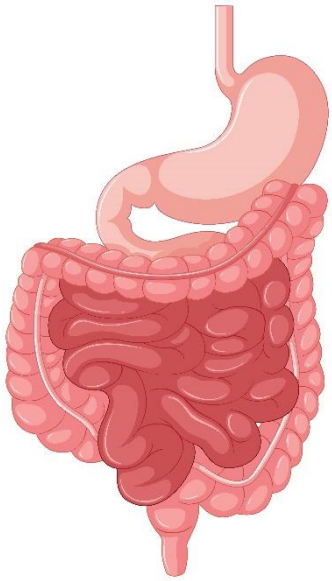


Figure 49. Main results from chapter 4



CHAPTER 5

Digestibility of optimized infant formulas and flours

Chapters 3 and 4 focus on the study of oxidative stability and optimization of the lipid profile of IFF and IF. Modulation of formulation parameters appear to be a promising strategy for improving nutrient and stability properties in both types of dispersed systems. But what is the impact of the optimization strategies on the bioaccessibility of nutriments?

In this context, the aim of this chapter is to evaluate the bioaccessibility of lipids (FA and vitamin E) and proteins in the IFF and IF that have demonstrated the most improved nutritional and stability properties, and to compare them with commercial products. An *in vitro* digestion process was adapted to the specific digestive conditions of 6-month-old infant and applied to a limited number of dispersed systems including three IFF (PO_L, DF_DPL_L and LIFF_L) and two IF (OIF_HT_PUFA_BRB and CIF). Characterization of the digesta enabled the assessment of the nutrients bioaccessibility.

This chapter is based on a research article entitled "Bioaccessibility of lipids and proteins in infant follow-on formulas and flours using a static in vitro digestion model for 6-month-old infants" in preparation for submission.

1. Introduction

The bioaccessibility of a nutrient is defined as the fraction released from food under the form of compound that can be absorbed through the intestinal barrier and used by the body. It depends on various factors, including the type of food matrix, its composition (interaction between nutrients promoting or limiting release) and also the physiological conditions of the digestive system (Hu, Lin *et al.* 2023). This notion is therefore essential for assessing the potential of different food matrices as nutrient carriers. Furthermore, digestive immaturity in infants during the first 1,000 days limits the efficiency of the enzymatic system and affects nutrients bioavailability (Bourlieu, Ménard *et al.* 2014). This digestive immaturity is quite strong over the first month of life, marked for gastric compartment with mature gastric lipase but immature pepsin secretions and limited acid secretion capacity, and for the duodenal compartment with mature protease secretions but lower amount of PLRP2 and biliary secretions. This immaturity rapidly disappears, however based on a previous infant digestive physiology review and previous neonatal model, we selected *in vitro* digestive parameters to mimic as closely as possible a 6-month old digestive model by reducing the amount of pepsin in gastric compartment and reducing biliary salt concentration in comparison with adult in the duodenal compartment.

In previous works, optimization of the lipid profile and oxidative stability of IFF and IF playing on formulation and structural parameters have been proposed (chapters 3 and 4). However, these strategies can impact the nutrients release capacity during the digestion process. For IFF, these parameters triggered the exploration of model systems based on quite large lipid droplets (around 1 µm) with a core either based on PO versus DF blended in both case with various vegetable oils. This micronic structure and its evolution through the upper gastro-intestinal system will influence the kinetics of lipolysis and proteolysis (Golding, Wooster *et al.* 2011, Bourlieu, Ménard *et al.* 2015). These systems are generally fortified with tocopherols but bioaccessibility of tocopherols along with FA and protein has never been compared in both types of systems although representative of very typical IFF products. Similar question can be set concerning the bioacessibility of optimized IF compared to a commercial reference of nutritional interest. IF have different structure depending on the type of flours that enters in their composition. Whole flour will bring peripheral tissues rich in fibers, and phenolic compounds but that can be digestive inhibitors.

The aim of this section is therefore to assess the bioaccessibility of lipids (FA and vitamin E) and proteins in IFF and IF with optimized lipid profiles and oxidative stability properties. The present paper proposed to i) compare the bioaccessibility of FA, vitamin E and proteins as function of the dispersed system type (IFF *versus* IF) and ii) for a given product type, determine the impact of optimization strategies of nutritional properties and oxidative stability on the bioaccessibility of these same nutrients. In this aim total of three IFF were selected: LIFF_L as the commercial reference, PO_L as the

model IFF reference and DF_DPL_L, which demonstrated the most important nutritional and oxidative stability properties. In addition, two IF were also selected: CIF as the commercial reference and OIF_HT_PUFA_BRB, which had the most optimized nutritional and stability properties.

In this context, a static *in vitro* digestion model was set up to simulate digestion conditions of full-term infants aged 6 months onward (Bourlieu, Ménard *et al.* 2014, Minekus, Alminger *et al.* 2014, Ménard, Bourlieu *et al.* 2018). As the residence time of IFF and IF under the form of infant gruel in the mouth is short, the oral phase was omitted. The *in vitro* static digestion process used included two stages *i.e.* gastric and intestinal phases during which various aliquots were collected in order to characterize nutrient digestion kinetics.

2. Materials and methods

All the experimental materials and techniques are detailed in Chapter 2. In this study, the following model IFF were investigated (see memento side A and B):

For IFF:

- LIFF_L → Named LIFF in this section
- PO_L → Named PO in this section
- DF_DPL_L → Named DF_DPL in this section

For IF:

- CIF
- OIF_HT_PUFA_BRB → Named OIF in this section

3. Results and discussion

3.1. Initial characterization and impact of baking

IFF subjected to the *in vitro* digestion process had similar lipid profiles with a majority of MUFA followed by SFA and PUFA (**Table 25**). However, the SFA profile varied according to the composition of the lipid phase, with the presence of short and medium chains in LIFF and DF_DPL provided by dairy lipids, in contrast to PO, which contained mainly palmitic acid as SFA (memento side A). Initial tocopherols content varied from 366.3 ± 10.8 to 863.2 ± 9.6 ppm, indicating a low control of normalization as we targeted 800 ppm in DF_PL and PO.

	Infant follow-on formulas			Infant gurels	
	LIFF	PO	DF_DPL	CIF	OIF_HT_PUFA_BRB
Fatty acid (g/100 mL of IFF or IF)					
Total lipids content	3.1	3.4	3.4	1.0	0.4
SFA	0.98 ± 0.05	1.22 ± 0.05	0.92 ± 0.05	0.40 ± 0.05	0.13 ± 0.00
MUFA	1.24 ± 0.07	1.58 ± 0.07	1.32 ± 0.07	0.23 ± 0.01	0.07 ± 0.00
PUFA	0.56 ±0.03	0.68 ± 0.02	0.58 ± 0.03	0.32 ± 0.01	0.18 ± 0.01
LA C18:2 ω6	0.46 ± 0.03	0.58 ± 0.02	0.49 ± 0.03	0.30 ± 0.01	0.11 ± 0.00
ALA C18:3 ω3	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
ARA C20:4 ω6	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	-	0.02 ± 0.00
DHA C22:6 ω3	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	-	0.03 ± 0.00
ω6/ω3	5.7	8.1	8.1	10.9	2.5
Total tocopherols (ppm or µg/g of lipids)					
Initial content	595.5 ± 18.8	863.2 ± 9.6	366.3 ± 10.8	1105.8 ± 13.9	1033.6 ± 17.2
Trypsin inhibitory activity (mg/g of IF dry base)					
Before baking	-	-	-	9.0 ± 0.1	55.2 ± 1.2
After baking	-	-	-	N.D.	N.D.
Structural parameters					
Droplet sizes (µm)	0.47 ± 0.00	0.84 ± 0.04	0.86 ± 0.08	-	-
Texture (mm/30 s)	-	-	-	112.7 ± 5.0	93.7 ± 10.0

Table 25. Initial characterization of IFF and infant gruel subjected to *in vitro* digestion process

Considering now the microstructure of these emulsions, droplet size distribution in the IFF was assessed by laser light diffraction technique (**Table 25** and **Figure 50**). The results indicate a s expected and targeted monomodal distribution centered in mode on average 0.85 µm for PO and DF_DPL and 0.47 µm for LIFF. In order to obtain physically stable IFF, industrial homogenization procedures using strong scales (in excess of 300 bars) leading to submicron droplet sizes are usually applied, explaining the nearly two-fold smaller size in the case of LIFF. However, such processes generate local heating that can lead to oxidation initiation and loss of thermolabile compounds such as the vitamin A. Reducing droplet size also leads to an increase in specific surface area, impacting lipid digestion kinetics and distancing the lipid structure from its native state by destructuration of dairy MFG (Bourlieu, Deglaire *et al.* 2017).

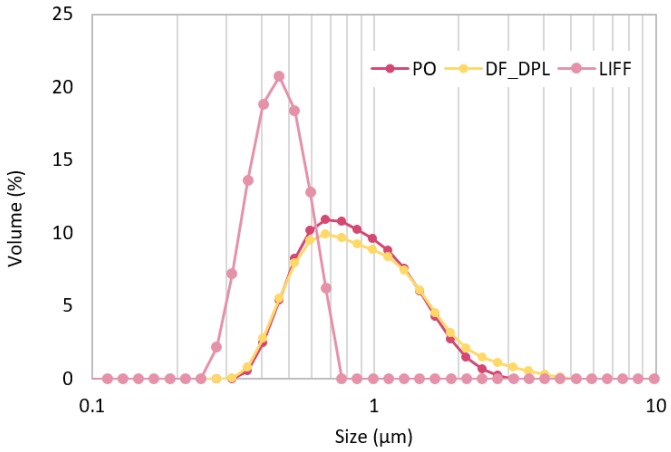


Figure 50. Droplet size distribution of IFF subjected to *in vitro* digestion process

Regarding IF, this infant food is consumed under the form of gruel. This involves diluting IF in an appropriate liquid (here rehydration to 9% using ultra-pure water) and baking (here boiling for 5 minutes) to obtain a porridge-like mixture. IF dilution consequently affects their total lipid content, which is lowered to 1.0 and 0.4% for CIF and OIF, respectively (**Table 25**). OIF gruel contained in addition to essential FA their long-chain derivatives including ARA and DHA, which were absent in CIF gruel. Initial tocopherol levels in both gruels were equivalent and averaged of 1069.7 ppm. Plant-food ingredients commonly contain anti-nutritional factors such as saponins, tannins, phytic acid and protease inhibitors. Trypsin inhibitory activity was measured to assess the content of anti-nutritional factors in both IF. The results show a significantly higher concentration in OIF than in CIF, probably due to the introduction of black rice bran (Kaur and Sharma 2011). However, the baking of the gruels results in the elimination of anti-trypsin factors which were then undetectable in both gruels (Irakli, Lazaridou *et al.* 2020). The texture of the gruels, characterized by flow rate measurement with a Bostwick consistometer, was around 100 mm/30 s, which is representative of the gruels usually consumed (Mouquet, Greffeuille *et al.* 2006).

3.2. Bioaccessibility of macro and micronutrients in infant follow-on formulas

Monitoring of lipid classes over the various digestion times showed that lipolysis for all IFF is almost complete after 120 minutes of intestinal phase (**Figure 51.a**). TAG hydrolysis, leading to the formation of DAG, MAG and FFA, occurred mainly during the intestinal phase. As expected, after 60 minutes of gastric phase, a majority of TAG remained due to the limited action of gastric lipase, which is responsible for only 10-30% of total lipid hydrolysis. This rate would depend on the type of meal, with a significantly higher rate of gastric lipolysis on infant formula than on breast milk that can be explained by the high capacity of gastric lipase to get inserted at interface and on its dependence on interfacial

surface available (Pafumi, Lairon *et al.* 2002, Bourlieu, Ménard *et al.* 2015, Liu, Lin *et al.* 2022). During intestinal phase, TAG are rapidly hydrolyzed (the majority of lipolysis occurs within the first 10 minutes), mainly by pancreatic lipase. The evolution of lipids classes profiles appears to be relatively similar for all IFF subject to the *in vitro* digestion (**Figure 51.a**). However, depending on IFF composition, monitoring of the released FFA profile indicates the stereoselectivity of gastric lipase (here mammalian analogue to human gastric lipase) and the regioselectivity of pancreatic lipase (**Figure 51.b**). Indeed, this point confronted to a different regiodistribution of FA in vegetal and dairy sources result in different profile of FFA release in digesta (Lecomte, Bourlieu et al. 2015). Short- and medium-chain FFA were released preferentially in LIFF and DF_DPL, which include dairy ingredients, whereas these FFA are little or not represented in PO. These results are in agreement with *in vitro* and *in vivo* data obtained on gastric phase by (De Oliveira, Bellanger *et al.* 2017, De Oliveira, Bellanger *et al.* 2017). DHA was released mainly in intestinal phase and to a similar extend in all IFF.

For proteins, the degree of hydrolysis was corrected by the rate of autoproteolysis, which may be overestimated on the short times and resulting in low rate in the gastric phase (**Figure 51.c**). The proteolysis rate was nevertheless expected to be low at this stage of digestion, although the maturity of pepsin and low HCl secretory capacity has probably evolved in the 6-month-old infant compared to 1-month-old. Indeed, this enzyme only reaches 30% of its adult activity four weeks after birth (Bourlieu, Ménard *et al.* 2014). These results suggest that the majority of proteins have undergone only minor hydrolysis during the gastric phase. During intestinal phase, the proteolysis rate accelerated over short timescales, with kinetics that appear similar across all IFF (results to be confirmed with statistical analysis) and reached an average hydrolysis degree of 26.9 ± 5.4% after 120 minutes of intestinal phase (**Figure 51.c**). These results indicate a total proteolysis degree lower than that estimated in adults, which can reach 52% at the end of the intestinal phase for the digestion of milk proteins (Mat, Le Feunteun *et al.* 2016).

The total tocopherols bioaccessibility was also estimated and ranged from 7.1 ± 3.1 to 15.4 ± 3.6% for DF_DPL and LIFF, respectively (**Figure 51.d**). This result seems particularly low in comparison with the study by Reboul, Richelle *et al.* (2006) in which the authors reported a bioaccessibility of 21.95 ± 1.60% and 6.76 ± 2.59% for α- and γ-tocopherol, respectively, in bovine milk (UHT) using an *in vitro* digestion model. The greater capacity for vitamin E release in LIFF compared with other PO and DF_DPL was probably due to the approximately twofold smaller droplet size in this IFF.

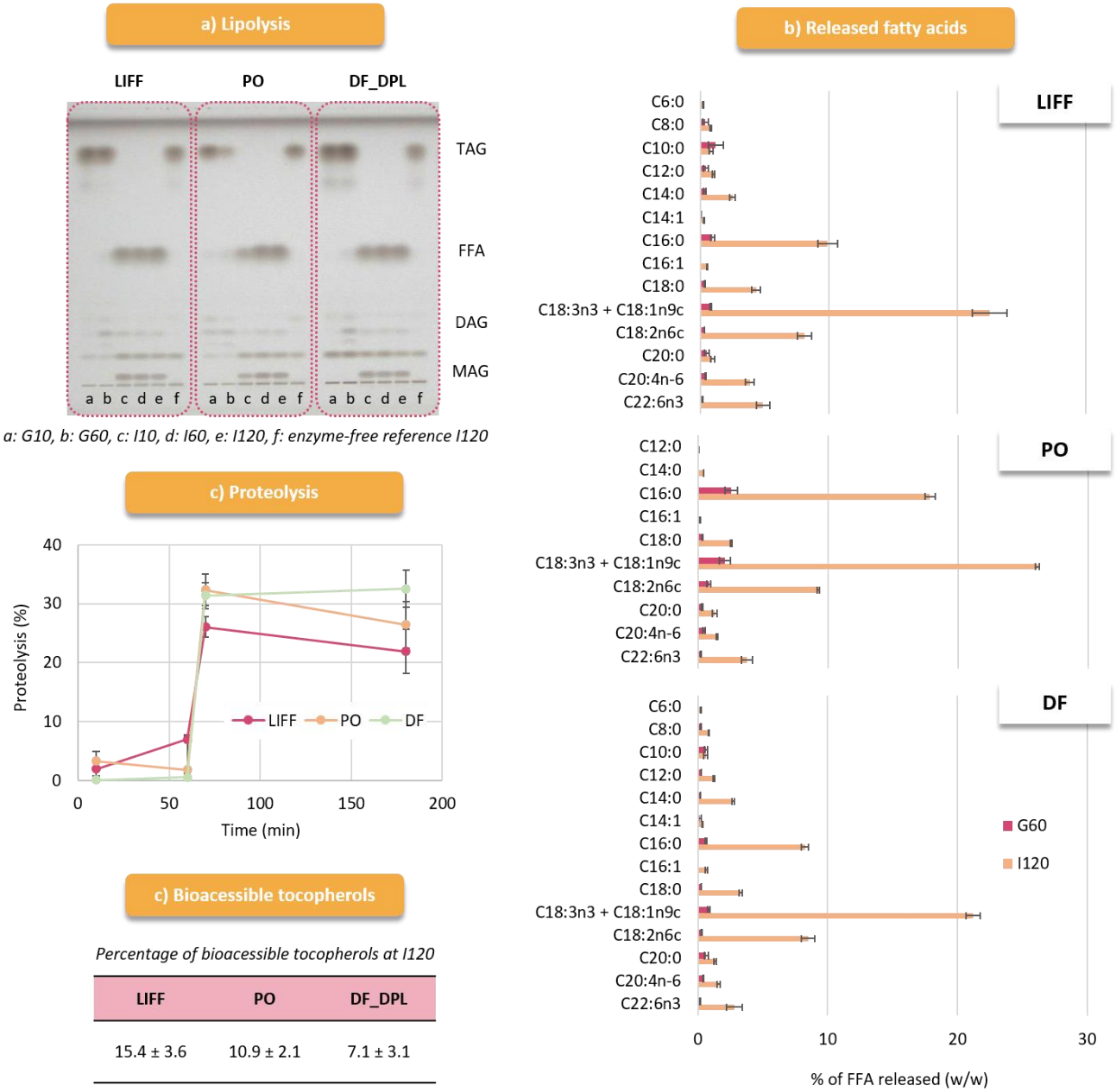


Figure 51. Bioaccessibility of lipid, protein and vitamin E in IFF

3.3. Bioaccessibility of macro and micronutrients in infant gruels

Similar to IFF, the rate of lipolysis in infant gruels was limited during the gastric phase and occurred mainly during the intestinal phase. From the first 10 minutes of the intestinal phase, lipid classes monitoring indicated almost complete TAG hydrolysis in both infant gruels (**Figure 52.a**). Spot intensity was lower in the case of OIF, whose lipid content was more than a factor two lower than that of CIF. Concerning the FFA released profile, the results confirm that lipolysis occurred mainly during intestinal phase with a significant increase between G60 and I120 (**Figure 52.b**). The percentage of FFA released were as expected significantly lower than for IFF due to the lower total lipid contents in infant gruels

(up to 20% in IFF and below 6% for infant gruels). The results also show that DHA and ARA begin to be released during the gastric phase and in higher amount compared to other FA and then their relative amount remain stable in the duodenal compartment at higher lipolysis degree.

The proteolysis degree increased significantly during intestinal phase similarly in both gruels and reached on average 24.9 ± 3.6% which was equivalent to the extent assessed in IFF. This proteolysis degree is quite low but difficult to compared with data from literature for which the overall *in vitro* digestibility is generally reported after longer time of digestion and ranged for various complementary porridges varying in formulation and processing from 37 to 58 % (Ejigui, Savoie *et al.* 2007).

The total tocopherols bioaccessibility was also estimated to 20.7 ± 0.7 in CIF and could not be determined in OIF (**Figure 511.d**).

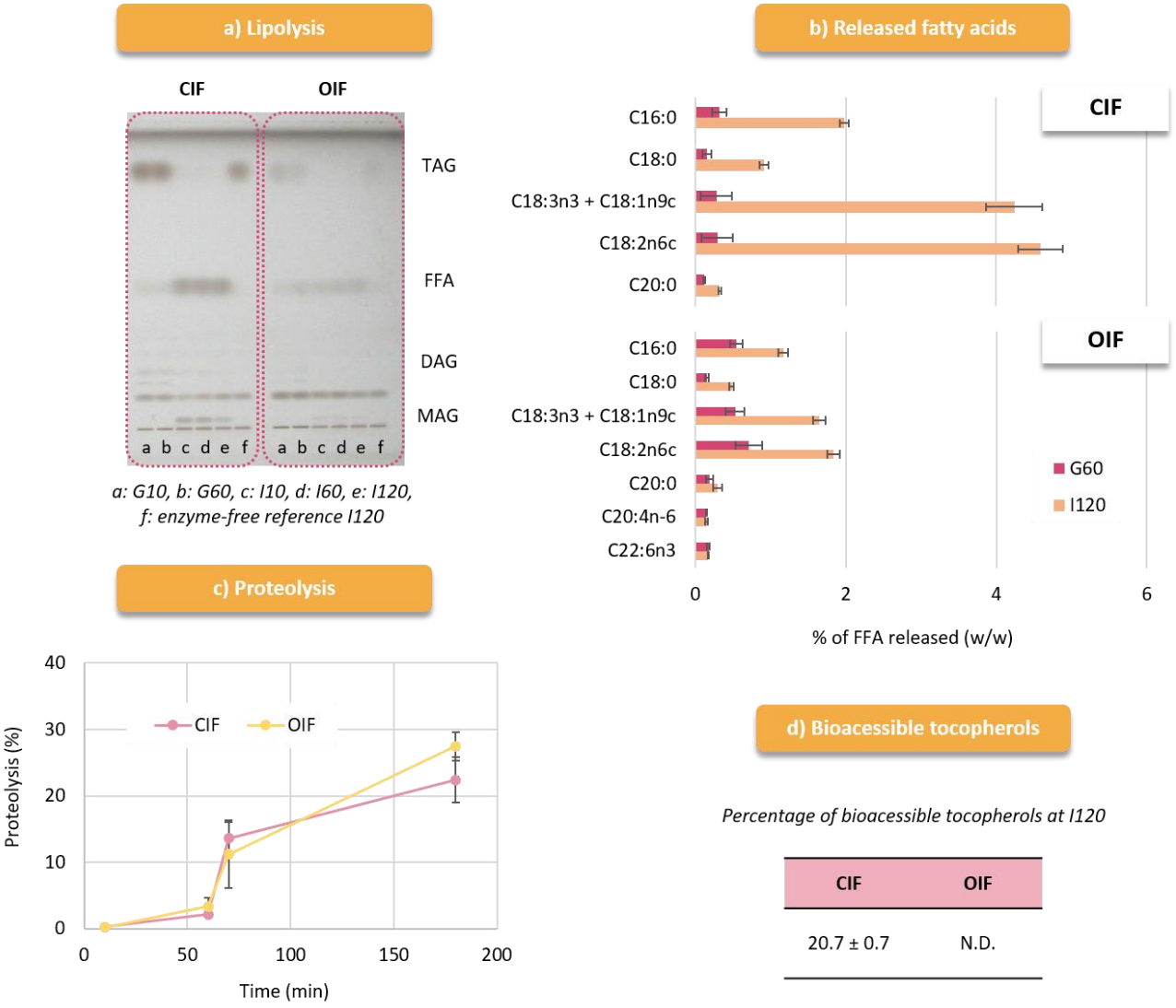


Figure 52. Bioaccessibility of lipid, protein and vitamin E in infant gruels

Comparison of both types of dispersed systems show that IFF and IF present in the selected subset similar digestive lipolysis, proteolysis extents and tocopherols bio accessibility (around 20 %). The released FA profiles however differ in gastric and intestinal phase for IFF dairy based (LIFF and DF_DPL). The quality of peptides released from IFF and IF could not be estimated but is certainly very different between IFF (containing important amount of dairy proteins) and IF (based on vegetal proteins).

4. Conclusions

The optimized IFF and IF subjected to the *in vitro* digestion investigation present overall good FA and LC-PUFA (including DHA) bioaccessibility. The results have shown a limited proteolysis in the *in vitro* condition used aspect and 7-20 % tocopherols bioaccessibility. This approach should be reinforced but has to be bear in mind to have global multicriteria optimization approaches when modifying infant foods.

MAIN RESULTS

- Fair bioacessibility of LC-PUFA in all IFF and IF tested
- High lipolysis degree at the end of the intestinal time with FA profile that reflect the regiodistribution of FA in the lipid source marked with high level of short to medium chain in DF-based IFF
- Limited proteolysis extent in all systems
- Fair tocopherol bioacessibility (around 20 %) in most systems



CHAPTER 6

General discussion



The aim of this chapter is to establish a comparison between the findings derived from investigations of the oxidative stability of IFF and IF and their digestive behavior.

In a first part, the comparison of the two types of dispersed systems aims to determine the main correlations and differences of their oxidative behaviors and to identify the key parameters to control oxidation in both wet and dry systems. In this part, mechanistic hypotheses for the diffusion of lipid oxidation regarding the type of system and its physico-chemical properties are proposed. A second section is dedicated to the issues related to the fortification of infant foods, with an overview of both nutritional properties and digestibility, and the challenges associated with maintaining oxidative stability. The importance of balancing these two aspects during infancy period is therefore discussed. Which matrices are most suitable for different nutrients? Is it necessary to enrich infant foods with LC-PUFA or are precursors sufficient? How can we manage the complementarity of foods in a diet over the 6 to 12 months period (IFF and IF) and cover all nutritional needs, in addition to breast milk?

1. Comparison of the oxidative stability of wet and dry dispersed systems

The main results obtained from the studies of the oxidative stability of IFF and IF are described in chapters 3 and 4. The major physico-chemical lipid levers identify as modulator of IFF and IF stability are summarized in **Table 26**.

1.1. Main similarities in lipid oxidation patterns between wet and dry dispersed systems

1.1.1. Vitamins: key levers to control lipid oxidation

The results show that vitamins play a crucial role in the lipid oxidation rate in IFF and IF. In both types of system, vitamins act as sacrificial agents for lipid oxidation. They are lost primarily, leading to an increase in the lag phase. When their consumption level is advanced, an increase in PV and a loss of FA (mainly LA in IF whereas LC-PUFA remained stable in our systems) is observed. The composition of vitamins and their chemical forms, conditioned by the choice of raw materials, influences their physico-chemical properties and consequently their effectiveness in delaying lipid oxidation.

VMC-enriched IFF and IF studied have a total tocopherols contents of approximately of 700 and 1800 ppm, respectively. Tocopherols are chain-breakers of lipid oxidation due to their ability to scavenge free radicals (Ma, Wang *et al.* 2023). This activity leads to the formation of tocopheryl radicals, which possess a resonance structure making them unreactive and therefore unable to propagate lipid oxidation. Moreover, tocopheryl radicals are capable of reacting with each other to form tocopherol dimers, or with lipid peroxyl radicals to form tocopherol-lipid peroxide complexes. Tocopherols also possess singlet oxygen quenching activity, although it is less important than that of carotenoids (Barouh, Bourlieu *et al.* 2021). The antioxidant capacity of tocopherols depends on their concentration (pro-oxidant effect above a threshold concentration) and tocopherol isomer distribution (Jerzykiewicz, Cwieląg-Piasecka *et al.* 2013). Our results have shown that in both oil-in-water emulsions and dry granular systems the α-isomer is lost first followed by γ-tocopherol. Thus, IFF with a predominance of the γ-isomer show lower PV evolution and total tocopherols loss than those with a majority of α-tocopherols. For IF, γ-tocopherol is systematically the main isomer, and similarly to what had been observed for IFF the α-tocopherol was totally lost after a 6-month storage. In bulk oil systems, the antioxidant capacity of tocopherols isomers based on dissociation energy of their phenolic O-H bond predicts the following order of antioxidant efficiency: α < β < γ < δ. However, in more complex dispersed systems such as ours, this ranking does not seem to be applicable, probably due to the chemical environment influencing the antioxidant efficiency of the different isomers. Some authors have reported that α-tocopherol can exhibited a pro-oxidant effect and may act as a chain transfer agent in the lipid oxidation mechanism leading to an acceleration of auto-oxidation (Nain, Berdal *et al.* 2021).

Vitamin A profile of the studied IFF and IF depends on the raw materials origin. Ingredients from vegetable origin (vegetable oils and flours) are carriers of carotenoids, while animal fats also provide retinol and its ester forms (retinyl palmitate and acetate). Vitamin A fortification, which constitutes the majority of the total content, was systematically performed under the form of retinyl acetate. The results show that in both wet and dry dispersed systems, vitamin A is particularly sensitive to oxidation and therefore undergoes significant degradation. Loss rates for vitamin A are almost systematically higher than that for vitamin E, which can be explained by the important reactivity due to the chemical structure of compounds belonging to the vitamin A family (preformed and pro-vitamin A). Indeed, tocopherols are composed of a chromanol ring and an isoprenic chain, while preformed and pro-vitamin A compounds have a carbon chain with minimum five double bonds. Vitamin A stabilization effects have been demonstrated in our work the presence of carotenoids in RPO based IFF and in IF including AL. In these systems, carotenoids are degraded first, thus delaying retinoid losses. Once carotenoid consumption was advanced, retinoid losses have been observed. The main functional carotenoids are β-carotene and lutein for the non-polar (carotenes) and polar (xantophylls) carotenoids, respectively. These compounds exhibit antioxidant activity due to their significant singlet oxygen quenching capacity. β-carotene and lutein have 10 and 11 conjugated double bonds respectively, giving them an effective capacity for energy transfer quenching (Ma, Wang *et al.* 2023). In addition, carotenoids have free radical scavenging activity *via* a hydrogen donation mechanism, slowing down the lipid peroxidation process (Edge and Truscott 2018). The resonance of carotenoid radical products ensures their stability. Similar results of vitamin A stabilization are observed in the presence of phenolic compounds provided by the addition of BRB in IF.

The results also show that vitamin C under the form of ascorbic acid provided by the addition of VMC is totally lost in IFF after a 20-days storage tests at 40°C and lost at approximately 50% in IF containing LC-PUFA after a 6 months of storage test at 25°C.

Overall, these results suggest that vitamin losses order depend on the system composition:

- In IF non-enriched with LC-PUFA: primarily losses of vitamins A and E followed by vitamin C.
- In IFF and IF enriched with LC-PUFA: primarily losses of vitamins A and C followed by vitamin E.
- In IFF and IF with vitamin A-stabilizing agents such as carotenoids or phenolic compounds: primarily losses of vitamins E and C and then vitamin A.

In addition, the presence of multiple compounds exhibiting antioxidant capacity leads to synergistic or antagonistic interactions between them depending on the nature of the interactions, which may explain these mechanistic changes.

1.1.2. Antioxidants synergies

IFF and IF contain multiple compounds with antioxidant activity. Some of these compounds are capable of interacting synergistically, thus developing a greater antioxidant capacity than when added individually. These synergies have been the subject of numerous studies and are therefore widely documented in the literature.

Tocopherols and ascorbic acid, for example, act synergistically *via* both combination of activities (metal chelating and free radical scavenging) and a regeneration of tocopherols by ascorbic acid through electron transfer (Barouh, Bourlieu *et al.* 2021). This synergy is particularly effective in a bulk oil system due to the polarity of ascorbic acid as describe by the "polar paradox effect" (Frankel, Huang *et al.* 1994).

Tocopherols can also have a synergistic effect with carotenoids such as β-carotene. Heinonen, Haila *et al.* (1997) and Zou and Akoh (2015) reported this effect in oil-in-water emulsion and suggest that β-carotene promotes the regeneration of tocopheryl radical back to their active form. The improved oxidative stability of the wet dispersed systems and the vitamin A stabilization in dry systems in the presence of carotenoids can be explained by this antioxidant synergy. In both systems losses in tocopherols could be due to the mobilization of tocopherols for the carotenoid regeneration.

Tocopherols exhibit synergism effect with PL. Samdani, McClements *et al.* (2018) reported significant synergy between δ-tocopherol and PS and suggested that this effect was linked to an interaction between the amino groups of PL and tocopherols. More recently, Tang, Cao *et al.* (2023) showed using electron paramagnetic resonance spectroscopy that the presence of PL had an impact on the localization of tocopherols, with an improved distribution in the interfacial area. The results obtained in this project show significant synergy between DPL and tocopherols in IFF. It can be explained by synergy between SM (24-29% of total dairy PL) and vitamin E (Suzuki-Iwashima, Iwasawa *et al.* 2021).

Physical effect cannot be discarded as SM contain very long chain PUFA (over C20 ; Bourlieu-Lacanal, Cheillan *et al.* 2018). Protein contained in DPL ingredient can also present antioxidant activity (Wong and Kitts 2003).

1.1.3. Effect of processing on IFF and IF oxidative stability

The results have shown that in both IFF and IF, the processing conditions impact the initial oxidative state of the systems, with a loss of vitamin A in particular retinol in IFF during homogenization and an increase in PV in IF including LC-PUFA and photosensitive compounds.

Homogenization, a necessary step to guarantee the physical stability of IFF, involves shearing forces of varying magnitude depending on the conditions applied, which induce local heating. Retinol, being particularly heat-sensitive, is partly lost during this stage of the process (Sachdeva, Kaushik *et al.* 2021).

On the other hand, the impact of homogenization is not significant on all the other indicators of oxidation monitored.

In the case of IF, the process involves milling and blending steps that cause cell destruction (release of cell content including enzymes such as LOX) and promote the contact between the unsaturated lipids and pro-oxidant compounds, light and oxygen. Thus, IF processing facilitates the initiation of oxidation, especially when the composition includes sensitive compounds (Lampi, Damerau *et al.* 2015). Results have shown that in the presence of LC-PUFA or photo-sensitive compounds, post-processing PV were increased. However, oxidation propagation was relatively slow during storage.

Processing conditions also determine droplet size in IFF and particle size in IF. The results have shown that in both types of dispersed system the impact of these parameters (on the tested size range) on oxidative stability during storage is either non-significant or limited, except for carotenoids in IFF, which presented loss twice higher in the case of 0.4 µm droplets as compared to 0.7 µm droplets. Such an effect has already been reported by (Zhang and Li 2021) and was attributed to the increase in specific surface area.

In the literature, the effect of droplet/particle size is highly controversial. Intuitively, the hypothesis concerning the effect of droplet size in emulsion would be that the increase in specific surface area due to a decrease in size would increase sensitivity to oxidation by offering a wider contact area between the unsaturated lipids and the pro-oxidants contained in the water phase (Berton-Carabin, Ropers *et al.* 2014, Berton-Carabin and Villeneuve 2023). However, these results are not always verified (Nakaya, Ushio *et al.* 2005, Let, Jacobsen *et al.* 2007, Atarés, Marshall *et al.* 2012). The results obtained in this project have shown that this effect is limited and may be hidden by more influential factors such as the composition of the interface (Sørensen, Baron *et al.* 2007). Regarding solid particles in dry dispersed systems, a greater reduction in size result in the release of cellular bioactive compounds such as antioxidants (Rosa, Barron *et al.* 2013), but this reduction also increases the exposure of lipids to oxidation-initiating factors (Rohfritsch, Canelli *et al.* 2021). It would therefore be interesting to confirm these results obtained in IF over a wider range of sizes.

1.2. Main differences in lipid oxidation patterns between wet and dry dispersed systems

1.2.1. Oxidation diffusion rate

The results have shown a significant increase in lag phase in IF compared with IFF (order of a few days *versus* months, respectively). In fact, oxidation mechanisms are slowed down in IF except for vitamin A without antioxidant fraction addition, where losses occurred over short times (about 50% loss have been observed during the first 15 days of storage). The lipid oxidation rate is associated with the

mobility capacity of compounds, which depends in particular on the Aw of the system (Barden and Decker 2016). A decrease in Aw up to 0.2 leads to limited diffusion of compounds, reducing contact and concomitantly lipid oxidation kinetics. Low-moisture systems with low Aw, such as IF, have a limited capacity for molecular mobility, compared with oil-in-water emulsions such as IFF, in which lipids, enzymes, antioxidants and pro-oxidants are highly mobile and can therefore interact (Nelson and Labuza 1992). Moreover, the protective effect observed at Aw levels of 0.2 to 0.4 is attributed to the formation of a bound water monolayer (unreactive). Below an Aw of 0.2, the water monolayer is no longer present, resulting in direct contact between lipids and oxygen, which explains the greater sensitivity to oxidation of systems with very low Aw.

The reduced mobility of compounds in IF compared to IFF also impacts the effectiveness of antioxidants according to their mechanisms of action and solubility properties. While in IFF the oxidation initiation pathway by pro-oxidant metals contributes to the increase in oxidation rate, in dry systems this mechanism seems particularly limited. Thus, metal-chelating antioxidants are effective in limiting oxidation in oil-in-water emulsions such as IFF, but are unlikely to be effective in dry systems. In dry systems, antioxidants such as free radical scavengers and oxygen quenchers seem to be more appropriate. However, their effectiveness also depends on their solubility properties and those of their environment. The theories of the polar paradox and the cut-off effect enable to predict the effectiveness of an antioxidant, particularly in emulsified systems (Frankel, Huang *et al.* 1994, Frankel, Huang *et al.* 1996, Laguerre, López Giraldo *et al.* 2009, Laguerre, López Giraldo *et al.* 2010). These theories are based on the partitioning of antioxidants between emulsion compartments. Thus, the antioxidant efficacy of a compound is enhanced when its solubility is inverse to that of its chemical environment. Moreover, at the CCL the antioxidant is localized at the interface and is therefore the most effective. These theories have proved particularly reliable for predicting antioxidant efficacy in oil-in-water emulsion systems. In dry systems several authors have suggested that the most effective antioxidants are the most fat-soluble (Barden, Barouh *et al.* 2015, Ten Klooster, Villeneuve *et al.* 2022). The most fat-soluble antioxidant compounds would have the same localization as lipids and could therefore guarantee a protective effect. However, the results obtained in this project, in correlation with studies by Van Wayenbergh, Struyf *et al.* (2020) and Van Wayenbergh, Coddens *et al.* (2023) have shown a significant antioxidant effect of cereal bran, attributed in particular to its water-soluble phenolic compound profile. Further research is therefore needed to understand the full descriptors (polarity, location) of an effective antioxidant in dry dispersed systems.

Another parameter explaining the significantly slower oxidation rate in IF compared to IFF is based on the difference in oxygen diffusion coefficients depending on the nature of the system. Indeed, oxygen diffusion is much significantly faster in a gaseous medium than in a liquid or solid one. In a medium of similar polarity, oxygen diffusion is faster in a liquid system than in a solid one: for non-oxidized liquid

olive oil, the oxygen diffusion coefficient is 7.5 x 10^{-10} to 9.3 x 10^{-10} m²/s, and is reduced by a factor two in solid copra oil with a coefficient of 2.2 x 10^{-10} to 4.8 x 10^{-10} m²/s (Chaix, Guillaume *et al.* 2014). Cuvelier, Soto *et al.* (2017) also reported i) that oxygen was up to 5 times more soluble in sunflower oil than in water and ii) a higher oxygen diffusion coefficient in oil than in water. All these physical parameters explain the lower oxygen diffusion capacity in IF compared to IFF, and contribute to slower oxidation kinetics in these dry systems at Aw > 0.2.

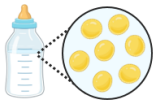
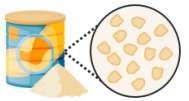
1.2.2. How does oxidation propagate depending on the dispersed system type?

Differences in physico-chemical properties, and in particular in the mobility of compounds in IFF and IF, instinctively impacts the modes of oxidation propagation.

The mechanisms involved in the propagation of oxidation in oil-in-water emulsions such as IFF are not yet elucidated, but several hypotheses have been put forward to explain the generalization of oxidation to the entire system (Laguerre, Bily *et al.* 2017, Laguerre, Tenon *et al.* 2020, Villeneuve, Bourlieu-Lacanal *et al.* 2021). One of these hypotheses involves micelle-assisted transfer. Micelles are presumed to act as carriers of radicals, facilitating their propagation between droplets. In addition, LOOH formed during lipid oxidation have surface-active properties, enabling them to aggregate onto the droplet surface and self-assemble into micelles (or co-micelles with emulsifiers). The colloidal structures thus formed would then be involved in the diffusion of oxidized compounds through the water phase to other droplets, contributing to the spread of oxidation.

Regarding dry systems, the results obtained suggest that oxidation is induced mainly during processing, by promoting contact between unsaturated lipids and pro-oxidant compounds (release of cellular contents), oxygen and light. Due to the reduced mobility at low Aw, oxidation propagation seems to occur from one point to another at a very slow rate. It has been suggested that in such systems, peroxyl radicals are rapidly neutralized by nearby antioxidant compounds, leading to the formation of antioxidant radicals. In this way, the conversion of peroxyl radicals to hydroperoxide and then to secondary compounds is slowed down (Wei, Zhu *et al.* 2021). In the case of the presence of photosensitive compounds such as chlorophyll, the results show faster propagation, which may be linked to the rate of photo-oxidation, which is up to 1,000 to 1,500 times faster than autoxidation (Ghanbari, Anwar *et al.* 2012).

Table 26. Main results of oxidative stability studies of IFF and IF

Dispersed liquid system - Oil in water emulsion *Infant follow-on formulas*		Dispersed dry system - Granular system *Infant flours*
		
Formulation levers		
Lipid sources *Different sources of SFA compared to palm oil* **Parameters** • Coconut oil → *Medium-chain SFA* • Red palm oil → *Provitamin A and tocotrienols* • Dairy fat → *FA profile more biomimetic of breast milk* **Oxidative stability** • ↗ oxidative stability with γ-tocoperol as the dominant isomer • Carotenoids and tocotrienol improved oxidative stability (synergy with tocopherols)		**Flours sources** *Different sources of flours* **Parameters** • Northern or southern IF (cereals versus legumes) → *Lipid profile imbalance in commercial IF* • Addition of ω3-source flours (cowpea and teff flours) → *Improve lipid profile with a lower ω6/ω3 ratio* • Addition of LC-PUFA powders → *Provides essential FA and their long- chain derivates* **Oxidative stability** • Acceptable stability of representative IF of commercial products • Acceptable stability (without LC-PUFA addition) compared to commercial products • Reduced oxidative stability in the presence of LC-PUFA with important loss of vitamin A
Vitamins and minerals fortification *Addition of a VMC and different chemical form of vitamin A* **Parameters** • Addition of a VMC → *Provides essential vitamins and minerals* • Chemical form of vitamin A (retinol, retinyl esters or carotnoids) → *Improved vitamin A status* **Oxidative stability** • VMC is an antioxidant • Retinyl acetate more stable than retinol • ↗ oxidative stability in presence of carotenoids • Small droplet size → Carotenoids destabilization		**Forrtification** *Addition of a VMC, different antioxidant fractions* **Parameters** • Addition of a VMC → *Provides essential vitamins and minerals* • Addition of antioxidant fractions (carotenoids from amaranth leaves powder or phenolic compounds from black rice brans) → *Provides bioactifs compounds* **Oxidative stability** Limited vitamin A degradation in the presence of antioxidant fractions
Interface composition *Different emulsifier source* **Parameters** Source of emulsifiers: • Soy lecithin • DPL → *DPL complexify interface* **Oxidative stability** ↗ oxidative stability in presence of DPL (synergy of SM with tocopherols + steric barrier)		
Structure/process levers		
Droplet core composition *Different droplet core composition* **Parameters** Droplet core composition: • Homogenous • Heterogeneous → *More biomimetic of breast milk* **Oxidative stability** No impact on oxidative stability		**Particle size** *Flours grains size* **Parameters** Particle size of IF: • D50: 184.5 µm (specific surface: 1006.6 cm²/mL) • D50: 102.2 µm (specific surface: 1306.3 cm²/mL) Particle size of black rice bran: *(data not show)* • D50: 67.8 µm (specific surface: 1599.5 cm²/mL) • D50: 49.7 µm (specific surface: 2082 cm²/mL) **Oxidative stability** No impact on oxidative stability on the range of size tested
Emulsifier addition process *Emulsifier addition process (in oil vs water phase)* **Parameters** Emulsifier addition process: • In oil phase • In water phase **Oxidative stability** Decreased oxidative stability in presence of colloidal strcutures (mass transport of pro-oxidant compounds)		
Droplet size *Different lipid droplet sizes* **Parameters** Lipid droplet size: • 0.4 µm • 0.7 µm → *More biomimetic of breast milk* **Oxidative stability** • ↘ vitamin A and carotenoids losses with smaller droplets • No impact on oxidative stability on the range on size tested		**Heat treatment** **Parameters** • Heat treatment of cowpea flour → Reduction of anti-nutritional factors **Oxidative stability** Reduction of LOX activity and delaying the loss of linoleic acid
Key stabilization levers		
• Oxidation rate relatively fast: Rapid diffusion, possible involvement of colloidal structures • Oxidation marked primarily by losses of fat-soluble vitamins: Impact of tocopherols isomers distribution (γ-tocopherol most stable) • Chemical form of preformed or pro-vitamin A: Carotenoids (synergy with tocopherols) and retinyl acetate most stable • Interface composition: DPL increases interface coverage and density and provides SM (synergy with tocopherols) potential effect of whey or milk fat membrane • Limited effect of droplet sizes (over the size range tested) • Process: Homogenization impact initial content of vitamin A and carotenoid loss rates during storage		• Oxidation rate relatively slow except for vitamin A: Limited diffusion (over the Aw range tested) • Oxidation marked primarily by losses of fat-soluble vitamins and change in phenolic compounds profile: Fat-soluble vitamin loss kinetics depend on chemical composition (presence or absence of antioxidants) • Vitamin A stability depend on antioxidants: Amaranth leaves and black rice bran are vitamin A-stabilizing agent (carotenoids and phenolic compounds) • Limited effect of particle sizes (over the size range tested) • Process: Impact on the initial oxidation level

2. Optimization of infant foods: how to find a balance between nutritional properties and oxidative stability

2.1. Which matrices are most suitable for different nutrients?

The challenges of meeting infant nutritional requirements are multiple. On the one hand, infant foods must be sufficiently stable to oxidation to provide nutrients in sufficient quantity and of good quality throughout their shelf life. In addition, these nutrients must be sufficiently bioaccessible to be absorbed and used by the body. The balance between oxidative stability and the nutritional properties of IFF and IF is therefore essential. The results obtained have shown that, overall, lipids (FA and fat-soluble vitamins) are more stable in IF than in IFF, due to the slower oxidation kinetics in dry systems. However, emulsified feeds such as IFF have the advantage of favoring micellarization of lipid compounds during the digestion process and thus promoting their absorption. The bioaccessibility of nutrients is linked to their structure in the food matrix, it has been reported that lipolysis and proteolysis kinetics were significantly different between breast milk and infant formulas, notably due to their different droplet sizes (Bourlieu, Ménard *et al.* 2015). In addition, IFF have a higher nutrient density than gruels prepared from IF. In order to obtain a sensorially acceptable texture (gruel flow rate around 100 mm/ 30 s), IF are diluted mainly with water to around 6 to 14% dry matter, which considerably reduces their nutritional density (Mouquet, Greffeuille *et al.* 2006). IF dilution also affects their lipid content. For carotenoids, it has been shown that a minimum lipid content of 1.55% is required for micellarization (Conboy Stephenson, Ross *et al.* 2021). Moreover, the anti-nutritional compounds contained in IF are also a limiting factor, but can easily be eliminated during baking. The accessibility of IFF and IF is also a point to consider. In northern regions, IFF are more consumed than IF, and inversely in southern regions, where the prevalence of malnutrition issues is the highest. Raising awareness and promoting good practices in the preparation of infant gruels are therefore key points in guaranteeing their nutritional properties. Cereal flours have already demonstrated their potential as micronutrient carriers, for example by fortifying wheat flour with retinyl palmitate (Rahman, Ahmed *et al.* 2015) or with iron (Harrison, Hays *et al.* 2022). In view of the results obtained, optimizing the lipid profile of IF by introducing flours that are sources of essential FA with a balanced ratio and adding vitamin A stabilizing agents could contribute to cover infant nutritional needs more effectively.

2.2. Is it necessary to enrich infant foods with ω3 LC-PUFA or are precursors sufficient?

In this work, that was triggered in a way by a regulatory evolution targeting IFF (compulsory fortification with DHA), the importance of LC-PUFA for infant development is emphasized. However, as shown in **Table 27**, and in line with the INCA3 survey of food consumption habits carried out in France between 2014 and 2015, for a combined consumption of 500 mL/d of IFF and 50 g/d of IF, DHA

intakes are below recommendations from 6 to 12 months of age. When the adequacy of average IFF from the global overview of marketed product or CIF in our study, with nutritional recommendations is questioned, it appear that combining both foods in recommended quantities provides 70% of daily total energy, 61 % of recommended lipids, all essential FA, 74 mg of DHA and almost all vitamins A and E (**Table 27**). It is important to underline that similar adequacy and higher health benefits would be reached if combining breast milk and IF. However, in southern regions, low intake of preformed LC-PUFA is common due to the poor accessibility of marine foods except in coastal areas. In Gambia, for instance, the DHA intake fell from 21 to 1 mg/kg body weight between 6 and 24 months (Prentice and Paul 2000). DHA status also depends on the bioconversion capacity of ALA, which is assumed to be limited during infancy (Hadley, Ryan *et al.* 2016). This capacity depends, among other factors, on the balance of LA and ALA levels in the diet, with a reduction in biosynthesis efficiency at higher ratios due to metabolic pathway competition effects. As shown in **Table 27**, cumulative daily LA levels are higher than recommended although not present in very excessive amounts.

Table 27. Adequacy of lipid profiles of IFF and IF with nutritional recommendations (FAO 2001, FAO and WHO 2004, EFSA 2017)

	IFF* *500 mL/d*	**IF**** *50 g/d*	**Total IFF + IF**	**Recommendations** *6 - 12 months*
Energy (Kcal/d)	336.0	202.5	538.5 *70% of recommended TE*	765
Lipids	16.5 g/d *i.e.* 148.5 Kcal/d	4.4 g/d *i.e.* 39.6 Kcal/d	20.9 g/d *i.e.* 188.1 Kcal/d	40% of TE *i.e.* 306.0 Kcal/d
LA	2.5 g/d *i.e.* 22.6 Kcal/d	1.9 g/d *i.e.* 16.7 Kcal/d	4.4 g/d *i.e.* 39.2 Kcal/d	4% of TE *i.e.* 30.6 Kcal/d
ALA	0.3 g/d *i.e.* 2.4 Kcal/d	0.2 g/d *i.e.* 1.8 Kcal/d	0.5 g/d *i.e.* 4.2 Kcal/d	0.5% of TE *i.e.* 3.1 Kcal/d
DHA (mg/d)	74.0	-	74.0	100
ARA (mg/d)	47.0	-	47.0	-
Vitamin A (µg/d)	307.5	49.1	356.6	400
Vitamin E (mg/d)	6.0	1.6	7.6	5

TE: total energy

**Mean IFF from the panorama*

***CIF*

The question of the essential nature of LC-PUFA during infancy therefore arises in a context where LA intakes are very often excessive and ALA deficient. The pre-clinical investigation conducted by Carlson,

O'Loughlin *et al.* (2019) demonstrated, on an animal model, that a diet comprising solely DHA and ARA as the sources of ω3 and ω6 FA, without the inclusion of any precursors, effectively supported the proper growth and development. However, a strategy to improve DHA status based solely on fortification with preformed LC-PUFA is difficult to envisage from an economic point of view (high value-added products), accessibility in southern regions in particular, and to guarantee product oxidative stability. Improving DHA status can also be achieved by rebalancing the LA/ALA ratio of infant foods, with a reduction in ω6 intake to ensure efficient bioconversion (Brenna, Akomo *et al.* 2015). In this sense, OIF_HT could be an interesting system. Regarding IFF, Delplanque, Du *et al.* (2013) demonstrated that dairy lipids could serve as a sacrificial agent for β-oxidation and lead to better elongation of ALA to DHA. Thus, model IFF based on DF seems of interest both from oxidative and nutritional point of view.

2.3. Are the IFF and IF complementary and do they cover all nutritional requirements?

Table 27 shows the cumulative daily nutritional intake resulting from the consumption of 500 mL IFF and 50 g IF. Lipid intake, which should represent 40% of total energy from 6 to 12 months, covers 35% of energy, assuming that all ingested lipids are absorbed. LC-PUFA are only provided by IFF at low levels, which are below recommended levels. In addition, vitamin A intake appears to be low, considering the percentage of loss during processing and storage (over 50% in two weeks for IF without added antioxidant fraction) and the percentage of absorption capacity ranging from 75 to 100% for retinol, and a significantly reduced capacity for carotenoid with 23.5 to 47.5% (Reboul 2013, Conboy Stephenson, Ross *et al.* 2021).

As highlighted in the overview of IF nutritional values and in agreement with Moustiés, Bourlieu *et al.* (2019) overages is usually applied particularly for micronutrient to guarantee adequate levels throughout the storage period. However, this practice raises questions about the toxicological effect of vitamin A and E oxidation products.



CHAPTER 7

General conclusions and perspectives

The aim of this thesis was to identify the key physico-chemical levers to enhance the oxidative stability of LC-PUFA enriched dispersed systems while maintaining acceptable nutrients bioaccessibility. While numerous studies in the literature focus on improving oxidative stability in simplified systems, few have been applied to more complex matrices that represent real consumed foods. Moreover, limited research has evaluated the impact of proposed strategies on lipid digestion. The originality of this work is therefore to propose a multi-approach of optimization to extend the lag phase of lipid oxidation and optimizing nutritional benefits of products consumed during the vulnerable period of dietary diversification.

The results provide a comparison of the behaviors and mechanisms of lipid oxidation and antioxidants in two types of dispersed systems: liquid systems in the form of oil-in-water emulsions represented by IFF, and solid granular systems represented by IF. It first demonstrates that the composition of commercial IFF is homogeneous and in line with regulations, although their lipid fractions mainly include vegetable oils which are not biomimetic of breast milk in terms of structure and composition. With regard to IF, market analysis shows considerable heterogeneity in nutritional values across the world, and a prevalence of imbalanced lipid profiles (excess ω6) and micronutrient overages. A current challenge is therefore to optimize the composition of IFF and IF to meet infant nutritional needs. The speed and mode of oxidation propagation depend on the physico-chemical properties of the matrices. Whereas in emulsified systems, molecule mobility leads to rapid oxidation propagation, probably involving droplet-to-droplet contact and colloidal structure-assisted transport, granular systems are characterized by significantly slower kinetics, except for vitamin A, which can be very rapidly degraded, probably involving local diffusion. In both types of system, oxidation primarily affects vitamins, notably A and E, while LC-PUFA remain relatively stable.

Formulation parameters played a key role in controlling lipid oxidation in both types of systems. For IFF, the re-introduction of dairy ingredients (DPL and DF) providing SM and the addition of carotenoids in the presence of tocopherols led to an improvement in oxidation resistance and optimization of the nutritional profile to meet infant needs. With regard to IF, the selection of alternative flours such as cowpea and teff (sources of ALA) rebalances the lipid profile without impacting oxidative stability. In addition, the results demonstrate the significant potential of black rice bran for providing phenolic compounds as a vitamin A-stabilizing agent. In both systems, the initial quality of the used raw materials and the processing conditions are determinant for oxidative stability during storage and in particular the stability of vitamin A. Flour characterization showed that, depending on the source considered, initial quality was variable and poorly controlled.

Overall, the effect of structural parameters on the oxidative stability of granular and emulsified systems was much more limited. In particular, the results show that droplet and particle size had, on the range of sizes investigated, no influence on the oxidation stability.

Regarding the bioaccessibility of lipids and proteins on subset of optimized IFF and IF, investigated in 6-month old static *in vitro* digestion model, results showed high FA release and DHA bioaccessibility, around 20 % tocopherol bioacessibility in most systems, and limited proteolysis. Introduction of DF, did modulate strongly the gastric and intestinal FA profile.

In this work, we focused on purpose on lipid formulation and physical parameter that modulate the systems reactivity as these aspects had not been deeply studied in the literature. But we bear in mind, that several non-lipid factors are extremely effective in stabilizing IFF and IF against oxidation. These factors gather protective headspace, introduction of antioxidant proteins or peptides for instance. In a way our lipid factors are less powerful but also more easy to be implemented in infant foods. We have in mind that global nutritional improvement of infant food should go through multicriteria approach and nutrients global optimization.

Project limitations and perspectives

In this work, the multiple approaches have enabled proposing IFF and IF formulations meeting several nutritional and lipid chemical stability criteria, while providing an understanding of the complex mechanism involved (although quite descriptive more than mechanistic). However, certain aspects of the project are yet to be explored and would benefit from further investigations, such as:

- **Oxidative stability of powdered IFF:** IFF can take the form of ready-to-eat liquid, as studied in this project, or concentrated liquid or reconstituted powder. Whatever the final form, the manufacturing process includes a wet-mix phase (ready-to-eat liquid IFF), which may undergo concentration (concentrated liquid IFF) and spray-drying (reconstituted powder). Ready-to-eat liquid IFF do not represent the majority of the market by volume, and are intended for rapid consumption. It would therefore be interesting to continue the IFF manufacturing process through to the spray-drying step, and then carry out a storage test on a powdered IFF. Such an experimental design would enable to compare liquid and dry IFF with strictly the same composition. The effect of process, matrix type and compound organization could be investigated. The bioaccessibility of lipids in a liquid IFF or a reconstituted IFF could also be compared.

- **Lipid and protein co-oxidation:** in this work, the focus was on the lipid fraction, but other constituents such as proteins also undergo oxidation phenomena. Therefore, it would have been interesting to carry out a simultaneous study of protein and lipid stability in both types of system. The development of a method for simultaneous quantification of protein and lipid



oxidation levels could be envisaged, using a Fourier transform infrared spectroscopymethod for example.

- **Combination of antioxidant synergies:** in this project, antioxidant synergies between SM, carotenoids or phenolic compounds were demonstrated with tocopherols. However, these synergies were investigated individually. It would be interesting to add several antioxidants simultaneously to explore the cumulative effect. In this way, it would be possible to identify whether, in combination, they enhance synergies or, on the contrary, compete with each other, leading to antagonistic effects.

- **Addition dairy raw materials:** in the literature, lactoferrin has been shown to have strong antioxidant properties in infant formulas. It would be interesting to evaluate the effect of these compounds in granular systems enriched in LC-PUFA. However, it should be bear in mind that lactoferrin has a high added value, its use on an industrial scale is limited. Would it be more effective in these systems than dry natural iron chelating systems such as phytic acid?

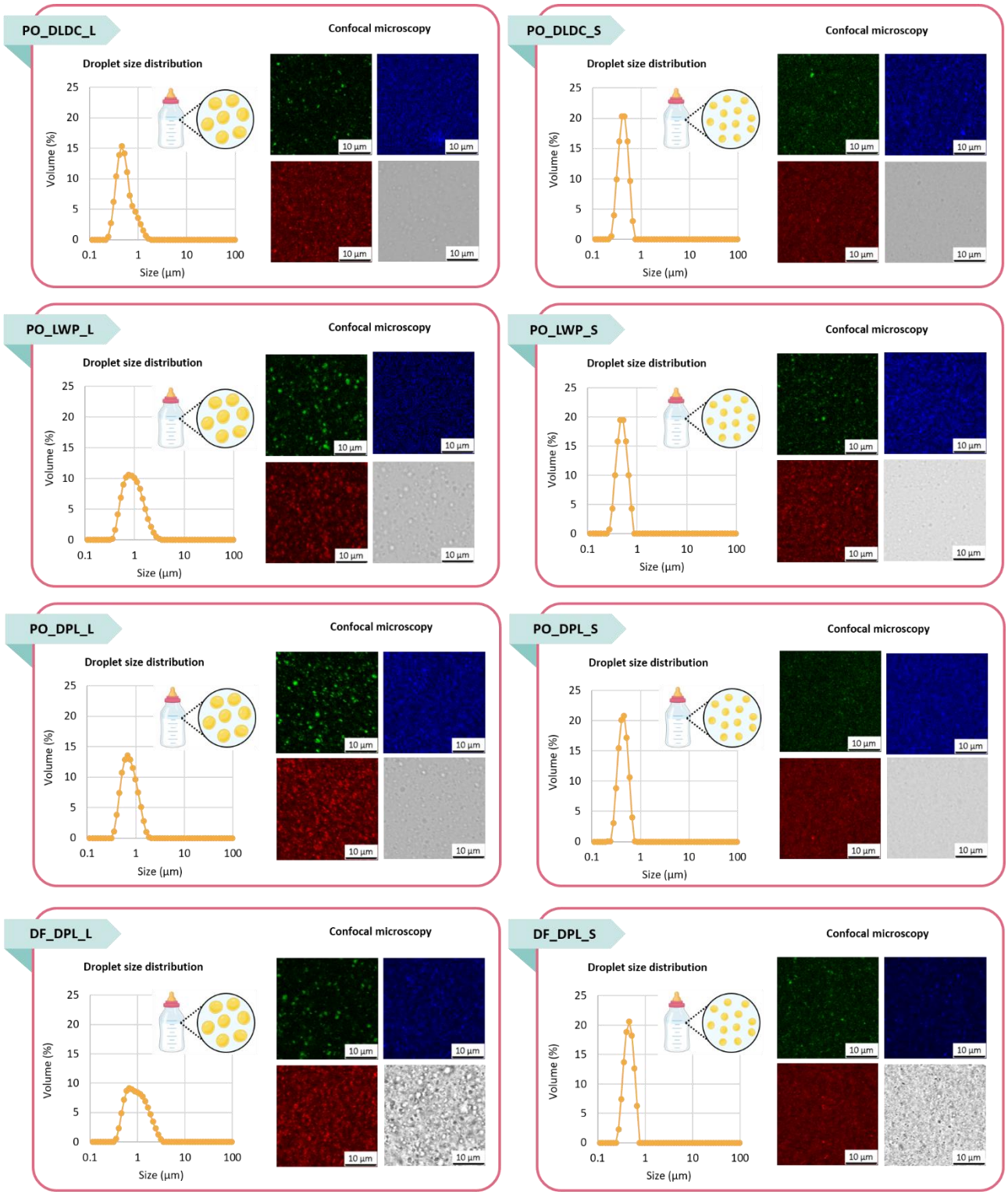
- **Oxidized matrix digestion:** the IFF and IF that underwent the *in vitro* digestion process in this work were non-oxidized. However, once initiated and sufficiently advanced, oxidation is associated with physical destabilization due to changes in the polarities of the oxidation products. In addition, some oxidation compounds have been shown to be potentially toxic. Thus, it would have been interesting to repeat the *in vitro* digestion process on oxidized matrices to assess the impact of oxidation on nutrient bioaccessibility. To go further an intestinal cell model or explants could be used to assess the absorption capacity of oxidized compounds. Using these approaches, absorption kinetics could be studied. It would also be interesting to demonstrate competitive digestion between these oxidized compounds and nutrients. These questions remain unanswered and could be the subject of further study in other PHD projects.

In terms, of perspectives, I will carry on with a postdoctoral study during which I will try to include in IFF natural seed lipoproteic assemblies (called oil body or oleosomes) and will try to shed light on the oxidative stability and bioacessibility of nutrients in such systems. I have also plan to shed light on the putative quantity of contaminants in the lipid mixtures used in my different IFF to again broaden the multi-objectives optimization of these products.

This work has contributed to the identification of key lipid parameters that determine the oxidative stability of IFF and IF enriched in LC-PUFA but opened new chapters in finalized research…



ANNEX



Particles size distribution was assessed by laser light scattering. Confocal laser scanning micrograph were collected using a 40× water-immersion with each time four micrographs: blue colored: proteins; red colored: amphiphiles compounds; green colored: lipids; white colored: transmission light micrograph.

Supplementary figure 1. Droplet size distribution of model IFF and physical organization

1. Accepted articles

Research article

M. Cancalon, Y. M. Hemery, N. Barouh, B. Baréa, C. Berton-Carabin, L. Birault, E. Durand, P. Villeneuve and C. Bourlieu-Lacanal (2023). "Comparison of the effect of various sources of saturated fatty acids on infant follow-on formulas oxidative stability and nutritional profile." Food Chemistry **429**: 136854. https://doi.org/10.1016/j.foodchem.2023.136854

Review article

M. Cancalon, N. Barouh, Y. Hemery, E. Durand, P. Guesnet, P. Villeneuve and C. Bourlieu-Lacanal (2022). "Supplémentation des formules infantiles en acides docosahexaénoïque et arachidonique : effets sur le développement de l'enfant et difficultés associées à leur introduction." Cahiers de Nutrition et de Diététique **57**(6): 370-383. https://doi.org/https://doi.org/10.1016/j.cnd.2022.04.007

Dataset

M. Cancalon, Y. M. Hemery, N. Barouh, E. Durand, P. Villeneuve and C. Bourlieu-Lacanal (2023). Overview of the nutritional composition of infant flours commercialized worlwide in 2021, Recherche Data Gouv. https://doi.org/10.57745/TUSCZF

M. Cancalon, Y. M. Hemery, N. Barouh, R. Thomopoulos, E. Durand, P. Villeneuve and C. Bourlieu-Lacanal (2023). Panorama of the nutritional composition of follow-on infant formula commercialized worldwide in 2021, Recherche Data Gouv. https://doi.org/10.57745/CLER60

Datapaper

M. Cancalon, Y. M. Hemery, N. Barouh, R. Thomopoulos, B. Baréa, E. Durand, P. Villeneuve and C. B. Lacanal (2023). "Dataset of the nutritional composition of follow-on infant formulas commercialized worldwide in 2021." Data in Brief: 109649.

2. Submitted manuscripts

Research article

M. Cancalon, N. Barouh, Y. M. Hemery, B. Baréa, E. Durand, C. Bourlieu-Lacanal and P. Villeneuve. "Stabilization of infant formulas against lipid oxidation: what are the key structural levers?"

Datapaper

M. Cancalon, Y. M. Hemery, N. Barouh, B. Baréa, E. Durand, P. Villeneuve and C. Bourlieu-Lacanal. "Dataset of the nutritional composition of infant flours commercialized worldwide in 2021."

3. Manuscripts in preparation for submission

Research articles

M. Cancalon, C. Bourlieu-Lacanal, P. Villeneuve, N. Barouh, , B. Baréa, E. Durand, Y. M. Hemery. "Influence of chemical forms and homogenization process on the stability of vitamin A in infant follow-on formulas"

M. Cancalon, Y. M. Hemery, N. Dormoy, N. Barouh, B. Baréa, E. Durand, R. Barbar, C. Antoine-Assor, M. Lebrun, L. Lhomond, A. Reau, P. Villeneuve and C. Bourlieu-Lacanal. "Optimization of the lipid composition and oxidative stability of infant flours."

M. Cancalon, M. Tournaux, Y. M. Hemery, P. Pinel, M. Robert, J. Claudel, N. Barouh, B. Baréa, E. Durand, P. Villeneuve and C. Bourlieu-Lacanal. "Bioaccessibility of lipids and proteins in infant follow-on formulas and flours using a static *in vitro* digestion model for 6-month-old infants"

3. Communications

3.1. Oral (N = 4)

Journée CNIEL by the French Society for the Study of Lipids (SFEL) - September 2022: « Les lipides laitiers : Dans les 1000 [1ers] jours et au-delà… » - Paris, France

Réintroduction de lipides laitiers dans les formules infantiles : impact sur la stabilité oxydative et sur le profil nutritionnel

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La période postnatale est caractérisée par une croissance et un développement rapide. Les lipides et plus particulièrement les acides gras polyinsaturés à longues chaînes (AGPI-LC) tels que les acides docosahexaénoïque (DHA) et arachidonique (ARA) jouent un rôle essentiel durant cette période. L'objectif principal des formules infantiles est d'être biomimétique de « l'étalon d'or » : le lait maternel, aussi bien en termes de composition que de structure, pour ainsi offrir la meilleure alternative possible à l'allaitement lorsqu'il a échoué et/ou n'est pas possible. Dans ce contexte, la réglementation européenne a évolué pour encourager une optimisation de la composition des préparations infantiles. Ainsi, depuis février 2020 la supplémentation des formules en DHA a été rendue obligatoire. Ces formules sont également fortifiées en ARA, autre AGPI indispensable pour le développement de l'enfant, bien qu'aucune obligation ne soit établie quant à sa teneur minimale. Leur important degré d'insaturation rend ces acides gras particulièrement sensibles à l'oxydation lipidique, complexifiant ainsi leur introduction dans les formules infantiles. Les lipides laitiers du fait de leur haute teneur en certains acides gras saturés constituent donc un atout pour la stabilisation contre l'oxydation. De plus, d'un point de vue structurel, des différences persistent entre le lait maternel et les formules infantiles ce qui a un fort impact sur les cinétiques de digestion et donc d'absorption des nutriments. Une fois de plus, les lipides laitiers (polaires ou apolaires) du fait de leur structure et composition complexe, présentent un intérêt particulier pour le développement de l'enfant notamment en modulant la digestion des protéines, le développement intestinal et en contribuant à l'éducation immunitaire et à celle du microbiote. L'objectif de cet exposé est de mettre en évidence, à travers une étude de stockage, l'intérêt technologique et nutritionnel de la réintroduction des lipides laitiers dans la composition des préparations de suite afin de les stabiliser contre l'oxydation.

Journées Chevreul of French Society for the Study of Lipids (SFEL) - January 2023: « 80 years of SFEL » - Paris, France

Optimization of the oxidative stability and lipid profile of DHA-enriched infant follow-on formulas by the introduction of dairy lipids

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Infancy period is a key window of development during which the requirements in lipids and more particularly in long-chain polyunsaturated fatty acids are specific. In order to abide by the recommendations, European regulation now requires a fortification of IFF with docosahexaenoic acid (C22:6, DHA) which strongly impacts their oxidative stability. The aim of this study was to optimize the structure and formulation of DHA-enriched IFF in order to improve their lipid profile and oxidative stability. To do so, a model IFF representative of marketed products was formulated and declined by varying the saturated fatty acids sources (refined (POM) or unrefined (RPOM) palm oil, coconut oil (COM), and dairy fat (DFOM)), the emulsifiers (soy lecithin or DPL) and the structure (droplet size). These model IFF were submitted to an accelerated storage test of 20 days at 40°C during which peroxide values (PV), tocopherols, vitamin A, and fatty acids profiles were monitored. Despite the addition of DHA, all model IFF had quite good oxidative stability with PV lower than 10 meqO2/kg of lipids until 9 days of storage. DFOM and POM had equivalent kinetics of PV increase and tocopherols degradation. Moreover, no significant differences were observed between these two IFF regardless of droplet size (0.4 or 0.7 µm). RPOM was more stable than POM, very likely due to its higher carotenoids content. Regarding emulsifiers, DFOM-DPL (stabilized with DPL) had a PV twice lower than POM (stabilized with lecithin) at 20 days and a tocopherols degradation of 67.5% *versus* 88.8% for POM. This result translates a more important resistance to oxidative degradation of DFOM-DPL. This favorable effect was even reinforced for smaller droplet sizes. The introduction of dairy lipids but also of carotenoids in DHA-enriched IFF composition are interesting levers of stabilization. Moreover, dairy lipids lead to a more complex lipid profile and a higher content of short and medium-chain saturated fatty acids which is beneficial for infant nutrition.

15th Congress of International Society for the Study of Fatty Acids and Lipids (ISSFAL) - July 2023 - Nantes, France

Effect of various saturated fatty acids sources on oxidative stability and nutritional profile of DHA-enriched infant follow-on formulas

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Infancy is characterized by significant growth and development that leads to very specific nutritional requirements. During this period, lipids and more particularly long-chain polyunsaturated fatty acids play an important role. To comply with the recommendations, European regulation have recently evolved and now requires fortification of IFF with docosahexaenoic acid (DHA) which strongly impacts their oxidative stability. The aim of this study was to improve the lipid profile and oxidative stability of DHA-enriched IFF by using structural and formulation lipid levers. To do so, a model IFF representative of marketed products was formulated and declined by varying the saturated fatty acids sources (refined (POM) or unrefined (RPOM) palm oil, coconut oil (COM), and dairy fat (DFOM)), the emulsifiers (soy lecithin or dairy phospholipids DPL) and the structure (droplet size). RPOM-IFF showed a higher stability than POM-IFF. This effect can be attributed to its content of provitamin A compounds (in the form of carotenoids) which act in synergy with tocopherols and have beneficial effects for infant nutrition. The combined use of DPL and DFOM led to a better oxidative stability with a peroxide value twice lower than POM (stabilized with lecithin) after 20 days of storage at 40°C and less important tocopherols degradation. This favorable effect was even reinforced for smaller droplet sizes. Dairy lipids also provide a complex lipid profile and high content of short and medium saturated chain fatty acids which is beneficial for infant nutrition. The introduction of dairy lipids and carotenoids in DHA-enriched IFF composition are interesting levers of stabilization.

19th Euro Fed Lipid Congress and Expo (EFL) - September 2023 - Poznan, Poland

Vitamin A stability in follow-on infant formulas depending on the chemical form and the homogenization process

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Vitamin A deficiency is the most widespread micronutrient deficiency worldwide leading to reduced immunity, enhanced morbidity and potentially to children blindness (250,000 to 500,000 children yearly; WHO). To ensure an adequate vitamin A status, IFF are usually supplemented with the addition of premixes containing different chemical forms of vitamin A. However, vitamin A is very sensitive to degradation which makes its inclusion in food matrices very challenging. This study aimed at investigating the vitamin A stability in IFF depending on the pressure of the homogenization process applied and on the chemical form of vitamin A. For this propose, a model IFF representative of commercialized products was formulated and processed at two different homogenization pressures and supplemented with three different chemical forms of vitamin A or its precursor (retinol, retinyl acetate or carotenoids). Loss of vitamin A in the IFF was assessed either before or just after the high pressures homogenization step or after an accelerated storage test of 20 days at 40°C. Considering loss during processing, the homogenization pressure had, as expected, a strong impact on vitamin A stability with loss of 2.5% at lower pressure (100/30 bars, 8 passes) *versus* 10% at higher pressure (350/40 bras, 8 passes). This most important degradation of vitamin A content for higher homogenization pressure (needed for instance to obtain 0,4 µm average droplet size) was very likely explained by higher shearing forces leading to local heating of molecules. Moreover, chemical form of vitamin A used for fortification also significantly influenced vitamin A loss, with 44.1% of degradation for IFF fortified with retinyl acetate *versus* 68.5% for IFF fortified with retinol after 20 days of storage. This result points out a more important sensitivity to degradation of retinol very likely due to its higher hydrophilicity in agreement with data already evidenced in other matrices by the Scientific Committee on Consumer Safety (2016). The results also indicated that the presence of carotenoids and especially β-carotene led to a stabilization effect, only 40.7% of vitamin A was degraded after 20 days of storage in the presence of carotenoids *versus* 75.9% in the absence of carotenoids. More generally, the presence of carotenoids provides a better resistance to lipid oxidation due to its synergetic effect with vitamin E. This study highlights that despite the important sensitivity of vitamin A, a good knowledge

of its reactivity during processing and selection of chemical form and potential synergy with other compounds can improve its stability in IFF emulsions.

3.2. Poster (N = 5)

2021 American Oil Chemists Society (AOCS) Annual Meeting & Expo - May 2021 - Online congress

Nutritional values of infant formulas and flours and their adequacy with the requirements of infants aged 6 to 12 months

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Neonatal and infancy period is characterized by significant growth and development. From a nutritional point of view, this translates into specific requirements, notably in long chain polyunsaturated fatty acid (LC-PUFA) such as docosahexaenoic acid. To ensure an adequate nutritional intake different types of products, such as infant formulas and flours are designed. The aim of the present work is to assess the adequacy of these products with the infants aged from 6 to 12 months' requirements and with recent changes in European regulations. To reach this objective, a meta-analysis of the nutritional values of 91 follow-on formulas and 68 infant flours from northern et southern hemispheres was carried out with special focus on lipid profiles. For follow-on formulas, a significant homogeneity of macro- and micronutrient contents is observed from one product to another, and a good similarity of macronutrients contents with mature breast milk despite the persistence of differences in the lipids organization and fatty acids profile. In the case of infant flours, the regulatory framework being less strict, an important heterogeneity of nutritional values is observed with, in particular, a significant difference in energy density among flours marketed in northern and southern countries. This analysis is part of a larger study which aims to understand the mechanisms of lipid oxidation in LC-PUFA-enriched infant foods.

Nutritional Values of Infant Formulas and Flours and their Adequacy with the Requirements of Infants Aged 6 to 12 Months

M. Cancalon[1,2,3*], Y. Hemery[4], N. Barouh[1,2], E. Durand[1,2], P. Villeneuve[1,2] and C. Bourlieu[3]

[1]CIRAD, UMR IATE, 34398 Montpellier, France. [2]Qualisud, Univ Montpellier, Avignon Université, CIRAD, Institut Agro, IRD, Université de La Réunion, Montpellier, France. [3]UMR IATE, INRAE, Montpellier France. [4]UMR Qualisud, IRD, Montpellier France. *mathilde.cancalon@cirad.fr



Introduction

The infancy period is characterized by a significant growth and development and can be divided into several stages due to infant's evolving nutritional needs. During the "second stage", from 6 to 12 months of age, dietary diversification begins, and infants have specific requirements, notably in LA, ALA but also in LC-PUFA such as DHA. These FA are involved in many physiological mechanisms (notably cognitive development). Infant nutritional needs are summarized by WHO (2008). To ensure an adequate nutritional intake at this stage, different types of products are designed, such as follow-on formulas and flours, which must cope with regulatory frameworks (*i.e. Codex Alimentarius*, or EU regulation). Among infant flours, those produced in northern/southern markets will be hereafter named "north" or "south", respectively.

Aim of the present work: to assess the adequacy of follow-on formulas and infant flours with the nutritional requirements of 6-12 months old infants, and with the regulations.

Methods

1. **Overview of nutritional values** of marketed products:
- **91** follow-on formulas
- **68** infant flours

Selection criteria: *Type of products, target age group, widely consumed products – worldwide market*

2. **Statistical data analysis** of the listed nutritional values (R software - R.2.13.0, http://cran.r-project.org):
- Descriptive statistics
- Principal component analysis

3. **Comparison** with:
- Infant requirements (WHO,2008)
- International and European regulations in force
- Golden standard: Human milk composition

Results

Follow-on formulas

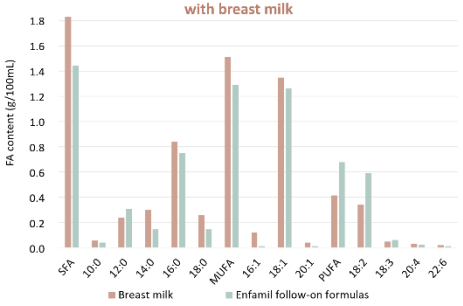
- Significant homogeneity of nutritional values from one product to another
- Good similarity of macronutrient contents with mature breast milk
- Compliance with regulatory standards based on recommendation

Average nutritional composition of follow-on formulas in comparison with breast milk composition, recommendations and regulations

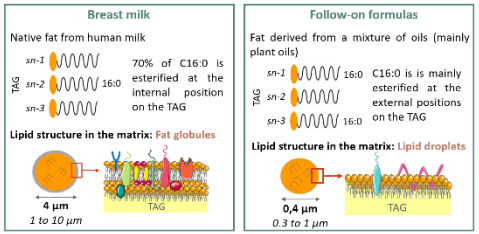
For 100mL of final preparation	Overview (mean ± sd)	Breast milk	Recommendations (6 - 12 months)	EU Regulation Min	EU Regulation Max
Energy (kcal)	67.2 ± 1.3	67	619 kcal/day	60	70
Fat (g)	3.3 ± 0.2	3.5	45-50% of energy	2.64	4.2
SFA (g)	1.1 ± 0.4	1.65	-	-	-
MUFA (g)	1.5 ± 0.4	1.54	-	-	-
PUFA (g)	0.6 ± 0.1	0.35	-	-	-
LA (mg)	502.2 ± 0.1	310	2.75 g/day	300	840
ALA (mg)	53.7 ± 12.6	28	345 mg/day	30	70
LA/ALA	*9.4*	*11.1*	*Between 5 to 15*	*10*	*12*
ARA (mg)	9.4 ± 5.6	18	-	-	42
DHA (mg)	14.8 ± 4.0	7 to 42	100 mg/day	12	35
Vitamin A (µg)	61.5 ± 6.4	-	400 µg/day	45	191.3
Vitamin D (µg)	1.4 ± 0.3	-	5 µg/day	0.6	2.55
Vitamin E (mg)	1.4 ± 0.4	-	-	0.28	-

Considering the oil composition of follow-on formulas, some differences remain with breast milk in their **FA profile** and their **regiodistribution on TAG**.

FA profiles of a representative follow-on formula in comparison with breast milk



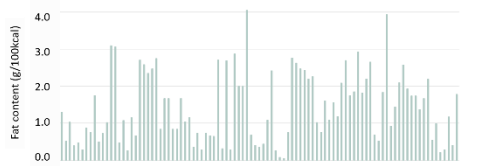
Lipid structure in follow-on formulas and breast milk



Infant flours

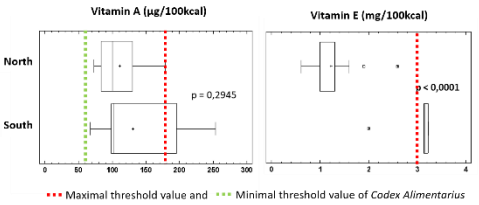
- Important heterogeneity of nutritional values among products (but PCA wasn't able to distinguish clusters of infant flours).
- A significant difference in fat content of infant flours, ranging from 0.1 to 4.0 g of lipids/100kcal.
- Difference in energy and nutritional density among flours produced in northern or southern countries.

Fat content (g/100kcal) of infant flours



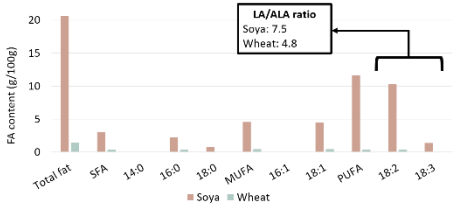
Overages of vitamins A and E were observed in infant flours produced in the South, compared to the limits imposed by the *Codex Alimentarius*.

Distribution of infant flours according to their vitamin A and E content



These North/South differences in infant flours composition are due to the use of different raw materials: cereal sources (in the North) *vs* legumes (in the South). An excess of LA was observed in several infant flours produced in southern countries, due to the use of Soya/Maize.

FA profiles of wheat and soya (g/100g)



Conclusion

This study analyzed the nutritional values of two categories of products targeting 6-12 months infants, i.e. follow-on formulas and infant flours, with special emphasis on lipid composition. It was observed that:

- Despite homogenous nutritional values of follow-on formulas, differences of FA profile and structure remains,
- Nutritional values of infant flours are heterogeneous due to their ingredients list, and vitamin-overages were observed.

This analysis is part of a larger study which aims at understanding the mechanisms of lipid oxidation in LC-PUFA-enriched infant foods and improve their stability.

References

- Innis (2011), Dietary triacylglycerol structure and its role in infant nutrition, Adv Nutr J.
- Cusick and Georgieff (2016), The Role of Nutrition in Brain Development: The Golden Opportunity of the "First 1000 Days", J. Pediatr
- Mäkelä, Linderborg et al. (2013), Breast milk fatty acid composition differs between overweight and normal weight women: the STEPS Study, Eur. J. Nutr
- WHO (2008), Indicators for assessing infant and young child feeding practices
- Commission Delegated Regulation (EU) 2016/127, September 25, 2015
- Delplanque, Gibson et al. (2015), Lipid Quality in Infant Nutrition: Current Knowledge and Future Opportunities, J. Pediatr. Gastroenterol. Nutr.
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- USDA, composition table, 2020

ANNEX – Thesis outputs

2022 American Oil Chemists Society (AOCS) Annual Meeting & Expo – May 2022 – Atlanta, United States

Lipid oxidation kinetics of model systems representative of follow-on formulas

Mathilde Cancalon[a,b], Nathalie Barouh[a], Youna Hemery[c], Erwann Durand[a], Pierre Villeneuve[a] and Claire Bourlieu-Lacanal[b]

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The main objective of follow-on formulas is to mimic as closely as possible the composition and structure of mature breast milk. In order to optimize the formulas, important regulatory changes have taken place, including the obligation to supplement the formulas with DHA. The oil phase is therefore formulated from a mixture of oils mainly of vegetable origin with palm oil as the main source of saturated fatty acids and enriched in long-chain polyunsaturated fatty acids. However, these oil blends are not optimal for nutritional needs of infants and are particularly sensitive to lipid oxidation. The aim of this study was to substitute palm oil with other sources of saturated fatty acids in order to ensure a more suitable nutritional profile for infant without impacting or improving stability against lipid oxidation. To reach this objective a model system representative of marketed follow-on formulas was formulated and declined into a panel of model systems depending on the source of saturated fatty acids used. The results showed that depending on the composition of the oil phase the stability against lipid oxidation could be improved. This study has thus highlighted the importance of the balance between nutritional profile and oxidation stability.

LIPID OXIDATION KINETICS OF MODEL SYSTEMS REPRESENTATIVE OF INFANT FOLLOW-ON FORMULAS

M. Cancalon[1,2,*], N. Barouh[1], B.Baréa[1], Y. Hemery[3], E. Durand[1], P. Villeneuve[1] and C. Bourlieu[2]

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Introduction

Follow-on formulas aims at mimicking as closely as possible the composition and structure of mature breast milk. In order to optimize the formulas, important regulatory changes have taken place, including the obligation to supplement the formulas with DHA[1]. The oil phase is therefore formulated from a mixture of oils mainly of vegetable origin with palmitic acid as the main saturated fatty acid (SFA) and enriched in long-chain polyunsaturated fatty acids[2]. However, these oil blends are not optimal for nutritional needs of infants[3,4] and are particularly sensitive to lipid oxidation[5].

OBJECTIVE **Substitute palm oil with other sources of saturated fatty acids in follow-on formulas in order to ensure a more suitable nutritional profile for infant without impacting their oxidative stability**

Lipids oxidation monitoring and experimental strategy

Samples preparation

Oil phases preparation
4 oils mixtures formulated by linear programming composed by sunflower, high oleic sunflower, rapeseed, ARA-rich fungal and DHA-rich algal oils and whose main source of SFA varies (palm, red palm or coconut oils or dairy fat)

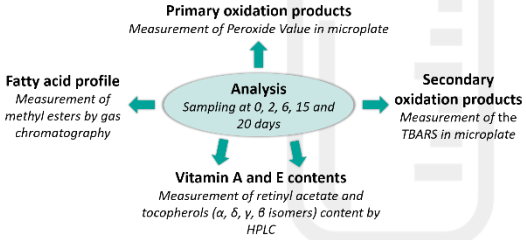
↓

Water phase preparation
Ultra pure water, whey protein, skim milk powder, lactose, maltodextrin

↓

Emulsification by high pressure homogenization
Average droplet size: 0.7 µm

Analyses over accelerated oxidative challenge test (40°C)



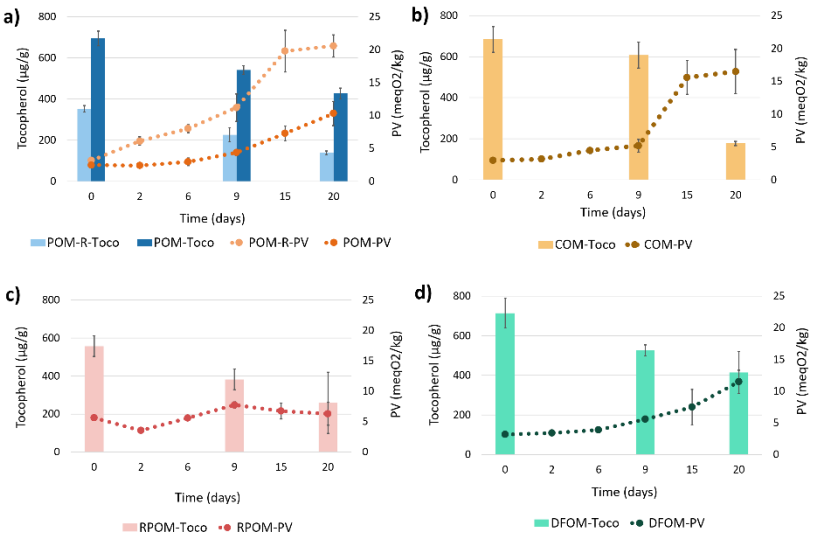
Results

	POM-R*	POM*	COM*	DFOM*	RPOM*
Oil phase composition (weight %)					
Palm oil	67.81	67.81			
Rapeseed oil	18.41	18.41	18.56	12.63	15.42
Sunflower oil	9.11	9.11	14.36	19.39	11.98
Oleic sunflower oil	3.09	3.09	34.91	11.14	
Coconut oil			30.59		
Dairy fat				55.26	
Red palm oil					71.02
ARA oil	0.61	0.61	0.61	0.61	0.61
DHA oil	0.97	0.97	0.97	0.97	0.97
Soy lecithin	0.20	0.20	0.20	0.20	0.20
VMC	-	+	+	+	+
Fatty acid profile (%)					
SFA	36.5 ± 0.6	35.9 ± 0.9	37.6 ± 3.4	35.0 ± 1.3	35.3 ± 0.1
C16:0	30.9 ± 0.5	30.3 ± 0.9	6.7 ± 0.1	16.6 ± 0.7	28.9 ± 0.1
MUFA	44.6 ± 0.4	45.2 ± 0.5	42.9 ± 3.0	42.9 ± 1.1	45.5 ± 0.2
PUFA	18.4 ± 0.2	18.4 ± 0.3	19.4 ± 0.4	21.4 ± 0.1	19.3 ± 0.1
LA (C18:2 ω6)	16.3 ± 0.2	16.3 ± 0.2	17.3 ± 0.4	17.9 ± 0.2	17.1 ± 0.2
ALA (C18:3 ω3)	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.7 ± 0.2	1.6 ± 0.2
ARA (C20:4 ω6)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
DHA (C22:6 ω3)	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
LA/ALA	11.0	11.0	11.3	10.8	10.7

*POM: Palm oil based oil mixture, COM: Coconut oil based oil mixture, DFOM: Dairy fat based oil mixture, RPOM: Red palm oil based oil mixture, VMC: Vitamin mineral complement

The oil phase compositions were fixed by linear programming in order that SFA, MUFA, PUFA, LA, ALA, ARA and DHA contents were equivalent in all systems at the initial point. The systems were also iso-Antioxidants considering VMC and intrinsic main antioxidants.

Evolution of total tocopherol contents and peroxide values at 40°C in a) POM-R *vs* POM, b) COM, c) RPOM and d) DFOM systems



- A statistical analysis showed that tocopherol loss and peroxide value were reliable indicators of oxidative degradation.
- The supplementation of the systems with a VMC consisting of vitamins A, C and D and iron sulfate reduces the lipid oxidation. The VMC has an antioxidant effect (POM-R *vs* POM).
- The substitution of palm oil by coconut oil improves the fatty acid profile thanks to its content of short and medium chain SFA (lauric acid C12:0) easily absorbed by infants but reduces oxidative stability.
- The substitution of palm oil by red palm oil improves the oxidative stability of infant formulas because of its composition in carotenoid and tocotrienol but does not mimic the fatty acid profile of breast milk.
- The substitution of palm oil with dairy fat improves the fatty acid profile by providing a greater diversity of short and medium chain SFA without impacting the oxidative stability.

Importance in balancing nutritional properties and oxidative stability to meet infants needs

FATTY ACID PROFILE	OXIDATIVE STABILITY
As close as possible to the fatty acid profile of breast milk	*Protect sensitive fatty acids (DHA) from oxidation to ensure adequate nutritional intake*

Conclusion

This study shows that tailoring the oil phase composition while keeping compliant with nutritional recommendations, can help stabilizing formulas against lipid oxidation. The combined use of vegetable oils and dairy fat would improve both nutritional profile and oxidative stability. A re-introduction of dairy fat in infant formulas is therefore strongly encouraged due to its wide array of nutritional and functional benefits.

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[5] Cancalon et al. (2022), Infant formula supplementation with DHA and AA: effects on child development and difficulties associated with their introduction. CND.

15th Congress of International Society for the Study of Fatty Acids and Lipids (ISSFAL) - July 2023 - Nantes, France

Oxidative stability and nutritional profile of omega-3 enriched flours optimized for infant nutrition

Mathilde Cancalon[a,b], Youna Hemery[c], Nathalie Barouh[a], Bruno Baréa[a], Erwann Durand[a], Valérie Micard[b], Pierre Villeneuve[a] and Claire Bourlieu-Lacanal[b]

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Dietary diversification, which starts between 4 and 6 months, is a pivotal period in the infant development. After this diversification, complementary foods such as infant flours are therefore widely consumed throughout the world, particularly in southern countries. However, infant flours have often an unbalanced lipid profile in favor of omega 6 and overages in certain vitamins content (especially A and E). The aim of this study was to optimize the formulation of enriched infant flour in order to improve its nutritional profile and oxidative stability. For this purpose, the nutritional values and ingredient lists of 96 infant flours were analyzed to formulate model flours representative of the marketed products. The lipid profile of these model flours was then optimized by including flours with balanced omega 6/omega 3 ratios, *i.e.* teff and cowpea flours, and/or by adding long-chain polyunsaturated fatty acid (LC-PUFA) powder (DHA and ARA). After 3 months of storage, the optimized flour showed a quite good resistance to oxidation with a low evolution of peroxide values and a low tocopherols degradation. However, the inclusion of LC-PUFA significantly increased the oxidation sensitivity of the flours. In order to counteract this phenomenon, phenolic compounds were added *via* the addition of brown rice bran which did not seem to have a positive effect on stabilization regardless the particle size. Nevertheless, optimized omega 3 enriched and fortified infant flours could be proposed with fair stability over 3 months of storage.

N°267

Oxidative stability and nutritional profile of omega-3 enriched flours optimized for infant nutrition

Mathilde Cancalon[a,b,c], Noémie Dormoy[a,b], Youna Hemery[b,d], Nathalie Barouh[a,b], Bruno Baréa [a,b], Erwann Durand[a,b], Valérie Micard[c], Pierre Villeneuve[a,b] and Claire Bourlieu Lacanal[c]*

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Context



4/6 months
Transition from an exclusively milk-based diet to a diversified diet





Infant flours
Complementary food widely consumed throughout the world especially in southern countries

(Cancalon et al., CND, 2022)

MAIN ISSUES
(Moustiès et al., EJLST, 2019)



Disbalances in lipid profile



Overage in certain micronutrients *(vitamins E & A)*

Aim of the study

Optimization of omega-3 enriched infant flours formulation in order to improve their lipid profiles and identification of key levers that increase their oxidative stability

Key steps of the study

1. Infant flours formulation with a fatty acid profile adapted to nutritional needs of infants
2. Storage test of infant flours for 3 months
3. Monitoring of several indicators of the oxidation rate

Materials and methods

Infant flours formulation



Analysis of the nutritional values of **96 infant flours** on the world market

Formulation of the optimized infant flours
by introducing ω3 flours sources



41.6% Teff flour | **33.7 %** Cowpea flour | **24.7%** Soy flour

Studied optimized infant flours



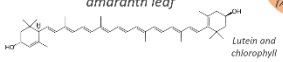
OF
Optimized flour



Algae powder rich in PUFA

OF_PUFA
Optimized flour enriched in PUFA

OF_PUFA_AL
Optimized flour enriched in PUFA and amaranth leaf



Lutein and chlorophyll

Addition of antioxidants fraction (AOX-F)

OF_PUFA_PT
Optimized flour enriched in PUFA and peripheral tissus of black rice

(Barros Santos et al., Metabolites, 2021)



Phenolic compounds including flavonoids

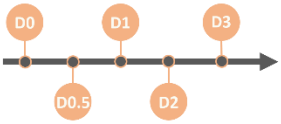
Storage test



25°C

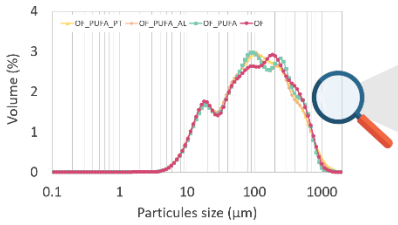


3 months

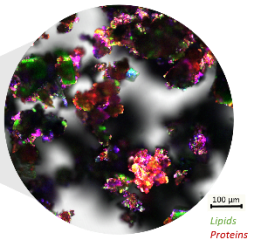


Physical characterization

Particules size distribution



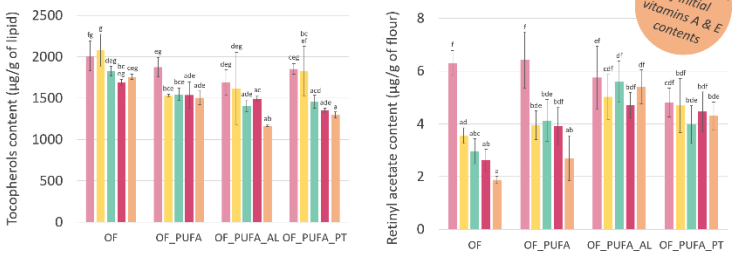
Typical confocal laser scanning microscopy observation



All the infant flours studied showed similar multimodal particules size distribution

Monitoring lipid oxidation rate

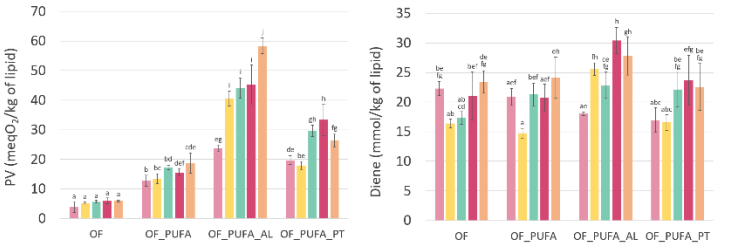
Evolution of total tocopherols and retinyl acetate contents



The degradation kinetics of fat-soluble vitamins are dependent on the addition of antioxidants fraction

AOX-F *Retinyl acetate is rapidly degraded, while tocopherols slowly decrease* vs AOX-F *Tocopherols are progressively degraded, while retinyl acetate remains relatively stable*

Evolution of primary oxidation compounds (peroxide value and conjugated dienes)



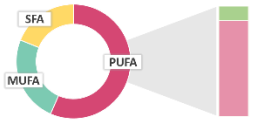
Primary oxidation compounds evolved through different mechanisms (auto or photo-oxidation) depending on the added antioxidants fraction composition

AOX-F / PUFA *No significant evolution of primary oxidation compounds* vs AOX-F / PUFA *Higher level of primary oxidation compounds post-processing, then limited evolution with time* vs AOX-F / PUFA *Increase of primary oxidation compounds sharper in the presence of photo-sensitive compounds (amaranth leaves)*

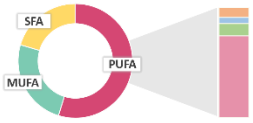
Fatty acid profile

Linoleic acid | α-linolenic acid | Arachidonic acid | Docosahexaenoic acid

Non-enriched flours
OF



Enriched flours
OF_PUFA ; OF_PUFA_AL ; OF_PUFA_ PT



SFA: Saturated fatty acid ; MUFA: Monounsaturated fatty acid ; PUFA: Polyunsaturated fatty acid

	Non-enriched flour	Enriched flours	Marketed infants flours
Omega 6 / Omega 3	6.6	3.9	Up to > 30

The fatty acid profiles were adapted to the nutritional needs of infants and remain stable over the first 3 months of storage

CONCLUSIONS

This study showed that OF had a quite good overall oxidative stability after 3 months of storage and a lipid profile adapted to the nutritional needs of infants with much improved ω6/ω3 ratio compared to commercial products. Depending on the chemical composition of the flours, and in particular their intrinsic antioxidants (AOX-F), different oxidation mechanisms appear to occur, with degradation primarily of vitamin A with a dominant auto-oxidative pathway (without AOX-F) or vitamin E with a dominant photo-oxidative pathway (with AOX-F).

The authors thank the PLANET facility (DOI: 10.15454/1.5572338990609338E12) run by the IATE joint research unit for providing process experiment supports

19th Euro Fed Lipid Congress and Expo (EFL) - September 2023 - Poznan, Poland

Optimization of the oxidative stability and lipid profile of omega-3 enriched infant flours

Mathilde Cancalon[a,b], Youna Hemery[c], Reine Barbar[b], Nathalie Barouh[a], Bruno Baréa[a], Leslie Lhomond[c], Adrien Reau[c], Erwann Durand[a], Valérie Micard[b], Pierre Villeneuve[a] and Claire Bourlieu-Lacanal[b]

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Dietary diversification, which starts between 4 and 6 months, is a pivotal period in the infant development. Complementary foods such as infant flours are gradually introduced into the infant diet and are widely consumed especially in southern countries. However, infant flours have often an unbalanced lipid profile in favor of omega 6 and overages in certain vitamins content (especially A and E). The aim of this study was to optimize the formulation of vitamin A and E-fortified infant flours in order to improve its nutritional profile and oxidative stability. For this purpose, model infant flours were formulated by optimizing the lipid profile through the inclusion of flours with balanced omega 6/omega 3 ratios, *i.e.* teff and cowpea flours, and/or by adding long-chain polyunsaturated fatty acids (LC-PUFA) powders. In order to limit their sensitivity to oxidation, two strategies were studied, namely the addition of amaranth leaf powder for their carotenoid content and brown rice peripheral tissues for their phenolic compounds composition. These model infant flours were submitted to storage test during 6 months at 25 or 40°C. As expected the inclusion of LC-PUFA significantly increased the oxidation sensitivity of the flours. The use of raw materials with balanced fatty acid profiles resulted in contrasted omega 6/omega 3 ratio of 6.7 in un-enriched in LC-PUFA model infant flours *versus* 3.9 for the LC-PUFA enriched model infant flours. As expected, the addition of LC-PUFA increased the sensitivity to oxidation with a significantly higher increase of peroxide value for the flours enriched with these fatty acids. Despite a significant increase of peroxide value, the fatty acid profiles and vitamin E contents remained stable after 1 month of storage. A significant degradation of vitamin A was however observed mainly on short time with up to 50% of retinyl acetate degraded after 2 weeks of storage. This degradation was significantly delayed for model infant flours enriched with amaranth leaf or black rice peripheral tissue. In conclusion optimized omega 3 enriched infant flours could be proposed with fair stability over 3 months of storage.

19th Euro Fed Lipid Congress and Expo 17-20 September 2023 Poznań - Poland

Optimization of the oxidative stability and lipid profile of omega-3 enriched infant flours

OXI-006

Mathilde Cancalon[a,b,c], Youna Hemery[b,d], Noémie Dormoy[a,b], Nathalie Barouh[a,b], Erwann Durand[a,b], Bruno Baréa [a,b], Reine Barbar[c], Leslie Lhomond[c], Adrien Reau[c], Valérie Micard[c], Pierre Villeneuve[a,b] and Claire Bourlieu Lacanal[c]*

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CONTEXT



4/6 months
Transition from an exclusively milk-based diet to a diversified diet





Infant flours
Complementary food widely consumed throughout the world especially in southern countries

MAIN ISSUES

(Moustiès et al., EJLST, 2019)



Disbalances in lipid profile



Overage in micronutrients *(vitamins E & A)*

AIM OF THE STUDY

Optimization of omega-3 enriched infant flours formulation in order to improve their lipid profiles and identification of key lipid levers that increase their oxidative stability

EXPERIMENTAL STRATEGY

Infant flours formulation



Analysis of the nutritional values of **96 infant flours** on the world market

(Cancalon et al., Dataverse, 2023)

Important heterogeneity of nutritional values

Southern regions	Northern regions
Legums (soy, groundnut)	Cereals (rice, wheat, oat and corn)

Formulation of the optimized infant flours

by introducing ω3 flours sources



41.6%	33.7 %	24.7%
Teff flour	Cowpea flour	Soy flour



OIF
Optimized infant flour

Enzymatic inactivation and inhibition of antinutritional factors by heat treatment

Normalization of initial vitamins A & E contents

Infant flours preparation



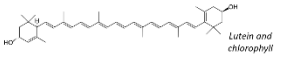
OIF
Optimized Infant Flour

Algae powder rich in PUFA

OIF_PUFA
Optimized Infant Flour enriched in PUFA

Addition of antioxidants fraction (AOX-F)

OIF_PUFA_AL
Optimized Infant Flour enriched in PUFA and Amaranth Leaf



Lutein and chlorophyll

OIF_PUFA_BRB
Optimized Infant Flour enriched in PUFA and Black Rice Bran



Phenolic compounds including flavonoids

(Barros Santos et al., Metabolites, 2021)

Storage test



25°C



6 months

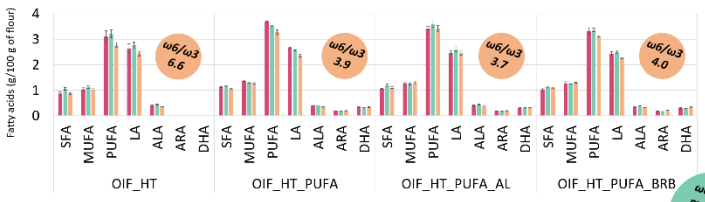
Sampling times

D0 | D0.5 | D1 | D2 | D3 | D6 *months*

RESULTS

Fatty acid profiles

SFA: Saturated fatty acid, MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acid, LA: Linoleic acid, ALA: α-linolenic acid, ARA: Arachidanic acid, DHA: Docasahexaenoic acid

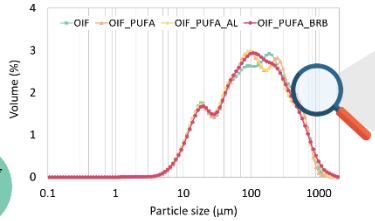


✓ **The fatty acid profiles were adapted to the nutritional needs of infants**

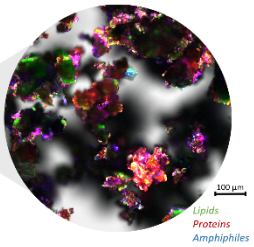
ω6/ω3 of marketed infants flours Up to > 30

Physical characterization

Particle size distribution



Typical confocal laser scanning microscopy observation

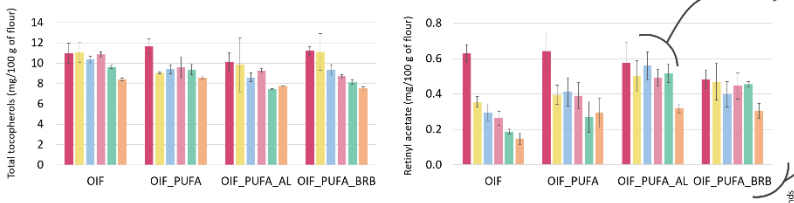


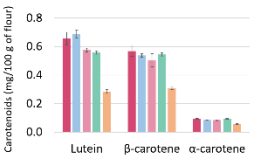
✓ **All the infant flours studied showed similar multimodal particle size distribution**

Monitoring lipid oxidation rate

D0 D0.5 D1 D2 D3 D6

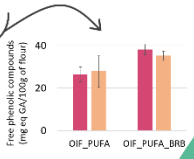
Evolution of total tocopherols and retinyl acetate contents





Carotenoids and cereal bran act as stabilizing-vitamin A agents

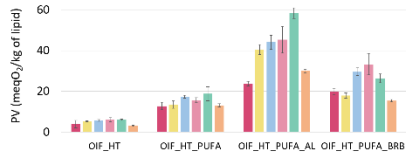
(Van Wayenbergh et al., FRI, 2023)



✓ **The degradation kinetics of fat-soluble vitamins are dependent on the addition of antioxidants fraction**

AOX-F *Retinyl acetate was rapidly degraded, while tocopherols slowly decreased* vs AOX-F *Tocopherols were progressively degraded, while retinyl acetate remained relatively stable*

Evolution of oxidation compounds (peroxide value and volatiles compounds)



ng eq 3-heptanol/g	D3		D6	
	Hexanal	Pentanal	Hexanal	Pentanal
OIF	n.d.	n.d.	n.d.	n.d.
OIF_PUFA	n.d.	n.d.	4.6 ± 0.7	n.d.
OIF_PUFA_AL	5.9 ± 1.3	38.3 ± 3.7	12.8 ± 2.0	47.8 ± 4.4
OIF_PUFA_BRB	4.1 ± 0.7	n.d.	11.1 ± 0.9	n.d.

n.d. : not detected

✓ **Primary oxidation compounds evolved through different mechanisms depending on the added AOX-F composition**

AOX-F PUFA *No significant evolution of primary oxidation compounds* vs AOX-F PUFA *Higher level of primary oxidation compounds post-processing, then limited evolution with time* vs AOX-F PUFA *Increase of primary oxidation compounds sharper in the presence of photo-sensitive compounds (Amaranth Leaves)*

CONCLUSIONS & PERSEPECTIVES

This study showed that OIF had a quite good overall oxidative stability after 6 months of storage and a lipid profile adapted to the nutritional needs of infants with much improved ω6/ω3 ratio compared to commercial products. Depending on the chemical composition of the flours, and in particular their intrinsic antioxidants (AOX-F), different oxidation mechanisms appear to occur, with degradation primarily of vitamin A with a dominant auto-oxidative pathway (without AOX-F) or vitamin E with a dominant photo-oxidative pathway (with AOX-F).

The authors thank the PLANET facility (DOI: 10.15454/1.5572338990609338E12) run by the IATE joint research unit for providing process experiment supports

INRAE cirad IRD UMR QualiSud UMR IATE

Journées Francophones de Nutrition (JFN) 2023 - Décembre 2023 - Marseille France

Adéquation des valeurs nutritionnelles des préparations pour nourrissons et farines infantiles avec les recommandations et besoins des enfants de 6 à 12 mois

Mathilde Cancalon[a,b], Youna Hemery[c], Nathalie Barouh[a], Bruno Baréa[a], Valérie Micard[b], Erwann Durand[a], Pierre Villeneuve[a] et Claire Bourlieu-Lacanal[b]

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Une nutrition adéquate de la naissance jusqu'à l'âge de deux ans est essentielle pour soutenir la croissance et le développement important ayant lieu sur cette fenêtre. De la naissance à 6 mois, l'allaitement maternel exclusif est fortement recommandé par l'OMS. Cependant, il est estimé que seulement 48% des enfants de moins de 6 mois sont exclusivement allaités, et seulement 70% d'entre eux le sont encore jusqu'à l'âge de 12 mois. Les préparations pour nourrissons (PPN) sont alors très largement consommées. A partir de 6 mois, le lait maternel ou les PPN ne sont plus suffisantes pour couvrir l'ensemble des besoins énergétiques du nourrisson et garantir un apport complet en macro et micronutriments. Cette période spécifique du développement se caractérise par l'introduction progressive d'aliments de compléments tels que les farines infantiles (FI) (particulièrement consommées dans les pays du sud). La part lactée de l'alimentation reste néanmoins majoritaire jusqu'à l'âge de 12 mois. La diversification alimentaire marque donc la transition d'un régime exclusivement lacté à un régime diversifié. L'objectif de cette étude était d'évaluer l'adéquation des compositions et valeurs nutritionnelles des PPN et FI consommées entre l'âge de 6 et 12 mois avec les recommandations et les besoins nutritionnels des enfants de cette tranche d'âge.

Les listes d'ingrédients et les valeurs nutritionnelles de 91 PPN et 96 FI ont été collectées. La collecte des données a été réalisée entre 2020 et 2021 directement sur l'emballage des produits, sur les sites internet des marques ou extraites d'articles de recherche. Des analyses statistiques descriptives ont été réalisées ainsi qu'une analyse en composantes principales pour les FI. Les données ont été comparées avec la composition moyenne du lait maternel et les réglementations encadrant la composition des PPN et FI.

Le jeu de données concernant les PPN a montré une importante homogénéité des valeurs nutritionnelles due au cadre réglementaire strict concernant leur composition. Depuis février 2020, la réglementation impose la supplémentation en DHA à hauteur de 20 à 50 mg pour 100 kcal. L'ensemble des PPN appliquait ces changements récents, cependant, la teneur moyenne était de 22 mg/100 kcal soit proche de la limite règlementaire inférieure. Seul 14% des PPN incluaient de la matière grasse

laitière dans leur composition malgré le consensus scientifique encourageant sa réintroduction du fait de ces propriétés nutritionnelles (composition et structure plus adaptée aux besoins de l'enfant) et sa stabilité oxydative. Malgré des teneurs en macronutriments proches de celles du lait maternel, des différences persistent notamment au niveau de la composition en acides gras saturés et de la structure des lipides. Concernant les FI, le jeu de données a mis en évidence une importante hétérogénéité des valeurs nutritionnelles due à l'utilisation d'une grande diversité de matière premières. Il s'agissait principalement de produits céréaliers (riz, blé, avoine et maïs) pour les FI produites et commercialisées dans les pays du Nord alors que celles des pays du Sud incluaient des légumineuses (soja, arachide). Le choix des matières premières impactait également le profil lipidique avec dans certains cas d'importants déséquilibres caractérisés par des ratios ω6/ω3 jusqu'à 30, dépassant ainsi les recommandations internationales qui indiquent un ratio optimal compris entre 5 et 15. Des surdosages en vitamines notamment A et E ont été mis en évidence. Ces surdosages volontaires ont pour objectif de compenser les éventuels pertes lors du stockage, cependant, ils augmentent le risque d'exposition à des apports excédentaires pouvant conduire à des hypervitaminoses et une exposition à des produits d'oxydation.

Cette étude a mis en évidence de futurs challenges liés à l'optimisation des aliments destinés aux enfants de 6 à 12 mois. Pour les PPN un renforcement du bio-mimétisme avec le lait maternel doit être privilégié pour assurer une bio-accessibilité des nutriments suffisante sans impacter la stabilité oxydative. En ce qui concerne les FI une meilleure maitrise de la formulation et une compréhension plus approfondies de ces systèmes est nécessaire pour améliorer à la fois leurs propriétés nutritionnelles et leur conservation.

4. Awards

- European Travel grant, AOCS Annual meeting and expo - Student award winner - AOCS 2022
- Minerva Trophy Jury award in the Life and Environmental Sciences, Material Sciences and Chemistry section - Fondation F.iniciativas 2023

5. Supervisions

- Co-supervision of Marine Tournaux, Master student (Final year, ENSMAC – Graduate school of materials, food sciences and chemistry), from March to September 2023.
- Co-supervision of Noémie Dormoy, Bachelor student (Second year, IUT Montpellier-Sète – University Institute of Technology), from April to June 2023.

6. Training courses

Category: communication

- Annual ED GAIA meeting - 2 hours
- Poster presentation at the AOCS 2022 congress - 4 hours
- Writing a scientific article - 14 hours

Category: teaching

- Vacation at the University Institute of Technology (IUT) of Montpellier - Sète - 25 hours

Category: tools and methods

- Basics of statistics - 12 hours

Category: scientific and cultural awareness

- My thesis in 180 seconds - 20 hours

Category: responsible research

- MOOC Scientific integrity in the research professions - 15 hours

Total participation: 92 hours / 7 modules

1. Contexte et objectifs

« *Que ton alimentation soit ta première médecine* » - Hippocrate, 400 av. J.-C.

Cette citation datant de l'antiquité souligne l'importance de l'alimentation et de son effet sur la santé globale. Elle encourage à considérer les bonnes pratiques alimentaires comme une composante clé de la santé à court et long terme.

Ce principe est d'autant plus applicable pendant la période de l'enfance, une étape cruciale du développement. Le concept des 1000 jours, qui fait référence à la période critique du développement de l'enfant de la conception jusqu'à l'âge de deux ans, met l'accent sur l'importance de fournir une nutrition adéquate à ce stade de la vie. En effet, les apports nutritionnels sont essentiels pour soutenir le développement et la croissance rapide caractéristique de cette période et sont également impliqués dans les effets de programmation. Pendant les deux premières années de la vie, le développement est usuellement scindé en trois stades. Durant le premier âge qui s'étend de 0 à 6 mois, un allaitement exclusif est fortement recommandé par l'OMS. Cependant l'allaitement n'est pas toujours possible et/ou désiré, ainsi il est estimé que seul 48% des enfants de moins de 6 mois sont exclusivement allaités. Des préparations pour nourrissons (PPN) peuvent alors être choisies comme substitut. Le deuxième âge de 6 à 12 mois est une période charnière du développement durant laquelle le lait maternel et/ou les PPN ne sont plus en mesure de couvrir l'ensemble des besoins énergétiques ainsi qu'en macro- et micronutriments des nourrissons. Afin de compléter l'apport, des aliments complémentaires tels que les farines infantiles (FI) sont progressivement introduits. Enfin, pendant la troisième étape du développement, de 12 à 24 mois, la diversification alimentaire se poursuit jusqu'à devenir exclusive.

Parmi les macronutriments, les lipides sont les fournisseurs principaux d'énergie pendant la période de l'enfance avec un apport couvrant jusqu'à 55% des apports caloriques totaux. En plus d'être une forme de stockage de l'énergie, les lipides sont également impliqués dans la structuration membranaire ainsi que dans la génération et propagation de signaux physiologiques. Ils jouent par conséquent un rôle essentiel pendant la période de l'enfance et sont notamment impliqués dans le développement cognitif, visuel, immunitaire et digestif. Les lipides alimentaires sont majoritairement trouvés dans l'alimentation sous leur forme de triesters d'acides gras avec le glycérol (triacylglycérols - TAG). Cependant, d'autres formes sont également retrouvées comme les lipides polaires (PL) ou encore les sphingolipides. Ces différentes structurations confèrent aux lipides des propriétés physico-chimiques et nutritionnelles spécifiques et déterminent leur rôle biologique. Parmi les acides gras certains sont qualifiés d'indispensables du fait de l'incapacité de l'organisme humain à les synthétiser. C'est notamment le cas des précurseurs métaboliques et chefs de file des familles non-

interconvertibles que sont les oméga 6 (ω6) et les oméga 3 (ω3). Il s'agit de l'acide linoléique (LA, C18:2) et de l'acide alpha-linolénique (ALA, C18:3). Ces acides gras conduisent, par une voie spécifique de désaturations-élongations successives, à la biosynthèse d'autres acides gras polyinsaturés (AGPI) dits à longues chaînes (LC, chaîne carbonée supérieure ou égale à 20) tels que les acides docosahexaénoïque (DHA, C22:6 ω3) et arachidonique (ARA, C20:4 ω6) qui sont indispensables pour le développement cognitif et l'acuité visuelle de l'enfant. Une déficience en ces acides gras peut avoir d'importantes conséquences à long terme avec une augmentation des risques de développer certaines maladies mentales et/ou métaboliques à l'âge adulte. Il est donc reconnu qu'il existe une importance à la fois quantitative et qualitative de l'apport lipidique durant la période de l'enfance.

Afin de garantir un apport nutritionnel adéquat, une vigilance particulière doit être donnée quant à la composition des aliments infantiles. La fortification en AGPI des PPN et FI semble donc être une stratégie fiable pour garantir un apport nutritionnel adéquat. Cependant, ces acides gras du fait de leurs multiples insaturations sont particulièrement sensibles à l'oxydation, principal phénomène d'altération dans les produits alimentaires. Cette réaction radicalaire auto-catalytique initiée sous l'influence de facteurs extérieurs tels que l'exposition à la lumière, la température ou la présence d'oxygène et de métaux de transition peut se produire selon trois voies à savoir l'auto-oxydation, l'oxydation enzymatique ou la photo-oxydation. L'auto-oxydation, induite par l'oxygène triplet, est la voie d'oxydation majoritaire dans les produits transformés. Les réactions impliquées dans les mécanismes d'oxydation sont habituellement présentées selon un schéma simplifié en trois étapes incluant une phase d'initiation, de propagation et de terminaison. Elle conduit tout d'abord à la formation de composés d'oxydation primaires, les hydroperoxydes, qui réagissent ensuite pour former des composés d'oxydation secondaires correspondant à des substances volatiles (seuil de détection sensorielle très bas de l'ordre de la partie par million - ppm). L'oxydation lipidique est à l'origine d'une perte de propriétés nutritionnelles (perte d'acides gras insaturés et en vitamines liposolubles) et organoleptique (formation de composés volatiles) ainsi qu'à une exposition a des produits d'oxydation potentiellement toxiques.

Les travaux de cette thèse se sont focalisés sur l'optimisation à la fois de la qualité nutritionnelle et de la stabilité oxydative de la fraction lipidique de deux aliments infantiles consommées entre 6 et 12 mois, à savoir les PPN et les FI. L'objectif de ces travaux était de répondre à la question de recherche suivante :

Quels sont les leviers physico-chimiques clés pour contrôler la stabilité oxydative des systèmes dispersés enrichis en AGPI-LC tout en maintenant une bioaccessibilité acceptable des lipides ?

La stratégie globale mise en place pour répondre à cette problématique est présentée en **Figure 16**.

Le choix des matrices étudiées permet de couvrir deux types de systèmes dispersés : les systèmes émulsionnés liquides (émulsion huile dans eau) représentés par les PPN et les systèmes granulaires secs représentés par les FI.

La première partie de la stratégie a été consacrée à l'étude des PPN. Pour cela, une PPN modèle avec une phase aqueuse simplifiée a été formulée selon la composition des PPN commerciales et la réglementation Européenne. Afin d'améliorer le profil lipidique et d'identifier les leviers clés impactant la stabilité oxydative, la PPN modèle a été déclinée en un panel de systèmes selon différents paramètres de formulation et de structures. L'ensemble des PPN modèles a été soumis à des tests de stockage en conditions accélérées afin d'évaluer les cinétiques d'oxydation. Les PPN modèles ayant démontré une résistance à l'oxydation relativement importante et des propriétés nutritionnelles améliorées ont été soumises à un processus de digestion *in vitro* afin d'évaluer l'impact de l'optimisation sur la bioaccessibilité des nutriments. La seconde partie de la stratégie a été consacrée à l'étude des FI. Pour cela, les mêmes étapes que pour les PPN ont été appliquées. Ainsi, une optimisation du profil lipidique a d'abord été réalisée selon la composition des FI présentes sur le marché mondial et la règlementation internationale. Dans un second temps, des tests de stockage ont permis de mieux comprendre les mécanismes d'oxydation dans les systèmes granulaires secs et d'identifier les paramètres clés permettant de maitriser ce phénomène. Enfin, les systèmes possédant les propriétés nutritionnelles et de stabilité oxydative les plus importantes ont été soumis à une digestion *in vitro* pour évaluer la bioaccessibilité des nutriments dans ce type de système. La troisième partie de la stratégie a été dédiée à la comparaison des deux types de systèmes en termes de stabilité oxydative, de mécanismes de diffusion de l'oxydation impliqués, de propriétés nutritionnelles et de matrices « vecteurs » utilisées en fortification nutritionnelle.

2. Principaux résultats

Dans un premier temps les systèmes dispersés liquides sous forme d'émulsions huile dans eau représentatives de PNN ont été étudiés.

2.1. Stabilité oxydative des préparations pour nourrissons

2.1.1. Etude de marché des PPN

Cette section s'appuie en partie sur un jeu de données intitulé : "Panorama of the nutritional composition of IFF commercialized worlwide in 2021" publié dans le répertoire Dataverse de l'INRAE (Cancalon, Hemery *et al.* 2023) et un data paper intitulé : "Dataset of the nutritional composition of follow-on infant formulas commercialized worldwide in 2021" publié dans la revue Data in Brief (Cancalon, Hemery *et al.* 2023).

Les listes d'ingrédients et les valeurs nutritionnelles de 91 PPN commercialisées sur le marché mondial ont été collectées de décembre 2020 à avril 2021, directement sur l'emballage des produits ou *via* les sites internet des différentes marques. Ce jeu de donnée inclue uniquement les PPN de deuxième âge dites "classiques" (pas de formules anti-reflux ou hypoallergéniques...) qui sont largement consommées afin de couvrir plus de 60% des parts du marché en volume. L'analyse du jeu de données a montré une importante homogénéité des valeurs nutritionnelles des PPN commerciales et une bonne conformité avec les valeurs seuils imposées par le *CODEX Alimentarius* CX156/1987 au niveau mondial et le décret CE2016/127 au niveau Européen. Depuis le premier décret européen sur la composition des laits concentrés et en poudres destinés à la consommation humaine en 1961, la réglementation a été régulièrement mise à jour selon les recommandations nutritionnelles basées sur la composition du lait maternel (l'étalon-or) et les connaissances scientifiques. Depuis février 2020, le règlement européen CE 2016/127 impose par exemple la supplémentation des PPN en DHA à hauteur de 20 à 50 mg/100 Kcal (soit 0,5 à 1 % des AG totaux), jusqu'alors facultative. La teneur moyenne en DHA des PPN issues du jeu de données est de 22.0 ± 0.3 mg/100 Kcal, ce qui est conforme mais proche de la teneur règlementaire limite minimale. Ce jeu de données a également mis en évidence que la majorité des fractions lipidiques des PPN sont exclusivement basées sur des mélanges d'huiles d'origine végétale avec principalement les huiles de palme, coco, colza et tournesol. En effet, seul 17.6% des PPN dont les sources de matières grasses été mentionnées incluent des lipides d'origine animale (principalement des sources bovine). 78% des PPN incluent des huiles riches en AGPI de sources marine, algale et fongique. En ce qui concerne les tensioactifs, la lécithine de soja est présente dans 72.5% des PPN présentes dans le jeu de données.

Le jeu de données a permis la formulation d'une PPN modèle représentative des PPN commerciales par un système de programmation linéaire. La phase lipidique de la PPN modèle est composée d'un mélange d'huile de palme, de colza, de tournesol et de tournesol oléique et d'huiles riche en AGPI (ARA et DHA).

2.1.2. Impact des paramètres de formulation sur la stabilité oxydative

Une première étude visant à comparer l'impact de différentes sources d'acides gras saturés (AGS) sur la stabilité oxydative et l'amélioration du profil lipidique de PPN modèles a été réalisée. Cette partie est basée sur une article de recherche intitulé "Comparison of the effect of various sources of saturated FA on IFF oxidative stability and nutritional profile" paru dans le journal Food Chemistry (Cancalon, Hemery *et al.* 2023). La PPN modèle à base d'huile de palme (PO) précédemment formulée a été déclinée en utilisant différentes sources d'AGS (AGS représentent au minimum 45% des acides gras totaux) à savoir l'huile de palme rouge (RPO), de coco (CO) ou d'oléine de matière grasse laitière d'origine bovine (DF). La composition chimique (profils en acides gras et teneurs en vitamines A et E)

ainsi que la structure (taille de gouttelette de 0.7 µm) ont été normalisée dans l'ensemble des PPN modèles qui ont alors été soumises à un test de stockage en conditions accélérées (20 jours à 40°C ou 25°C dans des tubes ambrés clos avec ou sans agitation de 110 rpm).

Les résultats montrent que l'ensemble des PPN modèles formulés sont relativement stables à l'oxydation. Les mécanismes d'oxydation des lipides sont dans un premier temps marqués par des pertes en vitamines (notamment A et E) puis une augmentation des composés primaires d'oxydation évalués par la mesure de l'indice de peroxyde (IP). Cependant, des IP de maximum 30 meqO_2/kg ont été mesurés ce qui traduit un niveau d'oxydation relativement peu avancé. L'analyse statistique de l'ensemble des données expérimentales issues de cette étude a montré que dans ces PPN modèles l'évolution des IP et des teneurs en tocophérols sont les indicateurs les plus informatifs du niveau d'oxydation. De plus, cette étude a mis en évidence que la composition en isomères de tocophérols module la stabilité oxydative avec une amélioration pour les PPN ayant comme isomère majoritaire le γ-tocophérol. La perte en tocophérol est majoritairement due à une perte en isomère α (jusqu'à 89.5% de perte en tocophérols totaux après 20 jours de stockage). Ainsi les PPN modèles à base de CO, dans lesquelles l'α-tocophérol est l'isomère majoritaire, sont plus sensibles à l'oxydation comparées aux PPN à base de PO. L'abaissement du point de fusion de la phase lipidique par l'introduction de CO dans les PPN peut également contribuer à la déstabilisation du système. De plus, les résultats montrent un important effet antioxydant des caroténoïdes, apportés par la RPO, résultant de leur synergie avec les tocophérols et tocotriénols. Les lipides laitiers, apporté par l'ajout de DF, présentent un intérêt particulier pour l'optimisation des PPN à la fois du fait de leur profil nutritionnel et de leur stabilité à l'oxydation. En effet, en plus de complexifier le profil lipidique et d'apporter des AGS à chaine courtes et moyennes favorisant l'absorption des lipides. Ils permettent d'obtenir une stabilité oxydative équivalente aux PPN à base de PO. De plus, les PL laitiers conduisent à une amélioration de la stabilité oxydative comparée aux PPN modèles à base de PO notamment grâce à la synergie de la sphingomyéline (SM) avec les tocophérols. La SM, du fait de sa composition en AGS à très longues chaines (> 20 carbones), permet une rigidification locale de l'interface (barrière physique) limitant ainsi le contact des pro-oxydants de la phase aqueuse avec les lipides insaturés ce qui peut également contribuer à l'amélioration de la résistance contre l'oxydation. Cette étude a souligné l'importance de l'équilibre entre stabilité et propriétés nutritionnelles pour répondre aux besoins des nourrissons et montre que les paramètres de formulation lipidique sont des leviers importants dans la stabilisation contre l'oxydation.

2.1.3. Impact des paramètres de structure sur la stabilité oxydative

Une seconde étude visant à identifier les principaux leviers structurels permettant l'amélioration de la stabilité des PPN a été réalisée. Cette partie est basé sur une article de recherche intitulé " Stabilization

of infant formulas against lipid oxidation: what are the key levers?" soumis dans le journal European Journal of Lipid Science and Technology. La PPN modèle à base PO précédemment formulée a été déclinée selon différents paramètres de structure incluant la taille des gouttelettes (0.4 ou 0.7 µm), la composition du cœur des gouttelettes lipidiques (homogènes ou hétérogènes *i.e.* gouttelettes composées d'huiles à point de fusion élevé - l'huile de palme - et très bas - les huiles riches en AGPI - ou d'huiles à points de fusions intermédiaires), la source d'émulsifiant (PL laitier ou lécithine de soja) et son mode d'incorporation (dans la phase huileuse ou aqueuse). La composition chimique globale (profils en acides gras et teneurs en vitamines A et E) des différentes PPN modèles a été normalisée. Les PPN modèles ont alors été soumises à un test de stockage en conditions accélérées (20 jours à 40°C dans des tubes ambrés clos avec une agitation de 110 rpm). Sur la base de la cinétique de dégradation du tocophérol et de l'évolution du PV, les résultats ne montrent aucun effet significatif de l'hétérogénéité de la composition du cœur des gouttelettes lipidiques. De plus, la stabilité à l'oxydation des PPN modèle semble être davantage influencée par l'environnement chimique que par la taille des gouttelettes. Cependant, les conditions d'homogénéisation pour obtenir des tailles de gouttelettes de 0.4 µm entrainent une diminution de la teneur initiale en vitamine A plus importante que pour des gouttelettes de 0.7 µm. Les résultats montrent que la présence de structure colloïdales n'entraine pas une diminution de la stabilité oxydative probablement en favorisant la diffusion des composés oxydés (accélération de l'oxydation) ou en limitant la synergie avec les tocophérols (diminution de la protection). En revanche, la modulation de la composition de l'interface, largement influencée par la source d'émulsifiant, permet d'améliorer significativement la stabilité oxydative. Les PPN modèles avec des PL laitiers n'ont montré aucune perte de tocophérols et un IP jusqu'à deux fois inférieur comparé aux PPN modèles avec de la lécithine de soja après 20 jours de stockage. Cet effet est particulièrement prononcé pour les gouttelettes de 0,4 µm. Ces résultats suggèrent que le PPN modèles avec des PL laitiers présentent une plus grande stabilité oxydative, probablement en raison de la présence de SM et de la formation d'un domaine rigidifié à la surface des gouttelettes. Ainsi, en considérant à la fois les questions liées à l'oxydation des lipides et celles liées à la nutrition, les PPN enrichis en LC-PUFA et incorporant des lipides laitiers peuvent être une stratégie fiable pour garantir la stabilité oxydative et un profil nutritionnel adéquat.

2.1.4. Stabilité oxydative de la vitamine A

Dans un troisième temps, un focus a été réalisé sur la stabilité de la vitamine A dans les PPN avec une étude visant à évaluer l'impact du processus d'homogénéisation et les différentes formes chimiques de la vitamine A sur la stabilité dans des PPN modèles représentatifs des formules commerciales. Cette partie est basée sur une article de recherche intitulé "Influence of chemical forms and homogenization process on the stability of vitamin A in infant follow-on formulas" en préparation pour soumission. La

PPN modèle à base PO précédemment formulée a été déclinée selon différentes formes de vitamine A ajoutée (rétinol, esters de rétinyle ou caroténoïdes) et le processus d'homogénéisation appliqué (gouttelettes de 0.4 ou 0.7 µm). Les pertes de vitamine A ont été évaluées avant ou après le processus d'homogénéisation, ou après un stockage de 20 jours à 40°C des PPN modèles ayant un profil lipidique (profil d'acides gras et teneur en vitamine E équivalents) et des teneurs totales en rétinoïdes (mais différentes formes) normalisés. Le processus d'homogénéisation conduit à une perte de vitamine A jusqu'à 14,3% à haute pression (350/40 bars, 8 passages) expliquée par des forces de cisaillement importantes conduisant à un échauffement local des molécules. De plus, l'acétate de rétinyle présente une stabilité supérieure à celle du rétinol à la fois pendant l'homogénéisation et le stockage. Cependant, les résultats montrent qu'une augmentation de la teneur en acétate de rétinyle au-delà des valeurs règlementaires seuils n'a pas d'effet bénéfique additionnel et peut potentiellement avoir des effets délétères. La présence de caroténoïdes et surtout de β-carotène a eu un effet stabilisateur avec une perte de seulement 40,7% de la vitamine A pendant le stockage (jusqu'à 84,9% sans caroténoïdes). Les caroténoïdes offrent une meilleure résistance à l'oxydation des lipides grâce à leur effet synergique avec la vitamine E. Cependant, les pertes en caroténoïdes sont dépendantes de la taille de gouttelettes et sont deux fois plus importantes pour une taille de 0.4 µm comparé à 0.7 µm. Cette étude souligne qu'une bonne connaissance de la réactivité de la vitamine A au cours de la transformation et l'ajout de caroténoïdes permet d'améliorer la stabilité oxydative des PPN.

2.1.5. Comparaison avec préparations pour nourrissons commerciales

Les résultats obtenus dans ce chapitre ont montré une stabilité globale relativement bonne des PPN modèles, avec une possibilité d'optimisation en modulant les paramètres de formulation. Cependant, les PPN modèles étudiées sont représentatives en termes de composition en macronutriments des produits commerciaux mais ont une phase aqueuse simplifiée dans laquelle se trouvent les composés initiateurs de l'oxydation. Afin de valider les résultats, quatre PPN commerciales ont été sélectionnées et soumises à un test de stockage dans les mêmes conditions que les PPN modèles, c'est-à-dire 20 jours à 40°C avec une agitation constante à 110 rpm. L'objectif étant de comparer la stabilité à l'oxydation des PPN modèles et commerciales. Les PPN commerciales ont été sélectionnées en fonction de la composition de leur phase lipidique. Les résultats ont montré des cinétiques d'oxydation relativement similaires entre les PPN modèles et commerciales, ce qui a permis la validation des résultats et garantie la possibilité de les extrapoler à des systèmes réels.

2.2. Stabilité oxydative des farines infantiles

Dans un second temps les systèmes dispersés granulaires secs représentatifs de FI ont été étudiés.

2.2.1. Etude de marché des farines infantiles

Cette section s'appuie en partie sur un jeu de données intitulé : "Overview of the nutritional composition of infant flours commercialized worldwide in 2021" publié dans le répertoire Dataverse de l'INRAE (Cancalon, Hemery *et al.* 2023) et un data paper intitulé : "Dataset of the nutritional composition of infant flours commercialized worldwide in 2021" soumis dans le journal Data in Brief. Les listes d'ingrédients et les valeurs nutritionnelles de 96 FI commercialisées sur le marché mondial ont été collectées de décembre 2020 à avril 2021, directement sur l'emballage des produits, *via* les sites internet des différentes marques ou extraites d'articles de recherches. Le jeu de données inclus uniquement des produits transformés à base de céréales destinées à être reconstituées avec du lait ou d'autres liquides appropriés, et les céréales enrichies en protéines destinées à être reconstituées avec de l'eau ou des liquides sans protéines. L'analyse statistique des valeurs nutritionnelles rassemblées dans le jeu de données a révélé une variabilité importante des teneurs en macro- et micronutriments, caractérisée par des écarts types élevés et des écarts considérables entre les valeurs minimales et maximales. La teneur en lipides variait par exemple de 0,2 à 18,8 g/100 g de FI. La variabilité des valeurs nutritionnelles est associée à la diversité des matières premières entrant dans leur composition. Les FI produites et commercialisées dans les régions nord (FI Nord) sont composées en majorité de céréales, avec respectivement 51,8 ; 32,1 ; 30,4 et 23,2% des FI Nord comprenant des farines de riz, d'avoine complète, de blé et de maïs. Pour les FI Sud, les matières premières utilisées sont principalement des légumineuses, avec 57,5% de farine de soja et 45% de farine d'arachide, mais aussi des céréales, avec 30 et 55% des FI Sud composées de farines de millet et de maïs, respectivement. Les céréales, qui sont principalement utilisées dans les FI Nord, ont une densité nutritionnelle inférieure à celle des légumineuses en raison de leur plus faible teneur en lipides (1,7 % pour le blé contre 22,5 % pour le soja) et en protéines (13,1 % pour le blé contre 38,7 % pour le soja). Le profil des AG et le ratio LA/ALA varient également en fonction de la matière première utilisée, allant de 1,9 pour la farine de niébé à 49,8 pour la farine d'arachide. De plus, la comparaison des valeurs nutritionnelles des FI du jeu de données avec les seuils réglementaires imposés par le *CODEX Alimentarius* CXS 74-1981 a montré des surdosages pour certains micronutriments, notamment le fer et les vitamines A, E et C.

2.2.2. Stabilité oxydative de farines infantiles représentatives des produits commerciaux

Afin d'identifier les leviers clés permettant de contrôler la stabilité oxydative dans les systèmes secs, différents tests de stockage ont été réalisés. Le premier d'entre eux visait à évaluer la stabilité

oxydative d'une FI commerciale (CIF) en comparaison de deux FI formulées à partir des résultats issus du panorama afin d'être représentatives des produits présents sur le marché dans les régions sud (SIF) ou nord (NIF). Ces trois FI ont été stockées 5 mois avec des variations de température comme décrit dans le mémento B. Les résultats ont montré peu d'évolution des PV dans l'ensemble des FI (PV < 10 meqO_2/kg pendant les 5 mois de stockage). En revanche, des pertes en tocophérols ont été observées. Les résultats de ce premier test de stockage ont montré une stabilité relativement bonne, notamment pour les FI des régions du sud (CIF et SIF). Cependant, ces FI n'ont pas été enrichies en vitamine A, particulièrement sensible à l'oxydation. De plus, leur profil lipidique pourrait être encore optimisé pour répondre aux besoins du nourrisson.

2.2.3. Optimisation du profil lipidique des farines infantiles et impact des paramètres de structure et formulation sur la stabilité oxydative

Dans un second temps le profil lipidique des FI a été optimisé en introduisant dans leur composition des farines sources d'acides gras essentiels à des teneurs équilibrées. La FI OIF (Optimized Infant Flour) composées d'un mélange de farines de teff, niébé et soja a été formulée. Des ingrédients « sud » ont été privilégiés du fait de l'importante consommation de FI dans ces régions du monde.

2.2.3.1. Impact des paramètres de structure sur la stabilité oxydative de farines infantiles optimisées

Afin de réaliser un pré-criblage des leviers permettant de contrôler la stabilité oxydative dans les systèmes secs, OIF a été enrichie avec du son de riz noir (BRB), pour son importante teneur en composés phénoliques ayant une capacité antioxydante. De plus, l'impact de la taille des particules sur la stabilité oxydative a été étudié. Ainsi, dans cette section trois FI, OIF_NE, OIF_BRB_NE et OIF/RM_BRB_NE, enrichis en minéraux, notamment en calcium à une concentration de 0,324 mg par 100 g de FI, ont été soumis à un test de stockage de 9 mois avec des variations de température comme décrit dans le mémento B. Leur stabilité oxydative a été évaluée par le suivi de l'évolution des PV et des teneurs en tocophérols totaux. Les résultats ont montré une relativement bonne stabilité des IF pendant les cinq premiers mois de stockage puis une augmentation des PV à partir de 9 mois d'avantage marquée pour les IF incluant du son de riz noir ce qui pourrait être liée à une oxydation des composés phénoliques. Des pertes en tocophérols ont également été observées mais étaient relativement limitées. Les résultats ont mis en évidence que sur la gamme de tailles de particules testée ce paramètre n'avait pas d'influence sur la stabilité oxydative des IF.

2.2.3.2. Impact des paramètres de formulation sur la stabilité oxydative de farines infantiles optimisées

Cette partie est basés sur une article de recherche intitulé "Optimization of the lipid composition and oxidative stability of infant flours" en cours de préparation pour soumission. Dans cette étude OIF a subi différents traitements/fortifications dont un traitement thermique (inactivation enzymatique et inhibition des facteurs antinutritionnels), un enrichissement en AGPI-LC (ARA et DHA) et un enrichissement en fractions antioxydantes exogènes (sons de riz noir et poudre de feuilles d'amarante) pour améliorer à la fois son profil nutritionnel et sa stabilité à l'oxydation. Après un stockage de 6 mois à 40°C, les résultats ont montré que l'introduction de farines de teff et de niébé dans les FI a permis une optimisation du profil lipidique avec un abaissement du ratio ω6/ω3 proche de 5 tout en garantissant une stabilité oxydative acceptable (cinétiques relativement lentes) excepté pour la vitamine A qui était rapidement dégradée même lorsqu'un traitement thermique était appliqué. L'initiation de l'oxydation se produisait principalement pendant le procédé de production de la farine (broyage) puis se propageait lentement du fait de la diffusion limitée des composés à des Aw faibles. La présence de composés photosensibles conduisait à une augmentation plus rapide du niveau d'oxydation probablement due au fait que la photo-oxydation est 1000 à 1500 fois plus rapide que l'auto-oxydation. Les caroténoïdes apportés par les feuilles d'amarante et les composés phénoliques apportés par le son de riz noir agissaient comme des agents stabilisateurs de la vitamine A en retardant significativement sa dégradation

2.3. Digestibilité des préparations pour nourrissons et des farines infantiles optimisées

Un modèle de digestion in vitro a été employé pour évaluer la bioaccessibilité des lipides et protéines dans un nombre réduit de PPN et FI optimisés d'un point de vue de la stabilité oxydative. Les résultats ont montré un taux de lipolyse élevés dans les deux systèmes avec des profils en acides gras exprimant la régiosélectivité des lipases et la régiodistribution des lipides entrant dans les formulations. Les IFF incluant des ingrédients laitiers avaient la particularité de permettre la libération d'acides gras à chaines courtes et moyennes. La libération des AGPI-LC étaient bonnes dans tous les systèmes en fin de phase intestinales. La bioaccessibilité des tocophérols a également été évaluée et était autour de 20 % dans la plupart des systèmes. Enfin le taux de protéolyse atteignait des valeurs autour de 25% assez faibles donc mais sur des temps de digestion restreints (120 minutes en phase intestinale).
Aucun effet matrice fort n'a donc été observé confirmant que les aliments optimisés testés étaient d'assez bons vecteurs de nutriments.

3. Discussion générale

L'étude de la stabilité des PPN et FI a mis en évidence que :

- Dans les deux types de systèmes dispersés étudiés, les vitamines A et E sont des facteurs clés servant d'agents de régénération et permettant d'allonger les temps de latence de l'oxydation
- Dans des systèmes contenant ces vitamines et bien formulés les AGPI-LC sont stables à l'oxydation et bioaccessibles dans un modèle statique de digestion imitant un nourrisson de 6 mois
- Des synergies entre antioxydants ont été mises en évidence dans les deux types de systèmes (e.g. caroténoides / tocophérols)
- Dans les deux types de systèmes le procédé de préparation a un poids important sur l'état d'oxydation initial du système et sa stabilité oxydative à suivre

Des différences de comportements à l'oxydation ont été mises en évidence pour les deux systèmes, avec des cinétiques d'oxydation significativement plus lentes dans les systèmes secs comparés aux systèmes émulsionnés qui s'expliquent notamment par des différentes de cinétiques de diffusion de l'oxygène et des radicaux dans ces systèmes hétérogènes. Ces modes de propagation de l'oxydation ont été plus étudiés dans les systèmes émulsionnés que dans les systèmes secs. Des questions demeurent néanmoins dans les deux types de systèmes quant au rôle de la phase micellaire en systèmes émulsionnés et des antioxydants les plus efficaces en systèmes secs ainsi que leur mode d'action en phase solide (effet des composés phénoliques endogènes présent dans les sons par exemple).

4. Conclusion

L'objectif de ces travaux de thèse était d'identifier des leviers physico-chimiques clés pour améliorer la stabilité oxydative de systèmes dispersés enrichis en AGPI-LC tout en maintenant une bioaccessibilité acceptable des nutriments. L'originalité de ce travail est de fournir une comparaison des comportements et des mécanismes d'oxydation des lipides et des antioxydants dans deux types de systèmes dispersés : les systèmes liquides sous forme d'émulsions huile-dans-eau représentés par PPN, et les systèmes granulaires solides représentés par FI. Les résultats ont d'abord montré que, bien que la cinétique de propagation de l'oxydation soit significativement plus lente dans les systèmes granulaires que dans les systèmes émulsifiés, les paramètres de formulation jouent un rôle clé dans le contrôle de l'oxydation des lipides dans les deux types de systèmes. Pour les PPN, la réintroduction d'ingrédients laitiers (PL et MGLA) et l'ajout de caroténoïdes ont permis d'améliorer la résistance à l'oxydation et d'optimiser le profil nutritionnel pour répondre aux besoins des nourrissons. En ce qui

concerne les FI, la sélection de farines alternatives telles que le niébé et le teff (riche en d'ALA) a permis de rééquilibrer le profil lipidique sans impacter la stabilité à l'oxydation. En outre, les résultats ont démontré le potentiel significatif des sons de riz noir en tant qu'agent stabilisateur de la vitamine A. Dans les deux systèmes, la qualité initiale des matières premières utilisées est déterminante pour la stabilité oxydative pendant le stockage. La caractérisation des farines a montré que, selon la source considérée, la qualité initiale était variable et mal contrôlée. Globalement, l'effet des paramètres structurels sur la stabilité oxydative des systèmes granulaires et émulsifiés est plus limité. En particulier, les résultats ont montré que la taille des particules n'avait pas d'influence significative sur la stabilité à l'oxydation. Quant à la taille des gouttelettes de lipides, son effet était plus complexe à prédire et dépendait fortement de l'environnement chimique, la nature de l'interface étant le facteur le plus influent. Dans ce travail, l'approche multiple a permis de proposer des formulations PPN et FI répondant à plusieurs critères de nutritionnels et de stabilité chimique des lipides, tout en permettant une meilleure compréhension des mécanismes impliqués. Cependant, certains aspects du projet n'ont pas été explorés et mériteraient d'être approfondis, tels que l'implication des protéines dans les phénomènes d'oxydation ou encore l'ajout de corps gras issus d'assemblages naturels végétaux comme des oléosomes.



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UNIVERSITÉ DE MONTPELLIER

Titre : Étude des mécanismes de l'oxydation lipidique et des antioxydants dans des matrices alimentaires complexes en vue d'améliorer leur stabilité et la bioaccessibilité des lipides : Exemple des préparations pour nourrissons et farines infantiles enrichies en acides gras polyinsaturés à longues chaînes

Mots clés : Nutrition infantile, Oxydation des lipides, Antioxydants, Acides gras polyinsaturés, Digestion

Résumé : La diversification alimentaire, démarrant à partir de 4/6 mois, est une période clé du développement caractérisée par des besoins nutritionnels spécifiques couverts en majorité par le lait maternel et/ou les Préparations Pour Nourrissons (PPN) et l'introduction progressive d'aliments de complément tels que les Farines Infantiles (FI). Les lipides jouent un rôle crucial pendant cette période en tant que fournisseur principal d'énergie et vecteur d'acides gras essentiels et de leurs dérivés à longues chaînes contribuant au développement de l'enfant. Ces acides gras sont particulièrement sensibles à l'oxydation du fait de leurs multiples insaturations entraînant une perte de qualité nutritionnelle et la formation de composés potentiellement toxiques. L'oxydation lipidique est un mécanisme complexe multifactoriel difficile à prédire dans des aliments tels que les PPN et FI. L'objectif de ce projet était d'identifier des leviers physico-chimiques clés permettant d'optimiser le profil nutritionnel et la stabilité oxydative de systèmes dispersés représentatifs des PPN et FI enrichis en acides gras polyinsaturés (AGPI) tout en maintenant une bioaccessibilité acceptable des nutriments. Des tests de stockage allant de 20 jours à 9 mois à 25 ou 40°C ont été réalisés sur des systèmes modèles représentatifs des PPN et FI, déclinés en un panel de modalités selon des paramètres de formulation et structure. Les systèmes ayant démontré les meilleures propriétés de stabilité ont été soumis à un processus de digestion in vitro pour caractériser la bioaccessibilité des nutriments. Les résultats ont montré que la composition des PPN est homogène et acceptable alors que celle des FI est variable, avec des surdosages en oméga 6 et vitamines. Les mécanismes d'oxydation dans les PPN et FI sont tout d'abord marqués par des pertes en vitamines (A et E), les AGPI et leurs dérivés à longues chaines restent quant à eux, relativement stables. Les FI sont caractérisées par une cinétique de propagation de l'oxydation significativement plus lente que les PPN. Alors que les paramètres de structures (ex. taille de gouttelettes ou particules, ordre d'ajout des tensioactifs) ont un effet limité, les paramètres de formulation (ex. composition des matières premières, nature des tensioactifs) ont un important potentiel dans la maitrise de la stabilité oxydative. L'introduction de caroténoïdes en présence de tocophérols conduit à une amélioration de la stabilité oxydative et de la vitamine A. Cet effet synergique est également observé en présence de sphyngomyéline dans les PPN. La bioaccessibilité des lipides et protéines s'est avérée relativement acceptable et peu matrice dépendante. Ces résultats ouvrent la voie à une meilleure vectorisation des AGPI à longues chaines et vitamines dans les aliments infantiles.

Title: Study of lipid oxidation and antioxidants mechanisms in complex food matrices in order to improving their stability and lipid bioaccessibility: Example of infant formulas and flours enriched with long-chain polyunsaturated fatty acids

Keywords: Infant nutrition, Lipid oxidation, Antioxidants, Polyunsaturated fatty acids, Digestion

Abstract: Dietary diversification, from 4/6 months of age, is a key developmental period characterized by specific nutritional needs covered mainly by breast milk and/or Infant Follow-on Formulas (IFF), and the gradual introduction of complementary foods such as Infant Flour (IF). Lipids play a crucial role during this period, as the main energy supplier and carrier of essential fatty acids and their long-chain derivatives, which contribute to infant development. These fatty acids are particularly prone to oxidation due to their multiple double bonds, resulting in a loss of nutritional quality and the formation of potentially toxic compounds. Lipid oxidation is a complex, multifactorial mechanism that is difficult to predict in foods such as IFF and IF. The project aimed to identify key physico-chemical levers for optimizing the nutritional profile and oxidative stability of dispersed systems representative of IFF and IF enriched with polyunsaturated fatty acids (PUFA), while maintaining acceptable nutrients bioaccessibility. Storage tests ranging from 20 days to 9 months at 25 or 40°C were carried out on model systems representative of IFF and IF, and then declined in a panel of modalities according to formulation and parameters. Systems with the most improved stability properties were subjected to an in vitro digestion process to characterize nutrients bioaccessibility. The results showed that the composition of IFF was homogeneous and acceptable, whereas that of IF was variable, with overages in omega 6 and vitamins. Oxidation mechanisms are primarily characterized by losses of vitamins (A and E), while PUFA and their long-chain derivatives remain relatively stable. IF are characterized by slower oxidation propagation kinetics than IFF. While structural parameters (*e.g.* droplet or particle size, order of surfactant addition) have a limited effect, formulation parameters (*e.g.* raw material composition, nature of surfactants) have significant potential in controlling oxidative stability. The introduction of carotenoids in the presence of tocopherols leads to improved oxidative stability and vitamin A content. This synergistic effect is also observed in the presence of sphyngomyelin in IFF. The bioaccessibility of lipids and proteins is relatively acceptable and not matrix-dependent. These results pave the way for improved vectorization of long-chain PUFA and vitamins in infant foods.