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Influence of chemical forms and homogenization process on the stability of vitamin A in infant follow-on formulas

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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 infant follow-on formulas (IFF) depending on its chemical forms and on the homogenization process. For this propose, model IFF representative 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 compared to 84.9% without carotenoids. Carotenoids provide 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.

1. Introduction

Vitamin A is a crucial micronutrient that plays an essential role in the growth, development, and overall health of infants (O'Byrne & 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, 2016). 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 (Carazo et al., 2021). 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 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 & Zimmermann, 2007). In order to prevent vitamin A deficiency, various approaches aiming 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 et al. (2013) on powdered milk, or the study of Sandjaja et al. (2015) on oil, and which have been shown to be

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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 et al. (2022) the vitamin A content of breast milk decreased significantly as lactation progressed, and is estimated ranging between 339.4 and 431.3 μg/L for mature breast milk. Vitamin A is found under the predominant form of esters (palmitate mainly), the retinol to ester ratio is variable but around 10:90 w/w (Jensen et al., 1992). When breastfeeding is either not desired or not possible the most common alternative is the use of IFF. Their composition is governed by precise regulations in Europe, which 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 et al. 2023d), the main chemical form of vitamin A used 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 (Nieva-Echevarría et al., 2017; Palace et al., 1999; Ross, 2016). 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 thermosensitive and photosensitive and can be impacted by high pressure homogenization which triggers high local shear forces. High pressure homogenization is a classical step in IFF processing (Bourlieu-Lacanal et al., 2015a; Bourlieu et al., 2017). It allows the dispersion of oil phase under oil in water emulsion but also triggers the formation of neoformed interface in IFF that strongly differ from native milk fat globule (Bourlieu et al., 2020; Bourlieu & Michalski, 2015c) and modify its digestive behavior (Bourlieu-Lacanal et al., 2015a; Bourlieu et al., 2015b; De Oliveira et al., 2017) and metabolization (Bourlieu & Michalski, 2015c; Michalski, 2007).

Several physico-chemical parameters and processing factors can 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 et al., 2018). However, to the best of our knowledge, no study has 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.

In this context, the aim of the present study was to investigate the impact of homogenization process and of different chemical forms of vitamin A on its stability in model IFF representative of marketed formulas. Therefore, the strength of our study was to formulate eight model IFF with normalized lipid profile (equivalent fatty acid (FA) profile and vitamin E content) but with various droplet sizes and chemical forms of vitamin A (retinol or retinol esters or carotenoids) and compare vitamin A stability in order to improve it in commercialized IFF. The vitamin A stability was assessed by evaluating the content of each forms before and after homogenization process and during storage under accelerated conditions.

This article is part of a series of studies that aimed at improving the oxidative stability of model IFF, which are also the subject of the present study. The first focused on the impact of formulation parameters on oxidative stability (Cancalon et al., 2023b) and the second on the identification of key structural levers to limit lipid oxidation (Cancalon et al., 2023a). However, these articles highlighted the high sensitivity of vitamin A and the need to focus on this parameter alone in order to better understand the underlying mechanisms.

2. Materials and methods

2.1. Materials

Palm oil was kindly provided by Cargill (Paris, France). Red palm oil (Elaeis Guineensis) was purchased in specialised grocery shops (La Pangée, Montpellier, France). Sunflower (*Helianthus annuus*) and rapeseed (*Brassica napus*) oils were purchased in local supermarket (Casino, Saint-Etienne, France). High oleic sunflower oil was purchased from Cuisinor (Saint-Médard de Guizières, France). PUFA oils (ARASCO© and DHASCO©) were kindly provided by DSM Nutritional products France (*La Garenne Colombes*, France). Dairy fat was a gift from Corman (Limburg, Belgium) and soy lecithin by Novastell (Etrepagny, France). The skim milk powder and serum protein isolate were generous gifts from Ingredia (Arras, France). A vitamin and mineral complex (VMC), consisting of a blend 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), was kindly supplied by DSM Nutritional Products South Africa (*Isando*, South Africa). The contents of each mineral and vitamin of the VMC were set according to European regulations and the average contents of marketed IFF (Cancalon et al., 2022, 2022d).

All reagents, solvents and analytical standards including the retinol used for the content normalization were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.2. Model infant follow-on formulas formulation and preparation

The composition of the model IFF was determined according to the nutritional values of worldwide marketed IFF as described in a previous work (Cancalon et al., 2023a; Cancalon et al., 2023b; Cancalon et al., 2023c). The water and oil phases were prepared 24 h prior to model IFF preparation and stored at 4 °C. 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 phase represented 32.0 g/L (34.0 g/L with lecithin) and was based on three oil mixtures based on palm, red palm oils or dairy fat (oleic fraction) as shown in Table 1. The oil mixtures were formulated by linear programming to be representative of commercially available IFF. For this purpose, the nutritional values of 91 infant follow-on formulas were listed (Cancalon et al., 2023c; Cancalon et al., 2023d), enabling the definition of target values for saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, as well as for linoleic (LA), α-linolenic (ALA), arachidonic (ARA) and docosahexaenoic (DHA) acids. These target values were in line with European regulations CE 2016/127. Linear programming with Microsoft Excel Solver software (Excel 2016) was used to calculate the proportions of ingredients in each oil mixtures in order to achieve the target values set (Table 1). The oil phases were obtained by blending the fats in the proportions described in Tables 1 and 2 g/L of soy lecithin. To be representative of IFF industrial manufacturing processes, vitamin and mineral fortification and standardization was carried out just prior to preparation of the model IFF as described in Table 1. 20 mg/100 mL VMC was added to the oil phase. Vitamin E content was also 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 was added to the water phase previously heated to 70 °C. Both phases were pre-emulsified by two cycles of 5 min at 5000 rpm using a Silverson L5M (Silverson, Longmeadow, USA). Model IFF were homogenized with an APV-1000 lab homogenizer (SPXflow, Charlotte,

Table 1
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_RE/RA_L	PO_RE/RA_S	PO_RA_L	PO_3RA_L	RPO_RE/RA_L	RPO_RE/RA_S	DF_RA_L	DF_RA_S
Oil phase composition (%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	15.4	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	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Retinol	0.1	0.1	–	–	0.1	0.1	–	–
Retinyl acetate	–	–	0.1	0.3	–	–	–	–
Tocopherol	9.1	9.1	9.1	9.1	9.10	9.1	6.1	6.1
Vitamin E content (ppm of lipids)								
Total vitamin E	740.0 ± 18.6[a]	710.5 ± 17.7 [a]	828.3 ± 112.5 [a]	803.1 ± 34.1 [a]	726.7 ± 51.3 [a]	707.6 ± 25.4 [a]	764.9 ± 19.6 [a]	728.8 ± 54.4 [a]
γ-tocotrienol	–	–	–	–	170.2 ± 31.8	152.4 ± 7.3	–	–
Vitamin A content (ppm RE of lipids)								
Total vitamin A	14.3 ± 1.0 [a]	13.2 ± 0.7 [a]	14.5 ± 0.9 [a]	36.0 ± 2.4 [b]	63.4 ± 2.4 [d]	58.9 ± 0.8 [c]	12.8 ± 1.8 [a]	12.7 ± 0.9 [a]
Retinol	2.0 ± 0.20	1.8 ± 0.1	–	–	0.0 ± 0.0	0.0 ± 0.0	–	–
Retinyl acetate	12.3 ± 0.8 [ab]	11.4 ± 0.6 [a]	14 0.5 ± 0.9 [b]	36.0 ± 2.4 [c]	12.7 ± 0.5 [ab]	11.8 ± 0.4 [ab]	11.1 ± 1.3 [a]	11.0 ± 0.8 [a]
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	–	–

VMC: Vitamin and mineral complex.
ppm RE correspond to μg retinol equivalent/g of lipids.
Different letters indicate a significant difference ($P < 0.05$).

Table 2
Nomenclature, main component in Oil phase formulation, vitamin A forms and droplet size of the eight model IFF studied in terms of vitamin A stability.

Name of IFF[a]	Main component in oil phase formulation	Added chemical forms of vitamin A [b]	Size of droplets (μm)[a]
PO_RE/RA_L	Palm oil	RE and RA	0.7
PO_RE/RA_S	Palm oil	RE and RA	0.4
PO_RA_L	Palm oil	RE and RA	0.7
PO_3RA_L	Palm oil	RA (3 times higher than PO_RA_L)	0.7
RPO_RE/RA_L	Red palm oil	RE and RA	0.7
RPO_RE/RA_S	Red palm oil	RE and RA	0.4
DF_RA_L	Dairy fat	RA	0.7
DF_RA_S	Dairy fat	RA	0.4

RE is provided by the addition of a standard solution and RA by the addition of CMV and a standard solution to the oil phase.
[a] L stands for large and S for small droplet size of respectively 0.7 or 0.4 μm respectively.
[b] RE stands for Retinol and RA for retinyl acetate.

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 (*i.e.* 10/3 MPa) and resulted in droplets of 0.7 μm. The second homogenization process involved eight cycles at pressures of 350/40 bar (*i.e.* 35/4 MPa) and resulted in a droplet size of 0.4 μm. A total of eight model IFF were studied, including four based on palm oil, two based on red palm oil and two based on dairy fat (Table 2).

After homogenization, sodium azide was added to all model IFF at 0.02% w/w to prevent bacterial proliferation. Each model IFF was aliquoted in four replicates in 40 mL amber tubes and stored at 40 °C for 20 days with constant orbital stirring at 110 rpm with a IKA KS 4000 i control incubator (IKA, Staufen, Germany). Samplings were made before high pressure homogenization and after 0, 1, 3, 6, 9, 15 and 20 days of storage. Model IFF samples were flushed under nitrogen and stored at −20 °C until further analysis.

2.3. Droplets size distribution

The particle size distribution was determined by par laser light diffraction techniques (Malvern Mastersizer 2000; Worcestershire, UK) using a refractive index of 1.458 or the dispersed phase and 1.33 for the dispersant (water) (Bourlieu et al., 2012). Model IFF was deposited in a measurement cell to reach an obscuration rate between 5 and 10%. The surface-weighted mean diameter namely D[3,2] and the distribution mode were measured and the average mode was used as an approximation to summarize the size of the global distribution in a given model IFF.

2.4. Confocal laser scanning microscopy (CLSM)

Model IFF microstructure was observed by CLSM system (Leica SP8, Heidelberg, Germany) using an inverted microscope. Multiple images of a model IFF were obtained using two He-Ne lasers (excitation at 543 and 633 nm) and an argon laser (excitation at 488 nm) and a 40 × water-immersion objective (Kergomard et al., 2021). Three fluorescent dyes were added to model IFF to localize apolar 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 Rhod PE 1,2-dipalmitoyl-sn-glycero-3--phosphoethanolamin-N-(lissamine rhodamine B sulfonyl, λex 543 nm – λem 590 nm, Aventi polar lipids) and proteins with Fast green FCF (6:100 v/v−1, λex 633 nm – λem 655–755 nm, Invitrogen). The dyes were added to 200 μL of model IFF at least 10 min before observations. 10 μL of marked model IFF were then deposited on a glass slide. Leica LAS X software (Wetzlar, Germany) was used for the images collection and analysis.

2.5. Fatty acid profiles

The fatty acid profiles were determined by gas chromatography (GC) following a FA methylation as detailed in our previous work (Cancalon et al., 2023b). A Focus GC (Thermo Electron Corporation, Massachusetts, USA) 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) was used. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The initial column temperature was 150 °C and reach 225 °C with a rise of 5 °C.min^{-1}. Final temperature (225 °C) was maintained for 10 min. The injector and detector temperatures were 250 and 270 °C, respectively. Flame ionization detector was used and FA methyl esters (FAME) were identified using standard solution of methyl esters mixture. ChromCard software (version 2005, Thermo FisherScientific, Massachusetts, USA) was used to collect and analyze data.

2.6. Retinol, retinol esters and carotenoids contents

The different chemical forms of vitamin A (retinol, retinyl acetate, retinyl palmitate and carotenoids) were quantified by High performance liquid chromatography (HPLC) technique according to Montúfar et al. (2010) with slight modifications. 1 mL of model IFF was mixed with 2 mL of hexane/isopropanol (3:2 v/v) extraction solvent and centrifuged for 5 min at 1500 rpm with a CR412 centrifuge (Jouan Thermo Electron, Waltham, USA). The upper 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 and −9 °C for 20 min (Eppendorf 5810R, Hamburg, Germany). TAG were removed by rapid sampling of the upper acetone phase and filtration 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 using a YMC-30 column (250 × 4.6 mm, YMC) 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 et al. (2013): (i) 55% A and 45% B for 1 min, then (ii) a linear gradient from 55% A to 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 (A = methanol/water (60:40 v/v), B = methanol/methyl tert-butyl ether/water (28.5:67.5:4 v/v/v)). 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.

2.7. Statistical analysis

Model IFF were stored in quadruplicates. All analysis were performed on each aliquot with up to two technological repetitions of measurement. Results are presented as mean ± SD. Statistical significance was determined by analysis of variance (one-way ANOVA) using R software (R.2.13.0, http://cran.r-project.org). Differences at $p < 0.05$ were considered statistically significant.

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.

3.1. Structural and composition normalization of model IFF

Model IFF were designed to be representative of commercial formulas as described in a previous study (Cancalon et al., 2023a; Cancalon et al., 2023b). 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_RE/RA_L and RPO_RE/RA_S contained also in this total γ-tocotrienol representing 161.3 ± 19.6 ppm. Table 1 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 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_L 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_ RE/RA_L and PO_ RE/RA_S and absent in RPO_RE/RA_L and RPO_RE/RA_S. Depending on the source of fat constituting the lipid fraction, other chemical forms of vitamin A were present. Dairy-based model IFF (DF_RA_L and DF_RA_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.

The structure of the model IFF was also standardized. The typical droplet size distribution and structure of PO_RE/RA_L and PO_RE/RA_S at the initial point of storage (D0) assessed by granulometry and CLSM, respectively (Fig. 1). 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.

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. The results showed a non-significant loss of 2.6% in total retinoids for the homogenization process applied to obtain large droplets (Fig. 2a). 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 homogenization and the chemical forms used for content normalization on total vitamin A losses was assessed by comparing PO_RA/RE_L and PO_RA_L (Fig. 2b). The results showed no significant losses during the processing in these model IFF probably due to the low impact for the homogenization pressure used to obtain 0.7 μm droplets. It would have been interesting to test this effect at higher

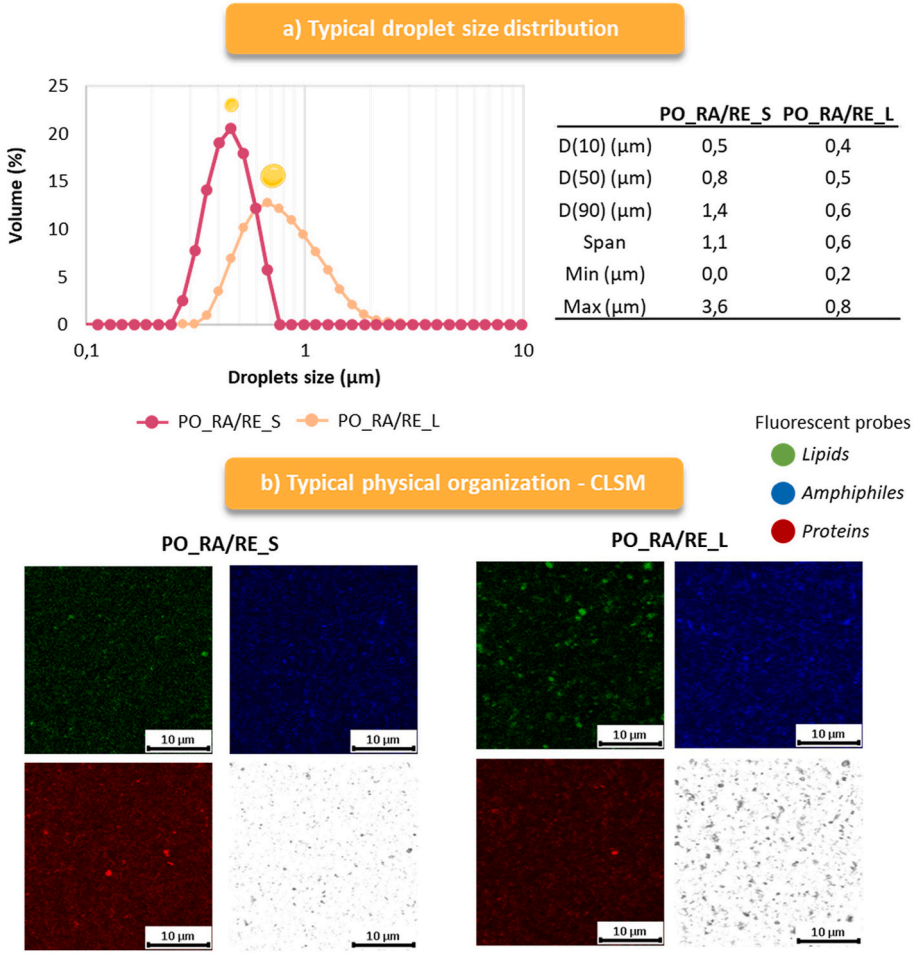

Fig. 1. Structural characterization by a) droplet size distribution and b) CLSM observations
Particles size distribution was assessed by laser light scattering.
Confocal laser scanning micrograph were collected using a 40 × water-immersion objective with each time four micrographs: blue colored marks proteins; red colored marks amphiphiles compounds, green colored marks lipids and white micrograph corresponds to transmission light. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

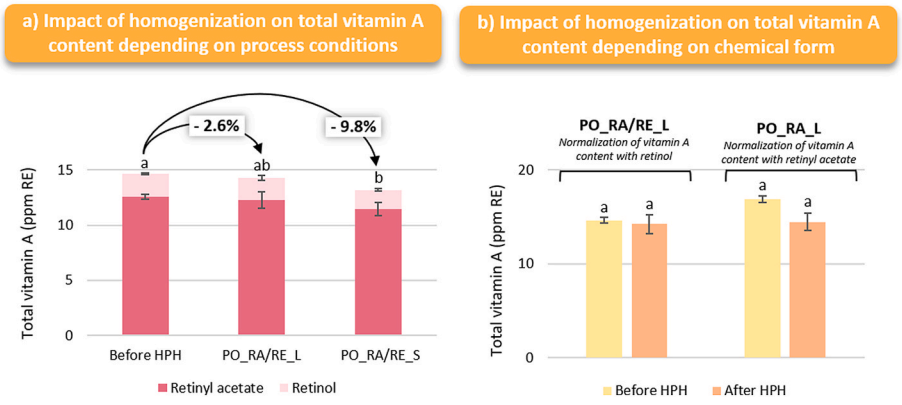

Fig. 2. Impact of the homogenization on total vitamin A content depending on a) process conditions and b) vitamin A chemical form
The percentages in the labels associated to the markdown arrows correspond to the global loss percentage of vitamin A for all the cumulated chemical forms (RE and/or RA) between before and after the homogenization process (eight cycles at 100/30 or 350/40 bars).
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.

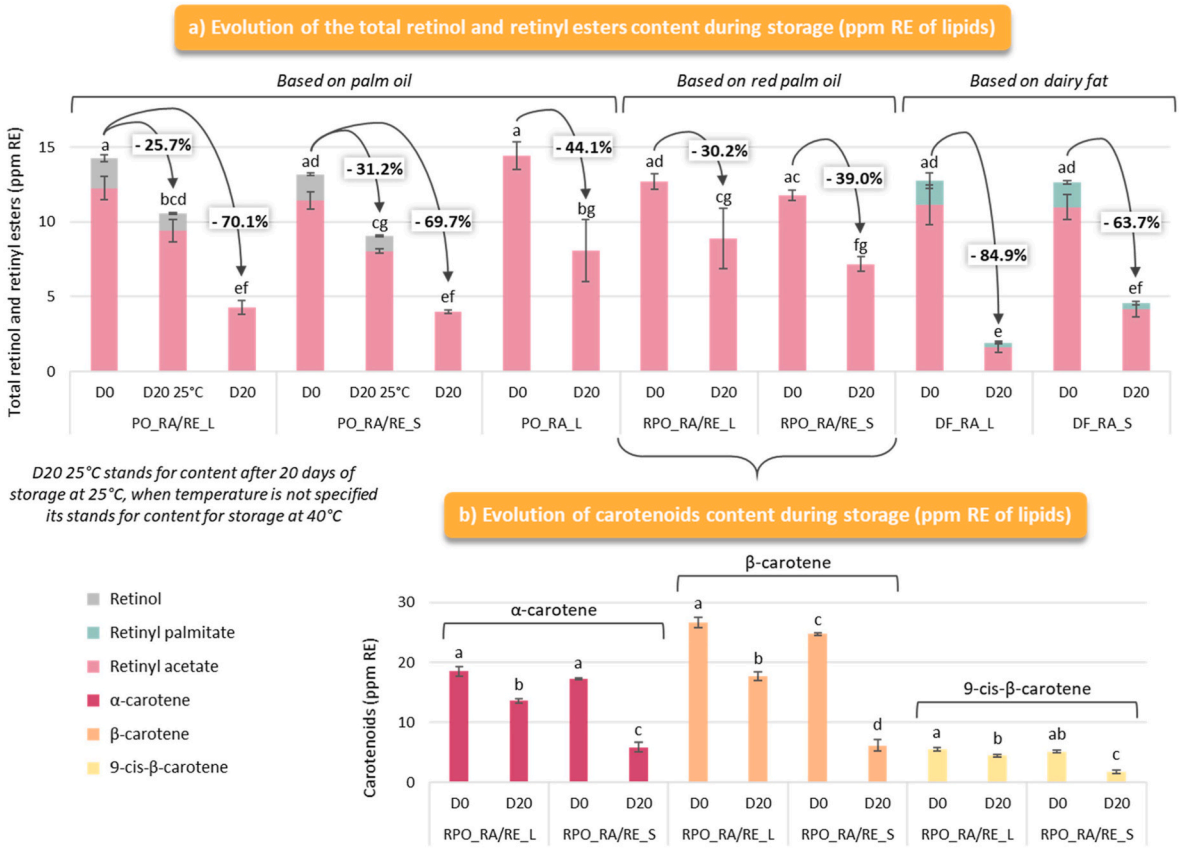

Fig. 3. Impact chemical forms of vitamin A on the evolution of a) the total retinoids content and b) carotenoids content during 20 days of storage
The percentages in the labels associated to the markdown arrows correspond to the global loss percentage of vitamin A for all the cumulated chemical forms (RE and/or RA) between after homogenization (D0) and after 20 days of storage at 25 or 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.

homogenization pressures.

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 (Fig. 3a). Vitamin A losses were more than twice as high, whatever the droplet size, for the model IFF stored at 40 °C compared to those stored at 25 °C. These losses were 70.1 and 69.7% at 40 °C *versus* 25.7 and 31.2% in PO_RE/RA_L and PO_RE/RA_S, respectively. 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_ RE/RA_L, respectively). After 20 days of storage, all retinol was lost in both model IFF.

The introduction of red palm oil into the lipid fraction led after 20 days 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. Red palm oil is an important source of carotenoids. Thus, three compounds belonging to the carotenoid class were present in RPO_RE/RA_L and RPO_RE/RA_S (Fig. 3b). 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_RE/RA_L *versus* 75.1% for RPO_RE/RA_S. Total retinoids contents in RPO_RE/RA_L and RPO_RE/RA_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_RE/RA_L and RPO_RE/RA_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_RA_L and DF_RA_S. The results show that losses of 84.9 and 63.7% of total retinoids occurred during storage for DF_RA_L and DF_RA_S, respectively. These losses were not significantly different according to droplet size.

3.4. Impact of overage of vitamin A during processing and storage

The impact of vitamin A acetate overage on its stability during the homogenization process leading to small droplets and storage was assessed (Fig. 4). 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

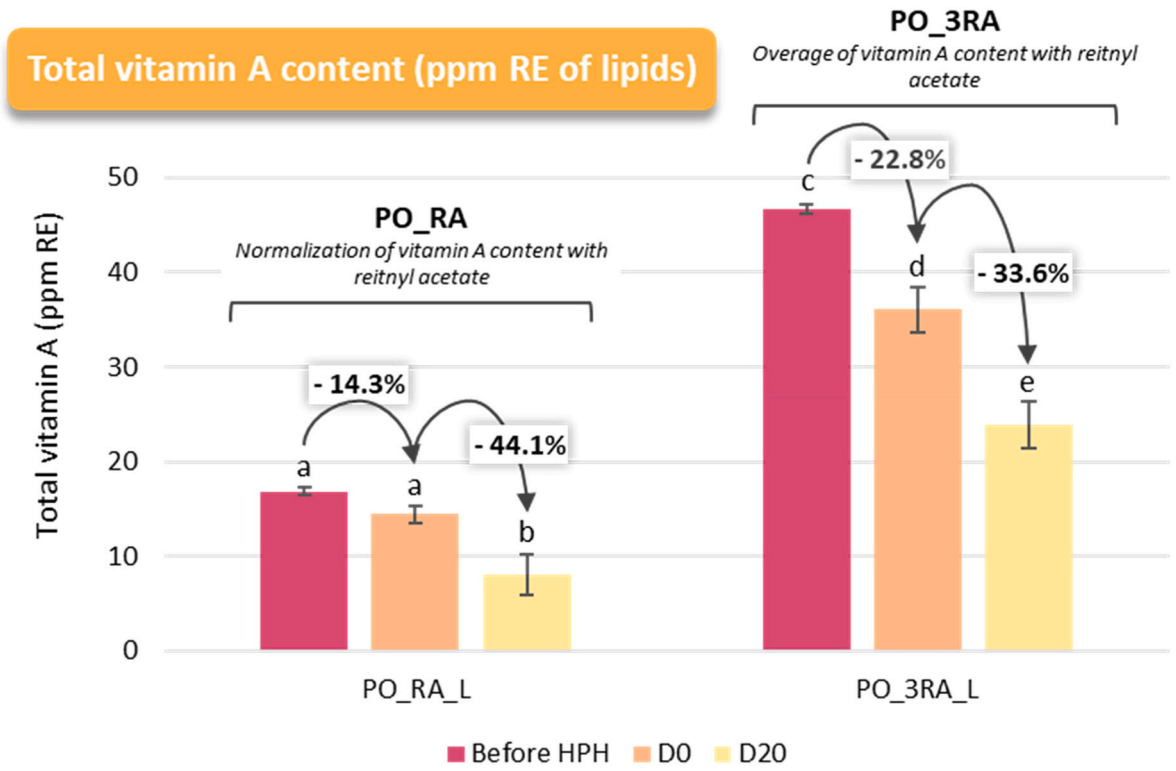

Fig. 4. Impact of overage of vitamin A as form of retinyl acetate on the total vitamin A content stability during homogenization process and storage in accelerated conditions

The percentages in the labels associated to the markdown arrows correspond to the global loss percentage of vitamin A for all the cumulated chemical forms (RE and/or RA) between before and after the homogenization process (D0) (eight cycles at 100/30 bars leading to a droplet size of 0.7 μm) and after 20 days of 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.

significantly different.

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.

4.1. 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. Tanglao 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 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 et al. (2021, 2022) who observed that vitamin A stability increased with homogenization pressure (up to 2000 bars) and α-tocopherol concentration (maximum effect for the highest of the concentrations tested, *i.e.* 1000 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 et al., 2012; Valencia-Flores et al., 2013). As described in a previous work (Cancalon et al., 2023a), the impact of droplet size alone is difficult to predict in such complex systems and is also linked to the chemical environment (Lethuaut et al., 2002).

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_RE/RA_S

and after 20 days of storage at 40 °C in PO_RE/RA_L and PO_RE/RA_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 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 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 & Allen, 2018).

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 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 et al. (2006) also demonstrated an increase in the oxidation induction phase in the presence of 1000 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_RE/RA_L and RPO_RE/RA_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 et al., 2017).

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 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 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 576–806 μg RE, considering only infant formulas intake (volume between 6 and 12 months of age of 500–700 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.

5. Conclusion

This study has shown 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 of up to 14.3% of total content for a homogenization process leading to a droplet size of 0.4 μm. Analyses performed after a storage test at 40 °C during 20 days showed a total loss of retinol and up to 85% loss of retinyl esters. The presence of carotenoids improved overall vitamin A stability with an even greater effect for larger droplet size (0.7 μm *vs* 0.4 μm). The antioxidant activity of provitamin A such as β-carotene can be associated with its anti-free radical activity and its synergy with tocopherols and could be an interesting ingredient in IFF to benefit from such synergy. 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. Although this study has been conducted on eight model IFF only and with a restricted number of vitamin A chemical forms, it has underlined important levers to stabilize this micronutrient. These include limiting homogenization pressure during processing, use retinyl esters instead of retinol and try to develop useful synergies such as the ones observed in breast milk between carotenoids and vitamin A using biomimetic approaches.

CRediT authorship contribution statement

Mathilde Cancalon: Writing – original draft, Methodology, Investigation, Formal analysis. **Pierre Villeneuve:** Writing – review & editing, Supervision. **Nathalie Barouh:** Writing – review & editing, Methodology. **Bruno Baréa:** Resources. **Erwann Durand:** Writing – review & editing. **Claire Bourlieu-Lacanal:** Writing – review & editing, Validation, Supervision, Methodology. **Youna Hemery:** Writing – review & editing, Validation, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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