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ORIGINAL ARTICLE

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Early detection of lipid oxidation in infant milk formula by measuring free oxylipins—Comparison with hydroperoxide value and thiobarbituric acid reactive substance methods

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Abstract: Infant milk formula was used as a model food to compare the sensitivity of thiobarbituric acid reactive substances (TBARS) and hydroperoxide methods to UPLC-MS/MS oxylipin analysis for detecting early lipid oxidation. Two different infant milk formulas were tested during 21 days of storage at 4°C. Formulas 1 and 2 contained canola oil and canola oil + 1% docosahexaenoic acid ethyl ester, respectively. Formulas were sampled up to 21 days of storage. Formula 2 had higher peroxide values than Formula 1 across all time points. However, no significant differences over time in TBARS and peroxide values in either formula were observed. Several oxylipins increased in both formulas starting on day 7 (linoleic acid and alpha-linolenic acid-derived oxylipins in Formula 1 and DHA-derived oxylipins in Formula 2). These results indicate that free oxylipins are effective in detecting early lipid oxidation and distinguishing between formulations containing different fatty acids.

KEYWORDS
hydroperoxide, lipid oxidation, milk formula, TBARs, UPLC-MS/MS

Practical Application: We have recently shown that primary oxidation products known as oxylipins can be measured in their free form by ultra-high pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to detect early lipid oxidation. However, a head-to-head comparison of the sensitivity of this approach to conventional spectrophotometric methods has not been evaluated. Our results indicate that free oxylipin measurements are better than conventional methods in detecting early lipid oxidation in milk infant formula distinguishing between different formulations.

1 | INTRODUCTION

Lipid oxidation is the major cause of rancidity and loss of nutritional quality in foods (German, 1999). It involves the oxidation of polyunsaturated fatty acids (PUFAs) by free radicals or singlet oxygen molecules to generate PUFA hydroperoxides, which can further oxidize to form more stable PUFA hydroxy or ketone molecules known as oxylipins or primary oxidation products (Richardson et al., 2017; Yang et al., 2009). Oxidized PUFAs can autodegrade via beta-scission into secondary volatile compounds (e.g., malonaldehyde and short-chain ketones) responsible for many of the sensory properties of foods (Ahmed et al., 2016; Christi & Harwoo, 2020; Dias et al., 2020; St. Angelo, 1996).

To date, conventional methods used to measure lipid oxidation in food matrices have relied on the quantification of free fatty acids, primary oxidation products such as hydroperoxides, or secondary oxidation products such as malonaldehyde (Cesa, 2004; Cesa et al., 2015; Dias et al., 2020; Drapala et al., 2018; Turoli et al., 2004). Some other analytical methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), or near-infrared reflectance (NIR) spectroscopy have also been used for determining some oxidation compounds in milk, including odorants (secondary fatty acid oxidation products), cholesterol oxidation products (COPs), and aldehydes (Daoud et al., 2020; Gorassini et al., 2017; Siefarth et al., 2013).

Recently, we reported the use of ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) for identifying and quantifying multiple primary oxidation products (i.e., oxylipins) in oils (Richardson et al., 2017), potatoes (Zhang et al., 2021), bovine milk (Dias et al., 2020; Teixeira et al., 2021), and human breast milk (Gan et al., 2020). A key advantage of UPLC-MS/MS is that multiple primary oxidation products can be measured simultaneously, thus providing detailed analytical information on the composition of oxylipins in food matrices and how they may change with processing (Dias et al., 2020; Zhang et al., 2021). Another advantage is that oxylipins can be detected at nanomolar concentrations, which is lower than the millimolar limits of detection of more conventional assays such as peroxide value and thiobarbituric acid reactive species (TBARS) for measuring PUFA hydroperoxides and malonaldehyde, respectively.

One unresolved question was whether the UPLC-MS/MS method employed by our group could be used to detect early lipid oxidation, compared to other conventional assays. In a prior study, PUFA-derived oxylipins decreased in milk following thermal processing, but hydroperoxide content and TBARS did not change, suggesting that oxylipins may serve as more sensitive markers of lipid oxidation compared to spectrophotometric assays (Dias et al., 2020).

In the present study, we tested the hypothesis that oxylipins measured with UPLC-MS/MS would readily detect early lipid oxidation in infant milk formula compared to standard conventional oxidation methods such as TBARS and hydroperoxide value, during refrigerated storage. A secondary aim was to evaluate the impact of milk formula PUFA composition on lipid oxidation, as current research has shown that the oxylipin profile of a sample is related to the precursor PUFA composition of the sample (Emami et al., 2020; Richardson et al., 2017). Infant milk formula was used as a model food representative of the main PUFAs in breast milk.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Cumene hydroperoxide (Cat#L06866) and n-Propyl 3,4,5-Trihydroxy-benzoate (Propil gallate [PG]; Cat#A10877) were purchased from Alfa Aesar (Haverhill, MA, USA). Barium chloride ($BaCl_2$; Cat #S25187), acetonitrile (Cat #A956-1), chloroform HPLC grade (Cat #C607-4), ethyl acetate (Cat #E196-4), iso-octane HPLC grade (Cat #0296-1), methanol (Cat #A454-4), methanol LC-MS grade (Cat #A456-4) were purchased from Fisher Scientific (Hampton, NH, USA). 1-butanol HPLC grade (Cat # AC393750010) and isopropanol (Cat #AC184130010) were purchased from Acros Organics (Morris, NJ, USA). Acetic acid (Cat #695092), butylated hydroxytoluene (BHT; Cat #W218405-SAMPLE-K), ethylenediaminetetraacetic acid (EDTA; Cat #EDS-100G), Iron (II) sulfate heptahydrate ($FeSO_4$; Sigma, Cat #F7002), 2-Thiobarbituric acid (TBA; Cat#T5500), trichloroacetic acid (TCA; Cat#91228), triphenylphosphine (TPP; Cat #3T84409), and 1,1,3,3-Tetramethoxypropane (TEP; Cat #108383) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Deuterated standards used as surrogates and unlabeled oxylipins used for compound identification and the external standard curve were purchased from Cayman Chemical (Ann Arbor, MI, USA). The nine deuterated compounds used in the surrogate mix were: d11-11,12-epoxyeicosatrienoic acid (EpETrE – Cat #10006413), d11-14,15-dihydroxyeicosatrienoic acid (DiHETrE – Cat #1008040), d4-6-keto-prostaglandin (PG) $F_{1\alpha}$ (Cat #315210), d4-9-hydroxyoctadecadienoic acid (HODE – Cat #338410), d4-leukotrine B4 (LTB4 – Cat #320110), d4-prostaglandin (PG) E2 (Cat #314010), d4-thromboxane B2 (TXB2 – Cat #319030), d6-20-hydroxyeicosatetraenoic acid (HETE – Cat #390030), and d8-5-hydroxyeicosatetraenoic acid (HETE – Cat #334230).

For the preparation of the infant milk formulas, instant nonfat dry milk, canola oil, and lecithin were purchased from a local supermarket in Davis, CA, USA. Maltodextrin was purchased from Grain Processing Corporation (Muscatine, IA, USA), and docosahexaenoic acid ethyl ester (DHA ethyl ester; Cat # 9090310) was acquired from Cayman Chemicals.

2.2 | Samples

Infant formulas must have an appropriate balance of water, carbohydrate, protein, fatty acids, vitamins, and minerals to provide sufficient growth and maintain the good health of infants. The three major classes of infant formulas are milk-based formulas, soy-based formulas, and specialized formulas, which are usually enriched with nutrients, such as DHA and AA. Children who receive formula need an exogenous supply of AA and DHA to achieve fatty acid levels in plasma like breastfed infants. There are beneficial effects of PUFA on the cognitive and visual development of infants (Fang et al., 2020; Guo & Ahmad, 2014; Mendonça et al., 2017; Nieto-Ruiz et al., 2020).

Hence, two different infant formulas were prepared, one containing canola oil as the lipid base (Formula 1) and the other containing both canola oil and DHA ethyl ester as the source of lipids (Formula 2). The composition of the formulas was as follows:

Formula 1: 86.69% water; 4% oil (100% canola oil), 8% maltodextrin, 1.3% milk protein concentrate 0.01% lecithin.

Formula 2: 86.69% water; 4% oil (99.7% canola oil and 0.3% DHA ethyl ester), 8% maltodextrin, 1.3% milk protein concentrate 0.01% lecithin.

The ingredients were mixed using a homogenizer system (PolyTron, PT-2500E, Kinematica) for 5 min at 20,000 rpm. The total volume for each formula was 2 L, which was further separated into four batches of 500 ml. Each formula was sampled at baseline (time point 0; 1 aliquot of 15 ml of each batch, $n = 4$ for each formula) and frozen at −80°C. The milk formulas were then stored at 4°C for 21 days. Aliquots ($n = 4$) of each sample were collected on 1, 3, 7, 14, and 21 days of storage and frozen at −80°C until further analysis.

2.3 | Free oxylipin measurements

Free oxylipins were extracted from 200 µl of infant formula. Ice-chilled methanol (600 µl) was used to precipitate proteins, and the samples were spiked with 10 µl of an antioxidant mixture containing 0.2 mg/ml of each ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), triphenylphosphine (TPP), and 10 µL of a 2-µM deuterated surrogate standard mix containing d11-11(12)-EpETrE, d11-14,15-DiHETrE, d4-6-keto-PGF1a, d4-9HODE, d4-LTB4, d4-PGE2, d4-TXB2, d6-20-HETE, and d8-5-HETE in LC-MS grade methanol. The antioxidant mix was prepared by mixing 0.6 mg/ml EDTA in water, 0.6 mg/ml BHT in methanol, and 0.6 mg/ml TPP in water:methanol (1:1; v/v) at a 1:1:1 ratio (v/v/v), and filtering the mixture through a 0.45-µm Millipore filter (Millipore Corporation, Bedford, MA, USA; Cat #SLHVM25NS) to remove solid particles.

Free oxylipin extracts were vortexed and centrifuged at 0°C at 15,000 × g for 10 min. The methanol content was adjusted to 15% methanol by mixing ~770 µl of supernatant with 3.3 ml of distilled water. Oxylipins were purified with 60 mg Oasis HLB solid-phase extraction (SPE) columns (3 cc, Waters Corporation, CA, USA; Cat #WAT094226), washed with one column volume of ethyl acetate and two column volumes of methanol, and conditioned with two column volumes of SPE buffer containing 0.1% acetic acid and 5% methanol in ultra-pure Millipore water.

The columns were dried for 20 min under vacuum (~15 psi), and the oxylipins were eluted with 1.5 ml of ethyl acetate and 0.5 ml of methanol (LC-MS grade). The samples were then dried using nitrogen and reconstituted with 100 µl of methanol. The reconstituted samples were transferred to Ultrafree-MC centrifugal filters (0.1 µm; Millipore Merck, #UFC30VV00), centrifuged at 11,998 × g for 20 min at 0°C and analyzed by UPLC-MS/MS.

2.3.1 | UPLC-MS/MS analysis

Oxylipins were analyzed on a 1290 Infinity ultra-high-pressure-liquid chromatography (UHPLC) instrument coupled to a 6460 QQQ MS/MS with electrospray ionization (ESI) and Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA). Oxylipins were separated using a ZORBAX Eclipse Plus C18 column (ID: 2.1 mm; length: 150 mm; particle size; 1.8 µm; Agilent Technologies). The mobile phases (A and B) consisted of: 0.1% acetic acid in ultrapure water and acetonitrile:methanol (80:15, v/v) with 0.1% acetic acid.

The samples were eluted according to the following gradient profile: 35% B as the initial condition; 40% at 3 min; 48% at 4 min; 60% at 10 min; 70% at 20 min; 85% at 24 min; 100% at 24.6 min, and 35% at 26.1 min. The total run time was 28 min. The flow rate was 0.3 ml/min from 0 min to 3 min, it was reduced to 0.25 ml/min between 3 min and 24.5 min, increased to 0.35 ml/min from 24.6 min to

27.5 min, and then reduced to 0.3 ml/min at 27.6 min until 28 min. The sample injection volume was 10 µl.

The triple quadrupole was operated in negative ESI mode. Oxylipins were identified under optimized dynamic multiple reaction monitoring (dMRM) conditions summarized in Table S1. The drying gas temperature was 300°C with a flow rate of 10 L/min, sheath gas temperature was 350°C at a flow of 11 L/min and the nebulizer pressure was 35 psi. Identification of compounds was achieved by plotting a standard curve for each analyte. Deuterated surrogates used to quantify detected oxylipins in the infant formula samples are shown in Table S2.

2.3.2 | Surrogate standard recovery

The percent recovery of each surrogate was determined as follows:

$$\text{Surrogate recovery (\%)} = \frac{\text{Peak area of surrogate in sample}}{\text{Peak area of surrogate in oxylipin standard mix}} \times 100 \quad (1)$$

2.3.3 | Ion suppression

Ion suppression was determined by preparing the following solutions:

(1) Solution I: 10 µl of a standard solution + 50 µl blank methanol sample.
(2) Solution II: 10 µl of methanol + 50 µl of oxylipin extract from each formula sample.
(3) Solution III: 10 µl of a standard solution + 50 µl of oxylipin extract from each formula sample.

The samples were analyzed using UPLC-MS/MS, and ion suppression was determined as follows:

$$\text{Ion supression (\%)} = \frac{\text{Peak area of solution III}}{\text{Peak area of solution I} + \text{Peak area of solution II}} \times 100 \quad (2)$$

2.4 | Quantification of hydroperoxide value by spectrophotometric method

Hydroperoxides were measured by mixing 300 µl of each infant formula with 1.5 ml of isooctane/isopropanol (3:1, v/v), using a vortex (10 s, three times) and then centrifugated at 14,600 × g for 10 min. The organic solvent phase (200 µl) was added to 2.8 ml of methanol/1-butanol (2:1, v/v), followed by the addition of 30 µl of the indicator solution. The indicator solution was prepared with a mixture of equal parts (1:1 v/v) of (i) 3.94 M ammonium thiocyanate and (ii) ferrous iron solution (prepared by mixing 0.144 M $FeSO_4.7H_2O$ in Mili-Q water and 0.132 M $BaCl_2.H_4O_2$ solution in HCl 0.4 M, 1:1, v/v). The absorbance of the solution was measured at 510 nm, 20 min after the addition of the iron. Hydroperoxide concentrations were determined using a cumene hydroperoxide (CHP) standard curve ($R^2 = 0.9998$) with concentrations varying from 0 µg.ml^{-1} to 18.26 µg.ml^{-1} (Shantha & Decker, 1994).

2.5 | Thiobarbituric acid reactive substance measurement

Lipid peroxidation was also determined using the thiobarbituric acid reactive substance (TBARS) assay as described by Papastergiadis et al. (2012) and Mendes et al. (2009). This method measures malondialdehyde (MDA), secondary oxidation products of lipid peroxides, using thiobarbituric acid (TBA) reagent. The reaction of MDA with TBA forms a pink-colored complex that is measured spectrophotometrically at 532 nm (Papastergiadis et al., 2012). The infant formulas were vortexed, and approximately 3 ml of each sample was weighed in a 50 ml falcon tube. A total of 6 ml of 7.5% trichloroacetic acid (TCA) (w/v) with 0.1% (w/v) of ethylenediaminetetraacetic acid (EDTA) and 0.1% (w/v) of PG was added. The mixture was centrifuged for 10 min at 6000 rpm. For the spectrophotometric determination, 1 ml of the supernatant and 1 ml of the TBA reagent (46 mM in 99% glacial acetic acid) were mixed in a test tube and heated in a boiling water bath for 35 min. The reaction mixture was chilled, and the absorbance was measured at 532 nm by spectrophotometry (Thermo Scientific, GENESYS 10S UV-Vis, USA). For quantification, a standard curve of 1,1,3,3-tetra-ethoxypropane (TEP; approximately 97%) ($R^2 = 0.9992$) ranging from 0.13 µg.g^{-1} to 2.2 µg.g^{-1} was prepared.

2.6 | Statistical analysis

Data were analyzed with a two-way repeated measures analysis of variance (ANOVA) followed by Dunnett's post-hoc test, using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, U). The sample size was 4 per formula, collected at multiple time points (0, 1, 3, 7, 14, and 21 days). Statistical significance was set at $p < 0.05$ (two-tailed). An unpaired t-test was used to compare the two different infant formulas at each time points.

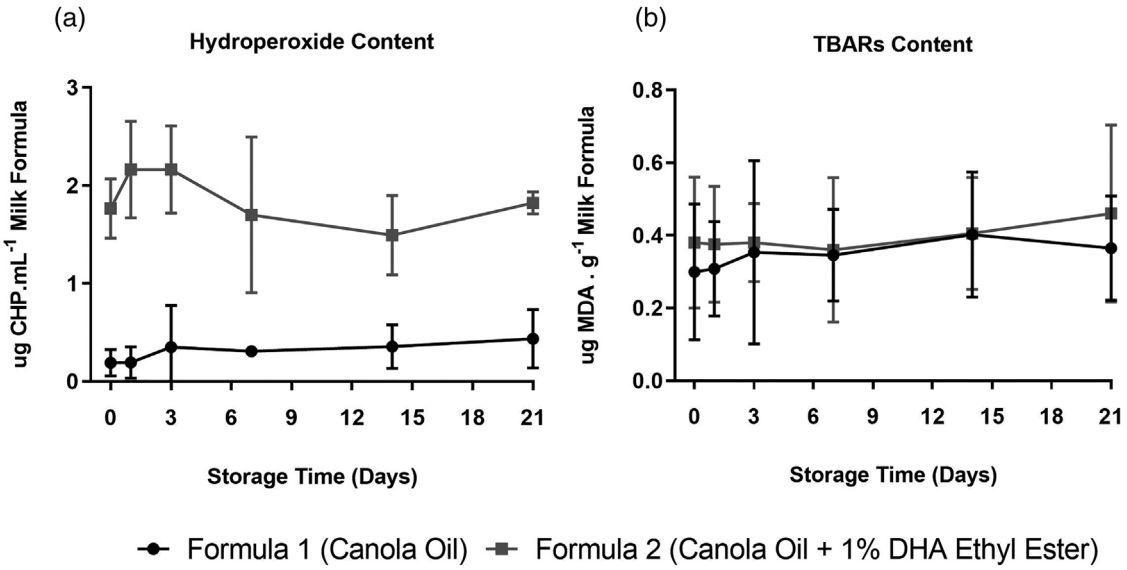

FIGURE 1 Hydroperoxide (a) and TBARs (b) content, in μg CHP ml^{-1} and μg MDA g^{-1} of Infant Milk Formulas, respectively. Notes: * Statistically different from the control (Initial storage time [0 days]) by two-way repeated measured ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 1. + Statistically different from the control (Initial storage time [0 days]) by two-way repeated ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 2

3 | RESULTS AND DISCUSSION

Lipid oxidation in food involves the continuous formation of hydroperoxides and unstable primary oxidation products, which are susceptible to decomposition into secondary oxidation products (Shahidi & Zhong, 2020). Therefore, we measured hydroperoxide and TBARS concentrations in two infant milk formulas with different PUFA compositions, stored at 4°C for 21 days.

Storage time did not significantly alter hydroperoxide concentrations for both formulas (Figure 1a). However, in Formula 2, hydroperoxide concentration was significantly higher than the hydroperoxide concentration of Formula 1, at all time points (Figure 1a). Hydroperoxide concentrations ranged between 0.19 μg CHP.ml^{-1} and 0.44 μg CHP.ml^{-1} and from 1.49 to 2.16 μg CHP.mL^{-1} in Formulas 1 and 2, respectively. Consistent with the hydroperoxide values, storage time did not alter TBARS concentrations (Figure 1b). However, unlike the hydroperoxide findings, no statistically significant effects were revealed between the types of formulas. TBARS values ranged from 0.30 to 0.40 and from 0.36 to 0.46 μg MDA.g^{-1} for Formulas 1 and 2, respectively (Figure 1b).

Differences in hydroperoxide content between formulas may be related to their PUFA composition. Formula 1 lacked DHA, whereas Formula 2 contained 1% DHA ethyl ester of total fatty acids. These findings suggest that the presence of longer-chain PUFA such as DHA in the sample may make the formula more susceptible to oxidation. This is consistent with a study by Ogasawara et al. (2020) which studied the development of human milk fat substitutes (HMFSs). They found that the oxidation stability depends on the molecular species of the lipid fraction since modifications in the PUFA-TAG molecules had a significant effect on the stability of HMFSs.

The reasons for the inconsistency between hydroperoxide and TBARS between formulas may relate to the specificity of the TBARS assay towards malonaldehyde (MDA). The degradation of hydroperoxides leads to the formation of hundreds of secondary volatiles which include short-chain (< 6 carbons) aldehydes, ketones, or alcohols (Shahidi & Zhong, 2020). The TBARS method used in the present study only measures one class (MDA) out of many volatiles. It is possible that the other secondary products of hydroperoxide degradation, not measured in the present study, differed between formulas. This can be further investigated in future studies with head-space GC-MS methods.

The lack of storage effect on milk samples agrees with other studies. Semeniuc et al. (2009) studied the oxidative status of powdered infant formula products stored at 15°C for 35 months and observed no significant effect on peroxide value and TBARS during storage. A similar trend was observed for the storage of milk formula emulsions for 3, 7, 11, and 14 days at 40°C (Drapala et al., 2018). This last study and ours presented a high standard deviation for hydroperoxide content and TBARS. The coefficient of variation (CV) varied from 0.6% to 139.1% for hydroperoxide and 0.0 to 119.4% for TBARS analysis, in the Semeniuc et al. (2009) study, and from 6.22 to 120.66 % for hydroperoxide and 28.23 to 71.27% for TBARS analysis, in ours. The high CV observed in both studies could be due to the relatively

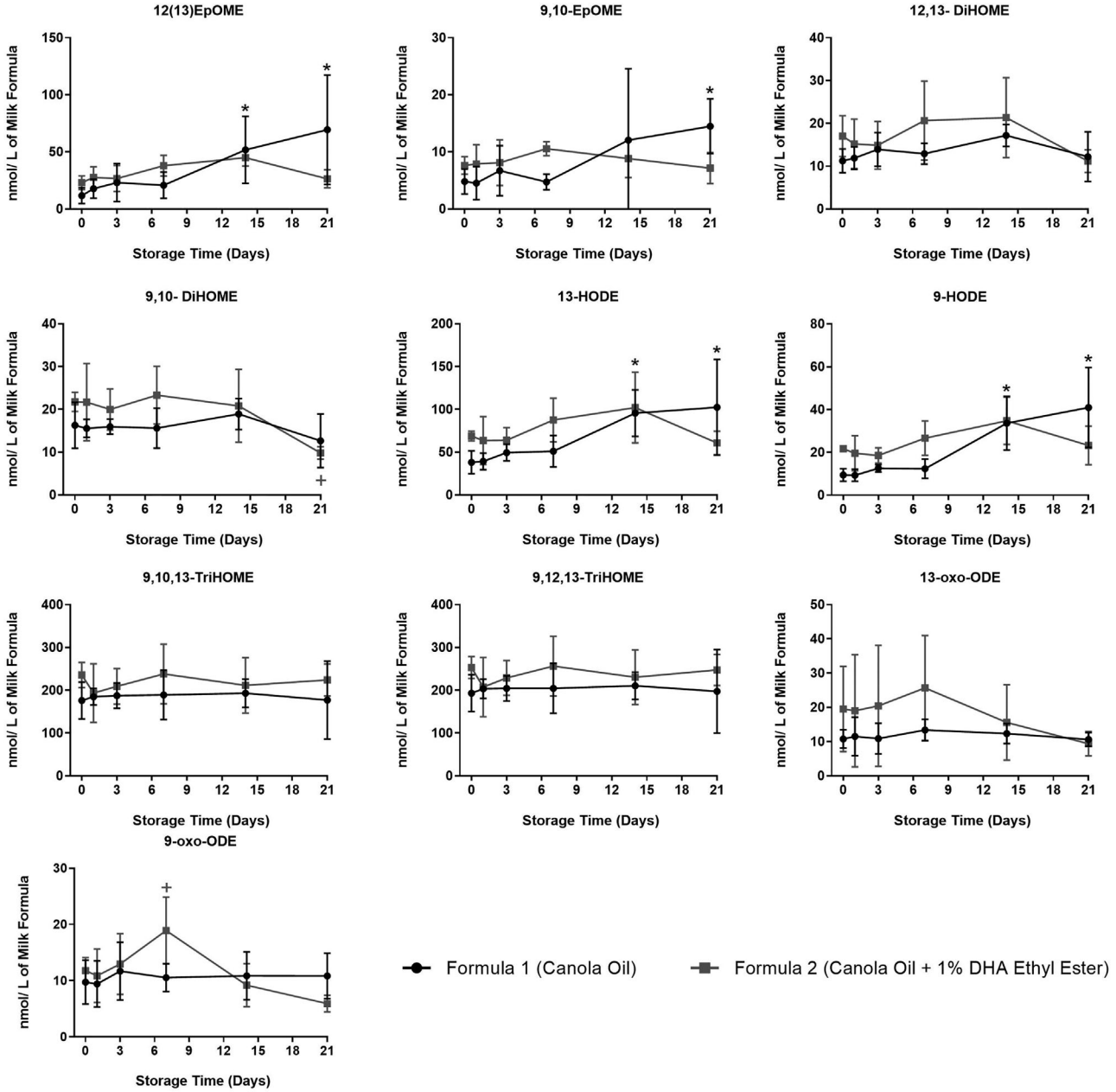

FIGURE 2 Linoleic acid (LA)-derived oxidized metabolites in infant milk formula (nmol L^{-1}). Notes: * Statistically different from the control (initial storage time [0 days]) by two-way repeated ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 1. + Statistically different from the control (Initial storage time (0 days)) by two-way repeated ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 2

low concentration of MDA, which typically forms during late-stage oxidation, the instability of hydroperoxides, or the matrix involved.

Because hydroperoxides are unstable, we measured the concentration of more-stable primary oxidation products (i.e., oxylipins) with UPLC-MS/MS. The concentration of free oxylipins derived from linoleic acid (LA), alpha-linolenic acid (ALA), and docosahexaenoic acid (DHA) are shown in Figures 2–4, respectively. The content of each compound in the different time points was compared with baseline (Time 0). As shown in the figures, PUFA-derived oxylipins increased with storage time for both formulas; however, those derived from DHA only increased in Formula 2, whereas those derived from LA and ALA increased, mainly, in Formula 1.

In Formula 1, LA-derived metabolites, 12(13)-epoxyoctadeenoic acid (12 (13)-EpOME), 13-hydroxyoctadecadienoic acid (13-HODE), 9-hydroxyoctadecadienoic acid (9-HODE), significantly increased by the 14th day of storage. Also, 9,10-epoxyoctadeenoic acid (9,10-EpOME) showed a significant increase from 4.81 nmol/L (day 0) to 14.49 nmol/L on day 21. Formula 2 only presented

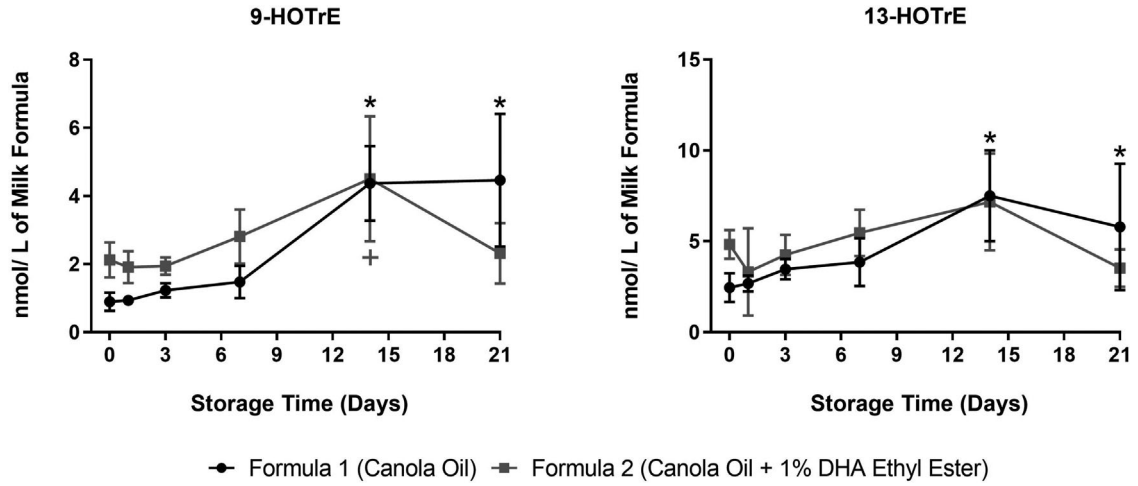

FIGURE 3 Alpha-linolenic acid (ALA)-derived oxidized metabolites in infant milk formula (nmol L^{-1}). Notes: * Statistically different from the control (Initial storage time [0 days]) by two-way repeated ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 1. + Statistically different from the control (Initial storage time [0 days]) by two-way repeated ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 2

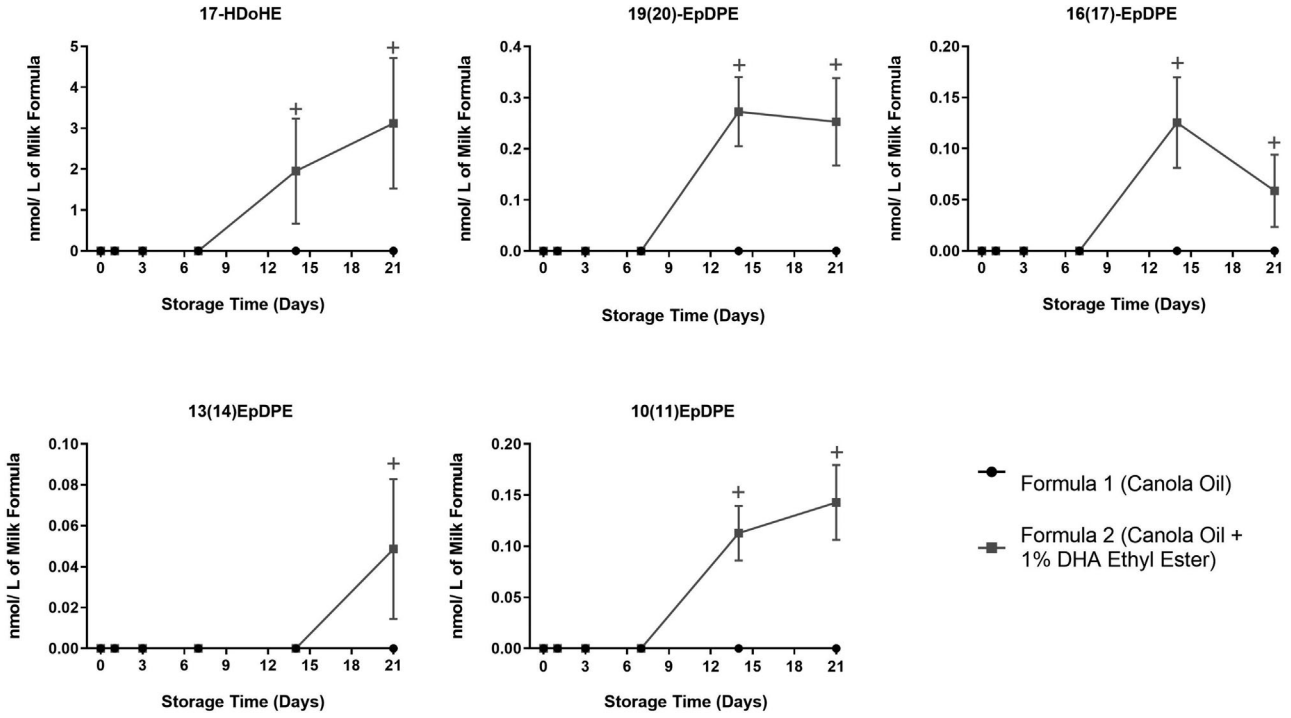

FIGURE 4 Docosahexaenoic acid (DHA)-derived oxidized metabolites in infant milk formula (nmol L^{-1}). Notes: Values before 14 days were below the instrument limit of detection (LOD; $LOD_{17HDoHE} = 0.83$ nmol/L; $LOD_{19(20)\text{-}EpDPE} = 0.69$ nmol/L; $LOD_{16(17)\text{-}EpDPE} = 0.17$ nmol/L; $LOD_{13(14)EpDPE} = 0.17$ nmol/L; $LOD_{10(11)EpDPE} = 0.17$ nmol/L). * Statistically different from the control (Initial storage time [0 days]) by two-way repeated ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 1. + Statistically different from the control (Initial storage time [0 days]) by two-way repeated ANOVA followed by Dunnett's post-hoc test with $p < 0.05$ for Formula 2

an increase in the 9-oxo-ODE content on the 7th day of storage.

Similarly, ALA-derived 9-hydroxyoctadecatrienoic acid (9-HoTrE) and 13-hydroxyoctadecatrienoic acid (13-HOTrE) present on Formula 1 increased after 14 days of storage (Figure 3). Formula 2 showed an increase only in the 9-HoTrE content on the 14th day, from 2.12 nmol/L (day 0) to 4.51 nmol/L (day 14).

TABLE 1 Ion suppression of oxidized metabolites in infant milk formula

Precursor	Compounds	IS	Ion suppression (%)
LA	9-HODE	d4-9HODE	96
	13-HODE	d4-9HODE	105
	9-oxo-ODE	d6-20-HETE	92
	13-oxo-ODE	d6-20-HETE	105
	12(13)-EpOME	d11-11(12)-EpETrE	88
	9(10)-EpOME	d11-14,15-DiHETrE	90
	12,13-DiHOME	d11-14,15-DiHETrE	109
	9,10-DiHOME	d11-14,15-DiHETrE	92
	9,10,13-TriHOME	d4-TXB2	88
	9,12,13-TriHOME	d4-TXB2	91
ALA	13-HOTrE	d4-9HODE	108
	9-HOTrE	d4-9HODE	97
DHA	19(20)-EpDPE	d11-11(12)-EpETrE	95
	16(17)-EpDPE	d11-11(12)-EpETrE	87
	13(14)EpDPE	d11-11(12)-EpETrE	88
	10(11)EpDPE	d11-11(12)-EpETrE	90
	17-HDoHE	d4-9HODE	103

Abbreviations: DiHETrE, dihydroxyicosatrienoic acid; EpETre, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; EpDPE, epoxydocosapentanoic acid; HDoHE, hydroxydocosahexaenoic acid; EpOME, epoxyoctadecenoic acid; DiHOME, dihydroxyoctadecenoic acid; TriHOME, trihydroxyoctademonoenoic acid; HODE, hydroxyoctadecadienoic acid; oxo-ODE, oxo-octadecadienoic acid; HOTrE, hydroxyoctadecatrienoic acid; IS, internal standard; TXB2, thromboxane B2.

TABLE 2 Recovery (%) of oxidized metabolites in milk Formula 1

Recovery (%)—Formula 1								
Precursor	Compounds	IS	T0	T1	T3	T7	T14	T21
LA	9-HODE	d4-9-HODE	81.31 ± 31.93	67.3 ± 9.27	66.03 ± 7.84	77.26 ± 20.51	78.29 ± 12	72.25 ± 30.91
	13-HODE	d4-9 -HODE	81.31 ± 31.93	67.3 ± 9.27	66.03 ± 7.84	77.26 ± 20.51	78.29 ± 12	72.25 ± 30.91
	9-oxo-ODE	d6-20-HETE	63.49 ± 33.51	57.19 ± 11.48	61.72 ± 10.87	70.87 ± 18.37	70.72 ± 18.69	68.5 ± 29.62
	13-oxo-ODE	d6-20-HETE	59.86 ± 31.6	53.92 ± 10.83	58.19 ± 10.25	66.81 ± 17.32	66.67 ± 17.62	64.58 ± 27.93
	12(13)-EpOME	d11-11(12)-EpETrE	68.26 ± 26.35	57.1 ± 8.48	57.1 ± 8.71	68.44 ± 16.48	63.6 ± 9.59	60.36 ± 23.41
	9(10)-EpOME	d11-14,15-DiHETrE	68.82 ± 26.57	57.57 ± 8.55	57.57 ± 8.78	69.01 ± 16.61	64.13 ± 9.67	60.86 ± 23.6
	12,13-DiHOME	d11-14,15-DiHETrE	119.59 ± 51.12	101.81 ± 15.24	95.19 ± 15.4	111.82 ± 25.3	116.36 ± 21.28	109.57 ± 53.12
	9,10-DiHOME	d11-14,15-DiHETrE	119.59 ± 51.12	101.81 ± 15.24	95.19 ± 15.4	111.82 ± 25.3	116.36 ± 21.28	109.57 ± 53.12
	9,10,13-TriHOME	d4-TXB2	90.91 ± 22.4	78.28 ± 1.12	73.67 ± 11.27	87.19 ± 20.71	81.54 ± 6.31	76.6 ± 23.43
	9,12,13-TriHOME	d4-TXB2	93.13 ± 22.94	80.19 ± 1.14	75.47 ± 11.54	89.32 ± 21.22	83.53 ± 6.47	78.47 ± 24
ALA	13-HOTrE	d4-9HODE	81.31 ± 31.93	67.3 ± 9.27	66.03 ± 7.84	77.26 ± 20.51	78.29 ± 12	72.25 ± 30.91
	9-HOTrE	d4-9HODE	81.31 ± 31.93	67.3 ± 9.27	66.03 ± 7.84	77.26 ± 20.51	78.29 ± 12	72.25 ± 30.91
DHA	19(20)-EpDPE	d11-11(12)-EpETrE	119.56 ± 51.11	101.79 ± 15.24	95.17 ± 15.39	111.8 ± 25.3	116.33 ± 21.27	109.54 ± 53.1
	16(17)-EpDPE	d11-11(12)-EpETrE	68.82 ± 26.57	57.57 ± 8.55	57.57 ± 8.78	69.01 ± 16.61	64.13 ± 9.67	60.86 ± 23.6
	13(14)EpDPE	d11-11(12)-EpETrE	68.82 ± 26.57	57.57 ± 8.55	57.57 ± 8.78	69.01 ± 16.61	64.13 ± 9.67	60.86 ± 23.6
	10(11)EpDPE	d11-11(12)-EpETrE	68.73 ± 26.53	57.49 ± 8.54	57.5 ± 8.77	68.92 ± 16.59	64.04 ± 9.66	60.78 ± 23.57
	17-HDoHE	d4-9HODE	81.31 ± 31.93	67.3 ± 9.27	66.03 ± 7.84	77.26 ± 20.51	78.29 ± 12	72.25 ± 30.91

Abbreviations: DiHETrE, dihydroxyicosatrienoic acid; EpETre, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; EpDPE, epoxydocosapentanoic acid; HDoHE, hydroxydocosahexaenoic acid; EpOME, epoxyoctadecenoic acid; DiHOME, dihydroxyoctadecenoic acid; TriHOME, trihydroxyoctademonoenoic acid; HODE, hydroxyoctadecadienoic acid; oxo-ODE, oxo-octadecadienoic acid; HOTrE, hydroxyoctadecatrienoic acid; IS, internal standard; TXB2, thromboxane B2.

TABLE 3 Recovery (%) of oxidized metabolites in infant milk Formula 2

Recovery (%)—Formula 2								
Precursor	**Compounds**	**IS**	**T0**	**T1**	**T3**	**T7**	**T14**	**T21**
LA	9-HODE	d4-9HODE	71.06 ± 21.07	54.37 ± 27.9	69.2 ± 6.39	61.61 ± 11.02	66.72 ± 15.4	69.59 ± 9.81
	13-HODE	d4-9HODE	71.06 ± 21.07	54.37 ± 27.9	69.2 ± 6.39	61.61 ± 11.02	66.72 ± 15.4	69.59 ± 9.81
	9-oxo-ODE	d6-20-HETE	62.77 ± 20.06	50 ± 25.75	55.92 ± 16.77	47.83 ± 15.35	61.16 ± 26.91	56.76 ± 5.69
	13-oxo-ODE	d6-20-HETE	59.18 ± 18.91	47.14 ± 24.28	52.72 ± 15.81	45.09 ± 14.47	57.66 ± 25.37	53.51 ± 5.36
	12(13)-EpOME	d11-11(12)-EpETrE	62.47 ± 16.57	42.6 ± 22.21	56.89 ± 7.39	50.9 ± 8.51	57.26 ± 11.78	70.04 ± 10.2
	9(10)-EpOME	d11-14,15-DiHETrE	62.99 ± 16.71	42.96 ± 22.39	57.36 ± 7.45	51.32 ± 8.58	57.73 ± 11.88	70.62 ± 10.28
	12,13-DiHOME	d11-14,15-DiHETrE	114.14 ± 36.36	81.38 ± 44.5	100.84 ± 15.89	91.32 ± 16.65	98.01 ± 23.6	99.65 ± 7.11
	9,10-DiHOME	d11-14,15-DiHETrE	114.14 ± 36.36	81.38 ± 44.5	100.84 ± 15.89	91.32 ± 16.65	98.01 ± 23.6	99.65 ± 7.11
	9,10,13-TriHOME	d4-TXB2	74.98 ± 14.27	61.19 ± 31.14	70.74 ± 5.2	70.12 ± 7.97	71.77 ± 9.52	76.6 ± 4.1
	9,12,13-TriHOME	d4-TXB2	76.81 ± 14.62	62.68 ± 31.9	72.46 ± 5.33	71.83 ± 8.16	73.52 ± 9.76	78.47 ± 4.2
ALA	13-HOTrE	d4-9HODE	71.06 ± 21.07	54.37 ± 27.9	69.2 ± 6.39	61.61 ± 11.02	66.72 ± 15.4	69.59 ± 9.81
	9-HOTrE	d4-9HODE	71.06 ± 21.07	54.37 ± 27.9	69.2 ± 6.39	61.61 ± 11.02	66.72 ± 15.4	69.59 ± 9.81
DHA	19(20)-EpDPE	d11-11(12)-EpETrE	114.11 ± 36.35	81.36 ± 44.5	100.82 ± 15.89	91.3 ± 16.65	97.99 ± 23.59	99.63 ± 7.11
	16(17)-EpDPE	d11-11(12)-EpETrE	62.99 ± 16.71	42.96 ± 22.39	57.36 ± 7.45	51.32 ± 8.58	57.73 ± 11.88	70.62 ± 10.28
	13(14)EpDPE	d11-11(12)-EpETrE	62.99 ± 16.71	42.96 ± 22.39	57.36 ± 7.45	51.32 ± 8.58	57.73 ± 11.88	70.62 ± 10.28
	10(11)EpDPE	d11-11(12)-EpETrE	62.91 ± 16.69	42.9 ± 22.37	57.29 ± 7.44	51.25 ± 8.57	57.66 ± 11.86	70.53 ± 10.27
	17-HDoHE	d4-9HODE	71.06 ± 21.07	54.37 ± 27.9	69.2 ± 6.39	61.61 ± 11.02	66.72 ± 15.4	69.59 ± 9.81

Abbreviations: DiHETrE, dihydroxyicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EpDPE, epoxydocosapentanoic acid; EpETre, epoxyeicosatrienoic acid; EpOME, epoxyoctadecenoic acid; HDoHE, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HOTrE, hydroxyoctadecatrienoic acid; oxo-ODE, oxo-octadecadienoic acid; TriHOME, trihydroxyoctademonoenoic acid; TXB2, thromboxane B2.

DHA-derived compounds were only detected in Formula 2, which contained added DHA. 17-HDoHE, 19(20)-EpDPE, 16(17)-EpDPE, and 10(11)-EpDPE increased beginning from day 14 of storage compared to day 0 (Figure 4). The results suggest that PUFA composition determines the type of oxylipins that change due to storage.

In addition, to assess the possibility of the matrix interfering with the detection of oxylipins, ion suppression and the percent of surrogate recovery were measured. Ion suppression ranged from 87% to 103% (Table 1), suggesting minimal matrix effects on the compounds measured (a value of 100% reflects no matrix effects). The surrogate standard recoveries ranged from 53.92 to 119.59% in Formula 1 (Table 2) and 42.60 to 114.14% in Formula 2 (Table 3), reflecting similar losses of some compounds during the extraction across all storage time points and between formulas. This means that the observed differences in oxylipin concentrations between Formulas 1 and 2 are real and unrelated to ion suppression or surrogate extraction recoveries.

An interesting but unexpected finding in the present study is that the addition of DHA (in Formula 2) spared the oxidation of LA and ALA. This suggests some form of competition among PUFA molecules for oxidation. In this particular case, DHA appears to have been preferentially oxidized over LA and ALA (Formula 2) which is in agreement with the notion that highly unsaturated fatty acids are more easily oxidized than less unsaturated fatty acids (Matsushita, 1990).

4 | CONCLUSION

The findings from this study demonstrate that changes in infant milk formula lipid oxidation during 4°C storage were detected by oxylipin analysis with UPLC-MS/MS but not hydroperoxide or TBARS measurement. Specific oxylipins were altered during storage, depending on the PUFA composition of the infant formula. When DHA was present, DHA-derived oxylipins changed over time (Formula 2). In its absence, mainly LA and ALA-derived oxylipins changed over time (Formula 1). These signatures could be used to identify the extent of lipid oxidation in specific food products based on their PUFA composition. Finally, UPLC-MS/MS analysis of oxylipins effectively detects lipid oxidation in the early stages, potentially allowing for more accurate shelf-life estimations.

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AUTHOR CONTRIBUTIONS

Bianca Ferraz Teixeira, Fernanda Furlan Gonçalves Dias, Ameer Y. Taha, Juliana Maria Leite Nobrega de Moura Bell, and Thais Maria Ferreira de Souza Vieira designed the study. Bianca Ferraz Teixeira and Fernanda Furlan Gonçalves Dias carried out the experiments, analyzed the data, performed the statistical analyses, and drafted the manuscript under the supervision of Ameer Y. Taha, Juliana Maria Leite Nobrega de Moura Bell, and Thais Maria Ferreira de Souza Vieira. The final version of the manuscript was read and approved by all the authors.

CONFLICT OF INTEREST

We declare no conflict of interest.

REFERENCES

Ahmed, M., Pickova, J., Ahmad, T., Liaquat, M., Farid, A., & Jahangir, M. (2016). Oxidation of lipids in foods. *Sarhad Journal of Agriculture*, *32*(3), 230–238. https://doi.org/10.17582/journal.sja/2016.32.3.230.238

Cesa, S. (2004). Malondialdehyde contents in infant milk formulas. *Journal of Agricultural and Food Chemistry*, *52*(7), 2119–2122. https://doi.org/10.1021/jf034446l

Cesa, S., Casadei, M. A., Cerreto, F., & Paolicelli, P. (2015). Infant milk formulas: Effect of storage conditions on the stability of powdered products towards autoxidation. *Foods*, *4*(3), 487–500. https://doi.org/10.3390/foods4030487

Christi, W. W., & Harwoo, J. L. (2020). Oxidation of polyunsaturated fatty acids to produce lipid mediators. *Essays in Biochemistry*, *64*(3), 401–421. https://doi.org/10.1042/EBC20190082

Daoud, S., Bou-Maroun, E., Waschatko, G., Horemans, B., Mestdagh, R., Billecke, N., & Cayot, P. (2020). Detection of lipid oxidation in infant formulas: Application of infrared spectroscopy to complex food systems. *Foods*, *9*(10), 1432. https://doi.org/10.3390/foods9101432

Dias, F. F. G., Augusto-Obara, T. R., Hennebelle, M., Chantieng, S., Ozturk, G., Taha, A. Y., de Vieira, S. T. M. F., & de Bell, M. J. M. L. N. (2020). Effects of industrial heat treatments on bovine milk oxylipins and conventional markers of lipid oxidation. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *152*, 102040. https://doi.org/10.1016/j.plefa.2019.102040

Drapala, K. P., Mulvihill, D. M., & O'Mahony, J. A. (2018). Improving the oxidative stability of model whey protein hydrolysate-based infant formula emulsions with lecithin. *International Journal of Dairy Technology*, *71*(4), 966–974. https://doi.org/10.1111/1471-0307.12538

Emami, S., Zhang, Z., & Taha, A. Y. (2020). Quantitation of oxylipins in fish and algae oil supplements using optimized hydrolysis procedures and ultra-high performance liquid chromatography coupled to tandem mass-spectrometry. *Journal of Agricultural and Food Chemistry*, *68*(35), 9329–9344. https://doi.org/10.1021/acs.jafc.0c02461

Fang, X., Sun, W., Jeon, J., Azain, M., Kinder, H., Ahn, J., Chung, H. C., Mote, R. S., Filipov, N. M., Zhao, Q., Rayalam, S., & Park, H. J. (2020). Perinatal docosahexaenoic acid supplementation improves cognition and alters brain functional organization in piglets. *Nutrients*, *12*(7), 1–21. https://doi.org/10.3390/nu12072090

Gan, J., Zhang, Z., Kurudimov, K., German, J. B., & Taha, A. Y. (2020). Distribution of free and esterified oxylipins in cream, cell, and skim fractions of human milk. *Lipids*, Advance online publication. https://doi.org/10.1002/lipd.12268

German, J. B. (1999). Food processing and lipid oxidation. *Advances in Experimental Medicine and Biology*, *459*, 23–50. https://doi.org/10.1007/978-1-4615-4853-9_3

Gorassini, A., Verardo, G., Fregolent, S. C., & Bortolomeazzi, R. (2017). Rapid determination of cholesterol oxidation products in milk powder based products by reversed phase SPE and HPLC-APCI-MS/MS. *Food Chemistry*, *230*, 604–610. https://doi.org/10.1016/j.foodchem.2017.03.080

Guo, M., & Ahmad, S. (2014). Formulation guidelines for infant formula. In *Human milk biochemistry and infant formula manufacturing technology* (pp. 141–171). Woodhead Publishing. https://doi.org/10.1533/9780857099150.2.141

Matsushita, S. (1990). Oxidation of food. In *Food packaging* (pp. 25–44). Academic Press. https://doi.org/10.1016/B978-0-08-092395-6.50006-4

Mendes, R., Cardoso, C., & Pestana, C. (2009). Measurement of malondialdehyde in fish: A comparison study between HPLC methods and the traditional spectrophotometric test. *Food Chemistry*, *112*(4), 1038–1045. https://doi.org/10.1016/J.FOODCHEM.2008.06.052

Mendonça, M. A., Coelho, W. M. A., Borgo, L. A., & de Alencar, R E. (2017). Lipid profile of different infant formulas for infants. *PLoS ONE*, *12*, e0177812. https://doi.org/10.1371/journal.pone.0177812

Nieto-Ruiz, A., Diéguez, E., Sepúlveda-Valbuena, N., Herrmann, F., Cerdó, T., López-Torrecillas, F., De-Castellar, R., Jiménez, J., Pérez-García, M., Miranda, M. T., Catena, A., García-Santos, J. A., Bermúdez, M. G., & Campoy, C. (2020). The effects of an infant formula enriched with milk fat globule membrane, long-chain polyunsaturated fatty acids and synbiotics on child behavior up to 2.5 years old: The COGNIS study. *Nutrients*, *12*(12), 3825. https://doi.org/10.3390/NU12123825

Ogasawara, S., Ogawa, S., Yamamoto, Y., & Hara, S. (2020). Enzymatic preparation and oxidative stability of human milk fat substitute containing polyunsaturated fatty acid located at sn-2 position. *Journal of Oleo Science*, *69*(8), 825–835. https://doi.org/10.5650/jos.ess19332

Papastergiadis, A., Mubiru, E., Van Langenhove, H., & De Meulenaer, B. (2012). Malondialdehyde measurement in oxidized foods: Evaluation of the spectrophotometric thiobarbituric acid reactive substances (TBARS) test in various foods. *Journal of Agricultural and Food Chemistry*, *60*(38), 9589–9594. https://doi.org/10.1021/jf302451c

Richardson, C. E., Hennebelle, M., Otoki, Y., Zamora, D., Yang, J., Hammock, B. D., & Taha, A. Y. (2017). Lipidomic analysis of oxidized fatty acids in plant and algae oils. *Journal of Agricultural and Food Chemistry*, *65*(9), 1941–1951. https://doi.org/10.1021/ACS.JAFC.6B05559

Semeniuc, C. A., Guş, C., Rotar, A. M., Socaci, S. A., Suharoschi, R., & Laslo, C. (2009). Effect of storage time on the oxidative status of infant formula. *Bulletin UASVM Agriculture*, *66*(2), 448–452. https://journals.usamvcluj.ro/index.php/agriculture/article/download/4051/3712

Shahidi, F., & Zhong, H. Y. J. (2020). Methods for measuring lipid oxidation. In *Bailey's industrial oil and fat products* (pp. 1–27). Wiley. https://doi.org/10.1002/047167849X.bio050.pub2

Shantha, N. C., & Decker, E. A. (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International*, *77*(2), 421–424. https://doi.org/10.1093/jaoac/77.2.421

Siefarth, C., Serfert, Y., Drusch, S., & Buettner, A. (2013). Comparative evaluation of diagnostic tools for oxidative deterioration of polyunsaturated fatty acid-enriched infant formulas during storage. *Foods*, *3*(1), 30–65. https://doi.org/10.3390/foods3010030

St Angelo, A. J. (1996). Lipid oxidation in foods. *Critical Reviews in Food Science and Nutrition*, *36*(3), 175–224. https://doi.org/10.1080/10408399609527723

Teixeira, B. F., Dias, F. F. G., Vieira, T. M. F., de, S., Bell, J. M. L. N., de, M., & Taha, A. Y. (2021). Method optimization of oxylipin hydrolysis in nonprocessed bovine milk indicates that the majority of oxylipins are esterified. *Journal of Food Science*, *86*(5), 1791–1801. https://doi.org/10.1111/1750-3841.15697

Turoli, D., Testolin, G., Zanini, R., & Bellù, R. (2004). Determination of oxidative status in breast and formula milk. *Acta Paediatrica, International Journal of Paediatrics*, *93*(12), 1569–1574. https://doi.org/10.1080/08035250410022495

Yang, J., Schmelzer, K., Georgi, K., & Hammock, B. D. (2009). Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Analytical Chemistry*, *81*(19), 8085–8093. https://doi.org/10.1021/ac901282n

Zhang, Z., Hennebelle, M., Emami, S., & Taha, A. Y. (2021). Effects of potato processing and frying on oxylipin concentrations. *ACS Food Science & Technology*, *1*(8), 1436–1443. https://doi.org/10.1021/ACSFOODSCITECH.1C00139

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