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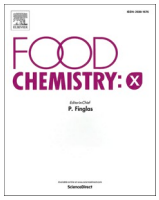

Effects of different reducing carbohydrate types on the physicochemical characteristics of infant formula food stored for special medical purposes

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ABSTRACT

The formula of food for special medical purpose has a direct impact on physicochemical stability, especially in hot climes and high temperature transport storage environments. An accelerated test (50 °C for 7 weeks) was used to analyze the mechanism of the physicochemical instability of formula A with lactose and maltodextrin, and formula B with maltodextrin. Deep dents and wrinkles were observed on the surface of the formula B, and more fat globules covered the surface of formula A particles after storage for a long time. Significantly higher amounts of furosine and N^{ε}-carboxymethl-L-lysine (**CML**) were formed and the loss of available lysine was greater in formula A than in formula B. No significant difference was observed in lipid oxidation indicators between the two formulas. The results of this research demonstrated lactose was more active than maltodextrin and led to physicochemical instability.

Introduction

Food for Special Medical Purpose (**FSMP**) for infants, is a type of nutritional formula manufactured to satisfy the special nutritional requirements of infants with medical conditions (Union, 2006). Hydrolysate formula is a kind of FSMP in which hydrolyzed protein was used as a source of protein, it was provided to infants with cow's milk protein allergy. Compared to intact proteins, hydrolyzed proteins have less secondary structure level and more amino acid group exposure, therefore they are more active to Maillard reactions (Murphy, Roos, Hogan, Maher, Flynn, & Fenelon, 2015). The shelf-life stability of this type of FSMP is obtaining more and more interest in the field since the nutrition and safety of the products are always the top consumers' needs. Different types of special medicinal purpose formulations may experience physical and chemical changes during storage, impacting product quality.

Maillard reaction can happen easily in infant formula which contains a large amount number of carbohydrates and protein when stored at high temperatures or other undesirable storage conditions. The reaction is detrimental to FSMP quality not only because of decreased nutritional quality and availability of essential amino acids but also produces plenty of harmful products, especially AGEs. After endogenous AGEs have built up to a certain degree, several chronic diseases, including type 2 diabetes, chronic kidney disease, atherosclerosis, and others, may become more common, which have a high rise for human health, let alone infants (Dong, Li, Chen, Liu, Qiao, Sang, et al., 2023; Lu, Li, Huang, Xie, Shen, & Xie, 2022). The Maillard reaction can also influence the color, taste, and organoleptic properties of the formula. Carbohydrates are a group of macronutrients in formulas, but only carbohydrates with reduced activity will participate in Maillard reactions. Major reducing carbohydrate types used in FSMPs include lactose, maltose, glucose, and maltodextrins (Masum, Chandrapala, Huppertz, Adhikari, & Zisu, 2021). According to regulations, the carbohydrate content of infant formula should range from 2.2 to 3.3 g/100 kJ. And lactose was generally the primary component, accounting for at least 1.1 g/100 kJ in formula based on cow's milk (Nasirpour, Scher, & Desobry, 2006). What's more, lactose intolerance was common in infants, so maltodextrin was used as a substitute for lactose. Maltodextrin is a kind of starch hydrolysis product whose dextrose equivalent (**DE**) value was lower than 20. DE value is the percent of reducing carbohydrates relative to glucose (expressed on a dry basis) and affected by the hydrolytic degree. Some researchers studied the relationship between the DE value of maltodextrin and the Maillard reaction degree in liquid nutritional products and whey protein-carbohydrate conjugates, results showed a

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degree of Maillard reaction intensified with the increase of DE value (Murphy, Roos, Hogan, Maher, Flynn, & Fenelon, 2015). Also, research about the formation of N^{ε}-carboxymethyl-L-lysine (**CML**) in the saccharide-lysine model system with different saccharides (lactose, glucose, and sucrose) has been reported (Zhang, Poojary, Rauh, Ray, Olsen, & Lund, 2020).

As aforementioned, plenty of research on the subject has reported the stability of FSMPs influenced by different types of protein, and most of the studies were researched in a model infant formula system. What's more, several studies have been paying attention to the impact of the type of reducing carbohydrate on infant formula, especially on FSMP for infants. However, to the best of our knowledge, little research has been done on the storage stability of milk powder with various nutritional components, but the results will directly influence the design of the milk powder formula and raise its marketability.

The aim of this study is to determine the mechanism underlying the physicochemical instability of FSMP derived from different carbohydrate sources during high temperature storage. Normally, the high temperature is not used during storage, but FSMP often reaches up to 60 °C for months in hot climes and throughout international transportation (Deeth & Lewis, 2017). The preliminary test results suggest that when the accelerated test temperature exceeds 50 °C, the sample reaction differs from that in normal temperature storage, hence the accelerated test temperature is set at 50 °C in this experiment. In this study, two FSMPs with different types of carbohydrates were used to conduct an accelerated storage study (50 °C, 7 weeks). We hope these properties could provide comprehensive data to describe how different types of reducing carbohydrates affect the physicochemical properties of nutrition formulas, and then support future design and shelf-life assessment of FSMP.

Materials and methods

Sample preparation and storage study design

Two nutritional formulas containing partially hydrolyzed milk protein with a different type of reducing carbohydrates (lactose and maltodextrins) were manufactured in the pilot plant following commercial production standards, and the macronutrient information of the two formulas is shown in Table 1.

80 g powder of the two nutritional formulas was sealed into laminated aluminum bags with nitrogen. The components of packaging from outside to inside are respectively Polyethylene Terephthalate (**PET**), Aluminium (**Al**), Nylon, and Polyethylene (**PE**). The composite flexible packaging has excellent air and moisture resistance, because of the air and moisture barrier properties of PET and PE. Two nutritional formulas were incubated at 50 °C for 7 weeks with sampling every week. After sampling, formulas were cooled down to room temperature for the following analysis.

Table 1
The major macronutrient constituent of two infant formulas with different type of reducing carbohydrate.

Macronutrient		Formula A (g/100 g)	Formula B (g/100 g)
hydrolyzed protein		11.75	11.75
lipid		27.50	27.50
reducing carbohydrate	lactose	22.22	-
	maltodextrine	22.10	42.32

Note: "-" indicates that the formula without this macronutrient. "Formula A" indicates that the formula of food for special medical purpose with lactose and maltodextrin, "Formula B" indicates that the formula of food for special medical purpose with maltodextrin, the same below.

Chemicals and reagents

Nile red, Nile blue A, and sodium hydroxide were purchased from Sigma-Aldrich Co. (Missouri, United States); 1,2-propanediol, hydrochloric acid, ammonium thiocyanate were obtained from Merck KGaA (Darmtstadt, Germany); potassium dihydrogen phosphate, sodium borate, sodium borohydride, sodium citrate dihydrate, barium chloride, ferrous sulfate, and ferrous iron were purchased from Sinopharm chemical reagent Co., Ltd (Shanghai, China); isopropanol, acetonitrile, pentane, ethyl acetate, hexane, isooctane, methol, and butanol were purchased from Thermo Fisher Scientific (Waltham, USA). Furosine*HCl was purchased from Iris Biotech GmbH (Marktredwitz, Germany); N^{ε}-carboxymethyl-L-lysine was purchased from Toronto Research Chemicals Inc. (Toronto, Canada); L-lysine was purchased from Anpel Laboratory Technologies Inc. (Shanghai, China); FMOC *N*-hydroxysuccinimide ester (≥98 %, chromatographic grade) and Cumene hydroperoxide (80 %, technical grade), propanal (97 %) and hexanal (98 %) were purchased from Sigma-Aldrich Co. (Missouri, United States); Propionaldehyde-2,2,3,3,3,-d_5 (98.7 %, chromatographic grade) and hexanal-d_{12} (98.5 %, chromatographic grade) were purchased from CDN isotopes Inc. (Quebec, Canada).

Physical properties analysis

Moisture content analysis. A Smart Turbo Microwave Moisture Analyzer (CEM, Matthews, USA) was used to measure the moisture content of formulas. Each sample was analyzed in triplicates, and the results were reported as the moisture percentage on a wet basis.

Water activity analysis. An AquaLab Series 4TE Water Activity Meter (Decagon Devices Inc., Pullman, USA) was used to measure the water activity of formulas at room temperature (25 ± 1 °C). Each sample was analyzed in triplicates.

Colorimetric analysis. The color of the formulas was tested by a colorimeter (ColourFlex EZ, HunterLab, USA) according to the method described by Zhu et al. (Zhu, Cheng, Li, Erichsen, Petersen, Soerensen, et al., 2018). Before the measurement, black tile, white tile, and green tile were used to do instrument calibration. About 10 g of formula was weighed into the glass sample cup and then covered with a black metal cover to avoid any straight light. The color of samples was expressed by L*, a*, and b* values. The increase of the value of L* means color changing from darkness to lightness; an increase of a* value represents color from greenness toward redness; an increase of b* value reflects color from blueness to yellowness. All the measurements were conducted in triplicates. The overall color change of formula samples was evaluated by their ΔE value, which was calculated by the equation below:

$$\Delta E = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$$

$$\Delta L^* = \Delta L_T^* - \Delta L_0^*,\ \Delta a^* = \Delta a_T^* - \Delta a_0^*,\ \Delta b^* = \Delta b_T^* - \Delta b_0^*,$$

where T means test sample and 0 means a sample of 0 weeks, and ΔL_T^* is the ΔL^* value of test sample, ΔL_0^* is the ΔL^* value of the sample at 0 weeks. So were the Δa^* *and* Δb^*.

Particle size analysis. The particle size of formulas was measured using an LS-13320 Laser Diffraction Particle Size Analyzer (Beckman Coulter, USA). The particle size distribution was expressed as D_{90}, which means 90 % of the powder particles were smaller than this value. All the measurements were conducted in triplicates.

Microstructure analysis. Scanning Electron Microscopy (**SEM**) (JCM-6000 Plus Neoscope ™, JEOL, LTD. Tokyo, Japan) was used to analyze the microstructure of formulas. Powders were attached to the sample mount with double-sided adhesive carbon tape and then sputter-coated with gold for 1 min (30 s once for twice) in DII-29030SCTR Smart Coater (JEOL LTD. Tokyo, Japan). The sample was then subjected to the SEM analysis with 15 kV accelerating voltage.

Confocal laser scanning microscopy (CLSM). The distribution of protein and fat was observed using confocal laser scanning microscopy (TCS SP2 AOBS, Leica Microsystem, Wetzlar, Germany) according to the method of Tham et al. with a slight modification (Tham, Yeoh, & Zhou, 2017). Nile red and Nile blue A were used as dyes to label fat (red fluorescence) and proteins (green fluorescence). The two dyes were dissolved in 1,2-propanediol and then mixed at the ratio of 1:1 (volume/volume). 0.3 mg of formula was weighed into a 2 mL centrifuge tube, added 200 μL of dye mixture, was vortexed, and stained for 10 min before imaging. 20 μL of the stained sample was dropped on a glass slide and a coverslip was covered. Ar/Kr laser was used to excite Nile red dye at 488 nm, the emission wavelength was 500–530 nm. He/Ne laser was used to excite Nile blue A dye at 633 nm, the emission wavelength was 5650–615 nm.

Maillard reaction analysis

Measurement of N^{ε}-furoylmethyl-L-lysine (Furosine). Furosine was measured according to the method proposed by Li et al. with a slight modification (Li, Jia, Wang, He, Zeng, & Chen, 2022). Two grams (2.0 ± 0.1 g) of nutritional formula (corresponding to 234 mg protein) was reconstituted in 25 mL of Milli-Q water (deionized, 25 ± 2 °C), and 0.20 ± 0.01 g of the reconstituted solution was accurately weighed into a 50 mL microwave vessel (to make the excellent sealing performance), followed by adding 8 mL of 6 M hydrochloric acid solution. The vessel was flushed with nitrogen for 5 min to keep an inert reaction environment inside of the vessel. Samples were then subjected to acid hydrolysis under 110 °C for 20 h. Before being injected into HPLC, 5 mL Sodium citrate was added to 2 mL hydrolysates, and the mixture was purified for analysis. All the measurements were conducted in triplicates.

The analysis of furosine was performed on an Agilent 1260 infinity reverse-phase high-performance liquid chromatography (**HPLC**) (Agilent Inc., Palo Alto, U.S.A.) equipped with an MWD detector. The separation was carried out on a YMC-Pack ODS-AQ column (4.6 × 250 mm, 5 μm) (YMC CO., LTD. Kyoto, Japan) with the mobile phase A and B. Phase A was potassium dihydrogen phosphate buffer solution (0.05 M, pH = 2.9). Phase B consists of potassium dihydrogen phosphate buffer (0.05 M, pH = 2.9), isopropanol, and acetonitrile (650:175:175, v/v/v). The gradient elution program was 100 % A (0.3 mL/min) for 20 min, and 100 % B (0.5 mL/min) for 10 min, 100 % A (0.3 mL/min) for 30 min. The injection volume was 5 μL, and the detection was at 280 nm. The furosine content was calculated by the comparison to the calibration curve prepared with the furosine hydrochloride reference standard, and the results were reported as mg furosine per 100 g of formula sample.

Measurement of N^{ε}-carboxymethl-L-lysine (CML) and available lysine. The nutritional formula (0.2 ± 0.01 g) was accurately weighed into a 50 mL microwave vessel, followed by adding 4 mL Sodium Borate buffer (0.2 M, pH = 9.5) and 2 mL sodium borohydride solution (1 M in 0.1 M NaOH). The mixture was whirled for 30 s and standing for 4 h at room temperature, then 10 mL hydrochloric acid (12 M) and 5 mL Milli-Q water were added into a vessel with hydrolysate, followed by flushed with nitrogen for 5 min then subjected to acid hydrolysis under 110 °C for 20 h. After hydrolyzed, 50 % sodium hydroxide was added to the hydrolyzed mixture to make the value of pH between 8 and 9, and Milli-Q water was added to 30 mL, then the mixture was purified by 0.45 μm filter member for the following step. 50 μL filtered sample (or standard solution of CML and L-lysine) was added into a glass tube with 250 μL Sodium Borate buffer (0.2 M, pH = 9.5), then 250 μL FMOC *N*-hydroxysuccinimide ester derivatization (10 mg FMOC/mL acetonitrile) were performed for 10 min at ambient temperature after vortexed. Before being injected into HPLC, the mixture was filtered into a vial by 0.45 μm filter member, and 500 μL pentane/ethyl acetate (9:1, v/v) was added and vortexed to extract excess FMOC to the upper organic phase. All the measurements were conducted in triplicates.

The analysis of CML and available lysine was performed on an Agilent 1260 infinity reverse phase HPLC (Agilent Inc., Palo Alto, U.S.A.) equipped with an FLD detector. Zorbax SB-C18 column (4.6 × 250 mm, 5 μm, Agilent Inc., Palo Alto, U.S.A.) was used for separation with the mobile phase A and B. Phase A was the mixture solution of Sodium citrate dihydrate (0.05 M, pH = 3.0) and acetonitrile. Phase B consists of acetonitrile and water (4:1 v/v). The elution program was gradient elute: 20 % A for 15 min, 100 % B for 45 min, 100 % A for 10 min, and the flow rate was 0.5 mL/min. The injection volume was 5 μL, the excitation wavelength of the flame ionization detector was 262 nm and the emission wavelength was 310 nm. The CML and available lysine content were calculated by the comparison to the calibration curve prepared with CML and L-lysine reference standard, and the results were reported as mg CML (or lysine) per 100 g of formula sample.

Lipid oxidation analysis

Free fat analysis. The free fat was fat located on the surface of powder particles, which can be dissolved easily into organic solvent in a short exposure time. Two grams (2.0 ± 0.1 g) of nutritional formula was accurately weighed into a folded filter paper (No.4, Whatman, UK), which was then put onto a glass funnel and extracted using the method of Kim et al. with slight modification (Kim, Chen, & Pearce, 2009). Formula samples were washed with hexane 4 times, and 5 mL each time. The filtrate was collected into an aluminum dish which was pre-weighed before use. The dish was then put on a heating plate (80 °C) (ER-35S, Huitai Equipment Manufacturing Co., Ltd, Shanghai, China) to remove the organic solvent. After removing most of the organic solvent, the dish was transferred to a vacuum drying chamber (VC50, Salvis, Rotkreuz, Switzerland) to eliminate any organic solvent residue until it obtained a constant weight. The free fat content was calculated by taking out the weight of the aluminum dish, and the results were reported as g free fat/100 g formula. All the measurements were conducted in triplicates.

Peroxide value (PV) assay. The measure of peroxide value used the method described by Tian et ta. with some modifications (F. Tian, Decker, & Goddard, 2012). One gram (1 ± 0.1 g) of nutritional formula was dissolved by 2 mL of Milli-Q water (deionized, 25 ± 2 °C), and 10 mL isooctane/isopropanol (3:1, v/v) was added to the solution and vortexed vigorously for 30 s to extract the generated lipid hydroperoxide products. The mixture was subjected to centrifugation (3400 × *g*; 5 min) to get layer separation (Multifuge X3R, Thermo Fisher Scientific, Waltham, USA). Aliquot (1 mL) of the supernatant was transferred to a 15 mL glass tube, followed by the sequential addition of 2.8 mL of mixed organic reagent (methyl/ butanol, 2:1, v/v), 15 μL of ammonium thiocyanate (3.94 M), and 15 μL of ferrous iron solution. The ferrous ion solution was prepared freshly every time before being used by mixing an equal amount of barium chloride (0.132 M, dissolved by 0.4 M hydrochloric acid) and ferrous sulfate (0.144 M). The reaction solution was vortexed for 30 s and then incubated at ambient temperature for 20 min. The absorbance of the reaction solution was detected at 510 nm. The content of lipid hydroperoxides was calculated by the comparison to the calibration curve prepared with cumene hydroperoxide reference standard curve with different concentrations, and the results were expressed as μmol/kg of the formula sample. All the measurements were conducted in triplicates.

Volatile oxidation compounds analysis. Propanal and hexanal were chosen as the main volatile oxidation compounds for analysis, the two compounds are important products of the second stage of lipid oxidation. Propanal is decomposed from n-3 PUFAs and hexanal is from n-6 PUFAs (Romeu-Nadal, Castellote, & López-Sabater, 2004). One gram (1 ± 0.1 g) of formula was transferred into a headspace clear crimp vial immediately after opening (22 mL, Perkin Elmer Inc., Waltham, Massachusetts, U.S.) and 2 mL Milli-Q water (deionized, 25 ± 2 °C) was used to reconstitute the formula. Propionaldehyde-2,2,3,3,3-d_5 and hexanal-d_{12} were used as the internal standard for the analysis of propanal and hexanal, respectively. Mixed internal standards solution (100 μL, 1 mg/mL in methanol) was added into headspace vials with the sample solution, and capped immediately for following gas chromatography-mass

spectrometry (**GC–MS**) analysis. All the measurements were conducted in triplicates.

GC–MS analysis was performed on a 7890B gas chromatograph equipped with a 5977A mass spectrometry detector (Agilent Inc., Palo Alto, U.S.A.) and a TurboMatrix headspace injector with trap (Perkin Elmer Inc., Waltham, Massachusetts, U.S.). Sample vials were pre-heated at 60 °C for 20 min to release volatile compounds from sample solutions to the headspace of vials. The separation was carried out on a capillary column (DB-5MS, 30 m × 250 μm × 0.25 μm, Agilent Inc., Palo Alto, U.S.A.) with helium (99.999 % purity) as the carrier gas and flow rate of 1.0 mL/min. The initial oven temperature was 35 °C for 5 min, ramped to 120 °C at a rate of 4 °C/min, then to 240 °C at a rate of 30 °C/min, and maintained for 1.35 min. Conditions of MS were as follows: the ionization energy was 70 eV, the electric current was 200 μA, the temperature of the ion source was 200 °C, the electron multiplier voltage was 1.2 kV, SIM scanning model, scanning from 30 to 500 *m/z* 30–500. The content was calculated by the comparison to the calibration curve prepared with propanal and hexanal reference standards, and the results were reported as μmol propanal (or hexanal) per 100 g of formula sample.

Statistical analysis

All the values were reported as the mean of three measurements, and results were presented as mean ± SD. One-way analysis of variance (ANOVA) was analyzed using SPSS 26.0 software (Chicago, Ill., U.S.A.). Differences were considered significant at p-value < 0.05.

Results and discussion

Physical properties

For physical properties, studies focused on moisture content, water activity, color, particle size, surface morphology, etc. The FSMP may be plasticized by an increase in moisture content, in addition, adhesive stress between particles could be increased then cause deterioration of FSMP (Tham, Wang, Yeoh, & Zhou, 2016). Water activity also can influence the rate of Maillard reaction and lipid oxidation (van Boekel, 2001; Zhu, et al., 2018). Storage for a long time could also modify the microstructure of particles, particle size increased even caking of formulas, further affecting functionality properties such as flowability and solubility (Kelly, O'Mahony, Kelly, & O'Callaghan, 2016). Therefore, physical properties such as water content, water activity, color, particle size, and particle surface morphology were monitored weekly during storage in this experiment.

Moisture content and water activity. Two samples showed different moisture content at the beginning of the research (the 0 week). The moisture content in formula A (with lactose) was lower than that in formula B (with maltodextrin) (Fig. 1a). This might be due to that maltodextrin can absorb the released water and hold it tightly so it can absorb more water than lactose (Masum, Chandrapala, Huppertz, Adhikari, & Zisu, 2020). Along with the storage, the moisture content in both samples all increased significantly with a similar increasing trend, especially from the 0 to the 4th week. Beyond storage for 4 weeks, the increase of moisture content became more gradual. The obvious increase in moisture content from 0 to the 4th week may be attributed to the Maillard reaction which will produce water at the initial stage of the reaction. The moisture content of formula A increased gradually after 4 weeks may be caused by the crystallization of amorphous lactose because compared to crystallographic lactose, more water could be held when lactose is in the stage of amorphous form (Tham, Wang, Yeoh, & Zhou, 2016).

The initial water activity of formula A (0.130) was slightly lower than that of formula B (0.145), which was consistent with the moisture content results (Fig. 1b). This might be because of that Maltodextrin has a stronger ability to absorb and bind water than lactose, which caused

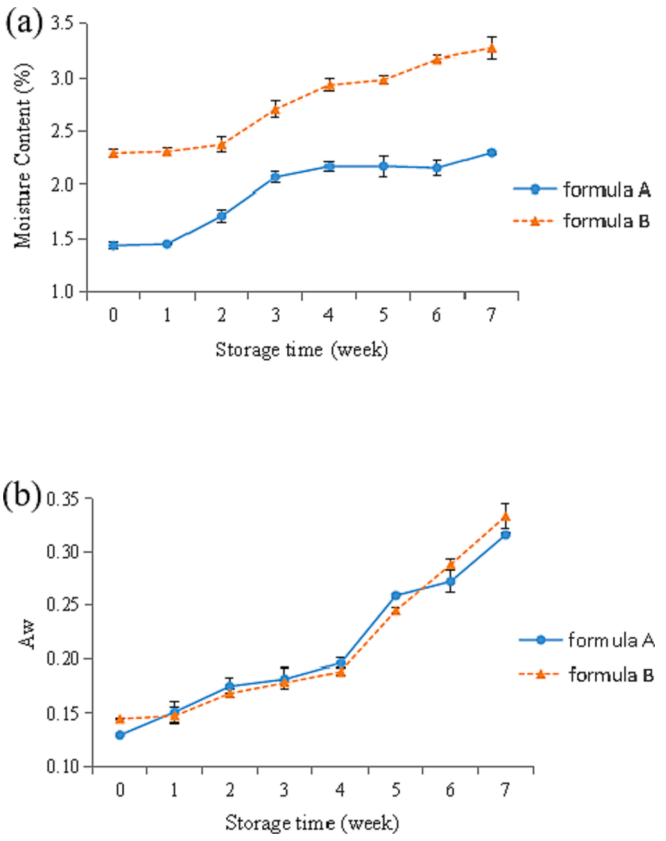

Fig. 1. Moisture content (a) and Water Activity (Aw) (b) of formulas with different types of reducing carbohydrates.

less free water in the powders and close to formula A of water activity (Masum, Chandrapala, Huppertz, Adhikari, & Zisu, 2020). Along with the accelerated temperature storage, the high temperature caused more mobility of molecules, leading to more free water (*i.e.* higher water activity) in the powders. The two samples showed a similar water activity increasing trend through the storage period (Fig. 1b).

Color. The freshly nutritional formula is light yellow, while the color of the formulas is not the same as it depends on the concentration of formula compositions (*i.e.*, fat, lutein, and carotene). And the higher the content of these formula compounds, the more yellow the formula was. Generally, the color of formulas will become darker along with storage time, showing up as a decrease of L* and an increase of a* and b*, and darker color could be shown under higher storage temperatures. The change of color may be attributed to the oxidation of PUFAs, attenuation of carotenoid, and browning process of the Maillard reaction during storage (Cheng, Zhu, Erichsen, Soerensen, Petersen, & Skibsted, 2017). Maillard reaction (also called non-enzymatic browning) produces brown nitrogenous polymers and co-polymers, which will cause discoloring of the formula from regular to yellow even brown, it was known as the major reaction which has a higher correlation with the change of color. The increase of the b* value represents a color turn to much yellower, so b* was commonly used to assess the changes in the color of formulas (Thomas, Scher, Desobry-Banon, & Desobry, 2004).

Two formulas showed similar change trends of the b* value (Fig. 2a). There was a dramatic increase of b* in the 4th week, and the b* value of the two samples had no significant difference ($p > 0.05$). After that, the changes were gently and the b* value of the formula with lactose (formula A) was higher than the lactose-free formula (formula B). These may be caused by formula with lactose presenting a more serious advanced Maillard reaction, which occurred in the later storage period. It was discovered that the extent of browning was connected with the amount and activity of reducing carbohydrates, higher concentration

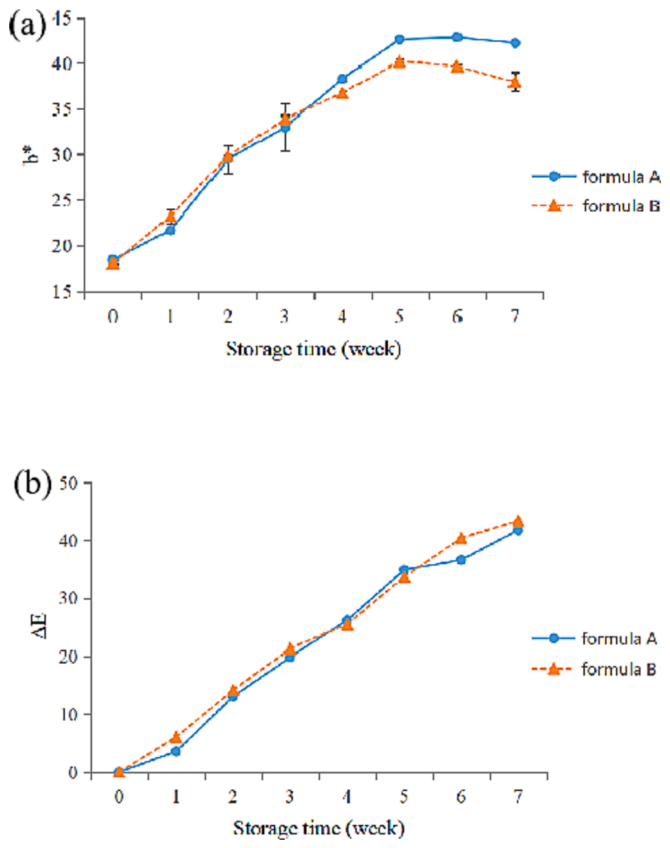

Fig. 2. B* (a) andΔE (b) of formulas with different types of reducing carbohydrates.

and more activity reducing carbohydrates would aggravate the Maillard reaction and then appear darker color (Ferrer, Alegría, Farré, Abellán, & Romero, 2002).

ΔE of the two formulas were increased rapidly throughout the storage period but there were no significant differences ($p > 0.05$) between them (Fig. 2b). The extent of browning of both the formulas increased rapidly along with the storage time, this may be because of the high temperature applied in this research, as temperature is the main influence factor of non-enzymatic browning. However, the rise of browning level is unlikely to be observed when formulas are stored at ambient temperature (25 or 30 °C), even storage for a long period (Cheng, Zhu, Erichsen, Soerensen, Petersen, & Skibsted, 2017).

Particle size. The D_{90} value was used to represent the particle size, results of Fig. 3 showed that formula A had a larger D_{90} than that of formula B. The same situation could also be seen from SEM (Fig. 4a) in the accumulation degree of formula A was larger than B. D_{90} of the two

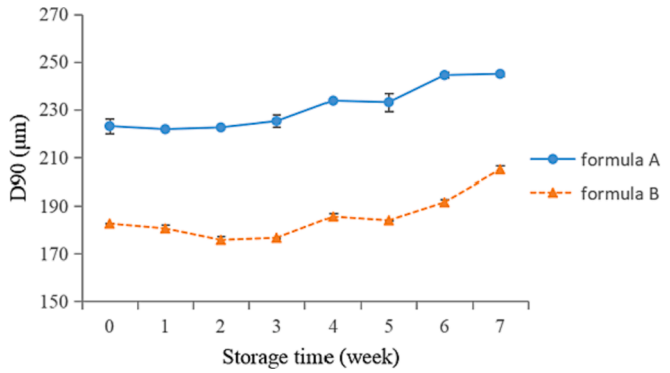

Fig. 3. Particle Size of formulas with different types of reducing carbohydrates.

formulas was increased as prolonged storage. In general, amorphous lactose is highly hygroscopic and readily absorbs moisture, which can result in the formation of liquid bridges between particles resulting in the formation of lumps. this means the size of most powder particles gradually gets larger. Larger particle size is probably due to the formation of fat bridges on the surface of particles during storage at high temperatures, fat bridges could hold particles together and increase the particle size. Meanwhile, more free fat from formula A could be observed by CLSM during storage (Fig. 4b), this may be attributed to milk formula A having a larger D_{90} than formula B. Saxena et al. discovered similar findings with dairy powders (Saxena, Adhikari, Brkljaca, Huppertz, Chandrapala, & Zisu, 2020).

Microstructure. Some obvious trends could be found by SEM from Fig. 4a, which shows the microstructure change of formula A and B after different storage times at 50 °C. At the beginning of the storage (the 0 week), most particles of formulas A and B had spherical shapes but the microstructure profiles of the two formulas were different. More pores were observed on the surface and inner of formula A whereas formula B showed deep dents and wrinkled surfaces. As well, Masum et al. observed the same phenomenon (Masum, Chandrapala, Huppertz, Adhikari, & Zisu, 2020). The microstructure of infant formula particles depends on many factors, including the composition of ingredients, processing, drying conditions, etc. All processing and composition of the two formulas were the same except for the type of the reducing carbohydrate, so maybe that is the reason for the different microstructure profiles. In the particles of the formula, lactose is present as a continuous matrix in which fat, protein, and air bubbles are dispersed. Water on the surface of particles evaporated when spray drying, whereas compared to formula with high lactose, low lactose caused water diffusion rapidly from inside and air bubbles shrinkage then pores were present (McEwen, McKenna, O'Kane, Phillips, & Johns, 2010).

The particles of the two formulas showed agglomeration with prolonged storage time, and the agglomeration degree of formula A was much stronger, this situation could be observed by particle size in Fig. 3 as well. A less smooth surface with more wrinkled particles of formula B was generated with the increase in storage time, the surfaces of formula A were still smoother than formula B. More solid particles were observed from formula A after the 4th week, particularly in the 7th week, and more wrinkly particles occurred at formula B. The formation of solid particles may be attributed to the crystallization of lactose (Tham, Yeoh, & Zhou, 2017).

Confocal laser scanning microscopy (CLSM) observation. The distribution of protein and fat of formula A and B were observed with CLSM, and images were shown as dark field and light field (Fig. 4b) at the same time to observe the diagrams much more clearly. For the fresh formulas A and B, particles were covered with green protein shells and had red fat inside. There was also a spot of the fat-covered surface of particles but they were invisible because the amount of fat was too poor compared with protein, another reason was the low resolution of CLSM. After storage for 7 weeks, a significant modification of protein and fat distribution could be seen. Plenty of fat globules were observed at the surface of formula A and B, which means fat released from core to surface during storage under high temperature, larger and more fat globules were distributed in the surface of formula A particles than formula B. The interparticle fat bridge could be formed by the free fat generated during storage, which helps powder particles bound together and then have larger particle sizes. These phenomena have also been reported by other authors that high temperature and long storage time will increase the content of free fat and then cause the formation of fat bridges (Kosasih, Bhandari, Prakash, Bansal, & Gaiani, 2016).

Maillard reaction analysis

In 1953, J.E. Hodge and others summarized and named a complex class of chemical reactions between amino compounds and carbonyl compounds the Maillard reaction or the carbon-ammonia reaction

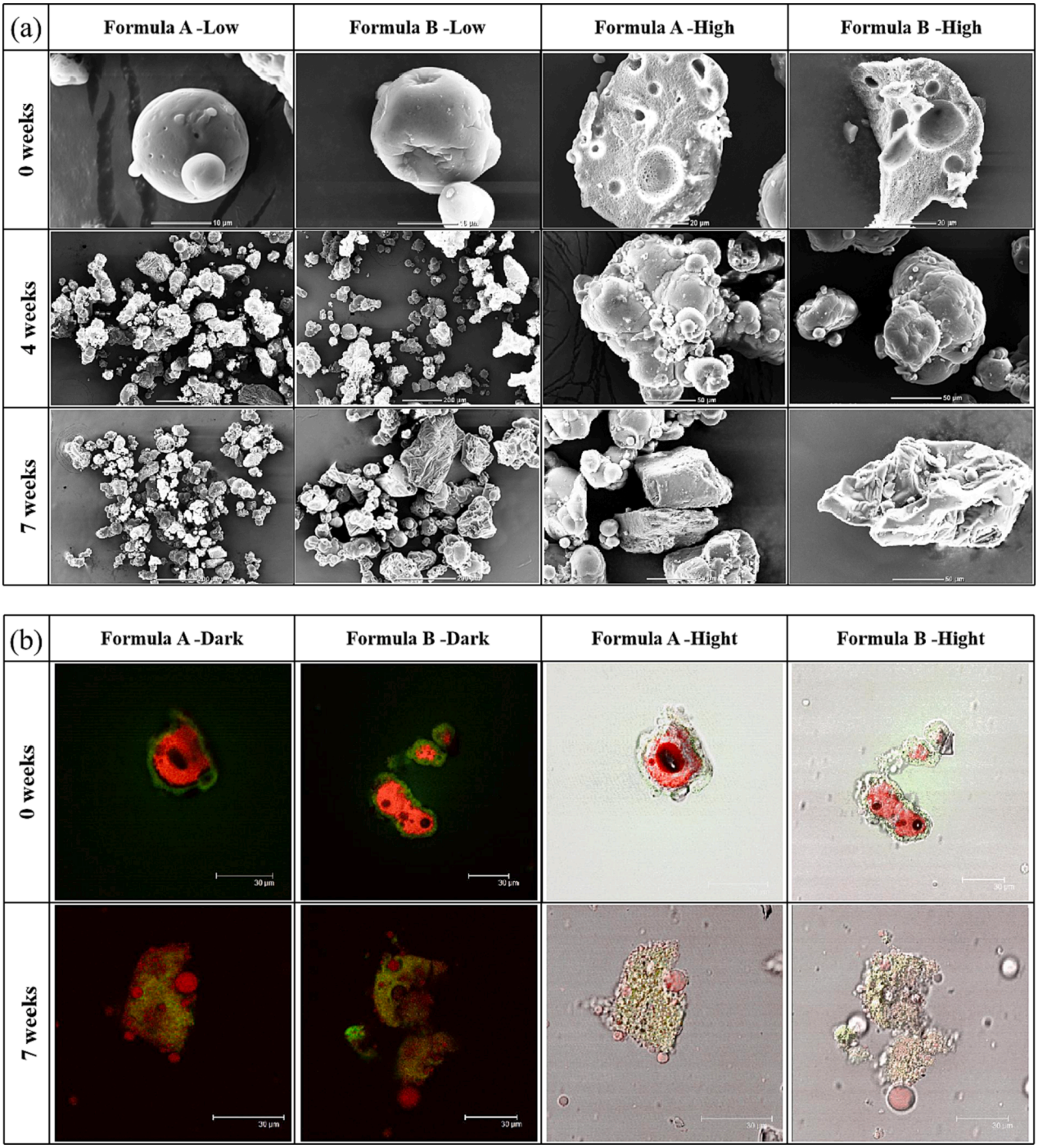

Fig. 4. Microstructure of two formulas under 50 °C at different storage times observed by Scanning Electron Microscopy (a) and Confocal Laser Scanning Microscopy (b). "Low" indicates that formula at low magnification; "High" indicates that formula at high magnification. Fat was labeled by red color and protein was labeled by green color. "Dark" indicates that formula at dark field diagrams; "Hight" indicates that formula at light field diagrams. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Hodge, 1953). Generally, the Maillard reaction was divided into initial stage, intermediate stage, and final stage. Firstly, the polymerization of carbonyl and amine moiety results in the formation of Schiff base, and then Amadori products are formed by Amadori rearrangement. Amadori products are 1,2 enolized to make Schiff's bases, and the residues are removed to form 3-deoxy glucuronide (**3-DG**) or degraded to form different furfural compounds, demonstrating that the Maillard reaction has entered the intermediate stage. The reaction proceeds to produce advanced glycation end products (**AGEs**). Finally, near the final stage of the Maillard reaction, the Amadori products' degraded molecules begin to polymerize, generating brown nitrogenous polymers and co-polymers known collectively as melanoidins (Z. Tian, Chen, Shi, Wang, Wu, & Li, 2023). Therefore, in this experiment, the Maillard reaction that occurred during the storage of samples was monitored by the content of furosine, CML, and available lysine.

Furosine determination. Amadori products originated in the condensation on reducing carbohydrates and protein, products of early Maillard reaction, were difficult to detect as they were easy to destroy during conventional acid hydrolysis. So furosine, a stable acid hydrolysis product of Amadori products, was widely used as an indirect marker of

early Maillard reaction in many food items, especially in infant formula.

The change in furosine concentration of two formulas during storage is shown in Fig. 5a. Initial furosine content of formula A and B were 39.58 and 32.11 mg/100 g of formula, much lower than that of milk powders reported by Cheng et al. (154 and 199 mg/100 g of powder) (Cheng, Zhu, Erichsen, Soerensen, Petersen, & Skibsted, 2017) and Le et al. (160 mg/100 g of protein) (Le, Bhandari, & Deeth, 2011). The amount of furosine raised rapidly and linearly during the initial storage, two formulas showed a similar variation trend and reached the highest value after storage for 4 weeks under high temperatures. In addition, more furosine was generated in formula A, values of formula A at the 4th week were 7.9 times as much as the amount at 0 weeks, and just 5.7 times as much for formula B. This can be explained as the yield of furosine depends on the ingredients of the formulas. Compared with the other reducing carbohydrates such as maltose and maltodextrin, lactose was more active and reacted with the amine group much more easily. After an increased period of furosine, formulas A and B followed by a gradual decrease. So furosine concentration was not increased linearly all the time, because intermediate and advanced products of the Maillard reaction were formed along with the lengthening of storage.

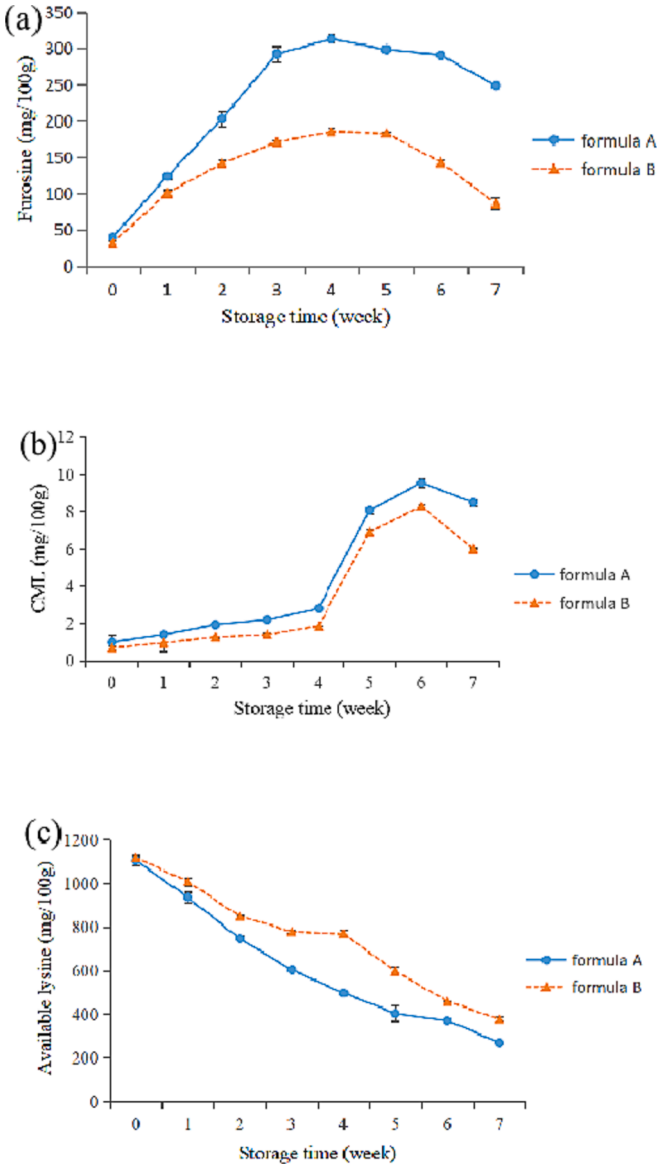

Fig. 5. Content of furosine (a), N^{ε}- (carboxymethyl) lysine (CML) (b), and available lysine (c) of formulas with different types of reducing carbohydrates.

According to the increase of furosine, moisture content increased rapidly as well (Fig. 1a). As formulas were sealed in aluminum foil bags, the increased moisture content may be correlated with the Maillard reaction, since water was one of the products of the early Maillard reaction (Thomsen, Lauridsen, Skibsted, & Risbo, 2005). Meanwhile, the increase in moisture content intensified the condensation of the amine group and carbonyl group, leading to obviously non-enzymatic browning. With the increase in storage time, the browning degree and color of the sample are higher (Fig. 2).

CML determination. Furosine was just a representative indicator to monitor the initial stage of the Maillard reaction, intermediate and advanced products were generated as the reaction progressed. CML was one of the most commonly studied AGEs since the potential is harmful to humans and has high stability, it was also considered as an indicator of the Maillard reaction advanced stage (Nguyen, van der Fels-Klerx, & van Boekel, 2014). Amadori Product and Glyoxal were important immediate precursors of CML. CML could provide more information after the content of furosine decreased, as the formation of CML needs more severe heat treatment and prolonged storage time, while this type of advanced product was not likely formed at ambient temperature or normal storage conditions (Cheng, Zhu, Erichsen, Soerensen, Petersen, & Skibsted, 2017). Therefore, combining the results of furosine and CML could analyze the progress of the Maillard reaction more comprehensively when formulas were stored at high temperatures.

The initial CML content of freshly trialed formulas was almost zero, 0.93 ± 0.002 mg/100 g of formula A and 0.78 ± 0.01 mg/100 g of formula B (Fig. 5b), the data of this study was by the other literature (Delatour, Hegele, Parisod, Richoz, Maurer, Steven, et al., 2009). The presence of CML at 0 week may be formatted during the thermal processing of formulas. A slight rise in CML was observed from 0 to the 4th week, while furosine increased rapidly at this time, as the initial stage of the Maillard reaction mainly occurred. Afterward, concomitant with furosine reduction, CML concentration rose promptly and suddenly in the next two weeks and was almost quadrupled until the 6th week, followed by a decline from the 6th to 7th week. The sudden increase in CML content may be due to the emergence of direct precursors of CML (Amadori products and glyoxal) with reaction time and the onset of the final stage of the Maillard reaction. The reduction of CML could be caused by the formation of some substances like melanoidin converted by CML, this situation was the result in raw milk (Niquet-Léridon, Jacolot, Niamba, Grossin, Boulanger, & Tessier, 2015) and the model system of reducing carbohydrate and protein (Nomi, Sato, Mori, & Matsumoto, 2022).

Along the storage period, the formation rate and concentration of CML of lactose-free formula were lower than that of formula with lactose (Fig. 5b). The different amounts of CML formed from the two formulas may be because of the type of reducing carbohydrate. Because lactose contains a greater fraction of open chains that can react with lysine than maltodextrin (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001). Other researchers support our view as well, the observations by Delatour et al. reported that the CML level of lactose-free formulas was lower than regular powder (Delatour, et al., 2009). It can be inferred that the formula with reducing carbohydrates appears higher CML than the free reducing carbohydrate formula, and lactose can react with the amine group to form the largest CML than the other reducing carbohydrates.

Available lysine determination. Lysine, the first limiting amino acid and one of the essential amino acids of humans, was one of the most dynamic amino acids as the primary amino group was in the side chain (Poulsen, Hedegaard, Andersen, de Courten, Bügel, Nielsen, et al., 2013). Therefore, the Maillard reaction easily occurred between lysine and reducing carbohydrates. So together with the detection of furosine and CML, available lysine was also used as a parameter to judge the degree of Maillard reaction. Significant differences could be observed in the concentration of available lysine between formula A and B after high temperature was carried out. Along with the increase of furosine and CML concentration, the amount of available lysine showed a rapid

decline and decreased as a function of storage time in two formulas (Fig. 5c), correlation coefficients of formula A and formula B were 0.96 and 0.98, respectively. Formula A has a higher value of blocked lysine than formula B during storage, the loss of available lysine of formula A was 75.57 %, and the value of formula B was 66.19 %. It means the Maillard reaction was more prone to occur in the system of formula A, which has supererogatory lactose as a reducing carbohydrate. Similarly, other researchers also reported a greater loss of available lysine detected from samples that contain more reducing carbohydrates, due to the extent of the Maillard reaction being much stronger (Choudhary, Arora, Kumari, Narwal, & Sharma, 2017).

There demonstrated a negative correlation between the content of furosine and available lysine from the 0 to 4th week in this study, and the value of the correlation coefficient was 0.99 for formula A and 0.94 for formula B. This significant negative function was because furosine is the acid hydrolysis product of Amadori products and almost no CML was generated at an early stage of storage. Also, ΔE has a significant linear negative correlation with available lysine during the whole storage, $R^2 = 0.976$ and 0.984 of formulas A and B, the same phenomenon has been reported in iron-fortified milk powder (Lee, Ho, Khoo, & Chow, 2012) and regular infant formulas (Emilia Ferrer, Alegría, Farré, Clemente, & Calvo, 2005), this means there was a correlation between color and Maillard reaction.

Lipid oxidation analysis

Except for protein and carbohydrates, lipid is another macronutrient in FSMP, and lipid oxidation is one of the major chemical reactions that would cause the quality deterioration of FSMP. In addition, there are interrelated between the Maillard reaction and lipid oxidation in the system of nutrition formula, the products of the two reactions affect each other (Vhangani & Van Wyk, 2016). Lipid hydroperoxides are formed from the early stage of lipid oxidation, then they can further break down and make off-flavor volatile compounds formation. Volatile compounds with off-flavor could be perceived by consumers easily, so it's a key indicator to estimate the deterioration of FSMP. Many external factors and internal factors could influence the extent of lipid oxidation, oxygen, temperature, humidity, and light are primary external factors. Internal factors mainly include composition and content of fatty acids, fat added to FSMPs which have a higher content of polyunsaturated fatty acid (**PUFA**), such as docosahexaenoic (**DHA**) and arachidonic acid (**AA**), will make the formula oxidized much easier than a formula with more saturated fatty acid. Therefore, lipid oxidation that occurred in samples was evaluated by determining the content of free fat, PV, propanal, and hexanal.

Free fat determination. Free fat refers to the fat located on the surface (involves pores and cracks) of particles that were unencapsulated into the inner. The concentration of free fat extracted from formula A was a little higher than formula B (Fig. 4b), the presence of pores on the surface of formula A (Fig. 4a) may be the reason, because as lactose crystallization advances, the structure of the powder particle is disrupted, allowing melted fat to flow from the particle's center to its surface and increasing the amount of surface free fat (Saxena, Adhikari, Brkljaca, Huppertz, Chandrapala, & Zisu, 2020). At high temperatures, more fat was presented as fluid fat globules, which also made fat migrate to the surface easily. As a result, fat bridges were formed among powder particles then powder agglomeration was found (Fig. 4b), and the more free fat, the bigger the aggregate particle was, this could also explain why formula A had a larger D_{90} than B (Fig. 3). During storage, a slightly increased content of free fat extracted by organic solvent was observed from formulas A and B, while more obviously accumulated free fat could be found from images of CLSM (Fig. 4b). This difference may be because some free fat was remained on the filter during extraction, and the content of free fat is pretty low, inaccurate results could be caused by operation factors.

In the production process, encapsulating profiles will affect encapsulation efficiency. Normally, fresh formula particles are spheres with fat globules inside and enveloped by protein and carbohydrates. In this research, the content of free fat was approximately 0.60 g/100 g of formula before storage, and the total fat was about 29 g/100 g of formula, there was only 2 % unencapsulated fat present on the surface (Fig. 6a). Thus, this study had a pretty good encapsulation during the spray-drying of food for special medical purpose. Meanwhile, the source of fat was another key factor that affect the encapsulation rate, when solid fat was used, free-fat content was difficult to increase (Kim, Chen, & Pearce, 2009). In the present research, a large amount of coconut oil (about 8 g/100 g of formula, 28 % of total fat) was used as one of the fat sources, this may be another reason why a slightly increased in free fat occurred during storage.

Lipid hydroperoxides and off-flavor volatile compounds like aldehydes and ketones could be produced by fat oxidation as free fat was easy to oxidation under high temperatures (Tham, Xu, Yeoh, & Zhou, 2017). More free fat covering the powder surface could reduce the solubility because of the hydrophobicity of fat. These phenomena ultimately lead to the loss of infant formula quality.

Peroxide value and volatile compounds determination. Lipids were easy to be oxidized under high temperatures. Peroxide value was used to indicate the level of lipid hydroperoxides, which are the major products of lipid oxidation's first stage. The value of PV increased gently before storage for 4 weeks, and formulas A and B increased from 106.05 and 101.26 μmol/kg to 112.82 and 107.14 μmol/kg (Fig. 6b). PV of formula A was slightly higher than formula B, this may be caused by a little bit higher free fat content of formula A, as the formula was prone to oxidation when more free fat existed. After that, a rapid increase occurred until reached a peak at the 6th week and then declined a slight bit at the end of the storage period.

As aforementioned, the PV level was used to measure the extent of the early stage of lipid oxidation. The second stage was the degradation of lipid hydroperoxides, which can bring volatile compounds with off-flavor (Francesca, Patrizia, Luca, Federico, & Annalisa, 2015). Propanal and hexanal are two important volatile products of the second stage of lipid oxidation, propanal was decomposed from n-3 PUFAs and hexanal was from n-6 PUFAs (Romeu-Nadal, Castellote, & López-Sabater, 2004). In this study, the degree of fat oxidation was improved and the amount of volatile compounds rose as storage duration increased. The propanal of formulas A and B reached maximum value after storage for the 3th and 4th week, and the hexanal of two formulas had the highest concentration after 1 week of storage (Fig. 6c and d). While the concentration of propanal and hexanal decreased along with the storage, maybe other products were formed by the degradation of the two off-flavor compounds. Similar results were also reported by other researchers, the concentration of off-flavor compounds produced by lipid oxidation increased rapidly during initial storage at high temperatures, but because of degradation to other products, even no off-flavor compounds could be detected after storage for a long time (Zhu, et al., 2018).

Based on the results, it can be assumed that the generation of lipid hydroperoxides and lipid hydroperoxides degraded into secondary products happened at the same time during the initial storage period, and lipid hydroperoxides were produced slower than they degraded. Therefore, the value of PV rose gradually while the increase rate of propanal and hexanal were rapid. Maillard reaction was considered accompanied by lipid oxidation, as researchers reported that substances produced by Maillard reaction have an action of anti-oxidation. The results of this research represented a rapid rise of PV that occurred after storage for 4 weeks, it is also the period the advanced stage of the Maillard reaction happened. Therefore, we supposed the rapid rise of PV may be a result of the suppression of secondary oxidation products generated from decomposed lipid hydroperoxides, which are caused by products of Maillard reaction advanced stage formation. Expect that, further decomposition of off-flavor lipid oxidation products happened and caused a decrease of propanal and hexanal.

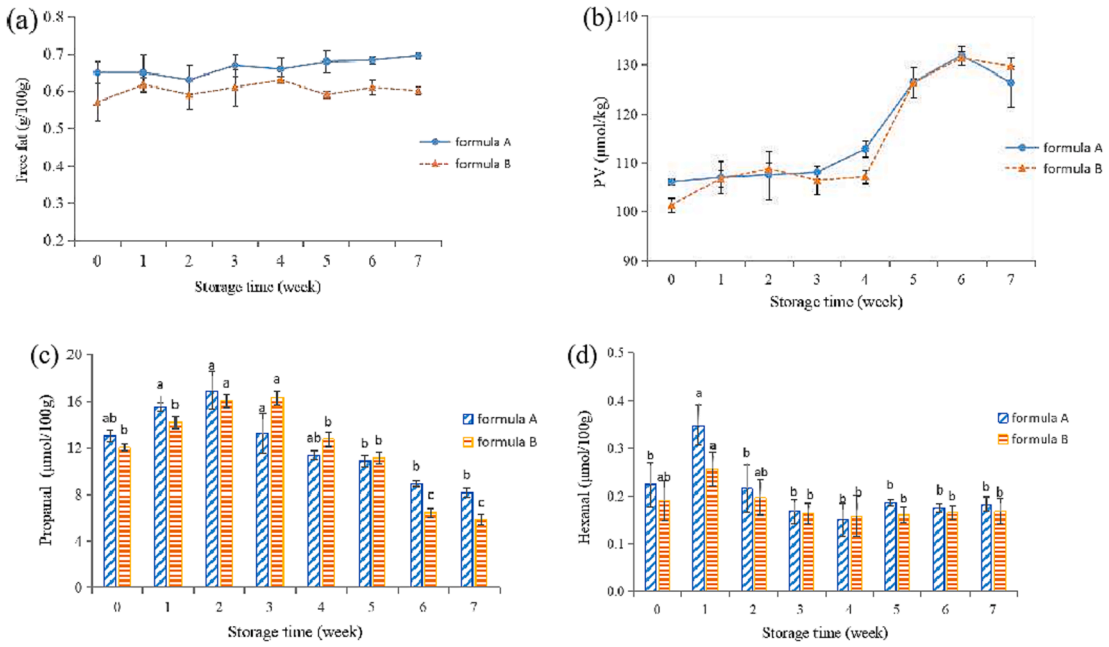

Fig. 6. Free fat (a), peroxide value (PV) (b), and volatile compounds (propanal (c) and hexanal (d)) quantification of formulas with different types of reducing carbohydrates. Different letters for the same index mean significant difference ($p < 0.05$).

Conclusions

In order to examine the mechanism of physicochemical property instability, two FSMPs with different types of reduced carbohydrates were exposed to accelerated experiments in this study. Formula A had lower moisture content and larger particle size than B, no difference in water activity and color between the two formulas, and all the values of physical properties increased along with the storage at high temperatures. Results of SEM presented formula with lactose has a smoother surface and CLSM showed more free fat covered on the particle surface after storage at high temperature for a long time. Higher intensity of the Maillard reaction occurred in formula A because lactose was more active than maltodextrin, more furosine and CML were produced and less available lysine remained. However, no significant difference in PV level and content of propanal and hexanal between the two formulas indicated lactose decreased chemical stability of FSMP through Maillard reaction primarily. In conclusion, reducing carbohydrate type affects the physicochemical characteristics of FSMP, compared with lactose, the formula with maltodextrin as reducing carbohydrate had better physicochemical stability when stored at high temperature. The results of this study are of critical value in formulation design and shelf-life in food for special medical purposes.

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.

Acknowledgments

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