# Redness generation via Maillard reactions of whey protein isolate (WPI) and ascorbic acid (vitamin C) in spray-dried powders

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## ARTICLE INFO

## ABSTRACT

Obvious redness generation has been observed in storage and processing for spray-dried WPI-ascorbic acid powders. The lower limit of detection by human observation is 0.001 g/mL for the concentration of ascorbic acid (AA:WPI ratio of 1:100). The redness is related to the adsorption of violet light (380 nm) and blue/green light (500 nm). Fluorescence analysis suggests the formation of formyl threosyl pyrroles and crosslinked poly(amino acids). DSC analysis shows that ascorbic acid peaks disappear as the Maillard reaction progress. The storage temperature has been found to significantly affect the Maillard reactions between WPI and ascorbic acid. Results show that the Maillard reaction rates between WPI and ascorbic acid are fast in spray-dried powders even at 20 °C. The formulated infant/baby milk powders on the market are suggested to require low temperatures (4 °C) or low oxygen ($N_2$ atmosphere) storage to reduce the extents of Maillard reactions (redness generation).

## 1. Introduction

Maillard reactions are a collection of chemical reactions between proteins (amino acids) and reducing materials that give brown products (Maillard, 1912). Monosaccharides, such as galactose (Liu et al., 2008), fructose (Zhang et al., 2015) and glucose (Song et al., 2018), and disaccharides, such as lactose (Wirth et al., 1998) and maltose (Li et al., 2011), have been typical reducing materials studied for Maillard reactions, since these sugars co-exist with proteins in foods. Many other ingredients, such as ascorbic acid (vitamin C), have been added to foods without thoroughly studying the possibility that toxin generation may occur at a low rate, such as through the Maillard reactions with proteins and amino acids (Ortwerth and Olesen, 1988; Troise et al., 2016). Ascorbic acid is an antioxidant, and it can be easily oxidised to dehydroascorbic acid and degraded to other products (Golubitskii et al., 2007), which are chemically active with proteins and amino acids, causing Maillard browning reactions (Min and Krochta, 2007).

In traditional cooking, Maillard reactions have been used for centuries as a household technique to give distinctive colours, aromas and flavours to cooked foods. However with the development of science, some of the end products that are generated in the late stages of Maillard reactions with foods and biological systems have been reported to have certain levels of toxicity to cells and tissues (O'Brien et al., 1989;

Van Nguyen, 2006). Typically, based on animal tests, such toxicity may result in damage to vascular and kidney tissues, several immune defects, insulin resistance and diabetic complications (Vlassara, 2005). To reduce the extent of Maillard reactions in foods, research has been carried out with respect to the chemical concentrations (amino acids, reducing materials and catalysts) and environmental conditions (temperature, humidity and atmosphere) over the past years (Ledl and Schleicher, 1990; Mildner-Szkudlarz et al., 2017; Sunds et al., 2018). Maillard reactions have also been reported as a key factor to increase the instability of many APIs (active pharmaceutical ingredients), such as amine chemicals, in oral medicines (Bharate et al., 2016; Chowdhury et al., 2018). Thus, it is reasonable to understand and control Maillard reactions in most edible products.

Regular milk contains milk sugar (lactose), milk proteins (whey protein and casein), fats, minerals and some other ingredients (Young et al., 1986). Since whey proteins are small and can be easily adsorbed by the intestinal tract, most infant/baby milk powders on the market have high contents of whey proteins in their formulae. To assist with human nutrition, ascorbic acid has been added as a functional ingredient in these milk powders. Table 1 lists the typical milk powders for infant or baby on the market with ratios between ascorbic acid and whey protein higher than 1:100 (the lower limit for a noticeable Maillard reaction reported in this work), while the highest ratio is 1:35 (Aptamil Gold + AR infant formula). However, ascorbic acid and whey have

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**Table 1**
List of typical milk powders for infant or baby use on the market, with ratios between ascorbic acid (AA) and whey protein higher than 1:100.

| Product | Sample size | Ascorbic acid (vitamin C) | Whey protein | AA:whey in powder |
|---|---|---|---|---|
| This work with noticeable redness | feed solutions | 0.001 g/mL | 0.1 g/mL | 1:100 |
| a2 Platinum Premium Infant formula | 100 mL prepared feed | 0.019 g | 0.88 g | 1:46 |
| a2 Platinum Premium Follow On Formula | 100 mL prepared feed | 0.016 g | 1.2 g | 1:75 |
| a2 Platinum Premium Toddler Milk Drink | 100 mL prepared feed | 0.012 g | 1.1 g | 1:92 |
| Aptamil Profutura 1 Premium Infant Formula | 100 mL prepared feed | 0.013 g | 0.84 g | 1:65 |
| Aptamil Profutura 2 Premium Follow On Formula | 100 mL prepared feed | 0.016 g | 0.77 g | 1:48 |
| Aptamil Gold + AR Regurgitation or Reflux | 100 mL prepared feed | 0.011 g | 0.38 g | 1:35 |
| Karicare Infant Formula | 100 mL prepared feed | 0.010 g | 0.84 g | 1:84 |
| Karicare Follow On Formula | 100 mL prepared feed | 0.012 g | 0.77 g | 1:64 |
| Nestle Nan Optipro 1 Gold | 100 mL prepared feed | 0.011 g | 0.91 g | 1:83 |
| Nestle Nan Optipro 2 Gold | 100 mL prepared feed | 0.011 g | 0.65 g | 1:59 |
| Nestle Nan L.I. Gold | 100 mL prepared feed | 0.012 g | 0.84 g | 1:70 |
| Nestle Nan Comfort 1 | 100 mL prepared feed | 0.014 g | 0.84 g | 1:60 |
| Nestle Nan Comfort 2 | 100 mL prepared feed | 0.012 g | 0.60 g | 1:50 |
| Bimbosan Organic Infant Formula | 100 g powder | 0.10 g | 7.1 g | 1:71 |
| Bimbosan Organic Follow On Formula | 100 g powder | 0.10 g | 5.0 g | 1:50 |
| Bimbosan Organic Growing Up Milk | 100 g powder | 0.10 g | 7.1 g | 1:71 |

been reported to give significant Maillard reactions, which may be unsafe especially for infants and babies. In the report by Min and Krochta (2007) a red pigment has been detected during the Maillard browning reaction (chemical reaction between amino acids) between dehydroascorbic acid (an oxidised form of ascorbic acid), which scavenges free radicals, and whey protein isolates (WPI). Moreover, some novel products (i.e. Nestle Nan Optipro HA 1 Gold infant formula) have hydrolysed whey proteins in their products to further improve intestinal adsorption, but the hydrolysed whey proteins have higher free amino contents and are actually more active with respect to the Maillard reactions (Mohan et al., 2015).

For milk preservation, as a reducing sugar and a typical component in milk, lactose has been extensively researched to reduce its Maillard reaction rate with milk proteins in the past decades (Aalaei et al., 2018; Jones et al., 1998; Morgan et al., 2005). Unlike lactose, the concentrations of ascorbic acid in the milk formula are very low, so that Maillard reaction may be hardly observed in this multicomponent mixture (milk). In this work, Maillard reactions of WPI and ascorbic acid have been observed in ratios similar to the commercial products in

spray-dried powders during aging and storage. The lower limit of ascorbic acid concentration has been studied according to the extent of redness generation by Maillard browning. Since some Maillard reaction products have safety risks, the results of this study may benefit the improvement of current milk products on the market, and provide some information for future toxicological works in related fields.

## 2. Experimental

### 2.1. Chemicals

Whey protein isolate (WPI) (Balance Ultra-Filtered Ion Exchange, unflavoured; Nutrition information per 100 g: 92 g protein, 0.4 g fat and 0.5 g carbohydrates) was purchased from Vitaco Health, Australia. L-Ascorbic acid (vitamin C, analytical reagent), hydrochloric acid (HCl, 32%, laboratory grade) and sodium hydroxide (NaOH, reagent grade) were purchased from Chem-Supply, Australia.

### 2.2. Spray drying

The clear feed solutions for spray drying were prepared by dissolving WPI and ascorbic acid in distilled water (same result were observed for using tap water) at the room temperature of 20 °C. The concentration of WPI was 0.1 g/mL, while the concentrations of ascorbic acid were 0, 0.001, 0.005, 0.01, 0.02 and 0.05 g/mL for ratios of ascorbic acid to whey protein of 0, 1:100, 1:20, 1:10, 1:5 and 1:2, noting that the ratios in commercial products are higher than 1:100 (Table 1). A Büchi Mini Spray Dryer B-290 (Büchi, Switzerland) was used for the spray drying. The operating conditions included a main air flow rate of 38,000 L/h (aspirator setting of 100%), a pump rate of 8 mL/min (25% of the maximum rate), a nozzle air flow rate of 540 L/h (45 on the nozzle rotameter scale) and an inlet/outlet temperature of 150/72 °C. Freshly spray-dried particles were collected from a vessel at the bottom of the cyclone, and immediately stored in a desiccator to avoid moisture adsorption. In the study for the effect of pH, original feed solutions were prepared with 0.1 g/mL WPI and 0.005 g/mL ascorbic acid. The pHs of the feed solutions were adjusted by adding HCl or NaOH to 6.6–4.0.

### 2.3. Maillard reactions during storage

The level of Maillard reactions between WPI and ascorbic acid (0–0.05 g/mL) was evaluated according to the colour change (redness generation) of the spray-dried powders during storage at a room temperature of 20 °C (stabilized by an air conditioner, the relative humidity of the air was 45 ± 5%). The aging time was 21 days (3 weeks) for each sample in order to attain a stable colour (redness). Material analysis was carried out to study the mechanism for the Maillard reactions between WPI and ascorbic acid. In the study on the effect of pH, an aging process for all the samples was performed at 60 °C for 1 h, and this process showed significant differences in their colours (redness).

To assess the effect of the storage temperature on the Maillard reaction rate, the colour changes (from the original white to a steady red) over time were recorded in terms of the colour parameters, L*, a*, b*, E* and C*, for the spray-dried WPI samples with low concentrations of ascorbic acid (0.005 g/mL for clear observations and 0.001 g/mL for the lower limit of detection) at storage temperatures of 20 °C, 40 °C, 60 °C and 80 °C. The elevated temperatures were achieved using a laboratory oven (Thermoline Scientific) at steady state before inserting the samples. The samples were heated until no further changes in colour were significantly observed.

In the study for product preservation, the redness generation was studied for the samples stored at a low temperature of 4 °C (in the refrigerator) or a $N_2$ atmosphere. The $N_2$ atmosphere was achieved by removing the air under a vacuum of under 0.05 bar (absolute) and re-fill-

ing with $N_2$ in a sealed container. The increases in a* values were recorded when all the samples could be observed to have red colours.

### 2.4. Instrumental analysis

#### 2.4.1. Colour analysis

Analysing the colour of the spray-dried powders was carried out through a standard lighting box with four fluorescent lamps (schematic diagram with positions shown in supporting Fig. S1). Samples were prepared by filling the spray-dried powders in plastic cuvettes with a size of 4.5 cm (height) × 1.2 cm (length) × 1.2 cm (width) for the colour analysis. The procedure for the colour analysis was as follows: turn on all four fluorescent lamps in the analysis box; place the camera on the clamp; fasten the clamp to the holding rod; place the sample in the centre of the analysing box; place a colour correction card near the sample; set a suitable self-timer (10 s) for the camera and close the analysis box; open the analysis box and upload the photo from the camera to the computer for MATLAB analysis. For the MATLAB analysis, three regions of 100 by 100 pixels were used to assess the L*a*b* values (colour parameters) for the collected photos according to the CIELAB sphere (Sant'Anna et al., 2013). The L*, a* and b* values are lightness (0–100 from black to write), redness/greenness (−100–100 from greenish to reddish colours) and yellowness/blueness (−100–100 from bluish to yellowish colours), respectively. Moreover, some other values also provide valuable information including the colour difference (E*) (Giangiacomo and Messina, 1988) and the chroma (C*) (Rhim et al., 1988), calculated from the colour values ($E^{*2} = L^{*2} + a^{*2} + b^{*2}$; $C^{*2} = a^{*2} + b^{*2}$). Among these colour parameters, the a* value is important for the WPI samples since redness generation shows the extent of Maillard reactions between WPI and ascorbic acid (Mohan et al., 2015).

#### 2.4.2. Particle morphology

The spray-dried WPI powders for SEM imaging were prepared by placing samples onto carbon tapes on aluminium sample stabs. After Au-coating for 2 min at 15 mA by a Quorum-SC7620 Mini Sputter Coater (Quorum Technologies, UK), the morphologies of samples were observed using a Phenom-Prox SEM (Phenom-World, Netherlands) in the detector mode for backscattered electrons with an operating voltage of 15 kV and an operating pressure of 1 Pa. The images were captured by the Phenom-World default software.

#### 2.4.3. Light adsorption and emission

A sample was prepared by dissolving 0.1 g spray-dried WPI-ascorbic acid (0–0.005 g/mL) powder in 10 mL distilled water for measurements by a UV–Vis spectrophotometer and a spectrofluorometer. A Cary 60 UV–Vis spectrophotometer was used in the scanning mode to find the adsorption peaks for the samples. For each scan, 1 mL sample was loaded in a 1-cm quartz cuvette and placed in the sample holder of the instrument. The range for scanning was set at 300–800 nm. The increment was 1 nm.

The wavelengths of lights at the adsorption peaks (380 nm and 500 nm) were used as the excitation wavelengths for fluorescence scanning to collect the emission spectra. A Horiba spectrofluorometer (FluoroMax-4) was used for the fluorescence scanning. The front entrance and exit slits were 4 nm. The range of emission spectra was 400–700 nm for the excitation wavelength of 380 nm, while the range was 520–800 nm for the excitation wavelength of 500 nm. The increment was 1 nm.

#### 2.4.4. Material analysis

DSC analysis was performed using a differential scanning calorimeter (TA Instruments Q1000) on the spray-dried pure WPI, the ascorbic acid, and the spray-dried WPI-ascorbic acid (0.01 g/mL; lower concentrations showed no signal) before and after aging to study the interaction between WPI and ascorbic acid. The samples for DSC measure-

ments were prepared following standard procedures using sealed pans. 2–5 mg of sample was used in each analysis. The samples were stabilized at 0 °C for 5 min and then heated to 300 °C using a ramp rate of 5 °C/min, with $N_2$ as the purge gas. Heat flow as a function of increasing temperature was recorded for the analysis of each sample.

Fourier transform infrared (FTIR) spectroscopy was used to investigate the change of functional groups in the spray-dried WPI-ascorbic acid (0.01 g/mL) powders before and after aging. The specimen was placed on the detector of a single bounce diamond ATR in a Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific, Australia). The FTIR spectra used a resolution of 4 cm$^{-1}$ with 128 scans and a scanning range from 600 to 2000 cm$^{-1}$.

Raman spectroscopy was used to complement FTIR spectroscopy for assessing the change in the functional groups. The analysis was performed using a Raman Station 400 F (PerkinElmer, USA) with a laser source at 785 nm. The laser power was set at 60%. The exposure time was 10 s for each scan (out of 64 scans per sample with a resolution of 2 cm$^{-1}$). The Raman spectra were analysed by Spectrum software (v6.3.4.0164).

## 3. Results and discussion

### 3.1. Redness generation from the spray-dried WPI-ascorbic acid powders

In this work, a simple mixture with only ascorbic acid and WPI has been studied in ratios of 1:100–1:2 (0 for the spray-dried pure WPI), since commercial infant/baby products have higher ratios than 1:100 (Table 1). As shown in Fig. 1, after aging, obvious redness can be seen from the sample with 0.001 g/mL ascorbic acid for a ratio of 1:100, bringing a potential safety concern for the commercial infant/baby products regarding the Maillard reaction (Vlassara, 2005). The detailed colour parameters are shown in the supporting Table S1. When the concentration of ascorbic acid increased from 0.001 to 0.01 g/mL, the redness (a* value) increased, corresponding to more ascorbic acid reacting with WPI. However, the redness decreased when the concentration of ascorbic acid further increased to 0.02 and 0.05 g/mL, showing that highly-concentrated ascorbic acid has a self-limiting effect on the Maillard reaction.

The reason for the self-limiting Maillard reaction is largely related to pH and solubility. The pHs of the WPI feed solutions were 6.6 ± 0.1, 6.2 ± 0.1, 5.6 ± 0.1, 4.9 ± 0.1, 4.5 ± 0.1 and 4.0 ± 0.1, corresponding to ascorbic acid concentrations of 0, 0.001, 0.005, 0.01, 0.02 and 0.05 g/mL, respectively. The isoelectric point of WPI is at a pH of 4.5, giving the lowest solubility of WPI (Pelegrine and Gasparetto, 2005). Due to the pH-controlled solubility of WPI, the particle morphologies are different for spray-dried WPI powders with different concentrations of ascorbic acid as shown in Fig. 2. The surfaces are more buckled for the WPI particles with low concentrations of ascorbic acid (0–0.01 g/mL for pHs of 6.6–4.9), while the surfaces are more crumpled for the
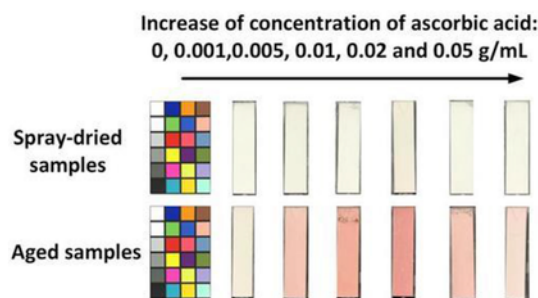


**Fig. 1.** Effect of the ascorbic acid concentration (0–0.05 g/mL) on the colour change by the Maillard reaction (aging) of WPI and ascorbic acid in spray-dried powders. Operating conditions of aging: temperature was 20 °C; relative humidity was 45 ± 5%; aging time was 21 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
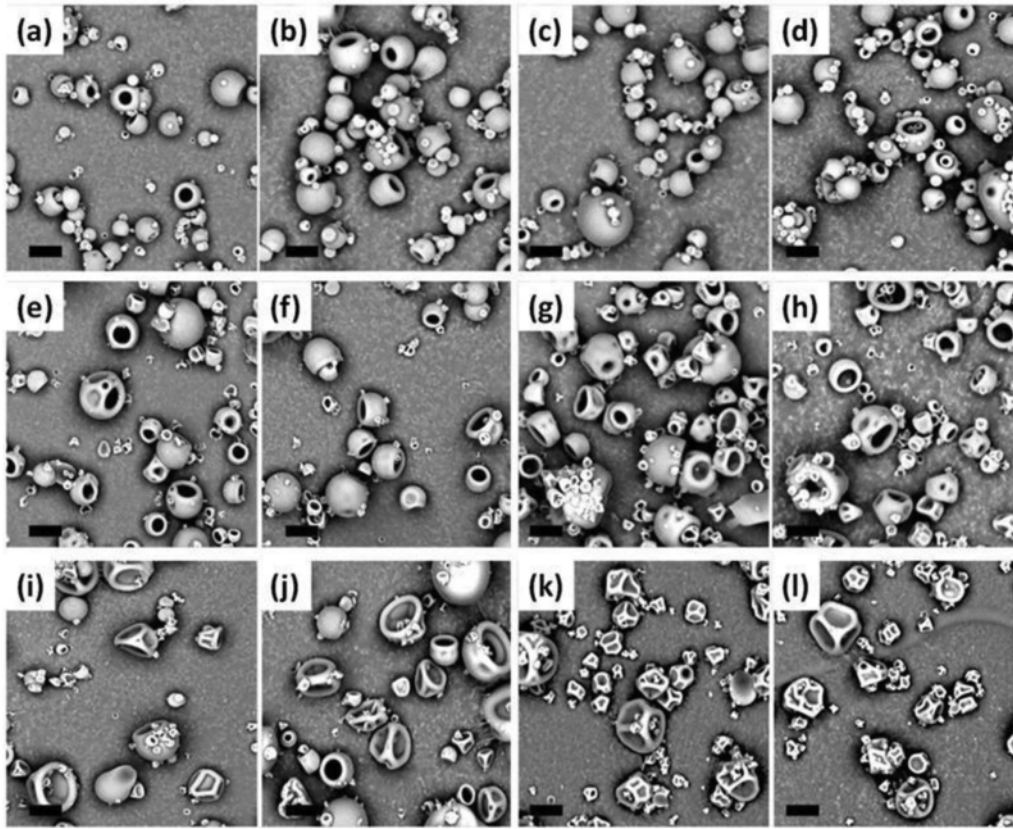
**Fig. 2.** SEM images of the spray-dried WPI powders with different concentrations of ascorbic acid: (a, b) 0, (c, d) 0.001, (e, f) 0.005, (g, h) 0.01, (i, j) 0.02 and (k, l) 0.05 g/mL before and after aging (Maillard reaction). Scale bars: 10 μm. Operating conditions of aging: temperature was 20 °C; relative humidity was 45 ± 5%; aging time was 21 days.

WPI particles with high concentrations of ascorbic acid (0.02–0.05 g/mL for pHs of 4.5–4.0), based on the description by Vehring (2008) for the following reason. WPI are protein molecules that are surface-active in the spray-dried particles, preferring to diffuse to the surface and precipitate. Since WPI can easily form aggregated structures (i.e. micelles and colloids) at pHs of around 4.5 that have a low solubility, the precipitation of WPI is more likely to happen during spray drying throughout the bulk of atomized droplets, where the mobility of WPI relies on not only the diffusion of molecules but also the dispersion (lower mobility) of aggregates. At the higher pHs, soluble WPI molecules can diffuse to the surface of the droplets more evenly, resulting in more surface precipitation rather than precipitation in the bulk of the particles. Morphological crumpling and buckling are then observed for spray-dried WPI particles at different pHs. With the high aggregation of WPI at lower pHs (higher ascorbic acid concentrations), the contact area between WPI molecules and ascorbic acid is geometrically smaller, decreasing the Maillard reaction rate.

Chemically, a key step for Maillard reactions is the reaction between the carbonyl groups (C=O) of degradation products from ascorbic acid and the amine groups ($-NH_2$) of WPI, forming imine groups (C=N) and releasing water molecules ($H_2O$) (Reihl et al., 2004). With high concentrations of $H^+$, low pHs can reduce the extent of the Maillard reaction by converting $-NH_2$ in WPI to $-NH_3^+$ (protonated amino group, insufficient free electrons in N for the formation of C=N). As indicated by Martins et al. (2000) the percentage of unprotonated amino group is a minimum at a pH of 4.0 and follows the equation $y = 3 \times 10^{-8}$ exp (2.3026x), where y is the ratio of unprotonated amino groups, while x is the pH. In agreement with Martins et al. (2000) as shown in Fig. 3a, the redness of the aged spray-dried WPI powders firstly increases with an increase in the concentration of ascorbic acid for pHs of 6.6–4.9, and then decreases with a further increase in the concentration of ascorbic acid for pHs of 4.5–4.0. For WPI, —

$NH_2$ groups become relatively inert when pHs are lower than 4.5, which is the isoelectric point for WPI (Morand et al., 2012). When pH is higher than 4.5, the amino acids in WPI are likely to have more $-COO^-$ and $-NH_2$ groups (rather than $-COOH$ and $-NH_3^+$ groups). The concentration of ascorbic acid, however, changes the pH of the WPI solutions. In the study with respect to only pH, the pH values were 5.6 ± 0.1 in the six original feed solutions containing 0.1 g/mL WPI and 0.005 g/mL ascorbic acid. The pHs of the five feed solutions were changed to 6.6 ± 0.1, 6.2 ± 0.1, 4.9 ± 0.1, 4.5 ± 0.1 and 4.0 ± 0.1 by adding HCl or NaOH. Fig. 3b shows that the redness (a* value) changes with respect to the pH. The redness significantly decreases when the pH decreases to 4.5 ± 0.1 (the isoelectric point) and further decreases at a lower pH (4.0 ± 0.1), showing that the activity of $-NH_2$ in WPI is important for the Maillard reaction. The related photos and other colour parameters are shown in the supporting Fig. S2 and Table S2, respectively. With pHs of around 6.6–4.9, the redness of the samples were almost the same values. The above results suggest that the Maillard reaction rate relies on both the reactant concentration and the pH.

### 3.2. Mechanism of the Maillard reaction between WPI and ascorbic acid

Fig. 4a shows the UV–Vis spectra for the aged WPI samples with the concentrations of ascorbic acid of 0, 0.001 and 0.005 g/mL. The spectra show significant absorption peaks at 380 nm (violet light) and 500 nm (blue or green light). The absorption peaks are stronger at greater ascorbic acid concentrations, showing increasing redness (~665 nm, a* value) and yellowness (~600 nm, b* value). In the fluorescence scanning, 380 nm and 500 nm are excitation wavelengths for the collection of the emission spectra. The change in fluorescence spectra suggests that a chemical reaction has taken place, altering the electronic states of molecules and the transitions between them (Jablonski,
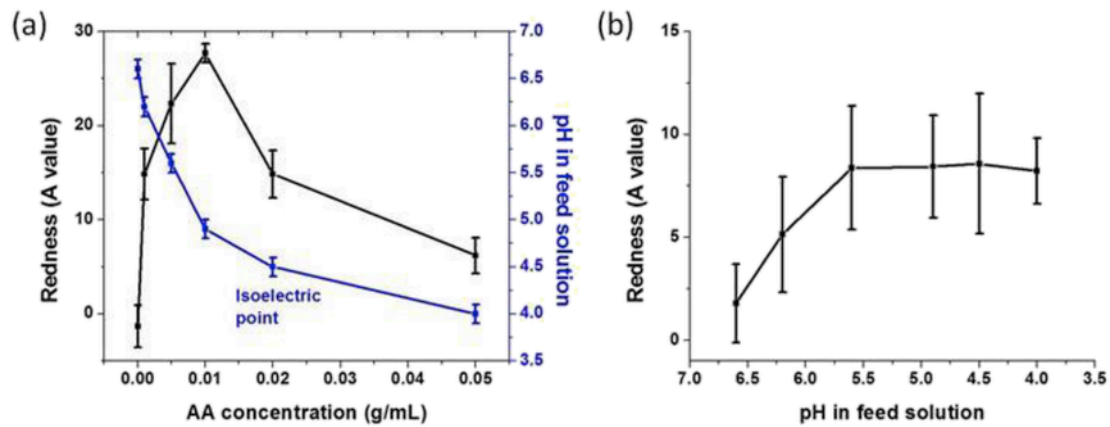
**Fig. 3.** (a) Effect of the concentration of ascorbic acid (0–0.05 g/mL) on the pH in the feed solution and the redness generation in the spray-dried powders after complete aging at room conditions. Operating conditions of aging: temperature was 20 °C; relative humidity was 45 ± 5%; aging time was 21 days. (b) Effect of pH on the redness generation from the spray-dried WPI-ascorbic acid (0.005 g/mL) powders after aging at 60 °C in oven for 1 h.
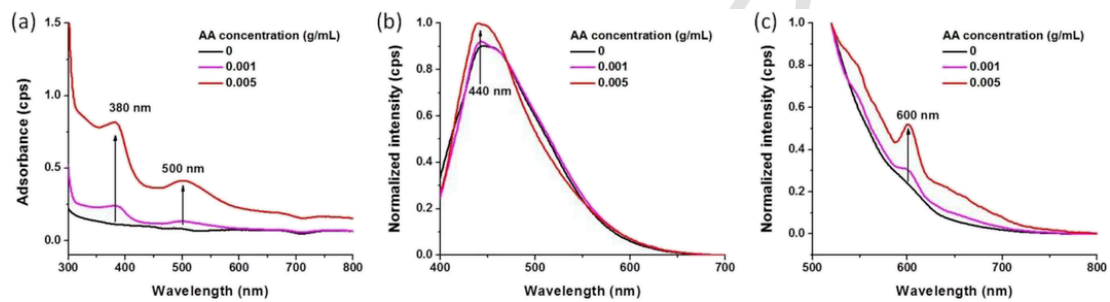


**Fig. 4.** (a) UV–Vis spectra, and fluorescence spectra with excitation wavelengths of (b) 380 nm and (c) 500 nm for the spray-dried WPI powders with ascorbic acid concentrations of 0, 0.001 and 0.005 g/mL after aging (Maillard reaction). Operating conditions of aging: temperature was 20 °C; relative humidity was 45 ± 5%; aging time was 21 days.

1933). Fig. 4b shows that the original WPI molecules have a broad fluorescence peak at around 450 nm with an excitation wavelength of 380 nm. After the Maillard reaction with ascorbic acid, a new peak at 440 nm is generated, causing the broad, main fluorescence peak to shift slightly to the left. The peak at 440 nm indicates the formation of formyl threosyl pyrroles, which are the typical products from Maillard reaction between amino acids and ascorbic acid (Matiacevich et al., 2005). For example, lysine (found in WPI) reacts with ascorbic acid forming 2-amino-6-(3-(1, 2-dihydroxyethyl)-2-formyl-4-hydroxymethyl-*l*-pyrrolyl)hexanoic acid, which is a formyl threosyl pyrrole (Bierhaus et al., 1998; Nagaraj and Monnier, 1995). The formation of formyl threosyl pyrroles also involves the intermediate generation of 1-deoxy-threulosyl-amino acids, called Amadori products. Fig. 4c shows that the original WPI molecules have no fluorescence peak at an excitation wavelength of 500 nm. After the Maillard reaction, another peak at 600 nm is generated, indicating none of the typical fluorescence products from Maillard reactions (Matiacevich et al., 2005). Such a high wavelength at 600 nm suggests the formation of complex molecular structures, such as poly(amino acids) (Karakisawa et al., 2012; Klymchenko and Mely, 2013), possibly formed through crosslinking between different amino acids and the degradation products of ascorbic acids, as indicated by Reihl et al. (2004) During the crosslinking reaction, one molecule of the degradation products containing several C═O bonds can react with two amino acids, while each amino acid from the peptide chains of WPI is linked with other amino acids. The formyl threosyl pyrroles and highly crosslinked poly(amino acids) are advanced glycation end-products with fluorescent structures that are capable of further reacting with blood sugars, binding proteins and inducing unexpected changes in structure and function (Bierhaus et al., 1998; Nagaraj and Monnier, 1995; Singh et al., 2001). The toxicologically accumulated effects from the advanced glycation end-products may create safety risks for protein aging, especially for long-lived pro-

teins such as collagen (Gautieri et al., 2017) and lens proteins (Linetsky et al., 2014), neurofibrillary disorder (Kuhla et al., 2015) and oxidative stress (Nowotny et al., 2015).

The lower limit of detection for the measurements using DSC, FTIR and Raman is 0.01 g/mL for an AA:WPI ratio of 1:10. Fig. 5a shows the DSC spectra of the spray-dried pure WPI and the ascorbic acid. The WPI sample has a dehydration and glass transition ($T_g$) peak at around 80 °C (Anandharamakrishnan et al., 2007; Pugliese et al., 2016), and a decomposition peak at around 250 °C (Cataldo et al., 2011). The melting point of ascorbic acid is 190 °C. Fig. 5b shows the DSC spectra of the spray-dried WPI-ascorbic acid (0.01 g/mL) powders before and after aging (due to the Maillard reaction). The peak for melting point of ascorbic acid is shown on the DSC spectrum of the spray-dried WPI-ascorbic acid powder before aging, while the peak disappears after aging, showing that the Maillard reaction has consumed ascorbic acid molecules. The Maillard reaction does not significantly affect the dehydration and decomposition peaks for the WPI molecules.

As shown in Fig. 5c, the FTIR spectra have many overlaps with the spectra for the spray-dried pure WPI (mainly at 1634 cm$^{-1}$, amide I) and the ascorbic acid (at 1651 cm$^{-1}$, stretching $\nu$ C═C). Notable peaks for the functional groups are shown on the FTIR spectra, including amide I (1634 cm$^{-1}$), amide II (1527 cm$^{-1}$), bending $\delta$ (CH$_2$) (1449 cm$^{-1}$), stretching $\nu$ (COO$^-$) or deformation $\gamma$ (CH$_3$) (1393 cm$^{-1}$), amide III (1306 cm$^{-1}$), stretching $\nu$ (PO$_2$) (1240 cm$^{-1}$) and stretching $\nu$ (CO─O esters) (1166 cm$^{-1}$) for WPI (Hasegawa et al., 2000; Joubran et al., 2013; Oleszko et al., 2017); and stretching $\nu$ (C═O) (1752 cm$^{-1}$), stretching $\nu$ (C═C) (1651 cm$^{-1}$), bending $\delta$ (C─H) (1313 cm$^{-1}$), stretching $\nu$ (C─O─C) (1111 cm$^{-1}$) and bending $\delta$ (C─O─C) (1023 cm$^{-1}$) for ascorbic acid (Panicker et al., 2006). Regarding the overlaps in the wavelength range from 1100 to 1800 cm$^{-1}$, only the significant peak at 1023 cm$^{-1}$ for $\delta$ (C─O─C) from ascorbic acid can be found on the spectrum of the spray-dried WPI-ascorbic acid powders
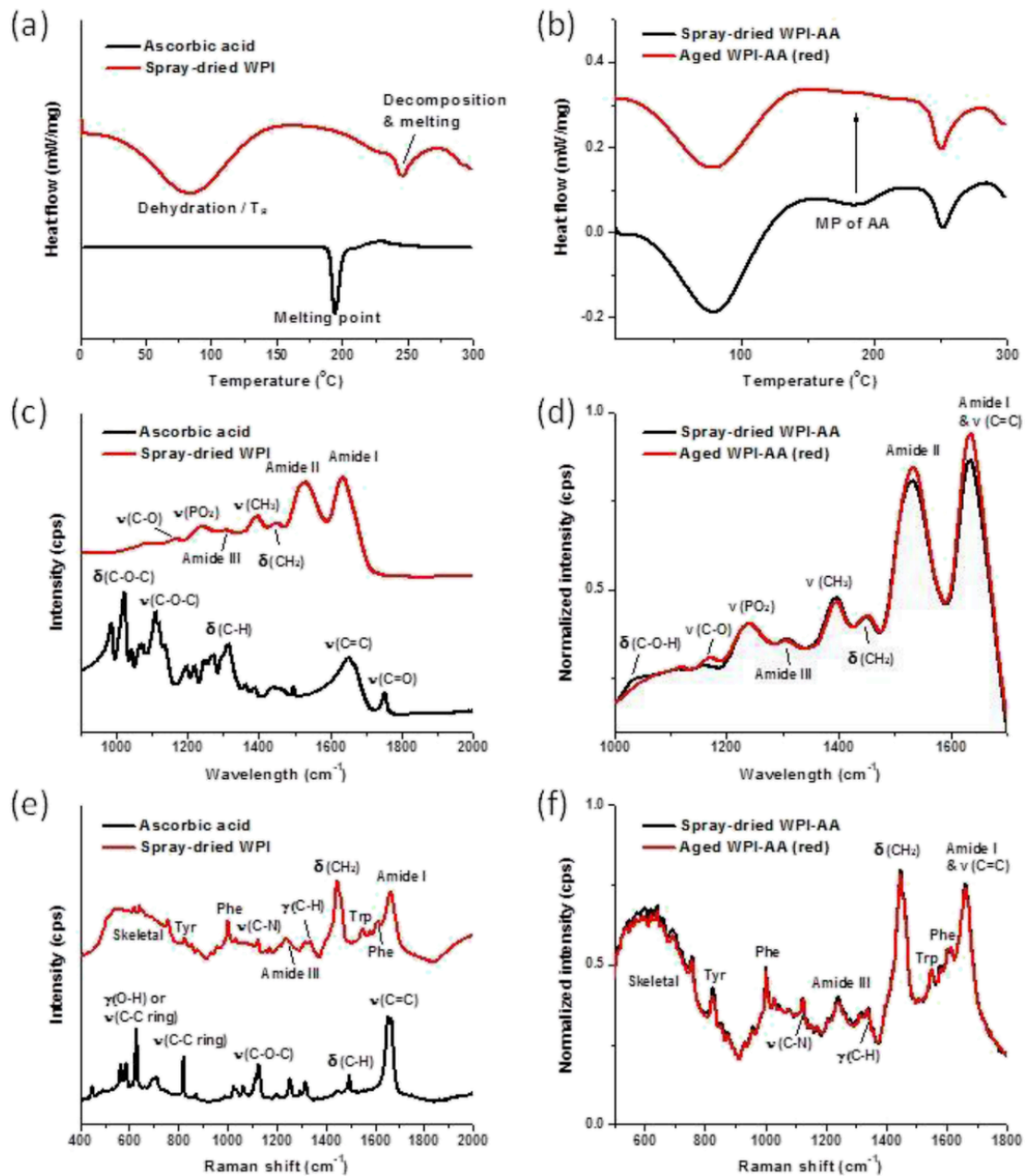
**Fig. 5.** (a, b) DSC, (c, d) FTIR and (e, f) Raman spectra for ascorbic acid, the spray-dried pure WPI, and the spray-dried WPI-ascorbic acid (0.01 g/mL) powders before and after aging (Maillard reaction). Operating conditions of aging: temperature was 20 °C; relative humidity was 45 ± 5%; aging time was 21 days.

before aging (Fig. 5d). The δ (C—O—C) peak disappears after aging, again showing that Maillard reaction consumes ascorbic acid molecules. Moreover, decreases in the amide I and amide II peaks have been observed after aging due to the consumption of functional groups and the conformational changes of proteins, agreeing with the observations of Ioannou and Varotsis (2017), Joubran et al. (2013) and Su et al. (2010) More overlaps are shown on the Raman spectra for the spray-dried pure WPI and the ascorbic acid (Fig. 5e). The functional groups from the Raman spectra that have been identified include amide I (1664 cm$^{-1}$), Phe (1614 and 1002 cm$^{-1}$), Trp (1550 cm$^{-1}$), bending δ (CH$_2$) (1446 cm$^{-1}$), deformation γ (C—H) (1340 cm$^{-1}$), amide III (1236 cm$^{-1}$), stretching ν (C—N) (1124 cm$^{-1}$), Tyr (826 cm$^{-1}$) and skeletal groups over the range of wavelengths from 400 to 800 cm$^{-1}$ for WPI; and stretching ν (C=C) (1652 cm$^{-1}$), bending δ (C—H) (1496 cm$^{-1}$), stretching ν (C—O—C) (1128 cm$^{-1}$), stretching ν (C—C

ring) (820 and 628 cm$^{-1}$) and deformation γ (O—H) (628 cm$^{-1}$) for ascorbic acid (Davidson et al., 2013; Oleszko et al., 2017; Panicker et al., 2006). Due to the overlaps, apart from the change of peak intensities for the skeletal structure (Fig. 5f), no other changes can be significantly observed for the spray-dried WPI-ascorbic acid powder before and after aging. However, the slight decreases in the peak intensities of amide I, ν (C=C), δ (CH$_2$), amide III, Tyr and skeletal groups may suggest the consumption of functional groups and the conformational changes of proteins, supporting the above FTIR results.

A schematic diagram is shown in Fig. 6 for the mechanism of Maillard reaction between ascorbic acid and WPI. Following several steps of oxidation and degradation, ascorbic acid can be converted through dehydroascorbic acid to multiple degradation products (Reihl et al., 2004). The degradation products (with C=O) can react with the amino acids (with —NH$_2$) in WPI via the formation of imine groups (C=N), resulting in molecular changes and protein deformation. There-
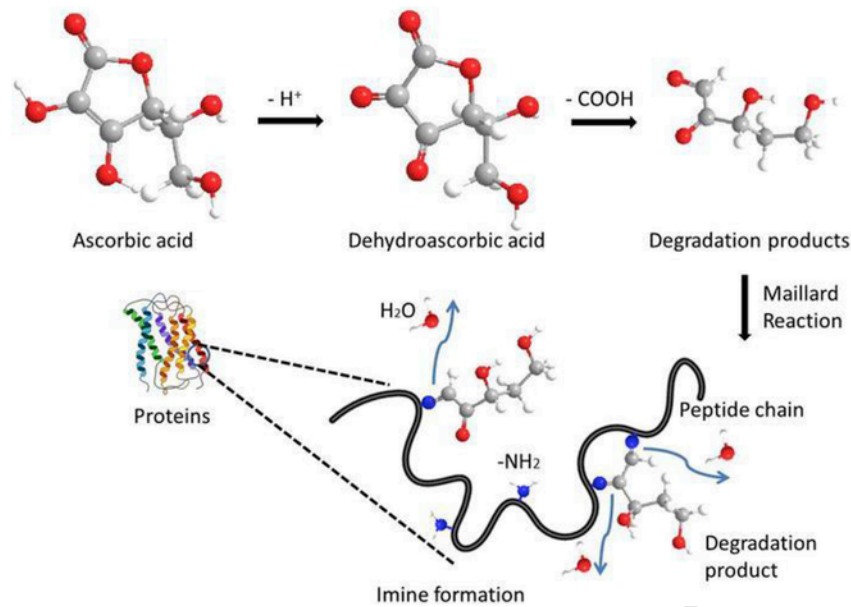
**Fig. 6.** Schematic diagram of the mechanism for the Maillard reaction between ascorbic acid and WPI.

fore, the effect of pH is significant in terms of the Maillard reaction between WPI and ascorbic acid, as discussed before, such that more —COO⁻ and —NH$_2$ groups in the amino acids will be converted to —COOH and —NH$_3^+$, respectively, at low pHs, limiting the formation of imine groups. For the Maillard reaction and the effect of ascorbic acid, the colour change (redness generation) of WPI molecules can be observed by the naked eye when the concentration of ascorbic acid is over 0.001 g/mL (with an AA:WPI ratio of 1:100). Lower concentrations (<0.001 g/mL) of ascorbic acid were tested, but no redness could be observed in the spray-dried WPI-ascorbic acid powders. This situation means that we are not claiming that lower concentrations of ascorbic acid are safe, only that the lower limit of detection by the naked eye is a concentration of 0.001 g/mL for ascorbic acid. This concentration for the AA:WPI ratio of 1:100 is also the lower limit of detection for the measurements by UV–Vis spectrophotometry and spectrofluorometry.

### 3.3. Aging as a function of time and temperature

The storage temperature (20–80 °C) has been found to significantly affect the Maillard reaction between WPI and ascorbic acid. The colour changes (from the original white to a steady red) have been analysed over time during the aging of the spray-dried WPI-ascorbic acid powders. The concentration of 0.005 g/mL for ascorbic acid has been used for clear observations, while the concentration of 0.001 g/mL is studied to represent the behaviour at the lower limit of detection. The images for the colour changes as functions of time are shown in the supporting Figs. S3–S10, with their colour parameters (L*, a*, b*, E* and C*) as obtained by the MATLAB analyses and shown in the supporting Tables S3–S10. These colour parameters have been plotted with respect to aging time in Fig. 7a-e (x-axis on a log$_{10}$ scale) to study the effect of storage temperature. Overall, both a higher concentration of ascorbic acid and a higher storage temperature have significantly increased the rates of colour change.

The final values of the colour parameters are limited by the concentration of ascorbic acid in the Maillard chemical reaction. The decreases in the L* values and the increases in the a* values for the 0.005 g/mL samples are both about three times greater than the decreases in the L* values and the increases in the a* values for the 0.001 g/mL samples (Fig. 7a and b), due to the light adsorption by the

aged WPI-ascorbic acid powders. However, as shown in Fig. 5a–c, the increases in the peak heights for the light adsorption (at 380 nm and 500 nm) and fluorescence emission (at 440 nm and 600 nm) are about five times greater for the 0.005 g/mL sample compared with the 0.001 g/mL sample, corresponding to the difference (five times) in the concentration of ascorbic acid. The reason for this result is that the spectral intensity (peak height) is generally directly proportional to the component concentration at the same light wavelength (peak position), according to the Beer-Lambert Law I = εcl (where I, ε, c and l are the spectral intensity, attenuation coefficient, concentration and cuvette length, respectively).

The temperature effect was studied by Maillard (1912) himself, finding that high temperatures increase Maillard reaction rates. High temperatures accelerate the formation of covalent bonds and cause unfolding of proteins, promoting the Maillard reactions chemically and physically (Teodorowicz et al., 2017), explaining the changes in the colour parameters apart from the b* value (yellowness). As shown in Fig. 7c, the changes in the b* values depend on the storage temperature more significantly than the concentration of ascorbic acid. The yellowness generation is less likely related to the Maillard reaction between WPI and ascorbic acid, but may due to the aging of the WPI itself at different temperatures. Moreover, the changes in the E* values that are calculated from the L*, a* and b* values show the overall colour differences between these samples (Fig. 7d), while the changes in C* values that are calculated from a* and b* values show the overall chroma (Fig. 7e), again supporting the above conclusions about the effects of temperature and concentration. The Maillard reaction rate is exponentially related to the temperature and can be described by the Arrhenius equation: k = A × exp(-E$_a$/RT), where k, A, E$_a$, R and T are the rate constant, the frequency factor, the activation energy, the universal gas constant and the absolute temperature, respectively (Martins et al., 2000). The times required for complete Maillard reactions (to a steady extent of redness) were 10080, 1080, 840 and 110 min for 0.005 g/mL ascorbic acid, and 30240, 4380, 240 and 70 min for 0.001 g/mL ascorbic acid at 20 °C (room temperature), 40 °C, 60 °C and 80 °C, respectively. Here, the Maillard reaction rates have been defined as the a* values divided by the times required for complete Maillard reactions. As shown in Fig. 7f, the fitted curves show an exponential relationship between the Maillard reaction rates and the storage temperature, where the Maillard reaction rates are higher for 0.005 g/mL ascorbic acid samples.
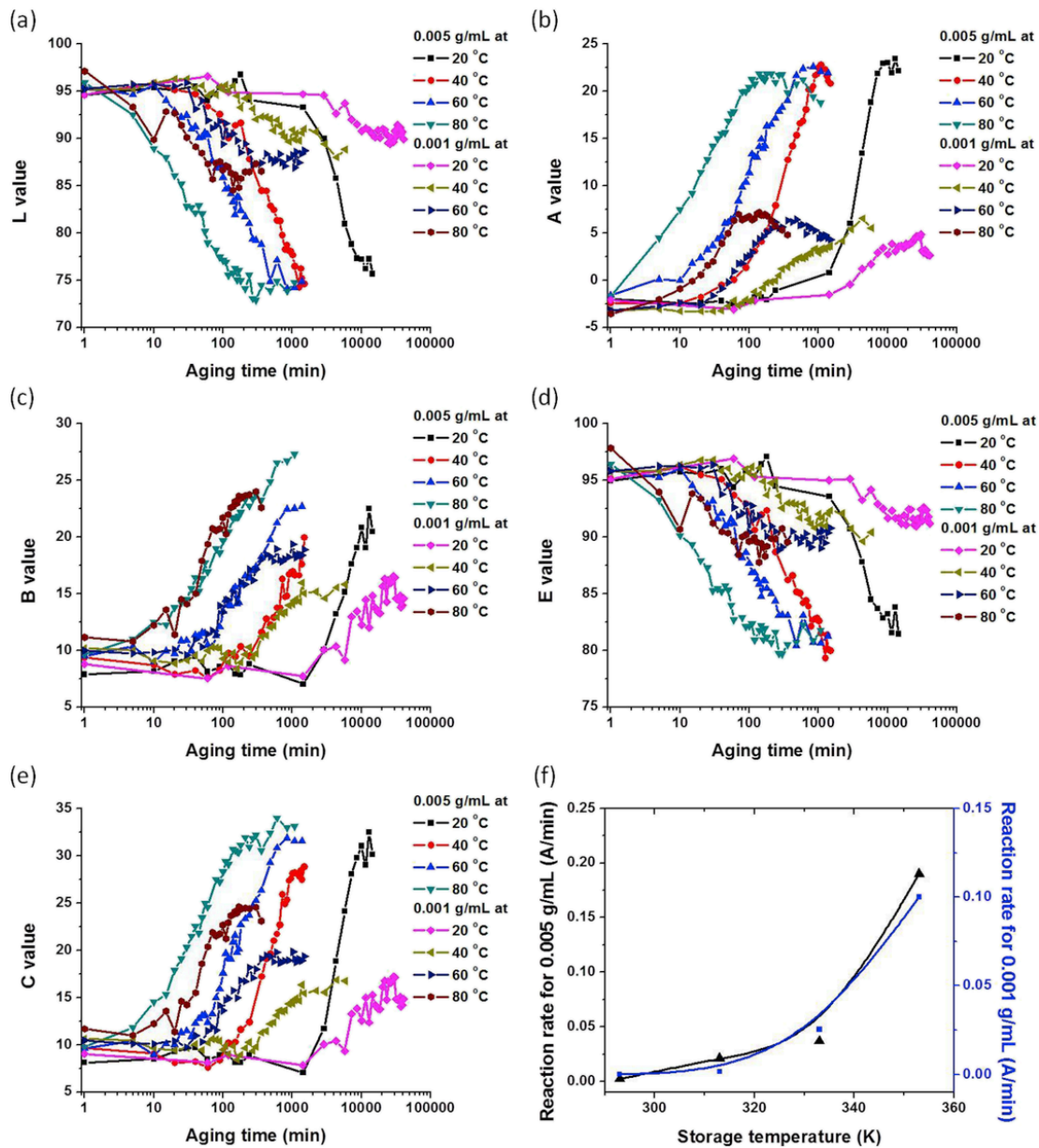
**Fig. 7.** Changes in the colour parameters of (a) L*, (b) a*, (c) b*, (d) E* and (e) C* as a function of time from the original white to a steady red colour for the spray-dried WPI-ascorbic acid (0.005 and 0.001 g/mL) powders stored at 20–80 °C. (f) Estimated Maillard reaction rate for the spray-dried WPI-ascorbic acid (0.005 and 0.001 g/mL) at 20–80 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The Maillard reaction for 0.001 g/mL ascorbic acid (AA:WPI ratio of 1:100) at 20 °C can be completed within one month (30240 min, equal to 21 days) and the detectable colour change (Maillard reaction) starts from the second day of aging (1440 min, equal to 24 h). For the preservation of the infant/baby milk powders listed in Table 1 (AA:WPI ratios over 1:100), low temperatures (4 °C in the refrigerator) or low oxygen levels (N$_2$ atmosphere) are suggested to be used for household or industrial storage according to the above investigations. As shown in Fig. 8 (images) and Table 2 (values), the samples sealed in air and stored at 20 °C can turn red from the original white colour by aging for one week, where the a* value increases (redness generation) are 25 ± 3 and 5 ± 1 for the samples containing 0.005 and 0.001 g/mL ascorbic acid, respectively. Since the Maillard reaction rate is exponentially related to the temperature (Arrhenius equation), low temperature storage at 4 °C shows significant decreases in the a* values compared with the room conditions. The redness can be hardly observed for the 0.001 g/mL ascorbic acid sample by the naked eye, but is detectable by
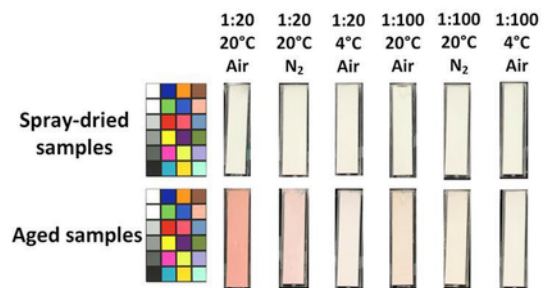


**Fig. 8.** Effect of the low temperature and the N$_2$ atmosphere on the colour changes by aging for one week. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**
The a* value increases (redness generation) by the aging for one week under different conditions.

| Conditions | 0.005 g/L ascorbic acid (clearer observation) | 0.001 g/L ascorbic acid (lower limit) |
|---|---|---|
| Seal in air and store at 20 °C | $25 \pm 3$ | $5 \pm 1$ |
| Seal in air and store at 4 °C | $2.8 \pm 0.9$ | $1 \pm 1$ |
| Seal in $N_2$ and store at 20 °C | $10 \pm 2$ | $2 \pm 1$ |

the MATLAB colour analysis. The a* value increases are only $2.8 \pm 0.9$ and $1 \pm 1$ for the 0.005 g/mL and 0.001 g/mL ascorbic acid samples, respectively. Since oxygen can promote the Maillard reaction (Reihl et al., 2004), $N_2$ atmosphere has been tested for the sample storage. The results show that the $N_2$ atmosphere storage can reduce the redness generation, where the a* value increases are only $10 \pm 2$ and $2 \pm 1$ for the 0.005 g/mL and 0.001 g/mL ascorbic acid samples, respectively. This work suggests that the low temperature storage at 4 °C in the refrigerator is necessary and effective (equal or better than the $N_2$ atmosphere storage) to reduce the Maillard reaction for household use, especially when the infant/baby milk powders are unpacked at home. The toxicological levels of the Maillard reaction products have not been assessed clinically for the spray-dried WPI-ascorbic acid powders. This situation means that we are not claiming that the Maillard reaction of WPI and ascorbic acid creates toxic products, only that the formulated infant/baby milk powders on the market with AA:WPI ratios higher than 1:100 (Table 1) may require low temperatures (4 °C) or low oxygen ($N_2$ atmosphere) storage to prevent Maillard reactions (redness generation). It is also not suggested that infant formulae for AA:WPI ratios lower than 1:100 have no Marillard reaction, although the redness generation cannot be observed by the naked eye.

## 4. Conclusions

This work shows obvious redness generation for spray-dried WPI-ascorbic acid powders. The lower limit of detection by human observation is 0.001 g/mL for the concentration of ascorbic acid. The redness is related to the adsorption of violet light (380 nm) and blue/green light (500 nm). Fluorescence analysis suggests the formation of formyl threosyl pyrroles and crosslinked poly(amino acids). DSC analysis shows that ascorbic acid peaks disappear as the Maillard reaction progress. FTIR and Raman studies mainly show the change of amide groups and skeletal structure during the aging of WPI. The storage temperature has been found to significantly affect the Maillard reactions between WPI and ascorbic acid. Results show that the Maillard reaction rates between WPI and ascorbic acid are fast in spray-dried powders even at 20 °C. The formulated infant/baby milk powders on the market are suggested to require low temperatures (4 °C) or low oxygen ($N_2$ atmosphere) storage to reduce the extents of Maillard reactions (redness generation).

## Nomenclature

| AA | ascorbic acid |
|---|---|
| ATR | attenuated total reflection |
| DSC | differential scanning calorimetry |
| FTIR | Fourier transform infrared |
| MATLAB | matrix laboratory |
| SEM | scanning electron microscopy |
| WPI | whey protein isolate |

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