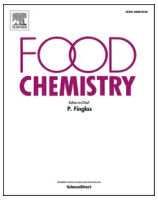
Food Chemistry 435 (2024) 137566

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Formation of di-Tyrosine in pasteurized milk during shelf storage

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ARTICLE INFO

Keywords:
Di-Tyrosine
Milk protein oxidation
Light exposure
Immune gold labelling
Pasteurized milk
Riboflavin

ABSTRACT

Formation of the protein crosslink di-Tyrosine was studied in PET-bottled pasteurized milk exposed to fluorescent light in a commercial display cabinet. An HPLC method with fluorescence detection was developed and intra-laboratory validated using pure di-Tyrosine synthesized on purpose. Di-Tyrosine was detected after 1-day lightening and increased up to 7 days, reaching around 250 and 320 μg/g protein in whole and partly skimmed milk, respectively. Afterward, a progressive decrease occurred. By transmission electron microscopy with specific immune gold labelling, presence of di-Tyrosine was observed for the first time on the surface of casein micelles of lightened milk. The crosslink formation, however, did not bring to protein aggregation phenomena detectable by laser light scattering measurements. Exposure to light also induced degradation of riboflavin and decrease of yellowness index. Di-Tyrosine proved to be a suitable indicator to evaluate the progress of protein oxidation in pasteurized milk during storage on the market.

1. Introduction

The shelf stability of pasteurized milk represents a major concern for the manufacturers since it involves microbiological, biochemical, and sensory issues. Due to both the minimal heating conditions adopted in industrial pasteurization and the refrigerated storage, this type of drinking milk keeps distinguished sensory traits that are appreciated by consumers looking for fresh tasting food products. As a drawback, pasteurized milk has a limited storage stability, normally one to two weeks, depending on processing conditions (IDF, 2022). The shelf-life of commercial pasteurized milk is primarily restricted by bacteria surviving the thermal process. It has been evidenced that *Pseudomonas* and *Bacillus* dominate among the post-pasteurization bacterial contaminants and continue to grow during milk storage due to their psychrophilic attitude (Martin et al. 2018). Heat-resistant enzymes, either native or microbial, may degrade milk components causing flavour impairment well before the acidification takes place (Ziyaina et al., 2019; D'Incecco et al., 2021). Furthermore, it has long been known that exposure of milk to light activates photo-degradative reactions associated to development of specific off-flavours (Hellwig, 2019; Meng et al., 2023). In a previous study, we have simulated the storage of pasteurized milk as it may occur in a real situation of retail (Limbo et al., 2020). Pasteurized milk packaged in clear-PET bottles was kept refrigerated in the dark for a total of 13 days but including temporary light exposure periods of 6, 12 or 18 h. Even though most of the evaluated chemical and enzymatic parameters remained substantially stable, light exposure negatively affected milk flavour. The presence of both oxygen and endogenous photosensitizers, namely riboflavin (RF), have been reported to be favourable conditions for promoting photo-oxidation reactions in milk whose target components can be unsaturated lipids, proteins, sugars and vitamins (Webster et al., 2009; Hellwig, 2019). The RF-mediated photo-oxidation reactions have been extensively investigated and two different mechanisms (Type I and Type II) were recognized with one prevailing over the other depending on environmental conditions (Cardoso et al., 2012; Pattison et al., 2012; Fuentes-Lemus et al., 2018). In milk under illumination, RF readily goes to the triplet state (^{3}RF) that can react with either oxidable components, principally tyrosine (Tyr) and tryptophan (Trp) residues, leading to formation of protein radicals (Type I), or with oxygen generating singlet oxygen (Type II). This last can react with proteins and generate endoperoxides (Fuentes-Lemus and Lopez-Alarcon, 2020). Tyr-derived phenoxyl radicals and Trp-derived indolyl radicals are able to dimerize by producing crosslinks such as di-Tyr, di-Trp or Tyr-Trp (Fuentes-Lemus and Lopez-Alarcon, 2020). Cys residues have been shown to react with oxidized Tyr residues to form covalent adducts (Rossi et al., 2022). It is noteworthy that, when crosslinks form at intermolecular level, protein molecules interactions result in stable aggregates progressively building three-dimensional networks.

Protein aggregation phenomena in milk have been mainly related to

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https://doi.org/10.1016/j.foodchem.2023.137566
Received 7 July 2023; Received in revised form 20 September 2023; Accepted 21 September 2023
Available online 26 September 2023

thermal treatments and hence assumed to be negligible in pasteurized milk (Renzone et al., 2022; Pellegrino & D'Incecco 2022). Beside the rearrangement of disulphide bonds, literature strongly supports the Maillard reaction to be the main responsible mechanism of protein aggregation in lactose-containing milk products, whereas crosslinked species formed *via* isopeptide bonds, such as lysinoalanine, prevail in products lacking lactose (Nielsen et al., 2022; Pellegrino et al., 2022). Relatively fewer studies have investigated milk protein crosslinking induced by oxidation phenomena (Hellwig, 2019). Furthermore, oxidation mechanisms have been mainly investigated in model systems where milk proteins were studied individually and where a thermal treatment was sometimes combined with the illumination (Dalsgaard et al., 2011; Østdal et al., 2000; Fuentes-Lemus et al., 2018; Rossi et al., 2022). Chen et al. (2019) and Scheidegger et al. (2010) studied oxidative modifications of proteins in dried dairy products, including whole and skimmed milk powders and infant formula, in terms of formation of protein carbonyls and di-Tyr. Havemose et al., (2006) evaluated the effect of cow feeding composition on the oxidative stability of milk proteins and lipids. To the best of our knowledge, however, no study has investigated formation of di-Tyr in commercial pasteurized milk exposed to light during refrigerated storage.

To fill this gap, the present work aimed to investigate the light-induced formation of di-Tyr in pasteurized milk. Commercial pasteurized milk in clear PET bottles was kept exposed to fluorescent light in a display cabinet for up to 14 days and bottles were sampled at defined times. The casein fraction was analysed for the formation of di-Tyr. To this aim, a direct HPLC method was preliminarily developed, and pure di-Tyr was synthesized for the quantification. Riboflavin (vitamin B2) and its degradation products were also determined by HPLC to evaluate their involvement in photo-oxidation of milk. Measurement of particle size distribution in milk was used to investigate possible formation of protein aggregates and transmission electron microscopy with immune gold labelling of di-Tyr residues was performed for their localization on casein micelles.

2. Materials and methods

2.1. Milk samples and light exposure trial

In a preliminary trial pasteurized milk was exposed to light to prepare samples suitable for developing an analytical method for di-Tyr measurement with the required performances. Subsequently, the core experiment was aimed to systematically study the trend of di-Tyr accumulation and other related changes induced by light exposure of milk. The experiment was repeated in the subsequent month and average data are presented. Single-lot 500-mL bottles of high temperature short time (HTST) pasteurized milk, including full fat (3.5 g/100 mL) and reduced fat (1.5 g/100 mL) milk, were collected at a local industrial plant and brought to the laboratory in a light-protecting refrigerated box. Bottles were in clear polyethylene terephthalate (PET); label sleeves were removed in order to achieve homogeneous exposure of bottled milk to light. Milk bottles were kept in a commercial vertical display cabinet (Costan, Italy) at 5 ± 1 °C under exposure to light of tubular fluorescent lamps TL-D super 58 W/840, 5.240 lm, (Philips, Italy) and were periodically turned and moved along the shelf. The characteristic emissions of the lamps were described in a previous study (Limbo et al., 2020). Specifically, irradiance values (W m^{-2}) were 1.1–2.9, 2.0–4.9 and 1.3–2.8 when measured in the ranges 360–490 nm, 491–590 nm and 591–780 nm of the visible spectrum. In the same ranges of the visible spectrum values of radiant exposure for 12 h (J cm^{-2}) were 4.3–12.5, 8.6–21.2, 5.6–12.0, respectively. To prepare control samples, milk bottles wrapped in an aluminium foil were kept in the same cabinet. At each sampling time (0, 1, 3, 7 and 14 days) and for each milk type (full fat and reduced fat), two bottles of light-exposed milk and one bottle of control sample were withdrawn from the cabinet and analysed. Milk pH value was measured in each bottle at opening and no significant changes were detected compared to the initial value (pH 6.72 ± 0.05) (data not shown).

2.2. Preparation of pure di-Tyrosine

Di-Tyrosine was synthesized in five steps from commercially available 3-iodo-L-tyrosine, by a slightly modified procedure of Skaff et al., 2005. Briefly, the amino function was protected with a *tert*-butyloxycarbonyl group (Boc), followed by benzylation with benzyl bromide and potassium carbonate. A one-pot, tandem Miyaura borylation-Suzuki coupling from the intermediate iodo-amino acid gave the protected di-Tyrosine. Eventually, hydrogenolysis with palladium-on-charcoal in methanol followed by acidic hydrolysis with 4 M HCl in dioxane afforded di-Tyrosine, whose spectroscopic data completely matched with those reported in the literature (Lygo, 1999; Takasaki et al., 2005). The synthetic procedure is described in Suppl. Material 1.

2.3. Di-tyrosine analysis by HPLC

Di-tyrosine (di-Tyr) content was determined in hydrolysed casein samples. Casein was obtained from milk by acidification to pH 4.6 and centrifugation at 3000*g* for 20 min. Casein pellet was kept at −20 °C until analysis. In addition, the di-Tyr content was determined in the whey protein fraction of milk exposed to light for 7 days and the respective control. This fraction was recovered from the whey obtained after acid casein precipitation, as described above. The whey was heated at 90 °C for 10 min in a thermostatic water bath, then was cooled to room temperature and centrifuged at 3000*g* for 20 min. The pellet was kept at −20 °C and analysed as described hereafter.

A 20-mg aliquot of protein, either casein or whey protein, was added with 8 mL of 6 N HCl in a glass vial and hydrolysed under vacuum at 110 °C for 23 h. After filtration, 2 mL hydrolysate was dried at 30 °C with a Rotavapor (Büchi Italia, Cornaredo, MI, Italy) and carefully resuspended with 250 μL 0.1 % (v/v) trifluoroacetic acid in milliQ water. The sample (10 μL) was analysed by HPLC using an Alliance 2695 equipment (Waters, Milford, MA, USA) and a fluorescence detector Hitachi L2480 (VWR, Milan, Italy) set at 285 nm e 400 nm for excitation and emission, respectively. The chromatographic column Platinum EPS C18, 4.6 mm i.d. × 150 mm, 100 Å pore size, 3 μm particle size (Grace Alltech, Italy) was maintained at 30 °C and eluted using a gradient of solvent A (0.1 % (v/v) trifluoroacetic acid in milliQ water) and B (0.1 % (v/v) trifluoroacetic acid in acetonitrile) at a flowrate of 0.8 mL/min. The gradient was as follows: 2 % B for 1 min, from 2 to 5 % B in 14 min, from 5 to 98 % B in 10 min and remaining at 98 % B for 5 min (washing step). The column was then re-equilibrated at 2 % B for 5 min prior the next injection. Chromatographic data were processed using Empower2 software (Waters). Di-Tyr content was calculated by the external standard method and expressed as μg/g protein. The exact protein content of the hydrolysate was determined in 2 mL using the Kjeldahl method (ISO 8968-1/IDF 20-1).

The HPLC method was validated in terms of linearity, limits of detection (LOD) and quantification (LOQ), and inter-day repeatability as summarized in Table S1. The linearity of the fluorescence response of di-Tyr was assessed on nine concentration levels in the range 0.01–14 μg/mL, approximately corresponding to 0.5–700 μg/g protein under the described conditions. The LOD and LOQ of di-Tyr, obtained as the signal-to-noise ratios of 3 and 10, respectively, were 0.004 μg/mL and 0.014 μg/mL. The repeatability was calculated as the relative standard deviation (RSD) value of peak area counts of a whole milk and a semi skimmed milk at two different levels of di-Tyr. For each sample, three replicates were carried out in two different days to calculate the overall (intra- and inter-day) repeatability.

2.4. Evaluation of proteolysis extent in milk

Selected milk samples were analysed by capillary zone

electrophoresis to evaluate the extent of casein degradation. The method described by Pellegrino et al. (2022) was followed. A P/ACE MDQplus system was used, including a Diode Array Detector (Sciex CE&Biopharma, Milan, Italy) with acquisition wavelength set at 214 nm, and equipped with a 50 cm fused silica capillary column (DB-WAX 126-7012, Agilent Technologies, Milan, Italy).

2.5. Transmission electron microscopy of milk with immune gold labelling of di-Tyrosine

Samples for transmission electron microscopy (TEM) were prepared avoiding alterations to native milk proteins. Both semi-skimmed milk after 7-day light exposure and its control were ultra-centrifuged (SW 28 rotor, L5-75 analytical ultracentrifuge, Beckman Coulter Inc., CA, USA) at 80,00*g* at 4 °C for 60 min to obtain the respective native casein micelles. The pellet was collected and embedded in resin for TEM as described by D'Incecco (2022). Briefly, humid pellet was mixed 1:1 (w/w) with low temperature gelling agarose (3 % in water, melted at 35–40 °C; VWR, Milan, Italy) and the suspension was layered onto a microscope slide, allowed to set, and then cut into 1-mm^3 cubes. Four cubes of each sample were fixed in 1 mL of fixative solution (glutaraldehyde 1 %, paraformaldehyde 4 % in Na cacodylate buffer, w/v; Agar Scientific, Stansted, UK). Cubes were washed in the same buffer for 30 min and then post-fixed in 1 % osmium tetroxide supplemented with 1 % potassium ferrocyanide in the dark (EMS, Hatfield, PA). A second 30-min washing was carried out with the same buffer. Dehydration of cubes was carried out in a series of ethanol solutions prior to embedding in London Resin White (EMS, Hatfield, PA, USA) into a mould, avoiding trapping of air, followed by curing at 60 °C for 24 h. Ultrathin sections, 70 nm thick, were cut from the resin blocks with a diamond knife using a ultramicrotome PowerTome XL (RMC Boeckler, USA). Sections were collected on 200-mesh nickel grids coated with forward film. Immune gold labelling was carried out letting the grid to float with the sections facing down on a drop of different solutions (D'Incecco, 2022). Sections were incubated in 50 mM Glycine in 20 mM PBS at room temperature for 20 min, and then rinsed with few drops of PBS. Sections were incubated on 50 mmol PBS containing 1 % BSA and 0.01 % Tween 20 (both from Aurion, Wageningen, NL). The sections were then incubated at 4 °C overnight in the primary monoclonal antibody to di-Tyr that had been raised in mouse (Biorbyt, Cambridge, UK); this antiserum was diluted 1:100 or 1:1,000 in PBS. After careful washing with PBS, the sections were incubated at room temperature for 2 h in a 15-nm colloidal-gold labelled secondary anti-mouse serum that had been raised in goat (Biorbyt, Cambridge, UK). Sections were washed with PBS and post-fixed with 0.2 % glutaraldehyde for 5 min. Finally, the grid was placed on a drop of distilled water and dried on a filter paper. Controls consisted of: i) pure di-Tyr solution (10 μg/mL); ii) sections incubated with a pre-immune primary antibody followed by the secondary gold-conjugate antibody; iii) sections incubated with the secondary antibody only. Prior observation, sections were stained with 2 % aqueous uranyl acetate and 2 % aqueous lead citrate. Stained sections were examined with a 120 kV TEM Talos L120C (Thermo Fisher Scientific, Waltham, US).

2.6. Particle size analysis

The particle size distribution in milk samples was measured by laser light scattering using a Malvern Mastersizer 3000 instrument (Malvern Instrument Ltd, Malvern, UK). Spherical particles were assumed and refractive indices of 1.47 and 1.33 were used for milk and dispersant (MilliQ water) respectively. Milk sample was added dropwise into the Hydro LV device filled with MilliQ water at 25 °C under stirring at 2300 rpm until ~ 8 % obscuration level was reached. The "general purpose" conditions based on volume distributions were used to evaluate data. Size parameters by percentiles (Dx_{10}, Dx_{50}, Dx_{90}) and mean ($D_{[4,3]}$) were derived from Mastersizer software (version 3.81). All samples were measured in duplicate, and each measurement was repeated 5 times.

2.7. Riboflavin and other flavin compounds analysis by HPLC

Riboflavin (RF), flavin mononucleotide (FMN), lumichrome (LC) and lumiflavin (LF) contents were determined by the HPLC method proposed by Fracassetti et al. (2018). Briefly, milk samples were skimmed by centrifugation and then ultrafiltered on Microcon disposable 10-kDa membrane filters (Millipore, Milford, MA, USA). The permeate (50 μL) was analysed using an Alliance 2695 HPLC equipment (Waters, Milford, MA, USA) and a Hitachi L2480 fluorescence detector (VWR, Milan, Italy) set at 420 nm excitation and 530 nm emission wavelengths, respectively. The chromatographic column Hypersil ODS (100 × 3 mm, 3 μm particle size) (CPS, Milan, Italy) was thermostated at 40 °C. Commercial RF, FMN (Sigma-Aldrich, St. Louis, MO, USA), LC and LF (Santa Cruz Biotechnology, CA, USA) were used for quantification by the external standard method. Analyses were conducted in triplicate.

2.8. Milk colour evaluation

Colour measurement was performed using a portable tristimulus colorimeter Chroma Meter CR-400 (Konica Minolta, Nieuwegein, Netherlands). The colour parameters (L*, a*, b*) were measured with three readings per sample (technical replicates) and different colour indexes were calculated as described by Milovanovic et al., (2020). Yellowness index (YI) and whiteness index (WI) were calculated using the following equations:

$$YI = 142.86\frac{b^*}{L^*}$$

$$WI = 100 - \left[(100 - L^*) + a^{*2} + b^{*2}\right]^{1/2}$$

The total colour difference (ΔE*) measured for the light-exposed milk and the corresponding control sample was obtained from the equation:

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

ΔE* is generally used for grading the colour difference perceived by human eye. Based on ΔE* values, colour differences were classified as not perceptible (ΔE* < 1), perceptible (1 < ΔE* < 3) and highly perceptible (ΔE* > 3) (Milovanovic et al., 2020).

2.9. Statistical treatment of data

Statistical differences were evaluated using the SPSS Win 12.0 software Version 27 (SPSS, IBM Corp., Chicago, IL). Three-way ANOVA was carried out to compare data of particle size after normality and homoscedasticity tests were verified. Differences at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant.

3. Results and discussion

3.1. Development of a direct HPLC method for di-Tyrosine analysis

Formation of di-Tyr has been widely investigated in human tissues, as it has been related to the onset of several neurological diseases (Al-Hilaly et al., 2016; Grune, 2020; Kehm et al., 2021), and in food products (Renzone et al., 2022). Therefore, various analytical approaches have been proposed for di-Tyr detection, including ELISA tests (Chen et al., 2019; Mo et al., 2022), LC-MS/MS (Fenaille et al., 2006; Nguyen et al., 2017), capillary electrophoresis (Tilley et al., 2006), or measurement of specific fluorescence (FL) of protein (Scheidegger et al., 2010). Due to the high fluorescence response of di-Tyr molecule and its stability to acid hydrolysis, direct HPLC-FL methods have been used to analyse this compound in hydrolysed protein samples (Havemose et al.,

2006; Fuentes-Lemus et al., 2018). However, to the best of our knowledge, no validated methods were produced with this technique and literature data are expressed in different ways making data comparison rarely feasible. Considering this, we have developed a direct HPLC-FL method and carried out an intra-laboratory validation. In parallel, pure di-Tyr molecule was synthesized on purpose (Suppl. Material 1).

Typical chromatograms obtained for pure di-Tyr solution, a SML sample exposed to light for 7 days and the respective control SMD are shown in Fig. 1. Di-Tyr elutes as a main peak with a shoulder, likely given by a minor stereoisomer, in a clean area of the chromatogram.

Unless differently indicated, di-Tyr data are expressed as μg/g protein. The calibration curve obtained the pure di-Tyr molecule was linear (r = 0.999) in the range 0.01–14.0 μg/mL. The method proved to have a good repeatability, with an inter-day RSD < 10 %, and allowed to quantify di-Tyr amount (LOQ) as low as 0.014 μg/mL, roughly corresponding to 0.7 μg/g protein. The fundamental parameters of the validated method are summarized in Table S1. Nguyen et al. (2017) used a LC-MS/MS method to quantify di-Tyr in zein protein with a LOQ of 0.14 μg/g protein and an inter-day RSD from 4.4 and 16.6 %.

3.2. Formation of di-Tyrosine in milk exposed to light

The trends of di-Tyr in milk samples during storage are shown in Fig. 2. No presence of di-Tyr was detected in freshly produced pasteurized milk (t = 0) as well as in the control samples (designed as WMD and SMD for whole and partly skimmed milk, respectively) up to 14 days of storage in the dark. On the contrary, di-Tyr formed in the samples exposed to light (WML and SML), with the highest levels observed at 7 days and, afterward, a slow decrease. The reason for this decrease remains to be directly investigated. However, given the high stability of di-Tyr molecule, we have hypothesized that casein degradation occurring in pasteurized milk during storage, mostly mediated by plasmin, may also result in the release of fragments bearing di-Tyr residues. Dalsgaard et al. (2008) reported that photooxidation of individual milk proteins affects their susceptibility to plasmin digestion. Considering this finding, we have evaluated the extent of proteolysis that occurred in both WM and SM during a 14-day storage, as a preliminary confirmation of our hypothesis. Individual casein fractions were separated by capillary zone electrophoresis and corrected peak areas were calculated (Fig. 3). The main α_s-caseins (α_s-1 and α_s-0) remained stable in milk stored in the dark, while both fractions decreased by 15–30 % in milk exposed to light. As expected, β-caseins (βA1 and βA2) decreased in milk stored in the dark, due to the plasmin activity, but a more intense degradation was observed in milk exposed to light. These trends were comparable for WM and SM. Although further investigation is needed, our data indicate an increased susceptibility to proteolysis of casein in milk exposed to light.

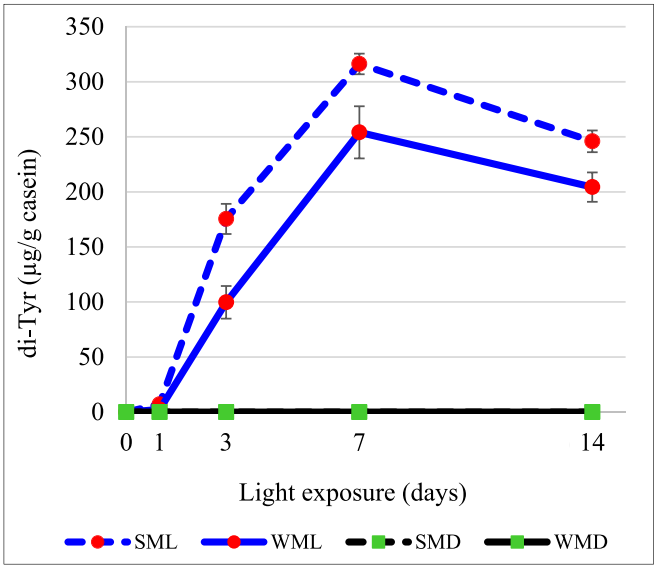

Fig. 2. Levels of di-Tyr in pasteurized milk during 14-day storage. SML = semi-skimmed milk exposed to light; WML = whole milk exposed to light; SMD = semi-skimmed milk kept in the dark; WMD = whole milk kept in the dark. The error bars indicate the standard deviation.

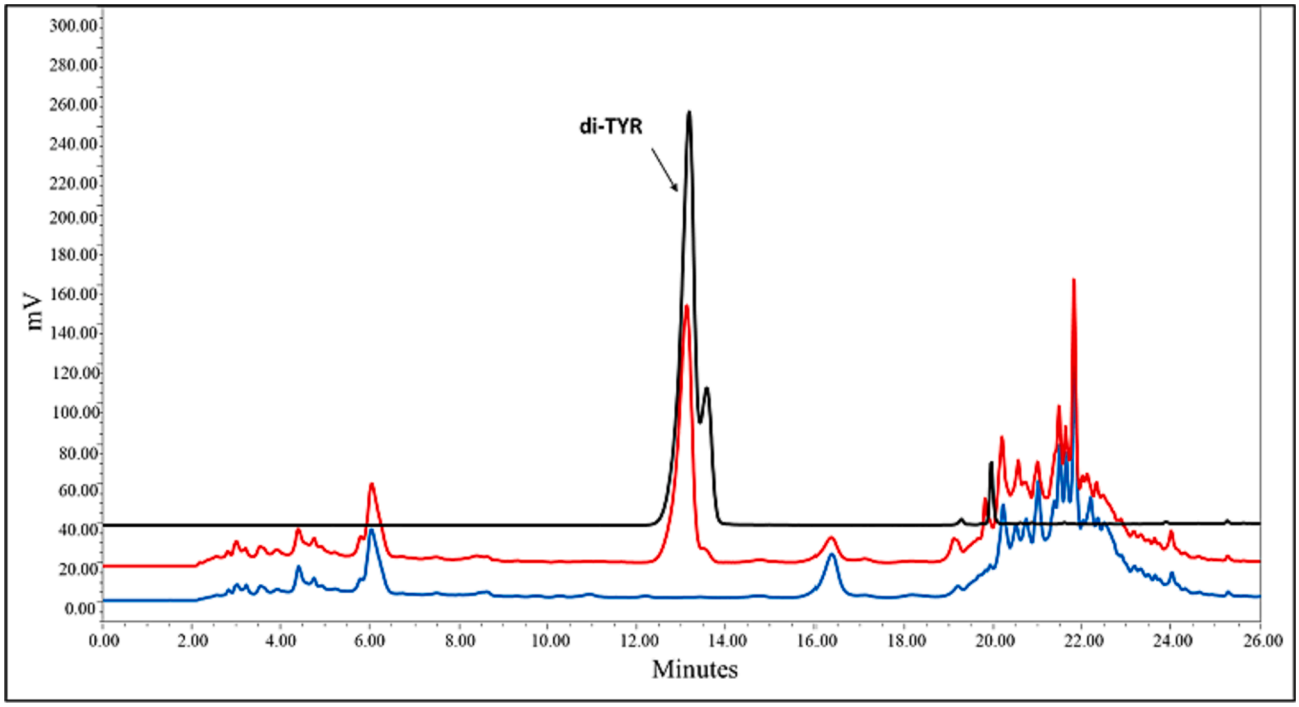

Fig. 1. HPLC chromatograms of di-Tyr in standard solution (black) and in pasteurized semi-skimmed milk exposed to light (red) or kept in the dark (blue) for 14 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

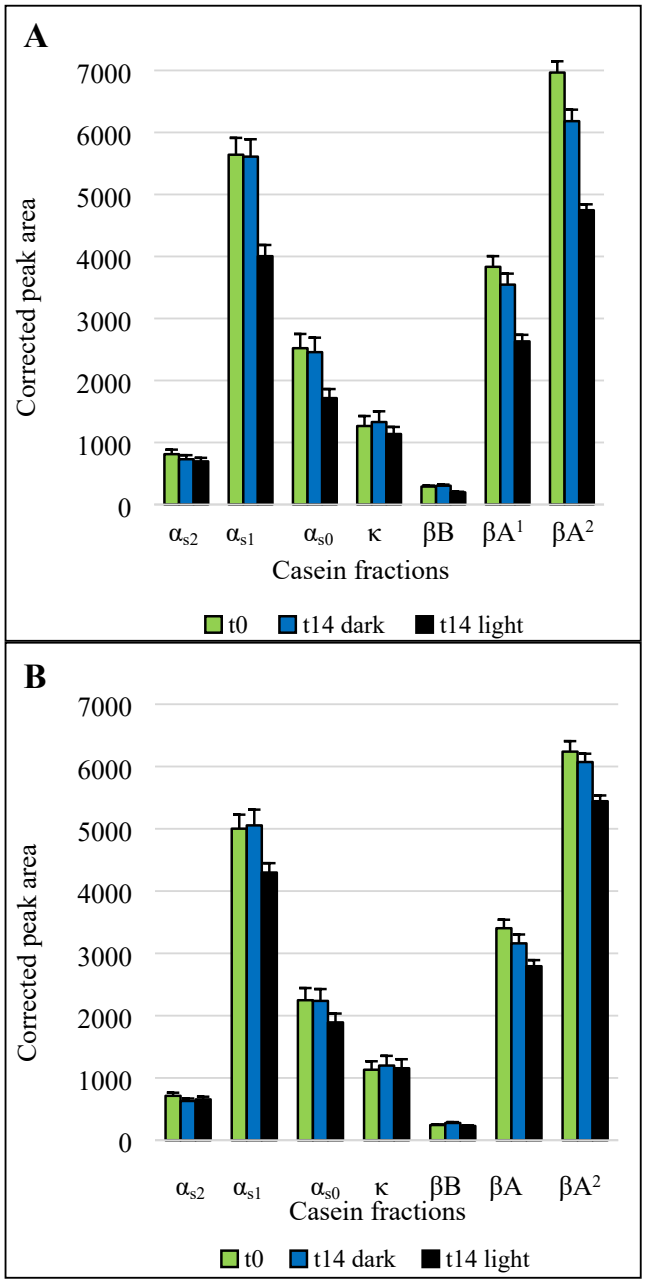

Fig. 3. Casein fractions in pasteurized semi-skimmed milk (A) and in pasteurized whole milk (B) determined by capillary zone electrophoresis at the beginning of storage (t0) and after 14 days of dark (t14 dark) or light (t14 light) storage. The error bars indicate the standard error of triplicate analysis.

Notably, di-Tyr levels were higher in SML samples than in the corresponding WML starting from the shortest illumination times. Indeed, presence of di-Tyr was already detected after 1 day of light exposure, with 2 and 7 μg/g protein in WML and in SML, respectively. At 7 days of illumination values were around 250 and 310 μg/g protein in WML and in SML, respectively. Assuming a casein content of 28 g/L milk, di-Tyr levels detected after 1-day illumination approximately correspond to 0.15–0.5 μmol/L milk while those detected after 7 days correspond to 19.4–24.1 μmol/L, depending on fat content. As previously mentioned, literature data on di-Tyr levels in liquid milk exposed to light are very few. Havemose et al. (2006) exposed to fluorescent light two milk samples obtained from cows' groups given a different diet. A progressive accumulation of di-Tyr was recorded, regardless the diet, and mean values 0.20–0.25 μmol/L were reached after 24-hour illumination. Those values are very close to ours for the same illumination time.

Scheidegger et al. (2010) exposed reconstituted milk powders, either full fat or skimmed, to fluorescent light. The authors observed a sharp increase in FL emission at the wavelength specific to di-Tyr during the first 8 h, with a slight prevalence in skimmed milk over full fat milk. Then values levelled off and kept constant until a total exposure of 24 h.

Fenaille et al. (2006) detected no presence of di-Tyr in commercial samples of both pasteurized and UHT milk, while levels in liquid infant formula ranged from 0 to 9.2 μg/g protein. Chen et al. (2019) reported levels of the same magnitude, i.e. 3–6.5 nmol/mg protein corresponding to 1–2.3 μg/g protein, in powdered infant formula. Although not indicated, all these samples were likely packaged in light-protective containers, thus presence of di-Tyr may derive from the protein ingredients used in product formulation. Dalsgaard et al. (2011) reported that the structural characteristics of protein molecules influence their susceptibility to formation of di-Tyr. The authors demonstrated that, when pure protein solutions containing RF were exposed to light, the amount of di-Tyr was approximately three times higher in the low structured β-casein than in globular proteins like β-lactoglobulin and serum albumin. The experimental conditions, however, were far from resembling the environmental conditions occurring in milk. To confirm the different yield of di-Tyr between casein and whey proteins, we have measured di-Tyr in the whey protein fraction recovered from milk samples exposed to light for 7 days as above described. The levels were 5.2 and 8.3 μg/g protein in WML and SML samples, respectively. These data confirmed that casein, even when assembled in micelles, is much more reactive than whey proteins in milk exposed to light. It has been suggested that Cys residues in whey proteins may scavenge reactive oxidant species, thus delaying oxidation of susceptible amino acids, including Tyr (Li et al., 2022). In particular, this activity was attributed to β-lactoglobulin that has a free thiol residue (Cys121). Nevertheless, presence of free thiols in milk depends on heating conditions, with HTST pasteurization inducing minimal denaturation of whey proteins. On the other hand, the unfolding of whey protein molecules may expose Tyr residues that are buried in native molecules. This thermal dependency of Tyr residue reactivity is not expected for casein that, however, had a much higher content of Tyr than whey proteins (6.3 vs 2.9 g/100 g protein) (Pellegrino et al., 2013). Therefore, further work is required to get more understanding of the actual contribution of whey proteins to di-Tyr formation in milk.

The role of fat in di-Tyr formation in milk is also unclear and, to the authors' knowledge, it was not systematically investigated. Chen et al. (2003) found that the total antioxidant capacity of bovine milk progressively decreased as the fat content was reduced from 3 % to 1.5, 0.5 and 0.1 %. This untargeted measurement may support the lower extent of protein oxidation, expressed as di-Tyr level, we observed in WML compared to SML samples.

Overall, our data indicate that the formation of di-Tyr evolves within the first 7 days in milk exposed to light. Thus, further analyses were carried out with a focus on this time period that, furthermore, corresponds to the actual storage period of the commercial pasteurized milk used in the present study.

3.3. *Localization of di-Tyr in milk*

The immunolocalization of di-Tyr on native casein micelles of milk exposed to light for 7 days was studied by transmission electron microscopy (TEM) with unconjugated monoclonal antibody and a secondary antibody conjugated with colloidal gold 15-nm in size. Ultrathin sections of pellet from ultracentrifugated milk showed well preserved casein micelles in both SML and SMD, as expected from conscious processing. Fig. 4A shows anti-di-Tyr labelling on SML revealing gold distributed at the edge of casein micelles, never inside. SMD did not label with the di-Tyr antibody (Fig. 4B) while positive control provided evidence of its presence (Fig. 4C). k-casein represents a preferential target for photo-oxidation reactions since it is located on the surface of

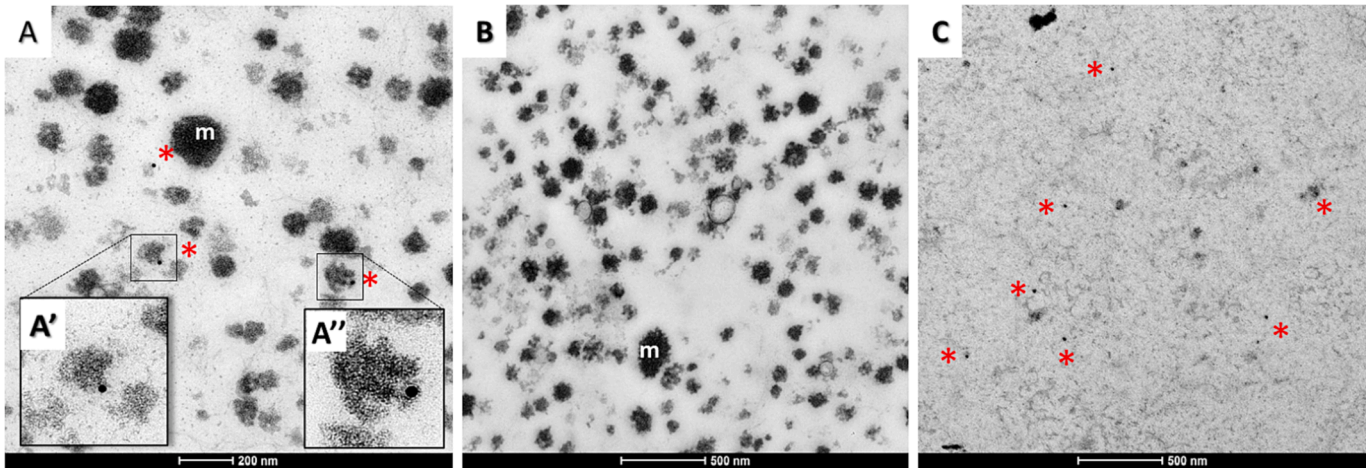

Fig. 4. Transmission electron micrographs of immunolocalization of di-Tyr in the pellet of pasteurized semi-skimmed milk exposed to light (A) or kept in the dark (B) for 14 days. Control is a solution of pure di-Tyr (C). Gold-labelled di-Tyr (black dots signalled by red asterisks for visibility) is localized at the periphery of casein micelle (m) as in the enlarged frame (A'). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the casein micelle. In addition, k-casein together with α_s-casein are richer in Tyr residues compared to β-casein, with this last mainly positioned inside the micelle. This could explain why gold particles distributed at the edge of casein micelle. The occurrence of di-Tyr was challenging to find in SML given that very few casein micelles were positive for di-Tyr in each grid. However, such a low incidence is not surprising when considering that the highest amount of di-Tyr found in milk after 7-day light exposure (2.1 μg/mL) corresponds to 0.024 μmol/mL that formed on 10^{14} casein micelles. TEM immunogold labelling of di-Tyr has been previously carried out on different tissues, often of human origin, such as Lewy bodies or neuroblastoma cells of the brain (Al-Hilaly et al., 2016) but never in milk.

3.4. Particle size analysis of milk

Light scattering analysis was performed to evaluate changes in particles size distribution in milk exposed to light up to 14 days. Particle size distribution and data of D_{10}, D_{50}, D_{90}, and $D_{[4.3]}$ measured by this technique are shown in Fig. 5 and Table 1, respectively. The average diameters by volume $D_{[4.3]}$ of were 0.76 and 1.49 μm at t = 0 for the SM and WM respectively, and decreased to 0.73 and 1.28 μm after 14-day storage. However, the decrease was equal for samples exposed to light and control samples. In fact, a slight increase observed in small particles in the range 100–200 nm for SM or 200–500 nm for WM during storage was independent of the exposure condition. Indeed, interactions among casein micelles were observed by TEM in both WML and WMD at t = 14 (Fig. 5, insert). The particle size distribution of milk was significantly (p

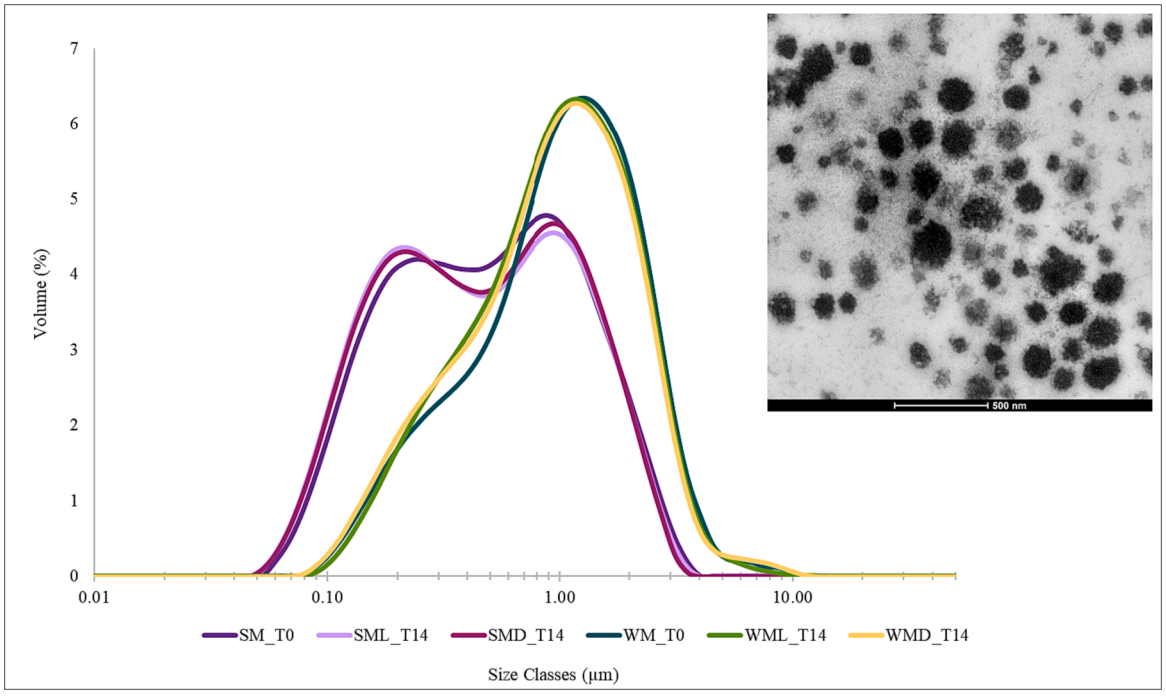

Fig. 5. Particle size distribution from light scattering analysis of pasteurized milk during light or dark storage of 14 days. SM_T0 = semi-skimmed milk analysed at the beginning of storage; SMD_T14 = semi-skimmed milk analysed after 14 days of dark storage; SML_T14 = semi-skimmed milk analysed after 14 days of light storage; WM_T0 = whole milk analysed at the beginning of storage; WMD_T14 = whole milk analysed after 14 days of dark storage; WML_T14 = whole milk analysed after 14 days of light storage. The insert shows interactions among casein micelles in WML_T14 observed by TEM.

Table 1
Parameters of D_{10}, D_{50}, D_{90} and $D_{[4,3]}$ from light scattering analysis of pasteurized milk.

Fat content	Storage time (days)	Exposure to Light	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	$D_{[4,3]}$ (μm)
SM	0	–	0.15 ± 0.00	0.53 ± 0.02	1.72 ± 0.03	0.76 ± 0.02
	14	Light	0.14 ± 0.00	0.48 ± 0.02	1.68 ± 0.02	0.73 ± 0.01
	14	Dark	0.14 ± 0.00	0.49 ± 0.02	1.67 ± 0.04	0.73 ± 0.02
WM	0	–	0.29 ± 0.02	1.12 ± 0.01	2.81 ± 0.02	1.49 ± 0.04
	14	Light	0.30 ± 0.02	1.04 ± 0.01	2.54 ± 0.04	1.28 ± 0.04
	14	Dark	0.27 ± 0.02	1.02 ± 0.01	2.54 ± 0.03	1.28 ± 0.03

SM = semi-skimmed milk; WM = whole milk.

< 0.05) affected by the fat content as a larger volume of particles 1 μm in size was found in WM, regardless the sampling time. At the beginning of storage, the size distribution was in the range 0.08–13 μm in WM and 0.06–4 μm in SM. After 14 days of storage the size distribution of both milk types significantly ($p < 0.05$) changed for their D_{50} and D_{90}, respect to the beginning. However, the light struck did not cause aggregation among particles since the size distributions of milk exposed to light and control in the dark fully overlapped, regardless the fat content. In fact, three-way Anova (Table S2) indicated the light exposure parameter and its interaction with all other parameters being not significant. These data agree with TEM micrographs showing no supramolecular organization of casein micelles related to light exposure.

Overall, our data showed no significant changes in the size distribution of milk particles as function of light exposure possibly because di-Tyr formed only within or among casein fractions that are part of the same micelle thus without inducing micelle aggregation. The significant ($p < 0.05$) role of fat content and storage time in determining changes in the particle size distribution of pasteurized milk was recently reported by Shao et al. (2023). Also these authors observed a decrease of the average diameter $D_{[4.3]}$ in whole pasteurized milk after one-week storage.

3.5. Levels of riboflavin and other flavins in milk

Riboflavin (RF) acts as a photosensitizer in milk, being theoretically involved in both type I and type II mechanisms. However, due to the low solubility of oxygen in milk, proteins and definitely Tyr and Trp residues become competitive quenchers of ^{3}RF, giving rise to a prevalence of type I mechanism (Cardoso et al., 2012). The levels of RF and other flavins we have measured in milk samples of the present study are shown in Fig. 6. Initial levels of these compounds in pasteurized milk were close to those previously reported, with RF largely prevailing over the others and lumiflavin (LF) content to be close to its LOQ (Fracassetti et al., 2018, Meng et al., 2023). Storage in the dark did not give rise to statistically significant changes of flavin levels, confirming the previously observed stability of these compounds in pasteurized milk when properly stored (Limbo et al., 2020). In contrast, during light exposure of milk, both RF and FMN levels gradually decreased while levels of main degradation

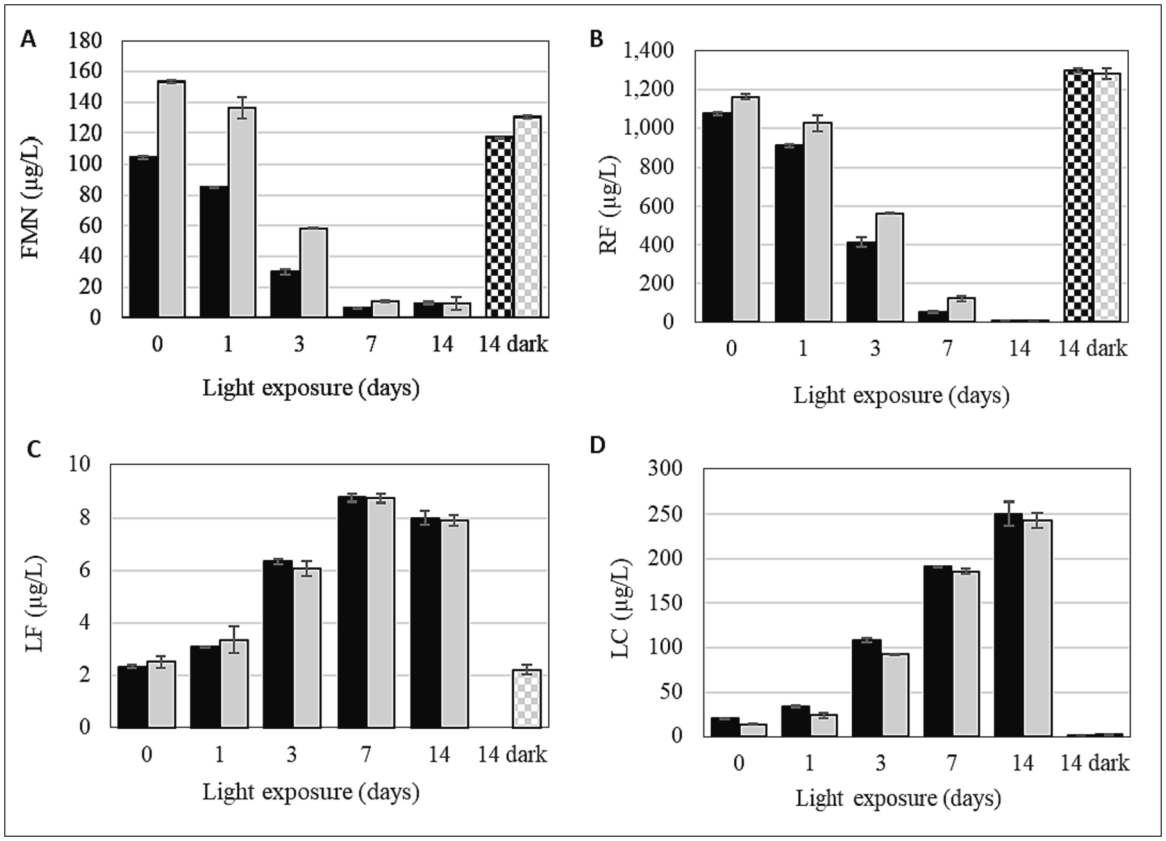

Fig. 6. Levels of riboflavin (RF, panel A), flavin mononucleotide (FMN, panel B), lumiflavin (LF, panel C) and lumichrome (LC, panel D) detected in pasteurized semi-skimmed milk exposed to light (SML, black bar) or in pasteurized whole milk exposed to light (WML, grey bar). Control samples (checked bar) were kept in the dark until the end of storage.

products of RF, i.e. LF and LC, increased. Around 10 % of initial levels of both RF and FMN were still present in milk after 7-day illumination. Other authors observed a gradual decrease of RF in ultrapasteurized milk (145 °C for 0.09 s) exposed to fluorescent light under conditions comparable to ours (Meng et al., 2023). Differently, Tan et al. (2021) reported a sharp decrease (around 85–90 %) of RF to occur in UHT milk after 1-day exposure to fluorescent light, with residual levels still detected 5 days later. Our data also indicated degradation of both RF and FMN to be faster in PS milk than in whole milk, despite the very close initial contents. No published studies were found that compared RF degradation in milk with different fat content. Whited et al. (2002) observed that the light-induced percent degradation of vitamin A was inversely correlated with milk fat content and thus a protective effect of fat was suggested. More specifically, a reduced light penetration in whole milk can be hypothesized. Given that fat in milk is initially protected within globular structures and then may undergo modifications induced by technological processes, clarifying the competing role of fat in photo-oxidation of proteins in different milk types is undoubtedly challenging.

3.6. Milk colour measurement

As a sudden change in colour was observed for milk exposed to light, we have measured it instrumentally to have an objective evaluation. The data of colour coordinates (a*, b*, L*), obtained directly from the colorimeter, are shown in Fig. S1. The trends of the derived whiteness index (WI) and yellowness index (YI) were more informative (Fig. 7A). Initial differences for both indices were observed between WM and SM, with values being higher for the former. This was expected because colour of fresh milk is due to the fat-soluble carotenoids and, to a lower extent, to RF (Kneifel et al., 1992). Values remained rather stable for samples stored in the dark. Differently, upon light exposure, major changes were observed for YI, regardless the fat content of milk, while WI did not change.

The values of total colour difference (ΔE*) (Milovanovic et al., 2020) indicated that, regardless the fat content, the effect of light exposure on milk colour is perceived by consumers after 3 days and highly perceived (ΔE* > 3) after 14 days (Fig. 7B).

4. Conclusions

Pasteurized milk takes a few days to deliver from the manufacturing facility to market and consumers, and consumers normally drink it through some more days. During these periods, exposure to light of milk is almost unavoidable. Studying protein photooxidation mechanisms is tremendously complex in milk system due to the possible involvement of other components playing either pro- or anti-oxidant roles. Here we have given evidence for the spontaneous formation of di-Tyr in pasteurized milk exposed to light in a retail display case. Both casein and whey protein fractions proved to be less reactive in whole milk than in reduced fat milk. Other light-dependent changes already described in literature have been evaluated to corroborate our findings. Although PET bottles are fully recyclable, and thus more environment friendly than other packaging materials, their inefficacy in protecting milk from light-induced damages during storage may undermine efforts of manufacturers to provide consumers with minimally processed drinking milk. While development of off-flavours and degradation of some vitamins are well-established negative effects of light exposure of milk, protein oxidation has resulted to be a further change that begins immediately and develops during the entire shelf-life. Our results indicated that mechanisms leading to di-Tyr formation in milk are worth of further investigation. Due to the extreme complexity of milk as reaction system, studies carried out on simpler models should necessarily be confirmed on the real product.

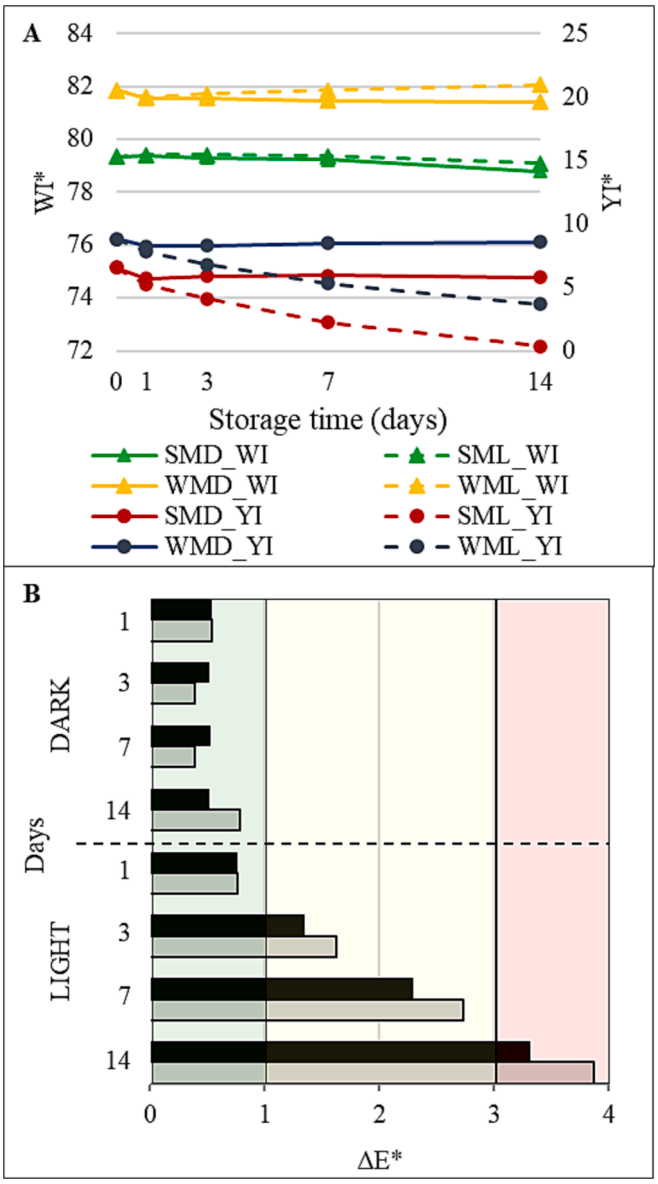

Fig. 7. A) Whiteness index (WI) and Yellowness index (YI) of pasteurized milk during 14-day storage. SMD = semi-skimmed milk, dark storage; WMD = whole milk, dark storage; SML = semi-skimmed milk, light storage; WML = whole milk, light storage. B) Total colour difference (ΔE*) between milk at t = 0 and at the end of 1-, 3-, 7- and 14-day storage. Grey bars refer to semi-skimmed milk and black bars to whole milk, either exposed to light or kept in the dark.

CRediT authorship contribution statement

Paolo D'Incecco: Conceptualization, Writing – original draft, Writing – review & editing. **Sabrina Dallavalle:** Conceptualization, Writing – original draft, Writing – review & editing. **Loana Musso:** Data curation. **Veronica Rosi:** Data curation. **Marta Sindaco:** Data curation. **Luisa Pellegrino:** Conceptualization, Writing – original draft, Writing – review & editing.

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

Microscopy observations were carried out at The Advanced Microscopy Facility Platform - UNItech NOLIMITS - Università degli Studi di Milano.

Founding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137566.

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