ORIGINAL ARTICLE

Effect of milk fat and its main fatty acids on oxidation and glycation level of milk

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Abstract Milk is a highly nutritional food rich in protein and fat that is prone to deterioration by oxidation and glycation reactions at storage and processing. In this study, glycation products and lipid oxidation products contents in skim milk, whole milk, and milk fat simulation groups were determined to evaluate the effect of milk fat components on glycation at 120 °C for 60 min. The increase rate of carbonyl compound, main advanced glycation end products (AGEs) levels, and glycation sites number of α-casein and β-casein are higher in whole milk than that in skim milk, indicating that milk fat promoted protein glycation significantly. In milk fat simulation groups, oleic acid and linoleic acid (LA) were added to milk fat in skim milk proportionally, promoting the formation of glycation products; however, palmitic acid had no such effect. LA exhibited strong promotion on AGEs formation. Lipid oxidation radicals, protein carbonyl amine condensation, and carbonyl compound formation were critical factors for milk glycation, according to OPLS-DA results. Therefore, radicals of fat oxidation are speculated to trigger the early glycation, and carbonyl compounds of fat oxidation act as important intermediates of glycation, fat type, form, and its degradation rate, thus play essential roles in milk glycation.
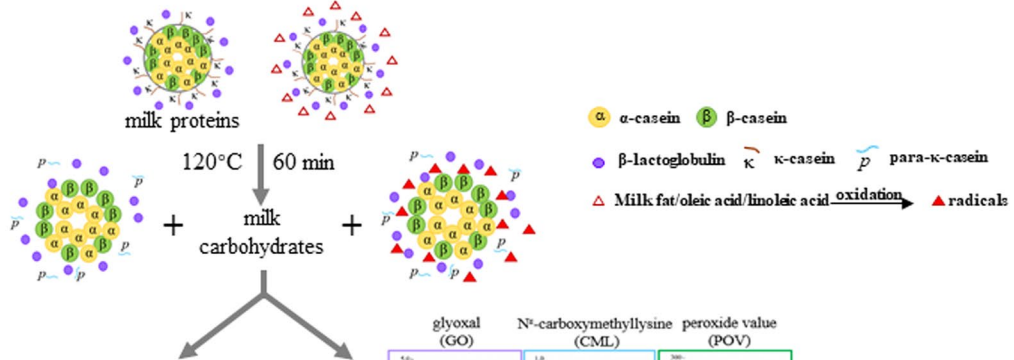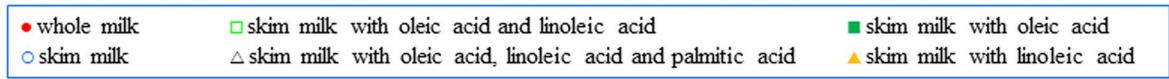
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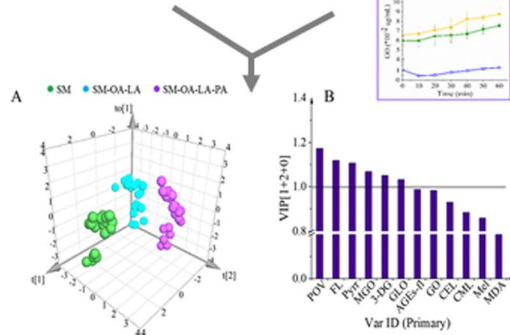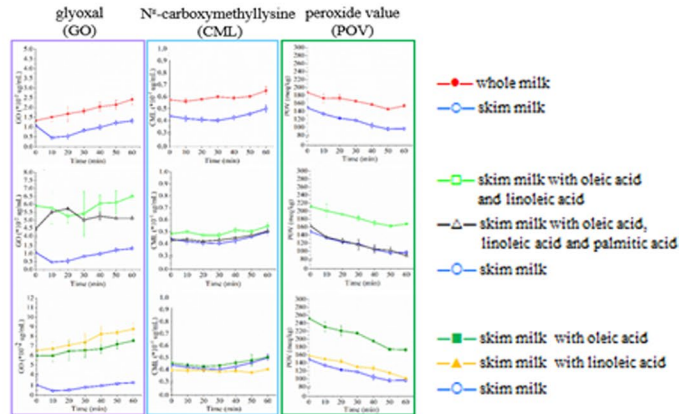
Graphical abstract

Keywords Glycation products · Maillard reaction · Milk fatty acids · Glycation sites · Lipid oxidation · OPLS-DA

Abbreviations

OA	Oleic acid
LA	Linoleic acid
PA	Palmitic acid
GO	Glyoxal
MGO	Methylglyoxal
3-DG	3-Deoxyglucosone
GLO	D-glucosone
FL	Fructosyllysine
CML	N^{ε}-carboxymethyllysine
CEL	N^{ε}-carboxyethyllysine
Pyrr	Pyrraline
AGEs	Advanced glycation end-products

AGEs-fl	Fluorescent-Advanced glycation end-products
POV	Peroxide value
MDA	Malondialdehyde
WM	Whole milk
SM	Skim milk
SM-OA-LA	Skim milk with oleic acid and linoleic acid
SM-OA-LA-PA	Skim milk with oleic acids, linoleic acid and palmitic acid
SM-OA	Skim milk with oleic acid
SM-LA	Skim milk with linoleic acid

OPLS-DA	Orthogonal projection to latent structures discriminant analysis
VIP	Variables important in the projection

Introduction

Milk and milk powder are widely used in cooking and baked foods, and their nutrients, such as protein, fat, and carbohydrates, are prone to chemical reactions, including milk fat oxidation and Maillard reaction during long-term heating or storage. These chemical reactions reduce the nutritional value of milk and generate some harmful substances. In recent years, research on glycation products in milk has increased. Meyer et al. (2012) detected N^ε-carboxymethyllysine (CML) in heated milk, and the study of Zhang et al. (2019) further analyzed glycation intermediates dicarbonyl compounds and advanced glycation end products (AGEs) in ultra-high temperature milk. AGEs are a complex and heterogeneous group of compounds that have been implicated in food and diseases, the accumulation of dietary-AGEs is considered to induce oxidative stress and inflammation in *vivo*, further increase the risk of diabetes and its complications and other chronic diseases (Li et al. 2021; Singh et al. 2001). Food rich in proteins, carbohydrates, and lipids are more likely to produce AGEs during heating or storage, including CML, N^ε-carboxyethyllysine (CEL), and pyrraline (Pyrr). Furthermore, dicarbonyl compounds such as glyoxal (GO), methylglyoxal (MGO), 3-deoxyglucosone (3-DG), and D-glucosone (GLO) are important intermediates to form AGEs.

Besides Maillard reaction, oxidation of lipids also leads to the formation of AGEs because Maillard reaction and lipid oxidation share some intermediates, especially in high-fat food (Zamora and Hidalgo 2005). Some unsaturated fatty acids with simple structure, such as oleic acid (OA) and linoleic acid (LA), significantly affect AGEs formation (Han et al. 2013a). OA and LA are the primary unsaturated fatty acids of triglyceride in milk (Haruna et al. 2020; Yang et al. 2020). Due to the heat sensitivity of milk, the physical and chemical properties of milk change with the heating process, reducing the nutritional value of milk. For example, Maillard reaction caused structure change of milk protein, and the oxidation of lactose and fat caused milk deterioration. Maillard reaction and lipid oxidation are considered together to trigger AGEs formation at storage and heating of milk. However, research on milk glycation is currently focused on the reaction between protein and carbohydrate (Tu et al. 2017; Wu et al. 2020), and there is no systematic study on the effect of milk fat on protein glycation. When the milk or milk powder is used as the main base material in heating food, these reactions in the milk have a great impact on the nutritional value of the food. Thus, this study aimed to quantify the glycation and oxidation products levels in real milk and milk fat simulation groups at 120 ºC based on considering the milk product as the materials in heating food, further explore the effect of milk fat components on glycation.

Material and methods

Chemicals

The fresh ultra-high temperature milk, skim milk and whole milk, were obtained from the Mengniu Dairy. These two kinds of milk only differ in milk fat, and the content of milk fat was 3.7 g/mL in whole milk. OA (90%) and 1,1,3,3-Tetra-ethoxypropane (97%) were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). LA (95%), Palmitic acid (PA, 95%), GO (40%), MGO (40%), and methanol (99%) were obtained from Sigma-Aldrich (St Louis, MO, USA). 3-DG (95%), GLO (95%), fructosyllysine (FL, 95%), CML (99%), CEL (96%), and Pyrr (98%) standards were obtained from Toronto Research Chemicals, Inc. (Toronto, Canada). O-phenylenediamine, trichloroacetic acid, 2-thiobarbituric acid, potassium thiocyanate, ferrous sulfate, hydrochloric acid, octanol, and ethylene diamine tetraacetic acid were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shenyang, LN, China). All other reagents were of the highest purity available.

Reaction model groups

Milk fat simulation groups were established as previously described by Zhao et al. (2018), with minor modifications. Model groups were classified as follows: (i) milk: skim milk (SM) and whole milk (WM); (ii) milk fat simulation group: skim milk with OA and LA (SM-OA-LA group), skim milk with OA, LA, and PA (SM-OA-LA-PA group), fatty acids account for 27%, 3%, 50% of milk fat respectively (Table S1); (iii) skim milk with fatty acids OA (SM-OA group) or LA (SM-LA group), fatty acids account for 100% of milk fat respectively (Table S1).

Milk fat simulation groups (SM-OA-LA and SM-OA-LA-PA) were prepared by dissolving OA (27% of the total milk fat) and LA (3% of the total milk fat) with or without PA (accounting for 50% of the total milk fat) in 25 mL skim milk, and stirred to ensure complete dissolution. Subsequently, the solution was homogenized at a pressure of 50 MPa (repeated 3 times) to obtain a homogeneous emulsion. In milk fat simulation SM-OA and SM-LA groups, OA or LA accounted for 100% of the total milk fat.

The model groups used to detect glycation and lipids oxidation products were incubated in an oven at 120 °C for 60 min and then analyzed at 10 min intervals. All samples

were prepared in triplicate, and prepared solutions were immediately cooled on ice until used. All aqueous solutions were prepared using ultrapure water from Heal Force Instrument Co., Ltd. (Shanghai, China).

The change rate of glycation product contents is calculated using Eq. (1),

The change rate (%) $= \dfrac{(A - B)}{B} \times 100$ (1)

where A is the content of glycation product at 60 min; B is the content of glycation product at 0 min.

Preparation of samples

Derivatization of α-dicarbonyl products

α-Dicarbonyl products (GO, MGO, GLO, and 3-DG) were analyzed for their corresponding quinoxaline derivatives after incubation with O-phenylenediamine. The experiments were conducted as described in previous studies with slight modifications (Hellwig et al. 2010; Zhao et al. 2020). Firstly, 200 μL of the samples were mixed with 400 μL of methanol and centrifuged at 12,000 r/min, 4 °C for 10 min. Secondly, 0.5 mL of supernatant or standards and 0.5 mL of O-phenylenediamine (60 mg/mL) were added to a capped brown bottle and incubated for 12 h at 4 °C in the dark. The mixtures were diluted 5 times, purified by 0.22 μm filters, and then analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (Shimadzu, Japan).

Acid hydrolysis

The acid hydrolysis samples were prepared as previously described (Hegele et al. 2008), with slight modifications. Briefly, 4.5 mL of hydrochloric acid (6 mol/L) and 50 μL of octanol were added to 0.5 mL samples, and the samples were hydrolyzed for 24 h at 110 °C. The hydrolysates were diluted twice and purified by 0.22 μm filters. Then 200 μL of the hydrolyzed and purified samples were N_2 evaporated to dryness. Then, the samples were reconstituted to 500 μL with ultrapure water, purified by 0.22 μm filters, and analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (Shimadzu, Japan).

Analysis of glycation products

Measurement of glycation products was performed using ultra-performance liquid chromatography-tandem mass spectrometry incorporated with electrospray ionization interfaces in the positive ion mode, and separation of glycation products was achieved using a reversed-phase C_{18} column (2.1 × 50 mm, 1.7 μm; Waters, USA). The flow rate was 0.2 mL/min with isocratic elution of methanol and 0.1% formic acid–water (70:30), and the injection volume was 5 μL. The analyses were conducted using a multiple-reaction monitoring scan mode, and the transitions monitored were m/z 131 → 77 for GO; m/z 145 → 77 for MGO; m/z 235 → 199 for 3-DG; m/z 251 → 173 for GLO; m/z 309 → 84 for FL; m/z 205 → 84 for CML; m/z 219 → 84 for CEL; m/z 255 → 84 for Pyrr. Furthermore, the characteristic transition of each product was monitored to improve selectivity by a triple quadrupole mass spectrometer (Shimadzu, Japan). Optimal ionization was selected as follows: electrospray source temperature, 110 °C; desolvation temperature, 350 °C; and capillary voltage, 3 kV.

The optimal conditions of all precursor and product ions were determined by optimizing standards or standard derivatives. The samples were quantified based on the external calibration curves, and the correlation coefficient (R^2) ≥ 0.9992. The mass chromatograms of glycation products standards are shown in Fig. S1. The calibration curves were achieved with at least 5 points with a good linear range. Linear range of MGO was 0.001–0.01 μg/mL, GO, 3-DG, and GLO were 0.01–0.1 μg/mL, FL was 1–50 μg/mL, CML and CEL were 0.01–0.1 μg/mL, and Pyrr was 1–50 μg/mL.

Determination of glycated sites

Sample digestion

Milk proteins were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to a revised method (Meltretter et al. 2008). Samples were diluted 10 times and boiled for 5 min to denature the protein completely, then separated in a 5% stacking and a 10% separating gel (sample load 15 μL). The gels were stained by Coomassie Brilliant Blue R250 staining for 30 min, and decolorized with a decolorizing solution containing 25% (v/v) methanol and 7% (v/v) acetic acid until the brands were clear. Gels images were analyzed with Gel Imaging System (GE AI600, USA).

The milk protein bands were cut into small pieces and destained with 30% acetonitrile. After, in-gel proteins were reduced with 10 mmol/L dithiothreitol for 30 min at 56 °C, then alkylated with 200 mmol/L iodoacetamide in the dark at room temperature for 30 min. Gel pieces were washed with 100 mmol/L ammonium bicarbonate and acetonitrile, respectively. A 40 μL of 12.5 ng/μL trypsin buffer was added in 25 mmol/L ammonium bicarbonate for digesting. The peptides were extracted 3 times with 60% acetonitrile, and the extracts were pooled and dried completely by a vacuum centrifuge.

Analysis by high performance liquid chromatography-tandem mass spectrometry

High performance liquid chromatography-tandem spectrometry analysis was conducted on a Q Exactive mass spectrometer coupled to Easy-nLC (Thermo Fisher Scientific) by a method previously described with some modification (Chen et al. 2017). The peptide mixtures were loaded onto a reversed-phase C_{18} column (0.075 × 150 mm, 5 µm; Thermo Fisher Scientific) for high-performance liquid chromatography-tandem mass spectrometry. The mobile phase consisted of solvent A (0.1% formic acid–water) and solvent B (0.1% formic acid in 84% acetonitrile) with a linear gradient at a flow rate of 250 nL/min controlled by IntelliFlow technology over 60 min. The gradient elution was 0–50 min, 4–50% solvent B gradient; 50–54 min, 50–100% solvent B gradient; 54–60 min, 100% solvent B. A data-dependent top10 method with Orbitrap high-energy C-trap dissociation/electron transfer dissociation fragmentation modes were used to acquire tandem mass spectra. Further detection of the target value was enabled with predictive automatic gain control and 20 s for dynamic exclusion. The normalized collision energy was 27 eV, and the underfill ratio was 0.1%.

Determination of fluorescent AGEs (AGEs-fl)

A Hitachi FL-4600 fluorometer (Tokyo) was used to analyze the fluorescence of samples (dilution 200 times). According to a previous description, the analysis method was performed with some modifications (Skrt et al. 2012). Excitation and emission wavelengths were 347 nm and 440 nm, respectively.

Melanoidin evaluation

A TU-1810 ultraviolet–visible spectrophotometer (Beijing, China) was used to analyze the UV-absorbance of samples (dilution 100 times). The UV-absorbance of samples was 420 nm, which correlated with the high molecular weight polymeric brown products formation, such as melanoidin (Lerici et al. 1990).

Analysis of lipid oxidation products

Peroxide value

Peroxide value was assessed by the ferric thiocyanate method (Mahmoodani et al. 2018). A 50 µL sample was dissolved in a 950 µL mixture of chloroform and methanol (v/v) (70:30), and the sample was mixed for 30 s before centrifugation at 12,000 r/min, 4 °C for 10 min. Then, 100 µL of the lower phase was dissolved in 900 µL mixture of chloroform and methanol (v/v) (70:30) followed by the addition of 50 µL potassium thiocyanate (300 g/L) and 50 µL ferrous sulfate (3.5 g/L). The samples were left to react for 20 min at room temperature before the 500 nm absorbance was measured in a microplate (96 cells) using a microplate reader (BioTek Instruments Co., Ltd., American). Peroxide induces iron (II) oxidation to iron (III), forming red compounds with the thiocyanate. The reference curves calculated the ferric ion content of samples in µg, and the peroxide value were expressed as milligram equivalents of oxygen per kilogram of fat.

Malondialdehyde content

The method used for detecting thiobarbituric acid reactive substances in milk is described in previous study with some modifications (Sugiarto et al. 2009). Briefly, 0.5 mL of the sample were dissolved in 1 mL trichloroacetic acid mixture in which trichloroacetic content was 150 g/L and ethylene diamine tetraacetic acid content was 2 g/L, and the samples were mixed for 30 s before centrifugation at 12,000 r/min, 4 °C for 10 min. Then, 1 mL of supernatant was mixed with 1 mL of 2-thiobarbituric acid (0.288 g/L) in a centrifuge tube, then immediately placed in a boiling water bath for 40 min. After heating, the mixture was cooled in ice and measured absorbance at 532 nm using a TU-1810 ultraviolet–visible spectrophotometer.

The 1,1,3,3-tetraethoxypropane was used as a standard due to the instability of malondialdehyde. A malondialdehyde stock solution (100 µg/mL) was prepared by dissolving 0.315 g 1,1,3,3-tetraethoxypropane in 1000 mL water and diluted further with water to form malondialdehyde solutions of various concentrations (from 0.01 to 0.25 µg/mL). 1 mL malondialdehyde solutions was reacted with 1 mL 2-thiobarbituric acid (as described above), and the absorbance of the mixtures was measured at 532 nm. These results showed a linear relationship between the absorbance and the concentration of the malondialdehyde standards.

Statistical analysis

ANOVA analyzed data. The significant difference between the control and experimental groups was assessed by orthogonal projections to latent structures discriminant analysis (OPLS-DA, SIMCA-P, version 14.0) and t-tests (SPSS, version 25.0). Correlation between the different groups was evaluated by Pearson's correlation (SPSS, version 25.0). The values were considered statistically significant at $P < 0.05$.

Results and discussion

Change of glycation products in milk

The carbonyl compounds in milk are mainly formed from carbohydrate oxidation, lipid oxidation, and protein glycation (Ruiz-Matute et al. 2014). As shown in Fig. 1a–d, the contents of four carbonyl compounds gradually increased in whole milk and skim milk, and their contents in whole milk were significantly higher than that in skim milk ($P < 0.01$). In Fig. 1a, the increase rate of GO content (81.80%) in whole milk was higher than that in skim milk (22.35%), which is related to the formation of carbonyl compounds of milk fat oxidation. The increase rates of GLO contents in two types of milk were similar (Fig. 1d), and MGO (165.39%) and 3-DG (23.03%) showed higher increase rates in skim milk than MGO (60.52%) and 3-DG (10.87%) in whole milk. Because the unsaturated fatty acid can reduce carbohydrate oxidation in whole milk (Zhao et al. 2020), this reason

explains that the increase rate of 3-DG and MGO contents lower in whole milk than that in skim milk.

FL is an important product in initial Maillard reaction. As shown in Fig. 1e, FL generation decreased in two types of milk during heating process, and this decrease was attributed to that degradation amount of FL was higher than its formation amount. Compared with whole milk, skim milk showed a higher decrease rate of FL, a possible explanation for this is that the formation amount of FL is higher in whole milk than that in skim milk, because the FL accumulation in whole milk can be promoted by milk fat (Han et al. 2013b).

Compared with other amino acid, lysine is most easily glycated in milk (Li et al. 2021). Thus, some lysine-derived AGEs including CML, CEL, and Pyrr were selected as AGEs markers in this study. Previous studies have shown that CML was formed mainly via the FL pathway of Maillard (Ahmed et al., 1986). In this study, the increase rate of CML contents in whole milk and skim milk were almost same (Fig. 1f), it is therefore speculated that FL degradation
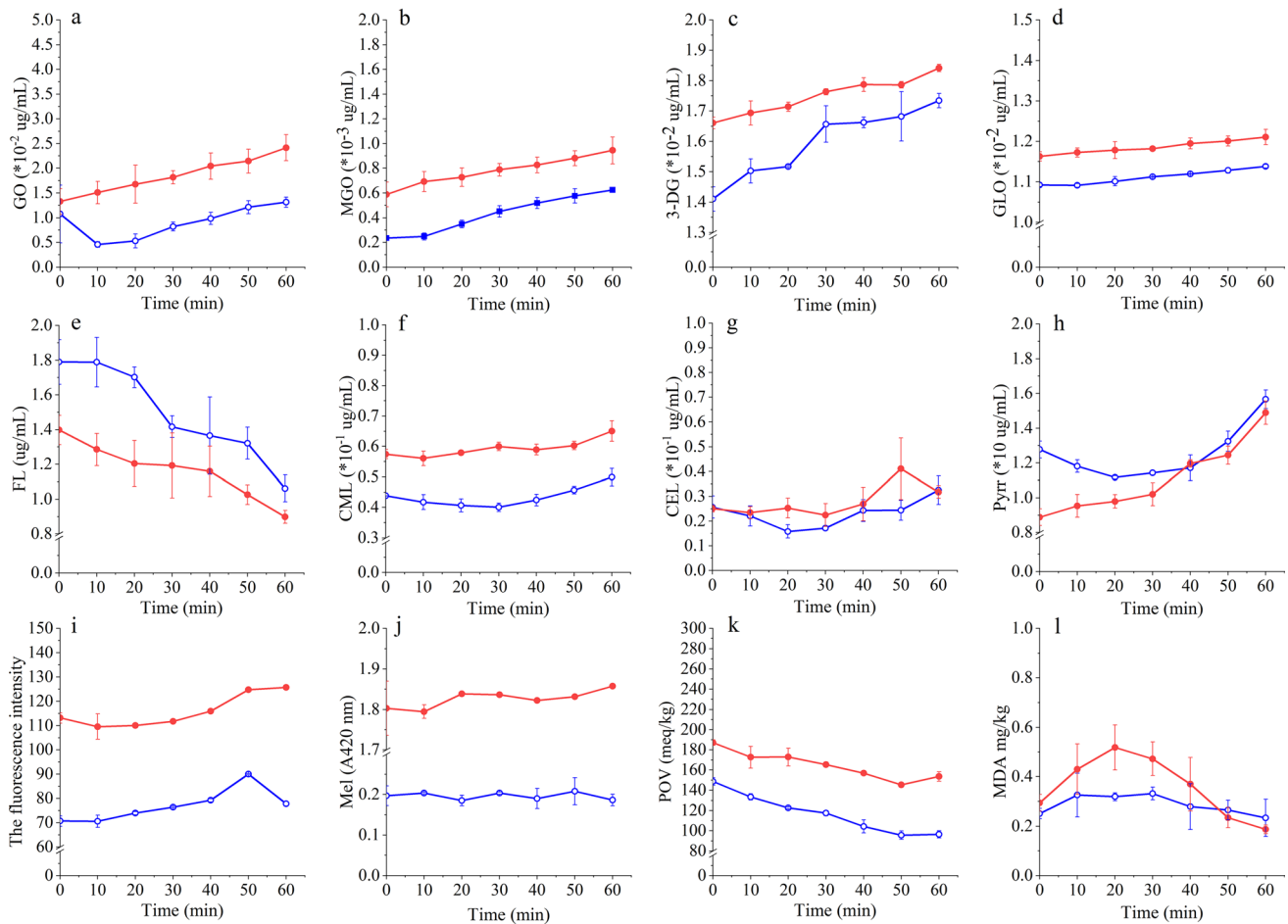

Fig. 1 Change of the contents of glycation products and lipid oxidation products in skim milk (SM; —O—) and whole milk (WM; —●—) heating at 120 ℃ for 60 min. **a**: glyoxal (GO), **b**: methylglyoxal (MGO), **c**: 3-deoxyglucosone (3-DG), **d**: D-glucosone (GLO), **e**: fructosyllysine (FL), **f**: N$^{\varepsilon}$-carboxymethyllysine (CML), **g**: N$^{\varepsilon}$-carboxyethyllysine (CEL), **h**: pyrraline (Pyrr), **i**: The fluorescence intensity, **j**: melanoidin (Mel), **k**: peroxide value (POV), **l**: malondialdehyde (MDA)

level was similar in two types of milk, then in contrast with Fig. 1e, it can be further speculated that more FL were formed in whole milk than in skim milk. These results are consistent with our previous research proving that lipid oxidation promotes FL formation in the Maillard reaction model at 70 °C for 40 h (Zhao et al. 2021). CEL, another AGE, is formed by the interaction of MGO and lysine (Nguyen et al. 2016). As depicted in Fig. 1g, the increase rate of CEL contents were almost same in whole milk and skim milk, although somewhat fluctuated at 20 min and 50 min. Pyrr was easy to be formed via 3-DG combination with lysine (Yu et al. 2020), seen from Fig. 1h, the increase rate of Pyrr content was much higher in whole milk than that in skim milk, speculating that more 3-DG was consumed to form Pyrr in whole milk than that in skim milk, which explains the reason that 3-DG accumulation is lower in whole milk than that in skim milk.

Rufián-Henares et al. (2007) proved that Maillard reaction products in milk correlates with fluorescence intensity and browning. Browning is closely related to melanoidin content. The increase rate of melanoidin and AGEs-fl levels in whole milk were higher than that in skim milk with time extended (Fig. 1i–j), which further proved that the glycation in milk can be promoted by milk fat. Wang's study (2019) already demonstrated the lipids promote the accumulation of AGEs by impacting the formation of Amadori products and α-dicarbonyl compounds, in line with this study of FL and carbonyl products in milk.

Milk is a typical high-nutrition food, and contains large amounts of the protein and lipid. In this study the role of lipids on the glycation of Maillard reaction was explored. Although the oxidations between proteins and between proteins and fat have some effects on glycation in milk, this study did not involve. Our main consideration is the Maillard interaction between protein and sugar, thus it is not convenient to design more influence factors. Of course, if the comprehensive factors of protein glycation are considered, a deep expansion of the analysis is further needed. The results obtained at current conditions demonstrate previous model conclusion about the mechanism of the fat promoting the glycation from real food systems.

Change of lipid oxidation products in milk

Lipids mainly react with proteins in form of peroxy free radicals in nonenzymatic browning (Zamora and Hidalgo 2000). Hydroperoxides are the initial oxidation products of the typical radical reaction in lipids oxidation, which are further converted into secondary oxidation products (Hedegaard et al. 2006; Zamora and Hidalgo 2000). As shown in Fig. 1k, peroxide value level in two types of milk gradually decreased, but malondialdehyde content increased initially and oxidation increased significantly within the first 20 min in whole milk (Fig. 1l, $P < 0.01$). According to correlation calculations, the glycation products levels were correlated to peroxide value and malondialdehyde in whole milk (Fig. S2). As shown in Fig. S2a, the generation of early and intermediate glycation products were significantly correlated to peroxide value in two types of milk, especially in whole milk, which means that peroxy free radicals have a more obvious effect on the early glycation period. The results are consistent with our previous model study that glycation was promoted by active radicals of lipid during early glycation (Zhao et al. 2021). Furthermore, in two types of milk, the malondialdehyde contents of later oxidation period were significantly correlated to AGEs contents (Fig. S2b), which can be explained that the decline in radical activity and further polymerization of radicals formed stable compounds at later period (Jiang et al. 2013).

Change of glycation sites of milk protein in milk

Proteomic analysis identified the glycation sites to study further the changes of α-casein and β-casein conjugation to carbohydrates. As depicted in Table 1, α-casein peptides 119–134, 38–51, and 19–49 were glycated in whole milk. In contrast, only α-casein peptide 38–51 was glycated in skim milk (Fig. 2). Similarly, the number of β-casein glycation sites in whole milk was also higher than that in skim milk (Table 2), proving that milk fat participated and promoted the glycation in milk.

Table 1 Glycated sites of α-casein in whole milk and skim milk at 120℃ after 60 min

sample	peptide location	m/z (glycated)	ΔM [ppm]	Sequence	glycated site
Whole milk	119–134	1022.48969	− 6.92	Y**k**VpQLEIVPNSAEEr	K_2
Whole milk	38–51	655.99548	− 0.68	FFVAPFPEVFG**k**E**k**	K_{12}, K_{14}
Whole milk	19–49	771.38318	− 13.28	HPI**kh**QGLPQEVLNEN-LLRf FVAPFPEVFG**k**	K_4, K_{31}
Skim milk	38–51	655.99609	0.25	FFVAPFPEVFG**k**E**k**	K_{12}, K_{14}

The bold k are the locations of lysine glycation sites

Fig. 2 The glycation sites of α-casein in whole milk **a** and skim milk **b** after heating at 120 ℃ for 60 min
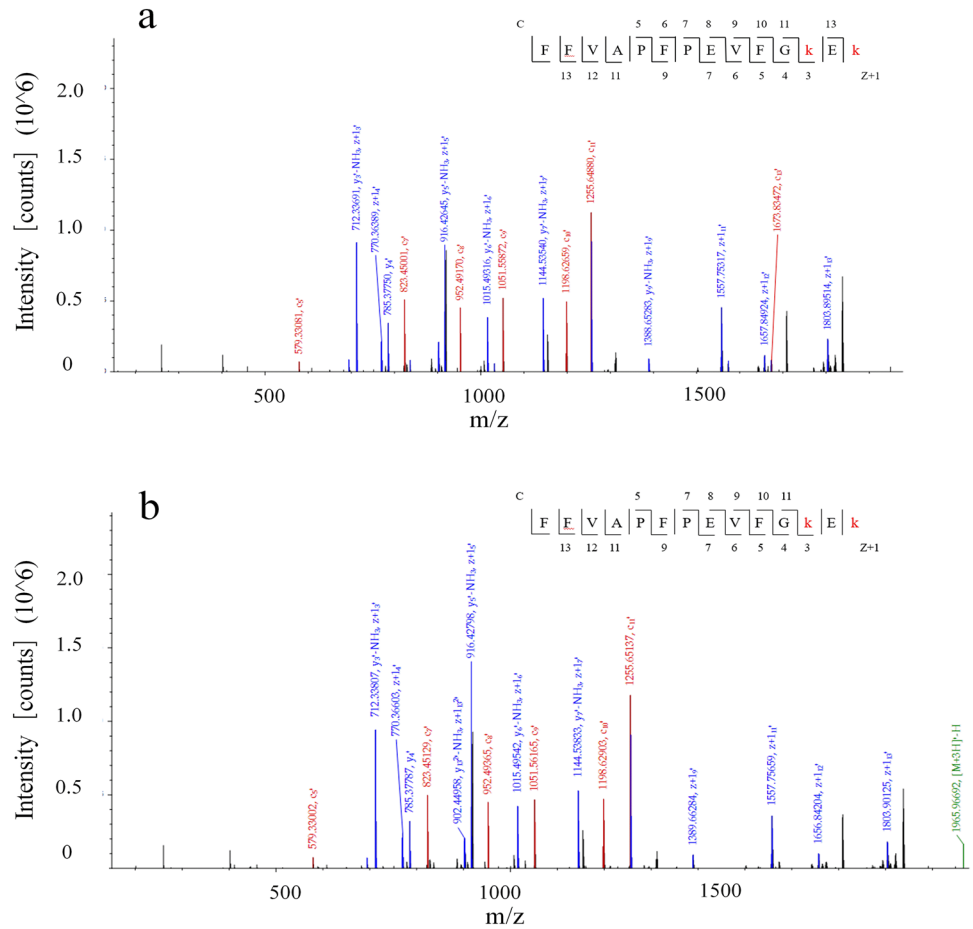

Table 2 Glycated sites of β-casein in whole milk and skim milk at 120 ℃ for 60 min

sample	peptide location	m/z (glycated)	ΔM [ppm]	Sequence	glycated site
whole milk	17–43	524.76611	12.4	ELEELNVpGEIVESLSSSEESItRIN**k**	K_{27}
whole milk	1–40	767.55542	− 10.91	M**k**VLILAcLVALALArELEEL-NVpGEIVESLSSSEESITr	K_2
skim milk	114–128	921.91486	− 3.47	EAMAPKHKEmpFp**k**	K_{14}

The bold k are the locations of lysine glycation sites

Change of glycation products in milk fat simulation groups

The simulation groups in Table S1 showed the type and proportion of fatty acids added. It can be seen from Fig. 3a–d, contents of GO, MGO, 3-DG, and GLO increased correspondingly in SM group, SM-OA-LA group, and SM-OA-LA-PA group, and carbonyl compound contents were higher in milk fat simulation groups than that in SM group ($P < 0.01$). Compared with SM-OA-LA group, SM-OA-LA-PA group did not show a significant difference in the content of carbonyl compounds ($P > 0.05$), indicating that unsaturated fatty acids have a more significant effect on carbonyl compounds than saturated fatty acids.

The intermediate product FL showed a decreased accumulation in SM, SM-OA-LA, and SM-OA-LA-PA groups (Fig. 3e). FL content reduced slower in SM-OA-LA and SM-OA-LA-PA groups than that in SM group ($P < 0.01$), which further supports the idea of the difference between two types of milk that FL content can be more accumulated in fat groups than that in SM group.

As seen from Fig. 3f–h, AGEs contents increased gradually in three groups ($P < 0.05$), the overall trends in these groups were similar to that in real milk, and CEL content was higher in SM-OA-LA group than that in
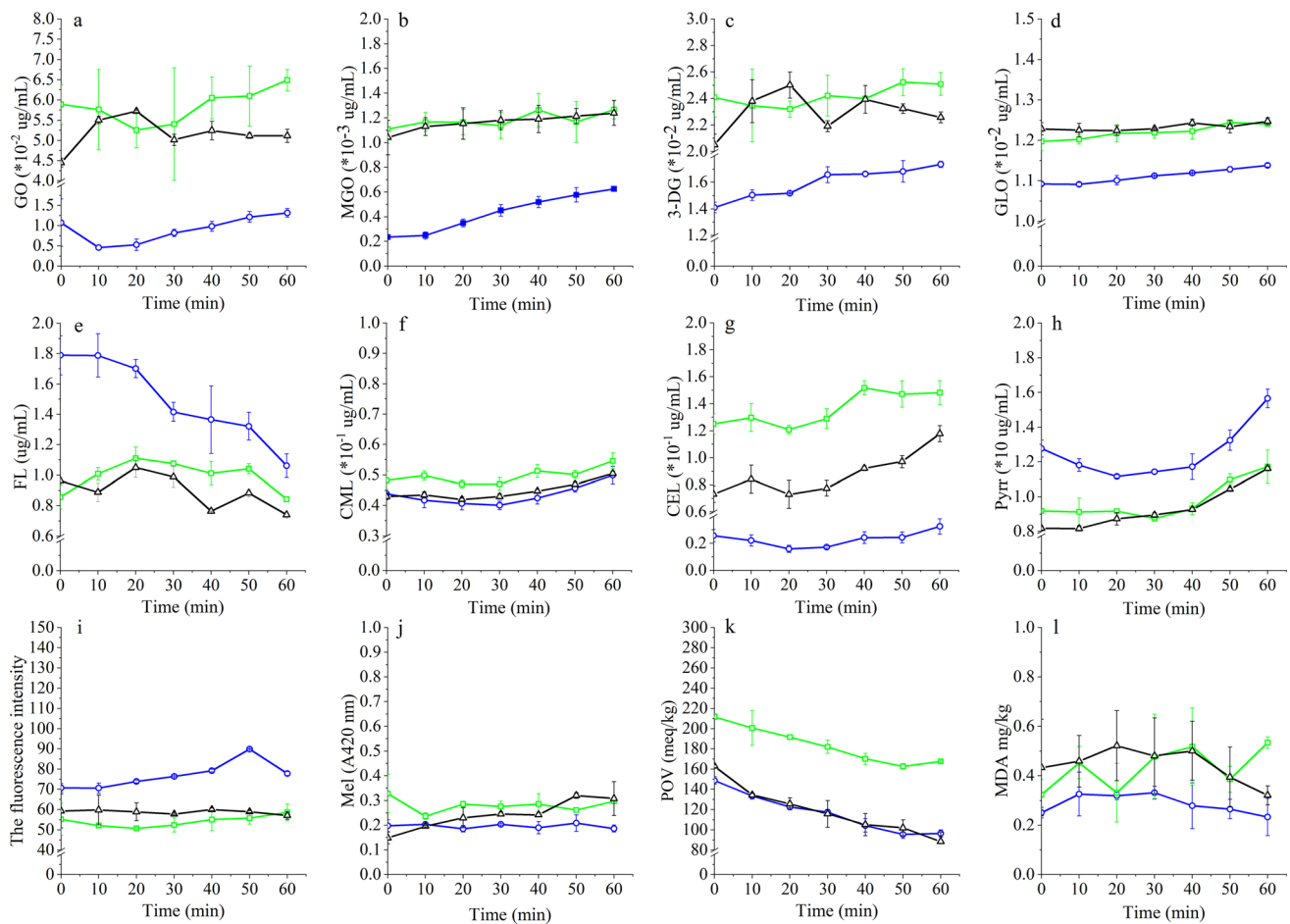
Fig. 3 Change of the contents of glycation products and lipid oxidation products in skim milk (SM; —O—), skim milk with oleic acid and linoleic acid (SM-OA-LA; —□—), and skim milk with oleic acid, linoleic acid and palmitic acid (SM-OA-LA-PA; —△—) groups heating at 120 ℃ for 60 min. **a**: glyoxal (GO), **b**: methylg-lyoxal (MGO), **c**: 3-deoxyglucosone (3-DG), **d**: D-glucosone (GLO), **e**: fructosyllysine (FL), **f**: N^{ε}-carboxymethyllysine (CML), **g**: N^{ε}-carboxyethyllysine (CEL), **h**: pyrraline (Pyrr), **i**: The fluorescence intensity, **j**: melanoidin (Mel), **k**: peroxide value (POV), **l**: malondial-dehyde (MDA)

SM-OA-LA-PA, but the growth rate was not faster in SM-OA-LA group.

In Fig. 3i, compares SM group with SM-OA-LA and SM-OA-LA-PA groups, the addition of fatty acids reduced the AGEs-fl level; however, contrast with Fig. 1i, it can be found that the milk fat in whole milk promoted AGEs-fl formation, which might be related to the fat form in milk.

Change of lipid oxidation products in milk fat simulation groups

Peroxide value decreased in SM, SM-OA-LA, and SM-OA-LA-PA groups ($P < 0.05$), and it was significantly higher in SM-OA-LA group than that in SM-OA-LA-PA group ($P < 0.01$; Fig. 3k). Furthermore, the increase rate of malondialdehyde content in SM-OA-LA group was higher

than that in SM-OA-LA-PA groups (Fig. 3l), one possible implication of this is that PA had almost no effect on milk fat oxidation ($P > 0.05$), this may be related to the fact that PA hardly generated active radicals as unsaturated fatty acid (Liu et al. 2019; Xie et al. 2019).

In this study, the effect of milk fat on the glycation of milk-based food model during heating was investigated. As shown in Fig. 1 and Fig. 3, not all product contents showed obvious difference, which may be due to the substrate concentration in models and milk. Compared with the Maillard model of amino acid and monosaccharide studied previously, neither the reactivity level nor the product concentration is predominant in this study. In addition, the composition and the interaction of real milk are complex, thus the relationship of the components needs to be further studied. Preliminarily the effect of fatty acid type on glycation has been studied in this study, but the effect of lipid

oxidation intermediates and by-products of oxidative radicals on glycation need be further considered in the future research.

Change of glycation and oxidation products in skim milk with OA or LA

To investigate the effect of unsaturated fatty acid of milk fat on glycation, milk fat groups of SM-OA and SM-LA were established (Table S1). As shown in Fig. S3a-d, GO, MGO, 3-DG, and GLO contents increased significantly in SM-OA and SM-LA groups ($P < 0.01$), and the increase rate of MGO and GLO contents were similar. LA showed more significantly promotion on the formation of carbonyl compounds, the increase rate of GO content (34.12%) and 3-DG content (18.02%) contents in SM-LA group were higher than that of GO (26.48%) and 3-DG (14.22%) in SM-OA group. This result may be related to the higher radical supplementation of LA than OA in Maillard reaction, in agreement with our previous research (Zhao et al. 2021).

As depicted in Fig. S3e, the decrease rate of FL content was higher in SM-LA group than that in SM-OA group, indicating that the amount of FL formation is greater in SM-LA group, thus it demonstrated a slower degradation trend than that in SM-OA group. Compared with Fig. 1e, FL showed the similar change trend in two types of milk.

In Fig. S3h, the increase rate of Pyrr content in SM-OA group was higher than that in SM-LA group. Liang's study (2016) already explicated that Pyrr can form dipyrraline from two Pyrr molecules, which may be the reason for low accumulation of Pyrr in SM-LA group. The increase rates of CEL were similar in SM-OA and SM-LA groups (Fig. S3g), however, the increase rate of CML was lower in SM-LA group than that in SM-OA group (Fig. S3f). The results showed that the level of glycation in SM-LA group was higher than that in SM-OA group, especially in the

increase rate of FL and GO, however, there is no corresponding increase rate of CML, a reasonable explanation was that CML maintained dynamic balance and the degradation of CML in SM-LA group was more than that in SM-OA group, resulting in a decrease in the accumulation of CML (Nguyen et al. 2016).

The change of melanoidin indicated that the glycation level in SM-LA group was significantly higher than that in SM-OA group (Fig. S3j). Moreover, the decrease rate of peroxide value in SM-LA group (38.88%) was higher than that in SM-OA group (32.83%), and the correlation between FL and peroxide value content was more significant in SM-LA group ($P < 0.01$; Table S2), these results were related to the higher oxidation activity of LA, and radicals are active in initial stages of Maillard reaction, demonstrated that LA has a significant effect on glycation.

Comparison of glycation products in three groups by OPLS-DA

The OPLS-DA was used to distinguish skim milk and simulation groups and identify potential key products. In Fig. 4a, the three simulation groups were distinguished significantly. As calculated, the R^2Xcum, R^2Ycum, and Q^2cum values were 0.851, 1, and 0.926, showing goodness of fit. The variable importance in projection (VIP) values in Fig. 4b were distinguishable in SM, SM-OA-LA, SM-OA-LA-PA groups. The products with VIP > 1 were peroxide value (1.17415), FL (1.1197), Pyrr (1.10757), MGO (1.06943), 3-DG (1.05239), and GLO (1.03406), and they were derived from the primary oxidation products, main intermediate products of Maillard reaction, glycation products, and dicarbonyl compounds, respectively.

The main factors causing milk glycation were oxidation radicals of lipids, carbonyl amine condensation of proteins, and carbonyl compound formation of carbohydrates, which

Fig. 4 OPLS-DA results, **a** The score plot; **b** variable importance in projection (VIP) values for discriminating skim milk (SM), skim milk with oleic acid and linoleic acid (SM-OA-LA), and skim milk with oleic acid, linoleic acid and palmitic acid (SM-OA-LA-PA) groups
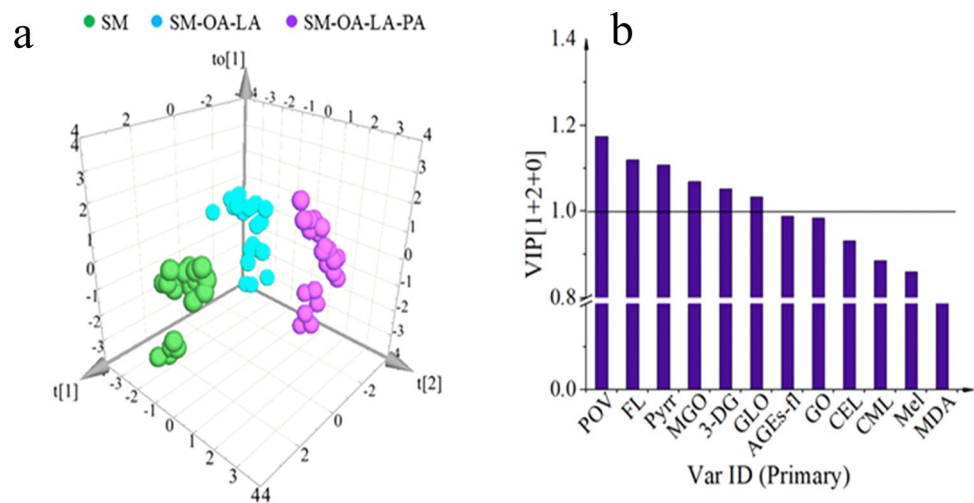
explained why saturated fatty acids play a smaller role than unsaturated fatty acid in glycation. The auto-oxidation of lipids occurred preferentially in unsaturated fatty acid, but the highest proportion of milk fats were saturated fatty acids, thus it is unusual for milk glycation to occur at room temperature in relatively short time. However, the percentage of acylglycerol and phosphatide content in milk fat and their degradation rate, should be considered during processing, which is determined by temperature and hence determines the rate of deterioration.

Conclusions

Milk fat promoted milk glycation, which mainly reflected in the increase rate of carbonyl compounds and AGEs accumulation, and the number of glycated sites. Two types of unsaturated fatty acid LA and OA are the main components in milk fat glycation, and LA's effect on glycation was more significant than OA. With the increase of free fatty acids via oxidation degradation, the glycation rate increased significantly in milk, especially LA exhibited better promotion on AGEs formation. The radicals triggered by unsaturated milk fat played an essential role in lipid oxidation and protein glycation, especially at heating or storage. OPLS-DA results showed that the key factors causing milk glycation were lipid oxidation radicals, protein carbonyl amine condensation, and carbonyl compound formation. Correlation analysis suggested that lipid oxidation was closely related to glycation products, indicating that the radicals formed by milk fat oxidation are the inducer of the initial glycation, and the carbonyl compounds formed by milk fat oxidation are also important intermediates of glycation in milk. These results further illustrated the correlation between milk oxidation and Maillard reaction.

Declarations

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