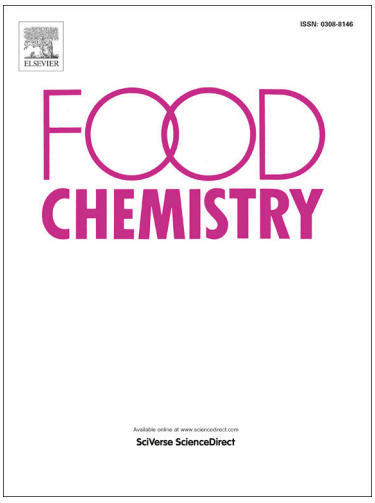

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The effect of Maillard reaction on flavour development of protein hydrolysates from cheese

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Abstract: This study aims to explore the effect of the Maillard reaction (MR) on flavour development of cheese protein hydrolysates. In addition, the effects of proteolysis, lipolysis, and the degreasing process on the MR have been explored. Cheese protein hydrolysates subjected to different treatments were heated with glucose and xylose, and their amino reactant components, colour parameters, and volatile compounds were determined. The results showed that the MR significantly affected the content of free amino acids, peptides, and volatile flavours of cheese protein hydrolysates. Peptides below 1500 Da and most of the free amino acids were the important amino reactants during the MR. 3-Ethyl-2,5-dimethylpyrazine, 2,5-dimethylpyrazine, 2-undecanone and 2-heptanone were the key volatile components of the MR products. The results also indicated that N-terminal amino acids of the peptide chain were easier to be reacted than C-terminal amino acids and thus produce a pyrazine-like flavour in the MR.

Keywords: Maillard reaction; Flavour development; Degrease; Lipolysis; Peptidomics

1 Introduction

Most cheeses have a high content of protein which can be hydrolysed by adding exogenous proteases, thereby producing a lot of free amino acids (FAAs) and low molecular weight peptides. These water-soluble components mainly contribute to the taste of food, while part of them undergo chemical reactions and produce some flavour compounds (Ardö, 2006). In general, protein hydrolysates have some defects such as a bland flavour and heavy bitter taste. Accordingly, the Maillard reaction (MR) is a non-enzymatic reaction that occurs between carbonyl groups (reducing sugars, aldehydes, or ketones) and amino compounds (proteins, peptides, or amino acids) (Ni, Wei, Zheng,

Thakur, Zhang, & Wei, 2022), which is usually used to increase the aroma of processed food. For example, Yang, Wang, Cao, Song, Xu, and Lin (2023) reported that pyrrole, furfural, 2-ethyl-3-methylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-6-methylpyrazine, methylpyrazine, and 5-methyl-2-furancarboxaldehyde are the key flavour compounds to strengthen the roasted attributes in Wuyi rock tea during the MR. It is also reported in the literature that sulphur-containing substances are mainly derived from the MR in Beijing roasted duck (Liu, Wang, Zhang, Shen, Hui, & Ma, 2020). These volatile compounds contribute a lot to the flavour development of the products. Therefore, research on the application of MR on cheese protein hydrolysates could be a potential strategy for reducing the hydrolysates' defects and producing key MR flavours.

Generally, cheese curd has a high content of crude fat, which contributes a lot to its aroma. During the heating process, the fat can degrade and produce more phospholipid moieties or cleavage products of fatty acid oxidation such as aldehydes and provide volatile compounds to improve the flavour of products (Cheraghi & Roozen, 1994). Therefore, the presence or absence of fat may affect the MR of cheese protein hydrolysates.

It is well known that the colour, taste, flavour, and quality of food can be fully affected after the MR. As a result, colour and flavour development can be used to evaluate the progress of the MR. The peptide chain length, peptide sequence and amino acid composition have been shown to have effects on the flavour characteristics for Maillard reaction products (MRPs) (Sun et al., 2022). Therefore, this study aimed to

explore the effects of the MR on the flavour development of cheese protein hydrolysates and the effect of the cheese fat on the MR. To achieve this, 6 samples were prepared, and the content of FAAs, peptide sequences, browning intensity, colour, and volatile compounds were measured. Subsequently, principal component analysis (PCA) between volatile compounds and FAA, peptide sequence was conducted. This study provides a novel insight for the application of the MR in dairy products.

2 Materials and methods

2.1 Materials

Immature cheese curd named 'Cagliata deep-frozen 48% fat i. d. m.' (with 23% protein and 26% fat) was purchased from the DMK Group and used as raw material. Three emulsifying salts were selected, disodium hydrogen phosphate (Na_2HPO_4) (Beijing Cuifeng Technology Co. Ltd., Beijing, China), sodium tripolyphosphate ($Na_5P_3O_{10}$) (Beijing Voge Oriental Technology Co. Ltd., Beijing, China), and sodium citrate ($C_6H_5Na_3O_7$) (Beijing Yishan Huitong Technology Co. Ltd., Beijing, China). Protease A 'Amano' 2SD (100,000 U/g) and Lipase DF 15 (not less than 150 u/mg) were kindly provided by Shanghai Amano Enzyme Manufacturing Co. Ltd. (Shanghai, China). Protease A 'Amano' 2SD has both endotangential and extangential activity. Food-grade D-xylose (99%) and glucose (99%) were purchased from Beijing Cuifeng Technology Co. Ltd. (Beijing, China). Dietary alkali was purchased from Beijing Shuntian Hengfeng Trading Co. Ltd. (Beijing, China) and used to adjust the pH of the system. The MR was carried out in a reversed-pressure high-temperature cooking pot (TS-25C, Beijing Landmaker Technology Development Co. Ltd., Beijing, China).

The FAA mixed standard was obtained from Sigma-Aldrich Co. Ltd. (MO, USA). The AccQ•Tag (mobile phase used for the determination of FAA) was purchased from Waters Technology Co. Ltd. (Shanghai, China). The n-alkane standards (C7-C40, ≥ 97%) for the linear retention indices were obtained from o2si smart solutions (Beijing, China).

2.2 Sample preparation

To prepare samples, 56.7% immature cheese curd, 41% water, and 2.3% emulsified salt ($C_6H_5Na_3O_7$: Na_2HPO_4 : $Na_5P_3O_{10}$ = 200 : 20 : 37) were mixed, sheared, and heated in a water bath at 80°C for 15 min. After rapidly cooling, the mixture was hydrolysed by 0.4% (w/w) protease 2SD for 4 h at 45°C, at 250 rpm. The proteolytic reaction was terminated at 90°C for 20 min and the obtained slurry underwent different treatments as shown in Figure S.1, as follows: proteolytic sample (P); proteolytic-MR sample (PH); proteolytic-degreased sample (D); proteolytic-degreased-MR (DH); proteolytic-lipolytic sample (L); proteolytic-lipolytic-MR sample (LH). Lipolysis was achieved by using 0.2% Lipase DF 15 (w/w) for 3 h at 40°C and 250 rpm, and the reaction was terminated at 90°C for 20 min. To remove the fat part of the proteolytic slurry, samples were centrifuged at 10,000 rpm at 4°C (Dallas, Guerrero, Khaldi, Castillo, & Lebrilla, 2013). The pH of samples P, D, and L was adjusted to 7 by using 13.8% dietary alkali to prepare the MRPs PH, DH, and LH. The MR conditions were as follows: ensure the protein content:sugar content = 2.576 : 1, xylose : glucose = 2 : 1, MR temperature: 101°C, MR time: 59 min. Each sample was produced in triplicate and stored at −18°C until analysis.

2.3 Chemical determination

Measurement of the fat and protein content of the 6 samples (P, PH, D, DH, L, and LH) followed the Gerber method (Chinese standard GB 5009.6–2016) and the Kjeldahl method (Chinese standard GB 5009.5–2016), respectively.

2.4 Determination of amino reactants

2.4.1 Determination of FAAs and molecular weight distribution

FAAs and molecular weight distribution (MWD) were determined by ultra-high-performance liquid chromatography (UHPLC) (Waters Technology Co. Ltd., Shanghai, China) according to the methods provided by the company. The quantification of FAAs was conducted using an Xbrigde BEH C18 column (kept at 37°C) and five different concentrations of amino acid standard solution were prepared and used to get the standard curve with $R^2 = 0.999$. A UV detector was used and the wavelength set at 248 nm. Mobile phase A was composed of a 1 : 10 diluted solution of AccQ•Tag A, and mobile phase B was acetonitrile. The flow rate was 1 mL/min. The gradient programme was as follows: mobile phase A was decreased from 100% to 99% during 0–0.5 min, then from 99% to 95% during 0.5–18 min, 95% to 91% during 18–19 min, 91% to 83% during 19–29.5 min, 83% to 0% during 29.5–33 min, and from 0% to 100% during 33–36 min.

The MWD of the samples was determined by a 2000 (300 × 7.8 mm) SWXL TSK gel filtration column (Tosoh Co. Ltd., Tokyo, Japan) and the sample held at a flow rate of 0.5 mL/min. Four standards (Shanghai Yuanye Bio-Technology Co. Ltd., Shanghai, China) were used for determination: aprotinin (6000 Da), bacitracin (1500 Da),

tetrapeptide GGTA (450 Da), dipeptide GS (150 Da).

2.4.2 Identification of peptide sequences by LC-MS/MS

Peptide sequences were identified by LC-MS (Thermo Fisher Technology Co. Ltd., Beijing, China). Pure peptides were prepared according to Dallas et al. (2013) with some modifications. Firstly, the sample solution was centrifuged several times to remove the fat; then the protein components were removed by precipitation with 200 g/L trichloroacetic acid (TCA) solution, and then the remainder was centrifuged and the supernatant collected. After that, the supernatant was purified by bed C18 column to remove the oligosaccharides and salt components. Pure peptide was obtained by further elution with 80% acetonitrile and 0.1% trifluoroacetic acid (TFA) solution. Finally, the pure peptide solution was concentrated by vacuum freezing to obtain the concentrated peptide fraction. Formic acid (0.1%) was used to reconstitute the peptides then the samples was passed through the Easy-nLC1200 system followed by a mass spectrometer (Orbitrap fusion lumos) according to Dingess et al. (2017) with some modifications. The mobile phase consisted of 0.1% formic acid/H_2O (solvent A) and 0.1% formic acid/80% acetonitrile/H_2O (solvent B). The gradient programme was as follows: 8% B was increased to 38% B for 50 min, 38% B to 63% B for 58 min, 63% B to 95% B for 59 min, then maintained for 70 min. The mass parameters were set as follows: Nanospray Flex™ (NSI) ion source; 600 nL/min mobile phase flow for 70 min; spray voltage, 2200 V; capillary temperature, 320°C; orbitrap resolution, 120,000 (200 m/z), with a scanning range of 400–1500 m/z; secondary ions, Ion Trap automatic scanning range; collision energy, 35%.

The raw files of peptidomics were analysed by Maxquant 1.6.3.4 and the peptidomics database was based on a previous study (338 milk proteins) (Boggs, Hine, Smolenski, Hettinga, Zhang, & Wheeler, 2016). Variable modifications were as follows: oxidation of methionine; N-terminal acetylation; phosphorylation of serine, threonine, or tyrosine; deamidation of asparagine or glutamine. The first search of mass tolerance was set at 20 ppm for MS peaks. The maximum false discovery rate (FDR) was set at 1% for both peptide and protein levels.

2.5 Browning intensity determination

The absorbance at 294 nm and 420 nm and the fluorescence intensity of the samples were determined by the method of Tan et al. (2021) with some modifications. Samples were diluted 200-fold with ultrapure water before measuring the absorbance at 294–420 nm using a spectrophotometer (Spark 20M, Tecan, Switzerland). Samples were diluted 100-fold to determine the fluorescence. The excitation wavelength was set at 347 nm, and a range of 300-400 nm was selected for the emission wavelength.

2.6 Colour determination

The colour of samples was determined using a colour meter (SPH 860, Colorlite, Germany). The colorimetric parameters were as follows: L^* (brightness), a^* (+a, redness; –a, greenness), b^* (+b, yellowness; –b, blueness), C^* (vividness of colour). Before the analysis, the reflection photometer was calibrated with a blank. After calibration, a sample was added to a 1 cm glass vial, and analysis of three replicates was carried out. ΔE, the total colour difference between two samples, was calculated with the following formula (Guo et al., 2022):

$$\Delta E = \sqrt{(L^*_1 - L^*_0)^2 + (a^*_1 - a^*_0)^2 + (b^*_1 - b^*_0)^2}$$

where L^*_1, a^*_1, b^*_1, L^*_0, a^*_0, and b^*_0 are the colour parameters of the respective sample.

2.7 Volatile compound determination

The volatile components of all samples were tested by solid-phase microextraction-gas chromatography and mass spectrometry (SPME-GC/MS) (GCMS-QP2010 Plus, Shimadzu, Japan) according to Bas, Kendirci, Salum, Govce, and Erbay (2019) with some modifications. To ensure optimal GCMS performance, auto-tuning of the GCMS was implemented before analysis. A 65 μm PDMS/DVB SPME fibre was used for extraction at 60°C for 30 min. The injection port temperature was set at 250°C, and a DB-WAX (30 m × 250 μm × 0.25 μm) column was used. The heating procedure was as follows: 40°C for 3 min; 200°C for 5 min, 5°C/min; 230°C and 10°C/min. Qualitative analysis was performed in accordance with a previous study by using two methods (NIST library and RI). The determined RI were checked against published studies which used the same column polarity.

The $rOAV_m$ (relative odour activity value) of the substance that contributed the most to the aroma of the sample was regarded as 100 (Wang et al., 2021). Therefore, the rOAV of the other main volatile components was calculated as follows:

$$rOAV_i = 100 \times C_i/C_m \times T_m/T_i$$

where C_i and T_i represent the relative peak intensity and odour threshold of i compound, respectively. C_m and T_m indicate the maximum odour activity value of the component.

2.8 Electronic nose (E-nose) determination and analysis

A PEN 3.5 portable E-nose (Winmuster, Version 1.6.2, Airsense Analytics GmbH,

Schwerin, Germany) equipped with 10 metal oxide semiconductor (MOS) sensors was used to explore the differences in odour of the samples. The 10 sensors and their descriptions are as follows: W1C – sensitive to aromatic constituents, benzene; W5S – sensitive to nitrogen oxides; W3C – sensitive to aroma, ammonia; W6S – sensitive to hydrides; W5C – sensitive to short-chain alkane aromatic components; W1S – sensitive to methyl; W1W – sensitive to sulphides; W2S – sensitive to alcohols, aldehydes, and ketones; W2W – sensitive to organic sulphides; W3S – sensitive to long-chain alkanes (Q. Chen, Hu, Wen, Wang, Qin, & Kong, 2021). Two grams of sample was added to a 10 mL glass vial and sealed. A needle was inserted and used to transfer the sample to the sensor chamber for 60 s, and the sensor signals were recorded.

2.9 Statistical analysis

The data are shown as mean ± standard deviation (SD). One-way ANOVA was used to analyse the significant difference ($p < 0.05$) among different treatments using IBM SPSS Statistics (Version 24, SPSS Inc., Chicago, IL, USA). Pos hoc Duncan tests were used for multiple comparisons. PCA analyses were performed using the R package ggplot2. The heatmap was made using TBtools (K. Chen, Yang, Hong, Feng, Liu, & Luo, 2020) and the peptidomics data visualisation was done using Peptigram (http://bioware.ucd.ie/peptigram/).

3 Results and discussion

3.1 Chemical analysis

Table S.1 shows the fat and protein content of the 6 samples. Samples D and DH had a low fat content (0.70% and 0.57%, respectively), which indicated a successful

degreasing process. Samples P, PH, L, and LH had a high fat content (> 11%). Basically, all of the samples had a high protein content (> 8%).

3.2 Analysis of amino reactants

3.2.1 Analysis of FAAs and MWD

The amino acid concentrations and MWD percentage of the samples are shown in Figure 1. According to Figure 1a and 1c, sample D contained the highest concentration of total FAAs and hydrophobic amino acids, followed by P, LH, L, and DH, while sample PH achieved the lowest total FAA and hydrophobic amino acid content. These results are in accordance with previous reports (Guo et al., 2022; Lotfy, Saad, El-Massrey, & Fadel, 2021) which mentioned decreased amounts of FAAs as the precursors to various volatile compounds in the MR and other processes (e.g. lipolysis). For example, free fatty acids containing four or more carbon atoms may come from lipolysis of milk fat or the breakdown of amino acids (Urbach, 1993). Furthermore, the carbonyl condensation of the MR is a complex reaction that can be analysed by the loss of amino acids during the reaction (Shang, Zhong, Zhu, Wang, Huang, & Li, 2020). Ma et al. (2020) reported that β-hydroxy amino acids can generate pyrazine and contribute a lot to the general flavour. Therefore, we deduced that some of the FAAs undergo chemical reactions and further transfer into a range of flavour compounds, resulting in a significant reduction ($p < 0.05$) in their concentration during lipolysis and the MR process. The results also showed that 17 amino acids were detected in all of the samples (Figure 1e). Leucine (2367.64 mg/L), phenylalanine (1627.51 mg/L), histidine (1212.61 mg/L), lysine (1107.33 mg/L), valine (1030.52 mg/L), isoleucine (984.12

mg/L), and glutamic acid (893.16 mg/L) were the most abundant in sample P. As for sample PH, for example, the concentrations of leucine (1747.02 mg/L), phenylalanine (1083.40 mg/L), and lysine (855.59 mg/L) were significantly decreased ($p < 0.05$) compared to those in sample P. This reveals that most of these FAAs may take part in the MR. Interestingly, the results showed that only the concentrations of histidine (1245.68 mg/L to 857.51 mg/L), threonine (438.04 mg/L to 381.88 mg/L), tyrosine (953.23 mg/L to 860.04 mg/L) and phenylalanine (1495.80 mg/L to 1303.64 mg/L) decreased from sample L to sample LH. Therefore, these 4 amino acids could be important amino reactants in the MR after lipolysis. From the above results, we deduced that different processing modalities may lead to different amino reactants during the MR. Under the same proteolysis process, sample defatting did not cause significant differences in the amino reactants during the MR, while lipolysis caused a significant reduction ($p < 0.05$) in amino acid content and affected the amino feedstock involved in the MR. In addition, compounds formed through lipolysis may interact with amino acids, resulting in an increase in the absorbance of samples LH and L.

Previous studies have reported that peptides can directly take part in the MR as the amino group (Hou, Mu, Ma, & Blecker, 2019), with even a higher ability to form specific flavours (especially pyrazines and ketones) than that of FAAs. In this study, the MWD was divided into 6 fractions (> 6000 Da, 3000–6000 Da, 1500–3000 Da, 450–1500 Da, 150–450 Da, < 150 Da) (Figure 1f). The percentages of 150–450 Da and > 3000 Da peptides were significantly increased ($p < 0.05$; Duncan's test results not shown) from P to PH (31.52% to 38% and 0.64% to 1.03%) and D to DH (29.31% to

33.08% and 0.56% to 1.01%), while the percentage of peptides below 150 Da was decreased from P to PH (35.89% to 29.81%) and D to DH (38.84% to 34.16%). These results indicate that peptides below 150 Da may be the main raw amino material for PH and DH. In addition, the study showed that the increase in proportion of Maillard peptides (< 1000 Da and 1000–5000 Da) may result from the cross-linking or polymerisation of low molecular weight peptides (< 1000 Da) (Chiang, Yeo, Ong, & Henry, 2022; Karangwa et al., 2015). In contrast, the proportion of peptides between 150 and 1500 Da was decreased from L to LH (65.94% to 62.53%), while the other fractions of peptides increased during the MR. This result is basically consistent with the above data for FAAs; similarly, 150–1500 Da peptides could be crucial reactants for LH samples. These results also reveal that the main contributor to cross-linking reactions varies with different products, especially for lipolysis. Generally, smaller peptides are likely to be the main reactive substances in the MR as reported by Chiang et al. (2022). Particularly, it has been reported that low molecular weight peptides (< 500 Da) are the most predominant cause of pyrazine production in the MR (Hrynets, Ndagijimana, & Betti, 2015). In summary, most FAAs and peptides below 150 Da are regarded as the essential amino reactants during the MR of the cheese proteolytic hydrolysates. Histidine, threonine, tyrosine, phenylalanine, and peptides with a molecular weight of 150–1500 Da were the main participants in the MR of cheese proteolytic-lipolytic samples.

3.2.2 Peptide sequence analysis

The volcano plots in Figure 2a illustrate the significant differences of peptides

between samples P and PH, P and DH, and PH and DH (|Log (fold change)| > 2, p < 0.05). It is clear that intensities for 11 peptides were significantly up regulated in sample PH compared to sample P (such as P02662_125_134, P02662_197_208, P02666_210_221, P02666_211_220, and P02662_200_211). Furthermore, the intensities of 12 peptides were significantly down regulated in sample PH. These include P02666_16_26, P02666_209_221, P02666_209_224, and P02662_137_155. The results showed that several up regulated peptides such as P02666_210_221 may be degraded from longer pepetides (P02666_209_224) during heat treatment and some of them were down regulated. And sample DH showed similar peptide intensities to sample PH compared to sample P, and only the intensity of P02666_166_175 peptide (LPPTVMFPPQ) was significantly higher in sample DH than sample PH. Accordingly, the results from peptidomics were similar to those for FAAs and MWD, which indicates that the degreasing process may have little impact on the amino reactants during the MR. Table S.2 list the peptides with significantly different (p < 0.05) intensities between sample P and sample PH, sample P and sample DH, and sample PH and sample DH. Figure 2b shows the peptigram for every precursor protein and the regions of sequence prone to aggregation for every sample. Precursor proteins including β-casein, αs1-casein, and αs2-casein are also presented in Figure 2b. It is clear that the amount and intensity of peptides from β-casein and αs2-casein at positions about 220 and 120 decreased after the MR, which suggests that these peptides may participate in the MR. Particularly, the intensity of peptides from αs1-casein at position 200 increased in the MRPs (PH and DH). Kalyankar, Khedkar, Patil, and Deosarkar (2016) reported that

αs2-casein is the most hydrophilic casein, while β-casein is the most hydrophobic of the caseins. Therefore, peptides from these most hydrophobic or hydrophilic proteins may be more prone to engage in the MR.

3.3 Browning intensity analysis

Figure 3 shows the absorbance and fluorescence intensity of all the samples. It has been reported that some products such as Amadori and Heyns compounds may be formed during the MR with a characteristic absorption wavelength of 294 nm (Chailangka et al., 2022). And the absorbance value at 420 nm was considered to represent the final browning products at the end of the MR(Ye et al., 2022). In this study, sample LH achieved the highest scores in the absorbance spectra, followed by L, PH, DH, and D, and the lowest scores were observed in sample P (Figure 3a). These results indicate that all of the MRPs (PH, DH, LH) achieved higher absorbance than did their corresponding unheated products (P, D, L). In addition, the absorbance value of sample LH was higher than that of sample PH, and even higher than that of sample DH, indicating that sample LH formed a browner polymer-melanoid than did sample PH or DH. This may be due to the interaction of fat hydrolysates with amino acids, or the thermal reaction of lipase inactivation. Moreover, the linear correlation of absorbance value between 294 nm and 420 nm indicates that some intermediate products may be converted into the final brown compounds. In addition, fluorescent compounds are formed in the early stages of the MR, which can be detected at an excitation wavelength of 347 nm (Lertittikul, Benjakul, & Tanaka, 2007). Clearly, the highest peak was observed at an emission wavelength of about 342 nm (Figure 3b).

3.4 Electronic eyes analysis

Brown colour compounds are usually generated from the degradation of Amadori products through furfural and reductone routes during the MR (Spotti, Perduca, Piagentini, Santiago, Rubiolo, & Carrara, 2013). Therefore, colour changes among samples were used to monitor MR progress by electronic eyes. As shown in Table 1, the parameter L^* was significantly decreased ($p < 0.05$) from P to PH, D to DH, and L to LH, respectively, suggesting that the MR is accompanied by a decrease in brightness of the samples. The above results are in accordance with those of Tan et al.'s (2021) study, as the formation of brown pigments during the MR may cause a decrease of the L^* value. On the contrary, substantial increases in the values of a^*, b^*, and C^* were detected after the MR; this is consistent with the results of Kaewtathip, Wattana-Amorn, Boonsupthip, Lorjaroenphon, and Klinkesorn (2022), who reported that a positive value of b^* represents a yellow colour with a strong correlation to colour formation in the MR. In addition, a^* values ranged from 2.27 to 18.51 between P and PH, 1.85 to 23.43 between D and DH, 2.67 to 14.15 between L and LH, respectively, which indicates that the sample went through a process from green to red. And the ΔE (total colour difference) value was measured. Obviously, the highest ΔE value (39.83) was achieved between D and DH, followed by the values between P and PH (30.82), L and LH (19.21), DH and LH (17.22), PH and LH (10.32), PH and DH (7.13), D and L (4.68), and P and D (3.09), and P and L (1.71) samples contained the smallest total colour difference (ΔE). These results indicate that the MR was the main contributor to the colour differences between samples. Comparing the colour parameters of the three MRPs (PH,

DH, and LH), the following results were obtained: for the values of parameter L^*, LH > PH > DH; in contrast, for the values of a^*, b^*, and C^*, LH < PH < DH. This indicates that the defatted sample (DH) may exhibit a stronger MR colour, followed by the non-defatted sample (PH) and lipolytic sample (LH). This is an interesting phenomenon worth exploring. Samples with fat present may be more inclined to produce flavour substances with a low threshold in the MR (Benet, Guàrdia, Ibañez, Solà, Arnau, & Roura, 2015), while samples with little or no fat content may be more likely to affect the colour of the product in the MR.

3.5 Volatile profiles of all samples

3.5.1 Differential volatile compounds of all samples

A heatmap of the volatile components of all samples is shown in Figure 4a. The Log scale method was used, as follows: base 2, Logwith 1. The redder the colour, the higher the content. A total of 51 volatile compounds were detected, 12 acids, 12 ketones, 4 aldehydes, 5 alcohols, 4 esters, 10 pyrazines and 4 others. It is clear that the volatile compound distributions of all samples were different from each other. Sample L showed the most kinds of volatile compounds (31), followed by LH (27) and PH (27), DH (24), and P (16), and sample D had the least variety of volatile substances (11). This result demonstrates that lipolysis and the heating process can increase the richness of volatile components, while degreasing may be conducive to the loss of some volatile substances. For example, butanoic acid and 9-decenoic acid were only detected in the lipolytic samples (L and LH), and all the pyrazine compounds (such as 2,5-dimethylpyrazine and trimethylpyrazine) were produced during the MR (PH, DH, LH).

According to previous studies (Khan, Jo, & Tariq, 2015; Ma et al., 2020), lipid oxidation and degradation results in the production of some volatile compounds and contributes to the overall odour of samples, and this may be a reason for the fewest kinds of volatile components in sample D (degreased). Furthermore, the interaction between lipid oxidation products and the MR can also form a series of reactions and accelerate the flavour development of samples (Sun et al., 2022). And the main effect of fat on the MR to produce aroma in the system is heating degradation and oxidation, which can produce various volatile compounds such as alkanes, aldehydes, alcohols, esters, carboxylic acids, etc. Some of these substances further participate in the MR to form more complex aroma substances (Cheraghi et al., 1994). In this study, hexanoic acid, octanoic acid, and n-decanoic acid were abundant in the lipolytic samples (L and LH), while the differences were that LH sample had a higher content of 2-heptanone, 2-nonanone, 2-undecanone, 2,5-dimethylpyrazine, and trimethylpyrazine than did the L sample. Therefore, lipolysis and the MR process play an important role in the flavour development of cheese protein hydrolysates.

Basically, a sample's flavour depends on the synergy and suppression of various odorants. Figure 4b shows the peak area percentage of all the samples: for sample P, the most abundant components were acids (33.90%), ketones (32.15%), alkanes (13.53%), alcohols (8.04%), aldehydes (6.25%), and esters (6.13%). After the MR, sample PH was significantly different and contained mostly pyrazines (54.86%) and ketones (35.54%). According to Negroni, D'Agostin, and Arnoldi (2001), the major carbonyl compounds (for example, 2-decanone and 2-nonanone) generated during

heating may be a result of unsaturated fatty acids. Sample DH showed a similar percentage of the 7 kinds of volatile compounds (pyrazine, esters, alcohols, alkanes, aldehydes, ketones, and acids) to sample PH. This indicates that the degreasing process has little effect on the overall percentage of various kind of flavour compounds during the MR based on cheese protein hydrolysates. This can be explained by the results for amino reactants (degreasing does not cause larger differences in the amino reactants during the MR). In addition, acids contributed most of the volatile compounds in samples L and LH, while part of the acids may be converted into ketones during the MR and make sample LH have a higher percentage of ketones. In general, the percentage of pyrazines and ketones significantly improved after the MR, while the percentage of acids decreased dramatically during the heating process.

An E-nose can analyse volatile compounds, and a clear discrimination is shown in Figure 4c. The x-axis and y-axis explained 83.77% and 11.29% of the variance, respectively, and the total contribution rate was above 95%. The separate circles indicate the difference of odour molecule composition and their concentration between the 6 samples. Significant differences were observed between the non-heated samples (P, D, L) and the Maillard reactants (PH, DH, LH); this indicates their largely different flavour and aroma.

3.5.2 Main volatile compounds of all samples

Different processes produced significant changes in essential flavour components correlated with some of the most enjoyable aromatic notes in the samples. As shown in Figure 4d, the top 10 volatile components in terms of rOAV value were the key

compounds for the flavour of all the samples (Table S.3). Generally, acetoin, acetophenone, n-decanoic acid, and benzaldehyde made a significant contribution to the flavour of sample P, while 2-undecanone, 3-ethyl-2,5-dimethylpyrazine, nonanal, 2,5-dimethylpyrazine, and 2-heptanone contribute a lot to the PH flavour. Nonanal, acetoin, and 2-undecanone were the main volatile components in sample D, while 3-ethyl-2,5-dimethylpyrazine, 2-undecanone 2,5-dimethylpyrazine, 2-acetyl-3-methylpyrazine, and 2-heptanone mainly impart the flavour of sample DH. In addition, components such as 2-undecanone, n-decanoic acid, hexanoic acid, heptanoic acid, and octanoic acid were the main flavours of sample L, but 2-undecanone, n-decanoic acid, 3-ethyl-2,5-dimethylpyrazine, 2,5-dimethylpyrazine, hexanoic acid and 2-heptanone were presented as the main volatile substances imparting the main flavours of sample LH. Therefore, 3-ethyl-2,5-dimethylpyrazine, 2,5-dimethylpyrazine, 2-undecanone, and 2-heptanone were the key volatile components of all MRPs (PH, DH, LH).

According to the literature, both differential and main volatile components can be defined as key volatile compounds (Yu, Xiang, Tan, Zhang, Shan, & Yang, 2021). Therefore, most of the above compounds can be referred to as the key volatile compounds in the 6 samples. These components are clearly associated with typical odours, for example, grassy, creamy, fruity, roasted, meaty, and fatty. Also, the MR involves a cascade of complex reactions and can generate various different flavour compounds (Scalone et al., 2020). In detail, acetoin constitutes the aroma of yogurt, butter and creamy, and this compound played an important role in the flavour of P, PH, D and DH, especially for P (100% of ROAV) and D (57.48% of ROAV). In addition,

acetophenone had a low odour threshold and contributed 43.79% ROAV to sample P with the flavour of salted egg yolk (Che, Yu, Sun, Lu, & Xie, 2021). 2-Undecanone constitutes an orange and fresh smell, which imparted the key flavour of PH, D, DH, L and LH. Nonanal is described as a citrus, grass, and fat scent with its low odour threshold; notably, it's the key compound for PH (54.38% of ROAV) and D (100% of ROAV) samples. Acids and esters are mainly responsible for fruity and rancid smells; these kinds of compounds were the key flavour contributions for samples L and LH in this study. Pyrazines strongly influence the flavour of some bakery foods due to their low odour detection thresholds. In this study, 3-ethyl-2,5-dimethylpyrazine, 2,5-dimethylpyrazine, and trimethylpyrazine were likely the most important contributors for the Maillard reactants (PH, DH, LH). According to Peña-Correa, Ataç Mogol, van Boekel, and Fogliano (2022), alkylpyrazines (methyl-, ethyl-, propyl-) are the key odour components and nitrogen-heterocyclics in cocoa derivatives and display sweet, potato, coffee, chocolate, cocoa, nutty, and earthy notes. Various amino acids and peptides can act as amino-group substrates to produce 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethyl-5-methylpyrazine, and 3-ethyl-2,5-dimethylpyrazine (Schieberle, 1990). ,Similarly, Scalone et al. (2020) reported that the addition of protein hydrolysates can facilitate the generation of 2,5-dimethylpyrazine. Pyrazine compounds may be also formed by the condensation of Strecker aldehydes and ketones (Zheng, Wei, Liu, Thakur, Zhang, & Wei, 2023). Another study reported that the peptide–sugar MR system and amino acid–sugar MR system showed different flavour profiles of MRPs. Dipeptides can produce more pyrazines (for example,

trimethylpyrazine and 2-dimethylpyrazine) than can amino acids (Van Lancker, Adams, & De Kimpe, 2010). Therefore, this study explored the correlations between the amino reactants and some key flavour compounds of the MRPs by PCA analysis, as shown in section 3.6.

3.6 PCA analysis

PCA plots for samples P, PH, and DH were created to discriminate the differences better visually between them, and the top 25 discriminators are shown in Figure 5. Generally, PC1 explained 52% of the variance which showed the significant differences between the proteolytic sample (P) and the MRPs (PH, DH), and PC2 explained 11.3% of the variance between the two MRPs (PH, DH). Sample DH and sample PH are located on the left side, while sample P is located on the right side. This indicates that the MR resulted in the large differences in volatile compounds, peptide distribution, peptidomics, and FAA, and the differences of peptide distribution, peptidomics, and FAAs may contribute to the differences in volatile compounds, too. Obviously, most of the FAAs (for example, glycine, methionine, valine, isoleucine, and threonine) and several peptides such as P02666_16_26, P02666_209_220, and P02666_182_191 were shown to be perfectly positively related to sample P, while negatively correlated to the MRPs (PH and DH). This reveals that most of these amino acids and peptides may be consumed during the MR. On the contrary, the concentrations of 2,5-dimethylpyrazine, trimethylpyrazine, 2-heptanone, arginine, and peptides (P02662_144_154 and P02662_200_214) were positively correlated to the MRPs (PH and DH), especially for 2,5-dimethylpyrazine, arginine, trimethylpyrazine, and 2-heptanone. This indicates that

the MRPs have the characteristic of a pyrazine flavour. Combined with the contributions of all the loading plots for each factor shown in Table S.4, it appears that the amino groups of glycine, threonine, valine, isoleucine, methionine, P02666_16_26, P02666_182_191, etc. play an essential role in the flavour development of the MRPs, with characteristics of 2,5-dimethylpyrazine, 2-heptanone, and trimethylpyrazine. Combined Table S.2, most of the contributing peptides had a mass less than 1500 Da, in common with the analysis of MWD during the MR. These peptides may play a more active role in the MR of cheese protein hydrolysates. Furthermore, as P02666_16_26 is near to the N-terminal amino acid of the peptide chain, and P02666_182_191 is near to the C-terminal amino acid of the peptide chain, and P02666_16_26 showed a greater contribution than P02666_182_191 in the flavour differences between sample P and the MRPs, we deduced that the N-terminal amino acid of the peptide chain plays a more important role than does the C-terminal amino acid in the MR to produce a pyrazine-like flavour, and this result was similar to that of the study of Van Lancker, Adams, and De Kimpe (2012). Overall, the results indicated that the MR process imparts a pyrazine-like flavour to samples based on cheese protein hydrolysates.

4 Conclusion

The present study utilised cheese protein hydrolysate, xylose and glucose as raw materials to prepare MRPs. Overall, most of the amino acids and low molecular weight peptides (< 1500 Da) mainly participate in the MR of cheese protein hydrolysates. Degreasing process did not cause significant differences in the amino reactants during the MR of cheese protein hydrolysates, while lipolysis process caused a significant

reduction ($p < 0.05$) in amino acid content and affected the amino feedstock involved in the MR. Volatile compound determination showed that lipolysis (more than 27 compounds) and the MR process (more than 24 compounds) process impart a significant effect on the overall flavour of samples based on cheese protein hydrolysates. Acetoin and acetophenone were the key volatile compounds in cheese protein hydrolysates. Acids were the key flavours for the lipolytic sample, while pyrazines and ketones were the key volatile components in all MRPs. This study provides a novel insight for the application of the MR in cheese, especially when it comes to some unavoidable hot processing of dairy products. In particular, it provides a new possibility for improving the flavour of dairy products by thermal reaction.

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Figure 1. Changes in (a) total free amino acids, (b) sulphur-containing free amino aicds (Cys and Met), (c) hydrophobic free amino acids (Pro, Tyr, Val, Met, Ile, Leu and Phe), (d) umami free amino acids (Glu and Asp), (e) free amino acids and (f) molecular weight distribution (MWD) of P, D, L and their Maillard reaction products (PH, DH, LH). Data represented the mean, and error bars represented the standard deviation of three replicates. (a-f; A-E; A*-F*; a*-d*): Different letters denoted significant differences ($p \leq 0.05$) for the 6 samples according to Duncan's test. P: proteolytic sample; PH: proteolytic-Maillard reaction sample; D: proteolytic-degreased sample; DH: proteolytic-degreased-Maillard reaction sample; L: proteolytic-lipolytic sample; LH: proteolytic-lipolytic-Maillard reaction sample.

Figure 2. (a): The volcano plot of the significant differences ($p < 0.05$) between sample P and PH, P and DH, and PH and DH. Down: significant decrease; Up: significant increase. (b): The peptide profile, peptide coverage visualisation with intensity values for every precursor protein of every sample. Dashed line represents no peptides detected in that sequence region. The darker green is, the higher intensities for peptide are. P: proteolytic sample; PH: proteolytic-Maillard reaction sample; DH: proteolytic-degreased-Maillard reaction sample.

Figure 3. (a): Absorbance values between 290 nm-420 nm, (b): fluorescence intensity of different samples. P: proteolytic sample; PH: proteolytic-Maillard reaction sample; D: proteolytic-degreased sample; DH: proteolytic-degreased-Maillard reaction sample; L: proteolytic-lipolytic sample; LH: proteolytic-lipolytic-Maillard reaction sample.

Figure 4. (a): Heatmap of the different volatile components; (b): peak area percentage of different chemical groups; (c): LDA plots of odour emissions from the 6 samples assessed by the PEN 3.5; and (d): bar graph of the main volatile compounds with the ROAV top 10 in 6 samples. P: proteolytic sample; PH: proteolytic-Maillard reaction sample; D: proteolytic-degreased sample; DH: proteolytic-degreased-Maillard reaction sample; L: proteolytic-lipolytic sample; LH: proteolytic-lipolytic-Maillard reaction sample.

Figure 5. PCA score plot and loading plot for sample P, PH and DH. The % of explained variance for each PC was shown in parentheses on the x and y axes. P: proteolytic sample; PH: proteolytic-Maillard reaction sample; DH: proteolytic-degreased-Maillard reaction sample.

Figure S.1 The preparation process flow chart of all samples.

Table 1. Electronic eyes analysis of the 6 groups.

Samples	L^*	a^*	b^*	C^*	ΔE_1^*	ΔE_2^*
P	92.78±0.17^{e}	2.27±0.05^{b}	19.00±0.19^{b}	19.13±0.19^{b}	^{1}30.82±0.21^{b}	^{4}3.09±0.11^{b}
PH	75.76±0.07^{b}	18.51±0.10^{e}	38.90±0.20^{e}	43.08±0.21^{e}		^{5}7.13±0.16^{d}
D	92.50±0.14^{de}	1.85±0.06^{a}	15.95±0.10^{a}	16.06±0.10^{a}	^{2}39.83±0.22^{c}	^{6}4.68±0.61^{c}
DH	71.96±0.16^{a}	23.43±0.02^{f}	42.39±0.18^{f}	48.43±0.16^{f}		^{7}17.22±0.34^{f}
L	92.22±0.05^{d}	2.67±0.24^{c}	20.55±0.53^{c}	20.72±0.56^{c}	^{3}19.21±0.73^{a}	^{8}1.71±0.61^{a}
LH	82.77±0.54^{c}	14.15±0.19^{d}	32.71±0.29^{d}	35.64±0.28^{d}		^{9}10.32±0.41^{e}

Note: P: proteolytic sample; PH: proteolytic-Maillard reaction sample; D: proteolytic-degreased sample; DH: proteolytic-degreased-Maillard reaction sample; L: proteolytic-lipolytic sample; LH: proteolytic-lipolytic-Maillard reaction sample. ΔE^*: colour difference; [1]Colour difference between P and PH; [2]Colour difference between D and DH; [3]Colour difference between L and LH; [4]Colour difference between P and D; [5]Colour difference between PH and DH; [6]Colour difference between D and L; [7]Colour difference between DH and LH; [8]Colour difference between P and L; [9]Colour difference between PH and LH; Means + standard deviations of triplicate determinations with different superscript letters [a-f] in the same column analysis are significantly different (Duncan's test, $p<0.05$).

Highlights

1. The Maillard reaction products were prepared based on cheese protein hydrolysates.
2. The amino components less than 1500 Da were the main amino reactants.
3. The Maillard reaction products showed pyrazine-ketone like flavour.

Author Contributions

Peng Gao: methodology, investigation, conceptualization, visualization, writing-original draft preparation. Wenyuan Zhang: Methodology, investigation, conceptualization, visualization, and editing. Xiaoxuan Zhao: Methodology, investigation, visualization. Chen Xu: Investigation, visualization. Xiaoyang Pang: Validation, funding acquisition. Fauconnier Marie-Laure: writing-review and editing. Shuwen Zhang: Conceptualization, funding acquisition, writing-review and editing. Jiaping Lv: Conceptualization, funding acquisition, writing-review and 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.

Figure 1

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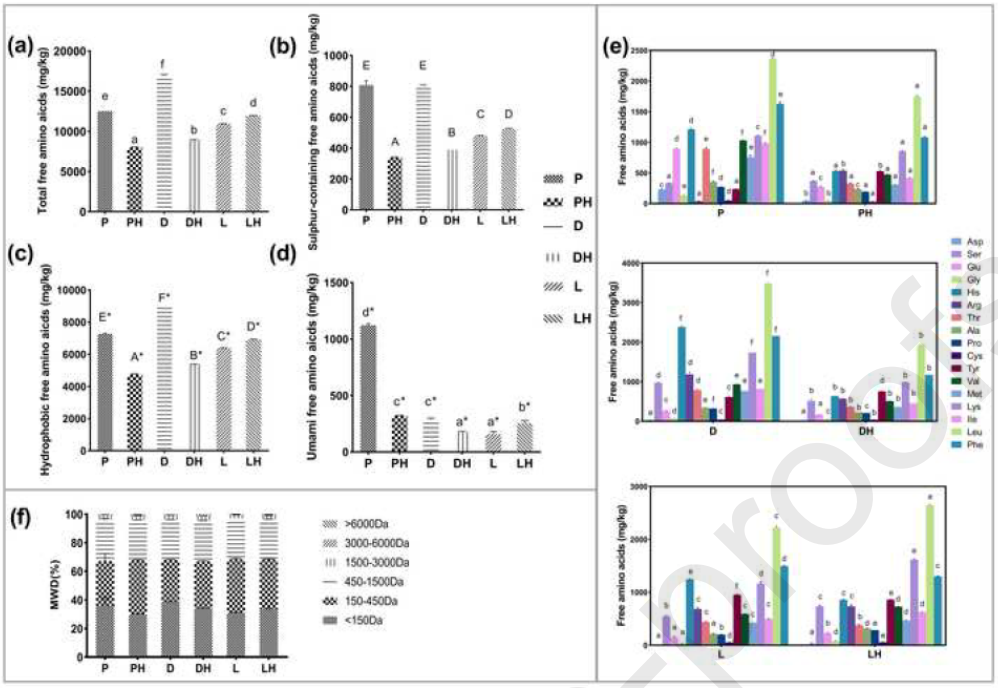

Figure 2

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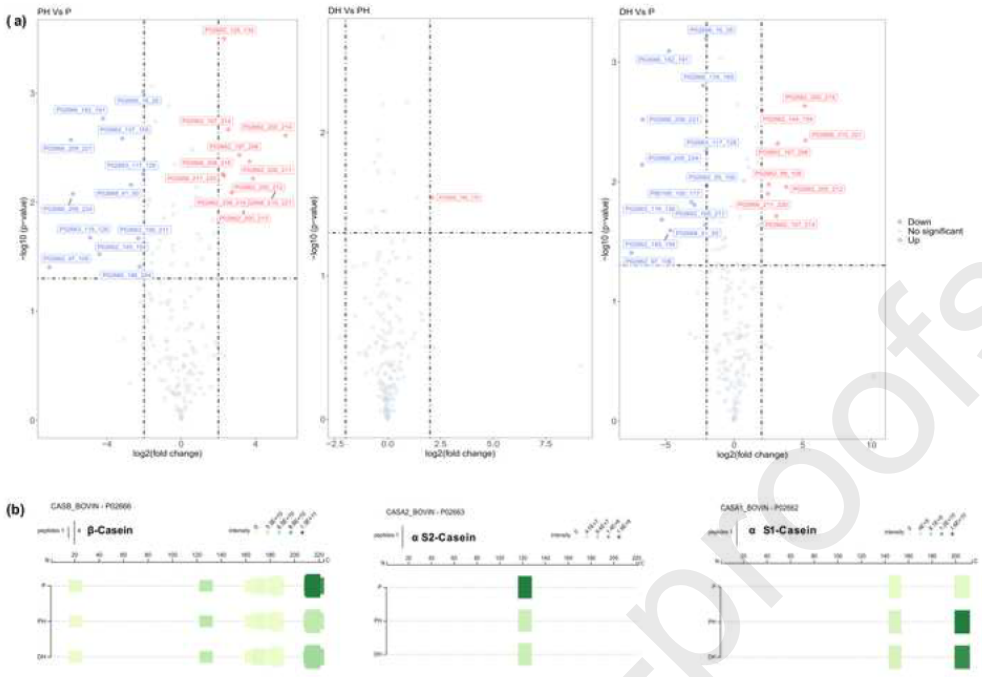

Figure 3

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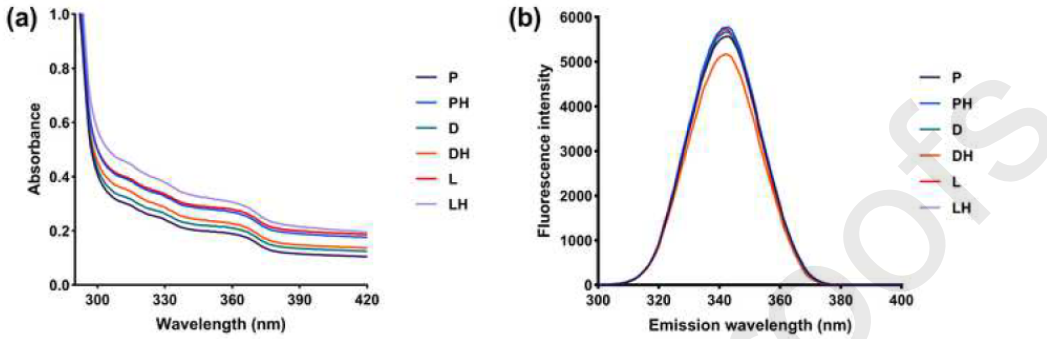

Figure 4

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