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Analysis of the non-volatile components and volatile compounds of hydrolysates derived from unmatured cheese curd hydrolysis by different enzymes

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ABSTRACT

Fifteen cheese protein hydrolysates were produced by using four different proteases. Then, the free amino acids (FAAs), molecular weight distribution (MWD), electronic tongue evaluation, and volatile compounds of the corresponding products were evaluated, respectively. The results suggested that 2SD had the strongest hydrolysis characteristic, followed by 6SD and FN. Samples hydrolyzed for less than 6 h or more than 18 h contained great defects of taste. Peptides with 150 Da–450 Da were mainly responsible for bitterness, saltiness, umami, and aftertaste in some enzyme hydrolysis. Under the same total enzyme concentration condition, the sample hydrolyzed by Flavourzyme and Neutrase for 18 h released more richness and less bitterness than the other systems, which were characterized by butter and cream odor. Notably, it was found for the first time that tetramethylpyrazine (TMP) was detected in cheese proteolysis with the highest content of 17.59 μg/g in Protease 2SD for 30 h. 2-Undecanone and acetoin played a key role in the flavor formation of the tested samples. Regarding the different chemical families of volatiles, acids were more abundant in the samples hydrolyzed by Protease 2SD and 6SD, while FN systems can achieve high ketone content.

1. Introduction

Cheese is rich in proteins and fats, which can be used as high-quality raw material for protease or lipase hydrolysis. During hydrolysis, complex interactions among milk proteins, fats, and carbohydrates contribute to the perceived sensory attributes (Forde & Fitzgerald, 2000; Khattab, Guirguis, Tawfik, & Farag, 2019). In the process, proteolysis plays an essential role in flavor development (Azarnia, Lee, Yaylayan, & Kilcawley, 2010), as it results in the production of water-soluble nitrogenous components, which directly contribute to cheese flavor, through the formation of peptides and amino acids, and indirectly act as precursors of volatile compounds (Molina, Ramos, Alonso, & López-Fandiño, 1999). Therefore, enzymes play a crucial role in hydrolysates' flavor development as they have diverse cleavage sites and properties, leading to different final sensory properties, while deeply enzymatic hydrolysis could reduce the bitter intensity (Fu, Liu, Hansen, Bredie, & Lametsch, 2018; Hou, Li, Zhao, Zhang, & Li, 2011). Water-soluble nitrogen (WSN) content and phosphotungstic acid-soluble nitrogen (PTA-N) content expressed as a percentage of the total nitrogen can roughly characterize the degree of proteolysis (Ali et al., 2017; Bas, Kendirci, Salum, Govce, & Erbay, 2019). Furthermore, basic tastes (umami, sweet, sour, bitter, and salty) are derived from a number of non-volatile compounds in terms of amino acids and peptides (Sabikun, Bakhsh, Rahman, Hwang, & Joo, 2020). For instance, peptides containing bulky hydrophobic amino acids result in a bitter taste in proteolysis, which leads to an unpleasant flavor (Polanco-Lugo, Dávila-Ortiz, Betancur-Ancona, & Chel-Guerrero, 2014), while umami flavor is mainly triggered by low molecular weight water-soluble

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substances (Nishimura et al., 2016; M. Yu et al., 2018).

On the other hand, the activity of free amino acids (FAAs) results in various flavor components (Smit, Verheul, Kranenburg, Ayad, & Engels, 2000), and the flavor characteristics are related to the types and concentrations of volatile compounds. Several groups of volatile compounds have been widely reported in cheese ripening, such as acids, ketones, alcohols, aldehydes, esters, and lactones. These volatile compounds can be grouped according to their possible origin-for example, aldehydes, alcohols, and ketones usually from lipid autooxidation, carbohydrate fermentation (e.g. phenylacetaldehyde), amino acid catabolism (e.g. 2, 3-butanediol, pyrazine), and Strecker and Maillard reaction (Pérez-Santaescolástica et al., 2018), and several of these volatiles may have more than one origin (Lorenzo, Bedia, & Bañón, 2013). Furthermore, milk fat improves the taste, flavor, and other sensory characteristics of processed food products, and it has flavor compounds such as free fatty acids (FFAs), carbonyls, and lactones (Sabikun et al., 2020). The characteristic food flavor is formatted from the interactions of several volatile (aroma) and non-volatile (taste) compounds, and these characteristics play a major role in consumers satisfaction.

To our knowledge, in the cheese industry, most of the literatures focused on proteolysis, lipolysis, volatile compound profile, and sensory characteristics of diverse ripened natural cheese (Lawlor, Delahunty, Sheehan, & Wilkinson, 2003; Salum, Govce, Kendirci, Bas, & Erbay, 2018). Although studies about the applications of exogenous proteases to accelerate cheese curd ripening have been reported, most of them focus on enzyme-modified cheese production (Bas et al., 2019; Kilcawley, Wilkinson, & Fox, 2006). The effects of protease application combined with high-temperature sterilization on the non-volatile components and volatile compounds of cheese hydrolysates have yet to be investigated. Furthermore, the information about the enzyme parameters that can be used to obtain a class of specific flavor compounds is very limited. Accordingly, a non-enzyme group and fifteen enzyme groups were studied to produce suitable hydrolysates as food ingredients or used for further processing. At the same time, the non-volatile components, taste evaluation, and volatile compounds analyses of hydrolysates were performed and elucidated. To better understand the relationships between non-volatile components (FAAs, MWD) and electronic taste, the principal component analysis (PCA) method was used.

2. Materials and methods

2.1. Materials

Immature cheese curd purchased from the DMK Group was used as the raw material. Disodium hydrogen phosphate (Beijing Cuifeng Technology Co., Ltd., China), sodium tripolyphosphate (Beijing Voge Oriental Technology Co., Ltd., Beijing, China), and sodium citrate (Biotopped, Beijing Yishan Huitong Technology Co., Ltd., Beijing, China) were adopted as emulsifying salts. Commercial proteases, including Neutrase (0.8 AU-N/g) and Flavourzyme (1100 LAPU/g) were provided by Novozymes., Ltd. (Beijing, China). Protease A "Amano" 2SD (100,000 U/g) and Protease P "Amano" 6SD (600,000 U/g) were provided by Shanghai Amano Enzyme Manufacturing, Ltd., (Shanghai, China). Flavourzyme, Protease 2SD, and Protease 6SD have both endo-tangential and extangential activities. Ultrapure water was prepared in the laboratory using a purifier (Millipore, Waltham, MA, USA). Heat sterilization was carried out in a reversed-pressure high-temperature cooking pot (TS-25C, Beijing Landmaker Technology Development Co., Ltd., Beijing, China).

2.2. Preparation of protein hydrolysates

The cheese curd was sheared and mixed with water and emulsified salt, with a percentage of 56.7%, 41%, and 2.3%, respectively. The mixture was heated in a water bath at 80 °C for 15 min, followed by sterilization at 121 °C for 15 min. After rapidly cooling, the mixture was hydrolyzed by four proteases in the incubator at 45 °C with 250 rpm (DJ5-2012R). Fifteen cheese hydrolysates were obtained by different enzyme treatment schemes, including 0.4% (w/w) Protease 2SD (2SD) for 0 h–30 h, 0.4% (w/w) Protease 6SD (6SD) for 0 h–30 h, and 0.1% (w/w) Flavourzyme combined with 0.3% (w/w) Neutrase (FN) for 0 h–30 h. Samples were taken at 6 h intervals and terminated at 90 °C for 20 min. All the enzyme concentration and incubation environments were selected according to the recommendations from suppliers. Each sample was produced in triplicate.

2.3. Chemical analysis

The measurement for fat, protein, moisture, and NaCl content of the proteolytic substrate was followed by the Gerber method (Chinese standard GB 5009.6–2016), the Kjeldahl method (Chinese standard GB 5009.5–2016), the direct drying method (Chinese standard GB 5009.3–2016), and potentiometric titration (Chinese standard GB 5009.44–2016), respectively. The pH was measured using the DELTA 320 pH meter (Mettler Toledo Co., LTD, Zurich, Swiss).

Nitrogen was determined via the macro-Kjeldahl method. The determination method of pH 4.6-WSN/TN% is described as follows: sample was mixed with distilled water at a ratio of 1:2, stirred with magnetic force at room temperature for 5 min, heated at 50 °C for 1 h, and then cooled with 1 mol/L HCl to adjust the pH to 4.6. After centrifugation at a speed of 6000 r/min for 20 min, a certain volume of supernatant was taken for Kjeldahl determination. Afterwards, 10 mL water-soluble nitrogen (WSN) was taken, and 7 mL 3.95 mol/L sulfuric acid and 3 mL 33.3% (W/V) PTA were added, mixed evenly, and placed overnight at 4 °C. Filtered by filter paper, a certain volume of the filtrate was taken for Kjeldahl determination.

2.4. Determination of non-volatile components

2.4.1. Determination of free amino acids (FAAs)

The relative content of FAA in the samples was analyzed via ultra-high-performance liquid chromatography (UHPLC) (Waters). Take part of the supernatant and add an equal volume of 12% trichloroacetic acid (TCA) to the supernatant, and then let it stand at room temperature for 1 h and centrifuge for 10 min at 6000 r/min. The supernatant was filtered through a 0.22 μm filter membrane and taken for derivatization. The derivatization steps are as follows: absorb 10 μL of supernatant, add 70 μL of LaccQ•FluorBuffer1 and 20 μL of AccQ•Fluor derivative (2A), vortex fully and place at room temperature for 1 min, and then put it in a water bath at 55 °C for 10 min, remove, and inject. An Xbrigde BEH C18 (4.6 mm × 100 mm, 2.5 μm) column was used and kept at 37 °C. The detection wavelength was 248 nm; a 1:10 diluted solution AccQ•Tag A was used as the mobile phase A, acetonitrile as mobile phase B, and ultra-pure water as mobile phase C. A mobile phase gradient elution procedure was performed as follows: 0 min–0.5 min, 100% - 99 %A, 0%–1% B. 0% C; 0.5 min–18 min, 99% - 95%A; 18 min–19 min, 95% - 91%A; 19 min–29.5 min, 91% - 83%A; 29.5 min–33 min, 83% - 0%A; 33 min–36 min, 0% - 100%A; 36 min - 45min 100% A. The flow rate was retained at 1 mL/min. A calibration curve was obtained using a standard amino acid mixture purchased from Waters Sigma-Aldrich Co. (St. Louis, MO, USA). Qualitative analysis was carried out based on retention time and the peak area of standard amino acids.

2.4.2. Determination of molecular weight distribution (MWD) of peptide

The molecular weight distribution (MWD) of the hydrolysates was analyzed via UHPLC with a Waters 600 liquid chromatography system (Waters Co., Milford, MA). The system was equipped with a Waters 2487 UV detector and an Empower work station on a 2000 (300 × 7.8 mm) SWXL TSK gel filtration column (Tosoh Co., Tokyo, Japan). The mobile phase used was a 55% (v/v) acetonitrile solution containing 0.1% trifluoroacetic acid, and equigradient elution was used. The samples were

eluted at a flow rate of 0.5 mL/min and monitored at 214 nm at 40 °C. Then 10 μL samples were injected into the HPLC system. The molecular weight calibration curve was obtained using four standards from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China): aprotinin (6000 Da), bacitracin (1500 Da), tetrapeptide GGTA (450 Da), and dipeptide GS (150 Da). The chromatogram was recorded using a UV detector at 220 nm.

2.4.3. Electronic tongue analysis

Electronic tongue analysis of the samples was performed with the SA402B Taste-Sensing System (INSENT SA402B, Tokyo, Japan). The taste analyzer consists of multi-channel lipid/polymer membrane electrodes, an Ag/AgCl reference electrode, which is used to measure the response intensity of each sensor, a 10-position autosampler, and a computer with an advanced chemometrics software package. Also, the E-tongue system was equipped with data acquisition and five different detecting sensors (CT0 specific for saltiness, AAE for umami, CA0 for sourness, C00 for bitterness, and aftertaste-bitterness (aftertaste-B), and AE1 for astringency and aftertaste-astringency (aftertaste-A)). Twenty-milliliter solutions of the samples were increased to 100 mL and taken for electronic tongue analysis. Measurement data were obtained for each flavor standard.

2.4.4. Sensory evaluation

As the bitter taste of protein hydrolysates is an important factor affecting consumer acceptance, sensory evaluation for the bitter taste in hydrolysates was conducted by three well-trained sensory evaluators. Different concentrations of tea alkaloids solution (0 g/L, 0.1 g/L, 0.2 g/L, 0.4 g/L, 0.8 g/L) were used as the standard to judge the bitter taste, which had been labeled as 1, 2, 3, 4, and 5 bitter scale (1, no bitterness; 2, weakly bitter; 3, mildly bitter; 4, bitter; 5, strongly bitter). During test, panelists were asked to rinse their mouths thoroughly with distilled water.

2.5. Volatile compounds analysis

2.5.1. Identification and quantitation of aroma compounds

The volatile compounds were determined via solid-phase micro-extraction-gas chromatography and mass spectrometry (SPME-GC/MS) (GCMS-QP2010 Plus, Shimadzu, Japan) according to Bas et al. (2019) with some modifications. Amounts of 2 g of samples were placed into 10 mL glass vials with a silicon septum, and 2.0 μL 2-octanol with 0.21 μg/μL was added as an internal standard (IS).The compounds in the samples were extracted and put into the vial using a 65 μm PDMS/DVB SPME fibre (Supelco., Ltd., USA) for 30 min at 60 °C. After extraction, the compounds were directly desorbed into the 250 °C injection port. The volatile compounds were separated using a capillary column (DB-WAX; 30 m × 250 μm ID × 0.25 μm film thickness; USP527752H, USA). The carrier gas was helium with a flow of 1.0 mL/min. The oven temperature was programmed initially at 40 °C for 3 min, and then the temperature was raised to 200 °C (5 °C/min, held 5 min) and to a final temperature of 230 °C (10 °C/min). After GC, the sample was subjected to MS. The temperature of the ion source and quadrupole were set to 230 °C and 150 °C, respectively. The ionization was electron impact mode, and electron energy of 70 Ev, with a mass scan range of 35–350 m/z.

The volatile compounds Were identified by comparing the mass spectra with those in the mass spectrometry library (NIST 17-1, 17-2, and 17s) and were confirmed by the retention indices (RI). A series of n-alkanes (C_7–C_{40}) were used as external references to determine the RI of each compound. The RI was calculated as follows: $RI = 100n + 100(t_i - t_n)/(t_{n+1} - t_n)$, where t_i, t_n and t_{n+1} were the retention times of compound i, alkane n and alkane n + 1 ($t_n < t_i < t_{n+1}$), respectively. Quantitative analysis was performed according to the peak area ratio of volatile compounds and the internal standard. All the samples were analyzed in triplicate.

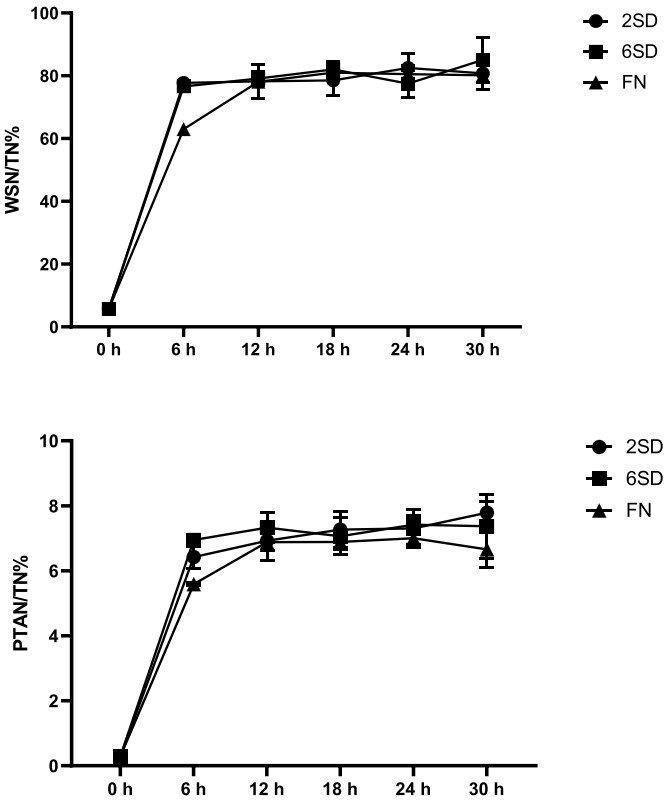

Fig. 1. The content of pH4.6WSN/TN and PTAN/TN in all samples. 2SD: Protease 2SD; 6SD: Protease 6SD; FN: the combination usage of Flavourzyme and Neutrase.

2.5.2. Odor activity value (OAV) and the contribution rate of aroma compounds

To understand the importance of a single odorant in the protein hydrolysate aroma profile, the OAVs and contribution rate were assessed. The compounds with OAVs >1 contributed significantly to the aroma profiles of hydrolysates. The compounds with OAVs <1 supplied a minor contribution. And the higher the contribution rate of an aroma compound, the greater its expected influence on the aroma distribution in the samples.

2.6. Statistical analysis

All the tests were run in triplicate and the results were presented as the mean value ± standard deviation (SD). The measured data were analyzed via one-way analysis of variance (ANOVA) using IBM SPSS Statistics (Version 24, SPSS Inc., Chicago, IL, USA). Duncan's test was used to identify significant differences ($P < 0.05$). The data matrix for PCA analysis has combined the results from free amino acids, molecular weight distribution, and electronic tongue. The pre-treatments of data matrix included mean centering and scaling. PCA was performed using language R (Version 4.1.2) including the packages of ggplot2, FactoMineR, and factoextra.

3. Results and discussion

3.1. Analysis of chemical components

The content of protein, fat, NaCl, moisture and pH of the control group (without enzymes) was 16.10%, 14.99%, 0.74%, 63.72%, and 6.40, respectively. The values of pH 4.6-WSN/TN% and 5% PTA-N/TN in the control group and hydrolysates are presented in Fig. 1. During the 0 h–6 h period, both of the values significantly increased in all

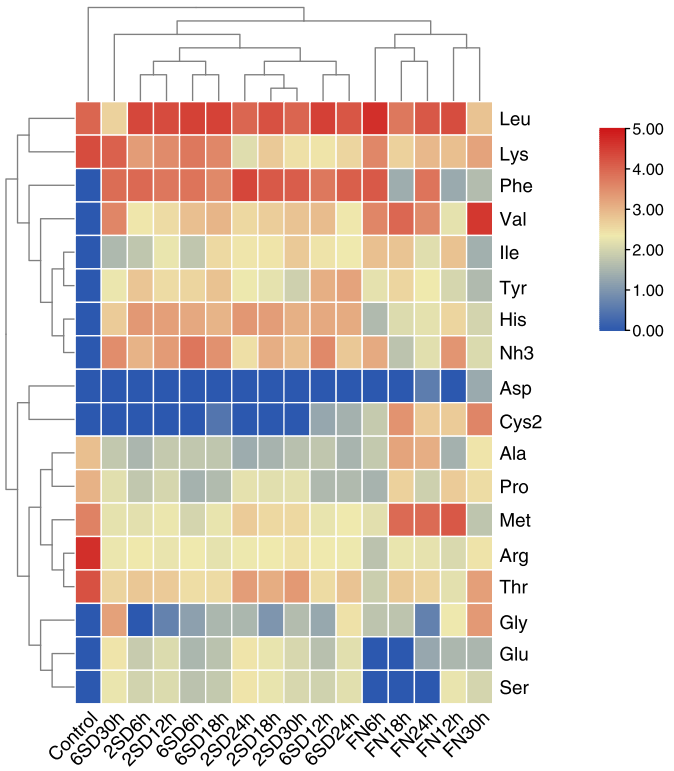

Fig. 2. The relative contents of free amino acids in the control group (without enzyme) and in the protein hydrolysates. Reaction conditions: add 0.4% (w/w), temperature of 45 °C, speed of 250 rpm, no pH control.

hydrolysates, especially the pH 4.6-WSN/TN% index. After hydrolysis for 18 h, the trend of the two values was basically stable. Evenly, the content of pH 4.6-WSN/TN can reach about 80%. These results suggested that proteolysis achieved saturation at about 18 h and underwent extensive protein hydrolysis, that is, the degree of protein hydrolyzed into polypeptides, short peptides, and FAAs. The content of 5% PTAN/TN can reach about 7% after 12 h of hydrolysis; this was 25 times higher than the control group (0.28%). It was an extensive proteolysis, which may affect the overall flavor of hydrolysates.

3.2. Analysis of non-volatile components

3.2.1. Analysis of free amino acids (FAAs)

The concentration of FAAs contributes greatly to the sensory properties of hydrolysates (Pérez-Santaescolástica et al., 2018). The FAAs relative contents of the control group and hydrolysates are listed in Fig. 2. The major FAAs were leucine, lysine, phenylalanine, and valine, which contribute multiple tastes of bitter/sweet/acid (Sabikun et al., 2020). Compared to the control group, the individual FAAs showed higher values in hydrolysates, except for aspartic acid and cysteine which presented low values. Notley, Protease 2SD, and 6SD showed relatively similar hydrolysis characteristics, while the FN combination system was slightly different. Phenylalanine and histidine presented high proportions in 2SD and 6SD hydrolysates, which is associated with acid taste, while in FN systems, valine, cysteine, alanine, and methionine characterized high proportions. Chen and Zhang (2007) reported that alanine and glycine contributed a significant sweet taste and showed a synergistic effect of umami-taste. The FAAs containing Sulphur atoms (cysteine and methionine) have a sulphury note. These differences in the individual FAAs content among the hydrolysates could induce differences in flavor.

3.2.2. Analysis of molecular weight distribution (MWD)

The MWD of the control group and hydrolysates are shown in Fig. 3. Significant changes in MW could be found after proteolysis. Accordingly, the hydrolysates prepared by three different commercial proteases had much higher content (nearly 80%) of oligopeptides with the molecular weight between 150 Da and 1500 Da, while 49.76% of the peptides in the control group were above 6000 Da. The MW of samples hydrolyzed by 2SD and 6SD was mainly distributed in 150 Da–450 Da, and gradually increased with the prolonged incubation time, while the change trend of peptides above 450 Da was opposite. It has been well documented that hydrolysates rich in low peptides (500 Da–3000 Da) would have high biological activities and nutritional value (Bhaskar, Benila, Radha, & Lalitha, 2008; Hou et al., 2011). Matoba and Hata (1972) reported that complete food proteins or large peptides (>10,000 Da) do not exhibit bitterness as most hydrophobic amino acids are intramolecular. As hydrolysis continues, more hydrophobic amino acid residues are exposed and the bitterness of the hydrolysates generally increases. Cho, Unklesbay, Hsieh, and Clarke (2004) discovered that hydrolytic peptides with a medium MW at 1000 Da–4000 Da isolated from soy protein had the most bitter taste while small peptide fractions below 1000 Da were less bitter. In this study, all the hydrolysates showed a high hydrolysis yield of small peptides (<1000Da). This phenomenon showed a successful breakdown of large dissolvable proteins and peptides during the hydrolysis course. Furthermore, the data showed that 2SD had the strongest hydrolysis characteristic, followed by 6SD, and FN had the smallest hydrolysis degree. The contribution of MWD of the samples to the taste characteristics is detailed below.

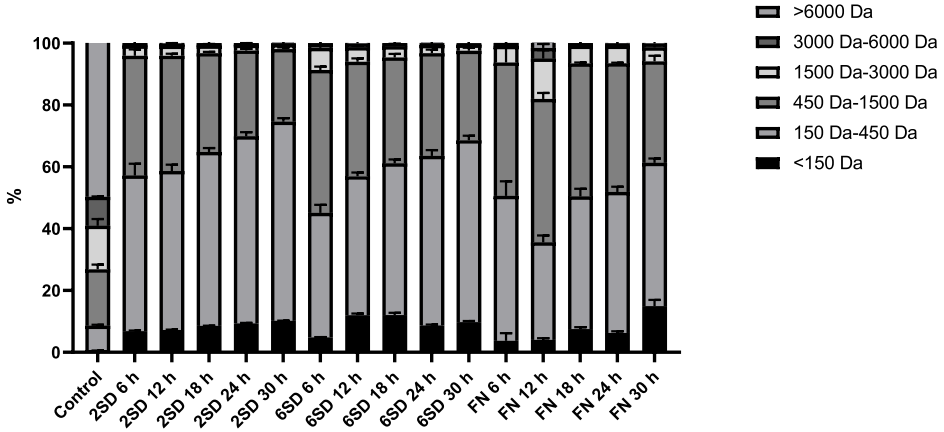

Fig. 3. The molecular weight distribution of peptides (%) in the control group (without enzyme) and in the protein hydrolysates. Reaction conditions: add 0.4% (w/w), temperature of 45 °C, speed up of 250 rpm, no pH control. *Values are represented as mean ± standard deviation (SD) of triplicate analysis.

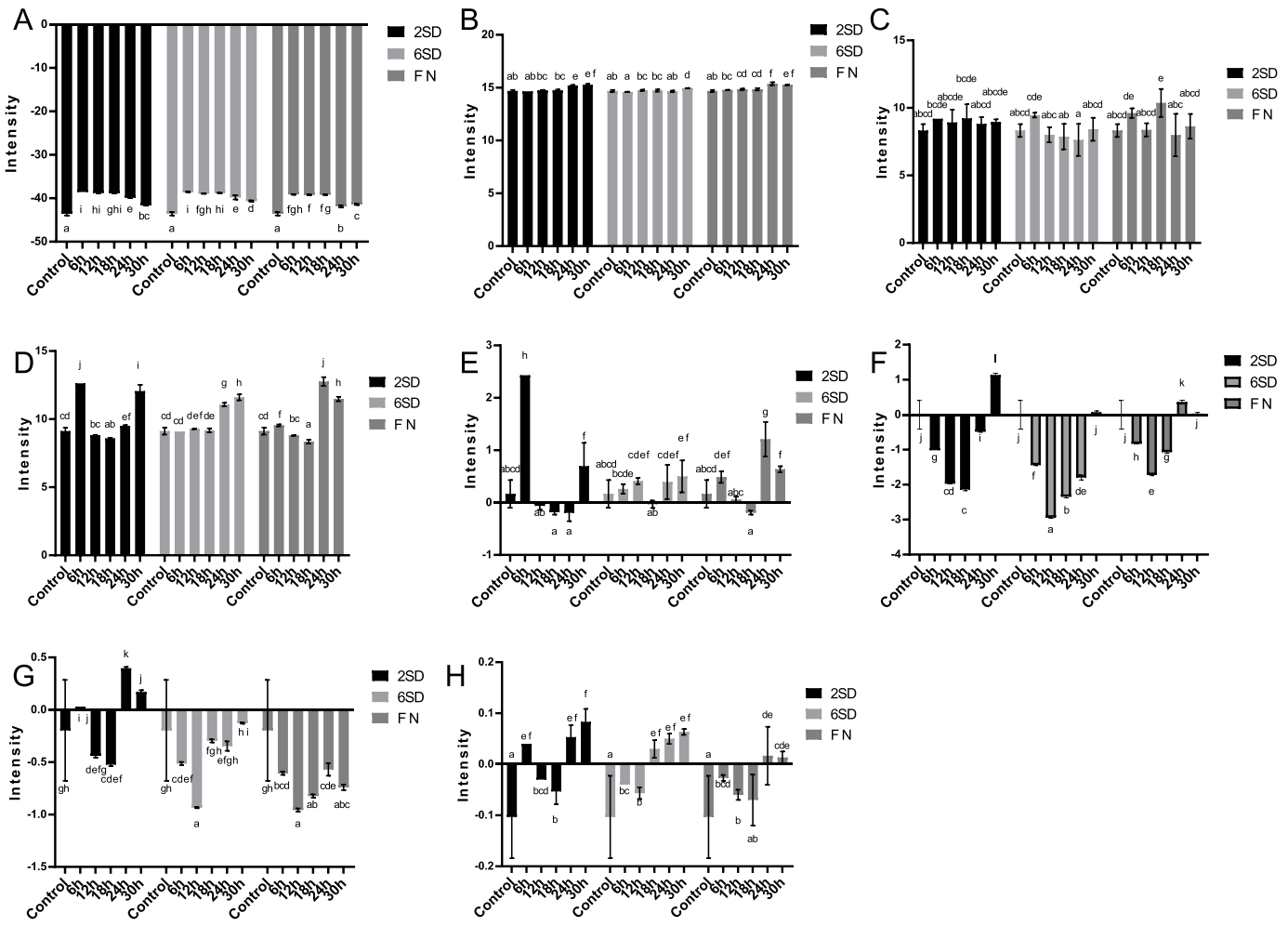

Fig. 4. Bar chart obtained from the control group (without enzyme) and the protein hydrolysates based on the electronic tongue system (mean ± SD). a–l: Different letters indicate significant statistical differences (Duncan, $p < 0.05$). Negative values indicate that the flavor contributes little to the overall flavor of the samples. The larger the positive value is, the greater the contribution of the flavor to the overall flavor of the samples. A: Sourness; B: Umami; C: Richness; D: Bitterness; E: Aftertaste-B; F: Saltiness; G: Astringency; H: Aftertaste-A.

3.2.3. Analysis of electronic tongue

The results, depicted in Fig. 4, showed that all the samples presented negative scores in sourness, and most of the samples had negative scores in astringency and saltiness, which indicated that both the control group and hydrolysates had low intensity of sourness, astringency and saltiness. In addition, when the initial sense output of the control group (non-enzyme treatment) was defined as a reference frame, the sourness of all the hydrolyzed samples significantly improved, with 2SD for 6 h and 6SD for 6 h having the highest sourness. The increase in acid taste may be related to the production of more acid amino acids during hydrolysis, such as phenylalanine and histidine. As for bitterness, during the 0–6 h period, the bitterness taste of 2SD and FN gradually improved, while with further hydrolysis, the degree of bitterness gradually decreased, and the lowest was reached at 18 h of hydrolysis. After 18 h, the bitterness began to increase again. As discussed previously, an excess of proteolysis causes a taste defect which may translate into non-acceptance by the consumers. This excess of proteolysis also entails an increase in the concentration of low molecular weight (peptides and FAAs); 2SD for 6 h and FN for 24 h achieved the highest bitterness taste, followed by 2SD for 30 h. Notably, FN 18 h had the least intensity of bitterness and aftertaste-B, while achieving the highest taste of richness. In general, when the samples were hydrolyzed for less than 6 h or more than 18 h, the taste showed great defect, especially in bitterness, astringency, and aftertaste.

Even though some low bitter protein hydrolysates have been studied,

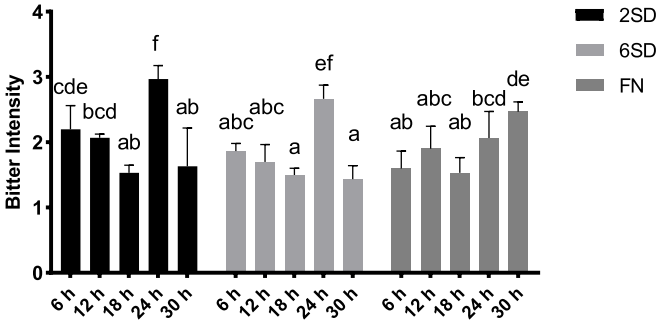

Fig. 5. Sensory evaluation of the control group (without enzyme) and the protein hydrolysates (mean ± SD). a–f: Different letters indicate significant statistical differences (Duncan, $p < 0.05$). Reaction conditions: add 0.4% (w/w), temperature of 45 °C, speed of 250 rpm, no pH control.

most of them prepared from animal or plant proteins (Fu et al., 2018; Hou et al., 2011), and scare information published on the preparation of protein hydrolysates by hydrolyzing cheese curd granules with exogenous proteases. This study produced a low bitter and high richness hydrolysate produced by combination usage of Flavourzyme and Neutrase for 18 h, the current approach is promising and the results can be applied in various ways. For example, the low bitterness and high

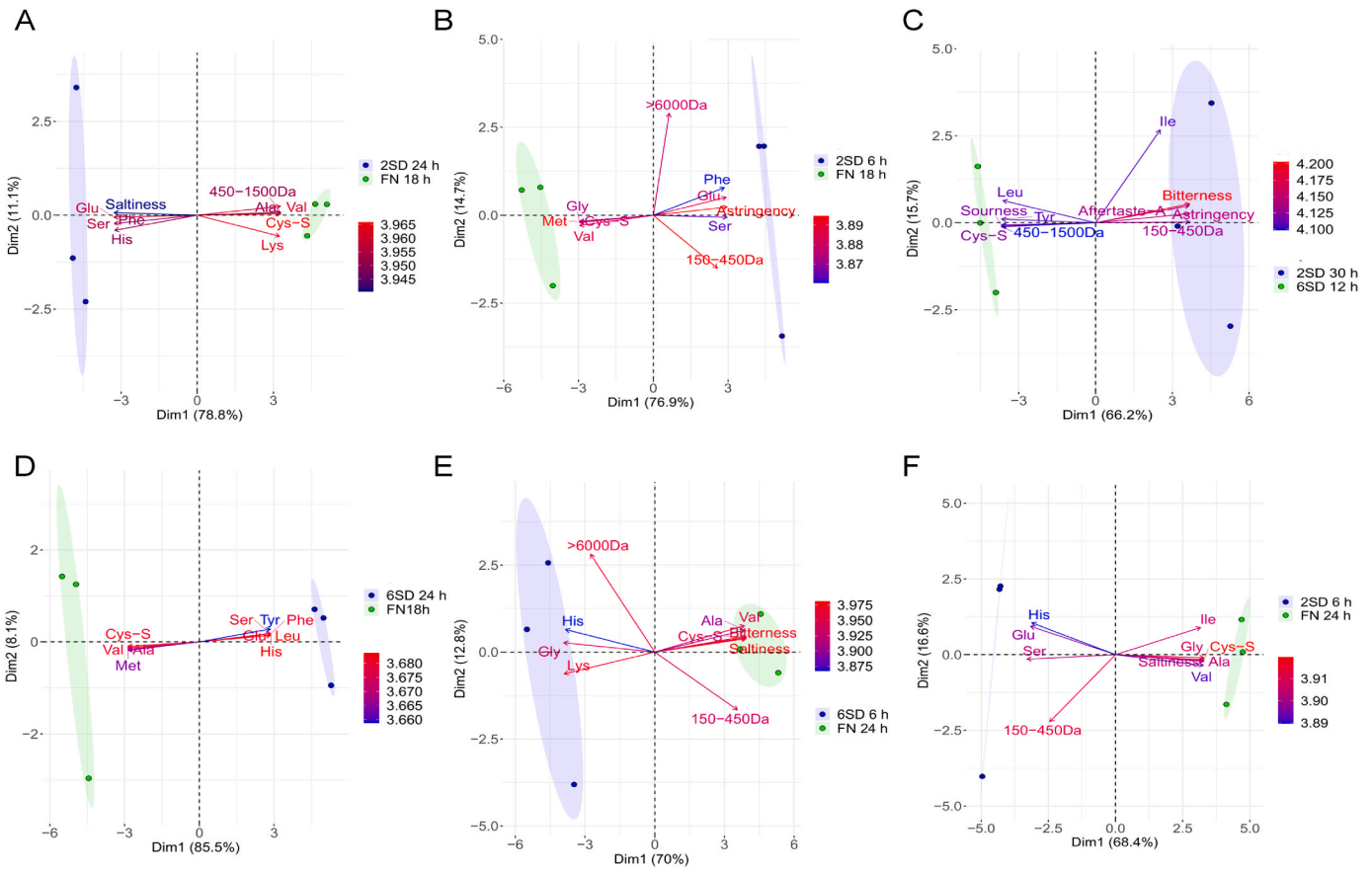

Fig. 6. Principal component analysis biplot (PCA-biplot) of samples with the greatest difference by electronic analysis. The contributions of all loading plots for each factor were shown in Table S1A: PCA analysis of astringency and aftertaste-A; B: PCA analysis of bitterness and aftertaste-B; C: PCA analysis of saltiness; D: PCA analysis of richness; E: PCA analysis of umami; F: PCA analysis of sourness.

richness hydrolysates have been produced and can be used for further lipolysis to get more enzyme modified cheese (EMC) as a flavor. Furthermore, the proteases also can be used to accelerate natural cheese ripening.

3.2.4. *Analysis of sensory evaluation*

Slightly different from the bitter taste results of the electronic tongue, as depicted in Fig. 5, sensory evaluations showed that samples hydrolyzed by 2SD for 24 h achieved the highest bitter taste, followed by 6SD for 24 h, and FN for 30 h. And FN for 18 h also contain low bitter intensity. All of the three proteases reached the lowest bitterness after hydrolysis for 18 h. With the incubation time prolong, the change trend of bitterness intensity was similar to that of electronic tongue, so the subsequent correlation analysis was carried out based on the results of electronic tongue measurement.

3.2.5. *Correlations between non-volatile compounds and taste attributes of hydrolysates*

PCA was carried out to establish the correlations of FAAs, peptide MWD, and taste characteristics of the samples with the greatest difference via electronic analysis. As indicated in Fig. 6, the redder the factor color, the greater the contribution to the taste difference. The PCA results clearly showed that different samples would be well distinguished in the distribution map within a relatively independent space. For astringency and aftertaste-A, the variance contribution rates of the first and second PCs were 78.8% and 11.1%, respectively. FN for 18 h showed lower astringency and aftertaste-A taste than the sample hydrolyzed by 2SD for 24 h. In particular, lysine, cysteine, valine, alanine, and peptides with 450 Da–1500 Da were on the same side as the sample hydrolyzed by FN for 18 h. This indicated that a certain proportion of these substances can effectively reduce the astringency and aftertaste-B intensity of FN 18 h sample, especially the relative high content of cysteine and lysine in FN 18 h sample. Likewise, the high proportion of serine, glutamic acid, histidine, and phenylalanine in 2SD 24 h sample were the main reason for its strong astringency and aftertaste-A intensity. As for bitterness and aftertaste-B taste, 2SD 6 h is located on the right side, and FN 18 h on the left side, indicating that the bitterness and aftertaste-B intensity of the two hydrolysates were significantly different.2SD 6 h showed strong bitterness and aftertaste-B characteristics, and it was significantly positively influenced by peptides with 150 Da–450 Da, >6000Da and glutamic acid. Although each tastant has a specific taste, tastant concentration, multiple taste stimuli, and other factors can enhance or suppress foodstuffs taste. From the point of taste-taste interactions, the high astringency intensity of sample hydrolyzed by 2SD for 6 h also contribute its bitterness and aftertaste-B taste. Notably, the occurrence of bitter peptides is a major obstacle for human to the utilization of protein hydrolysates. Hou et al. (2011) reported that the bitterness of protein hydrolysates was mainly caused by hydrophobic oligopeptides, and the usage of enzyme is decisive for the amount of such peptides occurring during hydrolysis. In this study, sample hydrolyzed by FN 18 h achieved the lowest bitter taste, and it had relatively high proportion of valine, methionine, cysteine, and glycine, accounting for 14.83%, 14.39%, 9.86%, 2.34%, respectively. Interestingly, the saltiness of all hydrolysates increased significantly after hydrolysis for more than 18 h or even 12 h, and the saltiness of sample hydrolyzed by 2SD for 30 h was the strongest. From the PCA analysis, the bitterness, astringency, and aftertaste-A characteristics of 2SD 30 h also made the saltiness even stronger. In addition, the 150 Da–450 Da

Table 1
The variety and concentration of volatile compounds in the control group and some hydrolysates.

Compounds (μg/g)	RI		Control	2SD 6 h	2SD 18 h	2SD 24 h	2SD 30 h	6SD 12 h	6SD 30 h	FN 12 h	FN 18h	FN 24 h
	Literature [x]	Calculation [y]										
Acids												
Acetic acid	1429	1432	2.19 ± 1.15^{b}	0.64 ± 0.19^{a}	0.90 ± 0.52^{a}	0.67 ± 0.51^{a}	0.36 ± 0.06^{a}	0.55 ± 0.17^{a}	0.88 ± 0.18^{a}	0.15 ± 0.01^{a}	2.63 ± 0.58^{b}	0.55 ± 0.28^{a}
Propanoic acid, 2-methyl-	1544	1548	–	–	–	0.76 ± 0.16^{a}	0.81 ± 0.17^{a}	–	–	–	–	1.02 ± 0.88^{a}
Butanoic acid	1628	1604	–	0.27 ± 0.06^{ab}	0.29 ± 0.05^{b}	0.28 ± 0.19^{ab}	0.26 ± 0.02^{ab}	0.14 ± 0.02^{a}	0.35 ± 0.03^{b}	–	0.78 ± 0.10^{c}	–
Hexanoic acid	1849	1822	3.48 ± 3.23^{ab}	7.50 ± 2.42^{bc}	14.35 ± 3.37^{d}	8.09 ± 5.08^{c}	8.71 ± 1.03^{c}	0.63 ± 0.07^{a}	4.67 ± 0.41^{abc}	0.27 ± 0.06^{a}	1.16 ± 0.77^{a}	0.85 ± 1.02^{a}
Heptanoic acid	1954	1920	–	0.10 ± 0.01^{ab}	0.26 ± 0.05^{c}	0.20 ± 0.20^{bc}	0.30 ± 0.04^{c}	–	–	–	–	–
Octanoic acid	2050	2028	3.12 ± 1.22^{a}	7.79 ± 1.09^{b}	15.65 ± 2.76^{c}	8.99 ± 5.60^{b}	17.62 ± 1.37^{c}	4.11 ± 0.35^{a}	9.46 ± 0.32^{b}	0.81 ± 0.25^{a}	0.75 ± 0.07^{a}	0.38 ± 0.03^{a}
Nonanoic acid	2144	2140	–	–	0.14 ± 0.02^{b}	–	0.14 ± 0.01^{b}	0.16 ± 0.03^{b}	0.11 ± 0.01^{a}	–	–	–
n-Decanoic acid	2279	2245	6.87 ± 1.97^{c}	7.01 ± 1.20^{c}	10.50 ± 1.30^{d}	7.79 ± 1.43^{c}	11.78 ± 0.17^{d}	4.44 ± 0.42^{b}	7.08 ± 1.02^{c}	1.41 ± 0.57^{a}	1.21 ± 0.20^{a}	0.81 ± 0.16^{a}
9-Decenoic acid	2335	2302	–	0.40 ± 0.07^{b}	0.68 ± 0.12^{c}	0.33 ± 0.23^{ab}	0.81 ± 0.05^{c}	0.18 ± 0.02^{a}	0.36 ± 0.07^{b}	–	–	–
Dodecanoic acid	2502	2451	4.25 ± 3.54^{c}	1.85 ± 0.36^{ab}	2.23 ± 0.51^{abc}	1.11 ± 0.15^{ab}	1.97 ± 0.17^{ab}	2.06 ± 0.27^{ab}	3.21 ± 0.82^{bc}	0.40 ± 0.05^{a}	–	–
Tetradecanoic acid	2684	2675	2.49 ± 0.91^{c}	1.07 ± 0.21^{b}	1.10 ± 0.29^{b}	0.77 ± 0.14^{ab}	0.69 ± 0.06^{ab}	2.94 ± 0.73^{c}	3.88 ± 0.67^{d}	–	–	–
Ketones												
Acetoin	1285	1268	1.84 ± 0.43^{a}	1.17 ± 0.15^{a}	0.85 ± 0.15^{a}	10.2 ± 3.48^{b}	13.11 ± 2.26^{b}	0.86 ± 0.07^{a}	0.68 ± 0.02^{a}	0.76 ± 0.02^{a}	17.32 ± 1.49^{c}	13.07 ± 5.09^{b}
2-Propanone, 1-hydroxy-	1275	1287	–	0.09 ± 0.03^{a}	0.10 ± 0.04^{a}	–	–	0.10 ± 0.01^{a}	–	–	–	–
2-Acetoxy-3-butanone	1358	1365	–	–	–	0.10 ± 0.03^{a}	0.09 ± 0.00^{a}	–	–	–	–	–
2,3-Butanedione	980	977	–	–	–	5.54 ± 0.92^{b}	3.95 ± 0.50^{a}	–	–	–	3.29 ± 0.70^{a}	5.41 ± 2.03^{b}
2-Heptanone	1180	1159	0.93 ± 0.33^{a}	2.51 ± 0.59^{cd}	2.84 ± 0.71^{d}	2.57 ± 1.08^{cd}	1.98 ± 0.47^{bcd}	1.79 ± 0.02^{abc}	1.12 ± 0.47^{ab}	4.37 ± 0.37^{e}	1.21 ± 0.03^{ab}	2.03 ± 0.30^{bcd}
2-Nonanone	1387	1369	3.21 ± 0.96^{d}	2.90 ± 0.16^{d}	3.13 ± 0.09^{d}	1.95 ± 0.23^{bc}	2.00 ± 0.07^{bc}	1.54 ± 0.14^{ab}	1.58 ± 0.03^{ab}	3.46 ± 0.23^{d}	1.20 ± 0.02^{a}	2.25 ± 0.14^{c}
2-Undecanone	1599	1580	4.22 ± 0.87^{d}	3.04 ± 0.20^{bc}	3.26 ± 0.40^{cd}	2.76 ± 0.27^{bc}	2.63 ± 0.12^{bc}	1.50 ± 0.13^{a}	1.50 ± 0.05^{a}	3.57 ± 0.07^{cd}	1.21 ± 0.11^{a}	2.02 ± 1.60^{ab}
2-Tridecanone	1814	1797	2.33 ± 0.48^{e}	1.62 ± 0.20^{cd}	1.88 ± 0.27^{d}	1.21 ± 0.06^{b}	1.38 ± 0.05^{bc}	1.15 ± 0.05^{b}	1.10 ± 0.09^{b}	1.89 ± 0.34^{d}	0.64 ± 0.03^{a}	1.34 ± 0.05^{bc}
2-Pentadecanone	2019	1998	–	0.44 ± 0.02^{a}	–	–	–	0.67 ± 0.05^{bc}	0.57 ± 0.04^{b}	–	–	0.71 ± 0.18^{c}
Alcohols												
Benzyl alcohol	1877	1847	–	0.14 ± 0.11^{b}	–	0.16 ± 0.14^{b}	0.07 ± 0.01^{ab}	–	–	0.09 ± 0.01^{ab}	–	0.10 ± 0.02^{ab}
Phenylethyl Alcohol	1912	1879	0.7 ± 0.11^{d}	0.22 ± 0.05^{bc}	0.23 ± 0.22^{bc}	0.59 ± 0.17^{d}	0.22 ± 0.04^{bc}	0.14 ± 0.02^{ab}	–	0.14 ± 0.00^{ab}	0.38 ± 0.01^{c}	0.32 ± 0.07^{bc}
2-Furanmethanol	1635	1640	–	0.14 ± 0.01^{a}	0.19 ± 0.01^{b}	–	–	0.13 ± 0.00^{a}	0.13 ± 0.00^{a}	–	–	–
2-Octen-1-ol	1617	1599	–	0.16 ± 0.04^{c}	0.13 ± 0.01^{b}	0.10 ± 0.01^{a}	–	–	–	–	–	–
2,3-Butanediol, [R-(R*,R*)]-	1573	1559	–	–	–	3.01 ± 0.09^{b}	0.10 ± 0.04^{a}	–	–	–	–	0.22 ± 0.08
3-Methyl-1-butanol	1185	1200	–	–	–	0.25 ± 0.02^{b}	–	0.16 ± 0.04^{a}	–	–	–	–
Pyrazines												
2,5-Dimethypyrazine	1314	1298	–	–	–	–	0.22 ± 0.01^{b}	–	0.18 ± 0.03^{a}	–	–	0.29 ± 0.03^{c}
Trimethylpyrazine	1395	1388	–	–	–	1.38 ± 0.49^{a}	3.59 ± 0.41^{c}	–	–	–	–	2.58 ± 0.15^{b}
Tetramethylpyrazine	1466	1462	–	–	–	4.38 ± 2.06^{a}	17.59 ± 2.05^{b}	–	–	–	–	4.30 ± 0.36^{a}
Aldehydes												
Benzaldehyde	1530	1529	1.64 ± 0.69^{ab}	1.6 ± 0.36^{ab}	1.76 ± 0.14^{ab}	0.94 ± 0.71^{ab}	1.67 ± 0.21^{ab}	1.06 ± 0.12^{ab}	0.99 ± 0.48^{ab}	1.16 ± 0.13^{ab}	0.31 ± 0.01^{a}	2.69 ± 0.06^{b}
Phenylacetaldehyde	1648	1611	–	0.16 ± 0.00^{b}	0.26 ± 0.01^{d}	0.21 ± 0.05^{c}	0.24 ± 0.01^{cd}	–	–	0.13 ± 0.01^{a}	0.13 ± 0.01^{a}	–
Lactones												
5-octanolide	1977	1925	–	0.20 ± 0.01^{bcd}	0.22 ± 0.02^{cd}	0.22 ± 0.04^{cd}	0.25 ± 0.02^{d}	0.15 ± 0.06^{b}	0.17 ± 0.05^{bc}	0.09 ± 0.01^{a}	–	0.09 ± 0.00^{a}
5-Decanolide	2179	2158	1.93 ± 0.32^{e}	0.90 ± 0.06^{cd}	0.89 ± 0.02^{cd}	0.91 ± 0.03^{d}	0.91 ± 0.04^{d}	0.69 ± 0.03^{abc}	0.62 ± 0.08^{ab}	0.57 ± 0.06^{a}	0.66 ± 0.03^{ab}	0.79 ± 0.12^{bcd}

(*continued on next page*)

Table 1 (*continued*)

Compounds (μg/g)	RI		Control	2SD 6 h	2SD 18 h	2SD 24 h	2SD 30 h	6SD 12 h	6SD 30 h	FN 12 h	FN 18h	FN 24 h
	Literature [x]	Calculation [y]										
4-Dodecanolide	2366	2333	–	0.09 ± 0.00^{a}	0.13 ± 0.01^{b}	0.13 ± 0.02^{b}	0.18 ± 0.01^{c}	0.27 ± 0.04^{d}	0.25 ± 0.02^{d}	–	–	–
γ-Tetradecanolide	2628	2567	–	–	–	–	–	0.34 ± 0.05^{a}	–	–	–	–
Others												
Trimethyloxazole	1200	1182	–	–	–	0.95 ± 0.99^{a}	1.82 ± 0.36^{b}	–	–	–	–	–

"-" means not detected (ND). Values are expressed as mean ± standard deviation (SD) of triplicate analysis. a–e Values bearing different superscript lowercase letters within the same row are significantly different (Duncan, $p < 0.05$). [x]: Reported data. [y]: Calculated data due to n-alkanes (C_7–C_{40}).

peptides had a certain contribution to the saltiness of sample hydrolyzed by 2SD for 30 h. In general, samples with high account of peptides between 150 Da and 450 Da achieved strong bitterness, aftertaste, umami, and saltiness. Peptides over 6000 Da contained low umami and more bitterness. This result was slightly different from that of Gao et al. (2021), who reported that peptides below 3000 Da (especially peptide between 1000 Da and 3000 Da) as well as eight FAAs (Glu, Pro, His, Thr, Asp, Ser, Gly, and Ala) were highly associated with umami and salty properties, while peptides in the range of 3000 Da–10000 Da were correlated to astringency and bitterness. The study is consistent with that of Fu et al. (2018), who found that a high proportion of low MW peptides (<500 Da) displayed strong umami taste or umami-enhancing attributes in some hydrolysates produced from plant or animal sources. Overall, the fraction containing peptides with MW of less than 1500 Da was found to be cheese protein hydrolysates most taste-active fraction. Sample hydrolyzed by FN systemfor18 h, showing more richness and umami, and less bitterness, aftertaste and astringency. Notably, all these characteristics were positively correlated to valine and cysteine. Due to the large number of factors, some factors cannot be displayed in the PCA biplot. Table S1 was used to display the contributions of all loading plots for each factor in the PCA biplot.

The above results suggest that the composition and concentration of FAAs as well as peptides' MWD are closely related to the taste of hydrolysates. In general, the intricate spatial structure of high peptide MW might prevent taste-active groups from approaching taste receptors, thereby contributing little to taste.

3.3. Analysis of volatile compounds

Based on electronic tongue analysis, in this experiment, samples with the largest difference in the same taste index were selected for volatile compounds analysis, and the amounts of these compounds (μg/g) are given in Table 1. In general, 36 compounds were detected, consisting of acids (11), ketones (9), alcohols (6), aldehydes (2), and others compounds (8). The total volatile content for the hydrolysates increased with the incubation time. Compared to the control group, 22 new volatile components were detected in hydrolysates, which contained acids (5), ketones (4), alcohols (5), aldehydes (1), and other compounds (7). Samples hydrolyzed by 2SD contained the largest variety of volatile substances, followed by 6SD, with FN being the lowest variety of volatile compounds. Furthermore, in general, the control group, Protease 2SD and 6SD achieved high total acid content than the FN system. Interestingly, the higher content of ketone compounds was recorded for samples hydrolyzed by 2SD over 24 h and FN over 18 h. The literature reports that most of the volatile compounds come from the chemical or enzymatic oxidation of unsaturated fatty acids and further interactions with proteins, peptides and FAAs. Other volatile compounds result from the Strecker degradation of FAAs and Maillard reactions (Merlo et al., 2021).

In this study, several volatile FFAs and related compounds were determined at the headspace. After extensive proteolysis, 2SD and 6SD contained higher total acid compound contents than the FN system. FFAs not only are aroma compounds by themselves but also serve as precursors of alcohols, lactones, methyl ketones, and esters (Curioni & Bosset, 2002). For example, phenylethyl alcohol arose from phenylalanine following the transamination of phenylpyruvate by a non-enzymatic breakdown and conferred a very pleasant rose aroma (Cincotta et al., 2021). Also, four lactones (4-dodecanolide, 5-decanolide, and 5-octanolide, γ-tetradecanolide) were identified, formed from hydroxylated FFA. While 4-dodecanolide has a fatty, peachy, somewhat musky odor and a buttery, peach-like flavor at a 1 ppm–10 ppm taste characteristic, which has been reported in blue cheese and Camembert cheese (Molimard & Spinnler, 1996). 4-Dodecanolide may be formed from hydroxydodecanoic acid by lactonization. In this study, the tested samples hydrolyzed by 2SD and 6SD contained this compound and its content was greater than 1 ppm (0.01 μg/g). Moreover, 5-decanolide and 5-octanolide also identified in the production of enzyme modified cheese with a ripened white cheese flavor (Bas et al., 2019).

Another important class of volatile compounds are ketones. In this study, four methyl ketones (2-heptanone, 2-nonanone, 2-undecanone, and 2-tridecanone) and acetoin were identified in all the tested samples. The sample hydrolyzed by FN for 24 h achieved the highest ketone content than the other samples. Methyl ketones are known to be formed from FFA via β-oxidation reactions (Collins, Mcsweeney, & Wilkinson, 2003). The content of acetoin was the highest by FN for 18 h 17.32 μg/g), followed by 2SD for 30 h and FN for 24 h. Acetoin can be formed by citrate metabolism as well as aspartic acid and alanine catabolism (Mcsweeney & Sousa, 2000; Terpou et al., 2017), and it has a central role in determining the flavor of immature fresh cheese. In this study, its amount exceeded its odor threshold of 0.8 μg/g (Natrella, Faccia, Lorenzo, De Palo, & Gambacorta, 2020) in most of the samples. Acetoin is characterized by buttery and woody sensory notes and is the main ketone in mozzarella (Cincotta et al., 2021). As previously mentioned, the origin of ketones can be diverse. However, it is well known that the main route of production of 2-ketones is the lipid oxidation of FFAs (Merlo et al., 2021). It is important to highlight that these types of ketones have great importance in the overall aroma of hydrolysates given their specific odor, for example, 2-heptanone contributes spicy, blue cheese and acorn aroma notes (García-González, Tena, Aparicio-Ruiz, & Morales, 2008).

Proteolysis plays an essential role in the generation of cheese flavor indirectly as the volatile compounds formed by FAA degradation reactions such as ammonia, aldehydes, amines, phenols, indole, and alcohols (Smit et al., 2000). Amines and α-keto acids are produced through FAA catabolism, and alcohols and acids are formed by oxidation-reduction reactions (Fox & Wallace, 1997; Yvon & Rijnen, 2001). In this study, benzaldehyde was detected in all samples, and phenylacetaldehyde was detected in 2SD for 6 h, 18 h, 24 h and 30 h and FN for 12 h, 18 h. Phenylacetaldehyde is one of the most important aromatic compounds and provides a honey-like odor taste to cheese (Curioni & Bosset, 2002). It was previously reported that this compound was formed by the degradation of phenylalanine (Fox & Wallace, 1997). Furthermore, benzaldehyde may be formed from the α-oxidation of phenylacetaldehyde or the β-oxidation of cinnamic acid and was

Table 2
The OAVs and contribution rate of the key aroma compounds in the control group and some hydrolysates.

Compounds	Odor description	OT (ug/g)		Control	2SD 6 h	2SD 18 h	2SD 24 h	2SD 30 h	6SD 12 h	6SD 30 h	FN 12 h	FN 18h	FN 24 h
Acetic acid	Sour	0.124[1]	OAV	17.661	5.161	7.258	5.403	2.903	4.435	7.097	1.21	21.21	4.435
			CR	1.7	0.63	0.78	0.38	0.17	1.06	1.6	0.15	1.36	0.33
Butanoic acid	Rancid, cheese, sweat	0.175[2]	OAV	0	1.543	1.657	1.6	1.486	0.8	2	0	4.457	0
			CR	0	0.19	0.18	0.11	0.09	0.19	0.45	0	0.29	0
Hexanoic acid	Sweat	0.42[2]	OAV	8.286	17.857	34.167	19.261	20.738	1.5	11.119	0.643	2.762	2.024
			CR	0.8	2.18	3.68	1.35	1.24	0.36	2.51	0.08	0.18	0.15
Octanoic acid	Sweat, cheese	0.5[2]	OAV	6.24	15.58	31.3	17.98	35.24	8.22	18.92	1.62	1.5	0.76
			CR	0.6	1.9	3.37	1.26	2.11	1.97	4.27	0.2	0.1	0.06
n-Decanoic acid	Rot acid	0.13[3]	OAV	52.846	53.923	80.769	59.923	90.615	34.154	54.462	10.847	9.308	6.231
			CR	5.08	6.57	8.7	4.19	5.43	8.19	12.29	1.34	0.6	0.46
Acetoin	Butter, cream	0.014[4]	OAV	131.429	83.571	60.714	728.571	936.429	61.429	48.571	54.286	1237.143	933.571
			CR	12.63	10.18	6.54	50.89	56.13	14.72	10.96	6.7	79.59	68.66
2-Heptanone	Soap	0.14[5]	OAV	6.643	17.929	20.286	18.357	14.143	12.786	8	31.214	8.643	14.5
			CR	0.64	2.18	2.19	1.28	0.85	3.06	1.81	3.85	0.56	1.07
2-Nonanone	Hot milk, soap, green	0.2[5]	OAV	16.05	14.5	15.65	9.75	10	7.7	7.9	17.3	6	11.25
			CR	1.54	1.77	1.69	0.68	0.6	1.85	1.78	2.13	0.39	0.83
2-Undecanone	Orange, fresh, green	0.0055[4]	OAV	767.273	552.727	592.727	501.818	478.181	272.727	272.727	649.091	220	367.273
			CR	73.75	67.32	63.87	35.05	28.66	65.37	61.56	80.07	14.15	27.01
Benzaldehyde	Almond, burned sugar	0.35[6]	OAV	0	40	65	52.5	60	0	0	32.5	32.5	0
			CR	0	4.87	7	3.67	3.6	0	0	4.01	2.09	0
Phenylacetaldehyde	Burnt	0.004[7]	OAV	4.686	4.571	5.029	2.686	4.771	3.029	2.829	3.314	0.886	7.686
			CR	0.45	0.56	0.54	0.19	0.29	0.73	0.64	0.41	0.06	0.57
5-Decanolide	Coconut fragrance	0.066[3]	OAV	29.242	13.636	13.485	13.788	13.788	10.454	9.394	8.636	10	11.970
			CR	2.81	1.66	1.45	0.96	0.83	2.51	2.12	1.07	0.64	0.88

OAVs: The ratio of the concentration of each compound to its perception threshold.
CR: Contribution rate - the ratio of OAVs of each aroma compound to the total compounds.
OT: Odor threshold. References: [1] (Frauendorfer & Schieberle, 2019); [2] (Pu et al., 2019); [3] (Wang et al., 2020); [4] (Natrella et al., 2020); [5] (Han et al., 2019); [6] (Lin et al., 2019); [7] (Kang et al., 2019).

characterized by bitter almond odor notes (Mcsweeney & Sousa, 2000). Moreover, some volatile pyrazines were detected in this experiment, including 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and tetramethylpyrazine (TMP); 2,5-dimethylpyrazine and 2,3,5-trimethylpyrazine have been identified in Camembert cheese, and 2,5-dimethylpyrazine imparts a characteristic earthy, potato-like odor, which could be due to threonine degradation (Molimard & Spinnler, 1996). To our knowledge, TMP is an important aroma component and functional substance in Chinese liquor, Japanese natto and fermented cocoa beans. In addition, TMP has various kinds of medical applications; for example, it is used for the treatment of cardiovascular problems as well as anti-inflammatory and analgesic effects (Lin, Wang, Zhou, Xu, & Yao, 2022). Cao et al. (2020) demonstrated that the effects of TMP on umbilical cord mesenchymal stem cells (ucMSCs) are dose dependent. At a low dose (<200 μmol/L, which was <27.24 μg/g), TMP protected the cells against H_2O_2-induced apoptosis. At a high dose (10 mmol/L), TMP improved the secretion of cytokines. This compound found for the first time in cheese hydrolysates, this would provide a reference for developing cheese functional products. TMP smells like chocolate when diluted to 20 μg/g; interestingly, 2SD for 30 h achieved 17.59 μg/g content of the compound and may be characterized by a rich chocolate flavor. According to the literature, the formation mechanism of alkylpyrazines involves two main controversial viewpoints: the Maillard reaction pathways and biogenic pathways (Xiao, Zhao, Tian, Wang, & Zhao, 2018). Acetoin and ammonium were deduced to be the precursors of TMP, and this reaction pathway co-existed with the Strecker degradation (H. Yu et al., 2021; Zhang et al., 2019). Interestingly, FN for 18 h achieved high acetoin content, while TMP was not detected, and this may be due to the lack of ammonium in the hydrolysis. Table 2 showed the possible aromatic backbone of the samples, most composed of the aroma compounds with OAV >1. Some of them (2-undecanone, acetoin, 2-nonanone, 5-decanolide et al.) exceeded the value in all samples. In particular, the cumulative contribution rate of 2-undecanone and acetoin was over 80%, indicating that they played a key role in the formation of the overall flavor of the samples. 2-Undecanone was responsible for orange, fresh, and green odor; acetoin has butter and cream notes. Generally, 2-undecanone, acetoin, 5-decanolide, n-decanoic acid, acetic acid were the key aroma compounds of the control group and 6SD hydrolysates. 2-Undecanone, acetoin, n-decanoic acid, benzaldehyde, 2-heptanone, hexanoic acid, octanoic acid were mainly responsible for 2SD hydrolysates. FN hydrolysates showed strong butter and cream flavor as a result of the high OAV of acetoin.

4. Conclusion

In conclusion, the present study suggested that different protein hydrolysates can be discriminated both by non-volatile components and volatile compounds. The interaction of several volatile (aroma) and non-volatile (taste) compounds determined the formation of characteristic protein hydrolysates flavor. With the same total enzyme concentration, a more richness taste, low bitterness, and butter and cream odor product was obtained by combining the enzyme of Flavourzyme and Neutrase for 18 h. TMP was firstly discovered in the cheese industry, which could exhibit functional properties. Peptides below 450 Da contributed significantly to the taste difference of hydrolysates. To sum, this study provides reference value for the use of exogenous protease and the understanding of its characteristics. The results obtained can be not only very useful in the application of exogenous protease to accelerate natural cheese ripening in cheese industry, but also help to develop medical value cheese product. In order to better identify the flavor peptides from cheese protein hydrolysates, the amino acid sequence of different peptides fractions would need to be further studied.

CRediT authorship contribution statement

Peng Gao: Methodology, Investigation, Conceptualization, Visualization, Writing – original draft, preparation. **Wenyuan Zhang:** Investigation, Conceptualization, Visualization, Writing – review & editing. **Miaohong Wei:** Methodology, Investigation, Visualization. **Baorong Chen:** Investigation, Visualization. **Huiquan Zhu:** Investigation, Visualization. **Ning Xie:** Validation, Funding acquisition. **Xiaoyang Pang:** Validation, Funding acquisition. **Fauconnier Marie-Laure:** Writing – review & editing. **Shuwen Zhang:** Conceptualization, Funding acquisition, Writing – review & editing. **Jiaping Lv:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no known competitive financial interests or personal relationships that could affect the work reported of this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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