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Pseudo-targeted metabolomics combined with a non-parametric discriminant model to distinguish commercial milk processed at different temperatures

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ABSTRACT: Thermal processing is essential for liquid milk quality and safety. However, relatively broad standard regulations and inefficient supervision methods encourage enterprises to use excessive heating to maximize commercial profits. Therefore, a pseudo-targeted metabolomics approach based on ultra performance liquid chromatography quadrupole time-of flight mass spectrometry (UPLC-QTOF MS) with multivariate statistical analysis was used to discover biomarkers that can distinguish among pasteurized (29 Pa, 72 °C, 75 °C, and 85 °C), extended shelf life (9 ESL, 121 °C), and ultra-high temperature sterilization (20 UHT, 138 °C) milk. Finally, 10 key metabolites were characterized, including peptides, nicotinamide, N^6-methyladenosin, and 2-hydroxycapric acid. The diagnostic performance of these candidate biomarkers was evaluated in 55 validation samples using receiver operating characteristic curve analysis and a non-parametric discrimination model, with 96.4% classification accuracy. These results demonstrated that the pseudo-targeted metabolomics method is reliable and provides effective biomarkers for evaluating the nutritional value of milk subjected to different thermal treatments.

Keywords: Milk; Heating process; Metabolomics; UPLC-QTOF-MS

1. Introduction

Thermal processing of liquid milk is essential for ensuring microbial safety and extending its shelf life while preserving its nutritional and sensory qualities[1]. Currently, pasteurization (Pa), extended shelf life (ESL), and ultra-high temperature (UHT) treatment are widely used as commercial thermal processes in many countries[2, 3]. According to European regulation EC 2074/2005, pasteurization must be performed at either 72 °C for 15 s (high-temperature short-time, HTST) or at least 63 °C for 30 min (low-temperature long-time, LTLT), and UHT treatment requires a minimum of 135 °C for an appropriate holding time[4]. By contrast, under Chinese Regulation NY/T 939-2016, pasteurization can be carried out under three alternative conditions: 80–85 °C for 10–15 s, 72–76 °C for 15 s, or 63–65 °C for 30 min. For UHT treatment, the domestic

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standard requires a minimum temperature of 132 °C with a relatively short holding time. Although existing regulations define general temperature ranges for milk processing, the wide sterilization temperature range and the absence of precise time–temperature limits can easily lead to milk overheating. Moreover, current regulations lack specific guidelines for ESL milk production in China, resulting in insufficient supervision and potential quality degradation owing to overheating. In addition, the price of pasteurized milk is typically two-to-three times higher than that of UHT milk[4]. Consequently, some manufacturers employ overheating treatment to extend the shelf life of products and maximize profits, which poses a significant threat to consumer rights. Excessive heat treatment significantly alters the nutritional quality of milk. For instance, ESL milk exhibits higher concentrations of lactulose and furosine and greater protein denaturation than Pa milk[1, 5]. Therefore, elucidating the thermal processing parameters and comprehensively understanding the metabolites produced during thermal processing are crucial for clarifying the nutritional, physiological, and technological implications of dairy products.

Time–temperature integrators (TTI) have been widely used to evaluate the heat treatment intensity of liquid milk[6]. These heat-sensitive markers are mainly divided into type I and type II indicators. Type I indicators detect the denaturation and degradation of thermally unstable components (e.g., whey protein, lactoperoxidase, and alkaline phosphatase)[7]. Type II indicators detect substances newly formed during thermal processing, such as Maillard reaction products (e.g., furosine, lactulose, or hydroxymethylfurfural)[8, 9]. However, type I biomarkers, such as alkaline phosphatase and whey protein, have limitations because of their heat resistance and potential reactivation after heat denaturation[10, 11]. Therefore, they are only suitable for low-temperature processes (such as pasteurization) because of their low inactivation temperatures [6, 12]. In contrast, Maillard reaction products in type II indicators can distinguish higher-temperature thermal processes, such as ESL and UHT, but studies have indicated that the levels of furosine and lactulose are affected not only by heating temperature but also by storage conditions[13]. Furthermore, the detection methods of these traditional indicators are relatively complex, and a single indicator is insufficient to effectively distinguish a broad range of heat treatments; thus, a combination of several indicators is often required[14]. Therefore, it is essential to develop highly sensitive and accurate biomarkers and discriminatory models to identify different thermal processes in liquid milk.

Omics technologies, including metabolomics, lipidomics, and proteomics, are powerful tools for discovering novel biomarkers and have been increasingly applied to identify and characterize different thermal treatment processes in milk[13, 15]. For instance, compounds such as N^{ε}-carboxymethyl lysine (CML), N^{ε}-carboxyethyl lysine (CEL), pentosidine, pyrraline, and lysinoalanine (LAL) have been identified as markers to distinguish between Pa and UHT milk[4]. CML and CEL are derived from the Maillard reaction, but their content is closely related to the storage period and humidity[16]. Seven oxylipids and one phospholipid were also identified as potential biomarkers for differentiating UHT milk from raw and Pa milk based on lipidomics[17]. In our laboratory, oxidized phosphatidylcholine and triacylglycerol were characterized and used to distinguish Pa and ESL milk from UHT milk[18]. Proteomics was used to identify specific peptides,

protein aggregation, and structural changes that characterize the extent of heat treatment. For instance, 16 marker peptides of β-lactoglobulin were identified to characterize thermal and non-thermal milk[19, 20]. Overall, these reported biomarkers are efficient for the identification of milk subjected to low- (Pa) and high-temperature (UHT) treatments, while there is a lack of reports for intermediate heating treatments (ESL) and precise identification of Pa temperature (85 °C, 75 °C, and 72 °C).

Pseudo-targeted metabolomics is a novel concept that effectively integrates the inherent strengths of both targeted and untargeted metabolomic approaches. High-resolution MS offers superior mass accuracy, resolving power, and coverage range for untargeted metabolites in food matrices and is constrained by high costs and low quantitative accuracy. In contrast, multipl e reaction monitoring (MRM) using low-resolution mass spectrometry (LC-MS/MS) provides a cost-effective and rapid method for targeted metabolite analysis[21]. Therefore, integrating these two techniques would facilitate both high coverage and high quantitative accuracy, enabling comprehensive data acquisition while constraining the dataset, thereby ensuring a more efficient and comprehensive analysis[22]. It has been proven reliable for analyzing metabolic changes, performing isotope tracing, and identifying potential biomarkers to distinguish between different sample groups[23].

Therefore, for the first time to our knowledge, we aimed to systematically describe the metabolic characteristics of milk heated under different conditions, using pseudo-targeted metabolomics. In this study, pseudo-targeted metabolomics approach based on ultraperformance liquid chromatography quadrupole time-of flight mass spectrometry (UPLC-QTOF-MS) was used to identify biomarkers to differentiate among Pa milk (72 °C, 75 °C, and 85 °C), ESL milk (121 °C) and UHT milk (137 °C). Furthermore, receiver operating characteristic (ROC) curves[24] and non-parametric discriminant models were used to evaluate the predictive performance of candidate differential metabolites. The established methodology and biomarkers not only provide a scientific basis for determining the thermal processing parameters of dairy products, but also offer a robust technical framework for optimizing industrial heat treatment technologies.

2. Materials and methods

2.1 Chemicals and reagents

HPLC-grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany) and HPLC-grade formic acid and ammonium acetate were supplied by DiKMA Technologies (Beijing, China). Ultrapure water was obtained using a Milli-Q system (Millipore, Billerica, MA, USA). The reference standards for Nicotinamide, Nicotinamide riboside (NR), and N^6-methyladenosine were purchased from Sigma-Aldrich (Shanghai, China).

2.2 Sample collection and preparation

In order to reduce bias arising from a limited sample source and account for inherent natural variation (e.g., seasonal changes, feeding model, and breeds), milk samples from different batches were provided from six independent farms of New Hope Dairy Company (Chengdu, China). According to the manufacturers'

specifications, milk samples were classified based on their sterilization conditions: milk sterilized at 72 °C, 75 °C or 85 °C for 15 s was identified as Pa milk; milk sterilized at 121 °C for 15 s, ESL milk; and milk sterilized at 137 °C for 4 s, UHT milk. All milk samples were collected and aliquoted on the day of production, then immediately stored at -20 °C and transported back to the laboratory via cold-chain transportation until analysis. The training dataset comprised 29 Pa (9 samples sterilized at 72 °C, 10 at 75 °C, 10 at 85 °C), 9 ESL, and 20 UHT milk samples. For the blind test set, 22 Pa, 11 ESL, and 22 UHT milk samples of various regional origins were obtained from local supermarkets. Detailed information on the milk samples is shown in Tables S1 and S2. A storage experiment was conducted on the collected samples. The samples were maintained at room temperature for 11 weeks to investigate the temporal patterns of metabolite variation. Detailed information on the milk samples is provided in Table S3.

Milk was pretreated as previously described [21] with some modifications. In brief, 2 mL of milk sample was taken into a centrifuge tube, and centrifuged at 12000 × *g* and 4 °C for 30 min to remove the upper fat layer. Subsequently, the skimmed milk was mixed with 4 mL of precooled acetonitrile and vortexed for 5 min. The protein was precipitated via centrifugation at 10000 × *g* and 4 °C for 30 min and the supernatant collected was filtered through 0.22 μm membrane. Ultimately, 5 μL supernatant was injected into UPLC-Q-TOF-MS system. Quality control (QC) samples were prepared as follows: 1 mL of each milk sample was mixed evenly. To monitor the stability of the instrument and ensure the stability and repeatability of the data, QC samples were analyzed after every five samples.

2.3 Metabolite analysis

The samples were analyzed by UPLC (ExionLC AC, AB Sciex, Framingham, MA, USA) and tandem QTOF mass spectrometry (Triple TOF 6600, Ab Sciex). The chromatographic separation column was ZORBAX Eclipse C18 (150 mm × 3.0 mm, 1.8 μm, Agilent, Santa Clara, CA, USA) and the column temperature was set to 40 °C. The injection volume was 5 μL and the flow rate was set to 0.4 mL/min. In positive ion mode, the mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program of mobile phase B was as follows: 0 min, 2%; 5 min, 2%; 8 min, 10%; 12 min, 95%; 18 min, 95%; 18.1 min, 2%; 20 min, 2%. In negative ion mode, the mobile phase was 5 mM ammonium acetate in water (A) and 5 mM ammonium acetate in acetonitrile (B). The gradient program of mobile phase B was as follows: 0 min, 2%; 5 min, 2%; 8 min, 10%; 12 min, 95%; 16 min, 95%; 16.1 min, 100%; 18 min, 2%.

The ion source of the mass spectrometer was an electrospray ionization (ESI) source, and the acquisition mode was information-dependent acquisition (IDA). The mass scanning range was 50–1000 m/z, and the collision energy (CE) was 35±15 eV. Under the positive ion mode, the parameters were set to: ion spray voltage floating (ISVF), 5500 V; ion source temperature, 550 °C; decluttering potential, 70 V; ion source gas 1 (GS1), 45 psi; ion source gas 2 (GS2), 65 psi; and curtain gas, 30 psi. Under the negative ion mode, the parameters were set to: ISVF, -4500 V; TEM, 55 °C; DP, -70 V; GS1, 45 psi; GS2, 65 psi; and CUR, 30 psi.

2.4 Statistical analysis

The raw data collected by UPLC-QTOF-MS were preprocessed (for noise setting, baseline correction, peak detection, and alignment) using the Peakview 2.2 software (AB Sciex). The following parameters were used to acquire the data: mass width, 0.02 Da; mass tolerance, 20 mDa. The extracted ion chromatogram (XIC) with an intensity above 100 counts or a signal-to-noise ratio above 3 was calculated. Finally, statistical analysis was performed using SIMCA 14.1 (Umetrics, Umea, Sweden). Principal component analysis (PCA) and loading plot analysis were performed for milk samples processed using different technologies. The variable importance in projection (VIP) values were set such that each latent variable (X) explained the amount of variance (Y) using orthogonal partial least squares discriminant analysis (OPLS-DA); larger VIP values indicated that the term X had a greater influence on the Y variables[21]. OPLS-DA was performed after Pareto scaling and log transformation. Student's t-test and *p*-values were analyzed using the MetaboAnalyst software (version 4.0; http://www.metaboanalyst.ca). The *p*-values of all metabolites were adjusted to account for multiple testing using the false discovery rate (FDR) method. The peak values of VIP > 1, fold change (FC) > 2 or < 0.5, FDR < 0.05, and $P < 0.01$ were considered as potential biomarkers[25].

2.5 Qualitative analysis

To qualify the metabolites, their molecular formula was deduced using a built-in element composition calculator (Masterview software; AB Sciex) according to the accurate mass and isotopic patterns of the metabolites. The feasible element compositions considered in the formula calculation were: C ($n \leq 150$), H ($n \leq 250$), O ($n \leq 50$), n ($n \leq 50$), s ($n \leq 5$), Cl ($n \leq 5$) and P ($n \leq 5$), and the mass accuracy threshold was set as 3 ppm. Molecular formula, accurate mass, and product ion information were used to search candidate databases, including Metlin (http://www.metlin.scripps.edu/), MassBank (http://www.massbank.jp/), HMDB (http://www.hmdb.ca), Lipidmaps (http://www.Lipidmaps.org), and MCDB (http://www.mcdb.ca). To better characterize the peptides in metabolites, the original data collected by mass spectrometry were imported into the ProteinPilot software to identify peptides with the following parameters: identification of sample type, no cysteine alkylation, no digestion, Triple TOF 6600 for instrument setting, *Bos taurus* for species (Fasta file was obtained from UniProt), biological modifications for ID focus, and rough ID for search workload. In addition, the result quality was set to 0.05% (10%) and a false discovery rate analysis was performed. Finally, the sequence of the marker peptide was identified with a 99% confidence interval.

2.6 Independent data acquisition (IDA) to multiple-reaction monitoring (MRM) workflow

The IDA-to-MRM workflow was as follows: First, untargeted metabolomics of Pa, ESL, and UHT milk samples were performed in the IDA acquisition mode. Second, the potential markers were screened using multivariate statistical analyses and pattern recognition (PR) method. Third, the ion pair information of the candidate markers, including precursor ions, fragment ions, and retention times, was collected from UPLC-QTOF-MS, and the MRM-targeted acquisition method was established by UPLC-MS/MS (AB Sciex 4500). The chromatographic separation and mass spectrometric detection parameters for UPLC-MS/MS were consistent with the untargeted metabolomic workflow, ensuring methodological continuity and comparability. Detailed information on the precursor ions, fragment ions, CE, and DP is provided in Table 1. To validate the

feasibility of the targeted method, extra milk samples were analyzed using nonparametric discriminant analysis.

2.7 Design and validation of the predictive model

To further evaluate the discriminative capability of the ten candidate metabolites, the MRM method and ROC analysis were used to quantify and define the best diagnostic markers. In general, the area of "AUC=1" indicates the best prediction accuracy, while "AUC=0.5" implied poor predictive ability[26]. In addition, the classification model was performed using non-parametric discriminant analysis in the SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). Subsequently, 55 extra milk samples (22 UHT, 11 ESL, and 22 Pa milk samples) were analyzed as a blind test set to evaluate the performance of the prediction model.

3 Results and Discussion

3.1 Untargeted metabolic profiling

To explore the metabolic differences in Pa, ESL, and UHT milk, an untargeted metabolomic analysis based on UPLC-QTOF-MS was performed. A total of 1574 and 605 compounds were detected in the positive and negative ion modes, respectively (Fig. S1). QC samples were analyzed to ensure signal correction, minimize variations, and assess analytical accuracy[27]. The superimposed TICs of the 13 QC samples showed consistent clustering, confirming the stability and repeatability of the instrument. Unsupervised PCA was used to distinguish milk samples, with a clear separation observed among the three milk types in both the positive (Fig. 1) and negative (Fig. S2A) ion modes. In the positive ion mode, the first five principal components accounted for 18.6%, 12.4%, 12.2%, 10.5%, and 7.9% of the grouping differences, with a cumulative contribution rate of 61.6%. In the negative ion mode, the first five principal components explained 33.3%, 11.4%, 10.6%, 8.9%, and 5.9% of the grouping differences, resulting in a cumulative variance contribution rate of 70.1%.

Supervised OPLS-DA was performed to maximize the separation among all types of samples and to screen for potential biomarkers (Fig. 1B-D and Fig. S2B-D). In positive ion mode, one predictive component and two orthogonal components were fitted. For Pa and ESL milk, 69.3% of the variables (R^2X) explained 99.2% of the variance (R^2Y), with a cross-validation predictive ability of 94.1% (Q^2cum), indicating that the OPLS-DA model had a good predictive capability. Similar results were observed for UHT versus ESL milk ($R^2X = 86\%$, $R^2Y = 97.3\%$, $Q^2cum = 89.5\%$) and UHT versus Pa milk ($R^2X = 84.3\%$, $R^2Y = 98.2\%$, $Q^2cum = 91.1\%$). The 200 permutation tests confirmed the validity of the model and did not overfit it (Fig. S3). Overall, the OPLS-DA model demonstrated high predictive capability and robust separation among milk types.

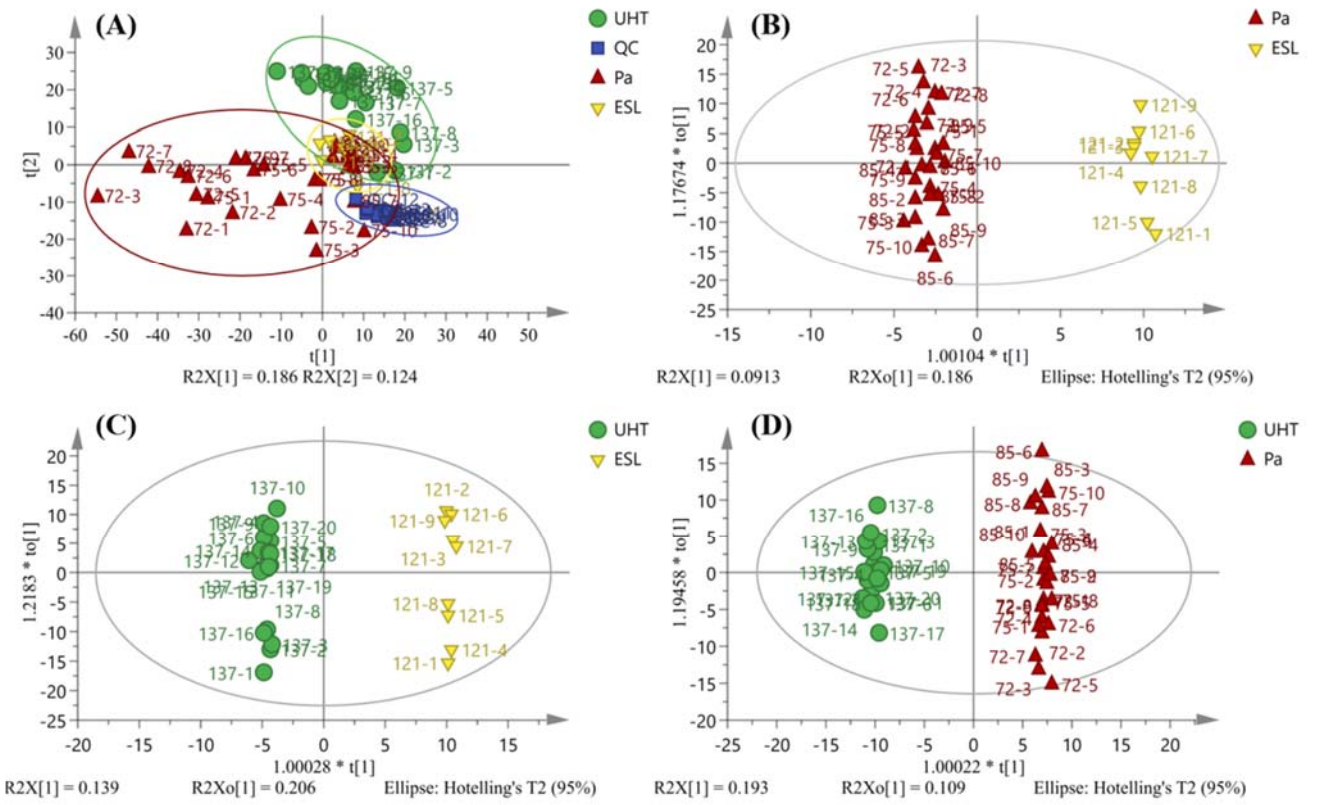

Fig. 1 (A) PCA score plot in positive ion mode. (B) OPLS-DA score plot of Pa milk and ESL milk in positive ion mode. (C) OPLS-DA score plot of ESL milk and UHT milk in positive ion mode. (D) OPLS-DA score plot of Pa milk and UHT milk in positive ion mode.

3.2 Screening and identification of differential metabolites

Differential metabolite biomarkers in Pa, ESL, and UHT milks were screened using a VIP > 1 threshold in the OPLS-DA model. Seventy metabolites were screened from the IDA dataset (39 positive and 31 negative ionization modes). To ensure the robustness of differential screening, Student's t-test with adjusted *p*-values and fold change (FC) analysis was applied. Metabolites meeting the criteria of FC > 2 or < 0.5, and $P < 0.01$ were retained in both ionization modes. All potential biomarkers were manually verified, and metabolites exhibiting low signal intensity, isotopic features, or lacking MS/MS fragmentation information were excluded. Ultimately, 19 differential metabolites in the positive ion mode and three in the negative ion mode were confirmed, as listed in Table S4.

Ten differential metabolites were identified by comparing the parent and fragment ion information from the purchased standards and reference standard databases, and the confidence level of all metabolites was above 99%. Nine metabolites in the positive ion mode and one metabolite in the negative ion mode were identified (Table 1), including six peptides, nicotinamide, N^6-methyladenosine, hydroxydecanoic acid, and their derivatives. Overall, the nicotinamide and nicotinamide riboside contents in Pa milk were higher than those in ESL and UHT milk, whereas the contents of N^6-methyladenosine, peptides and 2-hydroxycapric acid in Pa milk were lower than those in ESL and UHT milk. During the 11-week storage period, the FC of the 10 differential metabolites remained within a narrow range of 0.05 to 1.4. This stability suggests that these biomarkers were not influenced by the storage conditions.

Table 1 Qualitative and ROC curve results for differential metabolites

Number	Chemical Compound	Molecular Formula	Charge Number	Parent Ion	Fragment Ion[a]	Declustering Potential(eV)	Collision Energy(eV)	Retention Time	Error ppm	Fold Change			AUC		
										ESL/Pa	UHT/ESL	UHT/Pa	Pa vs ESL	Pa vs UHT	ESL vs UHT
1	Nicotinamide[b]	$C_6H_6N_2O$	1	123.0549	80.0510/78.0363	80	35	1.78	-1.0	0.16	0.06	0.01	1	1	1
2	RP[c]	$C_{11}H_{19}N_5O_2$	1	254.1614	195.1135/167.1179	80	35	4.94	-0.4	5.87	2.60	15.25	1	1	1
3	Nicotinamide riboside[b]	$C_{11}H_{15}N_2O_5$	1	257.2109	123.0552/57.0343	80	35	13.61	1.7	0.48	0.23	0.11	0.87	0.99	1
4	N^6-methyladenosine[b]	$C_{11}H_{15}N_5O_4$	1	282.1183	150.0778/94.0394	80	35	5.71	-1.3	12.81	3.36	43.04	1	1	1
5	SEKTTMPLW[c]	$C_{49}H_{77}N_{11}O_{15}S_1$	2	546.7726	133.0853/188.0714	80	50	9.65	-1.8	13.10	2.46	32.14	1	1	0.86
6	PVLGPVRGPFPIIV[c]	$C_{72}H_{117}N_{17}O_{15}$	2	730.9518	615.8726/559.3305	80	35	10.35	-0.1	66.01	4.17	275.09	0.79	0.96	1
7	QEPVLGPVRGPFPIIV[c]	$C_{82}H_{132}N_{20}O_{20}$	2	850.9896	437.2016/550.2850	80	35	10.64	-0.6	2.90	11.36	32.98	0.95	1	1
8	MPIQAFLLYQEPVLGPVRGPFPIIV[c]	$C_{136}H_{212}N_{30}O_{31}S_1$	3	932.1967	914.5220/801.4273	80	50	10.66	-0.6	12.33	2.38	29.33	1	1	0.98
9	MPIQAFLLYQEPVLGPVRGPFPIIV[c]	$C_{136}H_{211}N_{29}O_{33}S_1$	3	937.8706	914.5222/932.5368	80	50	10.65	-0.6	13.05	2.79	36.47	1	1	0.92
10	2-Hydroxycapric acid[b]	$C_{10}H_{20}O_3$	1	187.1336	141.1310/113.1008	80	20	10.09	-2.0	9.25	12.46	35.84	0.97	1	1

[a]Fragment ion (*m*/*z*): quantitative ion/qualifier ion

[b]Confirmed by reference standard

[c]Tentative

3.3 Performance of the biomarkers and validation of the model

Ten features (nine positive and one negative ion mode) with complete MS1, MS2, retention time, collision energy (CE), and declustering potential information were selected to set up the MRM workflow using UPLC-MS/MS. Detailed information is provided in Table 1. To assess the discriminatory power of biomarkers, receiver operating characteristic (ROC) curves were calculated based on the relative intensity of each candidate marker. The AUC values of 10 differential metabolites were in the range of 0.79–1.00, demonstrating the strong ability of these metabolites to differentiate between the three types of milk. However, the performance of the individual markers varied across milk type comparisons. For example, the AUC value of PVLGPVRGPFPIIV was 0.79 for discriminating Pa and ESL milk, which was significantly lower than for ESL/UHT (AUC=1) and Pa/UHT (AUC=0.96). In contrast, the AUC value of SEKTTMPLW was 0.86 for discriminating Pa and ESL milk, which was lower than its AUC values for UHT/Pa (AUC=1) and ESL/Pa (AUC=1). It can be assumed that different metabolites are differently sensitive to heating temperature.

For further validation, a blinded test was conducted on 55 samples (22 Pa, 11 ESL, and 22 UHT). Nonparametric discrimination analysis yielded a 96.4% classification accuracy, confirming that the selected biomarkers and discriminating model were reliable. All Pa milk and UHT milk samples were correctly classified. However, two ESL milk samples were misclassified as UHT milk. This discrepancy occurred because one of the misclassified ESL samples was sterilized via steam injection, a process that yielded a metabolic profile closely resembling that of UHT milk. The other ESL sample had ambiguously labeled brand information. To improve classification accuracy, future work will involve expanding the sample size to enhance model training.

3.4 Elucidation of biomarker mechanisms

Different heat processing technologies for raw liquid milk are regarded as efficient ways to stabilize or preserve samples and protect their nutritional properties[29]. In this study, ten metabolites, mainly nicotinamide and peptides, were identified, which can be effectively used to distinguish different heating temperature processes, especially pasteurized milk at different temperatures (72℃, 75℃, and 85℃).

3.4.1 Peptides

Milk protein structures such as denaturation, aggregation, and degradation are highly susceptible to heating during sterilization[13]. Thus, highly specific and sensitive peptides have great potential for monitoring sterilization during milk processing. In our study, six peptides were characterized, and their contents were significantly different in UHT, ESL, and Pa milk. Notably, the peptides from various sources exhibited individual differences at different heating temperatures.

MPIQAFLLYQEPVLGPVRGPFPIIV (m/z=937.8706 and 932.1967) originated from the C-terminal release of β-casein (Fig. 2). The peptide with m/z = 937.8706 was modified by oxidation at position 16 (P) and

deamidation at position 18 (R) compared to the unmodified peptide with m/z 932.1967. The semi-qualitative results showed that the two peptides had similar changing trends in the three milks, their average contents in ESL and UHT milk were significantly increased by 12.30, 12.66 and 33.87, 36.97 times compared with Pa milk, respectively. Further research found that the change was not significant (p>0.05) in the three pasteurization processes (72 °C, 75 °C, and 85 °C) (Fig. 3). Therefore, it was speculated that the release and degradation temperature was more than 85℃, and the rating was faster with the increase of heating temperature (Table 2). In addition, QEPVLGPVRGPFPIIV (m/z=850.9941) and PVLGPVRGPFPIIV(m/z=730.9518) were also derived from the C-terminal of β-casein (Fig. 2), and there were significant differences in UHT, ESL and Pa milk (Table 2). Among them, QEPVLGPVRGPFPIIV was identified as a strong antioxidant peptide and a weak ACE inhibitor by Eisele et al.[30]. The average content of PVLGPVRGPFPIIV in 85℃ was significantly higher than that in 72 °C and 75 °C milk (p<0.01), suggesting that it was released between 75 °C and 85 °C. Therefore, the PVLGPVRGPFPIIV can be considered a better biomarker for the identification of lower temperature processes (<85 °C) in comparison with MPIQAFLLYQEPVLGPVRGPFPIIV and QEPVLGPVRGPFPIIV (Fig. 3). The cleavage site (D_{184}-M_{186}) and (Y_{193}-Q_{194}) of MPIQAFLLYQEPVLGPVRGPFPIIV and QEPVLGPVRGPFPIIV are related to hydrolysis by cathepsin B and *Pseudomonas fluorescens*, respectively[13, 31]. This suggests that, to some extent, heating can affect protein cleavage by promoting enzyme activity; however, the rate of the enzymatic reaction needs to be investigated in the future. Overall, these peptides can be used not only as indicators to monitor processing temperature but also to characterize the residual activity of endogenous and exogenous proteases after heating.

Apart from β-casein, the peptides PR (m/z=254.1614) and SEKTTMPLW (m/z=546.7726) were derived from the C-terminal and N-terminal of α_{S1}-casein, respectively (Fig. 2). Their contents significantly increased with an increase in sterilization temperature among the three types of milk (Fig. 3Fig**. 3**. These peptides have been reported to correlate exclusively with heating temperature rather than with storage time[13, 32], which is consistent with our findings. Similar to PVLGPVRGPFPIIV, the relative content of RP in 85 °C was significantly higher than that in 72 °C and 75 °C milk (P < 0.01), suggesting that it can be used to monitor lower heat loading. Although six peptide markers were identified in this study, the experimental pre-treatment and instrumental methods were not specific to the peptide profiles. Therefore, a more comprehensive peptidomics approach should be implemented in future studies.

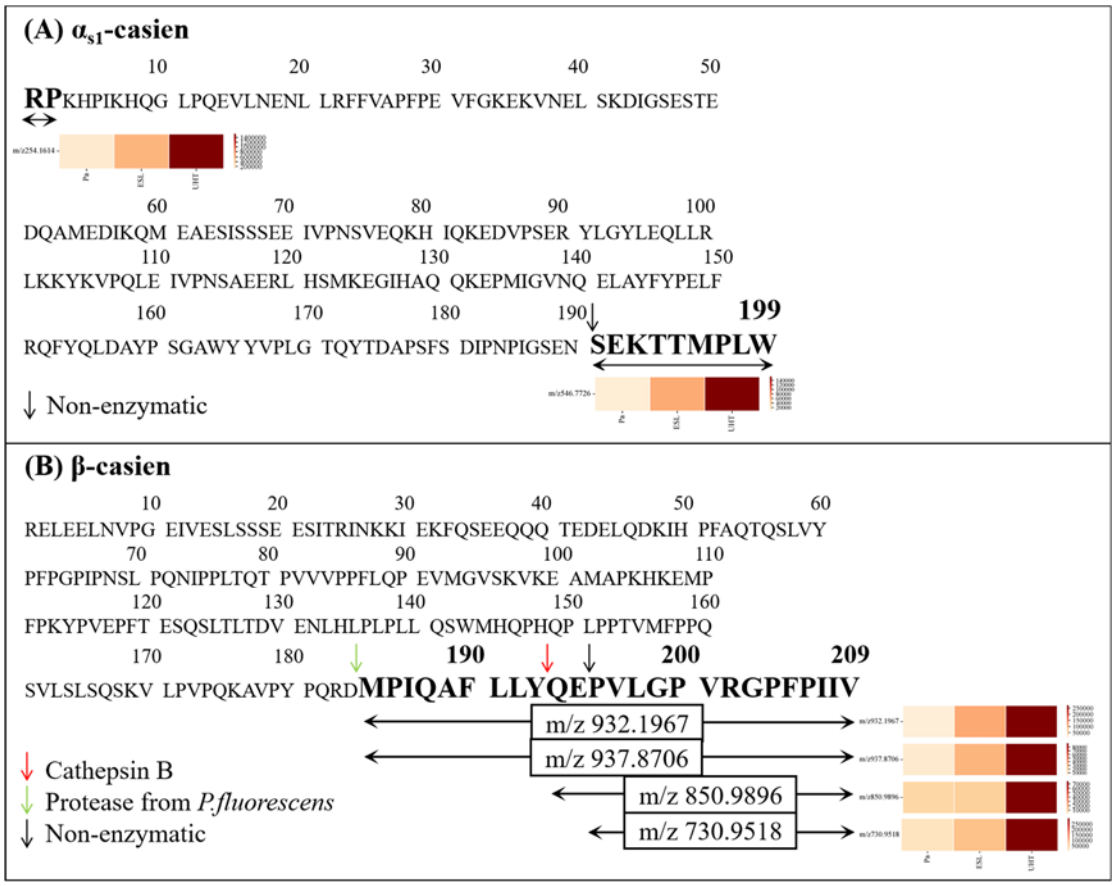

Fig. 2 Differential peptide sequences from α_{s1}-casein (A) and β-casein (B)

Table 2 Qualitative results of different marker peptides in milk heated at different temperatures

	Parent ion (m/z)	Sequence	Precursor protein	Modification	Position	Fold Change 72 °C	75 °C /72 °C	85 °C/72 °C	121 °C/72°C	137 °C/72°C
1	254.1617	RP	αs1-casein	/	1–2	1.00	0.83ns	1.26**	5.87**	16.80**
2	546.7726	SEKTTMPLW	αs1-casein	/	191–199	1.00	0.88ns	0.81ns	6.82**	26.31**
3	730.9518	PVLGPVRGPFPIIV	β-casein	/	196–209	1.00	0.71ns	2.96**	91.67**	361.96**
4	850.9896	QEPVLGPVRGPFPIIV	β-casein	/	194–209	1.00	0.85ns	1.22ns	3.05*	41.06**
5	932.1967	MPIQAFLLYQEPVLGPVRGPFPIIV	β-casein	/	185–209	1.00	0.96ns	1.37ns	13.74**	38.52**
6	937.8706	MPIQAFLLYQEPVLGPVRGPFPIIV	β-casein	Oxidated(P)@16 Deamidated(R)@18	185–209	1.00	1.03ns	1.50ns	14.97**	42.18**

$P > 0.05$ not significant (ns); P 0.01~0.05 *; $P < 0.01$**; The fold changes were normalized by the content of marker at 72 °C

3.4.2 Nicotinamide and their derivatives

In addition to peptides, other metabolites can be used to distinguish between different heating processes in commercial milk. As shown in Fig. 3, the nicotinamide and NR contents decreased significantly with increasing temperature. The nicotinamide content was reduced by 56.58% and 99.18% in ESL and UHT milk compared with Pa milk, respectively, and the NR content decreased by 59.53% and 90.95%, respectively. The

results indicated that nicotinamide and NR were sensitive to heat loading, but were almost completely lost after UHT processing. Although nicotinamide has been reported as a heat treatment indicator[21], there is still a lack of research on NR. Ummarino et al.[33] found that NR was degraded after 5 min at 95 °C but was stable at 75 °C in aqueous solution. To investigate whether similar results could be obtained in milk, the candidates were monitored in the 72 °C, 75 °C, 85 °C, 121 °C, and 135 °C milk samples. It was noteworthy that NR was not significantly different ($P > 0.05$) in the two pasteurized milk varieties (72 °C and 75 °C), whereas the content of nicotinamide decreased significantly at 75 °C ($P < 0.05$), suggesting that NR showed higher heat resistance in Pa milk than nicotinamide (Fig. 3A and C). Similar result was exhibited by 85 °C and 121 °C milk samples (Fig. 3A and C). It indicated that the nicotinamide had a lower initial decomposition temperature than NR, and that NR decomposition temperature range from 75 °C to 85 °C in milk. Therefore, nicotinamide and NR have been proposed as indicators to distinguish between different categories of heat-treated milk; however, nicotinamide could be used as a biomarker to identify lower sterilization temperature processes than NR.

Nicotinamide and NR are the amidated and pyridine nucleoside forms of vitamin B_3 respectively, which are widely present in daily products. Nicotinamide and NR participate in synthesis and catabolism[33]. It was found that about 60% of the nicotinamide adenine dinucleotide (NAD^+) precursor vitamin existed in the form of nicotinamide, while about 40% in the form of NR in milk[28]. In this study, the nicotinamide and NR contents in Pa milk were higher than those in ESL and UHT milk. In addition, the nicotinamide content decreased significantly in reconstituted milk compared to that in UHT milk because of the higher spray-drying temperature in our previous study[21]. In conclusion, these results imply that heating causes a significant loss of nicotinamide and NR in dairy products.

3.4.3 N^6-methyladenosine

As one of the products of ribonucleosides modification, N^6-methyladenosine (m^6Ado) is derived from the *Dimroth*-rearrangement of N^1-methyladenosine(m^1Ado) in heat-treated fluid milk[34, 35]. In this study, the m^6Ado content exhibited marked changes with increasing heating temperatures; the relative content increased by 10.70 times and 43.93 times in ESL and UHT milk compared with Pa milk, respectively. Meanwhile, there was no significant difference in the content of m^6Ado between 72 °C, and 75 °C, but a significant increase was observed at 85 °C (Fig. 3D). It could be interpreted as the reaction rate constants of the *Dimroth*-rearrangement were positively correlated with the heating loading (above 85 °C). This result is consistent with the *Dimroth*-rearrangement of m^1Ado to m^6Ado following first-order kinetics under thermal processing[36], but the detailed kinetic process still needs to be further explored. In contrast, the *Dimroth*-rearrangement has been shown to be resistant to enzymatic degradation (adenosine deaminase and alkaline phosphatase) in milk[36]. Thus, m^6Ado can be considered as an indicator for thermal processing above 85 °C.

3.4.4 2-hydroxycapric acid

2-hydroxycapric acid is derived from the mild oxidation of polyunsaturated fatty acids (PUFA), which are heat-unstable and easily oxidized[37]. Heat leading results in further lipid oxidation reactions and an increase in hexanal, pentanal, and malondialdehyde (MDA), which are markers of lipid oxidation[18, 38]. In our study, the average content of 2-hydroxycapric acid increased significantly with the increase of sterilization temperature, which was increased by 2.49 (ESL) and 37.09 (UHT) times compared with Pa milk, respectively. The results showed that the content in UHT milk was 73.85 times that in Pa milk. Therefore, 2-hydroxycapric acid can be used as an effective indicator to differentiate between UHT and Pa milk. Interestingly, the content of 2-hydroxycapric acid was not significantly different in Pa milk at three different temperatures (72 °C, 75 °C, and 85 °C) (Fig. 3J). It can be deduced that the temperature of 2-hydroxycapric acid generation was more than 85 °C. A previous study by our group found that the 2-hydroxycapric acid content in UHT milk was significantly higher than that in reconstituted milk[21]. Zhang et al. [39] also found significant differences in the number of seven types of oxidized lipids in UHT, raw, and Pa milk, proving that oxidized lipids are sensitive to heat. These results proved that higher heat loading could accelerate lipid oxidation and 2-hydroxycapric acid as markers to differentiate Pa, ESL, and UHT milk. More detailed oxidized lipidomic studies should be conducted in the future.

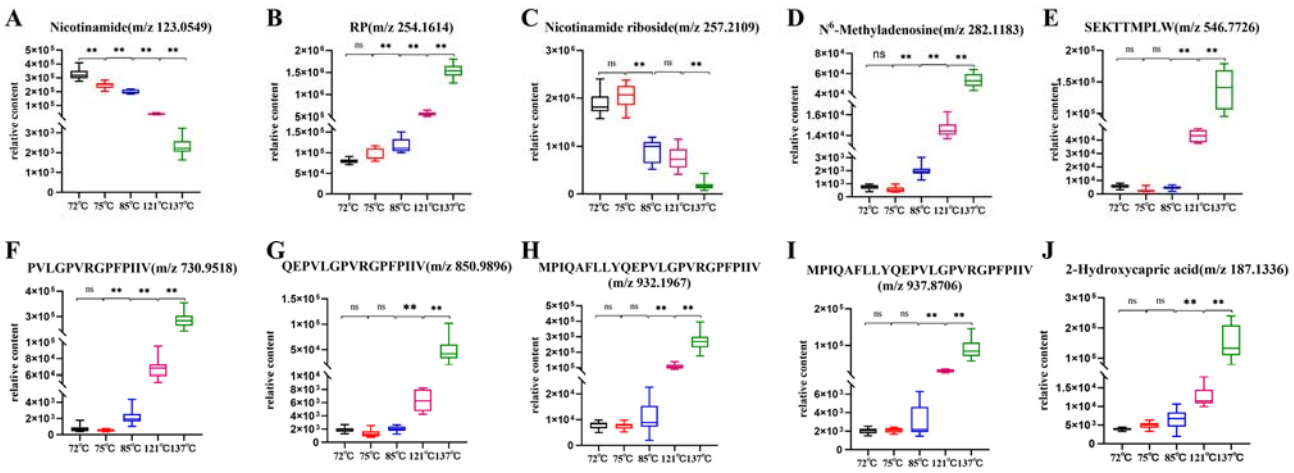

Fig. 3 Relative contents of 10 biomarkers at different heat treatment temperatures (72 °C, 75 °C, 85 °C, 121 °C, and 137 °C). ($P > 0.05$ not significant (ns); p 0.01~0.05 *; $P < 0.01$**)

4. Conclusion

In this study, a metabolomic technology based on UPLC-QTOF-MS and chemometrics was used to analyze three different heat-processed commercial milk samples: pasteurized, ESL, and UHT milk. A total of 22 different compounds were selected as feature metabolites to differentiate the three types of milk, of which 10 were characterized as peptides, nicotinamide, nicotinamide riboside, N^6-methyladenosin, and 2-hydroxycapric acid. Finally, 55 (22 UHT, 11 ESL, and 22 Pa milk) unknown milk samples were predicted correctly, and the accuracy reached 96.4% based on the ROC curves and non-parametric discrimination in the three types of milk. Notably, nicotinamide can be an excellent biomarker to characterize low-intensity thermal process (above 72 °C), while peptides RP and PVLGPVRGPFPIIV can be characterized at thermal process

above 85 °C. In conclusion, metabolomic technology was used to analyze the metabolites of different processing milks, and the 10 biomarkers could successfully distinguish different thermal processing intensities in commercial milk, providing effective biomarkers for the identification of different processing and adulterated milks. Importantly, the pseudo-targeted metabolomics approach can be directly applied for commercial detection. In future studies, we will further investigate the effects of microbial activity and packaging methods on biomarkers and establish a targeted metabolomic approach.

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Declarations of Competing Interests

The authors declare no competing financial interests.

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