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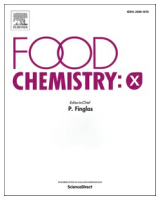

Unraveling the relationships between processing conditions and PhIP formation in chemical model system and roast pork patty via principal component analysis

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ABSTRACT

2-amino-1-methyl-6-phenylimidazole [4,5-b] pyridine (PhIP) is one of the higher levels of HAAs produced in protein foods during heating. The effects of heating temperature, time, and concentration of precursors on PhIP and related substances in the chemical model system and roast pork patty were studied using HPLC-Q-Orbitrap-HRMS and GC–MS. Results showed that the heating temperature, time, and concentration of four precursors significantly affected PhIP and its related substances ($P < 0.05$) in the chemical model system. Among them, PhIP production was greatest when heating at 200 min with 220 °C, and the concentrations of phenylalanine, creatinine, glucose, and creatine added were 10, 20, 20, and 20 mmol/L, respectively. Moreover, as the fat proportion of roast pork patties increased, PhIP and its intermediate-phenylacetaldehyde concentrations increased substantially ($P < 0.05$). PCA results showed that the samples of PhIP and related substances gradually dispersed as the temperature and time increased, and there were obvious effects among them.

1. Introduction

Heterocyclic aromatic amines (HAAs), a major category of carcinogenic and mutagenic substances, are generated while cooking protein-rich food materials derived from animals, including meat and fish (Wang et al., 2021). More than 30 HAAs have been identified and classified from food, and they are frequently present in fried and grilled animal items. Most HAAs found in processed foods are 2-amino-1-methyl-6-phenylimidazole [4,5-b] pyridine (PhIP), which is mainly formed by creatine, sugars, amino acids, or some nitrogenous bases and nucleotides. As a mutagen capable of binding human DNA and a potential animal carcinogen, PhIP has garnered considerable attention (Xu et al., 2023; Dong, Xian, Li, Bai, & Zeng, 2020; Yan et al., 2014.

At present, the formation path of PhIP is relatively clear (Fig. S1). It is proved by ^{13}C labeling that the benzene ring of phenylalanine is a constituent of PhIP, whereas the imidazole ring is derived from creatinine (Hidalgo & Zamora, 2022; Murkovic, Weber, Geiszler, Fröhlich, & Pfannhauser, 1999). Moreover, creatinine can produce phenylacetaldehyde, which is the byproduct of phenylalanine pyrolysis. After butyraldehyde products are produced by aldol condensation, butyraldehyde dehydration products are produced by dehydration (Hidalgo & Zamora, 2022). In this process, the formation of phenylacetaldehyde plays a crucial role, and phenylacetaldehyde and creatinine condensation are essential steps in forming PhIP.

The creation of PhIP is influenced by various elements, such as processing conditions, the kind of food, precursors, intermediates, and exogenous additives (Zhao et al., 2020). Among them, temperature and hot working time are the two most closely related parameters in the production of PhIP (Yan et al., 2021). In general, as the processing time duration increases, PhIP is gradually produced, eventually achieving the highest level of PhIP content. For example, a previous study (Hui et al., 2021) used the glucose/creatine/creatinine model to examine the development of PhIP at different processing times (10–90 min). The results showed considerable variances in the creation of PhIP, and the

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Table 1
Process conditions of the chemical model system.

Groups	Parameter					
	Heat temperature	Time	Glucose	Creatine	Creatinine	Phenylalanine
A	140 °C 160 °C 180 °C 200 °C 220 °C 240 °C	200 min	36 mg	52.5 mg	45.24 mg	66.08 mg
B	220 °C	100 min 120 min 150 min 180 min 200 min 220 min	36 mg	52.5 mg	45.24 mg	66.08 mg
C	220 °C	200 min	36 mg 72 mg 108 mg 144 mg 180 mg	52.5 mg	45.24 mg	66.08 mg
			36 mg	26.23 mg 52.5 mg 78.68 mg 104.9 mg 131.133 mg	45.24 mg	66.08 mg
			36 mg	52.5 mg	22.62 mg 45.24 mg 67.86 mg 90.48 mg 113.1 mg	66.08 mg
			36 mg	52.5 mg	45.24 mg	33.038 mg 66.08 mg 99.114 mg 132.152 mg 165.19 mg

content of PhIP reached 10 ng/mL after 90 min of processing. Furthermore, significant variations can be seen in the development of PhIP under various processing temperatures. Balogh et al. (Balogh, Gray, Gomaa, & Booren, 2000) observed that PhIP generated in meat products is almost undetectable at temperatures below 150 °C. However, samples processed at temperatures higher than 190 °C significantly increased PhIP content. These researches identified the influences of processing conditions (heating temperature and time) on PhIP in complex meat systems, recognizing them as crucial variables in the generation of PhIP in actual meat products (Wang et al., 2021). However, the research on the influence of multi-coupling process parameters such as temperature and time on PhIP generation is currently limited.

HPLC Q-Orbitrap-HRMS is suggested for the detection of known and unknown substances in diverse food types because of its benefits, which include excellent resolution, the ability to discriminate false positives from false negatives, fast speed determination, and good quantitative performance (Dong et al., 2020). However, HPLC Q-Orbitrap-HRMS analyses of HAAs produced in meat products need to be more frequently reported. Furthermore, a published method is lacking for detecting PhIP, its precursors, and intermediates. Therefore, a new HPLC Q-Orbitrap-HRMS method for the detection of PhIP and its related substances was established in our laboratory (Xu et al., 2021; Xu et al., 2023).

This study investigated the influence of different hot processing conditions (heating temperature, time, and precursors) on PhIP and related substances in the chemical model system and roast pork patties. The main influencing factors and rules for inhibiting the formation of PhIP were also revealed. Furthermore, the relationships between PhIP and its precursors and intermediates were investigated using multivariate statistical analysis technology. The influence rules of altered precursors, important intermediates, and amino acids in roast pork were also analyzed and determined. In contrast to other research on individual influences on the formation of PhIP, this study can provide a more precise depiction of PhIP formation by considering PhIP, precursors, and intermediates identified in authentic meat samples collectively as a comprehensive profile. In addition, this study aimed to establish a scientific and theoretical foundation for comprehending the process and implementing effective measures for controlling PhIP.

2. Materials and methods

2.1. Chemicals and reagents

PhIP standard was acquired from Toronto Research Chemicals (North York, Ontario, Canada). Glucose, creatinine, creatine, phenylalanine, and phenylacetaldehyde were obtained from Yuanye Biotechnology (Shanghai, China), of which the purity is over 98%. Methanol, acetonitrile, and formic acid were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), with a purity of 99.8%.

2.2. Chemical model system

2.2.1. Establishment of the chemical model system

The chemical model system was established using previous papers published in the laboratory (Xu et al., 2023). A 20 mL solution of diethylene glycol, with a water content of 50%, was injected into a reaction that contained glucose (0.2 mmol), phenylalanine (0.4 mmol), creatine (0.4 mmol), and creatinine (0.4 mmol). Then, it is heated at a certain temperature for a while.

2.2.2. Preparation of the precursors and PhIP

The pre-processing process for extracting PhIP, phenylalanine, creatine, and creatinine was as follows: Methanol, ultra-pure water, and a 1% formic acid solution of 3 mL each were added to the reactants of the system for activation and balance of the PCX column. Following the absorbance of 5 mL of the extract by the column, it experienced a series of washings using 1% formic acid, ultra-pure water and methanol of 3

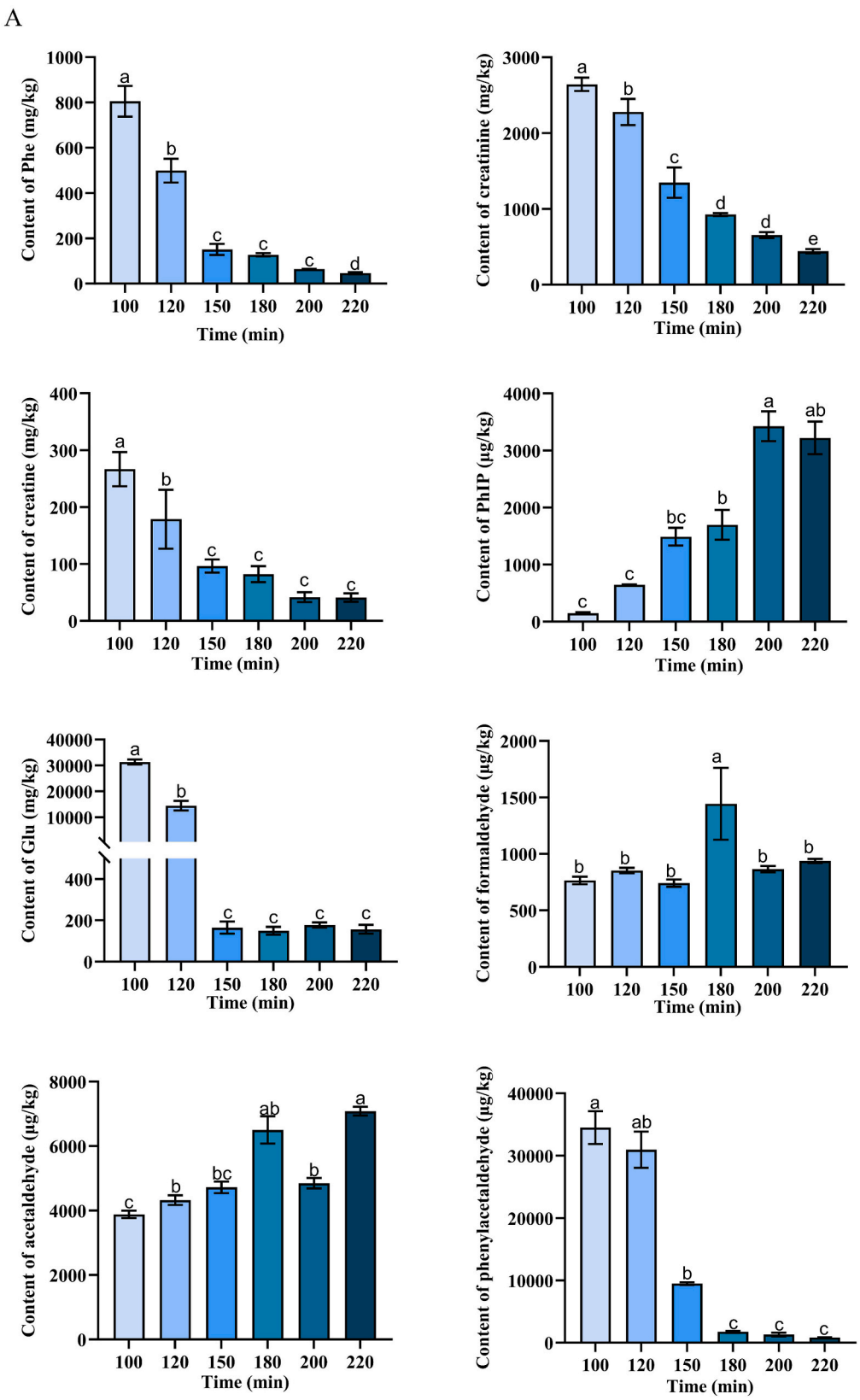


Fig. 1. (A) Effects of processing time on the phenylalanine, creatine, creatinine, glucose, PhIP, formaldehyde, acetaldehydeand, and phenylacetaldehyde contents of chemical model system, respectively ($n = 3$). Significant differences exist between results in the same series that have different lowercase letters ($P < 0.05$).
Fig. 1(B) Effects of processing temperature on the phenylalanine, creatine, creatinine, glucose, PhIP, formaldehyde, acetaldehyde, and phenylacetaldehyde contents of chemical model system, respectively (n = 3). Significant differences exist between results in the same series that have different lowercase letters (P < 0.05).

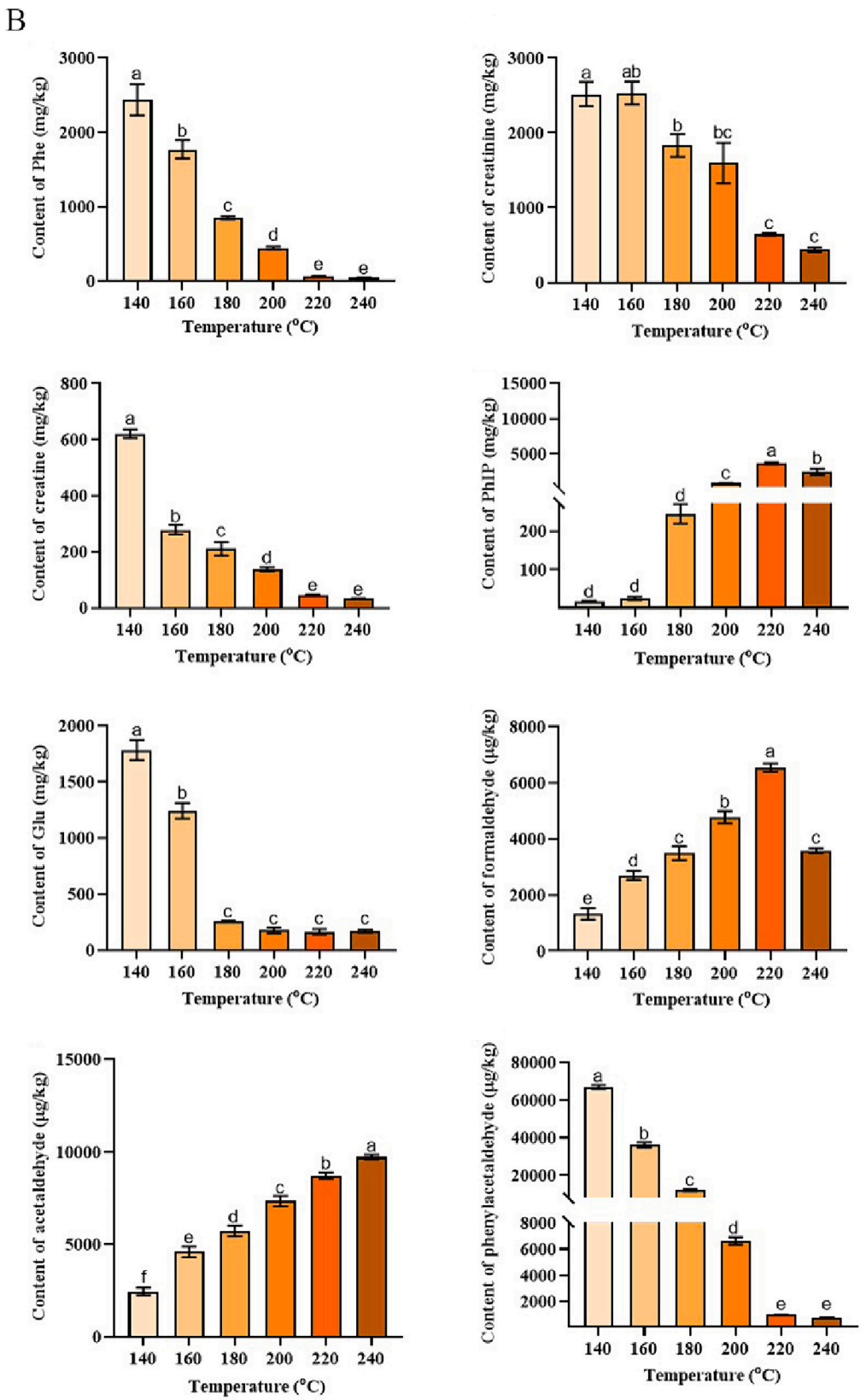


Fig. 1. (*continued*).

mL each. Finally, a methanol-ammonia eluent solution consisting of 4 mL (9:1; V:V) was mixed with 1.0 mL methanol after being re-eluted via nitrogen venting at 50 °C (whirled for 10 s). For determination, the reserve solution was collected using a 0.22 μm membrane.

The pre-processing process for extracting glucose from the chemical model system was as follows: Following the absorbance of 100 μL of the reactant, it was transported to a centrifuge tube. Then, adding 20 mL of the mobile phase, the mixture was vortically mixed. After mixing, the

reserve solution was collected using a 0.22 μm membrane for determination.

2.2.3. Preparation of the intermediates

To determine the content of formaldehyde and acetaldehyde, a derivative solution (2, 4-dinitrophenylhydrazine-acetonitrile solution: phosphate buffer solution =1:1 (V: V)) must be used to prepare. The pre-processing process for extracting formaldehyde and acetaldehyde was as follows: Following the absorbance of 100 μL of the reactant, it was transported to a centrifuge tube. Then, adding 20 mL of the derived solution was vortically mixed and placed in a water bath (60 °C) for 60 min. For determination, the reserve solution was collected using a 0.22 μm membrane.

The pre-processing process for extracting phenylacetaldehyde was as follows: Following the absorbance of 100 μL of the reactant, it was transported to a centrifuge tube. Then, adding 20 mL of n-hexane was vortically mixed. For determination, the reserve solution was collected using a 0.22 μm membrane.

2.2.4. Effect of precursors and processing conditions on the chemical model systems

The chemical model systems were divided into three groups: different heating temperatures (group A), different times (group B), and precursors with different concentrations (group C) to study their effects on the contents of PhIP and related substances. The specific parameter settings are shown in Table 1.

2.3. Roast pork patty system

2.3.1. Establishment of the roast pork patty system

The pork was offered from a Carrefour supermarket (Guangzhou, China). The visible fascia in the pork meat was removed and placed in a meat grinder to prepare minced meat. The minced meat was used in a round patty measuring 1 cm thick and 6 cm in diameter. Three meat patties were distributed to each group, and the experiment was conducted in triplicate. Then, the contents of PhIP, precursors, and intermediates were determined at different heating temperatures and times.

2.3.2. Preparation of the precursors and PhIP

The pre-processing process for extracting PhIP, phenylalanine, creatine, and creatinine from the roast pork patty system was as follows: the 0.1 g roast pork was transferred to a centrifuge tube. After adding 10 mL of diethylene glycol: water (1:1; V:V) was vortically mixed, ultrasound and centrifuged for 5 min each. Proceed through the previous steps once more. The column was then activated and balanced using the approach of 2.2.2. For determination, the reserve solution was collected using a 0.22 μm membrane. The concentrations of the standard solution were 2 g/L, 10 g/L, and 20 g/L.

The pre-processing process for extracting glucose from the roast pork patty system was as follows: the 0.1 g roast pork was transferred to a centrifuge tube. After adding 10 mL of diethylene glycol: water (1:1; V: V) was vortically mixed, ultrasound and centrifuged for 5 min each. Proceed through the previous steps once more. For determination, the reserve solution was collected using a 0.22 μm membrane. The concentrations of the standard solution were 20 μg/L, 100 μg/L, 200 μg/L.

2.3.3. Preparation of the intermediates

The pre-processing process for extracting formaldehyde and acetaldehyde from the roast pork patty system was as follows: the 0.1 g roast pork was transferred to a centrifuge tube. Then, 20 mL of derived solution was vortically mixed, ultrasound for 5 min, and placed in a water bath (60 °C) for 60 min. The concentrations of the standard solution were 20 μg/L, 100 μg/L, 500 μg/L.

The pre-processing process for extracting phenylacetaldehyde from the roast pork patty system was as follows: the 0.1 g roast pork was transferred to a centrifuge tube. After adding 10 mL of n-hexane was vortically mixed, ultrasound and centrifuged for 5 min each. Proceed through the previous steps once more. For determination, the reserve solution was collected using a 0.22 μm membrane. The concentrations of the standard solution were 50 μg/L, 100 μg/L, 200 μg/L.

2.4. HPLC-Q-Orbitrap-HRMS condition

The determination of PhIP, precursors and intermediates was carried out using previous papers published in the laboratory (Xu et al., 2023). The reference retention time, qualitative and quantitative ions of the target analyses are shown in Table S1.

2.5. GC–MS analysis condition

The determination of phenylacetaldehyde was carried out using previous papers published in the laboratory (Xu et al., 2023). The reference retention time, qualitative and quantitative ions of the target analyses are shown in Table S1.

2.6. Statistical analysis

One-way ANOVA was performed utilizing SPSS 21.0 software (IBM Corp., New York, USA). Multiple comparisons were assessed through the implementation of Duncan's method. The value of mean ± SD was obtained from a minimum of three repeated experiments.

3. Results and discussion

3.1. Effect of processing condition on PhIP and related substances in chemical model system

As shown in Fig. 1 (A), the heating time had an impact on the levels of PhIP, precursors, and intermediates. More precisely, the contents of creatine, phenylalanine, creatinine, glucose, and phenylacetaldehyde decreased significantly with the change of heating time in the range of 100–220 min at a constant temperature ($P < 0.05$). However, the glucose content was insignificant in 150–220 min. Similarly, phenylalanine, creatine, and phenylacetaldehyde were not significant at 150-220 min. Compared with phenylalanine, creatine, and phenylacetaldehyde, glucose is more sensitive to heating time and temperature. With the increase in heating time and temperature, the content of these substances gradually decreases and tends to be stable. This conclusion is consistent with the previous results, which may be caused by caramelization and Millard reaction. (Han et al., 2023; Yan et al., 2014). In addition, the highest content of PhIP was observed at 220 °C. When the heating process proceeds, the content of PhIP will trend downward after reaching a particular equilibrium point. This phenomenon occurred that creatine's cyclization product (creatinine) has an impact on the production of Maillard reaction products, including imidazolines and imidazolines formed from 2, 5-dimethylpyrazine and acetaldehyde (Cheng et al., 2023). Furthermore, the decrease in precursor and intermediate contents is primarily attributable to Strecker degradation (decarboxylation and deamination of amino acids) that occurs during the Maillard reaction between phenylalanine and glucose to produce phenylacetaldehyde, a crucial molecule in the reaction with creatinine to form the first intermediate of PhIP (Jinap et al., 2013; Jinap, Hasnol, Sanny, & Jahurul, 2018). Among them, the absence of a discernible trend in the concentrations of formaldehyde and acetaldehyde suggests that their respective concentrations were unrelated to the duration of heating. In the majority of instances, the formaldehyde content did not differ substantially ($P > 0.05$), but the concentration of acetaldehyde varied dramatically at various temperatures ($P < 0.05$).

As shown in Fig. 1 (B), the concentrations of phenylalanine, creatine, creatinine, glucose, and phenylacetaldehyde showed a significant decreasing trend with the increase of temperature ($P < 0.05$) under

A

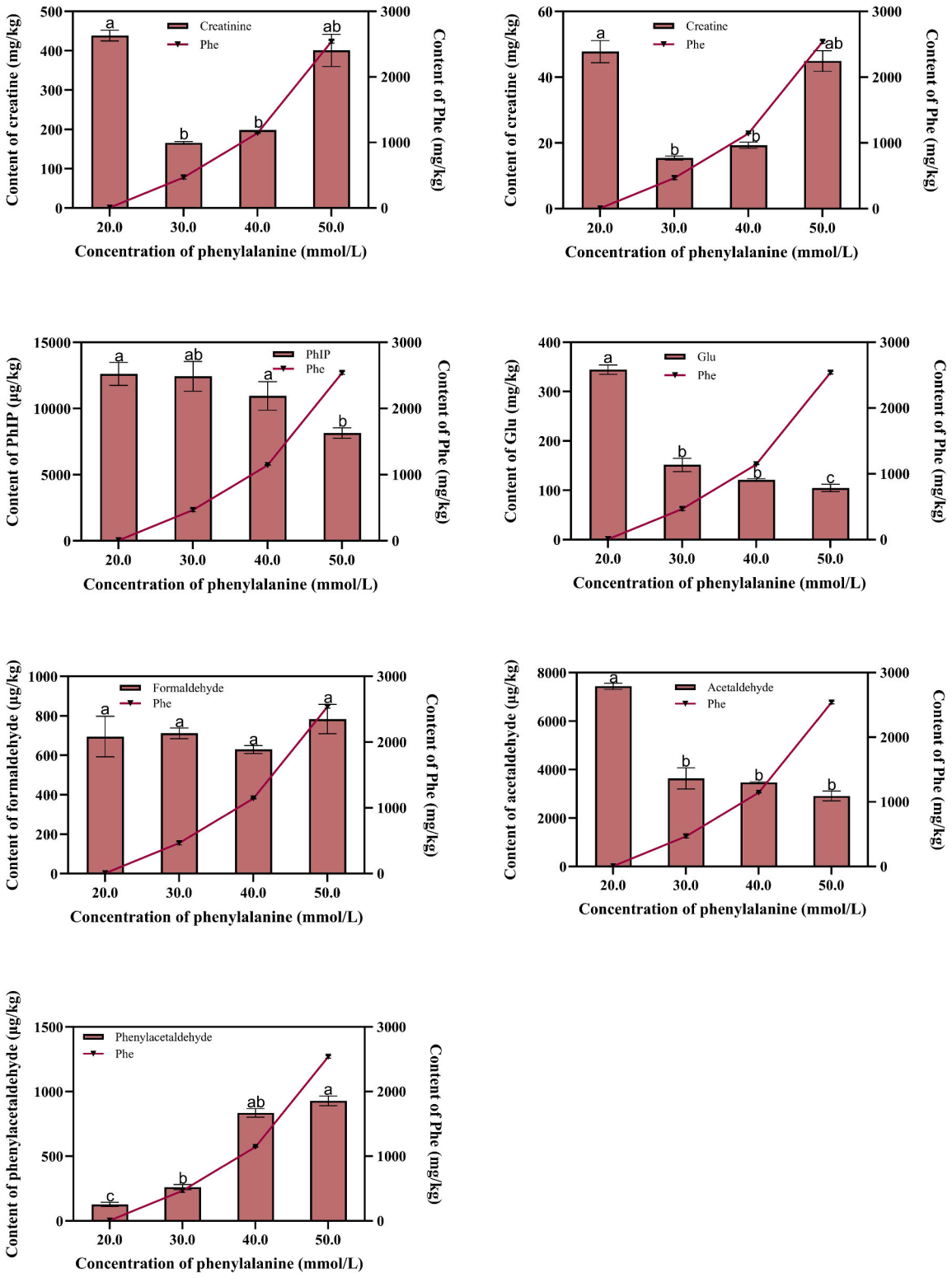


Fig. 2. (A) Effects of adding different phenylalanine contents on the creatine, creatinine, PhIP, glucose, formaldehyde, acetaldehyde, and phenylacetaldehyde contents of chemical model system, respectively (n = 3). Significant differences exist between results in the same series that have different lowercase letters ($P < 0.05$).

Fig. 2(B) Effects of adding different creatinine contents on the phenylalanine, creatine, PhIP, glucose, formaldehyde, acetaldehyde, and phenylacetaldehyde contents of chemical model system, respectively (n = 3). Significant differences exist between results in the same series that have different lowercase letters ($P < 0.05$).

Fig. 2(C) Effects of adding different creatine contents on the phenylalanine, creatinine, PhIP, glucose, formaldehyde, acetaldehyde, and phenylacetaldehyde contents of chemical model system, respectively (n = 3). Significant differences exist between results in the same series that have different lowercase letters ($P < 0.05$).

Fig. 2(D) Effects of adding different glucose contents on the phenylalanine, creatinine, creatine, PhIP, formaldehyde, acetaldehyde, and phenylacetaldehyde contents of chemical model system, respectively (n = 3). Significant differences exist between results in the same series that have different lowercase letters ($P < 0.05$).

B

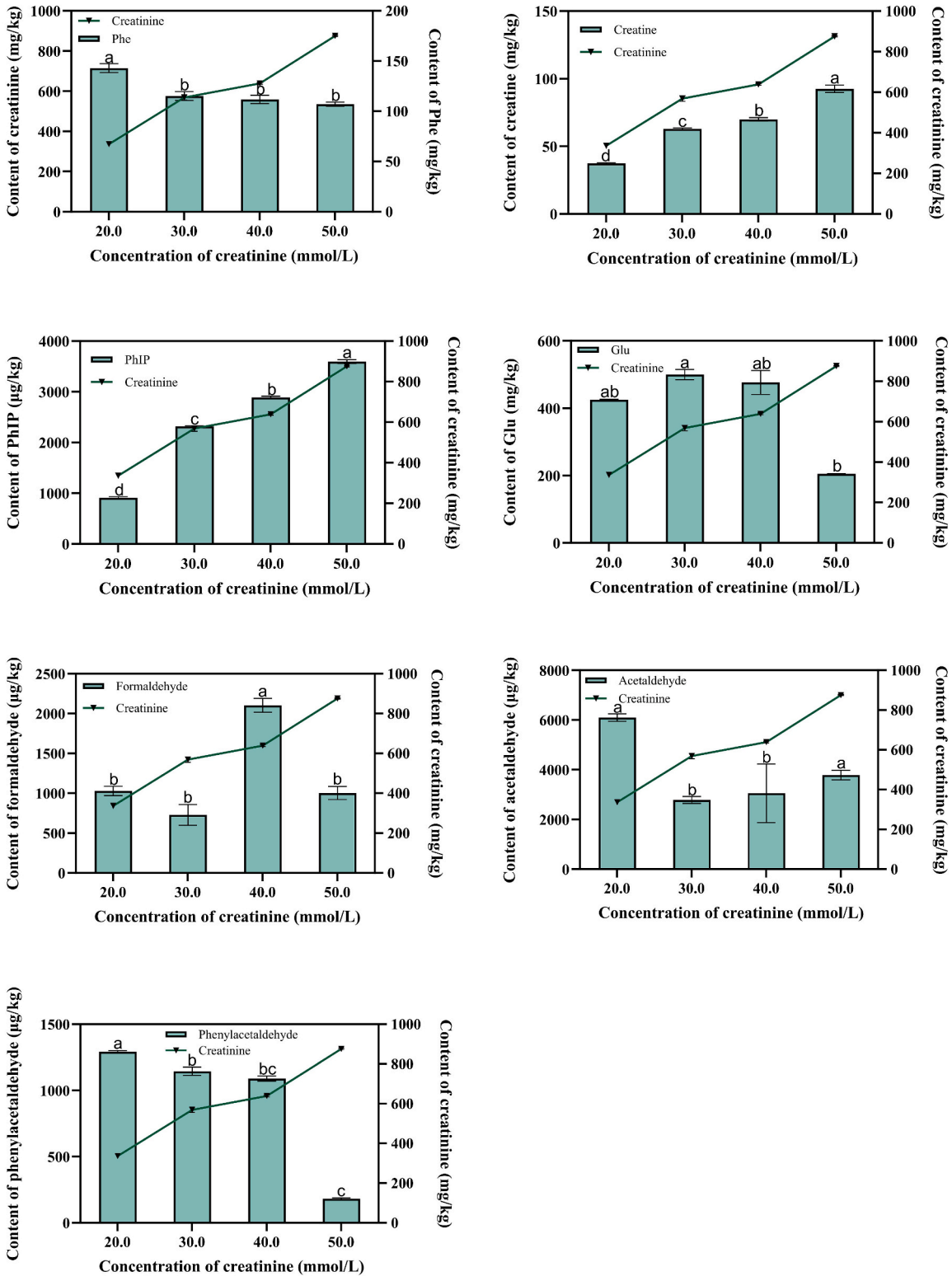
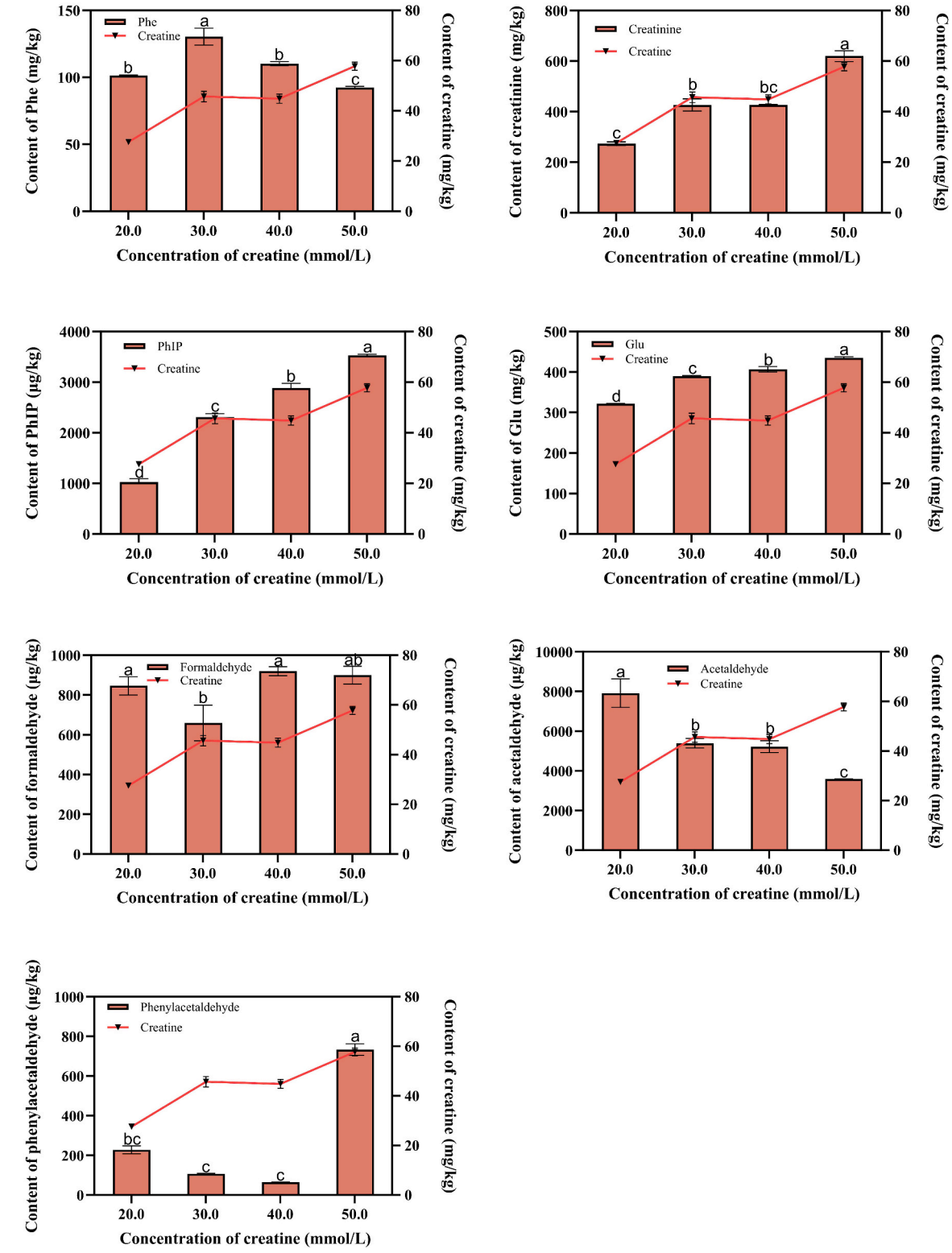


Fig. 2. (*continued*).

different processing temperatures. However, the glucose content was insignificant at 180–240 °C ($P > 0.05$). The content of formaldehyde is the highest at 220 °C, which is consistent with the content of PhIP, indicating that the content of PhIP will have a certain relationship with formaldehyde (Hidalgo, Navarro, & Zamora, 2018). It can also be observed in the figure that acetaldehyde content also increases with the increase in temperature ($P < 0.05$). In conclusion, the change in precursor content correlates with heating time and temperature. Furthermore, an association was observed between the concentration of phenylacetaldehyde and PhIP with heating time and temperature. Specifically, the contents of PhIP were the highest when the temperature was 220 °C and the time was 200 min. However, the change in

C



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formaldehyde and acetaldehyde content was irregular with heating time and temperature. This is consistent with previous findings that PhIP content increases significantly with increasing heating conditions (Wang, Cheng, et al., 2021; Wang, Li, et al., 2021). Gibis et al.(Gibis & Weiss, 2015) found that fried bacon (200–220 °C) had more HAA than the other bacon (150–170 °C). A further factor that significantly contributed to the development of PhIP was the enhanced weight reduction observed in fried roast pork. The moisture content of pork was very low at high frying temperatures and long durations, hence facilitating PhIP generation (Gibis, 2016). This may also be related to the type of meat, and subsequent research on different meats is necessary.

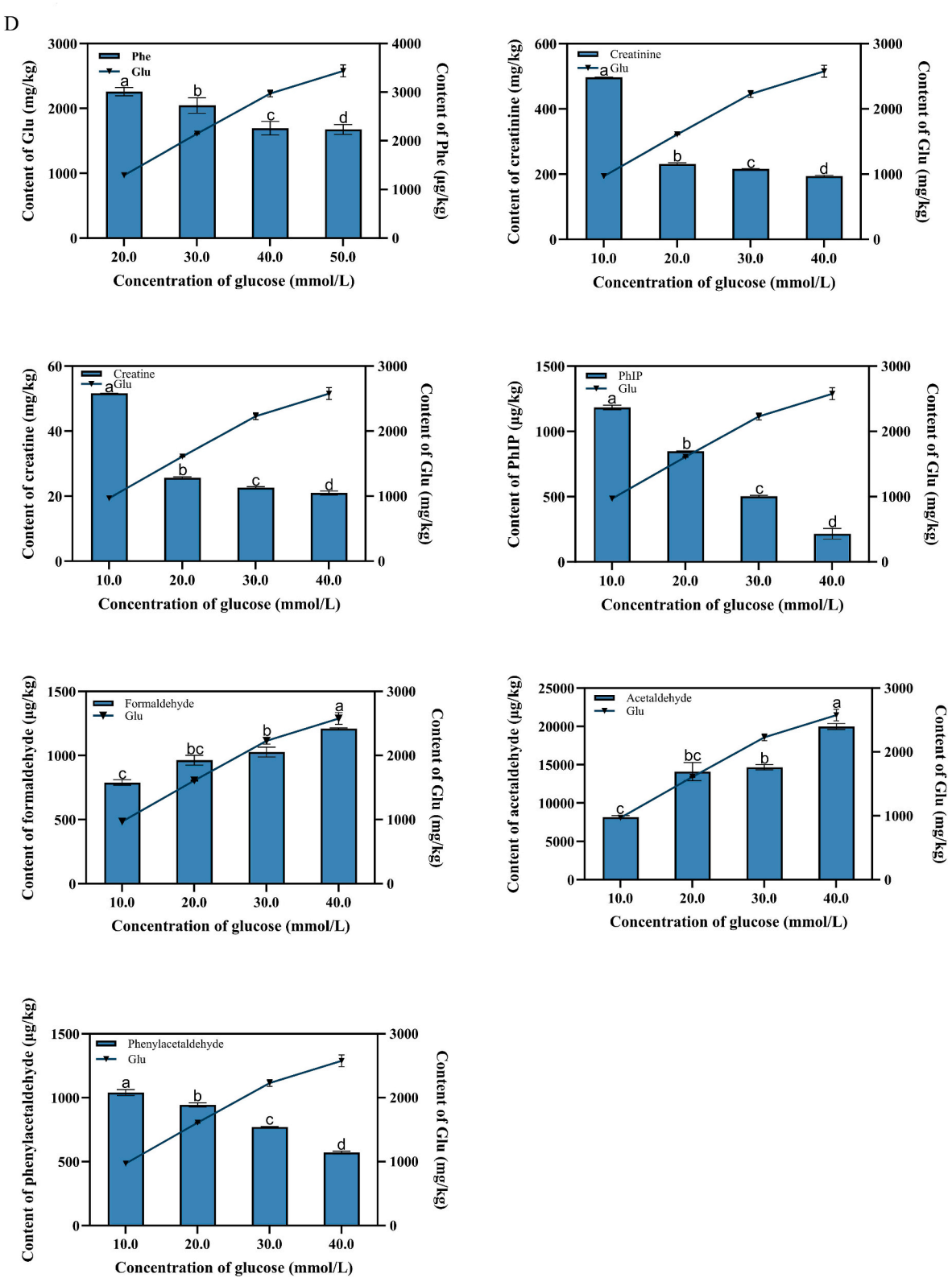

Fig. 2. (*continued*).

3.2. Effect of precursor concentration on PhIP and related substances in chemical model system

3.2.1. Phenylalanine

Regulating the concentration and type of precursors, such as glucose, phenylalanine, creatinine, and creatine, could inhibit the formation of PhIP. As shown in Fig. 2, the concentration of precursor in the chemical model system has an influence on PhIP and its associated substances. Fig. 2 (A) showed that the increased phenylalanine concentration resulted in a rise in the production of phenylacetaldehyde by Strecker degradation, but the concentration of PhIP declined. This phenomenon could be attributed to the equilibrium point of phenylalanine at 20

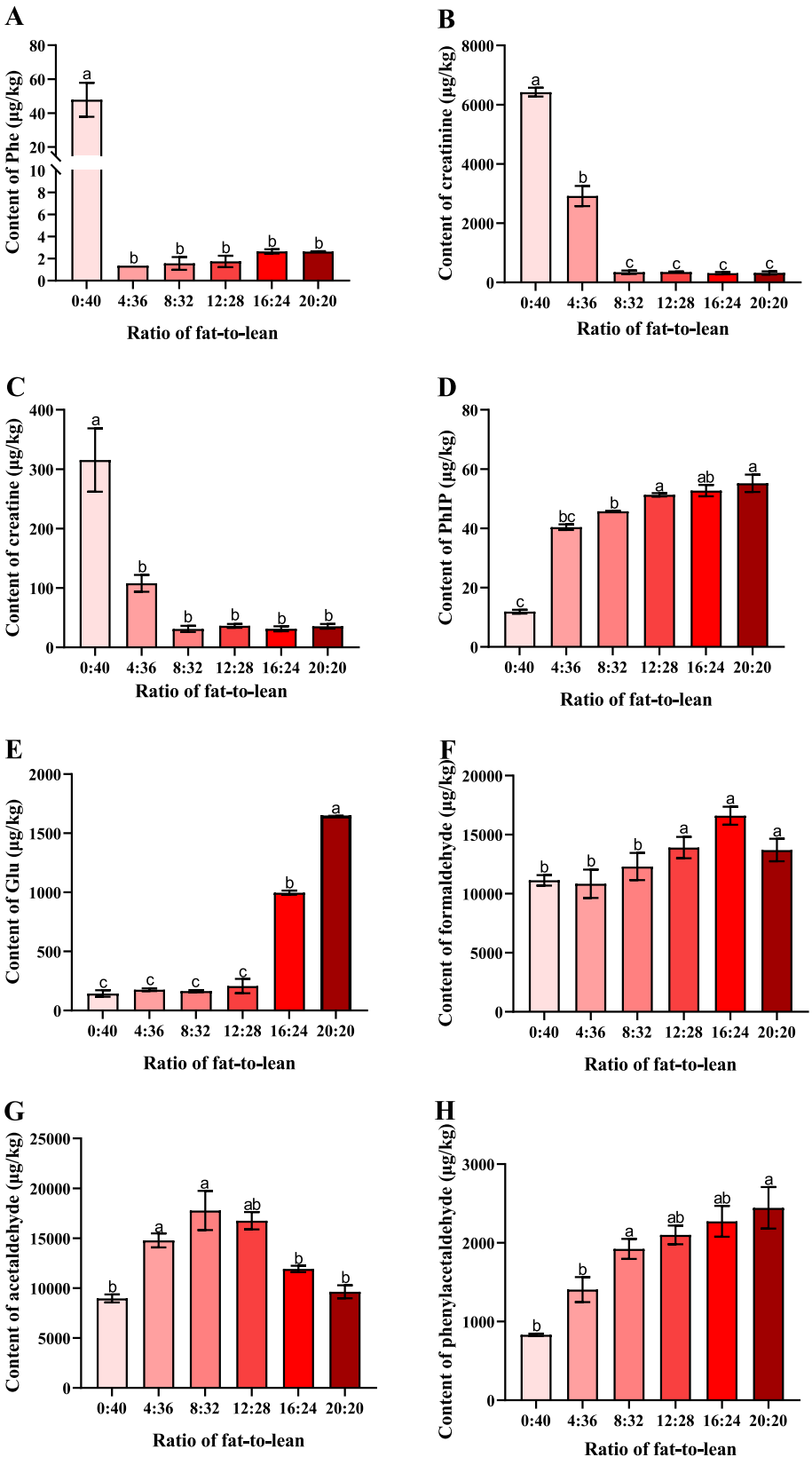

Fig. 3. Effects of fat-to-lean ratio (0:40, 4:36, 8:32, 12:28, 16:24, 20:20) on the phenylalanine (A), creatine (B), creatinine (C), glucose (D), PhIP (E), formaldehyde (F), acetaldehyde (G) and phenylacetaldehyde (H) contents of chemical model system, respectively (n = 3). Significant differences exist between results in the same series that have different lowercase letters ($P < 0.05$).

mmol/L, wherein both production and consumption are precisely equivalent. After this equilibrium point was exceeded, the amount of the initial reactant became less, and the amount of the required molecule decreased. At the same time, a portion of phenylalanine failed to react entirely and thus reacted with creatinine to form adducts (Delgado, Zamora, & Hidalgo, 2015). In addition, creatine and creatinine also showed the same trend at different phenylalanine concentrations. It can be considered that creatine cycled into creatinine without any reaction with other compounds (Jiang et al., 2022). Creatinine and creatinine were both at their highest point when the concentration was 20 mmol/L. The Maillard reaction is triggered by the heating of glucose, resulting in the formation of different carbonyl substances that facilitate PhIP production. Previous studies showed that glucose significantly affected the generation of PhIP. PhIP formation can be inhibited when its concentration exceeds that of creatine. This may be because excess glucose mainly generates 5-hydroxymethyl-2-furan aldehyde during the Maillard reaction, thereby impeding PhIP formation in the creatinine reaction (Gibis & Weiss, 2015; Jinap et al., 2018). In addition, glucose also plays an auxiliary role in the transformation of phenylalanine into phenylacetaldehyde (Cheng, Yu, Wang, Zhu, & Huang, 2021). By the prior discussion, the glucose content decreases with the addition of phenylalanine.

Formaldehyde and acetaldehyde are primarily produced by lipid oxidation, as previous studies have demonstrated (Zamora, Alcón, & Hidalgo, 2014). However, previous studies showed that formaldehyde is generated through the thermal breakdown of phenylacetaldehyde and the degradation of phenylalanine, phenylethylamine, styrene, and creatinine (Zamora et al., 2014). Furthermore, the PhIP concentration was greatly enhanced by adding formaldehyde to a solution of phenylacetaldehyde and creatinine. Previous research showed that the PhIP yield increased significantly with formaldehyde and ammonia added, while the activation energy of the reaction decreased significantly (Deng et al., 2022). These results suggested that formaldehyde and ammonia are crucial for forming PhIP in the chemical model system. It can also be observed from Fig. 2 (A) that acetaldehyde content decreased as phenylalanine concentration rose, but formaldehyde content was not regularly correlated with phenylalanine concentration. This is because acetaldehyde is generated from lipids and carbohydrates along with other dietary components, whereas formaldehyde is the second carbonyl compound responsible for PhIP formation (Zamora & Hidalgo, 2015).

3.2.2. Creatinine and creatine

As shown in Fig. 2 (B), the contents of phenylalanine and phenylacetaldehyde were decreased by adding different concentrations of creatinine, and the relationship was concentration-dependent. When the addition concentration was 30 to 50 mmol/L, the changing trend of phenylacetaldehyde and glucose content was similar. Moreover, the contents of formaldehyde and acetaldehyde were not consistent with the concentration of creatinine. Creatine and creatinine contents were concentration-dependent and they are also concentration-dependent with PhIP. Therefore, adding creatinine to phenylalanine and phenylacetaldehyde will result in the formation of additional creatinine condensates, aldol condensates, and PhIP (Zhang et al., 2020). In addition, creatine and creatinine contents changed similarly with different concentrations of creatine, as shown in Fig. 2 (C). They were dose-dependent with glucose, PhIP, and acetaldehyde. Furthermore, the concentrations of formaldehyde and creatine were irregular, while the contents of phenylalanine and phenylacetaldehyde showed an opposite trend. Previous studies also found that creatinine considerably affected the production of PhIP. The molecular docking results revealed the formation of hydrogen bonds between creatinine and PhIP in different ratios, namely 1:1 and 2:1. Furthermore, the major amino [N^2-] and sp^2 nitrogen atoms [N^3] of creatinine and PhIP, which serve as active sites for the formation of creatinine-PhIP adducts, coincide with active sites of PhIP metabolism and PhIP/lipid-derived active carbonyl adducts. This demonstrated that creatinine inhibits the synthesis of PhIP by forming adducts with hydrogen bonds (N^2 and N^3 sites) (Yu et al., 2018).

3.2.3. Glucose

As illustrated in Fig. 2 (D), the largest concentrations of PhIP and phenylacetaldehyde were found when glucose was 10 mmol/L and creatine, creatinine, and phenylalanine were 20 mmol/L. In addition, the changing trend of formaldehyde and acetaldehyde content is consistent with that of glucose content with different concentrations. It may be that glucose heating produces harmful substances, accompanied by the production of intermediate products, formaldehyde and acetaldehyde (Zhang et al., 2024). At the same time, the addition of different glucose concentrations showed opposite trends with creatinine, creatine, phenylalanine, phenylacetaldehyde, and PhIP. In addition, the significance of a low glucose concentration in the chemical model system for its contribution to the formation of PhIP was further illustrated. Previous studies have shown that chicken glucose concentrations were comparatively lower than those found in pork or beef, which range from 4 to 16 times greater in glucose concentration (Gibis & Loeffler, 2019). This explains that the prevention of PhIP formation due to raised glucose levels (the molar ratio of total creatine and glucose>0.5) can decrease PhIP content within the model system (Gibis & Loeffler, 2019).

In summary, phenylalanine, phenylacetaldehyde, PhIP, glucose, creatine, and creatinine were basically dose-dependent when different contents of precursors were added to the chemical model system. Among them, when glucose accounts for about half of creatine and phenylalanine, they will significantly increase the probability of PhIP formation. This is because a series of reactions will happen between the precursors to make adducts, which helps PhIP formation.

3.3. Effect of processing conditions on PhIP and related substances in roast pork patty

3.3.1. Fat and lean ratio

Free radicals are produced during lipid oxidation, which helps PhIP develop (Zamora, Alcón, & Hidalgo, 2012). Research has demonstrated that high-active substances interact with different substances, including proteins and amino acids in meat, during the cooking process without influencing lipid oxidation (Huang, Huang, & Dong, 2023). However, because of the differences in the meat patty's ratio of lean to fat exposed to air during baking, the pork patty will result in lipid oxidation. It was discovered that pork patties with different ratios of fat to lean also differed in the quantities of precursors, intermediates, and PhIP (Gibis, 2016; Oz et al., 2023). As shown in Fig. 3, the phenylalanine content in samples with a fat-to-lean ratio of 0:40 was significantly higher than in other samples, indicating that lipid oxidation can degrade phenylalanine (Hidalgo & Zamora, 2016). Studies have shown that lipid oxidation products, like carbohydrates at various stages, were produced by lipid oxidation. Furthermore, it can form adducts with phenylalanine, creatinine, and PhIP, reducing the remaining amounts of phenylalanine and creatinine (Zhang et al., 2020). Phenylalanine can be converted to phenylacetaldehyde via Strecker degradation helped by lipid oxidation products, which could facilitate PhIP formation. Previous studies have found that mild to moderate oxidation of soybean oil contributes to PhIP formation. In contrast, PhIP formation was inhibited by highly oxidized soybean oil in chemical models, and the lipid polymer produced by excessive lipid oxidation did not did not influence PhIP formation (Zamora et al., 2012). After heating treatment, unsaturated fats in food are vulnerable to rapid oxidation and degradation, which generates a range of oxidation byproducts such as reactive carbonyl species (RCS) and lipid hydroperoxides. With the increase of fat content, the oil content after heating increased. Moreover, the concentrations of phenylacetaldehyde and PhIP exhibited a rise, which is consistent with the findings of prior investigations.

It can be observed from Fig. 3(F)(G) that the content of formaldehyde and acetaldehyde in baked pork patty is generally high, which is consistent with the study of the production of intermediate aldehydes in

A

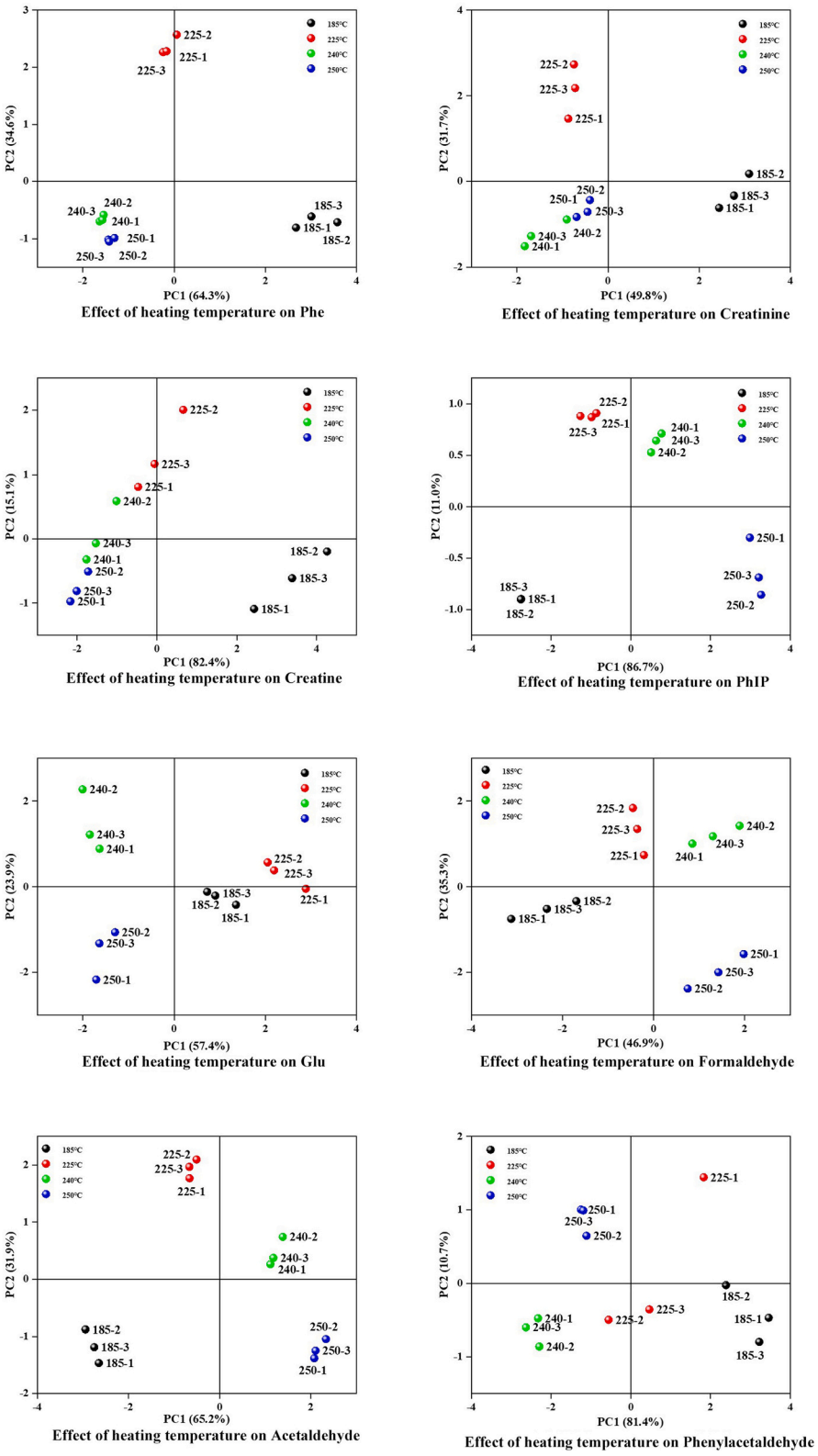

Fig. 4. (A, B) PCA score scatter plot of PhIP, precursors, and intermediates profiles in the roasted pork samples heated at different temperatures (I) (175 °C, 200 °C, 225 °C and 250 °C) for six periods of time (II) (10 min, 15 min, 20 min, 25 min, 30 min, 40 min).
Fig. 4(C) PCA score scatter plot of creatine, creatinine, phenylalanine, PhIP, glucose, formaldehyde, acetaldehyde, and phenylacetaldehyde profiles in the roasted pork patties heated for different temperatures and times.

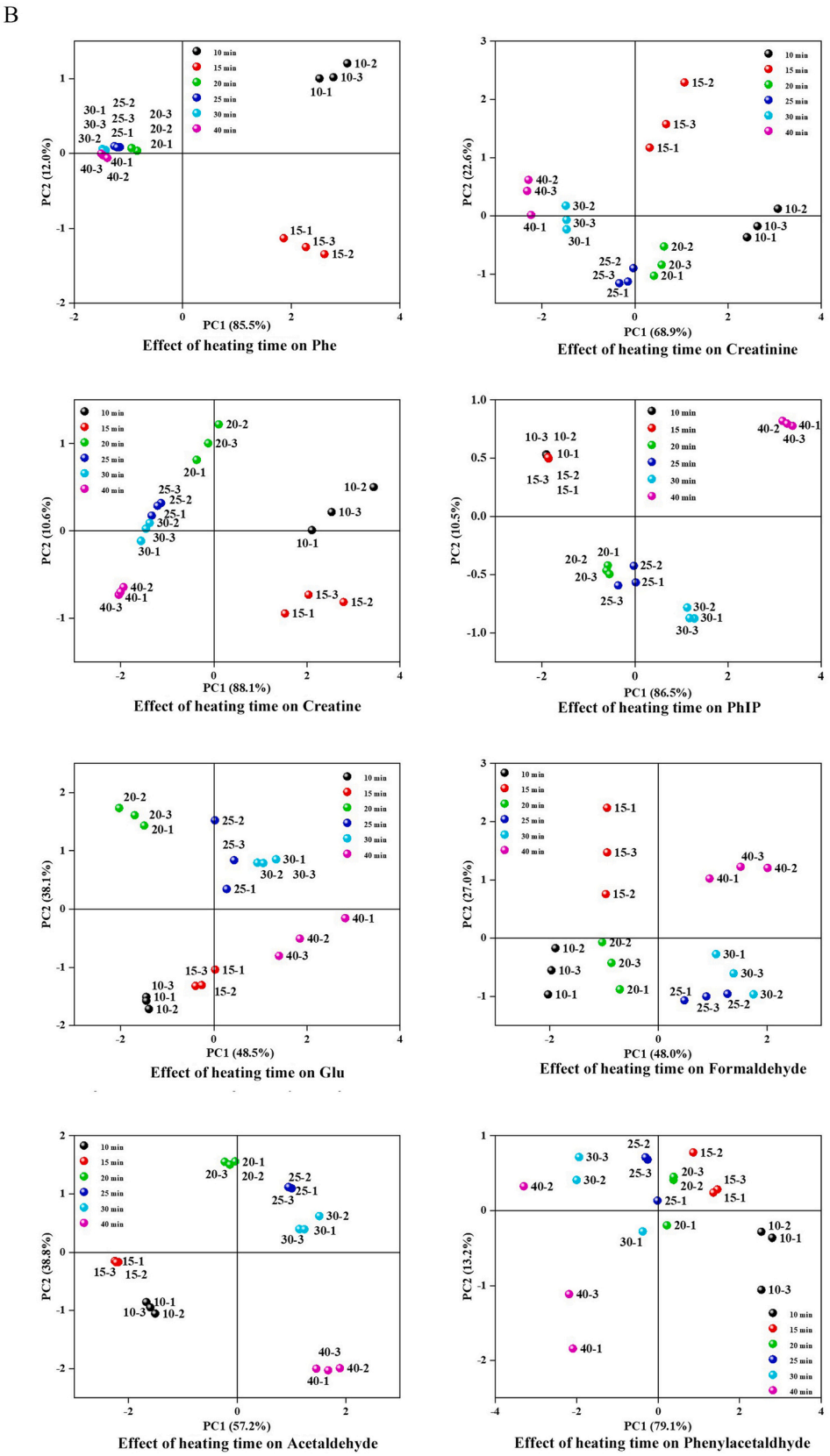

Fig. 4. (*continued*).

the process of lipid oxidation (Zhang et al., 2021). At the same time, it was also shown that acrolein generated via lipid oxidation exhibited a dose-dependent increase in PhIP formation. However, high concentrations of acrolein failed to result in further PhIP formation. This finding corresponds to the research result, which demonstrates a positive trend in the content curves of formaldehyde and acetaldehyde from

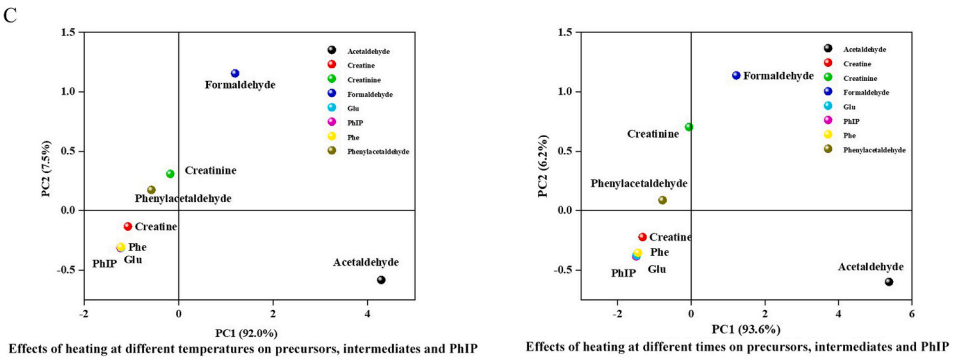

Fig. 4. (*continued*).

0:40 to 20:20 in the figure, followed by a negative trend beyond a certain altitude. Furthermore, with a fat-to-lean ratio ranging from 0:40 to 20:20, the trends of glucose, phenylalanine, and phenylacetaldehyde in the meat patty were comparable, and the trends of 16:24 and 20:20 were notably pronounced. This phenomenon might be attributed to the direct thermal decomposition of phenylalanine to phenylacetaldehyde, which could be facilitated by the different carbonyl compounds generated during the glucose decomposition (Chen et al., 2022).

In conclusion, the products resulting from lipid oxidation have the potential to facilitate the degradation of phenylalanine into phenylacetaldehyde through the Strecker pathway, thereby leading to an increase in the content of intermediates. Creatine is cyclized to produce creatinine, and the product of lipid oxidation can react with creatinine to produce stable adducts, while glucose plays an auxiliary role in this reaction, thus promoting the formation of PhIP.

3.3.2. *Processing temperature and time*

To explore the impact of the overall influence of temperature and time on PhIP in roast meat patties, the concentrations of diverse compounds were assessed at various heating times and temperatures were determined. Utilizing a principal component analysis (PCA) model, the effect of heating time and temperature on the spectra of PhIP and related substances was investigated. In the temperature model, the first main component (PC1) contributed 86.7%, while the second main component (PC2) contributed 11.0%. Similarly, for the time model, PC1 contributed 86.5% and PC2 contributed 10.5%. The cumulative contribution rates can reach 97.7% and 97.0%, better reflecting the information on the PhIP spectrum, as shown in Fig. 4(A, B). In addition, the contents of PhIP, precursors, and intermediates gradually changed at different rates under different times and temperatures. It can be seen from Fig. 4 (A) that samples under four different heating temperatures can be roughly separated in the PC1 direction. This demonstrates that the spectrum of PhIP in roast meat samples is significantly affected after roasting at different temperatures. In the PhIP score chart, the groups of 185 °C samples in pork patties were noticeably closer than groups at other temperatures. As the heating temperature increased, the distribution was increasingly spread out. Furthermore, at other temperatures, the sample groups that experienced reduced heating times exhibited a relatively narrow distribution, and the distribution became increasingly dispersed as the heating time was extended. Therefore, it can be fully demonstrated that the PhIP spectrum in roast samples with higher baking temperatures was more significantly affected by baking time. The sample points at 225 °C, 240 °C, and 250 °C deviating from the sample groups were all from samples heated for 10 or 15 min. These conclusions further indicate that the PhIP spectrum in the roast cooked quickly at a high temperature closely matched that of the roast simmered at a low temperature. However, a distinct separation was observed between the pork patty sample groups roasted at 250 °C for 40 min, indicating that PhIP concentrations in these samples were notably higher compared to the other sample groups. This is due to the formation of carbon bases during the thermal degradation of proteins or amino acids at high temperatures (Yan et al., 2021).

In the temperature and time models of the precursors (glucose, phenylalanine, creatinine, and creatine), their cumulative contribution rates could reach 81.3%, 98.9%, 81.5%, 97.5% and 86.6%, 97.5%, 91.5%, 98.7%, respectively. The cumulative contribution rate of formaldehyde, acetaldehyde, and phenylacetaldehyde can reach 82.2%, 97.1% and 92.1% and 75%, 96% and 92.3% respectively. In general, their accuracy and stability were good, and the visualization degree was high. As shown in Fig. 4(A)(B), the sample of precursors and intermediates can be roughly separated in PC1 and PC2 directions, indicating that the spectrum of precursors and intermediates of roast samples have obvious effects. In the glucose spectrum, samples at 185 °C, 10 min and 15 min clustered closely, but the aggregation of the samples progressively diminished as the conditions increased. In the spectrum of phenylalanine, creatine, creatinine and phenylacetaldehyde, samples at 240 °C and 250 °C and samples at 20–40 min were gradually loose, indicating that there was a significant influence between them as the temperature increased and the time extended. In the spectrum of formaldehyde and acetaldehyde, the dispersibility of temperature and time samples was better, indicating that it also has an obvious influence.

The precursor, PhIP, and intermediate contents of pork patties cooked at various temperatures and times were analyzed using PCA, as well as the correlation between precursors, intermediates, and PhIP (Fig. 4 (C)). Depending on the heating duration, the PC1 and PC2 of pork patties contributed 99.8% of the total change and 93.6% and 6.2% of the data change, respectively. The data variance at various thermal temperatures was calculated as PC1 variation was 92.0%, and PC2 was 7.5%, resulting in a total variation of 99.5%. The relationships between PhIP and precursors and intermediates were observed in Fig. 4 (C). When the heating time and temperature were varied, the precursors, intermediates, and PhIP were affected. More precisely, phenylacetaldehyde, creatine, creatinine, phenylalanine, glucose, and PhIP were found in higher quantities when PC1 was increased. On the other hand, formaldehyde and acetaldehyde were found in higher quantities when PC1 was decreased. The results of this investigation showed that PC1 had a negative correlation with PhIP, phenylacetaldehyde, creatine, creatinine, phenylalanine, and glucose. However, PC1 had a positive correlation with formaldehyde and acetaldehyde. At the same time, when PC2 was variable, formaldehyde, creatinine, and phenylacetaldehyde were positively correlated with PC2, while phenylalanine, creatine, glucose, PhIP, and acetaldehyde were negatively correlated. Moreover, the content of formaldehyde, acetaldehyde, creatinine, and phenylacetaldehyde in roast pork patty was higher.

The creatine concentration was reported to be inversely related to every HAA identified in smoked chicken legs. In addition, studies have shown that the production of all HAAs in pork is substantially correlated with glucose concentration (Buła, Przybylski, Jaworska, & Kajak-Siemaszko, 2019; Zhang et al., 2021). However, there was no

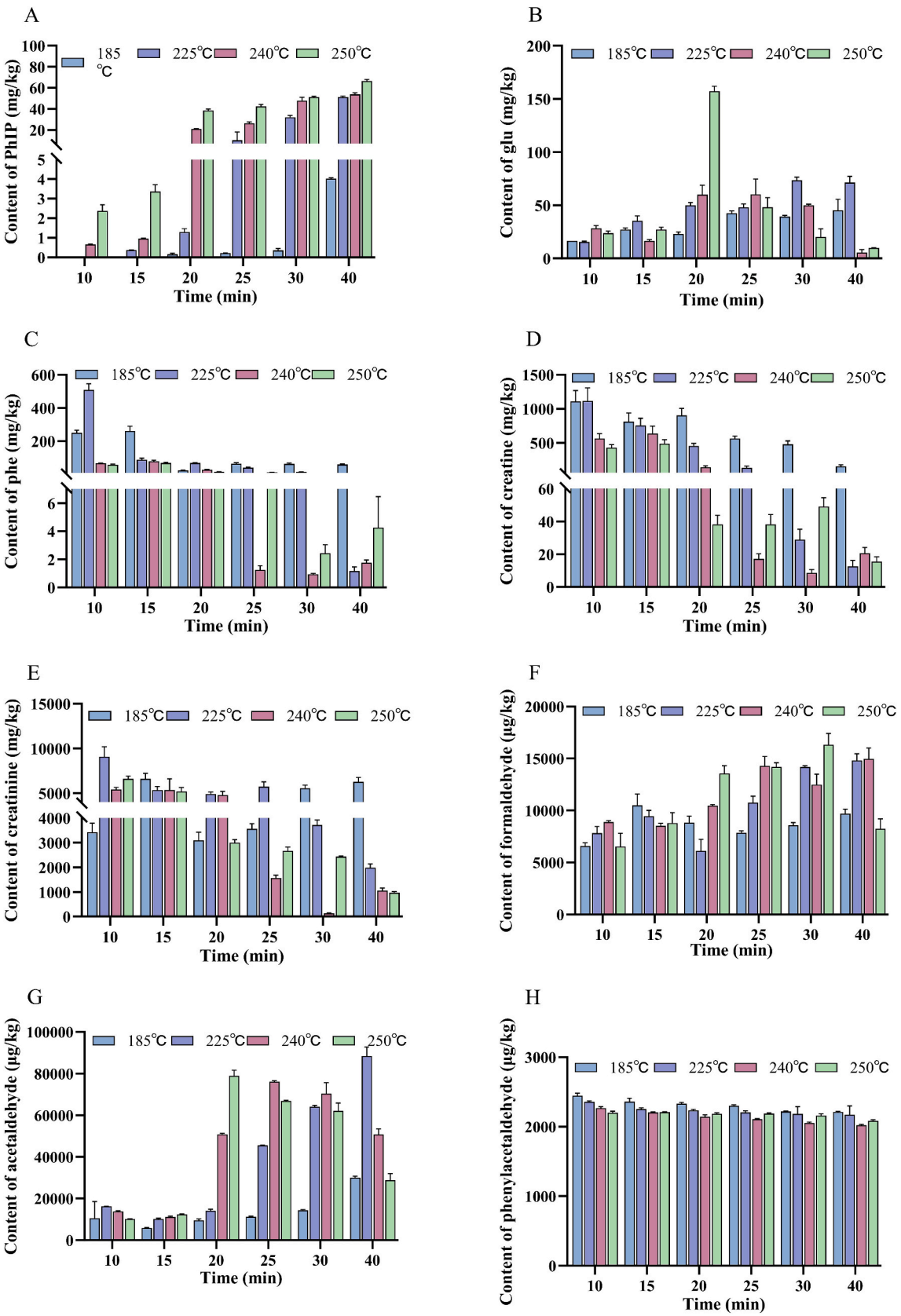

Fig. 5. Effects of heating temperature and time on the PhIP, glucose, phenylalanine, creatine, creatinine, formaldehyde, acetaldehyde, and phenylacetaldehyde contents of the roasted pork, respectively (n = 3).

significant correlation between the amounts of HAAs, creatine, and creatinine in spiced pork shoulder meat cooked at temperatures below 100 °C (Zhang & Zhou, 2022). As shown in Fig. 4(C), time and temperature were positively correlated, as indicated by the sample value being more consistent with the upper axis of PC2 and the right of PC1. After a long time of high-temperature baking, when PC2 was the variance, samples of phenylalanine, creatinine, and formaldehyde were in the positive part of PC2. When PC1 was the variance, samples of formaldehyde and acetaldehyde were positive, indicating that the variables in meat patty samples exhibited significant values. The study of Wang et al. (Wang, Cheng, et al., 2021; Wang, Li, et al., 2021) also showed that roast pork's HAA content would rise dramatically when the roasting procedure was extended from 2 min to 2.5 min, and the temperature was raised from 225 °C to 250 °C.

The results of PCA revealed that the heating conditions had an impact on the distribution of PhIP and related substances. The varied distribution patterns revealed that PhIP produced slowly at low temperatures in a short period was comparable to PhIP produced quickly at high temperature roast. Precursor and intermediate contents showed a similar pattern, changing significantly at high temperatures over a long period and insignificantly at low temperatures. As shown in Fig. 5, a significant increase in PhIP concentration was observed as the temperature was raised from 225 °C to 250 °C and the duration was extended from 20 to 40 min. The considerable rise in PhIP production observed at 250 °C may be attributed to the compound's formation mechanism, which potentially includes the thermal degradation of proteins or amino acids in high temperatures (Barzegar, Kamankesh, & Mohammadi, 2019; Fan et al., 2018). The changes in creatine and Phe are consistent with the previous findings (Yan et al., 2014), when heated to 195 °C at different times, the concentration of amino acids and creatine, in particular, will drastically decrease in the first five minutes, indicating an aggressive reaction process. The content changes then flattened out as the reaction developed. This difference in elimination kinetics between amino acids and creatine can be attributed to the fact that creatine is involved in the synthesis of creatinine and PhIP. The dehydration reaction from creatine to creatinine can be observed with a sharp rise in creatinine initially, followed by a gradual decline in subsequent reactions (Gibis & Weiss, 2015; Han et al., 2023). In conclusion, the effects of heating temperature, time, precursors, and intermediates on PhIP formation can be explained more easily by PCA profiling. Furthermore, it is advisable to select a reasonable baking temperature and duration for the meat patty in order to minimize the production of PhIP, and future research should consider the quality of roast meats in order to provide recommendations that correspond with both food standards and consumer preferences.

4. Conclusion

GC–MS and HPLC-Q-Orbitrap-HRMS were used to analyze and identify the influence rules of precursor substances and key intermediates in the roast pork patty. In addition, the influence of hot working conditions (heating temperature, time, and precursor contents) on PhIP and related substances in the chemical model system was studied, and the main influencing factors and rules for inhibiting the formation of PhIP were revealed. It was found that PhIP concentration was the highest when the temperature was 220 °C and the time was 200 min in the chemical model system. The change in formaldehyde and acetaldehyde content was basically irregular with heating time and temperature. After adding different contents of precursors in the chemical model system, precursors, phenylacetaldehyde, PhIP are basically dose-dependent. More precisely, when glucose is present in the system, constituting approximately half of the creatine and phenylalanine, it significantly enhances the likelihood of PhIP formation. Moreover, the content of PhIP and its intermediate phenylacetaldehyde increased significantly ($P<0.05$) as the fat proportion in the roast pork patty system increased. In addition, PCA analysis showed that samples of phenylalanine, creatine, creatinine, glucose, PhIP, formaldehyde, acetaldehyde and phenylacetaldehyde gradually dispersed as the temperature and time increased, and there were obvious effects among them. The results could serve as an experimental and theoretical foundation for the PhIP generation mechanism and control method.

CRediT authorship contribution statement

Qi Chen: Writing – review & editing, Writing – original draft. **Yan Xu:** Writing – review & editing, Writing – original draft, Data curation. **Hao Dong:** Writing – review & editing, Supervision, Funding acquisition. **Weidong Bai:** Validation, Supervision. **Xiaofang Zeng:** Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101404.

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