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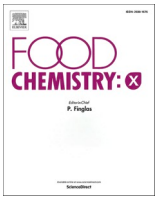

Effect of acid pretreatments with various acid types on gelling properties and identification characteristics of pigskin gelatin

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ABSTRACT

In order to study the effects of different types of acid pretreatment of pigskin on the gel characteristics and traceability aspects of the extracted gelatin, the molecular weight distribution and gelling properties of pigskin gelatin were studied using SDS-PAGE, texture analyzer, and rheometer. The characteristic peptides digested from pigskin gelatin were studied by HPLC-MS/MS technique. The findings revealed that gelatin extracted from pigskin pretreated with acetic acid showed the most typical bands. The gelatins extracted from pig skin pretreated with acetic acid and hydrochloric acid exhibited the highest gelation-melting transition temperatures (26.81/34.17 °C) and gel strength (605.278 g), respectively. 69 characteristic peptides were the same in all gelatins from pigskin pretreated by various acids. A further comparison of this work with prior studied revealed that 8 were detected under different extraction and processing conditions. These common characteristic peptides could be used as the foundation for pigskin gelatin traceability, boosting accuracy.

1. Introduction

Gelatin is an important biomass polymer material, derived from collagen through partial hydrolysis, mainly from mammals, of which pig gelatin and bovine gelatin account for more than 90 %(Alipal et al., 2021). Gelatin possesses diverse qualities including gelation, emulsification, and film-forming. Consequently, it finds extensive application in pharmaceuticals, photographic materials, food, chemical engineering, textiles, and cosmetics. The traceability of porcine gelatin has attracted much attention due to religious beliefs and foot-and-mouth disease, which have caused consumers to question its safety(Kaewruang et al., 2014). Enzyme-linked immune sorbent assay (ELISA) and polymerase chain reaction (PCR) are frequently used to detect mammalian gelatin sources due to their relatively fast and simple characteristics(Doi et al., 2009; Sultana et al., 2018). However, ELISA is prone to cross-reactivity with the matrix, leading to false positive and negative results. Meanwhile, for PCR detection, gelatin extraction often requires extreme conditions such as strong acids, alkalis, and high temperatures, which breaks the original DNA sample and reduces the accuracy of PCR detection results(Kleinnijenhuis et al., 2018). Therefore, it is difficult to confirm the traceability effect of gelatin using the above techniques. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) is a precise technique that may determine the source of gelatin by detecting characteristic peptides produced by enzymatic hydrolysis(Zhang et al., 2008). Guo successfully distinguished pig gelatin in different products using the Ultra performance liquid chromatography-mass spectrometry (UPLC-MS/MS), the characteristic characteristics of pig gelatin can also be recognized at low concentration levels(Guo et al., 2018). Furthermore, research findings suggest that HPLC-MS/MS is superior to ELISA and PCR in accurately identifying gelatin (Grundy et al., 2016).

In the extraction process of gelatin, the role of pretreatment is mainly to remove fat, minerals, non-collagen and other impurities in the raw material. Collagen's structure was destroyed, promoting gelatin extraction. Various types of acids could be used in the pretreatment process generally include hydrochloric acid, acetic acid, citric acid, or a combination of different acids(Cao et al., 2020). The relevant research found that gelatin extracted from African catfish skin under weakly acidic conditions produces higher molecular weight components(See et al., 2015). In comparison with gelatin from acetic acid pretreated skin of unicorn leatherjackets (*Aluterus monoceros*), gelatin from phosphoric acid pretreated skin showed a higher gel strength(M. Ahmad & Benjakul,

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2011). During rheological examination, gelatin derived from bovine bone and extracted using citric acid demonstrated the highest values for maximal elastic modulus, gelation and melting temperatures compared to the other two acids studied (hydrochloric acid and acetic acid)(Cao et al., 2020). However, the research on the gelling properties and traceability identification of pigskin gelatin by acid pretreatments with various acid types has not been reported yet, and an in-depth study is needed.

In this work, five acids containing strong acid, medium strong acid and weak acid, namely hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid and citric acid were selected to pretreat pig skin to prepare various gelatins. Gelatin extracted from pigskin pretreated with various acids was investigated using the shear stress rheometer and texture analyzer to measure gel strength, gelation and melting temperatures. Moreover, HPLC-MS/MS was used to trace and identify various gelatins to find out the common characteristic peptides. Finally, the comparison between this work and previous studies(Sha et al., 2019; Sha et al., 2020; Sha et al., 2022; Sha et al., 2023; Tang et al., 2024) was executed to obtain the constant characteristic peptides for the identification in pigskin gelatin. This study laid a foundation for the preparation of high gelling property pigskin gelatin and the establishment of pigskin gelatin traceability database.

2. Materials and methods

Porcine skin was obtained from Zhengbang Group (Nanchang, Jiangxi). NaOH was purchased from Xilong Chemical Co., Ltd. (Guangdong, China). Sequencing-grade modified trypsin (Frozen, V5113, 19,038 u/mg) was purchased from Promega Corporation (Madison, WI, USA), and it exhibited no other proteolytic activities. All other reagents used were of analytical grade.

2.1. Sample preparation

Referring to the method of Sha et al.(Sha et al., 2019) with slight modifications. Deionized water was used to rinse fresh pork skin after trimming off the greasy and fatty parts, washing, and cutting into 2 cm × 2 cm pieces. The fragments of skin were fully immersed in isopropyl alcohol for the entire night. Then rinsed with deionized water and soaked in anhydrous ethanol at a ratio of 1:3 for 10 min to remove the residual isopropanol, and then washed with water until odorless. And transferred to 0.1 M NaOH solution at a 1:10 (*w/v*) skin/solution ratio. Magnetic stirring was performed at 15–20 °C for two hours with replacement of alkaline solution every 40 min. After alkali treatment, the skin was washed to a pH of approximately neutral. The prepared skin was divided into 6 portions and pretreated by adding 0.2 M hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid and citric acid according to the material-liquid ratio of 1: 10 (w/v). For a duration of four hours, the mixture was stirred delicately, while the acid solution was substituted every 60 min. In the final step, a near neutral pH was obtained by rinsing the acid-treated skin, which was then used to extract gelatin. Pork skin gelatin was prepared by a material-liquid ratio of pigskin gelatin to ultrapure water of 1: 3 (g: mL), a heating temperature of 60 °C, with a duration of 12 h for the extraction process. Afterwards, the pig skin residue was removed by filtration, and the filtrate was freeze-dried to obtain pigskin gelatin.

2.2. Determination of molecular weight distribution of pigskin gelatin

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to ascertain the impact of acid pretreatment on the molecular weight distribution of pigskin gelatin. Following the method of Balti et al.(Balti et al., 2011), a 5 mg/mL gelatin solution was prepared and gel electrophoresis was performed using a concentration of 5 % stacking gel and 7.5 % separating gel. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. The molecular weight of the proteins was estimated by comparing the results with SDS-PAGE of standard proteins with molecular weights between 10 and 245 kDa.

2.3. Determination of gel strength

The gel strength of pigskin gelatin was measured using the TA-XT plus texture analyzer(Tu et al., 2015). The freeze-dried gelatin was prepared into 6.67 % gelatin solution, 15 mL of which was placed into a 25 mL beaker, and each sample was set up in 3 parallel groups and refrigerated at 4 °C for 18 h. After taking out the gelatin, the texture analyzer was used to determine the gel strength of the colloid. The parameters were configured as follows: the probe used was P/0.5R, the test speed was set to 1 mm/s, and the gel strength was defined as the greatest pressure exerted on the gelatin when the probe was pressed down by 4 mm.

2.4. Gelation-melting transition temperatures measurement

The gelation-melting transition temperatures of gelatin were determined by employing a shear stress-controlled rheometer, following the methodology established by Huang et al.(Huang et al., 2017). The solution underwent a chilling process, starting from an initial temperature of 40 °C and reaching a final temperature of 5 °C. Subsequently, it was subjected to a warming process, starting from 5 °C and reaching a final temperature of 40 °C, with a heating/cooling rate of 0.5 °C /min, and a strain of 0.5 % at a frequency of 1 Hz.

2.5. Sample enzymolysis

Referring to the previous experimental method of the group(Sha et al., 2020), the gelatin of pigskin was enzymatically digested, and the freeze-dried gelatin of pigskin was dissolved in SDT buffer (containing 4 % SDS, 1 mmol/L dithiothreitol (DTT), 150 mmol/L Tris-HCl, pH 8.0), boiled for 3 min, and then subjected to ultrasonic homogenization on ice. After centrifugation at 16000 ×*g* for 10 min at 25 °C, 250 μg of the supernatant was mixed with urea solution (containing 8 mol/L urea, pH 8.0), and then low molecular mass fractions such as DTT were removed by ultrafiltration. After collecting the concentrate, the operation was repeated. 50 M mol/L iodoacetamide (IAA) buffered with urea was added to 100 μL of the above-concentrated solution and centrifuged for 15 min after 20 min at room temperature and protected from light. This concentrated solution was then centrifuged with 100 μ L of urea buffer solution and the process was repeated three times. Afterwards, it was combined with 100 μL of a 25 Mmol/L NH_4HCO_3 solution and concentrated once again using centrifugation. This process was repeated two more times. The final concentrate obtained was warmed with 40 μL 25 Mmol/L NH_4HCO_3 containing 3 μg trypsin at 37 °C overnight and the filtrate was collected.

2.6. HPLC- MS/MS determination

The liquid portion of the experiment utilized a 0.1 % aqueous solution of formic acid as mobile phase A, whereas mobile phase B consisted of an 84 % aqueous solution of acetonitrile, which also included 0.1 % formic acid. The specimen was introduced into a C18 reverse phase chromatography column via an automated sampler, and subsequently partitioned by the column at a flow rate of 250 nL/min. The sample was eluted for 60 min, and the linear gradient of mobile phase A changed to mobile phase B. Collect the top 10 parent ions with the highest abundance in data dependent acquisition mode to obtain their primary mass spectrometry data, and obtain secondary mass spectrometry data through high-energy collision-induced dissociation (HCD).

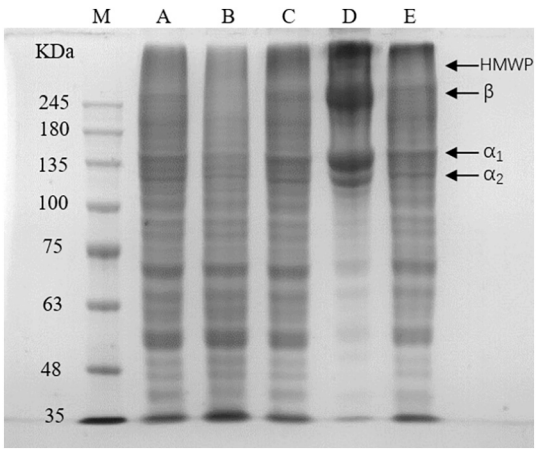

Fig. 1. Effect of acid pretreatments with various acid types on the molecular weight distribution of pigskin gelatin. (A): hydrochloric acid; (B): sulfuric acid; (C): phosphoric acid; (D): acetic acid; (E): citric acid.

2.7. Data analysis

All the experiments were conducted in triplicate. Significant differences between average values ($p < 0.05$) were studied by a one-way ANOVA test using SPASS 27.0.1.

The database was set up as a pig protein gelatin database and the obtained mass spectra were matched and analyzed using MASCOT software (Matrix Science, London, UK; version 2.2). The search parameters included: oxidation (K, M, P) was a variable modification; cysteine iodoacetamide (C) was a fixed modification. The maximum number of missing cutting positions was 2. The error rate of primary mass spectrometry was within a range of ±20 ppm. The error rate of secondary mass spectrometry was within a range of ±0.1 Da. When the Mascot score was greater than 40, the peptide peak was considered positive identification or had extremely high accuracy ($P < 0.05$) (Sha et al., 2022).

3. Results and discussion

3.1. Effect of acid pretreatments with various acid types on the molecular weight distribution of pigskin gelatin

Mammalian gelatin has a typical molecular mass distribution pattern of gelatin, including α-chain bands (α_1 and α_2), β-chain bands (α-chain dimers) and high molecular weight polymer (HMWP) bands(Lim & Mohammad, 2011). Various types of acid-pretreated pig skin had an impact on the structure of extracted gelatin. Previous research had shown that different extraction conditions could affect the molecular weight of gelatin. Therefore, the molecular weight distribution of gelatin extracted from various types of acid-pretreated pig skin was evaluated. Fig. 1 demonstrates the impact of different acid pretreatments on the distribution of molecular masses in gelatins derived from pig skin. Except for binary strong acid sulfuric acid, the gelatin extracted from pig skin pretreated with four various types of acids showed typical molecular weight distribution patterns, retaining the α_1, α_2, β and HMWP bands. Among them, the gelatin derived from pig skin pretreated with acetic acid revealed the most typical bands. The strength of the acid was determined by its composition and structure, manifested in its ionization ability. The dissociation constants of hydrochloric acid, sulfuric acid, acetic acid, citric acid, and phosphoric acid were − 8, −3, 4.76, 3.13, and 2.12, respectively. Strong acids were almost completely ionized in aqueous solution and their degree of ionization was so high that the corresponding dissociation constants were usually small. Under two strong acid conditions, the gelatin obtained from pig skin pretreated with sulfuric acid exhibited bands with lower molecular weights.

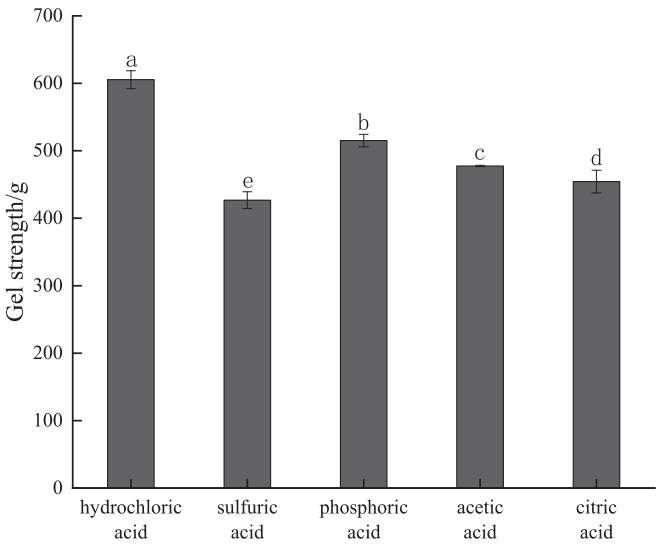

Fig. 2. Effect of acid pretreatments with various acid types on the gel strength of pigskin gelatin.

Perhaps because under the same acid concentration, strong acids could completely dissociate, and the concentration of hydrogen ions in sulfuric acid solution was higher, leading to excessive hydrolysis of collagen molecules(Liao et al., 2021). The smaller the dissociation constant, the more ionized the acid was, indicating a stronger acid. As the acidity of phosphoric acid, citric acid, and acetic acid decreased in sequence, the high molecular weight bands content of the sample increased. Perhaps this was due to the fact that weaker acidic treatment conditions resulted in less disruption of intermolecular cross-links in collagen. It resulted in less cleavage of intramolecular peptide bonds and produced components with higher molecular weight (α chain and β chain)(Hong et al., 2019). Simultaneously, the low molecular weight bands decreased. The lower dissociation constant, the higher degree of acid dissociation in aqueous solution, which intensified the depolymerization of gelatin. The results showed that pre-treatment of pig skin with different acids had various impact on the molecular mass distribution of extracted pigskin gelatin.

3.2. Effect of acid pretreatments with various acid types on the gel strength of pigskin gelatin

Gel strength is an important index for evaluating the performance of gelatin, which significantly affects its practical application(Kim et al., 2012). As shown in Fig. 2, when pigskin was separately pretreated by five types of acids, namely hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid and citric acid, the gel strength of prepared gelatin were 605.28, 426.75, 515.15, 477.51 and 454.33 g, respectively. The gel strength of gelatin was influenced by amino acid sequence, molecular weight size and distribution, cross-linking and bonding mode of molecular chain and other factors (Lueyot et al., 2021). Combined with the results of electrophoresis experiments in this work, it was found that the gel strength was not only affected by molecular weight. The gel strength of gelatin derived from pig skin that underwent pretreatment with hydrochloric acid was the highest, but its molecular weight distribution was not the most concentrated. The gel strength of gelatin derived from pig skin that underwent pretreatment with acetic acid was general, but its molecular weight was the most concentrated. It indicated that the gel strength was affected by many factors. When preparing the sample for measuring the gel strength, the collagen fibers forming helixes lost their conformation during heating. In the process of gel monitoring, it was placed at low temperature, and the collagen fiber would partially recover its structure during cooling, which had a positive effect on the process of gel formation(Duconseille et al., 2015). The gelatin derived from pig skin that was pretreated with hydrochloric acid had the highest

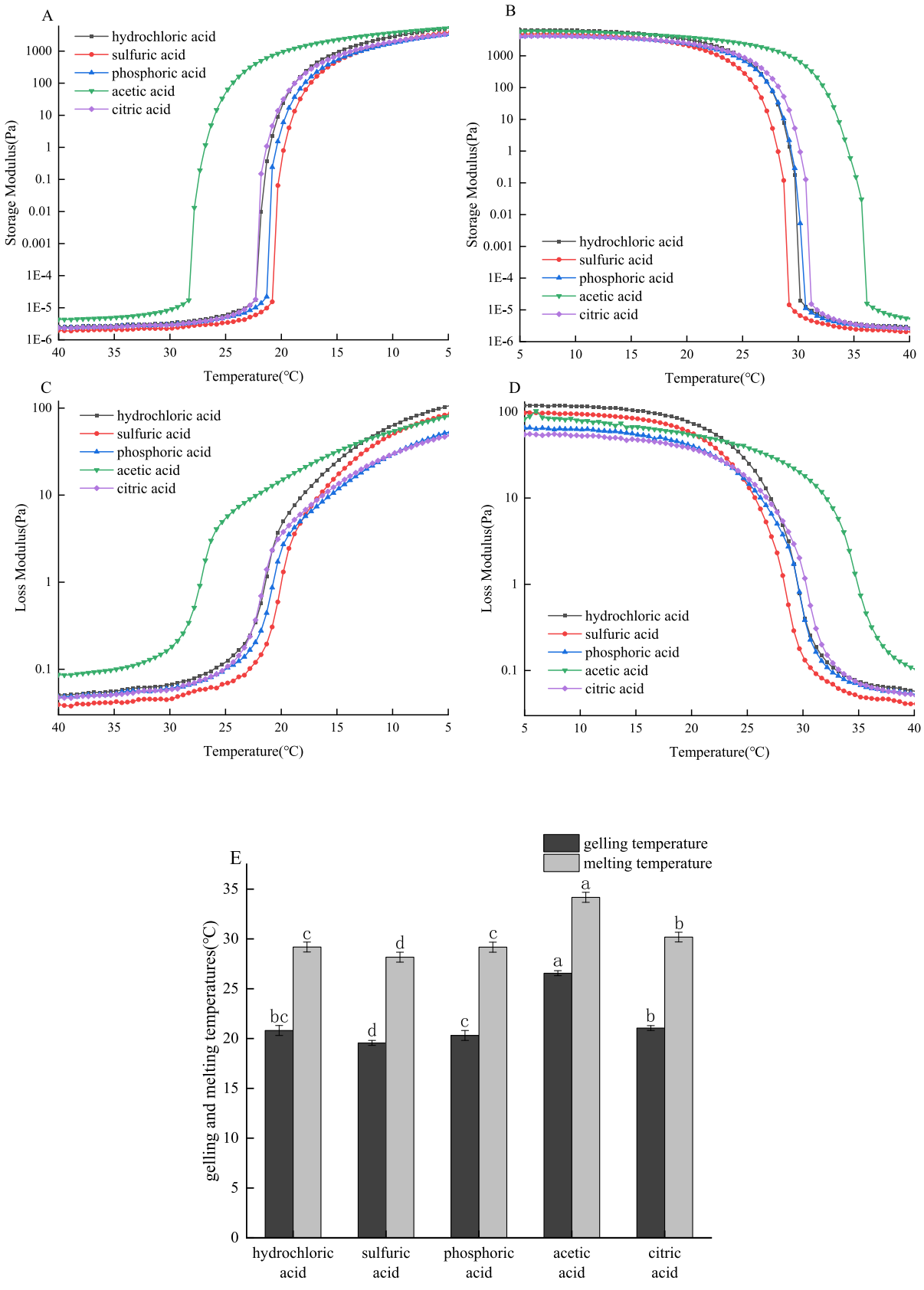

Fig. 3. Storage modulus (A) and loss modulus (C) of pigskin gelatin extracted at different temperatures upon cooling from 40 °C to 5 °C. Storage modulus (B) and loss modulus (D) of pigskin gelatin extracted at different temperatures upon heating from 5 °C to 40 °C (E) gelation and melting temperatures of pigskin gelatin.

Table 1
Unique characteristic peptides of pigskin gelatin pretreated with different acids.

Unique peptides from hydrochloric acid	Unique peptides from sulfuric acid	Unique peptides from phosphoric acid	Unique peptides from acetic acid	Unique peptides from citric acid
α_1:^{289}GEPGS*PGENGA*PGQMGPR^{306}	α_1:^{325}GNDGATGAAG*PPGPTGPAGPPGF*PGAVGA*K^{354}	α_1:^{325}GNDGATGAAGPPGPTGPAGP*PGF*PGAVGAK^{354}	α_1:^{289}GE*PGSPGENGA*PGQMGPR^{306}	α_1:^{325}GNDGATGAAGPPG*PTGPAGPPGFPGAVGA*K^{354}
α_1:^{325}GNDGATGAAGPPGPTGPAGPPGFPGAVGA*K^{354}	α_1:^{325}GNDGATGAAG*PPGPTGPAGP*PGF*PGAVGA*K^{354}	α_1:^{325}GNDGATGAAGPPG*PTG*PAG*P*PGFPGAVGAK^{354}	α_1:^{325}GNDGATGAAGPPG*PTG*PAGPPGFPGAVGA*K^{354}	α_1:^{373}GEPG*P*PG*PAGAAGPAGNPGADGQPGGK^{399}
α_1:^{325}GNDGATGAAG*PPGPTGPAGPPGFPGAVGA*K^{354}	α_1:^{373}GE*PG*PPGPAGAAGPAGNPGADGQ*PGGK^{399}	α_1:^{325}GNDGATGAAGPPG*PTG*PAG*P*PGF*PGAVGAK^{354}	α_1:^{325}GNDGATGAAGP*PG*PTG*PAGPPGFPGAVGA*K^{354}	α_1:^{373}GE*PG*P*PG*PAGAAG*PAGNPGADGQPGGK^{399}
α_1:^{325}GNDGATGAAGPPGPTGPAGP*PGF*PGAVGA*K^{354}	α_1:^{373}GE*PG*PPGPAGAAGPAGN*PGADGQ*PGGK^{399}	α_1:^{688}GVQGPPGPAGPR^{699}	α_1:^{325}GNDGATGAAGP*PG*PTG*PAG*PPGFPGAVGA*K^{354}	α_1:^{400}GANGA*PGIAGAPGFPGAR^{417}
α_1:^{325}GNDGATGAAGPPGPTG*PAG*PPGFPGAVGA*K^{354}	α_1:^{475}GE*PGPAGL*PG*PPGER^{489}	α_1:^{700}GANGA*PGNDGAK^{711}	α_1:^{400}GANGA*PGIAGA*PGFPGAR^{417}	α_1:^{451}GEPGPTGVQG*PPGPAGEEGK^{470}
α_1:^{373}GE*PG*PPGPAGAAGPAGN*PGADGQ*PGG*K^{399}	α_1:^{980}GP*PGPMGPPGLAGPPGESGR^{999}	α_1:^{805}GEPG*P*PG*PAGFAG*PPGADGQPGAK^{828}	α_1:^{418}GPSGPQGPSGP*PGP*K^{432}	α_1:^{475}GE*PGPAGLPG*P*PGER^{489}
α_1:^{373}GE*PG*P*PG*PAGAAG*PAGN*PGADGQPGGK^{399}	α_1:^{1000}EGAPGAEGSPGR^{1011}	α_1:^{1022}GESGPAGP*PGAPGAPGAPGPVGPAGK^{1047}	α_1:^{451}GEPG*PTGVQGPPGPAGEEGK^{470}	α_1:^{712}GDAGA*PGA*PGSQGAPGLQGMPGER^{735}
α_1:^{615}DGEAGAQGPPGPAGPAGER^{633}	α_1:^{1097}GFSGLQGP*PG*PPGSPGEQGPSGASGPAGPR^{1126}	α_1:^{1082}GETGEQGDR^{1090}	α_1:^{451}GE*PGPTGVQG*P*PGPAGEEGK^{470}	α_1:^{712}GDAGA*PGA*PGSQGA*PGLQGMPGER^{735}
α_1:^{615}DGEAGAQGP*PGPAGPAGER^{633}	α_1:^{1222}SLSQQIENIR^{1231}	α_1:^{1097G}FSGLQGPPG*P*PGS*PGEQGPSGASGPAGPR^{1126}	α_1:^{541}GLTGS*PGSPGPDGK^{554}	α_1:^{766}GLTGPIGPPGPAGAPGDK^{783}
α_1:^{805}GEPG*P*PGPAGFAGPPGADGQPGAK^{828}	α_2:^{199}GEPGA*PGENGTPGQTGAR^{216}	α_1:^{1127}GP*PGSAGAPGK^{1137}	α_1:^{712}GDAGAPGA*PGSQGAPGLQGM*PGER^{735}	α_1:^{805}GEPGP*PG*PAGFAGPPGADGQPGAK^{828}
α_1:^{805}GEPGP*PG*PAGFAG*PPGADGQPGAK^{828}	α_2:^{199}GEPGA*PGENGT*PGQTGAR^{216}	α_2:^{100}GPPGAVGAPG*PQGFQG*PAGE*PGE*PGQTGPAGAR^{132}	α_1:^{805}GEPG*P*PG*PAGFAGPPGADGQPGAK^{828}	α_1:^{868}VGPPG*PSGNAG*PPGPPGPAG*K^{888}
α_1:^{868}VG*PPGPSGNAGPPGP*PG*PAGK^{888}	α_2:^{283}GEVGL*PGVSGPVGP*PGN*PGANGL*PGA*K^{309}	α_2:^{235}GNDGSVGPVGPAGPIGSAGP*PGF*PGAPGP*K^{264}	α_1:^{920}GSPGADG*PAGA*PGTPGPQGIAGQR^{943}	α_1:^{956}GF*PGLPG*PSGEPGK^{969}
α_1:^{868}VGP*PG*PSGNAG*P*PGPPGPAGK^{888}	α_2:^{361}GEPGAAGPQGP*PGPSGEEGK^{380}	α_2:^{520}GA*PGPDGNNGAQG*PPGPQGVQGGK^{543}	α_1:^{920}GSPGADGPAGA*PGTPG*PQGIAGQR^{943}	α_1:^{1022}GESGPAGP*PGA*PGAPGAPGPVGPAGK^{1047}
α_1:^{920}GSPGADGPAGAPGT*PGPQGIAGQR^{943}	α_2:^{361}GE*PGAAGPQGP*PGPSGEEGK^{380}	α_2:^{622}GE*PGVLGA*PGTAG*PSGPSGLPGER^{645}	α_1:^{920}GS*PGADGPAGA*PGTPG*PQGIAGQR^{943}	α_1:^{1097}GFSGLQG*P*PG*P*PGSPGEQGPSGASGPAGPR^{1126}
α_1:^{1022}GESGPAGPPGA*PGA*PGAPGPVGPAGK^{1047}	α_2:^{487}GE*PGNIGF*PG*PK^{498}	α_2:^{715}GEVGPAGPNGFAGPAGAAGQPGAK^{738}	α_1:^{920}GS*PGADGPAGA*PGT*PGPQGIAGQR^{943}	α_1:^{1127}GPPGSAGA*PGK^{1137}
α_1:^{1022}GESGPAGPPGAPGA*PGA*PGPVGPAGK^{1047}	α_2:^{949}GYPGN*PG*PAGAAGA*PGPQGAVGPAGK^{974}	α_2:^{847}G*PSGEPGTAGPPGTPGPQGILGA*PGFLGL*PGSR^{879}	α_1:^{980}G*P*PG*PMGPPGLAGP*PGESGR^{999}	α_1:^{1138}DGLNGL*PGPIGPPGPR^{1153}
α_1:^{1052}GETGPAG*PAGPVGPVGAR^{1069}	α_2:^{997}GPSGPQGIR^{1005}	α_2:^{883}GLPGVAGSVGEPGPLGIAGPPGAR^{906}	α_1:^{1097}GFSGLQGP*PG*PPGSPGEQGPSGASGPAGPR^{1126}	α_1:^{1156}TGDAGPVGP*PG*PPGPPGPPGPPSGGFDFSFLPQPPQEK^{1193}
α_1:^{1156}TGDAGPVGPPGP*PG*P*PG*PPGPPSGGFDFSFLPQPPQEK^{1193}	–	α_2:^{907}GP*PGAVGNPGVNGA*PGEAGR^{926}	α_1:^{1097}GFSGLQG*P*PG*PPGSPGEQGPSGASGPAGPR^{1126}	α_2:^{100}GPPGAVGAPGPQGFQG*PAGE*PGE*PGQTGPAGAR^{132}
α_2:^{100}G*P*PGAVGAPGPQGFQGPAGEPGE*PGQTG*PAGAR^{132}	–	α_2:^{1081}GSQGSQGPAGP*PG*PPGPPGPPGPSGGGYDFGYEGDFYR^{1118}	α_1:^{1127}GPPGSAGAPGK^{1137}	α_2:^{100}G*PPGAVGAPGPQGFQGPAGEPGE*PGQTG*PAGAR^{132}
α_2:^{100}GP*PGAVGA*PG*PQGFQG*PAGEPGEPGQTGPAGAR^{132}	–	–	α_1:^{1156}TGDAGPVGPPGP*PGPPGPPGPPSGGFDFSFLPQPPQEK^{1193}	α_2:^{100}GPPGAVGA*PG*PQGFQG*PAGE*PGE*PGQTGPAGAR^{132}
unique peptides from hydrochloric acid	unique peptides from sulfuric acid	unique peptides from phosphoric acid	unique peptides from acetic acid	unique peptides from citric acid
α_2:^{265}GELGPVGNPGPAGPAGPR^{282}	–	–	α_1:^{1156}TGDAGPVGP*PG*PPGPPGPPGPPSGGFDFSFLPQPPQEK^{1193}	α_2:^{199}GE*PGA*PGENGTPGQTGAR^{216}
α_2:^{424}GPTGPAGVR^{432}	–	–	α_1:^{1156}TGDAGPVGPPGP*PG*P*PG*P*PGPPSGGFDFSFLPQPPQEK^{1193}	α_2:^{235}GNDGSVGPVGPAGPIGSAGP*PGF*PGA*PG*PK^{264}
α_2:^{487}GEPGNIGF*PGPK^{498}	–	–	α_2:^{100}GP*PGAVGA*PG*PQGFQG*PAGE*PGE*PGQTGPAGAR^{132}	α_2:^{265}GELGPVGN*PGPAGPAGPR^{282}
α_2:^{520}GAPG*PDGNNGAQGP*PGPQGVQGGK^{543}	–	–	α_2:^{199}GE*PGA*PGENGT*PGQTGAR^{216}	α_2:^{520}GAPG*PDGNNGAQGP*PGPQGVQGGK^{543}

(*continued on next page*)

Table 1 (*continued*)

Unique peptides from hydrochloric acid	Unique peptides from sulfuric acid	Unique peptides from phosphoric acid	Unique peptides from acetic acid	Unique peptides from citric acid
α_2:^{574}GI*PGEFGL*PG*PAGPR^{588}	–	–	α_2:^{235}GNDGSVGPVGPAGPIGSAGP*PGF*PGAPGPK^{264}	α_2:^{574}GI*PGEFGL*PGPAGPR^{588}
α_2:^{592}GPPGESGAAGPAGPIGSR^{609}	–	–	α_2:^{235}GNDGSVGPVGPAGPIGSAG*P*PGFPGAPGP*K^{264}	α_2:^{748}GENGPVGPTGPVGAAGPAGPNGPPG*PAGSR^{777}
α_2:^{592}GP*PGESGAAG*PAGPIGSR^{609}	–	–	α_2:^{592}G*PPGESGAAGPAGPIGSR^{609}	α_2:^{748}GENGPVGPTGPVGAAGPAG*PNG*PPGPAGSR^{777}
α_2:^{676}GAPGAVGAPGPAGANGDR^{693}	–	–	α_2:^{676}GA*PGAVGA*PGPAGANGDR^{693}	α_2:^{778}GDGGPPGATGF*PGAAGR^{794}
α_2:^{795}IGP*PGPSGISGPPGPPGPAG*K^{815}	–	–	α_2:^{847}G*PSGEPGTAGPPGTPG*PQGILGA*PGFLGL*PGSR^{879}	α_2:^{883}GLPGVAGSVGE*PGPLGIAGP*PGAR^{906}
α_2:^{795}IG*PPGPSGISGPPGP*PG*PAGK^{815}	–	–	α_2:^{883}GL*PGVAGSVGEPGPLGIAGP*PGAR^{906}	α_2:^{883}GL*PGVAGSVGE*PGPLGIAG*PPGAR^{906}
α_2:^{795}IG*P*PG*PSGISG*PPGPPG*PAGK^{815}	–	–	α_2:^{949}GYPGN*PGPAGAAGAPG*PQGAVGPAGK^{974}	α_2:^{907}GPPGAVGN*PGVNGA*PGEAGR^{926}
α_2:^{883}GL*PGVAGSVGE*PG*PLGIAG*PPGAR^{906}	–	–	α_2:^{1024}GHNGLQGL*PGLAGHHGDQGA*PGPVGPAGPR^{1053}	α_2:^{907}GP*PGAVGN*PGVNGA*PGEAGR^{926}
α_2:^{927}DGNPGSDG*PPGR^{938}	–	–	α_2:^{1024}GHNGLQGL*PGLAGHHGDQGA*PGPVGPAGPR^{1053}	α_2:^{927}DGNPGSDGP*PGR^{938}
α_2:^{979}GEPGPAGSVGPAGAVGPR^{996}	–	–	α_2:^{1081}GSQGSQGPAGPPGP*PGPPGPPGSGGGYDFGYEGDFYR^{1118}	α_2:^{1024}GHNGLQGLPGLAGHHGDQGA*$_{\text{PGPVGPAGPR1053}}$
α_2:^{1024}GHNGLQGLPGLAGHHGDQGA*$_{\text{PGPVGPAGPR1053}}$	–	–	α_2:^{1081}GSQGSQGPAGPPGP*PG*P*PGPPGPPGPSGGGYDFGYEGDFYR^{1118}	α_2:^{1081}GSQGSQGPAGP*PG*P*PGPPGPPGPSGGGYDFGYEGDFYR^{1118}
α_2:^{1024}GHNGLQGL*PGLAGHHGDQGAPG*PVGPAGPR^{1053}	–	–	α_2:^{1081}GSQGSQGPAGPPGP*PG*P*PGPPGPPGPSGGGYDFGYEGDFYR^{1118}	α_2:^{1081}GSQGSQGPAGPPG*P*PG*P*PGPPGPPGPSGGGYDFGYEGDFYR^{1118}
–	–	–	α_2:^{1141}SLNNQIETLLTPEGSR^{1156}	PPGPSGGGYDFGYEGDFYR^{1118}

* P indicates the hydroxylated proline.

gel strength in this investigation. Possibly, the hydrochloric acid broke down the amino acid polymer chain at the appropriate limit due to its low concentration. This enhanced the effect in the formation process of gel. Afterwards, amino acid monomer chains joined to form a continuous three-dimensional structure and bend water to form a compact gel structure. It was worth mentioning that the excessive hydrolysis of acid in the pretreated pigskin would have a negative impact on the gel strength of the sample. The gel strength of gelatin derived from pig skin that underwent pretreatment with sulfuric acid was the lowest. It might be that sulfuric acid was a strong binary acid. Under the same acid concentration, the concentration of hydrogen ions was higher, which over-hydrolyzed collagen molecules, resulting in a lower force required for gel formation. Furthermore, the gel strength of gelatin was found to be correlated with the concentration of its α component. The degradation of α components in gelatin (Fig. 1.), resulted in poor gel-forming ability(Duthen et al., 2021).

3.3. *Effect of acid pretreatments with various acid types on gelation and melting temperatures of pigskin gelatin*

The rheology of extracted gelatin was analyzed using temperature scanning to investigate the impact of different acid pretreatments on the storage modulus (G') and loss modulus (G"). Fig. 3 showed the variation curves of G' and G" of gelatin derived from pig skin that underwent pretreatment with various types of acids at 5–40 °C during heating and cooling processes, as well as the melting and gelation temperatures. G' and G" of all pigskin gelatin samples in the gelling and melting process decreased with the increase of temperature, and increased with the decrease of temperature. At the initial temperature of 40 °C, the values of G' and G" in gelatin were relatively low, and G" > G'. Those indicated that the gelatin molecules were arranged in a single chain and the samples were in a solution state. When the temperature dropped to 5 °C, both G" and G" increased sharply to remain stable. At this time, G" < G', the increase of G" was caused by the formation of a triple helix, which promoted gel formation.

During cooling and heating processes, there existed an intersection point of G' and G" and they were in equilibrium at that point, which corresponded to the gelation and melting temperatures of gelatin, respectively(Anvari & Chung, 2016). In this work, the gelation temperatures of gelatin derived from pig skin that underwent pretreatment with hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid and citric acid were 20.81, 19.56, 20.31, 26.81 and 21.05 °C, and the melting temperatures were 29.18, 28.17, 29.17, 34.17 and 30.18 °C, respectively. The two types of weak acids had the highest gelation and melting temperatures. Particularly worth mentioning was that the gelation and melting temperatures of gelatin derived from pig skin that underwent pretreatment with acetic acids were much higher than those of the other four samples. Probably because acetic acid could dissolve collagen better, but it was not easy to cause further degradation of collagen. Collagen was more stable, which led to the highest molecular weight integrity of gelatin, and thus made the cross-linking of the internal gel network increase(Felician et al., 2018). This was consistent with the result of SDS-PAGE in Fig. 1. The gelatin obtained from pig skin that was pretreated with acetic acid showed the most concentrated bands of typical molecular weight. This suggested that the distribution of molecular mass of pigskin gelatin would significantly affect the gelation and melting temperatures. The gelation and melting temperatures of gelatin derived from pig skin that underwent pretreatment with sulfuric acid were the lowest. According to Gilsenan and Ross-Murph, gelatin with low molecular-weight had a lower melting temperature compared to gelatin with high molecular weight(Gilsenan, 2000). The lower molecular weight, the more crosslinks per unit volume were needed to form a gel(Elharfaoui et al., 2007). Therefore, the gelatin derived from pig skin pretreated with sulfuric acid which displayed lower molecular weight, had lower gelling properties. The gelation and melting temperatures of gelatin derived from pig skin that underwent

pretreatment with hydrochloric acid and phosphoric acid were similar, perhaps due to having similar molecular weight distributions.

According to the analysis of the molecular weight distribution and gelling properties of different samples, it was found that pigskin gelatin extracted from pig skin pretreated with various acid types contained different properties. Therefore, in practical applications, different acids could be selected for pretreatment according to different needs. Pigskin could be pretreated with hydrochloric acid for high gel strength. In addition, acetic acid pretreatment could be used for high gelation and melting temperatures requirements. It provides a reference for the industrial production of pigskin gelatin with specific gelling properties.

3.4. Effect of acid pretreatments with various acid types on the identification of characteristic peptides of pigskin gelatin

3.4.1. Overall analysis of characteristic peptides from pigskin gelatin by various types of acid pretreatments

HPLC-MS/MS was used to identify the characteristic polypeptides of gelatin extracted from pig skin pretreated with various types of acids. Moreover, Venn diagrams were employed to analyze the similarities and differences between different pigskin gelatin samples. In this work, 248, 150, 213, 249, and 239 characteristic peptides were detected in gelatin derived from pig skin pretreated with hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, and citric acid, respectively. Among them, 69 characteristic peptides appeared simultaneously in gelatin samples extracted from pig skin pretreated with various types of acids, and these peptides were referred to as common characteristic peptides. Accordingly, the tryptic peptides obtained from one or two gelatin samples were defined as non-common characteristic peptides. 179, 81, 144, 180, 170 non-common characteristic peptides were detected in the gelatin derived from pig skin pretreated with hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid and citric acid. The number of characteristic peptides in the samples extracted from pig skin that underwent pretreatment with sulfuric acid was significantly lower than that of the other four samples. It was probably because sulfuric acid could aggravate the hydrolysis of gelatin, leading to high-degree hydrolysis of fragmented molecules to the small peptides that lost its characteristic properties(Derkach et al., 2019). The sample derived from pig skin that underwent pretreatment with acetic acid had the highest number of characteristic peptides, indicating that there was less cleavage of peptide bonds within collagen molecules. The structural stability of pig skin collagen was strong, and its peptide sequence could be better preserved during the extraction process. This was consistent with the previous trend of SDS-PAGE results.

3.4.2. Analysis of non-common characteristic peptides from pigskin gelatin pretreated with various types of acids

Many non-common characteristic peptides were detected in the gelatin derived from pigskin. As described above, 179, 81, 144, 180 and 170 non-common characteristic peptides were detected in gelatin derived from pig skin pretreated with hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid and citric acid, respectively. Table 1 showed 36, 17, 19, 37 and 37 characteristic peptides existed only in gelatin derived from pig skin pretreated with hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid and citric acid, respectively. Among them, the gelatin derived from pig skin that underwent pretreatment with sulfuric acids possessed fewer common characteristic peptides than gelatin samples from other groups. This was probably because sulfuric acid was unfavorable to the extraction of pigskin gelatin, and the least characteristic peptides of gelatin were detected under sulfuric acid treatment conditions resulting in a lower overlap of Venn diagrams. It showed 10 cases of non-common characteristic peptides of gelatin extracted from pig skin that existed in three kinds of acid pretreatment at the same time. In addition, it had five cases of non-common characteristic peptides of gelatin extracted from pig skin pretreated with four acids simultaneously. The number of characteristic peptide bars that they each share was shown in Fig. 4. The non-common characteristic peptides changed with the type of acid used for pretreatment, indicating that the existence of non-common characteristic peptides was unstable and could not be used as identification peptides.

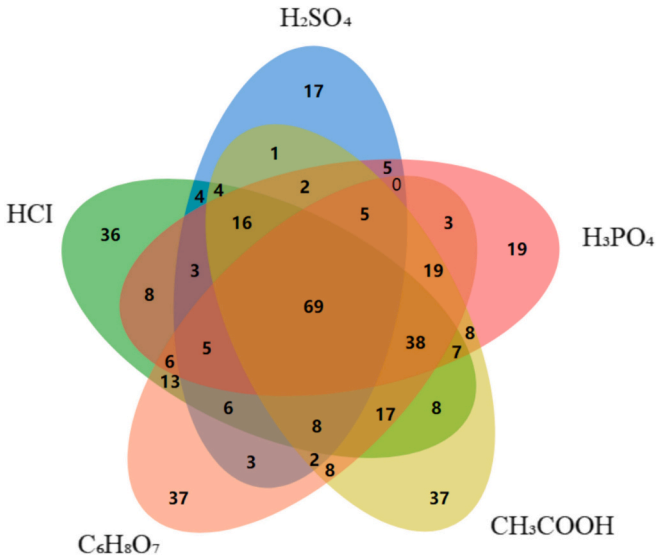

Fig. 4. Analysis of pigskin gelatins characteristic peptides from pigskin gelatin by various types of acid pretreatments.

3.4.3. Analysis of common characteristic peptides of pigskin gelatin pretreated with different acids

The 69 common characteristic peptides (44 from α_1 chain and 25 from α_2 chain) from five kinds of gelatins extracted from pig skin pretreated with various types of acids are shown in Table 2. These common characteristic peptides did not alter with the change of acid types, and they could be used as characteristic peptides for the traceability of pigskin gelatins. These common characteristic peptides were in two forms including unmodified and modified conditions There were 10 unmodified characteristic peptides out of 69 common characteristic peptides. Among them, 6 were from α_1 chain and 4 were from α_2 chain. Unmodified common characteristic peptides from α_1 chain contained ^{271}GFSGLDGAK^{279}、^{601}GVPGPPGAVGPAGK^{614}、^{920}GSPGADGPAGAP GTPGPQGIAGQR^{943}、^{956}GFPGLPGPSGEPGK^{969}、^{1052}GETGPAG-PAGPVGPVGAR^{1069}、^{1097}GFSGLQGPPGPPGSPG EQGPSGASGPAGPR^{1126}. Unmodified common characteristic peptides from α_2 chain were ^{694}GEAGPAGPAGPAGPR^{708}、^{715}GEVGPAGPNG-FAGPAGAAGQPGAK^{738}、^{831}TGETGASGPP GFAGEK^{846}、^{979}GEPGPAGSVGPAGAVGPR^{996}. The unmodified common characteristic peptides were easy to identify and match with fragment ions, and had an important application prospect in the identification of pigskin gelatin. In the second category, a total of 59 common characteristic peptides containing proline-hydroxylation modification were identified, of which 38 were from the α_1 chain and 21 were from the α_2 chain. Partial hydrolysis of collagen including hydroxylation of proline produce large amounts of hydroxyproline (Fernandes et al., 2011; Song & Mechref, 2013). Furthermore, there were characteristic peptide fragments with the same sequence but varying modifications. They were divided into different numbers of hydroxylation modification sites and the same number of modifications but different modification positions. This complex modification made the sequence recognition more difficult. Fig. 5. showed the secondary mass spectra of three common characteristic peptides from gelatin extracted from pig skin pretreated with various types of acids, which had the same amino acid sequence but different hydroxylation sites of proline. In other words, the peaks with *m/z* values of 1259.1155^{2+}, 1267.1130^{2+} and 1275.1105^{2+} were all from the α_1 chain of pigskin gelatin but with

Table 2
Common characteristic peptides of pigskin gelatin extracted at different acid pre-treatments.

Characteristic peptides of pigskin gelatin	*m/z*	*Ms*		
		theor	obsd	error (ppm)
α_1:^{271}GFSGLDGAK^{279}	426.7202^{2+}	851.4258	851.4224	3.9370
α_1:^{289}GE*PGS*PGENGA*PGQMGPR^{306}	872.3772^{2+}	1742.7399	1742.7344	3.1351
α_1:^{325}GNDGATGAAGPPGPTGPAGP*PGFPGAVGAK^{354}	1259.1155^{2+}	2516.2165	2516.2144	0.8299
α_1:^{325}GNDGATGAAG*PPGPTGPAGPPGF*PGAVGAK^{354}	1267.1130^{2+}	2532.2114	2532.2100	0.5564
α_1:^{325}GNDGATGAAGPPGPTGPAGP*PGF*PGAVGAK^{354}	1267.1130^{2+}	2532.2114	2532.2088	1.0299
α_1:^{325}GNDGATGAAG*PPGPTGPAGPPGF*PGAVGA*K^{354}	1275.1105^{2+}	2548.2063	2548.2040	0.9136
α_1:^{325}GNDGATGAAG*PPGPTGPAGP*PGF*PGAVGAK^{354}	1275.1105^{2+}	2548.2063	2548.2044	0.7568
α_1:^{325}GNDGATGAAG*PPGPTGPAGP*PGF*PGAVGAK^{354}	850.4094^{3+}	2548.2063	2548.2135	-2.8104
α_1:^{373}GE*PG*PPGPAGAAGPAGNPGADGQPGGK^{399}	1136.0289^{2+}	2270.0433	2270.0460	-1.2060
α_1:^{418}GPSGPQGPSGP*PGPK^{432}	667.3344^{2+}	1332.6543	1332.6510	2.4575
α_1:^{418}GPSGPQG*PSGP*PGPK^{432}	675.3319^{2+}	1348.6492	1348.6524	-2.3692
α_1:^{451}GE*PGPTGVQGP*PGPAGEEGK^{470}	925.9354^{2+}	1849.8563	1849.8530	1.7766
α_1:^{475}GE*PGPAGL*PGP*PGER^{489}	718.8479^{2+}	1435.6812	1435.6772	2.7892
α_1:^{496}GF*PGADGVAGPK^{507}	545.2759^{2+}	1088.5371	1088.5338	3.0443
α1:^{541}GLTGS*PGSPGPDGK^{554}	622.3054^{2+}	1242.5961	1242.5906	4.4191
α_1:^{601}GVPGPPGAVGPAGK^{614}	581.3284^{2+}	1160.6423	1160.6390	2.8039
α_1:^{601}GV*PGPPGAVGPAGK^{614}	589.3259^{2+}	1176.6372	1176.6342	2.5198
α_1:^{601}GV*PGP*PGAVGPAGK^{614}	597.3233^{2+}	1192.6321	1192.6284	3.0888
α_1:^{615}DGEAGAQGP*PGPAGPAGER^{633}	854.3937^{2+}	1706.7729	1706.7696	1.9136
α_1:^{712}GDAGAPGA*PGSQGA*PGLQGM*PGER^{735}	1092.4964^{2+}	2182.9782	2182.9762	0.9199
α_1:^{712}GDAGA*PGA*PGSQGA*PGLQGM*PGER^{735}	1100.4939^{2+}	2198.9731	2198.9666	2.9668
α_1:^{805}GE*PG*PPGPAGFAGP*PGADGQ*PGAK^{828}	1075.9966^{2+}	2149.9785	2149.9762	1.0874
α_1:^{851}GSAGP*PGATGF*PGAAGR^{867}	730.8535^{2+}	1459.6925	1459.6896	1.9566
α_1:^{868}VGPPG*PSGNAG*P*PGPPGPAGK^{888}	907.4511^{2+}	1812.8875	1812.8812	3.4933
α_1:^{920}GSPGADGPAGAPGTPGPQGIAGQR^{943}	1037.5127^{2+}	2073.0108	2073.0090	0.8819
α_1:^{920}GSPGADGPAGA*PGTPGPQGIAGQR^{943}	1045.5102^{2+}	2089.0058	2089.0022	1.6977
α_1:^{920}GS*PGADGPAGA*PGTPGPQGIAGQR^{943}	1053.5076^{2+}	2105.0007	2104.9976	1.4570
α_1:^{956}GFPGLPGPSGEPGK^{969}	649.3364^{2+}	1296.6583	1296.6536	3.6114
α_1:^{956}GFPGL*PGPSGEPGK^{969}	657.3339^{2+}	1312.6532	1312.6496	2.7459
α_1:^{956}GF*PGLPGPSGEPGK^{969}	657.3339^{2+}	1312.6532	1312.6510	1.6810
α_1:^{956}GF*PGL*PGPSGEPGK^{969}	665.3314^{2+}	1328.6481	1328.6450	2.3522
α_1:^{956}GF*PGL*PGPSGE*PGK^{969}	673.3288^{2+}	1344.6431	1344.6406	1.8193
α_1:^{980}GPPGPMGPPGLAGP*PGESGR^{999}	901.4422^{2+}	1800.8698	1800.8680	0.9762
α_1:^{980}G*PPGPMGPPGLAGP*PGESGR^{999}	909.4396^{2+}	1816.8647	1816.8616	1.6934
α_1:^{1022}GESGPAGP*PGA*PGA*PGAPGPVGPAGK^{1047}	1078.5280^{2+}	2155.0415	2155.0400	0.6815
α_1:^{1052}GETGPAGPAGPVGPVGAR^{1069}	774.4059^{2+}	1546.7973	1546.7938	2.2275
α_1:^{1097}GFSGLQGPPGPPGSPGEQGPSGASGPAGPR^{1126}	886.7641^{3+}	2657.2703	2657.2677	0.9811
α_1:^{1097}GFSGLQGPPGPPGS*PGEQGPSGASGPAGPR^{1126}	1337.6399^{2+}	2673.2652	2673.2644	0.3102
α_1:^{1097}GFSGLQGPPGPPGS*PGEQGPSGASGPAGPR^{1126}	892.0957^{3+}	2673.2652	2673.2637	0.5717
α_1:^{1097}GFSGLQGPPGP*PGS*PGEQGPSGASGPAGPR^{1126}	1345.6374^{2+}	2689.2602	2689.2566	1.3191
α_1:^{1097}GFSGLQGPPGP*PGS*PGEQGPSGASGPAGPR^{1126}	897.4274^{3+}	2689.2602	2689.2585	0.6129
α_1:^{1097}GFSGLQGP*PG*P*PGSPGEQGPSGASGPAGPR^{1126}	1353.6348^{2+}	2705.2551	2705.2466	3.1249
α_1:^{1097}GFSGLQGPPG*P*PGS*PGEQGPSGASGPAGPR^{1126}	1353.6348^{2+}	2705.2551	2705.2506	1.6474
α_1:^{1097}GFSGLQGPPG*P*PGS*PGEQGPSGASGPAGPR^{1126}	902.7590^{3+}	2705.2551	2705.2542	0.3175
α_2:^{166}GF*PGTPGL*PGFK^{177}	604.3150^{2+}	1206.6154	1206.6124	2.4739
α_2:^{265}GELGPVGN*PGPAGPAGPR^{282}	808.9167^{2+}	1615.8187	1615.8164	1.4278
α_2:^{328}GI*PGPAGAAGATGAR^{342}	620.8293^{2+}	1239.6440	1239.6388	4.2121
α_2:^{414}AGVMGP*PGSR^{423}	473.2382^{2+}	944.4618	944.4576	4.4798
α_2:^{451}GF*PGSPGNVGPAGK^{464}	629.8184^{2+}	1257.6223	1257.6202	1.6275
α_2:^{451}GF*PGS*PGNVGPAGK^{464}	637.8159^{2+}	1273.6172	1273.6154	1.3875
α_2:^{487}GE*PGNIGFPGPK^{498}	593.8022^{2+}	1185.5899	1185.5880	1.5746
α_2:^{487}GE*PGNIGF*PGPK^{498}	601.7997^{2+}	1201.5848	1201.5806	3.4812
α_2:^{574}GI*PGEFGL*PGPAGPR^{588}	727.8790^{2+}	1453.7434	1453.7384	3.4484
α_2:^{574}GI*PGEFGL*PG*PAGPR^{588}	735.8765^{2+}	1469.7383	1469.7364	1.3182
α_2:^{592}GP*PGESGAAGPAGPIGSR^{609}	776.3852^{2+}	1550.7558	1550.7538	1.2687
α_2:^{694}GEAGPAGPAGPAGPR^{708}	631.8215^{2+}	1261.6284	1261.6248	2.8252
α_2:^{715}GEVGPAGPNGFAGPAGAAGQPGAK^{738}	1018.5069^{2+}	2034.9992	2035.0002	-0.4958
α_2:^{748}GENGPVGPTGPVGAAGPAGPNGP*PGPAGSR^{777}	1283.6293^{2+}	2565.2441	2565.2408	1.2815
α_2:^{778}GDGGPPGATGF*PGAAGR^{794}	729.8457^{2+}	1457.6768	1457.6762	0.4247
α_2:^{778}GDGGP*PGATGF*PGAAGR^{794}	737.8432^{2+}	1473.6717	1473.6694	1.5857
α_2:^{795}IGPPG*PSGISG*P*PGPPGPAGK^{815}	921.9769^{2+}	1841.9392	1841.9370	1.2148
α_2:^{795}IG*P*PGPSGISGPPGP*PG*PAGK^{815}	929.9744^{2+}	1857.9342	1857.9286	2.9893
α_2:^{831}TGETGASGPPGFAGEK^{846}	732.3477^{2+}	1462.6809	1462.6798	0.7374
α_2:^{831}TGETGASGP*PGFAGEK^{846}	740.3452^{2+}	1478.6758	1478.6726	2.1544
α_2:^{883}GL*PGVAGSVGE*PGPLGIAGP*PGAR^{906}	1066.5644^{2+}	2131.1142	2131.1130	0.5813
α_2:^{949}GY*PGNPGPAGAAGA*PGPQGAVGPAGK^{974}	1103.5415^{2+}	2205.0683	2205.0666	0.7884
α_2:^{979}GEPGPAGSVGPAGAVGPR^{996}	767.3981^{2+}	1532.7816	1532.7804	0.7819
α_2:^{979}GE*PGPAGSVGPAGAVGPR^{996}	775.3956^{2+}	1548.7765	1548.7728	2.3988
α_2:^{1081}GSQGSQGPAGPPGP*PG*P*PG*PPGPSGGGYDFGYEGDFYR^{1118}	1228.2003^{3+}	3681.5790	3681.5838	-1.3000

* P indicates the hydroxylated proline.

1, 2 and 3 proline hydroxylation sites, respectively. A series of fragment ions generated by MS/MS accurately demonstrated the same sequence and different hydroxyproline numbers. As shown in Fig. 5A&C, fragment ions with y9-y10 were useful to confirm the common proline hydroxylation site. Meanwhile, y6-y7, y19-y20 ions in Fig. 5B&C indicated the second and third modified site in the peak of 1267.1130^{2+} and 1275.1105^{2+}. In addition, the mass spectrum peaks of tryptic peptides were detected in different charges, in which +2 and + 3 were the most frequent. Theoretically, the peaks with all charges could be used to identify and analyze proteins with high accuracy.

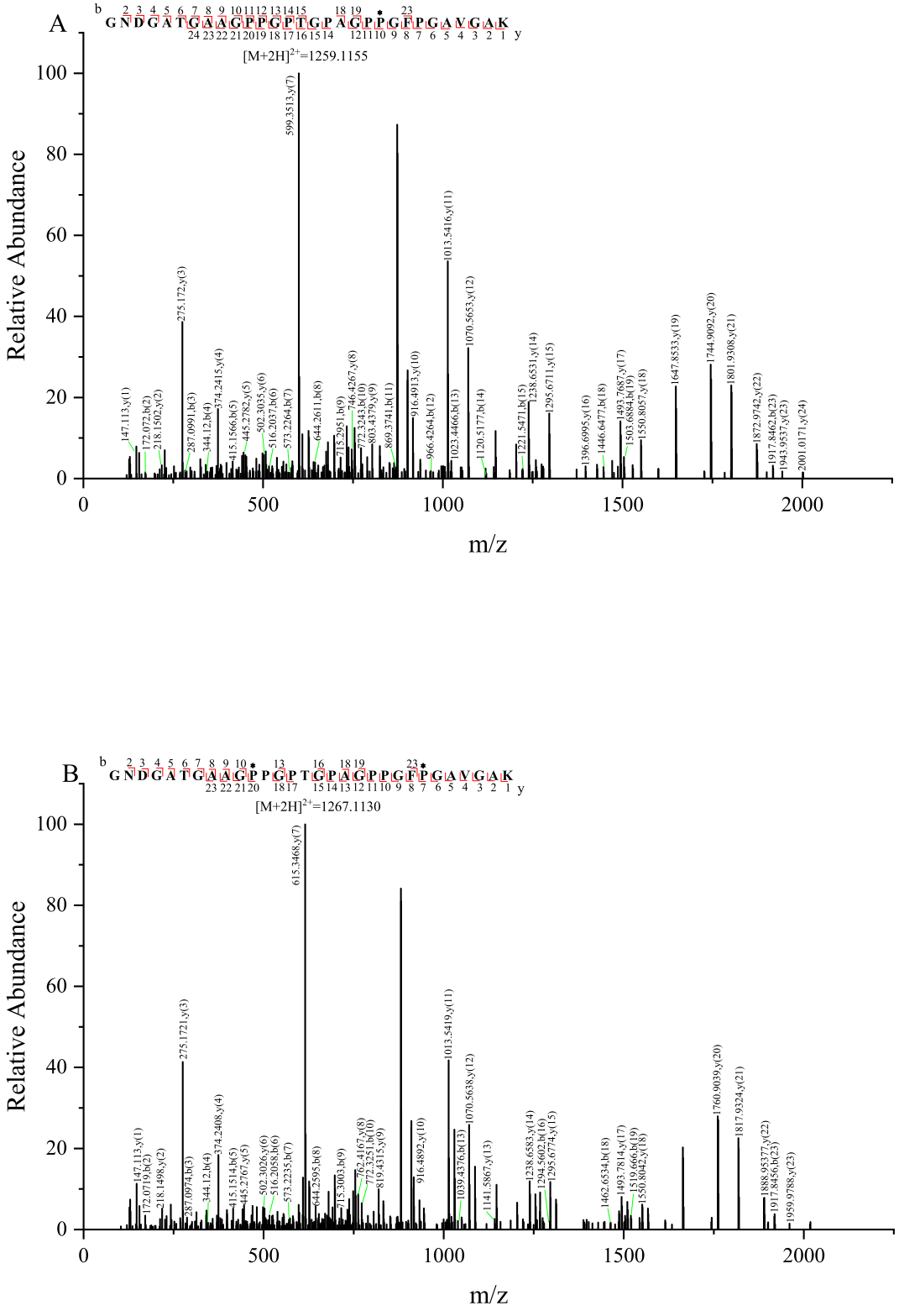

Fig. 5. Secondary mass spectra of characteristic peptides of pigskin gelatin. (A): Pigskin gelatin α_1 chain ^{325}GNDGATGAAGPPGPTGPAGP*PGFPGAVGAK^{354} (Pro345 was hydroxylated). (B): Pigskin gelatin α_1 chain ^{325}GNDGATGAAG*PPGPTGPAGPPGF*PGAVGAK^{354} (Pro335 and Pro348 were hydroxylated). (C): Pigskin gelatin α_1 chain ^{325}GNDGATGAAG*PPGPTGPAGP*PGF*PGAVGAK^{354} (Pro335, Pro345 and Pro348 were hydroxylated).

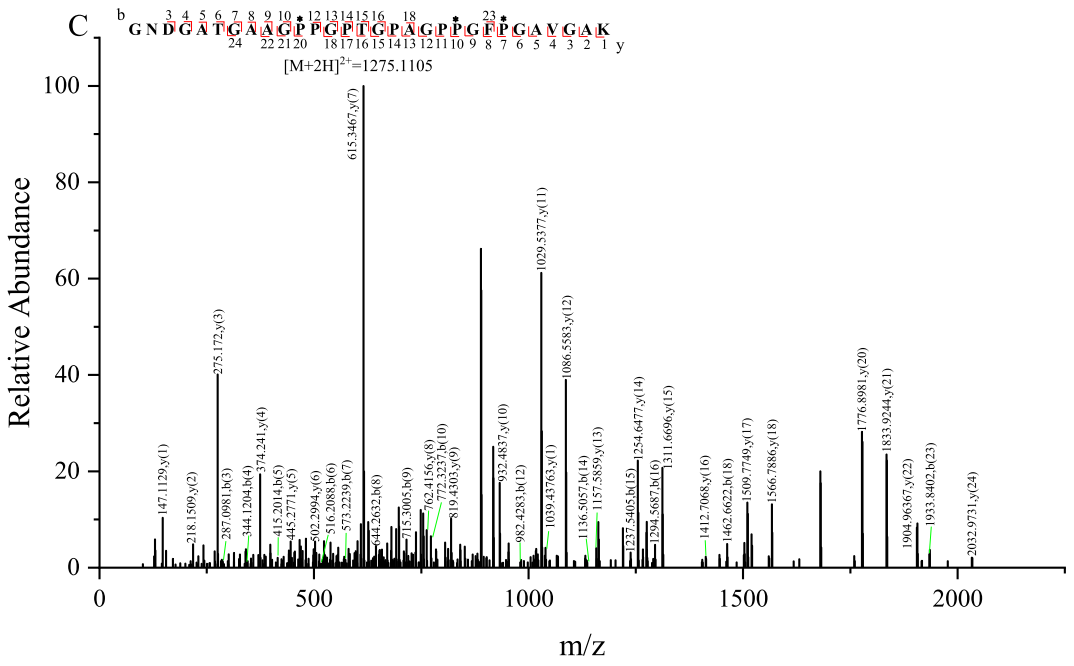

Fig. 5. (*continued*).

3.4.4. *Comparative analysis of common characteristic peptides in pigskin gelatin prepared from different conditions*

Our previous research had shown that extraction batch, temperature, time, pH, and glycation during gelatin processing significantly affected the identification of characteristic peptides in pigskin gelatin(Sha et al., 2019; Sha et al., 2020; Sha et al., 2022; Sha et al., 2023; Tang et al., 2024). Moreover, common characteristic peptides that did not vary with these extraction conditions, were identified and collected for the identification in pigskin gelatin. To further explore stable common characteristic peptides, a comparative analysis was conducted on characteristic peptides of pigskin gelatin prepared under different pretreatment conditions, extraction conditions, and processing processes to develop a more precise database of the identification in pigskin gelatin. Table 3 demonstrates the comparison of characteristic peptides extracted from pigskin gelatin under five distinct settings. A total of 8 common characteristic peptides were identified, comprising 2 α_1 chain peptides and 6 α_2 chain peptides. These common characteristic peptides were detected in trypsin-digested pigskin gelatin obtained under different conditions, suggesting their high stability. Thus, these characteristic peptides could offer important data support for the identification of pigskin gelatin. Prior literature also reported some characteristic peptides of pig gelatin. For example, Kleinnijenhuis identified the characteristic peptide ^{574}GI*PGEFGL*PGPAGPR^{588} (*P represented proline hydroxylation), which was also detected in this study (Table 3)(Kleinnijenhuis et al., 2018). In addition, Grundy et al. discovered five characteristic peptides from pig gelatin (Grundy et al., 2016). Among them, three characteristic peptides appeared in our final shared characteristic peptide table (Table 3) including ^{920}GS*PGADGPAGA*PGTPGPQGIAGQR^{943}(α_1), ^{1052}GETGPAGPVGPVGAR^{1069}(α_1) and ^{979}GEPGPAGSVGPAGAVGPR^{996} (α_2) (No modification showing proline hydroxylation). Similarly, Cheng et al. detected the fragment ^{451}GEPGPTGVQGPPGPAGEEGK^{470} (α_1) (Cheng et al., 2012) with an m/z of 925.43261^{2+}, which was retained in different extraction temperature treatments(Sha et al., 2019), but destabilized in different acid pretreatments, which might be due to the susceptibility of gelatin to acid pretreatment. Therefore, to improve the accuracy of pig gelatin identification, it was necessary to match the mass spectrometry results one by one and explored as many common characteristic peptides as possible. Among the 8 common characteristic peptides, there were 2 unmodified common characteristic peptides, including ^{1052}GETGPAGPVGPVGAR^{1069} from α_1 and was ^{979}GEPGPAGSVGPAGAVGPR^{996} from α_2. These unmodified characteristic peptides were readily identifiable and had minimal interference, making them of great significance for the actual identification of pigskin gelatin. Furthermore, Table 3 listed the theoretical m/z values corresponding to the valence states of common characteristic peptides of +1, +2, +3, and + 4, to assist in the rapid identification of specific peptides in subsequent practical applications.

Table 3
Common characteristic peptides of pigskin gelatin between this work and previous investigations.

Pigskin gelatin characteristic peptides	MH^{+1}	MH^{+2}	MH^{+3}	MH^{+4}
α_1:^{920}GSPGADGPAGA*PGTPGPQGIAGQR^{943}	2089.005 8	1045.006 5	697.006 8	523.006 9
α_1:^{1052}GETGPAGPAGPVGPVGAR^{1069}	1546.7973	773.902 3	516.270 6	387.4548
α_2:^{328}GI*PGPAGAAGATGAR^{342}	1239.644 0	620.325 7	413.886 2	310.666 5
α_2:^{452}GF*PGS*PGNVGPAGK^{464}	1273.617 2	637.312 2	425.210 6	319.159 7
α_2:^{574}GI*PGEFGL*PGPAGPR^{588}	1453.743 4	727.375 3	485.252 7	364.191 3
α_2:^{831}TGETGASGP*PGFAGEK^{846}	1478.675 8	739.841 5	493.563 4	370.424 4
α_2:^{979}GEPGPAGSVGPAGAVGPR^{996}	1548.776 5	774.891 9	516.930 3	387.949 6
α_2:^{979}GE*PGPAGSVGPAGAVGPR^{996}	1532.781 6	766.894 4	511.598 7	383.950 9

* P indicates the hydroxylated proline.

4. Conclusions

To summarize, pigskin pretreated with various types of acids had significant effects on the molecular weight distribution and gelling properties of the extracted gelatin. The gel strength of gelatin extracted from hydrochloric acid pretreated pigskin was the highest (605.28 g). Although sulfuric acid was the most acidic, gel strength was lowest for gelatin extracted from sulfuric acid pretreated pigskin (426.75 g). It was shown that gel strength was influenced by a number of factors. The proper breakdown of the amino acid polymer chains by the acid enhanced the effect of the gel formation process. Also, degradation of the α component of gelatin led to poor gel formation. The SDS-PAGE results showed that the stronger the acidic treatment conditions, the less high molecular weight bands the samples contained and more low molecular weight bands were produced. Low molecular weight gelatin had a lower melting temperature compared to high molecular weight gelatin. Acetic acid pretreatment resulted in the highest gelation and melting temperatures (26.81 °C, 34.17 °C). The gelatin extracted from sulfuric acid pretreated pigskin had the poorest gelling properties with the lowest gel strength, gelation and melting temperatures (426.75 g/ 19.56 °C/28.17 °C). HPLC-MS/MS results showed that variations in the acids used for pretreatment produced different characteristic peptides. 248, 150, 213, 249 and 239 characteristic peptides were identified from gelatin extracted from pig skin pretreated with hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, and citric acid, respectively, which contained 69 common characteristic peptides. By comparing the characteristic peptides extracted from pigskin gelatin under different acid pretreatments, extraction conditions and processing, a total of 8 common characteristic peptides were identified, consisting of 2 peptides from the α_1 chain and 6 peptides from the α_2 chain. These common characteristic peptide sequences could be used as the basis for the traceability of pigskin gelatin. The results of rheology, texture analysis and mass spectrometry showed that pretreated pig skin with different acids would affect the gelling properties and traceability of gelatin extracted from pig skin.

CRediT authorship contribution statement

Zi-Xuan Yang: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Xiao-Mei Sha:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hui Wang:** Supervision, Resources, Project administration, Conceptualization. **Ting Fang:** Writing – review & editing. **Sheng Shu:** Software, Data curation. **Zong-Cai Tu:** Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest to this work, and the manuscript is approved by all authors for publication.

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Data availability

Data will be made available on request.

References

Ahmad, M., & Benjakul, S. (2011). Characteristics of gelatin from the skin of unicorn leatherjacket (Aluterus monoceros) as influenced by acid pretreatment and extraction time. *Food Hydrocolloids, 25*(3), 381–388. https://doi.org/10.1016/j.foodhyd.2010.07.004

Alipal, J., Mohd Pu'ad, N. A. S., Lee, T. C., Nayan, N. H. M., Sahari, N., Basri, H., … Abdullah, H. Z. (2021). A review of gelatin: Properties, sources, process, applications, and commercialisation. *Materials Today: Proceedings, 42*, 240–250. https://doi.org/10.1016/j.matpr.2020.12.922

Anvari, M., & Chung, D. (2016). Dynamic rheological and structural characterization of fish gelatin – Gum arabic coacervate gels cross-linked by tannic acid. *Food Hydrocolloids, 60*, 516–524. https://doi.org/10.1016/j.foodhyd.2016.04.028

Balti, R., Jridi, M., Sila, A., Souissi, N., Nedjar-Arroume, N., Guillochon, D., & Nasri, M. (2011). Extraction and functional properties of gelatin from the skin of cuttlefish (Sepia officinalis) using smooth hound crude acid protease-aided process. *Food Hydrocolloids, 25*(5), 943–950. https://doi.org/10.1016/j.foodhyd.2010.09.005

Cao, S., Wang, Y., Xing, L., Zhang, W., & Zhou, G. (2020). Structure and physical properties of gelatin from bovine bone collagen influenced by acid pretreatment and pepsin. *Food and Bioproducts Processing, 121*, 213–223. https://doi.org/10.1016/j.fbp.2020.03.001

Cheng, X.-L., Wei, F., Xiao, X.-Y., Zhao, Y.-Y., Shi, Y., Liu, W., Zhang, P., Ma, S.-C., Tian, S.-S., & Lin, R.-C. (2012). Identification of five gelatins by ultra performance liquid chromatography/time-of-flight mass spectrometry (UPLC/Q-TOF-MS) using principal component analysis. *Journal of Pharmaceutical and Biomedical Analysis, 62*, 191–195. https://doi.org/10.1016/j.jpba.2011.12.024

Derkach, S. R., Kuchina, Y. A., Baryshnikov, A. V., Kolotova, D. S., & Voron'ko, N. G.. (2019). Tailoring cod gelatin structure and physical properties with acid and alkaline extraction. *Polymers, 11*(10), 1724. https://doi.org/10.3390/polym11101724

Doi, H., Watanabe, E., Shibata, H., & Tanabe, S. (2009). A reliable enzyme linked immunosorbent assay for the determination of bovine and porcine gelatin in processed foods. *Journal of Agricultural and Food Chemistry, 57*(5), 1721–1726. https://doi.org/10.1021/jf802733y

Duconseille, A., Astruc, T., Quintana, N., Meersman, F., & Sante-Lhoutellier, V. (2015). Gelatin structure and composition linked to hard capsule dissolution: A review. *Food Hydrocolloids, 43*, 360–376. https://doi.org/10.1016/j.foodhyd.2014.06.006

Duthen, S., Levasseur-Garcia, C., Kleiber, D., Violleau, F., Vaca-Garcia, C., Tsuchikawa, S., Delgado Raynaud, C., & Daydé, J. (2021). Using near-infrared spectroscopy to determine moisture content, gel strength, and viscosity of gelatin. *Food Hydrocolloids, 115*, Article 106627. https://doi.org/10.1016/j.foodhyd.2021.106627

Elharfaoui, N., Djabourov, M., & Babel, W. (2007). Molecular weight influence on gelatin gels: Structure. *Enthalpy and Rheology. Macromolecular Symposia, 256*(1), 149–157. https://doi.org/10.1002/masy.200751017

Felician, F. F., Xia, C., Qi, W., & Xu, H. (2018). Collagen from marine biological sources and medical applications. *Chemistry & Biodiversity, 15*(5). https://doi.org/10.1002/cbdv.201700557

Gilsenan, P. (2000). Rheological characterisation of gelatins from mammalian and marine sources. *Food Hydrocolloids, 14*(3), 191–195. https://doi.org/10.1016/S0268-005X(99)00050-8

Grundy, H. H., Reece, P., Buckley, M., Solazzo, C. M., Dowle, A. A., Ashford, D., … Collins, M. J. (2016). A mass spectrometry method for the determination of the species of origin of gelatine in foods and pharmaceutical products. *Food Chemistry, 190*, 276–284. https://doi.org/10.1016/j.foodchem.2015.05.054

Guo, S., Xu, X., Zhou, X., & Huang, Y. (2018). A rapid and simple UPLC-MS/MS method using collagen marker peptides for identification of porcine gelatin. *RSC Advances, 8*(7), 3768–3773. https://doi.org/10.1039/C7RA12539A

Hong, H., Fan, H., Chalamaiah, M., & Wu, J. (2019). Preparation of low-molecular-weight, collagen hydrolysates (peptides): Current progress, challenges, and future perspectives. *Food Chemistry, 301*, Article 125222. https://doi.org/10.1016/j.foodchem.2019.125222

Huang, T., Tu, Z.-C., Wang, H., Liu, W., Zhang, L., Zhang, Y., & ShangGuan, X.-C. (2017). Comparison of rheological behaviors and nanostructure of bighead carp scales gelatin modified by different modification methods. *Journal of Food Science and Technology, 54*(5), 1256–1265. https://doi.org/10.1007/s13197-017-2511-1

Kaewruang, P., Benjakul, S., & Prodpran, T. (2014). Characteristics and gelling property of phosphorylated gelatin from the skin of unicorn leatherjacket. *Food Chemistry, 146*, 591–596. https://doi.org/10.1016/j.foodchem.2013.09.111

Kim, H.-W., Song, D.-H., Choi, Y.-S., Kim, H.-Y., Hwang, K.-E., Park, J.-H., Kim, Y.-J., Choi, J.-H., & Kim, C.-J. (2012). Effects of soaking pH and extracting temperature on the physicochemical properties of chicken skin gelatin. *Korean Journal for Food Science of Animal Resources, 32*(3), 316–322. https://doi.org/10.5851/kosfa.2012.32.3.316

Kleinnijenhuis, A. J., van Holthoon, F. L., & Herregods, G. (2018). Validation and theoretical justification of an LC-MS method for the animal species specific detection of gelatin. *Food Chemistry, 243*, 461–467. https://doi.org/10.1016/j.foodchem.2017.09.104

Liao, W., Zhu, Y., Lu, Y., Wang, Y., Dong, X., Xia, G., & Shen, X. (2021). Effect of extraction variables on the physical and functional properties of tilapia gelatin. *LWT, 146*, Article 111514. https://doi.org/10.1016/j.lwt.2021.111514

Lim, Y. P., & Mohammad, A. W. (2011). Physicochemical properties of mammalian gelatin in relation to membrane process requirement. *Food and Bioprocess Technology, 4*(2), 304–311. https://doi.org/10.1007/s11947-009-0285-9

Lueyot, A., Rungsardthong, V., Vatanyoopaisarn, S., Hutangura, P., Wonganu, B., Wongsa-Ngasri, P., Charoenlappanit, S., Roytrakul, S., & Thumthanaruk, B. (2021).

Influence of collagen and some proteins on gel properties of jellyfish gelatin. *PLoS One, 16*(6), Article e0253254. https://doi.org/10.1371/journal.pone.0253254

See, S. F., Ghassem, M., Mamot, S., & Babji, A. S. (2015). Effect of different pretreatments on functional properties of African catfish (Clarias gariepinus) skin gelatin. *Journal of Food Science and Technology, 52*(2), 753–762. https://doi.org/10.1007/s13197-013-1043-6

Sha, X., Zhang, L., Chen, W., Wang, G., Li, J., Hu, Z., & Tu, Z. (2022). Characteristic tryptic peptides and gelling properties of porcine skin gelatin affected by thermal action. *International Journal of Food Science & Technology, 57*(3), 1573–1586. https://doi.org/10.1111/ijfs.15520

Sha, X.-M., Hu, Z.-Z., Xu, H., Zhang, L.-Z., & Tu, Z.-C. (2020). Identification and analysis of characteristic tryptic peptides from porcine gelatin extracted with multi-stage batch processing. *Food Hydrocolloids, 101*, Article 105540. https://doi.org/10.1016/j.foodhyd.2019.105540

Sha, X.-M., Hu, Z.-Z., Ye, Y.-H., Xu, H., & Tu, Z.-C. (2019). Effect of extraction temperature on the gelling properties and identification of porcine gelatin. *Food Hydrocolloids, 92*, 163–172. https://doi.org/10.1016/j.foodhyd.2019.01.059

Sha, X. M., Yan, N. Y., Chen, W. M., Xie, Z. H., & Lu, L. (2023). Effect of extraction pH on gelling properties and characteristic peptide identification of pigskin gelatin. *Food Science, 44*(18), 26–33. https://doi.org/10.7506/spkx1002-6630-20220903-027

Sultana, S., Hossain, M. A. M., Zaidul, I. S. M., & Ali, M. E. (2018). Multiplex PCR to discriminate bovine, porcine, and fish DNA in gelatin and confectionery products. *LWT, 92*, 169–176. https://doi.org/10.1016/j.lwt.2018.02.019

Tang, X.-Q., Wang, G.-Y., Hu, Z.-Z., & Sha, X.-M. (2024). The effect of glycation on the identification of characteristic tryptic peptides from porcine gelatin. *Food Hydrocolloids, 109909*. https://doi.org/10.1016/j.foodhyd.2024.109909

Tu, Z., Huang, T., Wang, H., Sha, X., Shi, Y., Huang, X., Man, Z., & Li, D. (2015). Physico-chemical properties of gelatin from bighead carp (Hypophthalmichthys nobilis) scales by ultrasound-assisted extraction. *Journal of Food Science and Technology, 52*(4), 2166–2174. https://doi.org/10.1007/s13197-013-1239-9

Zhang, G.-F., Liu, T., Wang, Q., Lei, J.-D., Ma, G.-H., & Su, Z.-G. (2008). Identification of marker peptides in digested gelatins by high performance liquid chromatography/mass spectrometry. *Chinese Journal of Analytical Chemistry, 36*(11), 1499–1504. https://doi.org/10.1016/S1872-2040(09)60003-7