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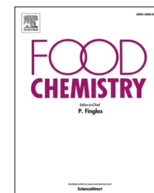

Top-down proteomics analysis revealed proteoform biomarkers for authenticating edible bird's nest

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ABSTRACT

Edible bird's nest (EBN), a prized delicacy valued for its nutrition and commercial significance, is often counterfeited. Authenticating its origin and verifying genuine EBN in processed products is essential for food safety and consumer trust. This study optimized sample preparation for top-down proteomic analysis to identify proteoforms as biomarkers for EBN authentication. Acidic mammalian chitinase proteoforms were consistently detected across EBNs from different origins, indicating their potential as authenticity markers. Among tested methods, chloroform/methanol precipitation produced the most reproducible proteoform profiles for distinguishing geographical origins. Additionally, thermostable proteoforms were found in hot aqueous extracts and instant EBN soups, supporting their use in verifying genuine EBN in processed foods. Our approach introduces a top-down proteomic method for authenticating EBN in raw and processed products, offering a promising tool for combating food fraud and enhancing food safety.

1. Introduction

Edible bird's nest (EBN), a prized delicacy in traditional Chinese medicine and cuisine, is primarily composed of salivary secretions of EBN-producing swiftlets (Kong et al., 2022). According to "Ben Cao Cong Xin", a new Chinese Compilation of Materia Medica, consumption of EBN can boost immunity, improve gastrointestinal health and restore stamina. Recent researches reported that extracts of EBN could stimulate growths of B lymphocytes and keratinocytes, resulting in enhancing productions of immunoglobulins and treating atopic dermatitis respectively (Lai et al., 2022; Zhao et al., 2016). Indonesia, Malaysia, Thailand, and Vietnam are major producers of EBN. The market price of EBN depends on its origin, colour, shape, size and cleanliness. In general, Indonesian EBN is the most affordable in Hong Kong market, followed by Thai and Vietnamese EBN. The price of Vietnamese EBN can be 4 to 6 times higher than the price of Indonesian EBN. Therefore, authenticating the geographical origin of EBN is essential not only for food traceability and protecting regional brandings, but also for quality assurance, food safety, and consumer trust.

Due to its high commercial value, EBN is often a target for food fraud and imitation. A few research attempted to develop methods for authenticating EBN from counterfeits using various approaches, like Fourier transform infrared spectroscopy (FTIR) (Guo et al., 2018), and nuclear magnetic resonance (NMR) spectroscopy (Yong et al., 2022). These methods differentiated EBN from counterfeits based on unique physical and chemical properties of EBN. Besides, authentications of EBN from counterfeits could be achieved by sequencing on mitochondrial or nuclear DNA sequences as well as real-time polymerase chain reaction (PCR)(Quek et al., 2018). These techniques could reliably differentiate genuine EBN from adulterated EBN and counterfeits, but failed to verify the origins of EBN. As the geographical origin of EBN is one of the determinants of its price, so there is a demand on methods for verifying the origin of EBN. Recently, metabolic and amino acid profiling using mass spectrometry (MS) were shown to be potential methods to differentiate the origins of EBN (Chua et al., 2014; Lee et al., 2022). These methods may significantly enhance the traceability of EBN and improving consumer trust. On top of that, verifying the presence of genuine EBN in processed food products poses a huge challenge. Instant

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Fig. 1. EBNs from different origins in South East Asia. The EBNs were directly purchased and imported from Indonesia, Thailand and Vietnam with the assistances from a local EBN vendor. As the EBNs received no chemical and physical treatments, so they still contained a few feathers (black in colour). Thus, only the white portions of the EBN were cut out for the top-down proteomic analysis to avoid contaminations from the feathers.

EBN soups are one of the popular products made of EBN. Due to the gelatinous appearance of processed edible bird's nest (EBN), the soup products are often susceptible to adulteration with gelatine, agar, peach gum or other substitutes with similar texture and appearance. This poses risks to consumer health and undermines product integrity. However, food processing and cooking significantly alter texture, appearance, as well as profiles of lipid, amino acid and metabolite of the food ingredients in the products (Wu et al., 2020; Xiao et al., 2022; Zhang et al., 2023). Although there were fingerprints for authenticating uncooked and unprocessed EBN from counterfeits as well as the geographical origins of EBN, these fingerprints may not be applicable in food products with processed EBN as the physical, chemical and metabolic features of EBN may be altered significantly during cooking and food processing. Lee and his research group attempted to detect mitochondrial cytochrome *b* gene sequence in 9 different processed commercial EBN products in Taiwan, but only 6 of them tested positive (Lee et al., 2019).

To address the need for developing reliable methods to authenticating the origin of EBN and the presence of genuine EBN in processed food products, the current study aimed to identify thermostable proteoforms in EBN using top-down proteomic approach to establish a fingerprint for verifying the presence of genuine EBN in processed food products. Previous proteomic study on EBN from different origins using 2-dimensional gel electrophoresis (2DGE) revealed that there were some differences in the proteomes of EBN originated from different areas (Seibold et al., 2009). The proteome of EBN may be served as a fingerprint for distinguishing EBNs from different origins and for other applications in the food industries. However, 2DGE is a demanding and tedious technique. Top-down proteomic technique is a relatively simple and rapid approach to identify proteoforms in food items and characterize the post-translational modifications of the proteoforms (Afzaal et al., 2022; Po & Eyers, 2023). Thus, it is possible to identify thermostable proteoforms in EBNs from different origins using top-down proteomic technique for authenticating the presence of genuine EBN in processed food products, like instant EBN soups.

2. Materials and methods

2.1. Sampling of EBN and instant EBN soup

EBNs originated from Indonesia, Vietnam and Thailand were kindly donated from a local EBN vendor who purchases and imports EBNs from Sout East Asia directly to Hong Kong (Fig. 1). The EBNs were harvested from the beams of the swiftlet houses, washed with water and air dried (Dai et al., 2021). At least 3 EBN samples from each origin have been used in the study. No chemical or physical treatment has been applied to the donated EBN samples. The EBN samples were stored in airtight dry box before analysis. Four brands of instant EBN soups which are commonly available in Hong Kong were selected for the study. Three batches of the instant EBN soups were purchased between September to November 2024. The instants EBN soups were stored at 4 °C before analysis.

2.2. Protein extraction and precipitations from EBN

White portions of the EBN cups were cut out and grinded into powder with quartz pestle and mortar in liquid nitrogen. The EBN powder was soaked with double distilled water (ddH_2O) at 4 °C for an hour in a ratio of 1 to 30 (weight per volume) following the previous study (Kong et al., 2016). After removing the soaking ddH_2O by centrifugating at 13000 g for 10 min at 4 °C, the EBN powder was stewed in ddH_2O at 100 °C for an hour to extract thermostable proteins from EBN. The proteins in the hot aqueous extract (HAE) were precipitated by 3 methods: 1) acetone (Ace) precipitation, 2) chloroform-methanol (CM) precipitation and 3) trichloroacetic acid (TCA) precipitation. For Ace precipitation, 1 portion of HAE was mixed with 4 portions of ice-cold acetone. After incubating at −20 °C overnight, the proteins were precipitated by centrifuging at 13000*g* for 10 min at 4 °C. The protein pellet was washed with ice-cold 80% Ace twice before air-dry. For CM precipitation, 100 μl HAE was mixed with 400 μl methanol thoroughly, followed by mixing with 200 μl chloroform thoroughly. Three hundred microliter ddH_2O was mixed thoroughly with the mixture before centrifuging at 14000*g* for 5 min at 4 °C. After removing the top aqueous layer, 500 μl methanol was then mixed with the protein flake and chloroform thoroughly before centrifuging at 20000*g* for 5 min at 4 °C. After removing the supernatant, the protein pellet was air dried at room temperature. For TCA precipitation, 1 portion of 20% TCA was mixed with 4 portions of HAE before incubating on ice for 30 min. The protein precipitate was harvested by centrifuging at 13000*g* for 20 min at 4 °C. The protein pellet was then washed with ice-cold 80% Ace twice before air-dry. The dried protein pellets were stored at −20 °C before top-down proteomic analysis.

2.3. Protein extraction and precipitations from instant EBN soup

The EBN residue in the instant EBN soup was removed by filtering through a sieve with 60 mesh (pore size: ∼ 250 μm). The filtrate was collected and quick frozen in liquid nitrogen. The filtrate was dried using a FD-12 freeze dryer (Labfreez, China). The dried filtrate was subsequently reconstituted in ddH_2O at one-tenth of its original volume. The proteins in the concentrate were extracted by Ace precipitation, CM precipitation and TCA precipitation as described previously for top-down proteomic analysis.

2.4. Top-down proteomic analysis

The air-dried protein pellets from different protein precipitation methods were incubated with 5 μl 80% formic acid (FA) at −20 °C for 15 min to dissolve the protein pellet for the top-down proteomic analysis (Donnelly et al., 2019). Afterwards, the samples were diluted with 15 μl mass spectrometry (MS) grade water. The samples were further diluted 10 times with 0.1% FA if necessary. The samples were analysed by Orbitrap Fusion Lumos Mass Spectrometer coupled with UltiMate™ 3000 RSLCnano system (ThermoFisher, USA) in data dependent acquisition (DDA) as described previously with modifications (Lau et al., 2025). Around 1 μg proteins were loaded into a BEH C_4 column (150

mm × 1 mm, 1.7 μm, 300 Å, ACQUITY, Waters, USA) which was equilibrated with 0.1% FA. The proteins were then fractionated by a linear elution gradient from 5% acetonitrile (ACN) to 50% for 45 min at the flow rate of 0.3 ml/ min. The Orbitrap was operated in positive ion mode. The spray voltage was 4000 V. The ion transfer temperature was 320 °C. The MS1 scan range was 500–2000 *m/z* with resolution at 60,000. The maximal injection time was 200 milliseconds. The MS/MS and scan range was 500–2000 m/z with resolution at 60,000. Isolations of the parent ions were carried out in quadrupole mode with isolation window of 10 m/z. The parent ions were fragmented by higher-energy collisional dissociation (HCD) with 25 to 35% of collision energy. After the fragmentation, the parent ion was excluded for 40 s. The raw data files generated from DDA mode were imported to BioPharma Finder v. 5.3 (Thermo Scientific, USA) for deconvoluting the mass

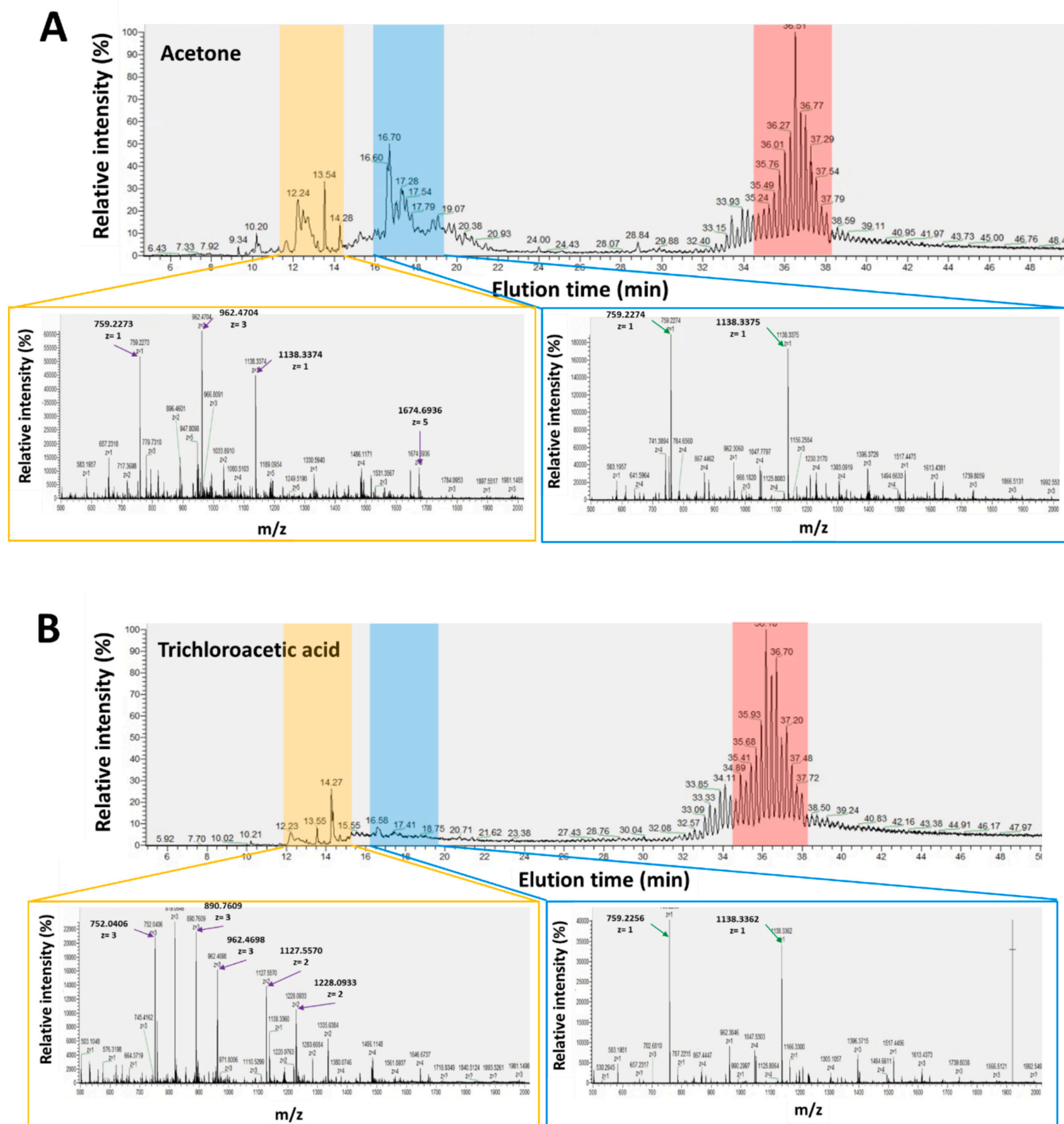


Fig. 2. The total ion chromatographs of HAEs of Indonesian EBN prepared with different precipitation methods. Ace (A) and TCA (B) precipitation resulted in similar peak profiles. The charges of the parent ions were around 1 to 5+ (indicated with purple and green arrows). Meanwhile, the CM precipitated samples contained multiple charged parent ions (C, indicated with purple and green arrows). The variations of the CM precipitated samples (blue) were found to be less compared to other precipitation methods (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

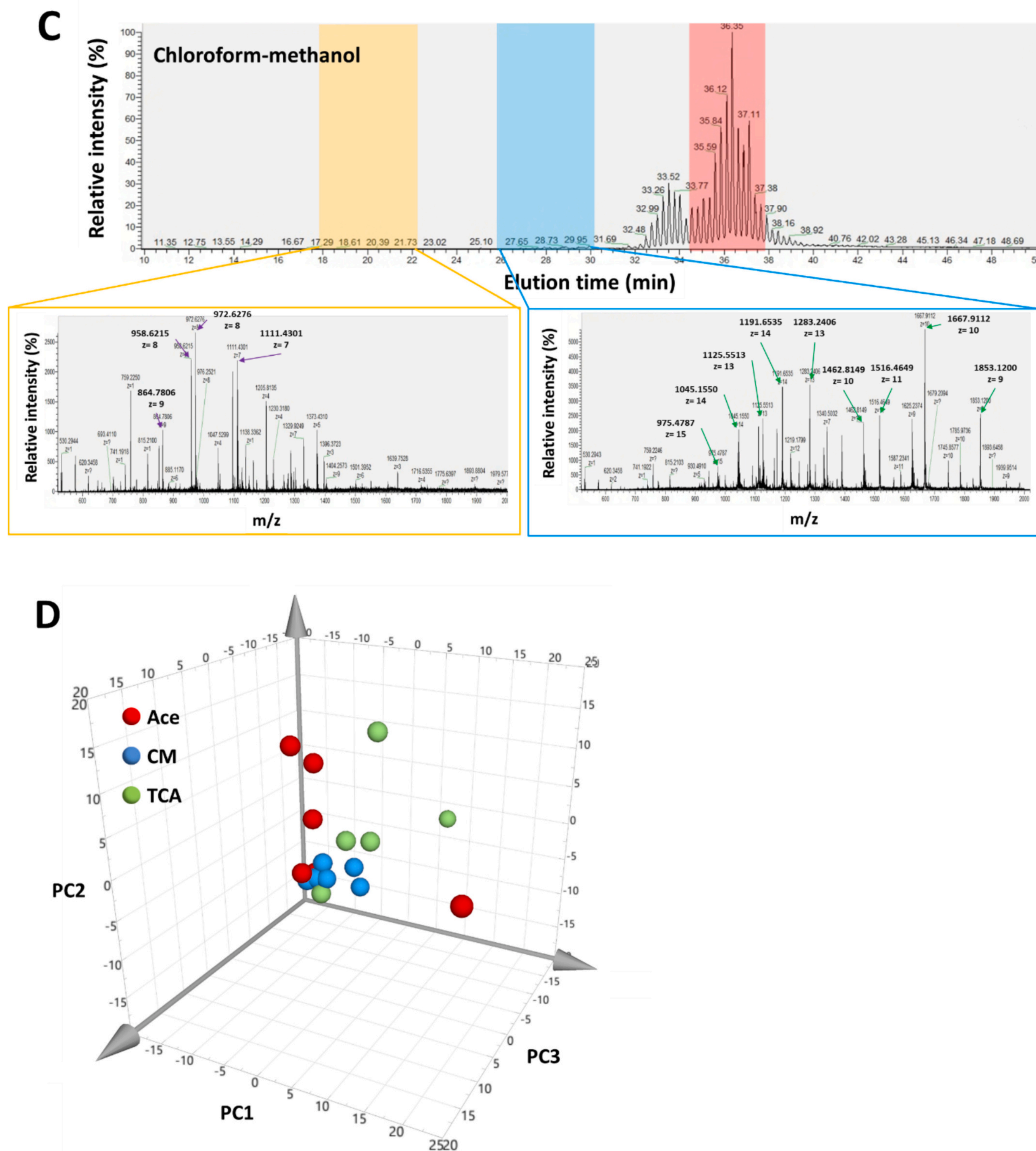


Fig. 2. (*continued*).

spectra using Xtract and ReSpect algorithms with default settings. The deconvoluted mass spectra were searched against an in-house database of coding sequences of EBN-producing swiftlet, *Aerodramus fuciphagus,* which contained 16,800 coding sequences using the top-down proteomic workflow from BioPharma Finder (Kong et al., 2022). The mass tolerance of parent and fragment ion was 20 ppm.

2.5. SDS-PAGE

Bradford protein assay and BCA protein assay were used to quantify the amounts of proteins in the HAEs of the EBNs from different origins. Ten micrograms of proteins from each HAE were mixed with 6 x SDS-PAGE sample loading buffer (75 mM TRIS-HCl pH 6.8, 9% SDS, 50%

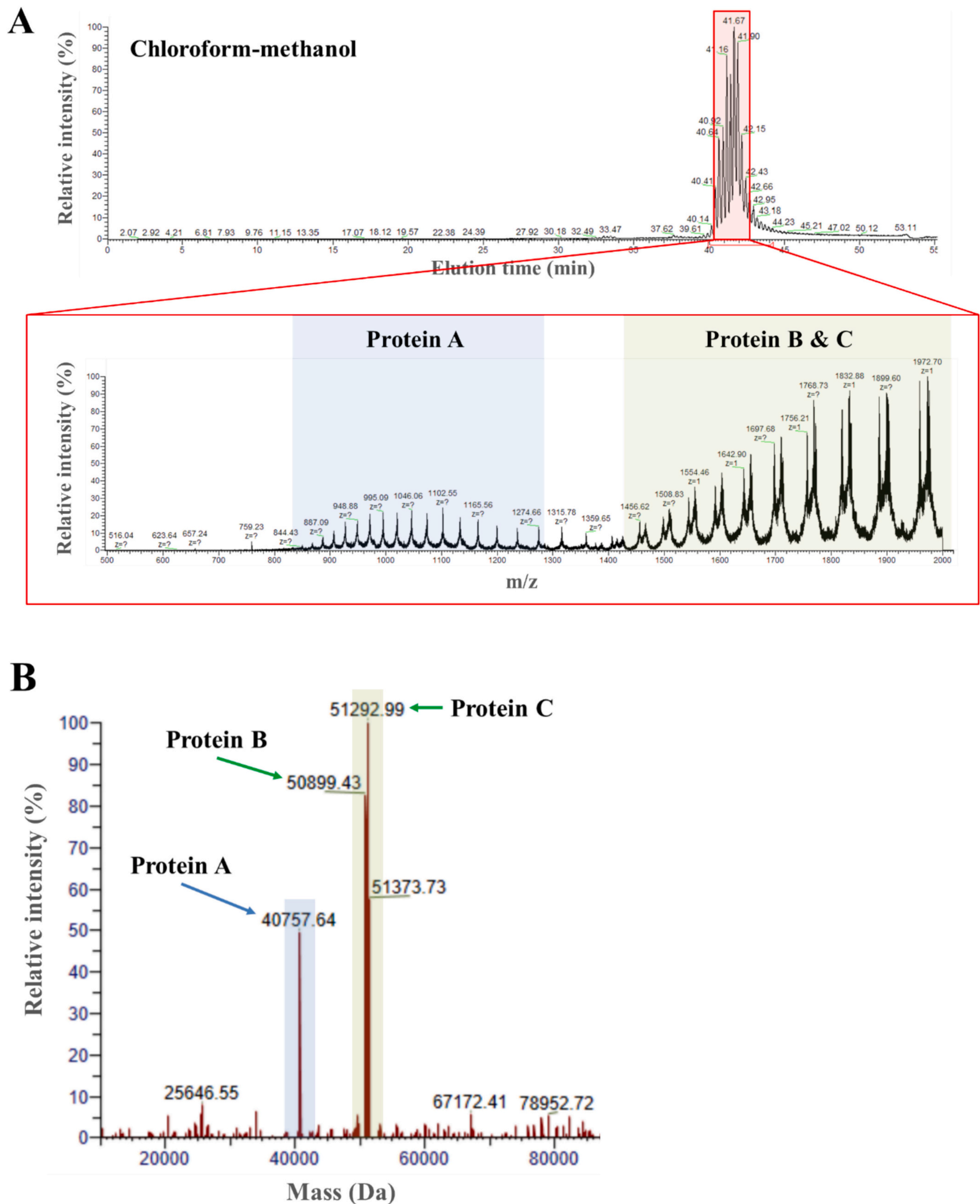


Fig. 3. Deconvolutions and identifications of proteins in HAEs of Indonesian EBN prepared with different precipitation methods. Three groups of ion clusters were commonly found in the Ace, TCA and CM precipitated samples (A). After the deconvolutions, the molecular weights of protein A, B and C were around 40. 76 kDa, 50.90 kDa and 51.29 kDa respectively (B). Protein bands with similar apparent molecular weights were observed in the SDS-PAGE (C).

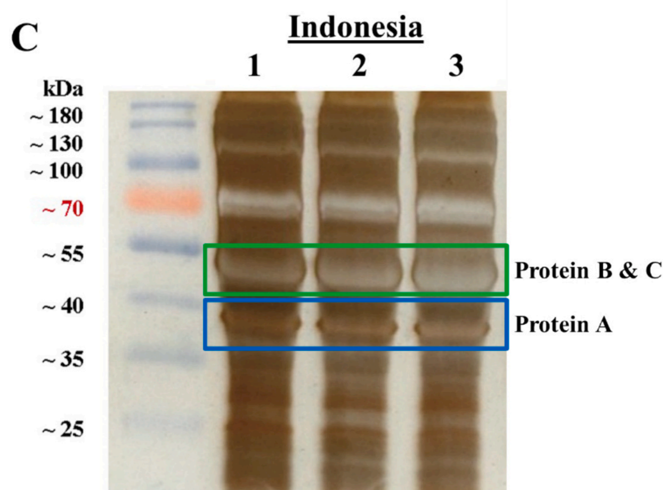


Fig. 3. (*continued*).

glycerol, and 0.03% bromophenol blue). After boiling for 5 min, the samples were loaded into 10% SDS-polyacrylamide gel and resolved with the aid of Mini-PROTEAN Tetra electrophoresis system (Bio-Rad, USA). The gel was then visualized using silver staining.

2.6. In-gel reduction, alkylation, tryptic digestion and peptide digestion

Protein bands of interest were cut out from the SDS-PAGE and cut into small pieces. The SDS-PAGE pieces were destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. After washing a few times with 25 mM ammonium bicarbonate, the SDS-PAGE pieces were dried with ACN. Subsequently, proteins in the dried SDS-PAGE pieces were reduced with 10 mM dithiothreitol (DTT) at 56 °C followed by alkylation with 55 mM iodoacetamide (IAA) in the dark. The proteins were then digested with sequencing-grade modified trypsin (Promega, USA) at 37 °C overnight. The tryptic peptides were extracted from the SDS-PAGE pieces with 50% ACN in 0.1% FA under ultrasonication. The tryptic peptides were dried under vacuum using CentriVap Centrifugal Concentrator (Labconco Corporation, USA) and redissolved in 10 μl 0.1% FA. The tryptic peptides were further cleaned up by Pierce™ C_{18} Spin Columns (ThermoFisher, USA) following the protocols from the manufacturer. The tryptic peptides were eluted with 200 μl 80% ACN in 0.1% FA. The elute was subsequently dried under vacuum and stored at −80 °C before the bottom-up proteomic analysis.

2.7. Bottom-up proteomic analysis

The dried tryptic peptides were re-dissolved 20 μl 0.1% FA for the bottom-up proteomic analysis. The samples were further diluted 10 times with 0.1% FA if necessary. The samples were analysed by Orbitrap Fusion Lumos Mass Spectrometer coupled with UltiMate™ 3000 RSLCnano system (ThermoFisher, USA) in data dependent acquisition (DDA). Around 1 μg peptides were loaded into a nanoflow UHPLC C_{18} column (15 cm × 75 μm, 1.7 μm, Aurora Eilte from Ionopticks, Australia) with equilibrated with 0.1% FA. The proteins were then fractionated by a linear elution gradient from 5% ACN to 30% for 60 min at the flow rate of 0.3 μl/ min. The Orbitrap was operated in positive ion mode. The spray voltage was 2000 V. The ion transfer temperature was 300 °C. The MS1 scan range was 400–1500 *m/z* with resolution at 60,000. The maximal injection time was 50 milliseconds. The MS/MS scan range was 100–1500 m/z with resolution at 30,000. Isolations of the parent ions were carried out in quadrupole mode with isolation window of 1.6 m/z. The parent ions were fragmented by HCD with 30% of collision energy. After the fragmentation, the parent ion was excluded for 40 s. The raw data files generated from DDA mode were imported to Proteome Discover v. 2.5 (Thermo Scientific, USA) for quantifying the relative abundances of the proteins in the samples with the mass tolerance below ±5 ppm.

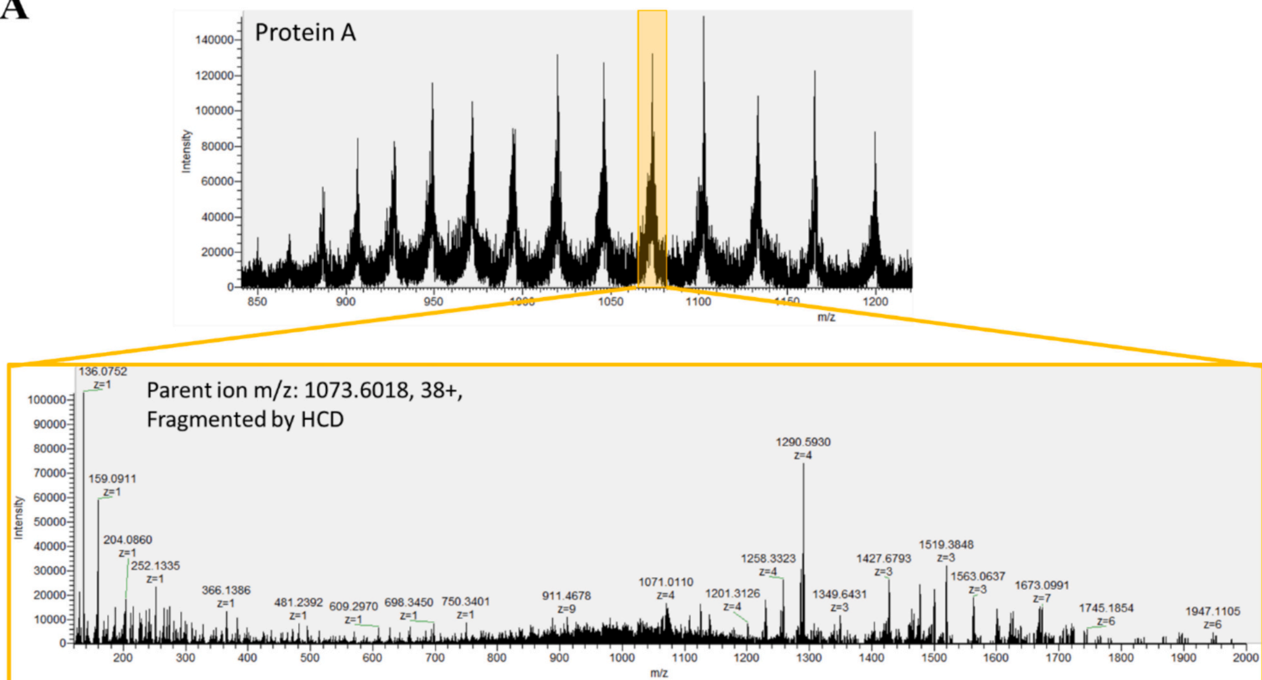
2.8. Statistical analysis

The peak lists of the top-down proteomic analysis were imported to SIMCA (Soft Independent Modeling of Class Analogies) for principal component analysis (PCA). At least 6 samples from each group were included in the PCA. Hotelling's T^2 and DModX (Distance to the Model in X-space) were used to identify the outliers in the datasets.

3. Results and discussion

3.1. Protein precipitation method played a critical role in the top-down proteomic analysis of EBN

The proteins in the Indonesian EBN were extracted using ddH_2O at 100 °C. Subsequently, the EBN proteins in the HAE were precipitated with 3 different methods before the top-down proteomic analysis. Ace and TCA precipitations yielded similar total ion chromatograms (TICs) and mass spectrum, but apparently different from CM precipitation (Fig. 2). When eluting from 10 to 30% ACN at around 10 to 30 min, multiple parent ions with +1 to +5 charges were found in the Ace and TCA precipitated samples (Fig. 2A & 2B). The relative intensities of these parent ions were relatively high. These parent ions were suspected to be peptides around 1.5 to 5 kDa. For the CM precipitation, the charges of the parent ions eluted from 10 to 30% ACN were found to be +6 or above (Fig. 2C). However, the relative intensities of these multiple charged parent ions were relatively low. Based on the mass spectrum from the CM precipitation, these parent ions were likely to be proteoforms around 10 kDa or above. Previous studies have reported that Ace, TCA and CM precipitations can precipitate small proteins (around 15 kDa) from aqueous samples with different efficiencies (Jiang et al., 2004; Pérez-Rodriguez et al., 2020). In general, Ace and TCA precipitations are more efficient in precipitating small proteins than CM precipitation. However, those proteoforms with molecular size around 10 kDa were only detected in the CM precipitated samples in the top-down proteomic analysis. This may be due to both Ace and TCA precipitations not just favoured the precipitations of those proteoforms, but also peptides from the HAE of EBN. As peptides are known to be ionized efficiently via



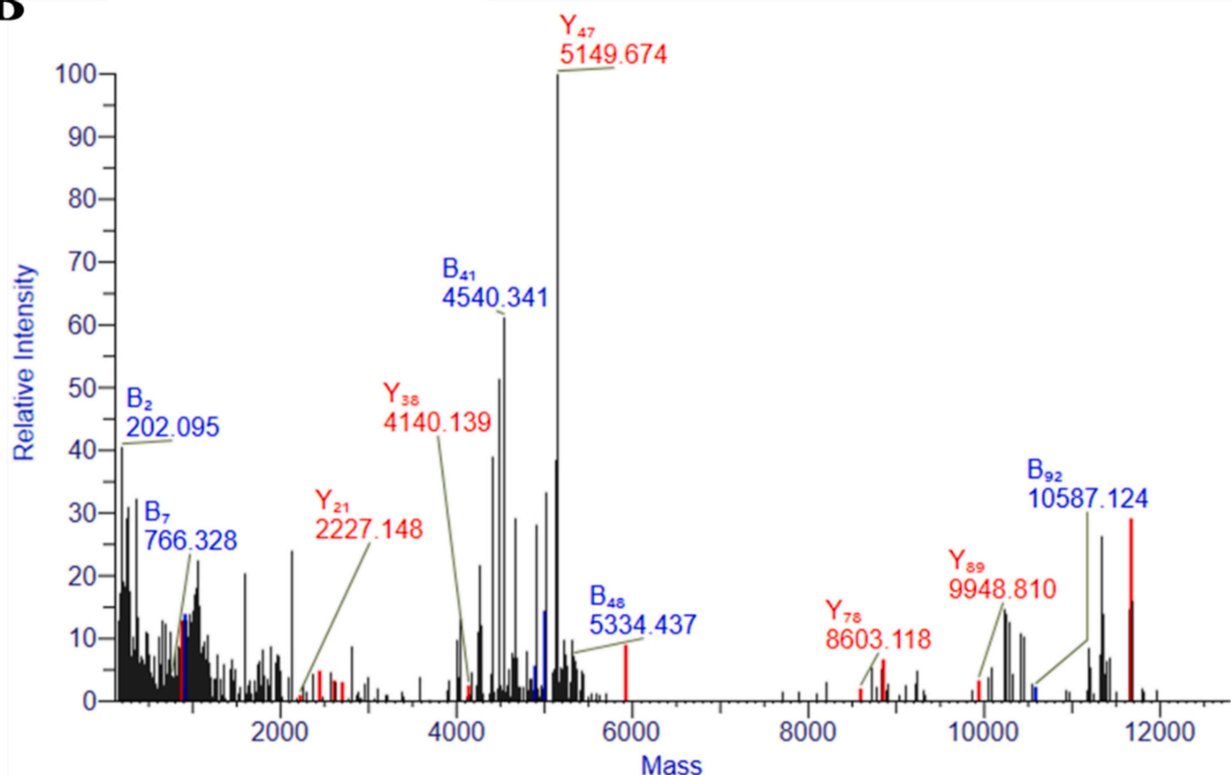


Fig. 4. Identification of Protein A, B and C. The multi-charged proteoform ions from Protein A were isolated and fragmented by HCD with 25% of collision energy (A). The MS/MS spectra were then deconvoluted and searched against the in-house database of coding sequences of EBN-producing swiftlet *A. fuciphagus* for identification. The identity of Protein A was found to be AMCase as multiple fragment ions from the corresponding deconvoluted MS/MS spectrum matched to the fragment ions of AMCase in the database (B). Around 7% of the fragment ions of AMCase were found in the deconvoluted MS/MS spectrum of Protein A (C). Similarly, the multi-charged proteoform ions from Protein B and C were isolated and fragmented by HCD with 35% of collision energy (D). The proteoforms of Protein B and C generated very similar MS/MS spectra. After deconvolution, the deconvoluted MS/MS spectra of Protein B and C were almost identical (E). Around 5% of the fragment ions of AMCase were found in the deconvoluted MS/MS spectra of Protein B and C (F). The b and y ions were indicated by Γ and Γ (blue in colour) respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

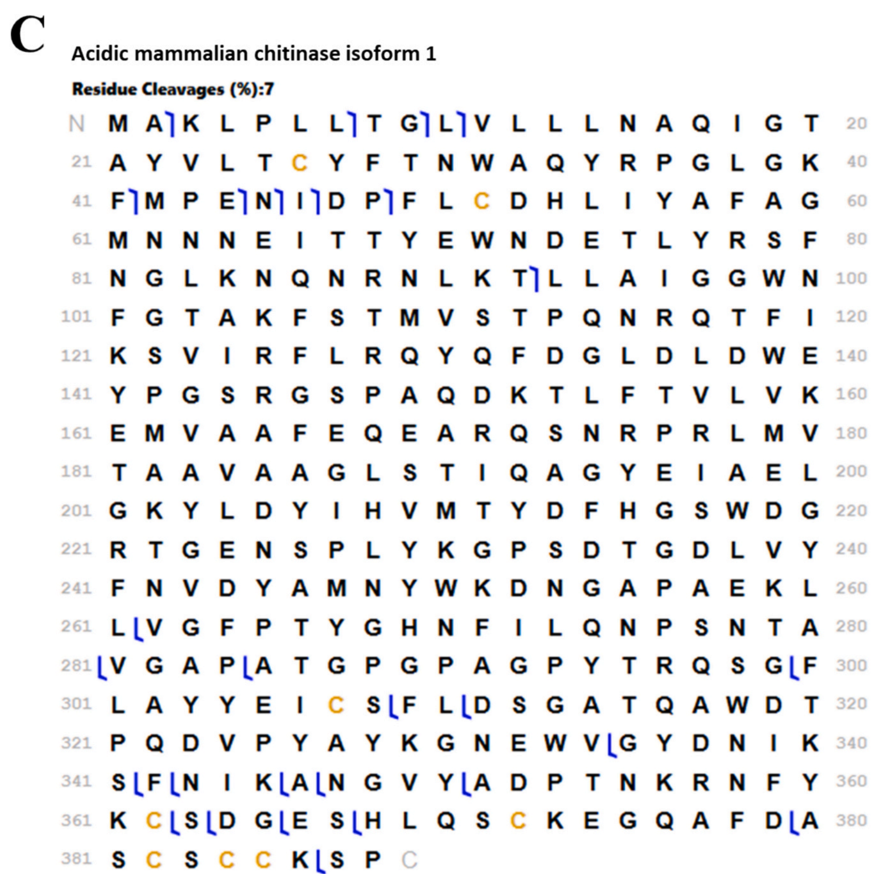
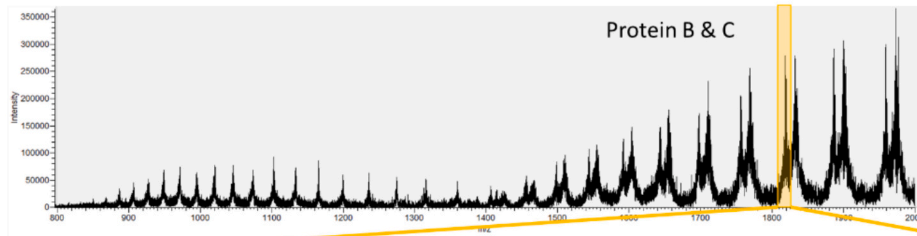
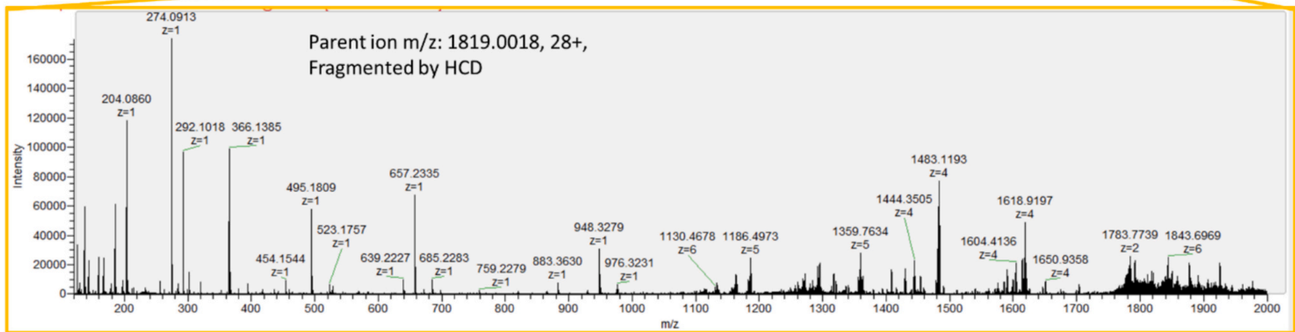


Fig. 4. (*continued*).

D





E

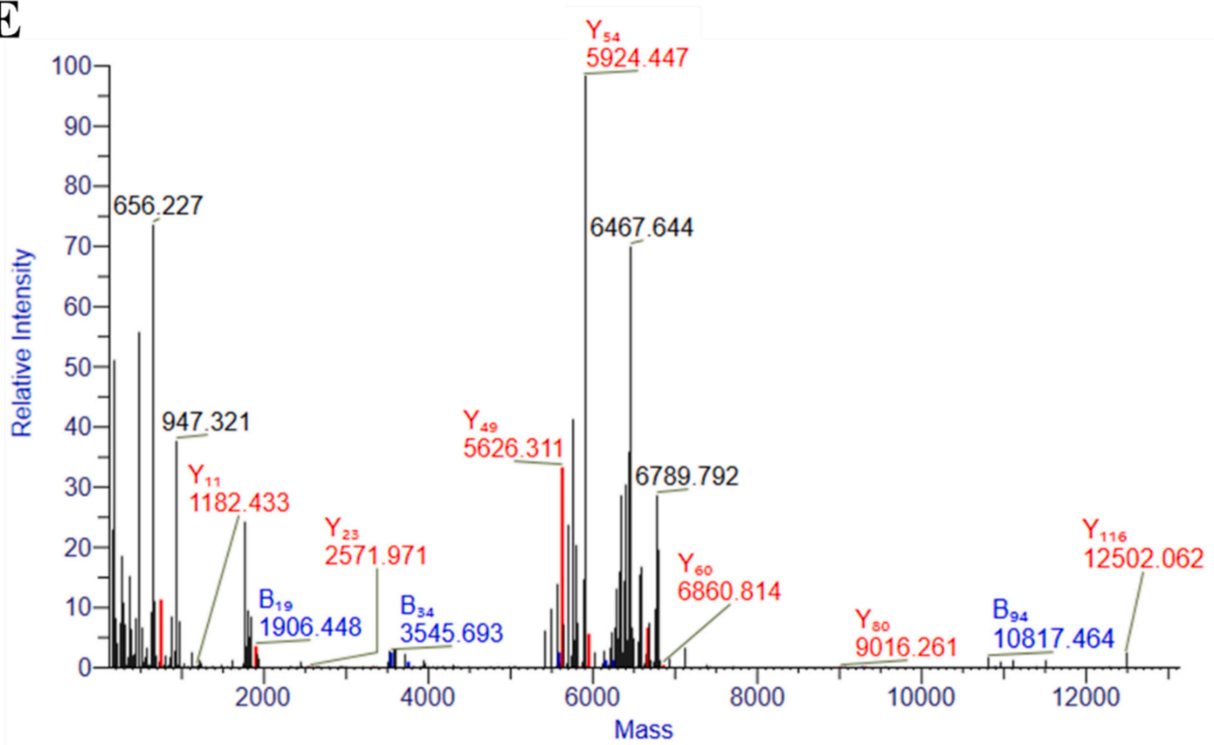


Fig. 4. (*continued*).

F

Acidic mammalian chitinase isoform 2

Residue Cleavages (%):5

N E G G L T P S G R R A A P G E R G E Q E 20
21 Q Q T G Q F T Q A G H A E Q D T G P L L 40
41 A Q D W H E I R G A Y K S G R Q A A T D 60
61 Q S W S K M A K L T L L T G L V L L L N 80
81 A Q I G T A Y V L S C Y F T N W A Q Y R 100
101 P G L G K Y M P D N I D P F L C N H L I 120
121 Y A F A G M N N N E I T T Y E W N D E T 140
141 L Y K S F N G L K N Q N R N L K T L L A 160
161 I G G W N F G T E K F S T M V S T P Q N 180
181 R Q T F I K S V I R F L R Q H Q F D G L 200
201 D L D W E Y P G A R G S P A Q D K T L F 220
221 T V L V K E M V A A F E Q E A R Q A N K 240
241 P R L M V T A A V A A G L S T I Q A G Y 260
261 E I A E L G K Y L D Y I H V M T Y D F Y 280
281 S A W D G R T G E N S P L H N G A N T Q 300
301 L S V E Y A M N Y W K N N G A P A R K L 320
321 L V G F P T Y G R S F T L Q N P S N T A 340
341 V G A P T S G P G P A G P Y T Q E A G L 360
361 L A Y Y E I C T L L D S G A T Q A W D A 380
381 S E D V P Y A Y K G S E W V G Y D D V K 400
401 S F K I K V D W L K K N N F G G A M V W 420
421 T L D M D D F T G S F C K Q G R Y P L I 440
441 T T L K N N L G C

Fig. 4. (*continued*).

electrospray ionization than proteoforms (Prabhu et al., 2023), so the peptides in the Ace and TCA precipitated samples were preferably ionized as the parent ions with relatively high intensities, and suppressed the ionizations of the small proteoforms in the top-down proteomic analysis. For the CM precipitation, the peptides were retained in the aqueous layer and removed during the precipitation. Thus, those proteoforms in the CM precipitated samples could be ionized in the top-down proteomic analysis in the absences of the peptides.

In the PCA, the CM precipitated samples were clustered tightly compared to the Ace and TCA precipitated samples (Fig. 2D). This suggested that the variations among the CM precipitated samples were less than those prepared by other precipitation methods. As CM precipitation was found to be more reproducible in extracting proteoforms from the HAEs of Indonesian EBNs, so it was considered as a more reliable method in preparing samples for the top-down proteomic analysis of EBNs.

3.2. Different isoforms of acidic mammalian chitinase can be served as proteoform biomarkers for authenticating EBN from different origins

One of the aims of the current study was to identify a few proteoform biomarkers for authenticating EBN from counterfeits using top-down proteomic analysis. To be a biomarker, it should be abundant in the EBNs from different origins. In addition, it should be easy to extract and detect by mass spectrometer. The top-down proteomic analysis revealed that 3 different proteoforms with relative high intensities were consistently detected in the Ace, TCA and CM precipitated samples (Fig. 2 & 3A). After deconvolution, the molecular sizes of these 3 proteoforms were around 40.76 kDa (Protein A), 50.90 kDa (Protein B) and 51.29 kDa (Protein C, Fig. 3B and Supplementary table S1). The HAEs of Indonesian EBN were then resolved by 10% SDS-PAGE and visualized by silver staining. Two protein bands with similar apparent molecular sizes to Protein A, B and C were observed in the resolved HAEs of Indonesian EBNs (Fig. 3C). To identify Protein A, B and C, the corresponding multi-charged proteoforms were isolated and fragmented by HCD (Fig. 4). After searching the MS/MS spectra against the in-house database of coding sequences of EBN-producing swiftlet, *A. fuciphagus*, Protein A, B and C were found to be different proteoforms of acidic mammalian chitinase (AMCase, Fig. 4C and F). To validate the top-down proteomics, the protein bands of interest were cut out from the SDS-PAGE for a bottom-up proteomic analysis (Fig. 3C). The bottom-up proteomic analysis of the tryptic peptides from the protein bands also revealed that the identities of Protein A, B and C were different proteoforms of AMCase. A few proteoforms of AMCase were identified in EBN by western blot in previous study (Kong et al., 2022). AMCase is an enzyme that can degrade chitin, a polysaccharide that composes fungal cell wall and arthropod exoskeleton to prevent invasions of chitinous pathogens (Hu et al., 2021). Human expresses various isoforms of AMCase with different molecular sizes (Seibold et al., 2009). The smallest AMCase isoform is around 33. 91 kDa (Uniprot ID: Q9BZP6–3), followed by 40.

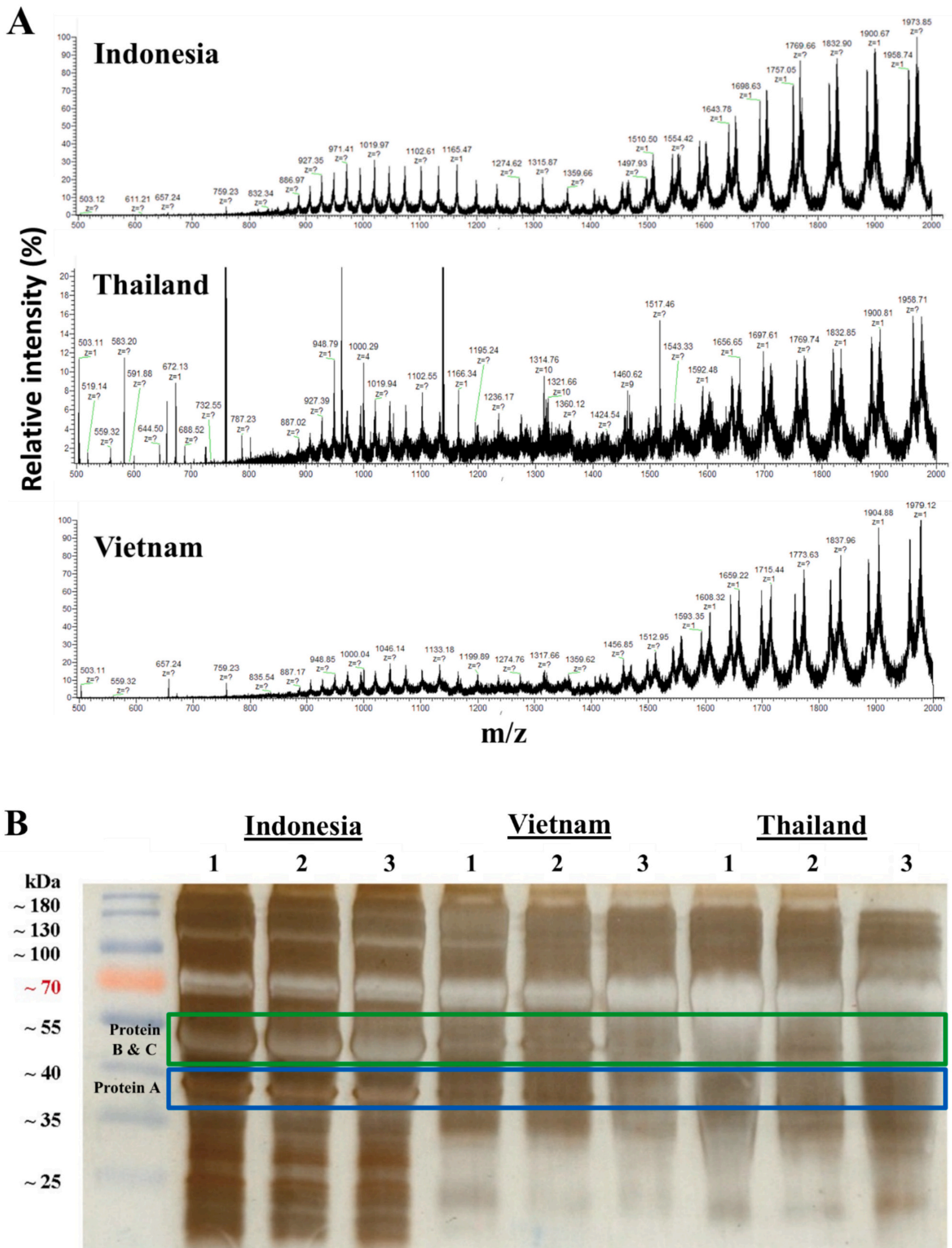


Fig. 5. Proteoform biomarkers of EBN from different origins. The proteoforms in the HAEs of Indonesian, Thai, and Vietnamese EBN were extracted by CM precipitation for top-down proteomic analysis. Protein A, B and C were consistently identified in the CM precipitated samples using Orbitrap (A) and SDS-PAGE (B). When prepared by CM precipitation, the Indonesian, Thai, Vietnamese samples were separated from each other (C). This indicated the proteoform profiles of HAEs of EBN from different origins were distinctive. Meanwhile, the variations among the proteoform profiles prepared by Ace precipitation were less (D).

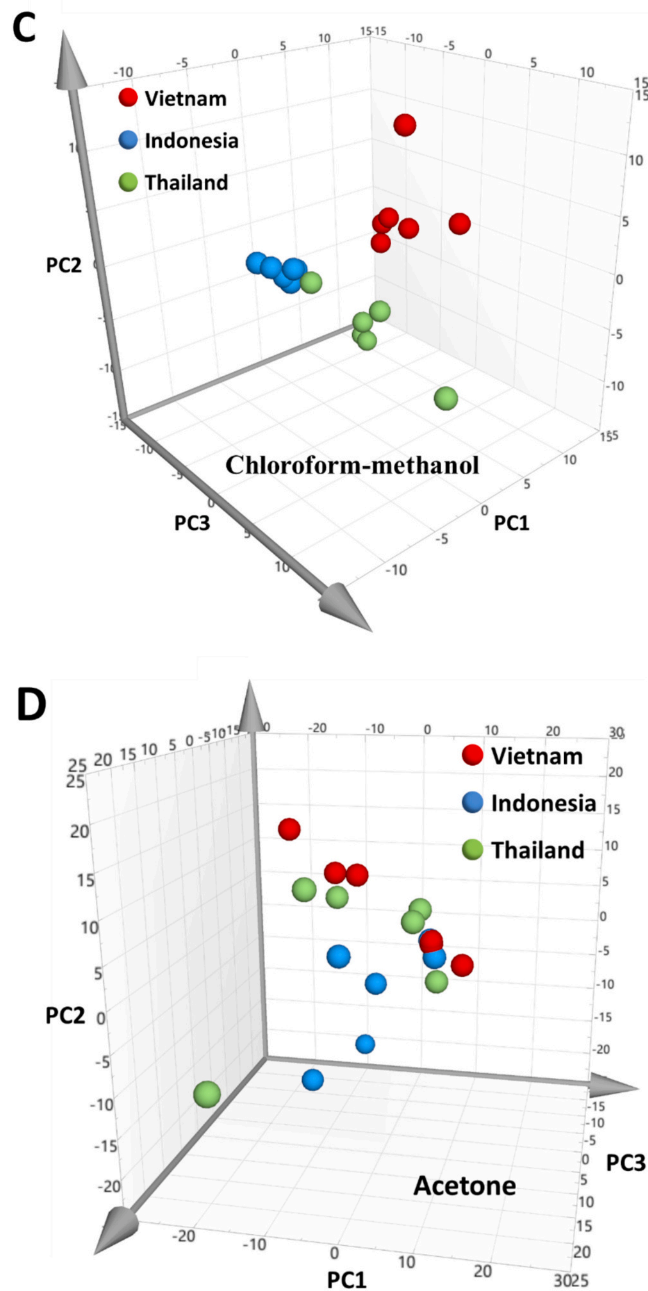


Fig. 5. (*continued*).

14 kDa (Uniprot ID: Q9BZP6–2), and 52.27 kDa (Uniprot ID: Q9BZP6). As the MS/MS spectra and the deconvoluted MS/MS spectra of protein B (50.90 kDa) and C (51.29 kDa) were almost identical (Fig. 4D & 4E), protein B and C was identified as the same AMCase isoform (Fig. 4F). The mass difference between protein B and C (around 393.5 Da) might be due to post-translational modifications, like glycosylation. However, the post-translational modification has not been identified yet. Thus, protein B and C might be the largest AMCase isoform with different post-translational modifications, meanwhile protein A might be the smaller AMCase isoform in EBN. As these AMCase isoforms were easily extracted from EBN and easily detected by the top-down proteomic analysis, they may serve as the proteoform biomarkers for authenticating EBN from counterfeits or substitutes.

Besides the Indonesian EBNs, the HAEs of Thai EBN and Vietnamese EBNs were subject to the top-down proteomic analysis. Different isoforms of AMCase were consistently detected in the Ace, TCA and CM precipitated samples prepared from the HAEs of the Thai and Vietnamese EBNs (Fig. 5A and Supplementary Fig. S1, S2 and S3). The HAEs of EBNs from Indonesia, Thailand and Vietnam were then resolved by 10% SDS-PAGE and visualized by silver staining. All the HAEs of EBNs from different countries had the protein bands with apparent molecular sizes similar as the molecular sizes of the isoforms of AMCase (Fig. 5B). However, the abundances of AMCase isoforms in the HAEs from different origins seemed to be varied. The top-down proteomes of the CM precipitated samples from 3 different origins were subject to PCA. The distinct clusters of the samples from different origins indicated that the CM precipitated samples from different origins were vary from each other (Fig. 5C). However, the Ace precipitated samples from different origins were overlapped with each other (Fig. 5D). This suggested that the variations of the top-down proteomes of the Ace precipitated

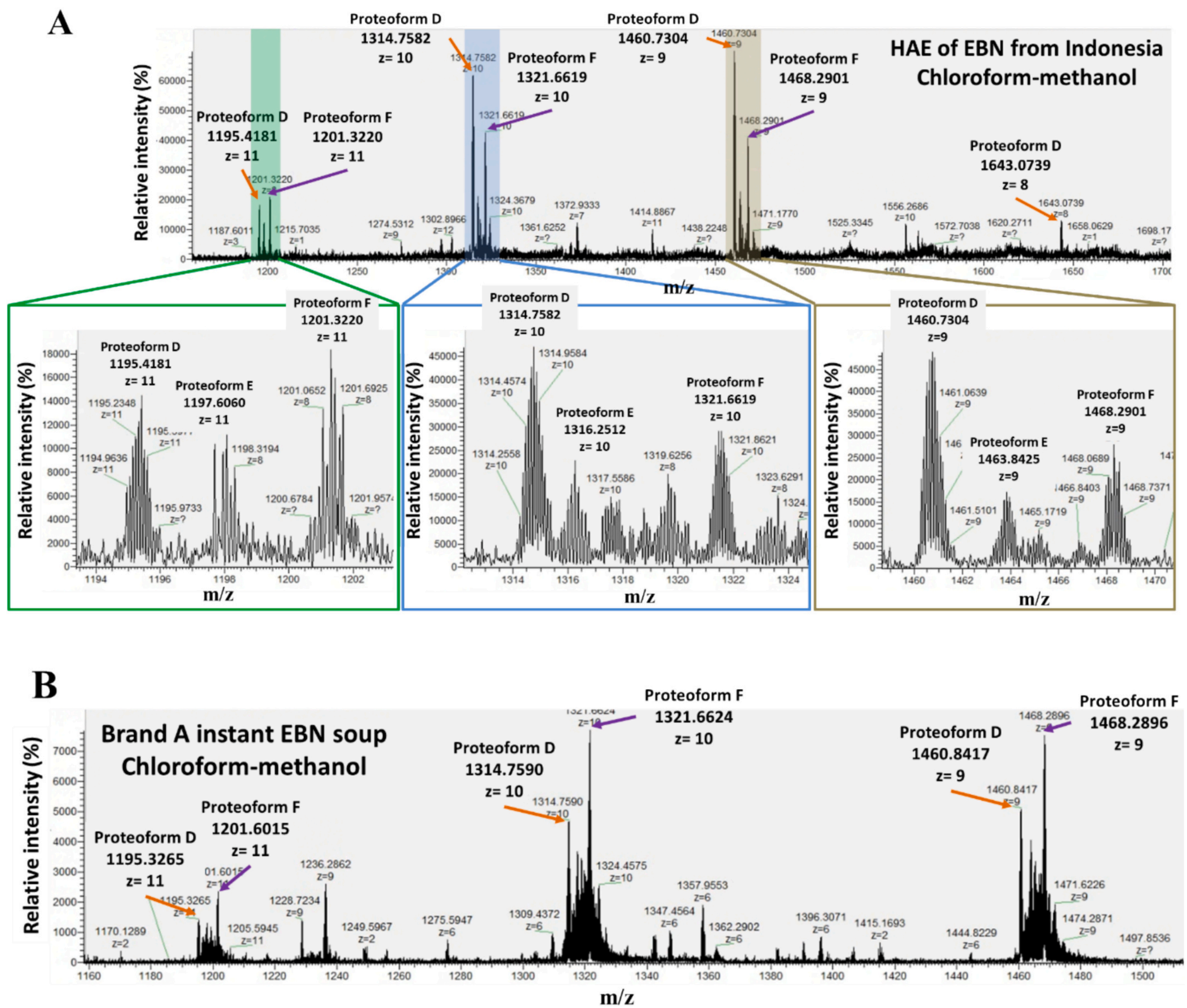


Fig. 6. Proteoforms identified in all the HAE of EBN from different origins as well as different brands of instant EBN soup available in Hong Kong. At least 3 groups of proteoforms (proteoform D, E and F) were consistently found in the HAEs of EBN prepared by CM precipitation as well as in the CM precipitated samples from different brands of instant EBN soup (C).

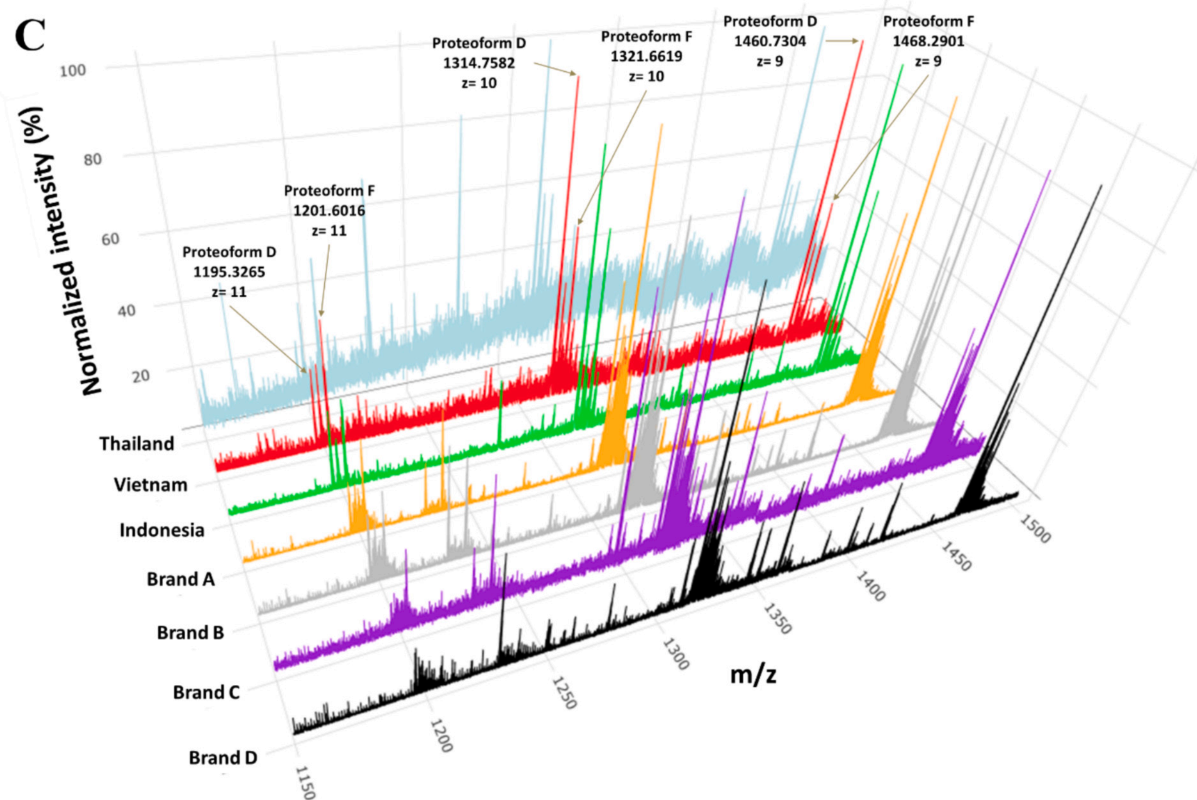


Fig. 6. (*continued*).

samples from different origins were less and indistinguishable from each other. As mentioned previously, Ace precipitation could extract both peptides and proteoforms from the HAE of EBN, meanwhile CM precipitation would mainly extract proteoforms from the HAE of EBN without peptides (Fig. 2A to 2C). Thus, our findings proposed that the top-down proteomic analysis on the proteoform profile of EBN might be a reliable method to differentiate the origins of EBNs, instead of the top-down proteomic analysis on the profiles of peptide and proteoform. This demonstrated that the importance of optimizing the sample preparations to extract a subset of proteoforms that was suitable for differentiating the origin of EBN using top-down proteomics.

3.3. *Potential proteoform biomarkers for authenticating EBN in different brands of instant EBN soups available in Hong Kong*

The instant EBN soups are the most popular food products made of EBN. The clean EBN was packed in a bottle with sugar and water, followed by autoclave which not just stews the EBN, but also sanitizes the soup product simultaneously (Dai et al., 2021). So, it was suspected that we should be able to detect the proteoform biomarkers of EBN in the instant EBN soups using the top-down proteomic approach. If so, the proteoform biomarkers of EBN could act as an indicator for authenticating the presence of genuine EBN in the soup products. In total, 4 different bands of instant EBN soup available in Hong Kong market were chosen for the study. After filtered out the EBN residue, the soup was concentrated and the proteins in the soup were extracted by CM precipitation before the top-down proteomic analysis. Different isoforms of AMCase were only found in some of the CM precipitated samples from instant EBN soup (Supplementary Fig. S3). The relative intensities of the AMCase proteoforms were low. It was suspected that manufacturing processes of the instant EBN soup might degrade the AMCase isoforms (Meade et al., 2005). As a result, it became difficult to detect the AMCase isoforms in the instant EBN soups. Thus, the biomarkers for authenticating the presence of real EBN in the soup products were needed to be resistant to the degradations during food processing steps. So that, the biomarkers can be consistently detected in the unprocessed EBN and in the instant EBN soups for authenticating purpose. Apart from AMCase proteoforms, the top-down proteomic analysis revealed another group of thermostable proteoforms consistently detected in both HAEs of EBN from different origins and the instant EBN soups available in Hong Kong market (Fig. 6). The molecular sizes of the proteoforms were around 13.137 kDa (Proteoform D), 13.165 kDa (Proteoform E) and 13.206 kDa (Proteoform F, Fig. 6A). It was suspected that these proteoforms represented the same small protein differing by specific post-translational modifications. The mass difference between of around 28 Da suggested that proteoform E might be a formylated proteoform D (Tan et al., 2024). Similarly, the mass increase of around 42 Da from proteoform E to F implied that proteoform F might be an acetylated derivative of proteoform E (Shang et al., 2022). Moreover, the relative intensities of proteoform D were found to be higher than proteoform E and F in the HAEs of the Indonesian EBN (Fig. 6A). The same group of proteoforms has been identified in the instant EBN soups and the relative intensities of proteoform F were found to be higher than proteoform D and E in the soup sample (Fig. 6B). This implied that either proteoform D was degraded or modified to proteoform F during the manufacturing steps of the instant EBN soups. Although proteoform D, E and F could be detected in all the CM precipitated sample from the HAEs of EBN from different origins and the instant EBN soups, their identities remained unknown (Fig. 6C). Further investigations were required to identify the protein family affiliations of these thermostable proteoforms before they can serve as the biomarkers for authenticating the presence of genuine EBN in the food products.

4. Conclusion

Using top-down proteomic approach, the current study revealed that the proteoforms of AMCase can be served as a biomarker for authenticating unprocessed or uncooked EBN from other food items. Moreover, it was possible to verify the geographical origins of EBNs by profiling the proteoforms prepared by CM precipitation using top-down proteomic analysis. This will enhance the traceability of EBN to increase consumer trust and protect the regional brands. Furthermore, our findings proposed that the thermostable EBN proteoforms could be acted as a fingerprint to authenticate the presence of genuine EBN in the instant EBN soups available in Hong Kong. Finally, our findings demonstrated the importance in protein extraction and precipitation in top-down proteomic analysis. Optimized sample preparation allowed extracting subset of proteoforms from EBN which was suitable for authentication.

CRediT authorship contribution statement

Anson Hin-chuen Lau: Investigation. **Victoria Tsz-ki Tsui:** Methodology, Investigation. **Kylie Ip Hung:** Investigation. **Tiffany Man-hei Le:** Investigation. **Sirius Pui-kam Tse:** Methodology. **Ka-hing Wong:** Funding acquisition, Conceptualization. **Hang-kin Kong:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hang-kin KONG reports financial support was provided by The Hong Kong Polytechnic University. Ka-hing WONG reports financial support was provided by The Hong Kong Polytechnic University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.148020.

Data availability

Data will be made available on request.

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