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Fast differentiation of meats from fifteen animal species by liquid chromatography with electrochemical detection using copper nanoparticle plated electrodes

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Abstract

A simple, rapid and reliable method based on high-performance liquid chromatography with electrochemical detection was developed to routinely differentiate among meat products from fifteen food animal species. Samples from cattle, pigs, goats, deer, horses, chickens, ducks, ostriches, salmon, cod, shrimp, crabs, scallops, bullfrogs and alligators each exhibited unique electrochemical profiles. Species-specific markers exhibited reproducible peak retention times with coefficients of variation less then 6% across different runs, body regions and subjects. The method requires no derivatization or extraction steps and may be applicable to fresh or cooked meats. Incubation of fresh beef, pork or chicken at room temperature for 24 h or repeated freezing and thawing changed the intensity but not the pattern of species-specific peaks. In conclusion, this method appears suitable for rapid differentiation of meats from various food animal species and demonstrates the utility of electrochemical detection to supplement existing immunochemical and molecular biological methods. The possibility of using this method to detect adulteration and degradative changes of meat proteins is discussed.
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Keywords: Meat differentiation; Protein degradation; HPLC-EC; Copper nanoparticle electrode; Adulteration

1. Introduction

Accurate identification of the origin of meat species presents a considerable challenge for food inspectors, animal feed analysts, game enforcement authorities and individuals seeking to comply with certain religious regulations. Consumers demand quality products that are labeled honestly in order to assure meat safety and fair pricing. Therefore, there has been a need for a fast and routinely applicable meat species identification system. Traditionally, species identification has been established through one of three approaches: molecular biology-based methods, enzymatic immunological methods or chromatographic methods. Molecular biology-based methods use techniques such as polymerase chain reaction (PCR) [1–5], restriction-enzyme fragment length polymorphism (RFLP) [6–10] to identify species-specific nucleotide sequences or variations within the mitochondrial DNA for the basis of species recognition. Genetic techniques are the most specific and sensitive methods for species identification, however, they require expensive laboratory equipment and a high degree of technical expertise. The genetic techniques also suffer from higher false-positive rates that come with the high sensitivity. The finding of species-specific sequences takes significant time and requires a long validation process. In addition, one primer is usually specific to only one species, which renders it more useful for the purpose of ruling out or confirming the presence of meat from a single species. Enzyme-linked immunosorbent assay (ELISA) [11,12], on the other hand, requires production of high titer antisera with specific

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antibodies for each meat species. The development process is time-consuming and the resultant assays detect only one target at a time.

Chromatographic methods such as high performance liquid chromatography (HPLC) [13–17], gas chromatography [18] and capillary electrophoresis [19,20] have all been reported for meat identification. While gas chromatography and capillary electrophoresis are generally disadvantageous in terms of expensive instrumentation and poor reproducibility, HPLC is a method with high sensitivity and reproducibility that is suitable for routine analysis. Most HPLC methods developed for meat identification are based on the differentiation of profiles of proteins [13,14], peptides [15,16,21] and/or amino acids [17] within different meats. The most widely used detection mode for proteins and peptides is ultraviolet (UV) absorbance [13–17,19–21]; however, the separation of protein peaks is typically unsatisfactory and the weak UV absorbance of peptides and amino acids necessitates cumbersome sample derivatization [15–17,21]. Mass spectrometry (MS) is a powerful tool with great sensitivity for structural identification. Although identification of trace evidence of drugs and toxins in biological matrices using MS detection are common, its application has not been extensively applied to the direct detection of biological tissues [22]. In addition to its high cost, MS may produce too many signals to allow for efficient differentiation of meat sources. The major disadvantages of published HPLC methods include the requirements for tedious extractions and long analysis times, which significantly limits widespread use of this methodology. Species that have been identified successfully by liquid chromatography include beef, pork, lamb, veal, chicken, turkey, duck and fish [13–17]. However, to our knowledge, no more than seven species can be differentiated simultaneously by a single chromatographic method.

The goal of this study was to develop an HPLC method with electrochemical detection (HPLC-EC) that is fast, economic and reliable for identification of meats from multiple species. A major advantage of EC detection is its ability to directly detect peptides and amino acids that exhibit little or no chromogenic or fluorescent properties [23,24]. In addition, under appropriate chromatographic conditions and simultaneous use of a copper nanoparticle-plated electrode, reliable detection is feasible without sample pretreatment [24]. Therefore, HPLC-EC detection represents a very attractive analytical scheme for the detection of electroactive peptides/amino acids as a basis for species differentiation. In the study described here, we report the first HPLC-EC method that is suitable for routine differentiation of food-meat species. The availability of this method provides a suitable EC application to supplement current methods for differentiation of meat species and provides a possible basis for monitoring degradative changes in meat-derived proteins.

2. Materials and methods

2.1. Meat samples

Species included in this study include food-meat species that are commonly available in Taiwan, including mammals such as cattle (*Bos taurus*), pig (*Sus domesticus*), and goat (*Capra hircus*); avian such as chicken (*Gallus domesticus*), duck (*Anas platyrhynchos*) and ostrich (*Struthio camelus*); fishes such as salmon (*Salmo salar*) and cod (*Gadus morhua*); arthropods such as shrimp (*Penaeus monodon*) and crab (*Scylla paramamosain*); scallops (*Tridacna gigas*); and species consumed less commonly or species may be substituted inappropriately for other species, including amphibians such as bullfrog (*Rana catesbeiana*); reptiles such as alligator (*Alligator mississippiensis*); deer (*Cervus nippon taiouanus*) and horse (*Equus caballus*) meats. To insure the freshness of tissues, raw meats of cattle, swine, goat, bullfrog, chicken and duck breast were purchased from local traditional markets in Taichung, Taiwan less than three hours after slaughter. Shrimp and crab were purchased live and sacrificed at the market. Meat from alligators, scallops, cod and salmon were purchased frozen from a national chain supermarket while fresh ostrich and deer meats were obtained from donation farms. Horse meat was obtained fresh from the necropsy room at the College of Veterinary Medicine, National Chung-Hsing University. Cuts of all fresh meats were processed immediately while frozen meats were thawed at 4 °C before processing. Unless stated otherwise, all remaining unused meat parts were stored at −20 °C. Each sample was prepared from 10 g of meat that was cut into small pieces and ground with a bio-homogenizer (M133, Biospec Product Inc., Bartlesville, OK, USA) in 10 mL of mobile phase (0.01 M phosphate buffer, pH 7.0 or PB, 0.866 g Na_2HPO_4 and 0.468 g NaH_2PO_4 in 1 L HPLC grade water). Unhomogenized meat fragments were removed by filtration through gauze and the crude filtrate was centrifuged for 5 min at 25 °C and 10,000 rpm. The resultant supernatant was filtered sequentially through 0.44 and 0.22 μm Millipore syringe filters (Millipore Corp., Billerica, MA, USA) and an aliquot of 20 μL of final filtrate was analyzed by HPLC-EC.

2.2. HPLC-EC methods

For the purpose of the study, HPLC-EC conditions were modified from our previous report detecting amino acids [23]. Chronoamperometric experiments were carried out with a CHI 721B electrochemical workstation (CH Instruments, Austin, TX, USA) and amperometric LC-4C detector (Bioanalytical system. Inc., USA). The detector is a three-electrode system consisting of a working electrode (Cu^n-SPE, copper nanoparticle-plated screen-printed electrode, geometric area = 0.2 cm^2), an Ag/AgCl reference electrode and a platinum auxiliary electrode (geometric area = 0.07 cm^2). The disposable Cu^n-SPE electrode (Zensor R&D, Taichung, Taiwan) was placed in the center of the flow cell where electrochemical detection occurs (Fig. 1). Chromatographic separations were performed using a silica-based HPLC column (Prevail organic acid, 5 μm particle size, 100 mm × 4.6 mm, Alltech, State College, PA, USA) with mobile phase. The analytical conditions were: detection potential −0.05 V, range 1 μA, run time 15 min, injection volume 20 μL. All experiments were done in triplicate for 3 meat cuts obtained from different venders. Data were analyzed with Clarity system software (DataApex. Inc., Prague, The Czech Public) with the retention times of all major peaks recorded as

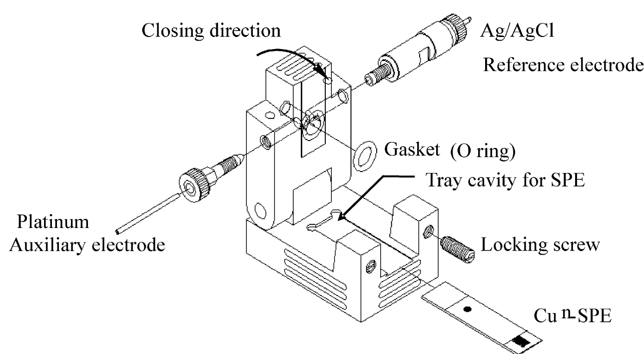

Fig. 1. A schematic depiction of the three-electrode detector system. Disposable Cu^n-SPE is incorporated in the center of the flow cell. After closing the top, sample flows in through the gasket, analytes are reacted with the circular dot plated with copper nanoparticles on Cu^n-SPE and reacted sample flows out through the channel of the auxiliary electrode.

the chromatographic profile for internal sample comparisons. A major peak was routinely defined as any peak with an area that exceeded 10% of the total peak areas of the entire sample chromatogram.

2.3. Evaluation of meat degradation

In order to evaluate the utility of the HPLC-EC method for determination of meat degradation, beef, pork and chicken were obtained from local slaughter houses fresh and either kept at room temperature (25 °C) for 24 h or going through two freeze (at −20 °C) and thaw (at 4 °C) cycles. Each meat species was cut into three equivalent parts, wrapped in plastic bags to prevent evaporative water loss, and analyzed immediately (fresh), and after 12 and 24 h storage at ambient temperature or after thawing the frozen sample at 4 °C. Chromatographic profiles of each meat were evaluated for changes in retention time, number and area (concentration) of all major peaks.

2.4. Evaluation of heat treatment, different meat area and adulteration

Fresh beef, pork, chicken and duck were used to evaluate if heat treatment or commonly consumed different meat areas would affect the electrochemical chromatographic profile. For heat treatment, 10 g of meat (beef, pork and chicken) were placed in 10 mL of mobile phase in a 50 mL beaker and boiled (100 °C) for 5 min in a water bath (Hipoint Inc., Kaohsiung, Taiwan). The resultant meat juice was then filtered and injected for analysis as described above. Chromatographic profiles of the same species before and after heat treatment were compared. For variations between different meat areas, different cuts of pork (round and flank) and duck (leg and breast) were obtained from a single animal ($n = 3$) and processed and analyzed in the manner described above. Chromatographic profiles of meat cuts from the above mentioned areas were compared within a single species. For evaluation of adulterations, 5 g each of beef, pork and/or horse meat were intermixed at 1:1 ratio (total of 10 g) to provide mixtures of beef/pork, beef/horse, and pork/horse. The individual meat extracts and mixtures were then processed and analyzed in the manner described above. Chromatographic profiles of beef, pork, horse and their mixtures were compared.

2.5. Statistics

Multiple comparisons of the peak areas from each treatment or condition (time and area of meat) were statistically analyzed using ANOVA (SAS 8.2 for Windows; SAS Institute, Cary, NC, USA) followed by Duncan's multiple comparison procedure. Statistical differences were set at $P < 0.05$ level.

3. Results

3.1. The chromatographic profiles of meat from 15 animal species

Fig. 2 depicts a representative chromatogram for a complex mixture of amino acids standards at various concentrations (50–350 μM). Under the analytical conditions described here, 11 amino acids including those with non-polar side chains (Pro, Met, Leu and Phe) as well as amino acids with polar side chains that are charged (Asp, Lys, His and Arg) or neutral (Gly, Cys and Tyr) were separated within 15 min. Since no major peaks were found between 15 and 25 min in the meat extracts in the preliminary tests, the detection time for meat extracts was set at 15 min. The chromatographic profiles of meat from 15 different animal origins are shown in Figs. 3–6. Chromatograms for selected species (Fig. 6) demonstrated significantly lower peak heights and, therefore, are shown on a more sensitive scale. Meat extracts from each species yielded a distinct chromatographic profile that could be differentiated readily by the number of major peaks and the peak retention times (Table 1) during a 15 min run. Among the 15 species tested, 9 (deer, chicken, ostrich, cod, crab, salmon, shrimp, alligator and bullfrog) exhibited a three-peak pattern, 4

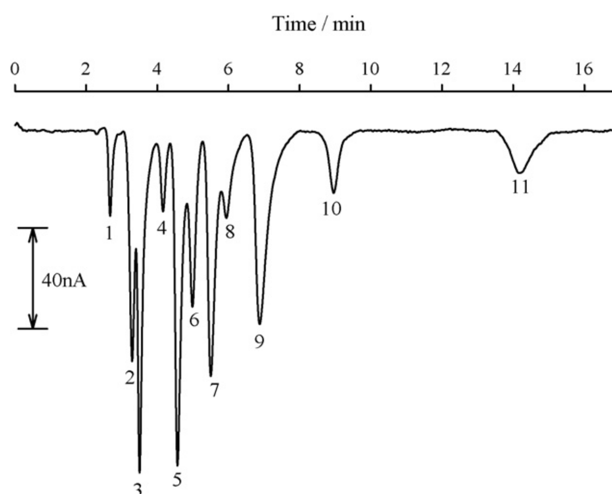

Fig. 2. Standard chromatogram of 11 amino acids at 50–350 μM. Analytical conditions: 10 mM PB pH 7, detection potential −0.05 V, range 1 μA, flow rate: 500 μL/min. Peak identity: (1) Asp; (2) Gly; (3) Cys; (4) Val; (5) His; (6) Met; (7) Lys; (8) Leu; (9) Arg; (10) Tyr; (11) Phe.

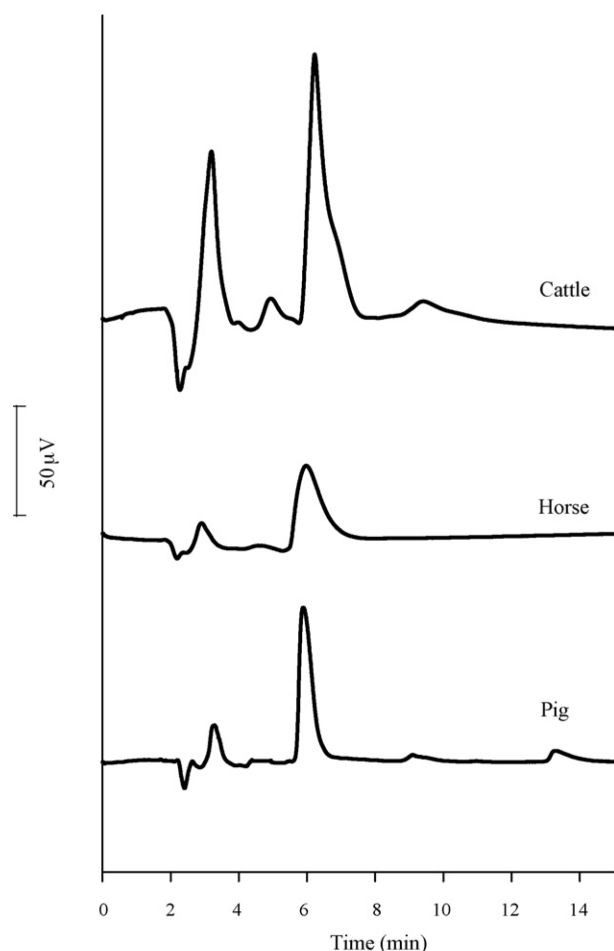

Fig. 3. Representative HPLC-EC chromatograms for meat extracts from three mammalian species. Analytical conditions: 10 mM PB, detection potential −0.05 V, range 1 µA, injection volume 20 µL.

Table 1
Average retention time (min) for major peaks in extracts of 15 meat species as identified by HPLC-EC method

	Peak1	Peak 2	Peak 3	Peak 4
Mammals				
Beef	3.2 ± 0.08	4.9 ± 0.01	6.2 ± 0.02	9.5 ± 0.06
Deer	2.9 ± 0.05	6.1 ± 0.11	13.1 ± 0.14	
Goat	3.3 ± 0.01	5.1 ± 0.05	6.4 ± 0.02	9.9 ± 0.06
Horse	2.9 ± 0.01	6.0 ± 0.06		
Pork	3.2 ± 0.01	6.0 ± 0.05	9.2 ± 0.05	13.3 ± 0.53
Avian				
Chicken	3.0 ± 0.04	5.8 ± 0.02	8.3 ± 0.04	
Duck	3.0 ± 0.12	4.8 ± 0.04	6.1 ± 0.03	9.2 ± 0.04
Ostrich	3.0 ± 0.06	4.5 ± 0.03	9.4 ± 0.05	
Seafood				
Cod	3.2 ± 0.08	4.8 ± 0.03	10.0 ± 0.15	
Crab	2.2 ± 0.04	3.0 ± 0.02	6.9 ± 0.06	
Salmon	3.1 ± 0.01	4.6 ± 0.01	9.5 ± 0.02	
Scallop	2.3 ± 0.01	3.0 ± 0.02		
Shrimp	3.1 ± 0.01	4.6 ± 0.01	6.3 ± 0.03	
Amphibians and reptiles				
Alligator	3.0 ± 0.01	6.3 ± 0.01	9.5 ± 0.02	
Bullfrog	2.9 ± 0.01	4.9 ± 0.04	6.2 ± 0.01	

Data represent mean ± SEM, $n = 4$.

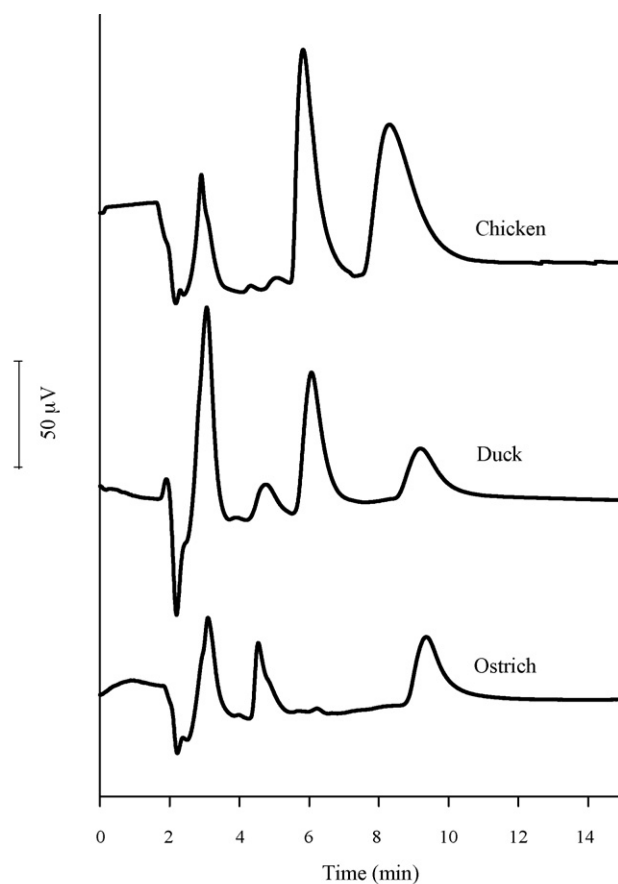

Fig. 4. Representative HPLC-EC chromatograms for meats from three avian species. Refer to Fig. 3 for analytical conditions.

(cattle, goat, pig and duck) exhibited a 4-peak pattern and 2 species (horse and scallop) exhibited a 2-peak pattern.

3.2. Evaluation of peak time as the primary determinant for meat differentiation

The reproducibility of chromatographic profiles, including peak numbers and retention times, were evaluated as primary determinants for species differentiation by comparing triplicate injections from a single meat filtrate as well as injections of extracts of three meat preparations from three different venders (i.e. the same cut of meat from three individual animals). The coefficients of variation (CV%) from these comparisons are shown in Table 2. Tabulated results indicate that the retention time for each peak was very consistent; with values of CV% being less than 3% for more than 80 of all major peaks in all 15 meat extracts (range 0.2–5.9%). Quantitatively similar results were obtained from comparisons of chromatographic peak patterns measured in comparable tissues from animals supplied from different venders (Table 2).

3.3. Use of HPLC-EC method for monitoring meat degradation

Chromatograms of meats incubated at room temperature (25 °C) for various amounts of time indicated no qualitative

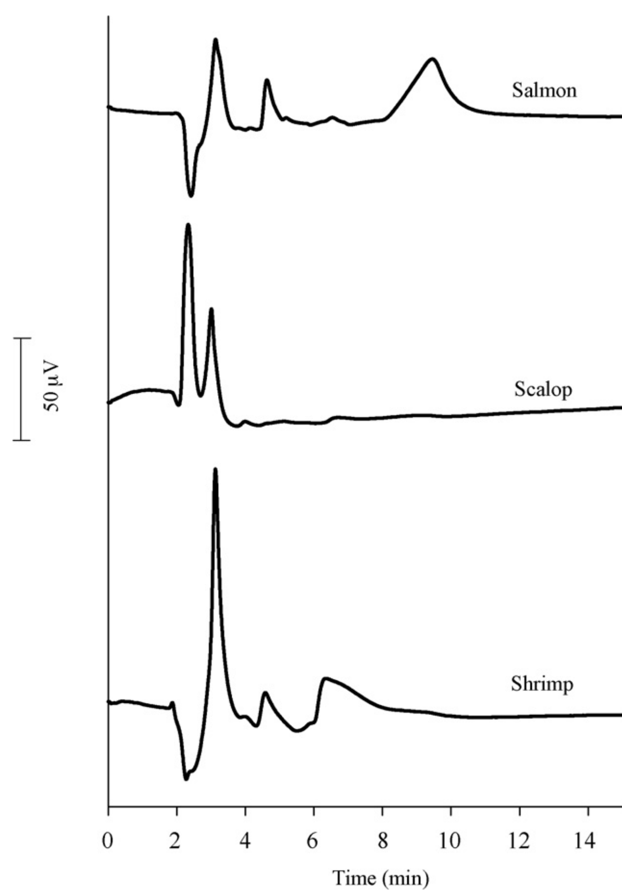

Fig. 5. Representative HPLC-EC chromatograms for meats from three marine species. Refer to Fig. 3 for analytical conditions.

Table 2
Coefficient of variation (CV%) for major peak times in extracts of 15 meat species

	Peak1	Peak 2	Peak 3	Peak 4
Mammals				
Beef	5.0 (3.2)	0.4 (0.7)	0.8 (2.6)	1.2 (0.8)
Deer	3.4 (0.2)	3.6 (0.2)	2.2 (2.1)	
Goat	0.6 (1.0)	2.0 (0.5)	0.6 (0.5)	1.2 (0.5)
Horse	0.6 (1.1)	2.0 (0.3)		
Pork	1.2 (3.0)	5.6 (5.9)	1.5 (2.1)	3.9 (1.4)
Avian				
Chicken	2.6 (0.5)	0.6 (2.6)	0.9 (1.1)	
Duck	4.0 (0.8)	1.7 (2.1)	1.0 (0.6)	0.8 (0.7)
Ostrich	4.0 (5.3)	1.6 (3.8)	1.5 (2.3)	
Seafood				
Cod	5.0 (0.2)	1.2 (1.9)	3.0 (0.8)	
Crab	3.6 (4.9)	1.4 (1.4)	2.0 (2.1)	
Salmon	0.6 (0.8)	0.4 (0.5)	1.4 (0.4)	
Scalop	1.8 (3.7)	1.4 (0.2)		
Shrimp	1.7 (4.1)	0.4 (0.5)	1.0 (0.9)	
Amphibians and reptiles				
Alligator	0.6 (0.8)	5.3 (3.5)	0.4 (0.9)	
Bullfrog	0.6 (5.9)	1.6 (0.4)	0.3 (0.6)	

Data shown are the CV% for the same cut of meat from three different venders. Values shown in parenthesis are CV% for triplicate injections from a single meat extract.

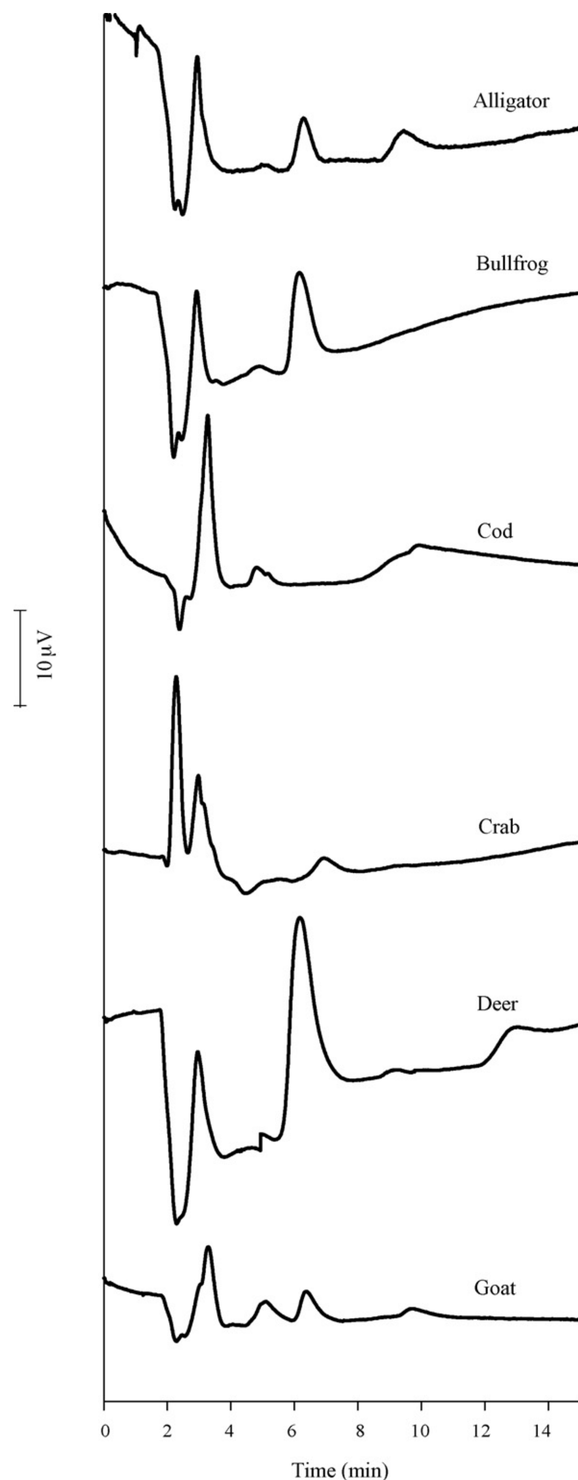

Fig. 6. Representative HPLC-EC chromatograms for miscellaneous meat species. All species listed here gave significantly weaker EC signals than species shown in Figs. 3–5.

difference in the chromatographic profiles with the same numbers of major peaks and retention times that were not different from fresh meat extracts. However, quantitative differences were noted for several major peaks as indicated by time-dependent changes in peak areas in several species (Fig. 7). In several cases, changes in peak area exhibited a marked temporal depen-

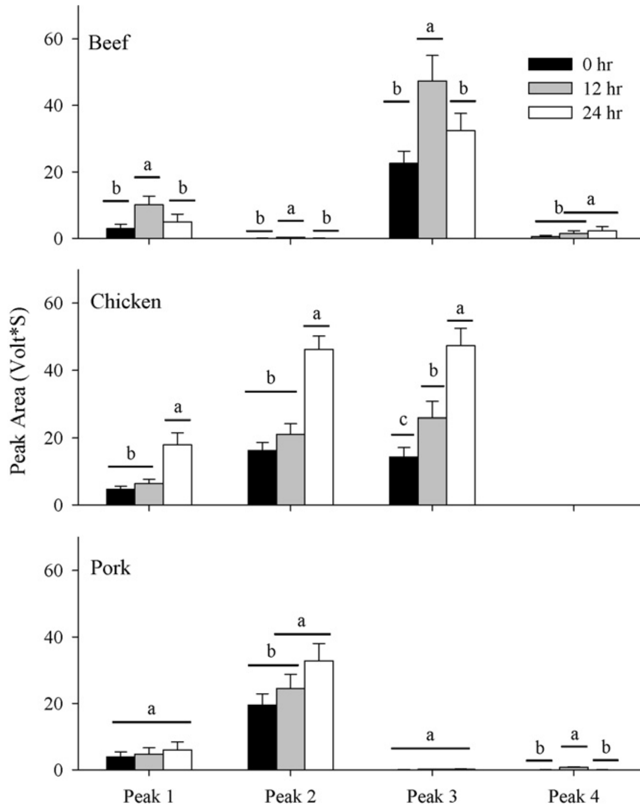

Fig. 7. Quantitative changes in major peak areas in extracts of beef, chicken and pork following incubation (12 h vs. 24 h) at room temperature. Refer to Table 1 for peak times. Data represent mean ± SEM, $n = 4$. Columns with different letter and bar are significantly different ($P < 0.05$).

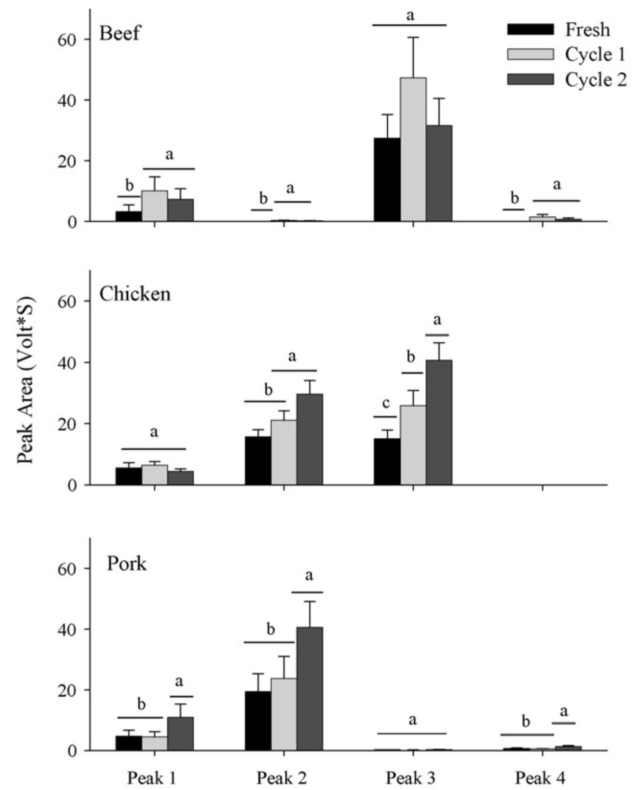

Fig. 8. Quantitative changes in major peak areas in extracts of beef, chicken and pork following two freeze (−20 °C) and thaw (4 °C) cycles. Refer to Table 1 for peak times. Data represent mean ± SEM, $n = 4$. Columns with different letter and bar are significantly different ($P < 0.05$).

dence with progressive increases noted between fresh versus 12 h samples and 12 h versus 24 h samples. The concentration change exhibited two distinct patterns in different species, with major peaks showing an initial increase and later decrease in beef extracts, while major peaks increased progressively with time in chicken and pork extracts, although the change in chicken was considerably less evident within the first 24 h. Following two free–thaw cycles, the major peak profiles and peak sizes were similar to those obtained after 24 h at room temperature. Both treatments demonstrated similar trends with respect to changes in the sizes of various peaks, although some differences in peak area were noted (Fig. 8).

3.4. *Effect of heat treatment, different meat area and adulteration*

Chromatograms of meat juice produced by boiling of meats in PB revealed only quantitative changes in peak area, with no changes detected in peak number or peak retention time (Fig. 9). Major peaks in boiled beef (peaks 1 and 3), chicken (peaks 1, 2 and 3) and pork (peak 2 and 4) all exhibited significant increases when compared to fresh (uncooked) meat samples. In contrast, the smaller major peaks in each species showed little or no difference in size or retention time when compared to fresh meat samples. The ratios of major peak areas in each species exhibited different changes following heat treatment as evidenced by increases (e.g., peak3/peak2 in chicken and peak2/peak1 in pork) as well as decreases (e.g., peak3/peak1 in beef and peak2/peak1 in chicken). With respect to the profiles obtained for different tissues (cuts) from a single species, nearly identical patterns of peaks were evident between leg and breast meats from duck and between round and flank cuts from pigs (Fig. 10). These observations suggest that differences in these two areas of the body are not significant ($P > 0.05$) in these two species. Mixing of beef, pork and horse meat could be distinguished readily by comparison of peak(s) patterns (area, retention time, etc.) for the individual species (Table 3). At low detection

Table 3
Areas and retention times for major peaks in extracts from beef, pork, horse meats and mixtures thereof at 1:1 ratio

Retention time (min)	Peak area (C)					
	Beef (B)	Pork (P)	Horse (H)	B/H	P/H	B/P
3.0	9.9	11.0	15.9	4.4	4.4	4.7
5.0	1.0	–	–	–	–	0.8
6.0	30.8	50.8	54.8	35.2	55.4	41.6
7.6	–	–	2.9	1.6	1.5	–
9.5	0.6	0.4	–	–	–	1.9
12.1	–	–	2.6	1.2	2.2	–
13.3	–	5.4	–	–	2.1	1.4

Values of peak area are expressed in coulombs (C). Retention times for common peaks are approximate.

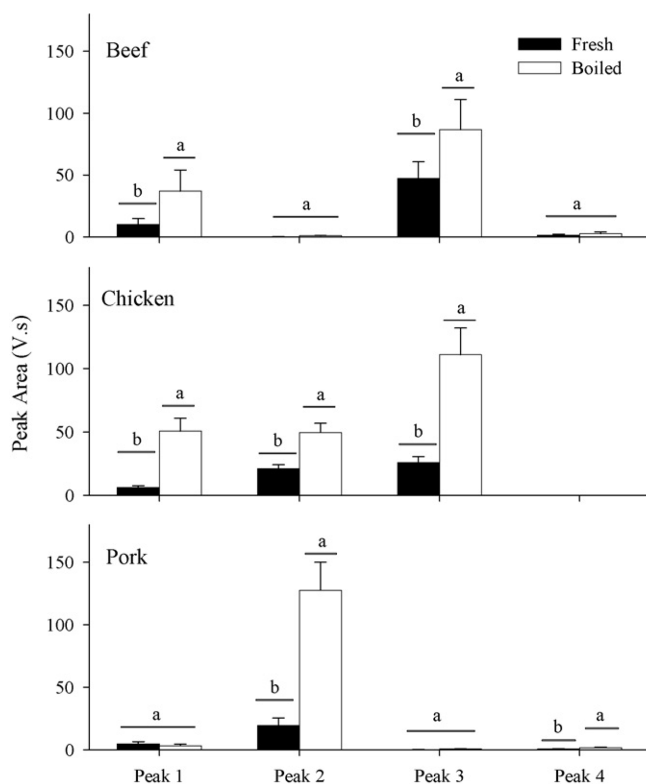

Fig. 9. Qualitative and quantitative changes in EC chromatograms for beef, chicken and pork extracts following high heat exposure (5 min at 100 °C in PB). Refer to Table 1 for peak times. Data represent mean ± SEM, $n = 3$. Columns with different letter and bar are significantly different ($P < 0.05$).

sensitivity (8 V full-scale setting), mixing at 1:1 ratio did not affect the appearance of any specific peaks. However, at higher detection sensitivity (2 V full-scale setting), equine-derived peaks (7.6 and 12.1 min) were observed thereby providing evidence for the presence of horse meat in the mixtures. At this sensitivity, no interfering peaks from beef or pork were detected.

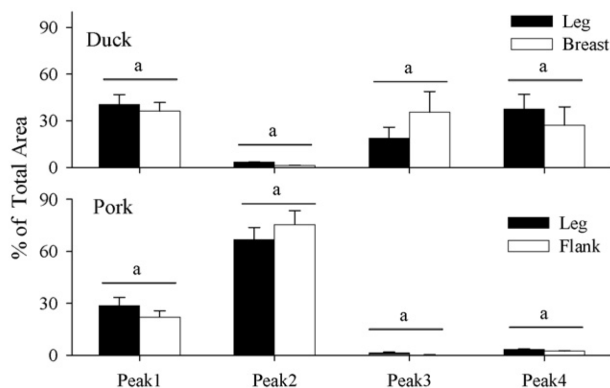

Fig. 10. Qualitative and quantitative comparisons of EC chromatograms for meat extracts of pork and duck collected from two meat areas. Refer to Table 1 for peak times. Data represent mean ± SEM, $n = 4$. Columns with different letter and bar are significantly different ($P < 0.05$).

4. Discussion

In 1988, Ashoor published an HPLC-UV method to differentiate raw beef, pork, veal, lamb chicken, turkey and duck [14]. Since then, very few chromatographic methods based on HPLC-UV has been developed or modified for the purpose of identifying and differentiating among different meat sources in single or mixed samples. The major limitations of this approach have included significant obstacles, including the challenge to interpret and distinguish among the complex chromatographic profiles of different species, the lengthy procedure to extract and prepare the sample, and the necessity to derivatize peptides and amino acids in order to achieve suitable detection sensitivity. In the study described here, we have used HPLC with EC detection to demonstrate that crude meat extracts from fifteen species can be differentiated without derivitization or time-consuming sample processing. By utilizing the characteristics of 2–4 major peaks in each chromatogram, we have demonstrated that it is feasible to differentiate clearly among meats from these species. This is the first report using a single analytical method to differentiate meat extracts from this many species and, to our knowledge, the first EC method designed for the differentiation of multiple meat species in (mixed) samples. One of the biggest advantages of this method is the ease and simplicity of sample preparation with no need of organic solvents or pre-column derivatization. The analysis time is also greatly reduced (from about 1 h to 15 min). Therefore, the developed HPLC-EC method has great potential to provide a simple, rapid and routinely applicable tool for differentiation of common and rare meat species.

The rationale of employing EC detection with Cu^n-SPE for meat differentiation is based upon the electrode's insensitivity to large proteins (see Fig. 11 and below for mechanisms). As a result, the chromatographic profile is greatly simplified (from >10 peaks in Ashoor's to ≦4 peaks in this study), yet each species still has a unique HPLC-EC chromatographic profile. Analyses of the profiles reveals that some peaks are common to many species while others are more specific to one or two species. For example, a peak around 3 min was found in all examined meat species while peaks around 4.8, 6.2 and 9.5 min were shared by 7, 10 and 6 examined species, respectively. While the current data prevented us from defining group – specific and/or species-specific peaks, animals with closer taxonomic relationships did appear to show more chromatographic similarities. For instance, all tested mammals showed a peak around 6.2 min, while tested avian species have a peak around or close to 9 min. We did not find any single peak that was clearly species specific and can be used as sole marker for identification of certain species; however, certain peaks (such as 2.2 min for crab and scallop, 13.1 min for deer and pig, 8.3 min for chicken) appear to be more closely associated with certain species, which might be useful for narrowing down these species in mixed samples. The results are in general agreement with the concept that some components (especially proteins, peptides/amino acids) are common in many fresh meats. The fact that different chromatographic patterns exist is likely attributable to differences in meat composition as well as differences in the amounts and/or ratios of each component.

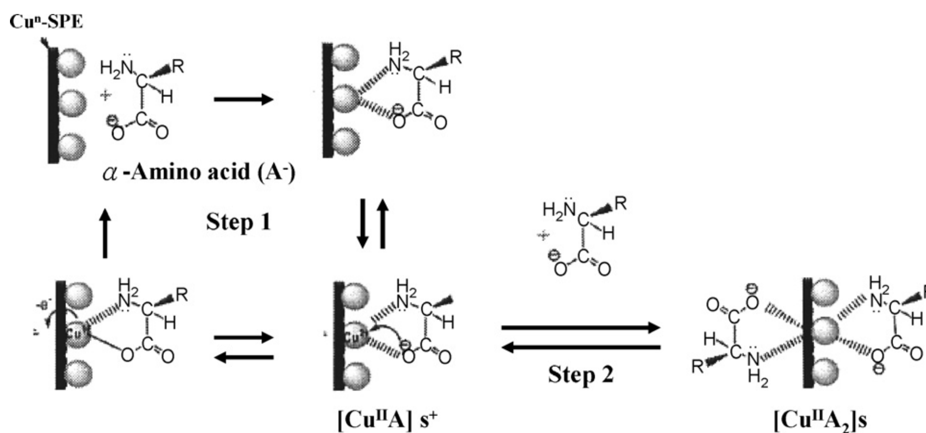

Fig. 11. Reaction mechanism for the copper(II)-amino acid complexation at the Cu^n-SPE.

For the purpose of this study, identification of each peak was not attempted; however, for the reasons stated below, it is plausible to assume that most detected peaks are likely amino acids/peptides and/or some small proteins from meat protein degradation. Proteins are the second largest components behind water in meat, evidence regarding the nature of the peaks could be deduced from the reaction mechanism of Cu^n-SPE. A two-step electrochemical mechanism has been proposed [24,25] for amino acid interactions with Cu electrodes (see Fig. 10). Normally, amino acids possess a bi-dentate ligand, where the $-COO^-$ and –N terminals function as the chelating site. The two-step process for the Cu^{II} (metal ion)-amino acid complexation could be diagramed as $Cu^{II} + A^- \leftrightarrow CuA^+$ (step 1) + $A^- \leftrightarrow$ CuA2 (step 2) where A denotes an α-amino acid. On the Cu^n-SPE surface, in step 1, the bi-dentate amino acid ligand is first chelated with Cu^n, followed by reversible reduction of $Cu^{II}O \rightarrow Cu_2{}^{I}O$. As soon as the reduced $Cu_2{}^{I}O$ is regenerated back to $Cu^{II}O$, the same cycle can be repeated. The reaction mechanism implies a weak adsorption of amino acid on the Cu^n during the complexation process, where the $Cu^{II}O$ is electrogenerated on the surface. The adsorbed amino acid can be easily desorbed as the $Cu^{II}O$ layer is reduced into $Cu_2{}^{I}O$. The rate of adsorption/desorption will significantly determine the reaction rate and resultant EC signal. The geometric structure of the chelation on Cu^n is also essential since $Cu^{II}O$ is not as free on the electrode surface as Cu^{II} metal ion in aqueous solution. Based on these mechanisms, free amino acids will be more steadily accessible to the $Cu^{II}O$ reaction site than peptides and proteins, whose density of functional $-COO^-$ and –N terminals, size and geometrical folding could sterically hindered the $Cu^{II}A2$ structure at the Cu^n-SPE surface (step 2). Ultimately, the overall reactions for large proteins are not sensitive, resulting in a clean baseline and efficient chromatographic profile for species identification. Fatty acids are also excluded from possible component of the major peaks based on the proposed mechanism. Since no single species-specific peak was clearly identifiable, it would be premature to perform LC/MS/MS or other methods for protein analysis in order to attempt peak identification at this stage. Further information concerning the nature of the peaks was obtained from analysis of heated (100 °C for 5 min) beef, pork and chicken samples. The heated meat juice revealed chromatograms with major peaks identical to the fresh samples (Fig. 9), suggesting that the composition of major peaks were heat-stable, water-soluble compounds. In view of the fact that heated juice produced chromatographic profiles very similar to the fresh meats, the developed EC method is likely also applicable for differentiation of cooked meats in at least the three test species.

The reproducible retention time of major peaks helps to establish the validity of using chromatographic profiling as a reliable primary screening method for species differentiation. Nutritional contents of animal feeds, sex, age, breed and geographical locations of the meat have all been suggested as possible confounding factors that could affect the species-specific profiles of meats. In this study, there were no significant profile differences among meat samples purchased from different vendors; therefore, the combined effects of feed, gender, age and individual differences produce little interference to the HPLC-EC. Comparisons of chromatographic profiles between pig round (leg) and flank and between duck leg and breast revealed no qualitative and only insignificant quantitative differences in the area of major peaks (CV for retention times between two body areas of pork and duck samples were all less than 2.1%). These findings suggest that differences in the two most consumed meat parts have a minimal effect on EC detection of these species (Fig. 10). Whether the qualitative similarities hold true for all other test species remains to be determined, however, it is likely that many edible cuts or parts from the same species share very similar chromatographic profiles that will be recognized by this approach. Freshness status of meats was another important factor to be considered when validating our method, since protein degradation begins immediately following an animal's slaughter. The degree of meat freshness could affect the amount and varieties of peptides/amino acids present in the sample and thus might exhibit time-dependent changes in chromatographic profiles. The chromatographic profiles of the most consumed species (beef, pork and chicken) were evaluated following incubation at room temperature (12 versus 24 h) or two freeze–thaw cycles and results indicate that only quantitative differences in peak areas occurred (Figs. 7 and 8). In view of the fact that our

samples were either purchased fresh (less than 3 h after slaughtering) or frozen at catch (cod, salmon) and the main determinant for species differentiation in this study is the time-specific chromatographic profiles, the consistency in retention time helps to further validate our method as a reliable tool for the differentiation of species of origin. In addition to retention time, EC response (or peak area) could also assist in the differentiation of similar chromatograms, such as alligator versus chicken and goat versus duck. The chromatograms of alligator (5-fold magnification in scale in Fig. 6) and goat are shown in the "miscellaneous" group, in which EC signals are significantly weaker than other species. Therefore, species with weak EC signals are presented together (Fig. 6) since original EC signal strength might serve as the initial screening parameter for our method.

In the study of chromatographic profiles during extended room temperature incubation and free–thaw cycles, we found that the EC method could be useful for detection of meat degradation. The change in peak concentration (expressed by peak area) in the first 24 h after an animal was slaughtered or after free–thaw treatment of fresh meat was monitored successfully by this EC method (Figs. 7 and 8) and it was feasible to quantitate degradative changes. The temporal change in chromatograms followed two patterns. While peaks in beef extracts decline at 24 h, major peaks in chicken and pork increase progressively with time. The similarity in concentration changes among most peaks suggest that a continuous degradation process takes place. The degradation of larger protein/peptides also gave rise to smaller peptide/amino acids, we found new peaks around 4 and 5 min starting to occurred at very low concentrations in beef and chicken (data not shown in Fig. 7 due to large scale), suggesting that longer incubation time or different incubation temperature (such as 4 °C) warrant further study and different species may exhibit different degradation patterns. Another noteworthy point was that despite minor statistical differences in peak areas, a 24 h room temperature incubation and 2 freeze–thawed cycles shared very similar trend in peak growth and decline, indicating that the pattern and extent of protein/peptide degradation were comparable by EC detection in these two situations. Identification of the peaks should greatly facilitate the understanding of any degradation process. Nevertheless, the current EC method was capable of monitoring the dynamic changes of the meat degradative process at room temperature and freeze–thawed processes. Although the study of possible protein degradation was conducted in only 3 species in view of the large amount of animal origins involved in this study, these are the 3 most consumed meat products by humans; the potential to evaluate meat degradation is exciting and possibly a unique feature that has not been explored with other species-identification techniques. Identification of adulteration of beef and pork carries significant importance for people with religious restrictions. Adulteration of more valuable meats with cheaper meats (e.g., horse meat in beef) is also a common and pervasive problem. Results shown here suggest that this method was possible to detect two-meat adulteration at 1:1 ratio as indicated by our ability to detect horse-specific peaks (7.6 and 12.1 min at more sensitive scale) in mixed meats samples from horse, beef and pork. Differentiation of pork and beef component by its characteristic peak (5.0 min for beef and 13.3 min for pork) were also demonstrated in this study (Table 3). Mixing of different meats in various ratios should provide further information regarding the validity of this method for detection of meat adulteration. One interesting fact we discovered in the current adulteration study was that not all peak areas followed the (1:1) dilution factor. While almost all peaks at 6.0, 7.6, 12.1 and 12.3 min generally follow the dilution effect (i.e., peak size reduced by around 50% when 1:1 mixed), small peaks (<1 C) at 5.0, 9.5 and 12.1 min exhibited increases while large peaks (≧10 C) at 3.0 min exhibited significantly decreased peak areas when two meats were mixed. The reasons for increased peak size following mixing could be related to the accumulation of EC signal from unquantifiable peak in one meat. In addition, it should be noted that these were smaller peaks with signals generated from near the lower limit of linear dynamic response of detector; larger quantitative variations were possible and thus contributed to the less well correspondence to the dilution factor. The reason for decreased peak area at 3 min remained to be studied but could be explained by the integration/formation of peptide/amino acids that changes the signal strength and retention time of the peak. The results indicated that EC signals in a meat mixture are not all predictable by dilution factor, further studies are therefore warranted. Preliminary result with modified analytical condition (increase mobile phase pH to 8) has yielded similar but distinct chromatographic profiles with selectively improved sensitivity for minor peaks (data not shown). Therefore, the current method at least provided a solid foundation for plausible use of EC detection to uncover 2-meat adulterations at least at 1:1 ratio.

In conclusion, we have demonstrated a simple and versatile application using EC detection to supplement molecular biological and other techniques for meat/species differentiation. The EC method not only is ideal for routine analysis of food-meat origin, it also has great potential to extend its application to the evaluation of degradative changes of meat proteins. Detection of meat adulteration with species-characteristic peaks is also a plausible direction (Table 3). The method was evaluated for fresh meat samples, but likely also is suitable for use on cooked meats. Although the method was shown to be capable of differentiating 15 meat species commonly consumed in Taiwan, it should be noted that situations requiring the simultaneous differentiation of these 15 species are rare. Differentiation of 2–3 species in doubt is more likely to occur in the real world and the current method should be a practical tool for such purpose. Nevertheless, further studies are needed to identify the chemical nature of major peaks in these chromatographic profiles. In-depth evaluations are warranted to further elucidate the extent to which factors that might affect the pattern of the profiles, namely gender, breed, canned processing and multiple anatomical locations from the animal. Modification of sample pre-treatment such as enzyme digestion to take advantage of the method's ability to differentiate small peptides/amino acids may be worthwhile in order to further improve the sensitivity for meat freshness evaluation and detection of partial adulteration. Forensic application could be another interesting field this method could be applied to.

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