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Article

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Rapid identification of meat species by the internal extractive electrospray ionization mass spectrometry of hemoglobin selectively extracted on functionalized graphene oxide

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Abstract: Hemoglobin (Hb) present in the blood and meat juice samples was selectively adsorbed by graphene oxide (GO) particles functionalized with amylopectin (AP) and was sensitively detected by the direct internal extractive electrospray ionization mass spectrometry (iEESI-MS) analysis for the identification of meat type. Various samples including the whole blood samples of chicken, duck, sheep, mouse, pigeon, turtledove and meat juice mixtures were successfully identified based on the difference in molecular composition of Hb reflected in MS. The adulteration of sheep blood with only 2% chicken blood could be detected, which demonstrated the high chemical specificity of the approach. The established method is featured by the high speed of analysis (4 min per sample, including the analyte extraction and sample loading), high sensitivity, minimal sample preparation and low sample consumption (0.9 μL whole blood or 300 mg raw meat). In perspective, the reported method can be extended for the sensitive detection of trace analytes in complex matrices in broad molecular range by using the selective enrichment on functionalized graphene oxide particles followed by iEESI-MS analysis.

Keywords: Internal extractive electrospray ionization, mass spectrometry, meat species, hemoglobin, functionalized graphene oxide

Introduction

The emergence of ambient mass spectrometry about a decade ago has enabled the direct chemical analysis of raw samples with greatly increased speed and simplicity.[1-4] Pioneered with desorption electrospray ionization (DESI)[5] and direct analysis in real time (DART),[6] more than 60 ambient ionization methods have been developed for direct characterization of many types of raw samples,[7-10] particularly for solid surfaces.[11-15] Internal extractive electrospray ionization (iEESI) was introduced for the characterization of interior chemicals in a bulk volume rather than at the surface of solid samples.[16] In iEESI, a solvent charged by a high voltage is infused by a syringe pump at 2-10 μL/min through the 3-dimensional volume of a raw bulk sample. The analytes in the 3-D volume are extracted by the solvent and are driven with the flow toward the sample edge that faces the ion entrance of the mass spectrometer. Charged plume is formed at the edge of the sample, which ultimately gives rise to the gas-phase ions detected by mass spectrometric detection. The iEESI technique has recently been applied to characterize biological bulk samples.[17-18]

Meat products are frequently adulterated by the addition of meat of inferior quality.[19] The identification of meat species protects customers from economic losses, risk of poison and/or violation of religious belief. The detection of meat adulteration may also help against the killing of rare animals, by providing concrete molecular evidences. To date, meat identification has been primarily conducted based on protein or DNA analysis.[20-21] Among the DNA-based methods, Polymerase Chain Reaction (PCR) analysis was prevalently employed,[22-23] and several variants of PCR-based technique including PCR-restriction fragment length polymorphism (PCR-RFLP),[24] real-time

PCR,[25-26] specific PCR,[27] random amplified of polymorphic DNA-PCR (RAPD-PCR) fingerprints,[28-29] amplified fragment length polymorphism-PCR (AFLP-PCR)[30] and actin gene-related polymerase chain reaction[31] are now available to precisely identify the origin of meat. However, DNA extraction from the meat tissue/juice is a time-consuming and laborious step. As food is a chemically diverse matrix, it is difficult to design a facile and versatile extraction protocol for a broad range of samples. Thus the DNA extraction methods must be optimized for each particular case in order to ensure that enough DNA was obtained for the analysis.[32] Alternatively, a number of proteomic-based approaches have been developed for meat identification. For instance, detection of chicken in meat mixes was succeeded by profiling the peptides using mass spectrometry coupled with liquid chromatography.[32] After meat digestion, analysis of whole meat digestate by liquid extraction surface analysis (LESA) mass spectrometry has been reported, suggesting that a bunch of five proteins (myoglobin, troponin C, actin, bovine serum albumin, tropomyosin) would be useful molecular markers for differentiation of meat samples.[33] However, the approach is generally labor and time consuming, usually requiring about 10 min per analysis (excluding meat digestion).

In this study, hemoglobin (Hb) present in the blood and meat juice samples was selectively preconcentrated by graphene oxide particles functionalized with amylopectin (AP) and was sensitively detected by the direct internal extractive electrospray ionization mass spectrometry (iEESI-MS) analysis for the identification of meat type. The current work establishes a fast protocol for molecular identification of meat species with minimal sample pretreatment and motivates advanced applications based on the combination of selective adsorption with direct iEESI-MS analysis for the sensitive detection of trace analytes in complex matrices.

Materials and Methods

Materials and reagents

High purity graphite powder was purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China) and amylopectin (AP, from waxy maize, 99.7%) was from Derui Bio-technology Co. Ltd (Zhengzhou, China). Hydrazine hydrate (85%) was obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China) and dimethyl sulfoxide (99%) was from J&K Scientific Ltd (Shanghai, China). Methanol and formic acid of HPLC grade from Anaqua Co. Ltd (Houston, USA) were used. Whole bloods and raw meats of different animals were obtained locally and kept at -80 ^{o}C for analysis.

Preparation and characterization of the AP-rGO material

AP-rGO (Amylopectin-reduced Graphene Oxide) material was synthesized and characterized with the literature method.[34] Characterization was completed by fourier transform infrared (FT-IR), scanning electron microscope (SEM), surface charge property, N_2 adsorption-desorption isotherms and thermogravimetric measurement (TGA), which were performed with Nicolet 6700 spectrometer (Thermo Electron, USA), S-3400N scanning electron microscope (Hitachi High Technologies, Japan), ZEN3600 Nano Zetasizer (Malvern, UK), Micromeritcs Tristar 3000 analyzer (USA) and TGA/DSC 1 STAR^{e} system (Mettler-Toledo, Switzerland), respectively.

Experimental procedure

The experimental protocol of internal extractive electrospray ionization ion trap mass spectrometry (iEESI-IT-MS) was shown in Figure 1. The meat sample (*c.a.*, 300 mg) was flushed using deionized water (*c.a.*, 200 μL) to obtain the meat eluate. Sheep meat slice (500 mg) was boiled in water (400 μL) at 90 ^{o}C for 5 min. After heating, the solution was transferred for further analysis. The whole blood (0.9 μL), blood stain (0.9 μL whole blood dried on paper) or chicken and sheep whole blood mixtures in different proportions (0.9 μL) was directly diluted with 200 μL deionized water to prepare the sample mixtures. Fresh whole blood was split into three 1 mL fractions, which were inoculated with Klebsiella pneumoniae (KP) at the amount of one monoclonal colony. Storing at 35 ^{o}C for 4, 24, 48 and 72 hours, the mixtures were shaken up and small amount (0.9 μL) was diluted with deionized water (200 μL) for further analysis. Then the sample was loaded into an aspirator passing a filter to remove the deposit. Before that, powder material AP-rGO (2 mg) had already been transferred into the aspirator. Mixture of filtered sample and material was heavily vortexed for 60 s, ensuring the Hb could be captured by the AP-rGO completely. After that, the sample waste was discharged and the AP-rGO material loaded with Hb was gathered in a filter. With three times of washing by deionized water, most matrix adhering on the material was removed. Finally, Hb adsorbed on material was extractively ionized online by using extraction solution (methanol/H_2O/formic acid (v/v/v, 49/49/2)) at 5 μL/min for mass analysis. The whole process was accomplished within 4 min (including the analyte capture and sample loading).

All samples were analyzed employing a linear trap quadruple (LTQ) mass spectrometer (Thermo Scientific, U.S.A.) coupled with a homemade iEESI source. The mass spectra were collected in a positive-ion detection mode. Extraction solution was pumped with a

syringe pump (Harvard Apparatus, Holliston, MA, U.S.A.). Ionization voltage was set at +2.5 kV, and the LTQ capillary was heated to 300 ^{o}C. The pressure of nitrogen sheath gas was 1.0 MPa. Collision induced dissociation (CID) experiments were performed, in which the precursor ion of *m/z* 1815 was isolated with a mass-to-charge ratio window width of 1 Da and normalized collision energy (NCE) was set to 20%.

Results and Discussion

Capture and release of hemoglobin by the AP-rGO material

The property of the three-dimensional AP-rGO framework has been described in our recent report.[34] AP and rGO combine together by the formation of hydrogen bonds between the hydroxyl groups of AP and the oxygen-containing functional groups of GO. With the formation of AP-rGO, the characteristic 3D structure was observed instead of rGO sheets, confirming that the synthesis of AP-rGO was successful.

Hemoglobin is a tetramer of two α and two β subunit chains with four noncovalently bound Fe^{2+}-containing heme groups. With hydroxyl groups and π-conjugated system as the active sites, AP-rGO exhibit excellent adsorption performance toward Hb through coordination with the sixth vacant coordinating position of Fe^{2+} and combination with the aromatic groups. At the same time, it showed low adsorption capacity of bovine serum albumin in which no heme group was involved. Our previous study[34] indicated that favorable adsorption of Hb was achieved around its isoelectric point (pH 6.8) by AP-rGO. Therefore, pH 6.8 was adopted for the sample solution in adsorption steps and it was maintained by using deionized water. After the adsorption process, material was infused by water and no obvious signal loss of Hb was obtained. It indicated that Hb

was suitably captured by material.

We found that at the medium pH below 4.0 both the surface of the AP-rGO and hemoglobin (pI 6.8) were positively charged. The electrostatic repulsion between the positively charged AP-rGO and hemoglobin would facilitate desorption. Therefore, an acidic solution (methanol/H_2O/formic acid (v/v/v, 49/49/2); pH 2.4) was used as extraction/spray solvent for further experiments.

Detection of Hb from raw/boiled whole blood

The iEESI-IT-MS analysis was applied to the whole blood. Typical mass spectra obtained from the different animal bloods show Hb signals detected by iEESI-IT-MS (Figure 2 and Figure 3(a)). Chain assignment was achieved by spectral deconvolution from the *m/z* domain to the Da domain. The blood samples of chicken and duck were composed of two Hb components which were identical in β chains but different in the sequences of α subunits (α^A and α^D). Mass spectrum obtained from a local chicken blood sample is illustrated in Figure 2 (a), from which the molecular weights of 15688 Da and 16327 Da were obtained for α and β-chains, respectively. Mass spectral profiles of a local duck blood sample (Figure 2 (b)) gave the molecular weights of 15742 and 16318 Da for the hemoglobin α^D and β-chains. These data are in good agreement with the data reported in literature[35-36]. The identification of abundant signals (b_1 and b_2) with molecular weights of 15090 and 15246 Da is left for future studies. The calculated molecular weights of the α- and β-chains of Hb in sheep blood samples were 15046 and 16050 Da (Figure 3 (a)). The molecular weight of the α-chain (15046 Da) was in agreement with the value (15047 Da) previously reported.[37] However, the MW of β-chain differed from the literature ones (16067/16073 Da). Probably, this was attributed

173 to the subspecies in sheep. For example, different molecular weight for Hb α-chain in
174 Hereford and Jersey cattle[37] was reported. The difference was due to the subspecies
175 differentiation. According to the spectrum of Figure 2(c), the molecular weights of
176 mouse α- and β-chains were 14960 and 15712 Da, respectively. In the literature[38],
177 difference in the molecular weights of α and β chains was observed among several
178 mouse strains. In one strain α and β chains appeared at 14981 and 15618 Da, whereas in
179 another strain α and β chains appeared at 14996 and 15712 Da. The existence of Hb
180 difference in subspecies made it achievable for subspecies identification. Below we
181 discuss differences in the blood composition for different species belonging to the same
182 family. Both turtledove and pigeon belong to Columbidae. The spectra of Figure 2(d-e)
183 revealed that they had identical MW of β-chains (16168 Da) but different ones of α-
184 chains (15087 and 15121 Da). In summary, Hb from different species displayed unique
185 molecular weight specific to the species, which provided molecular evidence for meat
186 identification.

187 To simulate the meat of inferior quality, fresh blood was inoculated with Klebsiella
188 pneumoniae (KP). After a certain period of time, the simulated blood samples were
189 analyzed by iEESI-IT-MS technique. Each spectrum was compared with the spectrum
190 detected from the non-inoculated blood. The intensity of hemoglobin signal detected
191 from KP blood notably decreased with storage time. The hemoglobin signal almost
192 disappeared after 72 h storage. We speculate that KP consumed hemoglobin or caused
193 hemoglobin denaturalization and deactivation, which affected the adsorption of
194 hemoglobin on AP-rGO material.

195 Extra experiments were performed to test the iEESI-IT-MS method for the

differentiation of cooked samples such as boiled blood samples. The sheep whole blood boiled at 100 ^{o}C for 2 minutes offered a noisy spectrum (Figure 3(b)) showing the characteristic peaks of the α-chain of sheep Hb, including sodium adducts in the heated sample[37] with low signal-to-noise ratio. Note that no signal of β-chain was observed while the α-chain appeared as the major ion in Figure 3(b), indicating that the β-chain of sheep Hb was more sensitive than the α-chain to heating although the mass spectrum was still of diagnostic value. Once the sheep blood was boiled for more than 5 min, no α-chain signal of sheep Hb was detected. However, both α-chain and β-chain of sheep Hb were detected once freshly collected sheep blood was added into the boiled samples, indicating that the low signal levels of sheep blood Hb were probably caused by the heat induced dissociation of the Hb protein. These data revealed that boiling for 2-5 minutes did not affect the traces obtained by the method except for the noise increase, however, the method was not suitable for well-cooked samples, especially for the blood samples since the protein in a cooked blood sample was easily dissociated due to the lack of the support and protection by the tissue textures.

Detection of Hb from whole blood mixtures

Substitution of high quality meat with inferior meat results in an increased illicit profit. To simulate adulteration of meat, chicken whole blood was mixed with sheep blood in different ratios, and the mixtures were tested by iEESI-IT-MS. As an example, Figure 4 shows spectra obtained by analysis of the sample containing 50%, 10%, 5% and 2% chicken blood in the mixture. The β-chain of chicken Hb was traced for the specific detection. With the same doping volume, the intensities of the globins were closer. When the content of chicken blood was decreased, its β-chain signal became weaker.

With only 5% chicken blood was present in the sample, its β-chain carried 11, 10 and 9 charges were clearly detected at *m/z* 1485, 1634 and 1815 with good signal-to-noise ratio. While with 2% chicken in sheep whole blood, only a tiny peak at *m/z* 1815 was detected as shown in the inset of Figure 4(d). Since tandem MS (MS/MS) could provide valuable information for structure elucidation, CID experiments were performed to confirm the identity of the peak at *m/z* 1815. An averaged MS/MS spectrum of ions of *m/z* 1815 detected from a sample of 100% chicken blood (Figure 5(a)) and a mixed sample with 2% chicken in sheep whole blood (Figure 5(b)) were comparatively shown, in which the same product ions were detected with different intensities owing to the varied concentrations of the chicken Hb in the samples. Compared with the MS/MS spectrum of the *m/z* 1815 ion in 100% sheep blood (Figure 5(c)) and in blank sample (Figure 5(d)), significant signal differences were observed in Figure 5(b), indicating successful differentiation of adulterated sample. Our experimental data indicate that the iEESI-IT-MS approach allows the detection of 2% chicken blood in sheep blood.

Quantitative analysis of blood mixtures with different proportions of chicken and sheep blood was carried out. A series of blood mixtures (chicken/sheep blood, 0.9 μL) were employed as working samples for iEESI-IT-MS analysis. The fragment ion (*m/z* 1347) of *m/z* 1815 was used as a marker signal for the quantification of chicken blood by MS/MS. As shown in Figure 6, the intensity of *m/z* 1347 signal was found in a very good linear correlation with the percentage of chicken blood over the range from 0 (no chicken blood; only sheep blood) up to 100% (only chicken blood; no sheep blood) ($y=324.2x(\%)-3.87$, $R^2=0.9995$). Each data point designated six measurements, and the relative standard deviations (RSDs) were below 5.0% for all the data points. The error bars in Figure 6 indicated the RSDs of six replicates. The results showed that the

proportion of chicken blood mixed in the samples could be successfully detected using iEESI-IT-MS/MS.

Detection of Hb in raw meat

Extracted solution of sheep meat sample was obtained by washing the sheep meat with deionized water. As shown in Figure 3(c), prime signals of Hb were observed in the iEESI-IT-MS spectrum. The complex matrix such as physiological salts, various proteins, fat and other soluble compounds[39] contained in the raw meat showed no significant interference. The relative intensity of heme group was strongly higher compared with that of the whole blood sample. Besides hemoglobin, meat contained myoglobin which was also a heme protein, probably because of the contribution of heme groups from myoglobin.

Detection of Hb in boiled meat

Apart from the raw meat samples, we also briefly tested the method performance for the analysis of cooked meat samples. No Hb signals could be detected from pork samples boiled over 5 min, whereas some new protein species with MW higher than that of globin chains were observed in the mass spectrum. The observed new proteins probably correspond to the adducts of Hb globin chains with other species from the meat and may potentially be useful as markers for the differentiation between cooked meat samples in future studies.

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Figure Captions

Figure 1. Schematic illustration of the concept and protocol of AP-rGO-iEESI-IT-MS technique for the analysis of meat and blood samples.

Figure 2. The iEESI-IT-MS spectra of hemoglobin detected from different whole blood samples: the multiply charged Hb ions determine the molecular weight of Hb protein. a-chicken, b-duck, c-mouse, d-turtledove, e-pigeon.

Figure 3. Mass spectrum of hemoglobin obtained by iEESI-IT-MS of various samples: a-sheep whole blood, b-sheep blood sample boiled for 2 min, c-sheep meat.

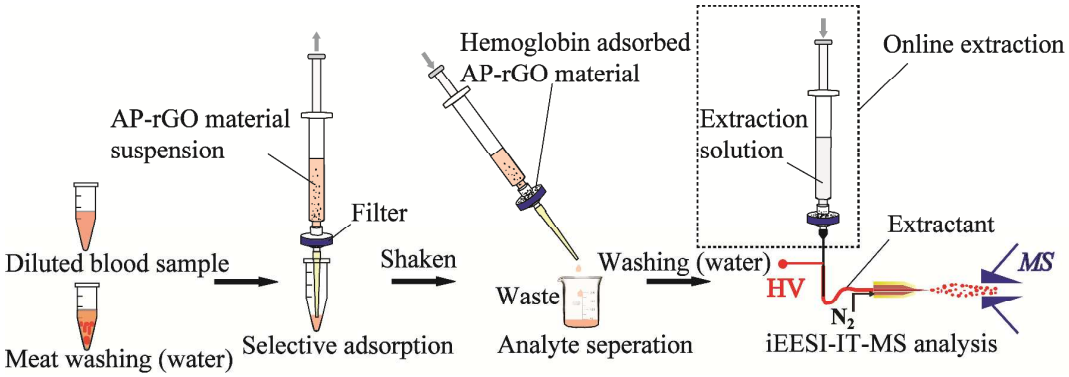
Figure 4. Mass spectra of hemoglobin extracted from the mixture of chicken in sheep blood obtained by iEESI-IT-MS. β^c-β chain of chicken Hb, α^s-α chain of sheep Hb, β^s-β chain of sheep Hb.

Figure 5. Averaged MS/MS spectra of ion *m/z* 1815 from whole blood samples. a-100% chicken whole blood, b-2% chicken in sheep whole blood, c-100% sheep whole blood, d-blank.

Figure 6. The intensity levels of the characteristic fragment (*m/z* 1347) against the percentage (%) of chicken blood in the blood mixtures.

Figures

Figure 1



413 **Figure 2**

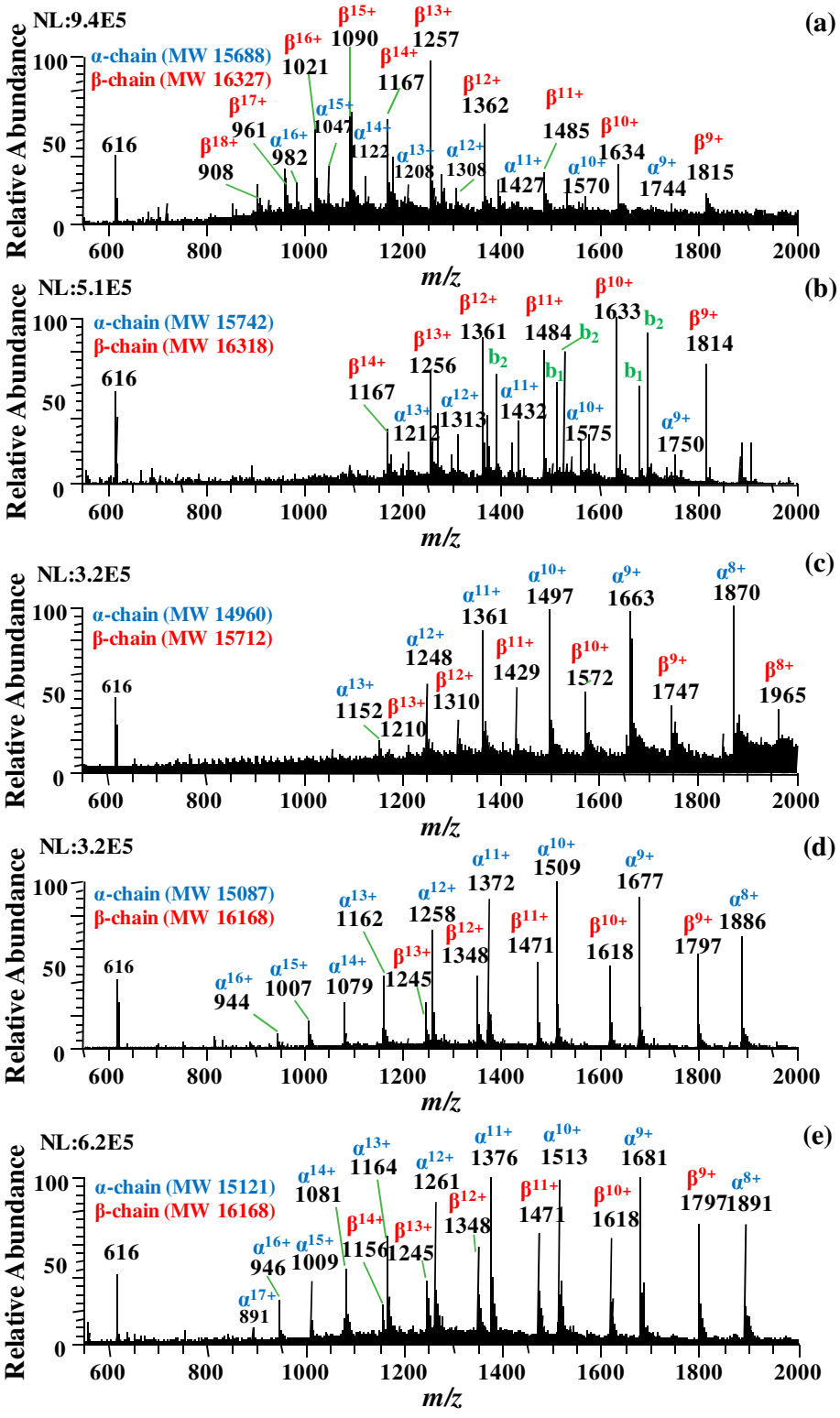


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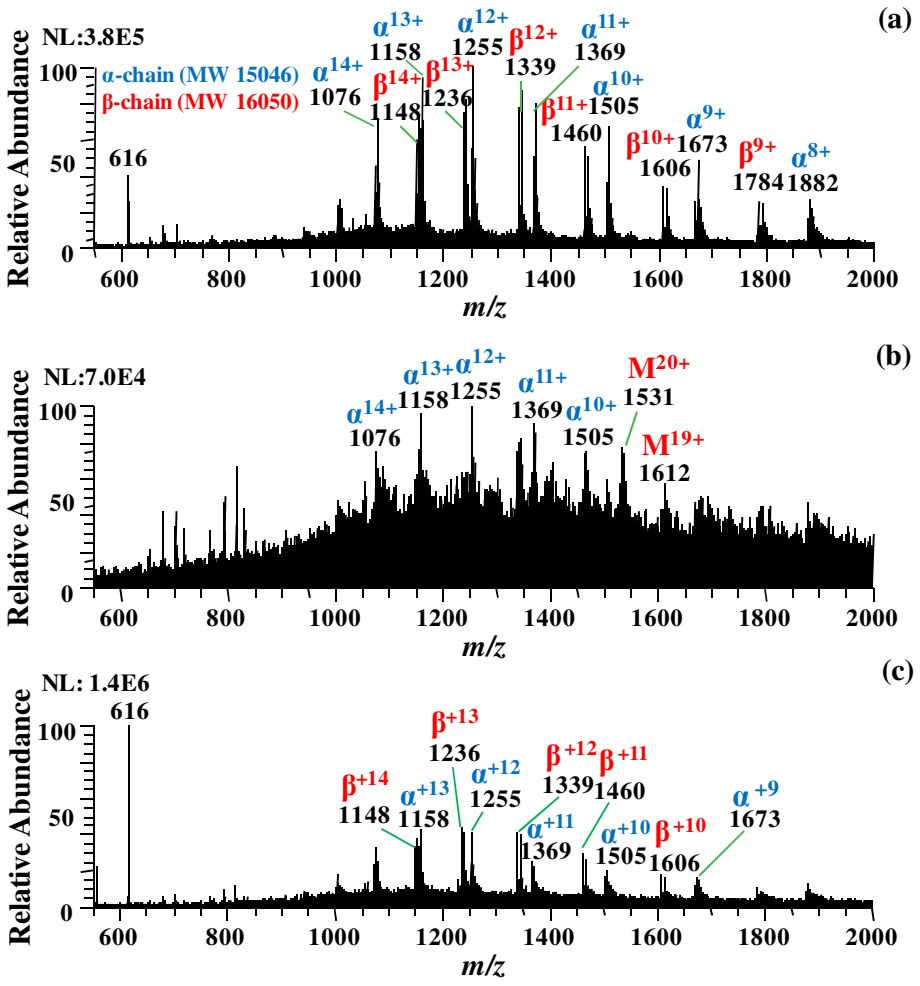
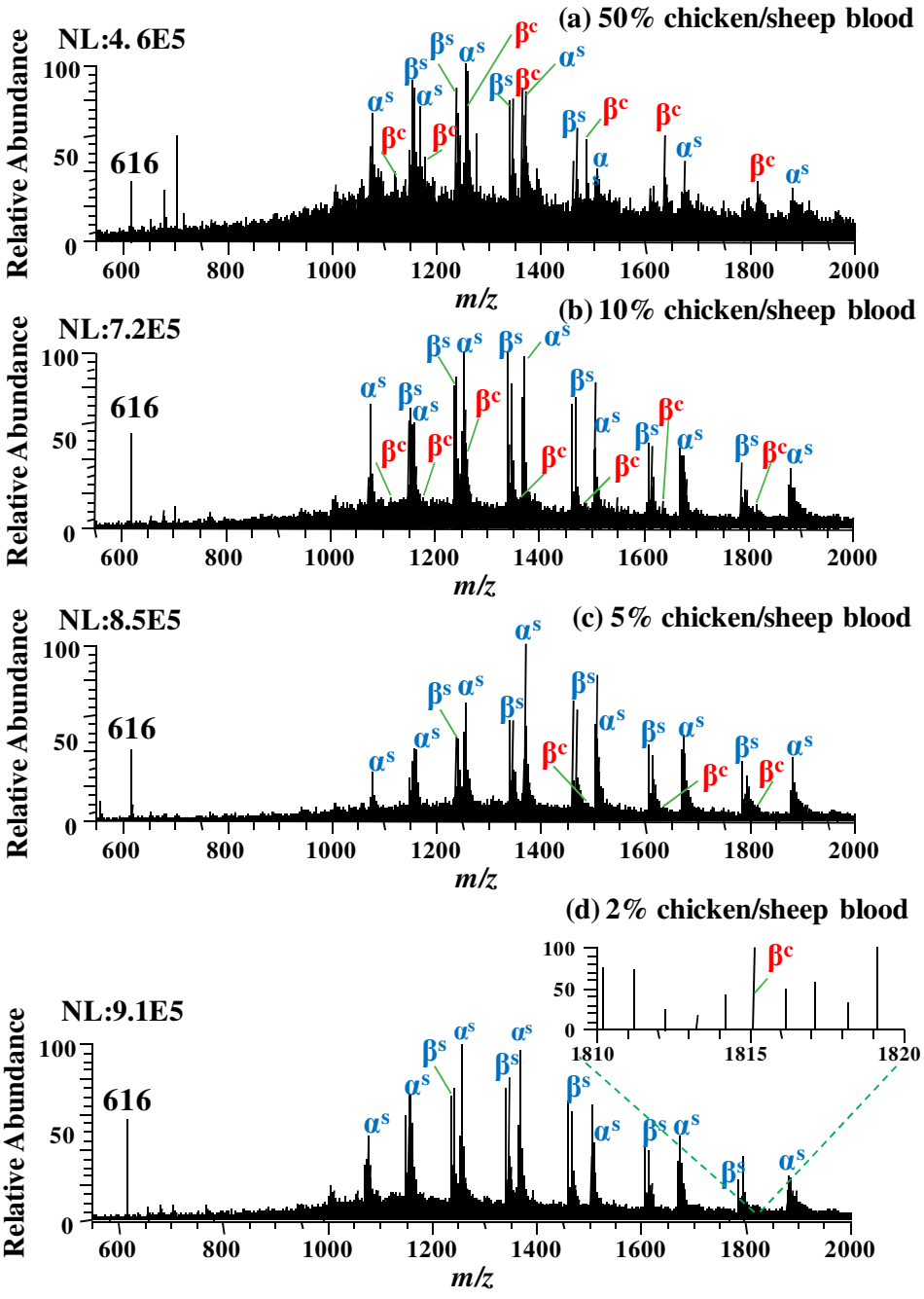
Figure 3


(a)
NL:3.8E5
α-chain (MW 15046)
β-chain (MW 16050)
α13+ 1158
α12+ 1255
β12+ 1339
α11+ 1369
α14+ 1076
β14+ 1148
β13+ 1236
α10+ 1505
β11+ 1460
α9+ 1673
β10+ 1606
β9+ 1784
α8+ 1882
616
Relative Abundance
m/z
(b)
NL:7.0E4
α13+ α12+ 1255
α14+ 1158
1076
α11+ 1369
α10+ 1505
M20+ 1531
M19+ 1612
(c)
NL: 1.4E6
616
β+13 1236
β+14 1148
α+13 1158
α+12 1255
β+12 1339
β+11 1460
α+11 1369
α+10 1505
β+10 1606
α+9 1673

418 **Figure 4**



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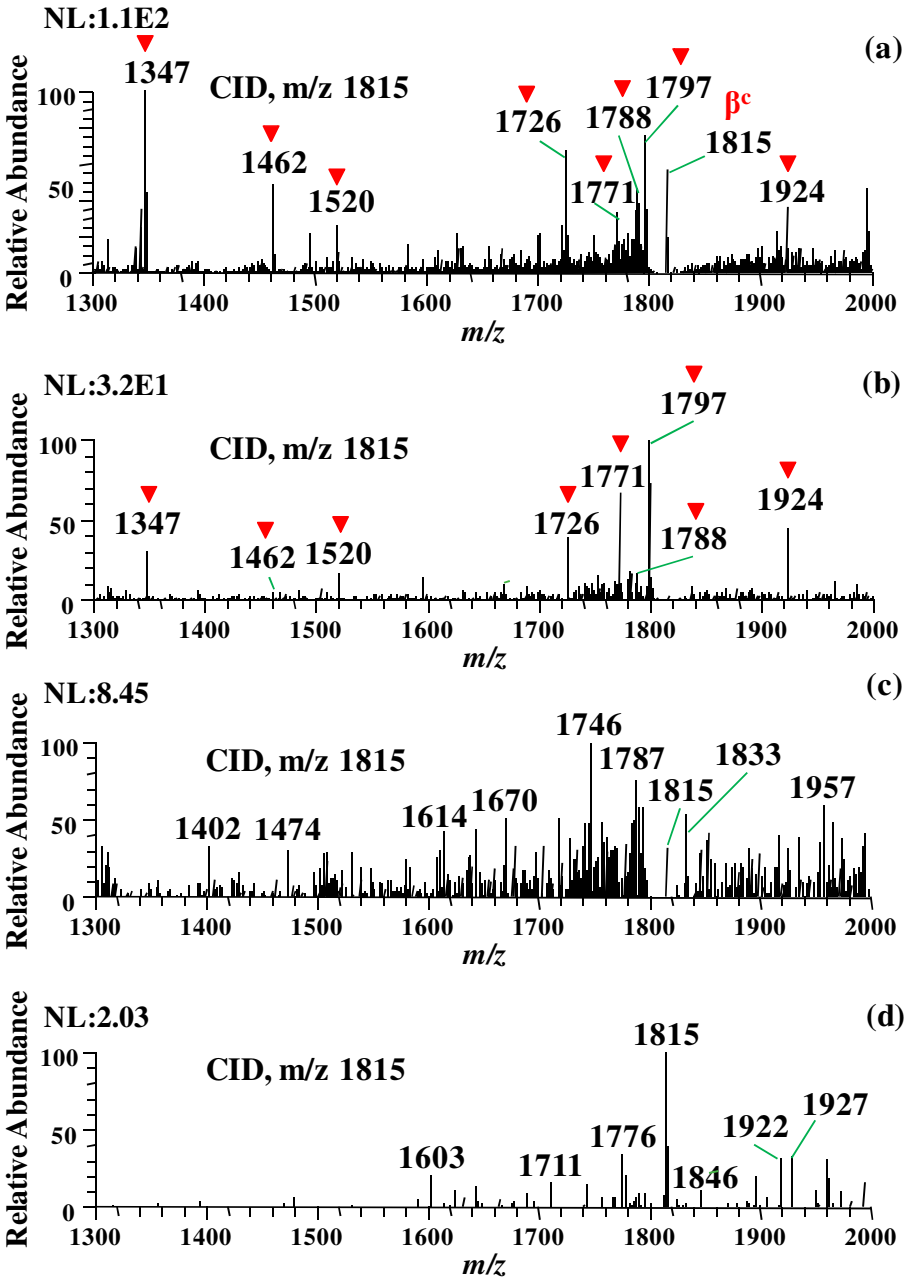
421 **Figure 5**



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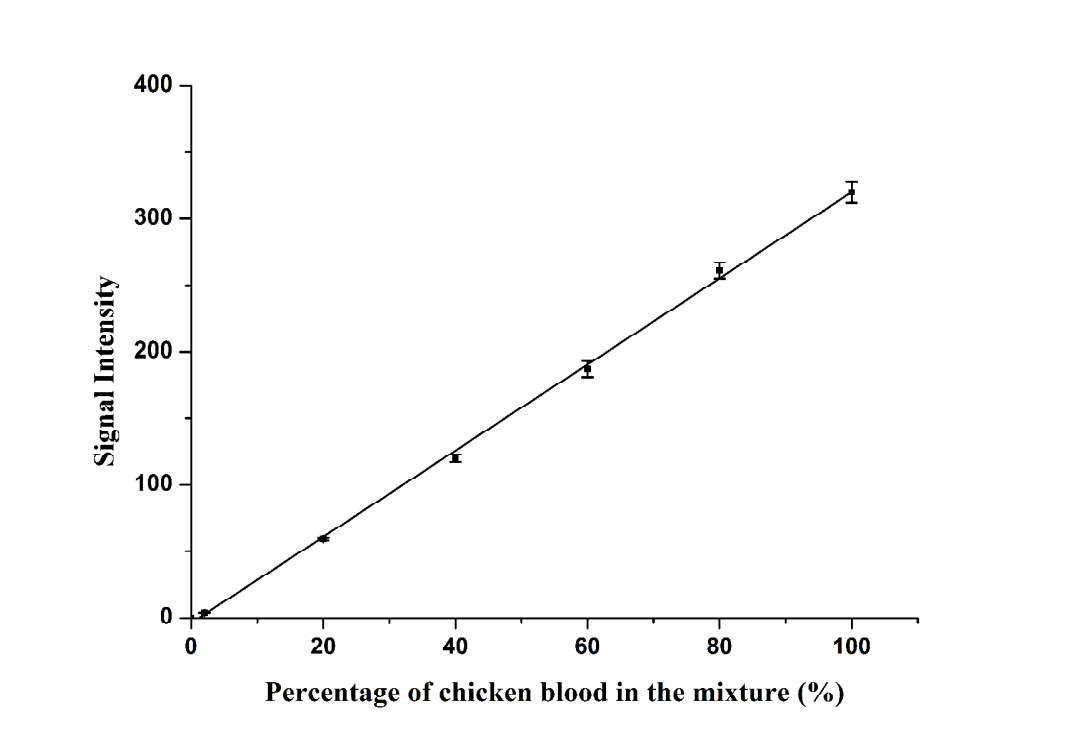
424 **Figure 6**



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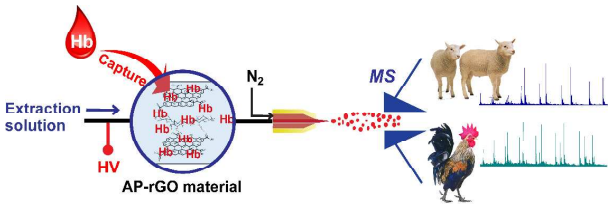
427 # TOC Graphic



428

429 8.47×3.03 cm (600 DPI)