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Authentication of meat species and net muscle proteins: updating of an old concept

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Abstract: The aim of the study was to develop an efficient method for assessment of meat origin and determination of net muscle protein (NMP; based on amino acids and β-alanylhistidine dipeptides) in meat products using the ultra-high performance liquid chromatography coupled with mass spectrometry. As an important result, a database of ratios of particular amino acids to 3-metylhistidine, applicable for muscle protein origin confirmation, was established. Based on the specific ratios of 1-methylhisitidine/3-methylhistidine, revealing of undeclared addition of 2% of chicken meat to the pork was enabled. Similar outcome was achieved by considering the ratios of β-alanylhistidine dipeptides. In the case of chicken-pork and pork-beef admixtures, as low additions as 0.5 and 2% of chicken or pork adulterant could be recognized. The ratio of 4-hydroxyproline/3-methylhisitidine was shown to be diagnostic for detection undeclared addition of 0.5 and 1.5% of connective tissues into the pork and chicken meat, respectively. On the basis of 3-methylhisitidine concentration, the conversion factor $F = 292 \pm 4$ was calculated for quantification of NMP (%) content.

Keywords: collagen; net muscle protein; 1-methylhisitidine; 3-methylhisitidine; β-alanylhistidine dipeptides

Counterfeiting of meat based products is a global problem (Ballin 2010; Manning *et. al* 2016). The most common types of fraud are partial substitution of muscle protein of a highly priced meet by a cheaper one, or addition of undeclared low grade proteins. Collagen-based connective tissues are used, to increase apparent 'total protein' content, alternatively, blood plasma or plant proteins are the options (Abbas *et al.* 2018).

Currently, the control of meat products quality and authenticity is based on 'net muscle protein' (NMP) content which is calculated as a difference between the 'total protein' determined by the Kjeldahl method (AOAC 991.22), and the amount of collagen that is calculated using determined 4-hydroxyproline (OH-Pro) concentration (AOAC 990.26). The main drawback of this 'official method' commonly used in routine practice, is an impossibility to detect addition any of adulterating proteins other than collagen (Benedict 1987; Ballin 2010).

Under these conditions, as a more specific way to categorize the quality of meat products might be considered a determination of amino acids occurring exclusively in muscle proteins such as 1-methylhistidine (1-MetHis) and 3-methylhistidine (3-MetHis) (Kvasnicka *et al.* 1999); alternatively,

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specific extracellular β-alanylhistidine dipeptides, carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-3-methylhistidine), and balenine (β-alanyl-L-1-methylhistidine) may be used (Mora 2007).

The amino acid 3-MetHis was proposed as a possible meat-protein indicator as soon as in 70th (Hibbert & Lawrie 1972); later on it was found that in almost all meat species the concentration of its content was comparable, approx. 5 mg/g of non-collagen nitrogen (Johnson and Lawrie 1988). On the other hand, contrary to 3-MetHis, the concentration of its isomer 1-MetHis is species dependent (Kvasnicka *et al.*1999).

As regards the above mentioned β-alanylhistidine dipeptides, carnosine, anserine, and balenine they also naturally occur in vertebrate animal tissues (Aristoy *et al.* 2004), and their amount varies with the animal species (Macià *et al*. 2012; Xie *et al.* 2013), age and/or diet (Chan & Decker 1994). The possibility differentiate between the animal species based on relative ratios of β-alanylhistidine dipeptides was proposed earlier, nevertheless, when used for recognition of meat product adulteration by undeclared meat, an addition as high as tens of percent could be only detected (Carneige *et. al.* 1984; Abe & Okuma 1995).

In the present study, new concept of meat quality and authenticity has been introduced employing information obtained by target analysis of 27 free amino acids and 3 β-alanylhistidine dipeptides, carnosine, anserine and balenine in experimental samples represented by chicken, beef and pork meet and their admixtures. Contrary to earlier studies concerned with analysis of these compounds, we were able to determine all these target analytes in a single run using a newly developed method based on hydrophilic interaction liquid chromatography (HILIC) in ultrahigh performance mode (U-HPLC) coupled with a simple, single quadrupole mass spectrometer (*MS*).

MATERIAL AND METHODS

Chemicals and reagents. Altogether analytical standards of 27 amino acids (Table S1, see electronic supplementary material; ESM) and 2 β-alanylhistidine dipeptides (L-anserine, L-carnosine) were obtained by Merck. The declared purity of all standards was > 98%. Acetonitrile was of HPLC grade and obtained from Merck. Other reagents and chemicals were of analytical grade. Formic acid, ammonium acetate, hydrochloric acid (35%), phenol, sodium metabisulfite, hydrogen peroxide and sodium carbonate were supplied by Merck.

Standards preparation

Amino acids. 17 amino acids (Solution I) were supplied by the manufacturer in acidified aqueous solution (0.1 mol/l HCl). Individual concentrations (mg/l) in the stock solution I are listed in Table S1 in ESM. Amino acids supplied in a solid form (Table S1 in ESM) were prepared at concentration of 200 mg/l in distilled water (Solution II). Working standards were prepared in 80% methanol, 100 µl of Solution I, 100 µl of Solution II and 800 µl of methanol were mixed in the vial giving a calibration point A (20 mg/l). Concentrations of all calibration standards B (10 mg/l) – M (0.002 mg/l) are summarized in Table S2 in ESM.

β-alanylhistidine dipeptides. Mixed stock solution of L-anserine and L-carnosine was prepared at concentration of 100 ng/ml in distilled water. Working standards were prepared at concentration range 10–0.001 mg/l in 80% methanol.

Analysed samples. Altogether, 36 authentic meat samples were obtained either directly from a slaughterhouse or from butcher. Homogenates of muscles from different parts of animals were analysed to recognize the variability of amino acids and β-alanylhistidine dipeptides composition across the animal body in the each species. The samples were as follows: chicken breast (n = 5), chicken leg (n = 5), pork leg (n = 4), pork choke (n = 4), pork neck-end (n = 4), pork shoulder (n = 4), beef chuck (n = 5) and beef round (n = 5). The same samples were used for characterizing the protein amino acid composition of proteins of animal species tested.

Preparation of model meat admixtures for the purpose of animal species authentication. The first set of model admixtures was prepared from chicken breast with pork leg. Homogenate of chicken meat was added to 20 g of pork to obtain final concentrations of 0.5; 1; 1.5; 2; 4; 6; 10; 15; 30 and 50% (w/w).

The second set of model admixtures simulating potential substitution scenario was prepared from beef round with pork leg. The pork meat was added to 20 g of beef to obtain concentration levels of 0.5, 1, 1.5, 2, 4, 6, 10, 15, 30 and 50% (w/w).

Model mixtures of meat and foreign proteins for the purpose of revealing their undeclared addition. To determine whether the addition of foreign

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protein is detectable based on amino acid composition ratios, two model admixtures containing either pork leg or chicken breast meat with added powdered collagen protein were prepared. To 20 g of homogenized pork or chicken meat, collagen was added to gain concentration levels of 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4; to 5% (w/w). Sample of dry collagen powder (designated as K4065) was supplied by State Veterinary Administration, Prague. The collagen content (OH-Pro x 8) determined by U–HPLC-MS method was 24% for all proteins.

Sample preparation. The sample preparation consisted of the three main steps: (1) extraction of homogenized sample to isolate free β-alanylhistidine dipeptides carnosine, anserine and balenine, (2) oxidation of sample to prevent the losses of sensitive sulphurous amino acids during protein hydrolysis, and (3) the acidic hydrolysis of proteins.

The weight of 1 g of homogenized sample was placed into the 50 ml centrifuge tube and extracted with 30 ml of 80% methanol by shaking with the laboratory shaker (240 rpm). The suspension was then centrifuged at 10 000 rpm for 5 minutes. The supernatant was then micro-filtered (2 μm), and 10 ×, 100 × diluted aliquot was used for the β-alanylhistidine dipeptides determination. The solid residue containing meat proteins was rinsed 2 × with 10 ml of 80% methanol, centrifuged again and then transferred into hydrolysis flask.

Prior to proteins hydrolysis by 6 mol/l hydrochloric acid, sensitive sulfur containing amino acids were oxidized by performic acid to prevent their losses. (Spindler *et al.* 1984*).* After completion of the hydrolysis, the content of the hydrolysis flask was filtered and quantitatively transferred into a 100 ml volumetric flask. Subsequently, 6 ml of the filtrate was transferred to a 10 ml volumetric flask and neutralized with 1.5 mol/l sodium carbonate to pH ≈ 7. With regard to different amino acids concentrations, the samples were diluted 10 ×, 100 × and 1000 × with 80% methanol before the U-HPLC-MS analysis.

Ultra–high-performance liquid chromatography (U–HPLC). U–HPLC separation was performed using the Waters ACQUITY UPLCTM SYSTEM (Waters, USA) equipped with Waters Acquity UPLC BEH Amide column (100 × 2.1 mm, 1.7 μm). The mobile phase was composed of solvent A (0.2% acetic acid with 5 mmol/l ammonium acetate in water) and solvent B (acetonitrile) with a gradient elution: 0–0.5 min, 25% A; 0.5–5 min, 25–40% A; 5–7 min, 40–50% A; 7–7.1 min, 50–25% A; 7.1–11 min, 75% A. The flow rate was 0.35 ml/min, the column temperature was maintained at 40°C, and injection volume was 3 μl.

Mass spectrometry (MS). For the determination of target analytes, mass spectrometer ACQUITY QDa® Mass Detector (Thermo Scientific, USA) operated in the selective ion recording (SIR) mode was utilized. The instrument setting in a positive ionization mode was as follows: ESI capillary voltage of 0.8 kV, capillary temperature of 600°C and cone voltage 15 V. For data processing, MassLynx® Mass Spectrometry Software (Waters, USA), was used.

Method validation. The limits of quantification (LOQs) were determined as the lowest concentration levels of calibration batch. In order to determine the method repeatability (expressed as a relative standard deviation, RSD_r), repeated analyses (n = 6) of pork leg samples were performed. The efficiency of protein hydrolysis, was verified by repeated analyses of hydrolysates of reference material Peptan® (Darling Ingredients, USA) (n = 6). The recovery of β-alanylhistidine dipeptides was controlled by repeated extractions (4 ×) of one sample. The efficiencies of hydrolysis ranged from 79% to 116%. Recoveries of β-alanylhistidine dipeptides were 97% for carnosine and 95% for anserine. The method had good repeatability with RSD values of 4.5–14.6% for amino acids, 7% for carnosine and 9% for anserine. Validation parameters are shown in Table S3 and S4 in ESM of supplementary material.

RESULTS AND DISCUSSION

Characterization of amino acids composition of meat. In the first phase of the experiments, the variability of amino acids concentration within the meat muscle samples taken from different parts of particular animal species was checked and the differences were very small. On the other hand, in line with expectation, concentrations of some amino acids differed between the tested animal species (chicken, pork and beef). The concentration ranges and mean values are summarized in Table 1 and are in line with the data derived from Czech Centre for Food Composition Database (2016). The most distinct difference was observed for 1-MetHis concentration of which was approx. one order of magnitude higher in chicken meat when compared to pig meat, and twenty times higher when compared to beef. As it was the only amino acid with such a different representation in the muscle protein across all of

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Table 1. The database of mean concentrations and concentration ranges of amino acids occurring in chicken, pork and beef meet (g/100 g of fresh meat)

Amino acid	Chicken		Beef		Pork	
	range	average (n = 10)	range	average (n = 10)	range	average (n = 16)
L-Alanine	1.0–1.3	1.2	1.1–1.3	1.2	0.77–1.3	1.0
L-Arginine	1.6–1.8	1.7	1.6–1.9	1.8	0.85–1.8	1.5
L-Asparagine + L-aspartic acid	1.4–1.7	1.6	1.1–2.1	1.6	1.1–2.2	1.9
Cystine	0.25–0.32	0.28	0.23–0.34	0.27	0.10–0.46	0.28
L-Phenylalanine	0.22–0.62	0.35	0.25–0.37	0.30	0.43–0.90	0.73
L-Glutamine + L-glutamic acid	3.2–3.6	3.4	3.2–4.1	3.6	1.8–5.8	3.7
L-Glycine	0.39–0.46	0.42	0.44–0.56	0.48	0.41–1.1	0.76
L-Histidine	0.71–1.1	0.97	0.68–1.2	1.0	1.0–1.2	1.1
cis-L-3-Hydroxylysine	0.026–0.032	0.030	0.051–0.069	0.058	0.026–0.051	0.040
DL-4-Hydroxyproline	0.051–0.055	0.053	0.080–0.13	0.10	0.025–0.094	0.068
L-Leucine + L-isoleucine	1.6–1.7	1.7	1.6–1.9	1.7	1.3–1.8	1.6
L-Lysine	2.1–2.3	2.3	2.2–2.6	2.4	1.6–2.4	2.4
1-Methyl-L-hisitidine	0.22–0.69	0.47	0.034–0.072	0.048	0.013–0.022	0.017
3-Methyl-L-hisitidne	0.059–0.071	0.064	0.064–0.070	0.066	0.064–0.072	0.066
L-Methionine	0.055–0.11	0.088	0.17–0.33	0.23	0.22–0.58	0.40
L-Proline	0.20–0.61	0.31	0.20–0.47	0.30	0.46–1.0	0.69
L-Serine	0.74–0.93	0.88	0.80–1.1	0.93	0.75–1.0	0.91
L-Threonine	1.11–1.14	1.1	1.16–1.21	1.2	0.88–1.2	1.1
L-Tyrosine	0.90–0.95	0.93	0.97–1.1	1.0	0.76–1.0	0.92
L-Valine	1.19–1.27	1.2	1.2–1.4	1.3	0.98–1.4	1.2

the animal species tested, 1-MeHis was considered as a diagnostic marker of animal species in following studies. In the case of 3-MetHis constant concentrations 0.064 g/100 g in chicken meat and 0.066 g/100 g in pork and beef meat. This concentration was in line with the recent study (Steinhufer *et. al.* 2003).

Authentication of poultry, pork and beef NMP. For calculation of NMP in meat products, the content of 3-MetHis multiplied by the calculated factor 292, can be used (i.e. NMP (%, w/w) = 3-MetHis (%, w/w) × 292). The factor 292 corresponds to a relative content of this amino acid in all tested meat species, where the overall protein content was determined by the Kjeldahl method, as well as by using the sum of all present amino acids (both of the approaches generated comparable results). The NMP content calculated using the equation above was compared with the sum of amino acids and the values were comparable as can be seen in Table 2. Standard uncertainty of factor F (uF = 2) was calculated from the relative standard deviation of an average content of 3-MetHis in all tested meat samples (n = 36). The uncertainty of the calculated NMP (uNMP) can be estimated as the combination of the uncertainty uF and the uncertainty of 3-MetHis analysis (e.g. NMP = 16.0 ± 2.2% is recommended for the result expression).

Table 2. Comparison of the calculated values of NMP from the 3-MetHis content with the sum of all amino acids after collagen subtraction (%, w/w)

	Chicken	Pork	Beef
NMP calculated from 3-MetHis	18.7	19.3	19.3
NMP (sum of all amino acids)	18.6	19.8	18.8

*??amount of amino acids after collagen subtraction (OH-Pro x 8)

When the NMP content in the sample is lower than declared, an additional step in authentication of the origin of NMP can be undertaken. A normalized database of ratios of individual amino acids to the 3-MetHis was established (Table S5 in ESM), and can be used for assessment of the compliance with unknown samples. The compliance with the database

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indicates addition of protein-free adulterant, e.g. water, whereas the non-compliance in amino acids rations suggests using of unknown protein source, e.g. blood plasma, collagen, or low-priced meat. Diagnostics of origin of some of the protein-based adulterants is described in chapters below.

Authentication of undeclared protein-based admixtures by the amino acid ratios. To investigate the possibility to identify partial substitution more expensive meat by a cheaper one (in particular addition a chicken meat to pork, and/or pork meat to beef might be fraudulent practices), the experimental model mixtures were prepared and analysed for 1-MeHis and 3-MeHis content. Although the ratio of these amino acids was proposed as indicator enabling recognition of muscle protein origin two decades ago (Kvasnicka *et. al.* 1999), the applicability of this approach has never been documented for meat admixtures. The ratio of 1-MeHis to other amino acids than 3-MeHis was tested, too, nevertheless, it was not found to be diagnostic. The ranges of 1-MeHis / 3-MeHis ratios in muscle protein calculated for particular meat species were 7.6–10; 0.20–0.29; and 0.62–0.73 for chicken, pork and beef, respectively (Table S6 in ESM). Figure 1 documents the increase of 1-MeHis / 3-MeHis values with growing content of chicken meat in the pork. Addition of chicken meat as low as 1.5% could be recognized as statistically significant ($\alpha = 0.05$). Unfortunately, the difference in 1-MetHis between pork and beef is not high enough to allow the same approach for their admixtures testing.

The possibility to detect addition of collagen protein to chicken and pork meat was tested, too. As OH-Pro is known to be abundant amino acid in connective tissues, it is commonly used as a marker of collagen presence. To quantify its addition to NMP, the ratio of OH-Pro/3-MetHis should be determined. As illustrated in Figure 2, collagen addition as low as 0.5% could be detected in the pork admixtures and 1.5% in case of chicken ones, see Figure 3.

Authentication of undeclared meat admixtures based ty the rations of β-alanylhistidine dipeptides. Authentication NMPs based on determining β-alanylhistidine dipeptides, concentration profile of which is characteristic for tested animal species was investigated (Table 3). The differences in anserine content were shown to be the most pronounced; its concentration in chicken meat was 5.2 mg/g, while in beef it was approx. 5-times lower (1.1 mg/g), and even approx. 1 × lower in pork (0.32 mg/g). The

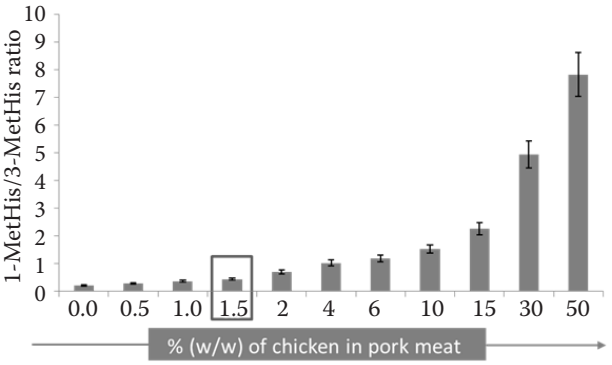

Figure 1. The rations of amino acids of 1-MetHis to 3-MetHis in the analysed pork mixtures containing the increasing addition of chicken meat

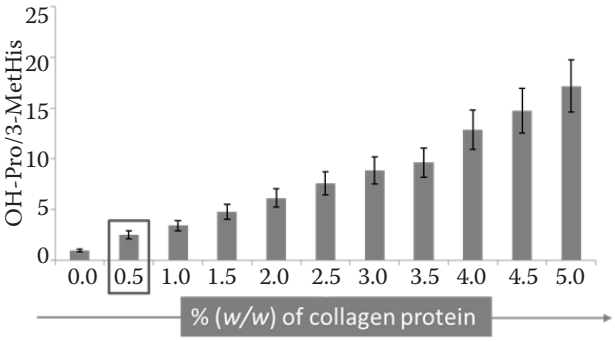

Figure 2. Rations of 4-hydroxyproline to the 3-MetHis in samples of pork meat with added collagen proteins

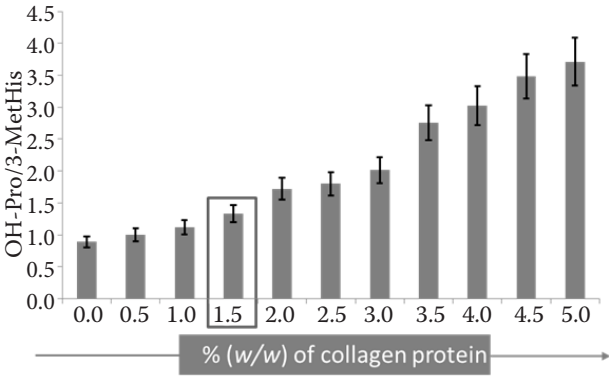

Figure 3. Ratios of OH-Pro to the 3-MetHis in the samples of chicken meat with added collagen proteins

Table 3. Carnosine and anserine content in chicken, beef and pork meat (mg/g)

Type of meat	Carnosine		Anserine	
	ratio	average	ratio	average
Chicken (n = 10)	1.29–2.77	2.1	3.79–7.73	5.2
Beef (n = 10)	2.59–2.61	2.6	0.84–1.38	1.1
Pork (n = 16)	1.98–4.68	2.9	0.2–0.41	0.32

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Table 4. The ratios of detection responses of β-alanylhistidine dipeptides in chicken, pork and beef meat (n = 10)

Type of meat	Anserine/balenine		Carnosine/balenine		Carnosine/anserine	
	range	average	range	average	range	average
Chicken	26–38	33	9.0–18	13	0.32–0.52	0.38
Beef	11–28	20	83–126	105	4.5–7.4	6.0
Pork	0.31–1.4	0.71	9.9–26.4	14	13–31	21

variability in concentrations of carnosine was not so pronounced; the mean contents were 2.6; 2.9 and 2.1 mg/g for pork, beef and chicken meat, respectively. As concerns balenine, its content could not be determined because of lack of the analytical standard. Nevertheless, based on detected signals of targeted β-alanylhistidine dipeptides (corresponding to their concentrations) respective ratios could be calculated.

As results from the Table 4, in case of chicken meat, high ratio of anserine to balenine and low ratio of carnosine to anserine are typical. The plot of these ratios shown in Figure 4 confirms the possibility to find out undeclared substitution of pork NMP even by low additions chicken when using anserine/balenine ratio 2 and 0.5% in case of carnosine/anserine. Analysis of β-alanylhistidine dipeptides and calculation their ratio may also help to recognize addition of 2% pork to beef. In pork the anserine/balenine ratio is low while it is high for carnosine/anserine. Based on these indicators,

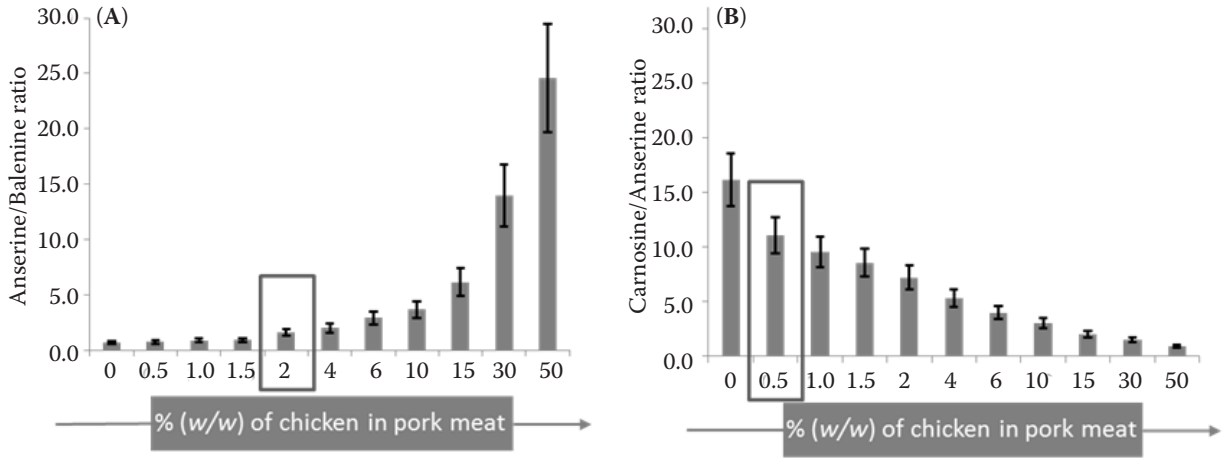

Figure 4. The ratios of anserine/balenine (**A**) and carnosine/anserine (**B**) in model mixtures of chicken meat added to pork

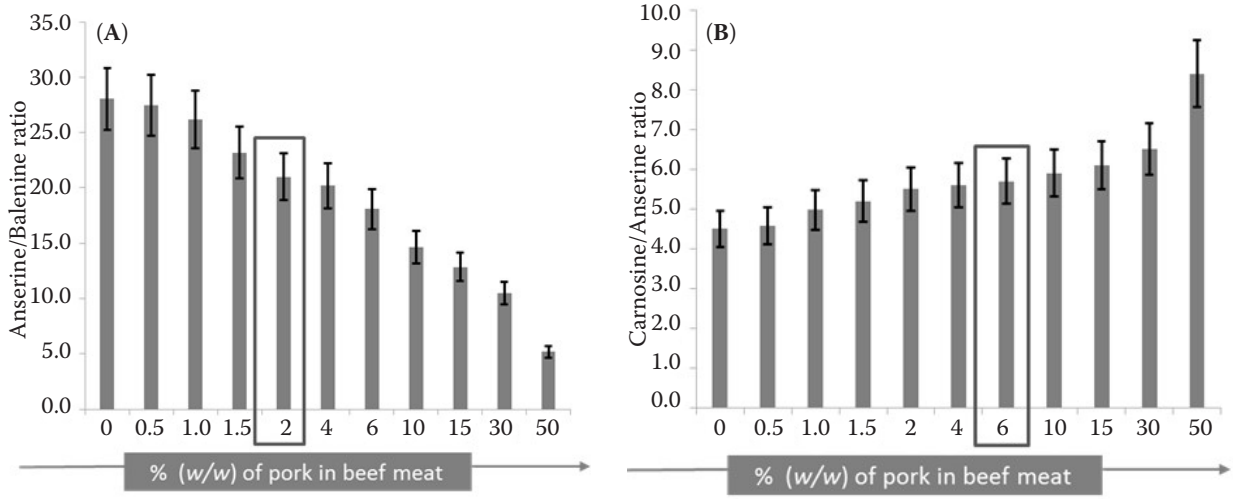

Figure 5. The ratios of anserine/balenine (**A**) and carnosine/anserine (**B**) in model mixtures with added pork meat to beef

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distinguishing of adulteration of beef meat with the cheaper pork was feasible from 2% of pork addition, Figure 5. Worth to notice, that compared to older study (Abe & Okuma 1995) in which, when using analysis of β-alanylhistidine dipeptides for similar admixtures characterization, additions approx. ten percentage were recognizable only, fairly better results were achieved here.

CONCLUSIONS

For the purpose of this study focused on evaluation of muscle protein quality and meat species authentication, simple, fast and easy-to-use analytical procedure employing U–HPLC-MS, enabling characterization of pattern amino acids and β-alanylhistidine dipeptides was introduced. The achieved results can be summarized as follows: (1) Specific amino acid/3-MetHis ratios shown to be diagnostic for assessment of the quality of net muscle proteins, and revealing of undeclared admixtures in order of units of percent. (2) The conversion factor F = 292 ± 4 was calculated for quantification of NMP (%) content on the basis of 3-MetHis concentration. (3) 1-MeHis/3-MeHis ratio enabled to detect as low as 1.5% addition of chicken meat to the pork. (4) Low collagen addition as 0.5% could be detected in the pork meat based on OH-Pro/3-MetHis. (5) For the chicken meat, the collagen was detectable at the concentration level 1.5% also on OH-Pro/3-MetHis ratio basis. (6) Based on carnosine/anserine ratio, addition of 0.5% of chicken could be detected. Based on anserine/balenine ratio, the addition of 2% pork meat to beef can be detected.

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