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Monitoring of imidazole dipeptides in meat products by capillary zone electrophoresis

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Abstract: A simple capillary zone electrophoretic technique (CZE) was developed for the determination of carnosine and anserine, and the main analytical performance characteristics were determined. The method was used for an analysis of raw meat samples, heat treated as well as high hydrostatic pressure (HHP) treated meat samples, and various meat products. The effect of heat treatment (10 min at 75 °C and 45 min at 90 °C) and HHP (100–600 MPa, 5 min) was investigated on pork *longissimus thoracis* muscle samples. With the exception of the milder heat treatment a slight decrease was detected in dipeptide contents of treated samples, but significant differences ($P < 0.05$) were not observed under any treatment. Thirty-two meat-based food products were also analysed. Imidazole dipeptides were detectable in all of them. The poultry products showed a characteristically low carnosine/anserine ratio. The data obtained were consistent with the food label information.

Keywords: carnosine; anserine; heat treatment; high hydrostatic pressure; meat products

There are many functional compounds found in the skeletal muscle of vertebrate animals. Imidazole dipeptides (carnosine and anserine) are prominent among them, because they have multifarious physiological functions and therapeutic effects, such as neurotransmitters in the brain, buffering capacities in the muscle, antiglycation and anti-ischemic effects, modification of enzymic activities, antineoplastic effects, antioxidant and membrane protective effect (Gariballa & Sinclair 2000). Meat is the main contributor to the supply of imidazole dipeptides in humans. The absorption of carnosine was investigated and verified in rat (Tomonaga et al. 2007) and pig models (Ma et al. 2010) and also in human studies (Park et al. 2005). Carnosine and anserine were suggested as biomarkers of meat intake (Dragsted 2010). The third in the family of beta-alanyl dipeptides is balenine (ophidine) which is predominant in snake and marine mammals like dolphins and whales (Dragsted 2010; Aristoy & Toldrá 2004a). In most other meats balenine may be found only at a low concentration, however Aristoy & Toldrá (2004b) detected considerable levels in pork, beef, and poultry samples.

These dipeptides, as natural antioxidants in meat, are effective in preventing oxidative rancidity and undesirable colour changes during the storage of meat (de Castro & Sato 2015) so they could be used as potential markers of meat quality. D'Astous-Pagé et al. (2017) established that high muscle carnosine is associated with improved pork meat quality. They observed greater pH 24 h, better water-holding capacity and improved meat colour values in pigs with high muscle carnosine content. The imidazole dipeptide content of meat

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varies from a few hundreds to several thousands of mg/kg depending on the species of the animal, metabolic type of muscle, gender, age, breeding, and others (Harris et al. 2012). The low carnosine/anserine ratio is typical of poultry meat, in contrast with pork and beef meat. It was established that the ratio of these dipeptides enables to detect the mammalian origin. Aristoy and Toldrá (2004b) found that feeds having carnosine/anserine molar ratios higher than 0.3 were strongly suspected of containing banned mammalian proteins. In addition to the carnosine/anserine ratio Abe & Okuma (1995) took into account the balenine/anserine ratio, and their results indicated that beef, pork, horse, deer, chicken and turkey meat samples can be correctly discriminated from these ratios. Jiru et al. (2019) used anserine/balenine, carnosine/balenine and carnosine/anserine ratios for the authentication of animal species in meat mixtures. Balenine content was not determined, but it could be calculated based on detected signals of targeted β-alanylhistidine dipeptides. According to their results based on a carnosine/anserine ratio the addition of 0.5% chicken meat and based on an anserine/balenine ratio the addition of 2% pork meat to beef is detectable. Imidazole dipeptides are fairly heat stable and unlike other endogenous polypeptides, they are relatively resistant to the hydrolytic breakdown of many common proteases (Maikhunthod & Intarapichet 2005). The HHP shows a big potential for the innovative development of new products also in the meat industry, but very limited data are available on the stability of these compounds during the HHP treatment (Hugas et al. 2002). In contrast with raw meat samples, only few studies have described the effect of food technologies on imidazole dipeptides and the carnosine and anserine contents of processed food products (Hermanussen et al. 2010).

For the analysis of imidazole dipeptides in tissues of different animal species several HPLC methods have been described (Kantha et al. 2000; Mora et al. 2007; Tian et al 2007; Mori et al. 2015) while capillary electrophoretic techniques are also used (Huang et al. 2005; Zunic & Splasic 2008; Staňová et al. 2011; Zinellu et al. 2011; Jozanović et al. 2017).

The aim of this study is to assess the contents of imidazole dipeptides in selected meat samples, and the effect of heat treatment and HHP on the dipeptide profile, in addition to obtain data on the carnosine and anserine contents of food products. A fast and simple capillary electrophoresis method was used for determination of the carnosine and anserine level in meat samples and meat-based foods.

MATERIALS AND METHODS

Meat and food samples. The *longissimus thoracis* (LT) and *masseter* (MS) muscles of individually slaughtered pigs (n = 10) from the same farm (of the same genetics, sex, management and diet) obtained from IRTA (Spain) were used. Chicken breast and thigh, turkey breast, pork loin, and beef sirloin meat samples as well as processed meat products were purchased from local supermarkets in Budapest. Thirty-two meat products which are frequently consumed were chosen for analysis (Table 2); 20 of them were made in Hungary and 12 originated from other European countries.

Chemicals. L-carnosine (CAR) (~99% purity, crystalline form) and L-anserine (ANS) (≥ 98% purity, nitrate salt) standards were from Sigma, (USA), the HPCE phosphate buffer solution (100 mmol L^{-1}, pH 2.50) used as a running electrolyte was purchased from Fluka (Germany).

Heat and HHP treatment. The pork LT samples were sliced (2 mm), vacuum-packed and cooked at 75 °C for 10 min (H1) or at 90 °C for 45 min (H2) in water bath. The HHP treatment was carried out in the Resato FPU-100-2010 equipment (Resato International BV, Netherlands) between 100 and 600 MPa by steps of 100 MPa for 5 min.

Extraction of imidazole dipeptides. Meat and food samples were finely ground by a meat cutter. An aliquot of 5 g of this sample was homogenized with 10 mL of distilled water. The homogenates were centrifuged at 20 000 g for 30 min. The supernatant was deproteinized by treatment in boiling water for 10 min, then centrifuged at 5 000 g for 10 min and filtered through a 0.45-μm membrane. The extracts were diluted tenfold with the running electrolyte solution before injection.

CZE (capillary zone electrophoresis) separation of carnosine and anserine. A BioFocus 2 000 System (Bio-Rad) with UV detector (Bio-Rad Laboratories, USA) was used for the experiments. The instrument was used in a pressure injection mode (constant injection pressure × time) at 10 psi × sec. The sample holder was thermostated at 18 °C. The sample was separated in an uncoated fused-silica capillary thermostated at 38 °C with dimensions of 50 μm I.D. (inner diameter) and effective length of 45.5 cm, under voltage of 15 kV. As a carrier electrolyte 10, 50, or 100 mmol L^{-1} phosphate buffer pH 2.5 was used. The dipeptides were detected without derivatization at 200 nm. Treated and raw meat samples and meat products were extracted and analysed in triplicate except the pork loin and chicken breast. For the repeatability study of the method six samples from

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Table 2. Carnosine and anserine contents, the ratio (and molar ratio) of dipeptides in meat products

No.	Meat product	Carnosine mean[a] ± SD	Anserine mean[a] ± SD	CAR/ANS
1	stuffed pork chop[H]	4 323 ± 190	430 ± 15	10.05 (10.68)
2	salami (pork)[H]	2 502 ± 125	236 ± 17	10.59 (11.25)
3	Debrecen sausage[H]	1 023 ± 31	141 ± 9	7.26 (7.70)
4	smoked ham[H]	5 205 ± 202	364 ± 32	14.30 (15.19)
5	Prague ham[H]	1 818 ± 168	257 ± 25	7.07 (7.51)
6	liverwurst 1[H]	112 ± 7	20 ± 2	5.60 (5.95)
7	lunch ham[H]	2 068 ± 79	178 ± 9	11.62 (12.34)
8	baked ham[H]	3 898 ± 192	312 ± 12	12.49 (13.26)
9	chop ham[H]	2 328 ± 115	401 ± 20	5.80 (6.16)
10	Wienerwurst[H]	1 254 ± 102	253 ± 11	4.96 (5.26)
11	liverwurst 2[H]	424 ± 25	72 ± 3	5.88 (6.25)
12	lunch meat[H]	171 ± 8	25 ± 3	6.84 (7.26)
13	Bologna sausage[H]	557 ± 39	66 ± 4	8.44 (8.96)
14	turkey breast ham 1[H]	664 ± 29	2 653 ± 141	0.25 (0.27)
15	turkey breast ham 2[H]	94 ± 6	861 ± 21	0.11 (0.12)
16	chicken breast ham 1[H]	193 ± 11	700 ± 17	0.28 (0.29)
17	chicken breast ham 2[H]	374 ± 15	1 342 ± 69	0.28 (0.29)
18	turkey ham[H]	95 ± 6	556 ± 10	0.17 (0.18)
19	smoked poultry sausage[H]	131 ± 9	422 ± 29	0.31 (0.33)
20	poultry hot dog sausage[H]	123 ± 7	577 ± 17	0.21 (0.23)
21	chorizo sausage[E]	3 754 ± 3	386 ± 5	9.72 (10.32)
22	traditional salami (pork)[E]	2 823 ± 6	220 ± 8	12.83 (13.63)
23	cooked Tuscan ham[I]	3 445 ± 4	377 ± 4	9.14 (9.70)
24	bresaola (dry cured beef)[I]	4 519 ± 5	833 ± 5	5.42 (5.76)
25	smoked cured bacon[D]	4 462 ± 5	399 ± 4	11.18 (11.88)
26	smoked turkey salami (turkey, pork)[D]	723 ± 7	1 386 ± 8	0.52 (0.55)
27	Sous-vide goose thigh[PL]	178 ± 7	1 456 ± 6	0.12 (0.13)
28	poultry cabanossi sausage[PL]	166 ± 6	604 ± 9	0.27 (0.29)
29	premium pork ham[CZ]	4 417 ± 4	484 ± 4	9.13 (9.69)
30	smoked chicken breast ham[CZ]	301 ± 4	1 100 ± 7	0.27 (0.29)
31	Bologna sausage (pork, beef)[A]	778 ± 4	112 ± 9	6.95 (7.38)
32	smoked cabanossi (pork, beef)[A]	4 029 ± 3	573 ± 5	7.03 (7.47)

[a]Means expressed as mg kg^{-1} of meat product; each value represents the mean of three samples from the same product; made in [H]Hungary, [E]Spain, [I]Italy, [D]Germany, [PL]Poland, [CZ]Czech Republic, [A]Austria

the same pork loin and six samples of the same chicken breast were processed to estimate the mean concentrations of carnosine and anserine together with coefficients of variation (*CV*). Pork loin and chicken breast samples were also used for the recovery tests by enriching with standard solutions of dipeptides.

Statistical analysis. The results are expressed as the mean ± standard deviation (SD). Data were subjected to Student's t-test for determining significant differences between control and treated meat samples using the Minitab Release 13 software (Minitab LLC., USA). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Method evaluation. The optimum resolution of carnosine and anserine was found at 100 mmol L^{-1} buffer concentration. Under the described conditions the resolution of the examined compounds was 1.48. The calibration equations (Y is the peak area and x is the compound

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concentration) were obtained from points resulting from the mean values of five measurements per point, corresponding to seven different concentrations between 10 and 5 000 mg L^{-1}. The linear regression equation for carnosine was $Y = 235x + 12\,693$ ($R^2 = 0.9995$) and for anserine $Y = 254x + 7\,415$ ($R^2 = 0.9975$) in the concentration range of 50–5 000 mg L^{-1}. The limits of detection (LOD) and limits of quantification (LOQ) of the compounds are based on a signal-to-noise ratio of 3 and 10, respectively. LOD (and LOQ) of the solution for carnosine corresponded to 1.58 (and 5.27) mg L^{-1} and for anserine 1.64 (and 5.47) mg L^{-1}, respectively. In the repeatability study of the method six samples from the same pork loin and six samples of the same chicken breast were processed to estimate the mean concentrations of carnosine and anserine together with coefficients of variation. In the case of pork loin the mean was 49 589 mg kg^{-1} (*CV* 3.75%) for carnosine, and 248 mg kg^{-1} (*CV* 7.34%) for anserine. In the chicken breast sample the mean was 1 177 mg kg^{-1} (*CV* 5.44%) for carnosine, and 4 620 mg kg^{-1} (*CV* 6.28%) for anserine. Migration time of carnosine and anserine was 7.52 min (*CV* 0.93%), and 8.11 min (*CV* 1.35%), respectively. Recovery was determined in two sets of pork loin and chicken breast, one set for carnosine, and the other for anserine. Samples were enriched with standard solutions of dipeptides to yield concentrations approximately equivalent to 0.5, 1, and 1.5 times the main value obtained in repeatability studies. At each level the analysis was performed in triplicate. The percentage recovery was 96.9 (*CV* 2.57%) for carnosine and 96.8 (*CV* 5.97%) for anserine.

Imidazole dipeptides in raw meat samples. Carnosine and anserine contents of fresh meat samples of different animal species are presented in Table 1. Dipeptide content of glycolytic type muscle samples (pork longissimus thoracis muscle and chicken breast) was substantially higher compared with that of oxidative type muscles (pork masseter muscle and chicken thigh). The distinctively low carnosine/anserine ratio in the poultry samples was also clearly observable. These findings are consistent with the previous studies by Aristoy & Toldrá (2004b) as well as by Maikhunthod and Intarapichet (2005).

Effect of HHP and heat treatment on carnosine and anserine contents of pork *longissimus thoracis* muscle. The detectable contents of dipeptides decreased slightly in the HHP treated samples (100–600 MPa, 5 min), but significant differences ($P < 0.05$) were not observed in any treatment (Figure 1). Subtle changes in carnosine and anserine concentrations may depend on the alterations in extractability related to the effect of HHP on the meat structure. A similar result was reported by Suzuki and co-workers for beef meat (Suzuki et al. 1994).

Most of the literature data confirms that imidazole dipeptides are fairly heat stable (Maikhunthod & Intarapichet 2005) as confirmed by the first finding in Liebig's meat extract (Gulewitch & Amiradzibi 1900). Jayasena et al. (2015) compared the amounts of imidazole dipeptides in breast and leg samples from Korean native chickens and commercial broilers and found out the changes in the concentration of these compounds during moist heat cooking to a core temperature of 72 °C. Individual comparisons between raw and cooked meat in each meat portion of each breed showed that the cooking effect on carnosine and anserine contents was significant only in the breast meat of Korean native chickens. In our experiment no considerable deviation was detected in carnosine and anserine contents of pork *longissimus thoracis* samples as a result of heat treatment

Table 1. Carnosine and anserine contents, the ratio (and molar ratio) of dipeptides in raw meat samples

Meat sample	Carnosine (mg kg^{-1}) mean[a] ± SD	Anserine (mg kg^{-1}) mean[a] ± SD	CAR/ANS
Pork LT	4146 ± 262	213 ± 52	19.46 (20.67)
Pork MS	320 ± 12	25 ± 4	12.80 (13.59)
Pork leg	2556 ± 157	143 ± 11	17.87 (18.98)
Pork loin	4959 ± 186	249 ± 18	19.92 (21.15)
Beef bottom sirloin	3212 ± 111	287 ± 18	11.19 (11.89)
Chicken breast	1178 ± 64	4621 ± 290	0.25 (0.27)
Chicken leg	334 ± 35	1391 ± 198	0.24 (0.26)
Turkey breast	1438 ± 125	5376 ± 416	0.27 (0.28)

CAR – carnosine; ANS – anserine; [a]means expressed as mg kg^{-1} of muscle; each value represents the mean of three samples (six samples in the case of pork loin and chicken breast) from the same meat cut

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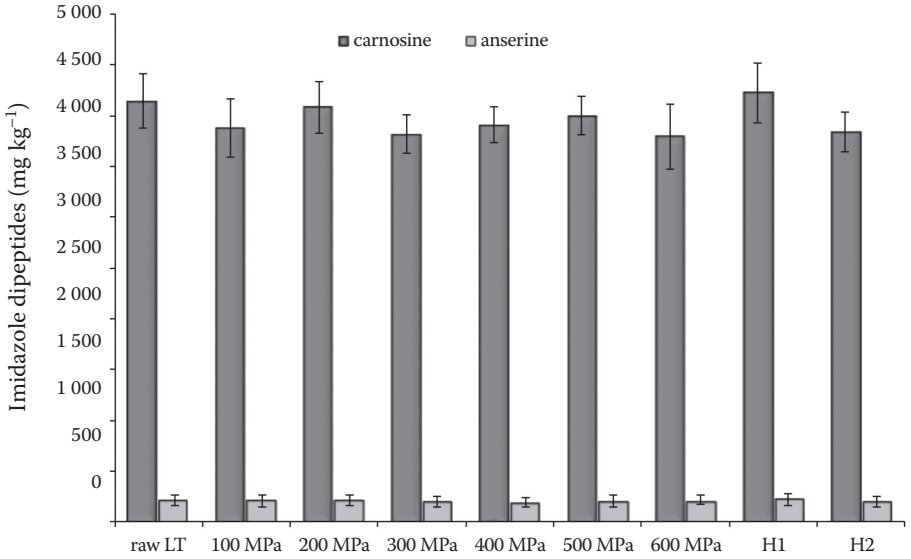

Figure 1. Levels of imidazole dipeptides (mg kg^{-1}) in HHP treated (0–600 MPa) and heat treated (H1 and H2) pork *longissimus thoracis* samples determined by capillary zone electrophoresis.

Each value represents the mean of three different extracts from the sample, and labelled error bars indicate the standard deviation for each measurement

(Figure 1). After treatment for 10 min at 75 °C (H1) a slight increase was observed, and after treatment for 45 min at 90 °C (H2) some decrease was noticed, but none of these was significant ($P < 0.05$).

Dipeptide patterns of meat-based food products. Most of the meat products contained significant amounts of imidazole dipeptides, depending on the lean meat content and quality of raw meat. Two typical electrophoregrams are shown in Figure 2. The largest peak on electrophoregrams of stuffed pork chop corresponds to carnosine, but in the case of chicken breast ham the anserine peak dominates. Table 2 summarizes the imidazole dipeptide contents of 32 frequently purchased meat-based foods. Obvious mismatch between dipeptide patterns

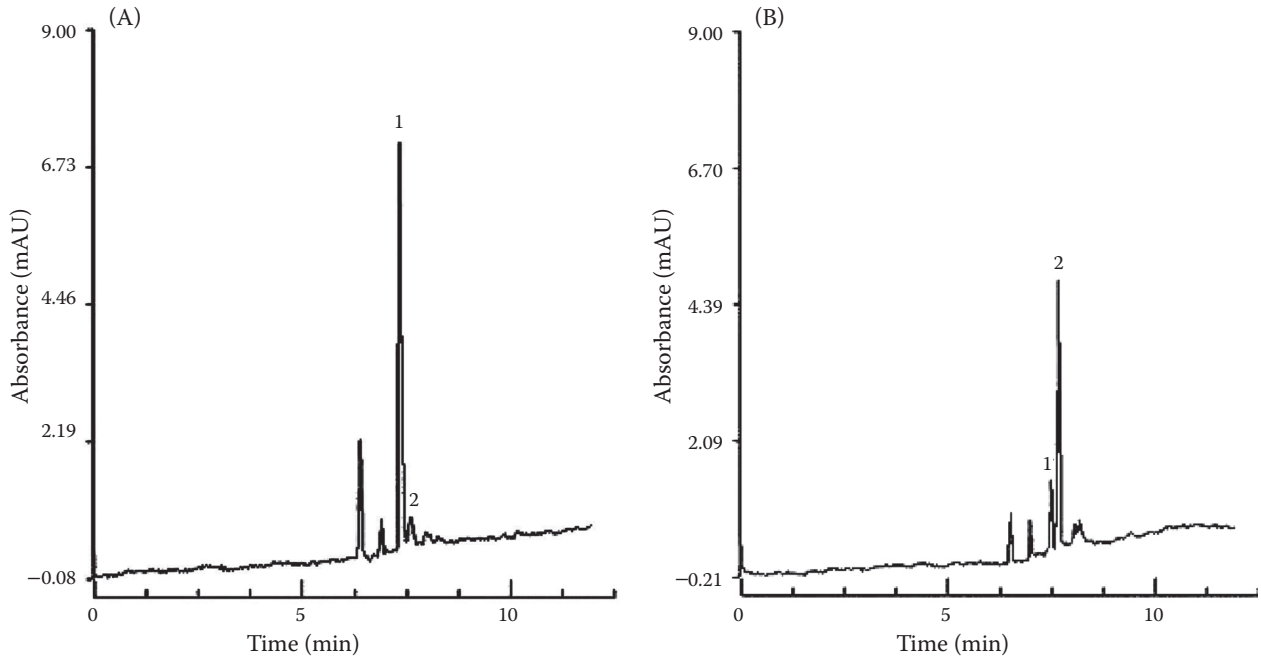

Figure 2. Electrophoregrams corresponding to the separation of the "stuffed pork chop" (A) and "chicken breast ham 2" (B)

Carnosine (peak 1) and anserine (peak 2) were detected at 200 nm

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and food label information was not found. Carnosine and anserine were detected in each of the tested products. The highest level of imidazole dipeptides was measured in smoked ham (sample 4), and the lowest in one of the liverwursts (sample 6). The low carnosine/anserine molar ratio was found to be characteristic of the chicken or turkey meat-based food products. This ratio was lower than 0.3 in poultry products except the smoked poultry sausage, where it was 0.33 (sample 19). For a product called smoked turkey salami (sample 26) the carnosine/anserine molar ratio was higher than 0.5. This can be explained by the detailed composition data on the packaging, which indicates that the product contains 15% of pork meat besides turkey meat.

CONCLUSION

The developed CZE method allows a relatively fast and simple determination of imidazole dipeptides in meat and meat products. Detectable carnosine and anserine contents of pork *longissimus thoracis* muscle samples are not significantly influenced by the applied HHP and heat treatments, suggesting that these compounds are quite resistant to the effects of food processing. In the case of meat-based food products there was no conflict between the dipeptide patterns and composition data indicated on food labels on the packaging. The results may be helpful in development of high-quality meat products which fit into a healthy diet.

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