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Determination of carnosine, anserine, homocarnosine, pentosidine and thiobarbituric acid reactive substances contents in meat from different animal species

Pier Giorgio Peiretti[a,*], Claudio Medana[b], Sonja Visentin[c], Valeria Giancotti[b], Valentina Zunino[d], Giorgia Meineri[d]

[a] Institute of Science of Food Production, National Research Council, via L. da Vinci, 44, 10095 Grugliasco, Torino, Italy
[b] Department of Analytical Chemistry, University of Torino, Via P. Giuria, 5 - 10125 Torino, Italy
[c] Department of Drug Science and Technology, University of Torino, Via P. Giuria, 9 – 10125 Torino, Italy
[d] Department of Animal Production, Epidemiology and Ecology, University of Torino, via L. da Vinci, 44, 10095 Grugliasco, Torino, Italy
[*] Corresponding author. Tel.: +39 011 6709230; fax: +39 011 6709297. E-mail addess: piergiorgio.peiretti@ispa.cnr.it (P.G. Peiretti).

Abstract

The aim of this research was to determine the content of the histidinic antioxidants, advanced glycation end products (pentosidine) and thiobarbituric acid reactive substance (TBARS) in the meat from different animal species. Carnosine, anserine, homocarnosine and pentosidine were quantified by HPLC/MS, while TBARS was determined by photometric measurements. The total CRCs (carnosine + anserine + homocarnosine) content was in the increasing order: beef < rabbit < pork < horse < chicken < turkey. The analysis showed traces of pentosidine above the instrumental determination limits in all the meat samples, while the susceptibility of these meat to lipid oxidation decreased from beef to chicken, with the exception of turkey meat, which presented a high TBARS content towards even though its total CRCs was the highest. The structure of homocarnosine was elucidated by high resolving power multistage mass spectrometry.

Key words: Meat, Carnosine, Pentosidine, Lipid oxidation.

1. Introduction

Recent scientific findings have highlighted the important role played by advanced glycation end products (AGEs) and oxidation in the pathogenesis of the degenerative processes related to ageing, diabetes, kidney disease, Alhzeimer's disease and some forms of cancer such as colorectal cancer (Johnson & Lund, 2007; Gunter et al., 2007). The glycation process begins with a non-enzymatic interaction between simple sugars (glucose, fructose and ribose) and protein amino groups. When oxidation accompanies glycation, extremely harmful glycoxidation end products are formed; in fact, the phenomenon of glycoxidation accompanies oxidative chemical processes that cause the production of unstable molecules, called "free radicals", that include malondialdehyde and peroxides (Bucala & Cerami, 1992). The acronym AGEs refers to a class of products that are the final and irreversible result of chain reactions that begin with an initial glycation (Chellan & Nagaraj, 1999). The term non-enzymatic glycation or glycosylation refers to a post-translational modification of a protein that consists of the addition of one or more sugars to the peptide chain; N-glycosylation or O-glycosylation, depending on whether the sugar binds with a nitrogen or oxygen atom. The chemical structures that can be considered as a indexes of glycoxydative stress are pentosidine, glycated hemoglobin and the carbonilic groups (Sell & Monnier, 1989; Singh, Barden, Mori, & Beilin, 2001).

In addition to their presence in body tissues, AGEs have been identified in food in Western countries, particularly in heat treated foods (O'Brien & Morrissey, 1989; Ledl & Schleicher, 2003).

AGEs can be formed during the cooking of food, in which case their intake is exogenous, or there can also be an endogenous formation of these compounds. In both cases, the reaction mechanism leading to the formation of these compounds is the same; AGEs are in fact formed through the Maillard reaction, a widely described and very important chemical process.

Recently, studies conducted on humans have confirmed that more than 10% of dietary AGEs are absorbed in the gut, released into the bloodstream and transported to different organs of the body, where they settle and accumulate over the years (Bucala et al., 1994). The intake of food origin AGEs is an important risk factor for tissue damage and restriction of the introduction of AGEs into the diet can therefore be considered a prevention factor of the previously mentioned chronic degenerative diseases.

However, our body has defense mechanisms against glycation and oxidation; one of these is represented by carnosine, a dipeptide which is present in the skeletal muscle (Babizhayev et al., 2001).

Carnosine reacts with simple sugars and blocks the glycation process of proteins, and therefore behaves like a competitive acceptor in the glycoxidation reaction.

In addition to carnosine there are also molecules indicated by the acronym CRCs (Carnosine Related Compounds) in vertebrates and these include anserine or β-alanyl-1-methyl-l-histidine and homocarnosine or amminobutirril-γ-L-histidine.

The carnosine present in the human body comes from endogenous and exogenous sources.

The endogenous part is synthesized from the essential amino acids alanine and histidine through the action of a specific synthetase, carnosinase; exogenous carnosine is instead introduced with the diet, which can introduce an amount of carnosine of approximately 50/250 mg/day (with at least one portion of beef, pork or chicken per day).

As far as CRCs in humans are concerned, homocarnosine is mainly found in the cerebrospinal fluid. Anserine is mainly found in the brain, while it is absent in the skeletal muscle of humans; it is instead present in high concentrations in the muscles of some animals.

Some studies have shown that carnosine and its related molecules are capable of acting as antioxidants (Quinn, Boldyrev, & Formazuyk, 1992; Kohen, Yamamoto, Cundy, & Ames, 1988). These histidine dipeptides have been shown to be particularly effective in preventing damage and/or the death of neurons caused by exposure to oxidative stress; this activity can be confirmed by the fact that, in humans, carnosine, anserine, and homocarnosine are found in the greatest concentrations in the central nervous system. It should be pointed out that the CRCs are also present in significant quantities in the skeletal muscle, which also consists of excitable tissues that are subject to intense oxidative stress.

A mechanism for the antioxidant action of histidine derivatives has been proposed in literature.

The study conducted by Kohen, Yamamoto, Cundy & Ames (1988) was performed by recreating systems as similar as possible to physiological ones. The antioxidant capacity of CRCs has tested against lipid peroxidation and their ability to prevent the formation of 8-hydroxy-2'-deoxy-guanosine symptoms that cause damage to DNA. A radical reaction initiator was added to these systems to trigger oxidation chain reactions. They then measured the amount of hydroperoxides formed in the reaction in the presence and absence of carnosine. These results have shown that carnosine, homocarnosine and anserine are able to slow down the degenerative processes triggered by free radicals; their activity was also found to be concentration dependent. Various histidine derivatives were analyzed in order to define the action mechanism of these molecules, and it was concluded that the active element is the imidazole ring of this amino acid.

Currently, the availability of data on glycoside and oxidation end product contents in the diet is extremely limited (Chuyen, 2006). One of the most important causes of the deterioration of meat is lipid oxidation and the thiobarbituric acid reactive substances (TBARS) test seems to be an appropriate method for the routine assessment of secondary oxidation in meat samples.

The aim of this research was to determine the carnosine, anserine, homocarnosine, pentosidine and TBARS contents of raw meats obtained from different species and whether there is a relationship between the total histidinic antioxidants and the glycosides and oxidation end products content of these samples.

2. Materials and methods

2.1. Chemicals

The L-Carnosine, heptafluorobutanoic acid, trichloroacetic acid and 2-thiobarbituric acid were purchased from Sigma-Aldrich (Milan, Italy) and used as received. The HPLC grade water was from MilliQ System Academic (Millipore, Vimodrone, Italy). The HPLC grade acetonitrile was from VWR (Milan, Italy). The pentosidine was synthesized in our laboratory according to the method reported in literature (Visentin, Medana, Barge, Giancotti, & Cravotto, 2010).
Standard solutions were prepared, using a starting eluent as a solvent, from 1 g/L water-methanol 1:1 stock solutions at 6 concentration levels (1, 5, 10, 50, 100, 200 mg/L). For each meat type (beef, rabbit, pork, horse, chicken and turkey) a standard addition calibration curve was built at 3 concentration level (0, 10, 100 mg/L) in order to exclude ion suppression effects due to the matrix.

2.2. Sample collection and processing

Three meat samples of cuts of beef (fillet), horse (fillet), pork (loin), chicken (breast), turkey (breast) and rabbit (hindleg) were purchased directly from three local supermarkets in Turin, Italy, for a total of 18 meat cut samples. These were immediately frozen at -20°C and then freeze-dried. When necessary, the bones and fat were removed. Three samples of each meat were analysed. Each sample was weighted (50 mg) in a 10 mL polyethylene tube together with the internal standard (phenylephrine hydrochloride, 2 ng/sample) and treated for 5 minutes with 3 mL of 0.6 M trichloroacetic acid (TCA) in order to denaturate the proteins. After centrifugation (15 minutes, 9000*g*), 500 µL of supernatant solution was diluted ten fold, using 5 mM heptafluorobutanoic acid, and then transferred into vials for analysis.

2.3. LC-MS analysis (carnosine, anserine, homocarnosine and pentosidine)

The chromatographic separations were run on an Ultimate 3000 HPLC (Dionex, Milan, Italy) coupled to a high resolving power mass spectrometer LTQ Orbitrap (Thermo Scientific, Rodano, Italy), equipped with an atmospheric pressure interface and an ESI ion source. Samples were analyzed using an RP C18 column (Phenomenex Synergi 150 × 2.1 mm, 3 µm particle size) at a 200 µL/min flow rate. A gradient mobile phase composition was adopted: 95/5 to 40/60 in 25 minutes, 5 mM heptafluorobutanoic acid/acetonitrile. The injection volume was 5 µL. The tuning parameters adopted for the ESI source were: source voltage 4.5 kV, capillary voltage 17.00 V, and tube lens 45 V. The heated capillary temperature was maintained at 265°C. The mass accuracy of the recorded ions (vs the calculated ones) was ± 5 mmu (milli-mass units). Analyses were run using both full MS (50-700 *m/z* range) and MS/MS acquisition in the positive ion mode. CRCs were quantified in the full MS mode while pentosidine in MS/MS mode (379 *m/z* ion, fragmented with a 30% collision energy).

2.4. Susceptibility to lipid oxidation

The thiobarbituric acid reactive substance (TBARS) assay was modified from that of Witte Krause, & Bailey (1970) and was performed for each meat sample; 10 g of freeze-dried meat were homogenised for 30 sec at a high speed with 20 mL of 10% trichloroacetic acid (TCA) using a Polytron tissue homogenizer (Type PT 10-35; Kinematica GmbH, Luzern, Switzerland). After centrifugation of the homogenate (600 rpm for 5 min at 4°C), the supernatant was filtered through Whatman #1 filter paper. One mL of filtrate was combined with 1 mL of 0.02 M acqueous 2-thiobarbituric acid solution (TBA), heated in a boiling water bath for 20 min together with a blank

containing 1 mL of a TCA/water mix (1/1) and 1 mL of TBA reagent and subsequently cooled under running tap water. The samples were analysed in triplicate and the results were expressed as mM MDA kg^{-1} DM, using a standard curve that covered the 1 mM to 10 mM 1,1,3,3-tetramethoxypropane concentration range (Sigma-Aldrich, Steinheim, Germany). The absorbance was measured at 532 nm with a Helios spectrophotometer (Unicam Limited, Cambridge, UK) against a blank that contained all the reagents but no meat.

3. Results and Discussion

3.1. Chromatographic analysis

Carnosine and similar molecules were identified in the meat on the basis of the preliminary separation/fragmentation study and quantified in the different livestock species using calibration curves obtained from the standard carnosine. We used an ion pair reversed phase chromatographic method because of very low hydrophobicity of these molecules, which were eluted with heptafluorobutanoic acid in the mobile phase. Detection limits (according to the S/N = 3 criterion) were 11 ng/mL for CRCs and 1 ng/mL for pentosidine; CRCs were quantified in the range 1-200 mg/L, so choosing a limit of quantification 1000 fold higher than detection limit. The correlation coefficient of the calibration curve was $9.91 \times 10^7 \pm 3.65 \times 10^6$ (RSD% = 3.7). We excluded matrix effects applying the standard addition procedure to each meat type at three concentration levels. The obtained slope ratios between standard addition curve and calibration curve were 0.98 (beef), 1.10 (rabbit), 0.95 (pork), 0.98 (horse), 0.99 (chicken) and 0.94 (turkey).

3.2. Interpretation of MS data

The protonated molecular ions were 227.1182 *m/z* for carnosine, 241.1300 *m/z* for anserine and homocarnosine and 379.2094 *m/z* for pentosidine. An example of carnosine and CRCs separation is shown in figure 1.

Figures 2 and 3 show the MS^2 spectrum of carnosine and the relative fragmentation interpretation. All of the proposed pathways were confirmed by MS^3 analysis. Ammonia loss is the most intense process; the $[MH-17]^+$ ion obtained originates in this way three main product ions by eliminating formic acid, water and cycloprop-2-en-1-one.

The anserine and homocarnosine isobaric compounds protonate to give two ions at 241.1300 *m/z,* with different structures and spectra. Their MS^2 spectra are shown in figure 4. The main differences in the fragmentation pathways are shown in figure 5: the N-methylation that occurs in anserine makes $NHCH_2$ loss and decarboxylation possible, both from the precursor ion and from the first generation product ions; the homocarnosine spectrum shows the same pattern as carnosine, but shifted by a methylene unit (14.0157 u). The multistage mass spectrometry study using the orbitrap technology allowed us to clarify that homocarnosine is a homohistidine derivative.

The chromatographic separation and MS^2 spectrum of pentosidine are shown in figure 6. The fragmentation study of the 379.2094 *m/z* ion is described in literature (Visentin et al. 2010).

3.3. Carnosine, anserine and homocarnosine

The final w/w percentages of carnosine, anserine and homocarnosine present in each sample are shown in table 1. The obtained results show that carnosine and anserine were measured in all the samples, while homocarnosine was only present in the pork meat samples. All of the quantitative determinations were done using carnosine calibration curves.

The mean percentage content of carnosine vs. total CRCs is higher in horse meat (98.9%), which is followed by pork loin (89.3%) and beef (88.0%), while turkey, rabbit and chicken have a lower percentage (19.9%, 23.3% and 30.6 %, respectively). As far as anserine is concerned, an almost inverse trend was observed; the meats with the highest carnosine content were those that had the lowest anserine levels. Turkey meat was very rich in anserine, with a percentage that was about four times higher than that of carnosine. Anserine was also found in the chicken and rabbit meat in

amounts that were more than double those of carnosine. Carnosine was instead present in the horse meat in very high amounts compared to anserine (about tenfold higher) and there was an even greater quantity of carnosine than anserine in the beef and pork. Normally homocarnosine is present in a small percentage respect to anserine (3.4% for rabbit, 7.1% for turkey, 7.7% for chicken, 10.9% for beef); in horse meat this percentage goes up to 30%. Only in the case of pork loin homocarnosine surpasses anserine more than two times.

After turkey, the species with the greatest amount of meat antioxidants was chicken (table 2), while rabbit and beef had the lowest percentage content of total CRCs (under 2%).

Considering the work by Gil-Agustì, Esteve-Romero, & Carda-Broch (2008), the data obtained by these authors are lower than those found in our study. However, there is a similar trend for the carnosine content. In fact, considering the types of meat analyzed in their study (pork, beef and turkey), the observed trend reflects our data: the highest carnosine content was present in pork, followed by beef and turkey. As far as anserine is concerned, the results are in agreement for turkey meat, which contained the largest percentage, as in our study.

The same trend can be seen for the carnosine/anserine ratio; the ratio is very high in pork (49:1), while it is 8:1 in beef. The anserine in the turkey breast is about six times more abundant than the carnosine.

When comparing these data, we should however also consider the fact that the study by Gil-Agustì, Esteve-Romero, & Carda-Broch (2008) used minced meat containing only a certain percentage of the meat in question.

The research by Maikhunthod & Intarapichet (2005) found values of carnosine in chicken breast of about 0.29 g/100 g. It is interesting to note that significant differences can be observed in the content of carnosine within the same chicken meat if different parts of the animal are examined. For example, the breast has a carnosine content that is about seven times higher than that of the thigh.

The carnosine contents also differ according to sex and genotype (Intrapichet & Maikhunthod, 2005).

The work conducted by Mora, Sentandreu, & Toldra (2008) examined the carnosine content in porcine muscles belonging to different metabolic types and the differences can be explained by the fact that the carnosine content differs according to the type of muscle metabolism; glycolytic muscles in general (such as the *longissimus dorsi*) have a higher carnosine content than those with an oxidative metabolism.

Purchas, Rutherfurd, Pearce, Vather, & Wilkinson (2004) found carnosine levels that are comparable with those observed in our experiment for beef.

3.4. Pentosidine (Advanced Glycation End Product)

The analysis showed traces of pentosidine above the instrumental determination limits (1 ng/mL equivalent to 20 µg/100 g meat) in all the meat samples.

Pentosidine is one of the Maillard reaction products (MRPs). The Maillard reaction is a chemical reaction that occours between an amino acid and a reducing sugar. Temperature and time are the most significant processing factors that can influence the Maillard reaction (Friedman, 1996) and pentosidine is formed in greater quantities in reactions of pentoses with lysine and arginine (Dyer, Blackledge, Thorpe, & Baynes, 1991).

Chao, Hsu, & Yin (2009) analyzed the content of MRPs (including pentosidine) in different types of raw or processed (boiled, fried and baked) meat (beef, chicken and pork). The level of pentosidine in the raw samples were very low, but they increased with cooking. Baked and fried meat had the greatest amount of pentosidine (10-20 µg/100g) and other AGEs, while boiling led to a lower increase in the pentosidine content.

The work conducted by Golberg et al. (2004) analyzed another MRP as a glycoxidation marker, N-carboxymethyllysine (CML), in boiled meat and other types of food. As the boiling time increased, the amount of CML in the meat samples increased.

3.5. Susceptibility to lipid oxidation

Lipid oxidation was monitored in each type of meat sample by measuring the TBARS (Table 2).

Our results have shown that beef fillet had the greatest amount of TBARS and this was followed by turkey breast, while the horse fillet, pork loin and rabbit hindlegs had similar values (between 14 – 18 nmol MDA/g) of TBARS and chicken breast had the lowest measured value.

The same trend was observed in the work conducted by Kim, Nam, & Ahn (2002), who analyzed TBARS levels in pork, beef and turkey meat; the highest TBARS level was measured in beef and this was followed by turkey and pork.

Tang, Sheehanm, Buckley, Morrissey, & Kerry (2001) reported that the susceptibility of untreated minced muscle to lipid oxidation was in the decreasing order: beef > duck > ostrich > pork ≥ chicken.

They regarded these differences in lipid stability as the result of different contents of total fat, iron and fatty acid composition between species. The obtained data showed that the susceptibility to lipid oxidation in red meat and poultry is species dependent.

The reason for the different susceptibility to lipid oxidation might be due to the fat content of the meat, feeding rations (including antioxidant status and unsaturated fatty acid contents) and storage conditions. These factors could explain the differences observed among the different types of meat and also, together with the time of hanging, the great variability of the data concerning the same animal that we can be found in many works in literature.

It is known from literature, that beef in general has higher lipid oxidation values than other types of meat, even though there is great variability, in absolute terms, in each work.

Turkey meat is also rich in TBARS and our values are comparable with those observed in the Nam & Ahn (2003) study, which demonstrated an increase in TBARS level over ten days of refrigerated storage.

Our result for beef is in the range of values obtained by Min, Cordray, & Ahn (2010), who quantified the amount of TBARS for 10 days of refrigerated storage (4°C); our data is slightly lower than that of the 5 days storage meat.

The same work analyzed chicken breasts which had much lower TBARS level than beef (more than sixfold) and even lower than the TBARS measured in the chicken in our study. Our results for chicken breast are in agreement with Du, Cherian, Stitt, & Ahn (2002).

The fact that beef is more vulnerable to lipid oxidation than chicken was also observed in the study by Mitsumoto, O'Grady, Kerry, & Buckley (2005).

TBARS are more abundant in chicken and turkey tights than in the respective breasts (Du, Nam, Hur, Ismail, & Ahn, 2002; Nam & Ahn, 2003),

Pork is one of the least susceptible meats to lipid peroxidation. The pork loin data measured in our tests are in the range found by Tang, Sheehanm, Buckley, Morrissey, & Kerry (2001) and are greater than those found by Joo, Lee, Ha, & Park (2002).

The data on rabbit meat are very variable; we found very similar values to those of Corino, Mourot, Magni, Pastorelli, & Rosi (2002), but higher than the TBARS observed by Badr (2004), even after 7 days of refrigerated storage.

4. Conclusions

The LC-MS analysis of meat from different animal species enables one to clearly determine the structure of the histidinic antioxidants that are present and to quantify them. The structure of homocarnosine was elucidated by high resolving power multistage mass spectrometry, showing that it is a homohistidine derivative. The total CRCs content was in the increasing order: beef < rabbit < pork < horse < chicken < turkey, while the susceptibility of these meat to lipid oxidation decreased

from beef to chicken, with the exception of turkey meat, which presented a high TBARS content even though its total CRCs was the highest.

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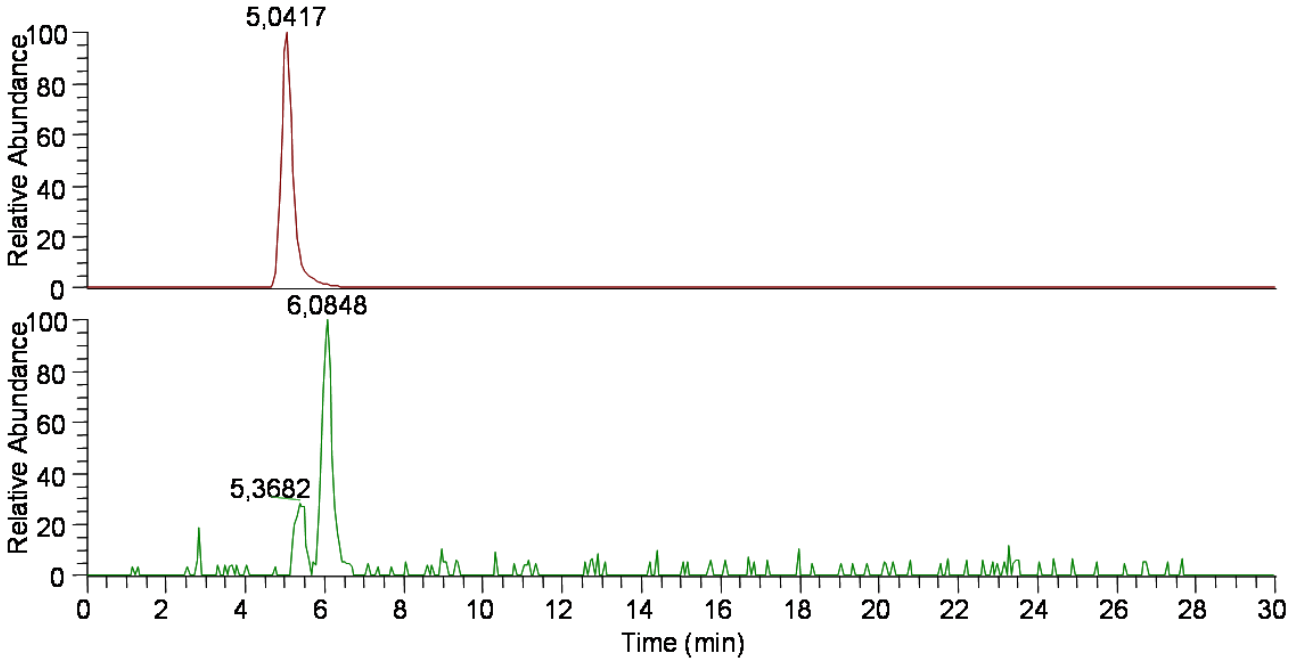


Fig. 1. Carnosine (top) and Carnosine Related Compound (bottom, anserine first and homocarnosine second eluted) chromatogram of a pork meat sample.

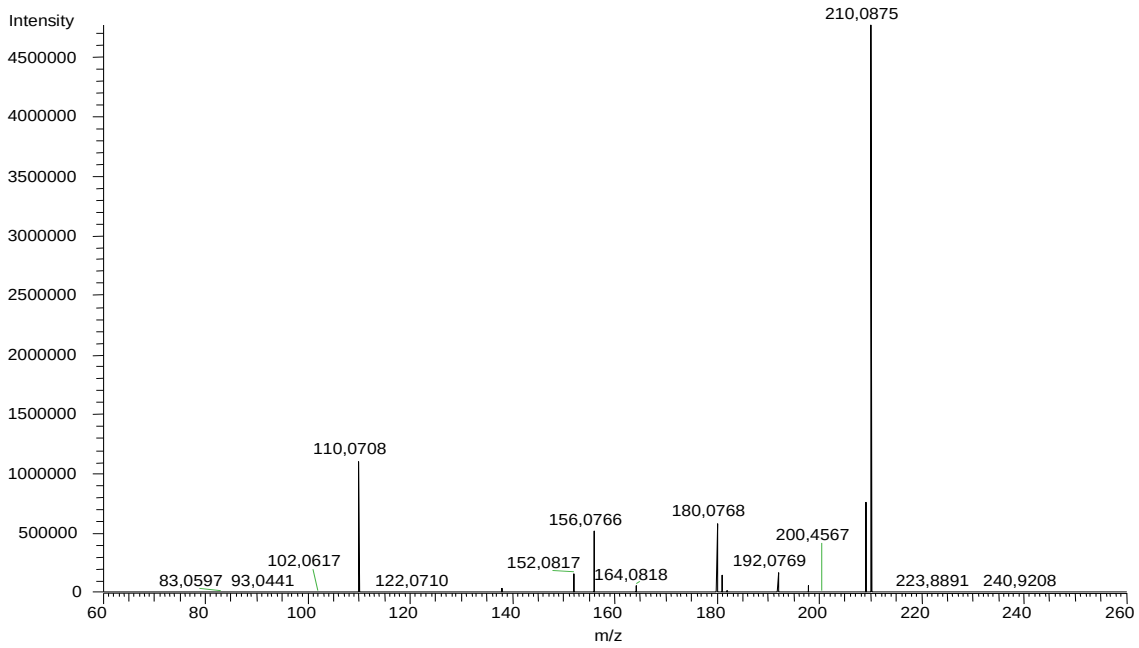


Fig. 2. MS^2 spectrum of carnosine protonated ion (227.1137 *m/*z).

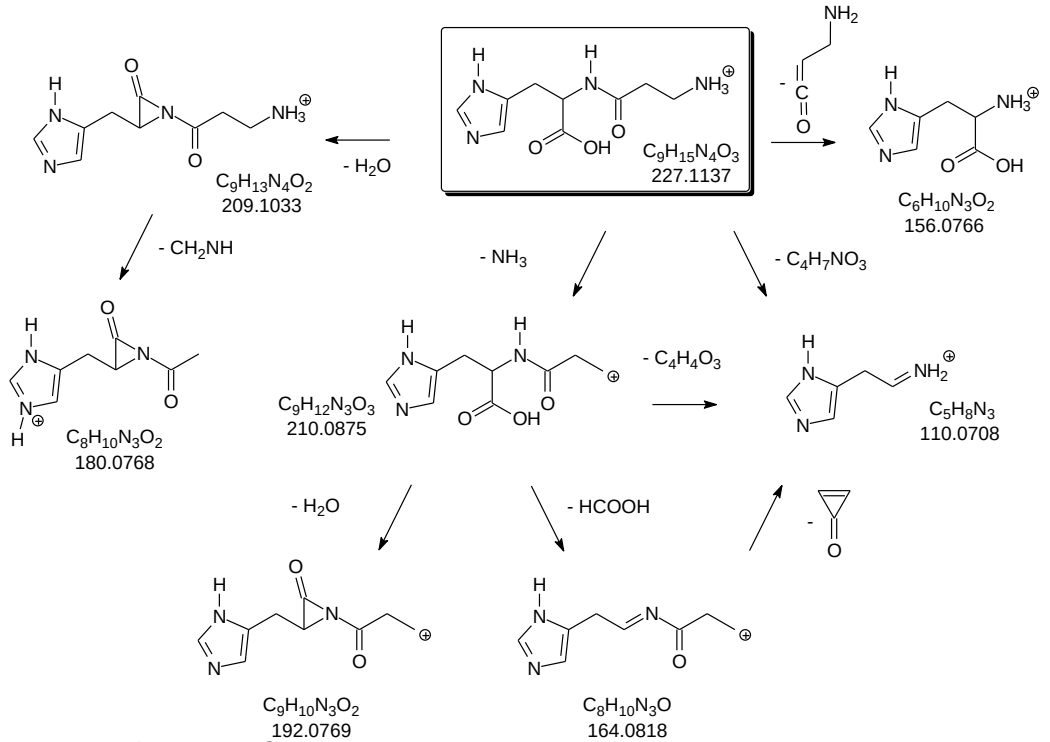
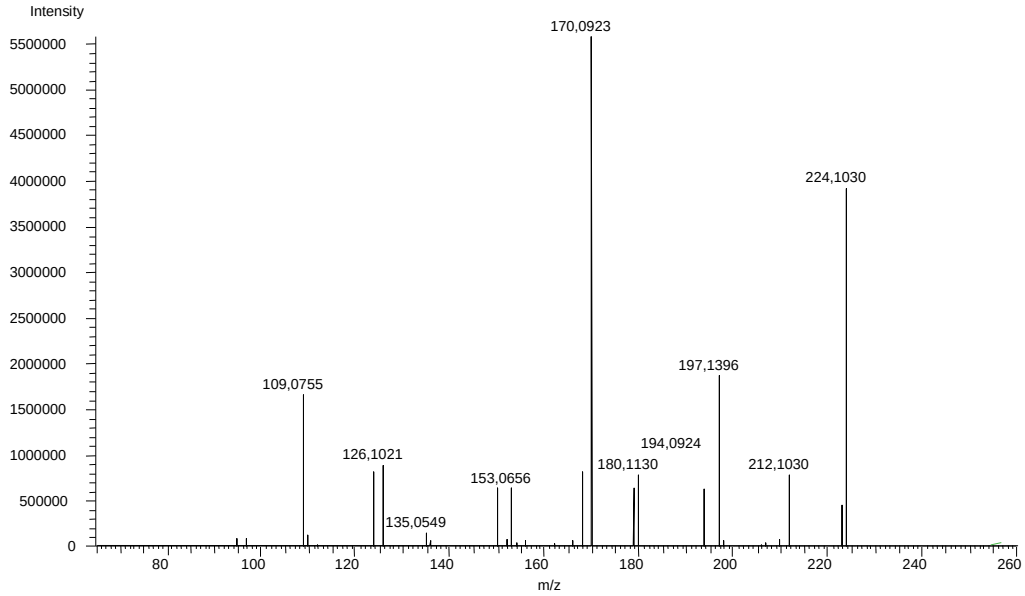
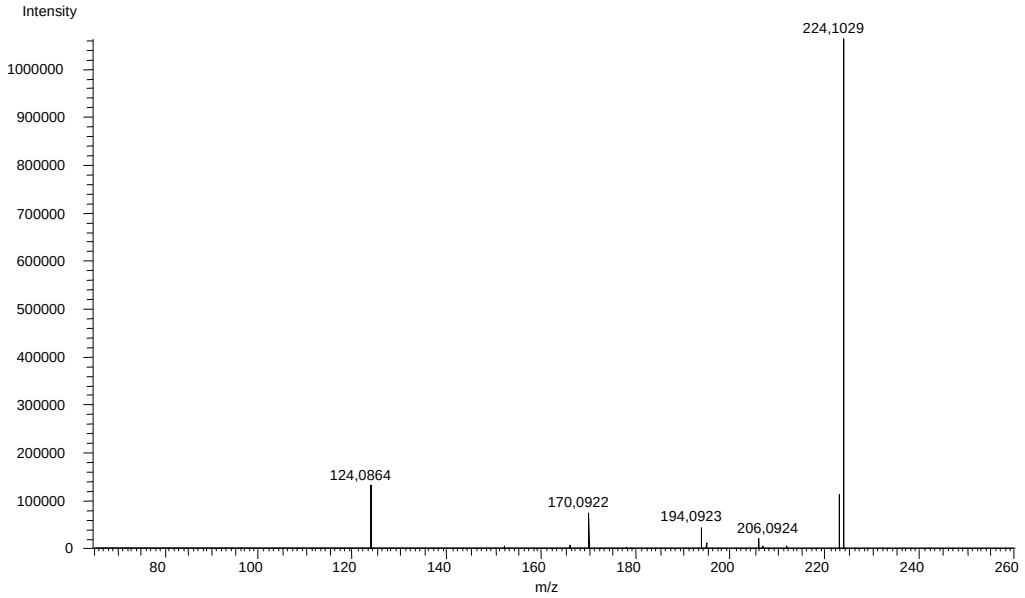


Fig. 3. Carnosine MS^2 and MS^3 fragmentation pathway. Multistage attributions were confirmed through MS^3 experiments.



A)



B)

Fig. 4. MS^2 spectrum of protonated ion of the anserine (A) and homocarnosine (B) isobaric compounds (241.1294 *m/*z).

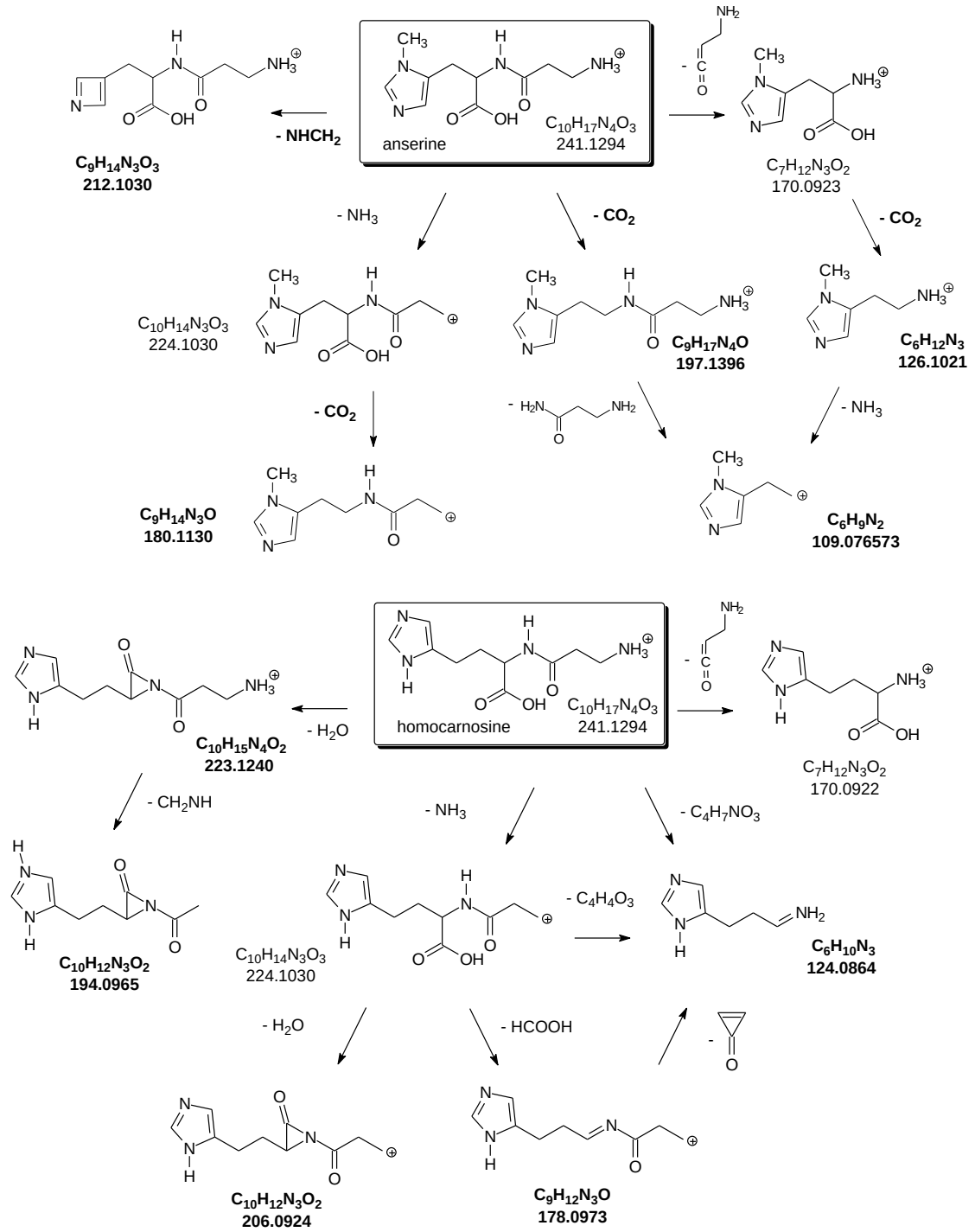


Fig. 5. The main anserine and homocarnosine MS^2 and MS^3 fragmentation pathways. The ions that were useful for characterization are in bold.

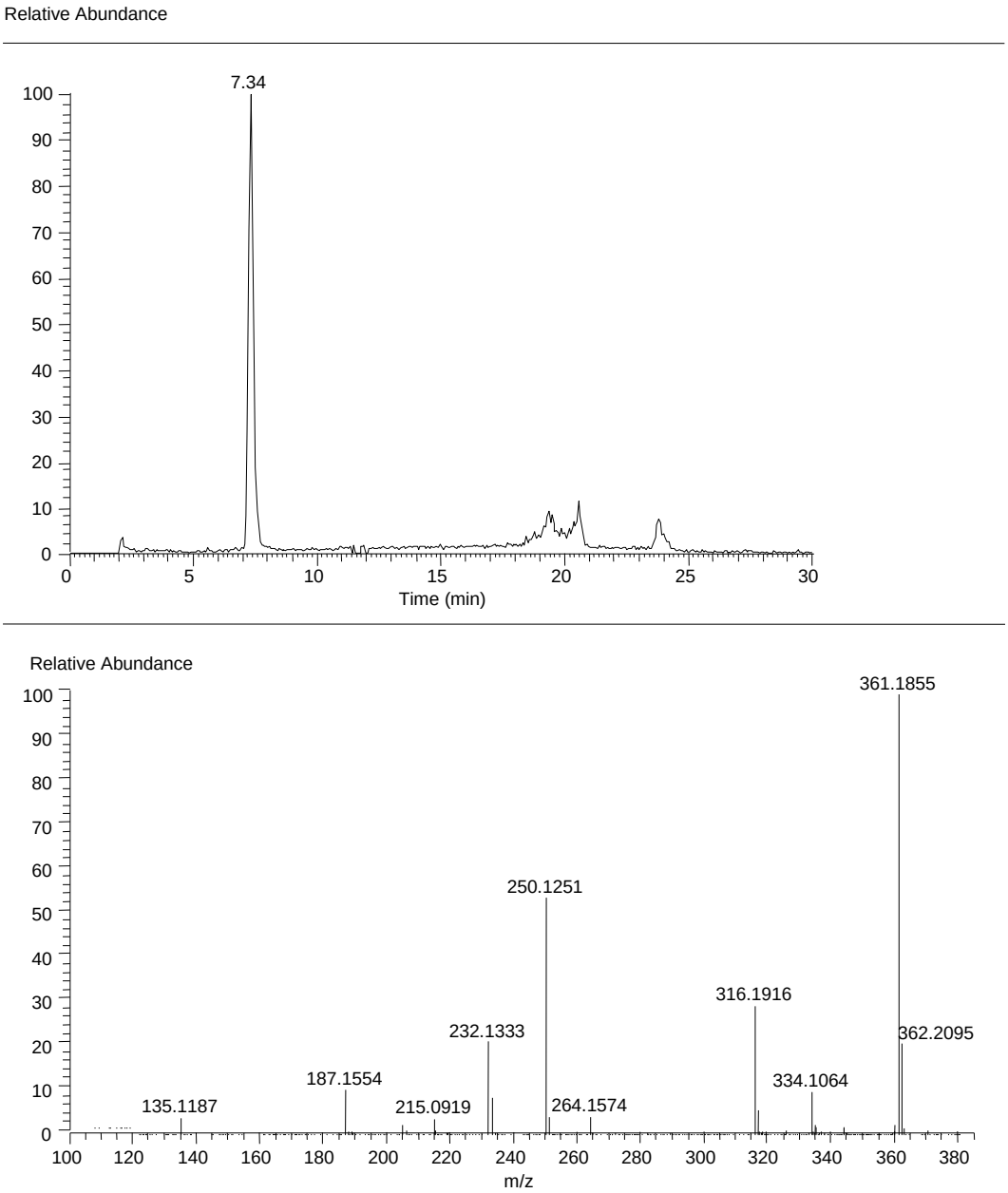


Fig. 6. Chromatogram and MS^2 spectrum of a pentosidine standard solution.

Table 1

Carnosine, anserine and homocarnosine contents (%w/w) in raw meats obtained from different species (triple extraction, means ± S.D.).

Meat cut	Supermarket	Carnosine	Anserine	Homocarnosine
Horse fillet	A	1.98 ± 0.62	0.020 ± 0.0044	0.0065 ± 0.0032
Horse fillet	B	2.54 ± 0.13	0.020 ± 0.0018	0.0040 ± 0.0018
Horse fillet	C	1.73 ± 0.083	0.010 ± 0.0026	0.0037 ± 0.00093
Pork loin	A	1.80± 0.15	0.059 ± 0.0093	0.16 ± 0.037
Pork loin	B	1.57± 0.39	0.041 ± 0.0084	0.14 ± 0.047
Pork loin	C	1.76± 0.015	0.050 ± 0.0031	0.18 ± 0.020
Beef fillet	A	1.49 ± 0.65	0.28 ± 0.14	0.024 ± 0.0085
Beef fillet	B	1.22 ± 0.40	0.098 ± 0.028	0.017 ± 0.0081
Beef fillet	C	1.43 ± 0.18	0.16 ± 0.015	0.014 ± 0.0036
Rabbit hindleg	A	0.46 ± 0.084	1.45 ± 0.18	0.042 ± 0.012
Rabbit hindleg	B	0.36 ± 0.085	1.25 ± 0.41	0.054 ± 0.040
Rabbit hindleg	C	0.39 ± 0.015	1.12 ± 0.12	0.027 ± 0.0016
Chicken breast	A	0.70 ± 0.20	1.70 ± 0.53	0.16 ± 0.056
Chicken breast	B	0.92 ± 0.36	1.67 ± 0.68	0.093 ± 0.059
Chicken breast	C	0.76 ± 0.25	1.46 ± 0.43	0.11 ± 0.059
Turkey breast	A	0.36 ± 0.13	2.11 ± 0.82	0.15 ± 0.032
Turkey breast	B	0.79 ± 0.18	2.46 ± 0.69	0.12± 0.039
Turkey breast	C	0.62 ± 0.15	2.19 ± 0.78	0.19 ± 0.062

Table 2

Total Carnosine Related Compounds (CRCs) and Thiobarbituric Acid Reactive Substances (TBARS) contents in raw meats (means ± S.D.) obtained from different species.

Meat cut	Sample (n.)	Total CRCs (%)	TBARS (mM MDA/kg)
Rabbit hindleg	3	1.71± 0.36	17.70 ±1.47
Beef fillet	3	1.53 ± 0.50	34.18 ± 20.98
Horse fillet	3	2.08 ± 0.51	14.11 ± 2.74
Pork loin	3	1.91 ± 0.29	13.41 ± 1.78
Chicken breast	3	2.53 ± 0.76	5.58 ± 0.60
Turkey breast	3	2.99 ± 0.89	27.81 ± 7.88