JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2004; **39**: 1417–1428
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.696

JMS

Profiling histidine-containing dipeptides in rat tissues by liquid chromatography/electrospray ionization tandem mass spectrometry†

Giancarlo Aldini,* Marica Orioli, Marina Carini and Roberto Maffei Facino

Istituto Chimico Farmaceutico Tossicologico, Faculty of Pharmacy, University of Milan, Viale Abruzzi 42, 20131 Milan, Italy

Received 10 May 2004; Accepted 15 July 2004

The histidine-containing dipeptides carnosine (CAR) and structurally related anserine (ANS) and homocarnosine (HCAR), widely distributed in vertebrate organisms, have recently been proposed as endogenous quenchers for highly cytotoxic α,β-unsaturated aldehydes generated by peroxidation. A sensitive, selective, specific and rapid liquid chromatographic/electrospray ionization tandem mass spectrometric assay was developed and validated for the simultaneous determination of these peptides in biological matrices in order to establish their plasma/tissue distribution. Samples (plasma or tissue homogenates from male rats) were prepared by protein precipitation with $HClO_4$ (1 : 1, v/v) containing H-Tyr-His-OH as internal standard. The supernatant was separated on a Phenomenex Sinergy polar-RP column with a mobile phase of water–acetonitrile–heptafluorobutyric acid (9 : 1 : 0.01, v/v/v) at a flow-rate of 0.2 ml min^{-1}, with a run time of 10 min. Detection was effected on an ion trap mass spectrometer equipped with an electrospray ionization interface operating in positive ionization mode. The acquisitions were in the multiple reaction monitoring mode using the following precursor → product ion combinations: H-Tyr-His-OH (internal standard) *m/z* 319 → 301; CAR *m/z* 227 → 210 + 209; ANS *m/z* 241 → 224 + 197 + 170; HCAR *m/z* 241 → 156. The method was validated over the concentration range 15–1000 nmol g^{-1} and the limit of quantification (LOQ) and limit of detection (LOD) were 12.5 and 4.2 pmol injected, respectively. The intra- and inter-day precisions were <10% (≤17.47% at the LOQ) and the intra- and inter-assay accuracies were within ±10% for all concentrations. The mapping profile in rat tissue gave the following results: the highest concentrations of CAR and ANS were found in skeletal muscles (soleus, gastrocnemius, tibialis), followed by the heart, cerebellum and brain (ANS below the LOQ). HCAR was found only in the brain and cerebellum. No histidine-containing dipeptides were detectable in plasma, liver, kidney and lung.

KEYWORDS: histidine-containing dipeptides; carnosine; anserine; homocarnosine; liquid chromatography/electrospray ionization tandem mass spectrometry; plasma and tissue profile

INTRODUCTION

Carnosine (CAR) (β-alanyl-L-histidine), homocarnosine (HCAR) (γ-aminobutyryl-L-histidine), anserine (ANS) (*N*-β-alanyl-3-methyl-L-histidine) and balenine (BAL) (*N*-β-alanyl-1-methyl-L-histidine) are histidine-containing dipeptides widely distributed in vertebrate animal tissues, especially skeletal muscles, heart and the central nervous system. Although their biological role is still not known, there is evidence that they act as intracellular buffers, immune modulators, neurotransmitters (GABA precursor), metal-ion chelators, antioxidants and free-radical scavengers.[1–6] It has been suggested that CAR could act as a detoxifying agent for cytotoxic reactive carbonyl species, especially α,β-unsaturated aldehydes (4-hydroxynonenal (HNE) and acrolein (ACR)) generated *in vivo* by metal-catalyzed oxidation of ω-6 polyunsaturated fatty acids such as arachidonic and linoleic acids.[7–9] We recently confirmed this[10–12] by studying the quenching capacity of CAR, HCAR and ANS towards HNE and ACR and elucidating the adduction reaction mechanism (mass spectrometric (MS) and NMR characterization of the conjugated products). The carbonyl quenching ability of CAR and ANS has been confirmed by liquid chromatographic/tandem mass spectrometric (LC/MS/MS) analysis (electrospray ionization (ESI) interface) in complex biological matrices (spontaneously oxidized rat skeletal muscle),[11] suggesting that this tissue, highly exposed to peroxidative attack, contains a histidine-dependent detoxification pathway against cytotoxic HNE alternative/concomitant to that involving thiol-containing peptides (i.e. GSH).

*Correspondence to: Giancarlo Aldini, Istituto Chimico Farmaceutico Tossicologico, Faculty of Pharmacy, University of Milan, Viale Abruzzi 42, 20131 Milan, Italy.
E-mail: giancarlo.aldini@unimi.it
†Paper presented at the 22nd Informal Meeting on Mass Spectrometry, Tokaj, Hungary, 2–6 May 2004.
Contract/grant sponsor: MIUR.

In view of the growing interest and the biological importance of histidine-containing dipeptides, there is a definite need for a sensitive, specific and simple analytical method to detect these compounds in biological matrices. Several methods, mainly high-performance liquid chromatographic (HPLC) (reversed-phase or ion-exchange), have been described for the analysis of histidine-containing dipeptides in matrices from different mammalian species. UV detection has been applied to underivatized peptides,[13,14] but on account of the low extinction coefficients, precolumn derivatization with different chromophores and fluorophores or post-column derivatization (by *o*-phthalaldehyde or ninhydrin) has been used more often.[15–21]

All these methods suffer from several drawbacks: low resolution, instability of derivatives, in some cases a high detection limit, and above all, they require complex and extensive sample processing and are tedious and time consuming. The most recent methods (HPLC) have only been used to search for histidine-containing peptides in feeds (to check for products of animal origin), and not in *ex vivo* animal tissues.[22–24] Surprisingly, MS has never been employed for this purpose, despite the well-known specificity and sensitivity of ESI for peptides.

We therefore designed this study to assess the potential of an LC/ESI-MS/MS approach for profiling histidine-containing dipeptides in the rat (plasma and tissues), a field not hitherto explored.

EXPERIMENTAL

Chemicals

All chemicals and reagents were of analytical grade, purchased from Sigma-Fluka-Aldrich Chemical (Milan, Italy). HPLC-grade and analytical-grade organic solvents were purchased from Merck (Bracco, Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Carnosine (*β*-alanyl-L-histidine) and the internal standard (IS) H-Tyr-His-OH were generous gifts from Flamma (Chignolo d'Isola, Bergamo, Italy). Homocarnosine (*γ*-aminobutyryl-L-histidine) and anserine (*N*-*β*-alanyl-3-methyl-L-histidine) and the protease inhibitor cocktail were purchased from Sigma (Milan, Italy) and heptafluorobutyric acid (HFBA) from Aldrich (Milan, Italy). Balenine (*N*-*β*-alanyl-1-methyl-L-histidine) was isolated from porcine muscle (Longissimus dorsi) as described by Aristoy *et al*.[23]

Instrumentation

LC/MS experiments were carried out on a Thermoquest Surveyor LC system equipped with a quaternary pump, a Surveyor UV/VIS diode-array programmable detector 6000 LP, a Surveyor AS autosampler, a vacuum degasser, Xcalibur software and connected to a Thermo Finnigan LCQ Advantage ion trap mass spectrometer (ITMS) (Thermo Finnigan Italia, Milan, Italy). Separations were performed by reversed-phase elution with a Phenomenex Sinergy polar-RP column (150 × 2 mm i.d., particle size 4 μm) (Chemtek Analytica, Anzola Emilia, Italy) protected by a polar-RP guard column (4 × 2 mm i.d., 4 μm) thermostated at 25 °C. Water–acetonitrile–HFBA (9 : 1 : 0.01, v/v/v) was the mobile phase delivered at a flow-rate of 0.2 ml min^{-1}; the samples rack was maintained at 4 °C. The ESI-MS source was set at capillary temperature 200 °C, spray voltage 4.5 kV and capillary voltage 21 V. The flow-rate of the nebulizer gas (nitrogen) was 5 l min^{-1}. MS conditions and tuning were achieved by mixing aqueous analyte solutions (flow-rate 5 μl min^{-1}) with the mobile phase through a T-connection at a flow-rate of 0.2 ml min^{-1}.

Collision-induced dissociation (CID) experiments in the positive ion mode were run by selecting the target ion with an isolation width of m/z ±0.5 and using helium as collision gas. The ion collision energy was adjusted by changing a percentage of the 5 V a.c. voltage which was applied to the end-caps of the ion trap at the resonance frequency of the selected ion and set at 30% (referred to as collision energy level).

Acquisitions were taken in the multiple reaction monitoring (MRM) mode using the following precursor → product ion combinations: H-Tyr-His-OH (IS) m/z 319 → 301; CAR m/z 227 → 210 + 209; ANS m/z 241 → 224 + 197 + 170; HCAR m/z 241 → 156.

Preparation of calibration standards and quality control samples

Two fresh stock solutions of 10 mM CAR, ANS, HCAR and the IS were prepared separately in 10 mM phosphate buffer solution (PBS, pH 7.4) and stored in liquid nitrogen. One solution was used to spike lung homogenate for calibration samples and the other to prepare the quality control (QC) samples (lung homogenate) and to spike tissue extracts. Stock solutions were diluted further with PBS and the solutions of each analyte were mixed together to obtain working solutions. Working solutions of 100 nmol ml^{-1} were analyzed by LC/MS/MS to ensure that the concentrations of the original solutions were within the limits of the maximum established error (≤3%). Calibration samples were prepared by spiking lung homogenate (1 : 3, w/v, in PBS) with each working solution to provide the following final concentrations: 15, 30, 150, 300, 450, 750 and 1000 nmol g^{-1} wet mass tissue. QC samples at three concentrations (15, 150, 750 nmol g^{-1}) were prepared in the same way. Each calibration sample was processed as described below.

Sample processing

Male Wistar rats (Charles River, Calco, Italy; body mass 250 ± 25 g) were maintained in compliance with the policy on animal care expressed in National Research Council guidelines (NRC 1985). Laboratory chow and drinking water were available *ad libitum*.

The animals were killed by decapitation and the blood was collected in heparinized test-tubes and tissues (soleus, gastrocnemius and tibialis skeletal muscles, heart, brain, cerebellum, kidney, lung, liver) were removed and stored in liquid nitrogen until processed. Blood was centrifuged at 1000 g for 15 min at 4 °C. The plasma was removed and stored in liquid nitrogen until analysis. Tissues were homogenized in cold PBS (1 : 3, w/v, heart, brain, cerebellum, kidney, lung, liver; 1 : 30, w/v, for skeletal muscles) containing protease inhibitor cocktail (50 μl g^{-1} wet tissue). Aliquots of 500 μl

of the homogenate were spiked with IS (50 µl of a 4 mM solution), deproteinized by addition of 500 µl of 700 mM perchloric acid (PCA) solution and vortex mixed for 1 min. After 15 min at 4 °C, the samples were centrifuged at 21 000 *g* for 10 min. The supernatant was diluted (1 : 1, v/v) with the mobile phase, filtered through 0.2 µm nylon filters and the filtrate transferred to the autosampler vial insert (10 µl samples injected).

Validation of the method

Calibration standards were prepared and analyzed in duplicate in three independent runs. The calibration curves were constructed by weighted ($1/x$) least-square linear regression analysis of the peak area ratios of CAR, ANS and HCAR to the IS against nominal analyte concentration.

Intra- and inter-day precisions and accuracies of the method were determined by assaying four replicates of each of the QC samples (three concentrations in the low, intermediate and high concentration ranges) in three separate analytical runs. Precision and accuracy were determined by calculating the coefficient of variation (CV, %) and the relative error (RE, %). Precision and accuracy should be within 15% except at the limit of quantification (LOQ), where it should not exceed 20%.

The absolute recovery of the analytes after protein precipitation was determined by comparing the results for QC samples ($n = 3$) at the three concentrations with a corresponding set of spiked deproteinized lung tissues (containing 100% of the theoretical concentration).

The stability of the analytes was investigated in the stock and working solutions and in the final extract at 4 °C (rack temperature) up to 24 h. The results were compared with those for freshly prepared QC samples and the percentage concentration deviation was calculated. The analytes were considered stable when 90–110% of the initial value was found.

To verify whether different tissue matrices affect the ionization efficiency of the analytes, tissue extracts were spiked with CAR, ANS and HCAR at 15 and 750 nmol g^{-1} (brain, cerebellum, heart) and 1500 nmol g^{-1} (skeletal muscles) and the precision and accuracy (four replicates for each concentration) were calculated as above.

RESULTS AND DISCUSSION

Optimization of LC and MS conditions

Although acetonitrile–water spiked with 0.1% trifluoroacetic acid (TFA) as ion-pairing agent is commonly used as mobile phase to separate peptides by reversed-phase high-performance liquid chromatography (RP-HPLC), it was not suitable for histidine-containing dipeptide analysis since they co-elute near the void volume. Similar results were obtained by raising the final concentration of TFA to 0.2%. Perfluorinated carboxylic acids with longer *n*-alkyl chains, such as pentafluoropropionic, heptafluorobutyric, perfluoropentanoic, perfluorohexanoic and perfluoroheptanoic acid, have been employed as alternatives to TFA for the separation of polar peptides and proteins[25,26] and for natural amino acids[27] and dipeptides.[28] Thus, adequate results were obtained using 0.1% HFBA as ion-pairing agent: the histidine-containing dipeptides eluted at 4.54 min (CAR), 4.64 min (ANS, HCAR) and 5.06 min (BAL), with an excellent bell shape. H-Tyr-His-OH was chosen as IS for quantitative analysis because no isotopic derivatives of these compounds are commercially available, and it eluted at 8.06 min.

The full mass spectra of CAR, HCAR, ANS, BAL and IS in MS infusion experiments showed the protonated molecular ions $[M + H]^+$ at m/z 227, 241, 241, 241 and 319, respectively (Table 1). The effect of HFBA on the ionization efficiency is shown in Fig. 1. Although HFBA suppressed the ion current by almost 50% for CAR, ANS and BAL, the effect was much less than that of the common ion-pairing agent TFA (ion suppression >75%). By contrast, HFBA increased the HCAR ion current by 35%, whereas TFA reduced it by 35%.

MS/MS experiments were carried out to determine the optimal fragmentation reaction for MRM analysis. For CAR, the most abundant product ions were at m/z 210 ($[M + H - NH_3]^+$) and 209 ($[M + H - H_2O]^+$) and for HCAR at m/z 156 (y_1 ion) (Table 1). There were three main product ions for ANS and BAL, at m/z 224 ($[M + H - NH_3]^+$), 170 (y_1 fragment) and 197 ($[M + H - CO_2]^+$), so we cannot discriminate the two *N*-methyl derivatives of CAR. Since the ion at m/z 170 is attributed to the protonated *N*-methylhistidine moiety, and the fragmentation patterns of N^1-methylhistidine and N^3-methylhistidine differ significantly,[28] the ion at m/z 170 was submitted to CID (MS^3) in order to distinguish the two dipeptides. In agreement with Turecek *et al.*,[29] N^1-methylhistidine arising from BAL fragmentation showed a dominant loss of $H_2O + CO$ to form a diagnostic fragment ion at m/z 124. By contrast, the N^3-methylhistidine fragment from ANS showed a characteristic product ion at m/z 126 due to the loss of CO_2. The two isobaric species ANS and BAL can therefore be unequivocally distinguished on the basis of the MS^3 fragment ions at m/z 124 (BAL) and 126 (ANS). The positive full mass spectrum of the IS showed the protonated molecular ion at m/z 319 and the MS/MS analysis of the parent ion gave as base peak the ion at m/z 301 ($[M + H - H_2O]^+$) and a minor peak at m/z 156 (y_1 ion). On the basis of the MS^n fragmentation pathways, we conclude that this approach can selectively

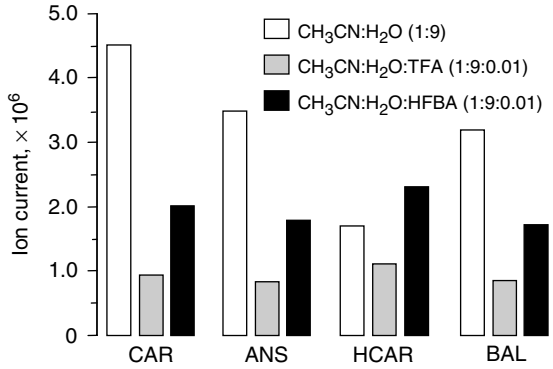
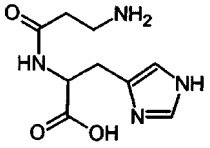
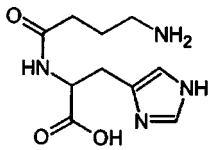
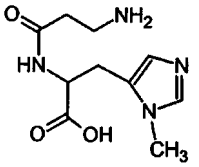
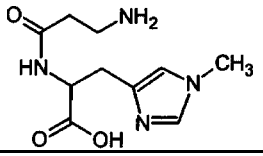


Figure 1. Effects of HFBA and TFA on ionization efficiency of CAR, HCAR, ANS and BAL. The experiments involved mixing the analyte water solution (100 nmol ml^{-1}, flow-rate 5 µl min^{-1}) through a T-connection with the mobile phase at a flow-rate of 0.2 ml min^{-1}.

Table 1. Chemical structures and MS and MS^n data for histidine-containing dipeptides[a]

Analyte	Structure	MS	MS^2	MS^3
CAR		227 $[M + H]^+ \rightarrow$	210 (100%) $[M + H - NH_3]^+$ 209 (40%) $[M + H - H_2O]^+$ 180 (10%) $[M + H - H_2O - CH_2NH]^+$ 156 (7%) (y1) 138 (10%) $[His + H - H_2O]^+$ 110 (8%) $[His + H - H_2O - CO]^+$	
HCAR		241 $[M + H]^+ \rightarrow$	156 (100%) (y_1)	
ANS		241 $[M + H]^+ \rightarrow$	224 (100%) $[M + H - NH_3]^+$ 223 (20%) $[M + H - H_2O]^+$ 212 (15%) $[M + H - CH_2NH]^+$ 197 (40%) $[M + H - CO_2]^+$ 194 (8%) $[M + H - H_2O - CH_2NH]^+$ 180 (12%) $[M + H - CO_2 - NH_3]^+$ **170 (95%) $[^3MHis + H]^+ \rightarrow$** 153 (10%) $[^3MHis + H - NH_3]^+$ 126 (13%) $[^3MHis + H - CO_2]^+$ 109 (20%) $[^3MHis + H - CO_2 - NH_3]^+$	(parent ion m/z 170): 153 (6%) $[^3MHis + H - NH_3]^+$ 126 (100%) $[^3MHis + H - CO_2]^+$ 125(24%) $[^3MHis + H - COOH]^{+\bullet}$ 109 (55%) $[^3MHis + H - CO_2 - NH_3]^+$
BAL		241 $[M + H]^+ \rightarrow$	224 (100%) $[M + H - NH_3]^+$ 223 (18%) $[M + H - H_2O]^+$ 197 (10%) $[M + H - CO_2]^+$ **170 (15%) $[^1MHis + H]^+ \rightarrow$**	(parent ion m/z 170): 124 (100%) $[^1MHis + H - H_2O - CO]^+$ 109 (18%) $[^1MHis + H - CO_2 - NH_3]^+$

[a] $^1MHis = N^1$-methylhistidine; $^3MHis = N^3$-methylhistidine.

JMS

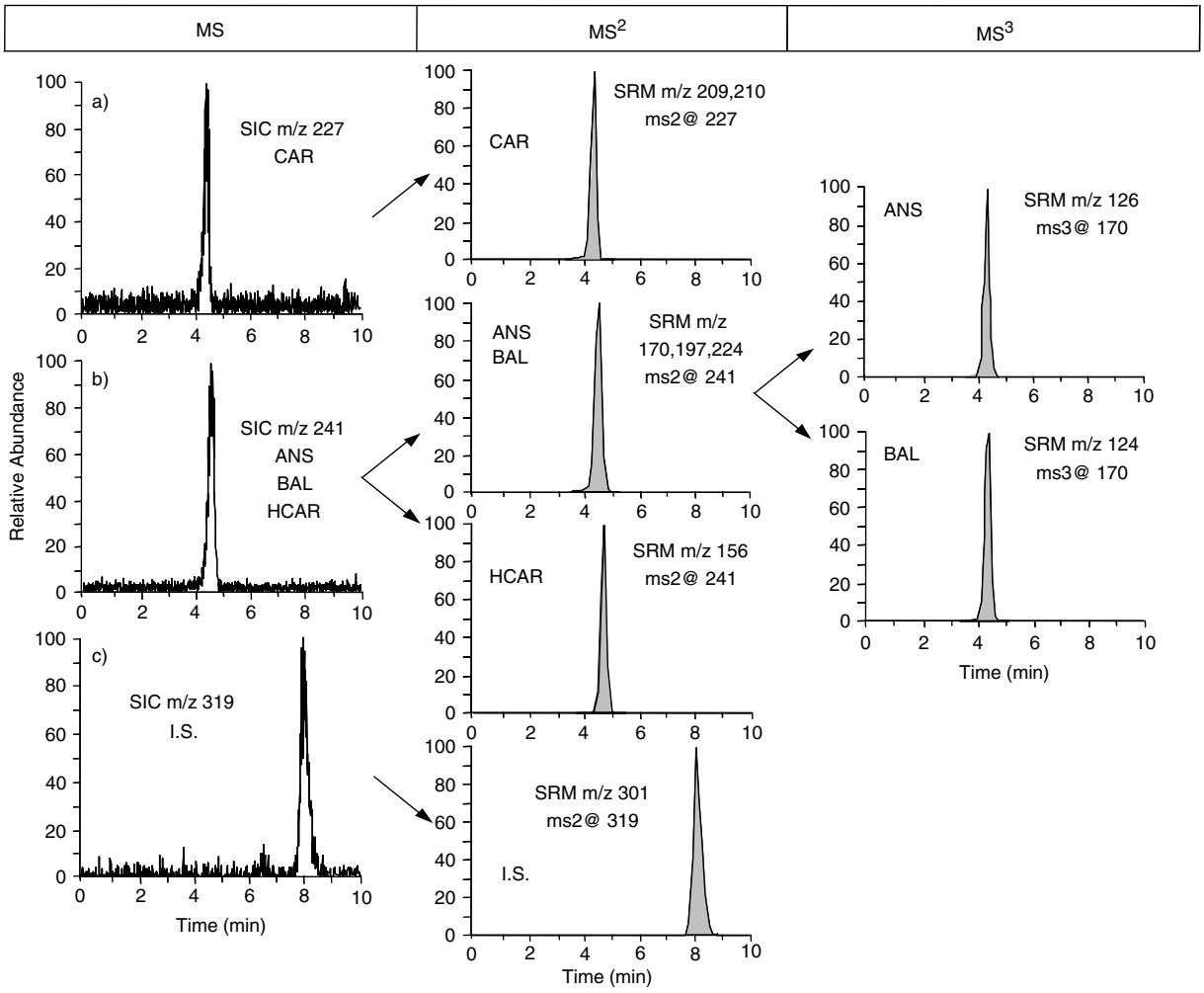


Figure 2. LC/MS and LC/MS^n analysis (positive-ion mode) of a mixture of standard histidine-containing dipeptides. MS panel: SICs at *m/z* 227 (a), 241 (b) and 319 (c). MS^n experiments: MS^2 was performed to detect and quantitate CAR (*m/z* 227 → 210 + 209) and HCAR (*m/z* 241 → 156) and MS^3 to discriminate between BAL (*m/z* 170 → 124) and ANS (*m/z* 170 → 126).

determine all the analytes, even if they are simultaneously present, have similar retention times and three of them are isobaric. This is clear from the LC/MS profiles of the four dipeptides (Fig. 2), which can be selectively detected by the following precursor–product ion combinations:

CAR: m/z 227 → 210 + 209;
HCAR: m/z 241 → 156;
ANS: m/z 241 → 170, 197, 224; 170 → 126 (MS^3);
BAL: m/z 241 → 170, 197, 224; 170 → 124 (MS^3);
IS: m/z 319 → 301.

Qualitative analysis in tissues

The method was employed, before quantitation, to detect the histidine dipeptides in rat tissues, starting from skeletal muscle (gastrocnemius), which contains large amounts of these derivatives. Figure 3 reports the results of injecting the crude homogenate after a simple deproteinization step by PCA: the total ion current (TIC) (a) shows a sharp peak between 4.2 and 5.1 min, whose full mass spectrum (b) contains two main peaks at m/z 227 and 241, eluting at 4.54 (c) and 4.64 min (d), respectively. These two peaks can thus be attributed to CAR (m/z 227) and to HCAR/ANS/BAL (m/z 241). As shown in Fig. 3, no interfering peaks in the mass and time ranges of interest are detectable, and no ionic species at m/z 319 ($[M + H]^+$ of the IS) in the crude biological matrix.

Confirmation was obtained by MS^2 and MS^3 analyses. Figure 4 unequivocally demonstrates CAR, since the full MS^2 spectrum (parent m/z 227) contains all the typical product ions of the compound (see Table 1). The full MS^2 spectrum of the parent ion m/z 241 (Fig. 4(d)) unequivocally excludes HCAR (lack of m/z 156), but does not discriminate between ANS and BAL. Again, this was achieved by MS^3 analysis: the full MS^3 spectrum (parent ion m/z 170; Fig. 4(e)) indicates the presence of ANS (diagnostic product ion at m/z 126) and the absence of BAL (no product ion m/z 124). This is confirmed by the corresponding selected reaction monitoring (SRM) traces (Fig. 4(f)).

To test the specificity of this MS^3 approach, pig ham, which contains similar amounts of ANS and BAL,[24] was analyzed in parallel. Figure 5(a) reports the TIC of the deproteinized muscle homogenate, showing a broad peak eluting between 4.01 and 5.50 min; its full mass spectrum (b) contains a prominent peak at m/z 227 attributed to CAR

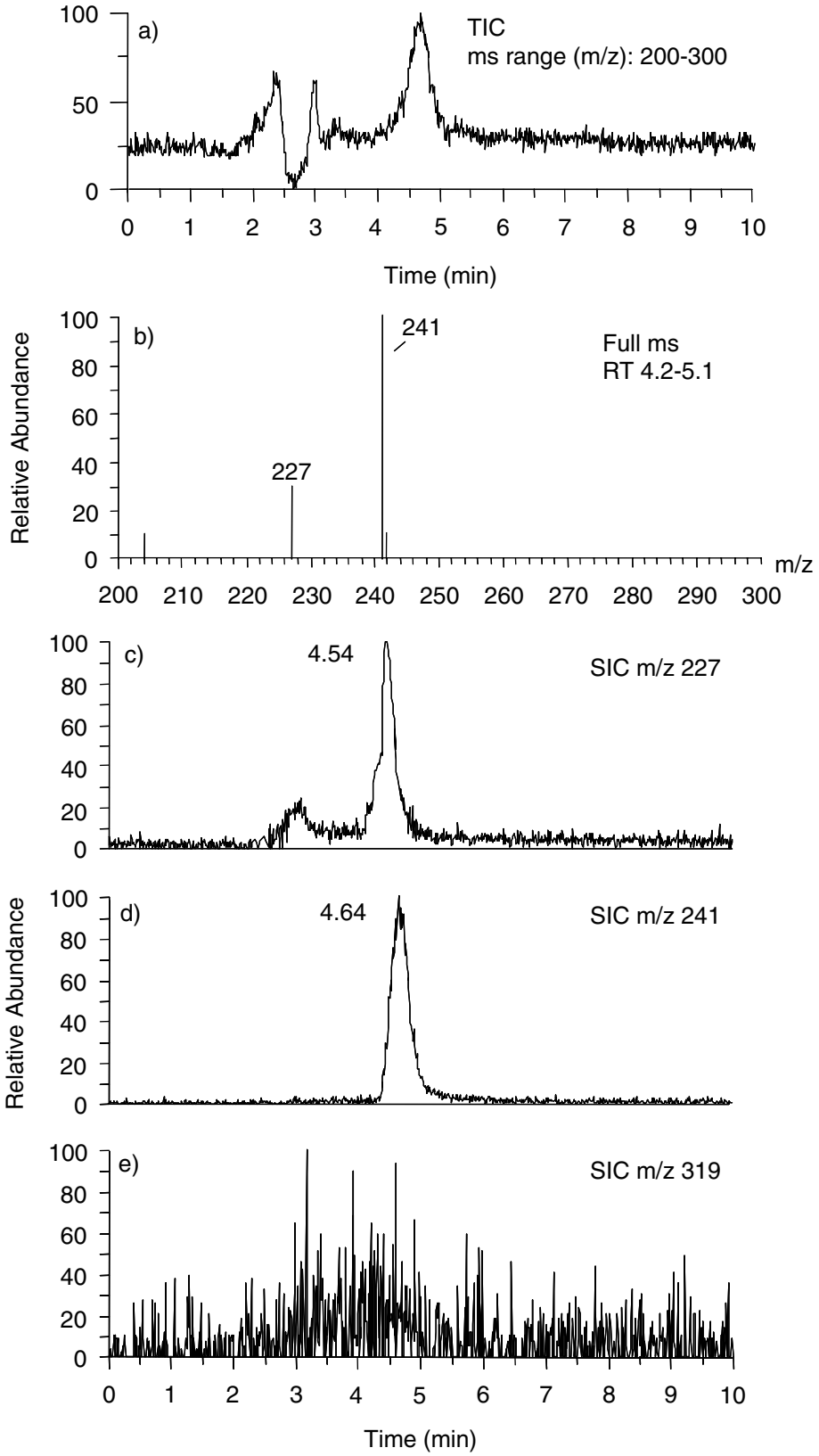


Figure 3. LC/MS analysis (positive-ion mode) of gastrocnemius skeletal muscle. (a) TIC; (b) full mass spectrum of the peak with retention time 4.2–5.1 min; (c) SIC *m/z* 227 (CAR); (d) SIC *m/z* 241 (ANS/BAL/HCAR); (e) SIC *m/z* 319 (IS).

in MS^2 experiments (data not shown) and a minor peak at *m/z* 241. MS^2 (c) and MS^3 (d) spectra indicate the presence of both ANS and BAL, confirmed by the corresponding SRM traces (e) at *m/z* 124 (BAL) and 126 (ANS).

The LC/MS/MS approach was then applied to the target rat tissues (data not shown), and the results can be summarized as follows: CAR and ANS were detected in all the skeletal muscles (gastrocnemius, tibialis and soleus),

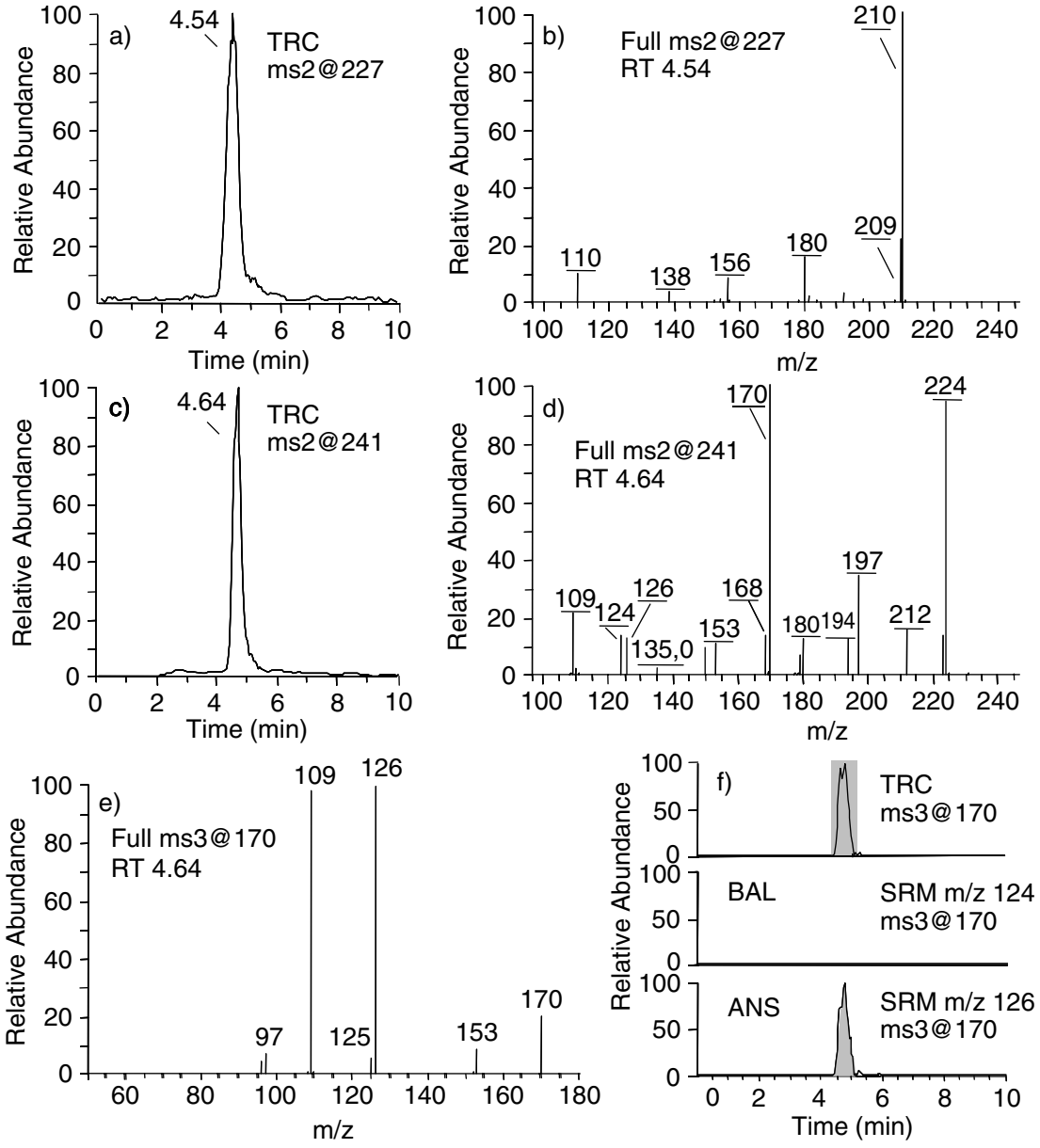


Figure 4. LC/MS^n analysis (positive-ion mode) of gastrocnemius skeletal muscle. (a) Total reaction chromatogram (TRC) of the ion at *m/z* 227 and (b) full MS^2 spectrum of the peak at 4.54 min; (c) TRC of the ion at *m/z* 241, (d) full MS^2 and (e) full MS^3 (parent ion *m/z* 170) spectra of the peak at 4.64 min; (f) TRC of the ion at *m/z* 170 (upper panel) and SRM traces at *m/z* 124 (BAL) and *m/z* 126 (ANS).

and in myocardium and cerebellum, but only CAR in brain. HCAR was present only in brain and cerebellum and BAL was never found. None of the histidine-containing dipeptides was detected in liver, lung and plasma.

Validation

Because of the absence of BAL in all rat tissues, quantitative analysis was done only for CAR, HCAR and ANS. It was impossible to validate the assay according to the FDA guidelines for Bioanalytical Method Validation because of the endogenous content of the analytes in the target tissues (http://www.fda.gov/cder/guidance/4252fnl.htm). Therefore, we executed a full validation procedure (linearity, limit of detection (LOD), LOQ, intra- and inter-day precision and accuracy, recovery and stability) on lung tissue, selected as a reference blank matrix since it contains no CAR, ANS or HCAR (see qualitative analysis). Standard curves for the analytes constructed on different working days showed good linearity over the entire calibration range (15–1000 nmol g^{-1}), with correlation coefficients (r^2) >0.998. The equations for the calibration lines were as follows:

$$y = 0.003751\ (\pm 0.000037)x - 0.004342\ (\pm 0.004539)\ \text{CAR}$$

$$y = 0.003473\ (\pm 0.000027)x - 0.005390\ (\pm 0.003296)\ \text{ANS}$$

$$y = 0.000992\ (\pm 0.0000089)x - 0.004724\ (\pm 0.001096)\ \text{HCAR}$$

The LOD was 5 nmol g^{-1} for all the analytes (4.2 pmol injected) and the LOQ was 15 nmol g^{-1} (12.5 pmol injected). The intra- and inter-assay precision and accuracy of the method were determined on QC samples by analysing four replicates at three concentrations, and the data are reported in Table 2. The intra-day precision (CV, %) was <14%

Table 2. Intra- and inter-day accuracy and precision for CAR, ANS and HCAR

Analyte	Nominal concentration (nmol g^{-1})	Mean measured concentration ±SD (nmol g^{-1})	CV (%)	Accuracy (RE, %)
Intra-day[a] —				
CAR	15	14.01 ± 1.02	7.28	−6.60
	150	145.65 ± 4.89	3.36	−2.91
	750	733.50 ± 19.29	2.63	−2.20
ANS	15	14.58 ± 2.04	13.99	−2.80
	150	149.34 ± 14.88	9.96	−0.44
	750	754.44 ± 14.10	1.87	+0.59
HCAR	15	14.61 ± 1.29	8.83	−2.60
	150	146.70 ± 11.04	7.52	−2.20
	750	757.74 ± 17.34	2.29	+1.03
Inter-day[b] —				
CAR	15	14.25 ± 2.49	17.47	−5.08
	150	147.24 ± 7.50	5.09	−1.84
	750	745.77 ± 24.03	3.22	−0.56
ANS	15	15.27 ± 2.58	16.89	+1.80
	150	145.38 ± 9.75	6.71	−3.08
	750	746.52 ± 13.68	1.83	−0.46
HCAR	15	16.59 ± 2.01	12.12	+10.08
	150	142.23 ± 9.84	6.92	−5.18
	750	746.91 ± 19.65	2.63	−0.41

[a] Four replicates at each concentration.

[b] Three runs, four replicates at each concentration over three days ($n = 12$).

and accuracy ranged from −6.60 to +1.03% of nominal concentrations; the inter-day CV values were <17.5% and accuracy was in the range −5.18 to +10.08%. Recoveries at the two extremes of the calibration ranges were satisfactory, ranging from 96.2 to 101.3% for all the compounds (data not shown).

The stability of CAR, ANS and HCAR in aqueous solutions and tissue extracts at room and higher temperatures (up to 105 °C) has already been reported.[23] We therefore checked the stability of the analytes in QC samples and in the spiked tissue extracts only under the experimental conditions used. The stability was guaranteed for at least 24 h at 4 °C and no significant differences (*t*-test) were found between freshly prepared homogenates and homogenates prepared from tissues stored in liquid N_2 (data not shown).

As shown in Table 3, precision and accuracy were satisfactory in spiked deproteinized samples of all the target tissues containing significant endogenous amounts of the analytes. These results indicate that there are no significant differences in the ionization efficiency between lung and the tissue extracts considered, so calibration curves prepared by spiking lung homogenate can be used successfully to quantitate the analytes in the other target tissues. Taking into account the dilution factor (1 : 10) for skeletal muscle homogenate in comparison with lung homogenate, the method allows quantitation up to 10 000 nmol g^{-1}.

Profile of histidine-containing dipeptides in rat tissues

The method was then applied to profile the dipeptide content in rat tissues (Fig. 6). Table 4 summarizes these results: as reasonably expected, owing to the presence of specific hydrolytic enzymes such as carnosinase,[30] plasma, liver and kidney do not contain histidine-containing dipeptides, which were also below the LOD in lung. There were large amounts of CAR and ANS in all the skeletal muscles, in agreement with previous findings in the rat[31] and other animal species (horse, camel, cattle).[18,24] It is worth noting that both of these dipeptides, but not HCAR, are present in rat myocardium at significant levels (~50 nmol g^{-1}). This finding, not reported before, is extremely important in the light of the well-documented anti-ischemic activity of carnosine, demonstrated in the isolated rat heart and *in vivo*.[32–33] In addition, this is the first study, to our knowledge, that unequivocally demonstrates the presence of HCAR in brain and cerebellum by an MS approach, since up to now it has only been detected by HPLC with UV detection[34] and by an immunochemical approach.[31] Immunochemical and radioimmunoassay techniques, because of their high sensitivity, are widely employed for detection and quantitation of peptides in biological matrices; anyway, as pointed out by Desiderio[35,36] in two papers reviewing the analysis of peptides in tissue extracts by different methodological approaches, the MS/MS technique furnishes the highest level of molecular specificity.

CONCLUSIONS

The proposed LC/MS/MS method, never before applied to detect histidine-containing dipeptides in biological matrices, requires minimal sample manipulation, including a very

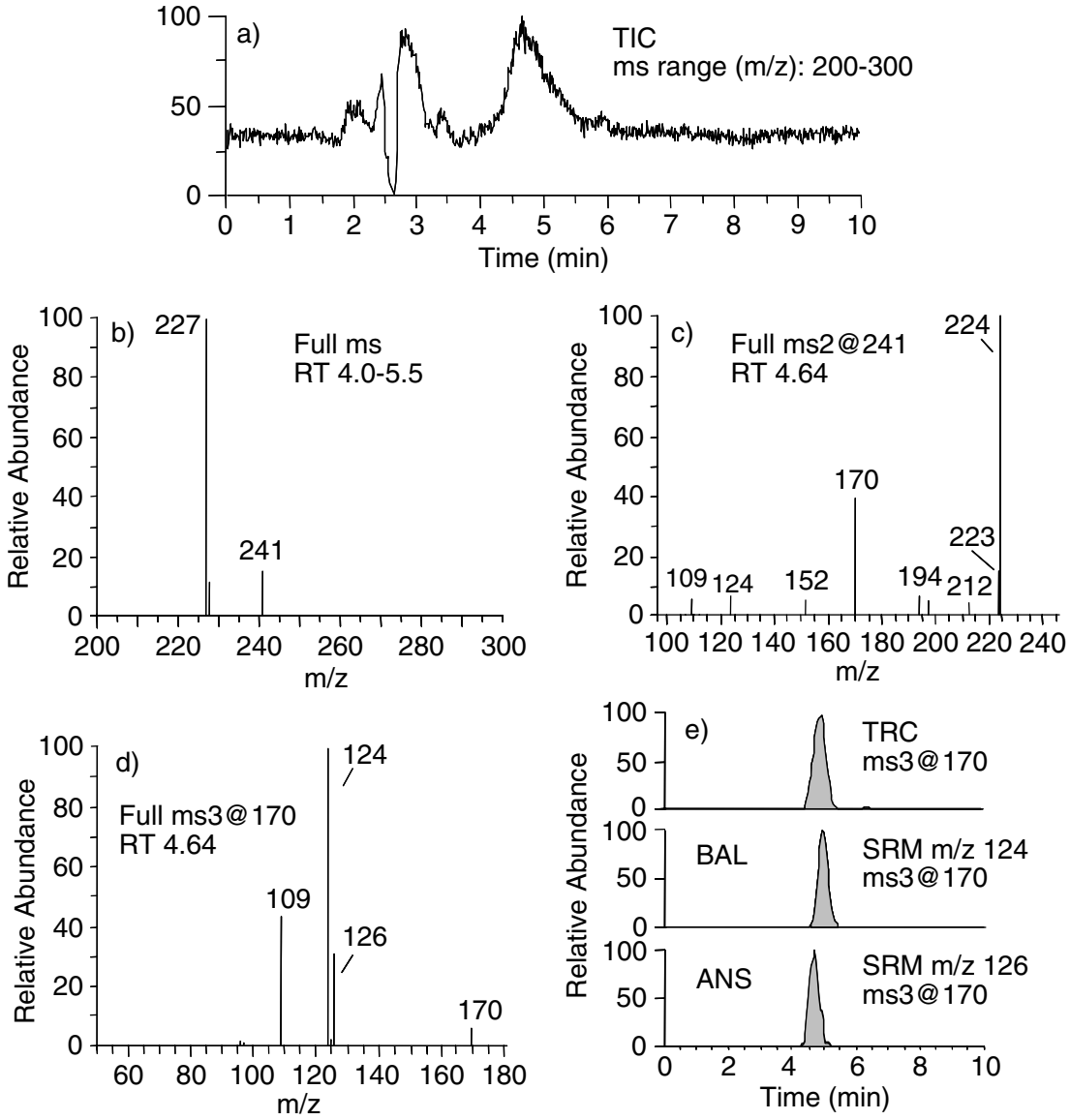


Figure 5. LC/MS^n analysis (positive-ion mode) of ham. (a) TIC; (b) full mass spectrum of the peak with retention time 4.0–5.5 min; (c) full MS^2 (parent ion *m/z* 241) and (d) MS^3 (parent ion *m/z* 170) spectra; (e) TRC of the ion at *m/z* 170 (upper panel) and SRM traces at *m/z* 124 (BAL) and *m/z* 126 (ANS).

simple deproteinization step without further purification. The method is fast (total analysis time 30 min, including tissue homogenization, sample preparation and LC/MS analysis) and sufficiently reliable, taking into account that ion trap mass spectrometry is not the technique of choice for quantitative analysis, providing less satisfactory reproducibility (although always within the accepted criteria in our case) than a three-stage quadrupolar system.

From a biological point of view, an analytical method specifically intended to profile the levels of histidine-containing dipeptides in the rat should help us to understand better their biochemical role in different physio-pathological conditions and to set up appropriate animal experiments, to demonstrate their carbonyl quenching ability *in vivo*. In this context, it is important that for the first time homocarnosine (a potential neuroprotective agent and neurotransmitter in human brain) was unequivocally detected and quantitated in rat brain.

Another important field of application of this simple approach would be the analysis of products of animal origin in feeds (meat meal, meat bone meal, fish meal), commonly used as valuable protein sources. In addition, the analysis of histidine-containing dipeptides has been proposed as an official method to detect products of animal origin in feed for ruminants, forbidden in many jurisdictions because of the increased risk of bovine spongiform encephalopathy (BSE). To be successful for regulatory control of fraudulent/illegal use, analytical methods must give unequivocal structure identification and be sensitive and specific enough to allow the simultaneous detection of traces of dipeptides in different matrices. The LC/MS/MS approach described seems to fulfil all these requirements.

Finally, the method would also be helpful in experimental animals and humans, to correlate the tissue content of CAR, ANS and HCAR with physiological (aging) and pathological

Table 3. Accuracy and precision for CAR, ANS and HCAR in spiked tissue extracts

Target tissue	Nominal concentration (nmol g^{-1})	Analyte	Mean measured concentration[a] ±SD (nmol g^{-1})	CV (%)	Accuracy (RE, %)
Heart	15	CAR	14.12 ± 2.13	15.08	−5.87
		ANS	15.23 ± 1.98	13.00	+1.53
		HCAR	14.56 ± 1.87	12.84	−2.93
	750	CAR	744.05 ± 15.78	2.12	−0.79
		ANS	754.92 ± 6.81	0.90	+0.66
		HCAR	743.96 ± 8.32	1.12	−0.80
Brain	15	CAR	15.27 ± 1.77	11.59	+1.80
		ANS	14.65 ± 1.12	7.64	−2.33
		HCAR	14.34 ± 1.35	9.41	−4.40
	750	CAR	749.10 ± 16.12	2.15	−0.12
		ANS	760.72 ± 8.23	1.08	+1.43
		HCAR	758.72 ± 4.25	0.56	+1.16
Cerebellum	15	CAR	15.22 ± 1.23	8.08	+1.47
		ANS	14.56 ± 1.13	7.76	−2.93
		HCAR	14.61 ± 1.19	8.14	−2.60
	750	CAR	746.97 ± 2.81	0.38	−0.40
		ANS	758.72 ± 4.25	0.56	+1.16
		HCAR	761.00 ± 5.98	0.79	+1.47
Gastrocnemius skeletal muscle	1500	CAR	1483.10 ± 37.88	2.55	−1.13
		ANS	1503.20 ± 29.64	1.97	+0.21
		HCAR	1514.84 ± 15.76	1.04	+0.99
Tibialis skeletal muscle	1500	CAR	1526.44 ± 16.38	1.07	+1.76
		ANS	1512.44 ± 18.10	1.20	+0.83
		HCAR	1493.00 ± 20.08	1.34	−0.47
Soleus skeletal muscle	1500	CAR	1487.10 ± 12.68	2.20	−0.86
		ANS	1517.34 ± 17.18	1.13	+1.16
		HCAR	1498.20 ± 35.28	2.35	−0.12

[a] Four replicates at each concentration.

Table 4. Profile of histidine-containing dipeptides in rat tissues[a]

Tissue	CAR	ANS	HCAR
Brain	27.35 ± 7.56	n.d.[b]	51.14 ± 6.10
Cerebellum	48.41 ± 4.66	57.48 ± 10.92	77.64 ± 11.04
Kidney	n.d.	n.d.	n.d.
Liver	n.d.	n.d.	n.d.
Lung	n.d.	n.d.	n.d.
Myocardium	67.03 ± 3.45	64.50 ± 9.86	n.d.
Plasma	n.d.	n.d.	n.d.
Gastrocnemius skeletal muscle	5279.95 ± 1006.21	7861.13 ± 2599.12	n.d.
Tibialis skeletal muscle	4200.86 ± 1093.41	7419.34 ± 701.17	n.d.
Soleus skeletal muscle	2606.23 ± 487.08	5414.51 ± 1367.06	n.d.

[a] Values are mean ±SD and are expressed as nmol g^{-1} wet mass tissue.
[b] n.d., Below the LOD.

conditions (Alzheimer, atherosclerosis) involving a carbonyl-mediated degenerative process.

Acknowledgement

Financial support from MIUR (Cofinanziamento Programma Nazionale 2004) is gratefully acknowledged.

REFERENCES

1. Seewell DA, Harris RC, Marlin DJ, Dunnett M. Estimation of the carnosine content of different fiber types in the middle gluteal muscle of the thoroughbred horse. *J. Physiol.* 1992; **455**: 447.
2. Kohen R, Yamamoto Y, Cundy KC, Ames BN. Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. *Proc. Natl. Acad. Sci. USA* 1988; **85**: 3175.

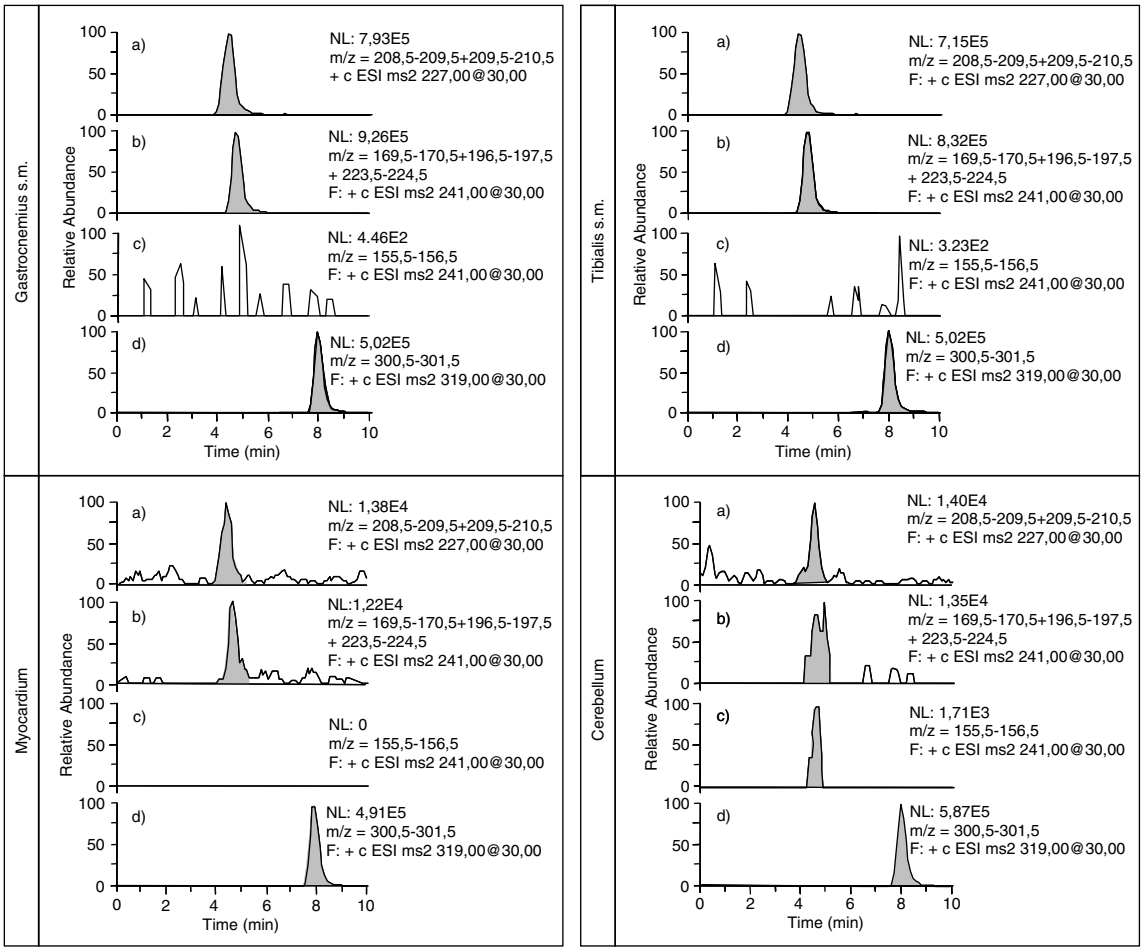


Figure 6. LC/MS^2 analysis (positive-ion mode) of rat tissues. (a) CAR, SRC of the ions at *m/z* 209 + 210 (parent ion *m/z* 227); (b) ANS, SRC of the ions at *m/z* 224 + 197 + 170 (parent ion *m/z* 241); (c) HCAR, SRC of the ion at *m/z* 156 (parent ion *m/z* 241); (d) IS, SRC of the ion at *m/z* 301 (parent ion *m/z* 319).

3. Chan KM, Decker EA. Endogenous skeletal muscle antioxidants. *Crit. Rev. Food Sci. Nutr.* 1994; **34**: 403.
4. Gopalakrishnan J, Decker EA, Means WJ. Antioxidant activity of mechanically separated pork extracts. *Meat Sci.* 1999; **52**: 101.
5. Flankbaum L, Fitzpatrik JC, Brotman DN, Marcoux AM, Kasziba E, Fisher H. The presence and significance of carnosine and histamine-containing tissues of several mammalian species. *Agents Actions* 1990; **31**: 190.
6. Petroff OAC, Hyder F, Rothman DL, Mattson RH. Homocarnosine and seizure control in juvenile myoclonic epilepsy and complex cortical seizures. *Neurology* 2001; **56**: 709.
7. Zhou S, Decker AA. Ability of carnosine and other skeletal muscle components to quench unsaturated aldehydic lipid peroxidation products. *J. Agric. Food Chem.* 1999; **47**: 51.
8. Hipkiss AR, Preston JE, Himsworth DT, Worthington VC, Keown M, Michaelis J, Lawrence J, Mateen A, Allende L, Eagles PA, Abbott NJ. Pluripotent protective effects of carnosine, a naturally occurring dipeptide. *Ann. N. Y. Acad. Sci.* 1998; **854**: 37.
9. Hipkiss AR. Carnosine and protein carbonyl groups: a possible relationship. *Biochemistry (Mosc.)* 2000; **65**: 771.
10. Aldini G, Granata P, Carini M. Detoxification of cytotoxic α,β-unsaturated aldehydes by carnosine: characterization of conjugated adducts by electrospray ionization tandem mass spectrometry and detection by liquid chromatography/mass spectrometry in rat skeletal muscle. *J. Mass Spectrom.* 2002; **37**: 1219.
11. Aldini G, Carini M, Beretta G, Bradamante S, Facino RM. Carnosine is a quencher of 4-hydroxy-nonenal: through what mechanism of reaction? *Biochem. Biophys. Res. Commun.* 2002; **298**: 699.
12. Carini M, Aldini G, Beretta G, Arlandini E, Maffei Facino R. Acrolein-sequestering ability of endogenous dipeptides: characterization of carnosine and homocarnosine/acrolein adducts by electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* 2003; **38**: 996.
13. Yokoyama Y, Horikoshi S, Takahashi T, Sato H. Low-capacity cation-exchange chromatography of ultraviolet-absorbing urinary basic metabolites using a reverse-phase column coated with hexadecylsulfonate. *J. Chromatogr. A* 2000; **886**: 297.
14. Dunnett M, Harris RC. Determination of carnosine and other biogenic imidazoles in equine plasma by isocratic reverse-phase high-performance liquid chromatography. *J. Chromatogr. A* 1992; **579**: 45.
15. Widemn J, Brink L, Stein S. New automated fluorimetric peptide micro-assay for carnosine in mouse olfactory bulb. *Anal. Biochem.* 1998; **86**: 670.
16. Kasziba E. Simultaneous determination of histidine-containing dipeptides, histamine, methylhistamine and histidine by high-performance liquid chromatography. *J. Chromatogr. A* 1998; **432**: 315.
17. Teahon K, Rideout JM. A sensitive and specific high-performance liquid chromatographic assay for imidazole dipeptides and 3-methylhistidine in human muscle biopsies, serum and urine. *Biomed. Chromatogr.* 1992; **6**: 16.
18. Dunnett M, Harris R. High-performance liquid chromatographic determination of imidazole dipeptides histidine, 1-methylhistidine and 3-methylhistidine in equine and camel

muscle and individual muscle fibers. *J. Chromatogr. B* 1997; **688**: 47.
19. Carnegie PR, Ilic MZ, Etheridge MO, Collins MG. Improved high-performance liquid chromatographic method for analysis of histidine dipeptides anserine, carnosine and balenine present in fresh meat. *J. Chromatogr.* 1983; **261**: 153.
20. Plowman JE, Close EA. An evaluation of a method to differentiate the species origin of meats on the basis of contents of anserine, balenine and carnosine in skeletal muscles. *J. Sci. Food Agric.* 1988; **45**: 69.
21. Carnegie PR, Hee KP, Bell AW. Ophidine (β-alanyl-L−3-methylhistidine, ''Balenine'') and other histidine dipeptides in pig muscles and tinned hams. *J. Sci. Food Agric.* 1982; **33**: 795.
22. Schönner J. Analysis of products of animal origin in feeds by determination of carnosine and related dipeptides by high-performance liquid chromatography. *J. Agric. Food Chem.* 2002; **50**: 1945.
23. Aristoy MC, Soler C, Toldrá F. A simple, fast and reliable methodology for the analysis of histidine dipeptides as markers of the presence of animal origin proteins in feeds for ruminants. *Food Chem.* 2004; **84**: 485.
24. Aristoy MC, Toldrà F. Histidine dipeptides HPLC-based test for the detection of mammalian origin proteins in feeds for ruminants. *Meat Sci.* 2004; **67**: 211.
25. Pearson JD, McCroskey MC. Perfluorinated acid alternatives to trifluoroacetic acid for reversed-phase high-performance liquid chromatography. *J. Chromatogr A* 1996; **746**: 277.
26. Naim JO, Desiderio DM, Trimble J, Hinshaw JR. The identification of serum tuftsin by reverse-phase high-performance liquid chromatography and mass spectrometry. *Anal. Biochem.* 1987; **164**: 221.
27. Petritis KN, Chaimbault P, Elfakir C, Dreux M. Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent. *J. Chromatogr. A* 1999; **833**: 147.
28. Petritis K, Brussaux S, Guenu S, Elfakire C, Dreux M. Ion-pair reversed-phase liquid chromatography–electrospray mass spectrometry for the analysis of underivatized small peptides. *J. Chromatogr A* 2002; **957**: 173.
29. Turecek F, Kerwin JL, Xu R, Kramer KJ. Distinction of *N*-substituted histidines by electrospray ionization mass spectrometry. *J. Mass Spectrom.* 1998; **33**: 392.
30. Teufel M, Saudek V, Ledig JP, Bernhardt A, Boularand S, Carreau A, Cairns NJ, Carter C, Cowley DJ, Duverger D, Ganzhorn AJ, Guenet C, Heintzelmann B, Laucher V, Sauvage C, Smirnova T. Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J. Biol. Chem.* 2003; **278**: 6521.
31. Jackson MC, Lenney JF. The distribution of carnosine and related dipeptides in rat and human tissues. *Inflamm. Res.* 1996; **45**: 132.
32. Lee JW, Miyawaki H, Bobst EV, Hester JD, Ashraf M, Bobst AM. Improved functional recovery of ischemic rat hearts due to singlet oxygen scavengers histidine and carnosine. *J. Mol. Cell Cardiol.* 1999; **31**: 113.
33. Stvolinsky SL, Dobrota D. Anti-ischemic activity of carnosine. *Biochemistry (Mosc)* 2000; **65**: 849.
34. O'Dowd JJ, Cairns MT, Trainor M, Robins DJ, Miller DJ. Analysis of carnosine, homocarnosine, and other histidyl derivatives in rat brain. *J. Neurochem.* 1990; **55**: 446.
35. Desiderio DM. Analysis of endogenous neuropeptides by reversed-phase high-performance chromatography and mass spectrometry. *Anal. Chim. Acta* 1996; **352**: 85.
36. Desiderio DM. Mass spectrometry, high performance liquid chromatography and brain peptides. *Biopolymers* 1997; **40**: 257.