JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Hydrophilic Chromatographic Determination of Carnosine, Anserine, Balenine, Creatine, and Creatinine

LETICIA MORA, MIGUEL ANGEL SENTANDREU,* AND FIDEL TOLDRÁ

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), P.O. Box 73, 46100 Burjassot, Valencia, Spain

A new HPLC procedure based on hydrophilic interaction chromatography (HILIC) has been developed for the simultaneous determination of carnosine, anserine, balenine, creatine, and creatinine in meat. This is the first time that HILIC has been directly applied to the study of meat components, having the advantage of not requiring complex cleanup and/or sample derivatization procedures. The chromatographic separation has been developed using a silica column (4.6 × 150 mm, 3 μm), and the proposed methodology is simple, reliable, and fast (<13 min per sample). The method has been validated in terms of linearity, repeatability, reproducibility, and recovery and represents an interesting alternative to methods currently in use for determining the mentioned compounds and other polar substances. The detection limits are 5.64, 8.23, 3.66, 3.99, and 0.06 μg/mL for carnosine, anserine, balenine, creatine, and creatinine, respectively.

KEYWORDS: Hydrophilic interaction chromatography; HILIC; meat; histidine dipeptides; carnosine; anserine; balenine; creatine; creatinine; peptides; polar compounds

INTRODUCTION

The imidazole dipeptide carnosine (CAR) and its methylated analogues anserine (ANS) and balenine (BAL) are histidine-containing dipeptides (**Figure 1**) widely distributed in vertebrate animal tissues, especially in skeletal muscle, the heart, and the central nervous system (*1*).

Creatine (Cr) (**Figure 1**) and its phosphorylated derivative phosphocreatine (PCr) are key components of the energy delivery process in several tissues, particularly those characterized by a high and/or fluctuating energy demand (*2*).

On the other hand, creatine turns into creatinine (Cn) (**Figure 1**) in muscle due to a nonenzymatic conversion by the removal of water and the formation of a ring structure. This nonenzymatic conversion takes place easily under heating conditions (*3*).

In view of the growing interest and the biological importance of biochemical compounds such as carnosine, anserine, balenine, creatine, and creatinine, attention has been focused on the development of analytical techniques to separate, characterize, and quantify these compounds.

Techniques such as thin-layer chromatography and colorimetry have been used in peptide separations for many years, but such procedures require long analysis times and have poor resolution and low sensitivity. However, the versatility, short analysis time, and high resolution of high-performance liquid chromatography (HPLC) have made this technique the method of choice for the analysis of histidine dipeptides. In fact, several HPLC methods have been described for the analysis of these compounds in tissues of different mammalian species. These methods include ion-exchange chromatography (IEC) applied to both underivatized (*4*) and postcolumn-derivatized peptides (*5*, *6*) with UV, fluorescence, or pulsed amperometric detection. Reverse-phase liquid chromatography (RP-HPLC) has been also employed for the analysis of histidine dipeptides in their precolumn derivatized forms (*8*).

Creatine and creatinine are commonly determined by using the Folin method, based on the Jaffé reaction, which is the basis of some official methods (*9*). The reaction of creatine with 1-naphthol and biacetyl (*10*), both through flow injection methodologies, has been used for the simultaneous determination of creatine and creatinine in meat and meat products (*11*, *12*). Alternative techniques include ion-pairing applied to reverse-phase chromatography (*7*, *13*), a capillary isotachophoretic method (*14*), and a spectrophotometric assay using enzyme-based systems (*15*).

RP-HPLC has been a very useful technique for the isolation of peptide compounds, having as its main advantage, apart from its powerful resolving capacity, the use of volatile mobile phases that avoid the need for sample desalting. Although RP-HPLC is a powerful separation technique, there is a major limitation consisting of the low retention of polar molecules. In this case, normal-phase liquid chromatography (NPLC) has been traditionally used to separate polar compounds with nonaqueous mobile phases, but an important limitation is the difficulty in dissolving hydrophilic compounds, such as peptides, in these phases. Moreover, this methodology is not environmentally friendly. For these reasons, the application of NPLC to biological

* Corresponding author (telephone +343900022, ext. 2113; fax +343636301; e-mail ciesen@iata.csic.es).

10.1021/jf0703809 CCC: $37.00
Published on Web 05/17/2007

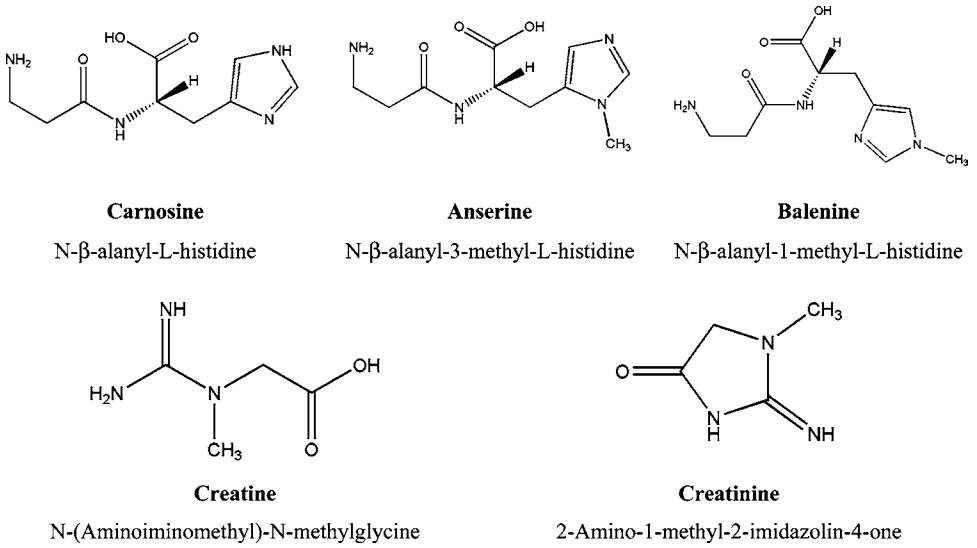

Figure 1. Chemical structures of carnosine, its methylated analogues anserine and balenine, creatine, and creatinine, the end product of creatine catabolism.

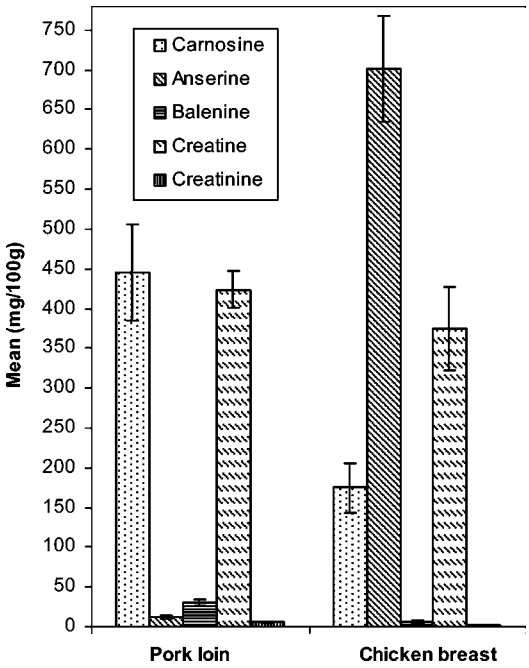

Figure 2. Levels of carnosine, anserine, balenine, creatine, and creatinine in pork loin and chicken breast determined by simultaneous separation using HILIC. Each value represents the mean of eight samples from different meat cuts, and labeled error bars indicate the standard deviation for each mesurement.

samples is limited and, thus, rarely used for separating peptide mixtures (*16*).

Hydrophilic interaction chromatography (HILIC) constitutes an interesting alternative to reverse-phase, ion-exchange, and normal-phase liquid chromatography for the separation of such polar compounds for many reasons: (i) it implies the use of volatile buffers, avoiding a desalting step; (ii) it has clear advantages with regard to the solubility of biological compounds such as peptides; and (iii) there is no need of compound derivatization. These advantages facilitate an easier sample processing and compatibility with further mass spectrometry analysis, where a low amount of volatile salt is allowed.

In HILIC, an initial mobile phase with a high content of an organic solvent is used to promote hydrophilic interactions between the analyte and the polar hydrophilic stationary phase. In this context, silica and amino columns with organic solvents compatible with aqueous mobile phases offer potential use in the HILIC mode (*17*). This chromatographic mode is similar to normal-phase chromatography because polar compounds are retained longer than the nonpolar ones and the polar component of the mobile phase (usually water) is the strong solvent used to elute the compounds. However, with regard to the solubility of analytes in the mobile phase and matrix compatibility, HILIC is superior to normal-phase chromatography, as the nature of the mobile phases that are used is comparable to reverse-phase separations (*16*).

The objective of the present study was to develop a new method based on HILIC for the analysis of small and highly hydrophilic relevant biochemical compounds such as carnosine, anserine, balenine, creatine, and creatinine present in meat. The results of this study demonstrate that a novel procedure based on hydrophilic chromatography directly applied to a meat extract yields a simultaneous isolation and quantification of the aforementioned compounds.

MATERIALS AND METHODS

Chemicals and Reagents. All chemical and chromatographic reagents used were of HPLC grade. Acetonitrile (ACN), ammonium acetate, glacial acetic acid, and hydrochloric acid 37% were purchased from Scharlau (Barcelona, Spain). Formic acid 50%, ammonium formate for mass spectrometry, trifluoroacetic acid, and standards of carnosine and L-anserine were from Sigma (St. Louis, MO). Creatine and creatinine were purchased from Fluka Chemie AG (Buchs, Switzerland).

Materials and Instrumentation. Pork loin and chicken breast were used to develop the method. These tissues were chosen because of the differences existing between them for the analyzed compounds (**Figure 2**). As has been demonstrated, anserine is predominant in non-mammalian species such as chicken, whereas pork is one of the species with a higher balenine content (*18*, *19*).

Chromatography was performed in a HPLC Agilent 1100 series system (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump (G1311A), an autosampler (G1313A), and a vacuum degasser (G1379A). Ultraviolet detection was made by a G1315B diode array detector, which was used in the range of 195−300 nm.

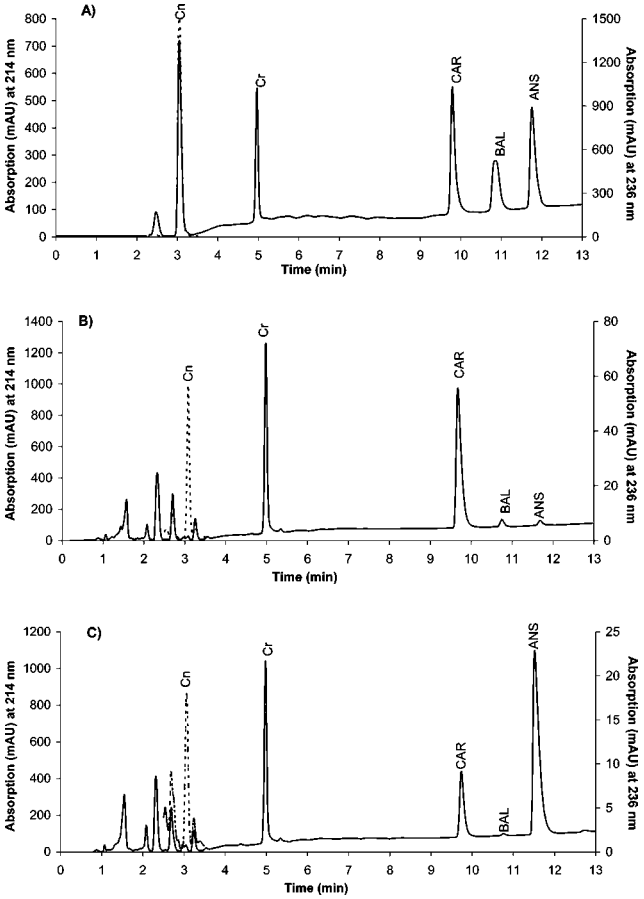

Figure 3. (**A**) Chromatogram corresponding to the separation of the standards of the five compounds determined with the HILIC method developed in this work. (**B**) Chromatogram corresponding to a pork loin sample. (**C**) Chromatogram corresponding to a chicken breast sample. In all cases carnosine (CAR), anserine (ANS), balenine (BAL), and creatine (Cr) were detected at 214 nm (solid line), whereas creatinine (Cn) was detected at 236 nm (dotted line).

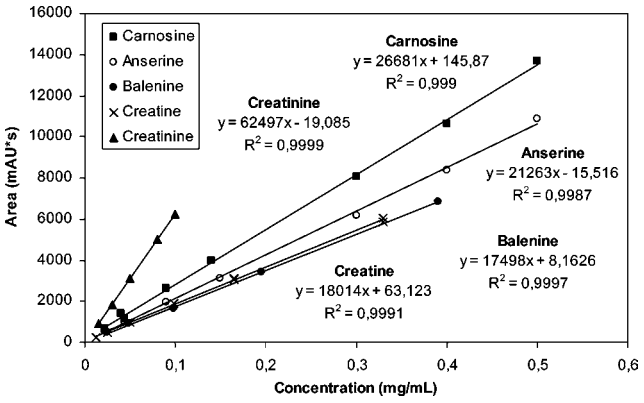

Figure 4. Linearity range and regression of carnosine, anserine, balenine, creatine, and creatinine.

Sample and Standards Preparation. Samples were extracted and deproteinized following the method described by Aristoy and Toldrá (*20*). Briefly, 5 g of sample tissue was homogenized with 3 volumes of 0.01 N HCl in a stomacher (Seward Laboratory) for 8 min and further centrifuged in the cold (4 °C) at 10000 rpm for 20 min. Supernatant was filtered through glass wool, and 250 μL of this solution was deproteinized by adding 3 volumes of ACN, standing at 4 °C for 20 min. Finally, the sample was centrifuged (10000 rpm) for 10 min at 4 °C and the supernatant directly analyzed. The rest of the meat extract was stored under vacuum at −20 °C until use.

The calibration ranges for the assayed compounds were established using a duplicate set of standards. Carnosine, anserine, and creatinine calibration standards were prepared by diluting a stock solution of 1 mg/mL. As balenine is not commercially available, pork meat was used as the source of this compound because of its high content. This standard solution was prepared according to the method of Aristoy et al. (*5*) and further quantified, obtaining a mean value of 0.83 mg/mL. A standard solution of creatine at 0.3 mg/mL was prepared because of the low solubility of this compound in the injection solution [0.01 N HCl in water/acetonitrile (25:75, v/v)]. Nevertheless, this concentration limit was enough to determine creatine levels normally found in muscle tissue. Working standard solutions were prepared over the appropriate concentration range by dilution of stock solutions. All stock solutions were stored at −20 °C until use.

Stability of the Compounds under Frozen Storage. The initial quantification was done immediately after extraction and deproteinization. The remaining pork loin extracts were kept under frozen storage (−20 °C) and further analyzed after 2, 3, and 8 days. Each analysis was done in triplicate.

Statistical Method. The ANOVA procedure was used to determine significant differences between initial and 2, 3, and 8 day quantifications using the software Statgraphics Plus (v 5.1). Each statistical analysis was $n = 3$, and the normality of the data was tested before application to the ANOVA procedure.

Description of the Chromatographic Method. Carnosine, anserine, balenine, creatine, and creatinine were analyzed by HILIC. Twenty microliters of each sample was injected into the HPLC system. The chromatographic separation was developed using an Atlantis HILIC silica column (4.6 × 150 mm, 3 μm) from Waters (Milford, MA) at room temperature. Mobile phases consisted of solvent A, containing 0.65 mM ammonium acetate, pH 5.5, in water/acetonitrile (25:75), and solvent B, containing 4.55 mM ammonium acetate, pH 5.5, in water/acetonitrile (70:30). The solvents were filtered through a 0.22 μm membrane filter and degassed prior to the analytical run. The separation conditions were a linear gradient from 0 to 100% of solvent B in 13 min at a flow rate of 1.4 mL/min. The separation was monitored using a diode array detector at a wavelength of 214 nm for creatine, carnosine, anserine, and balenine, whereas a wavelength of 236 nm was used for creatinine detection. Peak areas were correlated to compound concentration by interpolation in the corresponding calibration curve (**Figure 4**). The column was equilibrated for 5 min under the initial conditions before each injection.

RESULTS AND DISCUSSION

Development of the Chromatographic Method. To establish optimal conditions for the separation of carnosine, anserine, balenine, creatine, and creatinine by hydrophilic chromatography, the effects of ion-pairing, pH, and ACN gradient were examined.

Ion-Pairing and pH Selection. The use of a buffered mobile phase is crucial to achieving an acceptable repeatability in the chromatographic separation of charged compounds, because electrostatic interactions between the analyte and the stationary phase are controlled by the buffer nature. Therefore, an ion-pairing reagent is necessary because silanol groups, present with functionalized silica-based materials, can interact with ionic residues of compounds and then affect recovery and cause peak-tailing. For this reason, trifluoroacetic acid (TFA), ammonium formate, ammonium acetate, acetic acid, and formic acid were investigated to determine the appropriate reagent to eliminate ionic interactions.

TFA has been widely used as an ion-pairing reagent because of its volatility, transparency at 214 nm, and effectiveness for preventing ionic interactions. This acid not only acts as an ion-pair agent to improve peak shapes of basic compounds on silica-based column but also controls the pH of the mobile phases. In

fact, a good separation of standards of carnosine, anserine, balenine, creatine, and creatinine was achieved using this reagent, but a decrease of the selectivity was observed when these compounds were separated from a meat extract, possibly due to matrix effects. Two alternative volatile salts, ammonium formate and ammonium acetate, were evaluated in the solvent system. However, both of them are less volatile than TFA and both also absorb at 214 nm. To minimize absorption effects, lower concentrations of salt were tested (<6 mM). In the case of ammonium formate, variable retention times owing to an insufficient buffering effect of the mobile phase occurred at these low concentrations. Finally, a mobile phase containing ammonium acetate was found to provide the best results in selectivity and reproducibility and, thus, this salt was selected for the development of the method. Acetic acid and formic acid were also tested at a concentration of 0.1%, but this did not improve results obtained with ammonium acetate (results not shown).

In addition to the type of salt, the effect of salt concentration on the separation profile was also investigated by varying ammonium acetate concentrations along with the chromatographic gradient. In this way, HILIC retention was inversely proportional to the concentration of mobile phase, because increases in salt concentration resulted in lower hydrophilicity. A final gradient from 0.65 to 4.55 mM ammonium acetate was selected, providing an optimum analysis time of 13 min.

The pH value of the mobile phase has a significant impact on retention and selectivity in hydrophilic chromatography by influencing solute ionization. Low-pH conditions charge only basic and phosphorylated groups of peptides and amino acids. On the other hand, at higher pH values, both acid and basic residues are charged (*21*, *22*). Ideal pH conditions in HILIC separation were investigated by changing the pH of the mobile phase containing ammonium acetate. This pH was adjusted to 4.5, 5.0, and 5.5, using this factor to manipulate selectivity. In all cases, pH 5.0 offered the lowest retention times for carnosine, anserine, and balenine while keeping a good separation of these compounds. However, a pH of 5.5 was finally selected because it provided also the best selectivity for creatine and creatinine, which are eluted during the first 5 min of the chromatogram (results not shown).

Optimization of the Separation. In HILIC, peptide retention times increase with increasing the polarity of both peptides and the stationary phase, and with decreasing the aqueous content in the mobile phase (*16*). For these reasons, in this study, the effect of ACN concentration on peptide retention was also examined. The percentage of ACN was varied while keeping a gradient of ammonium acetate concentration from 0.065 to 4.5 mM, which was necessary to accommodate the low solubility of this salt in the high ACN content of the initial mobile phase.

The injection solvent is also an important parameter in HILIC because, as in reverse-phase chromatography, the injection solvent can strongly influence the peak shape of the analytes. The injection solvent should be as close as possible to the initial mobile phase, which must be adequate for solubilization of the compounds to analyze. In our case, this was achieved at an initial concentration of 75% acetonitrile/25% water solution.

Absorption at 214 nm was adequate for the determination of carnosine, anserine, balenine, and creatine, but meat extracts showed interferences in the detection of creatinine at this wavelength. To avoid this, creatinine was detected at 236 nm, where this compound has another maximum of absorption (**Figure 3B**,**C**).

Table 1. Repeatability ($n = 6$) for the Analysis of Carnosine, Anserine, Balenine, Creatine, and Creatinine in Pork Loin and Chicken Breast

	mean[a] (mg/100 g)	SD[b]	CV[c] (%)
	Pork Loin		
carnosine	462.33	16.86	3.65
anserine	10.76	1.23	11.44
balenine	33.40	1.88	5.62
creatine	427.32	12.66	2.96
creatinine	5.71	0.34	5.87
	Chicken Breast		
carnosine	149.89	6.05	4.04
anserine	677.49	28.61	4.22
balenine	5.41	0.67	12.31
creatine	336.24	12.38	3.68
creatinine	1.78	0.13	7.31

[a] Means expressed as mg/100 g of muscle. Each value represents the mean of six samples from the same meat cut. [b] Standard deviation. [c] Coefficient of variation.

Table 2. Reproducibility ($n = 8$) Obtained for the Analysis of Carnosine, Anserine, Balenine, Creatine, and Creatinine in Pork Loin and Chicken Breast

	mean[a] (mg/100 g)	SD[b]	CV[c] (%)
	Pork Loin		
carnosine	398.59	22.77	5.71
anserine	13.54	1.95	14.41
balenine	28.49	2.23	7.83
creatine	393.83	10.00	2.54
creatinine	4.63	0.15	3.18
	Chicken Breast		
carnosine	149.27	7.06	4.73
anserine	675.59	33.32	4.93
balenine	5.50	0.61	11.04
creatine	335.73	14.58	4.34
creatinine	1.76	0.14	7.94

[a] Means expressed as mg/100 g of muscle. Each value represents the mean of eight samples from the same meat cut. [b] Standard deviation. [c] Coefficient of variation.

The final chromatographic conditions (see Stability of the Compounds under Frozen Storage) resulted in a good separation of the five compounds, as can be observed in **Figure 3A**. Using these conditions, creatine and creatinine eluted at 4.4 and 3.1 min, respectively, whereas carnosine, balenine, and anserine eluted at 9.9, 10.9, and 11.9 min, respectively.

Method Evaluation. (a) Linearity and Calibration. Plots and respective regressions of carnosine, anserine, balenine, creatine, and creatinine are shown in **Figure 4**. As can be observed, the linearity of the method for all compounds was as good as results obtained with other methods (*5*, *8*, *23*). However, this method has the advantage that no initial cleanup and no derivatization steps are required for sample processing.

(b) Detection Limits (LOD). The LOD for the different compounds was calculated in two different ways: First, the LOD was established by analyzing 15 blank solutions without the analyte and calculating the respective signal-to-noise ratios at the time windows where the compounds are expected. For each compound, the LOD was set as 3 times the signal-to-noise ratio. Second, increasing dilutions of pork loin and chicken breast extracts were injected, confirming in this way, the detection limits calculated previously. Finally, a comparison between the two methods was done, and similar results were obtained. LODs obtained for carnosine, anserine, balenine, creatine, and creatinine were 5.64, 8.23, 3.66, 3.99, and 0.06 μg/mL (with relative

Table 3. Recovery of Carnosine (CAR), Anserine (ANS), Balenine (BAL), Creatine (Cr), and Creatinine (Cn) Obtained in Pork Loin and Chicken Breast

	pork loin ($n = 3$)				chicken breast ($n = 3$)			
	added[a]	theoretical value[a]	recovered[a]	recovery (%)	added	theoretical value	recovered	recovery (%)
CAR	0.1	0.309	0.308	99.67	0.05	0.162	0.160	98.89
	0.2	0.409	0.395	96.71	0.15	0.262	0.252	96.05
	0.35	0.559	0.531	95.13	0.225	0.337	0.339	100.64
ANS	0.05	0.063	0.065	103.71	0.2	0.584	0.577	98.87
	0.10	0.113	0.116	102.42	0.4	0.784	0.803	102.47
	0.20	0.213	0.203	95.49	0.8	1.184	1.165	98.42
BAL	0.0005	0.0261	0.0260	99.63	0.002	0.0066	0.0061	92.54
	0.0010	0.0266	0.0231	86.93	0.00	0.0086	0.0085	98.27
	0.0020	0.0276	0.0276	100.28	0.01	0.0126	0.0118	93.40
Cr	0.10	0.293	0.293	99.96	0.10	0.315	0.318	100.97
	0.26	0.622	0.674	108.40	0.26	0.480	0.432	90.17
	0.53	0.886	0.878	99.15	0.53	0.744	0.751	100.98
Cn	0.0015	0.0066	0.0066	100.21	0.0005	0.0021	0.0022	107.31
	0.0030	0.0081	0.0088	108.71	0.0010	0.0026	0.0028	107.81
	0.0060	0.0111	0.0106	95.42	0.0020	0.0036	0.0038	105.98

[a] Values espressed as mg of analyte/mL of injection solvent. Each value corresponds to three replicates at each concentration level.

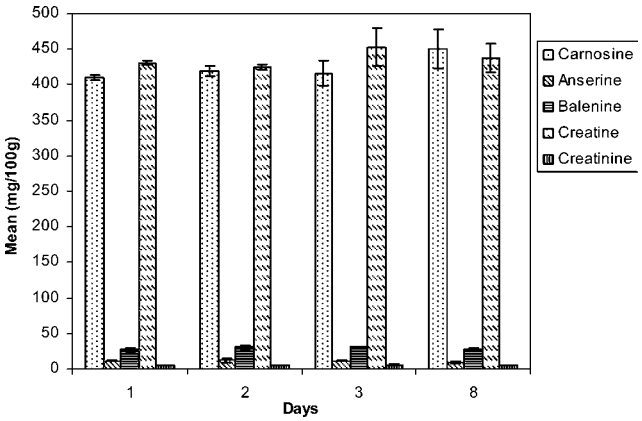

Figure 5. Stability of a pork loin sample within 8 days. Each value represents the mean of three samples from the same meat cut, and labeled error bars indicate the standard deviation for each measurement. No significant differences ($P < 0.05$) between initial and final conditions were found.

standard deviations of 17.68, 14.94, 19.98, 19.16, and 14.88, respectively).

(c) Repeatability. A repeatability study of the developed method was done. In this study, six samples from the same pork loin and six samples from the same chicken breast were processed to estimate the mean concentrations of carnosine, anserine, balenine, creatine, and creatinine, together with standard deviations and coefficients of variation (**Table 1**). The different measurements were done by the same analyst on the same instrument, on the same item, and under the same conditions. The standard deviation under repeatability conditions is often used as a measure of precision, and good results have been obtained in comparison to those reported previously using other techniques (*6*, *8*). Because of the lower mean concentration of anserine in pork loin and balenine in chicken breast, their coefficients of variation were slightly higher but always within acceptable values (**Table 1**).

(d) Reproducibility. Reproducibility of the method was tested by successive analysis of the five compounds of interest in eight samples from pork loin and eight samples from chicken breast within 30 days. Each analysis was taken on identical animal samples but under different conditions (different operators and apparatus, different times, and interday). Data, shown in **Table 2**, demonstrated a very good reproducibility from one analysis to another. Results obtained agree with those reported by many other authors when quantification of these histidine-containing dipeptides was done using other techniques (*5*, *18*, *19*, *24*) and are also comparable for those of creatine and creatinine (*3*, *15*).

(e) Recovery and Matrix Effects. Recovery was determined in five sets of pork loin and chicken breast, one set for each one of the studied compounds. Samples were enriched with standard solutions of the different compounds to yield concentrations equivalent to 0.5, 1, and 1.5 times the main value obtained in reproducibility studies. At each level, analysis were performed in duplicate, and recoveries were calculated by comparison with a blank meat sample. The percentage of recovery determined in each case is shown in **Table 3**.

In this way, the matrix effect of meat was evaluated on compound recoveries, and it was concluded that different meat matrices did not affect the quantification of the analytes, mainly because of the absence of intermediate steps in sample processing, which is a remarkable advantage with respect to previous existing methods used to quantify these compounds (*8*, *25*).

Stability of the Analyzed Compounds under Frozen Storage. The stability of the histidine dipeptides, together with creatine and creatinine, in a pork loin extract was investigated up to 8 days. A comparison between the obtained results is shown in **Figure 5**. As can be observed, when meat was stored for 8 days under freezing conditions, no significant differences ($P < 0.05$) were observed in the levels of the five compounds during this storage time.

Conclusion. HILIC constitutes a new and simple methodology for the separation of polar compounds. It requires minimal sample manipulation, thus avoiding the need for solid-phase extraction and derivatization steps. The method developed in this work for the analysis of carnosine, anserine, balenine, creatine, and creatinine is simple, fast (<13 min per sample), reliable, and easily applicable for any laboratory having conventional standard HPLC equipment. Furthermore, this method allows the use of low concentrations of salt, which avoids ionization suppression in ESI (*26*, *27*) and thus makes it suitable for further LC-MS analysis.

This is the first time that a separation using HILIC has been developed for the simultaneous determination of carnosine, anserine, balenine, creatine, and creatinine in meat samples. Adequate levels of linearity, recovery, reproducibility, and sensitivity prove that the proposed method is a good alternative for isolating and quantifying these compounds in complex matrices such as meat.

LITERATURE CITED

(1) Crush, K. G. Carnosine and related substances in animal tissues. *Comp. Biochem. Physiol.* **1970**, *34* (1), 3.

(2) Wyss, M.; Kaddurah-Daouk, R. Creatine and creatinine metabolism. *Physiol. Rev.* **2000**, *80* (3), 1107−1213.

(3) Pais, P.; Salmon, C. P.; Knize, M. G.; Felton, J. S. Formation of mutagenic/carcinogenic heterocyclic amines in dry-heated model systems, meats, and meat drippings. *J. Agric. Food Chem.* **1999**, *47*, 1098−1108.

(4) Carnegie, P. R.; Hee, K. P.; Bell, A. W. Ophidine (β-alanyl-L-3-methylhistidine, balenine) and other histidine dipeptides in pig muscles and tinned hams. *J. Sci. Food Agric.* **1982**, *33* (8), 795−801.

(5) Aristoy, M. C.; 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* (3), 485−491.

(6) Nardiello, D.; Cataldi, T. R. I. Determination of carnosine in feed and meat by high-performance anion-exchange chromatography with integrated pulsed amperometric detection. *J. Chromatogr., A* **2004**, *1035* (2), 285−289.

(7) Dunnett, M.; Harris, R. C.; Orme, C. E. Reverse-phase ion-pairing high-performance liquid-chromatography of phosphocreatine, creatine and creatinine in equine muscle. *Scand. J. Clin. Lab. Invest.* **1991**, *51* (2), 137−141.

(8) Schonherr, 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*, 1945−1950.

(9) Association of Official Analytical Chemists. Meat and meat products. In *Official Methods of Analysis of AOAC International*, 16th ed.; AOAC: Arlington, VA, 1984; Vol. II, pp 39-1−39-23.

(10) Mussini, E.; Colombo, L.; Deponte, G.; Marcucci, F. Determination of creatine in body-fluids and muscle. *J. Chromatogr.* **1984**, *305* (2), 450−455.

(11) Del Campo, G.; Irastorza, A.; Casado, J. A. Spectrophotometric simultaneous determination of creatinine and creatine by flow-injection with reagent injection. *Fresenius' J. Anal. Chem.* **1995**, *352* (6), 557−561.

(12) Dvorak, Z. Flow-injection determination of creatine in animal-tissues. *Anal. Chim. Acta* **1988**, *208* (1−2), 307−312.

(13) Tranberg, M.; Stridh, M. H.; Jilderos, B.; Weber, S. G.; Sandberg, M. Reversed-phase HPLC with UV detection for the determination of *N*-acetylaspartate and creatine. *Anal. Biochem.* **2005**, *343* (1), 179−182.

(14) Kvasnicka, F.; Voldrich, M. Isotachophoretic determination of creatinine in meat and meat products. *Electrophoresis.* **2000**, *21* (14), 2848−2850.

(15) Purchas, R. W.; Rutherfurd, S. M.; Pearce, P. D.; Vather, R.; Wilkinson, B. H. P. Concentrations in beef and lamb of taurine, carnosine, coenzyme Q(10) and creatine. *Meat Sci.* **2004**, *66* (3), 629−637.

(16) Yoshida, T. Peptide separation by hydrophilic-interaction chromatography: a review. *J. Biochem. Biophys. Methods* **2004**, *60* (3), 265−280.

(17) Alpert, A. J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic-acids and other polar compounds. *J. Chromatogr.* **1990**, *499*, 177−196.

(18) Aristoy, M. C.; Toldrá, F. Histidine dipeptides HPLC-based test for the detection of mammalian origin proteins in feeds for ruminants. *Meat Sci.* **2004**, *67* (2), 211−217.

(19) Cornet, M.; Bousset, J. Free amino acids and dipeptides in porcine muscles: differences between 'red' and 'white' muscles. *Meat Sci.* **1999**, *51* (3), 215−219.

(20) Aristoy, M. C.; Toldrá, F. Deproteinization techniques for HPLC amino acid analysis in fresh pork muscle and dry-cured ham. *J. Agric. Food Chem.* **1991**, *39* (10), 1792−1795.

(21) Mant, C. T.; Litowski, J. R.; Hodges, R. S. Hydrophilic interaction/cation-exchange chromatography for separation of amphipathic α-helical peptides. *J. Chromatogr., A* **1998**, *816* (1), 65−78.

(22) Zhu, B. Y.; Mant, C. T.; Hodges, R. S. Mixed-mode hydrophilic and ionic interaction chromatography rivals reversed-phase liquid-chromatography for the separation of peptides. *J. Chromatogr.* **1992**, *594* (1−2), 75−86.

(23) Huang, Y.; Duan, J. P.; Chen, H. Q.; Chen, M.; Chen, G. N. Separation and determination of carnosine-related peptides using capillary electrophoresis with laser-induced fluorescence detection. *Electrophoresis* **2005**, *26* (3), 593−599.

(24) Aristoy, M. C.; Toldrá, F. Concentration of free amino acids and dipeptides in porcine skeletal muscles with different oxidative patterns. *Meat Sci.* **1998**, *50* (3), 327−332.

(25) Dunnett, M.; Harris, R. C. High-performance liquid chromatographic determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine in equine and camel muscle and individual muscle fibres. *J. Chromatogr., B* **1997**, *688* (1), 47−55.

(26) Strege, M. A. Hydrophilic interaction chromatography electrospray mass spectrometry analysis of polar compounds for natural product drug discovery. *Anal. Chem.* **1998**, *70* (13), 2439−2445.

(27) Oertel, R.; Neumeister, V.; Kirch, W. Hydrophilic interaction chromatography combined with tandem-mass spectrometry to determine six aminoglycosides in serum. *J. Chromatogr., A* **2004**, *1058* (1−2), 197−201.

Received for review February 9, 2007. Revised manuscript received March 30, 2007. Accepted April 12, 2007. Scholarship FPU from Ministerio de Educación y Ciencia (Spain) to L.M. and an I3P-CSIC contract from the European Social Fund to M.A.S. are fully acknowledged. Work was supported by Agroalimed grant from Consellería de Agricultura, Pesca y Alimentación (Generalitat Valenciana, Spain).

JF0703809