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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

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Abstract

A simple methodology, consisting in cation exchange HPLC separation combined with OPA postcolumn detection, has been succesfully applied to the analysis and detection of histidine dipeptides in feeds. The use of the proposed methodology, which is very simple and relatively fast, allows the detection of 2.63 ppm and 0.58 ppm of carnosine and anserine, respectively, in cattle feed. As balenine (3-methylhistidine) was not commercialy available, it was isolated from pork muscle and used as standard for its quantitation in feeds. These dipeptides have shown good stability against intense heat treatments, even at temperatures as high as 120 °C for 20 min. So, these histidine dipeptides can be feasible used as markers of animal protein content in feeds.

Keywords: Histidine dipeptides; Carnosine; Anserine; Balenine; Animal proteins in feeds

1. Introduction

The histidine dipeptides; carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methylhistidine), and balenine or ophidine (β - alanyl - L - 3 - methylhistidine) are naturally occurring in vertebrate animal tissues (Boldyrev & Severin, 1990).

These dipeptides have been extensively studied because of their physiological properties such as the buffering (Abe & Okuma, 1991; Davey, 1960; Seewell, Harris, Marlin, & Dunnett, 1992; Suyama & Shimizu, 1982) and antioxidant capacity (Boldyrev, Dupin, Bunin, Babizhaev, & Severin, 1987; Chan & Decker, 1994; Chan, Decker, & Means, 1993; Decker, Chan, Livisay, Butterfield, & Faustman, 1995; Gopalakrishnan, Decker, & Means, 1999; O'Neill, Galvin, Morrissey, & Buckley, 1999), activity as neurotransmitter substance (Flancbaum, Fitzpatrik, Brotman, Marcoux, Kasziba, & Fischer, 1990; Ohtsuka & Takahashi, 1983), vasodilatory action (Ririe, Roberts, Shouse, & Zaloga, 2000), and their function as modulators for some enzymes (Gianelli, Flores, Moya, Aristoy, & Toldrá, 2000; Johnson, Fedyna, Schindzielorz, Smith, & Kasvinsky, 1982; Johnson & Hammer, 1989; Parkers & Ring, 1970). These peptides have also been related to the food sensory perception (MacLeod & Seyedain-Ardebili, 1981; Suyama & Shimizu, 1982; Pereira-Lima, Ordóñez, García de Fernando, & Cambero, 2000) or even to the generation of meat flavor compounds (Chen & Ho, 2000).

The amount of these dipeptides is much higher in the skeletal muscle than in other tissues, specially in muscles with a glycolytic metabolism (Aristoy & Toldrá, 1998; Cornet & Bousset, 1999; Davey, 1960). But it also varies with the animal species (Crush, 1970), age (Carnegie, Hee, & Bell, 1982; Chan & Decker, 1994), and/or diet (Chan & Decker, 1994; Chan, Decker, Chow, & Boissonneault, 1994). In this way, anserine is the predominant dipeptide in birds, while mammalians have higher amounts of anserine and carnosine, except humans that only have carnosine. Balenine is usually present in low amounts except in snake and marine mammalians, as dolphin and whales, where it is the predominant and, in some cases, the only present histidine dipeptide. Ratios between the dipeptides have been used to differentiate among animal species and to

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identify the origin of an animal feed (Carnegie et al, 1982; Carnegie, Collins, & Ilic, 1984; Plowman & Close, 1988).

Moreover, the analysis of these dipeptides have been proposed to detect the presence of products of animal origin in feeds for ruminants (Schönherr, 2002). This application is based on the exclusive presence of these dipeptides in animal tissues, and their absence in vegetables, and its usefulness has increased after the appearance of the bovine spongioforme encephalophathy and the recent governmental banning for the addition of animal source proteins to feeds for ruminants. The methodology developed by Schönherr (2002) was proposed as an easier and more convenient alternative to the microscopy technique which is time-consuming and requires experienced staff. But this method included a complex sample manipulation with losses in anserine and balenine dipeptides.

A new method for the detection of animal origin proteins in feeds, with minimal sample preparation, is proposed. This method is based on the analysis of 3 histidine dipeptides as markers.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile and methanol (HPLC ultragradient quality) were from Scharlau (Barcelona, España). Carnosine and Anserine were from Sigma (St Louis, Mo, USA). 5-Sulphosalicylic acid (SSA) was from Aldrich Chemical Co (Milwaukee, Wi, USA); perchloric acid (PCA) and 2-mercaptoethanol were from Merck (Darmstadt, Germany); phthaldialdehyde (OPA) was from Fluka Chemie AG (Buchs, Switzerland); boric acid, potassium hydroxide 85% pellets and polioxyethylen laurylic ether 30% p/v (Brij 30%) were from PanReac Química S.A. (Montcada i Reixac, Barcelona, España).

2.2. Materials

Pork muscle, *Longissimus dorsi,* was used for the isolation of balenine. Commercial feeds for dogs, cats, and bovine were used.

2.3. Sample preparation

Feeds or sample tissues were finely ground and 5 g was homogenised with 25 ml of re-distilled water in a polytron (3×15 s strokes) (Kinematica Gmbh. Berna. Switzerland). The homogenate was centrifuged at 10,000×*g* for 20 min in cold and the supernatant was filtered through glass-wool. 300 μl of supernatant was deproteinised by adding 3 vol (900 μl) of methanol (HPLC grade) and left into the freezer for 15 min. Sample was centrifuged (12,000 rpm) in an eppendorf centrifuge for 3 min and the supernatant directly analysed for their dipeptides content.

2.4. Heat dipeptides stability

Pork muscle *Longissimus dorsi* was cut into 1 cm width slices. 5 g of the inside portion of each slice were vacuum packaged into plastic bags and submitted to heating storage at 60, 75, 90, and 105 °C for 30, 60, 90 and 120 min at each temperature and to 120 °C for 20 min into the autoclave. After these treatments, the carnosine, anserine and balenine content of each sample (including the exudates) was analysed by cation exchange HPLC as described later.

2.5. Chromatographic analysis

The dipeptides were analysed by cation exchange-HPLC and OPA post-column derivatisation.

The HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisted in a model 1050 equipment with both a 1040 fluorescence detector and a variable wavelength ultraviolet detector. The equipment had both, manual (Rheodyne valve) and automatic injectors, and a column oven. For the post-column derivatisation, a T-zero death volume piece connects the outlet from the column with the OPA-derivatisation-reagent inlet and to a 200 cm long and 0.01 in. i.d. stainless steel reaction coil which takes the derivatising column effluent to the fluorescence detector. This post-column reaction system was maintained at room temperature. The OPA-reagent was pumped at a 0.5 ml/min with a single channel 1050 model pump (Agilent Tech.).

The separation was achieved by using a 4.6×250 mm column Waters Spherisorb SCX (Waters Corporation, Milford, MA, USA). Mobile phase was composed of: Phase A, 20% acetonitrile in 6 mM hydrochloric acid and phase B, composed by phase A with 0.8 M sodium chloride. The flow rate was 1 ml/min. The separation was achieved through a salt gradient as follows: after a 5 min isocratic elution with 20% B, a 10 min gradient to 50% B was applied followed by 10 min of a washing step with 100% B and returning to the initial conditions, waiting for 15 min before the next injection.

For the detection, an OPA-reagent was prepared as described by Hernández-Jover, Izquierdo-Pulido, Venciana-Nogues, and Vidal-Carou (1996), dissolving 15.5 g of boric acid and 13.0 g of potassium hydroxide in 500 ml of water and adding 1.5 ml of 30% Brij-35 solution and 1.5 ml of 2-mercaptoethanol. Then, 100 mg of OPA dissolved in 2.5 ml of methanol was added and the solution mixed. The pH of this reagent must range between 10.5 and 11. It must be protected from light and prepared fresh daily. The flow rate of the derivatisation

reagent was 0.5 ml/min. In the fluorescence detection, 340 and 445 nm were used as excitation and emission wavelengths, respectively.

2.6. Preparation and valoration of a balenine standard solution

The dipeptide balenine is not commercially available as a standard. Thus, its chromatographic identification and its response factor determination for its quantitation in samples required the previous isolation of this compound.

The balenine dipeptide was isolated from porcine muscle (*Longissimus dorsi*) as follows: Muscle was ground, weighed and 10 g extracted with 40 ml of 0.1 N hydrochloric acid and deproteinised with 120 ml of methanol (HPLC grade) as previously described. The deproteinised sample was vacuum-evaporated in a rotavapor and re-dissolved in 4 ml of 0.1 N hydrochloric acid. Four aliquots of 500 μl were injected, respectively by mean of a Rheodyne valve in a Spherisorb SCX of 250×10 mm (Waters Corp.) in the same conditions as previously described but with a flow rate of 4.2 ml/min. The detection was achieved at 214 nm.

The peak fraction eluted at 12 min (corresponding to 0.28 M of salt), was collected and pooled. Its identity as balenine was confirmed by hydrolysis as described below. This fraction was vacuum evaporated and redissolved in 4 ml of 50% methanol-water solution. This solution is a concentrated balenine solution that should be valorised to be used as a standard solution (BSS).

The BSS was valorised after quantitative hydrolysis of balenine to render its component amino acids, 3-methyl-histidine and β-alanine. These amino acids were analysed by reversed-phase HPLC and the molarity (concentration) of the BSS could be calculated by taking into account the following equation:

$$1\ \text{mol of balenine} = 1\ \text{mol of 3-methyl-histidine} + 1\ \text{mol of } \beta\text{-alanine}. \quad (1)$$

Their quantitation was possible because their standards are commercially available.

To this aim, 20, 50 and 100 μl of the BSS plus 50 μl of an hydroxyproline solution (1.325 μg/μl) used as internal standard, were taken up to 600 μl with re-distilled water respectively. Fifty micro litres of each of these blends (three replicates each) were submitted to 6 N hydrochloric acid vapor-phase hydrolysis at 110 °C for 22 h in the "Pico-Tag™ Work Station" (Waters Corp.) and the obtained free amino acids (3-methyl-histidine and β-alanine) analysed by reversed-phase HPLC in a 300×3.9 mm Nova Pack C18 column (Waters Corp.) through the method described by Bidlingmeyer, Cohen, Tarvin, and Frost (1987), and Flores, Aristoy, Spanier, and Toldrá (1997). The response factors of 3-methyl-histidine and β-alanine, relative to hydroxyproline were previously calculated being 1.410 and 0.658, respectively (data not shown).

3. Results and discussion

3.1. Preparation of a valorised balenine solution

In the revised literature on the histidine-related dipeptides balenine was obtained from Dr. Wolff from the National Institute of Health at Bethesda, Mo (USA), (Carnegie et al., 1982, Plowman & Close, 1988). In this report, a balenine standard solution (BSS) was prepared in the lab by a simple method, easy to afford in any chemical laboratory with standard equipment. Pork meat was used as a source of balenine because its balenine content was high enough. The feasibility of the obtained data on the concentration of the standard balenine solution was based on the complete hydrolysis reaction achieved by the described methodology.

The balenine solution was valorised using this methodology, obtaining a mean value of 190.8 ± 1.23 μg/ml With this balenine solution and the carnosine and anserine standards purchased from the commerce, it was possible to identify and calculate the response factor for each dipeptide using the cation exchange HPLC chromatographic method.

3.2. Analysis of dipeptides by cation exchange chromatography

3.2.1. Sample preparation

Sample preparation only includes two steps: extraction and deproteinization, and both are very simple and cheap when comparing with the methodology proposed by Schöneherr (2002). Different extraction solutions such as water, 80% ethanol, 80% methanol, 0.5 M perchloric acid and 5% 5-sulphosalycilic acid were assayed to optimise the dipeptides extraction. When water was used, a later deproteinization with organic solvents (3 volumes of methanol or ethanol) or strong acids (1 volume of 1 M perchloric acid or 10% (w/v) 5-sulphosalycilic acid) was required. The best recoveries for all dipeptides in a dog feed sample, were obtained by using water for extraction and methanol as deproteinizing agent (data not shown).

3.2.2. Chromatographic separation

The deproteinized sample was centrifuged and directly injected into the high performance liquid chromatograph. Cation exchange chromatography followed by OPA post-column derivatization has been traditionally used for the histidine dipeptide analysis (Carnegie, Ilic, Etheridge, & Collins, 1983; Carnegie et al., 1984;

Carnegie, Ilic, Etheridge, & Stuart, 1985). This technique is based on the strongly basic nature of these compounds and takes advantage of the high sensitivity of the OPA fluorescence detection. It has another important advantage consisting in the possibility of automation of the post-column derivatization reaction. The method proposed here uses a cheap and robust column (silica supported), where a slightly adjustment of the initial sodium hydrochloride content in the chromatographic phase A allows a very good reproducibility in the compounds retention times. With this precaution, more than five thousand injections have been achieved with the same column, keeping good performance yet. With the described method, the separation of these dipeptides could be achieved in just 12 min, as reflected in the chromatogram shown in Fig. 1.

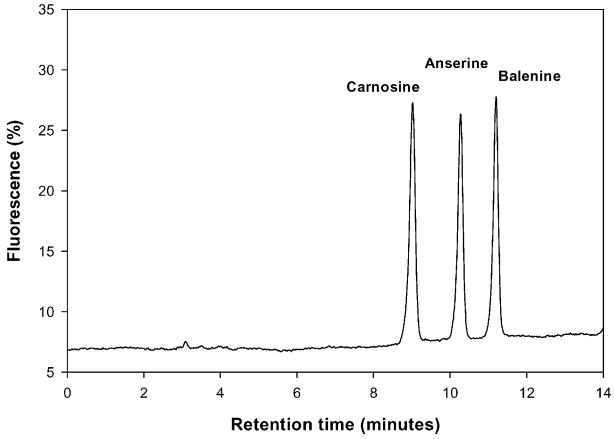

Fig. 1. Cation exchange chromatogram of carnosine, anserine and balenine. Chromatographic conditions are described in text.

3.2.3. Method evaluation

Linearity and calibration: detection limits and response linearity depend on the detector specifications and/or set conditions, but not on the derivatisation. The plots and respective regressions of carnosine, anserine and balenine are shown in Fig. 2A. Area data were obtained by optimising the detector parameters (gain = 12, lamp frequency = 110 Hz, time response = 2 s) to obtain the best signal to noise rate compatible with a wide lineal range. In these conditions, a lineal range up to 300 ng of carnosine and anserine and up to 400 ng of balenine were obtained. The regression lines tended to bias when area values exceeded 1000 to 1200.

The detection limits of standards were calculated by the successive injection of 5 μl of a decreasing dilute standard solution of 1 μM carnosine, anserine and balenine (see Fig. 2B for the lowest injected amounts). The dipeptide concentration giving a peak height double than noise was considered as the detection limit. Detection limits corresponded to 140, 218, and 250 pg of carnosine, anserine and balenine, respectively. These detection limits may be even lower depending on the specifications of the fluorescence detector used. A chromatogram of around 200 pg of injected carnosine, anserine and balenine, respectively is shown in Fig. 3.

The reproducibility of the method was evaluated for carnosine and anserine by successive analysis of both dipeptides in 8 replicates of a commercial cat food which contained poultry meat (40% declared). Data, shown in Table 1, demonstrated a very good reproducibility from one analysis to another.

The matrix effects in the dipeptide recoveries was evaluated by spiking anserine and carnosine on a feed previously to its analysis. The recovery was dependent on the matrix (feed). As an example, a cattle feed was spiked with around 0.5, 1, 2.5 and 5 ppm of anserine and carnosine and the obtained recoveries are shown in Table 2. When low amounts of carnosine were added (less than 2.63 ppm in the assayed case), the recoveries

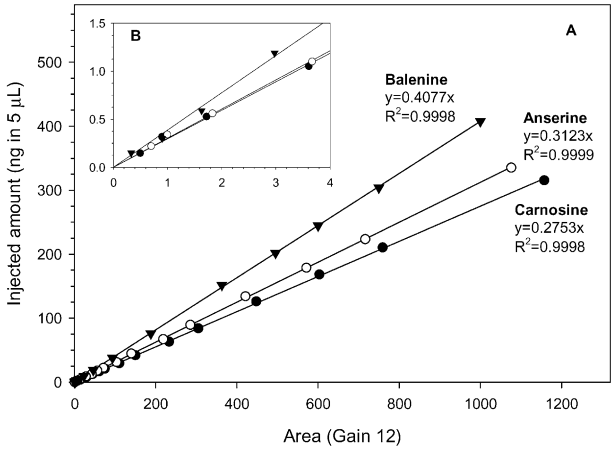

Fig. 2. Linearity range and regressions (A), and detection limit (B) of anserine (○), carnosine (●) and balenine (▼). Plot equations were calculated forcing to zero.

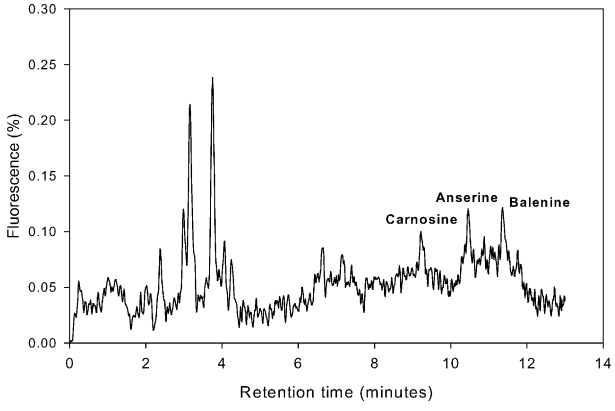

Fig. 3. Cation exchange chromatogram after injection of 140, 218, and 250 pg of carnosine, anserine and balenine, respectively.

were higher than 100% due to the presence of some feed components co-eluting with the carnosine peak. On the contrary, 0.58 ppm of anserine addition can be feasibly detected for this dipeptide because it elutes in a clean zone of the chromatogram. The chromatogram of the

Table 1
Reproducibility of the method: analysis of anserine and carnosine in a commercial cat feed containing poultry meat

	Carnosine (ppm)	Anserine (ppm)
	56.5	241.7
	58.7	247.9
	57.8	258.8
	56.5	240.0
	59.8	260.8
	57.5	241.9
	56.2	240.4
	57.5	246.8
	56.8	240.8
Mean	57.5	246.6
SD	1.1	7.6
CV (%)	1.9	3.1

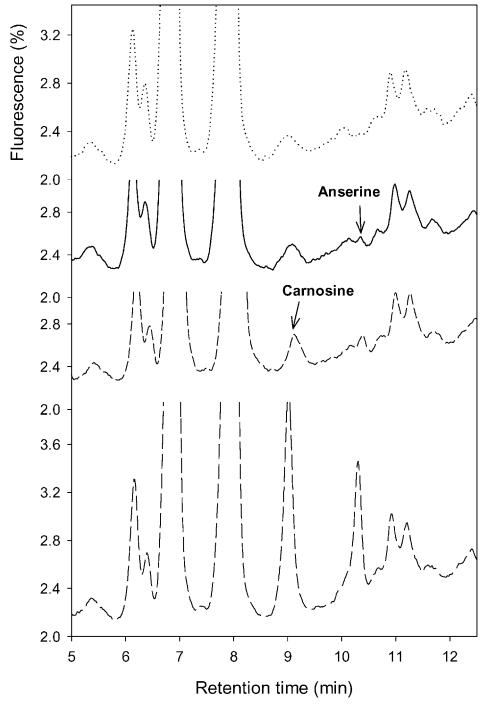

Fig. 4. Cattle feed cation-exchange chromatogram (...) and the same after addition 0.5 ppm (——), 1 ppm (– – –), and 5 ppm (— — —) of carnosine and anserine (for exact added amounts see Table 2).

cattle feed without any addition is shown in Fig. 4 together with those of the feed spiked with around 0.5, 1, and 5 ppm of anserine and carnosine. The arrows indicate the lower concentration with enough sensible detection for each dipeptide, being 0.58 ppm for anserine and 2.63 ppm for carnosine.

3.2.4. Heat dipeptides stability

In the feed manufacturing, the stabilisation of the product by means of intense heat treatment is required. Thus, the heat stability of the dipeptides was assayed by submitting *L. dorsi* pork muscle to different heating treatments. Results are represented in Fig. 5.

Table 2
Recovery of carnosine and anserine from cattle feed spiked with both dipeptides

Carnosine		Anserine	
Added (ppm)	Recovery (%)	Added (ppm)	Recovery (%)
0.53	221.38	0.58	88.86
1.05	139.47	1.17	90.38
2.63	103.6	2.91	98.99
5.26	99.31	5.86	100.4

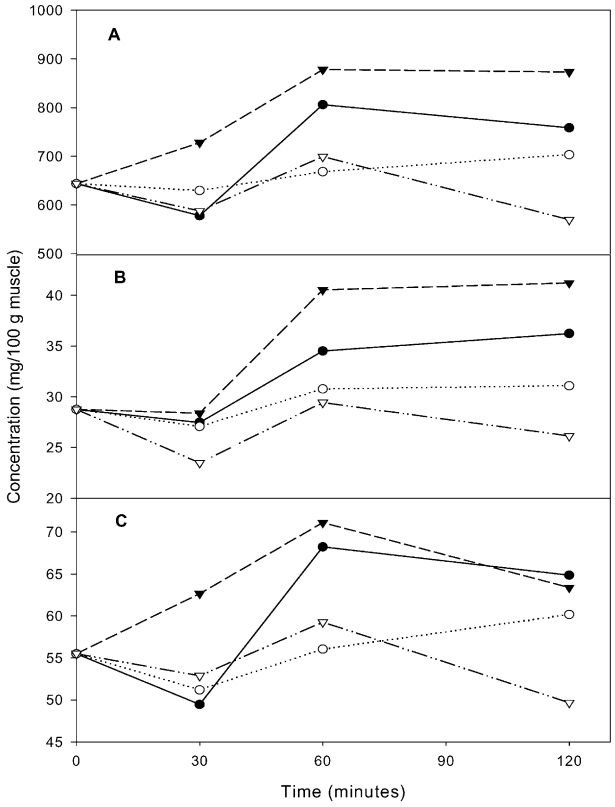

Fig. 5. Effect of the temperature treatment (——●—— 60°, · · · ·○· · · · 75°, – – –▼– – – 90°, – · ·▽– · · – 105°) on the carnosine (A) anserine (B) and balenine (C) content in *L. dorsi* pork muscle.

No remarkable degradation was observed in any of the heat treatments along time, for the three dipeptides (see Fig. 5) being the trends similar in all cases. The observed differences in the dipeptide amounts for each treatment may be due to the different extraction or solubilization of the dipeptides at different temperature/time treatment. The only remarkable effect was a trend for a slight increase in the dipeptides recovery after meat treatment at 90 °C.

Data extracted from the literature on the effect of heat treatment on the histidine dipeptides are contradictory. Pereira-Lima et al. (2000) observed an increase in the analysed dipeptides amount with temperature, probably due to a major solubilization or liberation of the dipep-

Table 3
Histidine dipeptides concentration[a] (mg/100 muscle) from porcine *L. dorsi* muscle after heat treatment at 120 °C for 20 min

	Carnosine	Anserine	Balenine
Initial	643.71a	28.76b	55.49c
Final	538.10a	20.63b	44.71c

[a] Data are means from three determinations. Same letters indicates no significant differences ($P < 0.05$) between initial and final treatment.

tides from the meat structure. On the contrary, a decrease in the carnosine content of mechanically separated pork extracts when submitted to heating treatments at 60, 70, and 80 °C was observed by Gopalakrishnan et al. (1999) and beef extracts heated at 60 and 90 °C was observed by Chan et al. (1993). In the same line, Kuroda, Ohtake, Suzuki, and Harada (2000), and Kuroda and Harada (2000, 2002) observed a decrease in the carnosine content during heating at 95 °C of beef and chicken meat extracts. They justified this fact by the incorporation of this dipeptide to the insoluble macromolecular fraction by formation of the tripeptide γ-glutamyl-β- alanyl-histidine. When meat was submitted to 120 °C for 20 min, a noticeable although not statistically significative ($P < 0.05$) decrease was observed for the three dipeptides as shown in Table 3. Thus, this severe heating treatment is not really affecting the determination of the three dipeptides.

4. Conclusions

The proposed method is based on minimal sample manipulation, including very simple extraction and deproteinisation steps without need for further purification before analysis. This method is fast (less than 3 h for eight and even more samples) and reliable. In fact, the reproducibility is very good, the recovery for the dipeptides is about 100% and they show good stability against heat treatments. In summary, the proposed method may constitute a valid alternative for detecting the presence of animal origin proteins in feeds.

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