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Analysis of Products of Animal Origin in Feeds by Determination of Carnosine and Related Dipeptides by High-Performance Liquid Chromatography

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Products of animal origin such as meat meal were commonly used as sources of protein and amino acids for the production of compound feeds. Because the feeding of such products is prohibited in Germany, the official feedstuff control of the government must evaluate feeds for the forbidden use of products of animal origin. Microscope examination is the official method to prove animal-originated adulterations of feeds. This paper proposes a high-performance liquid chromatography method for the determination of the dipeptide carnosine and related dipeptides (anserine and balenine) and shows the dependence of the contents of anserine, balenine, and carnosine in compound feeds on the content of meat meal in feeds. The presented method can complete and confirm the result of the microscopic method for evidence of components of animal origin in feeds.

KEYWORDS: Feeds; meat meal; high-performance liquid chromatography (HPLC); solid-phase extraction (SPE); dipeptides; carnosine; anserine; balenine

INTRODUCTION

Feeds of animal origin (meat meal, meat bone meal, fish meal, etc.) are valuable protein sources. The feeding of such feeds to pigs and poultry was generally common. As a consequence of the increased appearance of bovine spongioforme encephalopathy the feeding of animal-originated products, especially to ruminants, was forbidden by several laws (*1*−*3*). The authorities responsible for the rules of feeds must be able to detect admixes or displacments of products of animal origin in feeds.

The microscope examination is the official method to prove animal-originated adulterations of feeds. It is validated in three collaboration studies and manifested in the European law (*4*, *5*). Unfortunately, the microscopic technique is time-consuming and requires experienced staff and microscopic equipment. These suppositions do not exist in all laboratories that need to analyze products of animal origin in feeds. Furthermore, it is an estimation method and therefore its "subjective touch" is discussed again and again.

Analytical methods to prove parts of animal origin in feeds are in development but cannot be used yet for routine investigation. The Polymerase Chain Reaction (PCR) is a very sensitive method and will play an important role for species differentiation soon. Unfortunately, false-positive results occasionally occur. Many control reactions are nessecary to secure the result. Altogether the PCR is too time-consuming and expensive to become a routine method used for this purpose. The disadvantage of a proposed HPLC method, which determines the amino acid hydoxylysine, is the length of time needed for sample preparation and derivatization (*6*). The enzyme-linked immunosorbent assay is not validated for feeds and is insensitive within the trace area (*7*, *8*).

Therefore, the aim of this work was to develop a chemical method for the routine analysis of parts of animal origin in feeds as an alternative and supplement to the microscopic method, not to replace it. Because HPLC analyses are essential in most feed science laboratories, this simple and less expensive technique will be favorable.

The naturally occurring dipeptides carnosine (β-alanyl-L-histidine), balenine (β-alanyl-L-3-methylhistidine), and anserine (β-alanyl-L-1-methylhistidine) are found in animal tissue exclusively. Its presence in the fraction of the total water-soluble nitrogen-containing compounds (together with free amino acids, biogenetic amines, organic acids, nucleotides, and sugars) in animal tissues such as heart muscle, kidney, and liver, but in particularly high concentrations in muscle tissue, is described (*9*−*12*). Earlier papers mentioned the occurrence of anserine in red alga (*13*), but no information about the presence of these dipeptides in plants or bacteria could be found. The biological roles of these dipeptides are not fully explored. Numerous studies have demonstrated its strong and specific antioxidant properties (*10*, *14*), and a buffering effect is suggested (*9*, *15*). The role of the dipeptides as neurotransmitter substances (*9*) and their involvement in the modulation of enzymatic activities and during the heating of beef soup stock solution (*16*) are discussed. Therefore, there was an strong interest in analyzing these dipeptides in different matrices. Numerous methods were developed for it. In most cases, HPLC methods on reversed

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phase or ion-exchange columns have been used for the determination of the dipeptides in different tissues from several species. The underivatized dipeptides were detected by UV adsorbance (*15*, *17*−*20*), but frequently precolumn derivatization by phenylisothiocyanate (PITC) (*16*), 2-methoxy-2,4-diphenyl-3(*2H*)-furanone (MDPF) (*21*), or *o*-phthalaldehyde (OPA) (*22*−*24*) or postcolumn derivatization by OPA (*25*, *26*) or ninhydrin (*27*) was chosen due to its low UV absorbance. Some of these methods suffer from low resolution, unstable derivates, or high detection limit. Furthermore, only methods for determination of carnosine and related dipeptides in animal tissues (muscle, plasma, or other tissues) are described (*9*, *12*, *14*, *15*, *18*−*21*, *23*, *25*−*28*). No reports about analysis in plants or feeds could be found in the literature.

With the proposed HPLC method the animal-originated dipeptides anserine, balenine, and carnosine can be determined in feeds. It is shown that their detection in feeds correlated with the occurrence of products of animal origin in these samples.

MATERIALS AND METHODS

Principle. The sample is extracted with distilled water, and to precipitate proteins, it is diluted with sulfosalicylic acid. A part of the solution is cleaned and concentrated by a propanesulfonic acid solid-phase extraction cartridge (PRS-SPE cartridge). After derivatization with carbazole-9-carbonyl chloride (CFC), the sample is injected into the liquid chromatograph and detected by fluorescence detection.

Chemicals and Reagents. Distilled water was cleaned by ion exchange. Acetonitrile, methanol, and acetone were purchased from Merck Eurolab GmbH Deutschland (Darmstadt, Germany) in LiChrosolv quality. Tetrahydrofuran was purchased from Carl Roth GmbH & Co. (Karlsruhe, Germany) in RotiSolv HPLC quality. Carnosine, anserine, sarcosine, 1-methylhistidine, 3-methylhistidine, homocarnosine, taurine, γ-aminobutyric acid, amino acid standard solution (type AA-S-18), 5-sulfosalicylic acid (SSA), hydroxylamine hydrochloride (grade I, 99%), and 2-(methylthio)ethanol were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). CFC was purchased from Fluka Chemie AG (Buchs, Switzerland). All other reagents were of analytical grade.

Sample Preparation. *(1) Extraction and Precipitation with SSA.* Before assay, products were ground preferably to a particle size of <1 mm and afterward mixed thoroughly. Twenty-five grams of sample was weighed out into a 250 mL Erlenmeyer flask, and 100 mL of water was added. The sample was thoroughly mixed, stirred for at least 2 h at room temperature by using a magnetic stirrer, and afterward screened. A aliquot of 720 μL of sample extract was mixed with 720 μL of cold 10% SSA (w/v). After a settling step of at least 1 h at 4 °C, the solution was centrifuged.

(2) Cleaning and Concentration by PRS-SPE Cartridge. The precipitated extract obtained as described above was cleaned and concentrated by SPE using Bond Elut PRS cartridges (100 mg of resin; Phenomenex, Aschaffenburg, Germany). The PRS-SPE cartridge was conditioned by 4 × 1 mL of methanol followed by 4 × 1 mL of 1 M phosphoric acid. Five hundred microliters of the clear sample extract was applied to the cartridge. If the carnosine content is to be quantified, only 75 μL of sample extract was applied to the cartridge to avoid cartridge overloading. After washing with 1 mL of 1 M phosphoric acid, 500 μL of distilled water, and 250 μL of 0.4 M borate buffer, pH 9.5, the cartridge was air-dried under vacuum for 2 min. The fraction with carnosine was eluted with 450 μL of 0.4 M borate buffer, pH 9.5, and collected in an autosampler vial (at least 1.5 mL vial). For quantification, both the empty and the filled vial were weighed. The difference corresponds to the volume of solution.

(3) Derivatization of Samples with CFC. The fraction obtained by PRS-SPE (~320 μL) was mixed with 300 μL of freshly prepared CFC solution (2.5 mM in acetone) and gently shaken. After 90 s, 180 μL of freshly prepared cleavage solution [mixture of 1 mM EDTA in 0.1 N NaOH/0.5 M hydroxylamine hydrochloride in distilled water/2-(methylthio)ethanol; 34:15:1] was added and shaken again. After an additional 3 min, 420 μL of quench solution [20% (v/v) acetic acid in acetonitrile] was added, the vial was sealed, and the contents were finally mixed. Twenty microliters was injected for separation into the HPLC system.

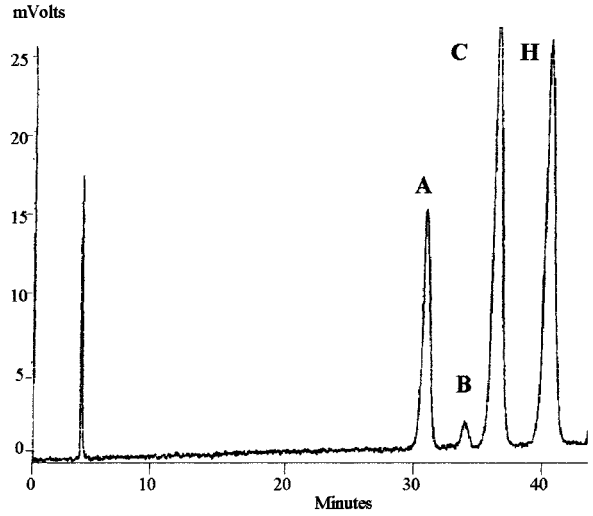

Figure 1. HPLC separation of standard solution. Per 20 μL injected: anserine (A), 3.23 ng; balenine (B), 0.37 ng; carnosine (C), 6.25 ng; and histidine (H), 5 ng. Conditions were as described under Materials and Methods.

(4) Derivatization of the Standard Solution with CFC. The derivatization of the standard solution was performed according to the derivatization of the samples. Thirty microliters of the standard solution (each 5 mg of anserine, balenine, carnosine, and histidine in 100 mL of 5% SSA) was mixed with 300 μL of CFC and gently shaken. After 90 s, 180 μL of cleavage solution was added and the mixture shaken again. After an additional 3 min, 420 μL of quench solution was added, the vial was sealed, and the contents were finally mixed. Twenty microliters was injected for separation into the HPLC system.

Chromatography. The HPLC system consists of a BT 8200 equipped with a BT 8310 ternary gradient degasser DG 1300 (all from Biotronik/Eppendorf, Maintal, Germany), an 851-AS intelligent autosampler, and an 821-FP intelligent spectrofluorometer (both from Jasco Deutschland, Gross-Zimmern, Germany). The samples were applied to a Jupiter column, C18, 300 Å, 5 μm particle size, 250 × 4 mm with guard column, widepore C18, 4 × 3 mm (both from Phenomenex, Aschaffenburg, Germany). The elution was performed at a flow rate of 1 mL/min and a column temperature of 23 °C and with fluorescence detection at λ_{ex} 287 nm/λ_{em} 340 nm or UV detection at 254 nm. The mobile phase consists of solution (A) 50 mM acetate buffer in distilled water, pH 4.37, and solution (B) a mixture of acetonitrile, methanol, and tetrahydrofuran 70 + 25 + 5 (v/v/v). The solutions were degassed before use or online. The profile of the binary gradient was the follows: 0−39 min, 18% B; 39−40 min, 18−100% B; 40−43 min, 100% B; 43−44 min, 100−18% B; 44−65 min, 18% B; after 65 min, the next injection follows.

RESULTS AND DISCUSSION

Chromatographic Separation of Carnosine and Its Related Dipeptides. Under the described conditions the retention times of the substances were as follows: anserine, 32.5 min; balenine, 35.7 min; carnosine, 38.8 min; histidine, 43.5 min (**Figure 1**). No proteinogenic amino acids were eluted near the anserine, balenine, and carnosine peaks and before histidine. The histidine-related amino acids 1-methylhistidine and 3-methylhistidine, the dipeptide homocarnosine, and the amino acids alanine, taurine, sarcosine, and γ-aminobutyric acid were eluted later than histidine. The retention times were strong depending on the mobile phase composition. Therefore, a daily analysis of a standard mixture or, better, a positive sample is suggested to control retention times. The HPLC separation was optimized for a baseline separation of carnosine from a small impurity

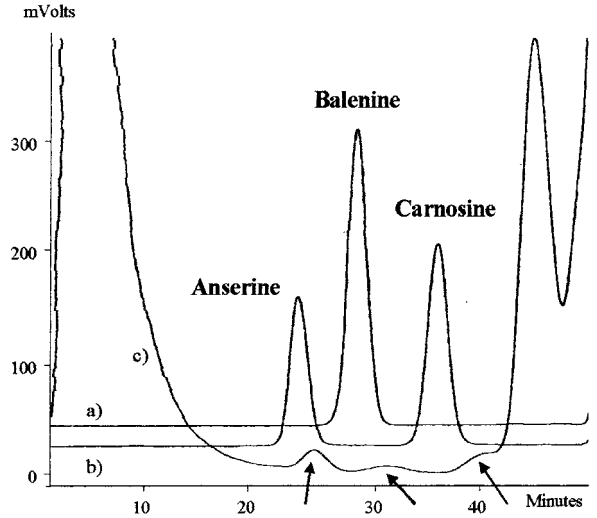

Figure 2. Comparison of HPLC runs of standards and shredded extracted soybean: (a) balenine standard; (b) anserine/carnosine standard; (c) shredded extracted soybean. The impurity peaks are marked by an arrow. Conditions were as described under Materials and Methods.

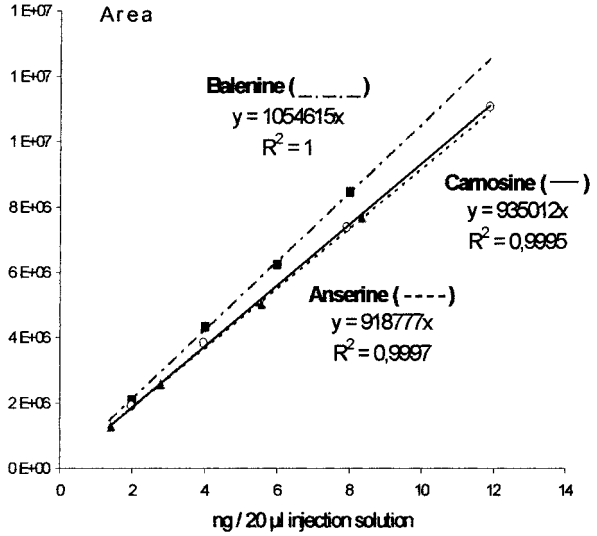

Figure 3. Linearity of the CFC derivatization of anserine, balenine, and carnosine.

Table 1. Calculation of the Detection Limit According to DIN 32 645

				calculated by	
ng/20 μL	area	height		area (ng/20 μL)	height (ng/20 μL)
0.05049	27823	611			
0.10098	90361	1425			
0.20196	195551	2793	decision limit	0.041	0.023
0.40392	369881	5364	detection limit	0.082	0.046
0.80784	755696	10684	determination limit	0.147	0.083
1.61568	1522808	21374			

peak appearing later than that of carnosine caused by shredded extracted soybean (**Figure 2**). As in shredded extracted soybean in most feeds occurred additionally impurity peaks from the matrix near anserine and balenine (**Figures 2** and **6**). In case of doubt, a standard addition could help to clear the peak position of carnosine and the other dipeptides. Independently of it, to answer the question if parts of animal origin are present in the feed or not, the presence of carnosine is crucial. Peaks of anserine and/or balenine could only support the decision. A detection of carnosine with liquid chromatography−mass spectrometry (LC-MS) would be still more elegant and unique. Because the device for this method does not belong yet to the standard equipment of feed laboratories in Germany, we did without it.

The same applies to more modern methods such as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), with which the dipeptides would be analyzable, too.

Extraction from Feed. Because of its good availability, simple management, and harmlessness, water was selected as extracting agent. Other extracting agents (0.1 N HCl, 80% ethanol, 5% SSA, or 0.5 M perchloric acid) do not furnish a higher yield of carnosine. An extraction time of 2 h is enough to solve the carnosine completely. Longer extraction times do not increase the carnosine content of the extraction solution (data not shown).

Solid-Phase Extraction by PRS Cartridge. The use of an PRS-SPE cartridge for cleaning and concentrating follows a method of Dunnett (*15*, *20*, *24*). It shows that a propanesulfonyl cation exchange bonded phase retains biogenic imidazoles effectively from acidic plasma extracts. We could show the possibility of retaining carnosine, balenine, and anserine from a sulfosalicylic acid water extract of feeds by a PRS-SPE cartridge. A comparison of the extract composition by HPLC before and after the PRS-SPE cartridge shows that the SPE is necessary. It leads to a concentration of dipeptides and to a cleaning of the extract (data not shown). The buffering of the extracts in one step was another advantage of the PSR-SPE cartridges. No further sample dilution was necessary to adjust the pH value for the derivatization conditions.

The recovery of anserine, balenine, and carnosine from the PRS-SPE cartridge was tested by PRS-SPE cleaning, derivatization, and HPLC determination of a standard solution containing 16.6 ng of anserine, 15 ng of balenine, and 23.76 ng of carnosine per 75 μL of solution applied to the PRS cartridge (n = 5). Carnosine was recovered at 100.8%, anserine at 80.2%, and balenine at 63.1%. The reason for lower recoveries of anserine and balenine compared to that of carnosine was its slightly slower elution from the PRS-SPE cartridge. The chosen elution volume of 450 μL of 0.4 M borate buffer is a compromise between recoveries of the dipeptides and detection limit. A higher elution volume leads to higher recoveries of anserine and balenine but, unfortunately, to a higher volume of eluate and more impurity peaks near carnosine in feed samples, too. This would be adisadvantage for the detection limit of the method.

Derivatization with CFC. Derivatization with CFC was performed according to the method of Rudolph (*29*) with slight modifications. CFC has several advantages as a precolumn labeling reagent of dipeptides. Within a short time and at ambient temperature, stable and highly fluorescent derivatives are formed. Due to the fluorescence activity of the CFC derivatives, the detection limit of the method is very low. The stability of the CFC peptide derivatives makes the preparation of sample series by autosamplers possible. The derivatives are stable for at least 40 h (*29*). Our results showed that the fluorescence does not change over 63 h (data not shown). The used cleavage reagent reacts with the excess of the CFC reagent. Thus, column pollution by CFC is avoided. The originated CFC hydroxylamine does not influence the HPLC separation, and an additional extraction step for removing the CFC excess is not necessary. The use of 0.4 M borate buffer, pH 9.5, instead of 0.2 M borate buffer, pH 9.5, for solving the sample was tested, and no difference in the derivative's fluorescence activity was noticed.

Table 2. Recoveries of Anserine, Balenine, and Carnosine from a Spiked Cattle Feed

anserine (spiked at 1.11 ppm)			balenine (spiked at 2 ppm)			carnosine (spiked at 1.58 ppm)		
area	μg/20 g	recovery (%)	area	μg/20 g	recovery (%)	area	μg/20 g	recovery (%)
113917	14.12	63.7	127814	20.20	50.5	148628	26.49	83.6
106812	12.49	56.3	107186	16.64	41.6	146483	25.65	81.0
101441	11.68	52.7	105449	16.50	41.3	135496	23.92	75.5
109126	12.97	58.5	120103	18.71	46.8	147504	25.91	81.8
105796	12.51	56.4	118748	18.63	46.6	139060	24.61	77.7
116295	14.33	64.6	132582	20.73	51.8	132376	23.35	73.7
	mean	58.7			46.4			78.9
	SD	4.6			4.4			3.9
	VC%	7.9			9.4			4.9

Statistical Data of the Method. *(1) The linearity of the CFC reaction* was very good, tested by derivatization of different amounts of dipeptide standards (**Figure 3**).

(2) The reproducibility of the CFC reaction and the HPLC determination was tested by derivatization of the same anserine/carnosine standard eight times within 54 days. The between-days coefficient of variation (CV) of the anserine peak area was 5.03%, and the CV of the carnosine peak area was 4.63%. Besides the good reproducibility, these results indicate that the standard solution is stable during this period.

(3) The detection limit (DL) was calculated with the indirect method (calibration line method; determination of the area/height) according to the German standard for the determination of the DL named DIN 32 645 (*30*). The calculated DL for carnosine was 82 pg/20 μL of injection volume (calculated by area). With the described method (75 μL was applied to the PRS-SPE cartridge) 0.54 ppm of carnosine in feed was detectable. If the DL was calculated by carnosine peak height, it was determined to be 46 pg/20 μL of injection volume (corresponding to 0.3 ppm) (**Table 1**). With this DL the evidence of parts of animal origin in feeds in traces far below 0.5% seems to be possible.

(4) Recoveries of the dipeptides influenced by feed matrix were tested by spiking 20 g of cattle feed with low quantities of anserine, balenine, and carnosine ($n = 7$; **Table 2**). Seventy-five microliters of extract from the spiked samples was applied to the PRS-SPE cartridge. If higher volumes of sample extracts were applied (up to 500 μL), recoveries decreased to ∼30% (data not shown), caused by overloading of the cartridge. Nevertheless, in relation to other substances in the sample, a maximum of carnosine is bound. When the method is used for screening, a quantification of carnosine will not be necessary so that this fact does not make a difference. At screening it depends on a yes/no decission. A quantification of animal-originated parts by means of carnosine contents is not possible, anyway, because the composition (muscle, bone, and other tissues, etc.) of animal-originated compounds in feed is unknown. Therefore, the concentration of carnosine or the other dipeptides does not correlate with the amount of animal-originated adulteration. The carnosine recovery from spiked feeds was slightly lower compared to the recovery of standard solution mentioned above. However, results from five spiked feeds of different kinds (**Table 3**) confirm the acceptable recovery of carnosine found before (78.9%).

(5) The repeatability of the method was tested by analysis of the dipeptides in a piglet feed produced with meat bone meal ($n = 8$; 500 μL of extract applied to the PRS cartridge). The within-day CV of carnosine area was 3.77% (**Table 4**). Because the area of balenine was lower, its CV was slightly higher but within acceptable values, too.

Table 3. Recoveries of Carnosine from Spiked Feeds

	carnosine (spiked at 3.37 ppm)		
feed	area	μg/20 g	recovery (%)
cattle feed	395395	70.14	104.2
cattle feed	377433	65.99	98.0
pig feed	380580	67.78	100.7
poultry feed	335744	59.22	88.0
poultry feed	389405	69.13	102.7

Table 4. Determination of Anserine, Balenine, and Carnosine in a Piglet Feed Produced with Bone Meal

anserine		balenine		carnosine	
area	ppm	area	ppm	area	ppm
7876784	8.63	870907	0.72	9812633	10.4
7862869	8.60	80391	0.70	10355537	11.0
8527286	9.39	874145	0.81	10730108	11.5
8189733	8.95	846811	0.70	10043235	10.6
8414882	9.25	899275	0.75	10588964	11.3
8069330	8.84	879361	0.73	9900333	10.5
8178120	8.95	849814	0.70	9963093	10.6
8378964	9.21	885148	0.74	10611447	11.3
mean	8.98		0.73		10.9
SD	0.29		0.04		0.41
VC%	3.22		5.15		3.77

Table 5. Contents of the Dipeptides in Feeds of Animal Origin

	n	anserine (ppm)	balenine (ppm)	carnosine (ppm)	carnosine/anserine
meat meal	14	131.5–862.4	29.0–100.8	555.5–1857.1	1.0–6.7
meat meal ?	1	497.0	nd[a]	9.4	0.02
meat bone meal	6	88.0–527.1	26.2–34.2	651.0–1088.3	2.1–9.0
fish meal	1	34.8	nd	6.2	0.2
feather meal	1	94.9	nd	21.2	0.2
blood meal	1	nd	nd	6.0	

[a] nd, not detectable.

Analysis of Carnosine and Related Peptides in Feeds. Twenty-three meat, meat bone, fish, feather, and blood meals were analyzed for their dipeptide contents (**Table 5**). In meat meal and bone meat meal concentrations of carnosine were higher or the same as those of anserine and much higher than those of balenine. Always, if balenine was detected in measurable concentrations, pork muscle was the cause. Other balenine sources such as whale and snake (*12*) could be excluded. According to the literature (*31*) the contents of the dipeptides in fish meal were lower and no balenine was found (**Figure 4**).

The contents in feather meal were amazing and could be explained by parts of muscle on the feathers. According to the

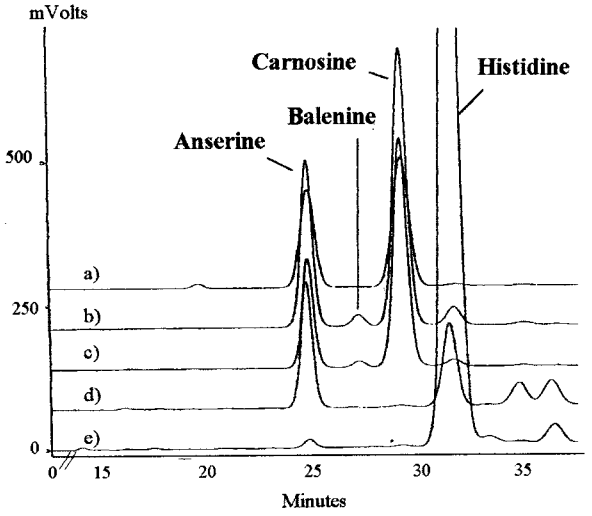

Figure 4. Comparison of HPLC runs of animal-originated feeds: (a) anserine/carnosine standard; (b) meat meal; (c) meat bone meal; (d) meat meal; (e) fish meal. Conditions were as described under Materials and Methods.

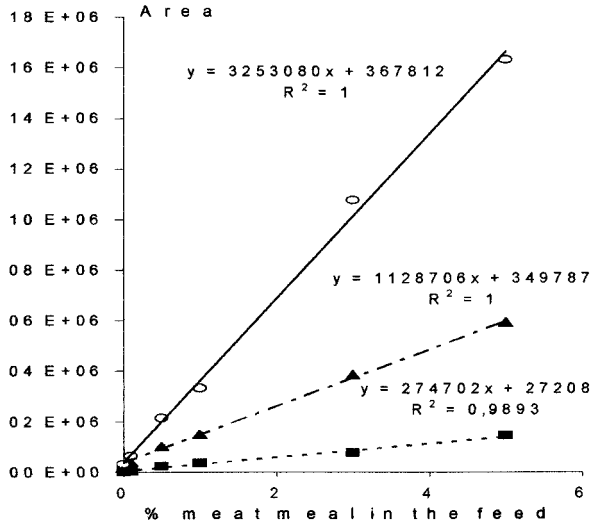

Figure 5. Contents of anserine, balenine, and carnosine in dependence on content of meat meal in defined mixed feeds: (—) carnosine; (– - –) anserine; (•••) balenine.

literature more anserine than carnosine was found in poultry (*10*, *11*). Because blood meal contains rests of blood plasma,the carnosine content is explainable thereby. The combination of carnosine amounts in feeds of animal origin and the DL mentioned above suggested that traces of animal-originated products (<0.5%) could be detected in compound feeds, too. To prove the relationships between contents of anserine, balenine, and carnosine and contents of products of animal origin, six compound feeds (from shredded extracted soybean, wheat, barley, mineral feed, and increasing parts of meat meal) were mixed. With increasing meat meal quantities the quantity of soybean was decreased proportionally to get the same protein content in each sample. One feed was mixed without meat meal (0-feed). Unfortunately, traces of animal origin (bones from land animals) were detected by the microscopic method in the 0-feed. For this reason, traces of anserine and carnosine were analyzed in the 0-feed, too. Nevertheless, our results have shown the dependence of the contents of anserine, balenine, and carnosine in the compound feed on the content of meat meal, as is shown in **Figure 5**.

The absence of the dipeptides in plants was proven by analysis of 15 samples of plant origin (shredded extracted rape, shredded extracted soybean, wheat and wheat bran, barley, oats, shredded extracted sunflower, shredded extracted palm kernel, maize, clover, pea, triticale, malt seed, beer yeast, and molasses shred). No anserine, balenine, or carnosine was found in any plant sample or in one analyzed mineral feed, too. More than 30 compound feeds for cattle, pig, and poultry were analyzed for their contents of the dipeptides. Some examples of HPLC runs are shown in **Figure 6**. At first a microscopic determination of animal-originated parts in all feeds was made. If there were found parts of animal origin (also in traces), a peak of carnosine was detected by the proposed HPLC method in each case. In some samples with a carnosine peak, anserine and balenine could be detected, too (Figure 6). In the reverse case the microscopy was negative each time if no carnosine was found by HPLC. These results showed that results analyzed by microscopy correspond to that received by the proposed HPLC method.

An HPLC method to analyze the contents of three dipeptides (anserine, balenine, and carnosine) in feeds was developed. These dipeptides occurs in high concentrations in animal muscle

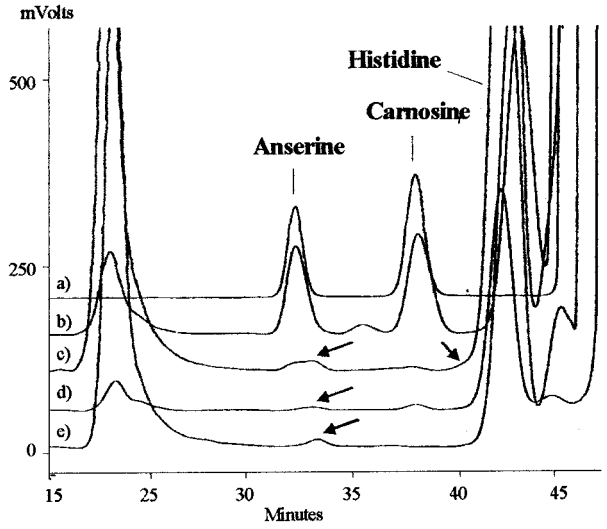

Figure 6. Comparison of HPLC runs of compound feeds: (a) anserine/carnosine standard; (b) piglet feed with meat bone meal; (c) cattle feed with fish meal; (d) cattle feed with traces of animal-originated parts (positive ion microscopy); (e) cattle feed without parts of animal origin (negative ion microscopy). The impurity peaks from the matrix are marked by an arrow. Conditions were as described under Materials and Methods.

and in other animal tissue, too. In the present study it has been shown that contents of the dipeptides in feeds were correlated with contents of meat meal in feeds and that the dipeptides are absent from plant material. Every laboratory with analytical equipment (HPLC) is able to apply the method. Compared with the microscopy, the new method is less time-consuming, particularly if many samples must be examined. The method is suitable for the analysis of >20 samples within a day (and a night) by one person. The proposed HPLC method sensitively detects dipeptides from the muscle part of animal-originated products in feeds. By the micoscopic method the recognition of muscles is more difficult than that of bone particles. Therefore, the presented method could be used as a screening method and can complete and confirm the statement of the microscopic method for evidence of parts of animal origin in feeds.

ABBREVIATIONS USED

CFC, carbazole-9-carbonyl chloride; CV, coefficient of variation; DL, detection limit; EDTA, ethylenediaminetetraacetic acid; LC-MS, liquid chromatography−mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MDPF, 2-methoxy-2,4-diphenyl-3(*2H*)-furanone; OPA, *o*-phthalaldehyde; PCR, Polymerase Chain Reaction; PITC, phenylisothiocyanate; PRS-SPE, propanesulfonic acid solid-phase extraction; SSA, sulfosalicylic acid.

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