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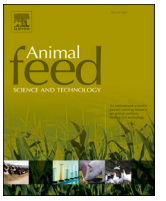

Feasibility of using free bioactive amines and amino acids for quality assessment and discrimination of animal meals

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ABSTRACT

The meat industry generates loads of residues, and its efficient utilization as animal meals is important from the environmental, economic, nutritional and safety points of view. The objective of this study was to investigate free amino acids and amines by ultra-performance liquid chromatography – UPLC™ in different types of animal meals and the feasibility of using them as quality and differentiation indices. Overall, 48 samples were analyzed; four types of meal [Feather meals (FM), meat & bone meals - bovine (MBM), pork/swine meals (SM), and Poultry meal (PM)] from 4 rendering plants; 3 different lots each). FM had higher protein and lower fat, whereas MBM) had higher ash, Ca and P ($P < 0.05$). Mercury was found in every sample (≤0.2 mg/kg). Total free amino acids were higher in MBM, and FM compared to the others ($P < 0.05$). FM had higher methionine, aspartic acid, and asparagine; SM had higher histidine ($P < 0.05$), which was not detected in MBM and FM. Phenylalanine was only found in MBM. Umami amino acids were prevalent in MBM; sweet amino acids in SM and FM; and bitter amino acids in SM and FM, thereby affecting palatability. PM had the widest diversity of amines (8), whereas MBM had the lowest (4). Higher amines levels were found in PM, and SM had the least ($P < 0.05$). Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) differentiated FM from MBM and from SM+PM, mainly by free amino acids and amines. The rendering plant D did not fit into any of the clusters, suggesting the poorest quality. Free amino acids and amines may be used as authenticity and quality indices for animal meals and rendering plants.

Abbreviations: FM, feather meals; HCA, Hierarchical Cluster Analysis; MBM, meat & bone meals; PCA, Principal Component Analysis; PM, poultry meals; SM, swine (pork) meals; TCA, trichloroacetic acid; UPLC, ultra-performance liquid chromatography; UV, ultraviolet.

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1. Introduction

Meat industries generate a large volume of by- and co-products (35–50% live weight) which are not prone for human consumption and require high costs to be treated and disposed of ecologically (Meeker, 2009; Toldrá et al., 2016; ABRA, 2021). The recycling of these by-products fits with sustainable agriculture (2030 United Nations' Agenda) by their transformation into valuable and nutritious ingredients or supplements for poultry, swine, aquaculture, and companion animals feed (Meeker, 2009; Poolsawat et al., 2021).

Animal meals are highly valued, rich in energy, protein, essential amino acids, B vitamins and minerals (Meeker, 2009; Jayathilakan et al., 2012; Toldrá et al., 2016; Callegaro et al., 2019; Sabbagh et al., 2019; Poolsawat et al., 2021). The high levels and quality of the protein represent an interesting protein source in animal feed, rich in essential amino acids and without antinutritional factors (Toldrá et al., 2016; Sabbagh et al., 2019). The raw materials for animal meals can be comprised of tissues, bones, feathers, depending on local traditions and specific legislations (Toldrá et al., 2016). Their processing, called rendering, involves heat and pressure treatments (115–145 ℃/40–90 min), for the inactivation of enzymes, bacteria, viruses, protozoa, and parasites, followed by fat removal and drying (den Brinker et al., 2003; Meeker, 2009; Feddern et al., 2019). Meat, meat & bone, poultry, hydrolyzed feather, blood, and fish meals, and animal fats are the primary products resulting from the rendering process.

Knowledge of the chemical composition of animal meals is important for the formulation of feed (Toldrá et al., 2016; Zinina et al., 2019). Information on protein, fat, ash, moisture, calcium, and phosphorus contents are available and must meet standards which are specific for each type of meal. They must also meet standards of quality and safety (Brasil, 2008).

The levels of total amino acids (free + protein) in animal meals are described in the literature (Toldrá et al., 2016; Zinina et al., 2019); however, scarce information is available regarding the occurrence and levels of free amino acids and free bioactive amines in animal meals. Free amino acids are naturally present or can result from proteolytic activity and heat treatment during processing (Piazza and Garcia, 2014; Toldrá et al., 2016). Free amino acids in the meals provide readily essential amino acids, required for the animals' nutrition and health. In addition, they contribute with umami, sweet or bitter taste to the feed, thereby affecting its acceptability (Dala-Paula et al., 2021). However, free amino acids can be decarboxylated by microbial enzymes or during heat treatment forming bioactive amines (Feddern et al., 2019). The presence of some amines in animal meals, e.g., spermine and spermidine, is desirable due to their relevance in animals' growth and health (Salazar et al., 2000; Dala-Paula et al., 2021). However, certain amines (histamine, tyramine) at high levels can be detrimental to the animals' health (Feddern et al., 2019). In addition, bioactive amines can be used as reliable food quality indexes from several points of view, including identity, nutritional, sanitary, and safety (Alvarez and Moreno-Arribas, 2014; Feddern et al., 2019; Wójcik et al., 2020; Dala-Paula et al., 2021). There is scarce information on the occurrence and levels of bioactive amines in animal meals. Therefore, the objective of this study was to investigate the profile and levels of free amino acids and bioactive amines in four different types of animal meals. Their role in the identity, quality and safety of animal meals was also investigated.

2. Material and methods

2.1. Material

2.1.1. Reagents

The reagents were of analytical grade, except ultra-performance liquid chromatography (UPLC) solvents which were liquid chromatography (LC) grade. Ultrapure water was from Milli-Q™ (Millipore Corp., Milford, MA, USA). Standards for bioactive amines (agmatine sulfate, cadaverine dihydrochloride, histamine dihydrochloride, 2-phenylethylamine hydrochloride, putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, serotonin hydrochloride, tryptamine, tyramine hydrochloride) and L-amino acids (alanine, arginine hydrochloride, aspartic acid, asparagine, cystine, glycine, glutamic acid, glutamine, histidine hydrochloride, isoleucine, leucine, lysine hydrochloride, methionine, norvaline, phenylalanine, proline, serine, threonine, tyrosine, valine) were from Sigma Chemical Co. (St. Louis, MO, USA). AccQ.Fluor™ pre-column derivatization kit was from Waters (Milford, MA, USA).

Trichloroacetic acid (TCA) was from Êxodo Química (Sumaré, SP, Brazil). Mercury (for Inductively Coupled Plasma – ICP, 99.8%) and acetonitrile (Chromasolv®, HPLC grade, 99.9%) were from Sigma-Aldrich (St. Louis, MO, USA).

2.1.2. Samples

Animal meals – poultry, pork (swine), meat & bone (bovine), and hydrolyzed poultry feathers – were obtained at three different production days in April of 2018 from different rendering plants in the South of Brazil. The plants operate under federal inspection and are responsible for the slaughter and industrialization of poultry, cattle, and pork. Overall, twelve rendering plants were involved (A to L) and the animal meals were from specific plants: poultry meals were from plants C, D, E and F; swine meals from A, B, C and D; meat & bone meals from I, J, K and L; and hydrolyzed feather meals from plants C, D, G and H. Therefore, 12 samples of each type of meal were analyzed (3 different days x 4 different plants, each), in a total of 48 samples analyzed in duplicates. The samples were taken from the processing line, packed in 200 g polyethylene bags, sealed, and sent immediately to the laboratory for analysis.

The samples were analyzed for free amino acids and bioactive amines. In addition, they were analyzed according to standards of identity (proximate composition, calcium, and phosphorus), and quality, e.g., *Salmonella* and mercury (safety), and peroxide value and titratable acidity (biochemical stability).

2.2. Methods of analysis

2.2.1. Determination of free amino acids and bioactive amines

Free amino acids and bioactive amines were determined after extraction, pre-column derivatization and analysis by reverse phase UPLC™ (Moreira et al., 2018; Reis et al., 2020). Briefly, 0.4 g of animal meal was extracted three consecutive times with 7 mL 5.0% TCA by shaking at 150 rpm for 5 min (orbital shaker TE-140, Tecnal, SP, Brazil), centrifugation at 11,180 x *g* at 4 °C for 21 min (Jouan SA MR23i, Saint Herblain, France) and filtration through qualitative filter paper (12.5 cm diameter, 80 g, 44 μm, Synth, Diadema, SP, Brasil). The supernatants were combined and brought to volume (25 mL). Norvaline was used as internal standard at 25 picomol *in column*. The extracts were neutralized (pH 7–8) with NaOH and AccQ.Fluor™ derivatized. The final extract was filtered through 0.22 μm HAWP pore size membranes (Millipore Corp., Milford, MA, USA) immediately prior to UPLC™ analysis.

The chromatographic analyzes of the AccQ derivatives were performed using the Waters Acquity Ultra Performance LC (UPLC™) System (Waters, Milford, MA, USA) equipped with an ultraviolet (UV) detector at 249 nm using Acquity UPLC® column (BEH C18, 50 × 2.1 mm, 1.7 μm). A gradient elution of (A) 0.1 mol/L sodium acetate in ultrapure water (pH 4.80) and (B) acetonitrile was used at a flow rate of 1.0 mL/min: initial 2.5 min/0–0% B; 2.5–4.0 min/0–3% B; 4.0–9.0 min/3–30% B; 9.0–9.5 min/30–100% B; 9.5–10.0 min/100–100% B; 10.0–10.5 min/100–0% B and equilibration at the initial conditions for an additional 1.5 min (12 min total run). The column temperature was 35 °C. The amino acids and amines were identified by comparison of retention times with standards and by spiking samples with standards. The levels of amino acids and amines were determined by interpolation in external calibration curves (9 concentrations – 0.1–12 mg/L) of nineteen amino acids, ammonia and nine amines ($r^2 \geq 0.9989$). The limit of quantification (LOQ) of the method varied from 2.2 to 13.1 mg/kg for amino acids and from 2.6 to 18.8 mg/kg for amines. The method was validated, and it was fit for the purpose for the analysis of nine amines (spermidine, agmatine, putrescine, cadaverine, histamine, tyramine, phenylethylamine, tryptamine, serotonin), 19 amino acids (aspartic acid, alanine, arginine, asparagine, cystine, glycine, glutamine+glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine) and ammonia.

The amino acids were also classified based on four categories: essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine); umami (aspartic acid, glutamic acid); sweet (alanine, glycine, proline, serine, threonine); and bitter (arginine, histidine, isoleucine, leucine, methionine, phenylalanine, valine).

2.2.2. Determination of the standards of identity and quality

The samples were analyzed for the parameters established by the Brazilian legislation (Brasil, 2008): contents of moisture, crude protein, fat, ash, calcium, and phosphorus. Near InfraRed Spectroscopy – NIRS was used for the analysis of these components following the official Association of Official Analytical Chemists (AOAC) method 2007.04 (Anderson, 2007). Water activity was analyzed directly using an Aqua-lab Decagon water activity measuring system (DirectIndustry, São Paulo, SP, Brasil). The peroxide value and total titratable acidity were determined according to the Brazilian Compendium of Animal Feed (BCAF, 2017), respectively, ISO 3960

Table 1
Parameters of identity, stability and safety of four types of animal meals – poultry, pork, meat & bone and hydrolyzed feather – from different rendering plants.

Parameters	Contents [mean ± standard deviation (range)] / Types of meal			
(wet weight basis)	Poultry	Pork	Meat & bone	Feather
Identity				
Moisture (g/kg)	40.1 ± 10.5^{c} (23.5–52.6)	41.5 ± 14.0^{bc} (23.9–68.7)	55.5 ± 13.2^{ab} (32.7–75.4)	69.4 ± 13.8^{a} (45.5–84.5)
Crude protein (g/kg)	591.5 ± 28.5^{b} (553.0–635.0)	554.1 ± 19.9^{c} (521.7–584.4)	478.2 ± 24.8^{d} (448.3–533.2)	890.3 ± 34.5^{a} (835.4–925.5)
Fat (g/kg)	140.2 ± 11.0^{a} (123.7–156.4)	130.7 ± 22.4^{a} (108.4–175.4)	104.4 ± 18.9^{ab} (80.1–145.3)	68.8 ± 19.3^{b} (42.2–104.1)
Ash (g/kg)	181.2 ± 21.2^{c} (157.4–221.6)	293.2 ± 20.7^{b} (258.7–330.9)	402.1 ± 32.0^{a} (324.8–436.1)	30.5 ± 6.2^{d} (22.9–42.1)
Minerals (g/kg)				
Calcium (Ca)	55.7 ± 9.7^{c} (35.3–70.6)	98.8 ± 11.4^{b} (80.9–115.2)	141.0 ± 13.3^{a} (109.7–156.0)	4.6 ± 2.7^{d} (2.3–9.8)
Phosphorus (P)	29.8 ± 2.6^{c} (26.0–35.4)	50.7 ± 5.2^{b} (40.6–57.9)	69.7 ± 6.4^{a} (56.9–79.9)	4.2 ± 1.2^{d} (3.1–6.7)
Ca/P	18.7 ± 2.2^{a} (12.7–21.8)	19.5 ± 1.0^{a} (17.5–21.0)	20.2 ± 0.5^{a} (19.3–20.8)	10.3 ± 2.8^{b} (6.6–14.8)
Stability				
Water activity	0.477 ± 0.019^{b} (0.441–0.511)	0.481 ± 0.054^{b} (0.417–0.563)	0.547 ± 0.130^{ab} (0.459–0.947)	0.577 ± 0.017^{a} (0.549–0.600)
Peroxide value (meq/kg)	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Total titratable acidity (mg NaOH/g)	1.31 ± 0.63^{a} (0.30–2.35)	1.38 ± 0.60^{a} (0.59–2.38)	0.79 ± 0.70^{a} (nd-2.21)	1.01 ± 0.33^{a} (0.52–1.56)
Safety				
Salmonella spp. (cfu/25 g)	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Total mercury (mg/kg)	0.08 ± 0.04^{a} (0.02–0.14)	0.05 ± 0.05^{a} (0.01–0.17)	0.09 ± 0.05^{a} (0.01–0.18)	0.08 ± 0.05^{a} (0.03–0.21)

n = 12, each type of animal meal.
Range = minimum-maximum values.
Mean values ± standard deviation with different superscripts in the same line are statistically different (Tukey test, $P \leq 0.05$).

(ISO, 2017a) and 016/IV Instituto Adolfo Lutz (IAL, 2008).

The presence of motile *Salmonella spp*. in 25 g sample was investigated (method 6579–1, ISO, 2017b) using the modified semi-solid Rappaport-Vassiliadis (MSRV) agar (Oxoid Brasil, São Paulo, SP, Brazil). The method included Salmonella detection, enumeration, and serotyping.

Total mercury was determined using a Direct Mercury Analyzer® (DMA-80, Milestone, Sorisole, Italy) according to USEPA (2007). The samples were homogenized, and 10–100 mg samples were weighed into nickel boats which were previously cleaned according to equipment guidelines. Compressed air was used as combustion and carrier gas; and quartz boats were used for standards. The drying and decomposition temperatures/times were 250 ºC/150 s and 650 ºC/60 s, respectively. The amalgamator heating time was 12 s, and absorbance readings were at 253.7 nm. The contents of total mercury (mg/kg fresh weight) were quantified by interpolation in analytical curves (1.0–8.5 ng, $r^2 \geq 0.9962$). A blank (empty sample boat) was analyzed periodically to make sure that mercury was not carried over between samples.

2.3. Statistical analysis

The experiment included 48 animal meal samples (4 types, 4 rendering plants, 3 sampling days), analyzed in duplicate. The results were submitted to the Ryan Joiner normality test. Means of parametric data (standards of identity and quality) were compared by the Tukey test, whereas medians of non-parametric results (bioactive amines and amino acids) were compared by the Kruskal Wallis test

Table 2
Median levels (range) of free amino acids in four types of animal meal – poultry, pork, meat & bone, and hydrolyzed feather – from different rendering plants.

Amino acid	Median levels (min-max) in mg/kg wet weight basis/ Meal type			
	Poultry	Pork	Meat & bone	Feather
Aspartic acid[1]	0[b] (nd–489)	0[b] (nd)	0[b] (nd)	1138[a] (nd–3662)
Alanine[2]	253[b] (52–409)	214[b] (nd–797)	1503[a] (nd–3423)	159[b] (nd–280)
Arginine[3]	2555[a] (412–5065)	1876[a] (731–2748)	2763[a] (1366–4849)	1667[a] (727–3251)
Asparagine	490[ab] (315–647)	398[b] (nd–14969)	0[bc] (nd–3116)	780[a] (472–85051)
Cystine	0[b] (nd)	408[a] (283–502)	0[b] (nd–2514)	0[b] (nd–482)
Glycine[2]	899[a] (562–2510)	1180[a] (505–4246)	0[b] (nd–6312)	2915[a] (nd–10597)
Glutamine+glutamic acid[1]	2683[ab] (1400–3870)	1178[b] (304–2131)	5555[a] (nd–19628)	1786[b] (136–4528)
Histidine[3] *	0[b] (nd–495)	248[a] (149–3267)	0[b] (nd)	0[b] (nd)
Isoleucine[3] *	263[b] (209–532)	415[a] (365–639)	521[a] (366–882)	522[a] (401–1212)
Leucine[3] *	128[b] (100–194)	194[b] (165–433)	188[b] (nd–267)	285[a] (231–592)
Lysine*	117[b] (97–223)	156[b] (87–916)	180[ab] (nd–325)	204[a] (185–463)
Methionine[3] *	2070[b] (537–2800)	269[c] (113–2130)	3137[b] (193–11770)	6788[a] (2664–15321)
Phenylalanine[3] *	0[a] (nd)	0[a] (nd–363)	0[a] (nd)	0[a] (nd)
Proline[2]	297[a] (191–331)	0[b] (nd)	382[a] (nd–588)	456[a] (150–2157)
Serine[2]	1648[c] (1338–2661)	2377[bc] (732–4147)	3719[a] (2090–5878)	3045[ab] (2002–7772)
Threonine[2] *	808[ab] (358–1531)	1121[ab] (582–4223)	454[b] (nd–2501)	1210[a] (569–3818)
Tyrosine	188[b] (161–412)	573[a] (286–6900)	579[a] (nd–1574)	580[a] (nd–1110)
Valine[3] *	265[c] (222–501)	1545[a] (398–3726)	734[ab] (617–8139)	430[bc] (387–952)
Total	11,657[b] (9114–18,928)	12,810[b] (5219–39,402)	29,092[a] (11,151–36,247)	33,931[a] (16,350–123,396)
NH_3	28[ab] (nd–41)	3[b] (nd–95)	125[a] (nd–410)	48[a] (35–134)

n = 12.
nd = not detected. Tryptophan was not analyzed.
Median values (calculated using nd=0) with different superscripts in the same line are significantly different (Kruskal-Wallis test, $P \leq 0.05$).
[1] umami; [2] sweet; [3] bitter; * essential amino acids.

(P = 0.05) using Minitab (2022). Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were used for the characterization and differentiation of the meat meals. The dataset consisted of 16 rows [4 meat meals (3 sampling days) from 4 rendering plants)] and 40 columns [identity and quality parameters, contents of individual free amino acids and amines, ammonia, total amines, total amino acids]. PCA and HCA analysis were performed using MatLab (2022).

3. Results

3.1. Standards of identity and quality

The animal meals had the following characteristics (Table 1): moisture < 100 g/kg for feather meal and < 80 g/kg for the other meals; and protein > 450 g/kg for meat & bone, > 460 g/kg for pork, > 550 g/kg for poultry and > 800 g/kg for feather meals. Meat & bone meals had mean calcium levels higher than 50 g/kg. *Salmonella spp*. was not present in any sample. Mercury was detected in every sample with no significant difference (P > 0.05) among types of meals. The water activity was low (≤ 0.578). Total titratable acidity was low (≤ 1.4 mg NaOH/g) and no peroxide was detected.

3.2. Profile and levels of free amino acids

The profile and levels of free amino acids in the different types of animal meals are given in Table 2. Glutamine and glutamic acid were not well resolved in the UPLC™ run; therefore, results were expressed as the sum of both. Sixteen free amino acids were detected in feather, poultry, and meat & bone meals, whereas pork meals had 15. Tryptophan was not detected in any sample. Phenylalanine was only detected in meat & bone meals. Aspartic acid was not detected in pork, and meat & bone meals; cystine was not detected in poultry meal; and proline was not detected in pork meal.

The free amino acids results followed non-parametric statistics; reason why median values were compared by the Kruskal-Wallis test. The median levels of free amino acids (P < 0.05) in feather, and meat & bone meals (~30,000 mg/kg), were approximately 2.5-fold higher than those in poultry and pork meals (~12,000 mg/kg). The different meals had similar median levels of arginine and glycine (P > 0.05). Feather meals had significantly higher median levels of methionine compared to the others. Meat & bone, and feather meals had higher median levels of lysine and serine. Pork meal had higher median levels of cystine, and histidine compared to the others.

When considering the contribution of each free amino acid to the total levels of free amino acids, poultry meals were characterized by high contribution of arginine and glutamine+glutamic acid (21% of total, each), followed by methionine (16%) and serine (13%). The prevalent amino acid in pork meals was serine (20%), followed by arginine (15%), valine (13%), and glycine and glutamine+glutamic acid (10%, each). Glutamine+glutamic acid were the predominant free amino acids in meat & bone meals (28%), followed by serine (19%) and by arginine and methionine (15%, each). In feather meals, methionine was the prevalent free amino acid (31%) followed by glycine and serine (13%, each).

All essential amino acids (Table 2), except tryptophan, were present in the meals. Histidine was only present in poultry and pork

Table 3
Median levels (range) of free bioactive amines in four types of animal meals – poultry, pork, meat & bone and hydrolyzed feather – from different rendering plants.

Amines	Median levels (min-max) in mg/kg wet weight basis/ Meal type			
	Poultry	Pork	Meat & bone	Feather
Spermidine	17.32[a] (nd–54.30)	4.01[b] (nd–26.14)	nd[b] (nd–4.99)	3.58[b] (nd–10.37)
Agmatine	1.39[a] (nd–13.00)	0.00[b] (nd–2.69)	nd[b]	0.80[a] (nd–2.90)
Putrescine	15.15[a] (1.55–182.43)	8.78[a] (0.85–18.60)	3.09[b] (nd – 72.15)	3.05[b] (nd–27.74)
Cadaverine	17.82[a] (3.27–223.75)	10.35[a] (2.97–43.15)	3.29[b] (nd –55.92)	4.00[b] (nd–11.02)
Histamine	4.60[a] (nd–23.09)	0.00[b] (nd–6.35)	0.00[b] (nd)	0.00[b] (nd–6.54)
Tyramine	8.49[a] (5.91–122.22)	20.56[a] (1.86–42.05)	11.05[a] (nd–24.83)	2.30[b] (nd–5.16)
Phenylethylamine	0.78[a] (nd–7.58)	0.00[a] (nd)	0.00[a] (nd)	0.00[a] (nd–2.40)
Tryptamine	22.57[a] (nd–65.39)	0.00[b] (nd)	0.00[b] (nd)	0.00[b] (nd)
Total	107.54[a] (15.08–668.69)	53.13[a] (8.86–101.64)	22.52[b] (nd–133.79)	18.03[b] (nd–49.28)

n = 12.
nd = not detected.
Median values (calculated using nd = 0) with different superscripts in the same line are significantly different (Kruskal-Wallis test, P ≤ 0.05).
Serotonin was not detected in any sample; Spermine was not analyzed.

meals; phenylalanine was only present in meat & bone meals; and tryptophan was not detected (Table 2). Among tasteful amino acids, the bitter ones were predominant in all meat meals, representing approximately 45% of the amino acids. Sweet amino acids were prevalent in pork meals (44%), followed by feather (39%), poultry (35%) and meat & bone (30%) meals. Umami amino acids were predominant in meat & bone meals (28%), followed by poultry (21%), feather (13%) and pork (10%). Ammonia was also detected in some meals, with higher median levels in meat & bone and feather meals compared to pork meals, whereas poultry meals showed intermediate levels.

3.3. Profile and levels of free bioactive amines

Spermidine, putrescine, cadaverine, and tyramine were present in all types of meals (Table 3). Serotonin was not detected in any sample and spermine was not quantified due to analytical conditions. Poultry meals had the largest diversity of amines (8), whereas meat & bone meals had the least (4).

Free bioactive amines results followed non-parametric statistics; therefore, medians were compared by the Kruskal-Wallis test. There was variability on the levels of amines in the meals. Higher total levels of free amines ($P < 0.05$) were found in poultry (107.54 mg/kg), followed by pork (53.13 mg/kg) and by meat & bone and feather meals (~20 mg/kg). Tyramine was the prevalent amine in meat & bone (63%) and pork meals (47%). However, in poultry and feather meals, several amines contributed in a similar way to total levels ($\leq$ 29%).

3.4. Discrimination of animal meals by multivariate analysis

In the Principal Components Analysis (PCA), the first two principal components, PC1 and PC2, were analyzed. PC1 explained 27.9% of X variance and it was possible to segregate feather (F) meals (negative PC1 values) from the other meals – poultry, pork and meat & bone (near 0 to positive PC1 values) (Fig. 1a). PC2 explained 21.5% of the total X variance, and mostly explained the difference between feather (F) and meat & bone (MB) meals (Fig. 1a). The Hierarchical cluster analysis (HCA) dendogram (Fig. 2) confirmed PCA

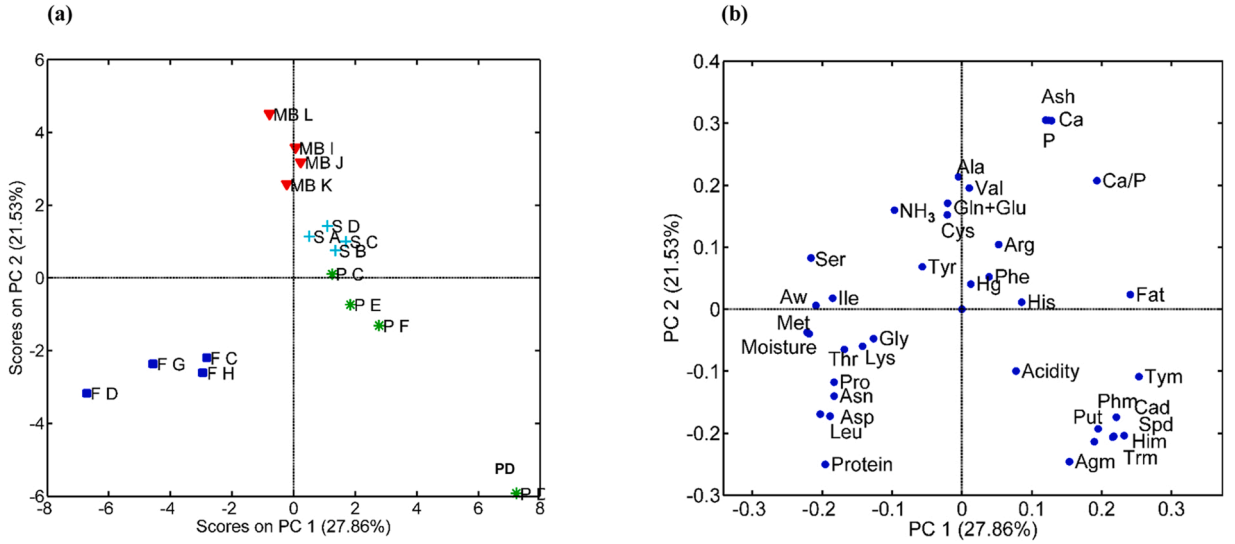

Fig. 1. Score (a) and loading (b) plots, obtained by Principal Component Analyses (PCA), for the means of proximate analysis (moisture, protein, fat, ash, calcium, phosphorus, water activity, acidity) and median levels of free amino acids and bioactive amines in four types of animal meals from different rendering plants. Based on Fig. 1a, we can see three major groups when analyzing all the parameters included in this study for the four different types of animal meal. Feather meals (F) from the rendering plants C, D, G & H are separated in the lower left quadrant and, thereby, show similar characteristics. When looking at the same quadrant in Fig. 1b, we can see the components which affect feather meals the most, including methionine, lysine, protein, moisture contents. However, FD and FG were further away at different distances from the others (FC & FH), indicating that these two plants provided somewhat different products. On the other hand, meat & bone meal (MB) from plants I, J, K & L are in the upper central part of the graphic (Fig. 1a) and are mainly affected by Ash, Ca, P, Ca/P, and some amino acids like alanine (Fig. 1b). Pork (S) and poultry (P) meals from the different plants did not separate well from each other (Fig. 1a), indicating that the same parameters (some amino acids and amines) affect both types of meals, and, therefore, they cannot be distinguished by these parameters. As observed for feather meal from plant D (FD), sample PD was located at a distance from the other poultry meal samples, indicating that, poultry meal from plant D had higher levels of amines, which could suggest poorest quality compared to the others. [F: Feather (C, D, G, H); MB: Meat & bone (I, J, K, L); S: Pork (Swine) (A, B, C, D); P: Poultry (D, E, C, F); Free amino acids: Ala - alanine, Arg – arginine, Asn – asparagine, Asp – aspartic acid, Gln – glutamine, Glu – glutamic acid, Gly – glycine, His – histidine, Ile - isoleucine, Leu – leucine, Lys – lysine, Met – methionine, Phe – phenylalanine, Pro – proline, Ser – serine, Thr – threonine, Tyr – tyrosine, Val – valine; NH_3 – ammonia; Bioactive amines, Agm – agmatine Cad – cadaverine, Him – histamine, Phm – 2-phenylethylamine, Put – putrescine, Spd – spermidine, Trm – Tryptamine, Tym – tyramine].

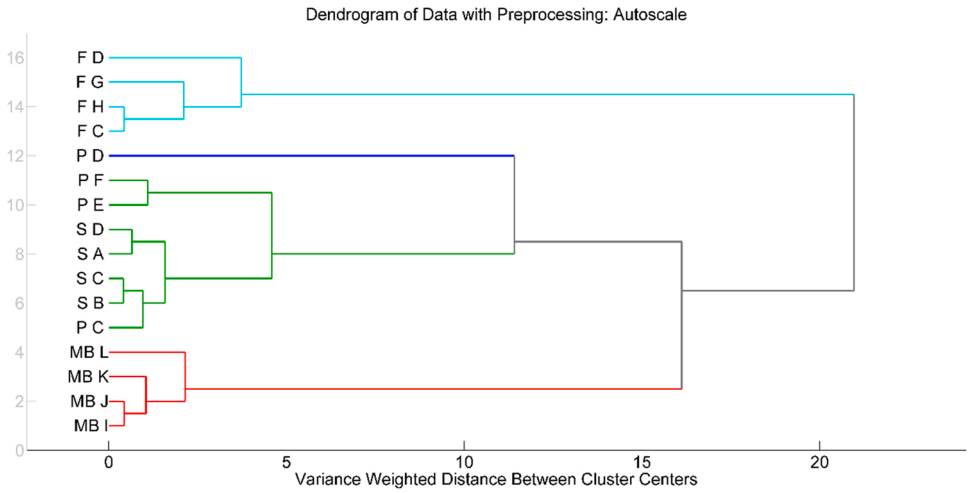

Fig. 2. Dendogram from Hierarchical Component (HCA) of animal meals from the different rendering plants. The HCA dendogram shows similarities among the four different meals (Meat and Bone, Poultry, Swine and Feather) from the different rendering plants. Like PCA results, HCA clustered the same three major groups, based on their similarities (weighted distance=5). However, an additional group was considered in HCA, the poultry meal from plant D (PD) which did not show similarity to the three main groups. The meals whose lines join together sooner are more similar. [Animal meals (rendering plants) – F: Feather (C, D, G, H); MB: Meat & bone (I, J, K, L); S: Pork (swine) (A, B, C, D); P: Poultry (D, E, C, F)].

results.

4. Discussion

4.1. Standards of identity and quality

Animal meals must comply with several criteria and conditions (Brasil, 2008): the raw material must originate from slaughterhouses under federal inspection; the use of some ingredients is forbidden, e.g., blood, stomach contents, feces, hooves, horn, hair, skin, fat; animal meals must be obtained by cooking, defatting, sterilizing, drying, grinding, and sifting. In addition, its use as feed for ruminant animals is prohibited. Among the animal meals included in this study, the ingredients allowed in poultry meal are deboned cuts, skin, and viscera (no bone, feather); in pork meal - deboned cuts and viscera; in meat & bone meal - carcass, meat, viscera and bones; and hydrolyzed feather meal - feathers from poultry slaughter submitted to cooking, hydrolysis under pressure and dehydration (Brasil, 2020).

The proximate composition (Table 1) of the meals complied with Brazilian legislation (Brasil, 2008), and are like literature values (Meeker, 2009; BCAF, 2017). Overall, animal meals were low in fat (60 – 120 g/kg), and rich in protein ($\geq$ 450 g/kg), especially feather meals which had 1.5–1.9 times higher protein compared to the others. Feathers have high protein (keratin) content but strong disulphide bonds; the reason why they must undergo hydrolysis, resulting in a more palatable and digestible protein source (Pacheco et al., 2016). The animal meals were source of calcium (60 – 130 g/kg), except feather meal. Meat & bone and pork meals were source of phosphorus (50 g/kg).

The absence of *Salmonella spp*. in the samples (Table 1), complied with legislation (Brasil, 2008). This assures the safe use of the meals as feed ingredients and can also suggest adequate processing (Meeker, 2009; Pulido-Landínez, 2019). Mercury, an important contaminant which could end up in agricultural animals, but seldomly investigated, was detected in every sample at similar levels among the types of meals ($P > 0.05$). Mean levels complied with the maximum tolerable limit in feed ingredients of 0.1 mg/kg (EFSA, 2008). However, some samples extrapolated the limit (8.3% feather, 16.7% poultry and pork, and 41.7% meat & bone meals); but it is likely that the final feed could comply with legislation taking into consideration the proportion of animal meal used. Inhalation of gaseous mercury from the ambient air, ingestion of contaminated water and contact with pesticides and fungicides can contribute to mercury exposure (EFSA, 2008).

The low water activity (Table 1) can prevent biochemical changes and microbial growth (Tapia et al., 2020). Low titratable acidity indicates that there was no contamination and bacteria growth in the raw material (Cypriano, 2016) or hydrolytic rancidity (Cypriano, 2016; BCAF, 2017). The absence of peroxide shows that lipid oxidation did not take place. In fact, antioxidants (BHT, 500 ppm) are added to the meals during processing to prevent oxidation and warrant a shelf life of 6 months. These results indicate that the animal meals were rendered under good manufacturing and hygienic conditions.

4.2. Profile and levels of free amino acids

To the best of our knowledge, this is the first report on free amino acids in animal meals. Tryptophan was not detected, probably

due to losses during analytical extraction (Moreira et al., 2018; Bellmaine et al., 2020). There was variability among results, perhaps due to differences on the proportions of raw materials, typical of animal meals processing (Meeker, 2009; Feddern et al., 2019). However, the differences in amino acids were evident among meals. The meals showed different profiles and levels of free amino acids, allowing applications for different purposes. For example, companion animals have unique dietary requirements, e.g., arginine for dogs and cats (Aldrich, 2008); therefore, all types of meals would be good sources of this amino acid. The prevalence of free amino acids varied with the type of meal: poultry – arginine and glutamine+glutamic acid; pork – serine; meat & bone – glutamine+glutamic acid; and feather – methionine. Poolsawat et al. (2021) found similar results for feather meals. Ammonia was detected in some meals; probably from the early stages of decomposition, providing additional evidence of raw material quality (Cypriano, 2016).

The animal meals were good sources of free essential amino acids, but histidine was only present in poultry and pork meals and phenylalanine in meat & bone meals. Therefore, when formulating animal feeds, one can choose a specific meal to obtain desirable characteristics. In addition, the type of meal can also be selected based on palatability by choosing free amino acids which contribute with bitter and sweet tastes or enhance meal taste with umami free amino acids (Dala-Paula et al., 2021). It is likely that by modulating free amino acids profile, the acceptability of the feed can be enhanced.

4.3. Profile and levels of free bioactive amines

This is the first study to report on the occurrence of nine amines simultaneously in four types of animal meals. Previous studies investigated only a few amines – putrescine, cadaverine and histamine (den Brinker et al., 2003) and phenylethylamine (Bedendo et al., 2018) in a limited number of samples. They found similar contents of these amines and high variability on amines levels ($\leq$ 70% coefficient of variation – CV – for all amines, except phenylethylamine (CV 135%). The high variability on amines levels in the meals is consistent with the possible variation in the proportion of ingredients (Meeker, 2009; Feddern et al., 2019).

Higher median spermidine levels ($P < 0.05$) were found in poultry meal ($\leq$54 mg/kg). Spermidine is relevant from the animal health and nutritional points of view. This amine is a growth factor (hormone like substance) and it is important in health maintenance (Gloria, 2006; Salazar et al., 2000). In addition, spermidine has antioxidant activity (Dala-Paula et al., 2021), also relevant for health and for the stability and longer shelf life of the meals.

Agmatine was present at higher levels ($P < 0.05$) in poultry and feather meals (1.39 and 0.80 mg/kg, respectively). Agmatine is produced from arginine decarboxylation. It is a central neurotransmitter. Therapeutic applications of agmatine have shown beneficial effects for treating depression, anxiety, neuropathic pain, cognitive decline and learning impairment, dependence on drugs, and metabolic diseases (diabetes and obesity) (Akasaka and Fujiwara, 2020). It also plays an important role in mammal feeding behavior (Lv et al., 2019), increasing feeding in a dose-dependent manner (Taksande et al., 2011).

Higher levels of putrescine and cadaverine ($P < 0.05$) were found in poultry ($\leq$15.15 and 17.82 mg/kg, respectively) and pork meals ($\leq$8.78 and 10.35 mg/kg, respectively). Putrescine, at low levels, is present in cells as a precursor of spermidine (Dala-Paula et al., 2020). However, at high levels it can be an index of spoilage. Cadaverine results from lysine decarboxylation, a reaction typical of Enterobacteria (Moreira et al., 2018). Therefore, high levels of these amines indicate the use of low-quality raw material or inadequate storage prior to processing (Montegiove et al., 2020). Putrescine and cadaverine, can impart a putrid flavor to the feed (Gloria, 2006). High levels of these amines in poultry meal have been reported previously (Salazar et al., 2000; Barnes et al., 2001; den Brinker et al., 2003).

Higher histamine levels ($P < 0.05$) were found in poultry meal (4.60 mg/kg), whereas higher tyramine ($P < 0.05$) was found in pork (20.56 mg/kg), meat & bone (11.05 mg/kg), and poultry meals (8.49 mg/kg) compared to feather meal. However, at high levels, these amines can suggest low quality raw material and/or inadequate storage prior to processing (Montegiove et al., 2020). In addition, histamine and tyramine, can be toxic depending on the amount present, and also the type of animal and health status, which may differ due to genetic reasons or illness (Feddern et al., 2019). The vasoactive and neuroactive amines phenylethylamine and tryptamine were present in poultry meals (0.78 and 22.57 mg/kg, respectively). Tryptamine was only detected in poultry meals, whereas phenylethylamine was sporadically found in poultry and feather meals. These amines play important roles in human health; however scarce information is available for animal health.

Amines in the diet are metabolized in the body by aminoxidases and converted into physiologically inactive products (Gloria, 2006; Wójcik et al., 2020). However, metabolizing enzymes can be inhibited in small animals by commonly used drugs (e.g., the antibiotic clavulanic acid and isoniazid) and by some detergents (Graig, 2019). Small intestine disorders can also decrease the synthesis of aminoxidases (Graig, 2019). Therefore, some amines, at high concentrations, can be harmful to some animals, and can limit the use of animal meals. For example, high levels of histamine and cadaverine in broiler feed can reduce body weight and feed conversion and increase carcass contamination from gastrointestinal rupture during processing. It can also increase the total number, incidence, and severity of gizzard erosion and proventricular ulcers, and can decrease the prevalence of gastric papillae (Barnes et al., 2001). In addition, high histamine levels can cause idiosyncratic reactions in histamine-sensitive cats (Graig, 2019) and regurgitation in Duroc pigs (Blonz and Olcott, 1978). Cadaverine, even at low levels, can potentiate histamine toxicity in guinea pig (Bjeldanes et al., 1978). Histamine can decrease arterial pressure, with larger effect in adult dogs (Privitera et al., 1969). Vasoactive amines can predispose dogs and cats to the development of allergic food reactions by lowering the tolerance threshold to certain food allergens (Graig, 2019; Montegiove et al., 2020). Tyramine can increase arterial pressure and heart rate in dogs (Privitera et al., 1969). Therefore, the knowledge of the profile and contents of free bioactive amines in animal meals is needed for adequate use in the feed to warrant animal's health.

4.4. Discrimination of animal meals by multivariate analysis

Multivariate statistics have been widely used as a tool to better separate multidisciplinary data and explore similarities and hidden patterns among samples (Moreira et al., 2018). Feather meals differed from the others mostly due to protein, moisture, water activity and the free amino acids serine, isoleucine, methionine, leucine, asparagine, aspartic acid, proline, threonine, lysine, and glycine (Fig. 1b). However, within the feather meals group, samples from rendering plant D (FD) had levels somewhat distant from the others (Fig. 1a), especially due to protein and to the free amino acids which are typical of feather. Sample FG also showed a different profile, intermediate to FD at one side, and both FC and FH at the other. Samples from rendering plant D also showed higher levels of free bioactive amines, suggesting that the quality of the raw materials or storage conditions were inadequate, which led to increased contents of amines.

Pork (S-swine) and poultry (P) meals were grouped near the center of the graph, which indicates that the measured parameters were not able to distinguish between these two meals. Meat & bone meals were highly influenced by calcium (Ca), phosphorus (P) and ash content, which can be related to the bone incorporated as raw material (Fig. 1b). They were also affected by some free amino acids – alanine, valine, glutamic acid+glutamine, and ammonia. Poultry meals from rendering plant D (PD), had a low PC2 value (Fig. 1a), mainly due to titratable acidity and contents of fat and free bioactive amines (all 8 amines), which could also suggest low quality raw materials or poor storage conditions.

The HCA dendogram (Fig. 2) clustered the meals in a similar way to PCA. The feather and the meat & bone meals were well characterized and grouped correctly by means of the parameters investigated, especially free amino acids and bioactive amines. HCA also confirmed that the parameters investigated were not able to differentiate poultry and pork meals, as most of the samples from this groups were considered similar.

Both the feather and poultry meals which showed an abnormal behavior were produced by the same rendering plant (D), which corroborates with the proposed theory of some flaws during processing or storage. Therefore, efforts should be undertaken by this rendering plant to revise procedures to improve the quality of the meals.

5. Conclusion

Free amino acids and bioactive amines were investigated for the first time in four types of animal meals from different rendering plants. Feather meals are rich in protein and low in fat, and meat & bone meals are rich in Ca and P. The meals showed good stability and were safe (Salmonella and mercury). In addition, each animal meal had its own profile of free amino acids and amines. This information can be useful for feed formulation to obtained readily available essential amino acids and palatability (bitter, sweet, umami). Amine's profile can be used in a similar way, to enhance desirable amines (spermidine, agmatine) and minimize those that can affect the flavor (putrescine and cadaverine), or that can cause adverse effect to the animal's health (histamine and tyramine). Free amino acids and amines are useful index of authenticity of animal meals (feather x meat & bone x poultry and pork). Ammonia, putrescine, cadaverine, histamine and tyramine can be used as an index of quality and safety, indicating poor quality of raw material or hygienic conditions during the production of animal meal. These parameters are also useful to attest the quality of animal meals and rendering plants.

CRediT authorship contribution statement

Douglas Evangelista Braga: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. **Audecir Giombelli**: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Writing – review & editing. **Bruno Gonçalves Botelho**: Data curation, Methodology, Resources, Validation, Visualization, Writing – original draft. **José Eduardo Gonçalves**: Data curation, Formal analysis, Methodology, Validation, Writing – review & editing. **Maria Beatriz A. Gloria**: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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