# Insights into the Potential of Pre-Processing of Ingredients to Improve their Economical Value to Aquaculture Species

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## Abstract

Processed animal protein ingredients are valuable ingredients for aquaculture feed formulations. However, the variability of the chemical composition of different batches of these ingredients and the relatively low digestibility of some of the nutrients (i.e. amino acids, phosphorus) occasionally represent significant limitations for these ingredients at high levels in the diet of some species.

Research efforts carried out at the University of Guelph explored the potential of simple and potentially cost-effective processing techniques to improve the digestibility and nutritive value of these ingredients. Processing aimed at improving digestibility of phosphorus showed that incubation with different organic acids and a chelating agent and fine grinding significantly improved *in vitro* bio-availability of bone phosphorus of high ash poultry by-products meal. However, this processing offered no advantage in terms of *in vivo* digestibility of phosphorus to rainbow trout, a species with an acid stomach. The technique may be useful for species lacking an acid stomach (e.g. carps, shrimp) but this hypothesis has not been verified. In another series of research efforts, incubation of feather meals with protease and a reducing agent, aiming to disrupt residual disulphide bonds and cross-linkage of keratin, significantly improved *in vivo* digestibility of protein and amino acids and bio-availability of arginine of this ingredient to rainbow trout.

The results illustrate the potential of simple processing techniques, based on sound chemical principles, to improve the bio-availability of nutrients of processed animal protein ingredients. However, careful animal assays need to be carried out to confirm the usefulness of these techniques in different species.

Keywords: Processing, ingredient, protein, phosphorus, fish

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## 1. **Introduction**

The aquaculture feed industry is one of the fastest growing animal feed industries in the world. It currently represents about 40 million metric tons (MMT) market which is expected to increase significantly over the next decades. Aquaculture feeds are the most expensive animal feeds available on the market and generally require the use of relatively high quality ingredients. There has been numerous calls to make better use of common by-products or under-valorized waste streams from the agriculture, food processing and industrial sectors in aquaculture feeds to improve sustainability of the aquaculture industry. Simple processing techniques could be used to improve the nutritive value and address the various limitations of by-products from various industries and ensuring that these adequately fit the needs of the different sectors of the aquaculture feed industry (e.g. high value feeds for highly carnivorous fish species vs. lower value feeds for omnivorous fish).

The rendering industry produce high quality feed ingredients, such as meat and bone meal, poultry by-products meal, feather meal and blood meal, from a variety of by-products from the animal agriculture and meat processing sectors. These ingredients are finding wide use in aquaculture feed formulations worldwide. The variability of the chemical composition of different batches of these ingredients and the relatively low digestibility of some of the nutrients (i.e. amino acids, phosphorus) occasionally represent significant limitations for these ingredients at high levels in the diet of some species.

The manuscript presents a brief overview of some of the research efforts that examined the potential of simple processing techniques, based on sound chemical principles, to improve the bio-availability of nutrients and nutritional value of rendered animal protein ingredients.

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## 2. Improving Digestibility of Phosphorus in High Ash Animal Protein Ingredients

Research efforts carried out at the University of Guelph explored the potential of simple and potentially cost-effective processing techniques to improve the digestibility of phosphorus (P) in high ash animal protein ingredients.

### 2.1 Digestibility of Phosphorus

Phosphorus (P) has an essential role in cellular functions. P, in the form of phosphate, is involved in numerous structural and metabolic roles, such as bone mineralization, components of phospholipids (DNA, RNA and nucleotides), and enzyme cofactor. However, phosphates are a problem when present in excess in the aquatic environment since they are a limiting factor for algal growth in freshwater, and can consequently aggravate the eutrophication process. As a result, the management of P waste outputs by freshwater fish culture operations has been a major area of focus (Bureau &Hua, 2010). Ensuring adequate supply of digestible P without great excess is an issue of increasing significance to aquaculture feed manufacturers.

Formulating feeds to a precise digestible P content can be a difficult task. The P concentration in ingredients commonly used in aquaculture diets is highly variable. In addition, P is a component of a very large variety of chemical compounds and is therefore found in many different chemical forms. These chemical forms of P have different digestion dynamics which are affected by different factors, and this results in variability in the digestibility of P contributed by feed ingredients to the diet (Hua & Bureau, 2006; Hua *et al.* 2010).

Hua Bureau (2006; 2010) developed models that estimated the digestibility of P compounds for three commercially important groups of fish species: salmonids (Hua & Bureau, 2006), tilapia (cichlids), and carp (cyprinids) (Hua & Bureau, 2010). These authors classified the different chemical forms of P into six broad categories: bone-P (hydroxyapatite), organic-P, phytate-P, phytase effect on phytate-P, and monobasic and dibasic inorganic P. Significant differences in the digestibility of the different P compounds for each fish species were observed in these studies. This modeling exercise showed that cyprinids had a very limited

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ability to digest low solubility P compounds. The digestibility of bone-P was estimated as nil to cyprinid fish species where it is about 60% to salmonid fish species and about 70% in tilapia (Hua & Bureau, 2010). Solubilization of compounds containing inorganic P in the stomach is the important factor for P digestibility (Nakamura, 1985). Bone P (hydroxyapatite) is the most stable and least soluble calcium phosphate and its digestibility is thus likely limited by gastric acid output.

Sustained interaction with feed formulators in Southeast Asia suggests that high levels of inorganic P supplements (approximately 20 kg/metric ton) are required to maximize weight gain and meet the digestible P requirement of cyprinids species. This need to supplement low value aquaculture feeds with high levels of P supplements is generally costly. There is great economical interest in increasing the digestibility of P already present in the feeds, notably the bone P contributed by animal protein ingredients.

## 2.2 Potential of Organic Acids

Organic acids have been used to improve P (and other mineral) utilization in fish. Dietary supplementation of citric acid, sodium citrate, and EDTA was able to improve P digestibility of fish meal to rainbow trout (Sugiura *et al*. 1998). Formic acid supplementation significantly improved P digestibility and retention in trout (Vielma & Lall, 1997). The positive effect of organic acid is probably due to the solubilisation of bone minerals, as well as a chelating effect that reduces the precipitation of Ca and P at the intestinal brush border (Sugiura *et al*., 1998; Sarker *et al.* 2005). However, high level of free form organic acids in feeds can results in reduced palatability and feed intake which could ultimately negatively affect the growth performance of the animal. An alternative approach may be to pre-treat high bone P ingredients with organic acids in order to improve their available mineral content prior to use in feeds. Pre-treatments could potentially be done with pure organic acids or through fermentation with micro-organisms. Soil fungus and bacteria, in association with the rhizosphere have developed mechanisms to solubilize inorganic phosphorus (Pi) and make it available to the plant. This unique ability by micro-organisms has been used in the agriculture industry to enhance soil Pi uptake by plants. The mechanism used by soil micro-organisms

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to solubilize Pi involves the production of organic acids that break down hydroxyapatite minerals into mono- or dibasic phosphates. These more soluble forms of Pi are therefore more available for uptake by plants. A variety of soil micro-organisms able to solubilize inorganic P have been described and a number of inoculants are available commercially. Fermentation of feed ingredients with microorganisms may be an option but this process is costly, complex and risky. Short incubation with organic acids may be the only viable option. One of the issues with the solubilisation of bone P with organic acids is the release of large amount of calcium ions concurrent to the release of phosphate ions. These calcium and phosphate ions have a natural tendency to precipitate together when the pH is neutral. Chelating calcium ions may prevent this precipitation and improve the effectiveness of the organic acid treatment.

### 2.3 Validation of an *in vitro* P Bio-Availability Assay

Examining the value of different processing and incubation techniques requires a reliable in vitro assay to predict the in vivo digestibility of P of processed ingredients. The *in vitro* phosphorous bio-availability assay (PBA assay) was identified as a potentially useful tool to rapidly screen and/or predict the *in vivo* digestibility of P of feed ingredients developed during the research efforts carried out at the University of Guelph.

Extensive testing indicated that the PBA assay was not suitable for estimating the bio-availability of P in low processed animal protein ingredients, such as blood meal and feather meal (results not shown). For high ash, high bone P, animal protein ingredients, preliminary results indicated that grinding had a very significant ($p<0.05$) positive effect on the PBA values (Table 1). This suggested that "particle size" has a significant effect on the estimate of P bioavailability obtained with the PBA assay. To confirm this, a comprehensive study was carried out to characterize the effect of particle size on estimates of P bioavailability obtained with the PBA assay.

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A very strong effect of grinding (particle size) within same batch of ingredient (Table 2). However, across batches of similar ingredients, the effect of particle size is not consistent. In the case of poultry by-products meals (meals with P content < 3%), there does not appear to be any significant relationship between particle size and *in vitro* P bioavailability (Figure 1). Conversely, for poultry bone meal (meals with P content > 6%), a very strong negative association between particle size and *in vitro* P bioavailability is noted (Figure 2).

Table 1. Results of preliminary study on the effect of grinding on the bioavailability P content (estimated with the *in vitro* PBA assay) of animal protein ingredients before or after grinding.

| Ingredients | Original Particle Size | After Grinding |
|---|---|---|
| | % | |
| Poultry bone meal (Batch 1) | $4.32\pm0.02^{B}$ | $6.53\pm0.07^{A}$ |
| Poultry bone meal (Batch 2) | $4.39\pm0.02^{B}$ | $5.59\pm0.27^{A}$ |
| Low Ash Poultry By-Products Meal | $1.37\pm0.03^{A}$ | $1.32\pm0.02^{A}$ |
| Regular Poultry By-Products Meal | $1.7\pm0.00^{A}$ | $1.69\pm0.00^{A}$ |

Values in the same row sharing the same subscript are not significantly different

This particle size effect is not expected to be physiologically meaningful since grinding has been shown to have no effect on apparent digestibility of P on *in vivo* digestibility trials with salmonids (Lall, 1991). However, it suggests that "methodological artifact" appear to severely limit the value of the PBA assay to predict the bio-availability of P *in vivo*. Taken alone, *in vitro* P bioavailability estimates may be misleading. However, it was concluded that the PBA assay could probably be used as a screening tool to determine the effectiveness of "treatment" within batches of the same ingredient. However, more efforts should be invested in *in vivo* assessment of P bioavailability, through digestibility or bio-availability (growth) trials) which should produce more reliable and meaningful results than *in vitro* assays.

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Table 2. Bio-availability of P (estimated with the *in vitro* PBA assay) of different animal by-products prior and after grinding

| Ingredient | Size | Total P % | Bioavailable P % | P Bioavailability % | Mean particle size $mm^2$ |
|---|---|---|---|---|---|
| Fish meal, herring W | Original | 2.75±0.12 | 1.93±0.12 | $70^{A}$ | 0.06 |
| | Ground | 2.78±0.06 | 2.02±0.03 | $73^{A}$ | 0.06 |
| Fish meal, herring Y | Original | 2.83±0.15 | 1.70±0.06 | $60^{B}$ | 0.22 |
| | Ground | 2.84±0.49 | 1.96±0.05 | $69^{A}$ | 0.18 |
| Fish meal, herring Z | Original | 2.76±0.18 | 1.70±0.04 | $65^{B}$ | 0.26 |
| | Ground | 2.72±0.01 | 1.91±0.02 | $70^{A}$ | 0.09 |
| Meat bone meal Q | Original | 3.75±0.48 | 2.27±0.23 | $61^{B}$ | 0.23 |
| | Ground | 3.68±0.06 | 2.70±0.06 | $73^{A}$ | 0.19 |
| Poultry bone meal L | Original | 7.04±0.30 | 5.12±0.13 | $73^{B}$ | 0.14 |
| | Ground | 7.00±0.11 | 5.42±0.09 | $77^{A}$ | 0.10 |
| Poultry bone meal M | Original | 6.56±0.37 | 4.49±0.29 | $68^{B}$ | 0.20 |
| | Ground | 6.85±0.16 | 5.72±0.12 | $83^{A}$ | 0.08 |
| Poultry bone meal N | Original | 6.91±0.36 | 4.66±0.19 | $67^{B}$ | 0.18 |
| | Ground | 7.11±0.02 | 5.95±0.10 | $84^{A}$ | 0.07 |
| Poultry by-products meal A | Original | 2.71±0.02 | 1.72±0.03 | $63^{B}$ | 0.14 |
| | Ground | 2.64±0.03 | 1.89±0.10 | $71^{A}$ | 0.12 |
| Poultry by-products meal B | Original | 2.56±0.49 | 1.58±0.07 | $62^{B}$ | 0.19 |
| | Ground | 2.53±0.01 | 1.92±0.06 | $76^{A}$ | 0.13 |
| Poultry by-products meal C | Original | 2.66±0.27 | 1.74±0.11 | $65^{B}$ | 0.17 |
| | Ground | 2.67±0.08 | 2.00±0.05 | $75^{A}$ | 0.16 |
| Poultry by-products meal D | Original | 2.88±0.10 | 1.87±0.04 | $65^{B}$ | 0.14 |
| | Ground | 2.70±0.07 | 1.96±0.02 | $73^{A}$ | 0.10 |
| Poultry by-products meal E | Original | 2.97±0.13 | 1.58±0.10 | $53^{B}$ | 0.12 |
| | Ground | 2.65±0.11 | 2.01±0.12 | $76^{A}$ | 0.07 |

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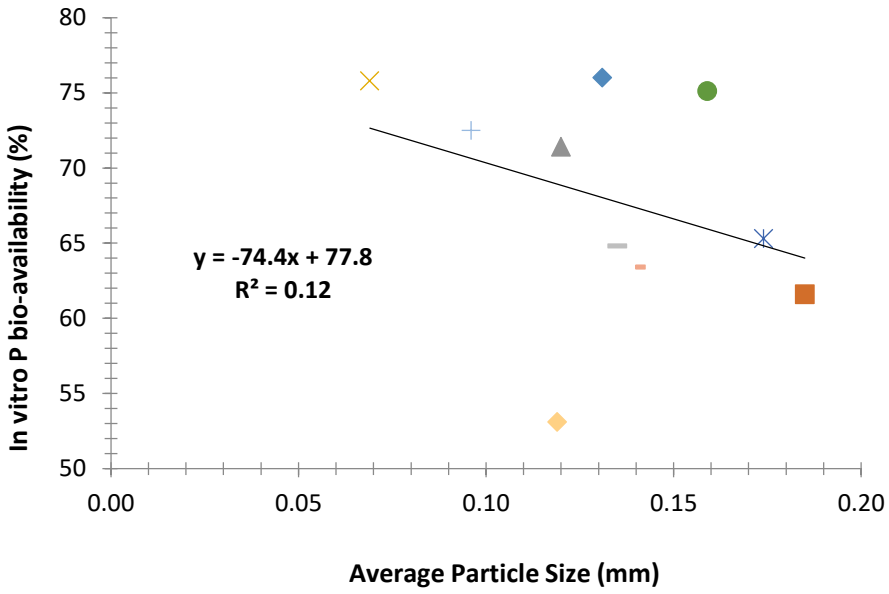


Figure 1. *In vitro* bio-availability of P of poultry by-products meals (Total P = 2.5 to 3.0%) with different average particle sizes

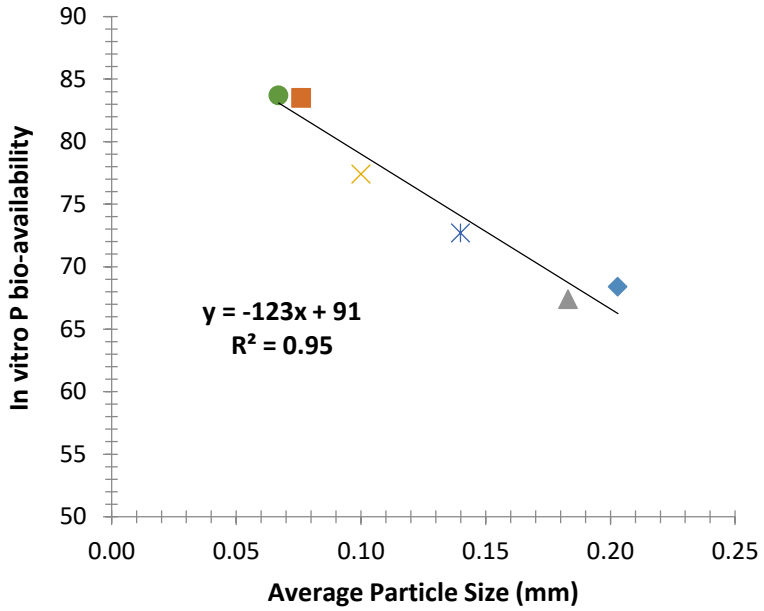


Figure 2. *In vitro* bio-availability of P of poultry bone meals (Total P = 6.6 to 7.1%) with different average particle sizes

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## 2.4 Laboratory Bench Scale Experiments to Examine Effects of Different Treatments on *in Vitro* Digestibility of P in Poultry By-Products Meal

A series of lab bench experiments, for which the general approach is described in Figure 3, were carried out to explore the effects of different factors and reagents on the bio-availability of P in poultry by-products meal (PBM). A simple water solubilisation technique to determine the amount of soluble P that was freed up by the different treatments since we believe that this technique may more realistically estimate the digestible (available) P content of the PBM and meat and bone meals for a gastric fish species (carps) than the PBA assay described above.

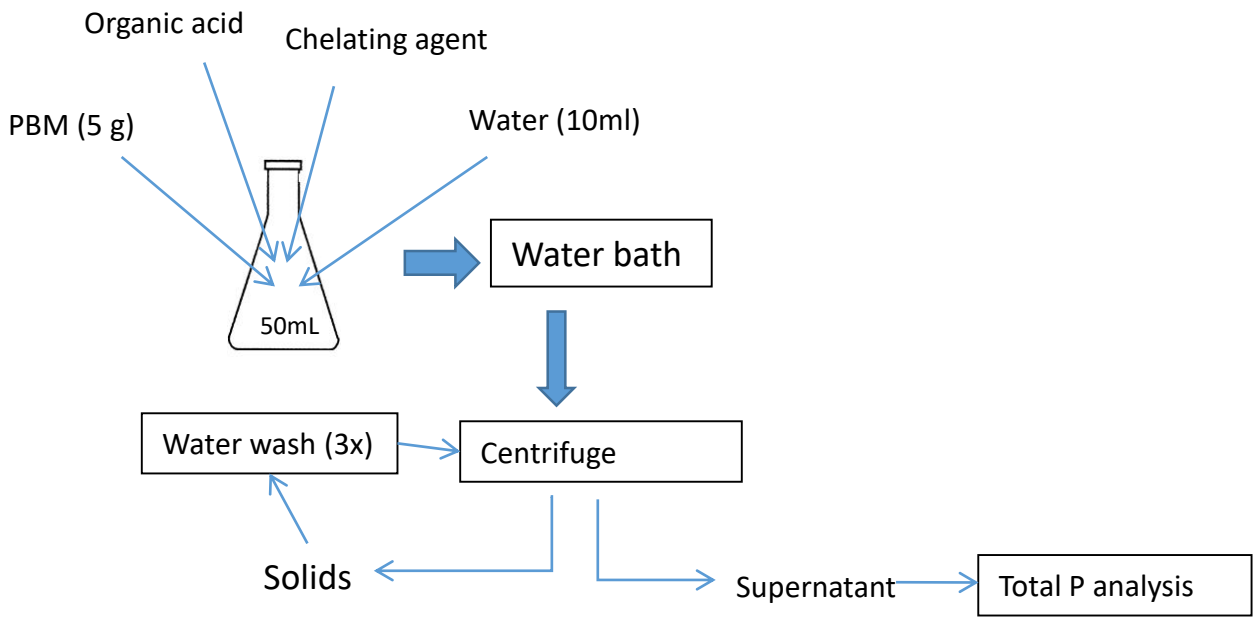


Figure 3. General approach used in the incubation trials .

This series of lab bench experiments used the same test ingredient, a relatively high ash PBM with approximately 2.6% phosphorus (as is basis) derived from a single production lot was obtained from a local rendered plant (Rothsay, Moorefield, ON, Canada). A very large proportion (> 80%) of the P content of this PBM is as estimated to be bone-P (hydroxyapatite).

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A first series of experiments examined the effect of different concentration of citric acids and of a calcium chelator (EDTA). The effect of incubation period (time) and temperature were examined. The results suggest that the use of EDTA in combination with citric acid appeared to be highly effective at solubilising P. A simple graphical analysis suggests that EDTA and citric acid do not appear to act in synergy but rather have additive effects (Figure 4). The use of combinations of citric acid (0 to 10% expressed as % of total PBM weight, as is) and EDTA (0 to 9%) resulted in an increase in the soluble P level of the PBM from 0.25% to about 1.1%. These results suggested that citric acid and EDTA combinations could potentially improve the availability of P in PBM to agastric fish species from less than 10% to more than 40%.

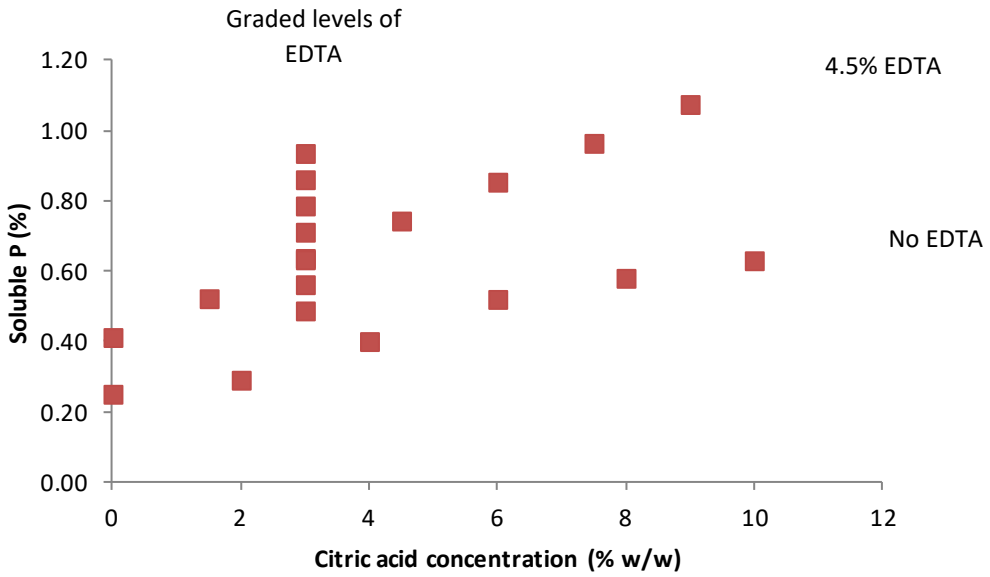


Figure 4. Effects of citric acid concentration (% w/w) on the amount of phosphorus solubilized from poultry by-products meal in the presence or absence of EFDA.

Another series of experiments compared different organic acids (or source thereof) under standardized conditions (3h at 50°C, 3.8% EDTA). These experiments suggest that citric, tartaric, oxalic formic acids were some the most effective organic acids for solubilizing bone P (Figures 5 and 6). Lactic, malic and acetic acid had moderate effects while benzoic and

ascorbic acids did not prove effective. Corn steep liquor, a source of lactic acid, did not prove effective at solubilising P in the PBM.

Of all the compounds tested, citric acid and EDTA appeared to have the greatest potential due to their reasonable cost since they are substances that have GRAS (Generally Recognized As Safe) classification by the US Food and Drug Administration (FDA).

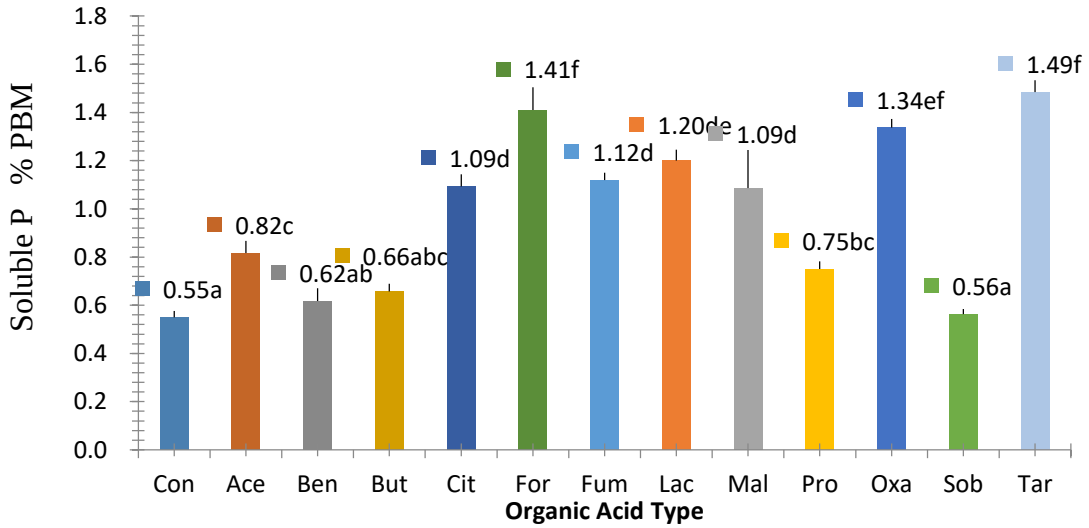


Figure 5. Soluble P obtained after incubation with 10 g 100 g-1 of acetic acid (Ace), benzonic acid (Ben), butyric acid (But), citric acid (Cit), formic acid (For), fumaric acid (Fum), lactic acid (Lac), malic acid (Mal), oxalic acid (Oxa), propionic acid (Pro), sorbic acid (Sob), and tartaric acid (Tar) respectively in condition of 3.8 g 100g-1 of EDTA and 65g 100g-1 of system moisture

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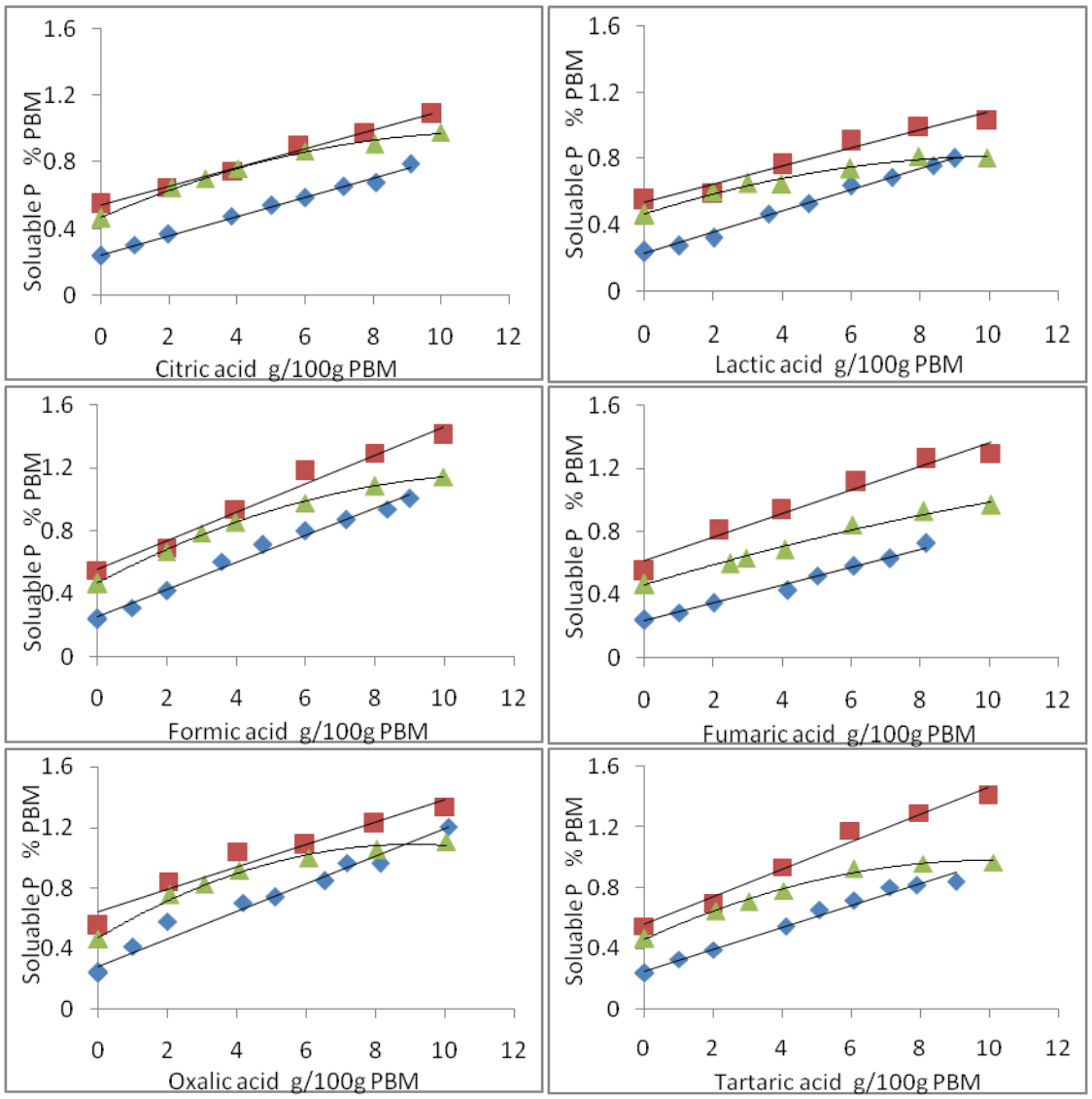


Figure 6. Effect of low molecular weight organic acids and moisture level on kinetic of phosphorus solubility from PBM.

The solubilisation of P by citric acid and EDTA treatment appeared to be very rapid (less than 1 h) (Figure 7). Increasing temperature (20-70°C) only had a very small effect on the solubilisation of P, regardless of the type of organic acid used (Figure 7) and long incubation periods (up to 200 h incubation time) did not yield any improvement in P solubility (results not shown). The incubation of PBM with a broad action protease in combination with citric acid and EDTA had no effect of the release of soluble P (results not shown).

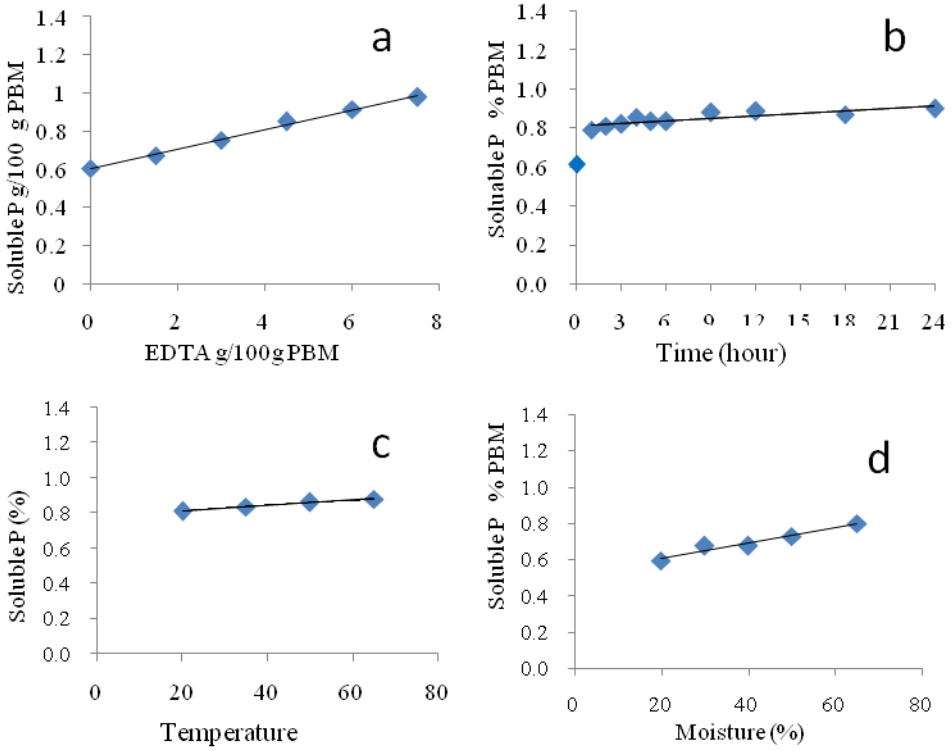


Figure 7. Effect of time, temperature, moisture and EDTA on bone P release from PBM

## 2.5 *In vivo* Assessment of the Digestibility of Incubated PBM and its Effects of Growth Performance

A pilot-scale trial to produce high bio-available phosphorus poultry by-products meals was conducted on the basis of the work described above. This involved producing several kg of processed PBMs with two different organic acids (formic acid and citric acid) and these two PBMs were used in a digestibility trial and a growth trial with rainbow trout carried out at the University of Guelph Fish Nutrition Research Laboratory.

### 2.5.1 Ingredients and Experimental Diets

The same batch of PBM described above was pre-treated by diluting 10 g citric acid or formic acid with 670 mL distilled water and adding this mixture to 1 kg PBM. After mixing thoroughly, the treated PBM was incubated at 50 °C for 3 h and then air dried. A PBM was also incubated with distilled water alone in order to have a “sham” treated PBM.

A reference diet (Table 3) was prepared and combined with each test ingredient (PBM pre-treated with either water (control), citric acid or formic acid) at a 70:30 ratio (as is basis) to produce three test diets. Yttrium oxide at an inclusion level of 100 ppm was added to the reference diet to serve as a digestibility indicator. The diets were mixed using a Hobart mixer and pelleted using a laboratory steam pellet mill. The feed pellets were subsequently dried using forced air at room temperature for 24 h. The diets were kept at 4 °C until used. The proximate composition of the PBM and the reference and test diets are shown in Table 4.

Table 3. Ingredient composition of the reference diet.

| Ingredients | % |
|---|---|
| Fish meal, herring, 70% CP | 18.5 |
| Soybean meal, dehulled | 6.0 |
| Blood meal, porcine, spray-dried | 6.0 |
| Corn gluten meal, 60% CP | 12.0 |
| Feather meal | 5.0 |
| Wheat middlings | 12.9 |
| Soy protein concentrate (HP300) | 12.0 |
| Vitamin premix , Martin Mills | 1.0 |
| Mineral premix· Martin Mills | 0.5 |
| Wheat gluten | 8.0 |
| Fish oil | 14.0 |
| Vegetable oil | 4.0 |
| Yttrium oxide | 100ppm |
| **Total** | 100 |

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Table 4. Proximate composition of the PBM, reference diet and test diets.

| PBM or Diet | DM (%) | Composition (dry matter basis) | | | | | |
|---|---|---|---|---|---|---|---|
| | | CP[a] (%) | Lipid (%) | Ash (%) | GE (kJ/g) | P (%) | Y[b] (µg/g) |
| PBM | 97.4 | 72.0 | 11.3 | 13.9 | 21.8 | 2.46 | - |
| Reference | 94.9 | 53.0 | 20.7 | 5.9 | 24.5 | 0.87 | 88 |
| Control | 95.0 | 55.8 | 18.1 | 8.7 | 23.7 | 1.37 | 59 |
| Citric acid | 95.0 | 58.5 | 17.7 | 8.6 | 23.6 | 1.26 | 58 |
| Formic acid | 95.0 | 58.3 | 17.8 | 8.6 | 23.7 | 1.37 | 60 |

[a]CP crude protein (N X 6.25)

[b]Yttrium

### 2.5.2 Fish and Experimental Conditions

Two trials were carried out with rainbow trout (*Oncorhynchus mykiss*) obtained from the Alma Aquaculture Research Station (Elora, Ontario). Fish were maintained in a flow-through system consisting of 60 L fiberglass tanks, individually aerated and supplied with well water at a rate of approximately 3 L/min and equipped with fecal settling columns (Guelph System) as described by Cho *et al*. (1982). Water temperature was maintained at 11.8 ± 0.5 $^{o}$C and 12.6±0.4 $^{o}$C for the digestibility and growth trials, respectively. Photoperiod was maintained at 12 h light: 12h dark in a windowless laboratory. The animals were kept in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1984). Fish in both trials were hand-fed to satiety three times daily on weekdays and once daily on weekends.

### 2.5.3 Digestibility trial

Groups of 15 fish with an initial average weight of 21 g/fish were randomly distributed into 24 tanks. The four experimental diets (reference, control, citric acid, formic acid) were

randomly allocated to two collection units each. The fish were acclimated to the experimental system and dietary regime for four days prior to collection. A total of four fecal samples per diet were collected. Two fecal samples per diet were collected during the first collection period (10 days). The experimental diets were then randomly re-allocated to new collection units for the second period and two additional fecal samples per diet were collected in the following 10-day period. One hour after the last daily meal, the drainpipe and the settling column were brushed out to remove feed residues and feces from the system. One-third of the water in the tanks was drained to ensure that the cleaning procedure was complete. At 08:30 h the following day, the settled feces and surrounding water were gently withdrawn from the base of the settling column into a large centrifuge bottle. These feces were free of uneaten feed particles and considered to be a representative sample of the feces produced throughout the 24 h period. The feces were centrifuged at 4000×$g$ for 10 min and the supernatant discarded. The feces were then freeze-dried, ground and stored at −20° C until analysis.

### 2.5.4 Growth trial

Groups of 15 fish with an initial average weight of 35.9 g/fish were randomly distributed into nine tanks, with 3 replicate tanks per diet (water, citric acid and formic acid treated PBM). Tank was considered the experimental unit. Fish were acclimated to the experimental conditions for one week prior to the start of the experiment. Throughout the duration of the experiment (58 days), feed intake was recorded weekly and fish were weighed every 28 days. At the beginning of the experiment, a pooled sample of 12 fish was taken for determination of initial carcass composition. At the end of the experiment, five fish per tank were sampled for carcass composition analysis and an additional 10 fish per tank were weighed and dissected in order to obtain the hepatosomatic index (HSI) and viscerosomatic index (VSI).

All results were assessed for normality by the Shapiro-Wilk test, homoscedasticity by SNHT and expressed as mean values. When the data did not show normality, transformation using Box-Cox was performed prior to analysis. The dependent variables were analyzed by one-

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way analysis of variance (ANOVA) followed by the Tukey's HSD test using the XLSTAT® software Version 2014.5.01. For all analyses, the level of significance adopted was P≤0.05.

### 2.5.5 Results

The results from the digestibility trial indicated no significant differences (P>0.05) in the ADCs of DM, CP, lipid, ash, GE or P among the pre-treated PBMs (Table 5). Pre-treatment of PBM with citric acid or formic acid did not significantly improve the digestibility of this ingredient in rainbow trout.

In the growth trial, inclusion of pre-treated PBM did not significantly affect growth, feed intake and feed efficiency of rainbow trout. Excellent growth rates (TGC) were achieved with all experimental diets (Table 6). Similarly, there were no significant effects of pre-treatment of PBM with citric or formic acid on proximate and mineral carcass composition or nutrient utilization efficiency of rainbow trout (results not shown). The pre-treatment methods employed in this study did not appear to improve the nutritive value of PBM for rainbow trout. Research needs to be carried out to determine if this treatment could improve the bio-availability of P to aquaculture species without acid stomach (carps, shrimp, etc.).

Table 5. Apparent digestibility coefficients of PBM pre-treated with water (control), citric acid or formic acid in rainbow trout.

| Ingredient | Apparent digestibility coefficients[1] | | | | | |
|---|---|---|---|---|---|---|
| | DM (%) | CP (%) | Lipid (%) | Ash (%) | GE (%) | P (%) |
| Control | 68[a] | 78[a] | 76[a] | 38[a] | 72[a] | 43[a] |
| Citric acid | 69[a] | 79[a] | 78[a] | 40[a] | 73[a] | 39[a] |
| Formic acid | 71[a] | 80[a] | 83[a] | 40[a] | 75[a] | 45[a] |
| **Significance[2]** | NS[4] | NS | NS | NS | NS | NS |
| S.E.M.[3] | 0.0101 | 0.0091 | 0.0178 | 0.0088 | 0.0210 | 0.0210 |

[1]Mean (n=4 replicates). Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.

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[2]Significance of the one-way ANOVA.

[3]S.E.M.=standard error mean.

[4]Not statistically significant (P≥0.05).

Table 6. Weight gain, growth rate, feed intake, feed efficiency (FE) and viscerosomatic (VSI) and hepatosomatic (HSI) indices of rainbow trout (initial average weight= 35.9 g/fish) fed the experimental diets for 58 days.

| Diet | Gain (g/fish) | TGC[1] | Feed intake (g/fish) | FE[2] (gain/feed) | VSI (%) | HSI (%) |
|---|---|---|---|---|---|---|
| Control | 143.0[a] | 0.311[a] | 122.6[a] | 1.16[a] | 20.4[a] | 1.36[a] |
| Citric acid | 140.0[a] | 0.313[a] | 125.7[a] | 1.12[a] | 19.8[a] | 1.46[a] |
| Formic acid | 138.3[a] | 0.304[a] | 120.7[a] | 1.13[a] | 18.8[a] | 1.39[a] |
| Significance[3] | NS[5] | NS | NS | NS | NS | NS |
| S.E.M. [4] | 2.711 | 0.005 | 2.085 | 0.01 | 0.38 | 0.02 |

[1]TGC=thermal-unit growth coefficient.

[2]FE=feed efficiency.

[3]Significance=significance of the one-way ANOVA. Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.

[4]S.E.M.=standard error mean.

[5]NS=not statistically significant (P≥0.05).

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## 3. Improving Digestibility of Protein and Amino Acids in Feather Meal

### 3.1 Overview

Hydrolyzed feather meal (FeM) is a relative economical feed ingredient with high protein content (78-87% CP). This ingredient is manufactured from feathers, a co-products of the poultry industry. In 2012 only, 60 billion chickens were raised and processed globally (FAO stats). Feathers represent from 5 to 7% percent of the chicken body weight (Williams et al. 1991). Consequently, extremely large volumes of feathers are generated annually around the globe (Poole *et al*. 2008; Huda & Yang 2009). Other keratinous material (hair, wool, horn, hoof, claws, etc.) also represent also very important protein resources globally.

Raw feathers are made of over 90% of keratin, a protein that is also the main structural component of hair, nails, wool, horns and claws. Keratin contains approximately 7% cysteine and this amino acid forms disulphide bridges with other cysteine molecules. Together with hydrogen bonds, these bonds tightly package the polypeptides in a filamentous structure to form a helical array of twisted β-sheets that gives strength to the keratin molecule (Fraser et al. 1972). The disulphide bonds also render keratin highly resistant to the action of proteases. Due to their very high keratin content, feathers are virtually indigestible in their natural state. Several studies have shown that raw feathers are indigestible by cats, dogs, chicks, owls and rats (Mangold & Dubiski 1930; Moran *et al*. 1966). The strong internal interactions make keratin extremely resistant to gastric solvents and proteolytic enzymes (Wrzesniewska-Tosik and Adamiec 2007).

Steam hydrolysis commonly be used to process raw feathers into feather meal. Exposing feathers to high moisture, pressure and heat creates conditions that promote the rupture of the disulphide bridges and of the hydrogen bonds, which dissociate the proteins, allowing to the amino acids in keratin to be accessible to protease and allow keratin to become digestible which free up amino acids and render them potentially bioavailable. Finally, the steam-treated feather biomass is then dried and grounded to produce feather meal. Feather meal is generally rich in arginine, cysteine and threonine, but deficient in methionine, lysine, histidine and tryptophan.

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The chemical composition, notably the high digestible protein and amino acid contents of feather meal makes it, in theory, a highly interesting ingredient for animal feeds, notably feeds for aquaculture species which are often formulated to very high (> 30%) protein levels (Bureau *et al*. 1999). However, negative perceptions exist among aquaculture feed manufacturers about the stability of the nutritive value of this ingredient. Some feed industry stakeholders believe that the composition, the *in vitro* digestibility (pepsin digestibility assay) and growth performance achieved with this ingredient are much too variable and relatively unpredictable to be used at a significant level in commercial feed formulations.

Processing equipment and conditions used by renderers are relatively highly diverse, each renderers using slightly different mixes of raw material (feathers, hog hair, etc.), cooking, drying and grinding equipment and operating conditions (temperature, pressure, duration, etc.). Variability in the nutritional value of FeM results from the use of different raw materials and processing conditions during the production of this ingredient (Latshaw, 1990; Latshaw *et al*. 1994; Wang and Parsons, 1997; Bureau *et al*. 1999). Variations of heat processing conditions create disparities of disulphide conformers, D-amino acids, and cross-linked amino acids contents, and these affect the nutritional value of protein ingredients. Better understanding of the chemical interactions occurring during the processing of FeM would enable the development of methods to improve and estimate the nutritional value of this ingredient, which could assure the quality of FeM to feed stakeholders.

### 3.2. Enzymatic Treatment and Fermentation to Improve Digestibility of Feather Meals

Over the past two decades, a great deal of effort was invested in improving the nutritional value of feed ingredients through enzymatic treatment. Most of the work involving the enzymatic treatment of feathers focused on the fermentation of raw material using keratinolytic bacteria such as *Bacillus subtillis* or *B. licheniformis* isolated from keratin wastes. Microbial fermentation requires cultivation of bacteria and incubation in a controlled environment which is logistically difficult to perform on a commercial scale. Studies suggested that the bacterial degradation of keratin is accomplished through of the combined action of disulfide reductases and proteases (Yamamura *et al*. 2002; Ramnani *et al*. 2005).

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The reductases break the disulphide bridges between the cysteine amino acids and allow to the proteases to cut down the peptides (Bockle and Muller 1997). Ramnani and Gupta (2007) almost completely degraded feather keratin (96%) using Savinase® (Novozymes), a serine protease from *Bacillus sp.*, and sodium sulfite as a reducing agent. It has been found that sodium sulfite can reduce the cystine-disulfide bonds abundant in keratin to produce a cysteine thiol and a Bunte salt (Elashi *et al*. 2013, Figure 8). This reduction of the cysteine-disulphide bonds destabilizes the protein and in theory render it more susceptible to proteolytic enzymes.

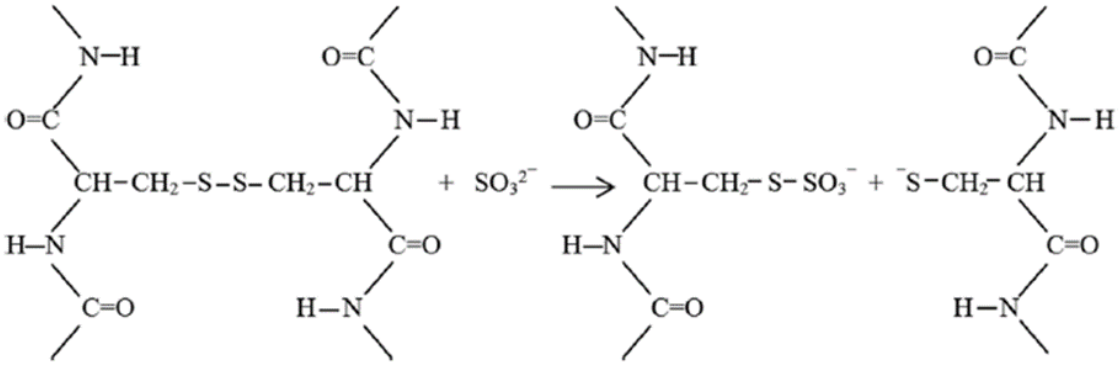


Figure 8 The addition of a sulfite to a cystine-disulfide bond to produce a cysteine thiol and a Bunte salt

Based on proprietary research and development (R&D) work around this process, Dupont Nutrition Biosciences APS (Copenhagen, Denmark) applied for US Patent Pub. No. US 2015/0197783 A1 "Method for the Degradation of Keratin and Use of the Keratin Hydrosate Produced". The work described in the patent is almost solely laboratory bench-scale studies with keratinous material and only theoretical use of the "keratin hydrolysate" in animal feeds is described. No pilot-scale trial and animal trials appeared to have been conducted in order to assess the nutritive value of the resulting material.

The procedure also substantially raises the cost of production as it requires expensive reagents (enzymes, buffers, and reducing agent), supplementary equipment, and addition of water, demanding additional energy for drying. There was consequently a need for validation

of this technology through animal assays. There is also a need to scale up the procedure and examine feasibility, cost and actual economic value of the processed ingredients.

## 3.3 Pre-Processing of Steam Hydrolyzed Feather Meal

### 3.3.1 Introduction

The purpose of a study conducted at the University of Guelph was to examine the effect of two different proteases and a chemical agent (sodium disulfite) able to reduce the cysteine-disulphide bonds abundant on the hydrolysis on the in vitro hydrolysis of keratin. The goal was to try to develop an optimized protocol for the pre-treatment of feather meal prior to its use in animal feeds. An *in vitro* estimate of digestibility, the degree of protein hydrolysis (degree of hydrolysis DH) was used as a measure of effectiveness for keratinolysis after enzymatic and reducing agent treatment.

### 3.3.2 Material and Methods

A full factorial model lab-bench study was conducted to investigate the effect of 1) protease level, 2) sodium sulphite level, and 3) digestion buffer level on the degree of hydrolysis of feather with using two different enzymes.

This study examined the effect of two different proteases: a fungal protease (Protease A) and a bacterial protease (Protease B), sodium sulfite and water on hydrolysis of protein of feather meal samples according to a multifactorial (2x3x3x4) design with 36 treatments (Table 7). Each hydrolysis trial were conducted on a bench scale basis on sample (approximately 20 g) of a commercial feather meal (Sanimax, Montreal, Qc, Canada). The trials were performed at the temperatures and pH recommended by the enzyme manufacturers for a period of 3h. The reaction will be stopped by adding one volume of 20% trichloroacetic acid (TCA). Each treatment combination was tested in three separate incubation (triplicates). In this study, a total of 216 feather meal samples were incubated.

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*Enzymatic Digestion*

The digestion buffer was prepared by mixing distilled water with Trizma base® (1.211 g/L) and sodium azide (0.10 g/L) thoroughly at room temperature (Coll *et al.* 2007). The pH of the digestion buffer was adjusted to meet the enzyme manufacturer's recommendations with a pH of 10.0 for Protease B and a pH of 7.5 for AG175. Each sample was incubated for a period of 3 hours in a shaking water bath at temperatures attuned to the reported optimal activity of the proteases, namely 37°C for the fungal protease (Protease A) and 55 °C for the bacterial enzyme (Protease B). The samples were centrifuged (4,000 rpm, 10 minutes) and the supernatant will be collected to determine degree of hydrolysis.

The efficiency of each combination of parameters to hydrolyze FeM was assessed as the percentage of nitrogen solubilized in trichloroacetic acid solution by the pre-treatment and expressed as degree of hydrolysis (DH).

Table 7. Experimental design of the hydrolysis trial

| **Independent Variables** | **Levels** | | | |
|---|---|---|---|---|
| P= Protease | A | B | | |
| A= Water:FeM ratio | 2:1 | 3:1 | 4:1 | - |
| B= Enzyme level (%FeM) | 0 | 1 | 2 | 3 |
| C= Sodium Sulfite Level (%FeM) | 0 | 1.5 | 3 | - |

### 3.3.3 Results

The results from this lab bench study showed that feather meal could be very effectively hydrolyzed in vitro with a mixture of protease and sodium sulfite. The addition sodium sulfite to the incubation mixture promoted the chemical reduction of the disulphide bridges and substantially unlocked the capacity of enzymes to hydrolyse FeM. Difference was found in the effectiveness of the proteases. Protease B showed a significantly greater ($P<0.0001$) hydrolyzing capacity in comparison to Protease A.

Enzyme level of 3% (%FeM w/w), reducing agent level of 3% (%FeM w/w), and water level of 500% (%FeM w/w) was found to be the optimal conditions to hydrolyze feather meal using both enzymes. Under optimal conditions, DH of 45% was reached with Protease B. The efficacy of the pre-treatment to improve the nutritive value of FeM in practical animal diets needs to be systematically investigated through digestibility and bioavailability trials with livestock species such as fish, poultry and swine. If proven efficient, the pre-treatment could enable a substantial increase in the incorporation of FeM in animal feeds.

Table 8. Actual levels of independent variables along with the observed values for the response variables, degree of hydrolysis for the Protease A and B.

| Run | Independent Variables | | | Degree of Hydrolysis (%) | |
|---|---|---|---|---|---|
| Number | *X1*[1] | *X2*[2] | *X3*[3] | Protease A[4] | Protease B[4] |
| 1 | 0 | 0 | 200 | 9.5 ± 0.36 | 9.3 ± 0.14 |
| 2 | 1 | 0 | 200 | 10.9 ± 0.32 | 16.1 ± 0.71 |
| 3 | 2 | 0 | 200 | 11.3 ± 0.33 | 17.3 ± 0.48 |
| 4 | 3 | 0 | 200 | 11.6 ± 0.10 | 18.5 ± 0.42 |
| 5 | 0 | 1.5 | 200 | 9.5 ± 0.19 | 9.7 ± 0.33 |
| 6 | 1 | 1.5 | 200 | 12.6 ± 0.05 | 22.4 ± 1.09 |
| 7 | 2 | 1.5 | 200 | 13.9 ± 0.41 | 25.7 ± 0.80 |
| 8 | 3 | 1.5 | 200 | 14.4 ± 0.77 | 27.1 ± 0.31 |
| 9 | 0 | 3 | 200 | 9.4 ± 0.74 | 9.8 ± 0.51 |
| 10 | 1 | 3 | 200 | 13.5 ± 0.70 | 27.4 ± 1.32 |
| 11 | 2 | 3 | 200 | 16.0 ± 0.87 | 31.2 ± 0.37 |
| 12 | 3 | 3 | 200 | 16.8 ± 0.84 | 32.9 ± 0.60 |
| 13 | 0 | 0 | 350 | 9.4 ± 0.16 | 9.4 ± 0.30 |
| 14 | 1 | 0 | 350 | 11.1 ± 0.29 | 17.0 ± 0.22 |
| 15 | 2 | 0 | 350 | 11.5 ± 0.21 | 18.7 ± 0.13 |
| 16 | 3 | 0 | 350 | 12.0 ± 0.27 | 21.2 ± 0.59 |
| 17 | 0 | 1.5 | 350 | 9.6 ± 0.27 | 10.2 ± 0.31 |
| 18 | 1 | 1.5 | 350 | 13.8 ± 0.04 | 27.4 ± 0.57 |
| 19 | 2 | 1.5 | 350 | 15.1 ± 0.64 | 30.7 ± 0.68 |
| 20 | 3 | 1.5 | 350 | 16.1 ± 0.08 | 31.8 ± 1.56 |
| 21 | 0 | 3 | 350 | 10.6 ± 0.28 | 10.3 ± 0.38 |
| 22 | 1 | 3 | 350 | 17.6 ± 0.76 | 36.2 ± 0.39 |
| 23 | 2 | 3 | 350 | 20.7 ± 1.16 | 40.1 ± 0.25 |
| 24 | 3 | 3 | 350 | 21.8 ± 1.16 | 43.5 ± 1.62 |
| 25 | 0 | 0 | 500 | 9.7 ± 0.44 | 10.0 ± 0.42 |
| 26 | 1 | 0 | 500 | 11.5 ± 0.36 | 18.6 ± 0.06 |
| 27 | 2 | 0 | 500 | 11.5 ± 0.22 | 20.4 ± 0.76 |
| 28 | 3 | 0 | 500 | 12.4 ± 0.25 | 22.0 ± 0.82 |
| 29 | 0 | 1.5 | 500 | 10.3 ± 0.35 | 10.2 ± 0.08 |
| 30 | 1 | 1.5 | 500 | 14.0 ± 0.27 | 28.8 ± 0.79 |
| 31 | 2 | 1.5 | 500 | 15.4 ± 0.17 | 30.5 ± 0.76 |
| 32 | 3 | 1.5 | 500 | 17.3 ± 0.55 | 33.2 ± 0.37 |
| 33 | 0 | 3 | 500 | 10.1 ± 0.27 | 10.7 ± 0.28 |
| 34 | 1 | 3 | 500 | 17.4 ± 0.65 | 38.3 ± 1.85 |
| 35 | 2 | 3 | 500 | 20.4 ± 0.28 | 39.3 ± 1.68 |
| 36 | 3 | 3 | 500 | 23.2 ± 0.22 | 45.1 ± 1.62 |

[1] *X1*=Enzyme level (%FeM w/w)

[2] *X2*=Sodium sulfite level (%FeM w/w)
[3] *X3*=Water level (%FeM w/w)
[4] Data are means (n=3) ± Standard deviation.

### 3.4 Assessing the Bio-availability of Arginine and Digestibility of Amino Acids in two Feather Meals and in their Pre-Treated Counterparts to Rainbow Trout (*Oncorhynchus mykiss*)

The effect of this pre-treatment on the bioavailability of arginine (Arg) and on the digestibility of amino acids of two commercial steam-hydrolyzed FeMs and their pre-treated counterparts (PTFeMs). To produce pre-treated FEMs, the two commercial FeMs were incubated in 200% distilled water (%FeM w/w), with 0.05% of a protease (Protease B, %FeM w/w) and 2% sodium sulfite (%FeM w/w) at 55ºC for 24 hours in a shaking incubator. The two PTFeMs were then lyophilized.

The relative bioavailability of Arg in two FeMs and their pre-treated counterparts (PTFeMs) were compared with that of crystalline L-arginine (L-Arg) in an 8-week growth trial with rainbow trout. An Arg deficient (1.2% Arg) basal diet was formulated and ten other diets were formulated to contain 1.35% or 1.5% Arg by adding increasing amounts of L-Arg, FeMs, or PTFeMs.

The results (Figure 9 and Figure 10) clearly indicated increased Arg bioavailability of the two PTFeMs compared to the two commercial FeMs. The two PTFeMs supported higher growth rates and Arg retention efficiency than their untreated counterparts. The PTFeM 1 supported an arginine retention efficiency of about 70% which is the maximum theoretical arginine retention efficiency predicted by factorial amino acid requirement models developed at the University of Guelph.

Two digestibility trials were carried out to examine the digestibility of diets formulated with the FeMs. In first trial using the diets used in the bio-availability trial, PTFeMs containing diets presented significantly ($P<0.05$) higher apparent digestibility coefficients (ADC) of crude protein (CP) and amino acids when compared to the diets containing the original FeM counterparts. In a digestibility trial carried out with the standard 70:30 indirect digestibility protocol, the ADC of CP in FeM1, PTFeM1, FeM2, and PTFeM2 were estimated to be 85%, 95%, 82%, and 96%, respectively. The pre-treatment of these FeMs significantly improved

($P < 0.05$) their ADC of CP and amino acids (with the exception of histidine, lysine and methionine).

These findings contradict those of Serwata (2007), Laporte (2007) and Davies *et al*. (2009) who did not observe a significant difference in ADC of CP between standard steam-hydrolyzed FeMs and enzyme-treated FeMs by rainbow trout, European sea bass, gilt-head sea bream, and turbot. This appears to indicate that the presence of both a reducing agent and disulphide reductase is essential to enable the proteolytic enzymes to efficiently hydrolyze keratin into peptides and amino acids.

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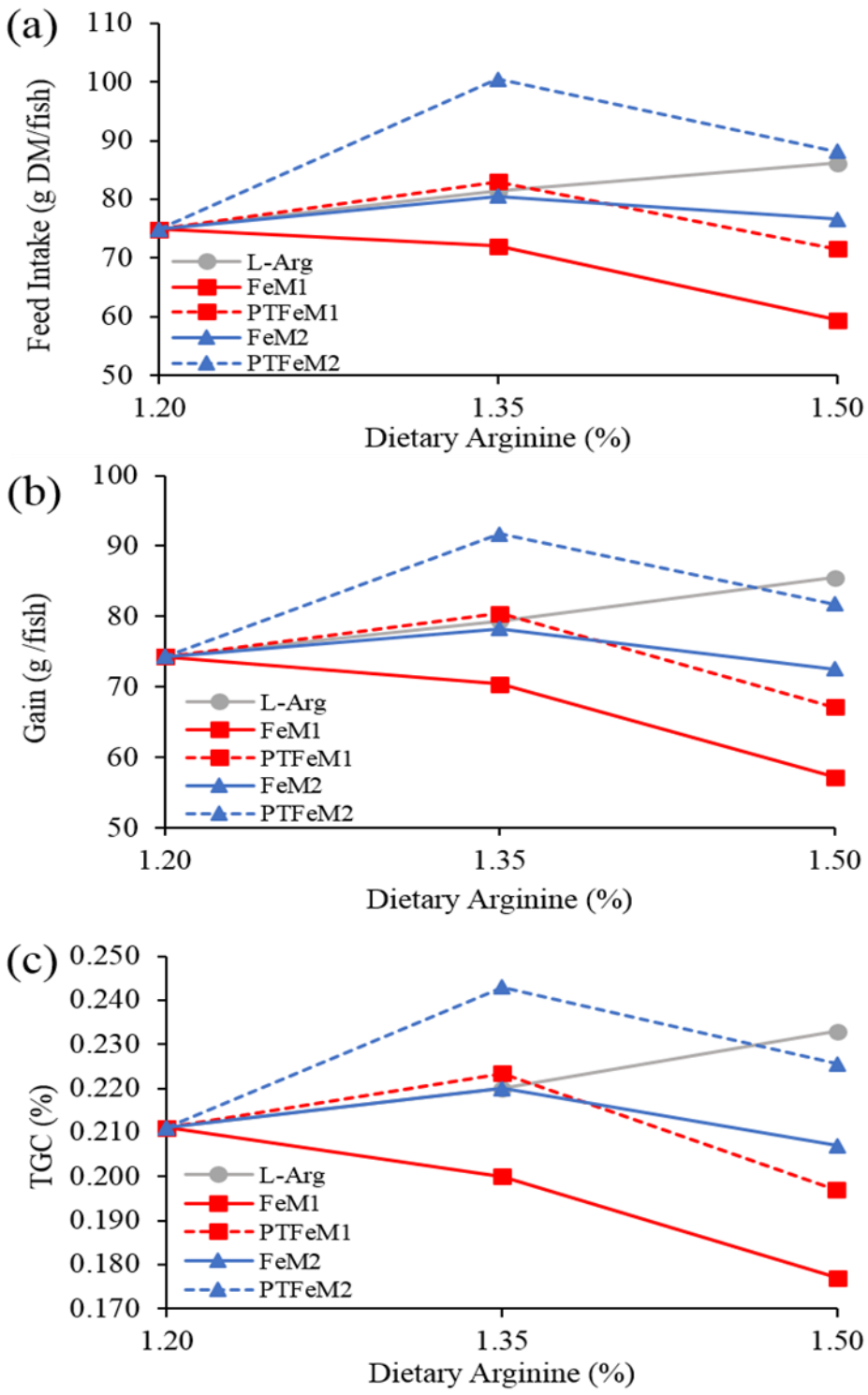


Figure 9. Feed intake (a), weight gain (b), and TGC (c) values of rainbow trout in response to being fed diets containing increasing arginine content supplied by L-arginine, FeM1, PTFeM1, FeM2 or PTFeM2. Values are mean (n = 3).

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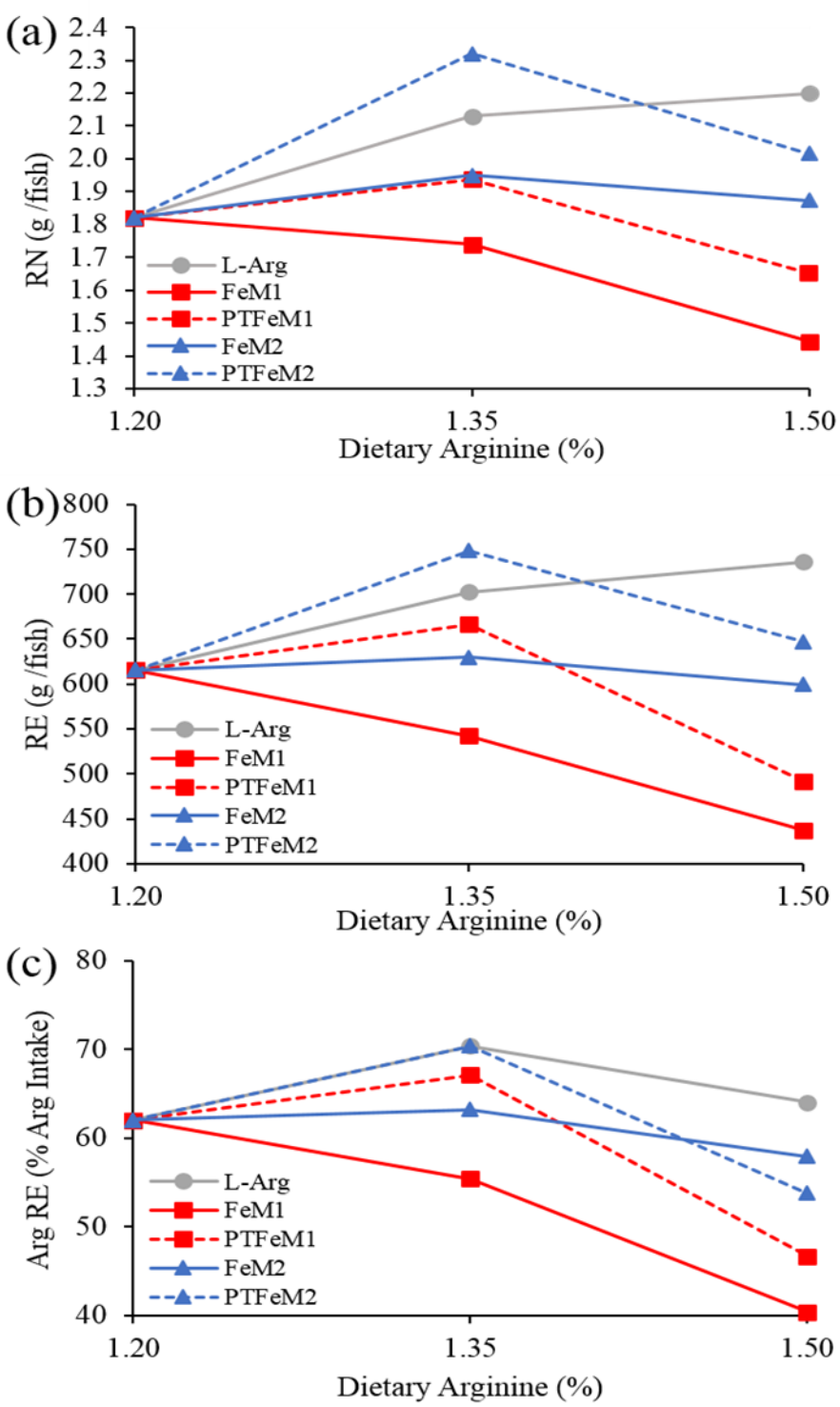


Figure 10. Retained nitrogen (a), recovered energy (b), and arginine retention efficiency (c) values of rainbow trout in response to being fed diets containing increasing arginine content supplied by L-arginine, FeM1, PTFeM1, FeM2 or PTFeM2. Values are mean (n = 3).

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