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Research Article

Evaluation of Acid-Insoluble Ash and Crude Fibre as Internal Markers in *In Vivo* Digestibility Studies: A Comparison in Three Cyprinid Species

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ABSTRACT

Though there are several reports of estimation of apparent nutrient digestibility values using different markers in fish nutritional studies, no study is available wherein the same nutrient digestibility was estimated using two or more markers. The study aimed at comparing the apparent Digestibility Coefficient (ADC) of nutrients, estimated using the markers-Acid-Insoluble Ash (AIA) and Crude Fibre (CF), in order to assess the suitability of digestibility markers. The apparent crude protein and fat digestibility values of experimental diets estimated in three indoor *in vivo* digestibility studies with cyprinids (*Labeo fimbriatus*, *L. rohita* and *Hypselobarbus pulchellus*) using AIA and CF are compared. In these studies, graded dietary levels of cottonseed meal, *azolla* meal and *moringa* leaf meal, respectively were evaluated. Apparent digestibility coefficients for protein and fat were calculated from protein and fat in feed and fecal matter, using analyzed AIA and CF contents as digestibility markers. In all the cases, ADC of both crude protein and fat were higher when estimated using AIA as compared to those estimated using CF. However, the general trend in the pattern of variation of ADC values among the different treatments remained almost the same, irrespective of the marker used. The results indicate that AIA as an internal marker is the most appropriate on the basis of the degree of precision that could be achieved in quantification of digestibility. The findings of this study are expected to help fish nutritionists in selection of suitable markers for *in vivo* digestibility studies.

Keywords: Acid-insoluble ash, Apparent digestibility coefficient, Crude fibre, Digestibility marker, Nutrient digestibility, Carp fish, Food quality, Nutritional food markers

Abbreviations: ADC: Apparent Digestibility Coefficient; AIA: Acid-Insoluble Ash; CF: Crude Fibre; HROM: Hydrolysis Resistant Organic Matter; CSM: Cotton Seed Meal; APD: Apparent Protein Digestibility; AFD: Apparent Fat Digestibility

INTRODUCTION

Determination of digestibility of different feed ingredients is significant for developing cost-effective diet formulas, assessing the quality of the ingredients, and preventing the release of nutrients into the environment that could harm the ecosystem [1,2]. Digestibility can be estimated both *in vitro* as well as *in vivo*. The former involves incubation of the test ingredient with the crude digestive enzyme extracted from the gut of the test fish [3]. Compared to *in vivo* procedures, *in vitro* techniques may be able to forecast changes in digestibility resulting from modifications in food processing more quickly and affordably. However, *in vivo* data on digestibility are needed to validate the data obtained through *in vitro* studies [4]. When using *in vitro* techniques, the responses are more extreme than when fish naturally digest food, and they release nutrients that wouldn't otherwise be available [5]. When comparing *in vitro* methods to *in vivo* approaches, Marletta L, et al. found that there is a propensity to overestimate the digestibility of leguminous plants [6]. Though *in vivo* estimation of digestibility is expensive and time-consuming, it is the most popular method to determine the bioavailability of nutrients from a feed stuff for a given species.

Different types of markers have been used in *in vivo* digestibility studies with fish. The external (exogenous) markers include chromic oxide, rare earth metal oxides such as ytterbium oxide and yttrium oxide, and hydrocarbon markers such as cholestane [7-9]. Acid-insoluble ash (AIA)/hydrolysis resistant ash, cellulose,

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Hydrolysis Resistant Organic Matter (HROM) and Crude Fibre (CF) have been used as internal (endogenous/natural/indigenous) markers for estimating nutrient digestibility [10-15]. There is ongoing debate about whether internal or exterior indicators are more suited and/or reliable and which specific indicators should be used. The majority of the research suggests that indigenous markers-which are present in significant amounts in the diet-are preferable to exogenous markers [16].

The use of internal markers such as AIA and CF has been recommended for the estimation of apparent digestibility coefficient (ADC) [17]. AIA has been reported to be an effective internal marker in digestibility studies with rainbow trout, *Oncorhynchus mykiss*; *Arctic charr*, *Salvelinus alpinus*; channel catfish, *Ictalurus punctatus*; and *tilapia*, *Oreochromis aureus* [18-21]. However, results obtained are not always consistent. When used with rainbow trout, AIA has been shown to provide digestibility coefficients that are comparable to those produced using chromic oxide, greater than those obtained using chromic oxide, or even lower [7,18,22]. Some researchers have opined limited use of AIA through their studies with different fish species [7,14,22].

CF has been found to be a more reliable marker than AIA for the cichlid *Etroplus suratensis* [11]. Several studies have shown CF as an effective marker [7,23].

Though AIA and CF have been used by several researchers as internal markers for digestibility estimations, no literature comparing the results obtained using these markers is available. The study aimed at comparing the ADC of nutrients, estimated using the marker-acid-insoluble ash and crude fibre, in order to assess the suitability of markers for *in vivo* digestibility studies. The present study compares the ADCs obtained for dietary protein and fat using AIA and CF as markers in three digestibility studies conducted with *Labeo fimbriatus*, *L. rohita* and *Hypselobabus pulchellus*, to find out the more appropriate one among them on the basis of the degree of precision that could be achieved in quantification. An effort has also been made to compare the ADC values obtained by several authors using these 2 markers.

MATERIALS AND METHODS

Applicable institutional guidelines for the care and use of animals were followed by the authors. In the present study, the digestibility values from three studies were taken for comparison. The digestibility values obtained using AIA as the marker were taken from two published papers and a communicated paper [24,25]. A brief explanation of preparation of experimental diets and conducting the digestibility trials is given for better understanding.

Preparation of experimental diets

The basal diet (control) for experiment 1 with *L. fimbriatus* was prepared with rice bran (45%) and groundnut cake (45%) (Table 1). The binder used was finger millet (9%). The diets had Cotton Seed Meal (CSM) at 10%, 20%, 30% and 40% levels replacing groundnut oilcake and rice bran in the basal diet [26]. For preparing the diets, the following steps were followed. All the ingredients other than rice bran were dried and powdered. After sieving the ingredients (0.5 mm), required quantity of the ingredients was mixed with hot water. A dough was made and passed through a pelletiser with 2 mm die. The pellets thus obtained were sun dried. The diets for the experiments 2 (*L. rohita*) and 3 (*H. pulchellus*) were also prepared in the same manner excepting that dried *Azolla* powder or *Moringa* leaf meal was used in place of CSM. The percentage incorporation of other ingredients in the 3rd experiment is not given since the whole experimental data is communicated to a journal.

Experimental design

Digestibility studies were conducted in 50 l, plastic tanks provided with aeration [24]. In the 1st study, 15 tanks were stocked with 10 advanced fingerlings of L. *fimbriatus* (*fimbriatus*) having body weight ranging from 7.39 g-10.88 g. After the initial acclimation for 10 days with control diet, fish were fed the 5 diets at 5% of body weight, every morning at 10:00 h, in triplicate tanks. The remaining pellets were siphoned out after 3 hours. The faecal matter from each tank was collected on the following day morning, by filtering the tank water with a nylon cloth (15 µm), dried and stored for proximate analysis. After faecal matter removal, nearly 50% of water from each tank was replaced with freshwater. This process was continued for a period of 45 days to obtain adequate quantity of dried faecal matter. Similar protocol was followed in the other 2 experiments. The fish used in the 2nd and 3rd experiment were *L. rohita* (rohu) and *H. pulchellus* (*pulchellus*) respectively, with stocking weight ranging from 8.50 g to 11.00 g and 3.25 g to 3.75 g, respectively [25].

Crude protein and fat content of the pelleted feed and faecal matter were analyzed [27]. Both AIA and CF were used as the reference markers [21,23,28,29]. AIA in diet and fecal matter was determined gravimetrically after drying, ashing, boiling of ash in hydrochloric acid (1:2), filtering and washing of the hot hydrolysate and re-ashing [18]. CF was estimated gravimetrically after chemical digestion and solubilization of other materials present. The fiber residue weight was then corrected for ash content after ignition [27].

Maynard et al, formula was used to calculate apparent nutrient digestibility as per the following equations [30].

Total dry matter digestibility (%)=100–[100 × % marker in feed/% marker in feces]

Nutrient digestibility (%)=100–[100 × %marker in feed/% marker in feces] × [%Nutrient in feces/% Nutrient in feed]

Statistical analyses

Analysis of variance was used for testing the data for statistical difference and Duncan's multiple range test (P=0.05) was applied to rank the treatment means tested for significance [31].

RESULTS

In all the digestibility trials, fish readily accepted the prepared diets and no mortality was observed. Table 2 gives the ADCs of crude protein and fat of CSM incorporated diets fed to *fimbriatus*.

The Apparent Protein Digestibility (APD) estimated using AIA varied between 83.01% and 86.69%, while that estimated using CF varied from 68.93% to 73.75%. The apparent fat digestibility (AFD) estimated using AIA ranged between 89.68% and 93.15%, while that estimated using CF fluctuated from 77.28% to 87.06%. It is evident that values were lower when CF was used as the marker compared to those obtained using AIA as the marker. However, the general response pattern in ADCs remained almost the same with both the markers. In the 2nd study with *Azolla*, the APD estimated in rohu using AIA varied between 73.45% and 87.97%, while that estimated using CF ranged between 67.28% and 82.31%. The AFD estimated using AIA fluctuated from 93.60% to 99.48%, while that estimated using CF varied between 92.13% and 99.28% (Table 3).

In this study also, the APD values were lower when CF was used as the marker compared to those obtained using AIA as the marker. However, the AFD values obtained with CF were almost equal to those obtained with AIA. In the 3rd experiment, the APD and AFD obtained with AIA in *pulchellus* varied between 87.23% and 94.75% and 91.55% and 98.16%, while the APD and AFD values calculated using CF ranged from 64.49% to 68.78% and from 86.91% to 91.01%, respectively (Table 4). Lower digestibility values with CF compared to AIA are apparent here also.

DISCUSSION

From the results of this study it is clear that ADC values obtained with AIA are higher compared to those obtained with CF which is evident from other studies as well. Tables 5 gives the ADC of protein and fat reported in earlier *in vivo* digestibility studies using AIA and CF as markers. Table 6 summarizes the APD and AFD values listed in Table 5. Among the 7 studies which used AIA as marker, 71% reported APD in the range of 90%-100%, whereas the rest 29% recorded APD of 80%-90%. Among the listed studies that used CF as marker, none reported APD ranging from 90%-100%. Moreover, a small percentage even reported APD in the lowest range of 50%-60%. With regard to AFD, studies with AIA recorded values ranging from 80-100%, while those with CF reported values varying from 70%-100%.

CF has been shown to be assimilated to a very small extent at least in certain fish species [32,33]. Although fish are unable to synthesize cellulase internally, their digestive tracts include microbial communities that aid in the digestion of plant materials [34,35]. The lower ADC values obtained in the present study with CF as the marker are attributable to the exogenous cellulase in fish gut.

Another important observation is the higher difference between the ADC values obtained through AIA and CF in the 3rd study with *pulchellus* compared to those obtained with the other two species. In nature, *pulchellus* is mainly a herbivorous, marginal submerged vegetative feeder subsisting on *Chara*, *Hydrilla*, *Vallisneria* and Ceratophyllum sp. In the gut analysis study by David A et al, decayed tissues of higher aquatic plants as well as grass blades were also detected [36]. Basavaraja N et al, observed that the captive stock of *pulchellus* very well accepts Napier grass, apart from artificial feed [37]. Hence, the species is expected to utilize plant material efficiently. Our study on the same species with dietary incorporation of aquatic plants-*Azolla sp*. and *Vallisneria sp*. has corroborated this hypothesis [38]. Being able to consume aquatic vegetation, *pulchellus* may be able to break down some quantity of fibre in the gut, leading to lower ADC values estimated through CF.

It is very unlikely that the AIA fraction can pass through the gut wall and hence, the higher ADC values. The advantages of using AIA as a marker includes the low cost and ease of measurement using basic laboratory equipment [10]. When utilizing AIA as a marker, it was suggested that it is preferable for the diet to contain AIA in excess of 0.75% [39]. In the present study, the AIA values ranged from 0.96% to 1.27 in the 1st study with CSM, 1.78% to 4.68% in the 2nd one with *Azolla* and 0.70% to 0.82% in the 3rd experiment with *Moringa* leaf meal. Sales J et al and Atkinson et al, opined that AIA is a trustworthy marker and could be used to accurately assess the digestibility of nutrients [18,40]. However, when pure ingredients are used for making experimental diets, AIA being primarily silica may not be present in sufficient quantity for accurate analysis. In such cases CF can be an alternative [41-54].

Table 1: Ingredient proportion (%) of Cotton Seed Meal (CSM) incorporated diets for evaluation in L. *fimbriatus*.

Ingredients	Control	10% CSM/*Azolla*	20% CSM/*Azolla*	30% CSM/*Azolla*	40% CSM/*Azolla*
Groundnut cake	45	40	35	30	25
Rice bran	45	40	35	30	25
Finger millet	8	8	8	8	8
CSM	0	10	20	30	40
Vitamin and mineral mixture	2	2	2	2	2

Note: CSM: Cotton Seed Meal.

Table 2: Apparent digestibility coefficient (%, Mean ± SD, n = 3) of crude protein and fat calculated using Acid-Insoluble Ash (AIA) and Crude Fibre (CF) in Cotton Seed Meal (CSM) incorporated diets fed to L. *fimbriatus*.

	Acid-Insoluble Ash (AIA) as the marker		Crude Fibre (CF) as the marker	
Diets	Protein	Fat	Protein	Fat
Control	83.01 ± 2.03[a]	91.60 ± 0.26[ab]	68.93 ± 0.79[a]	86.92 ± 0.23[d]
10% CSM	85.37 ± 1.49[ab]	93.15 ± 0.45[c]	72.38 ± 1.95[ab]	87.06 ± 0.44[d]
20% CSM	86.03 ± 0.78[b]	92.79 ± 0.79[c]	71.57 ± 0.70[bc]	82.96 ± 2.83[bc]
30% CSM	86.69 ± 0.28[b]	92.42 ± 1.40[bc]	73.75 ± 2.19[c]	83.35 ± 1.32[dc]
40% CSM	86.15 ± 1.73[b]	89.68 ± 0.80[a]	69.58 ± 1.13[bc]	77.28 ± 0.24[a]

Note: CSM: Cotton Seed Meal, [a,b,c]: Figures in the same column with different superscripts differ significantly (P<0.05).

Table 3: Apparent digestibility coefficient (%, Mean ± SD, n=3) of crude protein and fat calculated using Acid-Insoluble Ash (AIA) and crude fibre (CF) in *azolla* incorporated diets fed to *L. rohita*.

	AIA as the marker		CF as the marker	
Diets	**Protein**	**Fat**	**Protein**	**Fat**
Control	87.97 ± 0.40[c]	99.39 ± 0.12[c]	82.31 ± 0.52[c]	99.11 ± 0.19[cd]
10% *Azolla*	85.72 ± 0.47[c]	99.48 ± 0.03[c]	80.10 ± 2.06[c]	99.28 ± 0.06[d]
20% *Azolla*	85.17 ± 0.74[c]	96.76 ± 0.79[c]	80.13 ± 1.68[c]	95.71 ± 2.78[bc]
30% *Azolla*	77.59 ± 1.43[b]	93.60 ± 1.02[b]	74.14 ± 1.50[b]	92.63 ± 1.08[ab]
40% *Azolla*	73.45 ± 1.68[a]	93.61 ± 0.65[a]	67.28 ± 2.96[a]	92.13 ± 0.77[a]

Note: CF: Crude Fibre; AIA: Acid-Insoluble Ash, [a,b,c,d]: Figures in the same column with different superscripts differ significantly (P<0.05).

Table 4: Apparent digestibility coefficient (%, Mean ± Standard Deviation (SD), n = 3) of crude protein and fat calculated using Acid-Insoluble Ash (AIA) and Crude Fibre (CF) in *Moringa* Leaf Meal (MLM) incorporated diets fed to *H. pulchellus*.

	AIA as the marker		CF as the marker	
Diets	**Protein**	**Fat**	**Protein**	**Fat**
Control	92.34 ± 2.18[a]	97.83 ± 1.75[a]	65.43 ± 1.67[ab]	89.95 ± 0.30[a]
5% MLM	87.23 ± 1.74[a]	91.55 ± 1.39[a]	68.78 ± 1.24[c]	91.01 ± 0.33[a]
10% MLM	88.59 ± 1.71[a]	92.50 ± 1.94[a]	67.00 ± 1.16[bc]	90.12 ± 2.12[a]
15% MLM	94.75 ± 2.82[b]	98.16 ± 0.93[a]	67.57 ± 0.41[bc]	89.72 ± 1.56[a]
20% MLM	93.09 ± 1.69[a]	97.73 ± 1.71[a]	64.49 ± 0.89[a]	89.47 ± 1.59[a]
25% MLM	92.35 ± 1.01[a]	97.08 ± 1.85[a]	65.13 ± 1.81[a]	86.91 ± 0.66[a]
30% MLM	92.91 ± 2.67[a]	97.49 ± 1.84[a]	64.98 ± 0.41[a]	87.51 ± 0.40[a]

Note: MLM: *Moringa* Leaf Meal; [a,b,c]: Apparent digestibility coefficients; AIA: Acid-Insoluble Ash; CF: Crude Fibre.

Table 5: Apparent Digestibility Coefficient (ADC) of protein and fat reported in earlier *in vivo* digestibility studies using Acid-Insoluble Ash (AIA) and Crude Fibre (CF) as markers.

	Apparent Digestibility Coefficient (ADC) of protein (%, range)	Apparent Digestibility Coefficient (ADC) of fat (%, range)	Major ingredients	Species	Treatment	Reference
Acid-Insoluble Ash (AIA)	68.61-92.62	74.00-86.65	Fishmeal, blood meal, soybean meal, corn	*Clarias gaiepinus*	Dietary incorporation of brewer's spent grain replacing corn at 0 to 100%	[41]
Acid-Insoluble Ash (AIA)	82.96-89.98	87.97-91.13	Fishmeal, soybean meal, corn, cassava starch	*Oreochromis niloticus*	Dietary incorporation of Jackbean meal replacing SBM at 10 and 40%	[42]
Acid-Insoluble Ash (AIA)	78.04-92.22	74.76-91.24	Fishmeal, soybean meal, corn, starch	*Oreochromis niloticus*	Dietary incorporation of Jatropha seed meal replacing SBM at 20% and 40%	[43]
Acid-Insoluble Ash (AIA)	87.58-91.36	84.04-89.05	Fishmeal, soybean meal, maize, cassava starch	*Clarias gaiepinus*	Dietary incorporation of cooked sunflower seed meal replacing SBM at 15%, 30% and 45%	[44]
Acid-Insoluble Ash (AIA)	86.80-92.09	84.97-88.54	Fishmeal, soybean meal, corn meal, starch	*Clarias gaiepinus*	Dietary incorporation of toasted sunflower seed meal replacing SBM at 15%, 30% and 45%	[45]
Acid-Insoluble Ash (AIA)	82.7-92.5	82.7-92.5	Fishmeal, soybean meal, Corn, wheat middlings	*Oreochromis mykiss*	Dietary incorporation of sheep skin and alpaca skins hydrolysate at 30%	[46]

Acid-Insoluble Ash (AIA)	81.02-83.00	85.62-87.50	Wheat bran, rice bran, wheat middling, maize, sunflower meal, maize gluten meal, poultry meal, soybean meal	*Oreochromis niloticus*	Feeding balanced lysine diet and low lysine diet	[47]
Crude fibre	81.73-88.94	77.12-88.82	Fishmeal, groundnut cake, rice bran, tapioca	*Cyprinus carpio*	Dietary incorporation of Spirulina powder replacing FM at 25%-100%	[48]
Crude fibre	73.18-86.59	48.00-76.14	Fishmeal, groundnut cake, rice bran, tapioca	*Cyprinus carpio*	Dietary incorporation of 19 Norethindrone at 0.25 ppm-1.00 ppm	[49]
Crude fibre	50.14-58.32	54.07-70.47	Fishmeal, groundnut cake, rice bran, tapioca	*Cyprinus carpio*	Dietary incorporation of NaCl at 0.5%-2.0%	[50]
Crude fibre	66.09-78.39	50.29-74.98	Fishmeal, groundnut cake, rice bran, tapioca	*Cirrhinus mrigala*	Dietary incorporation of NaCl at 0.5%-2.0%	[50]
Crude fibre	74.22-83.29	66.70-79.34	Fishmeal, groundnut cake, rice bran, tapioca	*Labeo rohita*	Dietary incorporation of Spirulina powder replacing FM at 25%-100%	[51]
Crude fibre	82.76-88.28	78.39-83.66	Fishmeal, groundnut cake, rice bran, tapioca	*Catla catla*	Dietary incorporation of Spirulina powder replacing FM at 25%-100%	[51]
Crude fibre	59.57-74.84	59.97-90.63	Groundnut cake, rice bran, finger millet	*Labeo calbasu*	Dietary incorporation of *azolla* powder replacing basal diet at 10%-40%	[52]
Crude fibre	59.17-74.55	87.25-90.15	Groundnut cake, rice bran, finger millet	*Labeo fimbriatus*	Dietary incorporation of *azolla* powder replacing basal diet at 10%-40%	[53]
Crude fibre	47.63-69.56	77.07-81.42	Groundnut cake, rice bran, finger millet	*Cyprinus carpio*	Dietary incorporation of *azolla* powder replacing basal diet at 10%-40%	[53]
Crude fibre	72.27-83.35	84.31-94.73	Groundnut cake, rice bran, finger millet	*Labeo fimbriatus*	Dietary incorporation of soybean powder replacing basal diet at 10%-40%	[53]
Crude fibre	71.84-83.00	79.31-85.54	Groundnut cake, rice bran, finger millet	*Cyprinus carpio*	Dietary incorporation of soybean powder replacing basal diet at 10%-40%	[53]
Crude fibre	72.49-78.67	75.78-83.71	Groundnut cake, rice bran, finger millet	*Labeo fimbriatus*	Dietary incorporation of silkworm pupa powder replacing basal diet at 10-40%	[53]
Crude fibre	69.71-77.64	85.52-95.28	Groundnut cake, rice bran, finger millet	*Cyprinus carpio*	Dietary incorporation of silkworm pupa powder replacing basal diet at 10%-40%	[53]
Crude fibre	44.91-75.45	82.79-96.83	Groundnut cake, rice bran, finger millet	*Catla catla*	Dietary incorporation of *azolla* powder replacing basal diet at 10%-40%	[54]
Crude fibre	68.76-84.96	80.93-88.32	Groundnut cake, rice bran, finger millet	*Catla catla*	Dietary incorporation of soybean powder replacing basal diet at 10%-40%	[54]
Crude fibre	67.17-70.87	84.18-94.66	Groundnut cake, rice bran, finger millet	*Catla catla*	Dietary incorporation of silkworm pupa powder replacing basal diet at 10%-40%	[54]

Table 6: Range (%) of protein and fat Apparent Digestibility Coefficient (ADC) values obtained with Crude fibre (CF) and Acid-Insoluble Ash (AIA) as listed in Table 5.

	90%-100%	80%-90%	70%-80%	60%-70%	50%-60%
ADC of protein estimated with CF	0	43.75%	43.75%	6.25%	6.25%
ADC of fat estimated with CF	37.5%	37.5%	25%	0	0
ADC of protein estimated with AIA	71%	29%	0	0	0
ADC of fat estimated with AIA	43%	57%	0	0	0

Note: ADC: Apparent Digestibility Coefficient; CF: Crude Fibre; AIA: Acid-Insoluble Ash.

CONCLUSION

Though there are several reports of estimation of apparent nutrient digestibility values using different markers in fish nutritional studies, no study is available wherein the same nutrient digestibility was estimated using two or more markers. In the present study, the apparent crude protein and fat digestibility values of experimental diets estimated in three indoor *in vivo* digestibility studies with cyprinids (*Labeo fimbriatus*, *L. rohita* and *Hypselobabus pulchellus*) using AIA and CF are compared. The results of the present study indicate that usage of AIA as an internal marker is the most appropriate on the basis of the degree of precision that could be achieved in quantification of digestibility in carps. The findings of this study are expected to help fish nutritionists in selection of suitable markers for *in vivo* digestibility studies.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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