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Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids

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Abstract

Trivalent oxides of yttrium and rare earth metals were evaluated as inert markers in apparent digestibility studies with salmonids in four experiments. In Experiment 1, 100 mg kg^{-1} of each of 15 oxides (Dy_2O_3, Er_2O_3, Eu_2O_3, Gd_2O_3, Ho_2O_3, La_2O_3, Lu_2O_3, Nd_2O_3, Pr_2O_3, Sc_2O_3, Sm_2O_3, Tb(III and IV) oxide, Tm_2O_3, Y_2O_3 and Yb_2O_3) were included in a feed fed to rainbow trout. The ratio between each marker and Yb_2O_3 in stripped faeces was used as an indicator of recovery. Only Er_2O_3, Ho_2O_3 and Tm_2O_3 had lower recoveries than the other markers. Experiment 2 compared the excretion rates of Cr_2O_3 and of the selected alternative markers (La_2O_3, Y_2O_3 and Yb_2O_3). A feed with 7.5 g kg^{-1} of Cr_2O_3 and 750 mg kg^{-1} of each of the other markers was fed to Atlantic salmon for a period of 1 week. Thereafter, the fish were fed with a marker-free feed, and gastro-intestinal evacuation was evaluated by comparing the marker ratios in the feed and in the faeces sieved from the outlet water of the tanks. The results did not reveal any systematic differences in evacuation among the various markers. Experiment 3 compared in vitro solubility of Cr_2O_3, Dy_2O_3, La_2O_3, Y_2O_3 and Yb_2O_3 in weak acid (HCl, pH 3 as in stomach contents of Atlantic salmon), weak acid neutralised with NaOH, and in water. Cr_2O_3 was not dissolved. Only 1.3% of Yb_2O_3, 22% of Y_2O_3, 31% of Dy_2O_3, and 96% of La_2O_3 was soluble in weak acid, but more than 99% of the dissolved markers precipitated when neutralised, and none of the markers were soluble in water. Experiment 4 compared the estimates of apparent digestibility coefficients (ADCs) of nitrogen and fat in rainbow trout when using

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Cr_2O_3, La_2O_3, Y_2O_3 and Yb_2O_3 as markers. The feed contained 10 g kg^{-1} Cr_2O_3 and 100 mg kg^{-1} of each of the other markers. Markers in feeds and stripped faeces were dissolved for the analysis with both HCl:HNO_3 and H_3PO_4:$MnSO_4$. Except for Cr_2O_3, the markers gave similar ADCs within each acid solubilisation procedure. The ADCs of fat were similar with both procedures, but the ADCs of nitrogen were 0.2% lower with HCl:HNO_3 than with H_3PO_4. Cr_2O_3 was incompletely dissolved in HCl:HNO_3, resulting in low ADCs. With H_3PO_4, no differences were seen among the ADCs obtained with Cr_2O_3 and the other markers. In conclusion, trivalent metal oxides, such as La_2O_3, Y_2O_3 and Yb_2O_3, can substitute Cr_2O_3 in digestibility studies with salmonids, and can be used at lower concentrations without affecting accuracy.

Keywords: Digestibility; Inert marker — chromic oxide (Cr_2O_3)–lanthanium oxide (La_2O_3)–yttrium oxide (Y_2O_3)–ytterbium oxide (Yb_2O_3); Rainbow trout (*Oncorhynchus mykiss*); Atlantic salmon (*Salmo salar*)

1. Introduction

In the beginning of 1990s, several preliminary experiments with trivalent oxides of yttrium and rare earth metals (the III B and lanthanide series in the periodic system) as inert markers for digestibility estimation in fish were conducted at AKVAFORSK (Hillestad and Olafsen, 1990; Austreng and Thomassen, 1991; Austreng et al., 1994; Meland, 1991; Winther, 1991; Meland and Austreng, 1992; Monsås and Hustveit, 1994; Thomassen and Berge, 1994). The present study represents a continuation of this work, comparing the feasibility of yttrium and rare earth metal (lanthanide) oxides as inert markers compared with chromic oxide.

An inert marker for the estimation of digestibility should ideally be: (1) homogeneously incorporated into the feed and easily and accurately analysed, even at low concentrations; (2) indigestible and does not affect the metabolism of the animal; (3) pass through the gastro-intestinal tract at the same rate as the dietary nutrients; and (4) hygienic and harmless to people and the environment. The predominant inert marker in fish studies is chromic oxide (Cr_2O_3) (Nehring, 1963; Nose, 1967; Smith and Lovell, 1973; Austreng, 1978; Cho and Slinger, 1978, Windell et al., 1978), which was first used to estimate the digestibility in livestock at the beginning of the 20th century (Edin, 1918).

In fish, chromic oxide does not appear to satisfy the criteria listed above. Most important, dietary chromic oxide is not always totally recovered in the faeces (Bäckman, 1977; Lied et al., 1982). Thus, it must be included at relatively high concentrations (5–10 g kg^{-1} feed) in order to obtain homogeneous analytical results. High dietary levels of chromic oxide may disturb the absorption and metabolism of nutrients in fish. In studies with Arctic char, dietary chromic oxide (10 g kg^{-1} feed) reduced the concentration of fat in the faeces, and altered the aerobic bacterial flora in the intestine (Ringø, 1993a,b,c). Recent studies with hybrid tilapia also indicated that dietary chromic oxide (5–20 g kg^{-1} feed) affected the digestibility of carbohydrate, growth, and body composition (Shiau and Liang, 1995; Shiau and Shy, 1998). In contrast, Ng and Wilson (1997), Fernandez et al. (1999), and Hillestad et al. (1999) found no effect of dietary chromic oxide on the estimates of macronutrient digestibility in channel catfish, gilthead

sea bream, and Atlantic salmon, respectively. However, Fernandez et al. (1999) found that estimates of calcium and phosphorus absorption differed systematically with different concentrations of dietary chromic oxide. Furthermore, dietary chromic oxide has been reported to pass through the gastro-intestinal tract at a different rate than nutrients (Dabrowski and Dabrowska, 1981; Tacon and Rodrigues, 1984; Leavitt, 1985), hence, introducing variation to digestibility estimates. Also, the acceptability of feeds is influenced by the colour of the feeds in visual feeders like salmonid fish (Browman and Marcotte, 1987; Jakobsen et al., 1987). As chromic oxide colours the feed green, this may affect the feed consumption. Chromium may be toxic even at low concentrations and, in some cases, allergenic to man. This necessitates extra care during the manufacture, use, and/or analysis of feeds and faeces with high concentrations of chromium. Traditionally, perchloric acid has been used to solubilise chromic oxide in feeds and faeces (Petry and Rapp, 1971), but this is now limited in most laboratories because of the need for expensive hoods to prevent explosion.

Trivalent oxides of yttrium and lanthanides should theoretically satisfy the criteria listed above. They are slightly soluble in weak acid, but have a very low solubility under neutral conditions (Budavari et al., 1989). Rare earth metals are not known to be essential elements for fish, and should not affect the metabolism. They have an affinity for plant cell walls, and have been used in their soluble form to label indigestible plant fibre, in order to study digestion in ruminants (Smith, 1989a). They can be detected by plasma emission spectroscopy (Combs and Satter, 1992) and by atomic absorption, and thus, can be measured in feed and faeces at low concentrations (in the mg kg^{-1} range). Furthermore, the oxides of yttrium and lanthanides are white or grey in colour, and do not affect the colour of the feed when included at low concentrations.

Oxides of samarium, scandium, ytterbium and europium have been successfully used as inert markers to measure apparent utilization of nutrients in humans (Hutcheson et al., 1979). In salmonid fish, oxides of yttrium and lanthanides have already been used in several published digestibility studies with apparent success (Lanari and Turri, 1996; Bjerkeng et al., 1997; Hung et al., 1997; Refstie et al., 1997, 1998, 1999; Grisdale-Helland and Helland, 1998; Helland and Grisdale-Helland, 1998; Médale et al., 1998; Skrede et al., 1998; Storebakken et al., 1998a,b,c; Sugiura et al., 1998a,b; Hillestad et al., 1999). Combination of different trivalent oxides of yttrium and lanthanides has also been used to determine qualitative discrimination among different diets (Refstie et al., 1997, 1998), and gastro-intestinal evacuation rate (Storebakken et al., 1999). Oxides of samarium and lanthanum have also been used to monitor the movement of feedstuffs through the digestive tract of ruminants (Crooker et al., 1982), even though water soluble but poorly absorbable salts (acetates, chlorides and nitrates) of rare earth elements are more commonly used for this purpose (Dixon et al., 1983; Goetsch and Galyean, 1983; Coleman et al., 1984; Ortigues et al., 1990). Ytterbium acetate has also been evaluated as an inert marker to estimate protein digestibility in leader prawn, but gave different values than chromic oxide (Deering et al., 1984).

The aims of the present experiments are: to screen trivalent oxides of yttrium and different lanthanides with respect to faecal recovery to evaluate their potential in digestibility studies with salmonids (Experiment 1); to find out if yttrium and lanthanide oxides pass through the gastro-intestinal tract at the same rate as chromic oxide

(Experiment 2); to determine if the solubility of the markers in weak acid affects the faecal recovery of the markers (Experiment 3); and to test if the oxides of yttrium and lanthanides used as inert markers give digestibility estimates comparable to those obtained with the traditionally used marker, chromic oxide (Experiment 4).

2. Materials and methods

2.1. Common procedures

Four experiments were carried out at AKVAFORSK at Sunndalsøra, Norway. Two experiments were carried out with rainbow trout (*Oncorhynchus mykiss*), one with Atlantic salmon (*Salmo salar*), and one in vitro, based on the results obtained with Atlantic salmon. The experiments were carried out in 8–9°C freshwater, and the concentration of O_2 in the outlet water never fell below 7–8 mg l^{-1}.

The metal oxides (markers) were supplied by Sigma (St. Louis, MO, USA), with a purity $\geq 99.9\%$. Prior to inclusion in the feed mixes, mixtures of various inert markers were screened and premixed with wheat flour. Concentrations of the various markers in feeds and faeces were analysed on ICAP spectrometers (Thermo Jarrel/Ash, Franklin, MA, USA) at the Norwegian Agricultural Service Laboratory, Ås, Norway, or at the Institute of Occupational Health, Oslo, Norway. Two different acid solubilisation procedures were used (specified under each experiment).

The results were analysed statistically by one- and two-way analysis of variance (ANOVA), and significant ($P < 0.05$) differences among means or interactions were ranked by least-square means. The results from the screening, solubilisation, and digestibility experiments are presented as means $\pm$ s.e.m.

2.2. Screening of possible dietary markers (Experiment 1)

Ten rainbow trout with a mean weight of 0.3 kg were kept in each of the two tanks. The fish were fasted for 14 days prior to the experiment. The trout were fed a moist feed (Table 1), which was extruded through a meat-mincer with 5-mm dies, and kept frozen until the night before it was used. The feed contained a mixture of metal oxides: Dy_2O_3, Er_2O_3, Eu_2O_3, Gd_2O_3, Ho_2O_3, La_2O_3, Lu_2O_3, Nd_2O_3, Pr_2O_3, Sc_2O_3, Sm_2O_3, Tb (III and IV) oxide, Tm_2O_3, Y_2O_3, Yb_2O_3 and Cr_2O_3. Each marker was added at a concentration of 100 mg (kg DM)$^{-1}$.

The fish were fed by hand, to excess, eight times per day throughout a 9-day period before faeces were collected. Faeces were obtained by careful stripping from the posterior part of the intestine, as described by Austreng (1978). The faeces from all fish were pooled per tank prior to the analysis of markers. Samples of 150 mg faeces or 200–300 mg feed were combusted at 600°C for 1.5–2.0 h. Three milliliters *ortho*-phosphoric acid:$MnSO_4$ solution (30-ml 0.66 M $MnSO_4$, 485-ml H_2O and 485-ml *ortho*-phosphoric acid per liter) was added after cooling, together with 4 ml of 0.38-M KBr solution. Thereafter, the samples were diluted with distilled water to 250 ml, and kept refrigerated in darkness until analysed. This acid solubilisation procedure is hereafter referred to as the ''phosphoric acid solubilisation''.

Table 1
Formulation and composition of the feeds

Experiment	1	2	4
Formulation, g kg^{-1}			
Fish meal[a]	458.8	521.2	542.2
Wheat, extruded	120.0	225.0	239.5
Fish oil[b]	120.0	170.0	177.0
Cellulose powder[c]		40.0	
Alginate[d]	10.0		
PVP[e]		20.0	21.0
Vitamins and micro-minerals[f]	10.0	14.0	10.0
Marker premix[g]	1.2	9.8	10.3
Water	280.0		
Chemical composition			
DM, g kg^{-1}		903	885
Per kilogram of DM			
Crude protein (N×6.25), g		497	479
Crude fat, g		188	253
Ash, g		113	88
Gross energy, MJ		22.3	

[a]Norse LT, Norsildmel, Bergen, Norway.
[b]Different fish oils of unknown origin were used in each experiment.
[c]Nunc Inter Med, Roskilde, Denmark.
[d]Protanal 120, Na-alginate, Pronova, Drammen, Norway.
[e]Polyvinylpyrrolidone, ISP Europe, Guildford, England.
[f]Composition: Refstie et al. (1997).
[g]Experiment 1: 100 mg kg^{-1} (dry ingredients) of each of Dy_2O_3, Er_2O_3, Eu_2O_3, Gd_2O_3, Ho_2O_3, La_2O_3, Lu_2O_3, Nd_2O_3, Pr_2O_3, Sc_2O_3, Sm_2O_3, Tb(III and IV) oxide, Tm_2O_3, Y_2O_3,Yb_2O_3 and Cr_2O_3; Experiment 2: 750 mg kg^{-1} of each of La_2O_3, Y_2O_3 and Yb_2O_3, 7.5 g kg^{-1} of Cr_2O_3; Experiment 4: 100 mg kg^{-1} of each of La_2O_3, Y_2O_3 and Yb_2O_3, 10 g kg^{-1} of Cr_2O_3.

The ratios of marker in faeces and feeds were calculated as: CL_F/CL_D, where CL_F is the concentration of marker in faeces, and CL_D is the concentration in the feed. Each marker was rated relatively to Yb_2O_3, with its ratio set to 1.0, based on the low acid solubility of this marker in Experiment 3.

Based on a combination of high recovery figures (Table 2) and abundant supply, Dy_2O_3, La_2O_3, Y_2O_3 and Yb_2O_3 were compared with Cr_2O_3 in the subsequent experiments. Dy_2O_3 (data not shown) was excluded from Experiments 2 and 4 due to inconsistent analytic estimates of the dietary concentrations.

2.3. Uniformity of passage of markers through the gastro-intestinal tract (Experiment 2)

Three groups of Atlantic salmon (mean initial weight, 70 g; 20 fish in each tank) were kept in 40-l conical tanks, equipped with wire-mesh sieves to trap uneaten feed and faeces (Helland et al., 1996). The feed (Table 1) was cold-pelleted on a laboratory pellet mill (Simon Heesen, The Netherlands), with a pellet diameter of 5 mm. Dietary dry matter (DM, 105°C until stable weight), crude protein (Kjeldahl-N × 6.25), crude fat

Table 2
Concentration of inert marker in feeds and molar faeces:feed marker ratio, relative to the ratio found with Yb (Yb = 1.00). (Mean ± s.e.m, $n = 2$ tanks) (Experiment 1)

Marker	Dietary marker, mg (kg DM)$^{-1}$	Marker ratio (faeces:feed)
Yb_2O_3	102.2	1.00
Dy_2O_3	99.4	1.02 ± 0.04
Er_2O_3	107.6	0.86 ± 0.00
Eu_2O_3	99.3	0.97 ± 0.06
Gd_2O_3	98.8	0.94 ± 0.01
Ho_2O_3	107.0	0.86 ± 0.02
La_2O_3	100.2	1.00 ± 0.04
Lu_2O_3	98.3	0.94 ± 0.01
Nd_2O_3	99.3	0.96 ± 0.00
PrO	100.1	0.96 ± 0.03
Sc_2O_3	97.9	0.95[a]
Sm_2O_3	102.2	0.91 ± 0.03
Tb(III, IV) oxide	97.7	0.95 ± 0.01
Tm_2O_3	99.7	0.88 ± 0.02
Y_2O_3	101.3	1.04 ± 0.05

[a]No replicate.

(HCl-hydrolysis and ether extraction), and gross energy (bomb-calorimetry) were analysed. The fish were pre-adapted to the feed for 7 days prior to the experiment. The salmon were fed four meals (0800, 1400, 2000 and 0200) during the 24-h light-day. Then, the feed was replaced by a feed with similar composition, but without any marker added (0 h). Sieved faeces were collected from the wire mesh collector and pooled by tank every 2 h, dried, combusted in glass scintillation vials, digested by the ''phosphoric acid procedure'' described for Experiment 1, and analysed for the concentration of Cr, La, Y and Yb.

2.4. Solubility of markers under different pH conditions (Experiment 3)

Before the experiment, the pH of the stomach contents was measured in five Atlantic salmon kept in saltwater (salinity, 32 g l^{-1}; 7.4°C; weight, 0.14–0.32 kg; feed, 3-mm Smolt, Skretting, Stavanger, Norway) and five salmon kept in freshwater (5.2°C ; weight, 47–59 g; feed, 3-mm Sveve, Skretting). The pH was measured immediately after anaesthetising the fish with MS-222, dissecting the stomach open, inserting a Ross 8163 electrode (Orion Research, Berverly, MA, USA) connected to an Orion pH/ISE Meter Model 720A into the stomach content, and allowing the pH to stabilize for 30 s prior to monitoring the value. The pH range of the stomach contents was from 3.5 to 5.6 in saltwater and from 3.1 to 5.4 in freshwater.

Cr_2O_3, Dy_2O_3, La_2O_3, Y_2O_3 and Yb_2O_3 (100 mg of each) were accurately weighed into each of the twelve 500-ml flasks and filled to the mark with distilled water; eight of the flasks had the pH adjusted to 3.0 with 15–16 drops of 4 M HCl. The flasks were shaken every half-hour for 3 h. During that time, pH in the acidified flasks gradually increased from 3.0 to 5.7. The pH increased from 6.3 to 6.6 in the four flasks with

distilled water. At 3 h, the contents of four of the acidified bottles were neutralised with 6 M NaOH. The contents of the flasks were then immediately filtered (0.2 μm) and the liquid phase was kept at 4°C for 2 weeks before the analysis of concentration of dissolved marker by ICP.

2.5. Digestibility estimates obtained by using different markers and different acid solubilisation methods on feeds and faeces (Experiment 4)

A feed (Table 1) with a mixture of La_2O_3, Y_2O_3 and Yb_2O_3 (concentration 100 mg (kg DM)$^{-1}$ of each) and Cr_2O_3 (10.0 g (kg DM)$^{-1}$) was fed to trout in three tanks for 2 weeks prior to the stripping of faeces. The feed was cold-pelleted and its nutrient composition analysed as described for Experiment 2, and the fish were fed every 15 min, 24 h day^{-1}. Each tank (water volume 500 l) contained 45 fish with a mean initial weight of 0.65 kg. The faeces were freeze-dried and ground with a pestle and mortar, and fish scales were removed. The freeze-dried faeces were also analysed for nitrogen and fat by the same procedures as the feed.

Feeds and freeze-dried faeces were dissolved by two different methods: (1) phosphoric acid solubilisation, as in Experiments 1 and 2; and (2) hydrochloric:nitric acid solubilisation (developed for the elemental analysis in fish tissues by Shearer, 1984; first published for dissolving Y_2O_3 and Yb_2O_3 in feed and faeces for digestibility trials by Refstie et al., 1997) where freeze-dried samples (150–200 mg) were combusted in glass scintillation vials. When cooled, 5 ml of concentrated HCl:HNO_3 2:1 (v/v) was added and the samples were boiled until colourless. When cooled, a few drops of water were added, the sample was dissolved in 1.25 ml concentrated HNO_3 and diluted to 25 ml with distilled water prior to the concentration determination by ICAP-spectrometry.

3. Results

The results from the screening test (Experiment 1; Table 2), indicate that the recovery of several of the elements (Dy, Eu, La, Nd, Pr, Sc, Sm, Tb, Tm, Y) were 95% or more of the values obtained with Yb. The two oxides giving the lowest recovery, Er and Ho, had analysed dietary concentrations higher than what was actually added, indicating that the low recovery partly may have been due to incomplete mixing of those two markers in the feed samples.

In Experiment 2, the analysed dietary concentration (Table 3) of La_2O_3, Y_2O_3 and Yb_2O_3 ranged from 729 to 880 mg (kg DM)$^{-1}$, while that of Cr_2O_3 was 7.5 g kg^{-1}.

Table 3
Dietary marker concentration in Experiment 2

	Inert markers, (kg DM)$^{-1}$
Cr_2O_3, mg	7500
La_2O_3, mg	729
Y_2O_3, mg	880
Yb_2O_3, mg	856

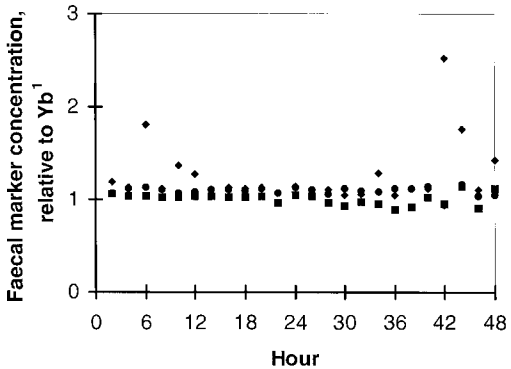

Fig. 1. Faecal concentration of Cr_2O_3 (♦), La_2O_3 (■) or Y_2O_3 (●), relatively to Yb_2O_3 in Atlantic salmon (Experiment 2). The results are corrected for differences in dietary concentration ($D_{Yb} / D_M * F_M / F_{Yb}$; D and F represent dietary and faecal concentrations, M is the actual marker, and Yb represents ytterbium oxide).

The initial (0- and 2-h samplings) concentration of Cr_2O_3 in the faeces was 59 g (kg DM)$^{-1}$. Corresponding values for La_2O_3, Y_2O_3 and Yb_2O_3 were 6.4, 5.7 and 6.3 g (kg DM)$^{-1}$, respectively. The marker concentrations then decreased after the switch to the feed without the marker added, and the faeces contained approximately 50% of the initial concentration at 20 h. The concentrations in the faeces fluctuated between 6% and 15% of the initial value from 40 h, and 8–10% of the initial concentrations were still recovered at 48 h. During the 48 h of faeces collection, the ratio among La_2O_3, Y_2O_3 and Yb_2O_3 in the faeces remained similar to that in the feed (Fig. 1), in spite of more than 90% dilution with faecal material from the marker-free feed. The ratio between Cr_2O_3 and Yb_2O_3, and thus, La_2O_3 and Y_2O_3, was similar to the feed in the beginning (0 and 2 h), middle (12–40 h) and end (46–48 h) of the sampling period; however, deviating results were seen at 6, 42 and 44 h.

In Experiment 3, both Dy_2O_3, La_2O_3, Y_2O_3 and Yb_2O_3, but not Cr_2O_3 dissolved in weak acid (Table 4). La_2O_3 dissolved almost completely, Dy_2O_3 and Y_2O_3 were intermediate, and only 1.3% of the Yb_2O_3 was dissolved. However, neutralisation with NaOH resulted in more than 99% of the dissolved La_2O_3 and 99.9% or more of the other markers precipitating. None of the markers were soluble in water.

Table 4
Solubility[a] (%) of different markers after 3 h in hydrochloric acid (pH 3), followed by neutralisation to pH 7 with NaOH, or 3 h in distilled water (pH 6.6) (Experiment 3)

	pH 3	pH 3 → pH 7	pH 6.6
Cr_2O_3	< 0.01	< 0.01	< 0.01
Dy_2O_3	31.2 ± 1.6^a	0.3 ± 0.2^b	0.03 ± 0.01^b
La_2O_3	96.3 ± 3.4^a	1.1 ± 0.7^b	0.05 ± 0.01^b
Y_2O_3	22.2 ± 0.3^a	0.2 ± 0.2^b	0.01 ± 0.00^b
Yb_2O_3	1.33 ± 0.08^a	0.02 ± 0.01^b	$< 0.01^b$

[a]Different superscripts[a,b] indicate significant ($P < 0.05$) differences among pH treatments, within marker.

Table 5
Analysed marker concentrations in feed and faeces in Experiment 4
Mean ± s.e.m., $n = 3$.

	Hydrochloric:nitric acid	Phosphoric acid
Feed, (kg DM)$^{-1}$		
Cr_2O_3, mg	5300	10,300
La_2O_3, mg	99	100
Y_2O_3, mg	107	104
Yb_2O_3, mg	110	106
Faeces, (kg DM)$^{-1}$		
Cr_2O_3, g	152 ± 2	447 ± 1
La_2O_3, mg	4313 ± 21	4502 ± 31
Y_2O_3, mg	4621 ± 32	4654 ± 36
Yb_2O_3, mg	4745 ± 35	4755 ± 43

In Experiment 4, the analysed concentration of La_2O_3, Y_2O_3 and Yb_2O_3 in the feed (Table 5) ranged from 99 to 110 mg (kg DM)$^{-1}$ when solubilised by hydrochloric:nitric acid, and 100–106 mg kg^{-1} when solubilised with phosphoric acid. Faecal marker concentrations followed a similar pattern as the dietary concentrations. The hydrochloric:nitric acid solubilisation resulted in poor solubilisation of Cr_2O_3, and only 5.3 g (kg DM)$^{-1}$ was recovered in the feed, in spite of an addition of 10 g kg^{-1}. The phosphoric acid solubilisation resulted in a dietary concentration of 10.3 g (kg DM)$^{-1}$.

Table 6
Apparent digestibility coefficients of nitrogen, fat and energy comparing different inert markers and solubilisation methods (Experiment 4)[a]

	Hydrochloric:nitric acid	Phosphoric acid	
ADC of N with different markers			
Cr_2O_3	83.1 ± 0.2[a]	88.7 ± 0.1[b]	
La_2O_3	88.9 ± 0.0[b]	89.1 ± 0.1[b]	
Y_2O_3	88.8 ± 0.1[b]	89.0 ± 0.2[b]	
Yb_2O_3	88.8 ± 0.1[b]	89.0 ± 0.2[b]	
ADC of fat with different markers			
Cr_2O_3	89.2 ± 0.3[a]	92.8 ± 0.3[b]	
La_2O_3	92.9 ± 0.2[b]	93.1 ± 0.3[b]	
Y_2O_3	92.9 ± 0.2[b]	93.0 ± 0.3[b]	
Yb_2O_3	92.9 ± 0.2[b]	93.0 ± 0.2[b]	
Two-way ANOVA[b]	Effect of marker	Effect of solubilisation	Interaction
ADC of N	46.2***	16.7***	36.3***
ADC of fat	43.2***	16.4***	33.2***

[a] Mean ± s.e.m., $n = 3$ tanks. Different superscripts[a,b] indicate significant ($P < 0.05$) differences among markers within digestion procedures, identified by one-way ANOVA.

[b] The values express the proportion (Type I SS) of the total variation in ADC of macronutrients explained by main effects and two-factor interactions.

*** $P < 0.001$.

One-way ANOVA revealed significant differences only among the apparent digestibility coefficient (ADC) estimates (Table 6) obtained by the hydrochloric:nitric acid solubilisation by using Cr_2O_3 and the other markers and solubilisation procedures. The two-way ANOVA revealed significant effects of marker, solubilisation method and interaction between the two on the ADC estimates for all nutrients. This was mostly ascribed to the low recovery of Cr_2O_3 dissolved by hydrochloric:nitric acid. When the results were analysed by two-way ANOVA excluding the results obtained with Cr_2O_3, no significant main effects or interactions were obtained among the three different markers for ADC of fat. For ADC of protein, solubilisation method resulted in a significant ($P = 0.02$) difference, but the estimates obtained by dissolving in hydrochloric:nitric acid were only 0.2% lower than the ones obtained using phosphoric acid.

4. Discussion

This study verified the feasibility of Y_2O_3 and oxides of lanthanides such as La_2O_3 and Yb_2O_3 as inert markers in the digestibility studies with salmonids. The results also showed that Y_2O_3, La_2O_3, and Yb_2O_3 gave accurate estimates of apparent digestibility at much lower dietary concentrations (100 mg kg^{-1}) than necessary when using Cr_2O_3 (10 g kg^{-1}).

The observed similar ratios in feeds and faeces of the various oxides of yttrium and lanthanides in Experiment 1 indicate that they were recovered at a similar rate after passing through the digestive tract. This is supported by the similar rate of faecal elimination of La_2O_3, Y_2O_3 and Yb_2O_3, found in Experiment 2. The observed rate of gastro-intestinal evacuation was slower than observed in a previous experiment with salmon of similar size and at similar water temperature, where 50% evacuation of a fish meal-based feed was reached at 18 h, and evacuation was almost complete at 20 h (Storebakken et al., 1999). The most plausible explanation to the difference is a lower feed intake in the present than in the previous experiment (Fänge and Grove, 1979; Smith, 1989b; Bromley, 1994).

No systematic trends were seen for the deviating results with Cr_2O_3 in the passage rate study. The deviation from the general pattern with Cr_2O_3 may have been due to incomplete mixing of the marker in the feed. The experiment does not provide any obvious explanation of the eventual differences in homogeneity of inclusion of Cr_2O_3 and other markers in the feed because all markers were sieved and mixed by the same procedure into the same premix.

The solubility of yttrium and lanthanide oxides in weak acid (Budavari et al., 1989) was confirmed in Experiment 3, but under pH conditions similar to the salmon stomach, this did not seem to be the case for Cr_2O_3. However, the similar ratios among the various markers in the faeces and the similar digestibility estimates obtained with the various markers show that the solubilised proportion of the markers was not absorbed in the stomach. The results demonstrate that they were precipitated when the digesta were neutralised in the intestine, and passed unabsorbed through the gastro-intestinal tract. The similar passage rate of all markers in Experiment 2 demonstrates that the solubility of the markers in the stomach did not affect their flow through the gastro-intestinal tract.

The lack of significant difference among ADC-estimates with the various markers when using the phosphoric acid method in Experiment 4, and the low random error of the ADC estimates (Searcy-Bernal, 1994), show that the oxides of yttrium, lanthanum and ytterbium can replace Cr_2O_3 in the digestibility studies with fish at an inclusion level as low as 100 mg kg^{-1} feed. This is in accordance with the findings of Hillestad et al. (1999). The lower ADC values obtained by using Cr_2O_3 and hydrochloric:nitric rather than phosphoric acid in Experiment 4 are ascribed to incomplete solubilisation of Cr_2O_3 by the hydrochloric:nitric acid method. This problem was more pronounced for faeces than feeds (Table 5), probably due to the higher marker concentration in faeces. This result shows that the hydrochloric:nitric acid method should not be used to dissolve Cr_2O_3 in feeds and faeces, and also, underlines the need for an extra precaution when employing new methods to replace perchloric acid (Petry and Rapp, 1971), which is now abandoned in many laboratories due to the hazards involved when using it.

The hydrochloric:nitric acid method also resulted in slightly lower recovery of the oxides of yttrium and lanthanides from faeces than the phosphoric acid method, with a subsequent difference in ADC of nitrogen of 0.2%. However, the practical consequence of this difference is minute, and both methods can be used to dissolve yttrium and lanthanide oxides. Furthermore, the hydrochloric:nitric acid method is preferred if the experiment is aimed at estimating the availability of elements, utilising the same solution as for assessing the marker (Storebakken et al., 1998b,c; Sugiura et al., 1998a,b), since only hydrochloric and nitric acids, and no other elements, are added to the sample. The phosphoric acid method or other methods verified to dissolve chromic oxide completely should, however, be employed when dissolving samples that contain Cr_2O_3.

In conclusion, uptake of Y_2O_3 and oxides of lanthanides in their soluble form does not seem to occur from the stomach, and the markers appear to be precipitated once entering the intestine. Yttrium and lanthanide oxides are more easily dissolved for analysis than Cr_2O_3, preventing possible errors due to low and uneven recovery of the marker in feed and faeces. Thus, they can be used accurately at much lower dietary concentrations. The estimates of the apparent digestibility obtained when using Y_2O_3 and oxides of lanthanides such as La_2O_3 and Yb_2O_3 as inert markers are similar to the estimates obtained with Cr_2O_3.

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