

In vitro digestion method to evaluate solubility of dietary zinc, selenium and manganese in salmonid diets

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ABSTRACT

Background: The determination of dietary mineral solubility is one of the main steps in the evaluation of their availability for a given species.

Methods: This study proposed an *in vitro* digestion method (acidic and alkaline hydrolysis). The method was applied to evaluate the solubility of inorganic and organic forms of zinc (Zn), selenium (Se) and manganese (Mn) in salmonid diets. An inorganic mineral (IM) diet was supplemented with zinc sulphate, sodium selenite and manganous sulphate and an organic mineral (OM) diet was supplemented with zinc chelate of glycine, L-selenomethionine and manganese chelate of glycine.

Results: The solubility of Zn was similar in both diets tested. The amount of soluble Zn was low in the acidic hydrolysis (3–8%) and lower in the alkaline hydrolysis (0.4–2%). The solubility of Se was higher in the OM diet (7–34%) compared with the IM diet (3–12%). Regarding Mn, after the acidic hydrolysis the solubility was higher in the IM diet (6–25%) than the OM diet (4–17%). The *in vitro* solubility were compared with *in vivo* availability of Zn, Se and Mn. Data obtained for solubility (%) of Zn, Se and Mn was lower when compared with apparent availability (%) of Zn, Se and Mn.

Conclusion: Data obtained demonstrated that solubility of Zn, Se and Mn was influenced by the mineral chemical form supplemented to the diet and by the gastrointestinal environment. The solubility of Zn, Se and Mn was not comparable with the apparent availability of Zn, Se and Mn. Nevertheless, the effect of the chemical form of the minerals was similar for the solubility of Zn, Se and Mn and the apparent availability of Zn, Se and Mn. Considering the overall results of this study, the *in vitro* method could replace some of the *in vivo* studies for a qualitative evaluation but not for a quantitative evaluation.

1. Introduction

For many years in the salmonid aquaculture industry, fish meal was used as main protein source. However, increasing demands and prices for fish meal has led the industry to increase the use of other protein sources [1]. These protein sources primarily include plant proteins, e.g. soybean meal and wheat gluten meal [2]. However, the market is changing rapidly and novel proteins sources (e.g. algae, yeast and insect meal) are used in commercial salmonid feeds. Before being used in feeds, novel ingredients and diets are evaluated for digestibility, growth performance and feed conversion. This evaluation is usually performed in feeding trials which are lengthy and hence costly. Thus, there is a big

incitement to develop tools for screening of the performance of novel ingredients and diets, which are less time consuming and costs less. *In vitro* methods can be applied to obtain more detailed knowledge on the influence of the different diet components, which is important in nutritional evaluation of ingredients and diets [3]. For salmonids, several *in vitro* digestion methods have been applied for studying protein digestibility and amino acid solubility [4–8]. In contrast, fewer studies have been dedicated to the study of *in vitro* mineral solubility in salmonids [7,9]. Applying *in vitro* digestion methods for the evaluation of mineral solubility can be challenging, as several factors need to be considered simultaneously. Morales and co-workers described an *in vitro* digestion model mimicking the conditions in the gut of rainbow

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trout (*Oncorhynchus mykiss*) and used this model to evaluate the bioavailability of phosphorus (P) in fish meal and soybean meal [7]. Weerasinghe and co-workers applied an *in vitro* method to study the solubility of P in 12 plant and animal feed ingredients [9].

Using mineral sources with higher availability in feed can reduce the level of mineral supplementation without compromising the dietary requirements of the fish [10]. In addition, the reduced amount of minerals in feeds will have an influence on mineral interactions and the mineral load in faeces [11]. Considering the three elements included in this study (*i.e.* zinc (Zn), selenium (Se) and manganese (Mn)), Se is the only element for which there is clear evidence of a higher availability of organic sources than inorganic forms in fish (as reviewed by Prabhu, Schrama and Kaushik [12]). Consequently, there is an increasing interest in comparing the availability of inorganic mineral salts with organic mineral forms. Thus, it was hypothesised in this study that dietary mineral solubility *in vitro* is affected by the chemical forms of Zn, Se and Mn. Minerals need to be released from the dietary matrix before becoming available for absorption [13]. Hence, determining the amount of soluble mineral can provide some information on mineral availability. For instance, Lall and co-workers reported significant differences in the availability of P, the more soluble the salt, the higher the availability of P [14]. Considering the successful use of an *in vitro* method to study the solubility of P, it was hypothesised in this study that an *in vitro* method can be useful when studying the solubility of Zn, Se and Mn in salmonid diets, and that the solubility of the Zn, Se and Mn could be used as a measure for Zn, Se and Mn availability.

The aims of the present work were to i) develop an *in vitro* digestion method to evaluate solubility of dietary Zn, Se and Mn in salmonid diets; ii) evaluate the influence of the gastrointestinal environment and the chemical form of the mineral supplemented in the diets on the solubility of Zn, Se and Mn, and iii) evaluate whether the solubility and apparent availability of Zn, Se and Mn are comparable.

2. Materials and methods

2.1. Chemicals and reagents

Analytical reagent grade chemicals and Milli-Q® water (18.2 MΩ cm) (EMD Millipore Corporation, Billerica, MA, USA) were used throughout the study unless otherwise stated. Hydrochloric acid (HCl, Emsure® ACS, ISO, 37% w/w), sodium hydroxide (NaOH, Emsure® ACS, ISO), hydrogen peroxide (H₂O₂, Emsure® ACS, ISO, 30% w/w) were obtained from Merck (Darmstadt, Germany). Nitric acid (HNO₃, trace select, ≥ 69% w/w) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Multielement (product number SS60835) and germanium (product number SS1230) standard solutions were obtained from Spectrascan TeknoLab (Drøbak, Norway). Pepsin (P7012, from porcine gastric mucosa), protease (P5147, type XIV from *Streptomyces griseus*), trypsin (T0303, type IX from porcine pancreas), α-chymotrypsin (C4129, type II from bovine pancreas) were purchased from Sigma Aldrich (St. Louis, MO, USA). Zinc sulphate monohydrate (ZnSO₄·H₂O, Zn 35%, Vilomix, Hønefoss, Norway), zinc chelate of glycine hydrate (Zn(x)₁₋₃·nH₂O, x = anion of glycine (C₂H₄NO₂⁻), Zn 26%, Phytobiotics, Eltville, Germany), sodium selenite (Na₂SeO₃, Se 4.5% BMP, DSM nutritional products, Basel, Switzerland), L-selenomethionine (C₅H₁₁NO₂Se, Se 0.16%, Orffa Additives, Werkendam, The Netherlands), manganous sulphate monohydrate (MnSO₄·H₂O, Mn 32%, Vilomix, Hønefoss, Norway) and manganese chelate of glycine hydrate (Mn(x)₁₋₃·nH₂O, x = anion of glycine (C₂H₄NO₂⁻), Mn 22%, Phytobiotics, Eltville, Germany) were used as mineral additive sources.

2.2. Experimental diets

The diets used in this study are described elsewhere [15]. In brief, an inorganic mineral (IM) diet was supplemented with zinc sulphate, sodium selenite and manganous sulphate and an organic mineral (OM)

Table 1

Formulation and proximate composition of the experimental diets. Values of proximate composition are presented as average ± standard deviation (n = 3). IM diet = diet supplemented with inorganic mineral sources (*i.e.* Zn sulphate, selenite and Mn sulphate). OM diet = diet supplemented with organic mineral sources (*i.e.* Zn chelate of glycine, L-selenomethionine, Mn chelate of glycine).

Ingredients (%)		
Wheat		8.3
Corn gluten		15.0
Hi-pro soya		14.4
Wheat gluten		20.0
Soya protein concentrate		10.0
Fish meal ^a		5.0
Fish oil ^a		9.9
Rapeseed oil ^b		12.3
Microingredients and premixes ^c		5.4
Experimental premixes (Zn, Mn and Se) ^d		0.6
Proximate composition, analysed (n = 3)		
Dry weight (%)	IM diet	OM diet
	91.0 ± 0.0	93.0 ± 0.0
Lipid (%)	22.0 ± 0.0	21.3 ± 0.6
Protein, analysed as N × 6.25 (%)	46.7 ± 1.5	43.7 ± 0.6
Ash (%)	3.9 ± 0.1	4.0 ± 0.0
Mineral concentration, analysed (n = 3)		
Zn (mg kg ⁻¹)	140 ± 11	152 ± 10
Se (mg kg ⁻¹)	0.54 ± 0.05	0.57 ± 0.01
Mn (mg kg ⁻¹)	27 ± 3	35 ± 13

^a North-Atlantic.

^b European, non-GM.

^c Contains monoammonium phosphate, histidine HCl, yttrium oxide, L-lysine and DL-methionine and astaxanthin; standard vitamin and mineral mix, excluding the target minerals Zn, Mn and Se.

^d The experimental premixes were manually prepared and added to the diets.

diet was supplemented with zinc chelate of glycine, L-selenomethionine and manganese chelate of glycine. In both diets, the nominal concentration of Zn, Se and Mn were 150 mg kg⁻¹ diet, 0.5 mg kg⁻¹ diet and 25 mg kg⁻¹ diet, respectively. The formulation, proximate composition and mineral concentration of the experimental diets are given in Table 1. The total concentrations of Zn, Se and Mn in the IM diet were 140 ± 11 mg of kg⁻¹ diet (n = 3), 0.54 ± 0.05 mg kg⁻¹ diet (n = 3) and 27 ± 3 mg kg⁻¹ diet (n = 3), respectively. In the OM diet, the total concentrations of Zn, Se and Mn were 152 ± 10 mg of kg⁻¹ diet (n = 3), 0.57 ± 0.01 mg kg⁻¹ diet (n = 3) and 35 ± 13 mg kg⁻¹ diet (n = 3), respectively.

2.3. Proximate composition analyses

The diets IM and OM were analysed in triplicate for dry matter, ash content, total lipid content and total protein content following standard procedures. Detailed information regarding the proximate composition analyses is described elsewhere [15]. Briefly, dry matter content was measured gravimetrically after drying at 104 °C for 24 h, ash content was determined by combustion in a muffle furnace flame combustion at 550 °C for 16–18 h, and total lipid was determined gravimetrically after acid hydrolysis and extraction with diethyl ether [16]. Total nitrogen (N) was measured with a nitrogen analyser (Vario Macro Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany) according to AOAC official methods of analysis [17], and protein calculated as N × 6.25.

2.4. Solubility of zinc, selenium and manganese

This method was developed based on principles described elsewhere [18–21]. The *in vitro* digestion method included two steps: acidic and alkaline hydrolysis, corresponding to the conditions in the stomach and the intestine, respectively. The choice of using pH 2.1 for acidic hydrolysis and pH 8 for alkaline hydrolysis was made, both taking in consideration physiological conditions [22] and optimal pH for enzyme

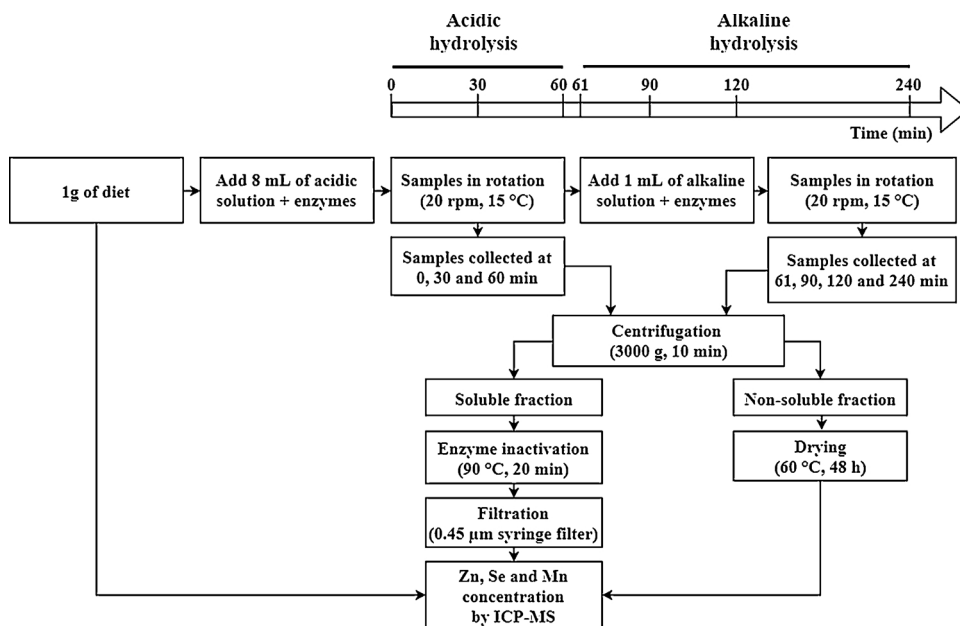


Fig. 1. Flowchart of the *in vitro* digestion method. The method includes two steps: acidic and alkaline hydrolysis. The acidic hydrolysis was initiated by adding the acidic solution (1 mg mL^{-1} pepsin in 10 mM HCl) and the alkaline hydrolysis followed the acidic hydrolysis was initiated by adding the alkaline solution (1 mg mL^{-1} protease, trypsin and α -chymotrypsin in 100 mM NaOH).

activity. An overview of the *in vitro* method can be seen in Fig. 1. The IM and OM diets were homogenised for 10 s at 3000 rpm using a knife mill (GM 300, Retsch GmbH, Haan, Germany) and kept at 4°C until further analysis. Mineral solubility was determined in triplicate and approximately 1 g of homogenised diet was weighed in round bottom tubes (13 mL). The acidic solution (10 mM HCl , pH 2.1) was prepared by diluting HCl in Milli-Q® water. The alkaline solution (pH 8) was prepared by dissolving an appropriate amount of NaOH to reach the desired ionic strength (100 mM) in Milli-Q® water. The acidic hydrolysis was initiated by adding 8 mL of the precooled (15°C) acidic solution (1 mg mL^{-1} pepsin in 10 mM HCl). Samples were collected at 0, 30 and 60 min. The alkaline hydrolysis followed the acidic hydrolysis was initiated by adding 1 mL of the precooled (15°C) alkaline solution (1 mg mL^{-1} protease, trypsin and α -chymotrypsin in 100 mM NaOH). Samples were collected at 61, 90, 120 and 240 min. All samples were kept in rotation (20 rpm , 15°C) and removed from the rotator according to the sampling time plan (0, 30, 60, 61, 90, 120 and 240 min). After removing the samples from the rotator they were submitted to centrifugation (3000 g , 10 min) and the soluble fractions were transferred to new tubes. The soluble fraction was submitted to heat (90°C , 20 min) for enzyme inactivation and subsequently filtered through a $0.45 \mu\text{m}$ disposable syringe filter (Sartorius, Göttingen, Germany) and transferred to new tubes. The non-soluble fractions were dried in an oven (TS 8136, Termaks, Bergen, Norway) for 48 h at 60°C . The soluble and non-soluble fractions ($n = 3$, %) were determined by calculating the ratio of Zn, Se and Mn obtained for each fraction compared, respectively, to total Zn, Se and Mn in diet. The variation obtained was acceptable taking into consideration the measurement uncertainty of the method, which is 20% for Zn and Mn, and 25% for Se.

2.5. Apparent availability of zinc, selenium and manganese in Atlantic salmon

The apparent availability of Zn, Se and Mn in diet IM and OM were determined in Atlantic salmon (*Salmo salar*) in a previous study [15]. Briefly, each diet was tested in triplicate tanks and the fish were fed the experimental diets for 11 days. A pooled sample of faeces from fish ($n = 20$) in the same tank was collected into a plate by stripping from the ventral fin to anus. The sample was collected in a 50 mL falcon tube and immediately stored at -20°C . The samples were kept at -20°C until further analysis. In this study, the results for the apparent availability of Zn, Se and Mn of diet IM and OM were used.

2.6. Mineral determination by inductively coupled plasma mass spectrometry

The concentration of Zn, Se, Mn and yttrium in diets and faeces were determined by inductively coupled plasma mass spectrometry (ICP-MS) as described elsewhere [15]. The Zn, Se and Mn concentrations in the soluble fractions ($n = 3$) and non-soluble fractions ($n = 3$) were also determined by ICP-MS using the following procedure. For the soluble fractions, approximately 1 mL of the soluble fraction was diluted in 9 mL of HNO_3 (5% w/w) prior to analysis by ICP-MS. For the non-soluble fractions, approximately 0.8 g of non-soluble fraction was digested using 10 mL of HNO_3 (69% w/w) and 10 mL of H_2O_2 (30% w/w) in an UltraClave (Milestone Inc., Shelton, CT, USA). The digested samples were subsequently diluted to 50 mL with Milli-Q® water. The samples were subsequently introduced into the nebulizer tube of the ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) equipped with an auto sampler (FAST SC-4Q DX, Elemental Scientific, Omaha, USA) and Zn, Se and Mn were detected at mass-to-charge ratios (m/z) 66, 78, 55 amu, respectively. The analysis was performed in the KED reaction mode using helium as collision gas. A solution of germanium (^{72}Ge) was added on-line for correction of instrumental drift during the analysis. The tuning of the ICP-MS was performed using a tuning solution (1 ppb tuning solution B, Thermo Fisher, in 2% HNO_3 and 0.5% HCl) prior to analysis. Data were collected and processed using the Qtegra ICP-MS software (Thermo Scientific, version 2.1, 2013). For the quantitative determination of Zn, Se and Mn an external calibration curve ($10\text{--}500 \text{ ng mL}^{-1}$) was used. Two certified reference materials (CRM) were included to assess the accuracy of the method, *i.e.* lobster hepatopancreas (TORT-3; National Research Council Canada, Ottawa, Ontario, Canada) and oyster tissue (SMR 1566b; National Institute of Standards and Technology, Gaithersburg, USA). The measured concentrations for the two CRMs ($n = 4$) were in agreement with the certified values.

2.7. Formulas and statistics

The solubility (%) and the apparent availability (%) were determined as described by Etcheverry and colleagues [23] (Eq. 1) and Cho and Slinger [24] (Eq. 2), respectively.

$$\text{Solubility (\%)} = \frac{\text{Zn or Se or Mn in the soluble fraction}}{\text{Zn or Se or Mn in diet}} * 100 \quad (1)$$

$$\text{Apparent availability (\%)} = 100 - \left(100 \frac{\text{yttrium in diet} * \frac{\text{Zn or Se or Mn in faeces}}{\text{Zn or Se or Mn in diet}}}{\text{yttrium in faeces}} \right) \quad (2)$$

2.8. Prediction of chemical species of zinc and manganese in aqueous conditions

The software Visual MINTEQ can be used for the prediction of chemical species and solubility of minerals in an aqueous conditions [25]. Visual MINTEQ (version 2.40b, 2006, KTH, Sweden) was run to predict the chemical species of Zn and Mn in an aqueous conditions. The media pH was set to 2.1 and 8, and temperature to 15 °C. Speciation was calculated based on ion concentration of 100 µg L⁻¹.

3. Results

3.1. Solubility of zinc, selenium and manganese

The soluble fraction recovered after applying the *in vitro* digestion method are presented in Table 2 and Fig. 2. The solubility of Zn (%) in diet IM ranged from 0.70 ± 0.03% to 8.3 ± 0.6% and in diet OM it ranged from 0.40 ± 0.06% to 7.6 ± 0.5%, between 0 and 240 min. The solubility of Se, between 0 and 240 min, ranged from 2.6 ± 0.5% to 11.8 ± 0.9% in the IM diet, and from 6.6 ± 0.3% to 34 ± 1% in the OM diet. The solubility of Mn ranged from 3.63 ± 0.06% to 25.1 ± 0.5% and from 3.8 ± 0.2% to 17 ± 3% in the IM and OM diets, respectively. The solubility of Zn (%) was low in both diets tested (Fig. 2A). The solubility of Zn decreased from 8 to 4 % between 0 and 60 min (diet IM) and from 8 to 3% (diet OM) in the acidic hydrolysis. In the alkaline hydrolysis, the solubility of Zn increased from 1.2 to 2% (diet IM) and from 0.4 to 1.2% (diet OM). The solubility of Se (%) was higher than the solubility of Zn (%). The solubility of Se, in the acidic hydrolysis, ranged from 3 to 28%, and in the alkaline hydrolysis the solubility ranged from 5 to 34% (Fig. 2B). The solubility of Se (%) was higher in the OM diet (7–34%) than for the IM diet (3–12%). As can be seen in Fig. 2B, the solubility of Se was initially similar in the two diets. However, between 0 and 30 min the solubility of Se increased from 7 to 25% in diet OM, while in diet IM the solubility of Se only increased from 3 to 5%. The solubility of Mn ranged from 4 to 25% in the acidic hydrolysis, and in the alkaline hydrolysis the solubility ranged from 4 to 8% (Fig. 2C). The solubility of Mn (%) was higher for the IM diet (6–25%) than for the OM diet (4–17%) during the acidic hydrolysis. However, similar solubility of Mn (%) was obtained during the alkaline hydrolysis in both diets. A decrease in the solubility of Mn was observed when changing from the acidic hydrolysis to the alkaline hydrolysis (see Fig. 2C). The non-soluble fraction recovered after applying the *in vitro* digestion method are described in Table 2. The recovered Zn in the non-soluble fraction, between 0 and 240 min, in diet IM ranged from 72 ± 2% to 88 ± 2% and in diet OM ranged from 66 ± 2% to 87 ± 2%. The recovered Se in the non-soluble fraction, between 0 and 240 min, in diet IM ranged from 67 ± 3% to 76 ± 3% and in diet OM ranged from 48 ± 1% to 68 ± 2%. The recovered Mn in the non-soluble fraction, between 0 and 240 min, in diet IM ranged from 62 ± 4% to 99 ± 7% and in diet OM ranged from 43 ± 6% to 71 ± 8%.

3.2. Prediction of chemical species of zinc and manganese in aqueous conditions

The Zn and Mn species in aqueous conditions were predicted by Visual MINTEQ [25]. At pH 2.1 and 15 °C, the Zn⁺² and Mn⁺² ions prevailed (100%). At pH 8 and 15 °C, Zn⁺² and Mn⁺² ions were the predominant species (Zn: 85.2%; Mn: 99.9%) but other less predominant Zn and Mn species such as ZnOH₂ (aq) (10.9%), ZnOH⁺ (3.9%) and MnOH⁺ (0.1%) were also present in solution.

Table 2

Soluble and non-soluble Zn, Se and Mn recovered after applying the *in vitro* digestion method. Soluble and non-soluble fractions (%) are expressed as average ± standard deviation (n = 3). Soluble and non-soluble fractions (%) were determined by calculation of the ratio of Zn, Se and Mn obtained for each fraction compared, respectively, to total Zn, Se and Mn in diet.

Time (min)	[Zn]			
	Diet IM		Diet OM	
	Soluble fraction (%)	Non-soluble fraction (%)	Soluble fraction (%)	Non-soluble fraction (%)
0	8.3 ± 0.6	88 ± 2	7.6 ± 0.5	87 ± 2
30	6.0 ± 0.4	72 ± 2	5.6 ± 0.5	66 ± 2
60	4.5 ± 0.1	72.3 ± 0.5	3.3 ± 0.1	66 ± 2
61	1.17 ± 0.08	86 ± 3	0.40 ± 0.06	85.4 ± 0.3
90	0.70 ± 0.03	82.3 ± 0.9	0.58 ± 0.02	78 ± 1
120	0.9 ± 0.1	79 ± 2	0.8 ± 0.1	78.2 ± 0.3
240	1.8 ± 0.2	77.5 ± 0.7	1.2 ± 0.1	73 ± 2

Time (min)	[Se]			
	Diet IM		Diet OM	
	Soluble fraction (%)	Non-soluble fraction (%)	Soluble fraction (%)	Non-soluble fraction (%)
0	2.6 ± 0.5	76 ± 3	6.6 ± 0.3	68 ± 2
30	5.1 ± 0.6	71 ± 2	24.8 ± 0.8	55.6 ± 0.6
60	5.8 ± 0.3	73 ± 2	27.9 ± 0.7	50.2 ± 0.6
61	4.9 ± 0.2	74 ± 2	27 ± 1	51 ± 1
90	7.7 ± 0.4	73 ± 2	30 ± 1	50 ± 1
120	8.2 ± 0.4	70 ± 1	30 ± 2	48 ± 1
240	11.8 ± 0.9	67 ± 3	34 ± 1	50 ± 6

Time (min)	[Mn]			
	Diet IM		Diet OM	
	Soluble fraction (%)	Non-soluble fraction (%)	Soluble fraction (%)	Non-soluble fraction (%)
0	6.4 ± 0.5	99 ± 7	3.8 ± 0.2	71 ± 8
30	25.1 ± 0.5	97 ± 3	17 ± 3	58 ± 26
60	20 ± 1	66 ± 7	16 ± 2	43 ± 6
61	3.63 ± 0.06	74 ± 5	5 ± 2	64 ± 22
90	6.0 ± 0.2	67 ± 3	6 ± 2	49 ± 18
120	7.3 ± 0.3	62 ± 4	7 ± 2	47 ± 10
240	7.9 ± 0.2	69 ± 5	7 ± 2	55 ± 13

3.3. Apparent availability of zinc, selenium, manganese in Atlantic salmon

The apparent availability of Zn, Se and Mn (average ± standard deviation, n = 3) in Atlantic salmon fed the IM diet were 32 ± 12%, 63 ± 4% and 31 ± 12%, respectively (Fig. 3). The apparent availability of Zn, Se and Mn (average ± standard deviation, n = 3) in Atlantic salmon fed the OM diet were 29 ± 13%, 67 ± 6% and 31 ± 17%, respectively (Fig. 3).

3.4. Comparison between of solubility and apparent availability of zinc, selenium and manganese

The solubility (%) at the end of the *in vitro* method (240 min) was compared with the apparent availability (%) (Fig. 3). The solubility (average ± standard deviation, n = 3) of the IM diet and the OM diet (shown as white bars) was compared with the apparent availability (average ± standard deviation, n = 3) of the IM diet and the OM diet (shown as blue bars), respectively. Overall, solubility (%) of Zn, Se and Mn is lower than the apparent availability (%) of Zn, Se and Mn. The apparent availability (%) has a larger variation when compared with the solubility (%).

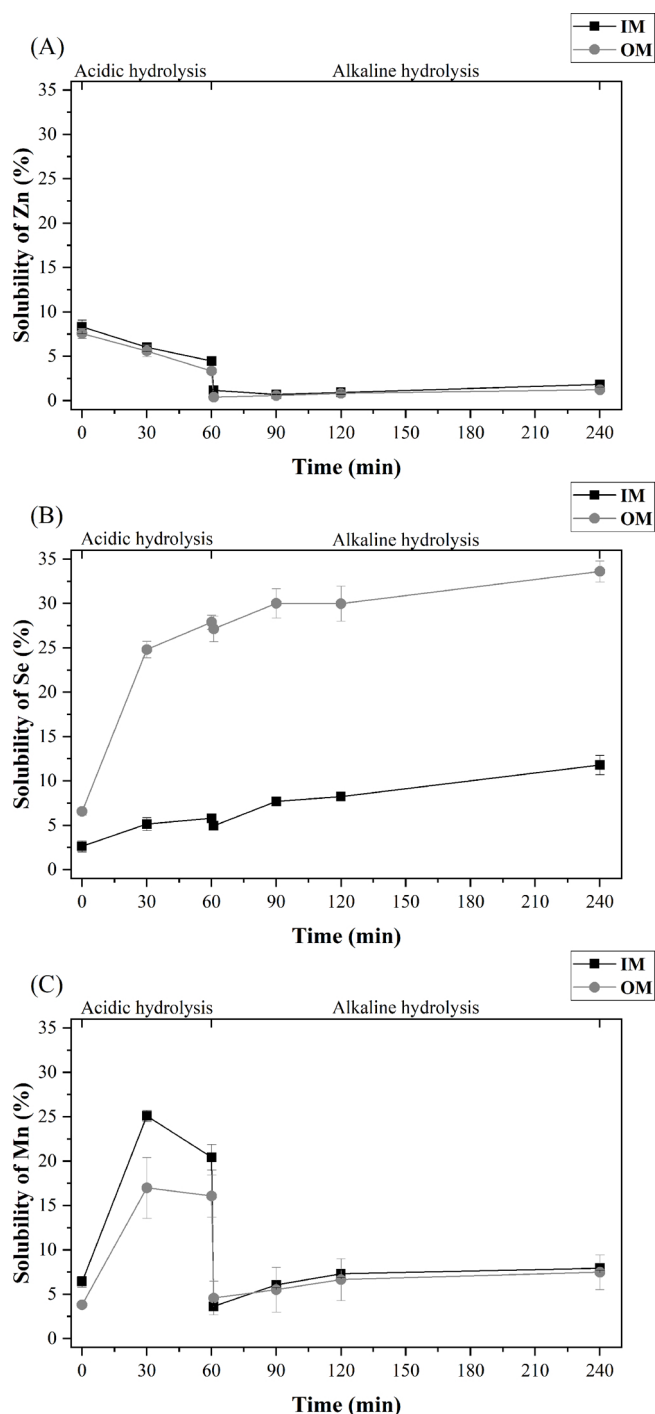


Fig. 2. Solubility of Zn (A), Se (B) and Mn (C) (%) calculated as solubility (%) = $100 \times (\text{Zn or Se or Mn in soluble fraction} / \text{Zn or Se or Mn in diet})$; the points represent the average with respective standard deviation ($n = 3$); the black line and the grey line represent the IM diet and OM diet, respectively; IM diet = diet supplemented with inorganic mineral sources (*i.e.* Zn sulphate, selenite and Mn sulphate); OM diet = diet supplemented with organic mineral sources (*i.e.* Zn chelate of glycine, L-selenomethionine, Mn chelate of glycine).

4. Discussion

4.1. An *in vitro* digestion method can be used to evaluate solubility of zinc, selenium and manganese in diets

In this study, one aim was to develop an *in vitro* digestion method to evaluate solubility of dietary Zn, Se and Mn in diets for Atlantic salmon.

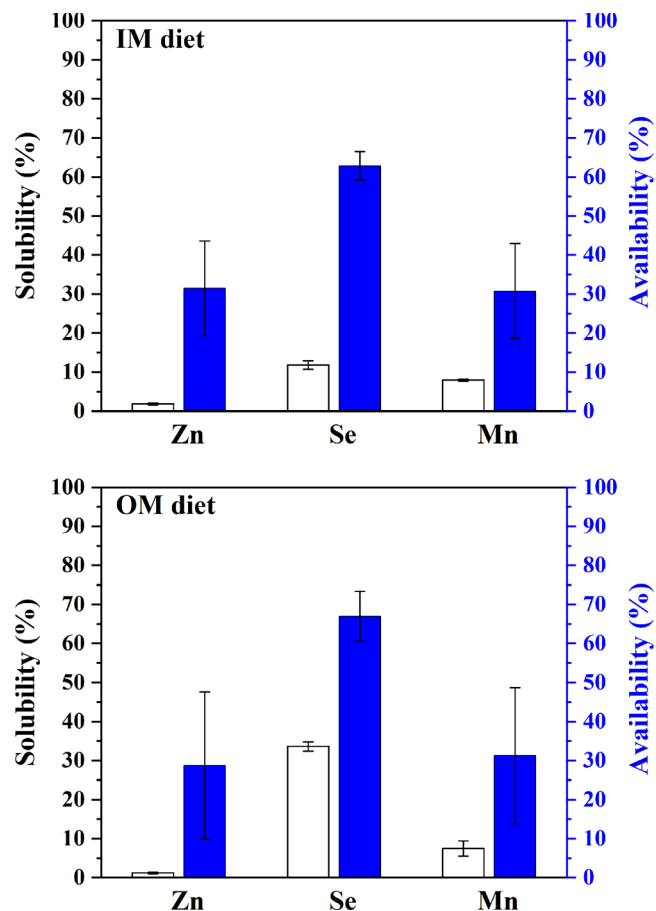


Fig. 3. Solubility (% shown in white bars) and apparent availability (% shown in blue bars) of Zn, Se and Mn; IM diet = diet supplemented with inorganic mineral sources (*i.e.* Zn sulphate, selenite and Mn sulphate); OM diet = diet supplemented with organic mineral sources (*i.e.* Zn chelate of glycine, L-selenomethionine, Mn chelate of glycine); Solubility of Zn, Se and Mn ($n = 3$, %) was calculated as solubility (%) = $100 \times (\text{Zn or Se or Mn in soluble fraction} / \text{Zn or Se or Mn in diet})$ and apparent availability ($n = 3$, %) of Zn, Se and Mn was calculated as apparent availability (%) = $100 - [100 \times (\text{yttrium in diet} / \text{yttrium in faeces}) \times (\text{Zn or Se or Mn in faeces} / \text{Zn or Se or Mn in diet})]$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The method consisted of two steps: acidic and alkaline hydrolysis, corresponding to the conditions present in the stomach and the intestine, respectively. *In vitro* simulation of digestion in fish has so far mainly been based upon methods developed for terrestrial animals [3]. Thus, the majority of the *in vitro* digestion studies in fish uses gastrointestinal conditions that are adapted for terrestrial animals (*e.g.* temperature, digestion period and enzymes) [3]. The temperature used during this *in vitro* digestion method was 15 °C, which is the approximate temperature of a Atlantic salmon body. The digestion period length and sampling time plan was based on principles described elsewhere [21]. Castillo-Lopez and co-workers have used a digestion period of 240 min and samples were collected gradually during this period. In several studies digestive enzymes have been extracted from the intestinal tract of salmonids and used in the digestion experiments [5,6,9,19,26]. One advantage of using this enzyme extract is the higher comparability of the results obtained *in vitro* and *in vivo* [26,27]. However, there are several drawbacks of using extract digestive enzymes from the intestinal tract of the fish, including high variability in enzyme activity, the need for purification and standardization of the enzymes and the need to sacrifice fish [28–30]. In this study, it was decided to use commercial enzymes from terrestrial sources (*i.e.* porcine

and bacterial sources) in order to ensure reproducible conditions.

It has been demonstrated that elements such Zn, Se or Mn can be associated with proteins or peptides [31,32]. Indeed, Zn-containing compounds were found in the soluble fraction of an Atlantic salmon feed and the data obtained suggested that the observed Zn-containing compounds could be metalloproteins [33]. Hence, mineral solubility can consequently be influenced by protein solubility [34]. It has been described that the use of acidic conditions have a positive effect on protein solubility in feedstuffs for ruminants [35]. Considering that the absorption of minerals is taking place in the small intestine, the successive gastrointestinal simulation and monitoring the change from stomach to intestinal conditions are important steps to understand mineral availability [36]. In light of this, it was decided to develop a sequential *in vitro* digestion method; *i.e.* stomach (acidic hydrolysis) and intestine (alkaline hydrolysis), which was successfully used to study mineral solubility.

4.2. The solubility of zinc, selenium and manganese is affected by the pH of the gastrointestinal environment and the mineral chemical form

The *in vitro* digestion method was applied to study the solubility of dietary Zn, Se and Mn in diets for Atlantic salmon. The solubility of Zn, Se and Mn was evaluated in regards to changes in the pH of the gastrointestinal environment and the chemical form of the mineral. The pH of the gastrointestinal environment did not affect the solubility of Se but affected the solubility of Zn and Mn in both IM and OM diets. For both Zn and Mn there was a clear drop in solubility when shifting from acidic to alkaline hydrolysis. However, the shift in pH when going from acidic to alkaline hydrolysis did not affect the solubility of Se, suggesting that the solubility of the Se compounds are not affected by the increased pH. Possibly, some of the solubilised species from the acidic digestion could have precipitated when the pH increased. As predicted by Visual MINTEQ at pH 2.1 the Zn^{+2} and Mn^{+2} ions prevailed (100%). At pH 8, Zn^{+2} and Mn^{+2} ions were the predominant species (Zn: 85.2%; Mn: 99.9%) followed by $ZnOH_2$ (aq) (10.9%), $ZnOH^+$ (3.9%) and $MnOH^+$ (0.1%). As predicted by a computational model [25] and reported by Krężel & Maret [37], $ZnOH_2$ was one of the predominant species at pH 8. Zinc hydroxide ($ZnOH_2$) has low solubility (13.2 mol of Zn per Kg of H_2O) at pH 7.96 and 12.5 °C [38] thus, partially explaining the decreased solubility of Zn when shifting from acidic to alkaline hydrolysis. The increased pH might also have promoted Zn and Mn ions to form chemical complexes that are less soluble. At pH 8, as predicted by a computational model, the most common Zn and Mn ions are positively charged (*i.e.* Zn^{+2} and Mn^{+2}) [25]. Unlike Zn and Mn, in aqueous solution, Se tend to form the oxyanions which are negatively charged (*i.e.* SeO_3^{-2} , SeO_4^{-2}) at pH 8 and 25 °C [39]. The effect of pH on the solubility of Zn and Mn, but not Se, might be explained by the ability of phytic acid (negatively charged compound) to bind positive cations as Zn and Mn ions. The phytic acid content in the IM and OM diets was 11.25 ± 0.05 ($\mu\text{mol g}^{-1}$) ($n = 2$). Phytic acid is commonly found in cereal grains. Consequently, phytic acid will be present in fish diets if plant-based ingredients are used [40].

The chemical form of the mineral supplemented in the diets affected the solubility of Se and Mn but not the solubility of Zn. The solubility of Zn for diet IM and OM was similar thus, the chemical form did not affect the solubility of Zn. A recent *in vivo* study using Atlantic salmon also demonstrated that there were no significant difference in Zn availability between an inorganic source (Zn sulphate) and an organic source of Zn (Zn chelate of glycine) [15]. Similar findings were reported by Maage and co-workers, where similar Zn availability was obtained in Atlantic salmon fed diets supplemented with an inorganic Zn source (Zn sulphate) and an organic Zn source (Zn gluconate) [41]. The solubility of Zn obtained in the present study (0.4–8%) was lower than the solubility of Zn (15–55%) of green beans evaluated by an *in vitro* method [42]. The reason for the differences seen between the two studies can be related to sample matrix effects. A salmonid feed contains both animal

and plant ingredients and it is a lipid rich sample (~20%), with phospholipids and triacylglycerols being the major lipid classes [43]. Lipids are mostly composed of non-polar hydrocarbon structures but there are some lipids which can contain charged chemical groups [44]. For instance, fatty acids and phospholipids are negatively charged. Hence, fatty acids and phospholipids tend to bind divalent cations. A recent study described the ability of divalent minerals (*e.g.* Zn) to bind to free fatty acids [45]. These chemical reactions can limit the solubility of Zn due to formation of poorly soluble soaps and salts in aqueous solutions [45]. The solubility of Se obtained for diet IM and OM was lower (3–34%) than the solubility of Se obtained in pelleted food for domestic animals (62–101%) evaluated by an *in vitro* method simulating digestion [46]. The differences seen between the two studies can be related with diet composition. A clear difference was seen in the solubility of Se in the IM and OM diets. The solubility of Se was higher in diet OM than diet IM suggesting that the chemical form of Se affected the solubility. The results showed that organic Se (SeMet) had higher solubility than inorganic Se (selenite). Thus, considering that the more soluble the Se source, the higher the availability of Se, it is expected that organic Se is more available than inorganic Se. This is in agreement with other studies, which have demonstrated that organic sources of Se (*e.g.* SeMet) are more available than selenite to fish [15,47–52]. Regarding salmonid studies, SeMet was found to be the most available form of dietary Se to Atlantic salmon when compared with SeCys and selenite [53], higher retention of Se was seen in Atlantic salmon fed diets supplemented with SeMet than with selenite [52], and in rainbow trout, Se yeast was more available when compared with selenite [54,55]. The solubility of Mn obtained for diet IM and OM (4–25%) was comparable with the solubility of Mn obtained in plant-based ingredients where the solubility of Mn was 13.1 ± 3.2 % in corn, 20.7 ± 4.16 % in wheat, and 15.6 ± 1.7 % in soybean meal [56]. The solubility of Mn was higher in diet IM than diet OM during the acidic hydrolysis but the solubility of Mn in diet IM and OM was similar during the alkaline hydrolysis. Our previous research showed that the inorganic source (Mn sulphate) had higher availability than the organic source (Mn chelate of glycine) in Atlantic salmon [15]. Conversely, a study in rainbow trout reported higher Mn availability when supplemented as organic source (Mn amino acid chelate) than inorganic source (Mn sulphate) [57]. Overall, these results suggested that the solubility of Zn, Se and Mn is affected by the pH of the gastrointestinal environment and the chemical form of the minerals supplemented in diets IM and OM.

4.3. Comparison between solubility and apparent availability of zinc, selenium and manganese

Data for obtained for solubility (%) and apparent availability (%) for Zn, Se and Mn were compared. Solubility (%) and apparent availability (%) of Zn, Se and Mn are not comparable. *In vitro* experiments can mimic the physicochemical conditions of the gastrointestinal tract only to some extent. The method developed gave low solubility, which can be related to reduced efficiency of the enzymes at 15 °C, as the optimal temperature of the enzymes is 37 °C according to the product description. Thus, it will be interesting to have access to commercial enzymes from fish to perform similar studies. Replacing the *in vivo* experiments by *in vitro* experiment has some drawbacks, which may affect the estimation of the availability such as difficulties to control the pH, use of enzymes that are not from fish and lack of certified reference materials to validate the methodologies [3].

Despite the weak comparability between solubility (%) and apparent availability (%), the effect of the chemical form of the minerals were similar for the solubility of Zn, Se and Mn and apparent availability of Zn, Se and Mn. The chemical form (Zn sulphate or Zn chelate of glycine) did not affect the solubility of Zn as shown in this study and the Zn apparent availability as shown elsewhere [15]. For Se, the organic form (SeMet) was more soluble and also had higher availability

[15]. Moreover, the Mn inorganic source (Mn sulphate) was more soluble and more available than the Mn organic source (Mn chelate of glycine) [15]. The results obtained in this study indicated that *in vitro* evaluation of the mineral solubility gives promising insights on mineral availability. Thus, this *in vitro* methods can be considered as screening method for evaluating mineral sources, replacing some of the *in vivo* feeding trials. However, more work needs to be done to improve the *in vitro* digestion method if one wishes to use this method for availability estimation. For instance, the *in vitro* method can be optimised by using larger digestion times and pH range.

5. Conclusions

The present work developed an *in vitro* digestion method to evaluate solubility of Zn, Se and Mn in salmonid diets. Data obtained demonstrated that solubility of Zn, Se and Mn was influenced both by the mineral chemical form supplemented to the diet and by the gastrointestinal environment. Regarding the mineral form supplemented to the diets, SeMet was more soluble than selenite, and Mn sulphate was more soluble than Mn chelate of glycine. Conversely, the Zn additive source did not influence the solubility of Zn. The gastrointestinal environment did not affect the solubility of Se but affected the solubility of Zn and Mn. For both Zn and Mn there was a clear drop in solubility when shifting from acidic to alkaline hydrolysis, suggesting that the solubility of the Zn and Mn compounds was affected by the increased pH.

The solubility of Zn, Se and Mn was not comparable with the apparent availability of Zn, Se and Mn. Still, the effect of the chemical form of the minerals were similar for solubility of Zn, Se and Mn and apparent availability of Zn, Se and Mn. Concerning the mineral form supplemented to the diets, SeMet was more soluble and available than selenite, and Mn sulphate was more soluble (during the acidic hydrolysis) and available than Mn chelate of glycine. Conversely, the Zn additive source did not influence the solubility or availability of Zn. Considering the overall results of this study, the *in vitro* method could replace some of the *in vivo* studies for a qualitative evaluation (*i.e.* to evaluate the effect of the chemical form), but not for a quantitative evaluation (*i.e.* comparable values for solubility and apparent availability).

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