Speciation of zinc in fish feed by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry – using fractional factorial design for method optimisation and mild extraction conditions

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ABSTRACT

Zinc (Zn) is an element essential to all living organisms and it has an important role as a cofactor of several enzymes. In fish, Zn deficiency has been associated with impaired growth, cataracts, skeletal abnormalities and reduced activity of various Zn metalloenzymes. Fish meal and fish oil traditionally used in salmon feed preparation are being replaced by plant-based ingredients. Zinc additives are supplemented to salmon feed to ensure adequate Zn levels, promoting good health and welfare in Atlantic salmon (*Salmo salar*). The main objective of the present study was to evaluate Zn species found in an Atlantic salmon feed. This work describes a Zn extraction method that was optimized using a fractional factorial design (FFD), whereby the effect of six factors could be studied by performing only eight experiments. The effects of the type of extraction solution and its molar concentration, pH, presence of sodium dodecyl sulphate, temperature and extraction time on Zn extraction were investigated. Mild extraction conditions were chosen in order to keep the Zn species intact. Total Zn (soluble fractions and non-soluble fractions) was determined by inductively coupled plasma mass spectrometry (ICP-MS). The highest Zn recovery was obtained using 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h where the total Zn in soluble fraction and non-soluble fraction was 9.9 ± 0.2% and 98 ± 6%, respectively. Zinc speciation analysis (on the soluble fractions) was further conducted by size exclusion inductively coupled plasma mass spectroscopy (SEC-ICP-MS). The SEC-ICP-MS method provided qualitative and semi-quantitative information regarding Zn species present in the soluble fractions of the feed. Four Zn-containing peaks were found, each with different molecular weights: Peak 1 (high molecular weight - ≥ 600 kDa), peak 2 and peak 3 (medium molecular weight – 32 to 17 kDa) were the least abundant (1–6%), while peak 4 (low molecular weight – 17 to 1.36 kDa) was the most abundant (84–95%).

1. Introduction

Zinc (Zn) is an element that occurs naturally in water, air and soil and it is essential to all living organisms [1]. Zinc plays an essential role as a cofactor of several enzymes and it has also paracellular and intracellular signalling functions [2]. In farmed fish, Zn deficiency has been associated with impaired growth, cataracts, skeletal abnormalities and reduced activity of various Zn metalloenzymes [3,4]. Feed consumption and waterborne mineral uptake are the main sources of Zn in Atlantic salmon (*Salmo salar*) [5]. The composition of salmon feed have during recent years changed from the use of mainly marine feed ingredients, such as fish meal and fish oil, to an increasing replacement with plant-based ingredients, e.g. soybean meal, maize gluten meal,

wheat gluten and rapeseed oil [6]. Zinc is naturally present in fish meal and in plant-based ingredients, with typical concentrations ranging from 64 to 74 mg kg^{-1} (data for 2008) [7] and from 35 to 48 mg kg^{-1} (data for 2016) [8], respectively. Zinc is added to feeds to prevent diseases and ensure animal welfare [9].

The European Union regulation EC No. 2003/1831 and amendments set the rules for the use of Zn additives in animal nutrition [10]. Examples of these additives are zinc acetate dihydrate, zinc chloride anhydrous, zinc oxide, zinc sulphate heptahydrate, zinc sulphate monohydrate, zinc chelate of amino acids hydrate, zinc chelate of protein hydrolysates, zinc chelate of glycine hydrate (solid) and zinc chelate of glycine hydrate (liquid). In the European Union, the current upper limit for total Zn in complete feed of all fish except salmonids is

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150 mg kg^{-1} and for salmonids feeds it is 180 mg kg^{-1} feed [11].

Elemental speciation analysis is the quantification or/and the identification of different chemical compounds, or element species [12]. There is limited knowledge on the chemical species of Zn in fish feeds. As the bioavailability of an element depends on its chemical form (i.e. its species) [13], Zn speciation analysis can provide valuable information with regards to fish nutrition studies. Zinc may be present in organic or inorganic forms. However, it is not so clear which forms have enhanced bioavailability [2,14]. Hence, development of proper analytical methods is needed to characterize Zn species present in feeds.

For separation of element species, high performance liquid chromatography (HPLC) is the traditional separation technique [15]. Other separation techniques, such as gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE) have also been used for the separation of element species [15,16]. Inductively coupled plasma mass spectrometry (ICP-MS) is the preferred detection method for elemental analysis as it gives high sensitivity and selectivity, provides isotope information and has multi-element capability [17]. For speciation analysis of Zn in plant-based matrices, samples are generally extracted using buffers, and subsequent analysis for Zn species is performed by size exclusion chromatography (SEC) coupled to ICP-MS [18–20]. Also, ion-exchange chromatography (IEC) coupled to ICP-MS [19] and CE-ICP-MS [21] have been used for Zn speciation in plant tissue and horse feed, respectively. So far, however, there is no reported study on Zn speciation in fish feed.

Traditionally, method development is performed using the one-factor-at-a-time (OFAT) strategy, which is a labour-intensive and material consuming approach. However, the use of design of experiments (DOE) is a much more efficient way to evaluate not only individual but also joint effects of the variables compared to the OFAT approach [22,23]. A DOE is selected based on experimental objectives, number of factors to be studied and on the amount of resources available. For screening purposes and a large number of factors to be studied, there are typically two types of design that are recommended, the Plackett-Burman and the fractional factorial design (FFD). A FFD is a design where the experiments conducted are only a subset of the runs in the full factorial design. The design can be expanded if needed [23,24].

The DOE has been applied in speciation studies of elements such as copper [25], selenium [26], mercury [27], chromium [28–30], arsenic [31,32] and antimony [32]. In speciation analysis, one of the most critical points is to keep the native structure of each chemical species intact along the extraction process and during the chromatographic separation [33,34]. For the extraction of Zn from a horse feed and tissues of barley grains, the use of ammonium acetate, Tris-HCl and NaCl in a range of concentrations from 10 to 100 mM as extraction solutions were reported [20,21]. In addition, different temperatures and extraction times were evaluated. Considering the lack of methods for Zn speciation in feed, the aim of the present study was to develop an extraction method for Zn in Atlantic salmon feed. The approach included (i) a FFD experimental setup, (ii) mild extraction conditions to keep chemical species intact, and (iii) a chromatographic method to characterize Zn species in Atlantic salmon feed.

2. Experimental

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 stated otherwise. Methanol (MeOH, LiChrosolv®, HPLC grade), acetic acid (CH$_3$COOH, Emsure® ACS, ISO, 96% w/w), hydrochloric acid (HCl, Emsure® ACS, ISO, 37% w/w), hydrogen peroxide (H$_2$O$_2$, Emsure® ACS, ISO, 30% w/w) were obtained from Merck (Darmstadt, Germany). Nitric acid (HNO$_3$, trace select, ≥ 69.0% 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). Tris(hydroxymethyl)aminomethane [Tris-HCl, NH$_2$C(CH$_2$OH)$_3$], ammonium acetate (NH$_4$CH$_3$CO$_2$), sodium dodecyl sulphate (SDS), thyroglobulin (T1001), glutathione peroxidase (G6137), superoxide dismutase (S7446), myoglobin (M1882) and vitamin B12 (V2876) were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Sample

The feed sample used (L1, 3 mm) is described elsewhere [35]. The feed was formulated based on commercial feed for Atlantic salmon, containing protein sources mainly from plant-based ingredients (i.e. 15% marine protein, 8% fish oil, 65% plant proteins and 10% plant oils). Zinc oxide was added to the feed. The feed was grinded by hand with a pestle and a mortar, and sieved to ensure a feed fraction with similar particle size (from 850 μm to 1.12 mm). To establish a target value for total Zn, the feed sample was analysed at the laboratory and two other accredited laboratories.

2.3. Experimental design

Based on previous Zn speciation studies [20,21], the factors included in the experimental design were type of extraction solution (A), molar concentration of the extraction solution, mM (B), pH (C), addition of 4% sodium dodecyl sulphate (SDS) solution (D), temperature, °C (E), and extraction time, hour/s (F) (Table 1). Factors were set at low (−1) and high (+1) levels. The experimental procedure was performed according to a 2^{6-3} fractional factorial design (resolution III). Eight experiments in triplicate ($n = 3$) and a blank for each experiment

Table 1
2_{III}^{6-3} fractional factorial design. The tested factors were type of extraction solution (A), concentration of the extraction solution, mM (B), pH (C), addition of 4% sodium dodecyl sulphate (SDS) solution (D), temperature, °C (E), and extraction time, hour/s (F). Factor level codes are shown as "−1" or "+1" followed by the real factor level shown between parenthesis. Concentration of soluble Zn is expressed as mean ± standard deviation (mg kg^{-1} feed, $n = 3$).

Exp.	Factors: Coded (real)						
	A: Extraction solution	B: Concentration (mM)	C: pH	D = AB: 4% SDS	E = AC: Temp. (°C)	F = BC: Time (h)	Soluble Zn (mg kg^{-1} feed)
1	−1 (Tris-HCl)	−1 (10)	−1 (6.5)	+1 (yes)	+1 (20)	+1 (24)	6.2 ± 0.2
2	+1 (Amm. Acetate)	−1 (10)	−1 (6.5)	−1 (no)	−1 (4)	+1 (24)	4.5 ± 0.3
3	−1 (Tris-HCl)	+1 (100)	−1 (6.5)	−1 (no)	+1 (20)	−1 (1)	5.66 ± 0.07
4	+1 (Amm. Acetate)	+1 (100)	−1 (6.5)	+1 (yes)	−1 (4)	−1 (1)	6.9 ± 0.1
5	−1 (Tris-HCl)	−1 (10)	+1 (8.5)	+1 (yes)	−1 (4)	−1 (1)	6.9 ± 0.2
6	+1 (Amm. Acetate)	−1 (10)	+1 (8.5)	−1 (no)	+1 (20)	−1 (1)	3.87 ± 0.09
7	−1 (Tris-HCl)	+1 (100)	+1 (8.5)	-1 (no)	-1 (4)	+1 (24)	10.9 ± 0.3
8	+1 (Amm. Acetate)	+1 (100)	+1 (8.5)	+1 (yes)	+1 (20)	+1 (24)	6.14 ± 0.05

($n = 1$) were performed (in total 32 experiments). Details about the conditions used for experiment 1–8 are presented in Table 1. The experimental design and analysis of data from experiments was performed using R commander plugin for DOE [R foundation for statistical computing, version 3.4, [36,37]. The main effect of each factor (A to F) was calculated using Eq. (1):

$$\text{effect of main factor} = \left(\frac{\sum Y+}{n+}\right) - \left(\frac{\sum Y-}{n-}\right) \tag{1}$$

where "Y +" refers to the responses at level (+1), the "Y −" to the responses at level (−1), the "n +" to the number of data points at level (+1) and "n −" to the number of data points at level (−1).

A two-tailed t-test was used to determine the statistical significance of the main effects at a confidence level of 95% using Eq. (2):

$$t - \text{value of the effect} = \frac{\text{effect of main factor}}{\text{standard error}} \tag{2}$$

Approximately 0.5 g of feed was extracted into 5 mL of extraction solution, for 1 or 24 h, at a temperature of 4 or 21 °C. The extraction solution applied was either Tris-HCl or ammonium acetate, with concentrations of 10 or 100 mM and pH values of 6.5 or 8.5. One milliliter of 4% of SDS was added to some samples (Table 1). The final volume was adjusted to 5 mL in all samples. The samples were extracted in a random order. After the extraction procedure, samples were centrifuged for 10 min at 3000g (Eppendorf® Centrifuge 5702, Hamburg, Germany). The samples were fractionated into soluble and non-soluble fractions using a Pasteur pipette. The soluble fractions were filtered through a 0.45 μm disposable syringe filter (Sartorius, Göttingen, Germany) and transferred to new tubes. The non-soluble fractions were dried in an oven for 24 h at 60 °C. The experimental outline of the study is presented in Fig. 1. Total Zn (soluble fractions and non-soluble fractions) was determined using ICP-MS and Zn speciation analysis (soluble fractions) was performed using SEC-ICP-MS.

2.4. Determination of total zinc by ICP-MS

For the determination of total Zn, the feed and feed fractions (i.e. the soluble and the non-soluble fractions) were decomposed using microwave assisted acid digestion based on the procedure previously described [38]. Briefly, approximately 0.2 g of feed was digested using

Table 2
The operating parameters for the ICP-MS and SEC-ICP-MS.

ICP–MS settings (iCapQ)	
Forward power	1550 W
Plasma gas flow	14.0 L min^{-1}
Carrier gas flow	1.02 L min^{-1}
Makeup gas flow	0.80 L min^{-1}
Dwell time	0.1 s per isotope
Isotopes monitored	^{66}Zn, ^{72}Ge

ICP–MS settings (7500cx)	
Forward power	1550 W
Plasma gas flow	15.0 L min^{-1}
Carrier gas flow	0.94 L min^{-1}
Makeup gas flow	0.25 L min^{-1}
Dwell time	0.1 s per isotope
Isotopes monitored	^{127}I,^{78}Se, ^{66}Zn, ^{59}Co, ^{57}Fe

HPLC settings	
Column	TSKgel G3000SWxl SEC column (30 cm × 7.8 mm, 5 μm particle size) + QC-PAK guard column (7 μm particle size)
Calibration range	1.0×10^4–5.0×10^5 Da
Mobile phase	50 mM Tris-HCl + 3% MeOH (pH 7.5)
Flow rate	0.7 mL min^{-1}
Injection volume	50 μL

2.0 mL of HNO$_3$ (69% w/w) and 0.5 mL of H$_2$O$_2$ (30% w/w) in a Milestone-MLS-1200 microwave oven (Milestone Inc., Shelton, CT, USA). The digested samples were subsequently diluted to 25 mL with Milli-Q® water. A similar procedure was applied to digest the entire dried non-soluble fractions (~0.5 g). The soluble fractions (500 μL) were digested using 2 mL of HNO$_3$ in an ultrawave digestion system (UltraWAVE, Milestone, Sorisole, Italy). The samples were capped and placed in the ultrawave system with a container of 130 mL Milli-Q® water and 5 mL H$_2$O$_2$. The extracts were then diluted to 25 mL with Milli-Q® water. The total Zn determination was performed by use of an ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) equipped with an autosampler (FAST SC-4Q DX, Elemental Scientific, Omaha,
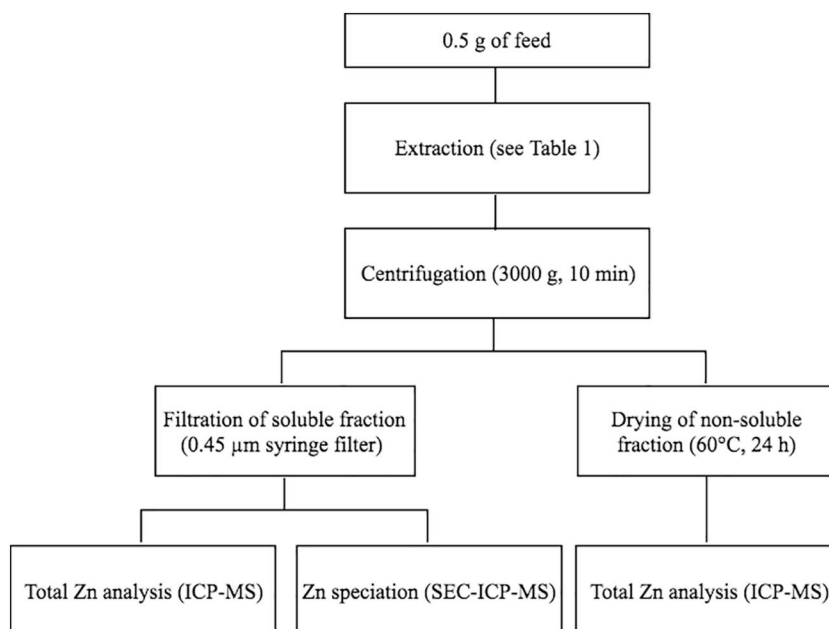


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                    ┌──────────────────┐
                    │  0.5 g of feed   │
                    └──────────────────┘
                              │
                    ┌──────────────────────┐
                    │ Extraction (see Table 1)│
                    └──────────────────────┘
                              │
                    ┌────────────────────────────┐
                    │ Centrifugation (3000 g, 10 min)│
                    └────────────────────────────┘
                              │
              ┌───────────────┴────────────────┐
    ┌─────────────────────────┐    ┌─────────────────────────┐
    │ Filtration of soluble fraction│  │ Drying of non-soluble   │
    │ (0.45 μm syringe filter)      │  │ fraction (60°C, 24 h)   │
    └─────────────────────────┘    └─────────────────────────┘
          │              │                      │
  ┌──────────────┐ ┌──────────────┐   ┌──────────────┐
  │Total Zn      │ │Zn speciation │   │Total Zn      │
  │analysis      │ │(SEC-ICP-MS)  │   │analysis      │
  │(ICP-MS)      │ │              │   │(ICP-MS)      │
  └──────────────┘ └──────────────┘   └──────────────┘
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Fig. 1. Methodology flowchart.

USA). The samples were analysed in a random order. A solution of germanium was added on-line for correction of instrumental drift during the analysis. The instrument was optimized using a tuning solution (1 ppb tuning solution B, Thermo Fisher, in 2% HNO_3 and 0.5% HCl) prior to analysis. The instrumental settings are presented in Table 2. Data were collected and processed using the Qtegra ICP-MS software (Thermo Scientific, version 2.1, 2013). For the quantitative determination of total Zn, an external calibration curve (10 to 500 ng mL^{-1}) was used and two certified reference materials were included to assess the accuracy of the method: 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 obtained values were in agreement with the certified values. The validated range for Zn determination is from 0.5 to 1400 mg kg^{-1} (DW).

2.5. Zinc speciation by SEC-ICP-MS

The SEC-ICP-MS method was developed based on principles described elsewhere [20,39]. Further optimisation was done in this study to the analysis of a fish feed. The soluble fractions were analysed using a 1260 HPLC coupled with a 7500cx ICP-MS (Agilent Technologies, Santa Clara, USA) and a SEC column (TSKgel G3000SWxl, Tosoh, Stuttgart, Germany). The mobile phase solution was prepared by dissolving an appropriate amount of tris(hydroxymethyl)aminomethane to reach the desired ionic strength (50 mM) in an aqueous 3% (v/v) MeOH solution, followed by adjustment of pH to 7.5 with HCl (37% w/w). The samples were analysed in a random order. The instrument was tuned according to manufacturer's instructions. The instrumental settings for the HPLC and ICP-MS are listed in Table 2.

Prior to speciation analysis of the soluble fractions, a molecular weight calibration was performed using thyroglobulin (660 kDa, monitoring ^{127}I), glutathione peroxidase (84 kDa, monitoring ^{78}Se), Zn/Cu superoxide dismutase (32 kDa, monitoring ^{66}Zn), myoglobin (17 kDa, monitoring ^{57}Fe), vitamin B12 (1.36 kDa, monitoring ^{59}Co). The standards were prepared with a concentration of 100 ng element mL^{-1} in Milli-Q® water. For the quantitative determination of Zn species an external calibration curve of the Zn/Cu SOD standard (5 to 200 ng Zn mL^{-1}) was applied, and species were quantified by peak areas. The calibration curve was analysed at the beginning and at the end of the analytical sequence. The 50 ng Zn mL^{-1} standard was analysed at the middle of the sequence. All sample extracts were spiked with 0.5 μL of vitamin B12 (1000 ng mL^{-1}) prior to analysis in order to correct for retention times shifts.

The chemical nature of the Zn species in the soluble fractions was further investigated. The soluble fractions of experiment 7 ($n = 3$) were split in two parts, one was heated and the other was kept as is. The soluble fractions were heated at 90 °C for 10 min using a heat block (Bibby Scientific Stuart, Stone, Staffordshire) as described by Temara and colleagues [40]. The heated and non-heated extracts were evaluated by the SEC-ICP-MS method as previously described.

3. Results and discussion

3.1. Total zinc in feed

The average total Zn concentration was 110 ± 8 mg kg^{-1} of feed ($n = 10$). The target value was used to calculate the recovery of the extraction experiments.

3.2. Effect of extraction factors by fractional factorial design

The concentration of soluble Zn was different in the various experimental runs proposed by the 2^{6-3} fractional factorial design (Table 1). The highest and lowest Zn recoveries were obtained under the conditions dictated by experiments 7 and 6 respectively (Table 1).
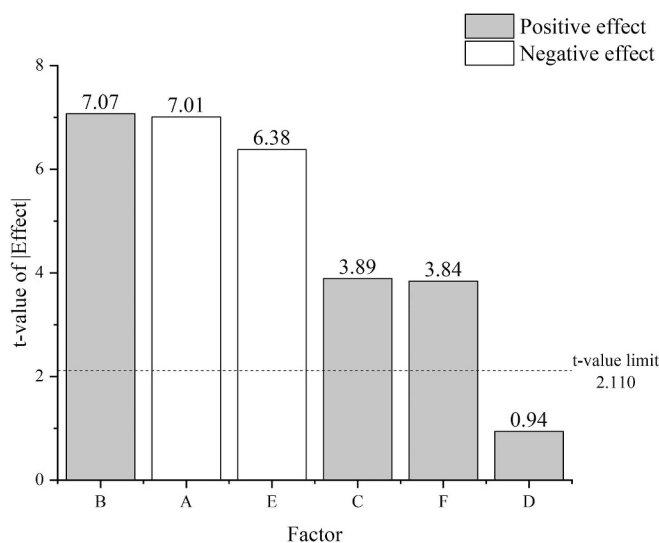

Fig. 2. Pareto chart with the magnitude of the main effects using the concentration of soluble Zn as response. Horizontal axis shows the factors ordered accordingly with their magnitude; type of extraction solution (A), concentration of the extraction solution, mM (B), pH (C), addition of 4% sodium dodecyl sulphate (SDS) solution (D), temperature, °C (E), and extraction time, hour/s (F); the vertical axis shows the *t*-value of the absolute effect. The reference line is the *t*-value limit (α = 0.05; d.f. = 17). A *t*-value of |effect| above the reference line indicates a significant contribution of this factor to the response ($p < 0.05$). In grey, the effects with positive *t*-value and, in white, the effects with negative *t*-value. The standard error was 0.1448 for each main factor.

The former condition consisted of 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h and the latter of 10 mM ammonium acetate, pH 8.5 at a temperature of 20 °C for 1 h.

The effect of the main factors on the response is presented in the form of a Pareto chart (Fig. 2). The results show that the factors having a statistically significant effect ($p < 0.05$) on Zn extraction were type of extraction solution (A), molar concentration of the extraction solution, mM (B), pH (C), temperature, °C (E) and extraction time, hour/s (F). The addition of 4% sodium dodecyl sulphate (SDS) solution did not have a significant effect on the Zn extraction and such finding was also reported elsewhere [41]. The same results suggest that to maximize the concentration of Zn extracted, factors such as type of extraction solution (A) and temperature, °C (E) should be kept at the low (−1) level, which implies using Tris-HCl as extraction solution and performing the extraction at 4 °C. Furthermore, factors such as molar concentration of the extraction solution, mM (B), pH (C) and time, hour/s (F) should be kept at the high (+1) level. This means the extraction should be performed using 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h. Altogether, these extraction conditions correspond to those described by experiment 7 (Table 1). As a set of experimental conditions was obtained in the initial fractional factorial design, no more experiments were performed.

3.3. Zinc extraction recovery

Zinc recovery (%) was determined for the soluble and non-soluble fractions by calculation of the ratio of Zn obtained for each fraction compared to total Zn in the feed (110 mg kg^{-1} feed) (Table 3). The variation obtained was acceptable taking into consideration the measurement uncertainty of the method, which is 20%. The sum of both fractions was calculated by adding the average value found in soluble Zn (%) and non-soluble Zn (%). The overall recovery of Zn ranged from 83 to 124%.

Between 4 and 10% of Zn was extracted into the soluble fraction of the feed (Table 3). The extraction method is a critical step in element

Table 3
Total Zn in the soluble and non-soluble fractions (%) and the calculated sum Zn (%). Soluble Zn and non-soluble Zn values are expressed as mean ± standard deviation (%, n = 3).

Exp.	Soluble Zn (%)	Non-soluble Zn (%)	Sum Zn (%)
1	5.6 ± 0.1	105 ± 12	111
2	4.1 ± 0.2	82 ± 19	83
3	5.15 ± 0.06	112 ± 5	117
4	6.3 ± 0.1	118 ± 6	124
5	6.2 ± 0.1	108 ± 14	114
6	3.52 ± 0.08	105 ± 3	109
7	9.9 ± 0.2	98 ± 6	108
8	5.58 ± 0.05	102 ± 7	108

speciation analysis. This is mainly due to the challenges of providing high extraction recovery as well as preserving the integrity of the original species during the extraction process simultaneously [42,43]. There is a number of speciation protocols, which include the use microwave and ultrasound assisted extraction [44,45]. However, our study focus on the use of mild extraction conditions to keep the chemical species intact. Microwave and ultrasound-assisted extraction could affect the species integrity [44,45]. Consequently, both microwave and ultrasound assisted extraction were not included in the extraction methodology.

Mild extraction conditions were applied to keep the integrity of the chemical species intact, which may compromise the extraction recovery in the soluble fraction. Furthermore, Zn ion (Zn^{+2}) can easily bind to other compounds which are less soluble in water (i.e. phytic acid, sulphides) [46,47]. The lower solubility found in this study could be due to Zn binding to other compounds present in the fish feed and thereby forming water insoluble Zn species.

3.4. Zinc speciation analysis of feed by SEC-ICP-MS

Different types of columns and mobile phases were tested in order to identify the most robust technique for Zn speciation analysis, i.e. a method that preserves the integrity of the metal binding species. First, various anion-exchange settings were applied for the separation of Zn species. However, the obtained chromatograms showed poor resolution and severe peak broadening. Hence, anion-exchange chromatography was disregarded as a chromatographic separation technique for Zn species in fish feed extracts. This finding is consistent with a previous study, according to Persson et al. (2009), anion-exchange chromatography showed poor chromatographic results for Zn compounds from barley grains [20]. Reversed phase chromatography (RPC) was applied for the separation of Zn species but the obtained chromatograms also showed poor resolution and peak broadening. Hence, RPC was also disregarded as a chromatographic separation technique for Zn species in fish feed extracts. In IEC and RPC, the separation is based on electrostatic forces [48] and this may cause effects on the native chemical structures in the separation creating artefacts and misleading information [49]. This may be due to de-stabilization of the metal binding species and the weak binding capacity of some metals, such as Zn [46]. When applying SEC, the stability of the Zn species markedly improved, and therefore SEC-ICP-MS was chosen as a method for Zn speciation. The SEC-ICP-MS method gave semi-quantitative results for the Zn species detected using the external calibration curve of the Zn/Cu SOD standard (Table 4). Furthermore, the method provided qualitative results regarding molecular size of Zn species present in the feed extracts, by comparison of elution times of Zn species with the elution times of the molecular weight calibration standards (Fig. 3 and Table 4).

The soluble fractions of experiment 1 to 8 were evaluated by SEC-ICP-MS. Both, number of peaks and total Zn in the soluble fraction were

used as parameters to select the set of Zn extraction conditions. The results from the SEC-ICP-MS analysis show the presence of several Zn species. The different extraction conditions affected the type and amount of species present in the extract (Table 3). Extraction conditions applied in experiment 1, 4, 5, 7 and 8 extracted peaks 1 to 4. However, when using the extraction conditions of experiment 2, 3 and 6, peak 1 was not detected. The ratio of each peak was calculated based on the sum of all peaks and it is presented in Table 3. Peak 1, peak 2, and peak 3 were the least abundant (1–6%) and peak 4 was the most abundant (84–95%). Fig. 3 shows the Zn profile of the soluble fraction of a feed extract using the extraction conditions of experiment 7 (*n* = 3) obtained by SEC-ICP-MS. The chromatograms from the three replicates of experiment 7 are overlapping, thus indicating good repeatability (Fig. 3).

The SEC-ICP-MS method gave qualitative information regarding the size range of the Zn species present in the soluble fraction. The molecular weight calibration was performed using thyroglobulin (660 kDa, Rt ~ 9.4 min), glutathione peroxidase (84 kDa, Rt ~ 12.9), Zn/Cu superoxide dismutase (32 kDa, Rt ~ 14.6), myoglobin (17 kDa, Rt ~ 17.2), vitamin B12 (1.36 kDa, Rt ~ 19.5). On the chromatograms, it was observed the elution of peak 1 (Rt ~ 8.6 min) and those Zn species have a high molecular weight (≥ 600 kDa). Additionally, peaks 2 and 3 peaks were observed (Rt ~ 15.7 and 16.6 min) and the Zn species in this case are medium molecular weight 1 (Mw ~ 32–17 kDa). Peak 4 (Rt ~ 18 min) indicates the presence of Zn species with low molecular weight (Mw ~ 17 kDa–1.36 kDa).

In SEC, the molecules separation is based on molecule size. Hence, each peak might contain several compounds with similar molecular weight [50].

Structural information about the Zn-containing compounds present in peak 1 to 4 would give complementary data about the Zn species. However, one limiting factor in further method development is the lack of standards to study Zn compounds [19,51]. The Zn compounds were further investigated providing complementary information of the chemical nature of the Zn species in the soluble fractions. The Zn compounds found in the soluble fraction originate from different sources, as the feed samples contain both animal and plant ingredients. The different ingredients and the molecular weight range of the Zn peaks suggest that the observed Zn peaks could be metalloproteins. One of the most studied metalloproteins is the ubiquitous metallothioneins (MTs). The MTs are thermally stable proteins, so a heating step is a commonly used protocol to confirm their presence [52]. Thus, the soluble fractions of experiment 7 (*n* = 3) were heated. The chromatographic profile obtained from heated and non-heated extracts were compared, and the chromatographic profiles were similar. The compounds eluting in peak 1, 2, 3 and 4 were heat stable, suggesting that the compounds are MTs. The MTs are known to be the only proteins which are heat stable and have metal association ability [53]. This supports our suggestion of the Zn compounds being MTs.

4. Conclusions

In the present study, the effect of different conditions for the extraction of Zn from fish feed was studied using a FFD approach. Eight experiments were carried out and the effect of six different factors on the extraction of Zn was determined. The highest recovery for Zn in fish feed was obtained when using 100 mM Tris-HCl, pH 8.5 at a temperature of 4 °C for 24 h and four peaks were found under these extraction conditions. The application of mild extraction conditions and SEC were found to be appropriate to keep the Zn species intact. The speciation profile of Zn in the soluble fractions was evaluated using a SEC-ICP-MS method developed to study Zn species in a fish feed. This analytical method will be used to characterize Zn species present in feeds.

Table 4

Zinc speciation analysis by SEC-ICP-MS (mean ± standard deviation, n = 3, mg kg $^{-1}$ feed).

Exp.	Peak 1 (≥ 600 kDa, Rt ~ 8.6 min)		Peak 2 (32–17 kDa, Rt ~ 15.7 min)		Peak 3 (32–17 kDa, Rt ~ 16.6 min)		Peak 4 (17–1.36 kDa, Rt ~ 18 min)	
	mg kg $^{-1}$ feed	Area (%)	mg kg $^{-1}$ feed	Area (%)	mg kg $^{-1}$ feed	Area (%)	mg kg $^{-1}$ feed	Area (%)
1	0.33 ± 0.03	4	0.505 ± 0.005	6	0.442 ± 0.004	6	6.75 ± 0.06	84
2	n.d.	0	0.263 ± 0.005	3	0.339 ± 0.002	4	8.6 ± 0.1	93
3	n.d.	0	0.44 ± 0.01	5	0.447 ± 0.003	5	8.7 ± 0.2	91
4	0.138 ± 0.002	1	0.349 ± 0.004	4	0.376 ± 0.004	4	8.9 ± 0.2	91
5	0.181 ± 0.004	2	0.278 ± 0.005	3	0.354 ± 0.002	4	8.3 ± 0.1	91
6	n.d.	0	0.294 ± 0.001	3	0.383 ± 0.001	4	9.0 ± 0.5	93
7	0.0635 ± 0.0004	1	0.171 ± 0.006	2	0.295 ± 0.001	3	9.7 ± 0.2	95
8	0.29 ± 0.06	4	0.326 ± 0.006	4	0.343 ± 0.005	4	7.13 ± 0.07	88

n.d. = not detected.
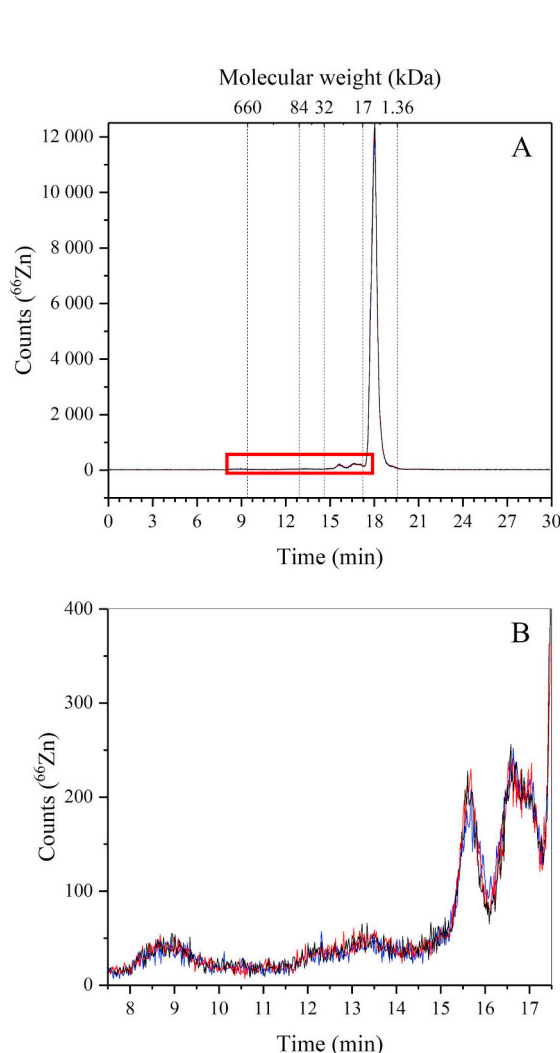
Fig. 3. Chromatogram of Zn species in the soluble fraction of feed, extracted according to experiment 7, and analysed by SEC-ICP-MS (*n* = 3); (A) Shows a complete Zn profile; (B) Shows an enlargement of Zn profile from 8 to 17 min; Blue, red and black lines represent the three replicates of experiment 7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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