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Analytical Methods

Measurement of haem and total iron in fish, shrimp and prawn using ICP-MS: Implications for dietary iron intake calculations

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ABSTRACT

Twenty-five species of fish, shrimp and prawn from local markets in Bangladesh were analysed for concentrations of total Fe, haem Fe and non-haem Fe by ICP-MS. Total Fe and non-haem Fe concentrations were measured in nitric acid-digested samples and haem Fe was extracted using acidified 80% acetone for 60 min. Total Fe concentrations ranged from 0.55–14.43 mg/100 g FW, and haem Fe% ranged from 18%–93% of total Fe. Repeat extractions with 80% acetone recovered additional haem Fe, suggesting that previous measurement by this technique may have underestimated haem Fe content. Calculation of Fe balance (summing Fe in acetone extracts and Fe in the residue after haem Fe extraction) was not significantly different from total Fe, indicating the two processes recovered the different forms of Fe with similar effectiveness.

1. Introduction

Anaemia is estimated to affect 1.6–2 billion people, while iron (Fe) deficiency may affect up to 40% of the global population (de Benoist, McLean, Egli, & Cogswell, 2008; McLean, Cogswell, Egli, Woidyla, & de Benoist, 2009; WHO, 2007). Even in the absence of anaemia (<110–150 g haemoglobin/L blood, depending on age and sex; (McLean et al., 2009), Fe deficiency can adversely affect health, reducing cognitive function and physical work capacity and ultimately economic productivity (Graham, Knez, & Welch, 2012). While some causes of anaemia include diseases such as thalassaemia or malaria, around 50% of the prevalence of Fe deficiency is due to inadequate dietary Fe intake or poor absorption of Fe from the diet. This is particularly problematic in many low-income countries where consumption of animal-source foods is low and diets are often predominantly plant-based. Food-based approaches to alleviating inadequate nutrient intake are generally preferable to supplementation or fortification (Miller & Welch, 2013) and therefore, the identification of foods of high iron content and high bioavailability is of significant importance. Mammal, bird and fish muscle tissues (meat) are considered good sources of Fe for their high total Fe concentration, as well as presence of haem Fe (Fe protoporphyrin IX). Haem Fe is found only in meat and has greater bioavailability than non-haem Fe that is the only form of Fe found in plant tissue, but which is also present in meat. The difference in bioavailability (15–35% for haem Fe versus 2–20% for non-haem Fe; (Cook & Monsen, 1976; Hurrell & Egli, 2010), is due to the different physiological mechanisms of transport across intestinal membranes; haem Fe is absorbed as an intact molecule (Shayeghi et al., 2005), whereas non-haem Fe is digested in the stomach and reduced to Fe^{2+} before absorption (Waldvogel-Abramowski et al., 2014).

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The classical methods of haem quantification e.g. phosphate (Drabkin, 1950), acidified acetone (Hornsey, 1956), ammonium sulphate (Brown, 1961), including their recent modifications (Chaijan & Undeland, 2015; Cross et al., 2012; Gomez-Basauri & Regenstein 1992) all rely on UV/Vis spectrophotometry, as do HPLC-based methods (Sato, Ido, & Kimura, 1994). However, interactions between the extraction medium, storage conditions of the muscle tissue and delays between extraction and measurement can influence the efficiency of haem extraction, greatly influencing the accuracy of each method (Chaijan & Undeland, 2015). Similarly, the iron concentration of the different fractions of meat has been measured by multiple methods, including UV/Vis, AAS or inductively coupled plasma (ICP) spectrophotometry methods (e.g. Carpenter & Clark, 1995; Valenzuela, de Romana, Olivares, Morales, & Pizarro, 2009). However, the iron concentration of the haem fraction has rarely been measured directly (Cross et al., 2012), but has usually been inferred from the molecular weight ratio of haematin (approximately 8.8 mg Fe/g haem; e.g. Kongkachuichai, Napatthalung, & Charoensiri, 2002). Alternative methods, such as subtraction of non-haem iron from total iron, leads to highly varied values of iron concentration in haem, especially when different analytical methods are used for the separate analyses (Lombardi-Boccia, Martínez-Dominguez, Aguzzi, & Rincón-León, 2002).

Numerous equations (Beard, Murray-Kolb, Haas, & Lawrence, 2007) have been devised to estimate dietary Fe absorption from haem and non-haem Fe components, yet most use a single generic value of 40% for the amount of haem Fe present in meats. This is usually based on a truncated value from the range of 30–40% (Cook & Monsen, 1976) which is treated almost as a constant, despite the variation and clear evidence that haem Fe represents 25–70% of the total Fe of red meat (Hurrell & Egli, 2010; Schönfeldt & Hall, 2011; Valenzuela, de Romana, Olivares, Morales, & Pizarro, 2009). Variation within individual species and even individual cuts of meat from the same animal exist which will influence dietary Fe intake and absorption (Rangan, Ho, Blight, & Binns, 1997; Schönfeldt & Hall, 2011; Valenzuela et al., 2009), as does the source of meat: mammal, bird, or fish.

Worldwide, fish provided 158×10^6 tons of food in 2012 from >400 species (FAO, 2014), yet the nutrient composition of only a fraction of these species is reported in the FAO/INFOODS database (173 entries; FAO, 2013) and the USDA National Nutrient Database (48 entries; USDA, 2014). In contrast, 21 and 29 analyses of Fe in various cuts of meat from a single livestock species (cattle, *Bos taurus*), are reported in these databases. Fish species diversity is high in Bangladesh, with over 267 freshwater fish species and other aquatic animals, contributing to a large proportion of the population intake of haem Fe (Thilsted, 2013). Few studies of haem Fe concentrations from different fish species have been published (Kongkachuichai et al., 2002; Turhan, Ustun, & Altunkaynak, 2004; Turhan, Sule Ustun, & Bank, 2006; Roos et al., 2007). Because different species were tested by a range of different analytical methods and Fe pool calculations, haem Fe concentrations in these papers are varied and inconsistent.

In order to improve procedures for haem Fe analysis in fish, and consequently, dietary recommendations of fish intake, we have developed a method for the analysis and calculation of total Fe, haem Fe and non-haem Fe in fish by ICP-mass spectrometry (ICP-MS) based on an ICP-optical emission spectroscopy (ICP-OES) analysis of beef (Cross et al., 2012). This paper reports on the total Fe, haem Fe and non-haem Fe in a number of small indigenous fish species (SIS; <25 cm in length), shrimp and prawn species commonly consumed in Bangladesh.

2. Materials and methods

2.1. Fish preparation

Samples of SIS were obtained from local markets and fish landing sites in Mymensingh, Sylhet, Khulna, Dinajpur and Cox's Bazar districts of Bangladesh in July–August 2014. Four replicates of 26 samples, comprised of 23 species of fish, one species of shrimp and one of prawn were all from capture fisheries, plus one fish species (*Amblypharyngodon mola*) was taken from both capture and a household culture pond (Table 1). All samples were cleaned, using non-metal equipment to obtain raw, edible parts, according to traditional practice. Samples were frozen, air-freighted on dry ice to Adelaide, South Australia, and re-frozen at −80 °C, then freeze-dried until all water was removed (>48 h). Samples were ground with an IKA 11 stainless steel mill (IKA, Staufen, Germany) until uniform particle size was achieved. Variations between species in terms of bone density and skin thickness contributed to between-species heterogeneity.

The reference material Dogfish protein DORM4 (National Research Council of Canada, Ottawa, Canada) was digested in duplicate with each batch to estimate the recovery of variability of the digestion efficiency. No certified reference material for haem Fe content is currently commercially available so locally purchased, imported salted dried prawn (*Metapenaeus ensis*) and anchovy (*Engraulis* ssp.) were dried and ground and used as additional check samples for between-batch repeatability.

2.2. Sample digestion for total Fe

Sub-samples of each SIS were digested in 15 mL polypropylene tubes (SC415, Environmental Express, Chapel Hill, USA) in a 96 well Hotblock® acid digestion block (Environmental Express). Approximately 0.1 g of each freeze-dried, ground sample was weighed into a digestion tube to ±0.0001 g on a Kern ABJ balance (Balingen-Frommern, Germany), and 2 mL of Baseline grade

Table 1
Fish, shrimp and prawn species names.

Scientific name	Local name	Common name
Fish		
Ailia coila	Kajuli, Bashpata	Gangetic ailia
Amblypharyngodon mola	Mola	Mola carplet
Amblypharyngodon mola (cultured)	Mola (cultured)	"
Botia dario	Rani	Queen loach
Chela cachius	Chela	Silver hatchet chela
Colisa fasciata	Boro Kholisha	Banded gourami
Corica soborna	Kachki	Ganges river sprat
Eleotris fusca	Kuli, Bhut Bailla	Dusky sleeper
Esomus danricus	Darkina	Flying barb
Glossogobius giuris	Bele, Bailla	Tank goby
Gudusia chapra	Chapila	Indian river shad
Heteropneustes fossilis	Shing	Stinging catfish
Hyporhamphus limbatus	Ekthute	Congaturi halfbeak
Lepidocephalichthys guntea	Gutum	Guntea loach
Macrognathus aculeatus	Tara Baim	Lesser spiny eel
Mastacembelus pancalus	Guchi	Barred spiny eel
Mystus cavasius	Golsha	Gangetic mystus
Mystus vittatus	Tengra	Striped dwarf catfish
Osteobrama cotio cotio	Dhela	Dhela
Pseudambassis ranga	Chanda	Indian glassy fish
Puntius sophore	Jat Punti	Pool barb
Puntius ticto	Tit Punti	Ticto barb
Stolephorus tri	Kata Phasa	Spined anchovy
Xenentedon cancila	Kakila	Asian needlefish
Prawn/Shrimp		
Macrobrachium malcolmsonii	Najari Icha	Monsoon river prawn
Metapenaeus monoceros	Harina Chingri	Speckled shrimp

HNO_3 (Seastar Chemicals, Sidney, Canada) was added and the screw top cap tightened firmly. Tubes were immediately placed in the Hotblock, which had been preheated to 50 °C. The block temperature was raised to 90 °C at 3 °C/min, held for 30 min, then increased to 110 °C and held for 120 min. During the 90 °C holding step, tube caps were unscrewed slightly to release excess pressure. Approximately 60 min into the 110 °C holding step, tubes were removed from the Hotblock in small batches, caps removed to add 0.25 mL of H_2O_2 to the sample, before replacement of the caps and reinsertion of the tubes into the Hotblock. The H_2O_2 oxidised most of the remaining organic material, leaving a pale yellow solution with minimal NO_x vapour above the sample at the completion of the digestion.

After the tubes had cooled to room temperature (20–23 °C), solutions were diluted to the 10 mL mark on the vials. The diluted solutions were mixed using a vortex and allowed to cool again, then a 0.25 mL sub-sample of each solution was diluted with 4.75 mL 2% HNO_3 (v/v) using a Microlab 600 Diluter (Hamilton, Reno, USA) in labelled 6 mL polypropylene screw cap tubes (TechnoPlas, Adelaide, Australia), prior to ICP-MS analysis (Section 2.3).

2.3. Haem Fe extraction

Haem Fe extraction was carried out using the method originally devised for spectrophotometric determination by Hornsey (1956) and modified by Cross et al. (2012). Approximately 0.1 g of freeze-dried, milled sample was weighed to ±0.0001 g in a 15 mL centrifuge tube (Greiner Bio-One, Kremsmunster, Austria) and 10 mL of an acetone:water:hydrochloric acid (80:10:10 v/v) mixture (HPLC grade acetone, Merck, Darmstadt, Germany: 18 MΩ·cm H_2O, Millipore, MA, USA: Baseline grade HCl, Seastar Chemicals), referred to as 80% acetone for brevity, was added to the sample and mixed using a vortex. The samples were placed in the dark for 60 min, vortexed again, then centrifuged at 885 g (2000 rpm) for 10 min in a Rotanta 460RC centrifuge (Hettich, Tuttlingen, Germany). The supernatant of each sample was sub-sampled by diluting 0.25 mL with 4.75 mL of 2% HNO_3 as before and analysed by ICP-MS. Blank samples and reference samples were also run to determine the variability of haem Fe extraction.

An additional experiment was carried out to measure completeness of extraction in a sub-set of eight samples plus reference materials by extracting the samples in three aliquots of 10 mL of 80% acetone for 60 min each. The 80% acetone mixture was subsampled as above, at the end of each centrifuge step. The remaining liquid was poured off and the residue re-extracted immediately. Sub-samples were diluted with 2% HNO_3 as above and analysed by ICP-MS.

2.4. Non-haem Fe digestion

After haem Fe extraction, the residual fish material (Section 2.3) was allowed to dry overnight in a fume cupboard after carefully decanting off the remaining acetone mixture. The residue was then digested as for total Fe (Section 2.2). Residue from reference materials and blank samples from the haem Fe extraction were also digested and analysed by ICP-MS.

2.5. ICP-MS analysis

Samples were analysed on an Agilent 7500ce ICP-MS equipped with AS100 model E auto-sampler, glass concentric nebuliser and Scott double pass spray chamber with 2.5 mm injector diameter torch, using the following operating conditions: spray chamber held at 2 °C, sample flow rate 350 μL/min, internal standard 15 μL/min, peristaltic pump 0.1 rps, plasma forward power 1500 W, sample depth 10 mm, nebuliser Ar flow 0.90 L/min, makeup gas flow 0.12 L/min and rinse time between samples 30 s at 0.3 rps. Before use, the ICP-MS was tuned with a 1 μg/L tuning solution (Li, Mg, Y, Ce, Tl and Co, Agilent part number 5185–5959) to within Agilent specifications. The He-mode tuning file was used for subsequent calibration and analysis. Internal standard elements In, Rh and Y (100 μg/L in 2% HNO_3 (w/w)) were added via a mixing T-piece. In addition to Fe, analysed elements included macro-elements Na, Mg, P, K, Ca, micro-elements Mn, Ni, Cu, Zn, Se, Mo and trace elements Ti, Cr, Co, As, Cd and Pb.

Calibration solutions were prepared in 2% HNO_3 for total Fe and non-haem Fe analyses, and 2% HNO_3 + 4% acetone (w/w) for haem Fe analyses. A Ni skimmer cone was used for all analyses as the organic solvent concentration was below the recommended maximum of 20%. However, as Cross et al. (2012) reported, the plasma was extinguished during Haem Fe analyses when the 2% HNO_3 + 4% acetone solution was aspirated at standard pump and gas flow settings. This was rectified by increasing plasma forward power to 1600 W, adjusting the Ar flow rate to the nebuliser to 0.92 L/min and makeup gas to 0.10 l/min and slowing the pump rate during rinses to 90 s at 0.2 rps.

Fe concentrations were recalculated from a freeze-dry weight basis (mg kg^{-1} DW) to edible portion fresh weight (mg 100 g FW) using moisture content data from Bogard et al. (2015) as follows:

$$\mathrm{Fe(mg/100gFW)} = \mathrm{Fe(mg/kgDW)} \times ((100 - [\mathrm{m\%}]) \div 100) \times 0.1$$

where [m%] is the percentage moisture content.

Other elements were recalculated on a mg/kg FW basis (Cu, Zn) or μg/kg FW basis (Cr, Ni, As, Se, Cd, Pb) as appropriate. Mean haem Fe% was calculated for each species using the three equations as follows:

$$\mathrm{HaemFe\%_{avg}} = (\text{average haem Fe})/(\text{average total Fe}) \times 100;$$

$$\text{Haem Fe\%}_{\mathrm{tot}} = (\text{haem Fe})/(\text{total Fe}) \times 100$$

$$\text{Haem Fe\%}_{\mathrm{bal}} = (\text{haem Fe})/(\text{Fe balance}) \times 100.$$

The first of these equations (Haem $Fe\%_{avg}$) is a simple ratio which does not consider replicate data, whereas Haem $Fe\%_{tot}$ and Haem $Fe\%_{bal}$ are averages of replicated data, hence paired *t*-tests can be applied. Statistical analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY).

3. Results

3.1. Elemental recovery and limits of detection

Analysis of acid digested DORM4 material achieved recovery of 314.6 ± 16.8 mg Fe/kg DW or 92% of the certified Fe concentration (Table 2). Of the other certified elements, Zn, As, Se and Cd were recovered with acceptable efficiency (83–102%), however, Ni, Cu, Cr, and Pb were not recovered efficiently (41–77% of certified concentrations; Table 3). Relative standard deviations (RSD%) for certified elements ranged from 5.3% for Fe to 20% for Ni and Se, while RSD% of non-certified elements in DORM4 was 7–10% (data not shown), indicating acceptable precision and repeatability given that the tested sample mass (0.1 g) was smaller than recommended (1 g). Similar RSD% results for macro-, micro- and trace elements from the in-house anchovy (5–10%, 7–25%, 5–60%) and prawn (2–4%, 7–28% and 5–53%; Table 3) suggest that the method is precise and repeatable for most elements, although results for Pb and Se in particular were highly variable.

Method detection limits (MDL) in Tables 2 and 3 are shown on a mg/kg DW basis and are at least an order of magnitude below the element concentrations of DORM4. Due to the lower ICP-MS

Table 2
Concentrations of total Fe, haem Fe, non-haem Fe and Fe balance recovery for fish, shrimp and prawn species, reference materials and method detection limits.

Species	Total Fe mg/100 g FW	Haem Fe mg/100 g FW	Non-haem Fe mg/100 g FW	Fe balance recovery (%)	P *t*-test[2]
Ailia coila	0.68 ± 0.2	0.50 ± 0.2	0.21 ± 0.1	110 ± 10	0.40
Amblypharyngodon mola	1.97 ± 0.3	1.17 ± 0.1	0.75 ± 0.2	81 ± 44	0.45
Amblypharyngodon mola (cultured)	14.43 ± 4.1	2.63 ± 0.5	11.79 ± 2.4	103 ± 24	0.55
Botia dario	1.66 ± 0.2	1.39 ± 0.1	0.51 ± 0.3	109 ± 6	0.32
Chela cachius	0.68 ± 0.1	0.57 ± 0.1	0.19 ± 0.1	110 ± 9	0.35
Colisa fasciata	11.76 ± 4.2	2.30 ± 1.7	9.32 ± 2.8	114 ± 15	0.56
Corica soborna	1.89 ± 1.3	0.74 ± 0.3	1.01 ± 0.8	100 ± 7	0.08
Eleotris fusca	0.55 ± 0.1	0.42 ± 0.1	0.24 ± 0.1	97 ± 14	0.23
Esomus danricus	3.14 ± 0.9	1.49 ± 0.5	1.36 ± 0.4	112 ± 10	0.89
Glossogobius giuris	1.34 ± 0.7	0.82 ± 0.3	0.74 ± 0.4	86 ± 25	0.46
Gudusia chapra	4.55 ± 1.6	1.82 ± 0.6	2.36 ± 0.9	103 ± 16	0.56
Hemirhamphus georgii	1.03 ± 0.2	0.64 ± 0.1	0.43 ± 0.1	96 ± 9	1.00
Heteropneustes fossilis	1.30 ± 0.1	1.04 ± 0.1	0.35 ± 0.1	105 ± 11	0.18
Lepidocephalichthys guntea	2.43 ± 0.8	1.64 ± 0.2	0.56 ± 0.3	107 ± 12	0.14
Macrognathus aculeatus	1.01 ± 0.2	0.51 ± 0.2	0.43 ± 0.1	107 ± 11	0.77
Mastacembelus pancalus	1.16 ± 0.4	0.88 ± 0.4	0.39 ± 0.2	77 ± 28	0.20
Mystus cavasius	1.74 ± 0.6	0.87 ± 0.1	0.69 ± 0.3	82 ± 50	0.36
Mystus vittatus	10.51 ± 4.3	4.87 ± 2.2	5.35 ± 3.4	90 ± 30	0.35
Osteobrama cotio cotio	0.87 ± 0.2	0.61 ± 0.1	0.40 ± 0.3	113 ± 10	0.42
Pseudambassis ranga	1.54 ± 0.2	1.03 ± 0.3	0.73 ± 0.1	109 ± 12	0.58
Puntius sophore	1.80 ± 0.8	1.34 ± 0.5	0.75 ± 0.5	103 ± 4	0.19
Puntius ticto	1.01 ± 0.1	0.94 ± 0.1	0.25 ± 0.1	115 ± 8	0.16
Stolephorus tri	1.52 ± 0.5	0.71 ± 0.1	0.83 ± 0.2	101 ± 20	0.17
Xenentedon cancila	0.59 ± 0.1	0.45 ± 0.1	0.12 ± 0.1	96 ± 5	0.79
Macrobrachium malcolmsonii	8.24 ± 1.0	4.91 ± 1.1	5.78 ± 1.4	122 ± 12	0.09
Metapenaeus monoceros	1.81 ± 0.2	0.71 ± 0.3	1.38 ± 0.5	109 ± 40	0.74
Reference materials (mg/kg DW)					
DORM4	314.6 ± 16.8	269.0 ± 47.5	62.6 ± 13.8	105	
In-house anchovy	20.7 ± 1.4	16.5 ± 0.8	19.1 ± 6.8	171	
In-house prawn	79.2 ± 7.0	28.1 ± 7.1	49.6 ± 11.9	98	
Method detection limit	1.82	2.51	1.82		

Concentrations given as average ± 1 SD ($n = 4$) for fish, shrimp and prawns (mg/100 g FW), reference materials (mg/kg DW) and Fe balance recovery (% of total Fe). P *t*-test indicates probability of Fe balance concentration differing from total Fe concentration.

sensitivity and higher SD of the acetone matrix blanks, MDL were 2–8 times higher in the 2% HNO_3 + 4% acetone matrix. The RSD% of haem Fe in 80% acetone extractions from DORM4 was 17.6% and from the in–house samples was extremely variable (0.6% for anchovy and 26% for prawn). RSD% for other elements in DORM4 extracted by 80% acetone was only slightly greater than in acid digested samples (data not shown).

3.2. Elemental concentrations in fish, shrimp and prawn species

Total Fe concentration showed considerable variation between species (Table 3), ranging from 0.55 ± 0.1 mg Fe/100 g in *Eleotris fusca* to 14.43 ± 4.1 mg Fe/100 g in *A. mola* (cultured). In comparison, *A. mola* from capture fisheries had a total Fe concentration of 1.97 ± 0.3 mg Fe /100 g. Variability of total Fe increased with concentration (Table 2). Data for other certified elements in DORM4 are shown in Table 4: Zn and Se were detected in all samples, whereas Cu was detected in less than half the samples, due to the high method detection limits (<0.60 mg kg^{-1}) and low recovery efficiency. Substantial Cu concentrations were observed in the crustacean species *Macrobrachium malcolmsonii* and *Metapenaeus monoceros*, as well as the in-house prawn. Three elements commonly of concern for human consumption were detected in many of the samples: As was found in all species, Cd in 20 and Pb in 23, with 19 species having all three elements present simultaneously. Chromium and Ni were found in low concentrations (<220 μg/kg FW) in many samples.

3.3. Haem Fe concentration

Haem Fe concentration in SIS (Table 2) ranged widely from 0.42 ± 0.1 mg Fe/100 g for *E. fusca* to 4.87 ± 2.2 mg Fe/100 g in *Mystus vittatus*. The shrimp and prawn species both had significant concentrations of Fe in haem extracts: 4.91 ± 1.1 mg Fe/100 g (*M. malcolmsonii*) and 0.71 ± 0.3 mg Fe/100 g (*M. monoceros*). CV% of Fe in 80% acetone extracts ranged from 2% in *Botia dario* to 46% in *Mastacembelus pancalus* and *M. vittatus*. The concentration of haem Fe across all species increased in a log–log relationship: Haem $Fe = 10^{(0.610\pm0.060\times\log10(\text{Total Fe})+0.513\pm0.057)}$, $R^2 = 0.607$, $n = 77$ (Fig. 1a).

3.4. Multiple haem Fe extractions

When eight fish species samples differing in Fe content were extracted with three changes of 80% acetone, approximately 87% of haem Fe was extracted in the first 60 min, 10% in the second 60 min and 3% in the third 60 min, regardless of individual total Fe concentration, suggesting that similar pools of Fe were mobilised by the 80% acetone in all species (Table 4). Other elements extracted in similar proportions to Fe were Mg, P, K, Mn, Ni, Zn, Se and Pb. In contrast, 100% of Cu and Ti was extracted in the first 60 min, whereas As, Se and Cd were fully extracted in the second 60 min. Extraction was less effective for Al, Ca and Co (62–82%) in the first 60 min but was nearly complete in the second 60 min. Because the anchovy and prawn in-house standards were highly salted, high concentrations of Na were found in both the first and second extracts, however most elements were extracted from the DORM4 and in-house standards in similar patterns to the fish samples (Table 4).

3.5. Non-haem Fe and Fe balance

Measurement of non-haem Fe from the sample residue after 80% acetone extraction showed average concentrations of 0.12–11.79 mg Fe/100 g (Table 2). In all cases, the concentration of non-haem Fe was less than the total Fe of the sample. The calculated Fe

Table 3
Concentrations of mineral elements in fish, shrimp and prawn species from acid digested samples.

	Concentration per kg FW							
	Cu mg	Zn mg	Cr µg	Ni µg	As µg	Se µg	Cd µg	Pb µg
Species								
Ailia coila		14.1 ± 2.5			42 ± 36	295 ± 12	14 ± 4	11 ± 7
Amblypharyngodon mola	0.25 ± 0.1	40.4 ± 5.2	16 ± 7	25 ± 7	226 ± 36	318 ± 202	60 ± 17	8 ± 4
Amblypharyngodon mola (cultured)	0.23 ± 0.0	38.9 ± 4.8	164 ± 52	155 ± 5	158 ± 8	217 ± 124	11 ± 3	93 ± 14
Botia dario	0.24 ± 0.0	31.0 ± 5.2		6 ± 6	102 ± 21	517 ± 41	9 ± 4	10 ± 8
Chela cachius		45.7 ± 4.1			105 ± 5	368 ± 150	11 ± 4	
Colisa fasciata	0.19 ± 0.1	20.0 ± 3.9	210 ± 105		115 ± 33	304 ± 86		85 ± 25
Corica soborna	0.15 ± 0.1	26.3 ± 3.3	41 ± 12	23 ± 1	119 ± 14	134 ± 50	229 ± 141	15 ± 4
Eleotris fusca		17.1 ± 3.1	27 ± 4		500 ± 53	648 ± 149	11 ± 5	21 ± 3
Esomus danricus	0.26 ± 0.0	41.4 ± 7.6	36 ± 9		194 ± 24	461 ± 83		181 ± 20
Glossogobius giuris		18.5 ± 2.0	13 ± 16	14 ± 1	90 ± 24	504 ± 166	6 ± 3	15 ± 8
Gudusia chapra	0.22 ± 0.2	31.5 ± 2.4	54 ± 31	51 ± 11	124 ± 14	301 ± 88	16 ± 8	34 ± 14
Heteropneustes fossilis		29.5 ± 5.2	33 ± 12		252 ± 20	510 ± 392	30 ± 12	22 ± 12
Hyporhamphus limbatus	0.13 ± 0.1	8.4 ± 1.3	23 ± 6	14 ± 7	40 ± 18	151 ± 165	11 ± 6	56 ± 3
Lepidocephalichthys guntea		19.4 ± 5.1	16 ± 14	20 ± 12	45 ± 14	388 ± 236	1 ± 1	8 ± 5
Macrognathus aculeatus	0.08 ± 0.0	10.6 ± 1.2	22 ± 27		24 ± 5	904 ± 134	28 ± 9	19 ± 4
Mastacembelus pancalus	0.18 ± 0.1	11.9 ± 1.0		5 ± 1	35 ± 9	586 ± 9	12 ± 3	18 ± 4
Mystus cavasius	0.06 ± 0.1	15.6 ± 2.5	28 ± 12	25 ± 9	29 ± 11	427 ± 230	8 ± 5	17 ± 3
Mystus vittatus		25.0 ± 4.1	187 ± 61		56 ± 7	695 ± 10	20 ± 5	79 ± 18
Osteobrama cotio cotio		36.8 ± 6.7			55 ± 7	370 ± 235		14 ± 2
Pseudambassis ranga		32.5 ± 6.3			128 ± 15	256 ± 125	12 ± 3	9 ± 2
Puntius sophore		30.8 ± 4.0			44 ± 19	758 ± 64	17 ± 1	25 ± 1
Puntius ticto	0.12 ± 0.1	36.5 ± 2.6	4 ± 3	12 ± 1	49 ± 14	246 ± 225	11 ± 3	8 ± 5
Stolephorus tri		28.4 ± 3.2	30 ± 13		545 ± 89	438 ± 65	16 ± 1	44 ± 9
Xenentedon cancila	0.1 ± 0.1	18.3 ± 3.5			19 ± 4	366 ± 231		
Macrobrachium malcolmsonii	6.76 ± 0.7	25.5 ± 2.9	99 ± 21	116 ± 35	238 ± 23	303 ± 22	57 ± 18	25 ± 7
Metapenaeus monoceros	2.46 ± 0.5	10.8 ± 1.5	18 ± 11		366 ± 56	450 ± 127	18 ± 9	15 ± 4
Reference materials:	Concentration per kg DW							
DORM4	9.8 ± 0.8	48.9 ± 5.7	1453 ± 177	988 ± 66	6775 ± 644	3630 ± 102	254 ± 21	171 ± 17
Certified value	15.9 ± 0.9	52.2 ± 3.2	1870 ± 160	1360 ± 220	6800 ± 800	3560 ± 340	306 ± 15	416 ± 53
Anchovy in-house	–	62.1 ± 8.5	–	–	4360 ± 434	2870 ± 230	646 ± 49	52 ± 23
Prawn in–house	2.8 ± 1.0	45.7 ± 4.4	689 ± 302	442 ± 8	5900 ± 336	2500 ± 1910	166 ± 15	115 ± 53
Method detection limit	µg/kg DW						–	
2% HNO_3 matrix	0.60	6.1	160	76	134	674	46	42
4% acetone, 2% HNO_3 matrix	2.9	2.5	502	258	430	1.857	110	358

Concentrations given as average ± 1 SD, (n = 4), on per kg FW basis for fish, shrimp and prawn and per kg DW basis for reference materials and method detection limits.

Table 4
Percentage of elements extracted by three sequential 60 min extractions in 80:10:10 acetone:H_2O:HCl.

Element	Fish (n = 8)			DORM (n = 2)			Anchovy (n = 2)			Prawn (n = 2)		
	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3
Na	84	14	2	84	14	2	64	23	13	55	29	17
Al	62	34	3	61	24	14	–	–	–	–	–	–
P	89	10	1	92	8	1	87	11	2	88	11	1
K	84	14	2	82	16	2	69	20	11	83	14	3
Mg	86	13	1	90	10	0	81	16	4	85	13	2
Ca	74	20	7	76	19	5	70	20	9	76	18	6
Mn	83	14	2	86	13	1	77	17	6	72	20	8
Fe	87	10	3	90	8	1	90	8	2	65	22	13
Cu	100	0	0	96	4	0	100	–	–	100	–	–
Zn	86	12	2	89	11	0	84	14	2	83	16	1
Mo	–	–	–	25	65	10	–	–	–	100	–	–
Ni	89	10	1	80	17	4	100	0	0	79	18	3
Ti	100	0	0	–	–	–	–	–	–	–	–	–
Cr	–	–	–	100	–	–	–	–	–	100	–	–
Co	79	20	1	79	17	4	72	20	8	79	18	3
As	93	7	0	90	9	1	82	15	2	88	11	1
Se	91	9	0	51	40	8	0	100	0	81	19	0
Cd	98	2	0	86	13	1	63	24	13	57	32	11
Pb	82	16	2	61	34	5	80	13	7	48	33	19

Data presented are percentage of total mass recovered from all three extractions per sample type (fish, DORM4, in-house anchovy, in-house prawn).

balance (sum of haem Fe + non-haem Fe) was close to total Fe concentration, generally exceeding total Fe by 1–15% (Table 5). Paired t-tests showed no significant difference between Fe balance and total Fe, indicating that analysis by ICP-MS of Fe extracted by 80% acetone accurately quantified the haem Fe pool of the samples.

3.6. Haem Fe% estimates

Average haem Fe% as calculated by three different equations yielded similar values in most species and ranged from 18% in *A. mola* (cultured) to 93% in *Puntius ticto* (Table 5). When calculated

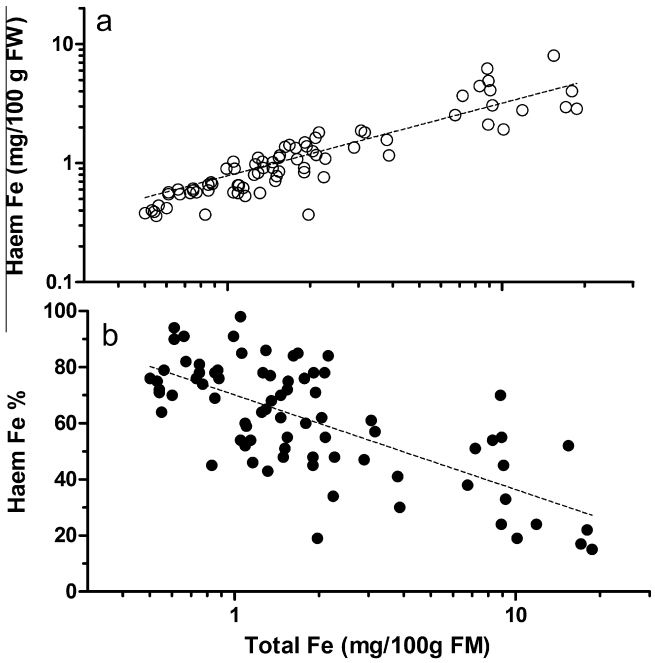

Fig. 1. Relationships between (a) haem Fe and total Fe and (b) haem Fe%$_{tot}$ and total Fe for all replicates of fish, prawn and shrimp species. Regression line equations: Haem Fe = $10^{(0.619\pm0.052+\log(\text{Total Fe})+(0.530\pm0.132))}$, R^2 = 0.607, n = 79. Haem% = $\log_{10}$(Total Fe) × (−33.73 ± 3.97) + (70.1 ± 1.96). R^2 = 0.491, n = 77.

as a simple ratio (haem Fe%$_{avg}$) the result was occasionally distinctly different to the values of haem Fe%$_{tot}$ or haem Fe%$_{bal}$; e.g. *Esomus danricus*, *E. fusca*, *M. pancalus*, *M. cavasius.* (Table 5). Paired *t*-tests indicated that haem Fe%$_{tot}$ and haem Fe%$_{bal}$ differed significantly only in *M. pancalus*, *P. ticto* and *A. mola* (capture). The particularly small CV% from *A. mola* may partly account for this difference, while heterogeneity in *M. pancalus* and *P. ticto* samples may have affected average haem results (Table 5). When plotted against total Fe, haem Fe%$_{tot}$ displayed an inverse semi-log relationship (Haem% = −33.73 ± 3.97 × $\log_{10}$(Total Fe) + (70.1 ± 2.0)). Although there was considerable variation (R^2 = 0.491, n = 77, Fig. 1b), haem Fe%$_{tot}$ declined as total Fe increased across all species.

4. Discussion

The ICP-MS method described here is able to detect metals in fish, shrimp and prawn samples from both acid digests and 80% acetone extractions. Minor modifications to the sample introduction parameters were required to ensure the plasma remained stable with 4% acetone in the matrix, slightly increasing method detection limits of most metals. Method detection limits were well below the observed concentrations of elements in the samples and reference materials in acid digested samples. A significant advantage of ICP-MS analysis over spectrophotometric methods based on the classical methods (Drabkin, 1950; Hornsey, 1956; Brown, 1961) is that Fe in the haem is analysed directly rather than inferred from haem calibration curves reducing some of the error propagated by combining results from multiple analyses (Lombardi-Boccia et al., 2002). Pigments extracted in buffers or solvents are subject to oxidation, pH and solvent concentration effects, degrading the colour of the extract, or altering background absorbance (Cross et al., 2012). In contrast, after extraction by acidified 80% acetone, the extracted iron remains dissolved in the solution, regardless of the condition of the haem Fe pigments. Elemental analyses (AAS, ICP) will not distinguish the form of haem iron that is present in an extract, requiring instead a hyphenated

Table 5
Haem Fe% values and bioavailable Fe in fish, shrimp and prawns.

Scientific name	Haem Fe%$_{avg}$	Haem Fe%$_{avg}$	Haem Fe%$_{bal}$	Bioavailable Fe (mg/100 g FW)	
	%	%	%	Measured Fe pools	Assuming 40% haem Fe
Ailia coila	74	81 ± 8.6	70 ± 11.7	0.12	0.07
Amblypharyngodon mola	59	61 ± 1.0^{c}	57 ± 0.8	0.30	0.22
Amblypharyngodon mola (cultured)	18	19 ± 3.5	18 ± 1.0	0.96	1.59
Botia dario	84	80 ± 7.7	74 ± 10.9	0.34	0.18
Chela cachius	84	85 ± 8.1	75 ± 9.4	0.14	0.07
Colisa fasciata	26	26 ± 5.9	26 ± 4.0	0.81	1.29
Corica soborna	39	45 ± 10.3	46 ± 10.7	0.20	0.21
Eleotris fusca	77^{ab}	69 ± 4.2^{a}	64 ± 7.7^{b}	0.10	0.06
Esomus danricus	47^{ab}	56 ± 1.3^{a}	56 ± 2.1^{b}	0.27	0.34
Glossogobius giuris	61	56 ± 11.4	54 ± 6.5	0.21	0.15
Gudusia chapra	40	42 ± 4.6	44 ± 3.7	0.49	0.50
Heteropneustes fossilis	80	80 ± 5.2	75 ± 4.4	0.16	0.11
Hyporhamphus limbatus	63	64 ± 12.5	61 ± 8.1	0.25	0.14
Lepidocephalichthys guntea	68^{a}	80 ± 3.5^{a}	75 ± 8.1	0.39	0.27
Macrognathus aculeatus	51	52 ± 10.0	56 ± 3.8	0.09	0.11
Mastacembelus pancalus	112*	77 ± 2.3^{c}	69 ± 2.2^{c}	0.21	0.13
Mystus cavasius	50^{ab}	66 ± 2.2^{a}	62 ± 0.7^{a}	0.15	0.19
Mystus vittatus	46^{a}	53 ± 2.1^{a}	50 ± 6.9	1.28	1.16
Osteobrama cotio cotio	70	68 ± 14.5	64 ± 18.9	0.15	0.10
Pseudambassis ranga	67	64 ± 10.3	58 ± 6.4	0.26	0.17
Puntius sophore	74	68 ± 5.8	66 ± 7.8	0.33	0.20
Puntius ticto	93^{b}	91 ± 6.4^{c}	79 ± 2.2^{bc}	0.22	0.11
Stolephorus tri	47	47 ± 15.3	46 ± 8.1	0.19	0.17
Xenentedon cancila	77	76 ± 5.0	80 ± 7.0	0.11	0.07
Macrobrachium malcolmsonii	60	56 ± 12.9	46 ± 9.0	1.30	0.91
Metapenaeus monoceros	39	37 ± 16.0	33 ± 7.7	0.20	0.20

Values are given as a ratio (haem Fe%$_{avg}$) or average ± 1 SD (n = 4, for haem Fe%$_{tot}$ and haem Fe%$_{bal}$). Superscripts indicate significant difference between Haem Fe%$_{avg}$, Haem Fe %$_{avg}$, Haem Fe%$_{bal}$ by one sided *t*-test (a, b) or paired *t*-test (c).
Bioavailable Fe was calculated similar to the method given in Schönfeldt and Hall (2011) using either the haem Fe%$_{tot}$ value (measured Fe pools) or 40% (Cook & Monsen 1976) for the proportion of haem Fe present and bioavailability of 23% (haem Fe) and 3% (non-haem Fe).
* No *t*-test was performed as 112% was assumed to be due to sampling error of haem Fe, total Fe or both.

method such as HPLC-ICP-MS to detect both the form of haem and the concentration of iron.

Although haem Fe extraction by acidified 80% acetone yields consistent results between samples differing in total Fe concentration (Table 2), the haem extraction process was incomplete after 60 min; further extractions recovered additional Fe from the second and third aliquots (Table 4). While it is possible that some non-haem Fe was solubilised by the 10% HCl present in the extraction solution, this was only small (1–2%) compared to the earlier extractions and the remaining Fe in the residue. Most procedures based on the Hornsey (1956) method utilise a single extraction, usually lasting 60 min (Carpenter & Clark, 1995; Harrington, Elahi, Merson, & Ponnampalavanar, 2001) and hence may have underestimated the amount of haem Fe in samples. A recent comparison of extraction of haem from fish tissue showed the standard Hornsey (1956) method was around 98% effective compared to a method combining SDS and heating (Chaijan & Undeland, 2015) but was still more effective than the buffer extraction methods of Brown (1961) and Drabkin (1950): 55% and 67% effective respectively. Storage of fish on ice for 5 days significantly reduced the recovery of haem by all four methods, suggesting that extended storage such as in the 25 species sampled in this paper was possibly also underestimating haem Fe concentrations (Chaijan & Undeland 2015). ICP analysis of multiple acetone extracts compared to the SDS/heat extracts may indicate whether the Hornsey method has consistently underestimated haem extraction in the past, and whether the iron concentration is also affected by the structure of haem.

Because the recovery of Fe from the DORM4 material by acid digestion was acceptable, and the Fe balance calculation checks showed no significant difference between total Fe and Fe balance values for any species (Table 2), the extraction of Fe in haem form by 80% acetone appears as effective as the recovery of total Fe by acid digestion (Cross et al. 2012), hence ICP-MS is suitable for the measurement of haem Fe from fish tissue.

Total Fe concentrations in Bangladeshi fish ranged from 0.55 to 14.43 mg Fe/100 g FW (Table 2) and are similar in magnitude to Fe concentrations of fish in the FAO/INFOODS database: 0.08–16.8 mg Fe/100 g FW, $n = 170$ and the USDA National Nutrient Database for Standard Reference; 0.09–3.25 mg/100 g FW, $n = 78$. Concentrations of other micro elements and trace elements (Table 3) were similar to those found in freshwater fish from capture fisheries in Bangladesh and elsewhere (Fallah, Saei-Dehkordi, Nematollahi, & Jafari, 2011), (Sharif, Alamgir, Mustafa, Hossain, & Amin, 1993). The range of concentrations of total Fe in Table 2 is somewhat greater than that reported for 16 Cambodian fish species (0.55–1.52 mg Fe/100 g FW equivalent), apart from *Esomus longimanus* (9.0 mg/100 g FW) (Roos et al., 2007). The related Bangladeshi species *E. danricus* had 3.14 ± 0.9 mg Fe/100 g FW (Table 2) and has been found previously to have high Fe concentrations (>10 mg/100 g FW; Roos, Islam, & Thilsted, 2003). The extent to which high Fe concentrations are species-specific or a product of the environment in which the fish live is unclear, however, the highest total Fe concentration was found in samples of cultured *A. mola* (14.43 ± 4.1 mg Fe/100 g FW), much higher than *A. mola* from capture fisheries (1.97 ± 0.3 mg Fe/100 g FW). This might be explained by the use of fertilizer in fish ponds which is sometimes practised to encourage plant growth (Roos et al., 1999).

The presence of Cu in total digest samples of *M. malcolmsonii*, *M. monoceros*, and the in–house prawn standard (Table 3), is presumably due to copper-containing haemocyanin which crustaceans use for oxygen transport (Terwilliger and Ryan, 2001). Surprisingly, all three samples showed considerable concentrations of Fe in the 80% acetone extracts (Table 2), which suggests the presence of haem Fe. The occurrence of dual oxygen transport mechanisms is uncommon, but has been observed previously in green shore crabs *Carcinus maenas* (Ertas, Kiger, Blank, Marden, & Burmester, 2011). Further study would indicate whether Fe and Cu are both associated with oxygen regulation in prawns or if the Fe was acquired through the prawns' diet.

The heavy metal elements that were detected in fish were generally well below acceptable levels (Table 3), although *C. soborna* (229 μg Cd kg^{-1} FW) had approximately half the acceptable European Commission Cd limit of 0.05 mg/kg FW (European Commission, 2006). Arsenic was present in all 25 species at low concentrations (<600 μg/kg FW). Because ICP-MS measures total As and does not distinguish between organic As and potentially toxic inorganic forms of As, additional analysis is required to evaluate whether consumption of these species is likely to be harmful. Lead was present in many of the sampled species, but was well below acceptable maximum levels (0.30 mg/kg FW, European Commission 2006).

Haem Fe% varied between fish species, ranging from <20% to >90% of total Fe (Table 5) and was inversely proportional to total Fe (Fig 1b). Previous estimates of haem Fe% in fish vary widely; 30–40% (Cook & Monsen, 1976; Kongkachuichai et al., 2002), 35–49% (Turhan et al. 2004, 2006) and 54–78% (Roos et al., 2007). However, as these values were derived by different methods such as summing inorganic Fe and haem Fe to derive the total Fe (Kongkachuichai et al. 2002) or imputing haem Fe% from the difference between total Fe and inorganic Fe (Roos et al. 2007), considerable ambiguity exists in the reliability of the derived values.

The data presented here support earlier findings that calculations of dietary intakes of Fe based on a single percentage value of total Fe inadequately express the variation present in animal-source foods (Carpenter & Clark, 1995; Schönfeldt & Hall, 2011). Following the example of Schönfeldt and Hall (2011), a simple estimate of Fe bioavailability based on the concentrations in Table 2, assuming bioavailability of 23% for haem Fe and 3% for non-haem Fe (Du, Zhai, Wang, & Popkin, 2000), and ignoring any enhancing or inhibiting factors from other foods, the fish species in this study provide between 0.09 and 1.30 mg of absorbed Fe per 100 g raw, edible portion. In contrast, assuming a constant of 40% total Fe for haem Fe% underestimates bioavailable Fe in fish by an average of 16% (Table 5).

5. Conclusions

ICP-MS analysis of acid-digested certified fish tissue (DORM4) yielded accurate results for Fe, Zn, As, Se and Cd with RSD% ranging from 3% (Se) to 12% (Zn). RSD% of total Fe (acid-digested) recovery was 5.3%. Fe balance calculations were not significantly different to total Fe measurements, hence the method effectively determines Fe concentration in fish, shrimp and prawn samples. Using a single analytical technique simplifies laboratory processes in contrast to methods which use a combination of different chemical extractions, instruments and calibrations. Method detection limits for most elements were at least an order of magnitude smaller than the certified element concentrations of DORM4 tissue. The 80% acetone extraction process is not complete within 60 min, as repeated extractions indicated only 87% of the extractable haem Fe was recovered in the first 60 min. This occurred regardless of total Fe concentration.

Concentrations of minerals in samples of fish, shrimp and prawn species from Bangladesh were similar to those found in other species in South East Asia. Total iron concentrations in samples ranged from 0.55 to 14.43 mg/100 g FW. The range of haem Fe% in fish, shrimp and prawn samples was much larger than previously reported, with higher values occurring in species with lower total Fe concentrations. The potential contribution of SIS of fish, shrimp and prawn to dietary Fe intake based on the conventional value of 40% haem Fe is likely to have been underestimated in the past.

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