Speciation of Cadmium in Crab Meat by Reversed-phase High-performance Liquid Chromatography With Electrothermal Atomisation Atomic Absorption Spectrometric Detection in a Model Gut Digestive System*

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The development of a model gut system has enabled the study of cadmium bioavailability to be carried out. The use of a biological membrane indicates that although soluble forms of cadmium may exist in the gut, only protein-complexed forms will pass through such a membrane. Chromatographic separation by high-performance liquid chromatography (HPLC) indicates that although cadmium - metallothioneine type complexes are present at the pH values of both the stomach and the intestine, their solubility is significantly reduced in the intestine. Experimental details are given for both the gut model and the speciation methodology used.

Keywords: *High-performance liquid chromatography; electrothermal atomistion atomic absorption spectrometry; model gut digestive system; cadmium; speciation*

The physico-chemical forms or species in which an element may exist, is now clearly understood to affect its mobility, availability and eventual fate in the environment. The need to identify not only the total elemental concentrations but also the component species present has led to a growing need to develop suitable methodology for species determination and quantification.[1–4] A report by the Analytical Methods Committee of the Royal Society of Chemistry[5] suggested the adoption of the two-stage enzymolysis procedure developed by Crews *et al.*[6] The models described in these references can be used to assess the bioavailability of trace components in foods. It is worth noting, however, that the models referenced and the one described in this work do not currently incorporate gut fauna and flora but represent only the abiotic chemical aspects of food digestion. To date only a limited amount of work has been reported for trace elemental speciation in foods.[1,7,8] This situation is mainly due to the complex nature and low elemental concentration associated with such systems. In this paper crab meat with naturally high levels of cadmium (1–2 μg^{-1}) has been used as the food component in a gut model system. The use of both filters and membranes to study the soluble fractions and subsequent high-performance liquid chromatographic (HPLC) separation with electrothermal atomisation atomic absorption spectrometry (ETAAS) for cadmium quantification will be discussed.

Experimental

Apparatus

Determination of cadmium was performed with a Thermo Electron Video II atomic absorption spectrometer fitted with a Thermo Electron 655 graphite furnace equipped with a Fastac 254 autosampler (Thermo Electron, Warrington, UK). Smith - Hieftje background correction was used throughout the analyses with cadmium absorbance measurements being made at 228.8 nm. Separation of the cadmium species was performed on a Shimadzu LC-4A high-performance liquid chromatograph fitted with a variable SPD 2AS UV - visible detector and printer (Dyson Instruments, UK). The analytical column was a styrene based reversed-phase C_{18} PRP-1 column (Anachem, Luton, Bedfordshire). The dialysis membrane and 0.45-μm Swinnek filters used were obtained from Scientific Suppliers (Park Royal, London, UK) and Millipore Water Associates (Harrow, UK), respectively.

The optimum operating parameters for the spectrometer and HPLC system are shown in Table 1.

Table 1. Instrumental conditions

Atomic absorption spectrometry—	
Lamp current	4.0 mA
Wavelength	228.8 nm
Ashing temperature	650 °C
Atomisation temperature	1600 °C
Tube clean	2300 °C
Purge gas	Argon
High-performance liquid chromatography—	
HPLC column	Anachem (PRP-1) (10-μm particle size, 25 cm × 5 mm i.d.)
Mobile phase	0.05% *V/V* orthophosphoric acid
Flow-rate	1.5 ml min^{-1}
Wavelength of detection	254, 280, 210 nm
Chart speed	3.0 cm min^{-1}
Injection volume	20 μl

Reagents

All glassware, silica crucibles and storage bottles used were soaked in 10% *V/V* nitric acid overnight and rinsed with fresh distilled de-ionised water prior to use. All the enzymes (Pepsin porcine Cat. No. P7000, Pancreatin porcine Cat. No. P1750, Amylase porcine Cat. No. A6880) and bile salts (Cat. No. B8756) were purchased from Sigma Chemicals (Poole, Dorset, UK). All other reagents were of AnalaR grade as supplied by BDH (Poole, Dorset, UK).

Sample Preparation

The ready to eat crab was purchased from a local market. It was dressed and the meat homogenised using a Hamilton Beach seven-speed blender. The homogenised samples were stored at −5° C in sealed polythene bags and used as required.

Enzymolysis Procedure and Trace Elemental Analysis

The enzymolysis procedure used is summarised in Fig. 1. It is an adaptation of the optimised procedure developed by Crews

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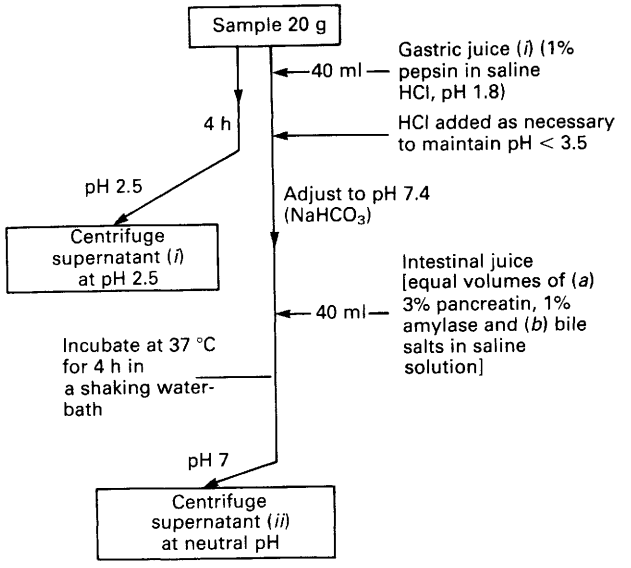

Fig. 1. Optimised enzymolysis procedure

Table 2. Determination of cadmium distribution in the gut digestion model

Sample	Cadmium content/μg per 20 g: Pepsin, pH 2	Pepsin - pancreatin, pH 7.4
Whole crab ($n = 5$) . .	24.5 (100%)	35.5 (100%)
Supernatant ($n = 4$) . .	18.0 (73%)	15.5 (44%)
Residue ($n = 4$)	6.5 (27%)	20.0 (56%)

et al.[6] Samples (20 g) were homogenised with the enzyme mixture in stoppered conical flasks and incubated at 37 °C in a shaking water-bath. All extractions were performed in duplicate together with corresponding blanks. Two digestion sets for each sample were prepared, the first set having only pepsin added to the samples whilst the second set had pepsin and pancreatin present. The sample pH's were adjusted to <2.0 by adding hydrochloric acid. After 4 h the first set (pepsin only) with the associated blanks were removed and aliquots (1 g) were taken and analysed for lead and cadmium. The remaining digest and blank were transferred into centrifuge bottles and centrifuged for 20 min. The supernatant liquids and residues were then removed separately and aliquots analysed for lead and cadmium. The second digest set was treated in a similar manner and analyses for lead and cadmium were carried out.

Total Elemental Analysis

For total eluted analysis, samples were dry ashed in a silica crucible to which 5 ml of 20% *V/V* sulphuric acid were added to aid ashing. The samples were then placed in an air oven to dry for 3 h after which time they were transferred into a muffle furnace and ashed at 600–650 °C overnight.[9] After dry ashing, the total cadmium determinations were carried out using ETAAS. At this stage 2.5 ml of a 1% ammonium dihydrogen phosphate solution and 125 μl of 0.2% *m/V* magnesium nitrate solution were added to 200 μl of the dry ashed digest. The results obtained for both the total cadmium concentration and the cadmium concentration in each fraction of the enzymolysis procedure are shown in Table 2.

Results and Discussions

The results obtained using the enzyme digestion procedure described indicate that the relative solubility of cadmium at pH 2.0 (73%) is significantly reduced at the intestinal pH of 7.4 (44%). It has been shown[6] that it is the acidic effect rather than enzyme action which is responsible for the higher solubility at pH 2.0 compared with pH 7.4.

It has been reported that the metal binding ligands (protein) in crabs have metallothioneine type properties.[10,11] The results obtained using HPLC separation suggest that at the stomach pH of 2.0 cadmium exists in the form of a soluble metallothioneine or single amino acid type of complex which becomes less soluble at pH 7.4.

The supernatant liquids (Fig. 1) were passed through both a 0.45-μm filter and a dialysis membrane. The filtrate (solution transferred) and the residues (sample left on the filter-paper and in the membrane, respectively) were dry ashed separately and analysed for cadmium (Table 3). The lower values for pepsin - pancreatin totals (filtration) was thought to be as a result of the high back-pressure developed in the sterile syringe fitted to the Swinnex filter. This occurred because of the highly gelatinous nature of the supernatant liquid, allowing only a small proportion of it, which was weighed by difference, to be filtered. Examination of the soluble supernatants showed that whereas 93 and 87% of cadmium passed through the filter from the pepsin and pepsin - pancreatin supernatants, respectively, only 53 and 8% passed through the dialysis membrane. The dialysis membrane is known to have a sheet-like structure with an average pore size of 6–10 μm consisting of lipid and protein bilayers.[12] Because of their composition, such membranes represent highly selective permeability barriers. The higher percentage of cadmium transported through the filter-papers could be attributed to the larger pore sizes whereas with the membrane, the transport of metals occurs via a carrier protein, *e.g.*, a metallothioneine type protein. The results obtained suggest

Table 3. Influence of filtration and dialysis membrane on cadmium concentrations (μg g^{-1})

Method	Pepsin: Residue	Pepsin: Transported	Pepsin - pancreatin: Residue	Pepsin - pancreatin: Transported
0.45-μm filter ($n = 4$)	0.08 (7%)	1.02 (93%)	0.03 (13%)	0.19 (87%)
Dialysis membrane ($n = 4$)	0.53 (47%)	0.59 (53%)	1.02 (92%)	0.08 (8%)

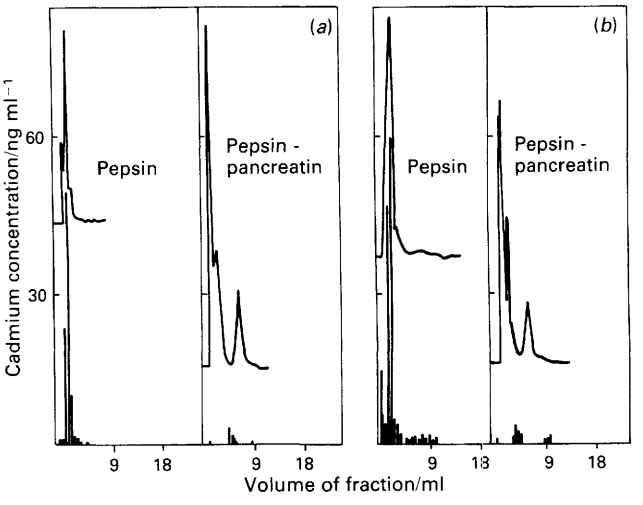

Fig. 2. Crab meat digest separated on a PRP-1 column using 5% *V/V* orthophosphoric acid, λ 254 nm. (*a*) Dialysis membrane and (*b*) 0.45-μm filter

that a transport effect depending on the size of the sample does not take place but that the selective nature of the dialysis membrane appears to dominate the transport of cadmium at both the stomach and intestinal pH conditions. The reduction in solubility of the cadmium - metallothioneine type complex at pH 7.4 is reflected in the significantly reduced transport of 8% observed for the dialysis membrane.

The cadmium profiles resulting from the chromatographic separation, using HPLC, of the transported fractions from both the filter and dialysis membrane are shown in Fig. 2. The results for UV are shown at only one wavelength (254 nm); chromatograms were, however, obtained at 210 and 280 nm. Fractions of 0.05 ml of eluant were collected into vials and their cadmium concentrations determined using ETAAS. Calibrations were carried out using standards made up in the HPLC eluent matrix. These experiments were repeated several times together with a similar procedure for blank digests and the appropriate corrections were made.

The results show one main difference between the filter [Fig. 2(*b*)] and the dialysis membrane fractions [Fig. 2(*a*)], namely the lack of the first cadmium peak in the membrane sample, clearly observed in the filtered sample for the pepsin fraction. From the various UV spectra obtained for the fractions, the second peak in the pepsin digest for the filter sample [Fig. 2(*b*)] and the only peak with a shoulder in the dialysis membrane sample [Fig. 2(*a*)] for the same digestion fraction appear to be associated with an organic group. It has been well documented that metallothioneine is the major cadmium binding protein in crab meat.[10,11] The protein contains neither disulphide nor free sulphydryl groups: instead all the cysteinyl side chains are bound to the metal ion in the mercaptide complex. It has been reported[13] that in invertebrates the cadmium binding protein exhibits high UV at 254 nm due to the cadmium - mercaptide shoulder (peculiar to metallothioneines). The result obtained suggests that the cadmium - mercaptide moiety is still intact at pH 2.0. The blank digests did not indicate any peaks for an organic species. The first peak in the pepsin fraction that is present only after filtration [Fig. 2(*b*)] appears to be due to a highly polar inorganic complex of cadmium. The interesting results observed from the two chromatograms of pepsin fractions are that the cadmium - metallothioneine remains intact and soluble at low pH. Thus the pepsin - pancreatin chromatogram confirms the earlier filter and membrane transport results indicating the reduced solubility of cadmium - metallothioneine at the intestinal pH of 7.4.

Conclusions

The use of a gut model for the study of cadmium bioavailability has been described. The results for the supernatant fractions from both the stomach (pepsin) and intestinal tract (pepsin - pancreatin) led to the following conclusions. Firstly, the bioavailability of cadmium cannot be determined from simply examining the soluble forms present, that is the fraction which passes through a 0.45-μm filter, as these results are significantly higher than the cadmium transported through a biological membrane. Secondly, the transport of highly polar inorganic complex does not appear to take place through the dialysis membrane, which only allows the transport of protein-complexed cadmium. Thirdly, the cadmium - metallothioneine complexes formed appear to be stable and highly soluble at pH 2.0 but are significantly reduced in solubility at pH 7.4. The results obtained confirm many of the *in vivo* results of earlier workers,[14] however, the gut model offers a simpler system by which to study the speciation and bioavailablity of trace elements.

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